Tulasi Satyanarayana
Sunil Kumar Deshmukh
Mukund V. Deshpande *Editors*

Progress in Mycology

Biology and Biotechnological Applications

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Preface

Fungi belong to a distinct, diverse, and ecologically important branch of Biology. These organisms play a vital and significant role in diverse ecosystems like soil, plant organic materials, rocks, and pelagic zones of the ocean. Fungi can be distinguished from plants by their heterotrophic nature, and distinct from animals too by their external rather than internal digestion. Fungi had diverged from their sister kingdom the animals 1.3 billion years ago. There is no trait that is uniquely shared by all fungi that defines the fungal kingdom. They are generally characterized by a chitinous cell wall and a form of nutrient uptake called osmotrophy whereby the enzymes breakdown larger substrates and molecules into smaller ones that can pass through the cell envelope. This mode of nutrition poses threat to ecosystems when exposed to pathogenic species that attack crops, wildlife, and humans. For outcompeting each other and other microbes, fungi are considered to have evolved several strategies to digest complex and insoluble substrates like lignin, cellulose, hemicellulose, and pectin which are major components of the plant organic matter, while fighting competitors using bioactive metabolites like antibiotics, ethanol, and organic acids.

Fungal diversity on Earth is estimated at 5.1–12 million species (M. Blackwell, 2011, Am J Bot, 98: 426–438; Wu, G. et al., 2014, Fungal Divers 69: 93–115). Among these, 1,40,000 fungal species have been described (Timothy, Y. et al., 2020, Annu Rev Microbiol 74: 291–313). Timothy et al. (2020) have recently organized 224 orders of fungal kingdom into 12 phyla (Ascomycota, Basidiomycota, Microsporidia, Chytridiomycota, Zoopagomycota, Cryptomycota, Mucoromycota, Neocallimastigomycota, Entorrhizomycota, Aphelidiomycota, Monoblepharidomycota, Blastocladiomycota). Genomics has a major impact on resolving the fungal tree of life. A great deal of progress has been made in elucidating the fungal tree using mitochondrial genomes, transcriptomics, a combination of a few common protein-coding genes (e.g. *RPB1*, *TEF*) and ribosomal RNA (rRNA) genes, or even just rRNA genes themselves.

The arrival of next-generation sequencing technology allows fungal genomes to be sequenced for as little as a few dollars now means that most phylogenetic studies up to species level can be conducted using genome-scale data. In recent years, two major separate efforts have been aimed at dramatically increasing the sequencing of fungal genomes. The first effort is the 1000 Fungal Genomes Project (http://1000.

fungalgenomes.org/home), which aims to sequence 1000 fungal genomes across the fungal tree of life by targeting taxa in lineages which were underrepresented or absent in genomic databases. This project has contributed more than 500 reference genomes towards more than 1500 now available for the kingdom. The second effort, known as the Y1000+ Project (https://y1000plus.wei.wisc.edu), is targeted to sequence the genomes of all ~1000 known species of budding yeasts (phylum Ascomycota, subphylum Saccharomycotina). The most recent output of this project has been an analysis of genomic data from 332 budding yeast species, including 220 new genomes.

Fungi have served a crucial role as model organisms for biological inquiry, such as brewer's yeast, *Saccharomyces cerevisiae*; and pink bread mould, *Neurospora crassa*. Major insights like the nature of the gene, autophagy, control of cell cycle, and how telomeres function have been made using morphologically simple organisms with complex cellular machinery similar to human cells. Because of their typically small genome sizes and life cycle stages with free-living haploid states, fungal genomes are easy to obtain, and fungi have served as models for genome evolution and reconstruction of phylogenetic relationships using genome-scale data.

Fungal biology has emerged in the last 10 years as a very strong scientific field with an enormous increase in the number of publications and understanding of basic and applied biological processes (Goldman, G.H, 2020, Front Fungal Biol. Vol. 1/article 596090, https://doi.org/10.3389/ffunb.2020.596090). Many fungal model systems have provided very important biological discoveries which go from George Beadle's hypothesis "one gene, one enzyme" using *Neurospora crassa* as a model system to the spectacular identification of essential components of the cytoskeleton, such as α-, β-, and γ-tubulin encoding genes in *Aspergillus nidulans*. This pioneering work has established the foundations for the introduction of molecular biology and genetic transformation systems in the Ascomycetes *N. crassa* and *A. nidulans*.

Although fungi are relatively understudied, these are an essential, fascinating, and biotechnologically useful group of organisms with an incredible biotechnological potential for industrial exploitation. Hyde et al. (Fungal Divers, 2019, 97: 1–136) have elegantly detailed 50 ways in which fungi can be exploited. The consumers around the world now increasingly prefer natural compounds over synthetic chemicals. Even in the industrial sectors that produce commodity chemicals, there is now an immense interest in developing sustainable biotechnological processes for obtaining new natural products that can eventually replace traditional synthetics. As compared to other biological sources, in particular plants, fungi have the great advantage that they can be grown in large bioreactors at an industrial scale, and suitable processes for their cost-effective fermentation have been available for many decades (e.g. for production of certain organic acids, enzymes, and antibiotics). The recent studies of Thai mycobiota by modern polyphasic approaches have been constantly revealing a plethora of new and undescribed species even in the fairly well-known genera of fungi like *Agaricus*. Even the majority of the known species in the fungal kingdom are virtually untapped as regards to potential applications since they have not yet been cultured and studied for their growth characteristics and

physiology. New methods and protocols have to be developed for this purpose, and this implies that substantial basic research must be carried out before the exploitation of the novel organisms can be envisaged.

Despite the fact that fungi have so many potential applications, research on their potential applications is in general poorly funded and much of the research is being carried out in academia is fundamental. For example, screening fungi for the production of antibiotics has often been reported, but it is unlikely to lead to industrial projects. Often, it will take over a decade even to bring a given project based on a novel fungal metabolite into the preclinics, and this is only possible by joint and interdisciplinary efforts of biologists, biotechnologists, pharmacists, and chemists. Furthermore, the Big Pharmaceutical industries have recently downsized their capacities for in-house research.

Several mycologists in India have made significant contributions in the diversity, ecology, and applied aspects of fungal strains isolated from a great variety of ecological niches spread throughout the country. This book entitled *Progress in Mycology: Biology and Biotechnological Applications* is an attempt to succinctly summarize the developments on various aspects of fungi encompassing diversity, ecology, and potential biotechnological applications. The book, with a major emphasis on bioprospecting, is divided into four parts. Each part contains 3–7 chapters contributed by experts with decade(s) of experience and praiseworthy expertise. Part I includes seven chapters on the developments in the production and potential biotechnologies related to lignin, starch, mannan, chitin, and phytate degrading extracellular fungal enzymes. Whereas Part II has three chapters on nanoparticles synthesized using various fungi and their applications in health care. Part III, containing six chapters, focuses on plant and human fungal pathogens and their control. The last Part IV consists of five chapters which deal with bioprospecting of fungi. We wish to thank all the contributors for putting in extensive and intensive efforts in writing and submitting the chapters in response to our request in the stipulated time.

Thanks are also due to Springer Nature for publishing the book in an efficient manner.

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About the Editors

T. Satyanarayana has been UGC-Faculty Fellow and Professor Emeritus at the Division of Biological Sciences and Engineering, Netaji Subhas University of Technology, New Delhi, after superannuating from the Department of Microbiology, University of Delhi South Campus, New Delhi, as Professor and Head, in June 2016. He has over 275 scientific papers and reviews, 11 edited books, and 3 patents to his credit. He is a fellow of the National Academy of Agricultural Sciences (NAAS), Academy of Microbiological Sciences (AMSc), Biotech Research Society (I) [BRSI], Mycological Society of India (MSI), and Telengana Academy of Sciences. He is a recipient of Dr. Manjrekar Award of AMI, Dr. Agnihotrudu Memorial Award of MSI, and Malaviya Memorial Award of BRSI for his significant contributions. He has over 45 years of research and teaching experience and has mentored 30 scholars for Ph.D. He was the president of AMI and MSI. He successfully executed 17 major research projects sanctioned by various Govt. agencies. He had collaborative Indo-German and Indo-Japanese research projects. His research efforts have been focused on understanding the diversity and applications of yeasts, thermophilic fungi and bacteria and their enzymes, metagenomics, carbon sequestration employing extremophilic bacterial carbonic anhydrases and bioethanol production from lignocellulosics employing microbial enzyme cocktails.

Sunil Kumar Deshmukh received his Ph.D. in Mycology from Dr. H.S. Gour University, Sagar (M.P.) in 1983. Veteran industrial mycologist who spent a substantial part of his career at Hoechst Marion Roussel Limited [now Sanofi India Ltd.], Mumbai, and Piramal Enterprises Limited, Mumbai, in drug discovery. He has to his credit 8 patents, 135 publications, and 13 books on various aspects of fungi and natural products of microbial origin. He was the president of the Mycological Society of India (MSI). He is a fellow of MSI, the Association of Biotechnology and Pharmacy, the Society for Applied Biotechnology, and Maharashtra Academy of Science. He was Fellow at Nano-Biotechnology Centre, TERI, New Delhi, and Adjunct Associate Professor in Deakin University, Australia, till Jan 2019 who had been working towards the development of natural food colours, antioxidants, and biostimulants through nanotechnology intervention.

Mukund V. Deshpande obtained his Ph.D. in 1982 in Biochemistry and D.Sc. in Microbiology of the University of Pune in 1994. His extensive work in the area of fungal biology especially fungal differentiation earned him D.Sc. Since then he has been working extensively on the use of fungi and fungal products in Biotechnology. Dr. Deshpande successfully completed more than 35 research projects funded by national and international funding agencies. Indo-Swiss Collaboration in Biotechnology (ISCB) Programme of the Department of Biotechnology (DBT), New Delhi, and Swiss Development Cooperation (SDC), Berne, Switzerland, on the development of mycoinsecticide, Indo-Belarus programme of DBT on biopesticides, Indo-Mexico programme of Department of Science and Technology, New Delhi, and CONACYT on fungal dimorphism, to name a few. Dr. Deshpande is an elected fellow of Maharashtra Academy of Sciences (FMASc, 1994) and the Society for Biocontrol Advancement (FSBA, 2010). He was also a recipient of the Department of Biotechnology Overseas (Short-term) Associateship (1995) and Commonwealth Science Council Fellowship (1998). He has to his credit more than 150 research papers, reviews, and chapters, 8 patents, 8 books, and a number of popular articles. He has his own start-up Greenvention Biotech located in Uruli Kanchan, Pune, for the translational activities in Agricultural Biotechnology.

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Part I

Fungal Enzymes

Progress in Fungal Mannanolytic Enzyme Research in India

1

Suresh Nath and Naveen Kango

Abstract

Apart from being the major constituent of the hemicellulosic fraction of lignocellulosic biomass, mannans are also found associated with some plant seeds as storage polysaccharide. Mannan degradation involves endo-β-mannanases and β-mannosidases along with β-glucosidases and α-galactosidases as accessory enzymes. Fungi, being natural lignocellulosic degraders, are being explored for the production of mannanolytic enzymes. Mannanases find a number of applications in various industries, and mannooligosaccharides (MOS) are being explored as health-promoting prebiotics. Looking at the quantum of mannan-rich agro-waste produced from copra and palm oil industries in south-bound states of India and huge amounts of guar gum produced in Rajasthan and Gujarat, it becomes immensely important for Indian scientists to develop enzyme-based technology for their value addition. The present chapter focuses on the contribution of Indian researchers in the area of production and application of mannanolytic enzymes from fungi.

Keywords

Mannans · β-Mannanase · β-Mannosidase · α-Galactosidases · Locust bean gum · Guar gum · Fungi

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1.1 Introduction

Mannans occur as structural and storage polysaccharides in a variety of plants. Apart from being part of the plant cell wall hemicellulose, mannans such as locust bean gum, guar gum, ivory gum, konjac gum, and fenugreek gum are found associated with plant seeds (Scheller and Ulvskov 2010). Mannans are composed of repeated mannose units linked by β-1,4-glycosidic bonds. Variation in the β-mannan structure due to glucose occurring in the main chain and galactose substitution in side chains results in glucomannan, galactomannan, and galactoglucomannan (Soni and Kango 2013; Singh et al. 2018). Sometimes, acetylation at C-2 or C-3 of mannose and glucose residues in the backbone is also present. Linear or homopolymeric mannans are found in the seeds of many plants, such as green coffee (*Coffea* spp.) and ivory nuts (*Phytelephas* spp.), and in the cell walls of some algae, e.g., *Codium* spp. (Chauhan et al. 2012). Galactomannans are derived from seed endosperms of some leguminous plants (e.g., guar), seaweeds, and some fungi in which the β-1→4-D-mannan backbone has galactose substituent at every C-6 position. Commercially available galactomannans with varying mannose/galactose ratios include fenugreek gum (~1:1), guar gum (~2:1), Tara gum (~3:1), and locust bean gum (~4:1). High number of galactosyl units makes Tara gum and fenugreek gum readily soluble even in cold water (Singh et al. 2018). Hydrophilic glucomannans consist of randomly arranged β-(1,4)-linked D-mannose chains and β-(1,4)-linked D-glucose residues in a ratio of 3:1, and the degree of polymerization (DP) is greater than 200 (Moreira and Filho 2008). Konjac gum is an important commercial source of glucomannan obtained from the *Amorphophallus konjac* plant. Ferns such as *Selaginella kraussiana*, *Equisetum giganteum*, *Microgramma squamulosa*, and *Adiantum raddianum* have glucomannans in cell walls (Singh et al. 2018).

Mannose, MOS, and partially hydrolyzed mannans have received attention for the generation of prebiotic oligosaccharides and animal feed additives (Jana et al. 2021a). Hydrolysis of plant mannans to useful mannose, prebiotic MOS, and partially hydrolyzed guar gum (PHGG) can be achieved using microbial mannanases. Major enzymes required for the breakdown of the mannan main chain made of mannose units include endo (1→4)-β-mannanase (EC 3.2.1.78) and exo (1→4)-β-mannanase or β-mannosidase (EC 3.2.1.25). Apart from these, β-glucosidase (EC 3.2.1.21) and α-galactosidase (EC 3.2.1.22) are also required to achieve complete hydrolysis of gluco-, galacto-, and galactoglucomannans (Fig. 1.1). The agroindustry by-products or wastes like copra meal (264 × 1000 MT/year) and PKC (10 × 1000 MT/year) are produced in large amounts in some southern states of India (https://www.indexmundi.com). Such mannan-rich agro-wastes can be exploited for the generation of value-added products such as MOS. Similarly, guar gum, a galactomannan produced at a very large scale in Rajasthan and Gujarat states of India, deserves special attention. It can be utilized for generation of value-added products using fungal mannanases.

Due to high titres, ease of production in solid-state cultivations, and choice of strains available, fungal mannanases predominate the scenario of mannanolytic enzymes (Soni and Kango 2013). This chapter gives an account of the important

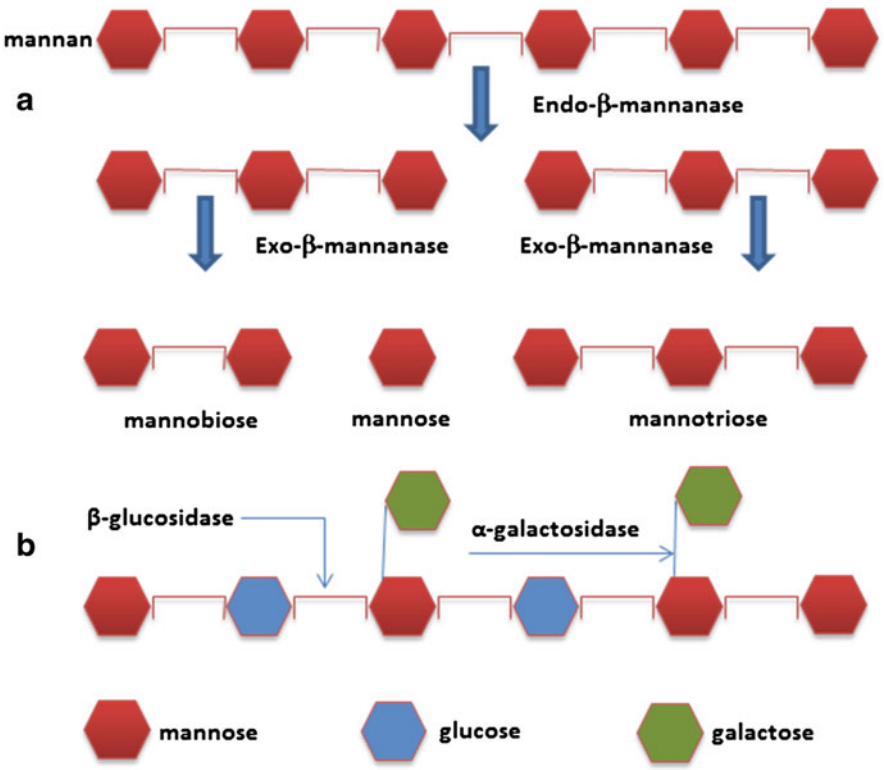

Fig. 1.1 Mannan being acted upon by mannanolytic enzymes (**a**) Linear mannan (ivory nut). (**b**) Galactoglucomannan (spruce tree)

milestones and noteworthy contributions of Indian scientists and mycologists in the area of fungal mannanases and their applications.

1.2 β-Mannanases

β-mannanases, the endo-acting hydrolases, attacking the internal glycosidic bonds of the mannan backbone chain, are commonly found as part of the hemicellulase repertoire produced by ascomycetous fungi (van Zyl et al. 2010). β-Mannanases have multifarious applications in food and feed processing, 2G biofuel generation, pulp and paper, and detergent industries (Fig. 1.2). Naganagouda et al. (2010) reported the production of β-mannanase from *Aspergillus niger* and demonstrated its applications in food processing. Similarly, β-mannanase of *A. niger* NS-2 was used for bioconversion of domestic waste residues into simple sugars for the production of 2G biofuel (Bansal et al. 2011). Both strains produced β-mannanases in submerged fermentation using locust bean gum as the substrate. Thermophilic fungi isolated from Indian habitats, such as *Thermomyces lanuginosus*

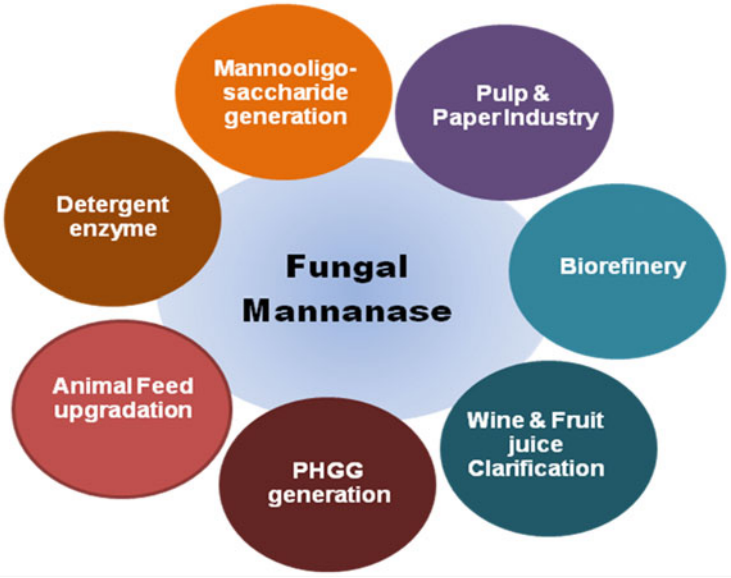

Fig. 1.2 Applications of fungal mannanases

MTCC 9331, *Myceliophthora fergusii* MTCC 9293, and *Malbranchea cinnamomea* MTCC 9294 along with thermotolerant *Aspergillus terreus* FBCC1369, were explored for the production of thermostable mannanase and accessory enzymes (Maijala et al. 2012). Soni et al. (2015) used rotatable central composite design (RCCD) to enhance the production of β-mannanase by ten times (417 U/gds) by *Aspergillus terreus* FBCC 1369 using palm kernel cake in solid-state fermentation. Use of β-mannanase was made to hydrolyze locust bean gum (LBG) and guar gum to produce MOS. In continuation, Soni et al. (2016) used different agro-wastes like wheat bran, wheat straw, rice husk, copra meal, palm kernel cake, fenugreek seed meal, and aloe vera pulp for the production of β-mannanase having utility in MOS (3–4) generation. Ahirwar et al. (2016a) have explored thermophilic fungi for β-mannanase production which could be used for applications in different areas, including in the pulp and paper, food and feed, and textile industries. Thermophilic *Malbranchea cinnamomea* NFCCI 3724 was used for the production of β-mannanase, which efficiently converted LBG and konjac gum into mannotetraose (M4).

Ahirwar et al. (2016b) also used palm kernel cake to produce thermostable β-mannanase from *M. cinnamomea* NFCCI 3724 for hydrolysis of a galactomannan (LBG) and a glucomannan (konjac gum) for production of MOS of 3–4 degree of polymerization (DP 3–4). Rastogi et al. (2016) have demonstrated the use of rice straw for low-cost production of β-mannanase by *Pyrenophora phaeocomes* S-1 with 10.45 IU/gds yield. Recently, Kaur et al. (2020) used a low-cost approach for production of β-mannanases (57 IU/g) of *A. niger* P-19 using rice straw as substrate.

Under optimized conditions, *Aspergillus oryzae* produced a multi-tolerant β-mannanase (434 U/gds) on copra meal, a low-cost agro-waste. The enzyme had MW ~34 kDa and specific activity of 335.85 U/mg (Jana et al. 2018). Apart from endocellulase (750 U/gds), *Aspergillus tubingensis* NKBP-55 produced 167 U/gds of xylanases, 1023 U/gds of mannanase, and 54 U/gds of α-galactosidase on copra meal in Solid state fermentation (SSF) (Prajapati et al. 2018). Zymography revealed the presence of six mannanases of varying molecular weights (Table 1.1).

The end-products of *A. oryzae* β-mannanase action on various mannans like, LBG, guar gum, and konjac gum were visualized by fluorophore-assisted carbohydrate electrophoresis (FACE) technique, which showed formation of sugars from DP 1–4 and some higher DP MOS from LBG, guar gum, and konjac gum (Jana et al. 2018). Suryawanshi et al. (2019) have demonstrated the use of glutaraldehyde-activated aluminum oxide pellets for immobilization of β-mannanase of *Aspergillus quadrilineatus* RSNK-1. Immobilization led to enhancement in pH and thermal stability of the enzyme with ten-cycle reusability for LBG hydrolysis. The preparation was used for clarification of fruit juices (apple, kiwi, orange, and peach) to enhance their clarity and to reduce sugar content. Recently, Jana and Kango (2020) reported that MOS derived from palm kernel cake using *A. oryzae* mannanase showed high cytotoxicity (74.19%) against the human colon adenocarcinoma cell line (Caco-2) in comparison to MOS derived from copra meal and guar gum. Similarly, Suryawanshi and Kango (2021) used 4 U/ml of β-mannanases of *A. quadrilineatus* to hydrolyze defatted copra meal, konjac gum, and LBG to produce partially hydrolyzed mannans that showed inhibition of Caco-2 cells and promoted generation of short-chain fatty acids (scFA) and film formation in probiotic *Lactobacillus delbrueckii* NCIM 2025 and *Lactobacillus acidophilus* NCIM 5306. The details regarding production and characteristics of endo-β-mannanases from fungal sources are presented in Table 1.1.

Srivastava and Kapoor (2016) have provided up-to-date information about the production strategies, biochemical and physical properties, and important applications of endo-mannanases. Some Indian workers have also explored bacterial mannanases for a variety of applications. Kaira et al. (2016) used solubilized-defatted flax seed meal for economic production of recombinant endo-mannanase (ManB-1601), which resulted in 3.25-fold (5926 U/ml) higher production and later used it for oil extraction from copra, which led to higher (18.75%) oil yield. Cross-linked enzyme aggregates (CLEAs) of ManB-1601 and novel chitosan magnetic nanocomposite CLEAs of ManB-1601 improved its activity by 73.2%, stability up to 83%, and reusability up to 12 cycles (Panwar et al. 2017). Recently, Kaira et al. (2019) studied the eight salt bridges in the GH26 endo-mannanase from *Bacillus* sp. and checked the effect of disruption of these salt bridges on its substrate affinity and catalytic efficiency. Simulation studies proved that salt bridges help in preserving the biological activity from thermal denaturation by rigidifying the active site.

Table 1.1 Production and characteristics of endo-β-mannanases reported by Indian mycologists

Fungal species	Substrate	Optimum activity		Molecular weight (kDa)	Mannanase activity	Potential application	Reference
		Temp (°C)	pH				
Aspergillus niger gr.	Copra meal	55	5.5	66	0.33 mg/ml	Food processing industry	Naganagouda et al. (2010)
Aspergillus niger NS-2	Locust bean gum	30	5	–	160 ± 6 IU/gds	2G biofuel generation	Bansal et al. (2011)
Aspergillus terreus FBCC 1369	Palm kernel cake (PKC)	50	8	–	417 U/gds	MOS generation	Soni et al. (2015)
Fusarium equiseti NFCCI 3284	Wheat bran	50	5	–	5945 nkat/gds	MOS generation	Soni et al. (2016)
Acrophialophora levis NFCCI 3286	Palm kernel cake	28	–	–	4726 nkat/gds	MOS generation	Soni et al. (2016)
Aspergillus terreus FBCC 1369	Copra meal	70	7	~49	5.9 mg/ml	MOS generation	Soni et al. (2016)
Malbranchea cinnamomea NFCCI 3724	Palm kernel cake	45	9	–	599 U/gds	MOS generation	Ahirwar et al. (2016a)
Pyrenophora phaeocomes S-1	Rice straw	55	5	–	10.45 IU/gds	Mannanase production on rice straw	Rastogi et al. (2016)
Aspergillus tubingensis NKBP-55	Copra meal	–	–	60, 40, 35, 30, 25, 20	1023 U/gds	Cellulase and hemicellulase production	Prajapati et al. (2018)
Aspergillus oryzae MTCC 1846	Copra meal	60	5	34 kDa	434 U/gds	MOS generation	Jana et al. (2018)
Aspergillus quadrilineatus RSNK-1	Copra meal	45	10	–	1021 U/gds	MOS generation Fruit juice clarification	Suryawanshi et al. (2019)
Aspergillus niger P-19	Rice straw	60	4	–	57 IU/g	Low-cost mannanase production	Kaur et al. (2020)

1.3 Accessory Mannanolytic Enzymes

Some mannans, being heteropolymeric, require accessory enzymes for complete degradation. β-Glucosidases cleave the glucose moieties from glucomannan and galactoglucomannan, while the removal of galactose residues is carried out by α-galactosidases and acetyl mannan esterases. α-Galactosidases remove the α-1,6-linked D-galactopyranosyl substituent attached to the mannan backbone, whereas acetyl mannan esterases release the acetyl groups from galactoglucomannan (van Zyl et al. 2010).

Among these accessory mannanolytic enzymes, α-galactosidases have been explored by some Indian researchers (Table 1.2). Kotwal et al. (1998) produced α-galactosidases of *Humicola* sp. NCIM 1252 on soy flour with 44.6 U/g and used it to hydrolyze raffinose and stachyose from soymilk. Thippeswamy and Mulimani (2002) immobilized α-galactosidases of *Gibberella fujikuroi* in polyacrylamide gel, which could retain 90% activity after 12 h of incubation. Similarly, Prashanth and Mulimani (2005) immobilized α-galactosidases of *A. oryzae* in calcium alginate and used it for reduction in the amount of raffinose family oligosaccharide content in soymilk. Naganagouda and Mulimani (2006) immobilized α-galactosidase of *A. oryzae* in gelatin-blended alginate hydrogel fiber and observed higher immobilization yield, greater storage stability, and better hydrolysis of oligosaccharides present in soymilk. Shankar and Mulimani (2007) scaled up α-galactosidase production of *A. oryzae* using a tray system for fermentation on red gram plant waste (RGPW) with wheat bran (WB). Aqueous two-phase system was used for partial purification and downstream processing of α-galactosidase from *A. oryzae* grown on guar gum, and a purification factor of 3.6 and 87.71% yield of enzyme activity in the bottom phase was obtained (Naganagouda and Mulimani 2008). Kumar and Mishra (2010) purified thermostable α-galactosidase from *Aspergillus parasiticus* MTCC-2796 with 2194.5 units of activity and observed a monomeric protein with 67.5 kDa molecular weight and used it to catalyze transglycosylation reaction for the synthesis of melibiose. *A. terreus* FBCC 1369 produced α-galactosidase and β-glucosidase on palm kernel cake, and the enzymes were used to digest locust bean gum and guar gum to produce MOS (Soni et al. 2015). *M. cinnamomea* NFCCI 3724 produced thermostable α-galactosidase, β-mannosidase, and β-glucosidase on copra meal, and the enzymes were used to hydrolyze konjac gum, locust bean gum, and guar gum to produce mannotetraose as the major product (Ahirwar et al. 2016a). Gajdhane et al. (2016) used central composite rotatable design (CCRD) for α-galactosidase production from *Rhizopus oryzae* SUK, resulting in 17.74-fold increase. Recently, Vidya et al. (2020) purified a thermostable α-galactosidase (118 ± 2 kDa and 22 U/mg) from *Aspergillus awamori* MTCC 548 on wheat bran supplemented with 6% defatted soy flour.

Table 1.2 Production and characteristics of accessory mannanolytic enzymes reported by Indian researchers

Fungal species	Substrate	Accessory mannanolytic enzyme	Optimum		Enzyme activity	Reference
			Temp (°C)	pH		
Humicola sp. NCIM 1252	Soy flour	α-Galactosidase	45	8.5	44.6 U/g	Kotwal et al. (1998)
Gibberella fujikuroi	Soymilk	α-Galactosidase	56	5.4	–	Thippeswamy and Mulimani (2002)
Aspergillus oryzae	Guar gum	α-Galactosidase	37	5	0.156 U/mg	Naganagouda and Mulimani (2008)
Aspergillus parasiticus MTCC-2796	Galactose	α-Galactosidase	50	5	2194.5 units	Kumar and Mishra (2010)
Aspergillus terreus FBCC 1369	Palm kernel cake	α-Galactosidase and β-glucosidase	37	6.5	13 U/gds and 21 U/gds	Soni et al. (2015)
Malbranchea cinnamomea NFCCI 3724	Copra meal	α-Galactosidase, β-mannosidase, β-glucosidase	45	7.75	0.017, 0.059, 0.044 U/gds	Ahirwar et al. (2016a)
Rhizopus oryzae SUK	Wheat bran with soybean meal and molasses	α-Galactosidase	50	5	218.45 U/g	Gajdhane et al. (2016)
Fusarium fusarioides NFCCI 3282	Locust bean gum	α-Galactosidase	50	5	20 ± 1.5 nkat/ml	Soni et al. (2017)
Fusarium equiseti NFCCI	Konjac gum	β-Glucosidase	50	5	7.8 ± 0.7 nkat/ml	Soni et al. (2017)
Cladosporium cladosporioides NFCCI 3285	Konjac gum	β-Mannosidase	50	5	0.12 nkat/ml	Soni et al. (2017)
Aspergillus awamori MTCC 548	30 g of wheat bran substituted with 6% defatted soy flour	α-Galactosidase	55–60	5.0–5.5	22 U/mg	Vidya et al. (2020)

1.4 Guar Gum: Indian Scenario

Guar gum, a gel-forming galactomannan obtained from *Cyamopsis tetragonolobus*, is a linear polysaccharide of 1,4-mannose residues linked to 1,6-galactose at every second mannose. A relatively less explored agro-produce, it has been used as a thickener and stabilizer in industries. Mainly implicated in cloth and paper industries, ionic guar gum derivatives are employed in oil and gas well stimulation. These applications have led to high commercial relevance of guar gum. More recently, guar gum derivatives PHGG and MOS are being explored for the treatment of health problems like irritable bowel syndrome, diabetes, heart disease, and colorectal cancer (Kango et al. 2019; Jana and Kango 2021; Jana et al. 2021b).

India, being the largest producer and exporter, predominates the guar gum market and is currently pursuing cost-effective technologies to manufacture value-added guar products to compete at the global level. Various reports have pointed out that the global guar gum market was valued at USD 708.6 million in 2016 and is projected to reach USD 1115.8 million by 2022, at a CAGR of 7.9% during the forecast period. India has exported 381,880.16 MT to the world in the year 2019–2020 (http://apeda.gov.in/apedawebsite/SubHead_Products/Guargum.htm). The future of Indian guar scenario largely depends on economic viability and advancements in the production and processing in guar gum industries. Development of processes for the production of value-added derivatives such as prebiotic MOS and PHGG through efficient extraction and enzyme-based processing is the need of the hour. As per the Technology Information Forecasting and Assessment Council (TIFAC) report 2015, improved gum qualities with high gum content above 35% with varied viscosity levels in guar seed are required for use in the manufacturing of different products of food and industrial grade. Of the total global production (15–20 lakh tons annually), India is the most important guar producer in the world, contributing almost 75–82% of global guar grain production. Rajasthan accounts for 65–70%, followed by Gujarat, Haryana, Punjab, Uttar Pradesh, and Madhya Pradesh. Looking at the quantum of guar production and its potential applications, it is very safe to conclude that it is highly socially relevant to farmers and guar industry persons. However, for various reasons, Indian guar export has shown instability in terms of volume and value. Thus, it invites attention for higher technology inputs, higher value realization, future potential, and sector performance. Guar gum is used in food, cosmetics, pharmaceuticals, and personal care preparations, employing water as the solvent. The demand for increasing quality, advanced processing, high-value guar derivatization, and developing alternate applications of guar is the recent spurt of interest. Enzyme technology employing mannanases from native microbial strains holds great potential in achieving these objectives. Enzyme-mediated bioprocessing has led to environment-friendly and cost-effective bioprocesses. Till now, there has been no study on enzyme-assisted extraction of guar gum. Enzyme pretreatment during guar extraction may lead to a significant gain in terms of quality and quantity. Mudgil et al. (2018) have demonstrated guar galactomannan hydrolysis by cellulase sourced from *A. niger* to generate PHGG, which can be utilized as a source of prebiotic for lactic acid

bacteria. Jana et al. (2018) have described the hydrolysis of guar gum and other mannan-containing substrates by *A. oryzae* β-mannanase and demonstrated the generation of PHGG and MOS. Soni et al. (2015) reported the generation of mannose, mannobiose (M2), and mannotetraose (M4) after enzymatic hydrolysis of guar gum by *A. terreus* FBCC 1369. Mudgil et al. (2016) have enzymatically hydrolyzed guar gum to lower its viscosity for possible use as dietary fiber. Shobha et al. (2014) have demonstrated the de-branching of guar gum by porcine pepsin for better solubility.

1.5 Conclusions and Future Perspectives

Among hemicellulases, mannanases are receiving increasing attention now. With the growing interest in potential functional food ingredients like MOS and feed components like PHGG, Indian scientists have started taking due interest in fungal mannanolytic enzymes. Mannanases are also being explored for their potential applications in diverse industries like detergent, pulp and paper, fruit juice, etc. As per reports, several *Aspergillus* species such as *A. oryzae*, *A. quadrilineatus*, *A. terreus*, and *A. tubingensis* stand out to be better mannanase producers. Genetic engineering of fungal mannanolytic enzymes in Indian context has been limited and remains a future prospect henceforth. Workers have found impact of mannanase supplementation on poultry as significant, while the effect of MOS on human and animal health is still being explored. Intensive research on these enzymes and their products is certain to bring out novel aspects of their utility.

References

Ahirwar S, Soni H, Rawat HK, Ganaie MA, Pranaw K, Kango N (2016a) Production optimization and functional characterization of thermostable β-mannanase from *Malbranchea cinnamomea* NFCCI 3724 and its applicability in mannotetraose (M4) generation. J Taiwan Inst Chem Eng 63:344–353

Ahirwar S, Soni H, Rawat HK, Prajapati BP, Kango N (2016b) Experimental design of response surface methodology used for utilisation of palm kernel cake as solid substrate for optimised production of fungal mannanase. Mycology 7:143–153

Bansal N, Tewari R, Gupta JK, Soni R, Soni SK (2011) A novel strain of *Aspergillus niger* producing a cocktail of hydrolytic depolymerizing enzymes for production of second generation biofuels. Bioresources 6:552–569

Chauhan P, Puri N, Sharma P, Gupta N (2012) Mannanases: microbial sources, production, properties and potential biotechnological applications. Appl Microbiol Biotechnol 93:1817–1830

Gajdhane SB, Bhagwat PK, Dandge PB (2016) Statistical media optimization for enhanced production of α-galactosidase by a novel *Rhizopus oryzae* strain SUK. Biocatal Agric Biotechnol 8:301–309

Jana UK, Kango N (2020) Characteristics and bioactive properties of mannooligosaccharides derived from agro-waste mannans. Int J Biol Macromol 149:931–940

Jana UK, Kango N (2021) Applications of fungal hemicellulases. In: Zaragoza O, Casadevall A (eds) Encyclopedia of mycology. Elsevier, pp 305–315

Jana UK, Suryawanshi RK, Prajapati BP, Soni H, Kango N (2018) Production optimization and characterization of mannooligosaccharide generating β-mannanase from *Aspergillus oryzae*. Bioresour Technol 268:308–314

Jana UK, Suryawanshi RK, Prajapati BP, Kango N (2021a) Prebiotic mannooligosaccharides: Synthesis, characterization and bioactive properties. Food Chem 342:128328

Jana UK, Kango N, Pletschke BI (2021b) Hemicellulose derived oligosaccharides: emerging prebiotics in disease alleviation. Front Nutr 8:427

Kaira GS, Panwar D, Kapoor M (2016) Recombinant endo-mannanase (ManB-1601) production using agro-industrial residues: Development of economical medium and application in oil extraction from copra. Bioresour Technol 209:220–227

Kaira GS, Usharani D, Kapoor M (2019) Salt bridges are pivotal for the kinetic stability of GH26 endo-mannanase (ManB-1601). Int J Biol Macromol 133:1236–1241

Kango N, Jana UK, Choukade R (2019) Fungal enzymes: sources and biotechnological applications. In: Satyanarayana T et al (eds) Advancing frontiers in mycology & mycotechnology. Springer Nature Pte Ltd, Singapore, pp 515–538

Kaur J, Chugh P, Soni R, Soni SK (2020) A low-cost approach for the generation of enhanced sugars and ethanol from rice straw using in-house produced cellulase-hemicellulase consortium from *A. niger* P-19. Bioresour Technol Rep 11:100–469

Kotwal SM, Gote MM, Sainkar SR, Khan MI, Khire JM (1998) Production of α-galactosidase by thermophilic fungus *Humicola sp*. in solid-state fermentation and its application in soyamilk hydrolysis. Process Biochem 33:337–343

Kumar S, Mishra SK (2010) Purification and characterization of a thermostable α-galactosidase with transglycosylation activity from *Aspergillus parasiticus* MTCC-2796. Process Biochem 45:1088–1093

Maijala P, Kango N, Szijarto N (2012) Characterization of hemicellulases from thermophilic fungi. Anton Leeuw Int J G 101:905–917

Moreira LRS, Filho EXF (2008) An overview of mannan structure and mannan-degrading enzyme systems. Appl Microbiol Biotechnol 79:165–178

Mudgil D, Barak S, Khatkar BS (2016) Development of functional yoghurt via soluble fiber fortification utilizing enzymatically hydrolyzed guar gum. Food Biosci 14:28–33

Mudgil D, Barak S, Patel A, Shah N (2018) Partially hydrolyzed guar gum as a potential prebiotic source. Int J Biol Macromol 112:207–210

Naganagouda K, Mulimani VH (2006) Gelatin blends with alginate: Gel fibers for α-galactosidase immobilization and its application in reduction of non-digestible oligosaccharides in soymilk. Process Biochem 41:1903–1907

Naganagouda K, Mulimani VH (2008) Aqueous two-phase extraction (ATPE): An attractive and economically viable technology for downstream processing of *Aspergillus oryzae* α-galactosidase. Process Biochem 43:1293–1299

Naganagouda K, Salimath V, Mulimani VH (2010) Purification and characterization of endo-β-1,4 mannanase from *Aspergillus niger* gr for application in food processing industry. J Microbiol Biotechnol 19:1184–1190

Panwar D, Kaira GS, Kapoor M (2017) Cross-linked enzyme aggregates (CLEAs) and magnetic nanocomposite grafted CLEAs of GH26 endo-β-1,4-mannanase: Improved activity, stability and reusability. Int J Biol Macromol 105:308–314

Prajapati BP, Suryawanshi RK, Agrawal S, Ghosh M, Kango N (2018) Characterization of cellulase from *Aspergillus tubingensis* NKBP-55 for generation of fermentable sugars from agricultural residues. Bioresour Technol 250:733–740

Prashanth SJ, Mulimani VH (2005) Soymilk oligosaccharide hydrolysis by *Aspergillus oryzae* α-galactosidase immobilized in calcium alginate. Process Biochem 40:1199–1205

Rastogi S, Soni R, Kaur J, Soni SK (2016) Unravelling the capability of *Pyrenophora phaeocomes* S-1 for the production of ligno-hemicellulolytic enzyme cocktail and simultaneous bio-delignification of rice straw for enhanced enzymatic saccharification. Bioresour Technol 222:458–469

Scheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol 61:263–289
Shankar SK, Mulimani VH (2007) α-Galactosidase production by *Aspergillus oryzae* in solid-state fermentation. Bioresour Technol 98:958–961
Shobha MS, Gowda LR, Tharanathan RN (2014) A novel catalysis by porcine pepsin in debranching guar galactomannan. Carbohydr Polym 102:615–621
Singh S, Singh G, Arya SK (2018) Mannans: an overview of properties and application in food products. Int J Biol Macromol 119:79–95
Soni H, Kango N (2013) Microbial mannanases: properties and applications. In: Shukla P, Pletscke BI (eds) Advances in enzyme biotechnology. Springer, New Delhi, pp 41–56
Soni H, Ganaie MA, Pranaw K, Kango N (2015) Design-of-experiment strategy for the production of mannanase biocatalysts using palm kernel cake and its application to degrade locust bean and guar gum. Biocatal Agric Biotechnol 4:229–234
Soni H, Rawat HK, Pletschke BI, Kango N (2016) Purification and characterization of β-mannanase from *Aspergillus terreus* and its applicability in depolymerization of mannans and saccharification of lignocellulosic biomass. 3 Biotech 6:136
Soni H, Rawat HK, Ahirwar S, Kango N (2017) Screening, statistical optimized production, and application of β-mannanase from some newly isolated fungi. Eng Life Sci 17:392–401
Srivastava PK, Kapoor M (2016) Production, properties, and applications of endo-β-mannanases. Biotechnol Adv 35:1–19
Suryawanshi RK, Kango N (2021) Preoduction of mannooligosaccharides from various mannans and evaluation of their prebiotic potential. Food Chem 334:127428
Suryawanshi RK, Jana UK, Prajapati BP, Kango N (2019) Immobilization of *Aspergillus quadrilineatus* RSNK-1 multi-enzymatic system for fruit juice treatment and mannooligosaccharide generation. Food Chem 289:95–102
Thippeswamy S, Mulimani VH (2002) Enzymic degradation of raffinose family oligosaccharides in soymilk by immobilized α-galactosidase from *Gibberella fujikuroi*. Process Biochem 38:635–640
van Zyl WH, Rose SH, Trollope K, Görgens JF (2010) Fungal β-mannanases: mannan hydrolysis, heterologous production and biotechnological applications. Process Biochem 45:1203–1213
Vidya CH, Gnanesh Kumar BS, Chinmayee CV, Singh SA (2020) Purification, characterization and specificity of a new GH family 35 galactosidase from *Aspergillus awamori*. Int J Biol Macromol 156:885–895

Thermophilic Fungal Lignocellulolytic Enzymes in Biorefineries

2

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Abstract

Lignocellulose-based (2G) ethanol plants have become operational at commercial and demonstration scale. Some of the major companies, such as Raizen-Iotech, Novozymes, Clariant, DSM POET, and PRAJ, have taken the lead in this venture. However, the research and development for reducing the cost of lignocellulosic ethanol is a hotly pursued area. Technological innovations for improved pretreatment of the lignocellulosic substrates and bioprospecting and developing robust catalytically active lignocellulolytic enzymes for efficient hydrolysis of cellulose are desired to make lignocellulose-based white biotechnology a reality in the near future. This chapter discusses the current scenario of two of the key components of the bioconversion platform, i.e., pretreatment and enzyme hydrolysis approaches, being followed in the existing facilities.

Keywords

Pretreatment technologies · Lignocellulolytic enzymes · LPMO · Secretome · Cellulose hydrolysis · Enzyme cocktail design

2.1 Introduction

Rapidly exhausted fossil fuel reserves, escalation of fuel prices, and their unfavorable environmental impacts are some of the global challenges that are compelling the world to switch toward sustainable lignocellulose-based biofuels. Substantial research and development on the large-scale production of second-generation cellulosic ethanol (2G) from lignocellulosic biomass (LCB) is now being considered

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worldwide. The goal of achieving 60 billion gallons of biofuel per year by 2030 has been set by the USA, whereas replacing 25% of transportation fuel with biofuel by 2030 has been set by the European Union (Himmel and Ding 2007). The production of first-generation biofuel from corn (in the USA) and sugarcane (in Brazil) is inadequate to meet the demand of such volumes. Constant debate on food versus fuel is also underway in several countries. As a result, lignocellulosic waste such as rice straw, wheat straw, sugarcane bagasse, and corncob has been found as the best suitable raw material for 2G ethanol production without compromising global food security (Bayer et al. 2007). LCB includes agricultural residues such as sugarcane bagasse, rice straw, wheat straw, corn stover, wood, grass, and dedicated energy crops such as *Miscanthus*, *Arundo*, and switchgrass, which are being targeted as abundant and renewable resources (Gomez et al. 2008). These LCB substrates, which are considered waste or low-value commodity, however, are rich source of complex carbohydrates consisting of cellulose (35–40%), hemicellulose (25–35%), and lignin (12–18%). Cellulose is a long-chain polymer of β-1,4-linked glucose units, which in turn form higher-order microfibrillar structure (Yang et al. 2011), while hemicelluloses constitute homopolymeric β-1,4-D-xylopyranosyl backbone substituted to variable degree with glucopyranosyl, 4-*O*-methyl-D-glucuronopyranosyl, α-L-arabinofuranosyl, and acetyl residues (Dodd and Cann 2009). Lignin, which consists of phenyl isoprenoid subunits (feruloyl or p-coumaroyl residues), is linked to α-L-arabinofuranosyl units of hemicellulose and is intertwined in between, making complex fibril macromolecular structures (Scheller and Ulvskov 2010). The chief limitations in developing enzyme-based bioconversion technologies are structural heterogeneity in the proportion and spatial distribution of cellulose, hemicellulose, and lignin in different lignocellulosics. The technological platform for lignocellulosic bioprocessing into ethanol involves pretreatment of lignocellulosics, enzymatic hydrolysis to unlock the sugars from the lignocellulosic substrates, fermentation of released sugars to ethanol, and its retrieval through distillation as the key steps (Payne et al. 2015). In brief, thermochemical pretreatment of LCB is carried out to dislodge the recalcitrant heteropolymer lignin in order to open up the compact cellulose fibril structure. The pretreated LCB is subsequently hydrolyzed with lignocellulolytic enzymes (glycosyl hydrolases and auxiliary enzymes) to release fermentable sugars, C_6 and C_5, from cellulose and hemicellulose fractions of the plant biomass, respectively, for subsequent fermentation of released sugars into ethanol (Chandel et al. 2012). Pretreatment and enzymatic hydrolysis are the two most cost-intensive steps in the bioconversion, and they contribute to 25–30% of the process cost. The high cost of lignocellulolytic enzymes and failure to produce all cellulolytic enzyme components in sufficient titers by any single microorganism are the main impediments for commercial production of 2G ethanol at industrial scale (Adsul et al. 2020; Jin et al. 2016). In the recent past, various attempts have been made to reduce the enzyme cost by optimizing the design of synergistic enzyme cocktails comprising of certain ratios of enzymes from various microbial strains that would be efficient in the hydrolysis of the pretreated biomass. Furthermore, in order to improve the economics of the process, nowadays, research is mainly focused on the complete conversion

of both hexose and pentose sugars during fermentation process to finally get the monomers as they also increase the theoretical yield (Merino and Cherry 2007). This has had an influence on the characteristics of the process like pretreatment method used and the enzymes essential for hydrolysis. Pretreatment is another challenging step in the bioconversion process for attaining cost-effective and competitive technology with low concentration of inhibitors. This chapter will discuss currently adopted pretreatment technologies in upcoming commercial and demonstration 2G ethanol plants and their effect on different LCBs. Further, significance of enzyme cocktails for efficient hydrolysis of LCB, factors affecting synergy, hydrolysis, and yield are also being discussed.

2.2 Pretreatment Technologies

Structural complexity and conformation, association of cellulose with hemicellulose and lignin, high lignin content, cellulose crystallinity, and acetyl groups in hemicelluloses are the major factors that hinder the use of lignocellulose for value addition (Chundawat et al. 2011; Singh et al. 2015). Addressing these lignocellulose structure-related issues holds key to efficient substrate hydrolysis. Pretreatment of lignocellulosics is the method of choice to overcome these limitations before saccharification. In pretreatment step, various physicochemical methods are employed to reduce the complexity and recalcitrance of biomass which leads to distortion and disengagement of lignin component and modifies the structure of cellulose and hemicellulose to an extent that makes the accessibility of lignocellulolytic enzymes easier (dos Santos et al. 2019). While pretreatment is an expensive process, the cost of not pretreating is even greater (Eggeman and Elander 2005). The main objective of a pretreatment technology is to enhance the enzymatic hydrolysis rate, to upgrade the yields of sugar monomers obtained from cellulose/hemicellulose, to minimize energy consumption, and to lower the capital, operational, and biomass cost. It is estimated that both pretreatment and hydrolysis cost may be up to 70% of the total bioprocessing cost (Satlewal et al. 2018). Different pretreatment methods result in different effects in the structure of substrates, and all these changes can lead to enhanced rate of hydrolysis. Pretreatment causes either degradation, disruption, re-organization of lignin structure, its disengagement with the rest of the biomass, or removal of some or all the lignin sheath, which leads to enhanced permeability in the substrate (Zhang and Lynd 2004). Changes that occur in the lignocellulosic biomass (LCB) after different pretreatments are depicted in SEM images (Fig. 2.1).

Pretreatment is a combination of physicomechanical and physicochemical steps. The first step in pretreatment is size reduction of lignocellulosic substrates (10–30 mm), which is essential prior to any other pretreatment. This is accomplished by using a number of physical pretreatment strategies such as milling, grinding, chopping, extrusions, freezing, and radiation, which results in reduction of particle size and crystallinity of the lignocellulosic biomass and enhanced specific surface area and porosity and also lessens the degree of polymerization (Rajendran et al.

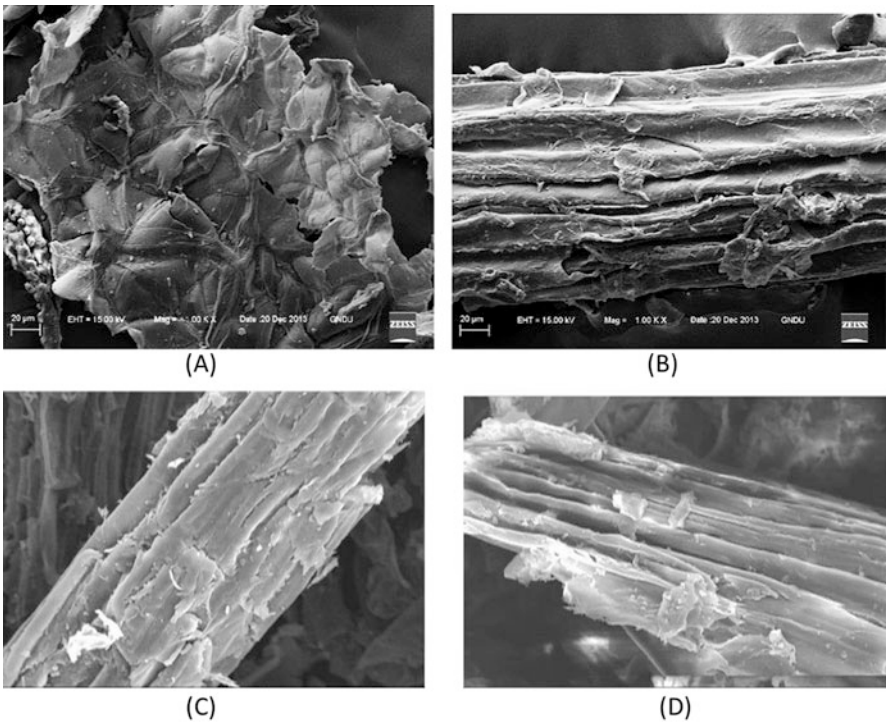

Fig. 2.1 Scanning electron microscopy (SEM) of substrates (**a**) native corncob; (**b**) acid-pretreated corncob (Brar et al. 2016); (**c**) native sugarcane bagasse; (**d**) acid-pretreated bagasse (Chandel et al. 2014)

2017). In the last few decades, a multitude of pretreatment techniques have been developed worldwide by various companies (Iogen (Canada), Abengoa (Spain), Blue Sugar Corporation (USA), Inbicon (Denmark), Clariant (Germany)), which are involved in developing 2G ethanol plants at demonstration/commercial scale. These companies use different lignocellulosic waste materials like corn stover, sugarcane bagasse, wheat, barley, rice and oat straw, energy crops, etc., which are structurally distinct, and hence, developing a universal technology is still not achievable. The selection of a pretreatment method is highly significant and must be based on several considerations as the type of method used influences the subsequent enzymatic hydrolysis (Romani and Garrote 2010). Pretreatment cost is chiefly based on the rate of consumption of energy and chemicals and recyclability of the chemicals. Often, chemical pretreatment must be headed by mechanical size reduction, which can have an extensive energy cost.

Different combinations of physical and chemical pretreatment technologies using acids, alkalis, solvents, and oxidants have been reported (Kumar et al. 2009). However, at industrial scale, pretreatment of lignocellulosic substrates uses dilute acid in combination with different process configurations. For example, Abengoa Bioenergy (Spanish global biotechnology company) uses dilute acid pretreatment

followed by steam explosion for biofuel production; DSM POET, Iowa, USA, also uses dilute acid hydrolysis in the presence of steam; similarly, Iogen-Raizen (Brazil) uses dilute acid hydrolysis in conjunction with steam explosion (Gandla et al. 2018) for the pretreatment of sugarcane bagasse. Steam explosion method (or autohydrolysis) is a promising thermo-mechano-chemical pretreatment method widely employed for wood-based lignocellulosics. Iogen Corporation biorefinery (Canada) developed this technique, which was then adopted by Beta Renewables Company in Italy. In this technique, pressurized steam (160–270 °C and 20–50 bar) is used to heat ground biomass for a short period of time, which brings about disruption of compact lignocellulosic structure (Kumar et al. 2009). The disruption is brought out by the released acetic acid from acetylated hemicelluloses as well as levulinic and formic acids formed during the process, resulting in autohydrolysis (Ramos 2003). BP biofuels (USA) employs modified steam explosion techniques for the pretreatment of corncob, husks, and leaves for efficient conversion of these substrates to 2G biofuels. Borregaard in Norway employs acidic calcium bisulfite cooking of spruce chips for pretreatment and has now established a BALI biorefinery (Sjöde et al. 2013). Liquid hot water (LHW) is another pH-controlled chemical-free hydrothermal pretreatment method that involves treatment of biomass using water maintained at high temperature (160–220 °C) with a residence time of 15 min without using any catalyst that results in fairly high xylose recovery of about 80%. In all of the above discussed methods, a major fraction of hemicellulose is removed as xylose or xylooligosaccharide-rich liquid stream. Some of the remaining xylan fraction gets realigned in the pretreated cellulose-rich solid residue. This method increases the cellulose digestibility by removing hemicellulose from the lignocellulosic substrates; however, the re-adsorption of the lignin has been observed (Laser et al. 2002).

Different acids have been used for dilute acid pretreatment of lignocellulosic biomass, which include hydrochloric acid, sulfuric acid, phosphoric acid, and oxalic acid (Pal et al. 2016). Among all, dilute sulfuric acid is the most commonly used because using concentrated acids poses technical challenges such as accumulation of inhibitors and corrosion of reaction vessel (Lee and Jeffries 2011), due to which dilute acid with low concentration (1–2% w/v) is usually preferred by most industries, which can be subsequently neutralized by lower amount of base (Jennings and Schell 2011).

In alkaline pretreatment, the use of alkalis such as sodium hydroxide, potassium hydroxide, calcium hydroxide, and aqueous ammonia (Sills and Gossett 2012) results in higher solubilization of lignin. However, the downside of using this method is that it cannot be used on a large scale as it generates black liquor which is difficult to handle and also results in loss of mass balance due to extraction of appreciable amounts of hemicellulose and cellulose to some extent. During the neutralization process, production of significant quantity of salt disturbs microbial growth and fermentation process and raises environmental concerns. Therefore, Chen et al. (2016) at NREL evolved a new promising method of deacetylation and mechanical refining (DMR) using dilute alkali (0.4%) for pretreatment of corn stover. An alternative approach is the use of alkali in the form of ammonia recovery percolation (ARP) method which can overcome the above drawbacks, but it has high

cost of ammonia recovery (Kim et al. 2008a). DuPont had employed ammonia-based pretreatment for its plant in Iowa. Ammonia fiber explosion (AFEX) pretreatment is one of the most widely reported physicochemical pretreatment methods (Zhao et al. 2014; Mathew et al. 2016). In this method, biomass is exposed to liquid ammonia at relatively mild temperature (60–100 °C) and high pressure for different time intervals. Rapid expansion of ammonia gas (due to release of pressure) leads to swelling and disruption of fibers of biomass and modification or effective reduction of lignin fraction, resulting in distortion of lignin-carbohydrate interactions (Mokomele et al. 2018), while hemicellulose and cellulose fractions may remain intact. Low temperatures used in the AFEX method reduce energy consumption and prevent the formation of inhibitory by-products. However, recovery of ammonia using evaporation is needed, which makes this process inconvenient, and requires more energy consumption and capital investment (Li et al. 2010). Balan and Sousa (2019) have devised modified AFEX process termed as COBRA (Compacted Biomass with Reduced Ammonia) process, which is much more efficient, wherein biomass pellets are supplemented with liquid ammonia in ratios lower than 1:1, allowing native cellulose I to be converted to highly digestible cellulose III at relatively low temperatures (~70 °C) with residence times of 3–4 h. COBRA pretreated biomass can be effectively digested with 60% less enzyme relative to the traditional AFEX pretreatment (Mokomele et al. 2018). Another process, namely, organosolv pretreatment developed at the University of British Columbia (Pan et al. 2006), was later endorsed by LIGNOL Innovations Ltd. in BC (Canada) for demonstration 2G ethanol plant. Recovery of high-grade lignin as a value-added product and the need for less energy are the advantages of this method.

2.3 Enzymes Involved in Lignocellulosic Degradation

After pretreatment, the enzymatic hydrolysis of pretreated substrates is carried out using a cocktail of lignocellulolytic enzymes. These enzymes have been clustered as carbohydrate-active enzymes (CAZymes) and is represented by six major enzyme groups (www.CAZy.org), including glycoside hydrolases, glycosyltransferases, polysaccharide lyases, carbohydrate esterases, auxiliary activity enzymes, and carbohydrate-binding modules (Lombard et al. 2014). These enzymes digest plant cell wall structure (including cellulose and hemicellulose) by cleaving glycosidic bonds in a hydrolytic and oxidative fashion. Based on their amino acid sequence, structural similarities, enzyme mechanism, and protein folding, these enzymes are represented in multiple families (Levasseur et al. 2013). In totality, 453 GHs were characterized, which were retrieved from 131 different fungal (mostly ascomycete) species, and represented 44 of the 115 CAZy GH families (Murphy et al. 2011). The annotated genes and proteins for more than 20 different thermophilic fungal strains have been assembled in a searchable online curated database of **c**haracterized **l**ignocellulose-**a**ctive **e**nzyme (CLAE). Recently, Kameshwar et al. (2019) have designed a web database "CAZymes Based Ranking of Fungi (CBRF)," for sorting and selecting fungal strains based on their genome-wide distribution of CAZymes by

retrieving complete annotated proteomic data of 443 published fungal genomes from JGI MycoCosm web repository for constructing CBRF-based web database.

The commercial lignocellulolytic enzyme cocktails primarily constitute cellulases and hemicellulases and recently discovered oxidative auxiliary activity enzymes termed as lytic polysaccharide monooxygenases (LPMOs) (Horn et al. 2012; Glass et al. 2013). Cellulases comprise a group of glycosyl hydrolases, which cleave β-1,4-glycosidic linkages of the integral cellulose and related cello-oligosaccharides derived in a synergistic manner (Horn et al. 2012). The components of cellulase complex, i.e., endoglucanases (EC 3.2.1.4), randomly cleave the β-1,4 linkages between adjacent glucose moieties, resulting in numerous reducing ends for the action of cellobiohydrolase I (EC 3.2.1.91) that processively cleave the cellulose chains from the reducing end, whereas cellobiohydrolase II tethers to the non-reducing end and carries out sequential hydrolysis. The synergistic action of these two cellobiohydrolases primarily results in formations of cellobiose moieties that are acted upon by β-glucosidase (EC 3.2.1.21) to produce glucose moieties (Payne et al. 2015).

2.3.1 Cellulases

Endoglucanases are categorized into 13 glycoside hydrolase (GH) families (Ezeilo et al. 2017), including GH5, GH7, GH12, GH44, and GH51 that carry out hydrolysis via retaining mechanism and EGs of GH6, GH8, GH9, GH45, GH48, GH74, GH124, and GH131 that operate via an inverting mechanism (Gao et al. 2017). The detailed analysis of these endoglucanases shows that the amino acid composition of EGs derived from thermophilic microbial strains differs significantly from their mesophilic counterparts. These include the presence of more charged amino acids (Arg, His, and Glu) crucial for imparting stability at higher temperatures in thermophilic EGs in addition to higher residue volume and residue hydrophobicity (Yennamalli et al. 2013). These differences in amino acid composition are specific to protein folds like $(\alpha/\beta)_8$ fold, β-jelly roll fold, and $(\alpha/\alpha)_6$ fold and enzyme families which impart variations in intramolecular interactions in a fold-dependent manner (Yennamalli et al. 2011). Cellobiohydrolase activity is attributed to glycosyl hydrolase families GH5, GH6, GH7, GH9, GH48, and GH74 (Poidevin et al. 2013). The industrially important CBHs primarily of fungal origin belong to GH6 and GH7. Aerobic bacterial CBHs are represented by GH6 and GH48, whereas those of anaerobic fungi and bacteria belong to family GH48. The hydrolytic mechanism of *Trichoderma reesei* cellobiohydrolase I (Tr Cel7A), an industrial rich source of CBHI, was demonstrated on cellulose fibers using atomic force microscopy by Igarashi et al. (2011). The tunnel-like configuration of active site in the catalytic domain of Tr Cel7A favors sliding of CBHI along the cellulose polymer in a processive fashion and results in cleavage of cellobiosyl units, which makes these enzymes proficient in hydrolyzing crystalline cellulose processively (Sweeney and Xu 2012). The presence of sharp twists (kinks) and other chemical moieties in the structure of pretreated cellulose fibers may result in fractal and "local jamming" effect (Xu and Ding 2007; Igarashi et al. 2011). B-glucosidases are included in

glycoside hydrolase families GH1, GH3, GH5, GH9, GH30, and GH116 (Lombard et al. 2014). GH3 has been found to be the most abundant family in CAZy database, and most of the fungal BGLs studied so far belong to this family of glycoside hydrolases (Singhania et al. 2013). Most of the BGLs have been reported to be vulnerable to the glucose inhibition (the major end-product in cellulose hydrolysis). Therefore, recent research has shown immense interest in the search for novel glucose-tolerant BGLs, as these enzymes are the key players in the hydrolysis of cellulose to monomer sugars (glucose), which can be successively fermented to 2G ethanol (Singhania et al. 2013; Ezeilo et al. 2017).

2.3.2 Hemicellulases

Hemicellulases exhibit activities similar to cellulases because of the presence of common β-1,4-glycosidic bonds in the hemicellulose backbone (Chang et al. 2011). Therefore, complete deconstruction of hemicellulose is also facilitated by the action of a variety of hemicellulases in a synergistic fashion. These include endo-xylanase (endo-1,4-β-xylanase, EC 3.2.1.8), as the major enzyme in addition to β-xylosidase (xylan-1,4-β-xylosidase, EC 3.2.1.37), α-arabinofuranosidase (α-L-arabinofuranosidase, EC 3.2.1.55), arabinase (endoα-L-arabinase, EC 3.2.1.99), α-glucuronidase (α-glucosiduronase, EC 3.2.1.139), acetyl xylan esterase (EC 3.1.1.72), and feruloyl xylan esterase (EC 3.1.1.73). Among these, endo-xylanase and β-xylosidase are the hemicellulases which are thoroughly studied, since xylan is the dominant constituent of hemicellulose from hardwood and agricultural biomass (Dodd and Cann 2009; Harris et al. 2014). Hemicellulases are mostly composed of catalytic domains to carry out enzyme functions, CBMs (used for docking with the substrates to perform enzyme functions), and other functional domains to carry out the hydrolysis of either glycosidic or esterified acid side groups (Dodd and Cann 2009). The glycosidic bonds of hemicellulosic substituents are hydrolyzed by α-glucuronidases, α-arabinofuranosidases, α-D-galactosidases, and mannanases, whereas acetyl or feruloyl esterases cleave the ester bonds of acetate or ferulic acid side groups. Mostly, hemicellulases act synergistically with cellulases in the deconstruction of lignocellulosic biomass to fermentable sugars. Arabinan side chains tethered to the xylan backbone are hydrolyzed by α-L-arabinases (EC 3.2.1.99) and α-L-arabinofuranosidases (EC 3.2.1.55) to arabinose. α-L-Arabinofuranosidases particularly cleave glycosidic bonds present between arabinofuranosyl substitutions and xylopyranosyl backbone residues of arabinoxylan. α-L-Arabinases show endo-α-1,5-linked L-arabinofuranosidase activity. Most of the commercial xylanases have been produced from *Trichoderma* strains, *Aspergillus niger*, and *Humicola insolens* (Polizeli et al. 2005). Diverse range of microbial strains produce xylanases, which include extremophilic bacteria, yeast, fungi, etc., and are categorized in various glycoside hydrolase (GH) families (5, 7, 8, 10, 11, 26, 30 and 43). For bioprospecting of thermostable xylanases, optimum temperature and pH are the two major criteria to be considered for industrial application (Chadha et al. 2019).

Many of these lignocellulolytic glycosyl hydrolases are multi-modular with catalytic function carried out by a single or multiple catalytic domains combined with one or more CBMs that are capable of binding to various carbohydrates without catalyzing substrate hydrolysis. These CBMs either present at the N- or C-terminus of the protein sequence were previously known as cellulose-binding domains (CBDs) (Lethio et al. 2001). Until now, 81 different families of CBMs have been characterized according to the CAZy database where family 33 CBMs are reclassified as oxidative enzymes (AA10) (Levasseur et al. 2013; Lombard et al. 2014). Those of lignocellulose-degrading fungi commonly implicate family 1 CBMs for biomass hydrolysis (Mello and Polikarpov 2014). The NMR spectroscopy revealed the presence of an irregular, three-stranded, antiparallel β-sheet arrangement, in which the hydrophobic flat face is present on the wedge part of the CBM, whereas a large hydrophilic flat face is constituted by three tyrosine residues and a huge number of polar amino acid residues, thereby exhibiting amphiphilic character. The most significant observation from this study was the occurrence of three conserved aromatic residues, which are present on the hydrophilic face of the CBM (Tyr5, Tyr31, and Tyr32 in the TrCel7A CBM) and are associated with the binding to crystalline cellulose. Apart from family 1 CBM, CBMs from other families were also studied, and 54 families have been categorized as type A, B, and C CBMs (Boraston et al. 2004; Ezeilo et al. 2017). Mostly, a catalytic domain and the CBM in an enzyme structure are attached together via highly glycosylated linker peptides of varying length and structure (Van Solingen et al. 2001). It has been found that cellulase linkers are inherently disordered proteins that are rich in Pro/Ser/Thr (Lima et al. 2013). It has also been suggested in a study that glycosylated linkers are directly involved in cellulose binding, like CBM but in a non-specific, dynamic manner (Payne et al. 2015). Glycosylation is known to facilitate protection to linker; moreover, the length of the linker is another factor that influences enzyme activity and the binding affinity, which vary within different GH families. It is usually seen that longer linker length in *T. reesei* cel6A promotes the search for hydrolytic sites, whereas linkers short in length promote processivity in Cel7A (Sammond et al. 2012).

2.4 Lytic Polysaccharide Monooxygenases (LPMOs)

In 2010, the study of LPMOs (a new class of enzymes) revealed an entirely novel mechanism for breaking glycosidic bonds present in cellulose and chitin, which uses oxidative mode for cleavage, differing from that of canonical glycoside hydrolases (Harris et al. 2010; Vaaje-Kolstad et al. 2010). LPMOs were previously thought to belong to family 33 CBMs (from non-fungal origin) or family 61 glycoside hydrolase (GH61) cellulases of fungal origin, which were reclassified in the CAZy database of carbohydrate-active enzymes (www.cazy.org) from families GH61 and CBM33 to "auxiliary activity" families AA9 and AA10, respectively (Levasseur et al. 2013). According to the sequence-based classification system of the CAZy database, LPMOs are currently categorized into six families of "auxiliary activities"

(AA9–11 and AA13–15). AA9–AA13 represent bacterial (AA10), viral (AA10), and fungal LPMOs (AA9, AA11, and AA13). However, in a recent study, a new family of LPMO named as AA16 was identified among secretomes of *Aspergillus* sp. (Filiatrault-Chastel et al. 2019). Crystalline structure of LPMOs studied thus far showed the presence of an active site located near the center of an extended flat face, which interacts with the crystalline surface of the substrate (Aachmann et al. 2012). In the active site of *T. aurantiacus* GH61 Cu (II), cations are present and display classic Jahn-Teller distortion with elongated coordination bonds in the axial positions, in which a single copper ion is chelated by two nitrogen atoms of the N-terminal histidine (through the NH2 terminus and an N-atom of the side chain) and a further nitrogen atom of another histidine side chain in an overall T-shaped N3 configuration coined as the histidine brace and is highly conserved in all reported LPMOs (Fig. 2.2, top row, stick representation) (Quinlan et al. 2011). The presence of Tyr residue in the axial position completes the geometry of histidine brace. Tyr residue is sometimes replaced with a Phe residue in some AA10 LPMOs (not shown). The protein structure of LPMOs displays a common immunoglobulin G (IgG)-like fold (Fig. 2.2 bottom row) with variation in surface topology (gray) depending on loop regions and helices. The catalytic copper-containing site is present at the surface of the interaction interface.

From lignocellulose-based biorefineries, LPMOs (AA9) are evolving as promising components of cellulase cocktails as these newly discovered enzymes are capable of cleaving the crystalline lattice of the polysaccharide chain by oxidation and disrupting the surface to such an extent that the polysaccharide chain becomes accessible to further attack by enzymes and eventual hydrolysis (Vaaje-Kolstad et al. 2010; Quinlan et al. 2011). The next-generation commercial cellulase preparations (Cellic CTec2 and Cellic CTec3) marketed by Novozymes have been designed by spiking the basal cellulase preparations with LPMOs derived from thermophilic fungal strains, *Thermoascus aurantiacus* and *Thielavia terrestris*. These cocktails exhibit much better improved catalytic performance and at lower enzyme loading rates are now being used in Raizen and Beta Renewables 2G ethanol plants (Harris et al. 2014; Johansen 2016). Some other important sources of AA9 (LPMOs) that have been discovered include those from *Malbranchea cinnamomea* (Huttner et al. 2019) and *Scytalidium thermophilum* (Agrawal et al. 2020). Another thermophilic fungus *Rasamsonia emersonii*, used for in-house production of lignocellulolytic enzymes by DSM POET 2G ethanol plant, is intrinsically loaded with LPMOs, constituting ~6% of the cellulase blend (Bevers et al. 2019). One of the technical issues during the use of preparations containing LPMO is that it requires a reducing agent, molecular oxygen as a co-substrate, and a copper ion in the active site (Langston et al. 2011). However, in a recent study, it was discovered that at low H_2O_2 feeding rates, saccharification rates and glucose yields are higher than those seen under standard aerobic conditions (Bissaro et al. 2017; Müller et al. 2018). These results indicate that H_2O_2, rather than O_2, is the co-substrate of LPMOs, and this new enzyme seems to be peroxygenase rather than monooxygenase (Forsberg et al. 2019). The reducing agent required for LPMO action can be mediated enzymatically by cellobiose dehydrogenase, which is often present in fungal

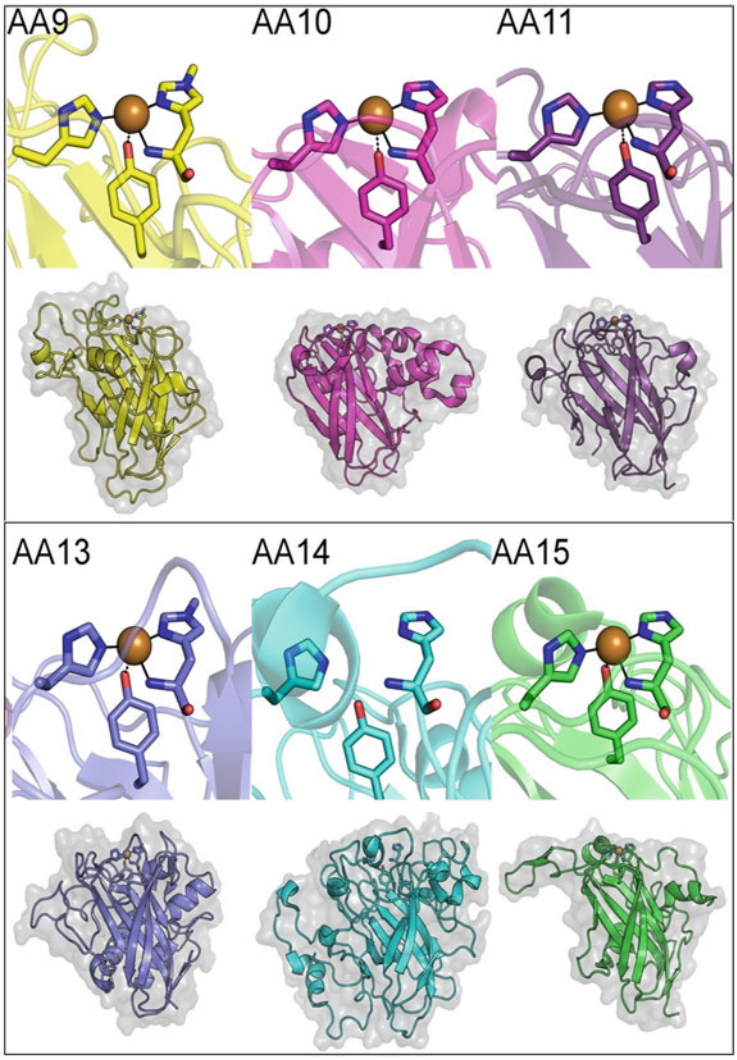

Fig. 2.2 Representative structures for LPMO families

secretomes or an externally added small molecule (ascorbate) that is required for priming the reduction of copper (Bissaro et al. 2017). Even the lignin-derived molecules that are found in biomass can act as a reducing agent (Langston et al. 2011).

Most of the studies on LPMOs are focused on the characterization of substrate specificity of these enzymes. Nearly all LPMOs reported to date were found to be active on β-1,4-linked polymers of glucose (e.g., cellulose), but LPMOs showing specificity toward soluble polysaccharides, such as β-glucans and xyloglucan, have

also been reported recently (Fanuel et al. 2017). LPMOs (AA9) from *M. cinnamomea* active on both cellulose and pure xylan have also been discovered (Basotra et al. 2019). On the basis of the principal site of oxidation (C1 or C4), LPMOs are also classified as Types 1, 2, and 3 (Vu et al. 2014). Types 1 and 2 AA9 catalyze the oxidation of C1 (reducing end) and C4 (non-reducing end) of cellulose, respectively, whereas Type 3 governs the oxidation of both C1 and C4 of cellulosic substrates (Vu et al. 2014). Incorporation of LPMOs in commercial enzyme preparations resulted in twofold reduction in both the total enzyme load and cost of cellulose degradation, which highlighted the industrial significance of these oxidative enzymes (Harris et al. 2010; Davis et al. 2013).

2.5 Secretome-Based Analysis of Lignocellulolytic Enzymes

The widely available genomic databases in tandem with fungal secretome analysis have led to profiling the expressed genes and identification of new enzymes (Kim et al. 2008b). Marx et al. (2013) utilized this approach to study diverse profiles of polysaccharide-degrading hydrolases in fungal secretome under the influence of different carbon and nitrogen sources. Kaur et al. (2013) also followed this approach to reveal the differential expression of proteins in the parent and developed heterokaryons. LC-MS/MS-based investigation of the secretome derived from commercial *T. reesei* strains revealed CBHI (Cel7A) as the most predominant protein followed by CBHII (Cel6A) (Chundawat et al. 2011). Ravalason and coworkers (2008) performed proteomic analysis to determine the compositional differences in the secretome of *Phanerochaete chrysosporium* CIRM-BRFM41 when grown under ligninolytic conditions and on soft wood chips under biopulping conditions. LC-MS/MS-based analysis of the secretomes obtained from *A. fumigatus* Z5 in the presence of glucose, avicel, and rice straw revealed that most of the lignocellulolytic GHs were upregulated when rice straw and avicel were used as carbon sources (Liu et al. 2013a).

The secretome analysis of *M. cinnamomea* revealed the production of a spectrum of metal-dependent GHs and other lytic polysaccharidases produced using sorghum straw as carbon source (Mahajan et al. 2016). The mass spectroscopy analysis of *Penicillium* sp. Dal 5 secretome identified a total of 108 proteins containing an array of GH, PL, CE, LPMOs, and swollenin, which makes it good enzymatic machinery for hydrolysis of lignocellulosics (Rai et al. 2016). The comparative analysis of *A. niger*, *T. reesei*, and *P. oxalicum* 114-2 revealed that cellulase system produced by *P. oxalicum* 114-2 is more balanced with diversity of enzymes involved in the degradation of xylan and β-D-glucans (Gong et al. 2015). The proteomic analysis of three commercial cellulase preparations, i.e., SP from *P. oxalicum* JU-A10T, ST from *T. reesei* SN1, and Celluclast 1.5 L from Novozymes, disclosed that both SP and ST are rich in carbohydrate-degrading enzymes and multiple non-hydrolytic proteins with a greater number of CBM1 in SP (Song et al. 2016). A comprehensive insight into the secretome of a hyper-cellulolytic *P. funiculosum* was provided by LC-MS/MS analysis performed by Ogunmolu et al. (2015). A detailed analysis of

secretome of thermophilic fungus *Mycothermus thermophilum* revealed a total of 240 different proteins with majority of glycosyl hydrolases belonging to 30 families. In addition, polysaccharide lyases, carbohydrate esterases, lytic polysaccharide monooxygenases, and a variety of carbohydrate-binding modules (CBM) were also identified, indicating the potential of this strain for lignocellulosic deconstruction (Basotra et al. 2016). Recent secretome analysis of *Talaromyces emersonii* has also revealed expression of diverse array of GHs and other auxiliary enzymes that were produced using a modified optimized industrial medium (Raheja et al. 2020).

2.6 Commercially Important Producers of Lignocellulolytic Enzymes

Development of different expression platforms is also an area of intense research and development. To date, several different expression hosts such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Kluveromyces lactis* (conventional yeasts), *Yarrowia lipolytica* and *Pichia pastoris* (unconventional yeasts), *T. reesei*, *Aspergillus* sp., and *Myceliophthora thermophila* have been reported for cloning and expression of thermophilic cellulases and xylanases (Boonvitthya et al. 2013; Lambertz et al. 2014). DSM POET in a joint venture "Project Liberty" has come up with the first successful commercial 2G ethanol plant in IOWA and is using indigenously developed hypercellulase-producing strain *Rasamsonia emersonii* through a combination of classical and molecular approaches (Perkins et al. 2012). The developed strains of *R. emersonii* are now used to produce in-house enzymes for efficient hydrolysis of acid pretreated corn stover. Another thermophilic strain *Myceliophthora thermophila* has been developed by Dyadic International Inc. for the production of cellulases (Visser et al. 2011). Similarly, a mesophilic fungal strain *Penicillium oxalicum* has been developed by a combination of system biology approaches targeting the regulatory network for producing high levels of cellulases (Gao et al. 2017). *Acremonium cellulolyticus* has also been developed in Japan, and the mutant strains are capable of producing high levels of filter paper activity at industrial scale (Fang et al. 2009).

In recent past, thermostable lignocellulolytic enzymes from thermophilic fungi (*Chaetomium thermophile*, *Myceliophthora*, *Scytalidium*, *Talaromyces emersonii*, *Thermoascus aurantiacus*, *Thermomyces lanuginosus*, and *Malbranchea flava*) (Morgenstern et al. 2014) have been studied widely for their application in biofuel industry, owing to the fact that at elevated temperatures, cellulose fibers swell up and become easily accessible for attack to hydrolytic enzymes (Timo et al. 2017). Thermophilic fungi are a potent source of thermostable enzymes and are being harnessed by various enzyme industries like DSM, Novozymes, DuPont, Genencor, etc. for the production of cellulases and hemicellulases beside auxiliary enzymes (Chadha et al. 2019). Thermostable enzymes, which work at elevated temperatures, offer potential benefits such as enhanced rate of catalysis, lower risk of microbial contamination, and improved mass transfer (Scully and Orlygsson 2015).

Among thermophilic fungi, *Talaromyces emersonii* (now renamed as *Rasamsonia emersonii*) (Murray et al. 2004; Morgenstern et al. 2014), *Myceliophthora* sp. (Visser et al. 2011) previously identified as *Chrysosporium lucknowense* (Emalfarb et al. 1998), and *Humicola insolens* (Syn. *Scytalidium thermophilum*) have been employed at industrial level for the production of cellulases. Cellulases of *Talaromyces emersonii* are known to be highly active and stable even at 70 °C (Murray et al. 2004). With the advancement in techniques like genome sequencing, annotation and analysis of thermophilic fungi reveals the presence of multiple xylanase-encoding genes (Hinz et al. 2009). Most of the xylanases from thermophilic fungi harbor genes either of GH10 or GH11 family (Chadha et al. 2019). The thermophilic fungal strains *Thermomyces lanuginosus*, *Melanocarpus albomyces*, *and M. cinnamomea* have been reported to produce copious amounts of xylanase and are being evaluated for their application in 2G ethanol plants (Winger et al. 2014; Chadha et al. 2019).

2.7 Designing Lignocellulolytic Enzyme Cocktails for 2G Ethanol Production

Efficient conversion of pretreated LCB into monomeric sugars requires synergistic action of endoglucanase, cellobiohydrolase, and β-glucosidase. Recently, the role of LPMOs and other hemicellulolytic enzymes (xylanase, β-xylosidase, and arabinofuranosidase) as accessory enzymes has also been implicated in enhanced hydrolysis of LCB (Hu et al. 2011; Gao et al. 2011; Laothanachareon et al. 2015). For the production of cellulosic ethanol at commercial level, the high cost of cellulases and failure of production of all cellulolytic components by a single microbial strain in sufficient titers are the major impediments. Although developed strains of *Trichoderma reesei* have been the industrial workhorse for production of cellulases, recent years have seen active research and development efforts for developing novel fungal strains such as *Acremonium cellulolyticus* (Fang et al. 2009), *Myceliophthora thermophila* (Visser et al. 2011), *Talaromyces emersonii* (syn. *Rasamsonia emersonii*) (Perkins et al. 2012), and *Penicillium decumbens* (Liu et al. 2013b) as enzyme platforms. However, the cost of lignocellulolytic enzymes in bioconversion process is still an area of concern as high amount of protein per gram substrate is needed for effective hydrolysis of differently pretreated cellulosic substrates due to lower catalytic efficiency of the existing enzyme preparations. The enzyme production by even the best of wild-type strains is low from commercial viewpoint, and for commercialization of these lignocellulases, potent microbial strains are required, which can produce high levels of enzyme titers besides exhibiting traits like thermostability, multifunctionality, and less sensitivity to product inhibition (Cheng et al. 2009). In spite of the exploitation of secretome from diverse microbial communities and also the availability of different pretreatment methods, successful bioconversion of LCB into monosaccharides in high yields is still a challenge that necessitates the discovery or construction of novel catalytically efficient enzymes in order to hydrolyze differentially treated LCB into fuels

(Banerjee et al. 2010). Since a broader suit of enzyme preparations are required to achieve high level of saccharification, which increases the cost and also influences the viability of the process, custom designing of cocktails by adding mono-components (either recombinant or purified from secretome) which can act on a wide array of agro-residues in order to lower the cost is one of the way to achieve high level of saccharification.

In the recent past, vigorous studies have been conducted on designing optimal synergistic enzymatic cocktails comprising of definite ratios of enzymes derived from different microbial sources, for improved hydrolysis of pretreated biomass and to reduce the enzyme cost. However, the mechanisms underlying the development and the approaches for production and evaluation of optimal cellulolytic cocktails are still not clear. However, best enzyme preparation, containing optimum level of all the enzyme components, is a key for effective hydrolysis of pretreated biomass. Therefore, designing prolific and cheap enzyme cocktail for cost-effective hydrolysis of LCB is one of the major research platforms in biofuel production. After a technical analysis, it has been suggested that the enzyme cost is greater than estimated before which ranges from $0.23 to $0.78 per gallon of ethanol (Liu et al. 2016), depending upon the countries and enzyme source, production approach (on-site, off-site, and integrated), and lastly the pretreatment method used.

Supplementation of xylanases and LPMOs in enzyme cocktails has been found to increase the sugar yield significantly (Hu et al. 2011; Basotra et al. 2018, 2019). Moreover, to economically convert pretreated LCB to ethanol, it must be fermented to a concentration of at least 4–5% (Jørgensen et al. 2007), which requires high sugar concentration, implying that a high substrate loading (~20%) will be required instead of lower substrate loading reported in different papers (Mokomele et al. 2018). However, at high-solids loadings, many problems are encountered, such as substrate recalcitrance, inefficient mass transfer (e.g., rheological problems), increased levels of enzyme inhibition due to accumulation of glucose and xylobiose, and various other degradation products that affect the dynamics of enzyme substrate interaction and negatively influences the enzyme catalysis (Zhang et al. 2009; da Silva et al. 2020), and also high enzyme/protein loadings are required while the yields were often around 60% after 72 h hydrolysis. Chylenski et al. (2017) achieved 100% conversion of Norway spruce after hydrolysis at enzyme loading rate of 16 mg/g of substrate by employing marginal enzyme cocktails consisted of several glycoside hydrolases from *T. reesei* and purified BGL from *A. niger*. In addition, an LPMO derived from *Streptomyces coelicolor* was also added in this cocktail. Further, by supplementing β-glucosidase in the hydrolysis reaction, end-product inhibition of cellobiose can be reduced, which is also known to affect xylanase-mediated catalysis.

The need to increase the concentration of monomeric sugars in hydrolysates has compelled to conduct studies on high substrate loadings for enzymatic hydrolysis. Under these conditions, a "high-solids effect" has been observed, which resulted in a decrease in glucose conversion yields (da Silva et al. 2020). To surpass high-solids effect, several scientific and technological approaches involve the formulation of more efficient enzyme cocktails, biomass and enzyme feeding strategies, reactor and

impeller designs, as well as process strategies to alleviate the end-product inhibition. In addition, pretreatment method that would fit better at high-solids loadings should also be explored as it has been frequently assumed that pretreatment methods would have comparable efficacies independently of the solids content in the hydrolysis media. In a recent study, Weiss et al. (2019) used a combination of pretreatment, wherein the wheat straw was initially pretreated using steam pretreatment which was followed by either delignification or incubation with xylanases and then compared the results obtained from enzymatic hydrolysis of pretreated substrate. It was found that at low substrate loadings, the delignified sample showed a better digestibility, while the substrate treated with xylanases resulted in better yields at high solids; these observations were then correlated with the variant capability of the pretreated biomass to restrain water at high solids. This study would allow a better choice of the pretreatment method and working conditions at high solids in order to reduce the high-solids effect.

Apart from research conducted on finding appropriate enzyme loadings, formulation of new combinations of enzymes with complementary activities (laccases, hemicellulases, pectinases, and LPMOs), and protein engineering to achieve resistance to inhibitors, studies have also been focused to counteract the high substrate effect (Kristensen et al. 2009; Hu et al. 2015; Raj and Krishnan 2019). At high substrate concentrations, cellulases would have greater difficulty accessing the cellulose component of LCB, and there would be high chances of nonproductive binding. This problem can be counteracted by using a combination of xylanase and AA9, which would remove the xylan fraction and punctures the crystalline cellulose, respectively (Hu et al. 2015). Hu et al. (2015) studied the boosting effects of using a combination of xylanase and lytic polysaccharide monooxygenase (AA9, formerly known as GH61) to check the hydrolytic efficacy of cellulase enzyme mixtures on steam-pretreated poplar and corn stover at high substrate concentrations (10–20% w/v). It was concluded that the overall protein loading needed to achieve effective hydrolysis of high substrate concentrations could be substantially reduced by optimizing the ratio of enzymes in the "cellulase" mixture.

Enzyme cocktails can also be customized by using a diverse library of cellulases along with other accessory enzymes (hemicellulases, LPMOs, etc.). Researchers have cloned and expressed mono-component enzymes for custom designing enzyme mixtures for the hydrolysis of lignocellulosic substrates used in various industries. The substrates include pretreated corn stover (Banerjee et al. 2010; Gao et al. 2010), wheat and barley straw (Billard et al. 2012; Kallioinen et al. 2014), and sugarcane bagasse (Kallioinen et al. 2014). The pretreatment technologies used in maximum of these experiments were either high pressure steam or ammonia fiber expansion (AFEX). The compositions of designed enzyme mixtures resulting from these studies varied, depending on utilized enzymes, type of biomass, and type of pretreatment, highlighting the importance of customizing enzyme mixtures for individual processes. Using the cocktail of cellulase-hemicellulase, Gao et al. (2011) reported appreciable quantities of reducing sugars from corn stover pretreated by ammonium fiber expansion (99% glucose and 55% xylose), ionic liquid (88% glucose and 53% xylose), and dilute acid (97% glucose and 68% xylose).

For the identification of efficient enzyme cocktails, high-throughput robotic approaches have been documented in the recent past (Gao et al. 2010). Using GENPLAT, a platform developed which employs robotic liquid handling and statistical design, synthetic mixture composed of commercial enzymes (Accellerase 1000, Multifect xylanase, and Spezyme CP Novozyme 188) was formulated and deployed to check the system that served as comparative benchmarks (Banerjee et al. 2010; Chundawat et al. 2017). The efficacy of these cocktails was examined against ammonia fiber expansion (AFEX)-pretreated corn stover, which showed improvement in hydrolysis using cocktails of pure enzymes of *T. reesei* expressed in *P. pastoris*. A four-component minimal enzyme cocktail was designed using enzymes from anaerobic fungus *Orpinomyces* sp. Strain C1A and it was observed that hydrolysis yield was comparable or slightly lower than the commercial Cellic CTec2 (Morrison et al. 2016). Table 2.1 represents the studies reported by a number of researchers on the pretreatment of lignocellulose substrates, the enzyme cocktails used for hydrolysis, and the yields obtained.

2.8 Approaches for Developing Cellulolytic Enzyme Cocktails

In order to develop the optimum cocktail, the best option is to first carry out the enzyme assay of base enzyme preparation and some accessory enzymes and then put the essential enzymes/proteins into the base preparation to custom design the cocktail based on hydrolysis performance. Also, as discussed in the above sections, the type of biomass and method of pretreatment are important parameters to be considered during cocktail designing. Biomass composition, structure, and region are also important factors to be taken into account. Different pretreatments result in variable texture/structure and compositions of biomass. Desired cellulolytic cocktail can be obtained for selected biomass/process by using the following approaches:

(a) By supplementing that the component of base enzyme preparation from other microbial source which is not secreted in sufficient amount, to make it optimal. Example: The industrial fungal strain *Trichoderma* lacks the ability to secrete sufficient titers of β-glucosidases, but it is a prolific producer of all other base enzymes; therefore, it is mandatory to supplement β-glucosidase from other microbial sources in order to achieve efficient rate of hydrolysis. Some enzyme preparations work better on one feedstock but do not work on other feedstocks. The analysis of the exact composition of feedstock would give us an idea of some feedstock-related enzyme requirement for cocktail preparation, e.g., mannose content is comparatively higher in case of spruce wood (10–15%) which necessitates the addition of mannanase for efficient deconstruction of spruce wood (Chylenski et al. 2017).
Similarly, the content of xylan in pretreated biomass depends on the type of pretreatment (e.g., during acid pretreatment more than 70% xylan hydrolyzed) and the determining factor for adding the amount of xylanase if it is lacking in base enzyme preparation. Non-specific enzyme components that can boost the

Table 2.1 Studies on lignocellulosic substrates explored for pretreatment and subsequent hydrolysis

Substrates	Pretreatment	Enzymes	Yield	Reference
Corncob	H_2SO_4 pretreatment	Crude cellulase from *Trichoderma reesei* ZU-02	83.90%	Chen et al. (2007)
		Cellobiase from *Aspergillus niger* ZU-07		
Rice straw	Alkali (NaOH) pretreatment	Crude extract from *Trametes hirsuta*	85% glc	Jeya et al. (2009)
	Acid pretreatment	Celluclast 1.5, Novozyme 188	85% glc	Hsu et al. (2010)
Wheat straw	H_2SO_4 (dilute) and steam explosion	Cellulases and xylanases from *Trichoderma reesei*, recombinant feruloyl esterase (FAE) from *Aspergillus niger*, and oxidoreductases (laccases from *Pycnoporus cinnabarinus*)	51.4% glc	Tabka et al. (2006)
Bagasse	Calcium hydroxide pretreatment	Arf, EM, EX	Not detected	Beukes and Pletschke (2010)
		Arf, EM, EX	Not detected	Beukes and Pletschke (2011)
	Steam pretreatment	Crude mixtures with Cel, EX, βG, and FaE	91% xyl, 80% glc	Gottschalk et al. (2010)
	AFEX, ammonium hydroxide	Spezyme (cel), Novozyme 188 (βG), Multifect xylanase		Prior and Day (2008)
Corn stover	AFEX	CBH1, CBH2, EG, EX, βG, βX	80% Glc, 56% xyl	Gao et al. (2010)
	Acid and alkaline pretreatment, AFEX	CBH (Cel7A) *Trichoderma reesei*, βG(BG)(A Niger) EX (XynA), βX (XlnD), and AXE (Axe1)		Selig et al. (2009)
	AFEX	Six core (CBH1, CBH2, EG, βG, EX, βX) and ten accessory enzymes	Core set alone—38.5% glucose released Core + 5 accessories—52.1% glucose released	Banerjee et al. (2010)
Poplar	Alkaline pretreatment (NaOH)	CBH, EG, βG from *Agaricus arvensis*	293 mg sugar/g substrate	Jeya et al. (2010)
	AFEX, ARP, controlled pH,		Best yield with dilute acid	

(continued)

Table 2.1 (continued)

Substrates	Pretreatment	Enzymes	Yield	Reference
	dilute acid, FT, lime, SO_2	Cel, βG, EX (commercial mixtures Spezyme, Multifect)	pretreatments (84.90%)	Kumar and Wyman (2009)
	Ionic liquid pretreatment	Spezyme CP	85% glc yield (Spezyme)	Samayan and Schall (2010)
		Novozyme 188		
		Primafast (supplemented with Multifect xylanase)		
Barley straw	Steam pretreated	Celluclast, Novozyme 188	78 g/L glucose Rosgaard	Rosgaard et al. (2007)
	Steam explosion at 210 °C	Cel, βG, EX (commercial mixtures) 10:5:1	84% Glc	Garcia-Aparicio et al. (2007)
Maize straw	NaOH	Crude cellulase from *Trichoderma reesei* ZU-02	83.30%	Chen et al. (2008)
		Cellobiase from *Aspergillus niger* ZU-07		

Abbreviations: *Cel* cellulase, *EX* endo-xylanase, *EM* endo-mannanase, *EG* endo-glucanase, *Arf* α-L-arabinofuranosidase, *RgaeA* rhamnogalacturonan acetyl esterase, *RhgA* rhamnogalacturonan hydrolase, *βG* β-glucosidase, *βX* β-xylosidase, *FaE* ferulic acid esterase, *FE* feruloyl esterase, *AFEX* ammonium fibre explosion, *ARP* ammonia recovery percolation

degradation of cellulosic materials with distinct mode of action different than regular the cellulo-/xylanolytic enzymes must also be considered. Example: Oxidative enzyme such as lytic polysaccharides monooxygenases/AA9 was found effective for quick liquefaction of biomass and it creates more access for other enzymes.

Next are the accessory enzymes (arabinofuranosidase, feruloyl esterase, acetyl xylan esterase) and non-catalytic proteins (CBM, swollenin, etc.), which are required at a very low amount and not present in base enzyme preparation. These enzymes also support hydrolysis of biomass. There might be some enzymes/proteins yet to discover and might contribute, in future, for the fast hydrolysis of biomass at a lesser amount (Kim et al. 2015). At present, addition of activator for oxidative enzymes, other enzymes such as cellobiose dehydrogenase (electron donor for AA9), and any other chemical species which helps in hydrolysis could be considered in this section. Some sterol compounds also found to increase the hydrolysis of cellulose could be considered. These compounds are generally added during saccharification; their addition in cocktail needed further study. The ultimate aim is to get maximum fermentable sugars within a short time. Short time is very important in industrial scale-up.

(b) Even though all the five enzyme components are present in base preparation, the mixing of enzyme preparation from different sources makes a good cocktail

(Peciulyte et al. 2017; Agrawal et al. 2018) and gives more hydrolysis of cellulosic materials at low protein level or FPU level.
Example: Different β-glucosidases from different sources has a different preference toward cello-oligosaccharides and cellobiose. Some are efficient to hydrolyze the cello-oligosaccharides from C2–C6, while some efficiently hydrolyze only C2 sugars (Adsul et al. 2020).

A vast array of cellulases are produced by most cellulolytic fungal strains, which can be detrimental for industrial enzyme cocktails. Therefore, extensive research was carried out to overcome this problem, and it was concluded that only a small segment of the enzymes in the whole cocktail are effective in performance under industrial conditions, and this issue can be resolved by deleting some cellulase-encoding genes in order to reduce the interference of less-contributive cellulases. For example, in *Myceliophthora thermophila*, some particular genes were deleted, which resulted in improved cellulolytic efficiency of the cocktail, validating the management of cellulase diversity as a strategy to obtain improved fungal cellulolytic cocktails (Reyes-Sosa et al. 2017). Another important aspect in this regard is to check the behavior of fungi when grown on a variety of biomass and detect the types of enzymes they secrete and to get the enzymatic sequences necessary to deconstruct each kind of biomass (Polizeli et al. 2017). Secretome obtained from *Aspergillus niger* and *Trichoderma reesei* grown on sugarcane bagasse showed that these two fungi use different enzymatic sequences to degrade the same biomass (Borin et al. 2015). In order to enhance the efficiency of cellulolytic cocktail, accessory enzymes such as LPMOs, peroxidases, catalases, and xylanases have been added to formulate effective cocktails (Xu et al. 2015). Novozymes formulated an efficient enzyme cocktail under the US Department of Energy-funded project DECREASE (Development of a Commercial Ready Enzyme Application System for Ethanol) for biomass conversion, where they conducted enzyme discovery program to identify the auxiliary proteins from thousands of fungal strains to boost the capability of benchmark enzymes. Recent studies have shown that xylanases play a vital role in hydrolysis (Gao et al. 2011; Hu et al. 2013; Laothanachareon et al. 2015). Novozymes also recommends the use of HTec3 along with Cellic CTec3 in the ratio of 1:9 to achieve higher hydrolysis. The major issue in using these commercial enzymes for bioethanol production is the high cost factor and the logistics that are involved, which motivates the upcoming biorefineries to go for in-house production of these enzymes for lowering the cost (Johnson 2016).

2.9 Statistical Optimization of Cellulolytic Cocktails

Formulation of synergistic enzyme cocktails is a promising approach for enhancing the hydrolysis rate of LCB (Peciulyte et al. 2017; Satlewal et al. 2018; Sanhueza et al. 2018). However, when the number of enzyme components is more, optimization for efficient cocktail preparation is needed for which statistical approach is a method of choice. First, to determine the effect of each enzyme component in a

mixture, the fractional factorial design could be used, and then further the optimization of more significant components can be performed using central composite design (CCD) and response surface analysis. Using statistical approach, optimum levels of a base or core enzyme preparation along with other accessory or base enzymes can be obtained. A high-throughput enzyme assay platform, called GENPLAT, was designed by Banerjee et al. (2010), for the optimization of a mixture of individual purified enzymes from ten "accessory" and six "core" enzymes. Optimization was carried out for the release of Glu, Xyl, or a combination of the two from ammonia fiber expansion (AFEX)-pretreated corn stover. Out of the ten tested accessory proteins, five enhanced Glu or Xyl yield compared to the core set alone, and five did not. An 11-component mixture containing the core set and five accessory enzymes optimized for Glu released 52.1% of the available Glu, compared to 38.5% with the core set alone. A mixture optimized for Xyl released 39.9% of Xyl, compared to 26.4% with the core set alone. In another study, Bussamra et al. (2015) used statistical approach for the optimization of concentration of six accessory enzymes (expansin, acetyl xylan esterase, α-L-arabinofuranosidase, endoglucanase, β-glucosidase, and endo-xylanase) supplemented to *T. reesei* extract as a base enzyme to achieve higher hydrolysis of pretreated sugarcane bagasse. The cocktail after optimization contained 80% extract from *T. reesei*, 10% endoglucanase, and 10% β-glucosidase. Enzyme preparation produced by *Penicillium funiculosum* ATCC 11797, *Aspergillus niger* ATCC 1004, and *Trichoderma harzianum* IOC 3844 optimized and resulted in a proportion of 50%, 35%, and 15%, respectively. Statistical methodology was also utilized by Kim and coworkers for rapid optimization of enzyme cocktails, which were specific to a variety of pretreated lignocellulosic substrates (Kim et al. 2015). Likewise, six enzymes, viz., Bgl3A, Cel5A, Cel6A, Cel7A, Cel7B, and Xyl10A, were purified from *Talaromyces cellulolyticus* culture supernatant. Among the six, the ratio of five (Bgl3A, Cel5A, Cel6A, Cel7A, and Xyl10A) enzymes was statistically optimized for the hydrolysis of dilute acid-pretreated corn stover. The sugar yield in case of optimized cocktail was found to be comparatively higher (Inoue et al. 2014). Chundawat et al. (2017) successfully generated several hundred unique cocktail combinations using statistical method. Fourteen commercially available cellulolytic and hemicellulolytic enzymes (procured from Novozymes®, Genencor®, and Biocatalysts®) were mixed together, and then the mixtures were assayed for activity on AFEX-treated corn stover (AFEX-CS). The enzyme blends screened after optimization were found to be enriched in various low-abundance hemicellulases and other accessory enzymes typically absent in most commercial cellulase cocktails. This simple approach resulted in fourfold reduction in total enzyme requirements (from 30 to 7.5 mg enzyme/g glucan loading) to achieve near-theoretical cellulose and hemicellulose saccharification yields.

2.10 Conclusions and Future Perspectives

Lignocellulose-derived bioethanol is one of the most flourishing bioenergy and renewable sectors with the maximum growth potential in the near future. Enzymatic cocktails comprise of a pool of enzymes with varying specificities and synchronize actions capable of deconstructing the LCB efficiently. Therefore, new strategies must be explored, such as enzymatic platforms focusing on the topography and molecular array of the substrate, making possible the utilization of a broad range of lignocellulosic substrates. Bioprospecting of novel lignocellulolytic genes from ever-expanding genome databases and metagenomes opens up new possibilities for designing a customized enzymatic cocktail. Searching for novel accessory enzymes using advanced biotechnological tools like metagenomics or proteomics or through microbial bioprospecting will also assist in lowering the cost of these enzymes. The development of more robust expression platforms is another area that needs to be addressed in the near future. Developing enzymes that can hydrolyze partially or untreated substrates is also a challenging area of future research.

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References

Aachmann FL, Sorlie M et al (2012) NMR structure of a lytic polysaccharide monooxygenase provides insight into copper binding, protein dynamics, and substrate interactions. PNAS 109:18779–18784

Adsul M, Sandhu SK et al (2020) Designing a cellulolytic enzyme cocktail for the efficient and economical conversion of lignocellulosic biomass to biofuels. Enzym Microb Technol 133:109442

Agrawal R, Semwal S et al (2018) Synergistic enzyme cocktail to enhance hydrolysis of steam exploded wheat straw at pilot scale. Front Energy Res 6:1–11

Agrawal D, Kaur B et al (2020) An innovative approach of priming lignocellulosics with lytic polysaccharide mono-oxygenases prior to saccharification with glycosyl hydrolases can economize second generation ethanol process. Bioresour Technol 308:123257

Balan V, Sousa LDC (2019) De-esterification of biomass prior to ammonia pretreatment and systems and products related thereto. US Patent Application 16/029,452

Banerjee G, Car S et al (2010) Synthetic enzyme mixtures for biomass deconstruction: production and optimization of a core set. Biotechnol Bioeng 106:707–720

Basotra N, Kaur B et al (2016) *Mycothermus thermophilus* (Syn. *Scytalidium thermophilum*): repertoire of a diverse array of efficient cellulases and hemicellulases in the secretome revealed. Bioresour Technol 222:413–421

Basotra N, Joshi S et al (2018) Expression of catalytically efficient xylanases from thermophilic fungus *Malbranchea cinnamomea* for synergistically enhancing hydrolysis of lignocellulosics. Int J Biol Macromol 108:185–192

Basotra N, Dhiman SS et al (2019) Characterization of a novel lytic polysaccharide monooxygenase from *Malbranchea cinnamomea* exhibiting dual catalytic behavior. Carbohydr Res 478:46–53

Bayer EA, Lamed R, Himmel ME (2007) The potential of cellulases and cellulosomes for cellulosic waste management. Curr Opin Biotechnol 18:237–245

Beukes N, Pletschke BI (2010) Effect of lime pre-treatment on the synergistic hydrolysis of sugarcane bagasse by hemicellulases. Bioresour Technol 101:4472–4478
Beukes N, Pletschke BI (2011) Effect of alkaline pre-treatment on enzyme synergy for efficient hemicellulose hydrolysis in sugarcane bagasse. Bioresour Technol 102:5207–5213
Bevers LE, Appledoorn M et al (2019) Enzyme composition. Patent Application WO 2018/096019 Al
Billard H, Faraj A et al (2012) Optimization of a synthetic mixture composed of major *Trichoderma reesei* enzymes for the hydrolysis of steam-exploded wheat straw. Biotechnol Biofuels 5:9
Bissaro B, Røhr AK et al (2017) Oxidative cleavage of polysaccharides by monocopper enzymes depends on H2O2. Nat Chem Biol 13:1123–1128
Boonvitthya N, Bozonnet S et al (2013) Comparison of the heterologous expression of *Trichoderma reesei* endoglucanase II and cellobiohydrolase II in the yeast *Pichia pastoris* and *Yarrowia lipolytica*. Mol Biotechnol 54:158–169
Boraston AB, Bolam DN et al (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem J 382:769–781
Borin GP, Sanchez CC et al (2015) Comparative secretome analysis of Trichoderma reesei and *Aspergillus niger* during growth on sugarcane biomass. PLoS One 10:1–20
Brar KK, Kaur S, Chadha BS (2016) A novel staggered hybrid SSF approach for efficient conversion of cellulose/hemicellulosic fractions of corncob into ethanol. Renew Energy 98:16–22
Bussamra BC, Freitas S, da Costa AC (2015) Improvement on sugar cane bagasse hydrolysis using enzymatic mixture designed cocktail. Bioresour Technol 187:173–181
Chadha BS, Kaur B et al (2019) Thermostable xylanases from thermophilic fungi and bacteria: current perspective. Bioresour Technol 277:195–203
Chandel AK, Chandrasekhar G et al (2012) The realm of cellulases in biorefinery development. Crit Rev Biotechnol 32:187–202
Chandel AK, Antunes FA et al (2014) Multi-scale structural and chemical analysis of sugarcane bagasse in the process of sequential acid–base pretreatment and ethanol production by *Scheffersomyces shehatae* and *Saccharomyces cerevisiae*. Biotechnol Biofuels 7:63
Chang L, Ding M et al (2011) Characterization of a bifunctional xylanase/endoglucanase from yak rumen microorganisms. Appl Microbiol Biotechnol 90:1933–1942
Chen M, Xia L, Xue P (2007) Enzymatic hydrolysis of corncob and ethanol production from cellulosic hydrolysate. Int Biodeter Biodegrad 59:85–89
Chen M, Zhao J, Xia L (2008) Enzymatic hydrolysis of maize straw polysaccharides for the production of reducing sugars. Carbohydr Polym 71:411–415
Chen X, Kuhn E et al (2016) DMR (deacetylation and mechanical refining) processing of corn stover achieves high monomeric sugar concentrations (230 g/L) during enzymatic hydrolysis and high ethanol concentration (>10% v/v) during fermentation without hydrolyzate purification or concentration. Energy Environ Sci 9:1237–1245
Cheng Y, Song X et al (2009) Genome shuffling improves production of cellulase by *Penicillium decumbens* JU-A10. J Appl Microbiol 107:1837–1846
Chundawat SP, Lipton MS et al (2011) Proteomics-based compositional analysis of complex cellulase–hemicellulase mixtures. J Proteome Res 10:4365–4372
Chundawat SP, Uppugundla N et al (2017) Shotgun approach to increasing enzymatic saccharification yields of ammonia fiber expansion pretreated cellulosic biomass. Front Energy Res 5:9
Chylenski P, Petrović DM et al (2017) Enzymatic degradation of sulfite-pulped softwoods and the role of LPMOs. Biotechnol Biofuels 10:177
da Silva AS, Espinheira RP et al (2020) Constraints and advances in high-solids enzymatic hydrolysis of lignocellulosic biomass: a critical review. Biotechnol Biofuels 13:58
Davis R, Tao L et al (2013) Process design and economics for the conversion of lignocellulosic biomass to hydrocarbons: dilute-acid prehydrolysis and enzymatic hydrolysis deconstruction of biomass to sugars and biological conversion of sugars to hydrocarbons. National Renewable Energy Laboratory, Golden, CO, NREL/TP-5100-60223

Dodd D, Cann IK (2009) Enzymatic deconstruction of xylan for biofuel production. GCB Bioenergy 1:2–17

dos Santos AC, Ximenes E et al (2019) Lignin–enzyme interactions in the hydrolysis of lignocellulosic biomass. Trends Biotechnol 37:518–531

Eggeman T, Elander RT (2005) Process and economic analysis of pretreatment technologies. Bioresour Technol 96:2019–2025

Emalfarb MA, Ben-Bassat A et al (1998) Cellulase compositions and methods of use. US Patent Application US5811381A

Ezeilo UR, Zakaria II et al (2017) Enzymatic breakdown of lignocellulosic biomass: the role of glycosyl hydrolases and lytic polysaccharide monooxygenases. Biotechnol Biotechnol Equip 31:647–662

Fang X, Yano S et al (2009) Strain improvement of *Acremonium cellulolyticus* for cellulase production by mutation. J Biosci Bioeng 107:256–261

Fanuel M, Garajova S et al (2017) The *Podospora anserina* lytic polysaccharide monooxygenases PaLPMO9H catalyzes oxidative cleavage of diverse plant cell wall matrix glycans. Biotechnol Biofuels 10:63

Filiatrault-Chastel C, Navarro D et al (2019) AA16, a new lytic polysaccharide monooxygenase family identified in fungal secretomes. Biotechnol Biofuels 12:55

Forsberg Z, Sørlie M et al (2019) Polysaccharide degradation by lytic polysaccharide monooxygenases. Curr Opin Struct Biol 59:54–64

Gandla M, Martín C, Jonsson L (2018) Analytical enzymatic saccharification of lignocellulosic biomass for conversion to biofuels and bio-based chemicals. Energies 11:2936

Gao D, Chundawat SPS et al (2010) Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover. Bioresour Technol 101:2770–2781

Gao D, Uppugundla N et al (2011) Hemicellulases and auxiliary enzymes for improved conversion of lignocellulosic biomass to monosaccharides. Biotechnol Biofuels 4:1–11

Gao L, Li Z et al (2017) Combining manipulation of transcription factors and over expression of the target genes to enhance lignocellulolytic enzyme production in *Penicillium oxalicum*. Biotechnol Biofuels 10:100

Garcia-Aparicio MP, Ballesteros M et al (2007) Xylanase contribution to the efficiency of cellulose enzymatic hydrolysis of barley straw. Appl Biochem Biotechnol 137:353–365

Glass NL, Schmoll M et al (2013) Plant cell wall deconstruction by ascomycete fungi. Annu Rev Microbiol 67:477–498

Gomez LD, Steele-King CG, McQueen-Mason SJ (2008) Sustainable liquid biofuels from biomass: the writing's on the wall. New Phytol 178:473–485

Gong W, Zhang H et al (2015) Comparative secretome analysis of *Aspergillus niger*, *Trichoderma reesei*, and *Penicillium oxalicum* during solid-state fermentation. Appl Biochem Biotechnol 177:1252–1271

Gottschalk LM, Oliveira RA, Da Silva Bon EP (2010) Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. Biochem Eng J 51:72–78

Harris PV, Welner D et al (2010) Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry 49:3305–3316

Harris PV, Xu F et al (2014) New enzyme insights drive advances in commercial ethanol production. Curr Opin Chem Eng 19:162–170

Himmel ME, Ding SY (2007) Biomass recalcitrance: engineering plants and enzymes for biofuel production. Science 315:804–807

Hinz SWA, Pouvreau L et al (2009) Hemicellulase production in *Chrysosporium lucknowense* C1. J Cereal Sci 50:318–323

Horn SJ, Vaaje-Kolstad G et al (2012) Novel enzymes for the degradation of cellulose. Biotechnol Biofuels 5:45

Hsu TC, Guo GL et al (2010) Effect of dilute acid pretreatment of rice straw on structural properties and enzymatic hydrolysis. Bioresour Technol 101:4907–4913
Hu J, Arantes V, Saddler JN (2011) The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Biotechnol. Biofuels 4:36
Hu J, Arantes V, Pribowo A, Saddler JN (2013) The synergistic action of accessory enzymes enhances the hydrolytic potential of a "cellulase mixture" but is highly substrate specific. Biotechnol Biofuels 6:112
Hu J, Chandra R et al (2015) The addition of accessory enzymes enhances the hydrolytic performance of cellulase enzymes at high solid loadings. Bioresour Technol 186:149–153
Huttner S, Várnai A et al (2019) Functional characterization of AA9 LPMOs in the thermophilic fungus *Malbranchea cinnamomea* reveals specific xylan activity. Appl Environ Microbiol. https://doi.org/10.1128/aem.01408-19
Igarashi K, Uchihashi T et al (2011) Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface. Science 333:1279–1282
Inoue H, Decker SR et al (2014) Identification and characterization of core cellulolytic enzymes from *Talaromyces cellulolyticus* (formerly *Acremonium cellulolyticus*) critical for hydrolysis of lignocellulosic biomass. Biotechnol Biofuels 7:151
Jennings EW, Schell DJ (2011) Conditioning of dilute-acid pretreated corn stover hydrolysate liquors by treatment with lime or ammonium hydroxide to improve conversion of sugars to ethanol. Bioresour Technol 102:1240–1245
Jeya M, Thiagarajan S et al (2009) Cloning and expression of GH11 xylanase gene from *Aspergillus fumigates* MKU1 in *Pichia pastoris*. J Biosci Bioeng 108:24–29
Jeya M, Nguyen NPT et al (2010) Conversion of woody biomass into fermentable sugars by cellulase from *Agaricus arvensis*. Bioresour Technol 101:8742–8749
Jin M, Sousa LC et al (2016) Toward lower cost cellulosic biofuel production using ammonia based pretreatment technologies. Green Chem 18:957–966
Johansen KS (2016) Discovery and industrial applications of lytic polysaccharide mono-oxygenases. Biochem Soc Trans 44:143–149
Johnson E (2016) Integrated enzyme production lowers the cost of cellulosic ethanol. Biofuels Bioprod Biorefin 10:164–174
Jørgensen H, Kristensen JB, Felby C (2007) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuels Bioprod Biorefin 1:119–134
Kallioinen A, Puranen T, Siika-aho M (2014) Mixtures of thermostable enzymes show high performance in biomass saccharification. Appl Biochem Biotechnol 173:1038–1056
Kameshwar AKS, Ramos LP, Qin W (2019) CAZymes-based ranking of fungi (CBRF): an interactive web database for identifying fungi with extrinsic plant biomass degrading abilities. Bioresour Bioprocess 6:1–10
Kaur B, Sharma M et al (2013) Proteome based profiling of hypercellulase-producing strains developed through interspecific protoplast fusion between *Aspergillus nidulans* and *Aspergillus tubingensis*. Appl Biochem Biotechnol 169:393–407
Kim SJ, Lee CM et al (2008a) Characterization of a gene encoding cellulase from uncultured soil bacteria. FEMS Microbiol Lett 282:44–51
Kim TH, Taylor F, Hicks KB (2008b) Bioethanol production from barley hull using SAA (soaking in aqueous ammonia) pretreatment. Bioresour Technol 99(13):5694–5702
Kim IJ, Jung JY et al (2015) Customized optimization of cellulase mixtures for differently pretreated rice straw. Bioprocess Biosyst Eng 38:929–937
Kristensen JB, Felby C, Jørgensen H (2009) Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol Biofuels 2:11
Kumar R, Wyman CE (2009) Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. Biotechnol Prog 25:302–314
Kumar P, Barrett DM et al (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48:3713–3729

Lambertz C, Garvey M et al (2014) Challenges and advances in the heterologous expression of cellulolytic enzymes: a review. Biotechnol Biofuels 7:135

Langston JA, Shaghasi T et al (2011) Oxidoreductive cellulose depolymerization by the enzymes cellobiose dehydrogenase and glycoside hydrolase 61. Appl Environ Microbiol 77:7007–7015

Laothanachareon T, Bunterngsoo B et al (2015) Synergistic action of recombinant accessory hemicellulolytic and pectinolytic enzymes to *Trichoderma reesei* cellulase on rice straw degradation. Bioresour Technol 198:682–690

Laser M, Schulman D et al (2002) A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. Bioresour Technol 81:33–44

Lee JW, Jeffries TW (2011) Efficiencies of acid catalysts in the hydrolysis of lignocellulosic biomass over a range of combined severity factors. Bioresour Technol 102:5884–5890

Lethio J, Wernerus H et al (2001) Directed immobilization of recombinant *Staphylococci* on cotton fibers by functional display of a fungal cellulose-binding domain. FEMS Microbiol Lett 195:197–204

Levasseur A, Drula E et al (2013) Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. Biotechnol Biofuels 6:41

Li BZ, Balan V et al (2010) Process optimization to convert forage and sweet sorghum bagasse to ethanol based on ammonia fiber expansion (AFEX) pretreatment. Bioresour Technol 101:1285–1292

Lima LHF, Serpa VI et al (2013) Small-angle X-ray scattering and structural modeling of full-length: Cellobiohydrolase I from *Trichoderma harzianum*. Cellulose 20:1573–1585

Liu D, Li J et al (2013a) Secretome diversity and quantitative analysis of cellulolytic *Aspergillus fumigatus* Z5 in the presence of different carbon sources. Biotechnol Biofuels 6:149

Liu G, Zhang L et al (2013b) Long-term strain improvements accumulate mutations in regulatory elements responsible for hyper-production of cellulolytic enzymes. Sci Rep 3:1569

Liu G, Zhang J, Bao J (2016) Cost evaluation of cellulase enzyme for industrial-scale cellulosic ethanol production based on rigorous Aspen Plus modeling. Bioprocess Biosyst Eng 39:133–140

Lombard V, Golaconda-Ramulu H et al (2014) The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 42:D490–D495

Mahajan C, Basotra N et al (2016) *Malbranchea cinnamomea*: a thermophilic fungal source of catalytically efficient lignocellulolytic glycosyl hydrolases and metal dependent enzymes. Bioresour Technol 200:55–63

Marx IJ, Van-Wyk N et al (2013) Comparative secretome analysis of *Trichoderma asperellum* S4F8 and *Trichoderma reesei* Rut C30 during solid-state fermentation on sugarcane bagasse. Biotechnol Biofuels 6:172

Mathew AK, Parameshwaran B et al (2016) An evaluation of dilute acid and ammonia fiber explosion pretreatment for cellulosic ethanol production. Bioresour Technol 199:13–20

Mello BL, Polikarpov I (2014) Family I carbohydrate binding-modules enhance saccharification rates. AMB Express 4:36

Merino ST, Cherry J (2007) Progress and challenges in enzyme development for biomass utilization. Adv Biochem Eng Biotechnol 108:95–120

Mokomele T, da-Costa SL et al (2018) Ethanol production potential from AFEX™ and steam-exploded sugarcane residues for sugarcane biorefineries. Biotechnol Biofuels 11:127

Morgenstern I, Powlowski J, Tsang A (2014) Fungal cellulose degradation by oxidative enzymes: from dysfunctional GH 61 family to powerful lytic polysaccharide monooxygenase family. Brief Funct Genom 13:471–481

Morrison JM, Elshahed MS, Youssef NH (2016) Defined enzyme cocktail from the anaerobic fungus *Orpinomyces* sp. strain C1A effectively releases sugars from pretreated corn stover and switchgrass. Sci Rep 6:29217

Müller G, Chylenski P et al (2018) The impact of hydrogen peroxide supply on LPMO activity and overall saccharification efficiency of a commercial cellulase cocktail. Biotechnol Biofuels 11:209

Murphy C, Powlowski J et al (2011) Curation of characterized glycoside hydrolases of fungal origin. Database 2011:bar020

Murray P, Aro N et al (2004) Expression in *Trichoderma reesei* and characterization of a thermostablefamily 3 β-glucosidase from the moderately thermophilic fungus *Talaromyces emersonii*. Protein Expr Purif 38:248–257

Ogunmolu FE, Kaur I et al (2015) Proteomics insights into the biomass hydrolysis potentials of a hypercellulolytic fungus *Penicillium funiculosum*. J Proteome Res 14:4342–4358

Pal S, Joy S et al (2016) Pretreatment and enzymatic process modification strategies to improve efficiency of sugar production from sugarcane bagasse. 3 Biotech 6:126

Pan X, Gilkes N et al (2006) Bioconversion of hybrid poplar to ethanol and co-products using an organosolv fractionation process: optimization of process yields. Biotechnol Bioeng 94:851–861

Payne CM, Knott BC et al (2015) Fungal cellulases. Chem Rev 115:1308–1448

Peciulyte A, Pisano M et al (2017) Hydrolytic potential of five fungal supernatants to enhance a commercial enzyme cocktail. Biotechnol Lett 39:1403–1411

Perkins JB, Kumar M et al (2012) *Talaromyces strains* and enzyme compositions. US Patent Application 13/381,871

Poidevin L, Feliu J et al (2013) Insights into exo- and endoglucanase activities of family 6 glycoside hydrolases from *Podospora anserine*. Appl Environ Microbiol 79:4220–4229

Polizeli ML, Rizzatti AC et al (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577–591

Polizeli MDLTM, Somera AF, Lucas RCD, Nozawa MSF, Michelin M (2017) Enzymes involved in the biodegradation of sugarcane biomass: challenges and perspectives. In: Buckeridge MS, Souza AP (eds) Advances of basic science for second generation bioethanol from sugarcane. Springer, Cham, pp 55–79

Prior BA, Day DF (2008) Hydrolysis of ammonia-pretreated sugar cane bagasse with cellulase, B-glucosidase, and hemicellulase preparations. Appl Biochem Biotechnol 146:151–164

Quinlan RJ, Sweeney MD et al (2011) Insights into the oxidative degradation of cellulose by metalloenzyme that exploits biomass components. PNAS 108:15079–15084

Raheja Y, Kaur B et al (2020) Secretome analysis of *Talaromyces emersonii* reveals distinct CAZymes profile and enhanced cellulase production through response surface methodology. Ind Crop Prod 152:112554

Rai R, Kaur B et al (2016) Evaluation of secretome of highly efficient lignocellulolytic *Penicillium* sp. Dal 5 isolated from rhizosphere of conifers. Bioresour Technol 216:958–967

Raj K, Krishnan C (2019) Improved high solid loading enzymatic hydrolysis of low-temperature aqueous ammonia soaked sugarcane bagasse using laccase-mediator system and high concentration ethanol production. Ind Crop Prod 131:32–40

Rajendran K, Drielak E et al (2017) Updates on the pretreatment of lignocellulosic feedstocks for bioenergy production—a review. Biomass Conver Biorefin 8:471–483

Ramos LP (2003) The chemistry involved in the steam treatment of lignocellulosic materials. Quim Nova 26:863–871

Ravalason H, Jan G et al (2008) Secretome analysis of *Phanerochaete chrysosporium* strain CIRM-BRFM41 grown on softwood. Appl Microbiol Biotechnol 80:719–733

Reyes-Sosa FM, Morales ML et al (2017) Management of enzyme diversity in high performance cellulolytic cocktails. Biotechnol Biofuels 10:156–156

Romani A, Garrote G (2010) Bioethanol production from hydrothermally pretreated *Eucalyptus globulus* wood. Bioresour Technol 101:8706–8712

Rosgaard L, Andric P et al (2007) Effects of substrate loading on enzymatic hydrolysis and viscosity of pretreated barley straw. Appl Biochem Biotechnol 143:27–40

Samayan IP, Schall CA (2010) Saccharification of ionic liquid pretreated biomass with commercial enzyme mixtures. Bioresour Technol 101:3561–3566

Sammond DW, Payne CM et al (2012) Cellulase linkers are optimized based on domain type and function: insights from sequence analysis, biophysical measurements, and molecular simulation. PLoS One 7:e48615

Sanhueza C, Carvajal G et al (2018) The effect of a lytic polysaccharide monooxygenase and a xylanase from *Gloeophyllum trabeum* on the enzymatic hydrolysis of lignocellulosic residues using a commercial cellulase. Enzym Microb Technol 113:75–82

Satlewal A, Agrawal R et al (2018) Natural deep eutectic solvents for lignocellulosic biomass pretreatment: recent developments, challenges and novel opportunities. Biotechnol Adv 36:2032–2050

Scheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol 61(1):263–289

Scully SM, Orlygsson J (2015) Recent advances in second generation ethanol production by thermophilic bacteria. Energies 8:1–30

Selig MJ, Adney WS et al (2009) The impact of cell wall acetylation on corn stover hydrolysis by cellulolytic and xylanolytic enzymes. Cellulose 16:711–722

Sills DL, Gossett JM (2012) Using FTIR spectroscopy to model alkaline pretreatment and enzymatic saccharification of six lignocellulosic biomasses. Biotechnol Bioeng 109:894–903

Singh S, Cheng G et al (2015) Comparison of different biomass pretreatment techniques and their impact on chemistry and structure. Front Energy Res 2:62

Singhania RR, Patel AK et al (2013) Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. Bioresour Technol 127:500–507

Sjöde A, Frolander A et al (2013) Lignocellulosic biomass conversion by sulfite pretreatment. Patent Application EP2376642 B1

Song W, Han X et al (2016) Proteomic analysis of the biomass hydrolytic potentials of *Penicillium oxalicum* lignocellulolytic enzyme system. Biotechnol Biofuels 9:68

Sweeney MD, Xu F (2012) Biomass converting enzymes as industrial biocatalysts for fuels and chemicals: recent developments. Catalysts 2:244–263

Tabka MG, Herpoël-Gimbert I et al (2006) Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulase xylanase and feruloyl esterase treatment. Enzyme Microb Technol 39:897–902

Timo S, Raphael G et al (2017) *Thermoascus aurantiacus* is an intriguing host for the industrial production of cellulases. Curr Biotechnol 6:89–97

Vaaje-Kolstad G, Westereng B et al (2010) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 330:219–222

Van Solingen P, Meijer D et al (2001) Cloning and expression of an endocellulase gene from a novel *Streptomycete* isolated from an east African soda lake. Extremophiles 5:333–341

Visser H, Joosten V et al (2011) Development of a mature fungal technology and production platform for industrial enzymes based on a *Myceliophthora thermophila* isolate, previously known as *Chrysosporium lucknowense* C1. Ind Biotechnol 7:214–223

Vu VV, Beeson WT et al (2014) Determinants of regioselective hydroxylation in the fungal polysaccharide monooxygenases. J Am Chem Soc 136:562–565

Weiss ND, Felby C, Thygesen LG (2019) Enzymatic hydrolysis is limited by biomass–water interactions at high-solids: improved performance through substrate modifications. Biotechnol Biofuels 12:1–13

Winger AM, Heazlewood JL et al (2014) Secretome analysis of the thermophilic xylanase hyperproducer *Thermomyces lanuginosus* SSBP cultivated on corn cobs. J Ind Microbiol Biot 41:1687–1696

Xu F, Ding H (2007) A new kinetic model for heterogeneous (or spatially confined) enzymatic catalysis: contributions from the fractal and jamming (overcrowding effects). Appl Catal A 317:70–81

Xu H, Xu F et al (2015) Process for increasing enzymatic hydrolysis of cellulosic material. Patent Application No. PCT/US2014/054067

Yang JS, Valente C, Polishchuk RS (2011) COPI acts in both vesicular and tubular transport. Nat Cell Biol 13:996–1003

Yennamalli RM, Rader AJ et al (2011) Thermostability in endoglucanases is fold-specific. BMC Struct Biol 11:10

Yennamalli RM, Rader AJ et al (2013) Endoglucanases: insights into thermostability for biofuel applications. Biotechnol Biofuels 6:136

Zhang YHP, Lynd LR (2004) Toward an aggregated understanding of enzymatic hydrolysis of cellulose: non-complexed cellulase systems. Biotechnol Bioeng 88:797–824

Zhang X, Qin W et al (2009) High consistency enzymatic hydrolysis of hardwood substrates. Bioresour Technol 100:5890–5897

Zhao C, Ding W et al (2014) Effects of compositional changes of AFEX-treated and H-AFEX-treated corn stover on enzymatic digestibility. Bioresour Technol 155:34–40

Fungal Glucoamylases: Developments in India and Recent Trends

3

Sanjeev Kumar, Rudrarup Bhattacharjee, and T. Satyanarayana

Abstract

Glucoamylases hydrolyze α-1,4-glycosidic and α-1,6-glycosidic bonds in soluble as well as raw starches. Other names of the enzyme include amyloglucosidase and γ-amylase. Its structure is highly conserved among fungal species and produced by several fungal species. Glucoamylases are industrially significant enzymes where they are used in producing crystalline glucose or glucose syrup from starch. Due to its high efficiency and industrial importance, glucoamylase is produced on a commercial scale by using fungal strains. Key factors behind its wide use are thermostability and optimal activity in acidic to neutral range of pH. This chapter provides a detailed overview of glucoamylases, including developments in India and recent advances.

Keywords

Fungal glucoamylase · Strain improvement · Production · Purification · Enzyme kinetics · Starch saccharification

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3.1 Introduction

Starch, cellulose, xylan, lignin, and pectin constitute a major part of biopolymers. A variety of microorganisms produce a large array of extracellular enzymes to degrade these substances (Antranikian 1992). The extracellular enzymes are of immense interest to enzyme industry. Starch is an abundant insoluble polysaccharide present in potato, maize, wheat, and several others and is considered as a principal food reserve in the plant kingdom to be used as a bulk nutrient in animal and human diets. Starch is a polymer of linear glucose residues connected by α-1,4-glycosidic bonds known as amylose (15–20%) and a branched polymer (having both α-1,4-glucan attached with α-1,6-linked branched junction), amylopectin (75–80%). Amylose and amylopectin possess different physical properties and are found in different ratios in natural starches. Usually, amylose-to-amylopectin ratios are between 1:3 and 1:4. Such a complex structure of native starches gives scope to various amylolytic enzymes for saccharifying starch to a number of different end products for various applications. Many different factors act upon and affect starch hydrolysis. The origins of starch and enzyme source were proved to be very important in starch hydrolysis. Temperature, substrate concentration, and pH are the important physical parameters that affect the starch hydrolysis to generate maximum end products (Tatsumi et al. 2007; Kumar et al. 2007, 2010).

Several mesophilic and thermophilic molds are known to secrete amylolytic enzymes (Fagerström and Kalkkinen 1995; Kumar et al. 2007; Kumar and Satyanarayana 2003, 2009a; Satyanarayana et al. 2004). Amylolytic enzymes hydrolyze the glycosidic linkages and are broadly categorized into endoamylases and exoamylases. Endoamylases randomly act on the starch molecule to release linear and branched oligosaccharides of varied length, while exoamylases act from the non-reducing end in a sequential manner to release defined end products. The most common amylases with higher potential for industrial uses are briefly described. α-Amylases (EC 3.2.1.1) hydrolyze α-1,4-glycosidic bond in starch randomly. These branched oligosaccharides are defined as α-limit dextrins. α-Amylases are further divided into saccharifying and liquefying α-amylases to produce free sugars and break down starch randomly to reduce the starch viscosity. The enzyme β-amylases (EC 3.2.1.2) hydrolyze the alternate α-1,4-linkages from the non-reducing end, thus releasing maltose. The end products are maltose and large random ß-limit dextrins. Glucoamylase/amyloglucosidase (EC 3.2.1.3) is a fungal enzyme that hydrolyzes α-1,4- and α-1,6-linkages to give glucose, in theory, a 100% yield. Amylases are frequently used to produce glucose and fructose syrups (Vihinen and Mäntsälä 1989; Jensen and Olsen 1999). α-Glucosidases (EC 3.2.1.20), often called as maltases, hydrolyze α-1,4- and/or α-1,6-linkages to produce α-D-glucose from the non-reducing end. Isoamylase (EC 3.2.1.68) hydrolyzes α-1,6-linkages in polysaccharides such as amylopectin, glycogen, and branched dextrins in an endo-fashion. Pullulanases are the debranching enzymes in amylopectin and pullulan. These are, however, inefficient in hydrolyzing α-1,6-glyosidic linkages in glycogen. Type I pullulanase (EC 3.2.1.41) are typical bacterial enzymes, also known as α-dextrin 6-glucanohydrolases, which specifically hydrolyze α-1,6-linkages but do

not hydrolyze α-1,4-linkages. It exclusively hydrolyzes α-1,6-linkages in pullulan to release maltotriose (Nisha and Satyanarayana 2013, 2016). Type-II pullulanases (EC 3.2.1.1/41) are bifunctional, endo-acting enzymes that hydrolyze both α-1,4- and α-1,6-glycosoidic bonds; these are also known as amylopullulanases. Except β-amylase, all other amylases are secreted by microbes to saccharify starch polymer. β-Amylase is produced in plants (Vihinen and Mäntsälä 1989; Antranikian 1992; Nigam and Singh 1995). Amylases are the second large group of enzymes of industrial significance; fungal glucoamylases contribute significantly in producing glucose and fructose syrups (Pandey 1995; Kumar et al. 2007; Reilly 2006).

3.2 Fungal Glucoamylase Research in India

Microorganisms possess a unique set of starch-hydrolyzing enzymes which are widely distributed. Indian scientists have made significant contributions in finding novel enzymes in order to meet the industrial demand. Several mesophilic as well thermophilic molds are known to produce glucoamylases. The data compiled on glucoamylases reflect the importance of fungal glucoamylases in meeting the industrial demand (Table 3.1). Prof. Satyanarayana and his team at the University of Delhi (South Campus) carried out investigations on screening a large number of thermophilic fungi for glucoamylase production. *Thermomucor indicae-seudaticae* was selected for a detailed study on the production of thermostable glucoamylase (Satyanarayana et al. 2004; Kumar and Satyanarayana 2003). A method was used to entrap sporangiospores of *T. indicae-seudaticae* in the alginate beads for glucoamylase production in submerged fermentation. The critical variables were optimized by response surface methodology (RSM). Higher enzyme titer was achieved by using alginate-immobilized spores (Kumar and Satyanarayana 2007a). The glucoamylase production was produced in molasses medium; a high enzyme production was achieved in comparison with that in other media (Kumar and Satyanarayana 2007b; Kumar et al. 2007). *Thermomucor indicae-seudaticae* was further taken up for mutagenic studies using nitrous acid and gamma radiation (^{60}Co) for enhancing glucoamylase production. The glucoamylase production doubled in mutants generated by sequential irradiation. Higher specific growth rate with improved productivity was attained. *T. indicae-seudaticae* CR19 mutant displayed high glucoamylase production (Kumar and Satyanarayana 2009b; Kumar et al. 2010). Genetic engineering tools were used to generate a novel chimeric biocatalyst (Amy-Glu) from α-amylase of *B. acidicola* and glucoamylase of *A. niger* to saccharify starch. Kinetic studies showed that the expressed chimeric biocatalyst yielded maltodextrins and glucose by saccharifying wheat and corn starches in an efficient manner (Parashar and Satyanarayana 2017; Kumar and Satyanarayana 2009a). Prof. Satyanarayana and his team focused on research to screen novel glucoamylase producers and developed a chimeric enzyme for single-step starch saccharification, which is considered as the noteworthy development in glucoamylase research.

Table 3.1 Fungal glucoamylase production in India

S. no.	Process	Organism	Production mode	References
1	Purification and enzyme kinetics	*T. indicae-seudaticae*	SmF	Kumar and Satyanarayana (2003)
2	Enzyme production using statistical mode	*T. indicae-seudaticae*	SmF	Kumar and Satyanarayana (2007a)
3	Enzyme production in cane molasses medium	*T. indicae-seudaticae*	SmF	Kumar and Satyanarayana (2007b)
4	Enzyme production and starch saccharification	*T. indicae-seudaticae*	SmF	Kumar et al. (2007)
5	Mutagenic	*T. indicae-seudaticae*	SmF	Kumar and Satyanarayana (2009b)
6	Enzyme production	*Fusarium solani*	SSF	Bhatti et al. (2007)
7	Chimeric biocatalyst and overexpression	*B. acidicola* and *A. niger*	SmF	Parashar and Satyanarayana (2017)
8	Purification and characterization	*A. niger*	SSF	Selvakumar et al. (1996)
9	Ethanol production	Yeast	SmF	Sharma et al. (2002)
10	Corn starch liquefaction	*A. niger* and *A. terreus*	SmF	Jain and Katyal (2018)
11	Rice beer production	*A. rouxii* and *R. oryzae*	SmF	Das et al. (2017)
12	Enzyme production from waste substrate	*Aspergillus* sp.	SSF	Anto et al. (2006), Prajapati et al. (2014)
13	Production of rice beer by ethnic community in Assam	*Amylomyces rouxii* and *Rhizopus oryzae*	SmF	Das et al. (2017)
14	Bioethanol from waste biomass	*Pogonatherum crinitum*	SmF	Waghmare et al. (2018)

In another study, four glucoamylases were purified by cultivating *A. niger* in solid-state fermentation (Selvakumar et al. 1996). Sharma et al. (2002) screened yeast strains for carrying out glucoamylase production and ethanol fermentation. An enhanced ethanol production up to 5.2% was achieved by enzymatic treatment of wheat flour (Sharma et al. 2002). A comparative study had been conducted to study the effect of laboratory-optimized glucoamylase production by two fungal strains; *Amylomyces rouxii* and *Rhizopus oryzae* were isolated and identified based on ITS sequence from traditional rice beer fermentation by Bodo and Deori communities in Assam. The fungus *A. rou*xii TU460 showed considerably high glucoamylase and α-amylase without any mycotoxin secretion that makes the process suitable for

further analysis. Glucoamylase was purified and characterized (Das et al. 2017). *A. niger* and *A. terreus* glucoamylases are commercially available for sugar production. Saccharification efficiency was enhanced by 53.2% (Jain and Katyal 2018). Recently, *A. terreus* C1 has been tested for the production of itaconic acid (IA) from potato starch waste. Cultural parameters were standardized using response surface designs and other statistical approaches to enhance the production of IA; 29.69 g/L production was attained in 3 L fermenter (Bafana et al. 2019).

3.3 Glucoamylase Production

The demand for glucoamylase in the food industry is paramount, and this raises its commercial demand (Ford 1999; Reilly 1999). Filamentous fungi like *Aspergillus* and *Rhizopus* are commonly utilized for commercial glucoamylase production by submerged fermentation. The typical medium used in the process contains 20% corn along with 2.5% corn steep liquor, and the reaction temperature is usually maintained at 30–45 °C (Gibbs et al. 2000). Among other strategies, batch, fed-batch, and continuous fermentations remain quite popular. These processes utilize a variety of vessels ranging from flasks at smaller level and airlift bioreactors (Merico et al. 2004; Kumar et al. 2007; Ganzlin and Rinas 2008).

Different strategies have been optimized and implemented for attaining higher yields of enzyme. Different substrates like sucrose, starch, maltose, dextrin, maltodextrins, dextrin, and amylopectin are used for amylase production. The choice of source was largely based on production system of the enzyme, i.e., whether constitutive or inducible (Chiquetto et al. 1992; Serour and Antranikian 2002). One of the earlier researchers explored the maltose impact on glucoamylase production by *A. niger*. When *A. niger* was grown on sorbitol, a low level of glucoamylase was produced. When the sorbitol was substituted with maltose and glucose, the synthesis of glucoamylase increased. Also, the use of yeast extract as a constituent of the medium led to the formation of both glucoamylase I and glucoamylase II. When ammonium chloride was used as a nitrogen source, glucoamylase I production was recorded. It was also reported that glucoamylase production using maltose was dependent on low pH but not dependent on the concentration of sulfate (Barton et al. 1972).When ammonium dihydrogen phosphate was used as a nitrogen source, *T. lanuginosus* produced a glucoamylase, having fourfold higher enzyme production in a fermenter by controlling pH (Haasum et al. 1991). Glucoamylase yield doubled upon optimizing yeast extract concentration in the fermentation media (Facciotti et al. 1991). Different nitrogen sources used included asparagine, meat extract, sodium glutamate ammonium nitrate, urea, corn steep liquor, and yeast extract (Nguyen et al. 2000; Kaur and Satyanarayana 2004; Metwally 1998; Kumar and Satyanarayana 2007a, b; Rajoka and Yasmeen 2005). Phosphorus was also found to be necessary for glucoamylase production. It has been reported that an increase in phosphate with controlled pH and a high yield of glucoamylase was achieved in *A. niger* (Zaldivar-Aguero et al. 1997).

Investigation was carried out for assessing the effect of environmental and nutritional parameters on the growth and production of glucoamylase in *Rhizopus microsporus* var. *rhizopodiformis*. High titers of amylolytic activities were observed when the medium was supplemented with oat meal, starch, and sugarcane bagasse at 45 °C. Catabolite repression was observed when supplemented with glucose. The enzyme production was reported on many substrates like amylose, maltose, starch, pullulan, amylopectin, and glycogen. The study suggested that the amylolytic activity was caused by a glucoamylase containing a low titer of α-amylase (Peixoto et al. 2003). Extracellular glucoamylase has also been produced by *Aspergillus fumigatus* isolated from goat rumen. The highest production of glucoamylase was reported at pH 7.0 and 37 °C in 30 days. A rapid increase in the production of glucoamylase was recorded upon using 4% starch and 0.25% $(NH_4)_2HPO_4$. The maximum glucoamylase production by *Aspergillus fumigatus* was attained at pH 7.0 and 35 °C (Cherry et al. 2004).

A process of reusability of inoculum was developed to produce glucoamylase by immobilization. Ca-alginate beads were used to entrap *Kluyveromyces lactis* cells for glucoamylase production in continuous stirred bioreactor. The entrapped Ca-alginate beads could retain the glucoamylase production and metabolic activity up to 4 months when stored at 4 °C (Alteriis et al. 2004). An improved glucoamylase production was recorded when *Thermomucor indicae-seudaticae* spores were immobilized in alginate beads. Parametric optimization was done in submerged fermentation using alginate-immobilized spores that supported enhanced glucoamylase production (Kumar and Satyanarayana 2007a, b). Extensive screening was conducted to find the best glucoamylase producer from 17 *Rhizopus* isolates; environmental variables were optimized for enhanced enzyme production. Some of the physical and chemical parameters (pH, incubation period, temperature, and nitrogen) were tested by one variable at time approach. The fermentation process utilized 10% potato starch as substrate. At 45 °C and pH 4.5, 0.3% yeast extract and 0.3% polypeptone supported high enzyme production (Nahar et al. 2008). Attempts were made toward an efficient ethanol fermentation. *Rhizopus oryzae* glucoamylase and *Aspergillus oryzae* glucoamylase encoded by *glaA a*nd *glaB* were expressed in yeast *Saccharomyces cerevisiae* MT8-1 and GRI 117 UK. The observations indicated that the transformant having *GRI 117-UK/pUDGAA glaA* glucoamylase gene produced the highest ethanol from liquefied starch, and the transformant MT8-1/pUDGAR displayed the highest activity of glucoamylase on its cell surface (Kotaka et al. 2008).

Bitter cassava (*Manihot esculenta*) was tried for the optimization of glucoamylase production by *A. niger* using response surface methodology (RSM) and face-centered central composite design for attaining the optimum level. It was indeed in agreement with the agitation workflow using low energy dissipation as suggested by Kelly et al. (2004). The medium contained bitter cassava as the main substrate, supplemented with various nutrients like maltose, yeast extract, NH_4NO_3, $MgSO_4$, FeSO4, $CaCl_2$, and KH_2PO_4. The effective values of parameters including inoculum concentration, pH, and agitation were 3.7% (v/v), 4.8, and 260 rpm for optimal production of glucoamylase (Alam et al. 2014). Another study investigated a novel

glucoamylase of *Aspergillus phoenicia* in submerged fermentation. Machado-Benassi (MB) medium was used with 1% maltose as a source of carbon at 30 °C in 4 days of incubation. The production was inhibited by different glucose concentrations and induced by maltose (catabolite repression). A combination of calcium and manganese was used to activate the produced glucoamylase (Benassi et al. 2014). Another study reported glucoamylase production by *A. niger* MENA1E and *Rhizopus* MENACO11A. The organisms were incubated with four types of substrates, viz.., rice bran, maize bran, wheat bran, and groundnut pod in solid-state fermentation. Although all the four substrates supported enzyme production, glucoamylase produced on wheat bran was very high (Nyamful et al. 2014). Recently, a black box model was constructed to study the relationship between the production of glucoamylase and the growth of *A. niger*; lower specific growth rate (<0.068/h) favored glucoamylase production. The model was generated by combining Pirt equation and Luedeking-Piret equation. The model may be useful in the optimization of enzyme production (Li et al. 2015). The radial turbine (RT) configuration formed heterogeneous mass transfer, while a wide-blade hydrofoil upward-pumping (WHu) supported homogeneities. The experimental setup indicated the relation between higher enzyme production and active mycelia. As a result, the axial flow impeller led to consistent mass transfer with improved glucoamylase production rate by 25% (Tang et al. 2015). There were many strategies applied for improving yield and reducing cost of production, keeping in mind the good quality of products. The study utilized fed-batch fermentation for glucoamylase synthesis. A varied substrate feeding strategy was utilized for efficient glucoamylase production. Corn starch hydrolysates were considered as the best carbon source by replacing the standard and expensive feeding medium to reduce the cost (Luo et al. 2015). Raw starch-digesting glucoamylase production by *Aspergillus niger* was enhanced significantly when the growth medium was supplemented with maltose and stearic acid ester (Sun and Peng 2017).

Solid-state fermentation (SSF) is an ideal process for fungi to grow, since it can grow on solids with low water activity. SSF is the favorite method among researchers using agro-residues (wheat bran, rice bran, and straw, various flours, sugarcane bagasse) that are readily available and yield higher enzyme titers (Pandey et al. 2001; Ellaiah et al. 2002; Kumar and Satyanarayana 2004a; Varzakas et al. 2008). All such solid agro-residues can further be moistened with various salt solutions and can be supplemented with various carbon sources to enhance the enzyme titer (Selvakumar et al. 1998; Kumar and Satyanarayana 2004a, b, 2007a; Bhatti et al. 2007). Pastry waste was utilized for SSF to economize the process, and the derived enzyme was successfully used in glucose production. The crude glucoamylase in the extract is active at 55 °C and pH 5.5 for optimal hydrolysis. Under optimal digestion conditions, it yielded around 53 g of glucose from 100 g of mixed food waste, showing its sustainable hydrolytic potential (Lam et al. 2013). Industrial starchy potato (mostly discarded as waste) was used as an inexpensive medium for increasing the production of glucoamylase. The mashed potato medium was supplemented with malt extract, $CaCl_2$, and $FeSO_4$, as per the optimal concentration suggested by the central composite design (Izmirlioglu and Demirci 2016).

Readily available agricultural wastes around Kolkata local areas with low-cost raw peel of garden pea as a substrate were used in SSF. Controlled process parameters like temperature, varying substrate amount, and fermentation time were optimized. The glucoamylase production was stable at pH 3.8, while the highest tolerated pH range was 5.4–6.2. The suitable temperature was 70 °C for 30 min. When incubated with magnesium, iron, lead, and copper, the enzyme showed higher catalytic efficiency. Glycogen was also used as a substrate with garden pea peel (Banerjee and Ghosh 2017). SSF is used for glucoamylase production from different agricultural residues (wheat bran, rice husk) at pH 4.0–5.0 in the temperature range of 30–40 °C in 4–5 days. As compared to the earlier strategies, the new approach was found suitable for glucoamylase production.

3.4 Mutation, Cloning, and Production of Recombinant Glucoamylase

With the advancements in modern molecular and bioinformatics tools, glucoamylase genes have been cloned and expressed in GRAS (generally recognized as safe) hosts for overexpression and enhanced production. Earlier, hyper-glucoamylase producers were generated through random mutagenesis and genetic recombination (Rubinder et al. 2000, 2002; Kumar and Satyanarayana 2009a). The genetic aspects of glucoamylase were a matter of interest since the early 1990s. A study determined the carbon source influenced the regulation of *Aspergillus awamori* glucoamylase gene expression (Nunberg et al. 1984). Soluble starch was a key regulator in encoding a 2.3 kb-long mRNA, which translates into the *A. awamori* glucoamylase; as compared to other substances like glucose, xylose displayed relatively low induction (Nunberg et al. 1984). Another study characterized the yeast (*S. fibuligera*) glucoamylase gene GLU1 and reported its complete nucleotide sequence. The gene encodes a single open reading frame translating into a 519-amino acid protein containing N-glycosylation sites. Sequence alignment studies showed that the glucoamylase from this study contains at least five regions which are highly conserved among other fungal glucoamylases (Itoh et al. 1987). The gene that encodes glucoamylase production was present on a 2.5 kb EcoRI-EcoRV fragment containing five intervening sequence. In case of glucoamylase I and glucoamylase II production, a 169 bp intron was involved along with four other very small introns, which were spliced via mRNA processing (Boel et al. 1984). The study highlighted the significance of genetic structure for glucoamylase gene and its potential in other studies involved in glucoamylase overexpression.

Subsequently, glucoamylase genes from *Aspergillus awamori* were cloned and analyzed, which led to the understanding of starch-binding domain and its mutation consequences (Goto et al. 1994). Also, glucoamylases types G1 and G2 were isolated from *A. niger* and, upon genetic studies, revealed the two coding mRNAs producing these enzymes being distinct yet closely related (Boel et al. 1984). The *glaA* gene of *A. niger* was identified for high levels of glucoamylase. Several mutagenesis studies using deletions were done to identify the key residues in gene

expression, and it has been understood that a region between the −562 and −318 sites is responsible for high expression, while a 5′ flanking sequence of 214 bp acts as initiator of transcription. When −562 alone was deleted, glucoamylase synthesis was normal. Along with −562, when another region −318 (present between *MluI* site and the *SmaI* site) was deleted, glucoamylase expression was hindered substantially. The "element 1" region was then attributed to the enhanced glucoamylase expression (Fowler et al. 1990). In a subsequent study, *glaA* gene from *A. oryzae* has been found to have 62% nucleotide level homology with *A. niger glaA* gene. It possesses high expression capacity and also contains four short introns, each having a length of 45–56 nucleotides (Yoji et al. 1991). Another research highlighted the regulation of glucoamylase production in *A. terreus* strain 4, where glucose catabolite repression was found as a key regulator, and the enzyme production was found to be maximum at 0.5% starch supplement (Ghosh et al. 1990). Some cloning studies have also been done with the *A. terreus* glucoamylase gene, which led to the understanding of its transcription framework. *A. terreus gla1* promoter contains some CREA consensus sequences similar to *A. nidulans.* The sequences contain a 13 bp separation in between. Importantly, these sequences are not at all similar to the consensus promoter sequences found in case of glucoamylase genes of *A. niger* and *A. oryzae*. The *gla1* gets induced majorly by starch; however, a limited amount of induction has also been detected with maltose, although much lesser than starch. The *A. terreus gla1* is a very high expressing gene and thus makes it industrially preferable to produce in large amounts. The major drawback lies in the fact that *A. terreus gla1* gene is repressed by both xylose and glucose, and the inhibition is not reversed by adding starch and maltose as well (Ventura et al. 1995). Glucoamylase gene from *Neurospora crassa gla1* has also been studied for screening *N. crassa* genomic DNA library, and it has also been compared with other fungal glucoamylase gene sequences that showed a high similarity (Stone et al. 1993). Apart from the above studies, one recent research identified glucoamylase gene, *gla1*, in basidiomycetous fungus *Lentinula edodes.* The gene was found to be of 3094 bp in length containing seven introns ranging from 46 to 55 bp in length. A comparative study of amino acid sequence of different fungal glucoamylases revealed that it contains an essential N-terminal catalytic domain required for enzyme function along with a C-terminal starch-binding domain (SBD). It also comprises a hinge region which is highly glycosylated. The sequence comparison also highlights its high level of similarity with a glucoamylase from another basidiomycetous fungus *Corticium rolfsii*. It however showed lesser sequence homology when compared to other fungal glucoamylases (Zhao et al. 2000).

The cloned and expressed gene of *Talaromyces emersonii* in *A. niger* showed higher specific enzyme activity and elevated thermal stability (Nielsen et al. 2002). The *Thielavia terrestris glaA* gene was cloned and heterologously expressed in *A. oryzae* (Rey et al. 2003). A 100-fold increase in glucoamylase secretion level was attained by genetic manipulation of *Rhizopus oryzae* enzyme. A modified signal peptide (MSP) associated with secretory vesicles showed 100-fold improvement in enzyme production (Liu et al. 2005). A hyperproducer *T. indicae-seudaticae* CR19

was isolated using gamma (^{60}Co) irradiation. The glucoamylase remained optimally active, though the mutant grew slowly (Kumar and Satyanarayana 2009a).

Point mutation study was carried out to decipher the conformation changes in α-helices of fungal glucoamylase of *A. awamori*. Point amino acid substitution mutation of G137A and A246C, I136L, and G139A evidently enhanced the thermostability of glucoamylase, which indeed is required for the industrial application. An enhanced thermostability was observed at 70 °C in a mutant of *A. awamori* glucoamylase gene. A point mutation was carried out at Ser54→Pro, Thr314→Ala, and His415→Tyr to enhance thermo-activation. The mutant displayed good hydrolyzing activity on potato, cassava, and corn starches (Pavezzi et al. 2011). In another mutagenic study, a total of five mutants were generated to test thermostability. Insertion of two extra disulfide bonds between 1 and 13 α-helices was explored in mutant and was found to show loss in thermostability. Loss in thermostability resulted from the destabilized protein structure due to an extra disulfide bond in the mutant. Recently, ion beam irradiation was used to produce *A. luchuensis* mutant U1. The mutant showed higher starch-hydrolyzing activity. Surprisingly, the mutant did not have any significant change in transcription of *glaA* or *amyA* gene, and higher production is independent of *glaA* gene transcription regulations. However, the RNA seq analysis revealed that there were 604 genes involved in metabolic processes, which might lead to hyperproduction of glucoamylase in the mutant strain U1 (Kojo et al. 2018).

Both α-amylase and glucoamylase co-express in *Saccharomyces cerevisiae* Y294 [AteA-GlaA] strain in cost-effective consolidated bioprocessing to degrade raw starch. The highest activity of amylase and glucoamylase was achieved for ethanol production (Sakwa et al. 2018). Nearly 14% enhanced glucoamylase activity was achieved by codon optimization, preferentially based on the highest codons expressed in *S. cerevisiae*. Five copies of α-amylases and glucoamylase genes were co-expressed in *S. cerevisiae* Y294. The engineered *S. cerevisiae* strains showed to possess potential to hydrolyze raw starch for industrial process (Cripwell et al. 2019). Genetic engineering tools were applied for designing to overexpress the glucoamylase (*glaA*) and α-amylase (*amyA*) genes in *A. niger* CICC2462. The recombinant *A. niger* strains demonstrated overexpression of *amyA* and *gla*A genes with improved yield of glucoamylase for industrial applications (An et al. 2019). Two mutant variants of *A. awamori* (Thr390Ala and Thr390Ala/Ser436Pro) were constructed by random mutagenesis for enhanced thermostability (Schmidt et al. 2019). A potential issue with glucoamylase was its thermal intolerance. A recent study of heterologous expression in *Pichia pastoris* tried to address this issue by producing a novel thermostable glucoamylase from *Aspergillus flavus* displayed optimal activity at around 70 °C, thus making it a suitable choice for large-scale industries of starch processing (Karim et al. 2016). Industrially important fungal glucoamylase sparked an immense interest in studying the enzymatic activities. However, recent advances in technological front and also development of molecular modification strategies greatly enhanced the production outcome of glucoamylase, as well as other commercial enzymes, while minimizing error rate.

3.5 Purification and Characterization

Fungal glucoamylases have been purified in simple steps:

1. Concentration of enzymes: the step aimed to have a working enzyme concentration from crude glucoamylase preparations from submerged as well as solid-state fermentations, involving precipitation/lyophilization/ultrafiltration.
2. The concentrated enzymes are then applied to ion-exchange column, gel filtration, and affinity column chromatography. The purified enzyme thus obtained, has been used for kinetic studies to determine K_m, V_{max}, pH and temperature optima, substrate specificity, and molecular mass. A recent and comprehensive data on purification and kinetic properties of fungal glucoamylases are summarized in Table 3.2.

One of the pioneering studies compared fungal glucoamylases from different strains using five commercial preparations. Three preparations were from *Aspergillus niger*, while one each from *A. candidus* and *A. foetidus*. After purification through Sepharose gel filtration, ultrafiltration, and DEAE-Sephadex chromatography, two types of glucoamylases were identified, viz., glucoamylase I and glucoamylase II. The specific activities of these enzymes were checked and found between 85- and 142-units/mg protein. The enzymes from *A. niger* and *A. foetidus* showed higher percentage of acidic amino acids as compared to the enzyme from *A. candidus*. The enzymes obtained from *A. nige*r were found to contain galactose, glucose, mannose, glucosamine, and xylose, while the enzyme from *A. foetidus* did not have xylose, and the enzyme from *A. candidus* lacked both glucose and xylose. This study highlights the variations in glycan structure of fungal glucoamylases, although they might appear homogeneous in electrophoresis and ultracentrifugation (Manjunath and Rao 1979).

Another study explored the properties of thermostable fungal glucoamylase from *Aspergillus niveus*. The enzyme in this case was produced by submerged fermentation. The conditions for the production were 40 °C for 72 h and pH 6.5; the enzyme was purified through ion-exchange chromatography. The molecular weight was around 76 kDa with an isoelectric point of 3.8 and carbohydrate content of around 11%. The enzyme was stable at pH 4.0–9.5 at-least for 2 h; it retained activity even after 6 h of incubation at 60 °C, showing strong evidence of its thermostability. Glucose was formed as a single product after the hydrolysis of starch for 120 min, confirming the enzyme as a glucoamylase. This study warranted having stability at various pH, temperatures, and solvents; the glucoamylase of *A. niveus is* a potential enzyme for downstream biotechnological process (da Silva et al. 2009). Another glucoamylase mutant with enhanced thermostability at 65 °C was purified using affinity chromatography-based acarbose-Sepharose. The purified enzyme was weighed 100 kDa and optimally active at pH 4.5. The study was significant in showing the enhanced thermostability and the ability to hydrolyze commercial as well raw starch from potato, cassava, and rice (Pavezzi et al. 2011). Meanwhile, a new type of glucoamylase was isolated *A. niger*, which was optimally active at 70 °C and pH 4.4–5.0, respectively. The thermostability of the glucoamylase was increased

Table 3.2 Production, purification, kinetic details, and starch digestibility of important fungal glucoamylases

Organism	Production mode	Purification method	Molecular mass (kDa)	pH	Temperature (°C)	K_m	Potential application	References
Thermomucor indicae-seudaticae	SmF/SSF	Anion exchange/gel filtration	42	7.0	60	0.4 mg/mL	Raw starch hydrolysis	Kumar and Satyanarayana (2003), Kumar et al. (2010)
Chaetomium thermophilum	SmF	Ammonium sulfate, anion exchange, affinity	64	4.0	65	–	Soluble starch	Chen et al. (2005)
Thermomyces lanuginosus	SmF	Ammonium sulfate and ion exchange	66	5.0	70	3.5 mM	Soluble starch	Thorsen et al. (2006)
Curvularia lunata	SmF	Gel filtration and ion exchange	66	4.0	50	–	Soluble starch	Feng et al. (2007)
Monascus purpureus RY3410	SSF: Red koji	Anion, cation, and hydrophobic chromatography	Two forms: 66 and 89	5.0 for both	50 and 65	4.0 and 1.1 mg/mL	Soluble starch	Tachibana and Yasuda (2007)
Paecilomyces variotii	SmF	Ion exchange and gel filtration	86.5	5.0	55	3.8, 4.1, 2.0, 2.5, 1.7 mg/mL	Starch (reagent), starch (sigma), amylopectin, amylose, and glycogen	Michelin et al. (2008)
Rhizopus microsporus var. *chinensis*	SSF	–	–		70		Raw corn starch	Li et al. (2010)
Rhizopus oryzae	SmF	Affinity	Two forms: 52 and 47	5.5 and 6.0	50	2.11 and 1.34	Soluble starch	Mertens et al. (2010)
Monascus anka	SSF	Anion exchange	Multiple forms	5.5	40	–	Soluble starch	Yoshizaki et al. (2010)

Endophytic fungus EF6	SmF	Anion exchange and gel filtration	62.2	6.0	60	2.63	Soluble starch	Tangngamsakul et al. (2011)
Rhizopus oligosporus	SSF	Activated charcoal	Two forms: 36 and 50	5.5	50	–	Soluble starch	Kareem et al. (2011)
Aspergillus phoenicis	SmF	–	–	5.0	65	–	Soluble starch	Benassi et al. (2014)
Penicillium verruculosum	SmF	Ammonium sulfate precipitation, anion exchange, and hydrophobic chromatography	70	4.0	50	0.13 mg/mL	Soluble starch	Volkov et al. (2013)
Aspergillus niger	SmF	Fractionation, anion exchange	62	5.0	70	0.33 mg/mL	Soluble starch	Bagheri et al. (2014)
Aspergillus flavus	SmF/ heterologous expression	Affinity chromatography	78	5.0	70	5.84 mg/mL	Soluble starch	Karim et al. (2016)
A. japonicus	SmF	Anion exchange	72	5.0	65	0.59 mg/mL	Potato starch	Pasin et al. (2017)
Tetracladium sp. (cold-adapted yeast)	SmF	Ammonium sulfate precipitation	84	6.0	30	4.5 mg/mL	Soluble starch	Carrasco et al. (2017)
Aspergillus tritici	SmF	Ammonium sulfate, ion exchange	51	5.0	45	1.5 mg/mL	Soluble corn starch	Xian and Feng (2018)
Tricholoma matsutake	SmF/ heterologous expression	Ammonium sulfate, gel filtration	63.9	5.0	60	–	Soluble starch	Onuma et al. (2018)
Leohumicola incrustata	SmF	Ion exchange, gel filtration	101	5.0	45	0.38 mg/mL	Soluble starch	Adeoyo et al. (2018)

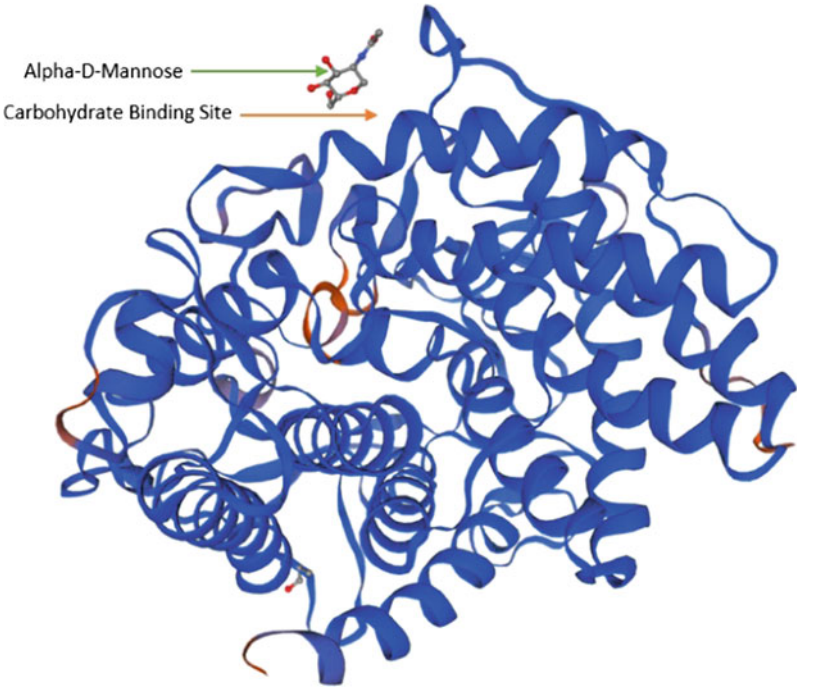
using starch and calcium ions, and its nature was identified by a specific substrate and thin-layer chromatography (Bagheri et al. 2014).

A new glucoamylase of *A. japonicus* was investigated by cultivating it in Khanna medium supplemented with maltose and potato starch in different concentrations. Enzyme production was higher when the substrate concentration was kept low. The most appropriate concentrations ranged between 0.5 and 0.75, which led to efficient enzyme production. Adding too much substrate caused a lower glucoamylase level, owing to the effect of catabolite repression. The favorable reaction conditions were 25 °C, pH 5.5, and 4 days of incubation. The enzyme was purified using DEAE-cellulose column. The kinetic parameters were also established for the enzyme; it showed a K_{cat} value of 369.58/s, while the catalytic efficiency, i.e., K_{cat}/K_m, was 626.40/s/mg/mL. The values of K_m (0.59 mg/mL) and V_{max} (308.01 U/mg) were calculated for the enzyme (Pasin et al. 2017).

Another study was performed on characterization and purification of a novel glucoamylase from cold-adapted fungus *Tetracladium* sp. using soluble starch as a substrate. Generally, glucoamylases are active at higher temperature and acidic pH with a high catalytic efficiency. However, this enzyme was isolated to perform optimal activity at ambient temperatures for industrial purposes. The enzyme was active at 30 °C and pH 6.0. Pervez et al. (2019) had shown significantly improved catalytic performance upon immobilizing the partially purified glucoamylase. A 5 °C shift was observed in temperature optima with doubling K_m and decreased V_{max}. Immobilized enzyme has been successfully reused six times efficiently (Pervez et al. 2019). A 42 kDa glucoamylase from *Thermomucor indicae-seudaticae* was active on raw as well as soluble starches at neutral pH and 60 °C with varied impact of metal ions. *Thermomucor* glucoamylase showed an enhanced thermostability in the presence of starch and released only glucose even at higher starch concentrations (Kumar and Satyanarayana 2003; Kumar et al. 2010). Glucoamylases from various other organisms have displayed a varied impact of metal ions (Kato et al. 2011; Kumar et al. 2010; Tangngamsakul et al. 2011). *Rhizopus microsporus* var. *chinensis* glucoamylase digest raw corn starch at 70 °C (Li et al. 2010). Two varied glucoamylases AmyC and AmyD from *R. oryzae* were purified and found to be active at pH optima of 5.5–6.0 and 60 and 50 °C with lowered K_m values (Mertens et al. 2010). Glucoamylase and α-amylase were isolated from red koji preparation. The purified glucoamylase was more active than amylase in acidic pH (Yoshizaki et al. 2010). Such characteristics make this enzyme suitable for industrial processes and biofuel production, which requires cold-active glucoamylases; such industrial enzymes can reduce the production cost by circumventing the heating requirement (Carrasco et al. 2017).

3.6 Structure of Fungal Glucoamylase

Molecular mass of fungal glucoamylases range between 48 and 90 kDa, with the exception of *Aspergillus niger*, which has a molecular mass of 125 kDa (Suresh et al. 1999). Most of the GAs are glycoproteins which contain 10–20% carbohydrate



Site	Position	Amino Acid Sequence
Substrate Binding site	144	Thr-Gly-Ser-Trp-Gly-Arg-Pro-Gln-Arg-Asp
Active Site-Proton Acceptor	200	Ala-Gln-Tyr-Trp-Asn-Gln-Thr-Gly-Tyr-Asp
Active Site-Proton Donor	203	Leu-Trp-Glu-Glu-Val-Asn-Gly-Ser-Ser-Phe

Fig. 3.1 *Aspergillus niger* glucoamylase 3D model (using SWISS-MODEL and Uniprot-KB) showing important amino acids at substrate binding site, active site with proton acceptor and donor

content by mass, and this may vary from species to species (Pazur et al. 1971; Ueda 1981). *Rhizopus niveus* produces two glucoamylases with carbohydrate contents of 14.9% and 12.7%, respectively (Ueda 1981). Glucoamylase from *Neurospora crassa* showed a lower (5.1%) carbohydrate content (Spinelli et al. 1996). *A. niger* glucoamylase 3D model was generated using Swiss model to demonstrate important amino acids in the binding site (Fig. 3.1).

Two very important forms of industrially important glucoamylase were produced from *A. niger* and *A. awamori* var. X100 (Lee and Paetzel 2011; Aleshin et al. 1992). Although many glucoamylases have been characterized till date, understanding these two forms of glucoamylase can build a framework to get the finer differences of existing glucoamylase enzymes.

A. niger glucoamylase contains catalytic domain of 458 amino acid residues. Ramachandran plot confirms the absence of residues from disallowed regions. There exist 374 residues, except glycine and prolines, which fall on the most favored regions, while other 30 residues can be confined to additional allowed regions. A hydrogen bond stabilizes the Asn337(313) residue, which remains present in a tight turn that falls on backbone atoms having flanking C-terminal Tyr335(311) and N-terminal Gly338(314). While Glu424(400) serves as the general base for the active site, there is a Ser435(411) residue having unusual conformation (Lee and Paetzel 2011).

The catalytic domain of glucoamylase enzyme is made up of α-helices arranged in secondary structure. A α/α barrel structure gets formed by 12 α-helices, which has 6 helices at the core and 6 other peripheral helices surrounding the core parallelly. The glucoamylase catalytic domain has a mostly α-helical secondary structure. The overall protein folding resembles with *A. awamori* glucoamylase (Aleshin et al. 1992). Three disulfide bonds are present. Two disulfide bonds form between Cysteine 234 and 237 and Cysteine 286 and 294. There is a third disulfide bond between Cysteine 246 and 473, which serves as an anchoring site for the α7 helix and the C-terminus of the protein having high glycosylation (Lee and Paetzel 2011). The catalytic domain of the *A. niger* glucoamylase contains an active site pocket corresponding to the subsites −1 and + 1 and contains a surface area of 198 Å accessible to solvents when ligand is bound, and in the absence of ligand, this surface area increases to 262 Å (Dundas et al. 2006).

Another extensively used glucoamylase coming from *A. awamori* was resolved to 2.2 Å by Aleshin and colleagues in 1992. The enzyme contains 13 α-helices to form core structure. The active site was found to be situated at inner α-helices. Amino acid residues from 430 to 440 blocks one side of the active site, making the opposite side open for substrate and solvent interactions. The active site is made up of certain loops, which connect the N-terminus of inner helices to the C-terminus of peripheral helices. This elucidated the fact that active sites in these enzymes remain present at the opposite ends of their respective barrel structure. Ten sites of O-linked glycosylation were present in a domain containing the last 30 amino acid residues of the enzyme. The glycosylation events include side chains having a serine or threonine residue linked to the α-anomer of a single mannose residue. Residues 440–470 attain an extended conformation, owing to O-glycosylation. The waist of the α/α-barrel structure gets curved by the polypeptide chain just like a belt. The glycosylation sites are of much significance as they provide additional structural support. This model of glucoamylase structure paved the path for elucidation of many more glucoamylases from other sources and helped in understanding their function (Aleshin et al. 1992). The 3D structure of GAs were determined from fungi, yeast, and bacteria (Aleshin et al. 1992, 1994, 1996, 2003; Harris et al. 1993; Stoffer et al. 1995; Sevcik et al. 1998, 2006).

3.6.1 Catalytic Domain

Substrate coordination at the site of catalysis is maintained by some conserved amino acid residues, viz., Arg78, Asp79, Leu201, Trp202, Glu204, and Arg329.

The proton gets donated to the glycosidic oxygen by Glu203, while Glu424 facilitates attack at C1 carbon via activating a nucleophilic water molecule. The oxocarbenium transition state serves as the rate-limiting step in such a reaction (Sauer et al. 2000). The catalytic domain becomes rigid upon low-density glycosylation around it. The linker with long amino acid residue rigidifies upon more glycosylation and hence distinguishes the catalytic and starch-binding domains physically. Another role of glycosylation was proposed to be the prevention of protein aggregation, and further, O-glycosylation protects the GAs from proteolytic cleavage, as low glycosylated GAs were found more prone to proteolytic hydrolysis (Coutinho and Reilly 1997; Le Gal-Coeffet et al. 1995).

3.6.2 Starch-Binding Domain (SBD)

SBD is important for raw starch degradation to maintain enzyme stability (Svensson et al. 1982; Southall et al. 1999; Goto et al. 1997). For dextrin hydrolysis, only catalytic domain of the enzyme is necessary (Libby et al. 1994). SBD is characterized by β-sheet structure (Jacks et al. 1995; Liu et al. 2007). The β-barrel is composed of eight anti-parallelly arranged β-strands (Jacks et al. 1995; Sorimachi et al. 1996, 1997; Juge et al. 2002). Hydrophobic and electrostatic interactions provide stability to SBD. It has been found that the first and seventh β-strands possess maximum stability, whereas strands 2 and 3 have the lowest stability. The reason for this low stability can be attributed to the fact that these strands act as initiation sites for the carbohydrate binding and, hence, help the SBD of the enzyme to open up, thus lowering the stability that helps in quicker unfolding action (Liu and Wang 2002). A semirigid "linker," composed of around 40 amino acid residues, has been found to exist between the SBD and catalytic domain of glucoamylase. This linker is responsible for maintaining a balance between the two domains, while some studies from *Rhizopus oryzae* suggested that the linker is involved in the expression of the enzyme (Williamson et al. 1992a, b; Lin et al. 2007). Studies from *Aspergillus niger* GA suggested the presence of a twisted sandwich domain containing more than one starch-binding sites in SBD domain (Sorimachi et al. 1997). Studies indicated that the catalytic domain and SBD lies apart by 90 Å in multiple orientations (Kramer et al. 1993; Sauer et al. 2000). The crystal structure of three industrially relevant glucoamylases from *Hormoconis resinae*, *Penicillium oxalicum*, and *Aspergillus niger* was revealed. The study highlighted that the carbohydrate-binding domain of the enzymes can exist in multiple relative orientations irrespective of the ligand bound in the active site. Such attribute enhances the substrate contact probability and increases the local substrate concentration, helping the catalytic site access multiple weak spots in starch, thereby bringing in efficient starch digestion (Roth et al. 2018). A schematic representation for catalytic domain and starch-binding domain for various species is shown in Fig. 3.2.

Being an exo-glycosyl hydrolase, GA breaks down α-1,4-glycosidic bonds sequentially beginning at the non-reducing ends of substrates, along with slower

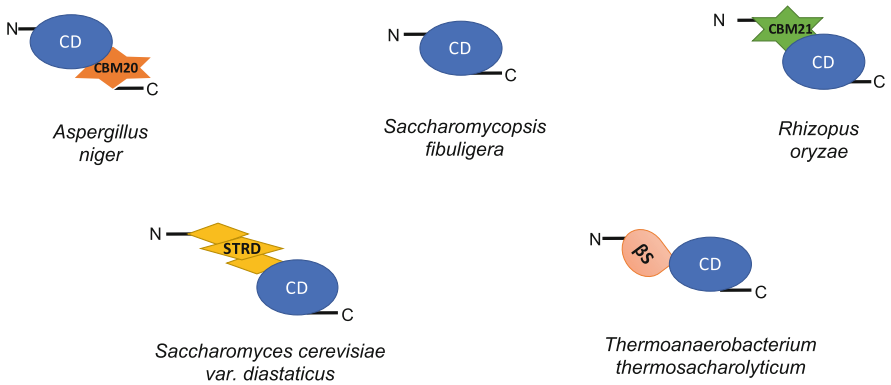


Fig. 3.2 Modular arrangements of glucoamylase from different species. *CD* GH15 catalytic domain, *CBM20* carbohydrate-binding domain family 20, *CBM21* carbohydrate-binding domain family 21, *STRD* serine/threonine-rich domain of Sta1 from *Saccharomyces cerevisiae* var. *diastaticus*, *βS* super-β sandwich domain found in prokaryotic enzymes

hydrolysis of α-1,6 (Pazur and Ando 1960). GAs also contain the ability to break all existing α-glycosidic bonds except that in α,α-trehalose (Pazur and Kleppe 1962; Meagher and Reilly 1989). In terms of Michaelis-Menten kinetics, the K_m of GAs gets lowered as the chain length of a substrate increases, while substrates having 4–5 glycosyl residues seem to increase the K_{cat} of the enzyme (Abdullah et al. 1963; Meagher et al. 1989; Reilly 2006). Glucoamylase catalytic mechanism involving the formation of oxocarbenium ion has been widely accepted (Chiba 1997). The reaction involves protonation of the glycosidic oxygen followed by water initiating a nucleophilic attack with the help of a base catalyst (Sinnott 1990; Tanaka et al. 1994; Sauer et al. 2000).

3.7 Applications of Glucoamylase

3.7.1 Production of Dextrose Syrup

Glucoamylase serves as a key enzyme in food and fermentation industries, which utilize the enzyme routinely for saccharification of starch and related oligosaccharides (Pavezzi et al. 2008). Coordinated action of various endo- and exo-amylases results in starch hydrolysis, and among them, glucoamylase is of great potential for commercial use, having the end product of reaction as glucose (Kumar et al. 2007). The glucose released from starch saccharification potentially can be used in various beverage industries and as food additives (Polakovič and Bryjak 2004). Glucoamylases gained immense attention and importance for use in the production of dextrose for commercial purpose. Commercial glucose yield via starch

saccharification requires a tremendous amount of glucoamylase and furnishes its commercial prominence. However, the first step in such a production pipeline is forming dextrin, which later is converted to glucose via saccharifying action of glucoamylase. The optimal conditions for such a reaction are acidic pH (4.0–4.5) and 60 °C temperature, under which approximately 96% glucose yields are attained after 3–4 days of saccharification (Crabb and Mitchinson 1997; Reilly 1999). Also, to hasten the production process and reduce by-products, isoamylase or pullulanase enzymes are often used to debranch the starch via breaking the α-1,6-glycosidic bonds (Reilly 2006).

3.7.2 Alcoholic Fermentation

Another potential utilization of glucoamylase lies in the ethanol production industry. Substances like wheat, corn, cassava, potato, etc. are utilized as starchy substrates for producing ethanol (Giordano et al. 2008). However, a limited number of yeasts like *Saccharomycopsis fibuligera*, *Schwanniomyces alluvius*, *and Candida tropicalis* are capable of fermentation (Nakamura 1970; Simoes-Mendes 1984; Jamai et al. 2007). Another setback comes from the fact that *S. cerevisiae* lacks the capacity to directly utilize starch, despite being the efficient producer in the fermentation industry. Thus, ethanol production is preceded by an initial breakdown of starch (by glucoamylases and other enzymes) to fermentable sugars, which is then further processed by yeast to yield ethanol (Panchal et al. 1984). However, scientists reconsider the ways to produce ethanol in a single step (Eksteen et al. 2003). Molecular cloning strategies direct the cloning of glucoamylase coding genes in *S. cerevisiae*, enabling it to directly utilize starch, thereby producing alcohol (Khaw et al. 2006). Yeast transformed with glucoamylase gene from *Hormoconis resinae* showed growth in 5% starch for ethanol conversion (Vainio et al. 1993, 1994). Yeast transformed with *S. diastaticus* glucoamylase together with mouse α-amylase showed to utilize 93% starch (Kim et al. 1988). Another recombinant yeast strain was developed by expressing α-amylase gene from *B. amyloliquefaciens*, glucoamylase gene from *S. diastaticus*, and pullulanase gene from *K. pneumoniae*, altering the combinations (Janse and Pretorius 1995). Another study used glucoamylase gene from *A. awamori* and isoamylase gene from *Pseudomonas amyloderamosa* to transform the yeast *S. cerevisiae* and express these genes under the alcohol dehydrogenase gene (adh1) promoter of the yeast, which was then capable of utilizing starch and producing alcohol with efficient rate of conversion of approximately 95% or more (Ma et al. 2000). Researchers cloned α-amylase of *B. subtilis* and glucoamylase of *A. awamori* to produce a recombinant yeast, viz., *S. cerevisiae YPB-G*, which was capable of generating large amounts of enzyme for ethanol fermentation (Altintas et al. 2002). Efforts had been made to produce recombinant α-amylase from *Lipomyces kononenkoae* and glucoamylase genes from *Lentinula edodes* into expression host to produce ethanol (Knox et al. 2004; Wong et al. 2007; Khaw

et al. 2006, 2007). Immobilized glucoamylases are used for the production of glucose to enhance alcohol yield for producing low-calorie beer (Synowiecki 2007).

3.7.3 Fabric Industry

In case of fabric industry, a recombinant yeast containing glucoamylase from *Rhizopus oryzae* along with two cellulose-binding domains of *Trichoderma reesei* has been utilized to manufacture fabric from starched cotton. A recombinant glucoamylase and cellulose-binding domain (CBD) were utilized for de-sizing cotton cloth. The yeast cells having both glucoamylase and CBD showed improved action as compared to yeasts displaying only glucoamylase. Also, the yeast which had glucoamylase with two CBDs (CBD1 and CBD2) showed highest activity (4.36-fold higher than the control) than with single CBD (CBD1 or CBD2) as compared to only glucoamylase containing yeasts. Such modular activity of glucoamylase, when added with CBDs, can be utilized efficiently in the textile industry for de-sizing starched cotton textiles (Fukuda et al. 2008).

3.7.4 Raw Starch Digestibility

Raw starch degrading capability makes glucoamylase a suitable choice in low-cost biorefining using starch. Thus, a recent study utilized a strong promoter element pPoxEgCel5B from endoglucanase gene and a signal peptide from raw starch-degrading PoxGA15A glucoamylase gene to produce a robust enzyme in *Penicillium oxalicum* in large quantity, along with high starch-degrading capability that makes it an industrially relevant process. Successful implementation of this process over a large-scale production pipeline can greatly reduce the cost of raw starch-digesting enzymes in case of starch-based biorefining processes (Wang et al. 2018). Glucoamylase enzymes were also used as digestive aids, and there are a number of formulations commercially available in the market as presented in Table 3.3.

3.8 Conclusions

Glucoamylase is one of the enzymes used on a large scale in starch industry, and it is one of the high-demand industrial enzymes. As a result of increasing focus on clean energy and fuels, there has been an upsurge in the demand for enzymes in bioethanol production. Despite stress on the overexpression and pilot-scale studies, there is a scope for enhancing thermal stability and hydrolyzing raw starches for higher yields while minimizing by-products in the starch saccharification process. In view of the extensive applications of glucoamylases, investigations on extremophilic microbes become absolutely important for attaining higher thermal stability with catalytic and hydrolytic activities to digest raw starch from various sources. Advanced molecular and bioinformatics tools may be used in selecting

Table 3.3 Commercially available glucoamylases for industrial use

Serial no.	Product	Company
1	Novozymes Extenda® for low cost and high yield of saccharification product	Novozymes, Denmark
2	Glucoamylase from *Trichoderma reesei* (CTA, 2013), used for a variety of industrial process	Genencor, USA
3	DELTAZYM® GA L-E5, for saccharification of liquefied starch in case of fuel ethanol production	Verenium, USA
4	Glucoamylase L209+ for brewery, antibiotic production, starch industry, and gourmet power industry	Shandong Longda, China
5	Neozyme® GA for saccharification process: alcohol industry, spirits distillation, beer brewing, organic acid, etc.	Guangdong VTR Bio-Tech Co., Ltd., China
6	Sunson® GA for use in a variety of industries including brewing, distillation, organic acid, antibiotics, etc.	*Sunson* Industry Group Co., Ltd., China

glucoamylases from metagenomes identified in extreme environments and selecting glucoamylases with the desirable characteristics in order to meet the current demands.

References

Abdullah M, Fleming ID et al (1963) Substrate specificity of the amyloglucosidases of *Aspergillus niger*. Biochem J 89:35–36

Adeoyo OR, Pletschke BI, Dames JF (2018) Purification and characterization of an amyloglucosidase from an ericoid mycorrhizal fungus (*Leohumicola incrustata*). AMB Exp 8:154

Alam M, Khalaf A et al (2014) Process optimization of glucoamylase production by *Aspergillus niger* using bitter cassava (*Manihot esculenta*). Adv Environ Biol 8:42–47

Aleshin A, Golubev A et al (1992) Crystal structure of glucoamylase from *Aspergillus awamori* var. X100 to 2.2-A resolution. J Biol Chem 267:19291–19298

Aleshin AE, Firsov LM, Honzatko RB (1994) Refined structure for the complex of acarbose with glucoamylase from *Aspergillus awamori* var. X100-2.4 å resolution. J Biol Chem 269:15631–15639

Aleshin AE, Stoffer B et al (1996) Crystallographic complexes of glucoamylase with maltooligosaccharide analogs: relationship of stereochemical distortions at the nonreducing end to the catalytic mechanism. Biochemist 35:8319–8328

Aleshin AE, Feng PH et al (2003) Crystal structure and evolution of prokaryotic glucoamylase. J Mol Biol 327:61–73

Alteriis E, Silvestro G et al (2004) *Kluyveromyces lactis* cells entrapped in Ca-alginate beads for the continuous production of a heterologous glucoamylase. J Biotechnol 109:83–92

Altintas MM, Ulgen K et al (2002) Improvement of ethanol production from starch by recombinant yeast through manipulation of environmental factors. Enzyme Microb Technol 31:640–647

An X, Ding C et al (2019) Overexpression of amyA and glaA substantially increases glucoamylase activity in *Aspergillus niger*. Acta Biochim Biophys Sin 51:638–644

Anto H, Trivedi UB, Patel KC (2006) Glucoamylase production by solid-state fermentation using rice flake manufacturing waste products as substrate. Bioresour Technol 97:1161–1166

Antranikian G (1992) Microbial degradation of starch. In: Winkel G (ed) Microbial degradation of natural products. VCH Publishers, Weinheim, pp 27–51. ISBN: 3-527-28354-4

Bafana R, Sivanesan S, Pandey RA (2019) Optimization and scale up of itaconic acid production from potato starch waste in stirred tank bioreactor. Biotechnol Prog 35:e2774

Bagheri A, Khodarahmi R, Mostafaie A (2014) Purification and biochemical characterisation of glucoamylase from a newly isolated *Aspergillus niger*: relation to starch processing. Food Chem 161:270–278

Banerjee S, Ghosh U (2017) Production and characterization of glucoamylase by *Aspergillus niger*. Appl Food Biotechnol 4(1):19–26

Barton LL, Georgi CE, Lineback DR (1972) Effect of maltose on glucoamylase formation by *Aspergillus niger*. J Bacteriol 111:771–777

Benassi VM, Pasin TM et al (2014) A novel glucoamylase activated by manganese and calcium produced in submerged fermentation by *Aspergillus phoenicis*. J Basic Microbiol 54:333–339

Bhatti HN, Rashid MH et al (2007) Optimization of media for enhanced glucoamylase production in solid-state fermentation by *Fusarium solani*. Food Technol Biotechnol 45:51–56

Boel E, Hansen MT et al (1984) Two different types of intervening sequences in the glucoamylase gene from *Aspergillus niger*. EMBO J 3:1581–1585

Carrasco M, Alcaíno J et al (2017) Purification and characterization of a novel cold adapted fungal glucoamylase. Microb Cell Factories 16:75

Chen J, Li D et al (2005) Purification and characterization of a thermostable glucoamylase from *Chaetomium thermophilum*. J Gen Appl Microbiol 51:175–181

Cherry H, Hossain M, Anwar M (2004) Extracellular glucoamylase from the isolate *Aspergillus fumigatus*. Pak J Biol Sci 7:1988–1992

Chiba S (1997) Molecular mechanism in alpha-glucosidase and glucoamylase. Biosci Biotechnol Biochem 61:1233–1239

Chiquetto ML, Facciotti MCR et al (1992) Influence of carbon and nitrogen sources on glucoamylase production by *Aspergillus* in batch process. Biotechnol Lett 14:465–470

Coutinho PM, Reilly PJ (1997) Glucoamylase structural, functional, and evolutionary relationships. Proteins Struct Funct Genet 29:334–347

Crabb WD, Mitchinson C (1997) Enzymes involved in the processing of starch to sugars. TIBTECH 15:349–352

Cripwell RA, Rose SH et al (2019) Improved raw starch amylase production by *Saccharomyces cerevisiae* using codon optimisation strategies. FEMS Yeast Res 19(2):1–14

da Silva TM, Maller A et al (2009) Properties of a purified thermostable glucoamylase from *Aspergillus niveus*. J Ind Microbiol Biotechnol 36:1439–1446

Das AJ, Miyaji T, Deka SC (2017) Amylolytic fungi in starter cakes for rice beer production. J Gen Appl Microbiol 63:236–245

Dundas J, Ouyang Z et al (2006) CASTp: computed atlas of surface topography of proteins with structural and topographical mapping of functionally annotated residues. Nucl Acids Res 34: W116–W118

Eksteen JM, Rensburg P et al (2003) Starch fermentation by recombinant *Saccharomyces cerevisiae* strains expressing the α-amylase and glucoamylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*. Biotechnol Bioeng 84:639–646

Ellaiah P, Adinarayana K et al (2002) Optimization of process parameters for glucoamylase production under solid state fermentation by a newly isolated *Aspergillus* species. Process Biochem 38:615–620

Facciotti MCR, Wuhstrack GH et al (1991) Effect of yeast-extract on glucoamylase synthesis by *Aspergillus awamori* NRRL 3112. Biotechnol Lett 13:547–552

Fagerström R, Kalkkinen N (1995) Characterization, subsite mapping and partial amino acid sequence of glucoamylase from the filamentous fungus *Trichoderma reesei*. Biotechnol Appl Biochem 21:223–231

Feng B, Hu W et al (2007) Purification, characterization, and substrate specificity of a glucoamylase with steroidal saponin-rhamnosidase activity from *Curvularia lunata*. Appl Microbiol Biotechnol 76:1329–1338

Ford C (1999) Improving operating performance of glucoamylase by mutagenesis. Curr Opin Biotechnol 10:352–357

Fowler T, Berka RM, Ward M (1990) Regulation of the glaA gene of *Aspergillus niger*. Curr Genet 18:537–545

Fukuda T, Kato-Murai M et al (2008) Improvement in enzymatic desizing of starched cotton cloth using yeast codisplaying glucoamylase and cellulose-binding domain. Appl Microbiol Biotechnol 77:1225–1232

Ganzlin M, Rinas U (2008) In-depth analysis of the *Aspergillus niger* glucoamylase (glaA) promoter performance using high-throughput screening and controlled bioreactor cultivation techniques. J Biotechnol 135:266–271

Ghosh A, Chatterjee B, Das A (1990) Induction and catabolite repression of high affinity glucoamylase in *Aspergillus terreus* strain 4. J Gen Microbiol 136:1307–1311

Gibbs PA, Seviour RJ, Schmid F (2000) Growth of filamentous fungi in submerged culture: problems and possible solutions. Crit Rev Biotechnol 20:17–48

Giordano RLC, Trovati J, Schmidell W (2008) Continuous production of ethanol from starch using glucoamylase and yeast co-immobilized in pectin gel. Appl Biochem Biotechnol 147:47–61

Goto M, Semimaru T et al (1994) Analysis of the raw starch-binding domain by mutation of a glucoamylase from *Aspergillus awamori* var. kawachi expressed in Saccharomyces cerevisiae. Appl Environ Microbiol 60:3926–3930

Goto M, Ekino K, Furukawa K (1997) Expression and functional analysis of a hyperglycosylated glucoamylase in a parental host, *Aspergillus awamori* var kawachi. Appl Environ Microbiol 63:2940–2943

Haasum I, Erickson SH et al (1991) Growth and glucoamylase production by a thermophilic fungus *Thermomyces lanuginosus* in a synthetic medium. Appl Micobiol Biotechnol 34:656–660

Harris EMS, Aleshin AE et al (1993) Refined structure for the complex of 1-deoxynojirimycin with glucoamylase from *Aspergillus awamori* var. X100–2.4 å resolution. Biochemist 32:1618–1626

Itoh T, Ohtsuki I et al (1987) Nucleotide sequence of the glucoamylase gene glu1 in the yeast *Saccharomycopsis fibuligera*. J Bacteriol 169:4171–4176

Izmirlioglu G, Demirci A (2016) Strain selection and medium optimization for glucoamylase production from industrial potato waste by *Aspergillus niger*. J Sci Food Agric 96:2788–2795

Jacks AJ, Sorimachi K et al (1995) 1H and 15N assignments and secondary structure of the starch-binding domain of glucoamylase from *Aspergillus niger*. Eur J Biochem 233:568–578

Jain D, Katyal P (2018) Optimization of gluco-amylase production from *Aspergillus* spp. for its use in saccharification of liquefied corn starch. 3 Biotech 8(2):101

Jamai L, Ettayebi K et al (2007) Production of ethanol from starch by free and immobilized *Candida tropicalis* in the presence of α-amylase. Bioresour Technol 98:2765–2770

Janse BJH, Pretorius IS (1995) One-step enzymatic hydrolysis of starch using a recombinant strain of *Saccharomyces cerevisiae* producing α-amylase, glucoamylase and pullulanase. Appl Microbiol Biotechnol 42:878–883

Jensen B, Olsen J (1999) Amylases and their industrial potential. In: Johri BN, Satyanarayana T, Olsen J (eds) Thermophilic moulds in biotechnology. Kluwer Academic Publishers, Dordrecht, pp 115–137

Juge N, Le Gal-Coeffet MF et al (2002) The starch binding domain of glucoamylase from *Aspergillus niger*: overview of its structure, function, and role in raw-starch hydrolysis. Biol Bratisl 11:239–245

Kareem SO, Akpan I et al (2011) Activated charcoal—a potential material in glucoamylase recovery. Enzyme Res 2011:483943

Karim KM, Husaini A et al (2016) Heterologous, expression, and characterization of thermostable glucoamylase derived from Aspergillus flavus NSH9 in *Pichia pastoris*. BioMed Res Int 164:5962028

Kato M, Kitajima T, Iefuji H (2011) Isolation and characterization of glucoamylase from a wastewater treatment yeast *Hansenula fabianii* J640, and construction of expression vector. Appl Microbiol Biotechnol 90:981–987

Kaur P, Satyanarayana T (2004) Production and starch saccharification by a thermostable and neutral glucoamylase of a thermophilic mould *Thermomucor indicae-seudaticae*. World J Microbiol Biotechnol 20:419–425

Kelly S, Grimm LH et al (2004) Agitation effects on submerged growth and product formation of *Aspergillus niger*. Bioprocess Biosyst Eng 26:315–323

Khaw TS, Katakura Y et al (2006) Evaluation of performance of different surface-engineered yeast strains for direct ethanol production from raw starch. Appl Microbiol Biotechnol 70:573–579

Khaw TS, Katakura Y et al (2007) Enhancement of ethanol production by promoting surface contact between starch granules and arming yeast in direct ethanol fermentation. J Biosci Bioeng 103:95–97

Kim K, Park CS, Mattoon JR (1988) High-efficiency, one-step starch utilization by transformed *Saccharomyces* cells which secrete both yeast glucoamylase and mouse alpha-amylase. Appl Environ Microbiol 54:966–971

Knox AM, Preez JC, Kilian SG (2004) Starch fermentation characteristics of *Saccharomyces cerevisiae* strains transformed with amylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*. Enzyme Microb Technol 34:453–460

Kojo T, Kadooka C et al (2018) Characterization of amylolytic enzyme overproducing mutant of *Aspergillus luchuensis* obtained by ion beam mutagenesis. J Gen Appl Microbiol 63:339–346

Kotaka A, Bando H et al (2008) Direct ethanol production from barley β-glucan by sake yeast displaying *Aspergillus oryzae* β-glucosidase and endoglucanase. J Biosci Bioeng 105:622–627

Kramer GFH, Gunning AP et al (1993) Scanning tunnelling microscopy of *Aspergillus niger* glucoamylases. J Chem Soc Faraday Trans 89:2595

Kumar S, Satyanarayana T (2003) Purification and kinetics of a raw starch-hydrolyzing, thermostable, and neutral glucoamylase of the thermophilic mold *Thermomucor indicae-seudaticae*. Biotechnol Prog 19:936–944

Kumar S, Satyanarayana T (2004a) Statistical optimization of a thermostable and neutral glucoamylase production by a thermophilic mould *Thermomucor indicae-seudaticae* in solid-state fermentation. World J Microbiol Biotechnol 20:895–902

Kumar S, Satyanarayana T (2004b) Production of thermostable and neutral glucoamylase by a thermophilic mould *Thermomucor indicae-seudaticae* in solid-state fermentation. Ind J Microbiol 44:53–57

Kumar P, Satyanarayana T (2007a) Optimization of culture variables for improving glucoamylase production by alginate-entrapped *Thermomucor indicae-seudaticae* using statistical methods. Bioresour Technol 98:1252–1259

Kumar P, Satyanarayana T (2007b) Economical glucoamylase production by alginate-immobilized *Thermomucor indicae-seudaticae* in cane molasses medium. Lett Appl Microbiol 45:392–397

Kumar P, Satyanarayana T (2009a) Microbial glucoamylases: characteristics and applications. Crit Rev Biotechnol 29:225–255

Kumar P, Satyanarayana T (2009b) Overproduction of glucoamylase by a deregulated mutant of a thermophilic mould *Thermomucor indicae-seudaticae*. Appl Biochem Biotechnol 158:113–125

Kumar S, Kumar P, Satyanarayana T (2007) Production of raw starch-saccharifying thermostable and neutral glucoamylase by the thermophilic mold *Thermomucor indicae-seudaticae* in submerged fermentation. Appl Biochem Biotechnol 142:221–230

Kumar P, Islam A et al (2010) Characterization of a neutral and thermostable glucoamylase from the thermophilic mold *Thermomucor indicae-seudaticae*: activity, stability, and structural correlation. Appl Biochem Biotechnol 160:879–890

Lam W, Pleissner D, Lin C (2013) Production of fungal glucoamylase for glucose production from food waste. Biomol Ther 3:651–661

Le Gal-Coeffet MF, Jacks AJ et al (1995) Expression in *Aspergillus niger* of the starch-binding domain of glucoamylase: comparison with the proteolytically produced starch-binding domain. Eur J Biochem 233:561–567

Lee J, Paetzel M (2011) Structure of the catalytic domain of glucoamylase from *Aspergillus niger*. Actacrystallogr Sect F Struct Biol Cryst Commun 67(2):188–192

Li YN, Shi GY et al (2010) A newly isolated *Rhizopus microsporus* var. *chinensis* capable to secrete amylolytic enzymes with raw-starch-digesting activity. J Microbiol Biotechnol 20:383–390

Li L, Lu H et al (2015) Construction and application of black-box model for glucoamylase production by *Aspergillus niger*. Chin J Biotechnol 31:1089–1098

Libby CB, Cornett CAG et al (1994) Effect of amino acid deletions in the O-glycosylated region of *Aspergillus awamori* glucoamylase. Protein Eng 7:1109–1114

Lin SC, Liu WT et al (2007) Role of the linker region in the expression of *Rhizopus oryzae* glucoamylase. BMC Biochem 8:1–12

Liu HL, Wang WC (2002) The predicted unfolding order of the beta-strands in the starch binding domain from *Aspergillus niger* glucoamylase. Chem Phys Lett 366:284–290

Liu SH, Chou WI et al (2005) Improved secretory production of glucoamylase in *Pichia pastoris* by combination of genetic manipulations. Biochem Biophys Res Commun 326:817–824

Liu YN, Lai YT et al (2007) Solution structure of family 21 carbohydrate-binding module from *Rhizopus oryzae* glucoamylase. Biochem J 403:21–30

Luo H, Liu H et al (2015) Efficient and cost-reduced glucoamylase fed-batch production with alternative carbon sources. J Microbiol Biotechnol 25:185–195

Ma YJ, Lin LL et al (2000) Efficient utilization of starch by a recombinant strain of *Saccharomyces cerevisiae* producing glucoamylase and isoamylase. Biotechnol Appl Biochem 31:55–59

Manjunath P, Rao MRR (1979) Comparative studies on glucoamylases from three fungal sources. J Biosci 1:409–424

Meagher MM, Reilly PJ (1989) Kinetics of the hydrolysis of di- and trisaccharides by *Aspergillus niger* glucoamylases I and II. Biotechnol Bioeng 34:689–693

Meagher MM, Nikolov ZL, Reilly PJ (1989) Subsite mapping of *Aspergillus niger* glucoamylases I and II with malto- and isomaltooligosaccharides. Biotechnol Bioeng 34:681–688

Merico A, Capitanio D et al (2004) How physiological and cultural conditions influence heterologous protein production in *Kluyveromyces lactis*. J Biotechnol 109:139–146

Mertens JA, Braker JD, Jordan DB (2010) Catalytic properties of two *Rhizopus oryzae* 99-880 glucoamylase enzymes without starch binding domains expressed in *Pichia pastoris*. Appl Biochem Biotechnol 162:2197–2213

Metwally M (1998) Glucoamylase production in continuous cultures of *Aspergillus niger* with special emphasis on growth parameters. World J Microbiol Biotechnol 14:113–118

Michelin M, Ruller R et al (2008) Purification and biochemical characterization of a thermostable extracellular glucoamylase produced by the thermotolerant fungus *Paecilomyces variotii*. J Ind Microbiol Biotechnol 35:17–25

Nahar S, Hossain F et al (2008) Production of glucoamylase by *Rhizopus* sp. in liquid culture. Pak J Bot 40:1693–1698

Nakamura LK (1970) Influence of the acceptor during transglycosylation by transglucosylamylase of *Candida tropicalis*. Can J Biochem 48:1260–1267

Nguyen QD, Rezessy-Szabo JM, Hoschke A (2000) Optimisation of composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* ATCC 34626. Food Technol Biotechnol 38:229–234

Nielsen BR, Lehmbeck J, Frandsen TP (2002) Cloning, heterologous expression, and enzymatic characterization of a thermostable glucoamylase from *Talaromyces emersonii*. Prot Exp Purif 26:1–8

Nigam P, Singh D (1995) Enzyme and microbial systems involved in starch processing. Enzyme Microb Technol 17:770–778

Nisha M, Satyanarayana T (2013) Recombinant bacterial amylopullulanases: developments and perspectives. Bioengineered 4:388–400

Nisha M, Satyanarayana T (2016) Characteristics, protein engineering and applications of microbial thermostable pullulanases and pullulan hydrolases. Appl Microbiol Biotechnol 100:5661–5679
Nunberg JH, Meade JH et al (1984) Molecular cloning and characterization of the glucoamylase gene of *Aspergillus awamori*. Mol Cell Biol 4:2306–2315
Nyamful A, Moses E et al (2014) Solid state fermentation by *Aspergillus niger* MENATE and *Rhizopus* MENACO11 for glucoamylase production on agriculture residue. Int J Sci Res Pub 4:1–4
Onuma H, Uchiyama H et al (2018) Enzymatic characterization of an extracellular glucoamylase from *Tricholoma matsutake* and its cloning and secretory expression in *Pichia pastoris*. Biosci Biotechnol Biochem 82:2180–2190
Panchal CJ, Russell I et al (1984) Genetic manipulation of brewing and related yeast strains. Food Technol 38:99–111
Pandey A (1995) Glucoamylase research: an overview. Starch-Starke 42:439–445
Pandey A, Soccol CR et al (2001) Solid-state fermentation in biotechnology. Asiatech Pub, Inc., New Delhi
Parashar D, Satyanarayana T (2017) Engineering a chimeric acid-stable α-amylase-glucoamylase (Amy-Glu) for one step starch saccharification. Int J Biol Macromol 99:274–281
Pasin TM, Benassi VM et al (2017) Purification and functional properties of a novel glucoamylase activated by manganese and lead produced by *Aspergillus japonicus*. Int J Biol Macromol 102:779–788
Pavezzi FC, Gomes E, da Silva R (2008) Production and characterization of glucoamylase from fungus *Aspergillus awamori* expressed in yeast *Saccharomyces cerevisiae* using different carbon sources. Braz J Microbiol 39:108
Pavezzi FC, Carneiro AA et al (2011) Influence of different substrates on the production of a mutant thermostable glucoamylase in submerged fermentation. Appl Biochem Biotechnol 163:14–24
Pazur JH, Ando T (1960) The hydrolysis of glucosyl oligosaccharides with α-d-(1,4) and α-d-(1,6) bonds by fungal amyloglucosidase. J Biol Chem 235:297–302
Pazur JH, Kleppe K (1962) The hydrolysis of α-D-glucosides by amyloglucosidase from *Aspergillus niger*. J Biol Chem 237:1002–1006
Pazur JH, Knull HR, Cepure A (1971) Glycoenzymes: structure and properties of the two forms of glucoamylase from *Aspergillus niger*. Carbohydr Res 20:83–96
Peixoto SC, Jorge J et al (2003) *Rhizopus microsporus* var. *rhizopodiformis*: a thermotolerant fungus with potential for production of thermostable amylases. Int Microbiol 6:269–273
Pervez S, Nawaz MA et al (2019) Improvement of catalytic properties of starch hydrolyzing fungal amyloglucosidase: utilization of agar-agar as an organic matrix for immobilization. Carbohydr Res 486:107860
Polakovič M, Bryjak J (2004) Modelling of potato starch saccharification by an *Aspergillus niger* glucoamylase. Biochem Eng J 18:57–63
Prajapati VS, Trivedi UB, Patel KC (2014) Kinetic and thermodynamic characterization of glucoamylase from *Colletotrichum* sp. KCP1. Ind J Microbiol 54:87–93
Rajoka MI, Yasmeen A (2005) Induction and production studies of a novel glucoamylase of *Aspergillus niger*. World J Microbiol Biotechnol 21:179–187
Reilly PJ (1999) Protein engineering of glucoamylase to improve industrial performance—a review. Starch-Starke 51:269–274
Reilly PJ (2006) Glucoamylase. In: Whitaker JR, Voragen AGJ, Wong DWS (eds) Handbook of enzymology. Marcel Dekker, Inc., New York, pp 727–738
Rey MW, Brown KM et al (2003) Cloning, heterologous expression, and characterization of *Thielavia terrestris* glucoamylase. Appl Biochem Biotechnol 111:153–166
Roth C, Moroz OV et al (2018) Structural insight into industrially relevant glucoamylases: flexible positions of starch-binding domains. Acta Crystallogr Sect D Struct Biol 74:463–470
Rubinder K, Chadha BS et al (2000) Amylase hyper-producing haploid recombinant strains of *Thermomyces lanuginosus* obtained by intraspecific protoplast fusion. Can J Microbiol 46:669–673

Rubinder K, Chadha BS et al (2002) Amylase hyperproduction by deregulated mutants of the thermophilic fungus *Thermomyces lanuginosus*. J Ind Microbiol Biotechnol 29:70–74
Sakwa L, Cripwell R et al (2018) Consolidated bioprocessing of raw starch with *Saccharomyces cerevisiae* strains expressing fungal alpha-amylase and glucoamylase combinations. FEMS Yeast Res 18(7):1–10
Satyanarayana T, Noorwez SM et al (2004) Development of an ideal starch saccharification process using amylolytic enzymes from thermophiles. Biochem Soc Trans 32:276–278
Sauer J, Sigurskjold BW et al (2000) Glucoamylase: structure/function relationships, and protein engineering. Biochim Biophys Acta 1543:275–293
Schmidt A, Shvetsov A et al (2019) Thermostability improvement of *Aspergillus awamori* glucoamylase via directed evolution of its gene located on episomal expression vector in *Pichia pastoris* cells. Prot Eng Des Sel 32:251–259
Selvakumar P, Ashakumary L et al (1996) Purification and characterization of glucoamylase produced by *Aspergillus niger* in solid state fermentation. Lett Appl Microbiol 23:403–406
Selvakumar P, Ashakumary L, Pandey A (1998) Biosynthesis of glucoamylase from *Aspergillus niger* by solid-state fermentation using tea waste as the basis of solid substrate. Bioresour Technol 65:83–85
Serour E, Antranikian G (2002) Novel thermoactive glucoamylases from the thermoacidophilic Archaea *Thermoplasma acidophilum, Picrophilus torridus* and *Picrophilus oshimae*. Antonie Van Leeuwen 81:73–83
Sevcik J, Solovicova A et al (1998) Structure of glucoamylase from *Saccharomycopsis fibuligera* at 1.7 å resolution. Acta Crystallogr D Biol Crystallogr 54(5):854–866
Sevcik J, Hostinova E et al (2006) Structure of the complex of a yeast glucoamylase with acarbose reveals the presence of a raw starch binding site on catalytic domain. FEBS J 273:2171–2171
Sharma S, Pandey M, Saharan B (2002) Fermentation of starch to ethanol by an amylolytic yeast *Saccharomyces diastaticus* SM-10. Ind J Exp Biol 40:325–328
Simoes-Mendes B (1984) Purification and characterisation of the extracellular amylases of the yeast *Schwanniomyces alluvius*. Can J Microbiol 30:1163–1170
Sinnott ML (1990) Catalytic mechanisms of enzymatic glycosyl transfer. Chem Rev 90:1171–1202
Sorimachi K, Jacks AJ et al (1996) Solution structure of the granular starch binding domain of glucoamylase from *Aspergillus niger* by nuclear magnetic resonance spectroscopy. J Mol Biol 259:970–987
Sorimachi K, Le Gal-Coeffet MF et al (1997) Solution structure of the granular starch binding domain of *Aspergillus niger* glucoamylase bound to beta-cyclodextrin. Structure 5:647–661
Southall SM, Simpson PJ et al (1999) The starch-binding domain from glucoamylase disrupts the structure of starch. FEBS Lett 447:58–60
Spinelli LBB, Lourdes TM et al (1996) Biochemical characterization of glucoamylase from the hyperproducer exo-1 mutant strain of *Neurospora crassa*. FEMS Microbiol Lett 138:173–177
Stoffer B, Aleshin AE et al (1995) Refined structure for the complex of D-gluco-dihydroacarbose with glucoamylase from *Aspergillus awamori* var. X100–2.2 å resolution: dual conformations for extended inhibitors bound to the active site of glucoamylase. FEBS Lett 358:57–61
Stone PJ, Makoff AJ et al (1993) Cloning and sequence analysis of the glucoamylase gene of *Neurospora crassa*. Curr Genet 24:205–211
Sun H, Peng M (2017) Improvement of glucoamylase production for raw-starch digestion in *Aspergillus niger* F-01 by maltose stearic acid ester. Biotechnol Lett 39:561–566
Suresh C, Dubey AK et al (1999) Characterisation of a starch-hydrolysing enzyme of *Aspergillus niger*. Appl Microbiol Biotechnol 51:673–675
Svensson B et al (1982) Characterization of two forms of glucoamylase from *Aspergillus niger*. Carlsberg Res Commun 47:55–69
Synowiecki J (2007) The use of starch processing enzymes in the food industry. In: Polaina J, Mac Cabe AP (eds) Industrial enzymes, structure, function and applications. Springer, Dordrecht, pp 19–34

Tachibana S, Yasuda M (2007) Purification and characterization of heterogeneous glucoamylases from *Monascus purpureus*. Biosci Biotechnol Biochem 71:2573–2576
Tanaka Y, Tao W et al (1994) Transition state structure for the hydrolysis of α-D-glucopyranosyl fluoride by retaining and inverting reaction of glycosylases. J Biol Chem 269:32306–32312
Tang W, Pan A et al (2015) Improvement of glucoamylase production using axial impellers with low power consumption and homogeneous mass transfer. Biochem Eng J 99:167–176
Tangngamsakul P, Karnchanatat A et al (2011) An extracellular glucoamylase produced by endophytic fungus EF6. Prik Biokhim Mikrobiol 47:455–461
Tatsumi H, Katano H, Ikeda T (2007) Kinetic analysis of glucoamylase-catalyzed hydrolysis of starch granules from various botanical sources. Biosci Biotechnol Biochem 71:946–950
Thorsen TS, Johnsen AH et al (2006) Identification and characterization of glucoamylase from the fungus *Thermomyces lanuginosus*. Biochim Biophys Acta 1764:671–676
Ueda S (1981) Fungal glucoamylases and raw starch digestion. Trends Biochem Sci 6:89–90
Vainio AEI, Torkkeli HT et al (1993) Cloning and expression of *Hormoconis resinae* glucoamylase P cDNA in *Saccharomyces cerevisiae*. Curr Genet 24:38–44
Vainio AEI, Lantto R et al (1994) Production of *Hormoconis resinae* glucoamylase P by a stable industrial strain of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 41:53–57
Varzakas TH, Roussos S, Arvanitoyannis IS (2008) Glucoamylases production of *Aspergillus niger* in solid state fermentation using a continuous counter-current reactor. Int J Food Sci Technol 43:1159–1168
Ventura L, Gonzalez-Candelas J et al (1995) Molecular cloning and transcriptional analysis of the *Aspergillus terreus* gla1 gene encoding a glucoamylase. Appl Environ Microbiol 61:399–402
Vihinen M, Mäntsälä P (1989) Microbial amylolytic enzymes. Crit Rev Biochem Mol Biol 24:329–418
Volkov PV, Rozhkova AM et al (2013) Comparative study of biochemical properties of glucoamylases from the filamentous fungi *Penicillium* and *Aspergillus*. Biochemist 78:1180–1189
Waghmare PR, Watharkar AD et al (2018) Bio-ethanol production from waste biomass of *Pogonatherum crinitum* phytoremediator: an eco-friendly strategy for renewable energy. 3 Biotech 8(3):158
Wang L, Zhao S et al (2018) Secretory overproduction of a raw starch-degrading glucoamylase in *Penicilliumo xalicum* using strong promoter and signal peptide. Appl Microbiol Biotechnol 102:9291–9301
Williamson G, Belshaw NJ et al (1992a) O-glycosylation and stability: unfolding of glucoamylase induced by heat and guanidine hydrochloride. Eur J Biochem 207:661–670
Williamson G, Belshaw NJ, Williamson MP (1992b) O-glycosylation in *Aspergillus* glucoamylase: conformation and role in binding. Biochem J 282:423–428
Wong DWS, Robertson GH et al (2007) Synergistic action of recombinant α-amylase and glucoamylase on the hydrolysis of starch granules. Prot J 26:159–164
Xian L, Feng JX (2018) Purification and biochemical characterization of a novel mesophilic glucoamylase from *Aspergillus tritici* WZ99. Int J Biol Macromol 107:1122–1130
Yoji H, Kozo T et al (1991) Nucleotide sequence and expression of the glucoamylase-encoding gene (glaA) from *Aspergillus oryzae*. Gene 108:145–150
Yoshizaki Y, Susuki T et al (2010) Characterization of glucoamylase and α-amylase from *Monascus anka*: enhanced production of α-amylase in red koji. J Biosci Bioeng 110:670–674
Zaldivar-Aguero JM, Badino AC Jr et al (1997) Influence of phosphate concentrations on glucoamylase production by *Aspergillus awamori* in submerged culture. Braz J Chem Eng 14:4
Zhao J, Chen YH, Kwan HS (2000) Molecular cloning, characterization, and differential expression of a glucoamylase gene from the basidiomycetous fungus *Lentinula edodes*. Appl Environ Microbiol 66:2531–2535

Developments in Fungal Phytase Research: Characteristics and Multifarious Applications

4

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Abstract

Phytases, a class of phosphatases, catalyse the stepwise removal of soluble phosphate from phytates. Plants contain phytates, which are a major organic form of phosphorus. Phytates act as an antinutrient and is not utilizable by monogastrics. Human and animal diets can therefore be supplemented with phytases to ameliorate their nutritional status. Phytases also have potential utility in aquaculture, plant growth promotion, soil amendment and environmental pollution control and as therapeutics. Among microbes, fungi are the predominant phytase producers with potential applicability in various areas due to their characteristic features of broad substrate spectrum and specificity and broad pH and temperature ranges for their activities. This chapter focuses on the various types, methods of production, characteristics and potential utilities of fungal phytases. Developments in fungal phytase research in India are specially highlighted. This chapter focuses on reviewing the significant work done on fungal phytases in India.

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Phytate · Antinutrient · Phytase · Eutrophication · Food and feed additive · HAP · *Myo*-inositol · Fungi · Yeast

4.1 Introduction

A major form of organic phosphorus in plant cells is phytic acid. Chemically it is a *myo*-inositol hexakisphosphate, a derivative of *myo*-inositol, a cyclic alcohol phosphorylated at all six hydroxyl groups (Fig. 4.1). Its molecular weight and formula are 660.03 and $C_6H_{18}O_{24}P_6$, respectively. Phytic acid reduces the absorption of cations like iron, zinc, calcium and magnesium in the gastrointestinal tract because of its chelating property (Iwai et al. 2012). It makes proteins and carbohydrates difficult to digest by forming complexes with them (Sapna and Singh 2014). All these factors make phytic acid an antinutritional factor.

The absence of phytases in the digestive tracts of monogastric animals, including humans, leaves phytates in foods and feeds unutilized (Cominelli et al. 2018). Consequently, the concentration of unutilized phytates increases in their faecal excreta, which leads to aquatic pollution known as eutrophication. Also, this necessitates the need to supplement phosphorus to the diets. Using chemical and physical methods, the phytic acid levels can be reduced, but this may adversely affect the nutritional content of foods (Singh et al. 2011).

Phytases are a special class of phosphatases, which release phosphate from phytic acid by hydrolysing it in a stepwise manner, by cleaving in general, five of the six phosphates (Wyss et al. 1999; Konietzny and Greiner 2002). Phytases are found in

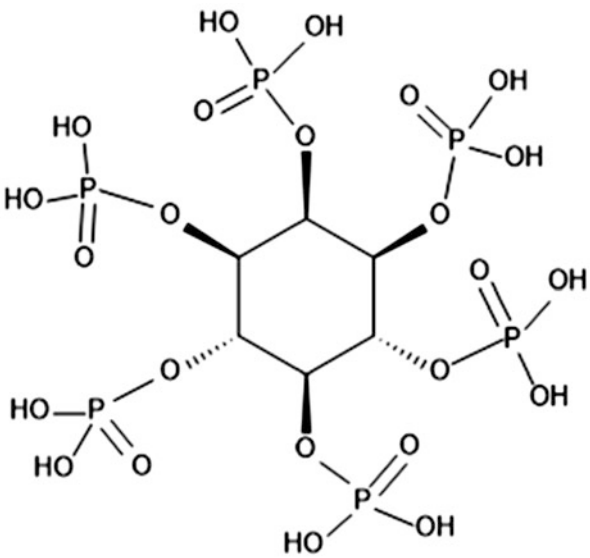

Fig. 4.1 Structure of phytic acid (IP_6, IUPAC)

animals, plants and microorganisms (Pandey et al. 2001; Vohra and Satyanarayana 2003; Yao et al. 2012) and have potential applications in the animal feed industry (Greiner and Konietzny 2012; Dersjant-Li et al. 2015; Jatuwong et al. 2020).

Phytases can be classified on the basis of a number of criteria, such as stereospecificity of phytate hydrolysis, optimal pH and their catalytic mechanisms. Phytases are classified as 3-phytases (EC 3.1.3.8), 4-phytases (EC 3.1.3.26), 5-phytases (EC 3.1.3.72) and 6-phytases (EC 3.1.3.26), depending on which carbon molecule present in phytic acid is attacked where dephosphorylation is initiated (Gontia-Mishra and Tiwari 2013). The 6-phytases, found in *E. coli* and plants, result in complete removal of phosphate from phytic acid. The 3-phytases form L-*myo*-inositol-2-phosphate (Gessler et al. 2018) and are found mainly in fungi and bacteria. Depending on the pH for their optimal activity, phytases can be grouped as acidic, alkaline and neutral. Acidic phytases, i.e. in the pH range of 2.0–6.0, are predominantly produced by fungi (Singh et al. 2011; Singh and Satyanarayana 2015). A cell-bound phytase optimally active at pH 4.0 is produced by the yeast *Pichia anomala* (Vohra and Satyanarayana 2003). Bacteria like *Bacillus subtilis* subsp. *subtilis* JJBS250 (Jain et al. 2018) and fungi like *Aspergillus flavus* and *Penicillium oxalicum* EUFR-3 have been reported to produce neutral phytases (Gaind and Singh 2015; Kaur et al. 2017). Alkaline phytases require Ca^{2+} for their activity. The first alkaline phytase recognized was that from *Bacillus licheniformis* (Azeem et al. 2015). Extracellular alkaline phytases are secreted by the soil bacteria *Bacillus amyloliquefaciens* (Idriss et al. 2002) and *B. laevolacticus* (Gulati et al. 2007c).

There are four classes of phytases on the basis of catalysis and structural information: histidine acid phosphatases (HAPs), β-propeller phytases (BPPs), purple acid phosphatases (PAPs) and cysteine phytases (CPs). HAPs are acidic phytases with optimal activity in the range of pH 4.5–6.0, which work efficiently in the gastrointestinal tracts of pigs and poultry. The structure of HAP consists of two folds, which are a larger α/β-domain and a smaller α-domain with motifs of RHGXRXP and HD present in the active site. HAPs are known to be produced by bacteria such as *Escherichia coli* (*Ec*Phy), *Klebsiella pneumoniae*, *Hafnia alvei* and *Yersinia kristensenii* (Ariza et al. 2013) and in fungi such as *Cladosporium* sp., *A. niger*, *A. awamori*, *Fusarium oxysporum*, *Debaryomyces castellii* (Ariza et al. 2013) and *Sporotrichum thermophile* (Singh et al. 2018). *Aspergillus fumigatus* produces a HAP, which retains >80% residual activity after being subjected to 100 °C for 20 min (Chen et al. 2015).

BPP is a group of alkaline phytases active at pH > 7.0. The structure of this enzyme is like a propeller having six blades, each having a calcium-binding site. These calcium-binding sites are responsible for the enhanced thermal stability. Alkaline phytases play an important role in soil and aquatic environments in phosphorus recycling (Huang et al. 2009). BPPs are present in bacteria such as *Bacillus licheniformis* (Tye et al. 2002), *B. amyloliquefaciens* and *B. laevolacticus* (Idriss et al. 2002; Gulati et al. 2007c) and in fungi such as *Arthrobotrys oligospora* (Hou et al. 2020).

Cysteine phytases or cysteine phosphatases (CP), also called protein tyrosine phosphatase-like phytases (PTPLPs), are optimally active at pH between 4.0 and 6.0

(Chen et al. 2015). PTPLPs have been reported from bacteria such as *Selenomonas ruminantium*, *Megasphaera elsdenii* (Puhl et al. 2008, 2009) and *Lactobacillus fermentum* NKN51 (Sharma et al. 2018).

PAPs have a pink or purple colour, which is due to the presence of binuclear Fe (III)-Me(II) centres, where Me is Fe, Mn or Zn (Schenk et al. 1999). Plants possess purple acid phosphatases with phytase activity (Yao et al. 2012; Singh and Satyanarayana 2015). This chapter reviews the developments in fungal phytase research in India and elsewhere.

4.2 Developments in Fungal Phytase Research in India

Since the first report of phytase in 1907 (Suzuki et al. 1907), these enzymes have been found to be widespread in nature in several plants, animals and most importantly innumerable microbes (Pandey et al. 2001; Vohra and Satyanarayana 2003; Kaur et al. 2007a). In India, one of the first reports on fungal phytase was on the screening of fungi from soils of Delhi and Ludhiana, whereby 12 isolates of the genera *Acrothecium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Phoma* and *Rhizoctonia* were found to be significantly effective in solubilizing calcium phytate, which was attributed to the expression of phytase in these fungi (Sethi and Subba-Rao 1968). Tarafdar and his co-workers undertook several studies on fungal phosphatases, and in one of them, they reported enhanced utilization of Na-phytate in plants upon inoculation with both *A. fumigatus* and *Glomus mosseae* (VAM) (Tarafdar and Marschner 1995). In a later study, they isolated efficient phytase- and phosphatase-producing fungi from soils of Rajasthan and tested their efficiency in hydrolysing different organic P compounds including phytin. The fungi were identified as the genera of *Aspergillus*, *Emericella* and *Penicillium*, and their potential to serve as seed inoculants to exploit soil organic P for nutrition was established, thus minimizing the need for P-based fertilizers (Yadav and Tarafdar 2003). Apart from these general reports, there are several researches that focused on one or more high phytase-producing fungal isolates (Table 4.1).

Researchers at the National Chemical Laboratory (NCL, Pune) isolated a thermotolerant extracellular phytase-producing fungus *Aspergillus niger* NCIM 563, and exhaustive research was carried out. It was cultivated in solid-state fermentation (SSF) on agricultural residues (Mandviwala and Khire 2000; Bhavsar et al. 2008), and the production was optimized by statistical approaches followed by scale-up (Bhavsar et al. 2011). It was also produced in submerged fermentation (SmF); and the physicochemical properties of the purified and partially purified enzymes indicated the possibility of two distinct forms of phytase, Phy I and Phy II, which were characterized (Soni and Khire 2007; Soni et al. 2010; Bhavsar et al. 2012). The fungal strain was improved by mutagenesis, and its production was scaled up (Shah et al. 2009; Bhavsar et al. 2013a). *Aspergillus niger* NCIM 563 was reported to produce dissimilar phytase isozymes in SSF and SmF, and their biochemical characteristics were compared, with SSF phytase proving to be less thermostable (Bhavsar et al. 2013b). The applicability of the phytase was reported in plant growth

Table 4.1 An exhaustive list of research work done on fungal phytases in India

Scientist and affiliation	Researchers	Fungi	Intracellular/ extracellular	References
Dr. Jayant MalharKhire, NCIM Resource Center, National Chemical Laboratory, Pune	T.N. Mandviwal, S.K. Soni, K. Bhavsar, P. Shah, P. Buddhiwan, V.R. Kumar, P. Gujar	*Aspergillus niger* NCIM 563	Extracellular	Mandviwala and Khire (2000), Soni and Khire (2007), Soni et al. (2010), Bhavsar et al. (2008, 2011, 2012, 2013a, b), Shah et al. (2009, 2017), Gujar et al. (2013), Buddhiwant et al. (2016)
Dr. U. C. Banerjee, Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, Mohali, Punjab, and Institute of Microbial Technology (IMTech), Sector 39A, Chandigarh	P. Vats	*Aspergillus niger* van Tieghem	Extracellular	Vats and Banerjee (2002, 2005, 2006), Vats et al. (2004, 2009)
Prof. H. Saini and Prof. B.S. Chadha, Department of Microbiology, Guru Nanak Dev University, Amritsar	H. Gulati, M. Minhas	*Rhizomucor pusillus*	Extracellular	Chadha et al. (2004)
		Mucor indicus MTCC 6333	Extracellular	Gulati et al. (2007a)
		Thermomyces lanuginosus	Extracellular	Gulati et al. (2007b)
Prof. Ashok Pandey, Biotechnology Division, Regional Research Laboratory, Council of Scientific and Industrial Research (CSIR), Trivandrum	P. Gautam, A. Sabu, S. Sarita, S. Ramachandran, K. Roopesh, K.M. Nampoothiri, G.J. Tomes	*Aspergillus ficuum* and *Rhizopus oligosporus*	Extracellular	Gautam et al. (2002)
		Rhizopus oligosporus	Extracellular	Sabu et al. (2002)
		Rhizopus sp.	Extracellular	Ramachandran et al. (2005)
		Thermoascus aurantiacus	Extracellular	Nampoothiri et al. (2004)
		Mucor racemosus	Extracellular	Roopesh et al. (2006)

(continued)

Table 4.1 (continued)

Scientist and affiliation	Researchers	Fungi	Intracellular/ extracellular	References
Prof. T. Satyanarayana, Department of Microbiology, University of Delhi, South Campus, New Delhi	A. Vohra, P. Kaur, S. Joshi, D. Verma	*Pichia anomala*	Intracellular	Vohra and Satyanarayana (2001, 2002a, b, 2004), Vohra et al. (2006, 2011), Kaur and Satyanarayana (2005, 2010), Kaur et al. (2010, 2011), Verma and Satyanarayana (2012), Joshi and Satyanarayana (2014, 2015a, b)
	B. Singh, B. Ranjan, A.K. Maurya, D. Parashar	*Sporotrichum thermophile*	Extracellular	Singh and Satyanarayana (2006a, b, 2008a, b, c, 2009, 2010, 2012), Ranjan et al. (2015), Ranjan and Satyanarayana (2016), Maurya et al. (2017)
Dr. Sanjoy Ghosh, Bioprocess Engineering Laboratory, Department of Biotechnology, Indian Institute of Technology Roorkee, Uttarakhand	R. Rani, S. Arora	*Rhizopus oryzae*	Extracellular	Rani and Ghosh (2011), Rani et al. (2014a, b), Arora et al. (2017)
Dr. Bijender Singh, Laboratory of Bioprocess Technology, Department of Microbiology, Maharshi Dayanand University, Rohtak, Haryana and Department of Biotechnology, Central University of Haryana, Mahendergarh, Haryana.	Sapna, A. Bala, A. Kumari, N. Singh	*Aspergillus oryzae*	Extracellular	Sapna and Singh (2013, 2014, 2015, 2017a, b)
		Humicolanigrescens	Extracellular	Bala et al. (2014)
		Aspergillus niger	Extracellular	Singh et al. (2015)
		Sporotrichum thermophile	Extracellular	Kumari et al. (2016)
Dr. S. Das, Food Technology & Biohemical Engineering	U. Ghosh	*Aspergillus niger* NCIM 612	Extracellular	Das and Ghosh (2014)

Department, Jadavpur University, Kolkata, West Bengal				
Dr. Jyotirmoy Ghosh and Dr. A.V. Elangovan, ICAR-National Institute of Animal Nutrition and Physiology, Bangalore	S. Ajith, D. Shet	*Aspergillus foetidus* MTCC 11682	Extracellular	Ajith et al. (2018, 2019)
Dr. R. Joseph and Dr. D. Somashekar, Department of Food Microbiology, Central Food Technological Research Institute, Mysore	S. Bindu	*Rhodotorula gracilis*	Intracellular	Bindu et al. (1998)

promotion and soil amendment (Gujar et al. 2013), as an additive to animal feedstocks (Buddhiwant et al. 2016), and in the degradation of organophosphorus pesticide (Shah et al. 2017). This group has also screened and isolated phytase-producing yeast with the ability to degrade phytic acid, thereby aiding in mobilization of minerals and degrading phytic acid in chickpea flour (CPF), suggesting its potential usage in feed and food industry (Pable et al. 2014).

At Chandigarh, an extracellular phytase overproducing strain of *Aspergillus niger* van Tieghem was isolated from rotten wooden logs, and its production in shake flasks (Vats and Banerjee 2002) and laboratory scale fermenter (Vats et al. 2004) were studied, as well as its detailed biochemical and catalytic characteristics (Vats and Banerjee 2005, 2006). Its applicability in livestock feed for dephosphorylation was also reported (Vats et al. 2009).

At Guru Nanak Dev University (GNDU, Amritsar), several phytase-producing thermophilic/thermotolerant fungi were isolated from composting soil, and the cultural conditions were optimized on solid media; phytase was purified and characterized. The partially purified phytase isolated from *Rhizomucor pusillus* exhibited broad substrate specificity, with a temperature and pH optima of 70 °C and 5.4, respectively (Chadha et al. 2004). The phytase from *Mucor indicus* MTCC 6333 had optimum pH and temperature of 6.0 and 70 °C, respectively. This phytase was thermostable under acidic conditions and displayed broad substrate specificity. In vitro feed trials were also performed (Gulati et al. 2007a). In *Thermomyces lanuginosus*, UV was used for developing phytase hyperproducing mutants. The purified phytase was thermo-acidstable, being optimally active at pH 5.0 and 70 °C, with broad substrate specificity (Gulati et al. 2007b).

Among several studies on phytase at Biotechnology Division, RRL-CSIR, Trivandrum, strains of *Aspergillus ficuum* and *Rhizopus oligosporus* were shown to produce extracellular phytase using polystyrene as inert support in SSF, and their cultural conditions were optimized (Gautam et al. 2002). The fungus *R. oligosporus* was reported to produce phytase in SSF using coconut oil cake (Sabu et al. 2002) and mixed oil cakes as substrates (Ramachandran et al. 2005). Phytase production by *Mucor racemosus* was conducted on both wheat bran and oilcakes in SSF, with sesame oil cake being the better substrate (Roopesh et al. 2006). The cultural conditions for a thermophilic fungus, *Thermoascus aurantiacus*, were optimized for the production of a thermostable phytase in submerged fermentation (Nampoothiri et al. 2004).

Our group at the University of Delhi (South Campus) has done some of the most extensive studies on fungal phytases in India. The unconventional yeast, *Pichia anomala*, isolated from dried flower buds of *Woodfordia fruticosa* was a source of cell-bound phytase (Vohra and Satyanarayana 2001). Extensive studies were performed on this phytase, owing to its desirable characteristics of acid stability, thermostability, resistance to digestive proteases, non-requirement of metal ions and broad substrate specificity (Vohra and Satyanarayana 2002b). Cultural optimizations were done using conventional and statistical approaches in complex as well as low-cost media (Vohra and Satyanarayana 2002a, 2004; Kaur and Satyanarayana 2005). Fed-batch fermentation in airlift fermenter enhanced phytase production

(Vohra et al. 2011); spent medium could also be recycled (Kaur et al. 2011), and cyclic fed-batch was used in economic cane molasses medium (Verma and Satyanarayana 2012). To further ameliorate the phytase production, the whole cells were permeabilized (Kaur and Satyanarayana 2010). *P. anomala* phytase gene was heterologously expressed in *Hansenula polymorpha* to characterize the recombinant phytase (Kaur et al. 2010) and in the methylotrophic yeast *P. pastoris* which led to high secretion (Joshi and Satyanarayana 2014). Phytase 3D structure was proposed by homologous modelling (Vohra et al. 2011). Native as well as recombinant phytases could be supplemented in animal feeds (Vohra et al. 2006), aquaculture (Hassan et al. 2009) and foods (Kaur and Satyanarayana 2010; Joshi and Satyanarayana 2014, 2015a) apart from other novel applications (Joshi and Satyanarayana 2015b).

In the same laboratory, a thermophilic mould *Sporotrichum thermophile* BJTLR50 was selected from among numerous thermophilic fungal isolates from various environmental samples by screening on phytase screening medium (PSM) agar plates, followed by cultivation in PSM broth (Singh and Satyanarayana 2012). Statistical optimization resulted in enhanced phytase secretion by this mould in SmF as well as in SSF, which was amenable to scale up (Singh and Satyanarayana 2006a, b, 2008a, b, c). The enzyme was purified and characterized (Singh and Satyanarayana 2009) and found to have potential application in decreasing phytic acid in food ingredients, bread-making and plant growth promotion (Singh and Satyanarayana 2010). An enhanced phytase production was achieved by cloning and expression of this phytase in heterologous hosts like *E. coli* (Ranjan et al. 2015) and *Pichia pastoris* (Ranjan and Satyanarayana 2016); this also allowed better understanding of its structural and biochemical characteristics (Maurya et al. 2017).

At IIT-Roorkee, linseed oil cake was used as a substrate to produce phytase in SSF by *Rhizopus oryzae*; media optimization was done using statistical approach. Strain improvement was achieved by a novel approach, and the enzyme was then purified and characterized (Rani and Ghosh 2011). An aqueous extraction process was optimized using response surface methodology combined with artificial neural network to enhance phytase production by *R. oryzae* (Rani et al. 2014a); statistical approaches were used for medium optimization (Rani et al. 2014b), and enzyme production was also carried out in a novel SSF bioreactor (Arora et al. 2017).

In the Microbiology Laboratory of Maharshi Dayanand University (MDU, Rohtak), soil samples were collected from different regions of Haryana, and nearly 300 fungal isolates were screened for extracellular phytase production on phytase screening medium; ten potent phytase-producing fungi were selected belonging to the genera *Aspergillus*, *Penicillium* and *Trichoderma*. Among them, *Aspergillus* sp. 50 was found to be the best phytase producer in liquid medium with protease resistance (Sapna and Singh 2013). The production was also optimized in SSF, and its applicability in dephytinization of wheat bran was demonstrated (Sapna and Singh 2014, 2015). Further, the production of this protease-resistant and thermostable phytase was enhanced in SmF using Taguchi design, and the fungus was immobilized in 4% agar, resulting in sustained phytase production (Sapna and Singh 2017a). The phytase was purified, characterized and found useful as a feed

supplement (Sapna and Singh 2017b). The work was also done on *Humicola nigrescens*, which produced an extracellular phytase in SSF, and *Aspergillus niger* in SSF and SmF, partial purification, characterization and application in dephytinization of cereal flours (Bala et al. 2014; Singh et al. 2015). Phytase production by thermophilic mould *Sporotrichum thermophile* in SSF in mixed substrate fermentation resulted in enhanced yields and was useful as an additive to poultry feed (Kumari et al. 2016).

Some standalone studies on fungal phytases have also been reported from different parts of the country. For example, the effect of nutritional supplementation on the production of extracellular phytase by *Aspergillus niger* NCIM 612 was studied in SSF using rice straw as the substrate (Das and Ghosh 2014). A novel phytase-producing strain of *Aspergillus foetidus* MTTC 11682 was immobilized on polyurethane cubes, after the optimization of cultural conditions, to ameliorate the yield, economics and continuous phytase production (Ajith et al. 2018); the enzyme was also partially purified and characterized (Ajith et al. 2019).

Rhodotorula gracilis, a red yeast, was shown to produce phytase, and the cultural conditions were standardized for the enzyme production, followed by the assessment of the efficacy of various permeabilizing agents for in situ determination of its phytase activity. The yeast suspension was subjected to 15 cycles of freezing and thawing to enhance the phytase activity (Bindu et al. 1998). A phytase-producing yeast *Candida parapsilosis* was isolated, its production optimized, and the enzyme characterized (Ranjan and Sahay 2013). The production of extracellular phytase from a nonpathogenic *Candida tropicalis* (NCIM 3321) was optimized and evaluated for plant growth-promoting potential (Puppala et al. 2018).

4.3 Brief Description of Methods of Phytase Production

4.3.1 Phytase Production by Microbes

Phytases are present in a large number of microorganisms (bacteria and fungi) as well as plants. It is preferred to use microbial phytases over others due to several reasons. Higher growth rates, simple media requirements, higher yields, consistency, ease of product modification and optimization, stability and greater catalytic activity are the attributes of microbial phytases (Gurung et al. 2013).

Yeasts are a source of both extracellular and cell-bound phytases. Five potential phytase producers (*Schizosaccharomyces octosporus* NCIM 3297, *Zygosaccharomyces bisporus* NCIM 3265 and 3296, *Williopsis saturnus* NCIM 3298 and *Z. priorionus* NCIM 3299) were selected after screening 600 yeast strains (Pable et al. 2014). Yeasts such as *Saccharomyces cerevisiae* (Klosowski et al. 2018), *Schwanniomyces occidentalis* and *Candida parapsilosis* (Ranjan and Sahay 2013), *Arxula adeninivorans* (Olstorpe et al. 2009) and *Pichia anomala* (Vohra and Satyanarayana 2001) are also known to produce phytases.

Fungi have been found to be the most active producers of extracellular phytases. Phytase from *Aspergillus ficuum* NRRL 3135 has been most commonly used

commercially (Chelius and Wodzinski 1994). Using different fermentation methods, phytases have also been produced from *A. carbonarius*, *A. fumigatus*, *A. niger*, *A. oryzae*, *Cladosporium* spp., *Mucor piriformis*, *Rhizopus oligosporus* and thermophilic fungus *Sporotrichum thermophile* (Howson and Davis 1983; Quan et al. 2004; Singh and Satyanarayana 2008a). Phytase activity has been reported in some edible mushrooms, such as *Agaricus bisporus*, *Agrocybe pediades*, *Ceriporia* sp., *Ganoderma stipitatum*, *Grifola frondosa*, *Lentinula edodes*, *Peniophora lycii*, etc. (Lassen et al. 2001; Collopy and Royse 2004); in mycelial fungi like *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); and in nematophagous fungus *Arthrobotrys oligospora* (Hou et al. 2020). For application in food and feed industry, phytases must be resistant at high temperatures, acidic pH and the action of the proteases present in the intestinal tract. The fungus *A. niger* having a GRAS status is known to be a producer of highly active phytase extracellularly.

Different methods of phytase production by fungi can be used such as the solid-state fermentation (SSF), semisolid fermentation and submerged fermentation (SmF) (Han et al. 1987; Shivanna and Venkateswaran 2014). Extracellular phytases are mostly produced in SmF. In this method, there is a better process control of parameters like mass transfer, heat transfer and oxygen supply. As compared to SSF, there are low labour costs and low scale-up requirements in SmF (Singhania et al. 2010). However, low product yields, enhanced costs and larger volumes of wastewater effluents are some of the disadvantages of SmF technique (Abd-Elhalem et al. 2015). A stirred tank and airlift bioreactors can be used to produce phytase (Maller et al. 2014). A phytase selective medium containing sodium phytate as a substrate has been optimized to investigate production by *Aspergillus ficuum* NRRL 3135 (Coban and Demirci 2014). A mixture of rice straw powder and soybean curd residue has been used as the substrates to produce phytase in SmF by *Aspergillus niger*, *Neurospora sitophila* and *Rhizopus oryzae* (Kanti and Sudiana 2018). Phytase from the yeast *Pichia anomala* has been produced in submerged fermentation using YPD medium (Vohra and Satyanarayana 2002a).

In SSF, a solid substrate, having very little free-flowing water, is utilized slowly and efficiently over a long period of fermentation (Chow and Ting 2015). SSF is the most commonly used process for phytase production from filamentous fungi such as *A. niger*, *A. flavus*, *A. ficuum*, *A. tubingensis*, *M. racemosus*, *R. oligosporus*, *R. oryzae*, *Thermomyces lanuginosus, Ganoderma stipitatum*, *Grifola frondosa*, *Penicillium purpurogenum*, *S. commune* and *Trametes versicolor*, using agricultural residues and other inexpensive natural substrates like peels of citrus fruits, wheat straw, soybean meal, wheat bran and rice bran for producing phytase (Awad et al. 2014; Huang et al. 2018). For the evaluation of phytase production by *A. niger*, triticale waste was used as a substrate (Neira-Vielma et al. 2018). *Rhizopus oligosporus* produced phytase on coconut oil cake in SSF (Sabu et al. 2002).

4.3.2 Production of Phytases by Recombinant Microbes

The industrial and biotechnological importance of phytase has increased considerably in the recent years. Wild-type strains give very low yield of phytase, but using processes such as mutagenesis, protoplast fusion and recombinant DNA technology, improvement in phytase levels has been attained (Bhavsar et al. 2013a; Murlidhar and Panda 2000).

The first phytases to be cloned were from *Talaromyces thermophilus* and *Emericella nidulans* (Pasamontes et al. 1997). *Fusarium venenatum* was used as a host to express phytase gene cloned from *T. lanuginosus* (Berka et al. 1998). The activity of the enzyme was highest at 65 °C as optimum temperature and could retain activity even at 75 °C. A 2060-bp-long sequence from rice (*Oryza sativa* L.) could be cloned, and it produced a phytase of 519 amino acids (Li et al. 2012). Eukaryotic organisms, particularly yeasts, have been used as hosts to study the effects of glycosylation on phytase activity and thermostability. *S. cerevisiae* and *Pichia pastoris* have expressed phytase genes (*phyA*) from *A. niger* and *Bacillus subtilis* (Han and Lei 1999; Guerrero-Olazarán et al. 2010). The expression of *Bacillus subtilis* phytase genes in *P. pastoris* cells resulted in increased glycosylation and thermal stability of the recombinant enzyme, whereas neither the pH nor the thermal optima of the enzyme changed (Guerrero-Olazarán et al. 2010).

Plants have been considered as alternate biofactories for phytase production due to their ability to be a source of low-priced protein sources. Several plants such as *Arabidopsis*, rice, maize sesame and rapeseed have been used as hosts to express phytase genes (Wang et al. 2013, 2017; Belgaroui et al. 2016; Chen et al. 2008; Jin et al. 2005). Since phytases from *A. niger* are stable over a broad temperature and pH ranges, Phy genes from these fungi are widely used in transformation studies (Pen et al. 1993; Gontia et al. 2012).

4.4 Purification and Characteristics of Phytase

Various methods have been used to purify phytases, including pretreatment, precipitation or chromatographic methods and salt precipitation (Bhavsar and Khire 2014). In order to release intracellular enzyme from the cell, it is subjected to treatments that permeabilize the cell, such as the use of detergents, enzymes and organic solvents (Bindu et al. 1998). For the separation of extracellular phytase, centrifugation and decantation techniques can be used. Concentration of culture filtrate can be done by using precipitating agents like salt, acetone and ultrafiltration (Bhavsar and Khire 2014). This can be followed by affinity chromatography, ion-exchange chromatography, gel filtration and hydrophobic interaction. Phytase has been purified by methods such as ultrafiltration, diafiltration, ion exchange, gel filtration and hydrophobic interaction (Konietzny et al. 1995). A 51-fold purification of extracellular phytase from *A. niger* 11T53A9 was attained by ammonium sulphate precipitation, ion chromatography and gel filtration (Greiner et al. 2009). The recombinant phytase of *Sporotrichum thermophile* was purified by anion-exchange chromatography in a

fast protein liquid chromatography (FPLC) system (AKTA prime PLUS, GE Healthcare, Bio-Sciences, Uppsala, Sweden) using Q-Sepharose (GE Healthcare) (Ranjan and Satyanarayana 2016). A two-step process of acetone precipitation followed by anion-exchange chromatography using DEAE-Sephadex was used to purify the native phytase of *Pichia anomala* (Vohra and Satyanarayana 2002b).

Phytases with a high temperature optimum are desirable so that they can withstand very high temperatures of 70–90 °C during the feed pelleting process (Doyle and Erickson 2006; Dersjant-Li et al. 2015). Stability in the presence of proteolytic enzymes and activity at a wide pH range are some of the other parameters important to withstand conditions in the gastrointestinal tract (Walton and Gray 1979; Schröder et al. 1998). Majority of phytases produced by fungi are stable at high temperatures, optimally active in the temperature range of 37–67 °C in a wide pH range (Simon and Igbasan 2002). Phytase from *Sporotrichum thermophile* was optimally active at pH 6.0 and 45 °C (Singh and Satyanarayana 2006a). *Myceliophthora thermophila*, when grown on sugarcane bagasse at 45 °C and 70% moisture level, exhibited high yield of phytase in 36 h (Hassouni et al. 2006). Phytase from *Thermomyces lanuginosus* has the optimum temperature of 65 °C and is eight times more thermostable than *A. niger* phytase when incubated at 65 °C for 20 min (Berka et al. 1998).

Most of the fungal phytases are active in the acidic pH of 2.0–6.0 and belong to the class of histidine acid phosphatases (HAPs) (Vohra and Satyanarayana 2003; Singh et al. 2011), but phytases of *Agaricus bisporus* and *Rhizopus microsporus* var. *microsporus* have optimum activity at pH 5.0–8.0 and 9.5, respectively (de Oliveira Ornela and Guimaraes 2019). *P. anomala* phytase worked best at 60 °C and pH 4.0. Phytase from this yeast could be used as an animal feed supplement since it had the requisite thermostability and acid stability, with a half-life of 7 and 8 days at 60 °C and pH 4.0, respectively (Vohra and Satyanarayana 2002b). *Penicillium oxalicum* EUFR-3 isolated from Himalayan soil produced phytase that retains high activity and remains stable at a wide range of pH values (pH 3–8), with maximum activity at 40 °C and pH 7.0 (Kaur et al. 2017), making it a promising candidate for usage in the food industry (Gessler et al. 2018). *A. niger* van Tieghem phytase showed a high degree of specific activity at a temperature of 52–55 °C along with a pH of 2.5 (Vats and Banerjee 2005). The various characteristics of several known fungal phytases are listed in Table 4.2.

4.5 Multifarious Applications of Fungal Phytases

Highlights of the diverse applications of the fungal phytases are presented in Fig. 4.2.

4.5.1 Ameliorating the Nutritional Status of Foods and Feeds

Utility in animal nutrition is the best-known biotechnological application of phytases especially in the nutrition of monogastrics (single-stomached animals including

Table 4.2 Characteristics of fungal phytases

Fungal taxa	Specific activity	Mol. mass (kDa)	pH_{opt}	T_{opt} (°C)	K_m (μM)	References
Acremonium zeae	–	–	7.0	50	–	Pires et al. (2019)
Agaricus bisporus	14.7 U/mg	14	5.0–8.0	>60	–	Collopy and Royse (2004)
Agrocybe pediades	400 U/mg	59	5.0–6.0	50	–	Lassen et al. (2001)
Arthrobotrys oligospora	74.71 U/mg	84.2	7.5	50	1015	Hou et al. (2020)
Aspergillus foetidus	12.6 FTU/mg	129.6	5.5	37	–	Ajith et al. (2019)
Aspergillus ficuum AS3.324	–	68.5	2.0, 5.5	50	750	Zhang et al. (2003)
Aspergillus ficuum NTG-23	150.1 U/mg	65.5	1.3	67	295	Zhang et al. (2010)
Aspergillus ficuum	178.76 U/mg	67.5–81.6	5	58	0.124	Ullah et al. (2003)
Aspergillus flavus ITCC 6720	46.53 U/mg	30	7	45	–	Gaind and Singh et al. (2015)
Aspergillus fumigatus	0.23 U/mg	118	6	40	7200	Sanni et al. (2019)
Aspergillus niger van Tieghem	22,592 U/mg	353	2.5	52–55	0.606	Vats and Banerjee (2005)
Aspergillus niger ATCC 9142	89.6 U/mg	84	5	65	100	
Aspergillus niger 307	339.72 U/mg	39	2.62, 5.05	55–58	0.929	Casey and Walsh (2003)
Aspergillus niger CFR 335	32.6 ± 3.1 U/mg	66	4.5	30	80 ± 0.1	Sariyska et al. (2005)
Aspergillus niger 7A-1	8.38 U/mg	89	5.3	56	220	Gunashree and Venkateswaran (2008)
Aspergillus oryzae		74	5.5–6.0	50	–	
Ceriporia sp.	2 U/ml	59	5.5–6.0	55–60	–	Neira-Vielma et al. (2018)
Cladosporium sp. FP-1	700 ± 80 U/mg	32.6	3.5	40	15.2 ± 3.1	
Flammulina velutipes	3.4 U/mg	14.8	5	45	–	Zhu et al. (2011)
Lentinus edodes	3.11 U/mg	14	5	37	–	Zhang et al. (2013b)
Mucor hiemalis	46.7 U/mg	45		55	–	

(continued)

Table 4.2 (continued)

Fungal taxa	Specific activity	Mol. mass (kDa)	pH_{opt}	T_{opt} (°C)	K_m (μM)	References
			5.0–5.5			Boyce and Walsh (2007)
Peniophora lycii	1080 ± 110 U/mg	72	4.0–5.0	50–55	–	Lassen et al. (2001)
Penicillium simplicissimum	3245 U/mg	65	4	55	–	Tseng et al. (2000), Buckle (1988)
Rhizopus oligosporus	9.47 U/mg	–	4.5	55	150	de Oliveira Ornela and Guimaraes (2019)
Rhizopus microsporus var. *microsporus*	0.8 U/mg	55	9.5	65	413	Zhang et al. (2013a)
Schizophyllum commune	5260.5 U/mg	72.5	4.6	50	248	Lassen et al. (2001)

humans). This is due to their ability to release phosphorus from phytin/phytates, thus allowing their assimilation, concurrently reducing the antinutrient phytate content from foods and feeds. The supplementation of phytase enzyme in foods emerged as one of the most lucrative and effective additives.

4.5.1.1 Applications in Animal Nutrition

Animal feeds, especially swine and fowl diets, are plant-based with phytate phosphorus accounting for more than 65% of the phosphorus of feedstuffs (Tyagi et al. 1998). This phytate phosphorus is unavailable to single-stomached animals under most of the dietary conditions (Nelson 1967), so there is a need for dietary supplementation with inorganic phosphate, which adds to the cost of the animal feed due to the limiting rock phosphorus supplies. Furthermore, the unutilized phytate can cause phosphorus pollution as it is excreted in the faeces of animals (Kaur et al. 2007b; Dersjant-Li et al. 2015). The phytates also chelate several vital minerals, thus reducing their availability to the animals feeding on such diet. Phytases are incorporated into the feed of non-ruminants or monogastric animals mainly to reduce the need for supplementation of phosphorus, but at the same time, trace minerals become available to the animal, and degradation of *myo*-inositol phosphates release *myo*-inositol, an important growth factor (Dailin et al. 2019). This enhances the digestibility and the nutritive content of the feeds, thereby improving the growth rate of animals. This decreases the amount of phosphorus excreted, thereby mitigating pollution of the environment. The nutritional value as well as the environmental significance of the usage of phytases in releasing phosphorus from plant-based

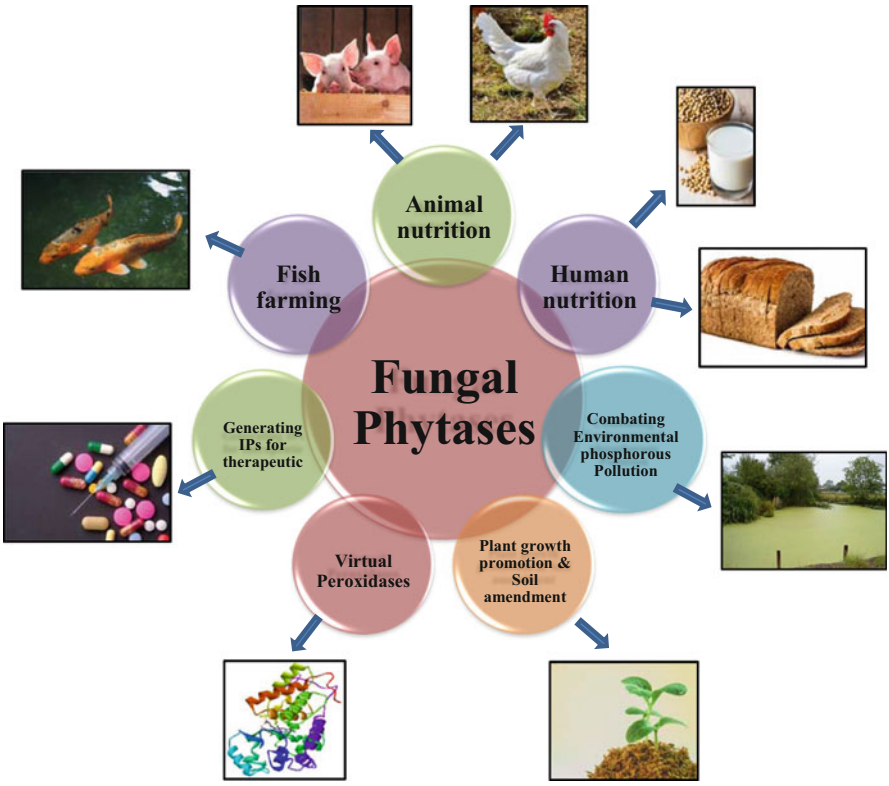

Fig. 4.2 Multifarious applications of fungal phytases

animal feeds is very well documented (Kaur et al. 2007b; Singh and Satyanarayana 2015).

Phytases can be used in feeds in two basic ways: (a) replacement of the supplementation of inorganic phosphorus with phytase to make soluble phosphorus available from phytates. However, as the reaction conditions (pH, temperature, incubation time, moisture) are not optimal in the animal stomach or intestine, the second method of phytase use, (b) pretreatment of feed with phytase to reduce their phytate content, seems to be a better option (Simell et al. 1989). Based on in vitro feed experiments, it has been observed that for animal nutrition, phytases with broad substrate specificity are better than those with narrow substrate specificity, like acid phytases from *A. niger* and *A. terreus* (Wyss et al. 1999). For poultry feed, an ideal phytase is one which is resistant to acidic pH and proteolytic enzymes in the animal stomach and small intestine (where P absorption takes place), economical in production and resilient to high temperatures (65–80 °C) that are encountered during feed pelleting (Lei and Stahl 2001), and HAPs are the most widely used. Supplemental phytase in animal feed has been shown to improve calcium, zinc and iron utilization (Lei et al. 1993; Jondreville and Dourmad 2005). Feed trial experiments

with chicks and hogs have established the option of replacing Pi (inorganic phosphorus) supplementation with the use of microbial phytase in the diet rich in phytate for monogastrics. Around 1000 units of phytase supplementation can decrease the release of phosphorus in the environment by 30–50% and can be a substitute of 1 g of Pi supplementation (Kemme et al. 1997; Yi et al. 1996).

The first phytase investigated for reducing the phytate content in poultry feed was that of *Aspergillus* (Nelson et al. 1968, 1971). However, the first phytase feed enzyme became commercially available only in 1991. The *A. niger* phytase under the trade name of Natuphos (BASF) was permitted as a feed additive in a number of Asian, European and North and South American countries, including Canada and the United States (Wodzinski and Ullah 1996).

Phytase from *A. niger*, when added to maize and soybean meal-based diets, led to enhanced growth and bone mineralization in broiler chicks along with a better relative retention of minerals P and Ca (Ahmad et al. 2004). Similarly, supplementing *A. niger* phytase to diets of pigs brought about an enhanced body weight and higher P in their bones (Sands et al. 2009). The phytase from *A. niger* van Tieghem efficiently hydrolysed phytate in numerous commercial livestock feeds (Vats et al. 2009). An enhanced growth performance of broilers was observed when their P-deficient diets were supplemented with *A. niger* phytase and evaluated till 35 days after hatching (Srikanthithasan et al. 2020). A novel fungal beta-propeller phytase has been recently reported from the nematophagous *Arthrobotrys oligospora* with probable application in animal feed due to its ability to release phosphorus from soybean meal and minerals from durum wheat and finger millet flour (Hou et al. 2020).

Apart from filamentous moulds, several studies have been done on the role of yeast phytases as a feed additive. Phytase from the yeast *S. castellii* could be successfully used as feed supplements as it can efficiently degrade phytic acid from wheat bran and glandless cotton flour (Segueilha et al. 1993). This yeast phytase also increased the availability of P in pigs, but *A. niger* phytase was found to give better results (Matsui et al. 2000).

The cell-bound phytase of *Pichia anomala*, when incorporated into the feed of broiler chicks, resulted in their enhanced growth, improved phosphorus retention in their body and reduced excretion of phosphorus in the faeces and was found to be commercially viable as poultry feed supplement (Vohra et al. 2006). A number of studies have been conducted to analyse the effect of *S. cerevisiae* phytase supplementation (Ayanwale et al. 2006; Haraldsson et al. 2005).

Subsequent to the first commercialization efforts by Gist-Brocades Europe (1993–1994), several companies like Alltech (USA), BASF (USA) and Alko Co. (Finland) started the production of phytase at industrial scale and sold them under the trade names Allzyme, Natuphos and Finase, respectively, which are effectively added in animal feeds. These are all produced from filamentous fungi. The phytase preparation has been approved as GRAS (generally regarded as safe) by the FDA (Food and Drug Administration) (Wodzinski and Ullah 1996). However, phytase production at industrial scale is expensive, thus limiting the widespread use of these feed additives. Generating transgenic plants and animals expressing phytase

have been considered as an alternative. Creation of transgenic animals expressing phytase in their digestive tracts and supplementation of phytase-expressing transgenic plants to animal diet have both shown growth-enhancing effects comparable to that of using phytase concentrates as feed additives. There are innumerable reports of phytase expression in various transgenic plants, including tobacco (Ullah et al. 1999), soybean (Li et al. 1997), alfalfa (Ullah et al. 2002), canola (Ponstein et al. 2002), wheat (Brinch-Pedersen et al. 2000, 2003), potato (Ullah et al. 2003), rice (Hong et al. 2004), legume *Medicago truncatula* (Abranches et al. 2005), *Brassica napus* (Wang et al. 2013) and others. The focus of all these reports was on expressing and accumulating phytase in the transgenic plants to be used as feed additives, and mainly *phyA* from *Aspergillus niger* was integrated. Supplementation of broiler diets with phytase-expressing transgenic seeds was shown to result in an improved growth rate (Pen et al. 1993). Rice plants transformed with *Schwanniomyces occidentalis* phytase gene supported enhanced phytase levels (Hamada et al. 2005), and these transgenic plants could be stockpiled as silage without any loss of activity until their use as feed additives (Hamada et al. 2006). These reports present a promising picture of using transgenic technology for phytase production through bio-farming.

4.5.1.2 Applications in Aquaculture/Fish Farming

Phytic acid acts as a major antinutrient in the diets of fishes due to their smaller GIT (Richardson et al. 1985), and this hampers the use of plant-based feed for fish. In aquaculture, the cost of feed is around 70% of the cost to produce fish (Rumsey 1993). Phytases play a dual role here, cheaper plant meals can be used as food, and lesser phosphorus is released into the water (Cao et al. 2007). Aquatic phosphorus pollution caused by mainly fish excreta is a serious concern, which has resulted in the enhanced use of phytases in fish feed in the past decades. The use of microbial phytase in fish feed enhances the bioavailability of phosphorus from phytate, and subsequently less P is discharged into the aquatic environment (Rodehutscord and Pfeffer 1995).

The effect of *A. niger* phytase (Natuphos) was investigated on common carp (*Cyprinus carpio*), and it resulted in enhanced utilization of phytate P, reduced phosphorus excretion as well as improved body weight and body phosphorus content (Schafer et al. 1995). Most of the available reports on application of phytase in aquaculture are using the commercially available sources, i.e. fungal phytases. But yeasts have additional probiotic effects besides being a source of phytase, due to which they provide additional advantage in fresh water and marine aquaculture. Supplementation of plant-based low-phosphorus diets for Nile tilapia (*Oreochromis niloticus*) with a consensus phytase SP1002 (produced by a genetically modified strain of *Hansenula polymorpha*) at 750 U/kg diet significantly ameliorated digestibility of protein, phosphorus and calcium (Liebert and Portz 2007). During fish feeding trials, on juvenile milkfish, *Chanos chanos*, the cell-bound phytase of *P. anomala* was supplemented to soybean meal and used to substitute fish meal; the observations on fish growth were promising, suggesting their potential use in environment-friendly mass culturing of milkfish (Hassan et al. 2009).

The phytase gene of *E. coli* was overexpressed in the salivary gland of pigs to generate the phytase-transgenic animal Enviropig. The faecal phosphorus levels were reduced by 75% in these animals as salivary phytases facilitated the assimilation of phytates from the fodder (Golovan et al. 2001). On the similar lines, transgenic fish (*Oryzias latipes*), electroporated with phytase gene from *A. niger*, have been developed (Hostetler et al. 2005). These transgenic studies suggested the possibility of developing eco-friendly animal and fish varieties with improved phytate digestion by overexpressing phytase genes.

4.5.1.3 Applications in Human Nutrition

The occurrence of phytates in plant-based foods including cereal flours is well recorded (Greiner and Konietzny 2006). A major proportion of our world population suffers from mineral deficiencies (mainly iron and zinc) because of consumption of plant diets which are rich in phytates (Bentley et al. 1997; Tatala et al. 1998), and studies in human subjects have irrefutably shown that iron and zinc absorption from a diet corresponds inversely to its phytate levels (Brune et al. 1992; Navert et al. 1985).

Cooking inactivates the dietary phytase, resulting in poor phytate digestion, thus affecting mineral absorption in the small intestine. The consequence of several food processing and preparation approaches, including germination, soaking, cooking and fermentation, is only a partial reduction in the phytate content of the treated food. The phytases have been found to be valuable as food additive during fermentation, food processing and bread-making, where they perform a significant part in human nutrition by carrying out the degradation of phytates during processing as well as in the gastrointestinal tracts.

Phytases improve proofing time, volume and crumb texture during bread-making. Phytase from *Aspergillus niger* was added to the bread dough, and this resulted in hydrolysis of phytates (Turk and Sandberg 1992), enhanced absorption of iron (Sandberg et al. 1996), increased volume of bread and improved crumb texture (Haros et al. 2001). The phytase of *A. niger* when added to bread during freezer storage could eliminate the adverse effects of bran on the availability of minerals (Rosell et al. 2009). Supplementing phytase of the thermophilic mould *S. thermophile* in bread improved the bread quality, bringing about a concomitant reduction in phytates (Singh and Satyanarayana 2008a), releasing inorganic phosphate from calcium, magnesium and cobalt phytates (Singh and Satyanarayana 2010), and could also effectively dephytinize sesame oil cake and soymilk (Singh and Satyanarayana 2006a, 2008b).

A number of investigations have been carried out on the baker's yeast *Saccharomyces cerevisiae* phytase (Greiner et al. 2001; Haraldsson et al. 2005), whereby the role of yeast phytase in bread-making has been emphasized by the researchers. During whole meal bread-making, phytate level reduction was due to the dual action of yeast and wheat phytases (Turk et al. 1996). In the conventional bread-making process, it is recommended to use high amounts of yeast and longer fermentation time to obtain bread with lesser phytates and increased fibre (Harland and Harland 1980). Rapid degradation of phytic acid could be attained in 24 h using two

commercially available strains of *S. cerevisiae* (Turk et al. 2000). A high phytase-expressing strain of *S. cerevisiae* (without the use of any heterologous DNA) has been proposed to be useful in the food industry as a source of food-grade phytase (Veide and Andlid 2006). Whole wheat dough supplemented with rPPHY (*P. anomala* phytase overexpressed in *P. pastoris*) brought about effective dephytinization of bread with no alteration in texture (Joshi and Satyanarayana 2015a).

Fungal phytases have also been found to be useful in reducing phytates in other plant-based products. Reduction in phytic acid levels up to 90% were attained after treating *Icacina mannii* (a shrub used as a source of starch-based food during periods of famine in Africa) with *S. cerevisiae* for 6 days (Antai and Nkwelang 1998). Chapathi, a traditional staple food in parts of India and some Asian countries, is prepared from whole wheat flour. Phytic acid levels in this flour could be reduced by 10–45% after treating it with the mutated strain of the yeast *Candida versatilis* (Bindu and Varadaraj 2005). The recombinant phytases from the mould *S. thermophile* and yeast *P. anomala* could also be used in mitigating phytates from Indian baked breads (Tandoori and Naan) (Ranjan et al. 2015; Joshi and Satyanarayana 2015a). The *S. cerevisiae* MTCC 5421 phytase could efficiently dephytinize pearl millet flour (94%) and refined wheat flour (100%) as well as completely eliminate phytate in wheat-based (Naan) and pearl millet-based (Rabadi) fermented products (Roopashri and Varadaraj 2015).

Similarly, baker's yeast as a source of phytase was investigated for reduction in phytic acid content in Tarhana, a traditional Turkish fermented and dried cereal food (Bilgicli et al. 2006). Oriental foods normally employ moulds for fermentations, which have the additional advantage of degrading phytates. A popular oriental fermented food Tempeh, made from soybeans, is inoculated by mould (*Rhizopus oligosporus*) in the koji process, and the fermentation improves the digestibility, vitamin content and flavour of soybean (Fardiaz and Markakis 1981). Phytates form complexes with proteins present in soybeans. Consequently, phytases find utility in the production of phytate-free soybean milk (Anno et al. 1985; Khare et al. 1994). The cell-bound phytase of *Pichia anomala* was reported to be useful in dephytinization of soymilk (Kaur and Satyanarayana 2010). The rPPHY (phytase of *P. anomala* overexpressed in *P. pastoris*) was also found to be useful in fractionating allergenic glycinin from soy protein and, thus, has potential utility in producing speciality foods for people allergic to some components of soy protein (Joshi and Satyanarayana 2014). Similarly, the role of phytase from *A. ficuum* in legume dephosphorylation was investigated; a 78% phytate reduction was attained upon treating soybean meal for 15 h (Han and Wilfred 1988).

In contrast to several transgenics developed for animal feed application so far, only a single transgenic plant expressing phytase heterologously has been developed to deal with the issue of dietary phytate in human nutrition: a transgenic rice plant overexpressing phytase genes from *A. fumigatus* to improve iron bioavailability from rice (Lucca et al. 2002). However, this enzyme got completely inactivated on cooking. Due to the specific health benefits of *myo*-inositol derivatives, special functional foods can be produced using phytases (Konietzny and Greiner 2003).

4.5.2 Mitigation of Environmental Phosphorus Pollution

Since phosphorus is an essential nutrient for living systems, its deficiency or excess can both will be problematic. Supplementing phytases to animal feed is intended to overcome the problem of underutilization of phytates and the cost of adding inorganic P. This unutilized phytin phosphorus has a tendency to accumulate in the environment as faecal material (Mullaney et al. 2000) and is ultimately released due to the phytase activity of soil and water-borne microbes, whereby the surplus inorganic phosphorus flows into water bodies, which causes problems of eutrophication and algal growth (Vohra and Satyanarayana 2003). The pretreatment of animal feed with phytase provides double advantage of enhancing inorganic phosphorus availability, thus helping in nutrition and concurrently mitigating the problem of phosphorus pollution of water bodies.

The addition of *A. niger* phyA to a corn-soybean-based feed having low phosphorus content enabled weanling pigs to better utilize the dietary phytate, whereby the retention of dietary phosphorus by the pigs was improved by 50% and the simultaneous faecal excretion of phosphorus was reduced by 42% (Lei et al. 1993). Similarly, the supplementation of broiler chick feed with *P. anomala* phytase (cell-bound) led to enhanced body weights and better retention of phosphorus in their bodies as well as decreased faecal excretion of phosphorus (Vohra et al. 2011). This cell-bound phytase was also found to be a valuable supplement to plant-based feed for the environment-friendly farming of marine (*Chanos chanos*) and freshwater (*Labeo rohita*, *Clarias batrachus*) fishes with decreased excretion of phosphorus in the water bodies (Hassan et al. 2009; Joshi and Satyanarayana 2017). In fact, the application of phytase as animal feed supplement was first proposed for eco-friendly reasons but, soon after their nutritional and health benefits were uncovered, made them lucrative.

4.5.3 Plant Growth Promotion and Soil Amendment

Of the total organic phosphorus in the soil, roughly 10–50% is present as phytic acid (Mullen 2005). However, this is not readily available to plants as it forms complexes with cations or gets adsorbed to various soil components. Therefore, there is a need to mobilize this phosphorus source (Richardson et al. 2001). The presence of plant growth-promoting fungi (PGPF) in the rhizosphere partly improves this availability by solubilizing/mineralizing phosphorus in the rhizosphere (Zhang et al. 2016; Hossain et al. 2017). The presence of several extracellular phytase producing fungi in the rhizosphere, such as *A. flavus*, *A. fumigatus* and *A. rugulosus*, has been implicated in improving the growth of plants by improving phosphorus nutrition (Tarafdar and Rao 1996; Gaind and Singh 2015). Moreover, species of *Trichoderma* and *Penicillium* have been reported to increase the extractable organic P under alkaline soil conditions (Gaind and Nain 2015; Singh and Satyanarayana 2010). These studies clearly indicated that fungal phytases resulted in enhanced plant growth and productivity levels.

Another approach to improve the utilization of phytate phosphorus from soil is to generate transgenic plants expressing phytase, which would help in reducing the dependency on phosphorus fertilizers in agriculture. Such transgenic plants will lead to an increased level of phosphorus availability for plants by secreting extracellular phytases into the rhizosphere. The fungal *phyA* gene from *A. niger* NRRL 3135 was expressed in *Arabidopsis*, which resulted in a transgenic line with improved phosphorus nutrition and growth as compared to a control (Richardson et al. 2001). Similarly, transgenic *Brassica napus* was created that expressed either the *phyA* or *appA* genes, which showed improved biomass yield when phytates were provided as the only source of phosphorus (Wang et al. 2013). Experiments aimed at obtaining plants with increased phytase activity were also carried out with mustard, tobacco, soya, wheat, sweet potato and canola (Balaban et al. 2017). The phytase of *Peniophora lycii* was more effective in hydrolysing phytate than *A. niger* phytase as the former has a lower pI (3.6) compared to the latter which has a pI of 5.0, thus allowing it to remain more soluble in the soil environment. This shows that certain properties of phytase are important in determining phytate hydrolysis, such as its isoelectric point (pI). In acidic soils, low-pI phytases work more efficiently, whereas high-pI phytases are preferred in basic soils (George et al. 2007; Lei et al. 2013). Extracellular phytase released from the roots of transgenic plants also improved the overall plant growth and created favourable conditions for adjacent plants due to an increased availability of phosphorus and minerals (Reddy et al. 2013). This further eliminated the need to use phosphorus fertilizers in agriculture.

4.5.4 Generating Specific *Myo*-inositol Phosphates for Use in Therapeutics

Myo-inositol phosphates have a key role to play in transmembrane signalling and to mobilize calcium from reserves present intracellularly. This has generated an enhanced interest in the production of inositol phosphates from phytic acid (Billington 1993). The stepwise degradation of phytic acid by phytases releases various lower inositol phosphates sequentially; this enables to study their individual effects on animal physiology. Phytase immobilized in a bioreactor can be useful for producing lower isomers of phytate. Phytase-producing *Candida krusei* cells were immobilized in Ca-alginate gel beads and used for the preparation of various IPs; the pure isomers obtained were then isolated by ion-exchange chromatography (Quan et al. 2003).

There can be several applications of the inositol phosphate derivatives such as enzyme stabilizers, inhibitors and substrates (Siren 1986a), thus showing their role as probable drugs and as chiral building blocks (Laumen and Ghisalba 1994). Some of these derivatives are beneficial to health, where their physiological functions are dependent on the number and position of the phosphate groups on the *myo*-inositol ring. Specific inositol triphosphates have found application in medicine as pain killers and for their antiviral effects especially against HIV (Siren 1995). Also in diseases such as cancers, renal stone and heart ailments, these myo-inositol

phosphates have proved to be helpful (Potter 1995; Modlin 1980; Graf and Eaton 1993; Shamsuddin and Vucenik 1999).

Specific novel phytate derivatives, such as D-*myo*-inositol 1,2,6-triphosphate, D-*myo*-inositol 1,2,5-triphosphate, etc., can also be prepared with the help of phytases from *S. cerevisiae* (Greiner and Konietzny 1996; Siren 1986b). Using chemical processes, the synthesis of these compounds is difficult (Billington 1993) as compared to the enzymatic process, since the latter offers the advantage of stereospecificity and mild reaction conditions. The *A. niger* phytase was reported to effectively break down phytic acid to lower phosphorylated derivatives, advocating its potential utility (Dvorakova et al. 2000).

4.5.5 Virtual Peroxidises Derived from Phytases

Peroxidases catalyse varied oxidation reactions with hydrogen peroxide being the main oxidant (Van de Velde et al. 2000). Fungal phytases and acid phosphatases exhibit a structural similarity with the active site of vanadium-dependent haloperoxidases, and considering this, a semisynthetic peroxidase was designed (Van de Velde et al. 2000). A vanadate ion was incorporated into the active site of *A. niger* (*ficuum*) NRRL 3135 phytase by the Delft group, which transformed the native phosphohydrolase activity of phytase into virtual peroxidase, with the capability to catalyse enantioselective oxidation of prochiral sulphides. Upon incorporation of vanadate ion into the active site of histidine acid phosphatase, it behaves as a peroxidase; vanadate serves as a phosphate analogue inhibiting the phytate-hydrolysing activity of phytase, thus making the enzyme to act as a vanadate-dependent haloperoxidase (Ullah et al. 2011). The diverse applications of vanadate haloperoxidases include the use in immunoassays, as antimicrobial agents, in diagnostics and others (Joshi and Satyanarayana 2015b; Ranjan and Satyanarayana 2016). The recombinant phytases from *P. anomala* and *S. thermophile* have been verified to have haloperoxidase activities (Joshi and Satyanarayana 2015b; Singh et al. 2018).

4.5.6 Other Applications of Fungal Phytases

Fungal phytases have been used in some novel and previously unexplored areas like in biosensors. Phytic acid plays a significant environmental role in animal feed industry besides influencing health in food industry; this prompted the development of a phytase-based biosensor for a simple, single-step quantifiable detection of phytic acid based on the sequentially acting enzyme phytase and pyruvate oxidase (POD) (Mak et al. 2004). Subsequently, a novel yeast cell-based assay was developed for the detection of estrogenic activity in wastewater using phytase enzyme, where the recombinant strains of *Arxula adeninivorans* were generated, under the glucoamylase promoter (GAA), simultaneously expressing human estrogen receptor α (hERα) and *Klebsiella*-derived phytase (*phyK*) reporter gene (Hahn et al. 2006).

A fairly recent application is in biofuel production, where a high phytase activity yeast strain, *Candida melibiosica* 2491, was used as a biocatalyst in microbial fuel cell (MFC), by coupling the consumption of inorganic phosphates with the electrochemical process (Hubenova and Mitov 2010).

Another novel potential utility of phytase is in the decomposition of organophosphorus pesticides, which are chemically phosphorus acid esters and destroy pests by inhibiting acetylcholine esterase. Nevertheless, these pesticides are not easy to remove from the environment, resulting in their bioaccumulation. Very recently, phytase from the fungus *A. niger* NCIM 563 was discovered to be capable of degrading organo-phosphorus pesticides like chlorpyrifos, monocrotophos and methyl parathion (Shah et al. 2017). This application can find potential utility in management as well as control of the leading global concern of environmental phosphorus pollution.

The use of phytases in nanotechnology-based drug delivery system is another innovative approach. It was discovered that ionic liquid-mediated self-assembled phytase nanospheres exhibited anti-tumour properties whose effectiveness could be boosted by the addition of platinum ions on their surface and loading the anti-tumour drug curcumin (present in turmeric, an Indian spice) inside them. The phytase nanospheres themselves exhibited 25% anticancer effect, which enhanced to 90% in platinum-phytase-curcumin nanospheres, thus showing potential in therapeutics for the development of multifunctional drug delivery systems. The platinum-phytase spheres were also suitable as an efficient, eco-friendly and recyclable phytate degradation systems. Phytase I was sourced from the fungus *A. niger*. As many therapeutic proteins approved by the FDA are glycosylated, phytase which is a highly glycosylated was preferred for use in this study (Soni et al. 2015).

4.6 Future Perspectives

Microbial phytases are finding applications in many industries. In 2015, they were responsible for annual sales of US$ 350 million which accounted for 83.6% of the revenue share of the total industry. With the rapid growth of poultry, swine and aquaculture farming along with increased awareness about the negative environmental impacts of phosphorus release, the usage of phytase in animal feed is bound to increase. Commercial production of phytase started in 1991, when the BASF Company (Germany) developed methods to obtain phytase based on *A. niger*. Currently, fungal phytases are being produced by a number of companies, such as Allzyme, Finase, Natuphos, Ronozyme, BASF and others (Table 4.3).

With their immense applications in feed and food industries, the demand for fungal phytases is increasing. However, only a few phytase-producing fungal strains have been studied. Therefore, there is a need to discover new fungal species with ameliorated phytase properties. Another area of phytase research, which needs to be emphasized upon, is the screening of phytases of higher stability at elevated temperatures. Research conducted till now reports of only a few phytases with the optimal temperature/stability exceeding 70 °C. Focus on protein engineering to

Table 4.3 Commercially available fungal phytases

Product	Company	Phytase source	Fungal strain used for production
Allzyme® SSF	Alltech	*Aspergillus niger*	*Aspergillus niger*
Finase® P/L	AB Vista	*Aspergillus niger* PhyB	*Trichoderma reesei*
Natuphos®	BASF	*Aspergillus niger* PhyA	*Aspergillus niger*
Ronozyme® P	Novozyme/DSM	*Peniophora lycii* PhyB	*Aspergillus oryzae*
Rovabio	Adisseo	*Penicillium funiculosum*	*Penicillium funiculosum*
Optiphos®	Huvepharma	*E. coli*	*Pichia pastoris*
Avizyme	Finnfeeds International	*Aspergillus awamori*	*Trichoderma reesei*
Bio-Feed Phytase	DSM	*Peniophora lycii*	*A. oryzae*
Phyzyme	Fermic	*A. oryzae*	*A. oryzae*
SP,TP,SF	Alko Biotechnology	*Aspergillus oryzae*	*A. oryzae*

improve desirable properties of phytase, for their usage in the feed industry, is gaining momentum. In addition, the development of overproducer strains should be attempted by using recombinant DNA technology. An area for future research is the emphasis on the application of heterologous expression systems to integrate the thermostable phytase gene in plants. Using genetic engineering and heterologous gene expression, transgenic plants expressing phytase can be generated; this could mitigate the need to supplement phosphorus or phytase to the feeds of monogastric animals (Chen et al. 2008). In addition, the cloning and protein engineering of potential phytase producing fungal species will also be highly useful.

4.7 Conclusions

The fungal phytases have gained immense interest in the past few decades as ideal enzymes for various biotechnological applications, owing to their desirable properties of broad substrate range, thermostability and acid stability. Moreover, many moulds and yeast are GRAS organisms or have probiotic potential, making them safe for use. The phytases bring about the generation of green phosphate and largely find uses in ameliorating the nutritional status of animal feed, due to the growing apprehensions about phosphorus pollution problems in the regions of intense animal farming. However, their potential commercial utility in several other areas including human nutrition, environmental phosphorus pollution abatement and soil fertility as well as in newer fields makes them the enzymes of choice. The discovery of new fungal phytases with higher yields and desirable

characteristics especially improved stability is an ongoing process. Simultaneously, bioengineering is enabling the use of desirable properties for generating phytate-hydrolysing transgenic plants and animals as well as for creating consensus phytases with improved and desirable properties for direct application.

References

Abd-Elhalem BT, El-Sawy M et al (2015) Production of amylases from *Bacillus amyloliquefaciens* under submerged fermentation using some agro-industrial by-products. Ann Agric Sci 60 (2):193–202

Abranches R, Marcel S et al (2005) Plants as bioreactors: a comparative study suggests that *Medicago truncatula* is a promising production system. J Biotechnol 120:121–134

Ahmad F, Rahman MS et al (2004) Performance of broiler on phytase supplemented soybean meal based diet. Int J Poult Sci 3:266–271

Ajith S, Shet D et al (2018) Immobilised phytase production from *Aspergillus foetidus* MTCC 11682 using an optimized media. Biotechnol J Int 21(3):1–13

Ajith S, Ghosh J et al (2019) Partial purification & characterization of phytase from *Aspergillus foetidus* MTCC 11682. AMB Exp 9:3

Anno T, Nakanishi K et al (1985) Enzymatic elimination of phytate in soybean milk. J Jpn Soc Food Sci Technol 32:174–180

Antai SP, Nkwelang G (1998) Reduction of some toxicants in *Icacina mannii* by fermentation with *Saccharomyces cerevisiae*. Plant Foods Human Nutr 53:103–111

Ariza A, Moroz OV et al (2013) Degradation of phytate by the 6-phytase from *Hafnia alvei*: a combined structural & solution study. PLoS One 8(5):e65062

Arora S, Dubey M et al (2017) Effect of mixing events on the production of a thermo-tolerant & acid-stable phytase in a novel solid-state fermentation bioreactor. Process Biochem 61:12–23

Awad GEA, Helal MMI et al (2014) Optimization of phytase production by *Penicillium purpurogenum* GE1 under solid state fermentation by using Box–Behnken design. Saudi J Biol Sci 21:81–88

Ayanwale BA, Kpe M, Ayanwale VA (2006) The effect of supplementing *Saccharomyces cerevisiae* in the diets on egg laying and egg quality characteristics of pullets. Int J Poult Sci 5:759–763

Azeem M, Riaz A et al (2015) Microbial phytase activity & their role in organic P mineralization. Arch Agron Soil Sci 61:751–766

Bala A, Sapna et al (2014) Production of an extracellular phytase from a thermophilic mould *Humicola nigrescens* in solid state fermentation & its application in dephytinization. Biocatal Agric Biotechnol 3:259–264

Balaban NP, Suleimanova AD et al (2017) Microbial phytases & phytate: exploring opportunities for sustainable phosphorus management in agriculture. Am J Mol Biol 7:1–29

Belgaroui N, Berthomieu P et al (2016) The secretion of the bacterial phytase PHY-US417 by *Arabidopsis* roots reveals its potential for increasing phosphate acquisition and biomass production during co-growth. Plant Biotechnol J 14(9):1914–1924

Bentley ME, Caulfield LE et al (1997) Zinc supplementation affects the activity patterns of rural Guatemalan infants. J Nutr 127:1333–1338

Berka RM, Rey MW et al (1998) Molecular characterization & expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginosus*. Appl Environ Microbiol 64:4423–4427

Bhavsar K, Khire JM (2014) Current research and future perspectives of phytase bioprocessing. RSC Adv 4:26677–26691

Bhavsar K, Shah P et al (2008) Influence of pretreatment of agriculture residues on phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. Afr J Biotechnol 7(8):1101–1106

Bhavsar K, Kumar RV, Khire JM (2011) High level phytase production by *Aspergillus niger* NCIM 563 in solid state culture: response surface optimization, up scaling & its partial characterization. J Ind Microbiol Biotechnol 38:1407–1417

Bhavsar K, Kumar RV, Khire JM (2012) Downstream processing of extracellular phytase from *Aspergillus niger*: chromatography process vs. aqueous two-phase extraction for its simultaneous partitioning and purification. Process Biochem 47:1066–1072

Bhavsar K, Gujar P et al (2013a) Combinatorial approach of statistical optimization & mutagenesis for improved production of acidic phytase by *Aspergillus niger* NCIM 563 under submerged fermentation condition. Appl Microbiol Biotechnol 97:673–679

Bhavsar K, Buddhiwant P et al (2013b) Phytase isozymes from *Aspergillus niger* NCIM 563 under solid state fermentation: biochemical characterization & their correlation with submerged phytases. Process Biochem 48:1618–1625

Bilgicli N, Elgun A, Turker S (2006) Effects of various phytase sources on phytic acid content, mineral extractability & protein digestibility of Tarhana. Food Chem 98:329–337

Billington DC (1993) The inositol phosphates. In: Chemical synthesis & biological significance. Verlag Chemie, Weinhiem, p 153

Bindu S, Varadaraj MC (2005) Process for the preparation of Chapathi dough with reduced phytic acid level. US Pat. #20050048165. Dated 3 Mar 2005

Bindu S, Somashekar D, Joseph R (1998) A comparative study on permeabilization treatments for in situ determination of phytase of *Rhodotorula gracilis*. Appl Microbiol Lett 27:336–340

Boyce A, Walsh G (2007) Purification & characterisation of an acid phosphatase with phytase activity from *Mucor hiemalis*. Wehmer J Biotechnol 132:82–87

Brinch-Pedersen H, Hatzack F et al (2000) Generation of transgenic wheat (*Triticum aestivum* L.) for constitutive accumulation of an *Aspergillus* phytase. Mol Breeding 6:195–206

Brinch-Pedersen H, Hatzack F et al (2003) Concerted action of endogenous and heterologous phytase on phytic acid degradation in seed of transgenic wheat (*Triticum aestivum* L.). Transgenic Res 12:649–659

Brune M, Rossander-Hulten L et al (1992) Iron absorption from bread in humans: inhibiting effects of cereal fibre, phytase and inositol phosphates from different numbers of phosphate groups. J Nutr 122:442–449

Buckle KA (1988) Characterization of extra- and intra-cellular phytases from *Rhizopus oligosporus* used in tempeh production. Int J Food Microbiol 6:67–79

Buddhiwant P, Bhavsar K et al (2016) Phytase production by solid-state fermentation of groundnut oil cake by *Aspergillus niger*: A bioprocess optimization study for animal feedstock applications. Prep Biochem Biotechnol 46(6):531–538

Cao L, Wang W et al (2007) Application of microbial phytase in fish feed. Enzyme Microb Technol 40:497–507

Casey A, Walsh G (2003) Purification & characterization of extracellular phytase from *Aspergillus niger* ATCC 9142. Bioresour Technol 86:183–188

Chadha BS, Harmeet G et al (2004) Phytase production by the thermophilic fungus *Rhizomucor pusillus*. World J Microbiol Biotechnol 20:105–109

Chelius MK, Wodzinski RJ (1994) Strain improvement of *Aspergillus niger* for phytase production. Appl Microbiol Biotechnol 41:79–83

Chen R, Xue G et al (2008) Transgenic maize plants expressing a fungal phytase gene. Transgenic Res 17:633–643

Chen CC, Cheng KJ et al (2015) Current progresses in phytase research: three-dimensional structure & protein engineering. Chem Bioeng Rev 2:76–86

Chow Y, Ting AS (2015) Endophytic L-asparaginase-producing fungi from plants associated with anticancer properties. J Adv Res 6(6):869–876

Coban HB, Demirci A (2014) Screening of phytase producers and optimization of culture conditions for submerged fermentation. Bioprocess Biosyst Eng 37(4):609–616

Collopy PD, Royse DJ (2004) Characterization of phytase activity from cultivated edible mushrooms & their production substrates. J Agric Food Chem 52:7518–7524

Cominelli E, Confalonieri M et al (2018) Phytic acid transport in *Phaseolus vulgaris*: A new low phytic acid mutant in the PvMRP1 gene & study of the PvMRPs promoters in two different plant systems. Plant Sci 270:1–12

Dailin DJ, Hanapi SZ et al (2019) Fungal phytases: biotechnological applications in food & feed industries. In: Yadav A, Singh S, Mishra S, Gupta A (eds) Recent advances in white biotechnology through fungi. Springer, Cham, pp 65–99

Das S, Ghosh U (2014) Effect of nutritional supplementation of solid-state fermentation medium on biosynthesis of phytase from *Aspergillus niger* NCIM 612. J Sci Ind Res 73:593–597

de Oliveira Ornela PH, Guimaraes LHS (2019) Purification & characterization of an alkalistable phytase produced by *Rhizopus microsporus* var. *microsporus* in submerged fermentation. Process Biochem 81:70–76

Dersjant-Li Y, Awati A et al (2015) Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract & influencing factors. J Sci Food Agric 95:878–896

Doyle MP, Erickson MC (2006) Reducing the carriage of food-borne pathogens in livestock & poultry. Poult Sci 85:960–973

Dvorakova J, Kopecky J et al (2000) Formation of *myo*-inositol phosphates by *Aspergillus niger* 3-phytase. Folia Microbiol (Praha) 45:128–132

Fardiaz D, Markakis P (1981) Degradation of phytic acid in Omcom (fermented peanut press cake). J Food Sci 46:523–525

Gaind S, Nain L (2015) Soil-phosphorus mobilization potential of phytate mineralizing fungi. J Plant Nutr 38:2159–2175

Gaind S, Singh S (2015) Production, purification & characterization of neutral phytase from thermotolerant *Aspergillus flavus* ITCC 6720. Int Biodeterior Biodegradation 99:15–22

Gautam P, Sabu A et al (2002) Microbial production of extra-cellular phytase using polystyrene as inert support. Bioresour Technol 83:229–233

George TS, Simpson RJ et al (2007) Differential interaction of *Aspergillus niger* & *Peniophora lycii* phytases with soil particles affects the hydrolysis of inositol phosphates. Soil Biol Biochem 39:793–803

Gessler NN, Serdyuk EG et al (2018) Phytases & the prospects for their application. Appl Biochem Microbiol 54:352–360

Golovan SP, Meidinger RG et al (2001) Pigs expressing salivary phytase produce low-phosphorus manure. Nat Biotechnol 19:741–745

Gontia I, Tantwai K et al (2012) Transgenic plants expressing phytase gene of microbial origin & their prospective application as feed. Food Technol Biotechnol 50:3–10

Gontia-Mishra I, Tiwari S (2013) Molecular characterization & comparative phylogenetic analysis of phytases from fungi with their prospective applications. Food Technol Biotechnol 51:313–326

Graf E, Eaton JW (1993) Suppression of colonic cancer by dietary phytic acid. Nutr Cancer 19:11–19

Greiner R, Konietzny U (1996) Construction of a bioreactor to produce special breakdown products of phytate. J Biotechnol 48:153–159

Greiner R, Konietzny U (2006) Phytase for food application. Food Technol Biotechnol 44:125–140

Greiner R, Konietzny U (2012) Update on characteristics of commercial phytases. In: International phytase summit. AB Vista, Rome, pp 96–107

Greiner R, Alminger ML, Carlsson NG (2001) Stereospecificity of *myo*-inositol hexakisphosphate dephosphorylation by a phytate-degrading enzyme of baker's yeast. J Agric Food Chem 49:2228–2233

Greiner R, Silva LC, Couri S (2009) Purification and characterisation of an extracellular phytase from *Aspergillus niger* 11T53A9. Braz J Microbiol 40(4):795–807

Guerrero-Olazarán M, Rodríguez-Blanco L et al (2010) Expression of a *Bacillus phytase* C gene in *Pichia pastoris* and properties of the recombinant enzyme. Appl Environ Microbiol 76 (16):5601–5608

Gujar PD, Bhavsar KP, Khire JM (2013) Effect of phytase from *Aspergillus niger* on plant growth & mineral assimilation in wheat (*Triticum aestivum* Linn.) & its potential for use as a soil amendment. J Sci Food Agric 93:2242–2247

Gulati HK, Chadha BS, Saini HS (2007a) Production of feed enzymes (phytase & plant cell wall hydrolyzing enzymes) by *Mucor indicus* MTCC 6333: purification & characterization of phytase. Folia Microbiol 52:491–497

Gulati HK, Chadha BS, Saini HS (2007b) Production, purification & characterization of thermostable phytase from thermophilic fungus *Thermomyces lanuginosus* TL-7. Acta Microbiol Immunol Hung 54(2):121–138

Gulati HK, Chadha BS, Saini HS (2007c) Production & characterization of thermostable alkaline phytase from *Bacillus laevolacticus* isolated from rhizosphere soil. J Ind Microbiol Biotechnol 34:91–98

Gunashree BS, Venkateswaran G (2008) Effect of different cultural conditions for phytase production by *Aspergillus niger* CFR 335 in submerged & solid-state fermentations. J Ind Microbiol Biotechnol 35:1587–1596

Gurung N, Ray S et al (2013) A broader view: microbial enzymes and their relevance in industries, medicine, and beyond. Biomed Res Int 2013:329121

Hahn T, Tag T et al (2006) A novel estrogen sensor based on recombinant *Arxula adeninivorans* cells. Biosens Bioelectron 21:2078–2085

Hamada A, Yamaguchi K et al (2005) High-level production of yeast (*Schwanniomyces occidentalis*) phytase in transgenic rice plants by a combination of signal sequence & codon modification of the phytase gene. Plant Biotechnol J 3:43–56

Hamada A, Yamaguchi K et al (2006) Recombinant, rice-produced yeast phytase shows the ability to hydrolyze phytate derived from seed-based feed & extreme stability during ensilage treatment. Biosci Biotechnol Biochem 70:1524–1527

Han Y, Lei XG (1999) Role of glycosylation in the functional expression of an *Aspergillus niger* phytase (*phyA*) in *Pichia pastoris*. Arch Biochim Biophys 364:83–90

Han YW, Wilfred AG (1988) Hydrolysis of phytate in soybean & cottonseed meals by *Aspergillus ficuum* phytase. J Agric Food Chem 36:259–262

Han YW, Gallagher DJ, Wilfred AG (1987) Phytase production by *Aspergillus ficcum* on semisolid substrate. J Ind Microbiol 2:195–200

Haraldsson AK, Veide J et al (2005) Degradation of phytate by high-phytase *Saccharomyces cerevisiae* strains during simulated gastrointestinal digestion. J Agric Food Chem 53:5438–5444

Harland BF, Harland J (1980) Fermentative reduction of phytase in rye, white & whole wheat breads. Cereal Chem 57:226–229

Haros M, Rosell CM, Benedito C (2001) Fungal phytase as a potential breadmaking additive. Eur Food Res Technol 213:317–322

Hassan S, Altaff K, Satyanarayana T (2009) Use of soybean meal supplemented with cell-bound phytase for replacement. Pak J Nutr 8:341–344

Hassouni H, Alaoui MI et al (2006) Effect of culture media and fermentation parameters on phytase production by the thermophilic fungus *Myceliophthora thermophila* in solid state fermentation. Micologia Aplicada Int 18:29–36

Hong CY, Cheng KJ et al (2004) Production of two highly active bacterial phytases with broad pH optima in germinated transgenic rice seeds. Transgenic Res 13:29–39

Hossain M, Sultana F, Islam S (2017) Plant growth-promoting fungi (PGPF): phytostimulation & induced systemic resistance. In: Singh D, Singh H, Prabha R (eds) Plant-microbe interactions in agro-ecological perspectives. Springer, Singapore, pp 135–191

Hostetler HA, Collodi P et al (2005) Improved phytate phosphorus utilization by Japanese medaka transgenic for the *Aspergillus niger* phytase gene. Zebrafish 2:19–31

Hou X, Shen Z et al (2020) A novel fungal beta-propeller phytase from nematophagous *Arthrobotrys oligospora*: characterization & potential application in phosphorous & mineral release for feed processing. Microb Cell Factories 19:1–13

Howson SJ, Davis RP (1983) Production of phytate-hydrolysing enzyme by some fungi. Enzyme Microb Technol 5:377–382

Huang HQ, Shi PJ et al (2009) Diversity of beta-propeller phytase genes in the intestinal contents of grass carp provides insight into the release of major phosphorus from phytate in nature. Appl Environ Microbiol 75:1508–1516

Huang SJ, Chen CH, Tsai SY (2018) Phytase production by *Grifola frondosa* & its application in inositol-enriched solid-state fermentation brown rice. Int J Food Eng 4:263–267

Hubenova Y, Mitov M (2010) Potential application of *Candida melibiosica* in biofuel cells. Bioelectrochemistry 78:57–61

Idriss EE, Makarewicz O et al (2002) Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. Microbiology 148:2097–2109

Iwai T, Takahashi M et al (2012) Dynamic changes in the 333 distribution of minerals in relation to phytic acid accumulation during rice seed 334 develop. Plant Physiol 160:2007–2014

Jain J, Kumar A et al (2018) Purification & kinetics of a protease-resistant, neutral, & thermostable phytase from *Bacillus subtilis* subsp. *subtilis* JJBS250 ameliorating food nutrition. Prep Biochem Biotechnol 48:718–724

Jatuwong K, Suwannarach N et al (2020) Bioprocess for production, characteristics, & biotechnological applications of fungal phytases. Front Microbiol 11:188

Jin UH, Chun JA et al (2005) Sesame hairy root cultures for extra-cellular production of a recombinant fungal phytase. Process Biochem 40(12):3754–3762

Jondreville C, Dourmad JY (2005) Phosphorous in pig nutrition. INRA Prod Anim 18:183–192

Joshi S, Satyanarayana T (2014) Optimization of heterologous expression of the phytase (PPHY) of *Pichia anomala* in *P. pastoris* & its applicability in fractionating allergenic glycinin from soy protein. J Ind Microbiol Biotechnol 41:977–987

Joshi S, Satyanarayana T (2015a) Bioprocess for efficient production of recombinant *Pichia anomala* phytase & its applicability in dephytinizing chick feed & whole flat Indian breads. J Ind Microbiol Biotechnol 42:1389–1400

Joshi S, Satyanarayana T (2015b) Characteristics & applicability of phytase of the yeast *Pichia anomala* in synthesizing haloperoxidase. Appl Biochem Biotechnol 176:1351–1369

Joshi S, Satyanarayana T (2017) Characteristics and multifarious potential applications of HAP phytase of the unconventional yeast *Pichia anomala*. In: Satyanarayana T, Deshmukh S, Johri B (eds) Developments in fungal biology and applied mycology. Springer, Singapore, pp 265–278

Kanti A, Sudiana IM (2018) Production of phytase, amylase and cellulase by *Aspergillus*, *Rhizopus* and *Neurospora* on mixed rice straw powder and soybean curd residue. In: Proceedings of the IOP conference series: earth and environmental science. IOP Publishing, Bristol, pp 1–9

Kaur P, Satyanarayana T (2005) Production of cell-bound phytase by *Pichia anomala* in an economical cane molasses medium: optimization using statistical tools. Process Biochem 40:3095–3102

Kaur P, Satyanarayana T (2010) Improvement in cell-bound phytase activity of *Pichia anomala* by permeabilization & applicability of permeabilized cells in soymilk dephytinization. J Appl Microbiol 108:2041–2049

Kaur P, Kunze G, Satyanarayana T (2007a) Yeast phytases: present scenario & future perspectives. Crit Rev Biotechnol 27:93–109

Kaur P, Singh B, Satyanarayana T (2007b) Microbial phytases in combating environmental phosphorus pollution. In: Singh KK, Tomar A, Phogat V, Phogat S (eds) Air, water and soil pollution. M.D. Publications Pvt. Ltd., New Delhi, pp 150–190

Kaur P, Singh B et al (2010) *Pphy*-a cell-bound phytase from the yeast *Pichia anomala*: molecular cloning of the gene PPHY & characterization of the recombinant enzyme. J Biotechnol 149:8–15

Kaur P, Verma D, Satyanarayana T (2011) Recycling of spent medium from *Pichia anomala* MTCC-4133 phytase fermentation for the production of useful microbial products. Kavaka 39:19–24

Kaur R, Saxena A et al (2017) Production & characterization of a neutral phytase of *Penicillium oxalicum* EUFR-3 isolated from Himalayan region. Nusantara Biosci 9:68–76

Kemme PA, Jongbloed AW et al (1997) The efficacy of *Aspergillus niger* phytase in rendering phytate phosphorus available for absorption in pigs in influenced by pig physiological status. J Anim Sci 75:2129–2138

Khare SK, Jha K, Gupta MN (1994) Entrapment of wheat phytase in polyacrylamide gel & its application in soy milk phytate hydrolysis. Biotechnol Appl Biochem 19:193–198

Klosowski G, Mikulski D, Jankowiak O (2018) Extracellular phytase production by the wine yeast *S. cerevisiae* (Finarome Strain) during submerged fermentation. Molecules 23(4):848

Konietzny U, Greiner R (2002) Molecular & catalytic properties of phytate-degrading enzymes (phytases). Int J Food Sci Technol 37:791–812

Konietzny U, Greiner R (2003) Phytic acid: nutritional impact. In: Caballero B, Trugo L, Finglas P (eds) Encyclopedia of food science and nutrition. Elsevier, London, pp 4555–4563

Konietzny U, Greiner R, Jany KD (1995) Purification and characterization of a phytase from spelt. J Food Biochem 18:165–183

Kumari A, Satyanarayana T, Singh B (2016) Mixed substrate fermentation for enhanced phytase production by thermophilic mould *Sporotrichum thermophile* & its application in beneficiation of poultry feed. Appl Biochem Biotechnol 178:197–210

Lassen SF, Breinholt J et al (2001) Expression, gene cloning, & characterization of five novel phytases from four basidiomycete fungi: *Peniophora lycii*, *Agrocybe pediades*, a *Ceriporia* sp. & *Trametes pubescens*. Appl Environ Microbiol 67:4701–4707

Laumen K, Ghisalba O (1994) Preparative scale chemo enzymatic synthesis of optically pure D-*myo*-inositol 1-phosphate. Biosci Biotechnol Biochem 58:2046–2049

Lei X, Stahl C (2001) Biotechnological development of effective phytases for mineral nutrition & environmental protection. Appl Microbiol Biotechnol 57:474–481

Lei X, Ku PK et al (1993) Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. J Nutr 123:1117–1123

Lei XG, Weaver JD et al (2013) Phytase, a new life for an 'Óld' enzyme. Ann Rev Anim Biosci 1:283–309

Li J, Hegeman CE et al (1997) Secretion of active recombinant phytase from soybean cell-suspension cultures. Plant Physiol 114:1103–1111

Li R, Lu W et al (2012) Molecular characterization & functional analysis of OsPHY1, a purple acid phosphatase (PAP)-type phytase gene in rice (*Oryza sativa* L.). J Integr Agric 11:1217–1226

Liebert F, Portz L (2007) Different sources of microbial phytase in plant based low phosphorus diets for Nile tilapia *Oreochromis niloticus* may provide different effects on phytate degradation. Aquaculture 267:292–299

Lucca P, Hurrell R, Potrykus I (2002) Fighting iron deficiency anaemia with iron-rich rice. J Am Coll Nutr 21:184S–190S

Mak WC, Ng YM et al (2004) Novel biosensors for quantitative phytic acid & phytase measurement. Biosens Bioelectron 19:1029–1035

Maller A, Mota AMDO et al (2014) Fermentation pH in stirred tank and air-lift bioreactors affects phytase secretion by *Aspergillus japonicus* differently but not the particle size. Biocatal Biotransformation 32(1):39–44

Mandviwala TN, Khire JM (2000) Production of high activity thermo stable phytase from thermo tolerant *Aspergillus niger* in solid state fermentation. J Ind Microbiol Biotechnol 24:237–243

Matsui T, Nakagawa Y et al (2000) Efficacy of yeast phytase in improving phosphorus bioavailability in a corn-soybean meal-based diet for growing pigs. J Anim Sci 78:94–99

Maurya AK, Parashar DP, Satyanarayana T (2017) Bioprocess for the production of recombinant HAP phytase of the thermophilic mold *Sporotrichum thermophile* & its structural & biochemical characteristics. Int J Biol Macromol 94:36–44

Modlin M (1980) Urinary phosphorylated inositols & renal stone. Lancet 2:1113–1114

Mullaney EJ, Daly CB, Ullah AHJ (2000) Advances in phytase research. Adv Appl Microbiol 47:157–199

Mullen M (2005) Phosphorus in soils: biological interactions. In: Hillel D (ed) Encyclopaedia of soils in the environment. Elsevier Ltd., Oxford, pp 210–215
Murlidhar RV, Panda T (2000) Fungal protoplast fusion-a revisit. Bioprocess Biosyst Eng 22 (5):429–431
Nampoothiri KM, Tomes GJ et al (2004) Thermostable phytase production by *Thermoascus aurantiacus* in submerged fermentation. Appl Biochem Biotechnol 118(1–3):205–214
Navert B, Sandstrom B, Cederblad A (1985) Reduction of the phytate content of bran by leavening in bread & its effect on zinc absorption in man. Br J Nutr 53:47–53
Neira-Vielma AA, Aguilar CN et al (2018) Optimized production of phytase by solid-state fermentation using triticale as substrate & source of inducer. Afr J Biotechnol 17:81–90
Nelson TS (1967) The utilization of phytate phosphorus by poultry. Poult Sci. 46:862–871
Nelson TS, Shieh TR et al (1968) The availability of phytate phosphorus in soybean meal before & after treatment with a mold phytase. Poult Sci. 47:1842–1848
Nelson TS, Sheih TR et al (1971) Effect of supplemental phytase on the utilization of phytate phosphorus by chicks. J Nutr 101:1289–1294
Olstorpe M, Schnurer J, Passoth V (2009) Screening of yeast strains for phytase activity. FEMS Yeast Res 9:478–488
Pable A, Gujar P, Khire JM (2014) Selection of phytase producing yeast strains for improved mineral mobilization and dephytinization of chickpea flour. J Food Biochem 38(1):18–27
Pandey A, Szakacs G et al (2001) Production, purification & properties of microbial phytases. Bioresour Technol 77:203–214
Pasamontes L, Haiker M et al (1997) Cloning of the phytases from *Emericella nidulans* & the thermophilic fungus *Talaromyces thermophilus*. Biochim Biophys Acta 1353:217–223
Pen J, Verwoerd TC et al (1993) Phytase-containing transgenic seeds as a novel feed additive for improved phosphorus utilization. Nat Biotechnol 11:811–814
Pires EBE, de Freitas AJ et al (2019) Production of fungal phytases from agroindustrial byproducts for pig diets. Sci Rep 9:9256
Ponstein AS, Bade JB et al (2002) Stable expression of phytase (*phyA*) in canola (*Brassica napus*) seeds: towards a commercial product. Mol Breeding 10:31–44
Potter SM (1995) Overview of proposed mechanisms for the hypercholasterolemic effect of soy. J Nutr 125:606S–611S
Puhl AA, Greiner R, Selinger LB (2008) A protein tyrosine phosphatase-like inositol polyphosphatase from *Selenomonas ruminantium* subsp. *lactilytica* has specificity for the 5-phosphate of *myo*-inositol hexakisphosphate. Int. J Biochem Cell Biol 40:2053–2064
Puhl AA, Greiner R, Selinger LB (2009) Stereospecificity of *myo*-inositol hexakisphosphate hydrolysis by a protein tyrosine phosphatase-like inositol polyphosphatase from *Megasphaera elsdenii*. Appl Microbiol Biotechnol 82:95–103
Puppala KR, Naik T et al (2018) Evaluation of *Candida tropicalis* (NCIM 3321) extracellular phytase having plant growth promoting potential & process development. Biocatal Agric Biotechnol 13:225–235
Quan CS, Fan SD, Ohta Y (2003) Immobilization of *Candida krusei* cells producing phytase in alginate gel beads: an application of the preparation of *myo*-inositol phosphates. Appl Microbiol Biotechnol 62:41–47
Quan CS, Tian WJ et al (2004) Purification & properties of a low-molecular-weight phytase from *Cladosporium* sp. FP-1. J Biosci Bioeng 97:260–266
Ramachandran S, Krishnan R et al (2005) Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using oil cakes as substrates. Process Biochem 40(5):1749–1754
Rani R, Ghosh S (2011) Production of phytase under solid-state fermentation using *Rhizopus oryzae*: novel strain improvement approach & studies on purification & characterization. Biores Technol 102(22):10641–10649
Rani R, Kumar S, Ghosh S (2014a) Optimization of aqueous extraction process to enhance the production of phytase by *Rhizopus oryzae* using response surface methodology coupled with artificial neural network. Afr J Biotechnol 13(7):874–883

Rani R, Arora S et al (2014b) Optimization of medium components for the production of phytase by *R. oryzae* using statistical approaches. J Bioremed Biodegred. https://doi.org/10.4172/2155-6199.S18-003

Ranjan K, Sahay S (2013) Identification of phytase producing yeast & optimization & characterization of extracellular phytase from *Candida parapsilosis*. Int J Sci Nat 4:583–590

Ranjan B, Satyanarayana T (2016) Recombinant HAP phytase of the thermophilic mould *Sporotrichum thermophile*: expression of the codon-optimized phytase gene in *Pichia pastoris* & applications. Mol Biotechnol 58:137–147

Ranjan B, Singh B, Satyanarayana T (2015) Characteristics of recombinant phytase (rSt-Phy) of the thermophilic mould *Sporotrichum thermophile* & its applicability in dephytinizing foods. Appl Microbiol Biotechnol 177:1753–1766

Reddy CS, Vani K et al (2013) Manipulating microbial phytases for heterologous expression in crops for sustainable nutrition. Ann Plant Sci 2:436–454

Richardson NL, Higgs DA et al (1985) Influence of dietary calcium, phosphorus, zinc & sodium phytate level on cataract incidence, growth & histopathology in juvenile chinook salmon (*Oncorhynchus tshawytscha*). J Nutr 115:553–567

Richardson AE, Hadobas PA, Hayes JE (2001) Extracellular secretion of *Aspergillus* phytase from *Arabidopsis* roots enables plants to obtain phosphorus from phytate. Plant J 25:641–649

Rodehutscord M, Pfeffer E (1995) Effects of supplemental microbial phytase on phosphorus digestibility & utilization in rainbow trout (*Oncorhynchus mykiss*). Water Sci Technol 31 (10):143–147

Roopashri AN, Varadaraj MC (2015) Functionality of phytase of *Saccharomyces cerevisiae* MTCC 5421 to lower inherent phytate in selected cereal flours & wheat/pearl millet-based fermented foods with selected probiotic attribute. Food Biotechnol 29:131–155

Roopesh K, Ramachandran S et al (2006) Comparison of phytase production on wheat bran & oilcakes in solid-state fermentation by *Mucor racemosus*. Bioresour Technol 97(3):506–511

Rosell CM, Santos E et al (2009) Wholemeal wheat bread: a comparison of different breadmaking processes & fungal phytase addition. J Cereal Sci 50:272–277

Rumsey GL (1993) Fish meal & alternate source of protein in fish feeds: update. Fisheries 18:14–19

Sabu A, Sarita S et al (2002) Solid-state fermentation for production of phytase by *Rhizopus oligosporus*. Appl Biochem Biotechnol 102:251–260

Sandberg AS, Hulthen LR, Turk M (1996) Dietary *Aspergillus niger* phytase increases iron absorption in humans. J Nutr 126(2):476–580

Sands JS, Ragland D et al (2009) Response of pigs to *Aspergillus niger* phytase supplementation of low-protein or high-phytin diets. J Anim Sci 87:2581–2589

Sanni DM, Lawal OT, Enujiugha VN (2019) Purification & characterization of phytase from *Aspergillus fumigatus* isolated from African Giant Snail (*Achatina fulica*). Biocatal Agric Biotechnol 17:225–232

Sapna, Singh B (2013) Improved production of protease-resistant phytase by *Aspergillus oryzae* & its applicability in the hydrolysis of insoluble phytates. J Ind Microbiol Biotechnol 40:891–899

Sapna, Singh B (2014) Phytase production by *Aspergillus oryzae* in solid-state fermentation & its applicability in dephytinization of wheat ban. Appl Biochem Biotechnol 173:1885–1895

Sapna, Singh B (2015) Biocatalytic potential of protease-resistant phytase of *Aspergillus oryzae* SBS50 in ameliorating food nutrition. Biocatal Biotransformation 33:167–174

Sapna, Singh B (2017a) Free & immobilized *Aspergillus oryzae* SBS50 producing protease-resistant & thermostable phytase. 3 Biotech 7:213

Sapna, Singh B (2017b) Purification & characterization of a protease-resistant phytase of *Aspergillus oryzae* SBS50 whose properties make it exceptionally useful as a feed supplement. Int J Biol Macromol 103:458–466

Sariyska MV, Gargova SA et al (2005) *Aspergillus niger* phytase: purification & characterization. Biotechnol Biotechnol Equip 19:98–105

Schafer A, Koppe WM et al (1995) Effects of microbial phytase on the utilization of native phosphorous by carp in a diet based on soybean milk. Water Sci Technol 31(10):149–155

Schenk G, Ge Y et al (1999) Binuclear metal centers in plant purple acid phosphatases: Fe-Mn in sweet potato & Fe-Zn in soybean. Arch Biochem Biophys 370:183–189
Schröder B, Hattenhauer O, Breves G (1998) Phosphate transport in pig proximal small intestines during postnatal development: lack of modulation by calcitriol. Endocrinology 139 (4):1500–1507
Segueilha L, Moulin G, Galzy P (1993) Reduction of phytate content in wheat bran & glandless cotton flour by *Schwanniomyces castellii*. J Agric Food Chem 41:2451–2454
Sethi RP, Subba-Rao NS (1968) Solubilization of tricalcium phosphate & calcium phytate by soil fungi. J Gen Appl Microbiol 14:329–331
Shah P, Bhavsar K et al (2009) Strain improvement & up scaling of phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation conditions. J Ind Microbiol Biotechnol 36:373
Shah PC, Kumar VR et al (2017) Phytase production by *Aspergillus niger* NCIM 563 for a novel application to degrade organophosphorus pesticides. AMB Exp 7:1–11
Shamsuddin AM, Vucenik I (1999) Mammary tumor inhibition by IP6: a review. Anticancer Res 19:36–71
Sharma R, Kumar P et al (2018) A novel protein tyrosine phosphatase like phytase from *Lactobacillus fermentum* NKN51: cloning, characterization & application in mineral release for food technology applications. Bioresour Technol 249:1000–1008
Shivanna GB, Venkateswaran G (2014) Phytase production by *Aspergillus niger* CFR 335 & *Aspergillus ficuum* SGA 01 through submerged & solid-state fermentation. Sci World J 392615:1–6
Simell M, Turunen M et al (1989) Feed and food applications of phytase. In: Lecture at 3rd meet. Industrial applications of enzymes, Barcelona, Spain
Simon O, Igbasan F (2002) In vitro properties of phytases from various microbial origins. J Food Sci Technol 37:813–822
Singh B, Satyanarayana T (2006a) Phytase production by thermophilic mold *Sporotrichum thermophile* in solid-state fermentation & its application in dephytinization of sesame oil cake. Appl Microbiol Biotechnol 133(3):239–250
Singh B, Satyanarayana T (2006b) A marked enhancement in phytase production by a thermophilic mould *Sporotrichum thermophile* using statistical designs in a cost-effective cane molasses medium. J Appl Microbiol 101:344–352
Singh B, Satyanarayana T (2008a) Phytase production by *Sporotrichum thermophile* in a cost-effective cane molasses medium in submerged fermentation & its application in bread. J Appl Microbiol 105:1858–1865
Singh B, Satyanarayana T (2008b) Phytase production by a thermophilic mould *Sporotrichum thermophile* in solid state fermentation & its potential applications. Bioresour Technol 99:2824–2830
Singh B, Satyanarayana T (2008c) Improved phytase production by a thermophilic mould *Sporotrichum thermophile* in submerged fermentation due to statistical designs. Bioresour Technol 99:824–830
Singh B, Satyanarayana T (2009) Characterization of HAP-phytase from a thermophilic mould *Sporotrichum thermophile*. Bioresour Technol 100:2046–2051
Singh B, Satyanarayana T (2010) Plant growth promotion by an extracellular HAP-phytase of a thermophilic mould *Sporotrichum thermophile*. Appl Microbiol Biotechnol 160:1267–1276
Singh B, Satyanarayana T (2012) Production of phytate-hydrolyzing enzymes by thermophilic moulds. Afr J Biotechnol 11:12314–12324
Singh B, Satyanarayana T (2015) Fungal phytases: characteristics & amelioration of nutritional quality & growth of non-ruminants. J Anim Physiol Anim Nutr 99:646–660
Singh B, Kunze G, Satyanarayana T (2011) Developments in biochemical aspects & biotechnological applications of microbial phytases. Biotechnol Mol Biol Rev 6:69–87
Singh N, Kumari A et al (2015) Enhanced cost-effective phytase production by *Aspergillus niger* & its applicability in dephytinization of food ingredients. Microbiology 84:219–226

Singh B, Sharma KK et al (2018) Molecular modeling & docking of recombinant HAP-phytase of a thermophilic mould *Sporotrichum thermophile* reveals insights into molecular catalysis & biochemical properties. Int J Biol Macromol 115:501–508

Singhania RR, Sukumaran RK et al (2010) Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases: review. Enzyme Microb Technol 46:541–549

Siren M (1986a) Stabilized pharmaceutical & biological material composition. Patent no. SE 003 165

Siren M (1986b) New myo-inositol triphosphoric acid isomer. Patent no. SW 052 950

Siren M (1995) Method of treating pain using inositol triphosphate. US Pat. 5407924

Soni SK, Khire JM (2007) Production & partial characterization of two types of phytase from *Aspergillus niger* NCIM 563 under submerged fermentation conditions. World J Microbiol Biotechnol 23:1585–1593

Soni S, Magdum A, Khire J (2010) Purification & characterization of two distinct acidic phytases with broad pH stability from *Aspergillus niger* NCIM 563. World J Microbiol Biotechnol 26:2009–2018

Soni SK, Sarkar S et al (2015) Intrinsic therapeutic & biocatalytic roles of ionic liquid mediated self-assembled platinum-phytase nanospheres. RSC Adv 5(77):62871–62881

Srikanthithasan K, Macelline SP et al (2020) Effect of adding phytase from *Aspergillus niger* to a low phosphorous diet on growth performance, tibia characteristics, phosphorous excretion & meat quality of broilers 35 days after hatching. J Poult Sci 57(1):28–36

Suzuki U, Yoshimura K, Takaishi M (1907) Übereinenzym 'Phytase' das 'anhydro-oxy-methylendiphosphorsaure' spalter. Bull Coll Agric Tokyo Imperial Univ 7:503–512

Tarafdar JC, Marschner H (1995) Dual inoculation with *Aspergillus fumigatus* & *Glomus mosseae* enhances biomass production & nutrient uptake in wheat (*Triticum aestivum* L.) supplied with organic phosphorus as Na-phytate. Plant Soil 173:97–102

Tarafdar JC, Rao AV (1996) Contribution of *Aspergillus* strains to acquisition of phosphorous by wheat (*Triticum aestivum* L.) & chick pea (*Cicer arietinum* Linn.) grown in a loamy sand soil. Appl Soil Ecol 3(2):109–114

Tatala S, Svanberg U, Mduma B (1998) Low dietary iron availability is a major cause of anaemia: a nutrition survey in the Lindi district of Tanzania. Am J Clin Nutr 68:171–178

Tseng YH, Fang TJ, Tseng SM (2000) Isolation & characterization of a novel phytase from *Penicillium simplicissimum*. Folia Microbiol 45:121–127

Turk M, Sandberg AS (1992) Phytate degradation during breadmaking: effect of phytase addition. J Cereal Sci 15:281–294

Turk M, Carlsson NG, Sandberg AS (1996) Reduction in the levels of phytate during wholemeal bread making; effect of yeast & wheat phytases. J Cereal Sci 23:257–264

Turk M, Sandberg AS et al (2000) Inositol hexaphosphate hydrolysis by baker's yeast. Capacity, kinetics & degradation products. J Agric Food Chem 48(1):100–104

Tyagi PK, Tyagi PK, Verma SVS (1998) Phytate phosphorus content of some common poultry feed stuffs. Ind J Poult Sci 33:86–88

Tye AJ, Siu FK et al (2002) Molecular cloning & the biochemical characterization of two novel phytases from *B. subtilis* 168 & *B. licheniformis*. Appl Microbiol Biotechnol 59:190–197

Ullah AHJ, Sethumadhavan K et al (1999) Characterization of recombinant fungal phytase (*phy*A) expressed in tobacco leaves. Biochem Biophys Res Commun 264(1):201–206

Ullah AHJ, Sethumadhavan K et al (2002) Cloned and expressed fungal *phy*A gene in alfalfa produces a stable phytase. Biochem Biophys Res Commun 290(4):1343–1348

Ullah AHJ, Sethumadhavan K et al (2003) Fungal phyA gene expressed in potato leaves produces active & stable phytase. Biochem Biophys Res Commun 306:603–609

Ullah AH, Sethumadhavan K, Mullaney EJ (2011) Vanadate inhibition of fungal *Phy*A & bacterial *App*A2 histidine acid phosphatases. J Agric Food Chem 59(5):1739–1743

Van de Velde F, Konemann L et al (2000) The rational design of semisynthetic peroxidases. Biotechnol Bioeng 67:87–96

Vats P, Banerjee UC (2002) Studies on production of phytase by a newly isolated strain of *A. niger* van Tieghem obtained from rotten wood-logs. Process Biochem 38:211–217
Vats P, Banerjee UC (2005) Biochemical characterization of extracellular phytase (*myo*-inositol hexakisphosphate phosphohydrolases) from a hyperproducing strain of *A. niger* van Tieghem. J Ind Microbiol Biotechnol 32:141–147
Vats P, Banerjee UC (2006) Catalytic characterization of phytase (*myo*- inositol hexakisphosphate phosphohydrolases) from *A. niger* van Tieghem: glycosylation pattern, kinetics & molecular properties. Enzyme Microb Technol 39:596–600
Vats P, Sahoo DK, Banerjee UC (2004) Studies on the production of phytase (*myo*-inositol hexakisphosphate phosphohydrolase) by *A. niger* van Tieghem in laboratory scale fermenter. Biotechnol Prog 20:737–743
Vats P, Bhushan B, Banerjee UC (2009) Studies on the dephosphorylation of phytic acid in livestock feed using phytase from *Aspergillus niger* van Tieghem. Bioresour Technol 100:287–291
Veide J, Andlid T (2006) Improved extracellular phytase activity in *Saccharomyces cerevisiae* by modifications in the *PHO* system. Int J Food Microbiol 108(1):60–67
Verma D, Satyanarayana T (2012) Phytase production by the unconventional yeast *Pichia anomala* in fed batch & cyclic fed batch fermentations. Afr J Biotechnol 11(72):13705–13709
Vohra A, Satyanarayana T (2001) Phytase production by the yeast *Pichia anomala*. Biotechnol Lett 23:551–554
Vohra A, Satyanarayana T (2002a) Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomala*. Process Biochem 37:999–1004
Vohra A, Satyanarayana T (2002b) Purification & characterization of the thermostable & acid stable phytase from *Pichia anomala*. World J Microbiol Biotechnol 18:687–691
Vohra A, Satyanarayana T (2003) Phytases: microbial sources, production, purification, & potential biotechnological applications. Crit Rev Biotechnol 23(1):29–60
Vohra A, Satyanarayana T (2004) A cost-effective cane molasses medium for enhanced cell-bound phytase production by *Pichia anomala*. J Appl Microbiol 97:471–476
Vohra A, Rastogi SK, Satyanarayana T (2006) Amelioration in growth & phosphate assimilation of poultry birds using cell-bound phytase of *Pichia anomala*. World J Microbiol Biotechnol 22:553–558
Vohra A, Kaur P, Satyanarayana T (2011) Production, characteristics & applications of the cell-bound phytase of *Pichia anomala*. Antonie van Leeuwenhoek 99:51–55
Walton J, Gray TK (1979) Absorption of inorganic phosphate in the human small intestine. Clin Sci (Lond) 56:407–412
Wang Y, Ye X et al (2013) Overexpression of *phy*A & *app*A genes improves soil organic phosphorus utilization & seed phytase activity in *Brassica napus*. PLoS One 8(4):e60801
Wang ZP, Deng LH et al (2017) Transgenic rice expressing a novel phytase-lactoferricin fusion gene to improve phosphorus availability and antibacterial activity. J Integr Agric 16(4):774–788
Wodzinski RJ, Ullah AHJ (1996) Phytase. Adv Appl Microbiol 42:263–302
Wyss M, Brugger R et al (1999) Biochemical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolases): catalytic properties. Appl Environ Microbiol 65:367–373
Yadav RS, Tarafdar JC (2003) Phytase & phosphatase producing fungi in arid & semi-arid soils & their efficiency in hydrolyzing different organic P compounds. Soil Biol Biochem 35:1–7
Yao M-Z, Zhang Y-H et al (2012) Phytases: crystal structures, protein engineering & potential biotechnological applications. J Appl Microbiol 112:1–14
Yi Z, Kornegay ET et al (1996) Improving phytate phosphorus availability in corn & soybean meal for broilers using microbial phytase & calculations of phosphorus equivalency values for phytase. Poult Sci 75:240–249
Zhang L, An L et al (2003) Properties of *A. ficuum* AS3. 324 phytase expressed in tobacco. Process Biochem 40:213–216

Zhang GQ, Dong XF et al (2010) Purification, characterization, & cloning of a novel phytase with low pH optimum & strong proteolysis resistance from *Aspergillus ficuum* NTG-23. Bioresour Technol 101:4125–4131

Zhang GQ, Chen QJ et al (2013a) Purification & characterization of a novel acid phosphatase from the split gill mushroom *Schizophyllum commune*. J Basic Microbiol 53:868–875

Zhang GQ, Wu YY et al (2013b) A phytase characterized by relatively high pH tolerance & thermostability from the shiitake mushroom *Lentinus edodes*. Biomed Res Int 2013:1–7

Zhang F, Meng X et al (2016) Hydrolytic amino acids employed as a novel organic nitrogen source for the preparation of PGPF-containing bioorganic fertilizer for plant growth promotion & characterization of substance transformation during BOF production. PLoS One 11(3): e0149447

Zhu MJ, Wang HW, Ng TB (2011) Purification & identification of a phytase from fruity bodies of the winter mushroom, *Flammulina velutipes*. Afr J Biotechnol 10:17845–17852

Fungi in Biofuel Research

5

Nandita Pasari and Syed Shams Yazdani

Abstract

Increased energy consumption across the globe has led to a surge in the global demand for an alternative renewable energy source such as biofuels. Biofuels commonly include alcohol-based molecules such as bioethanol or esters of fatty acids called biodiesel. The production of the first and second generation of biofuels is often based on the principle of conversion of a long-chain glucose polymer to monomers, which can be fermented into ethanol. Fungi are primary decomposers that secrete exoenzymes to degrade complex biomass into monomeric sugars. These consortia of enzymes consist of all the hydrolytic enzymes such as cellulases, hemicellulases, ligninolytic enzymes, pectinases, amylases, and lipases. Fungi can secrete these enzymes in copious amounts in the supernatant, which are easier to recover and can be used for industrial purposes. Filamentous fungi such as species of *Trichoderma*, *Penicillium*, and *Aspergillus* have been used for cellulolytic enzyme production and biomass hydrolysis for a long time. Enzyme production in these fungi is closely regulated so that energy is not wasted by the cells. For industrial use and cheaper enzyme production, these strains are constantly being improved in terms of enzyme production using

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random as well as targeted approaches. India is becoming a hub for industrial enzyme manufacturing and marketing. Many companies like Novozyme, Advanced Enzymes, Aumgene Biosciences, and MAPS Enzymes Ltd. have been producing fungal enzymes in India. The Government of India is also sponsoring various projects for the development of enzyme technology for biofuel production. We will discuss in this chapter the utility of fungi globally in the development of first- and second-generation biofuels, with a specific focus on the Indian scenario.

Keywords

Biomass · Saccharification · Filamentous fungi · Industrial enzymes · Bioethanol · Biodiesel

5.1 Introduction

With the increase in world population and steep growth in industrialization, energy consumption has increased steadily over the last century. The United States was reported to be the largest consumer of crude oil followed by China and India in the 2019 energy statistics presented by the Government of India. The rate of energy consumption has been increasing at a fast pace in India due to population and economic growth. With the population moving towards urbanization and having improved living standards, the demand is likely to rise a lot in the coming years as well. In terms of crude oil production, India accounts for less than 1% of the world's total production but is the third-largest consumer after the United States and China. Because of this huge gap in production and consumption, India's energy need is mainly met by import. Also, the emissions and wastes associated with the burning of fossil fuels have seriously impacted the climate due to the greenhouse effect (Von Blottnitz and Curran 2007). Limited availability of fossil fuels and disruption in the supply of fuel has led to a surge in the demand for an alternate energy source (Gowen 1989). This increased energy demand has urged the scientific community to search for renewable and sustainable alternatives. One of the best alternative energy sources to petroleum-based fuels has been biofuels, which are produced using renewable resources. Biofuels are environment-friendly and are considered to be "green fuels." The utilization of biofuels will lead to reduced carbon emissions that will have a carbon-neutral impact (Graham-Rowe 2008). There is a positive correlation between the use of fossil fuels and the increase in atmospheric greenhouse gases such as carbon dioxide, which contributes to global warming. A country like India, where agricultural residues are available in surplus, should use plant biomass-derived biofuel because of the significant impact it will make in mitigating air pollution by lowering the greenhouse gas emissions (Matsuoka et al. 2009). Numerous other advantages are associated with biofuels that are likely to bridge the gap between food, fodder, and fuel security**.** The biofuel production process is labor-intensive and thus can bring about rural employment by generating new jobs. This will pave the

way for new areas of income for farmers bringing about economic development in rural areas. Eventually, investment in this sector will bring economic development with the creation of new jobs and income for farmers. Besides, the generation of biofuels from wastes helps in minimizing the waste and puts it into the best use, a good example of "wealth from wastes."

5.2 Types of Biofuels

Biofuels are of diverse kinds such as biodiesel, bioethanol, biohydrogen, biomethane, etc. As explained in Fig. 5.1, they have been categorized as conventional and advanced biofuels based on their production processes. Conventional biofuels are also known as the first generation of biofuels, which are produced by the conversion of starch into sugars primarily from edible grains. The sugars obtained are then fermented into bioethanol using yeast (Balan 2014). On the other hand, biodiesel is produced by the transesterification of vegetable oils. Since the production of biofuel involves edible feedstocks, vast swaths of farmlands would be utilized, which would drive up the food prices. This is especially true in the case

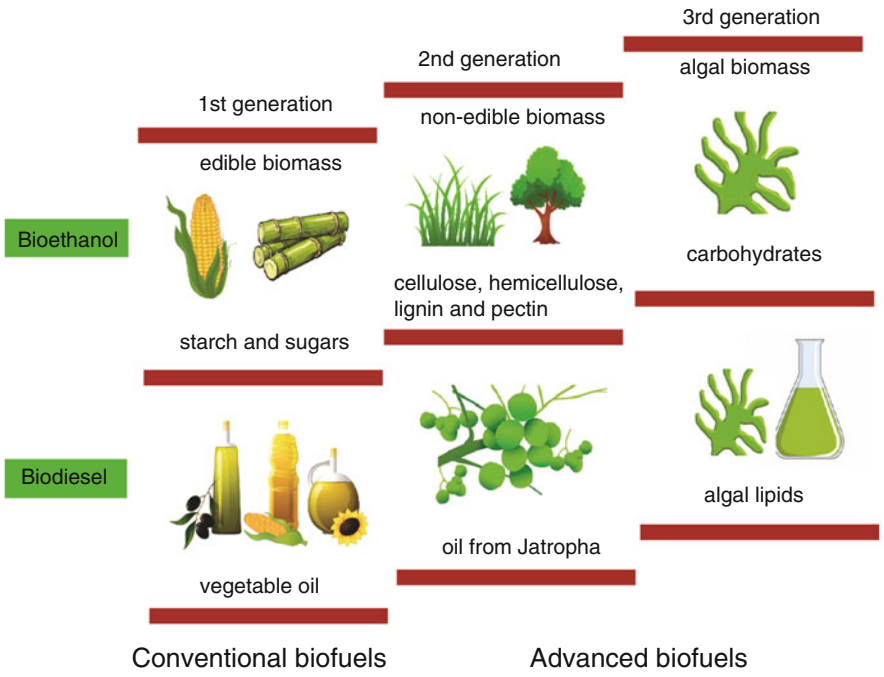

Fig. 5.1 Introduction to the different types of biofuels. Conventional fuels also called first-generation biofuels are generated from edible biomass, whereas second-generation biofuels are generated from nonedible biomass. The third-generation biofuel is recovered from photosynthetic microorganisms like algae

of India, where the land available for cultivation is limited and the majority of the country's population is dependent on it for food. This led to the production of advanced biofuels like the second and the third generation of biofuels. The second generation of biofuel is generated from nonedible feedstock such as lignocellulosic biomass or nonedible oils like *Jatropha* oil. Renewable lignocellulosic biomass, besides being cheap and abundant, also has the advantage that it does not compete with food production (DiPardo 2000; Yuan et al. 2008). They appear to be promising in terms of their net energy benefits and their ability to maintain CO_2 balance. The use of lignocellulosic polymers for bioethanol production will reduce not only CO_2 emissions but also the dependence on fossil fuels. Extensive research has led to the adoption of several methodologies to convert lignocellulosic materials to ethanol in the last two decades. Alternate studies for determining other potential feedstocks paved the way for the third generation of biofuel (Brennan and Owende 2010). Microalgae are photosynthetic microorganisms that require mainly light and carbon dioxide to produce a large number of lipids and carbohydrates (Nigam and Singh 2011). These carbohydrates and lipids can be processed into different biofuels and other valuable co-products. Microalgae thus have been used as feedstock for the production of the third generation of biofuels. In comparison with lignocellulosic biomass, the algal biomass has low lignin and hemicellulose content and is therefore considered suitable for bioethanol production (Behera et al. 2015; Chen et al. 2013).

5.3 National Policy

India is one of the fastest-growing economies in the world. With a steep rise in population and improving standards of living, energy consumption has been increasing at a relatively fast rate. A major requirement for transportation fuels is being met by petroleum-based oils (Kumar et al. 2015). Domestic crude oil can meet only about 23% of the demand. The rest of the oil is being imported, for which India has to spend hard-earned foreign exchange (Babu and Nautiyal 2012). To meet the rising demand, the Government of India made various plans and policies in the energy sector, including the consideration of renewable energy resources for power generation. India is bestowed with renewable energy resources which are abundant, sustainable, non-polluting, and inexhaustible. Human beings have been utilizing these energy sources for domestic purposes since prerecorded history, but a policy push happened about two decades ago.

With the drop in oil supplies after the global twin oil shocks in the 1970s, India started its biofuel production. The Government of India resolved to supply 5% ethanol-blended petrol in nine sugarcane-growing States and four Union Territories with effect from January 1, 2003 (Government of India 2018). The government policy promoted the production and use of biofuels as a substitute for petrol and diesel to meet the increasing demand. This policy also aimed to facilitate the development of biofuel by utilizing indigenous biomass feedstocks as depicted in Fig. 5.2, such as:

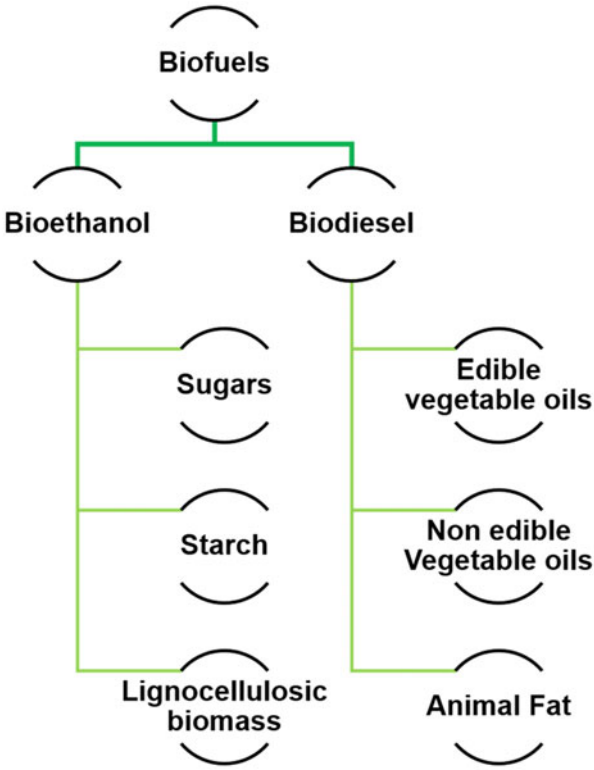

Fig. 5.2 Feedstock for two different forms of biofuels: bioethanol and biodiesel. The figure depicts different biological components used for biofuel production

(a) *Bioethanol*: Ethanol is produced mainly from the hydrolysis and fermentation process of sugar, starch, or cellulosic-containing materials. Sugar crops, such as sugarcane, sugar beet, and sweet sorghum, and starch-containing materials, such as corn and cassava, are used as feedstocks for conventional biofuel production. Biomass wastes such as bagasse, wood waste, and agricultural and forestry residues containing a complex mixture of cellulose, hemicellulose, and lignin are used for the production of advanced biofuels.
(b) *Biodiesel*: It is the fuel produced by transesterification of fatty acids obtained from both edible and nonedible vegetable oils, animal fat, or algal lipids.

Sugarcane, being a good source of renewable biofuel in terms of ethanol generated from its sugar, was developed as an alternative feedstock. Ethanol has been mainly produced from molasses, which is a by-product of the sugar industry. To further escalate ethanol production, sugarcane juice is now being used directly from the sugar industry (Talukdar et al. 2017). Oil Marketing Companies (OMCs) have already begun blending 5% of ethanol into gasoline in 20 States and 4 Union Territories. In October 2008, the Government of India made a 10% mandatory blending of ethanol with gasoline. However, policy-makers soon realized that using food crops as feedstock for biofuel production could shoot up the food prices

in India. Also, India's petrol demand rose to 27 billion liters in 2015–2016, for which 10% blending would be 2.7 billion liters. Since India did not want to divert farmland for growing feedstock for biofuel production, policy-makers made changes for considering renewable energy resources such as lignocellulosic biomass for energy generation. The Ministry of New & Renewable Energy of India has developed many projects and policies to promote the utilization of lignocellulosic biomass for biofuel production. Various subsidies and incentives have been provided by the government in this sector. This will not only secure our energy demands, but new employment opportunities will also be created. The current process for the conversion of lignocellulosic biomass to biofuels is expensive, but with technological advances, the costs will come down (Balan 2014).

5.4 Fungal Potential

Fungi are primitive organisms of great importance and have an immense role to play in different biofuel production processes. They are one of the most diverse kingdoms of eukaryotes with an estimated 5.1 million fungal species that exist on this planet (Chambergo and Valencia 2016; Hawksworth and Lücking 2017). Fungi can survive and adapt to extreme environmental conditions, which makes them widespread in different habitats and ecosystems on the Earth. The ability to survive odd environmental conditions is primarily due to the enclosure of fungal cells in thick and rigid cell wall made up of chitin. Chitin is a homopolymer of β-1-4-linked *N*-acetylglucosamine residues, and these β-linkages bring about the rigidity of the cell wall. Fungi are obligate heterotrophs and acquire carbohydrates and other nutrients from animals, plants, or decaying organic matter on which they live. It secretes a consortium of enzymes based on the requirement for the digestion of complex molecules such as starch, cellulose, lignin, or pectin present in its vicinity. It digests the complex molecules extracellularly and absorbs digested simpler products like glucose through its cell wall. They play a critical role in the global carbon cycle by recycling the photosynthetically fixed carbon (Buee et al. 2009). About 10^{11} tons of monosaccharides are released annually by this process, allowing the reflux of the fixed carbon in the environment (Brown and Jurasek 1979). Such inherent features make fungi a fascinating group of organisms whose potential should be exploited for industrial use (Hyde et al. 2019).

The importance of fungi in biotechnological processes dates back to prehistoric times. Fungi have been used in diverse industrial processes to obtain a variety of products. Fungal enzymes have attracted attention for industrial applications because of several reasons. Firstly, fungi secrete a copious amount of enzymes into the culture medium, making downstream processing easier and cost-effective (de Souza et al. 2015). Secondly, these extracellular enzymes are robust enough to survive harsh environmental conditions, making them ideal candidates for industrial use. Thirdly, most of the fungi grow in filamentous forms, allowing easy separation of cells from the supernatant. More than half of the enzymes used in biofuel, therapeutics, pulp and paper, textile, detergent, food, and feed industries are of fungal origin.

Table 5.1 Classification of industrially important fungal enzymes

Enzyme class	Function	Fungal enzymes
EC1	Oxidoreductases	Laccase, peroxidase, catalase, glucose oxidase
EC2	Transferases	Glucosyltransferase, transglutaminase
EC3	Hydrolases	Amylase, glucosidase, cellulase, β-glucanase, lactase, lipase, mannanase, pectin methyl esterase, pectinase, phospholipase, phytase, protease, pullulanase, xylanase
EC4	Lyases	Pectate lyase, α-acetolactate decarboxylase
EC5	Isomerases	Glucose isomerase
EC6	Ligases	Not used at present
EC7	Translocases	Not used at present

The majority of the industrially important enzymes are obtained from the fungi belonging to genera *Aspergillus*, *Trichoderma*, *Penicillium*, and *Rhizopus.* According to the Association of Manufacturers and Formulators of Enzyme Products (AMFEP), fungi belonging to the genus *Aspergillus* produce a maximum number of industrially important enzymes accounting for almost 25% of the total industrially important enzymes.

Some of the industrially important fungal enzymes have been classified according to the standard enzyme classification system maintained by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, which are presented in Table 5.1.

5.5 Fungal Secretory Enzymes

Fungi secrete copious amounts of hydrolyzing enzymes capable of breaking down polysaccharides, lipids, and proteins into smaller molecules. These hydrolytic enzymes are classified based on their function as depicted in Fig. 5.3. Cellulases hydrolyze the β-1,4-glycosidic linkages of the glucose polymer present in cellulose. Hemicellulases are a group of enzymes that can digest the heteropolysaccharides of xylose, mannose, glucose, galactose, and arabinose present in hemicellulose. Ligninolytic enzymes or lignin-degrading enzymes can modify and degrade the aromatic aldehydes of lignin. Accessory enzymes accelerate the cleavage of recalcitrant polysaccharides using an oxidative mechanism. Accessory enzyme GH61 and AA9 are exclusively present in fungi. Pectinases are involved in the breakdown of pectin that are polymers of poly-α-(1,4)-galacturonic acid. Amylases are starch-degrading enzymes that can hydrolyze amylose and amylopectin. Lipases are hydrolytic enzymes that can function as triacylglycerol acyl hydrolases. Proteases convert complex proteins into smaller peptides or amino acids. Phytases are a specific group of phosphatases that are capable of phytate hydrolysis with the formation of lower phosphorylated inositol derivatives.

The enzyme classes involved in biofuel production processes are described in more detail below.

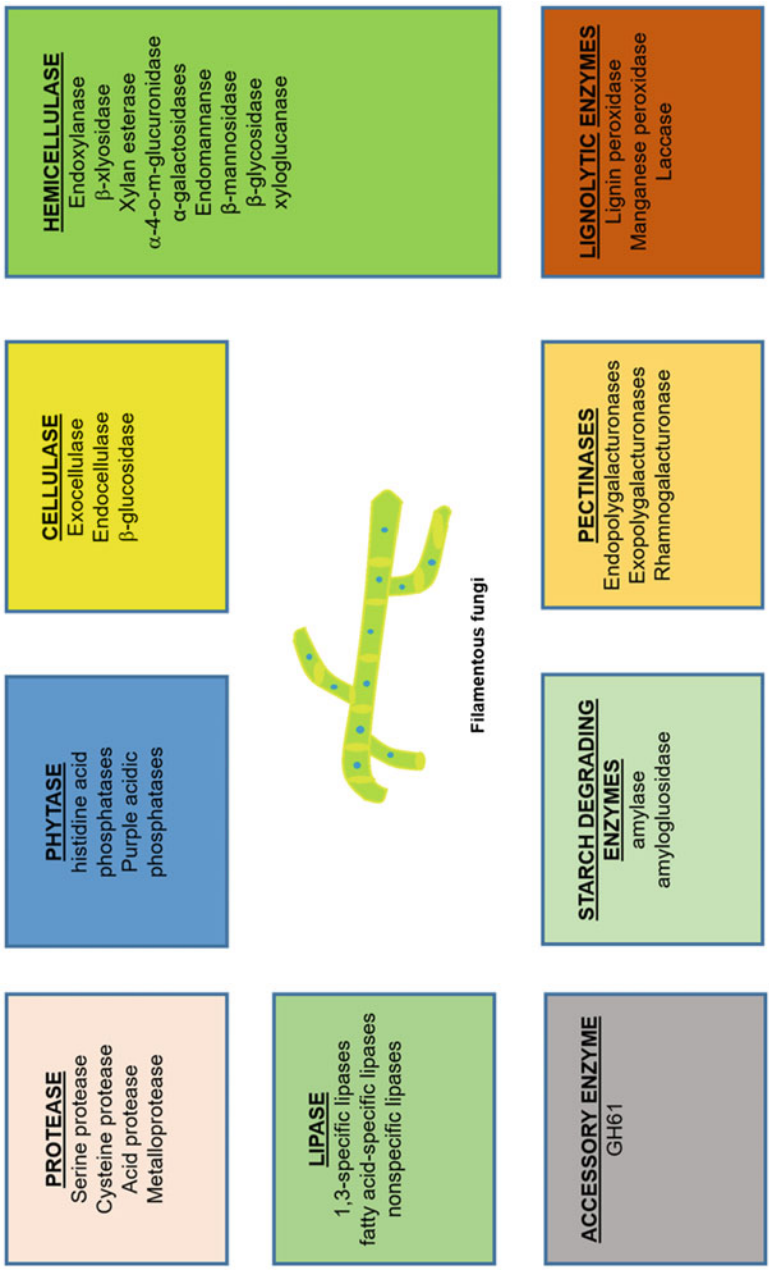

Fig. 5.3 Different classes of hydrolytic enzymes secreted by filamentous fungi

5.5.1 Cellulases

The major component of the plant cell wall is cellulose, which is a linear polysaccharide of β-1,4-linked glucose moieties. The complete digestion of cellulose to glucose requires the action of three main enzymes: cellobiohydrolases, endoglucanases, and β-glucosidases. Endoglucanase hydrolyzes the β1–4-linked internal bonds present in amorphous cellulose regions to release new terminal ends. Cellobiohydrolases (CBHs) act on the chain ends and efficiently degrade crystalline cellulose as compared to amorphous cellulose. The dimers of glucose (cellobiose) released by the action of CBHs and EGs are hydrolyzed by β-glucosidases. These enzymes along with the structural details and biological functions have been described below.

5.5.1.1 Cellobiohydrolase (CBHI)

One of the most important enzymes in cellulose hydrolysis has been CBHI. It is one of the major components of most of the commercially used fungal enzyme cocktails (Ogunmolu et al. 2017). One of the best-characterized cellulases has been *T. reesei* GH7 family cellobiohydrolase known as Cel7A. Cel7A has a modular organization and is composed of family 1 CBM linked to the GH7 catalytic domain via an O-glycosylated linker. The primary structure of TrCel7A (PDB ID: 1CEL) is a large β-sandwich formed by two large antiparallel β-sheets shown in blue in Fig. 5.4. The catalytic domain (CD) of Cel7a is a large structure containing a 50 Å tunnel for threading cellulose chains. Cel7a action on cellulose involves its binding

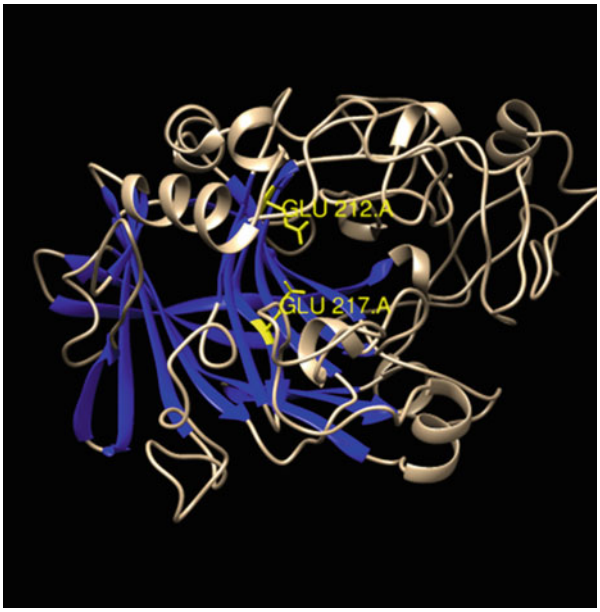

Fig. 5.4 Structural representation of *Trichoderma reesei* Cel7A (PDB ID: 1CEL). The β-sandwich formed by two large antiparallel β-sheets is shown in blue. The catalytic residues Glu^{212} and Glu^{217} are represented in yellow

to the biomass, hydrolysis of the chain end, and reformation of the catalytically active complex (Chundawat et al. 2011).

It employs a two-step retaining catalytic mechanism, which utilizes the glutamate residues—Glu^{212} and Glu^{217}—present at the catalytic site. Glu^{212} acts as a nucleophile for the first step of glycosylation. The other Glu^{217} residue donates a proton to the glycosidic oxygen obtained in the first step and in turn removes a proton from a water molecule. *Penicillium funiculosum* (NCIM 1228) Cel7A has been identified to be an important enzyme in terms of its hydrolytic activity and abundance in the secretome (Ogunmolu et al. 2015). Its structural comparison with TrCel7A showed the conservation of essential amino acid residues, though variations were observed in the regions enclosing the catalytic pathway, indicating the easier entrance of cellulosic substrates to the active site tunnel. Also, a higher tolerance level of PfCel7A toward its product cellobiose was observed (Ogunmolu et al. 2017). In terms of specific activity, catalytic efficiency, and tolerance to inhibitors, PfCel7A showed superiority. The studies show that PfCel7A can be a possible replacement for TrCel7A in industrial cellulase cocktails.

5.5.1.2 Endoglucanase

Endoglucanase is one of the key enzymes in the depolymerization of cellulose. It randomly cleaves the internal β-(1–4)-bonds of the cellulose chains, producing smaller polysaccharide units. In this process, they use a double-displacement retaining mechanism. These endoglucanases belong to the GH5 class, which includes enzymes with reported activities of 1,6-galactanase, 1,3-mannanase, 1,4-xylanase, and xyloglucanase.

The first solved structure of a fungal Cel5A was from *Thermoascus aurantiacus* (PDB ID: 1GZJ) (Leggio and Larsen 2002). It is a multimodular enzyme composed of a catalytic domain associated with the CBM via a glycosylated linker. Its tertiary structure comprising of the canonical $(\beta/\alpha)_8$ fold is depicted in Fig. 5.5, where the α-helices are represented in green and the β-sheets in red. The EGs are capable of binding up to celloheptaose oligomer within the −4 to +3 binding sites. The enzyme active site is a wide and shallow groove lined with aromatic residues Trp278, Trp279, Trp273, Trp170, Trp174, and Tyr200 represented in yellow in Fig. 5.5 (Leggio and Larsen 2002). Of these residues, Trp273 at the −1 binding site is strictly conserved, and the aromatic residues near the +1 and + 2 binding sites are spatially conserved throughout GH5s, suggesting functional relevance.

The major endoglucanase secreted by the model fungus *T. reesei* is *Cel5A*. It accounts for up to 55% of the total EG activity of *T. reesei* and is a key component of many industrial biomass conversion cocktails (Chen and Wang 2017). Structurally its closest homolog is *Thermoascus aurantiacus* Cel5A. In terms of structural stability, *T. reesei Cel5A* has a stable structure as it has four disulfide bonds compared to a single disulfide bond in *Thermoascus aurantiacus* Cel5A (Payne et al. 2015).

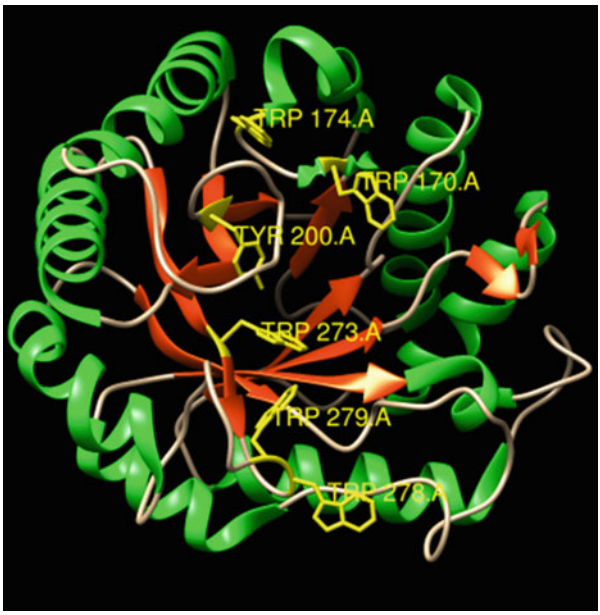

Fig. 5.5 Structural representation of *Thermoascus aurantiacus* Cel5A (PDB ID: 1GZJ). The α-helices are represented in green and the β-sheets in red. The aromatic residues of the active site are represented in yellow

5.5.1.3 β-Glucosidases

β-Glucosidases are key enzymes involved in the hydrolysis of β-linkages in cellobiose and short-chain glucose oligomers. They hydrolyze the ultimate step of biomass hydrolysis for producing monomeric sugars. Aspartate (D) is the key active site residue involved in catalysis. Fungal β-glucosidases are primarily placed in the GH3 family, and *Trichoderma reesei* produces three β-glucosidases: -Cel3A, Cel3B, and Cel3E. Cel3A secreted by *T. reesei* is only about 1% of the total set of enzymes, which is suboptimal for the degradation of cellulosic substrates. Its production has been enhanced by changing the native promoter with xylanase and cellulase promoters, which has led to a 4–7.5-fold increase in β-glucosidase activity. Efficient β-glucosidases have been obtained from other fungal species such as *Aspergillus* and *Penicillium* species.

The first crystal structure for β-glucosidase BGL1 of GH3 family was obtained from the filamentous fungus *Aspergillus aculeatus* (PDB ID: 4IIB) as represented in Fig. 5.6. It consists of a catalytic triosephosphate isomerase domain (Leu^{22}–Ser^{356}-depicted in red), an α/β-sandwich domain (Gln^{385}–Gly^{588} depicted in green), and a fibronectin type III domain (Tyr^{654}–Gln^{860}-depicted in cyan). These domains are connected with two linker regions (residues 357–384 and 589–653). The catalytic residues of AaBGL1 are the nucleophile Asp^{280} located at the barrel domain and the acid/base residue Glu^{509} at the α/β sandwich domains. A retaining double-displacement mechanism is followed for the hydrolysis of β-1,4-glycosidic bonds. At the catalytic active site, one carboxylic acid acts as a nucleophile and the other as an acid/base catalyst. These catalytic residues are not completely conserved but are rather phylogenetically variable (Sørensen et al. 2013).

Fig. 5.6 Structural representation of *Aspergillus aculeatus* BGL1 (PDB ID: 4IIB). The three domains are as follows: TIM barrel-like domain red, α/β-sandwich domain green, and FnIII domain cyan. The catalytic residues are represented in yellow

5.5.2 Hemicellulases

Hemicellulases are a group of enzymes capable of digesting heterogeneous polymers present in hemicellulose. Hemicellulose is a complex of heteropolysaccharides made up of xylose, mannose, or glucose chains modified with galactose, arabinose, and acetic/glucuronic acid ramifications (Saha 2003). Hydrolysis of β-1-4-linkages in xylan requires the cooperative action of endoxylanase and xylosidase (Jeffries 1994). Endomannase and β-mannosidase break down the mannose polymers present in hemicellulose (Pérez et al. 2002). Accessory enzymes such as xylan esterases, ferulic and *p*-coumaric esterases, α-L-arabinofuranosidases, and α-4-*O*-methylglucuronidase are involved in efficient hydrolysis of xylans and mannans. Another major hemicellulosic polysaccharide xyloglucan is acted upon by xyloglucan-specific β-1,4-glucanases, which has been designated as xyloglucanase (Benkő et al. 2008). Fungi belonging to *Aspergillus*, *Penicillium*, and *Trichoderma* are potent hemicellulase producers. *Penicillium funiculosum* synthesizes a consortium of hemicellulases that act synergistically for the hydrolysis of hemicellulose. It includes enzymes such as endoxylanase, β-xylosidase, endoglucanase, and arabinofuranosidase and accessory enzymes such as feruloyl esterase,α-D-galactosidase, β-D-mannosidase, and endomannanase (Karboune et al. 2008, 2009).

5.5.3 Ligninolytic Enzymes

Lignin-degrading enzymes are divided into two main groups—lignin-modifying enzymes (LME) and lignin-degrading auxiliary (LDA) enzymes (Fig. 5.7). LME

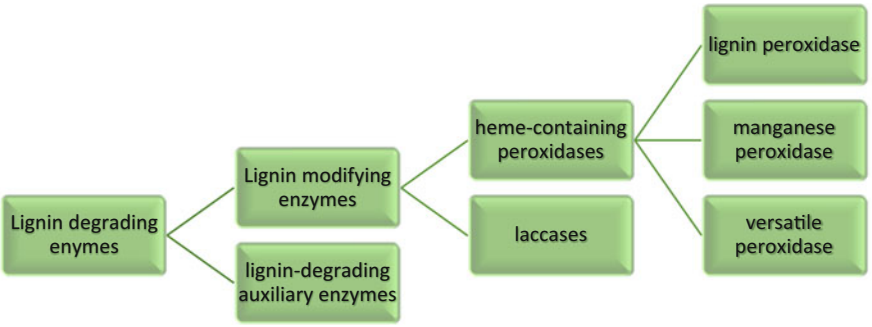

Fig. 5.7 Categorization of lignin-degrading enzymes

are further subcategorized as laccases and heme-containing peroxidases (POD), namely, lignin, manganese, and versatile peroxidase. Lignin peroxidases (LiPs) are relatively nonspecific to their substrates and can oxidize different phenolic aromatic compounds (Wong 2009). The enzymatic action of Mn-dependent peroxidases is similar to LiPs, except that it utilizes Mn(II) as the reducing substrate. Versatile peroxidases (VPs) have a combined molecular architecture of LiP and MnP. It can oxidize typical LiP substrates such as veratryl alcohol, methoxybenzenes, and non-phenolic model lignin compounds (Janusz et al. 2017). Auxiliary enzymes allow the lignin degradation process through the sequential action of several proteins such as glyoxal oxidase, aryl alcohol oxidases, pyranose 2-oxidase, cellobiose dehydrogenase, and glucose oxidase.

These lignin-degrading fungi have been categorized into three main groups—white-rot fungi, brown-rot fungi, and soft-rot fungi. White-rot fungi can degrade lignin completely by the action of three major oxidative enzymes LiP, MnP, and laccase (Levasseur et al. 2008; Eriksson et al. 2012). LiP participates in the slow oxidation of the cell wall by attacking at the lumen surface. It participates in the oxidative cleavage of non-phenolic aromatic lignin moieties. Manganese peroxidase aids in lignin degradation by catalyzing the chemical reaction that oxidizes numerous phenolic compounds in the presence of Mn^{2+}. MnP oxidizes Mn^{2+} to Mn^{3+}, which forms a complex with certain aliphatic organic acids such as malonate, oxalate, and lactate produced by white rot fungi. The complexed Mn^{3+} then diffuses into the cell wall and oxidizes phenolic lignin compounds. Laccases are copper-containing oxidases that use oxygen as an oxidant to degrade lignin. They act on phenols and similar molecules by performing one-electron oxidations. Unlike white-rot fungi, brown-rot fungi can only partially depolymerize the lignin. The action of brown-rot ligninolytic enzymes loosens up the structure of lignin in the cell wall, allowing easy access to hydrolytic enzymes for the degradation of carbohydrates (Arantes et al. 2012).

5.5.4 Auxiliary Activity Enzymes

Apart from the classical enzymes that are capable of cellulose degradation, cellulose hydrolysis is accelerated by the non-hydrolytic component. Lytic polysaccharide monooxygenases (LPMOs) are one such class of enzymes responsible for the oxidative cleavage of recalcitrant polysaccharides. The glycosidic linkages generated as the result of the action of LPMO make the polymer susceptible to hydrolysis by conventional cellulases. LPMOs employ copper at the catalytic site and are dependent on oxygen and reducing agents for activity. In the CAZy database, LPMOs are classified into four auxiliary activity (AA) enzyme families: AA9, AA10, AA11, and AA13. AA9 LPMOs (earlier classified as GH61) are exclusively found in fungi and act preferentially on cellulose. LPMOs have a β-sandwich fold structure with a large planar surface. The active site is present at the center of the flat surface where they interact with their substrates. Aromatic side chains present on the flat surface assist in binding to the cellulose. LPMOs are a key component of the enzyme cocktails used for the industrial production of lignocellulosic ethanol. AA9 from *Thielavia terrestris* (TtGH61E) and *Thermoascus aurantiacus* (TaGH61A) has been reported for promoting cellulase activity (Harris et al. 2010).

5.5.5 Pectinases

Pectins are methylated polysaccharides composed of α-(1–4)-linked galacturonic acid sometimes associated with L-rhamnose residue by α-1,2-glycosidic bond. Pectinases are a specialized group of enzymes capable of hydrolyzing the glycosidic bonds present in pectins. Rhamnogalacturonase cleaves the α-1,2-glycosidic bond, while pectin and pectate lyases cleave the methylated and the demethylated regions of pectin, respectively (Lombard et al. 2010). Generally, *Aspergillus*, *Penicillium*, *Rhizopus*, and *Fusarium* species are used for pectinase production.

5.5.6 Amylases

Amylase breaks down starch into glucose and simpler sugars. It is one of the most widely used commercial enzymes. Fungal amylases are secreted in abundant quantities, which makes their downstream processing easier. Due to this, they have received a great deal of attention in industrial use. Fungi belonging to the genus *Aspergillus* especially *Aspergillus niger* have been used for amylase production.

5.5.7 Lipases

Lipases are hydrolytic enzymes that can function as triacylglycerol acyl hydrolases. Based on their specificity, lipases have been divided into three groups, such as

1,3-specific lipases, fatty acid-specific lipases, and nonspecific lipases. Biodiesel production is one of the most important applications of lipase which was first reported by Mittelbach (1990). It has been described later in this chapter in a subsection on biodiesel. Lipases from *Aspergillus niger*, *Rhizopus oryzae*, and *Mucor miehei* have been used for the catalysis of transesterification reactions (Ribeiro et al. 2011).

5.5.8 Carbohydrate-Binding Modules

The lignocellulosic biomass has components that are insoluble, and for their degradation, enzymes have to bind to these components. To assist in hydrolysis, certain enzymes have acquired a modular organization (Gilkes et al. 1991). The catalytic modules of these enzymes are associated with a carbohydrate-binding module (CBM). These CBMs can bind to insoluble cellulosic components and assist the enzyme through the phase transfer from a soluble fraction (the enzyme) to an insoluble fraction (the substrate) (Shoseyov and DoI 1990). Also, by binding to the insoluble substrate, the CBMs disrupt the structure of the substrate and increase the accessibility and effective enzyme concentration on the substrate (Guillén et al. 2010). The importance of CBMs has been realized by the low activity of the associated catalytic module on insoluble substrates while the CBM was removed. *T. reesei* CBHI and CBHII showed a reduction in activity by about 85% and 50%, respectively, on insoluble substrate Avicel when its CBM was removed (Tomme et al. 1988). *Paenibacillus polymyxa* A18 xyloglucanase showed a drastic reduction in activity (about 70%) on the removal of its associated X2-CBM3 module (Pasari et al. 2017).

5.6 Classification in the CAZy Database

The carbohydrate-active enzyme (CAZy) database is a database of enzymes that specifically builds and breaks down complex carbohydrates and glycoconjugates. Based on the catalytic activity, the CAZymes have been subdivided into the following categories: glycoside hydrolase (GH), glycosyltransferase (GT), polysaccharide lyase (PL), carbohydrate esterase (CE), auxiliary activity enzyme (AA), and carbohydrate-binding module (CBM). Glycoside hydrolases (GHs) are responsible for the hydrolysis as well as transglycosylation of glycosidic bonds (Henrissat 1991). GH genes coding for both glycosidases and transglycosidases are abundant and present in the vast majority of the genomes. Polysaccharide lyases (PLs) cleave the glycosidic bonds of uronic acid-containing polysaccharides. They use a β-elimination mechanism in the process such that an unsaturated hexeneuronic acid residue and a new reducing end are generated (Yip and Withers 2006). Carbohydrate esterases (CEs) remove the ester-based modifications present in the carbohydrates. Glycosyltransferases (GTs) are responsible for the biosynthesis of glycosidic bonds from phospho-activated sugar donors. Carbohydrate-binding

Table 5.2 Domains present in some of the industrially important fungi

Fungus	GH	GT	PL	CE	AA	CBM
Trichoderma reesei QM6a	115	42	2	10	13	26
Trichoderma reesei RUT-C30	112	42	2	9	14	27
Talaromyces cellulolyticus	173	52	3	12	17	34
Penicillium funiculosum	158	50	3	12	16	30
Penicillium chrysogenum 54-1255	230	106	10	22	23	52
Saccharomyces cerevisiae s288c	55	68	0	3	5	14

modules (CBMs) are non-catalytic modules, but they are known to potentiate the activity of other enzymes (Cantarel et al. 2009; Pasari et al. 2017). A representation of the total numbers of the various domains present in some industrially important fungal genomes is presented in Table 5.2. A distinct variation in the number of enzymes belonging to each family of these fungi suggests a variation in the ability to hydrolyze different polymeric biomass (Pasari et al. 2019).

5.7 The Role of Fungi in Bioethanol Production

Fungi play an important role across the range of biofuel production processes. They are involved in the bioprocesses for producing ethanol, higher alcohols, as well as other hydrocarbons (Grigoriev et al. 2011). Fungi in their yeast form like *Saccharomyces cerevisiae* have been used for fermentation of sugars to ethanol for the production of first-generation bioethanol. Filamentous fungi can secrete copious amounts of hydrolytic enzymes and have been used for plant biomass saccharification aimed at producing second-generation bioethanol. Fungi also being oleaginous organisms have also been used as feedstock for the production of biodiesel. The role of fungi in the production of the first and second generation of biofuel and for biodiesel production has been elaborated further.

5.7.1 First-Generation Bioethanol

The first generation of biofuel relies largely on starch and sugar present in edible food crops. Starch from the food crops, such as wheat, corn, and barley, and sugars from sugarcane are used as feedstocks for ethanol production. These crops are first harvested for their sugar and starch content and then converted to biofuels using fermentation and distillation (Naik et al. 2010). Bioethanol production from sugarcane has been one of the commercially most successful biofuel generation systems in India and Brazil (Matsuoka et al. 2009; Yuan et al. 2008). There is a wide availability of sugarcane in both these countries as Brazil tops the annual production of sugarcane while India ranks second followed by China (Talukdar et al. 2017). The sugars are obtained from the sugarcane by crushing them in water in a step called liquefaction. The juice obtained by crushing contains mostly the disaccharide sucrose and

the monosaccharides glucose and fructose. Sucrose is hydrolyzed into monomers by the invertase enzyme. The other source of carbohydrates has been starch from corn. Starch is composed of polymers of glucose—amylose and amylopectin—which can be hydrolyzed to simpler sugars using amylases. The amylase enzyme is rather inexpensive and accounts for only US $0.04 per gallon of ethanol produced (Lee and Lavoie 2013; McAloon et al. 2000). Subsequently, the fermentable sugars obtained from sugarcane as well as corn are converted into ethanol and carbon dioxide by the process of fermentation. Generally, yeasts are used in this process, and they metabolize glucose to ethanol under anaerobic conditions. The yeast *Saccharomyces cerevisiae* is used for the production of ethanol and is the workhorse in the current biofuel industry (Buijs et al. 2013). Other yeasts like *Saccharomyces uvarum*, *Schizosaccharomyces pombe*, and *Kluyveromyces* species have also been used in the process of fermentation. The technology for the production of the first-generation biofuel is well established and is likely to dominate for few more years. Some of the leading players for the production of ethanol in the Indian market are India Glycols, HPCL Biofuels Limited, Bajaj Hindusthan Sugar, Mawana Sugars Ltd., Shree Renuka Sugars Ltd., and Jeypore Sugar Company Ltd.

5.7.2 Second-Generation Bioethanol

The second generation of biofuel is produced from lignocellulosic biomass that is inexpensive and available in abundant quantities. Agricultural and plantation residues such as leaves, bagasse, and straw are the major sources of lignocellulosic biomass. Some of the crops such as perennial grasses, short-rotation willows, hybrid poplar, and eucalyptus are also cultivated for second-generation biofuel production. Conversion of lignocellulosic biomass to ethanol uses advanced conversion technologies. The entire process of conversion comprises three essential steps—pretreatment, saccharification, and fermentation—as depicted in Fig. 5.8.

The lignocellulosic biomass is recalcitrant. To reduce the recalcitrance and make its cellulose and hemicellulose components accessible, the biomass is first pretreated. Pretreatment can be done using either thermal, chemical, or biological processes (Kumar et al. 2009). Once the biomass structure is loosened up, the easily

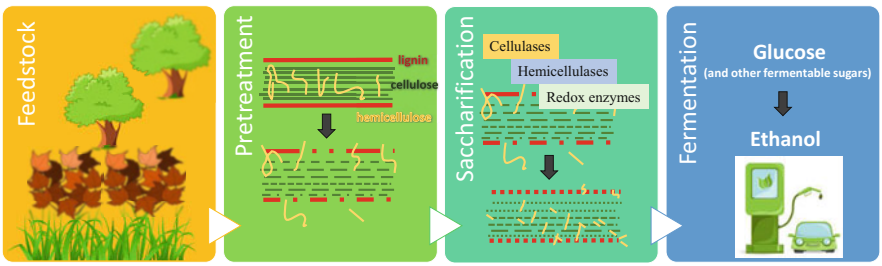

Fig. 5.8 Main steps involved in the conversion of lignocellulosic biomass to ethanol

accessible cellulolytic components are hydrolyzed to simpler sugars using enzymes in a process called saccharification. Majorly cellulases and hemicellulases, which have been described in the section on fungal secretory enzymes, are used for saccharification. The simpler sugars so obtained are converted into ethanol by fermentation in the third step. When the process of saccharification and fermentation is performed one after the other, the method of biofuel production is called separate hydrolysis and fermentation (SHF), whereas when both processes are performed simultaneously, the process is called simultaneous saccharification and fermentation (SSF). The bioethanol yield obtained by enzymatic hydrolysis and fermentation is reported in the range of 110–300 L per dry ton of feedstock (Ravindranath et al. 2011). Lignocellulosic ethanol is a renewable and cleaner-burning fuel due to which its use in the transportation sector will be very large soon.

Fungi can play major roles in the pretreatment and saccharification process as described below:

5.7.2.1 Pretreatment

The plant cell wall is a thick, protective, and rigid layer that provides support to the plant to maintain an erect structure. It is a highly recalcitrant structure composed of cellulose (40–50%), hemicelluloses (20–40%), and lignin (20–30%) (Yang and Wyman 2008). Lignin fills up the spaces between cellulose, hemicellulose, and pectin components of the cell wall. It is a complex polymer of aromatic aldehydes and is hydrophobic. It confers structural support to the cell wall and makes it resistant to microbial attack. Efficient degradation of biomass to simpler sugars requires penetration of the cell wall matrix by the hydrolyzing enzymes. To facilitate the binding of these enzymes to this crystalline matrix, pretreatment of lignocellulosic biomass is required. Pretreatment changes the supramolecular structure of the cell wall matrix and increases the accessibility for the enzymatic action. Pretreatment can be done by different methods such as steam explosion, ammonia fiber explosion (AFEX), alkaline hydrolysis, acid hydrolysis, and biological pretreatments. Once the physical structure of the biomass is made more accessible after pretreatment, it is hydrolyzed by the action of enzymes (Isikgor and Becer 2015).

Saprotrophic fungi play an important function in pretreatment as they have an inherent capacity to degrade dead plant organic matter. Wood-rotting fungi, a subcategory of saprotrophic fungi, are primary lignin degraders. They are capable of mineralization of this abundant plant product. Wood-rotting fungi have been divided into three main groups: white-rot, brown-rot, and soft-rot fungi. White-rot fungi can degrade lignin completely to CO_2 and H_2O. They are most effective in delignification due to their unique ligninolytic systems. They have provided a greener alternative to thermal and chemical pretreatment methods. Several white-rot fungi, such as *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Coriolus versicolor*, *Cyathus stercoreus*, *Lenzites betulinus*, *Dichomitus squalens*, and *Ceriporiopsis subvermispora*, have been studied for pretreatment of a wide range of biomass feedstocks.

Brown-rot fungi cannot degrade the lignin completely but only modifies it. In the plant cell wall degraded by brown-rot fungi, the remaining lignin contains a greater

number of ring hydroxyl groups. Brown-rot fungi such as *Serpula lacrymans* cause wood rot without destroying the lignin (Isaac 1997; Watkinson and Eastwood 2012). At the same time, lignin degradation by soft-rot fungi is limited. Members of soft-rot fungi typically attack the outer surface of the wood in relatively wet environments (Goodell et al. 2008). Members of Ascomycota such as *Penicillium chrysogenum*, *Fusarium solani*, and *F. oxysporum* mostly cause soft-rot decay but show lower degrees of lignin decomposition as compared to white-rot fungi (Kirk and Farrell 1987).

5.7.2.2 Enzymatic Saccharification

Pretreatment reduces the size of the biomass and makes the physical structure more accessible to enzymes. Depolymerization of pretreated biomass requires an array of hydrolyzing enzymes because of its heterogeneous nature. Many organisms across the kingdoms of life have acquired the ability to produce extracellular enzymes necessary for converting cellulose to soluble sugars. The organisms belonging to the kingdom Fungi have the inherent ability to produce secretory enzymes because of their heterotrophic nature. They acquire carbon from organic matter by producing a consortium of enzymes of which a large fraction is cellulolytic enzymes (Ogunmolu et al. 2015). Moreover, the fungal hyphae are filamentous, and their penetrative nature allows extracellular enzymes to reach quickly to the nearby tissues (Isaac 1997). Cellulase systems of fungi such as *Trichoderma reesei*, *Aspergillus niger*, *Phanerochaete chrysosporium*, *Fusarium solani*, and *Talaromyces emersonii* have been extensively studied. *Trichoderma reesei* is a leading commercial source of cellulolytic enzymes, and several of its mutant strains such as QM6a, QM9414 MCG77, and RUT C30 have been developed for industrial use (Gusakov 2011). *T. reesei* can secrete more than 40 g/L of cellulases, whereas in the case of certain industrial strains, a figure of 100 g/L has been reported (Martinez et al. 2008). Fungi belonging to other species of *Trichoderma* such as *T. koningii*, *T. longibrachiatum*, *T. harzianum*, and *T. viride* have also been reported to be potential cellulase producers. These strains are being used commercially for deconstruction of agriculture and forestry residues, woody biomass, and dead trees for the production of bioethanol (Raven et al. 2019). Another strain, *Penicillium funiculosum*, has been identified as a potential strain for the industrial production of cellulolytic enzymes. It secretes a repertoire of inducible CAZymes as well as non-hydrolytic accessory proteins, which synergistically mediate the action of biomass hydrolysis. CBHI from *P. funiculosum* has been identified as a viable alternative for *T. reesei* CBHI in industrial cellulase cocktails (Ogunmolu et al. 2015, 2017, 2018).

5.8 Fungi in Biodiesel Production

Diesel is a mixture of hydrocarbons obtained from the fractional distillation of petroleum. The increased rate of fossil fuel consumption has led to the depletion of liquid fuels like diesel too. To compensate for the fast depletion of diesel, exploring alternative sources that can replace the hydrocarbons present in diesel is

required. Fungi are oleaginous microorganisms exhibiting a good lipid profile for generating high-quality biodiesel. They have been explored to produce compounds such as alkanes, cyclohexanes, cyclopentanes, alkyl alcohols/ketones, benzenes, and polyaromatic hydrocarbons that are usually found in biodiesel (Strobel 2014). For industrial application, fungal strains containing high lipid content are required. So there is a need for bioprospecting fungi for increased lipid yields. These fungi can be cultivated in solid-state fermentation (SSF) using cheap carbon sources. So commercial production of biodiesel is possible by the cultivation of fungi in SSF such that both biomass and lipid yields can be increased simultaneously. The yeast strain S. *cerevisiae* 0A03 isolated from rice cakes in Assam (India) showed elevated proportions of saturated and monounsaturated fatty acids (Phukan et al. 2019). Another fungus *Gliocladium roseum* can convert plant material directly into hydrocarbons, which are found in petroleum (Strobel 2014). These fungal strains exhibit interesting features and can be exploited for bioenergy research.

Biodiesel is also produced using an enzymatic approach in a transesterification reaction catalyzed by lipase enzymes. Lipase is an important biocatalyst that catalyzes esterification that is the hydrolysis of the ester bond and transesterification reaction that is esterification with the second substrate to produce methyl esters. Lipases have been isolated from many sources, but fungi such as *Aspergillus niger*, *Rhizopus oryzae*, and *Mucor miehei* have been the preferred source for industrial production. Of these strains, *Aspergillus* and *Rhizopus* have been used as whole-cell biocatalyst (Yücel et al. 2012). Different varieties of feedstock ranging from edible to inedible vegetable oils, animal fats, waste oil, microbial oil, and microalgae oil have been used for enzyme-catalyzed transesterification (Demirbas 2009). For industrial-level biodiesel production using lipase transesterification reaction, significant cost reduction and improvement in enzyme activity are required.

5.9 Regulation of Enzyme Production in Fungus

Fungi have developed sophisticated mechanisms to sense and synthesize enzymes required for the hydrolysis of biomass present in its vicinity. The synthesis of these hydrolyzing enzymes is regulated by induction and catabolite repression mechanisms. Soluble derivatives from cellulose such as cellobiose, sophorose, and lactose have the ability to induce the cellulolytic enzyme system, whereas metabolized product such as glucose represses the synthesis of these enzymes (Fang et al. 2008; Mandels and Reese 1960). The signal transduction pathways confer the information for gene expression through the transcription factors (TFs) (Shelest 2008). Several TFs regulating the expression of cellulolytic enzymes have been identified. XlnR from *T. reesei* and XyR1 from *A. nidulans* were identified to be general inducers for the cellulolytic enzyme, and AraR was found to be an activator for arabinan-degrading enzymes (Battaglia et al. 2011). A transcriptional activator ACE2 was identified in *Trichoderma* sp. CreA/CRE1 protein, a Cys_2His_2-type transcription factor, is a key player in glucose repression (Dowzer and Kelly

1991) in *T. reesei* and *Aspergillus* species (Aro et al. 2005). Its homolog Mig1 was identified in *Penicillium funiculosum* (Randhawa et al. 2018).

Apart from the metabolizable sugars, the pH of the external environment also affects the enzyme secretion capacities of fungi. This regulatory system has been studied in detail in *A. nidulans*. PacC, a zinc-finger transcription factor, is the main player in this regulatory system. It acts as an activator for alkaline-expressed genes and prevents the expression of acid-expressed genes (Peñalva et al. 2008).

5.10 Strain Improvement

To improve the enzyme-producing capabilities of fungi, there is always a room for development and optimization. The strain improvement strategies used are either random or targeted. In the random approach, anywhere in the genome a mutation can occur, whereas in targeted approach, it is only at a defined place that the change can take place. The use of classical mutagenesis for the improvement of enzyme-producing fungi has a long and successful history. In this approach, the fungus is subjected to a nonlethal dose of a mutagen such as irradiation by UV light or addition of nitrosomethyl guanidine, and subsequently the survivors are screened for improved production of the desired product. *T. reesei* RUT-C30, one of the best cellulase producers currently used in the industry globally, has been obtained after several rounds of mutations (Peterson and Nevalainen 2012). Certain fungal strains have been improved by mutagenesis in India for increased cellulase production. The strain of *Penicillium janthinellum* NCIM 1171 was subjected to mutation using ethyl methylsulfonate (EMS) treatment and UV irradiation. Successive mutants showed enhanced cellulase production including increased exocellulase, endoglucanase, and filter paper activity. Another strain *Penicillium purpurogenum* P-26 was subjected to UV irradiation and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine treatment, and the mutants resistant to catabolite repression were isolated. These mutants were capable of synthesizing cellulolytic enzymes even under a high concentration of glucose (Anwar et al. 1996). *Aspergillus niger* NCIM 1207 has been reported as a hyperproducer of β-glucosidase and β-xylosidase. The strain was subjected to mutagenesis and genetic modifications to obtain improved strain producing high levels of cellulases for commercial use. But no commercially efficient enzyme complex has been produced as the high cost of cellulase production hinders the use of enzymes at an industrial scale for cellulose saccharification (Gokhale et al. 2011).

Strain improvement by a targeted approach involves the manipulation of the genome by recombinant DNA technology. Manipulation of the fungal genome is challenging as the outermost covering of fungi is of thick chitinous cell walls. Also, they lack any stable, natural extrachromosomally replicating DNA elements that could be manipulated for the introduction of a DNA element in the cell. Despite the challenges, PEG-mediated protoplast transformation has been used routinely for the introduction of genetic elements in fungi. In this method, the thick chitinous cell wall is first digested to obtain protoplasts. The linearized DNA is then introduced as Ca^{2+} salts on the cell surface to facilitate its uptake in the cells. The addition of

polyethylene glycol allows the protoplasts to glue together to facilitate the uptake of DNA by endocytosis. DNA received by the cells can integrate into the genome either randomly at nonspecific sites or by recombination at the homologous site (Hynes 1996). Protoplasts were then regenerated in the presence of a selection marker to facilitate the detection of positive clones (Turgeon et al. 2010)., Systematic attempts were made to introduce DNA into the filamentous fungi *Neurospora crassa* and *A. nidulans*. The principles developed were later applied to all other fungi including *T. reesei*, *Humicola insolens*, and *P. chrysosporium*. A major drawback of this technique is the low number of transformants obtained, which are as low as $<1/\mu g$ DNA (Shishido 1994). The introduction of improved techniques for transformation such as electroporation (Kapoor 1995) or the use of *Agrobacterium tumefaciens*-mediated DNA transfer (AMT) (De Groot et al. 1998) reduced these bottlenecks in most but not all fungi.

In India, the AMT method has been used by DBT-ICGEB Bioenergy Centre to create a repressor deficient strain of *P. funiculosum* (Randhawa et al. 2018), and the strain was further engineered to produce more potent enzymes by overexpression of cellobiohydrolase I (CBH1) and AA9 (Ogunyewo et al. 2020).

5.11 Research and Development in Academia and Industries

The Government of India has made a goal to achieve a 20% blending of biofuels in transport fuels by 2030. Since this is an ambitious target, the government has taken several steps to broaden the R&D base for improvements in the current technology and to explore newer options. The Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India, has established five Bioenergy Research Centres in the country that will integrate basic and translational science capabilities for biofuel development and scale-up (http://dbtindia.gov.in). These are DBT-ICT Centre for Energy Biosciences, DBT-IOC Centre for Advanced Bioenergy, DBT-ICGEB Centre for Advanced Bioenergy Research, DBT Pan-IIT Centre for Bioenergy, and DBT-TERI Centre of Excellence in Advanced Biofuel and Bio-commodities (recently established). The major focus of these centers is on technological development for cellulosic ethanol and algal biofuel. The government is making a concerted effort to enhance the pace of innovation for clean energy to meet the requirements for India's energy security on time. India via its nodal agency, DBT, is participating in the intergovernmental Mission Innovation clean energy program whose mandate is to double the funding support in cutting-edge clean energy projects for the development of environment-friendly energy molecules (http://mission-innovation-india.net/).

The production of ethanol from lignocellulosic biomass has been extensively studied and researched in the United States, Canada, and some European countries. International companies like Genencor and Novozymes are the major manufacturing units of commercial cellulases. NREL (USA) has already initiated projects with Genencor and Novozymes for reducing the cost of cellulases (Mathew et al. 2008). These enzyme majors have taken up research to understand the mechanism of

cellulase gene regulation and structure to function relationships, with the hope of reducing enzyme cost for lignocellulose ethanol production.

The Indian market has been putting efforts for a long time to obtain a dedicated enzyme preparation for biomass hydrolysis. Indian enzyme manufacturers, like Advanced Enzymes Ltd., MAPS India Ltd., and Zytex India Ltd., have been producing cellulases that were suitable for use in the textile industry. These cellulase preparations are much more expensive and hence were not suitable for bioethanol production. With increased government funding in the public and private sector for biofuel development, India emerged as a major hub for cutting-edge R&D. Multinational companies such as Novozymes (Denmark), Genencor, and Dyadic International (USA) started investing in India (Chandel et al. 2007). These companies have set up research centers for the development and optimization of potent cellulases for the efficient production of bioethanol. Novozymes recently opened its research and development center in Bangalore. Praj Industries, Pune, has developed the second-generation (2G) cellulosic ethanol technology "Enfinity." The Smart Biorefinery is based on its "Enfinity" technology, which aimed to process multiple feedstocks to produce multiple products like bioethanol, biobutanol, biochemicals, power, Bio CNG, CO_2, etc. DBT-ICT Centre developed 2G ethanol technology that produces ethanol from agricultural residue feedstock. The continuous processing technology converts biomass feed to alcohol within 24 h. The technology was proven at a 1 ton/day scale during Phase 1 at the India Glycols site. The DBT-ICT Centre, along with oil manufacturing companies, has plans to design and scale up the technology to a 400 ton/day scale. Indian Oil Corporation has also joined hands with Praj Industries to establish a commercial-level plant for second-generation bioethanol production. In all these cases, fungal enzymes are likely to play a major role in the economic viability of the 2G biofuels.

5.12 Conclusions and Future Perspectives

Increased energy demand has led to an escalated use of nonrenewable fossil fuels, in turn leading to the depletion of its reserves. The use of fossil fuels releases toxic substances, causing significant deterioration of the Earth's environment. Renewable sources of energy such as biofuels are an excellent source of green energy. Biofuels are derived from biomass using biological organisms and can severely reduce the dependence on fossil fuels.

Several classes of microorganisms like bacteria and fungi produce a plethora of enzymes capable of digesting the biomass. Fungi are saprophytic organisms and can secrete the enzymes in the environment to digest the available materials and absorb the digested matter. To exploit this property of fungi, these organisms have been used for biofuel production for a long time. For the production of the first generation of biofuel, starch and sugars from edible food sources are hydrolyzed to glucose using amylase. The glucose produced is then converted to bioethanol through fermentation. Yeasts such as *Saccharomyces cerevisiae* serve as an industrial workhorse for the production of the first-generation biofuel. The second generation of

biofuel is produced from nonedible lignocellulosic biomass. Filamentous fungi can secrete a wide diversity of enzymes ranging from cellulase, hemicellulase, ligninolytic enzymes, pectinases, and phytases. These enzymes work in a consortium to hydrolyze the recalcitrant biomass to simpler sugars, which can then be fermented to bioethanol. For the commercial use of these enzymes in the saccharification process, technological advancement in terms of yield, efficiency, and recovery is required to make the process economical. Approaches like bioprospecting for superior enzymes, reconstitution of enzyme mixtures, and enzyme engineering are gaining importance.

Apart from saccharification potential, fungi being oleaginous microorganisms can store lipids, which can serve as a precursor for biodiesel production. However, intensive research is required to optimize these technologies for cost-effectiveness and economic viability. Biodiesel can also be effectively produced from vegetable oils by the enzymatic action of lipases. For large-scale production of biodiesel, optimization of these lipases in terms of enzyme cost and efficiency is required. Research is underway to optimize the process conditions for biodiesel production.

India is still importing more than 70% of its crude oil requirements from Middle Eastern countries. If more attention is focused on research for the in-house production of biofuels, it will not only save the country from using the fossil fuel reserves but also create more employment and income. The Government of India through the Department of Biotechnology (DBT), the Department of Science and Technology (DST), and the Council of Scientific and Industrial Research (CSIR) has been funding projects related to biofuels and technology implementation in its production. This will help in the betterment of the technology for the production and hence reduction in the costs involved.

References

Anwar M, Suto M, Tomita F (1996) Isolation of mutants of Penicillium purpurogenum resistant to catabolite repression. Appl Microbiol Biotechnol 45(5):684–687

Arantes V, Jellison J, Goodell B (2012) Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. Appl Microbiol Biotechnol 94(2):323–338

Aro N, Pakula T, Penttilä M (2005) Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiol Rev 29(4):719–739

Babu M, Nautiyal S (2012) Socioeconomic and ecological consequences of biofuel development in India

Balan V (2014) Current challenges in commercially producing biofuels from lignocellulosic biomass. ISRN Biotechnol 2014:463074

Battaglia E, Hansen SF, Leendertse A, Madrid S, Mulder H, Nikolaev I, de Vries RP (2011) Regulation of pentose utilisation by AraR, but not XlnR, differs in Aspergillus nidulans and Aspergillus Niger. Appl Microbiol Biotechnol 91(2):387–397

Behera S, Singh R, Arora R, Sharma NK, Shukla M, Kumar S (2015) Scope of algae as third generation biofuels. Front Bioeng Biotechnol 2:90

Benkő Z, Siika-aho M, Viikari L, Réczey K (2008) Evaluation of the role of xyloglucanase in the enzymatic hydrolysis of lignocellulosic substrates. Enzym Microb Technol 43(2):109–114

Brennan L, Owende P (2010) Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev 14(2):557–577
Brown Jr RD, Jurasek L (1979) Hydrolysis of cellulose; mechanisms of enzymatic and acid catalysis
Buee M, Reich M, Murat C, Morin E, Nilsson R, Uroz S, Martin F (2009) 454 pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytol 184 (2):449–456
Buijs NA, Siewers V, Nielsen J (2013) Advanced biofuel production by the yeast Saccharomyces cerevisiae. Curr Opin Chem Biol 17(3):480–488
Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The carbohydrate-active EnZymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37(Suppl_1):D233–D238
Chambergo FS, Valencia EY (2016) Fungal biodiversity to biotechnology. Appl Microbiol Biotechnol 100(6):2567–2577
Chandel AK, Rudravaram R, Rao LV, Ravindra P, Narasu ML (2007) Industrial enzymes in bioindustrial sector development: an Indian perspective. J Commer Biotechnol 13(4):283–291
Chen H, Wang L (2017) Enzymatic hydrolysis of pretreated biomass. In: Technologies for biochemical conversion of biomass. Academic Press, London, pp 65–99
Chen C-Y, Zhao X-Q, Yen H-W, Ho S-H, Cheng C-L, Lee D-J, Bai F-W, Chang J-S (2013) Microalgae-based carbohydrates for biofuel production. Biochem Eng J 78:1–10
Chundawat SP, Beckham GT, Himmel ME, Dale BE (2011) Deconstruction of lignocellulosic biomass to fuels and chemicals. Annu Rev Chem Biomol Eng 2:121–145
De Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG (1998) Agrobacterium tumefaciens-mediated transformation of filamentous fungi. Nat Biotechnol 16(9):839–842
de Souza PM, de Assis Bittencourt ML, Caprara CC, de Freitas M, de Almeida RPC, Silveira D, Fonseca YM, Ferreira Filho EX, Pessoa Junior A, Magalhães PO (2015) A biotechnology perspective of fungal proteases. Braz J Microbiol 46(2):337–346
Demirbas A (2009) Progress and recent trends in biodiesel fuels. Energy Convers Manag 50 (1):14–34
DiPardo J (2000) Outlook for biomass ethanol production and demand. Available online at: http://www.ethanol-gec.org/information/briefing/6.pdf. Accessed Jul 2007
Dowzer C, Kelly JM (1991) Analysis of the creA gene, a regulator of carbon catabolite repression in Aspergillus nidulans. Mol Cell Biol 11(11):5701–5709
Eriksson K-EL, Blanchette RA, Ander P (2012) Microbial and enzymatic degradation of wood and wood components. Springer Science & Business Media, Berlin
Fang X, Yano S, Inoue H, Sawayama S (2008) Lactose enhances cellulase production by the filamentous fungus Acremonium cellulolyticus. J Biosci Bioeng 106(2):115–120
Gilkes N, Henrissat B, Kilburn D, Miller R, Warren R (1991) Domains in microbial beta-1, 4-glycanases: sequence conservation, function, and enzyme families. Microbiol Mol Biol Rev 55(2):303–315
Gokhale D, Khisti U, Bastawde K (2011) Hyper-production of βeta-glucosidase and βeta-xylosidase by Aspergillus niger NCIM 1207 in xylan containing media. Bioresources 6 (2):2066–2076
Goodell B, Qian Y, Jellison J (2008) Fungal decay of wood: soft rot—brown rot—white rot. ACS Publications, New York
Government of India (2018) National policy on biofuels
Gowen MM (1989) Biofuel v fossil fuel economics in developing countries: how green is the pasture? Energy Policy 17(5):455–470
Graham-Rowe D (2008) Electricity without carbon. Nature 454:816–823
Grigoriev IV, Cullen D, Goodwin SB, Hibbett D, Jeffries TW, Kubicek CP, Kuske C, Magnuson JK, Martin F, Spatafora JW (2011) Fueling the future with fungal genomics. Mycology 2 (3):192–209

Guillén D, Sánchez S, Rodríguez-Sanoja R (2010) Carbohydrate-binding domains: multiplicity of biological roles. Appl Microbiol Biotechnol 85(5):1241–1249
Gusakov AV (2011) Alternatives to Trichoderma reesei in biofuel production. Trends Biotechnol 29(9):419–425
Harris PV, Welner D, McFarland K, Re E, Navarro Poulsen J-C, Brown K, Salbo R, Ding H, Vlasenko E, Merino S (2010) Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family. Biochemistry 49(15):3305–3316
Hawksworth DL, Lücking R (2017) Fungal diversity revisited: 2.2 to 3.8 million species. In: The fungal kingdom. ASM Press, Washington, DC, pp 79–95
Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 280(2):309–316
Hyde KD, Xu J, Rapior S, Jeewon R, Lumyong S, Niego AGT, Abeywickrama PD, Aluthmuhandiram JV, Brahamanage RS, Brooks S (2019) The amazing potential of fungi: 50 ways we can exploit fungi industrially. Fungal Divers 97:1–136
Hynes MJ (1996) Genetic transformation of filamentous fungi. J Genet 75(3):297–311
Isaac S (1997) How do fungi degrade and obtain nutrients from cellulose? Mycologist 2(11):92–93
Isikgor FH, Becer CR (2015) Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. Polym Chem 6(25):4497–4559
Janusz G, Pawlik A, Sulej J, Świderska-Burek U, Jarosz-Wilkołazka A, Paszczyński A (2017) Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution. FEMS Microbiol Rev 41(6):941–962
Jeffries TW (1994) Biodegradation of lignin and hemicelluloses. In: Biochemistry of microbial degradation. Springer, Dordrecht, pp 233–277
Kapoor M (1995) Gene transfer by electroporation of filamentous fungi. In: Electroporation protocols for microorganisms. Springer, Totowa, NJ, pp 279–289
Karboune S, Geraert P-A, Kermasha S (2008) Characterization of selected cellulolytic activities of multi-enzymatic complex system from Penicillium funiculosum. J Agric Food Chem 56 (3):903–909
Karboune S, L'Hocine L, Anthoni J, Geraert P-A, Kermasha S (2009) Properties of selected hemicellulases of a multi-enzymatic system from Penicillium funiculosum. Biosci Biotechnol Biochem 73(6):1286–1292
Kirk TK, Farrell RL (1987) Enzymatic "combustion": the microbial degradation of lignin. Annu Rev Microbiol 41(1):465–501
Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48(8):3713–3729
Kumar A, Kumar N, Baredar P, Shukla A (2015) A review on biomass energy resources, potential, conversion and policy in India. Renew Sust Energ Rev 45:530–539
Lee RA, Lavoie J-M (2013) From first-to third-generation biofuels: challenges of producing a commodity from a biomass of increasing complexity. Anim Front 3(2):6–11
Leggio LL, Larsen S (2002) The 1.62 Å structure of Thermoascus aurantiacus endoglucanase: completing the structural picture of subfamilies in glycoside hydrolase family 5. FEBS Lett 523 (1–3):103–108
Levasseur A, Piumi F, Coutinho PM, Rancurel C, Asther M, Delattre M, Henrissat B, Pontarotti P, Asther M, Record E (2008) FOLy: an integrated database for the classification and functional annotation of fungal oxidoreductases potentially involved in the degradation of lignin and related aromatic compounds. Fungal Genet Biol 45(5):638–645
Lombard V, Bernard T, Rancurel C, Brumer H, Coutinho PM, Henrissat B (2010) A hierarchical classification of polysaccharide lyases for glycogenomics. Biochem J 432(3):437–444
Mandels M, Reese ET (1960) Induction of cellulase in fungi by cellobiose. J Bacteriol 79(6):816
Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, Chapman J, Chertkov O, Coutinho PM, Cullen D (2008) Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). Nat Biotechnol 26(5):553–560

Mathew GM, Sukumaran RK, Singhania RR, Pandey A (2008) Progress in research on fungal cellulases for lignocellulose degradation. J Sci Ind Res 67:898

Matsuoka S, Ferro J, Arruda P (2009) The Brazilian experience of sugarcane ethanol industry. In Vitro Cell Dev Biol Plant 45(3):372–381

McAloon A, Taylor F, Yee W, Ibsen K, Wooley R (2000) Determining the cost of producing ethanol from corn starch and lignocellulosic feedstocks. National Renewable Energy Lab, Golden, CO

Mittelbach M (1990) Lipase catalyzed alcoholysis of sunflower oil. J Am Oil Chem Soc 67 (3):168–170

Naik SN, Goud VV, Rout PK, Dalai AK (2010) Production of first and second generation biofuels: a comprehensive review. Renew Sust Energ Rev 14(2):578–597

Nigam PS, Singh A (2011) Production of liquid biofuels from renewable resources. Prog Energy Combust Sci 37(1):52–68

Ogunmolu FE, Kaur I, Gupta M, Bashir Z, Pasari N, Yazdani SS (2015) Proteomics insights into the biomass hydrolysis potentials of a hypercellulolytic fungus Penicillium funiculosum. J Proteome Res 14(10):4342–4358

Ogunmolu FE, Jagadeesha NBK, Kumar R, Kumar P, Gupta D, Yazdani SS (2017) Comparative insights into the saccharification potentials of a relatively unexplored but robust Penicillium funiculosum glycoside hydrolase 7 cellobiohydrolase. Biotechnol Biofuels 10(1):71

Ogunmolu FE, Kaur I, Pasari N, Gupta M, Yazdani SS (2018) Quantitative multiplexed profiling of Penicillium funiculosum secretome grown on polymeric cellulase inducers and glucose. J Proteome 179:150–160

Ogunyewo OA, Randhawa A, Gupta M, Kaladhar VC, Verma PK, Yazdani SS (2020) Synergistic action of a lytic polysaccharide monooxygenase and a cellobiohydrolase from Penicillium funiculosum in cellulose saccharification under high substrate loading. Appl Environ Microbiol 86:e01769-20

Pasari N, Adlakha N, Gupta M, Bashir Z, Rajacharya GH, Verma G, Munde M, Bhatnagar R, Yazdani SS (2017) Impact of module-X2 and carbohydrate binding module-3 on the catalytic activity of associated glycoside hydrolases towards plant biomass. Sci Rep 7(1):1–15

Pasari N, Gupta M, Eqbal D, Yazdani SS (2019) Genome analysis of Paenibacillus polymyxa A18 gives insights into the features associated with its adaptation to the termite gut environment. Sci Rep 9(1):1–14

Payne CM, Knott BC, Mayes HB, Hansson H, Himmel ME, Sandgren M, Stahlberg J, Beckham GT (2015) Fungal cellulases. Chem Rev 115(3):1308–1448

Peñalva MA, Tilburn J, Bignell E, Arst HN Jr (2008) Ambient pH gene regulation in fungi: making connections. Trends Microbiol 16(6):291–300

Pérez J, Munoz-Dorado J, De la Rubia T, Martinez J (2002) Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. Int Microbiol 5(2):53–63

Peterson R, Nevalainen H (2012) Trichoderma reesei RUT-C30–thirty years of strain improvement. Microbiology 158(1):58–68

Phukan MM, Bora P, Gogoi K, Konwar BK (2019) Biodiesel from Saccharomyces cerevisiae: fuel property analysis and comparative economics. SN Appl Sci 1(2):153

Randhawa A, Ogunyewo OA, Eqbal D, Gupta M, Yazdani SS (2018) Disruption of zinc finger DNA binding domain in catabolite repressor Mig1 increases growth rate, hyphal branching, and cellulase expression in hypercellulolytic fungus Penicillium funiculosum NCIM1228. Biotechnol Biofuels 11(1):1–22

Raven S, Francis A, Srivastava C, Kezo S, Tiwari A (2019) Fungal biofuels: innovative approaches. In: Recent advancement in white biotechnology through fungi. Springer, Cham, pp 385–405

Ravindranath N, Lakshmi CS, Manuvie R, Balachandra P (2011) Biofuel production and implications for land use, food production and environment in India. Energy Policy 39 (10):5737–5745

Ribeiro BD, de Castro AM, Coelho MAZ, Freire DMG (2011) Production and use of lipases in bioenergy: a review from the feedstocks to biodiesel production. Enzyme Res 2011:615803

Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30(5):279–291
Shelest E (2008) Transcription factors in fungi. FEMS Microbiol Lett 286(2):145–151
Shishido K (1994) Filamentous ascomycetes and basidiomycetes. In: Recombinant microbes for industrial and agricultural applications. Marcel Dekker, New York, pp 683–700
Shoseyov O, DoI RH (1990) Essential 170-kDa subunit for degradation of crystalline cellulose by Clostridium cellulovorans cellulase. Proc Natl Acad Sci 87(6):2192–2195
Sørensen A, Lübeck M, Lübeck PS, Ahring BK (2013) Fungal beta-glucosidases: a bottleneck in industrial use of lignocellulosic materials. Biomol Ther 3(3):612–631
Strobel G (2014) The use of endophytic fungi for the conversion of agricultural wastes to hydrocarbons. Biofuels 5(4):447–455
Talukdar D, Verma DK, Malik K, Mohapatra B, Yulianto R (2017) Sugarcane as a potential biofuel crop. In: Sugarcane biotechnology: challenges and prospects. Springer, Cham, pp 123–137
Tomme P, van Tilbeurgh H, Pettersson G, Van Damme J, Vandekerckhove J, Knowles J, Teeri T, Claeyssens M (1988) Studies of the cellulolytic system of Trichoderma reesei QM 9414: analysis of domain function in two cellobiohydrolases by limited proteolysis. Eur J Biochem 170(3):575–581
Turgeon BG, Condon B, Liu J, Zhang N (2010) Protoplast transformation of filamentous fungi. In: Molecular and cell biology methods for fungi. Springer, Totowa, NJ, pp 3–19
Von Blottnitz H, Curran MA (2007) A review of assessments conducted on bio-ethanol as a transportation fuel from a net energy, greenhouse gas, and environmental life cycle perspective. J Clean Prod 15(7):607–619
Watkinson S, Eastwood D (2012) Serpula lacrymans, wood and buildings. In: Advances in applied microbiology, vol 78. Elsevier, Burlington, pp 121–149
Wong DW (2009) Structure and action mechanism of ligninolytic enzymes. Appl Biochem Biotechnol 157(2):174–209
Yang B, Wyman CE (2008) Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels Bioprod Bioref Innov Sustain Econ 2(1):26–40
Yip VL, Withers SG (2006) Breakdown of oligosaccharides by the process of elimination. Curr Opin Chem Biol 10(2):147–155
Yuan JS, Tiller KH, Al-Ahmad H, Stewart NR, Stewart CN Jr (2008) Plants to power: bioenergy to fuel the future. Trends Plant Sci 13(8):421–429
Yücel S, Terzioğlu P, Özçimen D (2012) Lipase applications in biodiesel production. In: Biodiesel-feedstocks, production and applications. InTech, Rijeka, pp 209–250

Ligninolytic Fungi from the Indian Subcontinent and Their Contribution to Enzyme Biotechnology

6

Sonu Saini and Krishna Kant Sharma

Abstract

Lignin is the most abundant polyphenolic aromatic biopolymer on Earth, which is extremely recalcitrant toward biodegradation, owing to its heterogeneous structure and biochemical composition. Extensive research efforts have been made to understand the polymeric structure of lignin in a better way and develop a simple, cost-competitive, and eco-friendly method for its degradation. Over the past few years, wood-rotting fungi, especially white-rot fungi have emerged as a crucial group of microorganism capable of mineralizing lignin biopolymers more efficiently. Such fungi have evolved to produce a unique set of extracellular oxidative enzymes in different combinations. Further, they also produce enzymes in multiple isoforms and isozymes that catalyze ligninolysis using radical mediated oxidative reactions. The major ligninolytic enzymes include laccase, manganese peroxidase, lignin peroxidase, and versatile peroxidase. The ligninolytic activities of these enzymes can be enhanced by various natural and/or chemical redox mediators as well as some other auxiliary enzymes (aryl-alcohol oxidase, glyoxal oxidase, quinone reductases, aryl-alcohol dehydrogenases, and feruloyl esterase) to facilitate lignin degradation process. These enzymes have attracted attention of several researchers due to their broad substrate specificity, which make them readily available for numerous biotechnological and industrial applications including paper and pulp industry, food-feed and beverage industry, biofuel industry, bioremediation of hazardous pollutants, and degradation of toxic textile dye effluents. In this chapter, we appraise different ligninolytic fungi from Indian subcontinent and the research findings by native microbiologists and

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biotechnologists on the fungal enzymatic systems. Finally, the biotechnological and industrial applications of ligninolytic fungi and their enzyme arsenals are also discussed.

Keywords

Lignin · Ligninolytic fungi · Ligninolytic enzymes · Laccase · Lignin peroxidase · Manganese peroxidase · Lignin degradation

6.1 Introduction

The term "lignin" was derived from the Latin root "lignum," which means wood (Wong et al. 2020). It represents the second-most abundant renewable biopolymer on this planet and one of the major components of plant cell wall, besides cellulose and hemicellulose (Wang et al. 2019; Tao et al. 2020). The lignin polymer comprises of random aromatic molecules with estimated global presence around 300×10^9 tons (Becker and Wittmann 2019). It has been estimated that around 20×10^9 tons of lignin are produced annually by natural lignification processes (Li and Takkellapati 2018; Tribot et al. 2019). Lignin is an essential backbone of plant cell wall comprising up to 15–40% of plant biomass (Zhu et al. 2020). The biological function of lignin is to provide structural integrity in plant cell wall and resistance against pathogens by preventing enzymatic hydrolysis of the structural polysaccharides (cellulose and hemicellulose) (Mutuku et al. 2019; Vaahtera et al. 2019). Structure-wise, lignin is an irregular three-dimensional heterogeneous biopolymer of highly cross-linked aromatic alcoholic precursors (monolignols), which includes coniferyl alcohol, *p*-coumaryl alcohol, and sinapyl alcohol (Sharma et al. 2007; Ponnusamy et al. 2019; Liao et al. 2020). The oxidative coupling of the monolignols results in the formation of lignin subunits, viz., *p*-hydroxyphenyl subunit (H), guaiacyl subunit (G), and syringyl subunit (S), respectively (Vanholme et al. 2019). Although these subunits share the common feature of having basic phenylpropanoid structure, they vary in the number of methoxy groups in their aromatic rings (Sun et al. 2018; Lu et al. 2020). The H subunit is characterized by non-substituted phenoxide moiety, while G and S units contain monomethoxy phenoxide and dimethoxy phenoxide, respectively (Rinaldi et al. 2016; Gillet et al. 2017). Moreover, the ratio of these phenolic subunits varies among interspecies, as well as within the tissues of the same plant (Campbell and Sederoff 1996; Barros et al. 2015). Generally, softwood lignin is predominantly composed of G subunits (up to 90%), whereas lignin in Gramineae or Poaceae family roughly contains equal quantities of G, S, and H subunits (Lourenço and Pereira 2017). It has been shown that lignin from hardwood constitutes equimolar mixture of G and S unit, along with a small amount of H unit (Gellerstedt and Henriksson 2008; Sharma et al. 2020b). Numerous structural studies have shown that the highly cross-linked lignin structure is predominantly stabilized by various biological strong ether or ester linkages including β-aryl ether (β-*O*-4), β-β, β-5, 5–5, 5-*O*-4, and β-1 couplings (Giummarella

et al. 2019). Among these, β-*O*-4 bonds are the most frequent linkages formed during lignifications and may account for 50% and 80% in softwood and hardwood, respectively (Anderson et al. 2019; Zhu et al. 2019).

Not surprisingly, lignin is the most rebellious and recalcitrant structure among the lignocellulosic tissues; it resists biochemical degradation and valorization (Renders et al. 2017). The high structural complexity of lignin demands multiple biochemical reactions to achieve its efficient degradation that may occur simultaneously (Chio et al. 2019). These biochemical reactions include phenol oxidation, demethylations, hydroxylation of benzylic methylene groups, fission of inter-monomeric linkages, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, side-chain modifications, and cleavage of the aromatic ring (Sun et al. 2018), followed by the breakdown of complex aliphatic metabolites (Mobley et al. 2018). Generally, one of the most efficient ways of lignin valorization is the use of chemical and/or biological treatment (Cao et al. 2018). However, in comparison to biological treatment, the chemical tricks of lignin valorization demands high energy input and chemical agents, thereby significantly increasing the capital cost and causing environmental damage (Chen and Wan 2017). In this scenario, there is an increasing research interest in biological treatments employing natural lignin degraders, including both ligninolytic microorganisms and their enzymatic system, to break down the lignin polymers (Janusz et al. 2017). Under favorable conditions, the lignin biodegradation process is accomplished in two concomitant stages: (1) depolymerization of intact lignin polymer and (2) mineralization of the heterogeneous aromatics unit (Ruiz-Dueñas and Martínez 2009). A wide range of literature supports the efficient degradation of native lignin by fungal cultures, whereas some reports have shown the lignin degradation with bacterial cultures but with less efficiency (Bugg et al. 2011; de Gonzalo et al. 2016; Xu et al. 2018). Lignin is a macromolecule having high molecular weight (more than 100 kDa), which is hard to be assimilated and depolymerized inside the microbial cell; thus, the biological degradation is only possible by the action of extracellular ligninolytic enzymes (Tolbert et al. 2014). Generally, ligninolytic enzymes are divided into two major groups, namely, lignin-degrading accessory enzymes and lignin-modifying enzymes (Hatakka 1994; Wong 2009). The unique ability of these oxidative enzymes to use various phenolic and non-phenolic components of lignin as a substrate in the enzymatic reactions is also exploited in several biotechnological applications (Eriksson 2000; Sharma and Kuhad 2008). A considerable volume of literature is present on various biotechnological and commercial applications of ligninolytic fungi and their enzymatic systems including fuel (Saini et al. 2020b), agriculture, cosmetic and pharmaceuticals (Mohit et al. 2020), food and feed (Sharma et al. 2012), brewery and wine (Ghosh and Ghosh 2019), textiles and laundry (Manoharachary et al. 2005; Singh et al. 2014a, b), and pulp and paper (Dwivedi et al. 2009) as well as in research and development (Fig. 6.1). However, a cumulative study emphasizing detailed mechanisms and various applications of ligninolytic fungi and their ligninolytic machinery in Indian perspective is still scanty (Kuhad et al. 2007; Kuhar et al. 2007). Therefore, this book chapter aims to shed light on the diversity of ligninolytic fungi in the Indian subcontinent and the occurrence of several lignin degrading

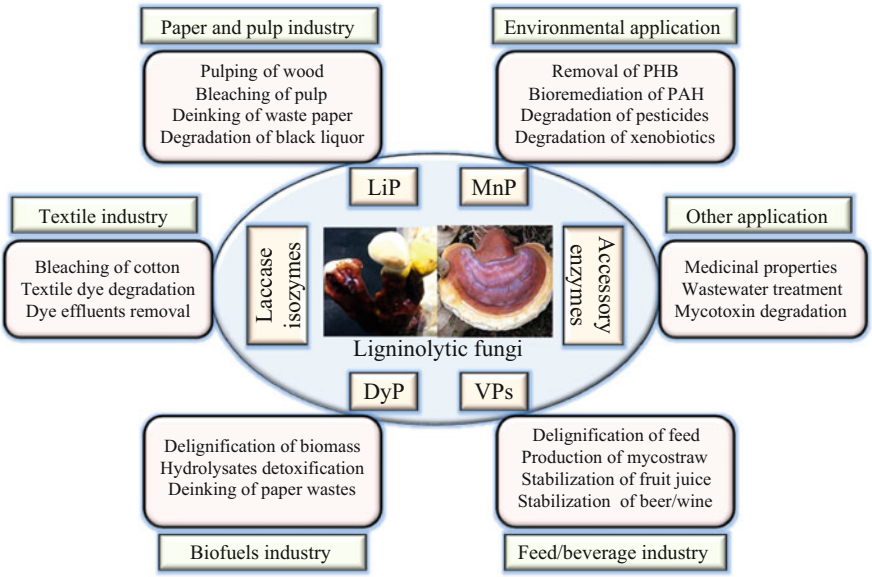

Fig. 6.1 An overview of biotechnological and industrial applications of ligninolytic fungi and their enzymatic system

enzymes. Further, the biotechnological applications of various ligninolytic enzymes are also discussed.

6.2 Lignin-Degrading Fungi

Lignin is the main component of the middle lamella and widely distributed among the secondary walls of the vascular plants (Barros et al. 2015). The mode of polymerization makes it optically inactive, amorphous, and insoluble in water, which is hard for microbes to penetrate and degrade it (Chen et al. 2012). However, in due course of evolution, several microorganisms including bacteria, actinobacteria, and fungi have evolved and adapted to oxidize and utilize lignin as a complex carbon source for their survival and cellular metabolism (de Souza 2013). Due to the unique lignin-degrading property, ligninolytic microorganisms are collected and identified, and a few have been intensively studied by several researchers in India (Sharma et al. 2005; Kuhar et al. 2007; Kumar et al. 2015a, b, 2019; Rudakiya and Gupte 2019). The exact mechanism by which lignin is degraded by microorganisms is still not yet unequivocally understood, but significant recent advances have been made to gain insight into their genomes incorporating modern-omics techniques (Sharma 2016; Shankar et al. 2019; Jain et al. 2019). The ability of microbes to disintegrate lignin polymers efficiently is thought to be attributed to their mycelial growth, which allows the organism to transport scarce

nutrients (Joutey et al. 2013). Over 100 species of the bacteria have been identified possessing the ability of lignin degradation but generally cause limited fraction and much slower degradation in comparison to ligninolytic fungi (Sharma and Kuhad 2009; Datta et al. 2017).

More than a million species of fungi are known to possess the lignin-degrading ability, majorly belonging to the phylum Basidiomycota and Ascomycota (Sigoillot et al. 2012). Further, they are grouped into brown-rot, soft-rot, and white-rot fungi according to the characteristics of the wood residues and degraded lignin (Kapoor et al. 2005; Kuhad et al. 2007). Soft-rot fungi generally cause incomplete and slow degradation of lignin and preferentially attack the hardwoods than softwoods (Hatakka and Hammel 2011). They belong to the largest division of the fungi, Ascomycota, and account for over 60% of all the fungi on Earth, but a few have been identified with lignin-degrading efficiency (Kang et al. 2019). It has been shown that some species of soft-rot fungi can dissolve the secondary cell wall of angiospermic wood and reduce the acid-insoluble Klason lignin content, eventually leading to the formation of microscopic pits and discoloration of wood (Shary et al. 2007). Previous studies revealed that soft-rot fungi can also catalyze the oxidative cleavage of Cα-Cβ and β-*O*-aryl linkages during lignin degradation (Kumar and Chandra 2020). A very few soft-rot fungi with lignin-degrading ability have been isolated from Indian continent, viz., *Lecythophora hoffmannii* (Bugos et al. 1988), *Chaetomium globosum* (Popescu et al. 2011), *Petrillidium boydii* (Kuhad et al. 2007), *Daldinia concentrica* (Nnagadesi and Arya 2015), and *Phialophora mutabilis* (Daniel and Nilsson 1988). On the other hand, brown-rot fungi selectively attack the conifers and cause limited lignin degradation, employing the action of nonspecific oxidants such as hydroxyl radicals (Arantes et al. 2012). Some species of brown-rot fungi are able to demethylate the aromatic and nonaromatic lignin moieties, consequently leading to ring splitting and chemical alteration in lignin structure (Venkatesagowda and Dekker 2020). Among the representatives of brown-rot lignin degraders of Indian origin, *Lentinus lepideus*, *Gloeophyllum trabeum*, *Fomitopsis pinicola*, *Pholiota adiposa*, *Poria placenta*, and *Tyromyces palustris* have been extensively studies (Goñi et al. 1993; Okeke et al. 1994; Wang et al. 2006). Unlike soft- and brown-rot fungi, white-rot fungi are the most intensively studied and are also the most efficient lignin-degrading fungi (Hatakka 1994; Kuhad et al. 2007; Kuhar et al. 2007). They predominately degrade lignin from deciduous trees, and also degrade some coniferous wood trees (Hastrup et al. 2012). The characteristic feature of white-rot fungi is the bleached appearance due to the large-scale degradation of lignin from the decomposed substrate (Eriksson et al. 1990; Kuhad et al. 2007). Further, they can be identified by their mode of lignin degradation and are grouped under two categories, namely, selective and nonselective delignifiers (Dashtban et al. 2010). The selective delignifiers predominantly degrade the lignin polymer and barely affect the structural carbohydrates of the wood material (Leonowicz et al. 1999), whereas the nonselective delignifiers synchronously degrade all the structural components of the attacked woody biomass (Janusz et al. 2017). More than 1000 species of lignin-degrading white-rot fungi have been recorded in the past few decades; further, the number is increasing due to

their various biotechnological and commercial applications (Maciel et al. 2010; Yadav and Yadav 2015). Numerous white-rot fungi have been isolated from different regions of India, for example, *Coriolus versicolor*, *Ceriporiopsis subvermispora*, *Coriolopsis caperata*, *Cyathus bulleri*, *Cyathus stercoreus*, *Daedalea flavida*, *Ganoderma austral*, *G. lucidum*, *Neurospora crassa* (discrete strains), *Pleurotus eryngii*, *Pleurotus florida*, *Pleurotus ostreatus*, *Phlebia brevispora*, *Phlebia radiata*, *Phlebia subserialis*, *Pycnoporus sanguineus*, *Pycnoporus cinnabarinus*, *Schizophyllum commune*, *Trametes ljubarskyi*, *Trametes hirsuta*, and *T. versicolor* (Sharma et al. 2005; Deswal et al. 2014; Sahu and Pramanik 2015; Kumar et al. 2015a, b, 2019; Meehnian and Jana 2016; Saha et al. 2016, 2017; Mustafa et al. 2016; Pamidipati and Ahmed 2017). Among these potent lignin degraders, *C. versicolor*, *T. versicolor*, *Phlebia radiata*, *Pleurotus* spp., and *Phanerochaete chrysosporium* have been extensively exploited for industrial applications worldwide and are presented as model organisms for studying lignin degradation and commercial applications, which are discussed in this book chapter. However, lignin degradation employing fungal cultures occurs at a slower pace, thus requiring a long incubation time (Schoenherr et al. 2017). Questions have been raised over industrial applications of fungal strains due to increased capital costs, owing to a very long degradation time (Madadi and Abbas 2017). To foil such challenges, crude or purified ligninolytic enzymes have been introduced as a versatile lignin degradation system in a relatively short incubation time (Mäkelä et al. 2017; Krumova et al. 2018; Kumar and Chandra 2020).

6.3 Lignin-Degrading Enzymes

Ligninolytic enzymes possess a critical role in the modification and degradation of lignin biopolymers (Kumar and Chandra 2020). Structural rigidity along with complex chemical composition of lignin mandates the ligninolytic microorganisms to produce an array of extracellular oxidative enzymes for efficient degradation (Kameshwar and Qin 2016; Ayeronfe et al. 2018). Broadly, these extracellular oxidative machineries, which include laccase (EC 1.10.3.2), lignin peroxidases (LiPs) [EC 1.11.1.14], manganese peroxidases (MnPs) [EC 1.11.1.13], and versatile peroxidases (VPs) [EC 1.11.1.16], play a direct role in the lignin modification and degradation (Krumova et al. 2018; Bilal and Iqbal 2020). Recently, a novel lignin-degrading peroxidase, namely, dye-decolorizing peroxidase (DyP) [EC 1.11.1.19], has been reported from several fungal cultures (Duan et al. 2018; Rajhans et al. 2020). In addition, several other ancillary enzymes have also been identified, such as glyoxal oxidases (EC 1.2.3.15) (Daou and Faulds 2017), feruloyl esterase (EC 3.1.1.73) (Andlar et al. 2018), alcohol oxidases (EC 1.1.3.13) (Carro et al. 2016), aryl-alcohol dehydrogenase (EC 1.1.1.90), and quinone reductase (EC 1.6.5.5), which enhance peroxidase activities and facilitate lignin degradation (Abdel-Hamid et al. 2013). Till date, numerous microbial communities including bacteria (de Gonzalo et al. 2016), fungi, and actinobacteria (Priya et al. 2018) have been reported for ligninolytic enzyme production (Kamimura et al. 2019). Among

these, white-rot fungi, having the ability to produce several different classes of oxidative enzymes, are always preferred for commercial scale production and applications (Mäkelä et al. 2017; Debnath and Saha 2020).

6.3.1 Laccases: The Leading Industrial Biocatalyst

Fungal laccases are glycosylated multicopper polyphenol oxidases that catalyze the degradation of lignin polymer through the reduction of oxygen molecule into water and concomitant oxidation of the aromatic and nonaromatic units (Tk et al. 1968; Thurston 1994; Sharma and Kuhad 2008). They are also known as benzenediol or blue multicopper oxidases or oxygen oxidoreductases or *p*-diphenol oxidases, which are superior to those of oxidoreductase class (Solomon et al. 1996). Yoshida was the pioneer in reporting laccase from the latex of Japanese lacquer tree *Rhus vernicifera* in 1883 (Yoshida 1883). Later, the presence of laccase was recorded in fungi by Laborde in 1897 (Maciel et al. 2010). Since then, laccase activity has been reported in higher plants, insects, few bacteria, and numerous fungal strains (Sharma et al. 2007; Sharma and Kuhad 2008, 2009; Senthivelan et al. 2016). Laccases are predominantly distributed in the wood-rotting basidiomycetes including *Cyathus stercoreus*, *Cerrena maxima*, *Daedalea flavida*, *Daedalea quercina*, *Pleurotus ostreatus*, *Pleurotus djamor*, *Lentinus squarrosulus*, *Lentinus tigrinus*, *Lentinula edodes*, *Pycnoporus cinnabarinus*, *Phlebia brevispora*, *Trametes villosa*, *T. versicolor*, *T. ochracea*, *Ganoderma lucidum*, *Trametes hirsuta*, *T. gallica*, *T. maxima*, and *T. ljubarskyi* (Arora and Rampal 2002, Sharma et al. 2005, Prasad et al. 2005, Patel et al. 2009, Dhakar and Pandey 2013, Kumar et al. 2015a, b, Singha and Panda 2015, Yadav 2018, Suman et al. 2018). However, some brown-rot fungi, such as *Postia placenta* (Yelle et al. 2011), *Fomitopsis* sp. (Nidadavolu et al. 2013), and *Coniophora puteana* (Shekher et al. 2011), and a few soft-rot fungi, including *Aspergillus nidulans* (Sahay et al. 2020), *Melanocarpus albomyces* (Pundir et al. 2016), *Neurospora crassa* (Chaurasia et al. 2013), *Trichoderma* spp. (Sadhasivam et al. 2010; Divya et al. 2014), *Fusarium* sp. (Chhaya and Gupte 2010), and *Penicillium chrysogenum* (Nayanashree and Thippeswamy 2015; Senthivelan et al. 2019), were also reported as potent laccase producer. The majority of laccases identified in wood-rotting fungi are extracellular glycoproteins; some reports have also reported intracellular laccase in different fungal cultures (Rigling and Alfen 1993; Nagai et al. 2003). Mostly, fungi secrete several isoforms of laccase having average molecular mass in the range between 38 and 150 kDa (Kumar et al. 2017b). These isoenzymes emerge from the same or different genes, and their number predominantly depends upon the source of enzyme (mycelia or fruiting body), species, and growth conditions (Kumar et al. 2017b, 2019; Jain et al. 2019).

Fungal laccases are inducible metalloproteins and monomeric glycoproteins with 10–20% carbohydrate content (Copete et al. 2015). The high level of glycosylation stabilizes the laccase structure and shelters them against thermal degradation (Arregui et al. 2019). Molecular characterization of laccase isozymes has uncovered that primary structure of enzyme constitutes 450–550 amino acid residues spaced in

three successive domains, with a β-sheet topology (Dedeyan et al. 2000). The first domain holds initial 150 amino acids; the second domain covers residues between 150 and 300, and the third domain extends between 300 and 500 amino acids (Agrawal et al. 2018a). Generally, the presence of two strong disulfide bridges confined between domains I and II sustain the enzyme structure (Schindl et al. 2019). Interestingly, earlier reports have also suggested the presence of the third disulfide bridge between domains II and III (Sharma et al. 2016).

The catalytic site in all the laccase isozymes is highly conserved and requires four copper (Cu) atoms arranged in two metallic active sites, which reduce the oxygen into water molecule (Kumar and Chandra 2020). Among these, one Cu molecule figures the substrates to be oxidized, owing to its redox potential, and the rest transfer the electrons to O_2 (Claus 2004). The four Cu molecules of laccases are distributed in three redox sites, i.e., T1Cu, T2Cu, and T3Cu, which differ in their spectroscopic and electronic paramagnetic properties (Chauhan et al. 2017). T1Cu (a type-I copper) has the highest redox potential and is bound to one cysteine and two histidine residues (Jones and Solomon 2015). T1Cu has a characteristic absorbance around 600 nm, responsible for the greenish-blue color of purified laccase (Gunne et al. 2014). Due to the absence of T1Cu, enzyme lacks its blue color and is therefore deemed as "yellow" or "white" laccase (Mot et al. 2020). T2Cu also coordinated with two histidine residues and is involved in the electron transfer (Gunne et al. 2014). On the other hand, T3Cu core are diamagnetic with strong absorption peak near 330 nm and participates in the fixation and reduction of dioxygen (Sitarz et al. 2016). Additionally, T2Cu/T3Cu redox sites may also engage in the enzyme inhibition by interacting with anions such as fluoride or cyanide (Thurston 1994).

Fungal laccases are broadly grouped into three classes depending upon the redox potential of the T1Cu: low (0.4–0.5 V), medium (0.5–0.6 V), and high (0.7–0.8 V) (Zimbardi et al. 2016). Owing to their low redox potential, laccases generally oxidize lignin model compounds having free phenolic groups such as mono-, di-, and polyphenols, arylamines, aminophenols, diamines and aromatic amines, and anilines (Sharma et al. 2016; Upadhyay et al. 2016). The oxidation reactions catalyzed by laccases involve the loss of a single electron and formation of free phenoxy radicals which can act as low molecular weight organic mediators for the enzymes (Shraddha et al. 2011). These intermediate mediators remain unstable; therefore, they leave the enzyme site and perform laccase-mediated oxidation or depolymerizing reactions (Munk et al. 2018). However, non-phenolic compounds cannot be directly oxidized by laccases due to their larger size or due to their relatively high redox potential (Bourbonnais and Paice 1990). Nevertheless, in the presence of suitable chemical mediators, laccases can also oxidize high redox potential non-phenolic model substrates (Bourbonnais et al. 1995; Morozova et al. 2007). The addition of artificial mediators (ABTS and HoBT) (Baiocco et al. 2003; Christopher et al. 2014) and lignin-derived natural mediators (acetosyringone and methyl syringate) (Johannes and Majcherczyk 2000) is a common practice employed in several biotechnological and industrial applications, including biomass delignification and detoxification for biofuel production, bioremediation of polluted soils, processing of food and beverages, degradation of xenobiotics and heavy metals, treatment of effluents

enriched with lignin derivatives, and degradation of hazardous textile dyes (Fig. 6.1) (Rodríguez Couto and Toca Herrera 2006; Morozova et al. 2007).

6.3.2 Lignin Peroxidases: General Properties and Mechanism

Lignin peroxidases were originally discovered in the mid-1980s, from the nitrogen- and carbon-limited medium of basidiomycetous fungus *P. chrysosporium* (Glenn et al. 1983). After the discovery of LiPs from *P. chrysosporium*, several other LiP-producing fungal cultures of Indian origin have been reported, for example, *Ganoderma lucidum*, *T. versicolor*, *Schizophyllum commune*, *Bjerkandera* sp., *Lentinus squarrosulus*, *Pycnoporus sanguineus*, *Phlebia floridensis*, *Phanerochaete sordida*, *Phlebia tremellosa*, and *Phlebia radiata* (Arora and Gill 2005; Bajwa and Arora 2009; Sharma et al. 2011; Selvam et al. 2012; Tripathi et al. 2012; Kaur et al. 2016; Shaheen et al. 2017). They are produced in several isoforms with a molecular mass ranging from 30 to 46 kDa (Vares et al. 1995). LiPs are heme-containing glycoproteins that genuinely catalyze the oxidation of lignin, therefore generally deemed as true ligninases with a high redox potential (Wang et al. 2018). In addition to their high redox potential, LiPs generally have very low pH optima near pH 3.0–4.5, which makes them superior to other classical peroxidases (Kersten et al. 1990). They are recognized as a family of extracellular monomeric glycosylated (up to 20–30%) enzymes having one ferric protoheme per molecule (Falade et al. 2016). The chemical structure of LiP constitutes 343 amino acids along with 370 water molecules, 4 carbohydrates, and 2 calcium ions (Choinowski et al. 1999). On the other hand, the globular structure of LiP is helicoidal in nature and composed of eight major and eight minor α-helices and two antiparallel beta-sheets organized into two domains at both sides of the heminic group (Hammel et al. 1994). This arrangement not only inlaid the protein but also forms an active center cavity to access the solvents via two small channels (Edwards et al. 1993; Bhaskar et al. 2006). The resulting heminic cavity constitutes 40 residues that bound the protein via hydrogen linkages (Poulos 2014). Moreover, the heminic group is interrelated with a water molecule and His amino acid that magnifies the redox potential of LiPs (Piontek et al. 2001). Some studies have also revealed that the presence of a tryptophan (Trp171) residue on the enzyme's surface also pumps the redox potential (Kamitsuji et al. 2005; Sáez-Jiménez et al. 2016).

Lignin peroxidases catalyze the monoelectronic and hydrogen peroxide (H_2O_2)-dependent oxidation depolymerization of lignin compounds through a multistep reaction (Falade et al. 2016). However, questions have been raised about the sensitivity of extracellular LiPs in the presence of H_2O_2, which can partially inactivate the activity of enzyme (Böckle et al. 1999). The addition of veratryl alcohol (VA) to the reaction mixture is a common practice employed to improve the enzyme production and stability (Alam et al. 2009; Falade et al. 2016). Some researchers have conceptualized that VA not only shield the enzyme from the action of H_2O_2, but also engage as a redox mediator between the enzyme and substrates which are

unable to get inside the heme iron center (Koduri and Tien 1995; Huang et al. 2003; Christian et al. 2005; Romero et al. 2019).

High redox potential enables LiPs to oxidize a wide variety of phenolic and non-phenolic lignin compounds (MacDonald et al. 2016). LiPs catalyze the oxidation of phenolic compounds more rapidly over non-phenolic units by removing one electron and creating intermediate free radicals such as phenoxy radicals and veratryl alcohol (Romero et al. 2019). Unlike classical peroxidases which can only act on strongly activated aromatic substrates, LiPs can also oxidize the moderately activated aromatic rings without involving the participation of redox mediators (Plácido and Capareda 2015). LiPs are highly active on a variety of phenolic (guaiacol, methoxybenzenes, vanillyl alcohol, syringic acid, and catechol) and non-phenolic lignin compounds (diarylpropane, VA, and β-*O*-4 lignin dimers) (Chan et al. 2020; Kumar and Chandra 2020). The oxidation of β-*O*-4 lignin dimer involves the generation of radical cations followed by a variety of pathways, including side-chain cleavage, demethylation, and phenol dimerization (Lange et al. 2013). LiPs catalyze several oxidation processes of lignin polymers, such as Cα-Cβ cleavage of the propyl side chains, phenol oxidation, oxidation of benzyl alcohols into aldehydes or ketones, hydroxylation of benzylic methylene groups, and ring cleavage of non-phenolic (aromatic) model compounds of lignin (Reddy et al. 2003; Mobley et al. 2018). LiPs also have great biotechnological potential and industrial applications (Fig. 6.1) due to their high redox potentials and low substrate specificity (Falade et al. 2016; Janusz et al. 2017; Chowdhary et al. 2020).

6.3.3 Manganese Peroxidases: Characteristics and Functions

Manganese peroxidases are extracellular heme-containing glycoproteins, secreted in several isoforms in wood-degrading basidiomycete fungi, including white-rot fungi (Dashtban et al. 2010). They are also termed as hydrogen-peroxide oxidoreductases, representing the most common type of lignin-modifying peroxidases and deemed as class II peroxidase (Morgenstern et al. 2008). Just after the discovery of LiP, MnP was also reported from the batch culture of *P. chrysosporium* in 1984 (Rivela et al. 2000). The production of MnP has been recorded from many other fungal cultures including *Panus tigrinus*, *Trametes versicolor*, *Lenzites betulinus*, *G. lucidum*, *Phanerochaete flavido-alba*, *Phanerochaete chrysosporium*, *Agaricus bisporus*, *Pycnoporus sanguineus*, *Bjerkandera* sp., *Schizophyllum commune*, *Nematoloma frowardii*, and *Phlebia* sp. (Gill and Arora 2003; Nazareth and Sampy 2003; Dhawan et al. 2005; Shanmugam et al. 2005; Padma and Sudha 2013; Pandey et al. 2018; Rao et al. 2019).The molecular mass of the most purified MnPs is around 45 kDa, whereas the mass of crude enzymes ranges between 38 and 62.5 kDa (Janusz et al. 2017). The globular structure of MnP comprises two domains with ten major helixes, a minor helix around the heme ion center (Bhaskar et al. 2006). Additionally, the active site of MnP is unique among peroxidases and comprises two Ca^{2+} ions, histidine and arginine residues, and five disulfide bridges (Kumar and

Chandra 2020). The chemical structure of MnP constitutes around 350 amino acid residues, of these 43% are identical with LiP sequence (Plácido and Capareda 2015).

MnPs are unique among heme peroxidases that catalyze peroxide-dependent oxidation of Mn^{2+} (primary reducing substrate) to Mn^{3+}, which is then released in the aqueous solution and remains quite unstable (Wong 2009). The stability of Mn^{3+} is instigated by chelation with simple organic acids such as malonate and oxalate, thus producing diffusible oxidizing chelate-oxalate (Grąz and Jarosz-Wilkołazka 2011). Indeed, many fungal cultures have been reported to secrete such organic acids along with MnP to stimulate the enzyme activity and Mn^{3+} stability (Martin 2002). The resulting Mn^{3+} chelator complex acts as a low molecular weight redox mediator, which can diffuse into the substrate and oxidize a wide range of phenolic substrates including amines, phenolic lignin substructures, phenols, phenolic lignin model compounds, and reactive dyes (Reddy et al. 2003). The oxidation of phenolic compounds is initiated by one-electron transfer to generate an intermediate phenoxy radical, which further helps in the dissolution of the compounds (Padma and Sudha 2013; Wang et al. 2018).

The oxidation capability of Mn^{3+} chelator complex is only restricted to phenolic lignin compounds (Datta et al. 2017). Unlike LiP, MnPs are not adequate for oxidizing the more fractious non-phenolic substrates (Higuchi 2004). However, it has been repeatedly theorized that MnP can impart the non-phenolic lignin structures, incorporating small mediators such as thiyl or lipid radicals (Qin et al. 2017). Therefore, the oxidation of non-phenolic substrates demands the generation of reactive radical species along with a second mediator (Higuchi 2004). Simple organic acids such as oxalate and malonate are the most commonly known second mediators to facilitate the production of reactive radical species including superoxide, carbon-centered radicals, lipid peroxyl, and formate radicals (Watanabe et al. 2001). The resulting reactive species are used by MnP as source of peroxides to facilitate the decomposition of non-phenolic lignin compounds in the absence of H_2O_2 (Pollegioni et al. 2015). Earlier studies have shown that, the strong MnP-lipid complex can also catalyze the dissolution of Cα-Cβ and β-aryl ether bonds of a non-phenolic lignin model (Urzúa et al. 1998; Ohashi et al. 2011).

6.3.4 Versatile Peroxidase: A Superior Lignin Degrader

Versatile peroxidases (VPs) are superior among the heme-containing ligninolytic fungal peroxidases, which were first discovered in wood-rotting fungus *Pleurotus eryngii* in 1996 (Sigoillot et al. 2012). Earlier, they were recognized as manganese peroxidase, having oxidative properties for aromatic compounds (Ruiz-Dueñas et al. 2009). Later, they were certified as a new peroxidase type belonging to oxidoreductase family (Ravichandran and Sridhar 2016), having a molecular mass between 40 and 45 kDa in several isoforms (Salame et al. 2012). Versatile peroxidases are majorly distributed among *Pleurotus* and *Bjerkandera* spp., such as *P. eryngii*, *P. pulmonarius*, *P. ostreatus*, *B. adusta*, and *B. fumosa* (Verma and Madamwar 2002; Tripathi et al. 2012; Pozdnyakova et al. 2018). Several other basidiomycetes,

including *Marasmiellus palmivorus*, *Physisporinus vitreus*, *Lentinus squarrosulus*, *Dichomitus squalens*, *T. versicolor*, and *Ganoderma* spp., have been also identified as potent producers of VPs (Kuhad et al. 2007; Aarthi et al. 2018; Ravichandran et al. 2019; Saikia et al. 2020).

Versatile peroxidases are attractive wild-type ligninolytic enzymes having characteristic bifunctional oxidative capability and a broad spectrum substrate preference (Camarero et al. 1999). The genomic studies have revealed that VPs share the catalytic properties of both MnP and LiP and are capable of oxidizing Mn^{2+} to Mn^{3+}, as well as degradation of high redox potential non-phenolic lignin substrates like MnP and LiP, respectively (Martínez 2002; Pérez-Boada et al. 2005). Unlike LiP and MnP, VPs are also competent in catalyzing the oxidation of complex lignin structures such as hydroquinone and substituted phenol units without any mediator (Ruiz-Dueñas et al. 2009). These hybrid peroxidases are also able to oxidize high redox dye such as dye Reactive Black 5 in the presence of VA (Pérez-Boada et al. 2005). Previous studies suggested that hybrid molecular structure of VPs, which provide multiple substrate binding sites along with their different pH optima (3.0 and 5.0), favors the degradation of these wide spectrum substrates (Ravichandran and Sridhar 2016; Knop et al. 2016; Gonzalez-Perez et al. 2016). These unique capabilities of versatile peroxidases have attracted lots of research attention and make them a potent "green catalyst" for numerous industrial applications (Fig. 6.1).

6.3.5 Dye-Decolorizing Peroxidases

Dye-decolorizing peroxidases (DyPs) constitute a newly reported superfamily of heme-containing peroxidases that do not show any phylogenetical homology with former fungal peroxidases including LiPs, MnPs, and VPs (Colpa et al. 2014). DyPs are bifunctional catalysts; besides their oxidative activity, they also possess additional hydrolytic activity (Singh and Eltis 2015). They were first identified in 1999 from fungus *Bjerkandera adusta* (formerly known as *Geotrichum candidum*) and named after their ability to catalyze the transformation of a wide range of industrial dye such as RB5, which are poorly converted by other peroxidases (Fernández-Fueyo et al. 2015; Amara et al. 2018). DyPs have a molecular mass of 40–65 kDa and are glycosylated up to 9–31% (Colpa et al. 2014). The molecular structure of DyP contains two domains, α-helices and antiparallel β-sheets, and a heme cofactor positioned in between the cavity of the two domains (Habib et al. 2019).

DyPs are important biocatalysts, which have attracted a great research interest due to their potential applications in lignin degradation (Abdel-Hamid et al. 2013). Compared with classical fungal peroxidases, DyPs work under much lower pH conditions and have wide substrate specificity (Duan et al. 2018). They can oxidize all typical substrates of other heme peroxidases including ABTS, anthraquinones, adlerol, and carotenoids (Shrestha et al. 2017). Several studies have shown that DyPs catalyze the oxidation of phenolic compounds, such as 2,6-dimethoxyphenol and guaiacol (Kim and Shoda 1999; Ogola et al. 2009; Duan et al. 2018). On the other

hand, some reports have emphasized the catalytic ability of DyPs to oxidize the non-phenolic lignin model compounds including β-*O*-4 dimeric lignin, VA, and Mn^{2+} (Liers et al. 2013). The ligninolytic activity of DyPs has been identified in very few fungal cultures, such as *Termitomyces albuminosus*, *Irpex lacteus*, and *Auricularia auricula-judae* (Salvachúa et al. 2013; Linde et al. 2014; Amara et al. 2018).

6.3.6 Accessory Enzymes for Lignin Degradation

In addition to these key ligninases, fungal ligninolytic machineries also include other extracellular enzymes which may act as auxiliary enzymes in the lignin degradation process (Andlar et al. 2018). These include hydrogen peroxide generating extracellular oxidases, which produce H_2O_2 mandatory for other peroxidases to catalyze the oxidative degradation of lignin-derived compounds (Falade et al. 2016; Datta et al. 2017). Examples include the aryl-alcohol oxidase secreted by various fungal cultures, including *Pleurotus eryngii*, *Pleurotus sajor-caju*, *Pleurotus ostreatus*, *Bjerkandera adusta*, and *Geotrichum candidum* (Ghosh et al. 1998, Singh et al. 2010a, b, Tripathi et al. 2011, Kumar and Rapheal 2011), and glyoxal oxidase found in few fungal cultures such as *P. chrysosporium*, *Pycnoporus cinnabarinus*, and *Myceliophthora thermophila* (Sharma et al. 2013b; Ansari et al. 2016). Moreover, fungi also secrete dehydrogenases such as aryl-alcohol dehydrogenases and quinone reductases, which are also involved in the degradation of lignin-derived compounds (Mathieu et al. 2016). Interestingly, some studies have shown that cellobiose dehydrogenase, which is a key member of cellulolytic system, also portrays an effective role in lignin degradation in the presence of H_2O_2 and chelated Fe ions (Temp and Eggert 1999; Harreither et al. 2009). However, the low production titer of these enzymes and inefficient fermentation condition is still a major obstacle that hampers the commercial scale production and application of these auxiliary enzymes (Gutiérrez et al. 2000; Kadowaki et al. 2018; Liu and Wilkins 2020). Nevertheless, with the advancement of modern-omics techniques besides the isolation of novel microbial strains capable of producing significant enzyme doses, the applicability of these enzymes may emerge in the near future.

6.4 Biotechnological and Industrial Applications of Ligninolytic Fungi and Enzymes

6.4.1 Biofuel Industry: Delignification and Detoxification

The conversion of lignocellulosic biomass to energy-enriched biofuels and value-added products is mainly achieved by three steps: (1) delignification of lignocellulose to release cellulose and hemicellulose fibers, (2) depolymerization of structural carbohydrates to produce fermentable sugars, and (3) fermentation of liberated sugars (Kumar et al. 2017a; Chandel et al. 2018). Biological treatments using

ligninolytic fungi and their enzymes have appeared as a new alternative to replace the physicochemical tricks (Sindhu et al. 2016; Ummalyma et al. 2019). In recent years, these biological agents have been extensively exploited for delignification and detoxification of lignocellulosic biomass prior to fermentation (Gupta et al. 2009; Kapoor et al. 2015; Bagewadi et al. 2017; Chandel et al. 2019).

Delignification of lignocellulosic biomass using ligninolytic fungi/enzymes can be categorized under four different methods: (a) fungal delignification, (b) enzymatic delignification, (c) enzyme-mediator system, and (d) integrated biological treatment (Wan and Li 2012, Singh et al. 2014a, b; Moreno et al. 2015). Fungal delignification is generally achieved by directly growing the microorganisms on the targeted biomass under a submerged or a solid-state condition (Chandel et al. 2015). However, the fungal pretreatment may result in loss of fermentable sugars and require a long treatment time (up to 20–50 days) to achieve high delignification percentages (Saha et al. 2017; Kainthola et al. 2019). The effectiveness of fungal pretreatment has been improved by employing an active fungal consortium and integration of fungal treatment with certain physical/chemical methods (Salvachúa et al. 2011; Shirkavand et al. 2016; Meehnian et al. 2017). Additionally, the use of ligninolytic enzymes with or without redox mediators has been accepted as an effective and simpler method to achieve high delignification percentage, consequently addressing the key limitations of fungal treatment (Table 6.1) (Gupta et al. 2009; Moilanen et al. 2014; Plácido and Capareda 2015; Saini et al. 2020b). For enzymatic delignification, laccase isozymes with or without redox mediators are always preferred, followed by MnP and LiP. The mixtures of ligninolytic enzymes have also been employed for some delignification treatments that have shown improved lignin removal efficiency in a short time (Heap et al. 2014; Kudanga and Le Roes-Hill 2014; Giacobbe et al. 2018).

In addition to delignification, enzymatic detoxification of biomass represents one of the few promising biotechnological applications of ligninolytic enzymes in the biofuel industry (Table 6.1) (Kapoor et al. 2015; Suman et al. 2018). The traditional physical and chemical pretreatment processes produce toxic compounds such as phenolic derivatives, furan, furfural, and weak acids (Gupta et al. 2009; Silveira et al. 2018; Saini et al. 2020a). Since these compounds strongly inhibit the cellulolytic enzymes and fermentation process, they need to detoxify in order to enhance enzyme and yeast performance (Tejirian and Xu 2011; Qin et al. 2016). Biological detoxification using ligninolytic fungi/enzymes have been shown to reduce the concentration of inhibitory compounds (Fang et al. 2015; Moreno et al. 2015). The toxic phenolic derivatives are predominantly detoxified by key ligninases (Rameshaiah and Jagadish Reddy 2015). Previous studies have shown that aryl-alcohol oxidases are capable of detoxifying furan derivatives such as 5-hydroxymethylfurfural and polyunsaturated alcohols (Carro et al. 2015; Feldman et al. 2015). Besides positive impacts such as high detoxification and delignification percentage, eco-friendly, and low sugar losses, fungal and enzymatic treatments have certain drawbacks such as high costs, low commercial availability, and long process duration (Ramarajan and Manohar 2017; Wagner et al. 2018). Nevertheless, delignification and detoxification capabilities of ligninolytic fungi/enzymes can be improved by using genomic and

Table 6.1 Recent applications of ligninolytic fungi and their enzymes in biofuel sector carried out by Indian mycologists

Culture	Substrate and process	Experimental condition	Results	References
Pleurotus florida, *Coriolopsis caperata*, and *Ganoderma* sp.	Sugarcane bagasse; delignification	Delignified at 30 °C for 15 days under SSF condition at 25% (w/v) moisture content	Reduced 7.91%, 5.48%, and 5.58% lignin, respectively	Deswal et al. (2014)
Coriolus versicolor	Sorghum bagasse; delignification	Treated at 27.5 °C in a mesh tray bioreactor with 75% relative humidity under SSF condition	Reduced 46.09% lignin after 20 days of treatment	Mishra and Jana (2019)
Daedalea flavida	Cotton stalk; delignification	Delignified at 28 °C with 75% moisture content under SSF condition	Degrade 27.83% lignin after 40 days of incubation	Meehnian and Jana (2016)
Lentinus squarrosulus	Kans grass; delignification	Treated at 35 °C with 15% (w/v) consistency and 3125 IU/g laccase	Removed 81.67% lignin after 6 h	Rajak and Banerjee (2016)
Trametes versicolor	Rice straw and poplar waste; detoxification	Detoxified the pretreated biomass at 28 °C with 1.2 U/ml laccase for 12 h	Reduced 76% and 94% phenolic inhibitors, respectively	Kapoor et al. (2015)
Trametes maxima	Sugarcane bagasse; detoxification	Detoxification was done with 0.1 U/ml laccase at 32 °C	Removed 66% of phenolics derivatives after 1.5 h	Suman et al. (2018)
Lentinus squarrosulus	Finger millet and paddy straw; delignification	Treated with 25 IU/g of enzyme at 60 °C for 24 h	Reduced 17 and 11% lignin from straws, respectively	Ravichandran et al. (2019)
Pleurotus ostreatus	Wheat straw and banana stem; delignification	Delignified at 30 °C with substrate to moisture ratio of 1:4 (w/v) under SSF condition	Degraded 40 and 29% lignin content after 32 days of incubation	Thakur et al. (2013)
Pleurotus ostreatus, *P. chrysosporium*, and *Ganoderma lucidum*	Rice straw; delignification	Delignification was done 30 °C with 70% relative humidity for 35 days	Reduced 21.85%, 38.74%, and 17.81% lignin content	Kainthola et al. (2019)
Coriolus versicolor	Sweet sorghum bagasse; delignification	Treatment was done at 27.5 °C with 70% (w/v) humidity	Reduced 24.8% lignin content after 20 days	Mishra and Jana (2019)

protein engineering approaches and appropriate interaction between different enzymes to evolve a dominant lignin degradation system for biofuel industry.

6.4.2 Food, Feed, and Beverage Industry

The direct use of lignocellulosic biomass as ruminant animal feed exhibits one of the oldest and most recent applications (Sharma et al. 2012). The abundance of poorly digestive lignin polymer hampers the use of such biomass by rumen microbes, thereby demanding lignin removal prior to feed application (Sheikh et al. 2018). Enzymatic delignification of lignocellulosic biomass has received considerable attention due to their efficiency in improving animal feed performance (Sridhar et al. 2015; Ravichandran et al. 2019). A number of extracellular enzymes including various cellulolytic, hemicellulolytic, and ligninolytic are extensively exploited for animal feed industry (Sharma et al. 2005; Thammaiah et al. 2016). Among these, ligninases have received much research interest due to their ability to enhance the digestibility, to improve the nutritional value, and to reduce the excretion of nitrogen and phosphorous (Table 6.2) (Kumar et al. 2015a, b; Chowdhary et al. 2019). Direct solid-state fermentation of biomass by selective and nonselective lignin-degrading white-rot fungi is also a very attractive alternative (Kuhad et al. 2013). Various Indian origin white-rot fungi, such as *B. adusta*, *P. chrysosporium*, *T. versicolor*, *Fomes fomentarius*, *Cyathus* sp., *Phlebia rufa*, *Coriolus hirsutus*, *Irpex lacteus*, *G. lucidum*, *G. applanatum*, *Dichomitus squalens*, *Pleurotus flabellatus*, *P. ostreatus*, *P. tuber-regium*, and *Lentinus subnudus*, have been studied for solid-state bioconversion of lignocellulosic biomass to enhance animal feed properties (Table 6.2) (Sharma et al. 2012, 2013a; Shrivastava et al. 2014; Raghuwanshi et al. 2014; Nayan et al. 2020). Solid-state fermentation of lignin-rich wheat straw with selective lignin degraders into high-energy cattle feed usually termed as "mycostraw" is a common practice to increase crude protein content and energy constituents for ruminant feed (Shrivastava et al. 2011, 2012; Nayan et al. 2018).

The utility of various ligninases has been reported in the processing of certain foods, feed, and beverages (Brijwani et al. 2010). Ligninases have been observed to eliminate phenolics saccharides from the fruit juices and fermented alcoholic beverages, which eventually reduce browning, haze formation, and turbidity (Minussi et al. 2002; Mäkelä et al. 2017). Ligninases not only stabilize the fruit juice and fermented alcohol beverages but also improve the color appearance, flavor, aroma, and taste of such beverages (Gassara-Chatti et al. 2013; Lettera et al. 2016; Ghosh and Ghosh 2019). Some previous studies have shown that LiPs and MnPs have the potential to generate natural aromatic flavors in various food products (dos Santos Barbosa et al. 2008, Kumar and Chandra 2020). Moreover, some white-rot fungi can be used to convert agricultural lignocellulosic wastes directly to human food, like edible fruit bodies of *Lentinus*, *Pleurotus sajor-caju*, *Agaricus bisporus*, *Cantharellus*, and *Volvariella* (Goyal et al. 2006; Ahlawat and Tewari 2007; Kumari et al. 2011; Thiribhuvanamala et al. 2012; Kumla et al. 2020).

Table 6.2 Ligninolytic fungi/enzymes application in food/feed/beverage industries explored in India

Culture	Feed/food/beverage	Research finding	References
Ganoderma sp. and *Crinipellis* sp.	Wheat straw	Fungal treatment increased the feed digestibility by degrading ether extract, neutral detergent fiber (NDF), and acid detergent fiber content (ADF)	Sharma et al. (2012)
Schizophyllum commune	Paddy straw, finger millet straw, wheat straws, etc.	Laccase treatment significantly reduced the indigestible NDF, ADF, and acid detergent lignin (ADL), consequently enhancing the in vitro dry matter digestibility (IVDMD)	Kumar et al. (2015a, b)
Ceriporiopsis subvermispora	Wheat straw	Fungal treatment preferred NDF, ADF, and ADL components and increased the carbohydrates-to-lignin ration (C/L ratio)	Nayan et al. (2017)
Coriolus versicolor and *Ganoderma lucidum*	Ragi straw	Ligninolytic enzymes significantly increased the protein content and dry matter digestibility (5%) and reduced the composition of lignin derived compounds	Sridhar et al. (2015)
Lentinus squarrosulus	Paddy straw, finger millet straw, little millet straw, etc.	Enzymes treatment reduced the NDF (2.6–4.2%) and ADL (2.9–5.2%) content and significantly increased the IVDMD (14–32%)	Ravichandran et al. (2019)
Ganoderma sp.	Wheat straw	Fungal treatment reduced the indigestible lignin constituents and increased the C/L ration (1.28–fold) with high IVDMD (32.2%)	Raghuwanshi et al. (2014)
Ganoderma lucidum	Pomegranate, lemon, and apple juice	Enzyme treatment significant reduced the toxic phenolics compounds (79–85%) and improved clarity, stability, and quality of the fruit juice	Manavalan et al. (2015)
Aspergillus flavus	Apple juice	Enzyme treatment brings a reduction in phenolic, flavonoid, and turbidity, which indicates the suitability of the juice	Ghosh and Ghosh (2019)

6.4.3 Paper and Pulp Industry: Biopulping, Biobleaching, and Deinking

Separation of lignin from the cellulosic fibers is a necessary step required during pulp and paper production from the woody substrates (Eugenio et al. 2019). Various woody materials including bamboo, eucalyptus, and leftover agricultural biomass have been exploited for paper manufacturing depending upon the type and quality of the paper (Trotter 1990; Eriksson 1998; Bajpai 2018a). Pulping and bleaching of

these woody substrates are the two important steps required for paper production (Bajpai 2018b). During pulping, the native substrates are subjected to different mechanical and chemical methods to reduce the fibrous state (Hubbe et al. 2007). Conventional and the present-day chemical methods involve consumption of enormous amounts of chlorine, sulfite, or oxygen-based chemical oxidants, which impose serious drawbacks to the natural environment (Leponiemi 2008; Liu et al. 2018). Besides environmental adverse effects, the chemical tricks are also cost-intensive processes, generating low pulp yield between 40% and 50% (Ince et al. 2011). To overcome these obstacles, microbial or enzymatic systems capable of delignifying woody material can be used (Bajpai 2018b). In this scenario, white-rot fungi and their oxidative enzymes are potentially useful agents because they not only diminish the energy and cost consumption but also improve the strength properties and are environmentally friendly due to reduced effluent toxicity (Singh et al. 2010a, b, Singh and Singh 2014, Janusz et al. 2017). The biopulping of woody material using white-rot fungi was first considered by Lawson and Still (1957) at West Virginia Pulp and Paper Company Research Laboratory (Akhtar et al. 1997). Since then, many white-rot fungi have been studied, and some of them were found to be efficient biobleachers, viz., *Bjerkandera* spp., *P. chrysosporium*, *Botrytis cinerea*, *Polyporus ciliates*, *Phlebia radiata*, *Lentinus tigrinus*, *Stereum hirsutum*, *Stropharia coronilla*, and *T. versicolor* (Table 6.3) (Kuhad et al. 2007; Kuhar et al. 2007; Saleem et al. 2018; Nathan et al. 2018; Chaurasia and Bhardwaj 2019; Sharma et al. 2019) in pulping processes and pilot mill trials. Previous studies have shown that ligninases such as laccase and LiP portray an important role in biopulping, but direct evidences for LiP are not available (Vrsanska et al. 2016; Damián-Robles et al. 2017). It has been theorized that biopulping using fungi and ligninolytic enzymes could save 30%–45% of the energy consumed in comparison to mechanical and chemical processes (Falah et al. 2011; Giles et al. 2015; Chukwuma et al. 2020).

Pulping is followed by bleaching of kraft pulp, which demands huge amount of chlorine and chlorine-derived chemicals, consequently leading to environmental disturbance (Kaur et al. 2019). To overcome these drawbacks, enzymatic processes have been envisaged to provide environmentally intact technology, and the use of laccase and laccase-mediator system remains the choice of technology to achieve the goals (Table 6.3) (Onysko 1993; Fillat and Roncero 2009; Singh et al. 2015, 2019). Previous studies have shown that laccase-mediator system such as violuric acid, ABTS, and HBT can enhance ISO brightness and reduce the kappa number and yellowness effect of the kraft pulp (Pala et al. 2004; Lee et al. 2011; Chutani and Sharma 2015; Tsatsis et al. 2017). Additionally, heme peroxidases such as MnP and LiP are also seen to be competent in decolorizing the kraft pulp mill effluents (Saraswathi and Saseetharan 2010; Chandra et al. 2018; Gaur et al. 2018). Although over 70% of the world's annual pulp is produced by chemical and mechanical processes, a large financial investment has been made to improve the biobleaching and biopulping processes using ligninolytic fungi and enzymes, which are likely to be accepted in the near future.

Beside biopulping and biobleaching, decolorization of paper and pulp mill effluents, and deinking of paper waste using ligninolytic enzymes to enhance the

Table 6.3 Ligninolytic fungi/enzymes from Indian subcontinent and their applications in paper and pulp industry

Fungal culture	Application	Research finding	References
Ganoderma sp., *Trametes* sp., and *Poria* sp.	Biobleaching of hard wood Kraft pulp	All fungal cultures significantly reduced the kappa number (5–12%) and increased the brightness of the pulp (30–45%)	Selvam and Arungandhi (2013)
Stereum ostrea and *Trametes versicolor*	Biobleaching of Kraft pulp	Kappa number was reduced to 17% after fungal treatment; addition of Mn^{2+} drastically enhanced the pulp brightness	Seshikala (2014)
Ganoderma lucidum MDU-7	Deinking of paper waste	Laccase-mediator system significantly reduced the ink particles (61%) and detoxified the paper hydrolysates	Saini et al. (2020b)
Nigrospora sp. and *Trametes* sp.	Decolorization of Kraft black liquor	Mixed fungal culture resulted in maximum decolorization (73%) and COD removal (71%)	Rajwar and Rai (2015)
Pleurotus ostreatus	Biobleaching of mixed wood pulp	Addition of xylanases to the culture extract of *P. ostreatus* significantly improved the pulp properties	Dwivedi et al. (2009)
Trichoderma harzianum	Biobleaching and bioremediation of paper industry effluent	*T. harzianum* was evaluated for ligninolytic activity; crude enzyme extract was found effective in biobleaching and bioremediation	Sadhasivam et al. (2010)
P. chrysosporium and *Pleurotus* sp.	Detoxification of paper industrial effluents	Both fungal strains were found to reduce the toxic chemicals mutagens and COD of the paper industrial effluents	Kulshreshtha et al. (2012)
Peniophora sp.	Deinking of paper waste	Laccase treatment improved the strength and brightness of recycled pulp, 1.7-fold and 6%, respectively	Shankar et al. (2018)
Fusarium equiseti	Biobleaching of waste newspaper	Enzymatic bleaching with laccase reduced the kappa number and increased the brightness (15%)	Nathan et al. (2018)
Daedalea flavida, *Polyporus hirsutus*, and *Phellinus* sp.	Bioremediation of pulp and paper mill effluents	Fungal cultures were shown to decolorize the waste effluents (62–66%) and reduce the COD (38–42%)	Selvam et al. (2011)

reusability of waste papers represent one of the few promising biotechnological applications of ligninolytic fungi (Kuhad et al. 2010; Chutani and Sharma 2015; Saini et al. 2020b; Chandranupap and Chandranupap 2020). Traditionally, deinking was performed under high temperature conditions, employing various chemical surfactants, which makes the process expensive and causes high environmental

damage (Pala et al. 2004; Virk et al. 2013; Chutani and Sharma 2016; Biswas et al. 2019). Therefore, to tackle these challenges, enzymatic methods are envisaged and proven to be economically feasible (Saxena and Chauhan 2017, Shankar et al. 2018, Valls et al. 2019, Sharma et al. 2020a). Among all, laccase-mediator system has been reported to be efficient in ink degradation and has been successfully scaled up to large-scale applications (Table 6.3).

6.4.4 Transformation and Degradation of Textile Dye Effluents

Different type of dyes and pigments are extensively used in the textile industries; the unadhered dye effluents from these industries are directly discharged to natural water bodies without any treatment (Chequer et al. 2013). The coarse textile effluents majorly constitute a large number of heavy metals such as Co, Cu, Cd, Mg, Fe, Cr, Ni, Hg, and Mn, which are highly pernicious and carcinogenic in nature (Carmen and Daniela 2012; Lellis et al. 2019). These unhealthy impurities have adverse impacts on the water quality in terms of biological oxygen demand, total organic carbon, chemical oxygen demand, pH, and color of the water resources (Tchounwou et al. 2012; Ali et al. 2019). Based on the chemical structure, the textile dyes are classified as anthraquinone, indigo, triarylmethane, phthalocyanine, and azo dyes; among these, azo dyes are deemed harmful due to their excessive utilization and high water solubility (Benkhaya et al. 2017, 2020; Ahlawat et al. 2019).

Traditionally, several attempts have been made to treat these hazardous effluents using various physicochemical processes, viz., filtration, precipitation, adsorption, coagulation, photolysis, and oxidation with H_2O_2 or ozone (Lee et al. 2006; Chutani and Sharma 2015; Hayat et al. 2015; Saini et al. 2020b). Unfortunately, the applications of these physicochemical methods are confined mainly due to excessive need of additional environmental hazardous chemical additives, incomplete and unsafe disposal of generated sludge, and high operating costs (Singh and Arora 2011; Pereira and Alves 2012; Jamee and Siddique 2019). In this context, biological methods have been shown as novel alternatives with low-cost and environmentally benign processes capable of degrading complete organic pollutants without involving the addition of other chemicals (Patel et al. 2013; Rajhans et al. 2020).

The biological degradation of hazardous textile dyes has been reported using various microorganisms including fungi, bacteria, and actinomycetes, employing single or complex microbial consortia (Singh et al. 2010a, b; Gao et al. 2018, Jamee and Siddique 2019, Varjani et al. 2020). Some of the modern molecular modelling and docking experiments have also been reported for the dye degradation potential of laccases from bacterial and fungal sources (Singh et al. 2014a, b; Ahlawat et al. 2019, Vani et al. 2020). Among these, white-rot fungi have been shown as better dye degraders over prokaryotes due non-specific nature of their extracellular ligninolytic enzymes capable of degrading a wide range of dyes (Table 6.4) (Gahlout et al. 2013; Patel et al. 2013; Srivastava et al. 2014). The degradation of textile dyes using a white-rot fungus, *P. chrysosporium*, was first reported by Glenn and Gold in 1983 (Glenn et al. 1983). Subsequently, numerous white-rot fungi, including *Bjerkandera*

Table 6.4 Applications of ligninolytic fungi/enzyme in bioremediation of organopollutants and hazardous dye effluents

Fungal culture	Pollutants	Result and findings	References
Ganoderma lucidum	PAH (phenanthrene and pyrene)	High degradation of phenanthrene (99.65%) and pyrene (99.58%) was achieved after 30 days of treatment at 27 °C	Agrawal et al. (2018b)
Pleurotus ostreatus	PAH (benzo (a) pyrene)	Addition of non-ionic surfactant (T-80) to the fungal treatment enhanced the degradation of PAH (1.18-fold)	Bhattacharya et al. (2014)
Trametes versicolor	Azo dyes and dye industry effluents	Batch mode fungal treatment removed 34.60% orange G, 98.27% Congo red, and 78.05% Amido black 10B, and 98.43% dye effluents	Selvam et al. (2012)
Ganoderma cupreum	Azo dyes (reactive violet 1)	Degradation was increased by adding nitrogen and carbon source to the decolorization medium	Gahlout et al. (2013)
Coriolopsis byrsina	PAH (pyrene)	High degradation of pyrene in vivo (96.1%) and in vitro (51.85%) condition was achieved, and final nontoxic compound were identified	Agrawal and Shahi (2017)
Phanerochaete chrysosporium	Reactive textile dye (yellow MERL and red ME4BL)	*P. chrysosporium* was found to produce laccase and MnP VP to catalyze the degradation of reactive textile dyes	Koyani et al. (2013)
P. flabellatus, *P. ostreatus*, and *P. citrinopileatus*	Azo dye (direct red)	Decolorization of dye was increased by adding 0.1% T-80, and laccase, MnP, and LiP were shown to catalyze the dye degradation	Srivastava et al. (2014)
Myceliophthora vellerea	Textile dye (reactive blue 220)	Fungal treatment using immobilized packed-bed reactor achieved 80% dye degradation and 50% decolorization up to the seventh cycle	Patel et al. (2013)
Trametes hirsuta	PAH (benzo (a) pyrene and phenanthrene)	Addition of rhamnolipid enhanced the fungal degradation efficiency (1.8-fold) and achieved high degradation of phenanthrene (91.26%) and benzo (a) pyrene (87.72%)	Rathankumar et al. (2020a)
P. ostreatus, *P. flabellatus*, *P. florida*, and *P. sajor-caju*	Azo dye (direct blue 14)	Up to 90% of dye degradation was achieved after 6–12 days of incubation, and all fungus culture were shown to produce laccase and MnP involved in dye decolorization	Singh et al. (2013)

(continued)

Table 6.4 (continued)

Fungal culture	Pollutants	Result and findings	References
Geotrichum candidum	Azo dye (methyl orange, Congo red, and trypan blue)	Significant degradation of azo was reported; methyl orange (94%), Congo red (85%), and trypan blue (70%). DyP, LiP, and laccase were identified to catalyze the degradation	Rajhans et al. (2020)
Ganoderma lucidum	Pesticide (lindane)	Pesticide degradation was found higher (75.5%) under SmF condition in comparison to SSF condition (37.5%)	Kaur et al. (2016)

adusta, *Coriolus versicolor*, *Lentinus edodes*, *Lentinus polychrous*, *Lentinus polychrous*, *Lenzites elegans*, *Laccaria fraternal*, *Hirschioporus larincinus*, *Funalia trogii*, *Fomes lividus*, *Inonotus hispidus*, *Pleurotus ostreatus*, *Pleurotus eryngii*, *Phlebia radiate*, *Phlebia tremellosa*, *Pycnoporus cinnabarinus*, *Sclerotium roysii*, *T. versicolor*, *Trametes modesta*, *Trametes hirsute*, and *Thelephora* sp., have been shown to decolorize various dyestuffs (Table 6.4) (Selvam et al. 2003; Singh and Pakshirajan 2010; Gulzar et al. 2020; Saha and Mukhopadhyay 2020; Singh et al. 2020; Munagapati et al. 2020; Vats and Mishra 2020; Zofair et al. 2020). Additionally, soft-rot fungi such as *Aspergillus flavus*, *Aspergillus terreus*, *Geotrichum candidum*, *Trichoderma harzianum*, *Mucor* spp., and *Penicillium* spp. have been identified to degrade several textile dyes including acid red, bromophenol blue, and Congo red (Ramya et al. 2007; Sadhasivam et al. 2010; Ranjusha et al. 2010; Kumar et al. 2012; Saroj et al. 2014; Rajhans et al. 2020; Singh and Dwivedi 2020). Dye degradation using white-rot fungi was largely attributed to LiP and MnP enzymes (Jayasinghe et al. 2008). In recent years, laccase and novel DyP enzymes have also gained a great research interest in relation to dye degradation (Lai et al. 2017; Yang et al. 2019; Iark et al. 2019). The utilization of laccase in combination with mediators and other auxiliary enzymes has been proved to be a valid alternative eco-friendly method with less capital-cost investment (Table 6.4).

6.4.5 Bioremediation of Hazardous Pollutants

Throughout the past century, human activities have released many hazardous organopollutants into the natural environment responsible for air, soil, and water pollution (Rodríguez Couto and Toca Herrera 2006; Álvarez-Rogel et al. 2007; Li et al. 2017). Some of these, including recalcitrant xenobiotic chemicals, pesticides, trinitrotoluene, synthetic dyes, polycyclic aromatic hydrocarbons, organochlorines, polychlorinated biphenyls, benzene, ethylbenzene, toluene, xylene, and munition wastes, remain persistent in the environment, which are known to be cytotoxic, carcinogenic, and mutagenic (McGuinness and Dowling 2009, Juwarkar et al. 2010, Varsha and Chenna 2011). Scientists are working toward decreasing these toxic

pollutants. The currently employed techniques are, however, either costly or time-consuming and may accumulate other hazardous by-products. In this sense, the use of white-rot fungi that produce ligninases is reported to be the most promising in detoxification and degradation of toxic pollutants (Torres et al. 2003; Viswanath et al. 2014; Deshmukh et al. 2016). Due to low substrate specificity of their oxidative enzymes and structural similarities between lignin and organopollutants, fungi are able to perform the breakdown of various recalcitrant effluents (Pointing 2001; Pozdnyakova 2012; Upadhyay et al. 2016). The degradation of organopollutants using white-rot fungi was first achieved by *P. chrysosporium* (Bumpus et al. 1985). Since then, numerous white-rot fungi, such as *Bjerkandera* sp., *Clavariopsis aquatic*, *Cyathus bulleri*, *G. lucidum*, *Pleurotus* sp., *Polyporellus brumalis*, *Phanerochaete sordida Schizophyllum commune*, *P. cinnabarinus*, *Pleurotus pulmonarius*, *T. versicolor*, *Trametes trogii*, *Trametes hirsutus*, *Coriolopsis* sp., and *Phlebia* sp., have been reported as potent degraders of various organopollutants (Table 6.4) (Skariyachan et al. 2016, Bhattacharya et al. 2017, Vaseem et al. 2017, Patil and Yadav 2018, Kumar et al. 2018, Navada and Kulal 2019, Chakraborty et al. 2019, Rathankumar et al. 2020b, Taha et al. 2020, Dhiman et al. 2020). Additionally, ligninolytic enzymes including laccases, LiP, MnP, and VPs from these fungi have also been successfully applied in the treatment of a wide range of toxic effluents (Bhattacharya et al. 2009; Rao et al. 2010; Rathankumar et al. 2020a). Enzyme-mediated degradation of organopollutants involves the polymerization of the pollutants themselves or copolymerization with other nontoxic substrates to facilitate their removal by means of filtration, sedimentation, or adsorption (Shen et al. 2011; Falade et al. 2016; Pathak and Navneet 2017). However, the majority of degradation studies have been conducted on artificially contaminated soils or on synthetic medium spiked with organic pollutants (Table 6.4). Therefore, it is important to investigate both fungal and enzyme performance under non-sterile conditions to transfer the laboratory technology to the actual large-scale contaminated field sites.

6.5 Concluding Remarks

In the foregoing discussion, we have emphasized on the current state of knowledge on lignin-degrading microorganisms, especially white-rot fungi. White-rot fungi have drawn enormous research interest due to their ability to produce vast amounts of extracellular oxidative ligninolytic enzymes. They are also selective lignin degraders and are responsible for efficient deconstruction of woody or lignocellulosic substrates. Since the discovery of ligninolytic fungi, extensive research efforts have been made on the isolation of novel ligninolytic microorganisms, characterization of novel ligninolytic enzymes, and cloning of genes encoding enzymes from the existing microbes. Currently, exploration of fungal proteome and secretome is an effective tool to identify novel enzymes. It can also be used to study the succession, interaction, and complementation between different ligninases involved in lignin degradation pathways. Lignin degradation is thought to proceed via multistep reactions involving an array of main oxidative ligninolytic enzymes in the first

step and several other accessory enzymes to help and achieve the degradation in later stages. However, the low specificity of ligninolytic enzymes engaged in the degradation pathways of lignin makes it difficult to understand the whole processes. Additionally, most of the lignin degradation pathway studies are carried out using lignin model compounds; still, comprehensive study using native form of lignin encountered by fungi/enzyme creating a natural environment in vitro is necessary. Therefore, future research should be aimed at determining the role and cooperation of enzyme consortia in lignin degradation, exploring the function of redox mediators and auxiliary enzymes in the catalytic mechanism of the ligninases, and reconstituting in vitro the actual environment for fungal/enzyme ligninolytic systems for lignin degradation.

The practical use of ligninolytic fungi and their enzymes for biotechnological and industrial applications holds a great potential. The advantages and disadvantages of ligninolytic fungi/enzymes should be evaluated before attempting industrial scale operations. Though biotechnological significance of these fungi/enzymes have increased drastically, there are still some aspects to be investigated, particularly process optimization and cost reduction. Both can be achieved by isolating native fungal stains and enzymes that are best suited for wide substrate spectrum and required narrow reaction conditions. Here genetic engineering such as protein engineering and gene editing technologies can have an important role to play. Searching for novel and native fungal strains and taking advantage of the enormous microbial diversity of the Indian subcontinent can enhance the choice for the customized industrial applications.

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References

Aarthi R, Rao RG, Thammaiah V, et al (2018) A novel versatile peroxidase from *Lentinus squarrosulus* (MH172167) towards enhanced delignification and digestibility of crop residues. bioRxiv 394098. https://doi.org/10.1101/394098

Abdel-Hamid AM, Solbiati JO, Cann IKO (2013) Chapter 1—Insights into lignin degradation and its potential industrial applications. In: Sariaslani S, Gadd GM (eds) Advances in applied microbiology. Academic Press, Amsterdam, pp 1–28

Agrawal N, Shahi SK (2017) Degradation of polycyclic aromatic hydrocarbon (pyrene) using novel fungal strain *Coriolopsis byrsina* strain APC5. Int Biodeterior Biodegrad 122:69–81. https://doi.org/10.1016/j.ibiod.2017.04.024

Agrawal K, Chaturvedi V, Verma P (2018a) Fungal laccase discovered but yet undiscovered. Bioresour Bioprocess 5:4. https://doi.org/10.1186/s40643-018-0190-z

Agrawal N, Verma P, Shahi SK (2018b) Degradation of polycyclic aromatic hydrocarbons (phenanthrene and pyrene) by the ligninolytic fungi *Ganoderma lucidum* isolated from the hardwood stump. Bioresour Bioprocess 5:11. https://doi.org/10.1186/s40643-018-0197-5

Ahlawat OP, Tewari RP (2007) Cultivation technology of paddy straw mushroom (*Volvariella volvacea*). National Research Centre for Mushroom, Solan

Ahlawat S, Singh D, Virdi JS, Sharma KK (2019) Molecular modeling and MD-simulation studies: fast and reliable tool to study the role of low-redox bacterial laccases in the decolorization of various commercial dyes. Environ Pollut 253:1056–1065. https://doi.org/10.1016/j.envpol.2019.07.083

Akhtar M, Blanchette RA, Kent Kirk T (1997) Fungal delignification and biomechanical pulping of wood. In: Eriksson K-EL, Babel W, Blanch HW et al (eds) Biotechnology in the pulp and paper industry. Springer, Berlin, pp 159–195

Alam MZ, Mansor MF, Jalal KCA (2009) Optimization of decolorization of methylene blue by lignin peroxidase enzyme produced from sewage sludge with *Phanerochaete chrysosporium*. J Hazard Mater 162:708–715. https://doi.org/10.1016/j.jhazmat.2008.05.085

Ali H, Khan E, Ilahi I (2019) Environmental chemistry and ecotoxicology of hazardous heavy metals: environmental persistence, toxicity, and bioaccumulation. J Chem. https://www.hindawi.com/journals/jchem/2019/6730305/. Accessed 14 Oct 2020

Álvarez-Rogel J, Jiménez-Cárceles FJ, Roca MJ, Ortiz R (2007) Changes in soils and vegetation in a mediterranean coastal salt marsh impacted by human activities. Estuar Coast Shelf Sci 73:510–526. https://doi.org/10.1016/j.ecss.2007.02.018

Amara S, Perrot T, Navarro D et al (2018) Enzyme activities of two recombinant heme-containing peroxidases, tvdyp1 and tvvp2, identified from the secretome of *Trametes versicolor*. Appl Environ Microbiol 84. https://doi.org/10.1128/AEM.02826-17

Anderson EM, Stone ML, Katahira R et al (2019) Differences in S/G ratio in natural poplar variants do not predict catalytic depolymerization monomer yields. Nat Commun 10:1–10. https://doi.org/10.1038/s41467-019-09986-1

Andlar M, Rezić T, Marđetko N et al (2018) Lignocellulose degradation: an overview of fungi and fungal enzymes involved in lignocellulose degradation. Eng Life Sci 18:768–778. https://doi.org/10.1002/elsc.201800039

Ansari Z, Karimi A, Ebrahimi S, Emami E (2016) Improvement in ligninolytic activity of *Phanerochaete chrysosporium* cultures by glucose oxidase. Biochem Eng J 105:332–338. https://doi.org/10.1016/j.bej.2015.10.007

Arantes V, Jellison J, Goodell B (2012) Peculiarities of brown-rot fungi and biochemical Fenton reaction with regard to their potential as a model for bioprocessing biomass. Appl Microbiol Biotechnol 94:323–338. https://doi.org/10.1007/s00253-012-3954-y

Arora DS, Gill PK (2005) Production of ligninolytic enzymes by *Phlebia floridensis*. World J Microbiol Biotechnol 21:1021–1028. https://doi.org/10.1007/s11274-004-7655-2

Arora DS, Rampal P (2002) Laccase production by some *Phlebia* species. J Basic Microbiol 42:295–301. https://doi.org/10.1002/1521-4028(200210)42:5

Arregui L, Ayala M, Gómez-Gil X et al (2019) Laccases: structure, function, and potential application in water bioremediation. Microb Cell Factories 18:200. https://doi.org/10.1186/s12934-019-1248-0

Ayeronfe F, Kassim A, Ishak N et al (2018) A review on microbial degradation of lignin. https://www.ingentaconnect.com/content/asp/asl/2018/00000024/00000006/art00125. Accessed 15 Sept 2020

Bagewadi ZK, Mulla SI, Ninnekar HZ (2017) Optimization of laccase production and its application in delignification of biomass. Int J Recycl Org Waste Agric 6:351–365. https://doi.org/10.1007/s40093-017-0184-4

Baiocco P, Maria Barreca A, Fabbrini M et al (2003) Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase–mediator systems. Org Biomol Chem 1:191–197. https://doi.org/10.1039/B208951C

Bajpai P (2018a) Value-added products from lignin. In: Bajpai P (ed) Biotechnology for pulp and paper processing. Springer, Singapore, pp 561–571

Bajpai P (2018b) Brief description of the pulp and papermaking process. In: Bajpai P (ed) Biotechnology for pulp and paper processing. Springer, Singapore, pp 9–26

Bajwa PK, Arora DS (2009) Comparative production of ligninolytic enzymes by *Phanerochaete chrysosporium* and *Polyporus sanguineus*. Can J Microbiol 55:1397–1402

Barros J, Serk H, Granlund I, Pesquet E (2015) The cell biology of lignification in higher plants. Ann Bot 115:1053–1074. https://doi.org/10.1093/aob/mcv046

Becker J, Wittmann C (2019) A field of dreams: lignin valorization into chemicals, materials, fuels, and health-care products. Biotechnol Adv 37:107360. https://doi.org/10.1016/j.biotechadv.2019.02.016

Benkhaya B, Harfi SE, Harfi AE (2017) Classifications, properties and applications of textile dyes: a review. Appl J Environ Eng Sci 3:311–320

Benkhaya S, M'rabet S, El Harfi A (2020) A review on classifications, recent synthesis and applications of textile dyes. Inorg Chem Commun 115:107891. https://doi.org/10.1016/j.inoche.2020.107891

Bhaskar B, Lad L, Poulos TL (2006) Iron: heme proteins, peroxidases, catalases & catalase-peroxidases. In: Encyclopedia of inorganic chemistry. https://doi.org/10.1002/0470862106.ia106

Bhattacharya SS, Karmakar S, Banerjee R (2009) Optimization of laccase mediated biodegradation of 2,4-dichlorophenol using genetic algorithm. Water Res 43:3503–3510. https://doi.org/10.1016/j.watres.2009.05.012

Bhattacharya S, Das A, Prashanthi K et al (2014) Mycoremediation of benzo[a]pyrene by *Pleurotus ostreatus* in the presence of heavy metals and mediators. 3 Biotech 4:205–211. https://doi.org/10.1007/s13205-013-0148-y

Bhattacharya S, Das A, Palaniswamy M, Angayarkanni J (2017) Degradation of benzo[a]pyrene by *Pleurotus ostreatus* PO-3 in the presence of defined fungal and bacterial co-cultures. J Basic Microbiol 57:95–103. https://doi.org/10.1002/jobm.201600479

Bilal M, Iqbal HMN (2020) Ligninolytic enzymes mediated ligninolysis: an untapped biocatalytic potential to deconstruct lignocellulosic molecules in a sustainable manner. Catal Lett 150:524–543. https://doi.org/10.1007/s10562-019-03096-9

Biswas P, Bharti AK, Kadam A, Dutt D (2019) Bio-deinking of mixed office waste paper by *Penicillium citrinum* NCIM-1398 and its comparative study with conventional chemical deinking. Bioresources 14:6544–6557

Böckle B, Martínez MJ, Guillén F, Martínez ÁT (1999) Mechanism of peroxidase inactivation in liquid cultures of the ligninolytic fungus *Pleurotus pulmonarius*. Appl Environ Microbiol 65:923

Bourbonnais R, Paice MG (1990) Oxidation of non-phenolic substrates. FEBS Lett 267:99–102. https://doi.org/10.1016/0014-5793(90)80298-W

Bourbonnais R, Paice MG, Reid ID et al (1995) Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization. Appl Environ Microbiol 61:1876–1880

Brijwani K, Rigdon A, Vadlani PV (2010) Fungal laccases: production, function, and applications in food processing. Enzym Res. https://www.hindawi.com/journals/er/2010/149748/. Accessed 23 Apr 2019

Bugg TDH, Ahmad M, Hardiman EM, Rahmanpour R (2011) Pathways for degradation of lignin in bacteria and fungi. Nat Prod Rep 28:1883–1896. https://doi.org/10.1039/C1NP00042J

Bugos RC, Sutherland JB, Adler JH (1988) Phenolic compound utilization by the soft rot fungus *Lecythophora hoffmannii*. Appl Environ Microbiol 54:1882–1885

Bumpus JA, Tien M, Wright D, Aust SD (1985) Oxidation of persistent environmental pollutants by a white rot fungus. Science 228:1434–1436. https://doi.org/10.1126/science.3925550

Camarero S, Sarkar S, Ruiz-Dueñas FJ et al (1999) Description of a versatile peroxidase involved in the natural degradation of lignin that has both manganese peroxidase and lignin peroxidase substrate interaction sites. J Biol Chem 274:10324–10330. https://doi.org/10.1074/jbc.274.15.10324

Campbell MM, Sederoff RR (1996) Variation in lignin content and composition (mechanisms of control and implications for the genetic improvement of plants). Plant Physiol 110:3

Cao L, Yu IKM, Liu Y et al (2018) Lignin valorization for the production of renewable chemicals: state-of-the-art review and future prospects. Bioresour Technol 269:465–475. https://doi.org/10.1016/j.biortech.2018.08.065

Carmen Z, Daniela S (2012) Textile organic dyes—characteristics, polluting effects and separation/elimination procedures from industrial effluents—a critical overview. Org Pollut Ten Years Stockh Conv—Environ Anal Update. https://doi.org/10.5772/32373

Carro J, Ferreira P, Rodríguez L et al (2015) 5-Hydroxymethylfurfural conversion by fungal aryl-alcohol oxidase and unspecific peroxygenase. FEBS J 282:3218–3229. https://doi.org/10.1111/febs.13177

Carro J, Serrano A, Ferreira P, Martínez AT (2016) Fungal aryl-alcohol oxidase in lignocellulose degradation and bioconversion. In: Gupta VK (ed) Microbial enzymes in bioconversions of biomass. Springer International Publishing, Cham, pp 301–322

Chakraborty S, Rene ER, Lens PNL (2019) Reduction of selenite to elemental Se(0) with simultaneous degradation of phenol by co-cultures of *Phanerochaete chrysosporium* and *Delftia lacustris*. J Microbiol 57:738–747. https://doi.org/10.1007/s12275-019-9042-6

Chan JC, Paice M, Zhang X (2020) Enzymatic oxidation of lignin: challenges and barriers toward practical applications. Chem Cat Chem 12:401–425. https://doi.org/10.1002/cctc.201901480

Chandel AK, Gonçalves BCM, Strap JL, da Silva SS (2015) Biodelignification of lignocellulose substrates: an intrinsic and sustainable pretreatment strategy for clean energy production. Crit Rev Biotechnol 35:281–293. https://doi.org/10.3109/07388551.2013.841638

Chandel AK, Garlapati VK, Singh AK et al (2018) The path forward for lignocellulose biorefineries: bottlenecks, solutions, and perspective on commercialization. Bioresour Technol. https://doi.org/10.1016/j.biortech.2018.06.004

Chandel AK, Albarelli JQ, Santos DT et al (2019) Comparative analysis of key technologies for cellulosic ethanol production from Brazilian sugarcane bagasse at a commercial scale. Biofuels Bioprod Biorefin 13:994–1014. https://doi.org/10.1002/bbb.1990

Chandra R, Sharma P, Yadav S, Tripathi S (2018) Biodegradation of endocrine-disrupting chemicals and residual organic pollutants of pulp and paper mill effluent by biostimulation. Front Microbiol 9. https://doi.org/10.3389/fmicb.2018.00960

Chandranupap P, Chandranupap P (2020) Enzymatic deinking of xerographic waste paper with non-ionic surfactant. Appl Sci Eng Prog 13:136–145

Chauhan PS, Goradia B, Saxena A (2017) Bacterial laccase: recent update on production, properties and industrial applications. 3 Biotech 7. https://doi.org/10.1007/s13205-017-0955-7

Chaurasia SK, Bhardwaj NK (2019) Biobleaching—an ecofriendly and environmental benign pulp bleaching technique: a review. J Carbohydr Chem 38:87–108. https://doi.org/10.1080/07328303.2019.1581888

Chaurasia PK, Yadav RSS, Yadava S (2013) A review on mechanism of laccase action. Res Rev Biosci 7:66–71

Chen Z, Wan C (2017) Biological valorization strategies for converting lignin into fuels and chemicals. Renew Sust Energ Rev 73:610–621. https://doi.org/10.1016/j.rser.2017.01.166

Chen S, Xu J, Liu C et al (2012) Genome sequence of the model medicinal mushroom *Ganoderma lucidum*. Nat Commun 3:913

Chequer FMD, de Oliveira GAR, Ferraz ERA et al (2013) Textile dyes: dyeing process and environmental impact. Eco-friendly text dye finish. https://doi.org/10.5772/53659

Chhaya U, Gupte A (2010) Optimization of media components for laccase production by litter dwelling fungal isolate *Fusarium incarnatum* LD-3. J Basic Microbiol 50:43–51. https://doi.org/10.1002/jobm.200900203

Chio C, Sain M, Qin W (2019) Lignin utilization: a review of lignin depolymerization from various aspects. Renew Sust Energ Rev 107:232–249. https://doi.org/10.1016/j.rser.2019.03.008

Choinowski T, Blodig W, Winterhalter KH, Piontek K (1999) The crystal structure of lignin peroxidase at 1.70 \AA resolution reveals a hydroxy group on the Cβ of tryptophan 171: a novel radical site formed during the redox cycle. J Mol Biol 286:809–827

Chowdhary P, More N, Yadav A, Bharagava RN (2019) Chapter 12—Ligninolytic enzymes: an introduction and applications in the food industry. In: Kuddus M (ed) Enzymes in food biotechnology. Academic Press, London, pp 181–195

Chowdhary P, Hare V, Mani S et al (2020) Chapter 1—Recent advancement in the biotechnological application of lignin peroxidase and its future prospects. In: Chowdhary P, Raj A, Verma D, Akhter Y (eds) Microorganisms for sustainable environment and health. Elsevier, Amsterdam, pp 1–16

Christian V, Shrivastava R, Shukla D et al (2005) Mediator role of veratryl alcohol in the lignin peroxidase-catalyzed oxidative decolorization of Remazol Brilliant Blue R. Enzym Microb Technol 36:327–332. https://doi.org/10.1016/j.enzmictec.2004.09.006

Christopher LP, Yao B, Ji Y (2014) Lignin biodegradation with laccase-mediator systems. Front Energy Res 2. https://doi.org/10.3389/fenrg.2014.00012

Chukwuma OB, Rafatullah M, Tajarudin HA, Ismail N (2020) Lignocellulolytic enzymes in biotechnological and industrial processes: a review. Sustainability 12:7282. https://doi.org/10.3390/su12187282

Chutani P, Sharma KK (2015) Biochemical evaluation of xylanases from various filamentous fungi and their application for the deinking of ozone treated newspaper pulp. Carbohydr Polym 127:54–63

Chutani P, Sharma KK (2016) Concomitant production of xylanases and cellulases from *Trichoderma longibrachiatum* MDU-6 selected for the deinking of paper waste. Bioprocess Biosyst Eng 39:747–758. https://doi.org/10.1007/s00449-016-1555-3

Claus H (2004) Laccases: structure, reactions, distribution. Micron 35:93–96

Colpa DI, Fraaije MW, van Bloois E (2014) DyP-type peroxidases: a promising and versatile class of enzymes. J Ind Microbiol Biotechnol 41:1–7

Copete LS, Chanagá X, Barriuso J et al (2015) Identification and characterization of laccase-type multicopper oxidases involved in dye-decolorization by the fungus *Leptosphaerulina* sp. BMC Biotechnol:15. https://doi.org/10.1186/s12896-015-0192-2

Damián-Robles RM, Castro-Montoya AJ, Saucedo-Luna J et al (2017) Characterization of ligninolytic enzyme production in white-rot wild fungal strains suitable for kraft pulp bleaching. 3 Biotech 7:319. https://doi.org/10.1007/s13205-017-0968-2

Daniel GF, Nilsson T (1988) Studies on preservative tolerant *Phialophora* species. Int Biodeterior 24:327–335. https://doi.org/10.1016/0265-3036(88)90018-8

Daou M, Faulds CB (2017) Glyoxal oxidases: their nature and properties. World J Microbiol Biotechnol 33:87

Dashtban M, Schraft H, Syed TA, Qin W (2010) Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol 1:36–50

Datta R, Kelkar A, Baraniya D et al (2017) Enzymatic degradation of lignin in soil: a review. Sustainability 9:1163. https://doi.org/10.3390/su9071163

de Gonzalo G, Colpa DI, Habib MHM, Fraaije MW (2016) Bacterial enzymes involved in lignin degradation. J Biotechnol 236:110–119. https://doi.org/10.1016/j.jbiotec.2016.08.011

de Souza WR (2013) Microbial degradation of lignocellulosic biomass. Sustain Degrad Lignocell Biomass Tech Appl Commer. https://doi.org/10.5772/54325

Debnath R, Saha T (2020) An insight into the production strategies and applications of the ligninolytic enzyme laccase from bacteria and fungi. Biocatal Agric Biotechnol 26:101645. https://doi.org/10.1016/j.bcab.2020.101645

Dedeyan B, Klonowska A, Tagger S et al (2000) Biochemical and molecular characterization of a laccase from *Marasmius quercophilus*. Appl Environ Microbiol 66:925. https://doi.org/10.1128/aem.66.3.925-929.2000

Deshmukh R, Khardenavis AA, Purohit HJ (2016) Diverse metabolic capacities of fungi for bioremediation. Indian J Microbiol 56:247–264. https://doi.org/10.1007/s12088-016-0584-6

Deswal D, Gupta R, Nandal P, Kuhad RC (2014) Fungal pretreatment improves amenability of lignocellulosic material for its saccharification to sugars. Carbohydr Polym 99:264–269. https://doi.org/10.1016/j.carbpol.2013.08.045

Dhakar K, Pandey A (2013, 2013) Laccase production from a temperature and ph tolerant fungal strain of *Trametes hirsuta* (MTCC 11397). Enzym Res. https://doi.org/10.1155/2013/869062

Dhawan S, Lal R, Hanspal M, Kuhad RC (2005) Effect of antibiotics on growth and laccase production from *Cyathus bulleri* and *Pycnoporus cinnabarinus*. Bioresour Technol 96:1415–1418. https://doi.org/10.1016/j.biortech.2004.11.014

Dhiman N, Jasrotia T, Sharma P et al (2020) Immobilization interaction between xenobiotic and *Bjerkandera adusta* for the biodegradation of atrazine. Chemosphere 257:127060. https://doi.org/10.1016/j.chemosphere.2020.127060

Divya LM, Prasanth GK, Sadasivan C (2014) Potential of the salt-tolerant laccase-producing strain *Trichoderma viride* Pers. NFCCI-2745 from an estuary in the bioremediation of phenol-polluted environments. J Basic Microbiol 54:542–547. https://doi.org/10.1002/jobm.201200394

dos Santos Barbosa E, Perrone D, do Amaral Vendramini AL, Leite SGF (2008) Vanillin production by *Phanerochaete chrysosporium* grown on green coconut agro-industrial husk in solid state fermentation. Bioresources 3:1042–1050

Duan Z, Shen R, Liu B et al (2018) Comprehensive investigation of a dye-decolorizing peroxidase and a manganese peroxidase from Irpex lacteus F17, a lignin-degrading basidiomycete. AMB Express 8:119. https://doi.org/10.1186/s13568-018-0648-6

Dwivedi P, Vivekanand V, Pareek N et al (2009) Bleach enhancement of mixed wood pulp by xylanase–laccase concoction derived through co-culture strategy. Appl Biochem Biotechnol 160:255. https://doi.org/10.1007/s12010-009-8654-4

Edwards SL, Raag R, Wariishi H et al (1993) Crystal structure of lignin peroxidase. Proc Natl Acad Sci 90:750–754. https://doi.org/10.1073/pnas.90.2.750

Eriksson K-EL (1998) Biotechnology in the pulp and paper industry: an overview. In: Enzyme applications in fiber processing. American Chemical Society, Washington, DC, pp 2–14

Eriksson K-EL (2000) Lignocellulose, lignin, ligninases. Encycl Microbiol 3

Eriksson K-EL, Blanchette RA, Ander P (1990) Microbial and enzymatic degradation of wood and wood components. Springer Science & Business Media, London

Eugenio ME, Ibarra D, Martín-Sampedro R et al (2019) Alternative raw materials for pulp and paper production in the concept of a lignocellulosic biorefinery. Cellulose. https://doi.org/10.5772/intechopen.90041

Falade AO, Nwodo UU, Iweriebor BC et al (2016) Lignin peroxidase functionalities and prospective applications. Microbiol Open 6. https://doi.org/10.1002/mbo3.394

Falah F, Fatrisari W, Ermawar RA et al (2011) Effect of corn steep liquor on bamboo biochemical pulping using *Phanerochaete chrysosporium*. J Ilmu Dan Teknol Kayu Trop 9:111–125

Fang Z, Liu X, Chen L et al (2015) Identification of a laccase Glac15 from *Ganoderma lucidum* 77002 and its application in bioethanol production. Biotechnol Biofuels 8:54. https://doi.org/10.1186/s13068-015-0235-x

Feldman D, Kowbel DJ, Glass NL et al (2015) Detoxification of 5-hydroxymethylfurfural by the *Pleurotus ostreatus* ligninolytic enzymes aryl alcohol oxidase and dehydrogenase. Biotechnol Biofuels 8. https://doi.org/10.1186/s13068-015-0244-9

Fernández-Fueyo E, Linde D, Almendral D et al (2015) Description of the first fungal dye-decolorizing peroxidase oxidizing manganese(II). Appl Microbiol Biotechnol 99:8927. https://doi.org/10.1007/s00253-015-6665-3

Fillat U, Roncero MB (2009) Biobleaching of high quality pulps with laccase mediator system: influence of treatment time and oxygen supply. Biochem Eng J 44:193–198. https://doi.org/10.1016/j.bej.2008.12.002

Gahlout M, Gupte S, Gupte A (2013) Optimization of culture condition for enhanced decolorization and degradation of azo dye reactive violet 1 with concomitant production of ligninolytic enzymes by *Ganoderma cupreum* AG-1. 3 Biotech 3:143–152. https://doi.org/10.1007/s13205-012-0079-z

Gao Y, Yang B, Wang Q (2018) Biodegradation and decolorization of dye wastewater: a review. IOP Conf Ser Earth Environ Sci 178:012013. https://doi.org/10.1088/1755-1315/178/1/012013

Gassara-Chatti F, Brar SK, Ajila CM et al (2013) Encapsulation of ligninolytic enzymes and its application in clarification of juice. Food Chem 137:18–24. https://doi.org/10.1016/j.foodchem.2012.09.083

Gaur N, Narasimhulu K, Pydi Setty Y (2018) Extraction of ligninolytic enzymes from novel *Klebsiella pneumoniae* strains and its application in wastewater treatment. Appl Water Sci 8:111. https://doi.org/10.1007/s13201-018-0758-y

Gellerstedt G, Henriksson G (2008) Chapter 9—Lignins: major sources, structure and properties. In: Belgacem MN, Gandini A (eds) Monomers, polymers and composites from renewable resources. Elsevier, Amsterdam, pp 201–224

Ghosh P, Ghosh U (2019) Statistical optimisation of laccase production by isolated strain *Aspergillus flavus* PUF5 utilising ribbed gourd peels as the substrate and enzyme application on apple juice clarification. Indian Chem Eng:1–12. https://doi.org/10.1080/00194506.2019.1684211

Ghosh M, Mukherjee R, Nandi B (1998) Production of extracellular enzymes by two *Pleurotus* species using banana pseudostem biomass. Acta Biotechnol 18:243–254. https://doi.org/10.1002/abio.370180309

Giacobbe S, Pezzella C, Lettera V et al (2018) Laccase pretreatment for agrofood wastes valorization. Bioresour Technol 265:59–65. https://doi.org/10.1016/j.biortech.2018.05.108

Giles RL, Zackeru JC, Galloway ER et al (2015) Single versus simultaneous species treatment of wood with *Ceriporiopsis subvermispora* and *Postia placenta* for ethanol applications, with observations on interspecific growth inhibition. Int Biodeterior Biodegrad 99:66–72. https://doi.org/10.1016/j.ibiod.2014.11.005

Gill P, Arora D (2003) Effect of culture conditions on manganese peroxidase production and activity by some white rot fungi. J Ind Microbiol Biotechnol 30:28–33. https://doi.org/10.1007/s10295-002-0002-4

Gillet S, Aguedo M, Petitjean L et al (2017) Lignin transformations for high value applications: towards targeted modifications using green chemistry. Green Chem 19:4200–4233. https://doi.org/10.1039/C7GC01479A

Giummarella N, Pu Y, Ragauskas AJ, Lawoko M (2019) A critical review on the analysis of lignin carbohydrate bonds. Green Chem 21:1573–1595. https://doi.org/10.1039/C8GC03606C

Glenn JK, Morgan MA, Mayfield MB et al (1983) An extracellular H2O2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 114:1077–1083. https://doi.org/10.1016/0006-291X(83)90672-1

Goñi MA, Nelson B, Blanchette RA, Hedges JI (1993) Fungal degradation of wood lignins: geochemical perspectives from CuO-derived phenolic dimers and monomers. Geochim Cosmochim Acta 57:3985–4002. https://doi.org/10.1016/0016-7037(93)90348-Z

Gonzalez-Perez D, Mateljak I, Garcia-Ruiz E et al (2016) Alkaline versatile peroxidase by directed evolution. Cat Sci Technol 6:6625–6636. https://doi.org/10.1039/C6CY01044J

Goyal R, Grewal RB, Goyal RK (2006) Nutritional attributes of *Agaricus bisporus* and *Pleurotus sajor-caju* mushrooms. Nutr Health 18:179–184. https://doi.org/10.1177/026010600601800209

Grąz M, Jarosz-Wilkołazka A (2011) Oxalic acid, versatile peroxidase secretion and chelating ability of *Bjerkandera fumosa* in rich and limited culture conditions. World J Microbiol Biotechnol 27:1885–1891. https://doi.org/10.1007/s11274-010-0647-5

Gulzar ABM, Vandana UK, Paul P, Mazumder PB (2020) The role of mushrooms in biodegradation and decolorization of dyes. Introd Mushroom. https://doi.org/10.5772/intechopen.90737

Gunne M, Höppner A, Hagedoorn P-L, Urlacher VB (2014) Structural and redox properties of the small laccase Ssl1 from *Streptomyces sviceus*. FEBS J 281:4307–4318. https://doi.org/10.1111/febs.12755

Gupta R, Sharma KK, Kuhad RC (2009) Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. Bioresour Technol 100:1214–1220

Gutiérrez A, Martínez MJ, Del Río JC et al (2000) Fungal pretreatment of eucalyptus wood can strongly decrease the amount of lipophilic extractives during chlorine free kraft pulping. Environ Sci Technol 34:3705–3709. https://doi.org/10.1021/es000938s

Habib MH, Rozeboom HJ, Fraaije MW (2019) Characterization of a new dyp-peroxidase from the alkaliphilic cellulomonad, *Cellulomonas bogoriensis*. Molecules 24. https://doi.org/10.3390/molecules24071208

Hammel KE, Mozuch MD, Jensen KA Jr, Kersten PJ (1994) H2o2 recycling during oxidation of the arylglycerol. Beta-aryl ether lignin structure by lignin peroxidase and glyoxal oxidase. Biochemistry (Mosc) 33:13349–13354

Harreither W, Sygmund C, Dünhofen E et al (2009) Cellobiose dehydrogenase from the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Appl Environ Microbiol 75:2750–2757. https://doi.org/10.1128/AEM.02320-08

Hastrup ACS, Howell C, Larsen FH et al (2012) Differences in crystalline cellulose modification due to degradation by brown and white rot fungi. Fungal Biol 116:1052–1063. https://doi.org/10.1016/j.funbio.2012.07.009

Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role from in lignin degradation. FEMS Microbiol Rev 13:125–135. https://doi.org/10.1111/j.1574-6976.1994.tb00039.x

Hatakka A, Hammel KE (2011) Fungal biodegradation of lignocelluloses. In: Industrial applications. Springer, Berlin, pp 319–340

Hayat H, Mahmood Q, Pervez A et al (2015) Comparative decolorization of dyes in textile wastewater using biological and chemical treatment. Sep Purif Technol 154:149–153. https://doi.org/10.1016/j.seppur.2015.09.025

Heap L, Green A, Brown D et al (2014) Role of laccase as an enzymatic pretreatment method to improve lignocellulosic saccharification. Cat Sci Technol 4:2251–2259. https://doi.org/10.1039/C4CY00046C

Higuchi T (2004) Microbial degradation of lignin: role of lignin peroxidase, manganese peroxidase, and laccase. Proc Jpn Acad B 80:204–214. https://doi.org/10.2183/pjab.80.204

Huang X, Wang D, Liu C et al (2003) The roles of veratryl alcohol and nonionic surfactant in the oxidation of phenolic compounds by lignin peroxidase. Biochem Biophys Res Commun 311:491–494. https://doi.org/10.1016/j.bbrc.2003.10.029

Hubbe MA, Venditti RA, Rojas OJ (2007) What happens to cellulosic fibers during papermaking and recycling? A review. Bioresources 2:739–788

Iark D, dos Reis Buzzo AJ, Garcia JAA et al (2019) Enzymatic degradation and detoxification of azo dye Congo red by a new laccase from *Oudemansiella canarii*. Bioresour Technol 289:121655. https://doi.org/10.1016/j.biortech.2019.121655

Ince BK, Cetecioglu Z, Ince O (2011) Pollution prevention in the pulp and paper industries. Environ Manag Pract. https://doi.org/10.5772/23709

Jain KK, Kumar A, Shankar A et al (2019) De novo transcriptome assembly and protein profiling of copper-induced lignocellulolytic fungus *Ganoderma lucidum* MDU-7 reveals genes involved in lignocellulose degradation and terpenoid biosynthetic pathways. Genomics. https://doi.org/10.1016/j.ygeno.2019.01.012

Jamee R, Siddique R (2019) Biodegradation of synthetic dyes of textile effluent by microorganisms: an environmentally and economically sustainable approach. Eur J Microbiol Immunol 9:114. https://doi.org/10.1556/1886.2019.00018

Janusz G, Pawlik A, Sulej J et al (2017) Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution. FEMS Microbiol Rev 41:941–962. https://doi.org/10.1093/femsre/fux049

Jayasinghe C, Imtiaj A, Lee GW et al (2008) Degradation of three aromatic dyes by white rot fungi and the production of ligninolytic enzymes. Mycobiology 36:114–120. https://doi.org/10.4489/MYCO.2008.36.2.114

Johannes C, Majcherczyk A (2000) Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Appl Environ Microbiol 66:524–528. https://doi.org/10.1128/AEM.66.2.524-528.2000

Jones SM, Solomon EI (2015) Electron transfer and reaction mechanism of laccases. Cell Mol Life Sci CMLS 72:869–883. https://doi.org/10.1007/s00018-014-1826-6

Joutey NT, Bahafid W, Sayel H, ElGhachtouli N (2013) Biodegradation: involved microorganisms and genetically engineered microorganisms. Biodegrad Life Sci. https://doi.org/10.5772/56194

Juwarkar AA, Singh SK, Mudhoo A (2010) A comprehensive overview of elements in bioremediation. Rev Environ Sci Biotechnol 9:215–288. https://doi.org/10.1007/s11157-010-9215-6

Kadowaki MAS, de Godoy MO, Kumagai PS et al (2018) Characterization of a new glyoxal oxidase from the thermophilic fungus *Myceliophthora thermophila* M77: hydrogen peroxide production retained in 5-hydroxymethylfurfural oxidation. Catalysts 8:476. https://doi.org/10.3390/catal8100476

Kainthola J, Kalamdhad AS, Goud VV, Goel R (2019) Fungal pretreatment and associated kinetics of rice straw hydrolysis to accelerate methane yield from anaerobic digestion. Bioresour Technol 286:121368. https://doi.org/10.1016/j.biortech.2019.121368

Kameshwar AKS, Qin W (2016) Lignin degrading fungal enzymes. In: Fang Z, Smith J, Richard L (eds) Production of biofuels and chemicals from lignin. Springer, Singapore, pp 81–130

Kamimura N, Sakamoto S, Mitsuda N et al (2019) Advances in microbial lignin degradation and its applications. Curr Opin Biotechnol 56:179–186. https://doi.org/10.1016/j.copbio.2018.11.011

Kamitsuji H, Watanabe T, Honda Y, Kuwahara M (2005) Direct oxidation of polymeric substrates by multifunctional manganese peroxidase isoenzyme from *Pleurotus ostreatus* without redox mediators. Biochem J 386:387–393. https://doi.org/10.1042/BJ20040968

Kang BR, Kim MS, Lee TK (2019) Unveiling of concealed processes for the degradation of pharmaceutical compounds by *Neopestalotiopsis* sp. Microorganisms 7:264. https://doi.org/10.3390/microorganisms7080264

Kapoor RK, Sharma KK, Kuhar S, Kuhad RC (2005) Diversity of lignin degrading microorganisms, ligninolytic enzymes and their biotechnological applications. In: Microbial diversity: current perspectives and potential applications. IK Int. Pvt. Ltd., New Delhi, pp 815–884

Kapoor RK, Rajan K, Carrier DJ (2015) Applications of *Trametes versicolor* crude culture filtrates in detoxification of biomass pretreatment hydrolyzates. Bioresour Technol 189:99–106. https://doi.org/10.1016/j.biortech.2015.03.100

Kaur H, Kapoor S, Kaur G (2016) Application of ligninolytic potentials of a white-rot fungus *Ganoderma lucidum* for degradation of lindane. Environ Monit Assess 188:588. https://doi.org/10.1007/s10661-016-5606-7

Kaur D, Bhardwaj NK, Lohchab RK (2019) Environmental aspect of using chlorine dioxide to improve effluent and pulp quality during wheat straw bleaching. Waste Biomass Valor 10:1231–1239. https://doi.org/10.1007/s12649-017-0193-6

Kersten PJ, Kalyanaraman B, Hammel KE et al (1990) Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. Biochem J 268:475–480

Kim SJ, Shoda M (1999) Purification and characterization of a novel peroxidase from *Geotrichum candidum* Dec 1 involved in decolorization of dyes. Appl Environ Microbiol 65:1029–1035

Knop D, Levinson D, Makovitzki A et al (2016) Limits of versatility of versatile peroxidase. Appl Environ Microbiol 82:4070–4080. https://doi.org/10.1128/AEM.00743-16

Koduri RS, Tien M (1995) Oxidation of guaiacol by lignin peroxidase role of veratryl alcohol. J Biol Chem 270:22254–22258. https://doi.org/10.1074/jbc.270.38.22254

Koyani RD, Sanghvi GV, Sharma RK, Rajput KS (2013) Contribution of lignin degrading enzymes in decolourisation and degradation of reactive textile dyes. Int Biodeterior Biodegrad 77:1–9. https://doi.org/10.1016/j.ibiod.2012.10.006

Krumova E, Kostadinova N, Miteva-Staleva J et al (2018) Potential of ligninolytic enzymatic complex produced by white-rot fungi from genus *Trametes* isolated from Bulgarian forest soil. Eng Life Sci 18:692–701. https://doi.org/10.1002/elsc.201800055

Kudanga T, Le Roes-Hill M (2014) Laccase applications in biofuels production: current status and future prospects. Appl Microbiol Biotechnol 98:6525–6542. https://doi.org/10.1007/s00253-014-5810-8

Kuhad RC, Kuhar S, Kapoor M et al (2007) Lignocellulolytic microorganisms, their enzymes and possible biotechnologies based on lignocellulolytic microorganisms and their enzymes. In: Lignocellulose biotechnology: future prospects. IK Int. Pvt. Ltd., New Delhi, pp 3–22

Kuhad RC, Mehta G, Gupta R, Sharma KK (2010) Fed batch enzymatic saccharification of newspaper cellulosics improves the sugar content in the hydrolysates and eventually the ethanol fermentation by *Saccharomyces cerevisiae*. Biomass Bioenergy 34:1189–1194. https://doi.org/10.1016/j.biombioe.2010.03.009

Kuhad RC, Kuhar S, Sharma KK, Shrivastava B (2013) Microorganisms and enzymes involved in lignin degradation Vis-à-Vis production of nutritionally rich animal feed: an overview. In: Kuhad RC, Singh A (eds) Biotechnology for environmental management and resource recovery. Springer, New Delhi, pp 3–44

Kuhar S, Kapoor M, Kapoor R et al (2007) Biodiversity of ligninolytic fungi. In: Kuhad RC, Singh A (eds) Lignocellulose biotechnology: future prospects. IK Int. Pvt. Ltd., New Delhi, pp 37–53

Kulshreshtha S, Mathur N, Bhatnagar P (2012) Aerobic treatment of handmade paper industrial effluents by white rot fungi. J Bioremed Biodegr 3:2

Kumar A, Chandra R (2020) Ligninolytic enzymes and its mechanisms for degradation of lignocellulosic waste in environment. Heliyon 6. https://doi.org/10.1016/j.heliyon.2020.e03170

Kumar VV, Rapheal VS (2011) Induction and purification by three-phase partitioning of aryl alcohol oxidase (AAO) from *Pleurotus ostreatus*. Appl Biochem Biotechnol 163:423–432. https://doi.org/10.1007/s12010-010-9050-9

Kumar CG, Mongolla P, Joseph J, Sarma VUM (2012) Decolorization and biodegradation of triphenylmethane dye, brilliant green, by *Aspergillus* sp. isolated from Ladakh, India. Process Biochem 47:1388–1394. https://doi.org/10.1016/j.procbio.2012.05.015

Kumar A, Sharma KK, Kumar P, Ramchiary N (2015a) Laccase isozymes from *Ganoderma lucidum* MDU-7: isolation, characterization, catalytic properties and differential role during oxidative stress. J Mol Catal B Enzym 113:68–75. https://doi.org/10.1016/j.molcatb.2015.01.010

Kumar VP, Naik C, Sridhar M (2015b) Production, purification and characterization of novel laccase produced by *Schizophyllum commune* NI-07 with potential for delignification of crop residues. Appl Biochem Microbiol 51:432–441. https://doi.org/10.1134/S0003683815040080

Kumar A, Singh D, Chandel AK, Sharma KK (2017a) Technological advancements in sustainable production of second generation ethanol development: an appraisal and future directions. In: Sustainable biofuels development in India. Springer, New Delhi, pp 299–336

Kumar A, Singh D, Sharma KK et al (2017b) Gel-based purification and biochemical study of laccase isozymes from *Ganoderma* sp and its role in enhanced cotton callogenesis. Front Microbiol 8. https://doi.org/10.3389/fmicb.2017.00674

Kumar R, Negi S, Sharma P et al (2018) Wastewater cleanup using *Phlebia acerina* fungi: an insight into mycoremediation. J Environ Manag 228:130–139. https://doi.org/10.1016/j.jenvman.2018.07.091

Kumar A, Arora S, Jain KK, Sharma KK (2019) Metabolic coupling in the co-cultured fungal-yeast suite of *Trametes ljubarskyi* and *Rhodotorula mucilaginosa* leads to hypersecretion of laccase isozymes. Fungal Biol 123:913–926. https://doi.org/10.1016/j.funbio.2019.09.013

Kumari D, Reddy MS, Upadhyay RC (2011) Nutritional composition and antioxidant activities of 18 different wild Cantharellus mushrooms of northwestern Himalayas. Food Sci Technol Int 17:557–567. https://doi.org/10.1177/1082013211427620

Kumla J, Suwannarach N, Sujarit K et al (2020) Cultivation of mushrooms and their lignocellulolytic enzyme production through the utilization of agro-industrial waste. Molecules 25:2811. https://doi.org/10.3390/molecules25122811

Lai C-Y, Wu C-H, Meng C-T, Lin C-W (2017) Decolorization of azo dye and generation of electricity by microbial fuel cell with laccase-producing white-rot fungus on cathode. Appl Energy 188:392–398. https://doi.org/10.1016/j.apenergy.2016.12.044

Lange H, Decina S, Crestini C (2013) Oxidative upgrade of lignin—recent routes reviewed. Eur Polym J 49:1151–1173. https://doi.org/10.1016/j.eurpolymj.2013.03.002

Lawson LRJ, Still CN (1957) The biological decomposition of lignin—literature survey. Tappi J 40 (9):56A–80A

Lee J-W, Choi S-P, Thiruvenkatachari R et al (2006) Evaluation of the performance of adsorption and coagulation processes for the maximum removal of reactive dyes. Dyes Pigments 69:196–203. https://doi.org/10.1016/j.dyepig.2005.03.008

Lee CK, Ibrahim D, Ibrahim CO, Daud WRW (2011) Enzymatic and chemical deinking of mixed office wastepaper and old newspaper: paper quality and effluent characteristics. Bioresources 6:3859–3875. https://doi.org/10.15376/biores.6.4.3859-3875

Lellis B, Fávaro-Polonio CZ, Pamphile JA, Polonio JC (2019) Effects of textile dyes on health and the environment and bioremediation potential of living organisms. Biotechnol Res Innov 3:275–290. https://doi.org/10.1016/j.biori.2019.09.001

Leonowicz A, Matuszewska A, Luterek J et al (1999) Biodegradation of lignin by white rot fungi. Fungal Genet Biol 27:175–185. https://doi.org/10.1006/fgbi.1999.1150

Leponiemi A (2008) Non-wood pulping possibilities—a challenge for the chemical pulping industry. Appita Technol Innov Manuf Environ 61:234

Lettera V, Pezzella C, Cicatiello P et al (2016) Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification. Food Chem 196:1272–1278. https://doi.org/10.1016/j.foodchem.2015.10.074

Li T, Takkellapati S (2018) The current and emerging sources of technical lignins and their applications. Biofuels Bioprod Bioref:1–32. https://doi.org/10.1002/bbb.1913

Li G, Lei Y, Ge J, Wu S (2017) The empirical relationship between mining industry development and environmental pollution in China. Int J Environ Res Public Health 14:254. https://doi.org/10.3390/ijerph14030254

Liao JJ, Latif NHA, Trache D et al (2020) Current advancement on the isolation, characterization and application of lignin. Int J Biol Macromol 162:985–1024. https://doi.org/10.1016/j.ijbiomac.2020.06.168

Liers C, Pecyna MJ, Kellner H et al (2013) Substrate oxidation by dye-decolorizing peroxidases (DyPs) from wood- and litter-degrading agaricomycetes compared to other fungal and plant heme-peroxidases. Appl Microbiol Biotechnol 97:5839–5849. https://doi.org/10.1007/s00253-012-4521-2

Linde D, Coscolín C, Liers C et al (2014) Heterologous expression and physicochemical characterization of a fungal dye-decolorizing peroxidase from *Auricularia auricula-judae*. Protein Expr Purif 103:28–37. https://doi.org/10.1016/j.pep.2014.08.007

Liu E, Wilkins MR (2020) Process optimization and scale-up production of fungal aryl alcohol oxidase from genetically modified *Aspergillus nidulans* in stirred-tank bioreactor. Bioresour Technol 315:123792

Liu Z, Wang H, Hui L (2018) Pulping and papermaking of non-wood fibers. Pulp Pap Process. https://doi.org/10.5772/intechopen.79017

Lourenço A, Pereira H (2017) Compositional variability of lignin in biomass. Lignin Trends Appl. https://doi.org/10.5772/intechopen.71208

Lu Y-C, Lu Y, Fan X (2020) Structure and characteristics of lignin. In: Sharma S, Kumar A (eds) Lignin: biosynthesis and transformation for industrial applications. Springer International Publishing, Cham, pp 17–75

MacDonald J, Goacher RE, Abou-Zaid M, Master ER (2016) Comparative analysis of lignin peroxidase and manganese peroxidase activity on coniferous and deciduous wood using ToF-SIMS. Appl Microbiol Biotechnol 100:8013–8020. https://doi.org/10.1007/s00253-016-7560-2

Maciel MJM, Silva AC, Ribeiro HCT (2010) Industrial and biotechnological applications of ligninolytic enzymes of the basidiomycota: a review. Electron J Biotechnol 13:14–15

Madadi M, Abbas A (2017) Lignin degradation by fungal pretreatment: a review. J Plant Pathol Microbiol 8:1–6

Mäkelä MR, Bredeweg EL, Magnuson JK et al (2017) Fungal ligninolytic enzymes and their applications. In: The fungal kingdom. John Wiley & Sons Ltd., pp 1049–1061

Manavalan T, Manavalan V, Thangavelu KP et al (2015) Characterization of a solvent-tolerant manganese peroxidase (MnP) from *Ganoderma lucidum* and its application in fruit juice clarification. J Food Biochem 39:754–764. https://doi.org/10.1111/jfbc.12188

Manoharachary C, Sridhar K, Singh R et al (2005) Fungal biodiversity: distribution, conservation and prospecting of fungi from India. Curr Sci 89:58–71

Martin H (2002) Review: lignin conversion by manganese peroxidase (MnP). Enzym Microb Technol 30:454–466

Martínez AT (2002) Molecular biology and structure-function of lignin-degrading heme peroxidases. Enzym Microb Technol 30:425–444. https://doi.org/10.1016/S0141-0229(01)00521-X

Mathieu Y, Piumi F, Valli R et al (2016) Activities of secreted aryl alcohol quinone oxidoreductases from *Pycnoporus cinnabarinus* provide insights into fungal degradation of plant biomass. Appl Environ Microbiol 82:2411. https://doi.org/10.1128/AEM.03761-15

McGuinness M, Dowling D (2009) Plant-associated bacterial degradation of toxic organic compounds in soil. Int J Environ Res Public Health 6:2226–2247. https://doi.org/10.3390/ijerph6082226

Meehnian H, Jana AK (2016) Cotton stalk pretreatment using *Daedalea flavida*, *Phlebia radiata*, and *Flavodon flavus*: lignin degradation, cellulose recovery, and enzymatic saccharification. Appl Biochem Biotechnol 181:1465–1484. https://doi.org/10.1007/s12010-016-2296-0

Meehnian H, Jana AK, Jana MM (2017) Pretreatment of cotton stalks by synergistic interaction of *Daedalea flavida* and *Phlebia radiata* in co-culture for improvement in delignification and saccharification. Int Biodeterior Biodegrad 117:68–77. https://doi.org/10.1016/j.ibiod.2016.11.022

Minussi RC, Pastore GM, Durán N (2002) Potential applications of laccase in the food industry. Trends Food Sci Technol 13:205–216

Mishra V, Jana AK (2019) Sweet sorghum bagasse pretreatment by *Coriolus versicolor* in mesh tray bioreactor for selective delignification and improved saccharification. Waste Biomass Valor 10:2689–2702. https://doi.org/10.1007/s12649-018-0276-z

Mobley JK, Jennings JA, Morgan T et al (2018) Oxidation of benzylic alcohols and lignin model compounds with layered double hydroxide catalysts. Inorganics 6:75. https://doi.org/10.3390/inorganics6030075

Mohit E, Tabarzad M, Faramarzi MA (2020) Biomedical and pharmaceutical-related applications of laccases. Curr Protein Pept Sci 21:78–98

Moilanen U, Kellock M, Várnai A et al (2014) Mechanisms of laccase-mediator treatments improving the enzymatic hydrolysis of pre-treated spruce. Biotechnol Biofuels 7:177. https://doi.org/10.1186/s13068-014-0177-8

Moreno AD, Ibarra D, Alvira P et al (2015) A review of biological delignification and detoxification methods for lignocellulosic bioethanol production. Crit Rev Biotechnol 35:342–354. https://doi.org/10.3109/07388551.2013.878896

Morgenstern I, Klopman S, Hibbett DS (2008) Molecular evolution and diversity of lignin degrading heme peroxidases in the Agaricomycetes. J Mol Evol 66:243–257

Morozova OV, Shumakovich GP, Shleev SV, Yaropolov YI (2007) Laccase-mediator systems and their applications: a review. Appl Biochem Microbiol 43:523–535. https://doi.org/10.1134/S0003683807050055

Mot AC, Coman C, Hadade N et al (2020) "Yellow" laccase from *Sclerotinia sclerotiorum* is a blue laccase that enhances its substrate affinity by forming a reversible tyrosyl-product adduct. PLoS One 15. https://doi.org/10.1371/journal.pone.0225530

Munagapati VS, Wen H-Y, Wen J-C et al (2020) Anionic congo red dye removal from aqueous medium using Turkey tail (*Trametes versicolor*) fungal biomass: adsorption kinetics, isotherms, thermodynamics, reusability, and characterization. J Dispers Sci Technol:1–14. https://doi.org/10.1080/01932691.2020.1789468

Munk L, Andersen ML, Meyer AS (2018) Influence of mediators on laccase catalyzed radical formation in lignin. Enzym Microb Technol 116:48–56. https://doi.org/10.1016/j.enzmictec.2018.05.009

Mustafa AM, Poulsen TG, Sheng K (2016) Fungal pretreatment of rice straw with *Pleurotus ostreatus* and *Trichoderma reesei* to enhance methane production under solid-state anaerobic digestion. Appl Energy 180:661–671. https://doi.org/10.1016/j.apenergy.2016.07.135

Mutuku JM, Cui S, Hori C et al (2019) The structural integrity of lignin is crucial for resistance against *Striga hermonthica* parasitism in rice. Plant Physiol 179:1796–1809. https://doi.org/10.1104/pp.18.01133

Nagai M, Kawata M, Watanabe H et al (2003) Important role of fungal intracellular laccase for melanin synthesis: purification and characterization of an intracellular laccase from *Lentinula edodes* fruit bodies. Microbiology 149:2455–2462. https://doi.org/10.1099/mic.0.26414-0

Nathan VK, Kanthimathinathan SR, Rani ME et al (2018) Biobleaching of waste paper using ligninolytic enzyme from *Fusarium equiseti* VKF2: a mangrove isolate. Cellulose 25:4179–4192. https://doi.org/10.1007/s10570-018-1834-z

Navada KK, Kulal A (2019) Enzymatic degradation of chloramphenicol by laccase from *Trametes hirsuta* and comparison among mediators. Int Biodeterior Biodegrad 138:63–69. https://doi.org/10.1016/j.ibiod.2018.12.012

Nayan N, Sonnenberg ASM, Hendriks WH, Cone JW (2017) Differences between two strains of *Ceriporiopsis subvermispora* on improving the nutritive value of wheat straw for ruminants. J Appl Microbiol 123:352–361. https://doi.org/10.1111/jam.13494

Nayan N, Sonnenberg ASM, Hendriks WH, Cone JW (2018) Screening of white-rot fungi for bioprocessing of wheat straw into ruminant feed. J Appl Microbiol 125:468–479. https://doi.org/10.1111/jam.13894

Nayan N, Sonnenberg ASM, Hendriks WH, Cone JW (2020) Prospects and feasibility of fungal pretreatment of agricultural biomass for ruminant feeding. Anim Feed Sci Technol 268:114577. https://doi.org/10.1016/j.anifeedsci.2020.114577

Nayanashree G, Thippeswamy B (2015) Natural rubber degradation by laccase and manganese peroxidase enzymes of *Penicillium chrysogenum*. Int J Environ Sci Technol 12:2665–2672. https://doi.org/10.1007/s13762-014-0636-6

Nazareth SW, Sampy JD (2003) Production and characterisation of lignocellulases of *Panus tigrinus* and their application. Int Biodeterior Biodegrad 52:207–214. https://doi.org/10.1016/S0964-8305(03)00051-9

Nidadavolu SVSSSLHB, Gudikandula K, Pabba SK, Maringanti SC (2013) Decolorization of triphenyl methane dyes by *Fomitopsis feei*. Nat Sci 2013. https://doi.org/10.4236/ns.2013.56A005

Nnagadesi PK, Arya A (2015) Wood decay fungi associated with Tamarind Tree in Gujarat, India. Int Lett Nat Sci 46. https://doi.org/10.18052/www.scipress.com/ILNS.46.84

Ogola HJO, Kamiike T, Hashimoto N et al (2009) Molecular characterization of a novel peroxidase from the cyanobacterium *Anabaena* sp. strain PCC 7120. Appl Environ Microbiol 75:7509–7518. https://doi.org/10.1128/AEM.01121-09

Ohashi Y, Uno Y, Amirta R et al (2011) Alkoxyl- and carbon-centered radicals as primary agents for degrading non-phenolic lignin-substructure model compounds. Org Biomol Chem 9:2481–2491. https://doi.org/10.1039/C0OB00797H

Okeke BC, Paterson A, Smith JE, Watson-Craik IA (1994) Relationships between ligninolytic activities of *Lentinula* spp. and biotransformation of pentachlorophenol in sterile soil. Lett Appl Microbiol 19:284–287. https://doi.org/10.1111/j.1472-765X.1994.tb00965.x

Onysko KA (1993) Biological bleaching of chemical pulps: a review. Biotechnol Adv 11:179–198. https://doi.org/10.1016/0734-9750(93)90040-T

Padma N, Sudha H (2013) Optimization of lignin peroxidase, manganese peroxidase, and lac production from *Ganoderma lucidum* under solid state fermentation of pineapple leaf. Bioresources 1:250–271

Pala H, Mota M, Gama FM (2004) Enzymatic versus chemical deinking of non-impact ink printed paper. J Biotechnol 108:79–89. https://doi.org/10.1016/j.jbiotec.2003.10.016

Pamidipati S, Ahmed A (2017) Degradation of lignin in agricultural residues by locally isolated fungus *Neurospora discreta*. Appl Biochem Biotechnol 181:1561–1572

Pandey RK, Tewari S, Tewari L (2018) Lignolytic mushroom *Lenzites elegans* WDP2: laccase production, characterization, and bioremediation of synthetic dyes. Ecotoxicol Environ Saf 158:50–58. https://doi.org/10.1016/j.ecoenv.2018.04.003

Patel H, Gupte A, Gupte S (2009) Effect of different culture conditions and inducers on production of laccase by a basidiomycete fungal isolate *Pleurotus ostreatus* HP-1 under solid state fermentation. Bioresources 4:268–284

Patel VR, Bhatt NS, Bhatt H (2013) Involvement of ligninolytic enzymes of *Myceliophthora vellerea* HQ871747 in decolorization and complete mineralization of Reactive Blue 220. Chem Eng J 233:98–108. https://doi.org/10.1016/j.cej.2013.07.110

Pathak VM, Navneet (2017) Review on the current status of polymer degradation: a microbial approach. Bioresour Bioprocess 4:15. https://doi.org/10.1186/s40643-017-0145-9

Patil PD, Yadav GD (2018) Comparative studies of white-rot fungal strains (*Trametes hirsuta* MTCC-1171 and *Phanerochaete chrysosporium* NCIM-1106) for effective degradation and bioconversion of ferulic acid. ACS Omega 3:14858–14868. https://doi.org/10.1021/acsomega.8b01614

Pereira L, Alves M (2012) Dyes—environmental impact and remediation. In: Malik A, Grohmann E (eds) Environmental protection strategies for sustainable development. Springer, Dordrecht, pp 111–162

Pérez-Boada M, Ruiz-Dueñas FJ, Pogni R et al (2005) Versatile peroxidase oxidation of high redox potential aromatic compounds: site-directed mutagenesis, spectroscopic and crystallographic investigation of three long-range electron transfer pathways. J Mol Biol 354:385–402. https://doi.org/10.1016/j.jmb.2005.09.047

Piontek K, Smith AT, Blodig W (2001) Lignin peroxidase structure and function. Biochem Soc Trans 29:111–116. https://doi.org/10.1042/bst0290111

Plácido J, Capareda S (2015) Ligninolytic enzymes: a biotechnological alternative for bioethanol production. Bioresour Bioprocess 2:23. https://doi.org/10.1186/s40643-015-0049-5

Pointing S (2001) Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 57:20–33. https://doi.org/10.1007/s002530100745

Pollegioni L, Tonin F, Rosini E (2015) Lignin-degrading enzymes. FEBS J 282:1190–1213. https://doi.org/10.1111/febs.13224

Ponnusamy VK, Nguyen DD, Dharmaraja J et al (2019) A review on lignin structure, pretreatments, fermentation reactions and biorefinery potential. Bioresour Technol 271:462–472. https://doi.org/10.1016/j.biortech.2018.09.070

Popescu C-M, Larsson PT, Vasile C (2011) Carbon-13 CP/MAS solid state NMR and X-ray diffraction spectroscopy studies on lime wood decayed by *Chaetomium globosum*. Carbohydr Polym 83:808–812. https://doi.org/10.1016/j.carbpol.2010.08.049

Poulos TL (2014) Heme enzyme structure and function. Chem Rev 114:3919. https://doi.org/10.1021/cr400415k

Pozdnyakova NN (2012) Involvement of the ligninolytic system of white-rot and litter-decomposing fungi in the degradation of polycyclic aromatic hydrocarbons. Biotechnol Res Int. https://www.hindawi.com/journals/btri/2012/243217/. Accessed 15 Oct 2020

Pozdnyakova N, Dubrovskaya E, Chernyshova M et al (2018) The degradation of three-ringed polycyclic aromatic hydrocarbons by wood-inhabiting fungus *Pleurotus ostreatus* and soil-inhabiting fungus *Agaricus bisporus*. Fungal Biol 122:363–372. https://doi.org/10.1016/j.funbio.2018.02.007

Prasad KK, Mohan SV, Rao RS et al (2005) Laccase production by *Pleurotus ostreatus* 1804: optimization of submerged culture conditions by Taguchi DOE methodology. Biochem Eng J 24:17–26. https://doi.org/10.1016/j.bej.2005.01.019

Priya S, Kumar G, Kumar S, Bharti SK (2018) Isolation, characterization and identification of lignocellulose degrading actinomycetes from litchi fruit orchard of Muzaffarpur, Bihar, India. Pharma Innov 7(8):463–467

Pundir RK, Mishra VK, Rana S, Lakhani M (2016) Screening of laccase producing fungi from soil samples—an in vitro study. Electron J Biol 12:254–257

Qin L, Li W-C, Liu L et al (2016) Inhibition of lignin-derived phenolic compounds to cellulase. Biotechnol Biofuels 9:70. https://doi.org/10.1186/s13068-016-0485-2

Qin X, Sun X, Huang H et al (2017) Oxidation of a non-phenolic lignin model compound by two *Irpex lacteus* manganese peroxidases: evidence for implication of carboxylate and radicals. Biotechnol Biofuels 10:103. https://doi.org/10.1186/s13068-017-0787-z

Raghuwanshi S, Misra S, Saxena RK (2014) Treatment of wheat straw using tannase and white-rot fungus to improve feed utilization by ruminants. J Anim Sci Biotechnol 5:13. https://doi.org/10.1186/2049-1891-5-13

Rajak RC, Banerjee R (2016) Enzyme mediated biomass pretreatment and hydrolysis: a biotechnological venture towards bioethanol production. RSC Adv 6:61301–61311. https://doi.org/10.1039/C6RA09541K

Rajhans G, Sen SK, Barik A, Raut S (2020) Elucidation of fungal dye-decolorizing peroxidase (DyP) and ligninolytic enzyme activities in decolorization and mineralization of azo dyes. J Appl Microbiol. https://doi.org/10.1111/jam.14731

Rajwar D, Rai JPN (2015) Kraft black liquor decolorization by fungi isolated from contaminated pulp and paper mill sludge. Int J Recent Sci Res 6:7770

Ramarajan R, Manohar CS (2017) Biological pretreatment and bioconversion of agricultural wastes, using ligninolytic and cellulolytic fungal consortia. Biorem J 21:89–99. https://doi.org/10.1080/10889868.2017.1282937

Rameshaiah GN, Jagadish Reddy ML (2015) Applications of ligninolytic enzymes from a white-rot fungus *Trametes versicolor*. Univ J Environ Res Technol 5

Ramya M, Anusha B, Kalavathy S, Devilaksmi S (2007) Biodecolorization and biodegradation of Reactive Blue by *Aspergillus* sp. Afr J Biotechnol 6. https://doi.org/10.4314/ajb.v6i12.57566

Ranjusha VP, Pundir R, Kumar K et al (2010) Biosorption of Remazol Black B dye (Azo dye) by the growing *Aspergillus flavus*. J Environ Sci Health A 45:1256–1263. https://doi.org/10.1080/10934529.2010.493812

Rao MA, Scelza R, Scotti R, Gianfreda L (2010) Role of enzymes in the remediation of polluted environments. J Soil Sci Plant Nutr 10:333–353. https://doi.org/10.4067/S0718-95162010000100008

Rao RG, Ravichandran A, Kandalam G et al (2019) Screening of wild basidiomycetes and evaluation of the biodegradation potential of dyes and lignin by manganese peroxidases. Bioresources 14:6558–6576

Rathankumar AK, Saikia K, Ponnusamy SK et al (2020a) Rhamnolipid-assisted mycoremediation of polycyclic aromatic hydrocarbons by *Trametes hirsuta* coupled with enhanced ligninolytic enzyme production. J Air Waste Manag Assoc. https://doi.org/10.1080/10962247.2020.1790443

Rathankumar AK, Saikia K, Ramachandran K et al (2020b) Effect of soil organic matter (SOM) on the degradation of polycyclic aromatic hydrocarbons using *Pleurotus dryinus* IBB 903-A microcosm study. J Environ Manag 260:110153. https://doi.org/10.1016/j.jenvman.2020.110153

Ravichandran A, Sridhar M (2016) Versatile peroxidase: super peroxidases with potential biotechnological applications. J Diary Vet Anim Res 4:00116

Ravichandran A, Rao RG, Thammaiah V et al (2019) A versatile peroxidase from *Lentinus squarrosulus* towards enhanced delignification and in vitro digestibility of crop residues. Bioresources 14:5132–5149

Reddy GVB, Sridhar M, Gold MH (2003) Cleavage of nonphenolic β-1 diarylpropane lignin model dimers by manganese peroxidase from *Phanerochaete chrysosporium:* evidence for a hydrogen abstraction mechanism. Eur J Biochem 270:284–292

Renders T, den Bosch SV, Koelewijn S-F et al (2017) Lignin-first biomass fractionation: the advent of active stabilisation strategies. Energy Environ Sci 10:1551–1557. https://doi.org/10.1039/C7EE01298E

Rigling D, Alfen NKV (1993) Extra- and intracellular laccases of the chestnut blight fungus, *Cryphonectria parasitica*. Appl Environ Microbiol 59:3634–3639

Rinaldi R, Jastrzebski R, Clough MT et al (2016) Paving the way for lignin valorisation: recent advances in bioengineering, biorefining and catalysis. Angew Chem Int Ed 55:8164–8215. https://doi.org/10.1002/anie.201510351

Rivela I, Rodríguez Couto S, Sanromán A (2000) Extracellular ligninolytic enzyme production by *Phanerochaete chrysosporium* in a new solid-state bioreactor. Biotechnol Lett 22:1443–1447. https://doi.org/10.1023/A:1005607000999

Rodríguez Couto S, Toca Herrera JL (2006) Industrial and biotechnological applications of laccases: a review. Biotechnol Adv 24:500–513. https://doi.org/10.1016/j.biotechadv.2006.04.003

Romero JO, Fernández-Fueyo E, Avila-Salas F et al (2019) Binding and catalytic mechanisms of veratryl alcohol oxidation by lignin peroxidase: a theoretical and experimental study. Comput Struct Biotechnol J 17:1066–1074. https://doi.org/10.1016/j.csbj.2019.07.002

Rudakiya DM, Gupte A (2019) Assessment of white rot fungus mediated hardwood degradation by FTIR spectroscopy and multivariate analysis. J Microbiol Methods 157:123–130. https://doi.org/10.1016/j.mimet.2019.01.007

Ruiz-Dueñas FJ, Martínez ÁT (2009) Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this. Microb Biotechnol 2:164–177

Ruiz-Dueñas FJ, Morales M, García E et al (2009) Substrate oxidation sites in versatile peroxidase and other basidiomycete peroxidases. J Exp Bot 60:441–452. https://doi.org/10.1093/jxb/ern261

Sadhasivam S, Savitha S, Swaminathan K (2010) Deployment of *Trichoderma harzianum* WL1 laccase in pulp bleaching and paper industry effluent treatment. J Clean Prod 18:799–806. https://doi.org/10.1016/j.jclepro.2009.11.014

Sáez-Jiménez V, Rencoret J, Rodríguez-Carvajal MA et al (2016) Role of surface tryptophan for peroxidase oxidation of nonphenolic lignin. Biotechnol Biofuels 9:198. https://doi.org/10.1186/s13068-016-0615-x

Saha R, Mukhopadhyay M (2020) Elucidation of the decolorization of Congo Red by *Trametes versicolor* laccase in presence of ABTS through cyclic voltammetry. Enzym Microb Technol 135:109507. https://doi.org/10.1016/j.enzmictec.2019.109507

Saha BC, Qureshi N, Kennedy GJ, Cotta MA (2016) Biological pretreatment of corn Stover with white-rot fungus for improved enzymatic hydrolysis. Int Biodeterior Biodegrad 109:29–35. https://doi.org/10.1016/j.ibiod.2015.12.020

Saha BC, Kennedy GJ, Qureshi N, Cotta MA (2017) Biological pretreatment of corn Stover with *Phlebia brevispora* NRRL-13108 for enhanced enzymatic hydrolysis and efficient ethanol production. Biotechnol Prog 33:365–374. https://doi.org/10.1002/btpr.2420

Sahay S, Chaurse V, Chauhan D (2020) Laccase from *Aspergillus nidulans* TTF6 showing pb activation for smaller substrates and dyes remediation in all climates. Proc Natl Acad Sci India B Biol Sci 90:143–150. https://doi.org/10.1007/s40011-019-01092-y

Sahu S, Pramanik K (2015) Delignification of cotton gin waste and its optimization by using white rot fungus *Pycnoporus cinnabarinus*. J Environ Biol 36:661

Saikia K, Vishnu D, Rathankumar AK et al (2020) Development of a magnetically separable co-immobilized laccase and versatile peroxidase system for the conversion of lignocellulosic biomass to vanillin. J Air Waste Manag Assoc. https://doi.org/10.1080/10962247.2020.1760958

Saini S, Chandel AK, Sharma KK (2020a) Past practices and current trends in the recovery and purification of first generation ethanol: a learning curve for lignocellulosic ethanol. J Clean Prod 268:122357. https://doi.org/10.1016/j.jclepro.2020.122357

Saini S, Chutani P, Kumar P, Sharma KK (2020b) Development of an eco-friendly deinking process for the production of bioethanol using diverse hazardous paper wastes. Renew Energy 146:2362–2373. https://doi.org/10.1016/j.renene.2019.08.087

Salame TM, Knop D, Levinson D et al (2012) Release of *Pleurotus ostreatus* versatile-peroxidase from mn2+ repression enhances anthropogenic and natural substrate degradation. PLoS One 7: e52446. https://doi.org/10.1371/journal.pone.0052446

Saleem R, Khurshid M, Ahmed S (2018) Laccases, manganese peroxidases and xylanases used for the bio-bleaching of paper pulp: an environmental friendly approach. Protein Pept Lett 25:180–186. https://doi.org/10.2174/0929866525666180122100133

Salvachúa D, Prieto A, López-Abelairas M et al (2011) Fungal pretreatment: an alternative in second-generation ethanol from wheat straw. Bioresour Technol 102:7500–7506. https://doi.org/10.1016/j.biortech.2011.05.027

Salvachúa D, Prieto A, Martínez ÁT, Martínez MJ (2013) Characterization of a novel dye-decolorizing peroxidase (dyp)-type enzyme from *Irpex lacteus* and its application in enzymatic hydrolysis of wheat straw. Appl Environ Microbiol 79:4316. https://doi.org/10.1128/AEM.00699-13

Saraswathi R, Saseetharan MK (2010) Investigation on microorganisms and their degradation efficiency in paper and pulp mill effluent. J Water Resour Prot 2:660–664. https://doi.org/10.4236/jwarp.2010.27076

Saroj S, Kumar K, Pareek N et al (2014) Biodegradation of azo dyes Acid Red 183, Direct Blue 15 and Direct Red 75 by the isolate *Penicillium oxalicum* SAR-3. Chemosphere 107:240–248. https://doi.org/10.1016/j.chemosphere.2013.12.049

Saxena A, Chauhan PS (2017) Role of various enzymes for deinking paper: a review. Crit Rev Biotechnol 37:598–612. https://doi.org/10.1080/07388551.2016.1207594

Schindl A, Hagen ML, Muzammal S et al (2019) Proteins in ionic liquids: reactions, applications, and futures. Front Chem 7. https://doi.org/10.3389/fchem.2019.00347

Schoenherr S, Ebrahimi M, Czermak P (2017) Lignin degradation processes and the purification of valuable products. Lignin Trends Appl. https://doi.org/10.5772/intechopen.71210

Selvam K, Arungandhi K (2013) Biobleaching and delignification of hard wood kraft pulp (HWKP) by *Trametes* sp., *Ganoderma* sp. and *Poria* sp. Int J Plant Anim Environ Sci 3:96–100

Selvam K, Swaminathan K, Chae K-S (2003) Microbial decolorization of azo dyes and dye industry effluent by *Fomes lividus*. World J Microbiol Biotechnol 19:591–593. https://doi.org/10.1023/A:1025128327853

Selvam K, Priya MS, Sivaraj C (2011) Bioremediation of pulp and paper mill effluent by newly isolated wood rot fungi from Western Ghats area of South India. Int J Pharma Biol Arch 2:1765–1771

Selvam K, Priya MS, Yamuna M (2012) Decolourization of azo dyes and dye industry effluents by lignin degrading fungus *Trametes versicolor*. Int J Pharm Biol Arch 3:666–672

Senthivelan T, Kanagaraj J, Panda RC (2016) Recent trends in fungal laccase for various industrial applications: an eco-friendly approach—a review. Biotechnol Bioprocess Eng 21:19–38

Senthivelan T, Kanagaraj J, Panda RC, Narayani T (2019) Screening and production of a potential extracellular fungal laccase from *Penicillium chrysogenum*: media optimization by response surface methodology (RSM) and central composite rotatable design (CCRD). Biotechnol Rep 23:e00344. https://doi.org/10.1016/j.btre.2019.e00344

Seshikala D (2014) Choosing of good white rot fungi for biobleaching of unbleached kraft pulp. Int J Life Sci Biotechnol Pharma Res 3:208–218

Shaheen R, Asgher M, Hussain F, Bhatti HN (2017) Immobilized lignin peroxidase from *Ganoderma lucidum* IBL-05 with improved dye decolorization and cytotoxicity reduction properties. Int J Biol Macromol 103:57–64. https://doi.org/10.1016/j.ijbiomac.2017.04.040

Shankar S, Shikha BC et al (2018) Laccase based de-inking of mixed office waste and evaluation of its impact on physico-optical properties of recycled fiber. Environ Sustain 1:233–244. https://doi.org/10.1007/s42398-018-0021-3

Shankar A, Ahlawat S, Sharma KK (2019) Exploring fungi-associated lignocellulose degradation: secretomic and proteomic approaches. In: Satyanarayana T, Deshmukh SK, Deshpande MV (eds) Advancing frontiers in mycology & mycotechnology: basic and applied aspects of fungi. Springer, Singapore, pp 251–277

Shanmugam S, Palvannan T, Sathish Kumar T, Michael A (2005) Biological decolourization of textile and paper effluents by *Pleurotus florida* and *Agaricus bisporus* (white-rot Basidiomycetes). World J Microbiol Biotechnol 21:1149–1151. https://doi.org/10.1007/s11274-005-0298-0

Sharma KK (2016) Fungal genome sequencing: basic biology to biotechnology. Crit Rev Biotechnol 36:743–759

Sharma KK, Kuhad RC (2008) Laccase: enzyme revisited and function redefined. Indian J Microbiol 48:309–316. https://doi.org/10.1007/s12088-008-0028-z

Sharma KK, Kuhad RC (2009) An evidence of laccases in archaea. Indian J Microbiol 49:142–150

Sharma KK, Kapoor M, Kuhad RC (2005) In vivo enzymatic digestion, in vitro xylanase digestion, metabolic analogues, surfactants and polyethylene glycol ameliorate laccase production from *Ganoderma* sp. kk-02. Lett Appl Microbiol 41:24–31. https://doi.org/10.1111/j.1472-765X.2005.01721.x

Sharma P, Goel R, Capalash N (2007) Bacterial laccases. World J Microbiol Biotechnol 23:823–832. https://doi.org/10.1007/s11274-006-9305-3

Sharma JK, Yadav M, Singh NP, Yadav KDS (2011) Purification and characterisation of lignin peroxidase from *Pycnoporus sanguineus* MTCC-137. Appl Biochem Microbiol 47:532. https://doi.org/10.1134/S0003683811050139

Sharma KK, Shrivastava B, Nandal P et al (2012) Nutritional and toxicological assessment of white-rot fermented animal feed. Indian J Microbiol 52:185–190

Sharma KK, Shrivastava B, Sastry VRB et al (2013a) Middle-redox potential laccase from *Ganoderma* sp.: its application in improvement of feed for monogastric animals. Sci Rep 3:1299. https://doi.org/10.1038/srep01299

Sharma KK, Singh D, Sapna et al (2013b) Ligninolytic enzymes in environmental management. In: Kuhad RC, Singh A (eds) Biotechnology for environmental management and resource recovery. Springer, New Delhi, pp 219–238

Sharma KK, Singh D, Kumar A (2016) Biochemical and structural studies of laccase isozymes from *Ganoderma lucidum* MDU-7. In: Microbes in the spotlight: recent progress in the understanding of beneficial and harmful microorganisms. BrownWalker Press, Boca Raton, pp 390–396

Sharma A, Jain KK, Srivastava A et al (2019) Potential of in situ SSF laccase produced from *Ganoderma lucidum* RCK 2011 in biobleaching of paper pulp. Bioprocess Biosyst Eng 42:367–377. https://doi.org/10.1007/s00449-018-2041-x

Sharma D, Chaudhary R, Kaur J, Arya SK (2020a) Greener approach for pulp and paper industry by xylanase and laccase. Biocatal Agric Biotechnol 25:101604. https://doi.org/10.1016/j.bcab.2020.101604

Sharma S, Sharma A, Mulla SI et al (2020b) Lignin as potent industrial biopolymer: an introduction. In: Sharma S, Kumar A (eds) Lignin: biosynthesis and transformation for industrial applications. Springer International Publishing, Cham, pp 1–15

Shary S, Ralph SA, Hammel KE (2007) New insights into the ligninolytic capability of a wood decay ascomycete. Appl Environ Microbiol 73:6691–6694. https://doi.org/10.1128/AEM.01361-07

Sheikh GG, Ganai AM, Reshi PA et al (2018) Improved paddy straw as ruminant feed: a review. Agric Rev 39:137–143

Shekher R, Sehgal S, Kamthania M, Kumar A (2011) Laccase: microbial sources, production, purification, and potential biotechnological applications. Enzym Res 2011:217861

Shen X, Zhu L, Wang N et al (2011) Molecular imprinting for removing highly toxic organic pollutants. Chem Commun 48:788–798. https://doi.org/10.1039/C2CC14654A

Shirkavand E, Baroutian S, Gapes DJ, Young BR (2016) Combination of fungal and physicochemical processes for lignocellulosic biomass pretreatment—a review. Renew Sust Energ Rev 54:217–234. https://doi.org/10.1016/j.rser.2015.10.003

Shraddha SR, Sehgal S et al (2011) Laccase: microbial sources, production, purification, and potential biotechnological applications. Enzym Res 2011. https://doi.org/10.4061/2011/217861

Shrestha R, Huang G, Meekins DA et al (2017) Mechanistic insights into dye-decolorizing peroxidase revealed by solvent isotope and viscosity effects. ACS Catal 7:6352. https://doi.org/10.1021/acscatal.7b01861

Shrivastava B, Thakur S, Khasa YP et al (2011) White-rot fungal conversion of wheat straw to energy rich cattle feed. Biodegradation 22:823–831

Shrivastava B, Nandal P, Sharma A et al (2012) Solid state bioconversion of wheat straw into digestible and nutritive ruminant feed by *Ganoderma* sp. rckk02. Bioresour Technol 107:347–351. https://doi.org/10.1016/j.biortech.2011.12.096

Shrivastava B, Jain KK, Kalra A, Kuhad RC (2014) Bioprocessing of wheat straw into nutritionally rich and digested cattle feed. Sci Rep 4:6360

Sigoillot J-C, Berrin J-G, Bey M et al (2012) Chapter 8—Fungal strategies for lignin degradation. In: Jouanin L, Lapierre C (eds) Advances in botanical research. Academic Press, London, pp 263–308

Silveira MHL, Vanelli BA, Chandel AK (2018) Chapter 6—Second generation ethanol production: potential biomass feedstock, biomass deconstruction, and chemical platforms for process valorization. In: Chandel AK, Luciano Silveira MH (eds) Advances in sugarcane biorefinery. Elsevier, Amsterdam, pp 135–152

Sindhu R, Binod P, Pandey A (2016) Biological pretreatment of lignocellulosic biomass—an overview. Bioresour Technol 199:76–82. https://doi.org/10.1016/j.biortech.2015.08.030

Singh K, Arora S (2011) Removal of synthetic textile dyes from wastewaters: a critical review on present treatment technologies. Crit Rev Environ Sci Technol 41:807–878. https://doi.org/10.1080/10643380903218376

Singh G, Dwivedi SK (2020) Decolorization and degradation of Direct Blue-1 (Azo dye) by newly isolated fungus *Aspergillus terreus* GS28, from sludge of carpet industry. Environ Technol Innov 18:100751. https://doi.org/10.1016/j.eti.2020.100751

Singh R, Eltis LD (2015) The multihued palette of dye-decolorizing peroxidases. Arch Biochem Biophys 574:56–65. https://doi.org/10.1016/j.abb.2015.01.014

Singh S, Pakshirajan K (2010) Enzyme activities and decolourization of single and mixed azo dyes by the white-rot fungus *Phanerochaete chrysosporium*. Int Biodeterior Biodegrad 64:146–150. https://doi.org/10.1016/j.ibiod.2009.11.003

Singh AP, Singh T (2014) Biotechnological applications of wood-rotting fungi: a review. Biomass Bioenergy 62:198–206

Singh AD, Sabaratnam V, Abdullah N et al (2010a) Decolourisation of chemically different dyes by enzymes from spent compost of *Pleurotus sajor-caju* and their kinetics. Afr J Biotechnol 9. https://doi.org/10.4314/ajb.v9i1

Singh P, Sulaiman O, Hashim R et al (2010b) Biopulping of lignocellulosic material using different fungal species: a review. Rev Environ Sci Biotechnol 9:141–151. https://doi.org/10.1007/s11157-010-9200-0

Singh MP, Vishwakarma SK, Srivastava AK (2013) Bioremediation of direct blue 14 and extracellular ligninolytic enzyme production by white rot fungi: *Pleurotus* spp. BioMed Res Int. https://www.hindawi.com/journals/bmri/2013/180156/. Accessed 13 Oct 2020

Singh D, Sharma KK, Jacob S, Gakhar SK (2014a) Molecular docking of laccase protein from *Bacillus safensis* DSKK5 isolated from earthworm gut: a novel method to study dye decolorization potential. Water Air Soil Pollut 225:2175. https://doi.org/10.1007/s11270-014-2175-7

Singh R, Shukla A, Tiwari S, Srivastava M (2014b) A review on delignification of lignocellulosic biomass for enhancement of ethanol production potential. Renew Sust Energ Rev 32:713–728. https://doi.org/10.1016/j.rser.2014.01.051

Singh G, Kaur K, Puri S, Sharma P (2015) Critical factors affecting laccase-mediated biobleaching of pulp in paper industry. Appl Microbiol Biotechnol 99:155–164. https://doi.org/10.1007/s00253-014-6219-0

Singh GK, Kaur S, Khatri M, Arya SK (2019) Biobleaching for pulp and paper industry in India: emerging enzyme technology. Biocatal Agric Biotechnol 17:558–565. https://doi.org/10.1016/J.BCAB.2019.01.019

Singh J, Das A, Yogalakshmi KN (2020) Enhanced laccase expression and azo dye decolourization during co-interaction of *Trametes versicolor* and *Phanerochaete chrysosporium*. SN Appl Sci 2:1095. https://doi.org/10.1007/s42452-020-2832-y

Singha S, Panda T (2015) Optimization of laccase fermentation and evaluation of kinetic and thermodynamic parameters of a partially purified laccase produced by *Daedalea flavida*. Prep Biochem Biotechnol 45:307–335. https://doi.org/10.1080/10826068.2014.887581

Sitarz AK, Mikkelsen JD, Meyer AS (2016) Structure, functionality and tuning up of laccases for lignocellulose and other industrial applications. Crit Rev Biotechnol 36:70–86. https://doi.org/10.3109/07388551.2014.949617

Skariyachan S, Prasanna A, Manjunath SP et al (2016) Environmental assessment of the degradation potential of mushroom fruit bodies of *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. towards synthetic azo dyes and contaminating effluents collected from textile industries in Karnataka, India. Environ Monit Assess 188:121. https://doi.org/10.1007/s10661-016-5125-6

Solomon EI, Sundaram UM, Machonkin TE (1996) Multicopper oxidases and oxygenases. Chem Rev 96:2563–2606

Sridhar M, Bhatta R, Dhali A et al (2015) Effect of exogenous ligninolytic enzyme-treated ragi straw on DM intake, digestibility, rumen fermentation and rumen enzymes in sheep. Indian J Anim Sci 85:1012–1016

Srivastava AK, Vishwakarma SK, Pandey VK, Singh MP (2014) Direct red decolorization and ligninolytic enzymes production by improved strains of *Pleurotus* using basidiospore derived monokaryons. Cell Mol Biol 60:15–21

Suman SK, Khatri M, Dhawaria M et al (2018) Potential of *Trametes maxima* IIPLC-32 derived laccase for the detoxification of phenolic inhibitors in lignocellulosic biomass prehydrolysate. Int Biodeterior Biodegrad 133:1–8. https://doi.org/10.1016/j.ibiod.2018.05.009

Sun Z, Fridrich B, de Santi A et al (2018) Bright side of lignin depolymerization: toward new platform chemicals. Chem Rev 118:614–678. https://doi.org/10.1021/acs.chemrev.7b00588

Taha AA, Hameed NJ, Ali FH (2020) Degradation of anthracene by immobilizing laccase from *Trametes Versicolor* onto chitosan beads and hyacinth plant. Al-Mustansiriyah J Sci 31:14–20. https://doi.org/10.23851/mjs.v31i3.670

Tao J, Li S, Ye F et al (2020) Lignin—an underutilized, renewable and valuable material for food industry. Crit Rev Food Sci Nutr 60:2011–2033. https://doi.org/10.1080/10408398.2019.1625025

Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ (2012) Heavy metals toxicity and the environment. EXS 101:133–164. https://doi.org/10.1007/978-3-7643-8340-4_6

Tejirian A, Xu F (2011) Inhibition of enzymatic cellulolysis by phenolic compounds. Enzym Microb Technol 48:239–247. https://doi.org/10.1016/j.enzmictec.2010.11.004

Temp U, Eggert C (1999) Novel interaction between laccase and cellobiose dehydrogenase during pigment synthesis in the white rot fungus *Pycnoporus cinnabarinus*. Appl Environ Microbiol 65:389

Thakur S, Shrivastava B, Ingale S et al (2013) Degradation and selective ligninolysis of wheat straw and banana stem for an efficient bioethanol production using fungal and chemical pretreatment. 3 Biotech 3:365–372. https://doi.org/10.1007/s13205-012-0102-4

Thammaiah V, Rao RG, Samanta AK et al (2016) Effect of lignin peroxidases obtained from white rot fungi in delignification of cereal crop residues for ruminant feeding: changes in chemical

composition and in vitro digestibility. IOSR-JAVS 09(09):47–58. https://doi.org/10.9790/2380-0909014758

Thiribhuvanamala G, Krishnamoorthy S, Manoranjitham K et al (2012) Improved techniques to enhance the yield of paddy straw mushroom (*Volvariella volvacea*) for commercial cultivation. Afr J Biotechnol 11:12740–12748

Thurston CF (1994) The structure and function of fungal laccases. Microbiology 140:19–26. https://doi.org/10.1099/13500872-140-1-19

Tk K, Jm H, Eb C (1968) Degradation of the lignin model compound syringylglycol-beta-guaiacyl ether by *Polyporus versicolor* and *Stereum frustalatum*. Biochim Biophys Acta. https://pubmed.ncbi.nlm.nih.gov/4970522/. Accessed 14 Oct 2020

Tolbert A, Akinosho H, Khunsupat R et al (2014) Characterization and analysis of the molecular weight of lignin for biorefining studies. Biofuels Bioprod Biorefin 8:836–856. https://doi.org/10.1002/bbb.1500

Torres E, Bustos-Jaimes I, Le Borgne S (2003) Potential use of oxidative enzymes for the detoxification of organic pollutants. Appl Catal B Environ 46:1–15. https://doi.org/10.1016/S0926-3373(03)00228-5

Tribot A, Amer G, Alio MA et al (2019) Wood-lignin: supply, extraction processes and use as bio-based material. Eur Polym J 112:228–240

Tripathi A, Upadhyay RC, Singh S (2011) Mineralization of mono-nitrophenols by *Bjerkandera adusta* and *Lentinus squarrosulus* and their extracellular ligninolytic enzymes. J Basic Microbiol 51:635–649. https://doi.org/10.1002/jobm.201000436

Tripathi A, Upadhyay RC, Singh S (2012) Extracellular ligninolytic enzymes in *Bjerkandera adusta* and *Lentinus squarrosulus*. Indian J Microbiol 52:381–387

Trotter PC (1990) Biotechnology in the pulp and paper industry: a review. Part 1: tree improvement, pulping and bleaching, and dissolving pulp applications. TAPPI J 73:198–204

Tsatsis DE, Papachristos DK, Valta KA et al (2017) Enzymatic deinking for recycling of office waste paper. J Environ Chem Eng 5:1744–1753. https://doi.org/10.1016/j.jece.2017.03.007

Ummalyma SB, Supriya RD, Sindhu R et al (2019) Chapter 7—Biological pretreatment of lignocellulosic biomass—current trends and future perspectives. In: Basile A, Dalena F (eds) Second and third generation of feedstocks. Elsevier, Amsterdam, pp 197–212

Upadhyay P, Shrivastava R, Agrawal PK (2016) Bioprospecting and biotechnological applications of fungal laccase. 3 Biotech 6. https://doi.org/10.1007/s13205-015-0316-3

Urzúa U, Kersten PJ, Vicuña R (1998) Manganese peroxidase-dependent oxidation of glyoxylic and oxalic acids synthesized by *Ceriporiopsis subvermispora* produces extracellular hydrogen peroxide. Appl Environ Microbiol 64:68–73

Vaahtera L, Schulz J, Hamann T (2019) Cell wall integrity maintenance during plant development and interaction with the environment. Nat Plants 5:924–932. https://doi.org/10.1038/s41477-019-0502-0

Valls C, Cusola O, Vidal T et al (2019) A straightforward bioprocess for a cleaner paper decolorization. J Clean Prod 236:117702. https://doi.org/10.1016/j.jclepro.2019.117702

Vanholme R, De Meester B, Ralph J, Boerjan W (2019) Lignin biosynthesis and its integration into metabolism. Curr Opin Biotechnol 56:230–239. https://doi.org/10.1016/j.copbio.2019.02.018

Vani V, Jayashree DJ, Navya KS, Zaman ST (2020) Homology modelling and in silico characterization of laccase from *Lentinula edodes*. Int J Pharm Invest 10:273–278. https://doi.org/10.5530/ijpi.2020.3.50

Vares T, Kalsi M, Hatakka A (1995) Lignin peroxidases, manganese peroxidases, and other ligninolytic enzymes produced by *Phlebia radiata* during solid-state fermentation of wheat straw. Appl Environ Microbiol 61:3515–3520

Varjani S, Rakholiya P, Ng HY et al (2020) Microbial degradation of dyes: an overview. Bioresour Technol 314:123728. https://doi.org/10.1016/j.biortech.2020.123728

Varsha YN, Chenna S (2011) An emphasis on xenobiotic degradation in environmental clean up. J Bioremed Biodegr 2:1–10. https://doi.org/10.4172/2155-6199.S11-001

Vaseem H, Singh VK, Singh MP (2017) Heavy metal pollution due to coal washery effluent and its decontamination using a macrofungus, *Pleurotus ostreatus*. Ecotoxicol Environ Saf 145:42–49. https://doi.org/10.1016/j.ecoenv.2017.07.001

Vats A, Mishra S (2020) Laccase isoform diversity on basal medium in *Cyathus bulleri* and role in decolorization/detoxification of textile dyes and effluent. World J Microbiol Biotechnol 36:164. https://doi.org/10.1007/s11274-020-02939-7

Venkatesagowda B, Dekker RFH (2020) Enzymatic demethylation of Kraft lignin for lignin-based phenol-formaldehyde resin applications. Biomass Convers Bioref 10:203–225. https://doi.org/10.1007/s13399-019-00407-3

Verma P, Madamwar D (2002) Production of ligninolytic enzymes for dye decolorization by cocultivation of white-rot fungi *Pleurotus ostreatus* and *Phanerochaete chrysosporium* under solid-state fermentation. Appl Biochem Biotechnol 102:109–118. https://doi.org/10.1385/ABAB:102-103:1-6:109

Virk AP, Puri M, Gupta V et al (2013) Combined enzymatic and physical deinking methodology for efficient eco-friendly recycling of old newsprint. PLoS One 8:e72346. https://doi.org/10.1371/journal.pone.0072346

Viswanath B, Rajesh B, Janardhan A et al (2014) Fungal laccases and their applications in bioremediation. Enzym Res 2014. https://doi.org/10.1155/2014/163242

Vrsanska M, Voberkova S, Langer V et al (2016) Induction of laccase, lignin peroxidase and manganese peroxidase activities in white-rot fungi using copper complexes. Molecules 21. https://doi.org/10.3390/molecules21111553

Wagner AO, Lackner N, Mutschlechner M et al (2018) Biological pretreatment strategies for second-generation lignocellulosic resources to enhance biogas production. Energies 11:1797. https://doi.org/10.3390/en11071797

Wan C, Li Y (2012) Fungal pretreatment of lignocellulosic biomass. Biotechnol Adv 30:1447–1457. https://doi.org/10.1016/j.biotechadv.2012.03.003

Wang W, Huang F, Lu XM, Gao PJ (2006) Lignin degradation by a novel peptide, Gt factor, from brown rot fungus *Gloeophyllum trabeum*. Biotechnol J 1:447–453. https://doi.org/10.1002/biot.200500016

Wang X, Yao B, Su X (2018) Linking enzymatic oxidative degradation of lignin to organics detoxification. Int J Mol Sci 19. https://doi.org/10.3390/ijms19113373

Wang H, Pu Y, Ragauskas A, Yang B (2019) From lignin to valuable products–strategies, challenges, and prospects. Bioresour Technol 271:449–461. https://doi.org/10.1016/j.biortech.2018.09.072

Watanabe T, Shirai N, Okada H et al (2001) Production and chemiluminescent free radical reactions of glyoxal in lipid peroxidation of linoleic acid by the ligninolytic enzyme, manganese peroxidase. Eur J Biochem 268:6114–6122

Wong DW (2009) Structure and action mechanism of ligninolytic enzymes. Appl Biochem Biotechnol 157:174–209

Wong SS, Shu R, Zhang J et al (2020) Downstream processing of lignin derived feedstock into end products. Chem Soc Rev 49:5510–5560. https://doi.org/10.1039/D0CS00134A

Xu R, Zhang K, Liu P et al (2018) Lignin depolymerization and utilization by bacteria. Bioresour Technol 269:557–566. https://doi.org/10.1016/j.biortech.2018.08.118

Yadav B (2018) Induction of laccase in fungus *Cyathus stercoreus* using some aromatic inducers. J Appl Nat Sci 10:445–447. https://doi.org/10.31018/jans.v10i1.1648

Yadav M, Yadav HS (2015) Applications of ligninolytic enzymes to pollutants, wastewater, dyes, soil, coal, paper and polymers. Environ Chem Lett 13:309–318. https://doi.org/10.1007/s10311-015-0516-4

Yang J, Gao T, Zhang Y et al (2019) Degradation of the phenolic β-ether lignin model dimer and dyes by dye-decolorizing peroxidase from *Bacillus amyloliquefaciens*. Biotechnol Lett 41:1015–1021. https://doi.org/10.1007/s10529-019-02696-0

Yelle DJ, Wei D, Ralph J, Hammel KE (2011) Multidimensional NMR analysis reveals truncated lignin structures in wood decayed by the brown rot basidiomycete *Postia placenta*. Environ Microbiol 13:1091–1100. https://doi.org/10.1111/j.1462-2920.2010.02417.x

Yoshida H (1883) LXIII.—chemistry of lacquer (Urushi). Part I. communication from the chemical society of Tokio. J Chem Soc Trans 43:472–486

Zhu X, Akiyama T, Yokoyama T, Matsumoto Y (2019) Stereoselective formation of β-o-4 structures mimicking softwood lignin biosynthesis: effects of solvent and the structures of quinone methide lignin models. J Agric Food Chem 67:6950–6961. https://doi.org/10.1021/acs.jafc.9b01968

Zhu J, Yan C, Zhang X et al (2020) A sustainable platform of lignin: from bioresources to materials and their applications in rechargeable batteries and supercapacitors. Prog Energy Combust Sci 76:100788. https://doi.org/10.1016/j.pecs.2019.100788

Zimbardi ALRL, Camargo PF, Carli S et al (2016) A high redox potential laccase from *Pycnoporus sanguineus* rp15: potential application for dye decolorization. Int J Mol Sci 17. https://doi.org/10.3390/ijms17050672

Zofair SFF, Arsalan A, Khan MA et al (2020) Immobilization of laccase on Sepharose-linked antibody support for decolourization of phenol red. Int J Biol Macromol 161:78–87. https://doi.org/10.1016/j.ijbiomac.2020.06.009

Fungal Chitinolytic Enzymes

7

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Abstract

Fungi contribute significantly in the production of industrially important enzymes like amylases, cellulases, and others. However, their roles in nature, except nutrition, are not described significantly. On the other hand, fungal cell wall polymers chitin/chitosan and chitinolytic enzymes, viz., endo-chitinase, *N*-acetylglucosaminidase, chitin deacetylase (CDA), and endo- and exo-acting chitosanases, play a significant role in fungal biology and also are gaining importance for their biotechnological applications. Furthermore, though cellulose, chitin, and chitosan are β-1,4-linked polymers, there are two pathways for chitin hydrolysis, which use either chitinases or CDA and chitosanases. As a result, chitinolytic enzymes are important in nutrition, growth and differentiation, dispersal, and interactions with other fungi, insects, and nematodes. This has prompted researchers to explore more about their applications in the biocontrol of insect pests, fungal pathogens, and nematodes and development of transgenic plants. Other than agriculture, healthcare and pharmaceutical industries also use these enzymes for low-volume high-cost products like chitooligosaccharides. The present chapter focuses on the fungal chitinolytic enzymes, their biochemical and molecular aspects, and their role in nature and potential applications.

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Keywords

Chitinases · Chitin deacetylase · Chitosanase · Role in fungal differentiation · Structure-function relationship

7.1 Introduction

A number of industrially important enzymes, such as amylases, cellulases-hemicellulases, isomerases, lipases, oxidases and peroxidases, phytases, proteases, and many others, have lots of applications. However, their roles in nature are not adequately described in the literature. This is not the case for chitinolytic enzymes, per se (Shaikh and Deshpande 1993; Patil et al. 2000).

Chitin, a polymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc), is abundantly present in marine invertebrates, insects, fungi, and algae as the main structural component. Marine organisms such as shrimps, crabs, squids, and others are rich in chitinous material (10–55% on dry weight basis), while cell walls of fungi contain 22–44% of chitin. It exists in three main forms based on different orientations of microfibrils: α-chitin has antiparallel chains, β-chitin is with parallel chains, and γ-chitin has the mixture of parallel and antiparallel chains. The β-chitin is relatively rare in living forms and present in squid pens, in the spines of diatoms, and in a few other organisms, while γ -chitin is present in the cocoon of the moth, *Orgyia dubia* (Deshpande 1986; Kaya et al. 2017). In most of the organisms, except spines of diatoms, *Thalassiosira fluviatilis*, deacetylated residues, i.e., glucosamine (GlcN) residues, are also present in chitin. When the % of deacetylated residues is >70%, then the polymer is called chitosan (Ghormade et al. 2017).

Recently, it has been suggested that fungi can be defined as the organisms which have a chitin/chitosan as the main structural component of cell walls. By this definition, oomycetous fungi such as *Phytophthora*, *Pythium*, and others, having cellulose as the main structural cell wall component, no longer belong to the Kingdom Mycota/Fungi, but they are placed in Kingdom Chromista/Stramnipila (Ho 2018). Zygomycetous fungi have more proportion of chitosan than chitin. A number of non-zygomycetous phyto- and entomopathogens too have high proportion of chitosan in the cell walls for the protection against their respective host chitinases (Nahar et al. 2004).

7.2 Chitin Metabolism in Fungi

7.2.1 Biosynthesis of Chitin and Chitosan

Chitin synthases as well as chitin deacetylases are important enzymes involved in chitin and chitosan synthesis. The sequential transfer of GlcNAc from UDP-GlcNAc to the non-reducing end of the growing polymer is the key step in chitin synthesis. The CS (chitin-(UDP-GlcNAc)-transferase, EC 2.4.1.16) catalyzes this glycosyl

transfer with inversion of the anomeric configuration. The CS is detected mainly in membrane fractions (Deshpande et al. 1997). Bartnicki-Garcia (2006) reviewed the past, present, and future of chitosomes, small secretory vesicles (ca. 100S) which carry latent enzyme to the growing tip of the fungus. As the substrate UDP-GlcNAc is present throughout the cytosol, zymogenicity is necessary to prevent the premature synthesis of chitin inside the cell. The zymogenic CS is delivered at the surface and activated by proteolytic cleavage for further chitin synthesis. According to Bartnicki-Garcia (1973), chitinases are important in plasticizing hyphal tip and also provide GlcNAc for the synthesis of a new cell wall, thus suggesting the lytic-synthetic hypothesis of cell wall growth (Ghormade et al. 2011). For chitosan synthesis, chitin is further deacetylated by chitin deacetylase (CDA, EC 3.5.1.41), an enzyme that catalyzes the hydrolysis of acetamido groups of GlcNAc (Ghormade et al. 2010).

7.2.2 Structure-Function Relationship of Fungal Chitinolytic Enzymes

In chitinolytic enzymes, a multi-enzyme complex is made up of randomly hydrolyzing endo-chitinases (EC 3.2.1.14) and dimer-hydrolyzing *N*-acetylhexosaminidases (EC 3.2.1.52). A third type is exo-chitinase, which removes a single unit of GlcNAc from the non-reducing end of the polymer. Most of the chitinases have overlapping mechanisms of actions depending on the nature of the substrate. The other pathway for chitin hydrolysis involves two enzymes, chitin deacetylase and chitosanase. Chitin deacetylase (CDA, EC 3.5.1.41) hydrolyzes the acetamido group in the GlcNAc units of chitin-producing GlcN by removing acetic acid (Chavan and Deshpande 2013). CDA from *Mucor rouxii* was reported to be exo-acting, while CDA of *Colletotrichum lindemuthianum* catalyzed the random hydrolysis of acetamido groups on chitin chain. Chitosanase (EC 3.2.1.132) performs endo-hydrolysis of β-1,4-linkages between GlcN residues in a partly acetylated chitosan, while exo-chitosanase (GlcNase, EC 3.2.1.165) removes successively GlcN residues from the non-reducing end of chitosan or chitosan oligosaccharides.

Both endo- and exo- chitinases and chitosanases are important in the processivity, i.e., the enzyme after one cleavage is not released but remains associated with the substrate for further action. The knowledge of structure-function relationship of chitinolytic enzymes, catalytic and biding site, and the role of chitin-binding proteins can give a better insight for the cost and benefits of processivity in chitin degradation.

A number of other enzymes such as chitinases, lysozyme, cellulases or hemicellulases, β-1,3/1,4-glucanases, proteases, and lipases were also reported to hydrolyze GlcN polymer or oligomers (Chavan and Deshpande 2013).

7.2.3 Classification of Chitinolytic Enzymes

According to the International Union of Biochemistry and Molecular Biology, there are two broad classes of chitinases based on their mechanism of action, viz., endo- and exo-hydrolyzing enzymes. Considering amino acid sequences, they are grouped into three families of glycosyl hydrolases (GH): GH18, GH19, and GH20. Fungal chitinases belong to family 18, while that from plants are in family 19. The bacterial chitinases are also grouped in family 18, while family 20 consists of *N*-acetylhexosaminidases from bacteria like *Vibrio* and *Streptomyces* and humans (Karthik et al. 2014). Seidl et al. (2005) carried out genome-wide analysis of *Trichoderma reesei* chitinase genes. According to them, fungal chitinases further can be divided into three groups: groups A (corresponding to class V chitinases) and B (class II chitinases) and group C, which is comprised of high molecular weight chitinases, that could be important in mycoparasitic interaction with *Rhizoctonia solani*. The total number of chitinases in fungi is highly variable, for example, 2 chitinases in *Ustilago maydis* to 27 in *Fusarium oxysporum.*

However, based on fungal genome sequencing, Seidl (2008) suggested that fungi have 10–25 different chitinases having different enzymatic properties, which are not necessarily understood by exo- and endo-classification. By studying the chitinolytic system of *Serratia marcescens*, chitinases are classified as processive and non-processive enzymes. The former group of enzymes does not release the substrate after hydrolytic cleavage, while the latter group of chitinases dissociates themselves from the substrate.

Goughenour et al. (2020) surveyed fungal chitinases (GH18) from 373 publicly available genomes, studied domain architecture, and analyzed their phylogenetic relationships. According to the authors, the large-scale analysis did not support the previous groups (clades A, B, C) as chitinases. A and C were not resolved as separate clades. Further, it was suggested that clade B is of bacterial origin, which might be due to horizontal gene transfer. The chitin binding domains are across the clades. They characterized eight chitinases from a dimorphic fungus, *Histoplasma capsulatum*: six from A clade, one from B clade, and one from C clade.

As there is structural similarity in cellulose, chitin, and chitosan, and chitinase, lysozyme, and *N*-acetylglucosaminidase act on partially acetylated chitosan, the chitosanases remained poorly defined. Henrissat and Bairoch (1996) proposed a classification of glycosyl hydrolases including chitosanases based on a hydrophobic cluster analysis for deduced amino acid sequences. According to this classification, bacterial chitosanases were classified into family 46, which is different than chitinase families. According to Zhang et al. (2000), fungal chitosanases are distinct from family 46, which could be attributed to the distinct evolutionary origin.

According to Grifoll-Romero et al. (2018), both chitin deacetylases (CDAs, EC 3.5.1.41) and chitooligosaccharide deacetylases (CODs, EC 3.5.1.105) are classified in the carbohydrate esterase family 4 (CE4) along with peptidoglycan *N*-acetylglucosamine deacetylases (EC 3.5.1.104), some acetyl xylan esterases (EC 3.1.1.72), and others. All these enzymes share a conserved region known as the NodB homology domain due to its similarity to the NodB oligosaccharide

deacetylase (Aragunde et al. 2018; Ghormade et al. 2010; Grifoll-Romero et al. 2018).

Fungal CDAs have been studied extensively than those from insects and from marine bacteria (Ghormade et al. 2010). The CDA was first identified from *Mucor rouxii.* In view of its significance in chitin hydrolysis, it has been reported from a number of fungi which include *Absidia coerulea*, *Aspergillus nidulans*, *Benjaminiella poitrasii*, *Colletotrichum lindemuthianum*, *Flammulina velutipes*, *Metarhizium anisopliae*, *Mucor racemosus*, *Rhizopus circinans*, *Rhizopus nigricans*, *Saccharomyces cerevisiae*, *Scopulariopsis brevicaulis*, and *Uromyces viciae-fabae* (Chavan and Deshpande 2013).

7.3 Biochemical Characteristics of Chitinolytic Enzymes

7.3.1 Chitinases

In the literature, a lot of reports on the production of chitinases from fungi are available; however, the data on purification and their biochemical characterization is sparse. Mycoparasitic *T. harzianum* is one of the favored organisms for chitinases. Several endo-chitinases, chitobiosidases, and *N*-acetylglucosaminidases from *T. harzianum* were reported. Other fungi studied for purification and characterization of chitinases are entomopathogenic *Beauveria bassiana*, *M. anisopliae*, mycoparasitic *Gliocladium virens*, nematophagous *Verticillium chlamydosporium*, thermophilic *Thermomyces lanuginosus*, and others like *Trichothecium roseum*, *Stachybotrys elegans*, and *Myrothecium verrucaria* to name a few (Li 2006; Vyas and Deshpande 1993). The chitinases from yeasts like *S. cerevisiae* and *Candida albicans* were also reported in the literature. *T. harzianum* are reported to produce seven individual chitinases: two *N*-acetylglucosaminidases, four endo-chitinases, and one chitobiosidase, while entomopathogenic *M. anisopliae* are found to produce six different chitinases. Usually molecular weights of fungal chitinases range between 27 and 190 kDa and are active toward acidic pH. The specific inhibitors for fungal chitinases are allosamidins (Chaudhary et al. 2013; Li 2006).

7.3.2 Chitosanases

A number of chitosanolytic enzymes from fungi were purified and characterized. Zhang et al. (2000) reported purification and characterization of chitosanase and exo-β-D-glucosaminidase from a koji mold, *Aspergillus oryzae*, having molecular weights 40 and 135 kDa, respectively. Earlier endo-type chitosanases were reported from *A. fumigatus*, *F. solani*, *Penicillium islandicum*, *M. rouxii*, and *T. reesei*. Exo-type chitosanase was also reported from *A. fumigatus*, which was of 108 kDa (Kim et al. 1998). Cheng and Li (2000) purified endo-acting chitosanase from *Aspergillus* species, having a potential to prepare chitosan oligosaccharides, with chitotriose, chitotetraose, and chitopentaose as the major products. Chen et al. (2005)

reported purification of two chitosanases from *Aspergillus* species. The chitosan hydrolysis product analysis suggested that one (ChiA) was an endo-acting enzyme and other one (ChiB) was an exo-acting enzyme. *Gongronella* sp. is a zygomycetous fungus used in chitosan production. Wang et al. (2008) reported purification of a 28 kDa chitosanase from *Gongronella* species. The enzyme produced chitobiose, chitotriose, and chitotetraose with chitotriose as the major product from colloidal chitosan.

7.3.3 Chitin Deacetylases

In general, chitin deacetylases are intracellular (or periplasmic) and isolated and purified from mycelium extracts of zygomycetous fungi, such as *Absidia coerulea* and *M. rouxii* and *Aspergillus nidulans* and *Colletotrichum lindemuthianum*. Extracellular CDA was purified from *Scopulariopsis brevicaulis.* Alfonso et al. (1995) reported purification of thermostable extracellular CDA from *A. nidulans.* Most CDA activities are either periplasmic or extracellular, which have different characteristics. For instance, acetate sensitivity is more for periplasmic CDA than extracellular ones. For example, extracellular CDA from *M. anisopliae* was not inhibited in the presence of inhibitors such as acetate and melanin (Ghormade et al. 2010; Nahar et al. 2004).

The enzymatic deacetylation has diverse deacetylation patterns, viz., endo- or exo-type, which can be attributed to different substrate specificities. For instance, *M. rouxii* and *C. lindemuthianum* CDAs had maximum affinity toward the substrates, tetra- and hexa- acetylglucosamine, and for *M. rouxii* CDA activity, the minimum degree of polymerization required was 4–7, while *M. anisopliae* CDA showed highest affinity toward polymeric ethylene glycol chitin. The mechanism of their action for CDAs from *M. rouxii* and *C. lindemuthianum* was studied. The former was found to be an exo-type enzyme, while the latter catalyzed the hydrolysis of acetamido groups (Chavan and Deshpande 2013).

Up to five isozymes of CDA were reported in several fungi. For instance, the broad bean rust *Uromyces viciae-fabae* has five isozymes with molecular weights ranging from 12.7 to 48.1 kDa. While zygomycetes *Mucor racemosus* showed the presence of three isozymes and *Rhizopus nigricans* had four isozymes, all were in the range of 26–65 kDa. The CDAs from *C. lindemuthianum* and *M. rouxii* had molecular weights around 33 and 275 kDa, respectively. Nahar et al. (2004) reported three isozymes for *M. anisopliae* with apparent molecular weights of 70, 37, and 26 kDa (Ghormade et al. 2010; Grifoll-Romero et al. 2018).

7.4 Molecular Studies of Chitinolytic Enzymes

A number of genes encoding chitin-metabolizing enzymes, viz., endo-chitinases, *N*-acetyl-ß-D-glucosaminidase, chitosanase, and chitin deacetylases, were isolated and analyzed from a wide range of fungi, which include generally saprophytic

Table 7.1 Functionally characterized fungal chitinase genes

Sr. no.	Fungus	Genes characterized	Associated chitinolytic activity
1.	*Aspergillus niger*	*Cfu1*	*N*-acetylglucosaminidase (NAGase)
2.	*Aspergillus fumigatus*	*ChiA1* and *ChiB1*	Endo-chitinase
3.	*Beauveria bassiana*	*Chit1*	Endo-chitinase
4.	*Candida albicans*	*Chit2* and *Chit3*	Chitinase
5.	*Coccidioides immitis*	*CiX1/Cts1*	Endo-chitinase/chitobiosidase
6.	*Histoplasma capsulatum*	*Cts1* to *Cts8*	Chitobiosidases, endo-chitinases, NAGase
7.	*Kluyveromyces lactis*	*Cts1*	Endo-chitinase
8.	*Metarhizium anisopliae*	*Chit30*, *Chit40*, *Chit42*, and *Chit46*	NAGase, endo-chitinase, chitobiosidase
9.	*Saccharomyces cerevisiae*	*Cts1*	Chitinase
10.	*Trichoderma harzianum*	*Chit33* and *Chit42, Ech30*	Endo-chitinase, chitobiosidase

Compiled from De Boer et al. (2007), da Silva et al. (2005), Deng et al. (2019), Dong et al. (2007), Fang et al. (2005), Fan et al. (2007), Fukamizo et al. (2001), Goughenour et al. (2020), Hoell et al. (2010), Rush et al. (2010), and van Munster et al. (2012)

(*A. nidulans*, *Aspergillus oryzae*, *Penicillium chrysogenum*, *S. cerevisiae*, *T. reesei*), mycoparasitic (*Stachybotrys elegans*, *T. harzianum*), entomopathogenic (*B. bassiana*, *M. anisopliae*, *N. rileyi*, *Verticillium lecanii*), nematophagous (*Paecilomyces lilacinus*), and human pathogenic (*A. fumigatus C. albicans*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Paracoccidioides brasiliensis*) as well as plant pathogenic (*Botrytis cinerea*, *Puccinia triticina*, *Ustilago maydis*) fungi to name a few. However, only a few are functionally characterized (Table 7.1).

The overexpression of the chitinase (*Chit1*) gene in *B. bassiana* improved its virulence by 23% (Fang et al. 2005). Similarly, *M. anisopliae* strain overexpressing chitinase (*Chi2*) was more effective against the cotton stainer bug, *Dysdercus peruvianus* (Merzendorfer 2013). Further, the *T. koningii* strain was engineered with *M. anisopliae Chit40*, which showed improved insecticidal potential against the silkworm (*Bombyx mori*) and Asian corn borer (*Ostrinia furnacalis*). Several fungal chitinase genes have also been used for transgenic expression in crop plants due to their broad spectrum of fungicidal and insecticidal activities. Fungal endo-chitinases have been transformed into plants, which imparted resistance against insects as well as plant pathogens (Bolar et al. 2000; Gentile et al. 2007; Okongo et al. 2019). For instance, the transgenic expression of chitinase gene in tomato plants inhibited the Colorado potato beetle (Lawrence and Novak 2006). Further, transgenic expression of a chitinase gene from *Pseudomonas fluorescens* protected the tea plants from *Helopeltis* spp. (Suganthi et al. 2017). Recently, Anwar et al.

(2019) transformed the chitinase gene (*Chit1*) of *M. anisopliae* into cotton plants, which improved its resistance against whitefly *Bemisia tabaci*. However, further studies are required to explore effectiveness of chitinases from different fungal strains against beneficial insect pests for their potential use as biopesticides.

The plant pathogenic *F. solani* f.sp. *phaseoli* extracellularly produced chitosanase in the absence of chitosan in the medium (Shimosaka et al. 1996). They further isolated and cloned chitosanase gene into *E. coli* for the overproduction of chitosanolytic activity. Wang et al. (2008) isolated the chitosanase gene (*Csn2*) from *Gongronella* sp., which was partly similar to chitosanase genes from *M. anisopliae* var. *acridum* and *A. fumigatus*. Based on sequence similarity, Csn2 was classified as a GH75 chitosanase. Zhu et al. (2012) cloned a chitosanase gene *csn* from *Penicillium* sp. into *E. coli*. The recombinant chitosanase showed catalytic activity over a broad pH range on colloidal chitosan, and according to the researchers, it had potential in recycling chitosan wastes.

The molecular characterization of the chitin deacetylase (*cda*) gene from different fungi, viz., *A. niger* (1.37 kb), *A. fumigatus* (1.46 kb), *A. nidulans* (0.75 kb), *C. lindemuthianum* (0.75 kb), *G. butleri* (1.29 kb), *M. anisopliae* (1.44 kb), *M. rouxii* (1.41 kb), *Puccinia graminis* (0.74 kb), *R. oryzae* (1.29 kb), *R. nigricans* (1.34 kb), *S. cerevisiae cda1* (0.9 kb), and *cda2* (0.94 kb), has been carried out to confirm their biological role or to further exploit their applications on a commercial scale. The conserved catalytic domain of the CDA gene from *M. rouxii* was heterologously expressed to obtain the active enzyme (Kafetzopoulos et al. 1993). In contrast, the *cda* gene from *G. butleri* was cloned and sequenced for its potential applications in chitosan production (Maw et al. 2002). Further, in *S. cerevisiae*, two *cda* genes (*cda* 1 and *cda* 2) were reported to be separated by a distance of 1.4 kb on chromosome XII. Both these genes were sporulation-specific, and the products of both the genes accounted for total CDA activity. The *cda*-double disrupted strains of *S. cerevisiae* showed lack of CDA activity (Christodoulidou et al. 1996). Similarly, two putative *cda* genes (*EhCDA1* and *EhCDA2*), each with one intron, were identified in the protist *Entamoeba histolytica*. In contrast, three *cda* genes (*EiCDA1–EiCDA3*) were identified in *Entamoeba invadens* and were reported to be responsible for modification of chitin to chitosan in the cyst wall (Das et al. 2006). In the deuteromycetous fungus *C. lindemuthianum*, the *cda* gene was sequenced and overexpressed in *E. coli* (Tokuyasu et al. 1999a). Further, Levitz et al. (2001) demonstrated that the *C. neoformans cda* gene stimulates T-cell responses and thereby showed its involvement in immunogenicity. The cloning and expression of the *cda* gene from other fungi have been carried out mainly to obtain the active recombinant enzyme for its extensive use (Matsuo et al. 2005; Mishra et al. 1997; Shrestha et al. 2004).

7.5 Roles of Chitinolytic Enzymes in Fungal Growth and Differentiation

The fungal cell wall structure, growth, and morphogenesis and enzymes involved in cell wall synthesis and degradation have been extensively reviewed by different researchers (Ghormade et al. 2011). Chitinase complex is important in nutrition, hyphal tip elongation and branching, separation of budding cells, differentiation into spores and their release from the conidiophore, morphological transition (yeast hypha) dimorphism, entomopathogenesis, and mycoparasitism (Patil et al. 2000; Yadav and Deshpande 2010). As filamentous fungi have chitin as a major component of their cell walls, they produce chitinases at all different stages of active growth, viz., during spore germination to form germ tubes, eventually during exponential growth and during development of mycelial mass (Gooday et al. 1992). Gooday et al. (1992) assessed the roles of chitinases by studying the effect of allosamidin, an endo-chitinase inhibitor, in these processes (Deshpande 1998a, b).

In a dimorphic fungus, *Benjaminiella poitrasii*, the endo-chitinase and *N*-acetylglucosaminidase activities increased during the hypha formation, and the reverse was true during budding yeast transition from hypha (Ghormade et al. 2000). The increase is up to 17-fold for *N*-acetylglucosaminidase activity during Y-H transition, whereas endo-chitinase activity increased by 12-fold. The isozyme studies showed that one of the isozymes of *N*-acetylglucosaminidase present in parent, both yeast and hyphal form, was absent in yeast-monomorphic mutant, suggesting its significant contribution in the morphological transition (Ghormade et al. 2000). *Ustilago maydis*, a smut fungus, shows dimorphic behavior during plant infection while proliferating as a mycelium in plant and producing teliospores for spreading. Langner et al. (2015) investigated the role of four chitinases produced by *U. maydis*. They did not observe any specific role of chitinases in morphogenesis but could identify that two chitinases were essential for the cell separation during saprophytic growth for nutrient access as well as for spreading yeast cells.

Usually, fungi cannot use exogenously supplied chitosan as a sole carbon source efficiently (Zhang et al. 2000). Chitosanases in fungi play a role in cell division or autolysis. Fungal extracellular chitosanolytic enzymes have been purified from *Aspergillus fumigatus*, *Fusarium solani*, *Mucor rouxii*, *Penicillium islandicum*, and *T. reesei* (Zhang et al. 2000). Most of them are endo-hydrolytic. Zhang et al. (2000) purified endo-acting chitosanase and exo-acting exo-glucosaminidase from *A. oryzae*.

Fungal CDAs are involved in nutrition, differentiation, and development, which include cell wall integrity and synthesis (especially in zygomycetous fungi), spore formation, appressorium formation, and fungal autolysis (Ghormade et al. 2010). One of the novel recently identified roles is in self-defense from plant chitinases, produced for defense against fungal pathogens as well as insect chitinases produced during molting (Ghormade et al. 2010; Nahar et al. 2004).

7.6 Application-Oriented Biological Roles of Fungal Chitinolytic Enzymes

The chitinases have a lot of roles to play in the fungal life cycle. In the subsequent sections, the applications of chitinases, CDA, and chitinases have been discussed.

7.6.1 Fungus-Fungus and Fungus-Insect Interactions

The mycoparasitic fungi can have antagonistic interactions either by direct contact with host or at a distance (Yadav and Deshpande 2010). In both cases, the release of non-enzymic components like antibiotics and enzymic components, such as cell wall lytic enzymes, mainly chitinases, is involved in the killing process. *Trichoderma*, one of the most studied mycoparasite, penetrates the host by secreting chitinases and other enzymes, which can hydrolyze cell wall of the host fungus.

In the fungus-insect interaction, the spore of an entomopathogen adheres to the cuticle, germinates, and, with the help of appressorium, invades the insect cuticle by a combination of mechanical pressure and extracellularly produced cuticle-degrading enzymes (CDEs), mainly chitinases, proteases, and lipases (Kapoor and Deshpande 2013; Yadav and Deshpande 2010). Kulkarni et al. (2008) isolated 63 *Metarhizium* strains from soil and different insect hosts and evaluated them for their potential to produce chitinolytic enzymes, viz., chitinases, CDAs, chitosanases, proteases, and lipases too, and to select the best strains for field studies to control different insect pests. Most of the strains exhibited high levels of induced chitinase activities, while few of them showed relatively higher constitutively produced CDA and chitosanase activities (Nahar et al. 2004). This indicated that either chitinase approach or CDA and chitosanase approach for cuticular chitin degradation was preferred by different *Metarhizium* strains. Furthermore, Nahar et al. (2008) observed that the repeated subculturing of *M. anisopliae* on the artificial media decreased the CDE activities, which showed correlation with the mortality of a dreadful pest *Helicoverpa armigera*. Significant decrease in chitinase, chitin deacetylase, and chitosanase activities during subsequent subculturing and increase after passage through *H. armigera* were reported. Melanized cuticle inhibits proteolytic and chitinolytic enzyme attack. It was reported that some *M. anisopliae* strains used another approach of CDA, which is not inhibited by melanin and chitosanase to enter into the insect body. It was earlier reported that the CDA activity contributed also to changing chitin/chitosan proportion in *Metarhizium* cell walls and thus helped in self-defense from the insect chitinases produced during molting process significantly (Nahar et al. 2004).

7.6.2 Chitinolytic Enzymes in Biocontrol of Plant Pathogenic Fungi and Insects

The mycoparasitic and entomopathogenic fungi produce chitinolytic enzymes for the entry into the respective hosts. It was reported that in fungus-insect interactions, production of cuticle-degrading enzymes, such as endo-chitinase, chitin deacetylase, chitosanase, alkaline protease, and lipase, by *M. anisopliae*, *M. flavoviride*, and *B. bassiana* played a significant role (Yadav and Deshpande 2010). As in both interactions, chitinolytic enzymes play a significant role, a number of mycopathogens and entomopathogens can show dual pathogenicity toward fungi and insects. For example, mycoparasitic *T. harzianum* can parasitize the elm bark beetle, *Scolytus*, while aphid pathogen *V. lecanii* can also attack rust fungi (Yadav and Deshpande 2010).

The chitinolytic enzymes are widely distributed in nature, and they play different roles in different organisms. For instance, plants produce chitinases as one of their defense mechanisms for the protection from fungal attack. Indeed there is no other "better proof" for the contribution of plant chitinases in self-defense from the pathogens than the increased deacetylation of chitin (i.e., more proportion of chitosan) in the cell wall by pathogens like *C. lindemuthianum* and *Uromyces viciae-fabae* on which plant chitinases cannot attack. Similarly, as mentioned above, the increase in the proportion of chitosan in *M. anisopliae* cell wall for self-defense from insect chitinases is produced during molting (Vidhate and Deshpande 2013).

The protective covers of insect pests and fungal pathogens, viz., cuticle and cell wall, respectively, have chitin as the common main structural component. Therefore, the enzyme mixture, which contains endo-chitinase, exo-*N*-acetylglucosaminidase, chitosanase, CDA, and other enzymes like proteases, lipases, and glucanases, can be used as wide-spectrum biocontrol agents in a single-crop system to control different insect pests and fungal pathogens. A number of fungi produce the complete chitinolytic complex. It was reported that fungi like *Myrothecium verrucaria* produce potent mixture of chitinolytic enzymes, which can be used to control different insect pests and fungal pathogens, singly and in combination with entomopathogenic fungi (Chandele et al. 2020; Chavan et al. 2009; Dandawate et al. 2014; Vidhate and Deshpande 2013; Vidhate et al. 2015).

Kapoor et al. (2013) evaluated biocontrol potential of *M. anisopliae* strains against larvae and adults of mosquito, *Aedes aegypti*. Similar to the agriculture pests, the mechanism of killing is the hydrolysis of a protective cover of mosquito. Earlier, *M. verrucaria* chitinase complex was found to kill both first (I) and fourth (IV) instar larvae of *A. aegypti* within 48 h. The purified endo-chitinase from *M. verrucaria* was also effective in killing both I and IV instar larvae with the lethal time (LT50) of 48 and 120 h, respectively (Mendonsa et al. 1996). This could be one of the potential areas where chitinase complex can be used in large quantities. Again, the cost and feasibility could be a major concern too. In this regard, an enzyme nanoformulation can be used to reduce the cost and to increase the stability. Additionally, the slow release of enzymes from nanoformulations could be

advantageous for on-field stability. *M. verrucaria* enzyme nanoformulation prepared using chitosan was reported to be useful to control insect pests and fungal pathogens (Ghormade et al., unpublished data; Chavan and Deshpande 2013).

7.6.3 Chitinases in Single-Cell Protein Production

For the effective utilization of chitinous waste, a single-cell protein (SCP) production can be a viable option. Earlier, chitinase from a bacterium *Serratia marcescens* was used to hydrolyze chitin to GlcNAc, which was further used to grow the yeast, *Pichia kudriavzevii*, as SCP. Vyas and Deshpande (1991) reported the use of *M. verrucaria* chitinase complex to hydrolyze chitin, and the hydrolysate was used to grow *S. cerevisiae* as SCP. The high levels of *N*-acetylglucosaminidase activity produced by *M. verrucaria* were useful to yield high concentration of GlcNAc for *S. cerevisiae* to grow.

7.6.4 Production of Chitooligosaccharides

In nature, chitooligosaccharides have role as elicitors of plant defense and in signaling for root nodule formation. Lipo-chitooligosaccharides (LCO) or Nod factors are signal molecules secreted by rhizobia. The applications of chitooligosaccharides in health care are inhibition of tumor growth and TH2-induced inflammation in asthma, as a bone-strengthener in osteoporosis, a carrier for gene delivery, an antimicrobial and an antimalaria agent, a hemostatic agent in wound-dressings, etc. To obtain the desired length of the oligosaccharides, a specific combination of endo-/exo-chitinolytic enzymes is necessary. Usually, high levels of endo-chitinases and low activity of *N*-acetylglucosaminidase and exo-chitinases are desirable to get higher oligomers. To obtain GlcNAc, a higher proportion of exo-chitinase and *N*-acetylglucosaminidase would be useful. Alternately, the transglycosylation activity of endo-chitinases can also be useful for generating desired chito-oligomers. Kadowaki et al. (1997) reported β-*N*-acetylhexosaminidase of *Penicillium oxalicum*, which possessed transglycosylation activity in addition to the hydrolytic activity. The enzyme was found to be useful to transfer GlcNAc to different alcohols useful in pharmaceutical industry.

The purified chitosanases produced by *Aspergillus* species as well as by *M. anisopliae* were found to be useful for the generation of chito-oligomers ($GlcN_{2-6}$) from chitosan (Cheng and Li 2000; De Asis et al. 2010). Similarly for the synthesis of (GlcNAc)-oligosaccharides, CDA from *C. lindemuthianum* was reported to be useful that acetylated $(GlcN)_{2-3}$, and the product formed was GlcNAc-GlcN exclusively (Tokuyasu et al. 1999b).

India is one of the richest countries for fungal diversity. The diversity from different states has been reviewed time to time by different researchers. Though the exploration of this kingdom started in 1901 by Sir E.J. Butler, the emphasis was mainly on disease-causing fungi. Research on mushrooms increased with the

establishment of the National Research Center at Solan in 1983. Bilgrami collated the fungal wealth of India (Bilgrami 1997). Earlier, Subramanian (1986) reviewed the progress and status of mycology in India. Regarding fungal physiology, biochemistry, and genetics, there were very few reports about cellulases from *Aspergillus* and other fungi.

Interestingly, chitin from fungi, such as *Agaricus*, *Hydnum*, and *Boletus*, was discovered 30 years before cellulose. However, as cellulosic material is the largest renewable resource available on earth, cellulose hydrolysis is extensively reported (Deshpande 1986). The preparative isoelectric focusing was employed to purify endo-chitinase and chitobiohydrolase (exo-hydrolytic) from *T. harzianum* to validate C_1-C_x concept like cellulases (Deshpande 1986). As chitin, especially from marine sources, is the second-best renewable resource, the researchers started working on chitinases for the effective utilization of marine waste for SCP production. Though a bacterium, *Serratia marcescens*, was the favored source for chitinases, fungi like *M. verrucaria* received attention too (Deshpande 1986; Patil et al. 2000; Shaikh and Deshpande 1993; Vyas and Deshpande 1991). The real boost for chitinolytic enzyme, especially CDA research in India, is due to their use in agriculture for the control of pests and pathogens using mycoparasitic and entomopathogenic fungi (Kulkarni et al. 2008; Ghormade et al. 2010; Nahar et al. 2004).

7.7 Epilogue

Fungi have different roles in nature and also have high potential in biotechnological applications. Since the characteristics required for any organism to become industrially important are speed of growth, post-translational modification, potential and cost for scale-up, and regulatory mechanisms, yeast and filamentous fungi are always a better option over bacteria, plant, and mammals, which are used in biotechnology. Further, chitinolytic enzymes, in spite of having significant contributions in nature, are somehow less explored for commercial ventures. One of the reasons could be the nature of application, which needs high volume with low cost. In the near future, hopefully fungal chitinolytic enzymes would be one of the important players in biotechnology.

References

Alfonso C, Nuero OM, Santamarfa F, Reyes F (1995) Purification of a heat-stable chitin deacetylase from *Aspergillus nidulans* and its role in cell wall degradation. Curr Microbiol 30:49–54

Anwar W, Javed MA, Shahid AA, Nawaz K, Akhter A, Ur Rehman MZ, Hameed U, Iftikhar S, Haider MS (2019) Chitinase genes from *Metarhizium anisopliae* for the control of whitefly in cotton. R Soc Open Sci 6:190412

Aragunde H, Biarnés X, Planas A (2018) Substrate recognition and specificity of chitin deacetylases and related family 4 carbohydrate esterases. Int J Mol Sci 19:412

Bartnicki-Garcia S (1973) Fundamental aspects of hyphal morphogenesis. Symp Gen Microbiol 23:245–267

Bartnicki-Garcia S (2006) Chitosomes: past, present and future. FEMS Yeast Res 6:957–965

Bilgrami KS (1997) Fungal wealth of India. Proc Indian Natl Sci Acad B63:269–280

Bolar JP, Norelli JL, Wong KW, Hayes CK, Harman GE, Aldwinckle HS (2000) Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigor. Phytopathology 90:72–77

Chandele AG, Pathan EK, Patil A, Borade S, Ghorpade S, Tupe SG, Deshpande MV (2020) Use of entomopathogenic *Metarhizium anisopliae* conidial formulation and cuticle degrading enzyme formulation of *Myrothecium verrucaria* for the control of thrips and jassids in grape fields. Biopestic Int 16:37–44

Chaudhary PM, Tupe SG, Deshpande MV (2013) Chitin synthase inhibitors as antifungal agents. Mini-Rev Med Chem 13:222–236

Chavan SB, Deshpande MV (2013) Chitinolytic enzymes: an appraisal as a product of commercial potential. Biotechnol Progress 29:833–846

Chavan S, Kolomiets E, Kuptsov V, Mandrik M, Kulkarni M, Deshpande MV (2009) Significance of cuticle degrading enzymes with special reference to lipase in biocontrol of sugarcane woolly aphids. J Mycol Plant Pathol 39:118–123

Chen XE, Xia WS, Yu X (2005) Purification and characterization of two types 358 of chitosanase from *Aspergillus sp.* CJ22-326. Food Res Int 38:315–322

Cheng CY, Li YK (2000) An *Aspergillus* chitosanase with potential for large-scale preparation of chitosan oligosaccharides. Biotechnol Appl Biochem 32:197–203

Christodoulidou A, Bouriotis V, Thireos G (1996) Two sporulation-specific chitin deacetylase-encoding genes are required for the ascospore wall rigidity of *Saccharomyces cerevisiae*. J Biol Chem 271:31420–31425

da Silva MV, Santi L, Staats CC, da Costa AM, Colodel EM, Driemeier D, Vainstein MH, Schrank A (2005) Cuticle-induced endo/exoacting chitinase *CHIT30* from *Metarhizium anisopliae* is encoded by an ortholog of the *CHI3* gene. Res Microbiol 156:382–392

Dandawate N, Kallure G, Vidhate R, Ghormade V, Chavan S, Deshpande M (2014) Cuticle degrading and mycolytic enzyme complex of *Myrothecium verrucaria* as a wide spectrum biocontrol agent to control pest and pathogens in grapes. In: Invited paper published in Proceedings of national conference on empowering mankind with microbial technologies (AMI-EMMT-2014), 55th Annual conference of AMI, Coimbatore, India

Das S, Van Dellen K, Bulik D, Magnelli P, Cui J, Head J, Robbins PW, Samuelson J (2006) The cyst wall of *Entamoeba invadens* contains chitosan (deacetylated chitin). Mol Biochem Parasitol 148:86–92

De Asis CF, Kelly AN, Binder Pagnoncelli MG, Silva da Pedrini MR, de Macedo GR, dos Santos ES (2010) Chitooligosaccharides enzymatic production by *Metarhizium anisopliae*. Bioprocess Biosyst Eng 33:893–899

De Boer W, Wagenaar AM, Klein Gunnewiek PJA, van Veen JA (2007) In vitro suppression of fungi caused by combinations of apparently non-antagonistic soil bacteria. FEMS Microbiol Ecol 59:177–185

Deng J-J, Shi D, Mao H-H, Li Z-W, Liang S, Ke Y, Luo X-C (2019) Heterologous expression and characterization of an antifungal chitinase (Chit46) from *Trichoderma harzianum* GIM 3.442 and its application in colloidal chitin conversion. Int J Biol Macromol 134:113–121

Deshpande MV (1986) Enzymatic degradation of chitin and its biological applications. J Sci Ind Res 45:273–281

Deshpande MV (1998a) Biochemical basis of fungal differentiation. In: Varma A (ed) Microbes: for health, wealth and sustainable environment. Malhotra Publishing House, New Delhi, pp 241–252

Deshpande MV (1998b) Chitin metabolism: a target for antifungal and insecticidal agents. In: Varma A (ed) Microbes: for health, wealth and sustainable environment. Malhotra Publishing House, New Delhi, pp 281–291

Deshpande MV, O'Donnell R, Gooday GW (1997) Regulation of chitin synthase activity in the dimorphic fungus Benjaminiella poitrasii by external osmotic pressure. FEMS Microbiol Lett 152:327–332

Dong LQ, Yang JK, Zhang KQ (2007) Cloning and phylogenetic analysis of the chitinase gene from the facultative pathogen *Paecilomyces lilacinus*. J Appl Microbiol 103:2476–2488

Fan Y, Fang W, Guo S, Pei X, Zhang Y, Xiao Y, Li D, Jin K, Bidochka MJ, Pei Y (2007) Increased insect virulence in Beauveria bassiana strains overexpressing an engineered chitinase. Appl Environ Microbiol 73:295–302

Fang WG, Leng B, Xiao YH, Jin K, Ma JC, Fan YH, Feng J, Yang XY, Zhang YJ, Pei Y (2005) Cloning of *Beauveria bassiana* chitinase gene *Bbchit1* and its application to improve fungal strain virulence. Appl Environ Microbiol 71:363–370

Fukamizo T, Sasaki C, Schelp E, Bortone K, Robertus JD (2001) Kinetic properties of chitinase-1 from the fungal pathogen *Coccidioides immitis*. Biochemistry 40:2448–2454

Gentile A, Deng Z, La Malfa S, Distefano G, Domina F, Vitale A, Polizzi G, Lorito M, Tribulato E (2007) Enhanced resistance to *Phoma tracheiphila* and *Botrytis cinerea* in transgenic lemon plants expressing a *Trichoderma harzianum* chitinase gene. Plant Breed 126:146–151

Ghormade VS, Lachke SA, Deshpande MV (2000) Dimorphism in *Benjaminiella poitrasii*: involvement of intracellular endo-chitinase and *N*-acetylglucosaminidase activities in the yeast-mycelium transition. Folia Microbiol 45:231–238

Ghormade V, Kulkarni S, Doiphode N, Rajamohanan PR, Deshpande MV (2010) Chitin deacetylase: a comprehensive account on its role in nature and its biotechnological applications. In: Mendez-Vilas A (ed) Current research, technology and education topics in applied microbiology and microbial biotechnology. Formatex Research Center, Badajoz, pp 1054–1066

Ghormade V, Pathan E, Deshpande MV (2011) Yeast-hypha dimorphism in zygomycetous fungi. In: Ruiz-Herrera J (ed) Dimorphic fungi: their importance as models for differentiation and fungal pathogenesis. Bentham Science Publishers, pp 118–139

Ghormade V, Pathan EK, Deshpande MV (2017) Can fungi compete with marine sources for chitosan production? Int. J Biol Macromol 104(B):1415–1421

Gooday GW, Zhi WY, O'Donnell RW (1992) What are the roles of chitinases in the growing fungus? FEMS Microbiol Lett 100:387–392

Goughenour KD, Whalin J, Slot JC, Rappleye CA (2020) Diversification of fungal chitinases and their functional differentiation in *Histoplasma capsulatum*. Mol Biol Evol 13:msaa293

Grifoll-Romero L, Pascual S, Aragunde H, Biarnés X, Planas A (2018) Chitin deacetylases: structures, specificities, and biotech applications. Polymers 10:352

Henrissat B, Bairoch A (1996) Updating the sequence-based classification of glycosyl hydrolases. Biochem J 316:695–696

Ho HH (2018) The taxonomy and biology of *Phytophthora* and *Pythium*. J Bacteriol Mycol 6:40–45

Hoell IA, Vaaje-Kolstad G, Eijsink VGH (2010) Structure and function of enzymes acting on chitin and chitosan. Biotechnol Genet Eng Rev 27:331–336

Kadowaki S, Saskiawan I, Watanabe J, Yamamoto K, Bunno M, Ichihara Y, Kumagai H (1997) Transglycosylation activity of β-*N*-acetylhexosaminidase from *Penicillium oxalicum* and its application to synthesis of a drug carrier. J Ferment Bioeng 83:341–345

Kafetzopoulos D, Thireos G, Vournakis JN, Bouriotis V (1993) The primary structure of a fungal chitin deacetylase reveals the function for two bacterial gene products. Proc Natl Acad Sci U S A 90:8005–8008

Kapoor M, Deshpande MV (2013) Development of mycoinsecticide for the control of insect pests: issues and challenges in transfer of technology from laboratory to field. Kavaka 40:45–56

Kapoor M, Pawar PV, Joseph M, Sen A, Deshpande MV (2013) Evaluation of biocontrol potential of *Metarhizium anisopliae* strains against larvae and adults of *Aedes aegypti* (L.). J Biol Control 27:194–203

Karthik N, Karthik A, Binod P, Pandey A (2014) Production, purification and properties of fungal chitinases—a review. Ind J Exp Biol 52:1025–1035

Kaya M, Mujtaba M, Ehrlich H, Martinez SA, Baran T, Amemiya C, Galli R, Akyüz L, Sargin I, Labidi J (2017) On chemistry of γ-chitin. Carbohydr Polym 176:177–186

Kim SY, Shon DH, Lee KH (1998) Purification and characteristics of two types of chitosanases from *Aspergillus fumigatus* KH-94. J Microbiol Biotechnol 8:568–574

Kulkarni SA, Ghormade V, Kulkarni G, Kapoor M, Chavan SB, Rajendran A, Patil SK, Shouche Y, Deshpande MV (2008) Comparison of *Metarhizium* isolates for biocontrol of *Helicoverpa armigera* (Lepidoptera: *Noctuidae*) in chickpea. Biocontrol Sci Tech 18:809–828

Langner T, Oztürk M, Hartmann S, Cord-Landwehr S, Moerschbacher B, Walton JD, Göhre V (2015) Chitinases are essential for cell separation in *Ustilago maydis*. Eukaryot Cell 14:846–857

Lawrence SD, Novak NG (2006) Expression of poplar chitinase in tomato leads to inhibition of development in Colorado potato beetle. Biotechnol Lett 28:593–599

Levitz SM, Nong S, Mansour MK, Huang C, Specht CA (2001) Molecular characterization of a mannoprotein with homology to chitin deacetylases that stimulates T cell responses to *Cryptococcus neoformans*. Proc Natl Acad Sci U S A 98:10422–10427

Li DC (2006) Review of fungal chitinases. Mycopathologia 161:345–360

Matsuo Y, Tanaka K, Matsuda H, Kawamukai M (2005) *cda1*, encoding chitin deacetylase is required for proper spore formation in *Schizosaccharomyces pombe*. FEBS Lett 579:2737–2743

Maw T, Tan TK, Khor E, Wong SM (2002) Complete cDNA sequence of chitin deacetylase from *Gongronella butleri* and its phylogenetic analysis revealed clusters corresponding to taxonomic classification of fungi. J Biosci Bioeng 93:376–381

Mendonsa ES, Vartak PH, Rao JU, Deshpande MV (1996) An enzyme from *Myrothecium verrucaria* that degrades insect cuticle for biocontrol of *Aedes aegypti* mosquito. Biotechnol Lett 18:373–376

Merzendorfer H (2013) Insect-derived chitinases. In: Vilcinskas A (ed) Yellow biotechnology II: insect biotechnology in plant protection and industry. Springer, Berlin, pp 19–50

Mishra C, Semino CE, McCreath KJ, De La Vega H, Jones BJ, Specht CA, Robbins PW (1997) Cloning and expression of two chitin deacetylase genes of *Saccharomyces cerevisiae*. Yeast 13:327–336

Nahar PB, Ghormade V, Deshpande MV (2004) The extracellular constitutive production of chitin deacetylase in *Metarhizium anisopliae*: possible edge to entomo-pathogenic fungi in the biocontrol of insect pest. J Invertebr Pathol 85:80–88

Nahar PB, Kulkarni SA, Kulye MS, Chavan SB, Kulkarni G, Rajendran A, Yadav PD, Shouche Y, Deshpande MV (2008) Effect of repeated *in vitro* sub-culturing on the virulence of *Metarhizium anisopliae* against *Helicoverpa armigera* (Lepidoptera: *Noctuidae*). Biocontrol Sci Tech 18:337–355

Okongo RN, Puri AK, Wang Z, Singh S, Permaul K (2019) Comparative biocontrol ability of chitinases from bacteria and recombinant chitinases from the thermophilic fungus *Thermomyces lanuginosus*. J Biosci Bioeng 127:663–671

Patil RS, Ghormade V, Deshpande MV (2000) Chitinolytic enzymes: an exploration. Enzyme Microbial Technol 26:473–483

Rush CL, Schüttelkopf AW, Hurtado-Guerrero R, Blair DE, AFM I, Desvergnes S, Eggleston IM, van Aalten DMF (2010) Natural product-guided discovery of a fungal chitinase inhibitor. Chem Biol 17:1275–1281

Seidl V (2008) Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions. Fungal Biol Rev 22:36–42

Seidl V, Huemer B, Seiboth B, Kubicek CP (2005) A complete survey of *Trichoderma* chitinases reveals three distinct subgroups of family 18 chitinases. FEBS J 272:5923–5939

Shaikh SA, Deshpande MV (1993) Chitinolytic enzymes: their contribution in basic and applied research. World J Microbiol Biotechnol 9:468–475

Shimosaka M, Kumehara M, Zhang X-Y, Nogawa M, Okazaki M (1996) Cloning and characterization of a chitosanase gene from the plant pathogenic fungus *Fusarium solani*. J Ferment Bioeng 82:426–431

Shrestha B, Blondeau K, Stevens WF, Hegarat FL (2004) Expression of chitin deacetylase from *Colletotrichum lindemuthianum* in *Pichia pastoris*: purification and characterization. Protein Expr Purif 38:196–204

Subramanian CV (1986) The progress and status of mycology in India. Proc Indian Acad Sci (Plant Sci) 96:379–392

Suganthi M, Senthilkumar P, Arvinth S, Chandrshakara KN (2017) Chitinase from *Pseudomonas fluorescens* and its insecticidal activity against *Helopeltis theivora*. J Gen Appl Microbiol 63:222–227

Tokuyasu K, Ohnishi-Kameyama M, Hayashi K, Mori Y (1999a) Cloning and expression of chitin deacetylase gene from a deuteromycete, *Colletotrichum lindemuthianum*. J Biosci Bioeng 87:418–423

Tokuyasu K, Ono H, Hayashi K, Mori Y (1999b) Reverse hydrolysis reaction of chitin deacetylase and enzymetic synthesis of β -DGlcNAc-(1,4)-GlcN from chitobiose. Carbohydr Res 322:26–31

van Munster JM, van der Kaaij RM, Dijkhuizen L, van der Maarel MJEC (2012) Biochemical characterization of *Aspergillus niger CfcI*, a glycoside hydrolase family 18 chitinase that releases monomers during substrate hydrolysis. Microbiology 158:2168–2179

Vidhate R, Deshpande MV (2013) Biocontrol of plant pathogenic fungi and insect pests: hydrolytic enzymes as biological control agents. SBSI Biopest News 17:3–5

Vidhate R, Singh J, Ghormade V, Chavan SB, Patil A, Deshpande MV (2015) Use of hydrolytic enzymes of *Myrothecium verrucaria* and conidia of *Metarhizium anisopliae*, singly and sequentially to control pest and pathogens in grapes and their compatibility with pesticides used in the field. Biopestic Int 11:48–60

Vyas PR, Deshpande MV (1991) Enzymatic hydrolysis of chitin by *Myrothecium verrucaria* chitinase complex and its utilization to produce SCP. J Gen Appl Microbiol 37:265–275

Vyas PR, Deshpande MV (1993) Purification and characterization of an endochitinase from *Myrothecium verrucaria*. J Gen Appl Microbiol 39:91–99

Wang J, Zhou W, Yuan H, Wang Y (2008) Characterization of a novel fungal chitosanase from CSn2 *Gongronella* sp. JG. Carbohydr Res 343:2583–2588

Yadav P, Deshpande MV (2010) Fungus-fungus and fungus–insect interactions. Biopestic Int 6:21–35

Zhang XY, Dai AL, Zhang XK, Kuroiwa K, Kodaira K, Shimosaka M, Okazaki M (2000) Purification and characterization of chitosanase and exo-β-D-glucosaminidase from a koji mold, *Aspergillus oryzae* IAM2660. Biosci Biotechnol Biochem 64:1896–1902

Zhu X-F, Tan H-Q, Zhu C, Liao L, Zhang X-Q, Wu M (2012) Cloning and overexpression of a new chitosanase gene from *Penicillium* sp. D-1. AMB Express 2:13. https://doi.org/10.1186/2191-0855-2-13

Part II

Production and Applications of Fungal Nanoparticles

8

Insight into Fungi-Mediated Nano-synthesis for Healthcare Applications: An Indian Perspective

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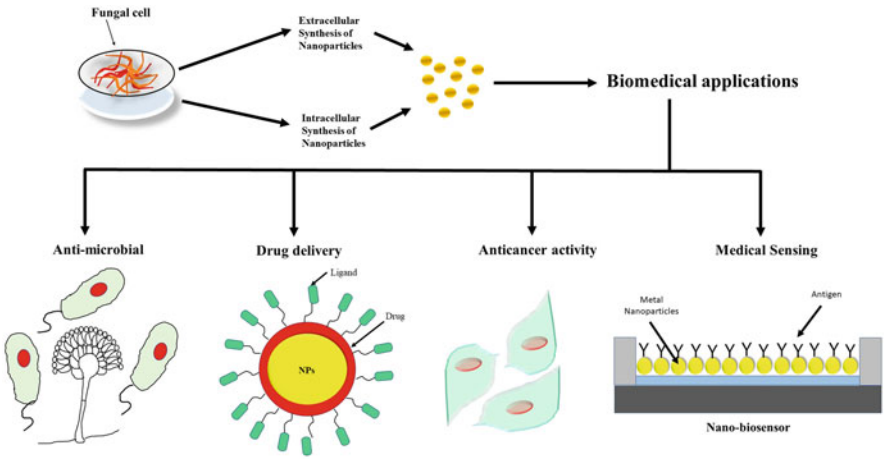
Abstract



Nanotechnology comprises the synthesis, manipulation, and utilization of nanosize materials in several areas. The chemical, physical, and biological methods are the ways to synthesize nanoscale material of the desired characteristics. However, the drawbacks of physical and chemical means of synthesis have switched the attention of researchers towards the environment-friendly and non-toxic greener approach which mainly involves the use of plants,

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bacteria, fungi, and viruses. Myconanotechnology is an emerging branch of nanotechnology which has attracted the interest of many researchers around the world and especially in India due to the availability of the wide diversity of fungi. The remarkable countenances of fungi, mainly easy cultivation, tolerance to extreme conditions, as well as high yield of nanoparticles, make them more a preferred biosource for nano-synthesis. Fungi can form nanoparticles either intracellularly by using whole cells or extracellularly by using cell-free extracts. Physiological factors like temperature, pH, and time account for a vital role in controlling the morphology and dispersity of mycosynthesized nanoparticles. This chapter explains not only the various dimensions of fungi-mediated nano-synthesis but also the proposed mechanisms. Further, this chapter illustrates the role of different types of fungi-derived metal, metal oxide, and semiconductor nanoparticles in the biomedical field. In the Indian context, nanoparticles synthesized using fungi are being explored for antimicrobial, antifungal, anticancer, and larvicidal properties. Apart from this, their use in sensing is getting much attention. The role of such nanoparticles in drug delivery is also giving exciting results. Fungi-mediated formation of nanoparticles and its numerous applications in healthcare have opened up many unexplored areas in myconanotechnology all over the world including in India. Myconanotechnology can play a crucial role in giving solutions to pandemic situations for mankind. There is a need to take up this research from lab to clinic so that we can see the commercially viable nanoproducts in the healthcare sector of India and the world.

Keywords

Mycosynthesis · Nanoparticles · Healthcare applications · Indian scenario

8.1 Introduction

Fungi are eukaryotic and non-phototrophic organisms that produce spores. They are mainly filamentous and consist of branched somatic structures along with hyphae and are enclosed by cell walls (Gade et al. 2010). The cell wall is largely composed of glucan and chitin. It provides mechanical strength and helps fungi in survival in osmotic and various environmental stress (Yadav et al. 2015). Fungi are particularly very important components of biodiversity on earth, with a reported 1.5 million species throughout the world. India has been the center of such diverse fungal species (Hasan and Gupta 2012). They are mainly found in moist soil, dead organic substrates (saprophytes), or as an endophyte in plants and parasites in animals (Arun et al. 2014). They are economically very important and have an extensive range of applications in various areas. In agriculture, they are employed as decomposers, biopesticides, and biofertilizers and also increases soil fertility. They also live as symbionts in various organisms. They have the potency to produce various industrially significant enzymes and fermented products (Singara Charya 2015; Hasan and Gupta 2012). They also help in environmental problems through their

bioremediation ability. The role of fungi in the medical field makes them vital organism for research; they are also capable of producing antibiotics and various medically important fungal products (Hasan and Gupta 2012).

Endophytic fungi are present asymptomatically inside the plant tissue (Danagoudar et al. 2020; Bhattacharjee et al. 2017). They complete a portion of their life cycle in various parts of the plant (Bhattacharjee et al. 2017). Most of them are competent to generate bioactive compounds that are the same as the host plants. They are a valuable source of medically important bioactive molecules that can be utilized as anticancer, antioxidant, antiparasitic, immunomodulatory, antimicrobial, antiviral, insecticidal, and anti-tuberculosis drugs (Danagoudar et al. 2020).

The traditional taxonomic system of classification of fungi divides them into four divisions: *Zygomycota* (zygomycetes), *Basidiomycota* (club fungi), *Ascomycota* (sac fungi), and *Deuteromycota* (fungi imperfecti). This classification is chiefly based on distinctions in sexual reproduction (Singara Charya 2015). Presently, the fungi are classified into seven divisions: *Chytridiomycota*, *Blastocladiomycota*, *Glomeromycota*, *Neocallimastigomycota*, *Ascomycota*, *Basidiomycota*, and *Zygomycota* (Hasan and Gupta 2012).

Nanotechnology is an area of modern research that encompasses mainly on the synthesis, the manipulation, as well as the application of particles varying in size from 1.0 to 100 nm (Ahmed et al. 2016; Karnani and Chowdhary 2013). The word "nanotechnology" was first coined by Prof. Norio Taniguchi of the Tokyo Science University in 1974. The term "nano" is a Greek word that means "dwarf" and also signifies 10^{-9} m (Khandel and Shahi 2016; Patel et al. 2017). Nanoparticles are the tufts of atoms with at least one dimension at the nanoscale (Devi and Joshi 2015; Baker et al. 2013). Nanomaterials develop chemical and physical properties that are different and more advantageous from the properties of their bulk material. High surface-area-to-volume ratio is one of the major characteristics of nanoparticles (Devi and Joshi 2015; Ahmed et al. 2016). The unique properties based on size, shape, composition, and other attributes of nanoparticles enhance their use in fields of agriculture, cosmetics, textiles, food, medicine, health, environment, chemical industries, electronics, space industries, and many other areas (Khandel and Shahi 2016; Ahmed et al. 2016). It can be produced by employing biological, physical, and also chemical modes. The biological model of synthesis bids good polydispersity, stability, and dimensions to nanoparticles (Ingale and Chaudhari 2013).

The connecting link between the fields mycology and nanotechnology is known as "myconanotechnology." It has tremendous potential due to the enormous range of fungi in the environment (Gade et al. 2010). The secretion of a large number of various proteins makes fungi a potent source for nano-synthesis (Gupta et al. 2012); therefore, they are considered as biofactories for nanoparticles synthesis (Kashyap et al. 2012). The fungal biosynthesis of nanoparticles is gaining importance due to easy handling, isolation, culturing, and downstream processing of fungi (Gupta et al. 2012). Hence, many nanoparticles such as silver, iron, silica, gold, platinum, titanium, and zirconium have been synthesized by employing numerous fungi (Kashyap et al. 2012) which can be explored for various healthcare applications.

8.2 Classification of Nanoparticles

Nanoparticles widely differ in shape, size, and dimensions from their bulk material (Ealias and Saravanakumar 2017). Based on dimensions, they are classified into zero-dimensional, one-dimensional, two-dimensional, and three-dimensional structures. Zero-dimensional (0D) nanoparticles possess all the three space vectors, length, breadth, and height in the range of 1–100 nm. Mainly spherical nanoparticles and quantum dots are the major examples of zero-dimensional nanomaterials. The shape of nanomaterials can also be cubic, clusters, or polygonal. In one-dimensional (1D) nanoparticles, only two space vectors are in the range of nanometers, and the third vector is allowed to grow outside the nanometer scale. Graphene is the apt example of 1D nanoparticles; this also includes nanowires and nanorods. Two-dimensional (2D) nanoparticles consist of nanostructures whose one vector is in the nanometer scale and two dimensions are away from the range of nanometers. Nanosheets, nanotubes, nanowalls, nanofilms, etc. are the good representatives of 2D nanostructures. Three-dimensional (3D) nanoparticles have all the dimensions that are length, breadth, and height away from the nanoscale, for example, bulk materials like fullerenes (Mazhar et al. 2017; Ealias and Saravanakumar 2017). These distinct shapes and small size imparts sensitivity, large reactivity, enhanced stability, and high strength to nanoparticles (Ealias and Saravanakumar 2017).

8.3 Types of Nanoparticles

The primary types of nanoparticles are organic, inorganic, and carbon-based nanoparticles.

8.3.1 Organic Nanoparticles

These are also referred to as polymeric nanoparticles and are very biocompatible with no toxicity and biodegradable. It consists of liposomes, dendrimers, micelles, and others. A few of these nanoparticles like liposomes and micelles have a hollow structure which makes them an ideal drug carrier, and this structure is also designated as nanocapsule. These nanoparticles are sensitized to electromagnetic radiation and also to thermal radiation (Ealias and Saravanakumar 2017). Polymeric nanoparticles are prepared by block copolymers comprising two or more chains of polymer with distinct hydrophilicity that are self-assembled. Further, hydrophilic or hydrophobic drugs, in addition to macromolecules like proteins, peptides, and nucleic acids, can be encapsulated in organic nanoparticles. Polymeric nanoparticles improve the stability of therapeutic molecules against enzymatic decay as well as help in acquiring desired therapeutic limits of agents in target tissues for the required duration (Patel et al. 2017); due to this, they are efficiently used for targeted drug delivery and other biomedical applications (Ealias and Saravanakumar 2017).

In, in vivo studies, the fate of organic nanoparticles mainly depends on its size and surface features (Patel et al. 2017).

8.3.2 Inorganic Nanoparticles

These are nanoparticles that are not composed of carbon and are chiefly divided into metal (platinum, copper, zinc, gold, and silver) and metal oxide (titanium dioxide, iron oxide, copper oxide, and zinc oxide) nanoparticles (Patel et al. 2017; Ealias and Saravanakumar 2017; Asmathunisha and Kathiresan 2013).

Metals like aluminum (Al), gold (Au), cobalt (Co), cadmium (Cd), copper (Cu), iron (Fe), zinc (Zn), lead (Pb), and silver (Ag) can be converted to their respective nanoparticles. The metallic nanoparticle develops distinguishing properties like the size in nanoscale, surface features like a high surface-area-to-volume ratio, surface charge, pore size, reactivity, crystallinity, different shapes, color, and sensitivity to different ecological attributes (Ealias and Saravanakumar 2017). The surface plasmon resonance property of metal nanoparticles is employed for bioimaging and other diagnostic applications (Kumar et al. 2020). It can be synthesized by various processes like photochemical, electrochemical, chemical, and green synthesis (Omar et al. 2019). Many metallic nanoparticles are extensively used in antimicrobial activity, making them an ideal alternative of antibiotics (Chauhan et al. 2015). Silver nanoparticles have a high antibacterial activity (Singh et al. 2017b; Balakumaran et al. 2015), antifungal and anticancer activity (Balakumaran et al. 2015), and wound healing activity (Thirumurugan et al. 2011). Gold nanoparticles are used in sensing (Tripathi et al. 2014) as an antimicrobial agent (Manjunath et al. 2017) and also as an antioxidant agent (Manjunath et al. 2016). Thus, metallic nanoparticles have extensive scope for use in a variety of applications.

The metal oxide nanoparticles have extraordinary properties related to their metal nanoparticles. It is prepared by modulating metal nanoparticles to metal oxide nanoparticles. For example, iron oxide (Fe_2O_3) nanoparticles of increased reactivity are formed by oxidation of iron nanoparticles in the existence of oxygen at the room temperature. The usually synthesized metal oxides are aluminum oxide (Al_2O_3), silicon dioxide (SiO_2), cerium oxide (CeO_2), iron oxide (Fe_2O_3), titanium oxide (TiO_2), magnetite (Fe_3O_4), and zinc oxide (ZnO). Moreover, ionic metal oxides are great antibacterial agents with a diverse range of physical and chemical properties. Zinc oxide (ZnO) nanoparticles have a significant antibacterial (Mustafa et al. 2011), antifungal, and UV filtering properties (Chauhan et al. 2015), while titanium oxide (TiO_2) nanoparticles also possess antibacterial property (Rajakumar et al. 2012). Oxide nanoparticles on photoirradiation produce reactive oxygen species (ROS) which enhance their use in different areas. Also, these particles can be applied for imaging (Kumar et al. 2020).

Quantum dots (QDs) have a wide variety of applications in nanobiotechnology (Mohanpuria et al. 2008) mainly in imaging (Kumar et al. 2020). The quantum confinement effect is the unique characteristic of quantum dots. They are semiconductors in nature (Uddandarao and Balakrishnan 2016). QDs consist of

several advantages like improved signal brightness, resistance to photobleaching, size, composition tunability, light emission, and simultaneous excitation of multiple fluorescence colors (Kumar et al. 2020). Semiconductor nanoparticles have novel optical and electronic properties, due to which, it has gained attention in fundamental research. For example, cadmium sulfide (CdS) nanocrystals can be applied in light-emitting diodes, solar cells, laser, and photoelectric devices (Sanghi and Verma 2009a).

8.3.3 Carbon-Based Nanoparticles

These nanoparticles are composed of carbon, mainly includes fullerenes, graphene, carbon nanofibers, carbon nanotubes (CNTs), and carbon black (Ealias and Saravanakumar 2017). It has enormous applications in agriculture, medicine, drug delivery, electronics, mechanics, environment, and optics (Omar et al. 2019). Fullerenes (C60) are formed due to the binding of about 28–1500 carbon atoms together through their sp2 hybridization. Fullerenes are spherical, with diameters around 8.2 nm for a single layer and multilayered fullerenes from 4 to 36 nm (Ealias and Saravanakumar 2017). It contains 20 hexagonal and 12 pentagonal rings. It also has a high antioxidant activity (Omar et al. 2019). Graphene is an allotrope of carbon, made up of the two-dimensional planar arrangement of carbon atoms into the hexagonal network of honeycomb lattice (Ealias and Saravanakumar 2017). The graphene sheet is around 1 nm thick. It is employed in various biomedical applications (Omar et al. 2019). Carbon nanotubes (CNTs) is a graphene nanofoil wound into hollow cylinders. Single-layered nanotubes have a diameter of around 0.7 nm, and multilayered CNTs have a diameter of 100 nm and length in the range of micrometers to several millimeters. The ends of CNTs are either hollow or closed with half fullerene molecules (Ealias and Saravanakumar 2017). It has attributes like stability, large surface area, and exceptional optical properties (Kumar et al. 2020). It can be used for bioimaging and drug delivery (Omar et al. 2019). In carbon nanofiber, graphene nanofoil is coiled into a cone or cup shape. On the other hand, a spherical amorphous material made up of carbon, with size from 20 to 70 nm, is called carbon black. This forms agglomerates of size around 500 nm due to high interactions among the particles (Ealias and Saravanakumar 2017). Carbon nanofiber can be applied for sensing, water treatment, wound dressings, antibacterial activity, etc. (Omar et al. 2019).

8.4 Synthesis and Characterization of Nanoparticles

The two major approaches that account for the synthesis of nanoparticles are top-down and bottom-up. The top-down or destructive approach includes the formation of the nanoparticle by the reduction of bulk material. This includes methods like nanolithography, thermal decomposition, sputtering, and laser ablation (Ealias and Saravanakumar 2017). As the name suggests, in bottom-up or constructive

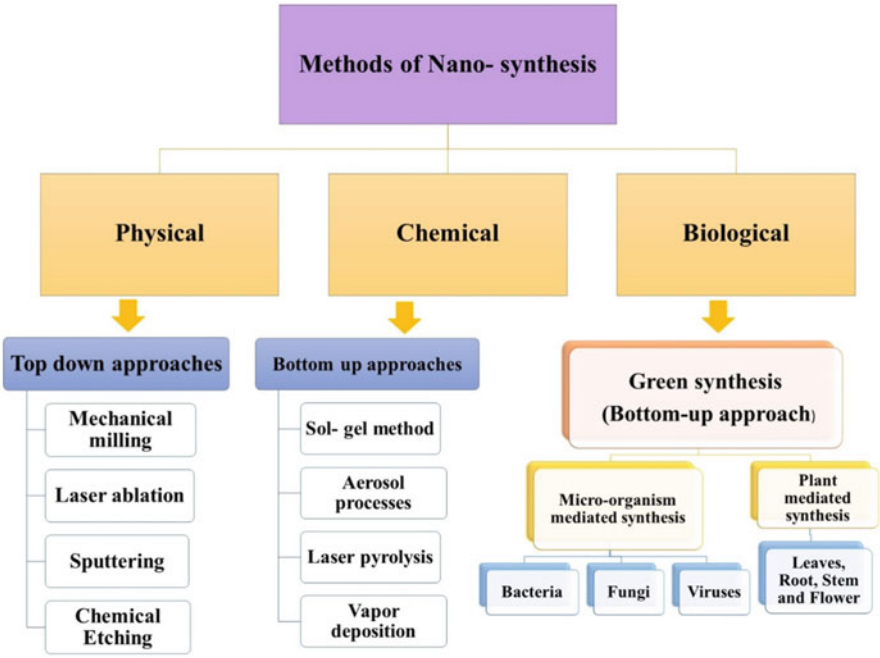

Fig. 8.1 Different methods for nano-synthesis

approach, atoms build up and grows into clusters to form nanoparticles (Mazhar et al. 2017; Ealias and Saravanakumar 2017), which mainly includes sol-gel, spinning, pyrolysis, chemical vapor deposition, and biological synthesis of nano-scale material (Ealias and Saravanakumar 2017). The bottom-up approach helps in the formation of nanomaterials with small defects and great uniformity because synthesis in this approach takes place near to thermodynamic equilibrium due to the dropping of Gibbs free energy (Saxena et al. 2014), whereas, in contrast to the constructive approach, the major limitation of the top-down approach is the formation of defective surface structures that affect the surface chemistry and physical properties of nanomaterials (Thakkar et al. 2010).

These two approaches involve various chemical, physical, and biological ways for the synthesis of nanoparticles of several dimensions and morphology (Fig. 8.1). The factors such as pH, temperature (Khandel and Shahi 2016), and time (Jain et al. 2011) affect the morphology of nanoparticles (Khandel and Shahi 2016) as well as the nanoparticles synthesis process (Jain et al. 2011).

The chemical method makes use of external stabilization and capping agents for the synthesis of nanoparticles. These agents stabilize and prevent the aggregation of nanoparticles (Vijayanandan and Balakrishnan 2018). This method saves time because in a small period, synthesis of nanoparticles occurs in the huge quantity (Ingale and Chaudhari 2013; Vijayanandan and Balakrishnan 2018). Further, via chemical synthesis, distinct morphologies and sizes of nanoparticles can be

obtained. Additionally, it is also an economical and easy process. Doping of foreign materials is also possible by this method (Mazhar et al. 2017). But there are some drawbacks associated with chemical synthesis like exerting synthesis procedures by maintaining specific conditions, the use of toxic chemicals, and difficulties in purification and extraction of nanoparticles (Vijayanandan and Balakrishnan 2018). It mainly includes the reverse micelle method, sol-gel method, sonochemical co-reduction (Mazhar et al. 2017), and photochemical reduction (Gahlawat and Choudhury 2019).

In the physical method, nanomaterials are synthesized at atmospheric pressure by evaporation–condensation which is achieved by using a tube furnace. Inward into a boat, centered at the furnace, the initial material is vaporized into the carrier gas (Ingale and Chaudhari 2013). Many materials like gold, silver, fullerene, etc. are converted to their respective nanoparticles by evaporation and condensation techniques (Ingale and Chaudhari 2013). This method eliminates the chance of contamination in synthesis and provides uniform nanoparticles (Mazhar et al. 2017). This method also has several drawbacks like it is expensive, the yield of nanoparticles is very less, and it requires a high amount of energy to maintain specific reaction conditions (Gahlawat and Choudhury 2019; Thakkar et al. 2010). Further, it is tough to achieve a narrow size distribution in nanoparticles via physical synthesis (Gade et al. 2010). Laser irradiation method, microwave irradiation (Mazhar et al. 2017), radiolysis, ultrasonication, spray pyrolysis, photoirradiation (Khandel and Shahi 2016), etc. are the different types of physical methods.

To overcome the drawbacks of chemical and physical methods, biological route of synthesis is employed. This has several advantages such as eco-friendly nature, no toxicity, cheaper, less time-consuming, less in pollution, easy to operate, safer (Mazhar et al. 2017; Vijayanandan and Balakrishnan 2018), and provides a high yield of nanoparticles (Ahmed et al. 2016). All these aspects make it the most sustainable method for the synthesis of nanoscale materials. Biological sources used in this method are plants, bacteria, algae, fungi (Karnani and Chowdhary 2013), and viruses (Mazhar et al. 2017). These prokaryotic and eukaryotic organisms help in the reduction of metal ions (Baker et al. 2013) to form stable nanoparticles (Vijayanandan and Balakrishnan 2018) and also control their size and shape by secreting proteins (Baker et al. 2013). Biosynthesis of nanoparticles can be intracellular or extracellular in microbes (Baker et al. 2013; Karnani and Chowdhary 2013). The ability of microbes to adapt to the heavy metals present in the environment, to sustain with other environmental stresses, and eventually to develop resistance against them makes them a noteworthy source of biosynthesis of nanomaterials (Baker et al. 2013). The biosynthesis of nanoparticles of diverse size and shape depends upon the biological entity exploited for synthesis along with pH, substrate concentration, and temperature (Hulkoti and Taranath 2014). The incredible features of biosynthesized nanoparticles make them an efficient candidate, which could be explored in various fields like agriculture, healthcare, environment, etc. (Ingale and Chaudhari 2013).

Characterization of nanoparticles is of immense importance to find out its morphology, chemical composition, surface area, and various other features (Khandel

and Shahi 2016). It also helps to regulate the synthesis of nanoparticles for different applications (Ingale and Chaudhari 2013). Scanning and transmission electron microscopy (SEM and TEM), energy dispersive spectroscopy (EDS), atomic force microscopy (AFM), Brunauer-Emmett-Teller (BET) analysis, X-ray powder diffraction (XRD), UV–visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance spectroscopy (NMR), X-ray photoelectron spectroscopy (XPS), and dynamic light scattering (DLS) are the various techniques utilized in the characterization of nanoscale materials (Ingale and Chaudhari 2013; Ealias and Saravanakumar 2017; Baker et al. 2013; Khandel and Shahi 2016). SEM and TEM are applied for the determination of size, morphology, and surface features of nanoparticles. These electron microscopy techniques give two-dimensional images of nanomaterials. Atomic force microscopy (AFM) helps in the qualitative speculation of surface roughness and other surface properties of nanoparticles (Khandel and Shahi 2016). It gives the three-dimensional image of nanoscale materials. Zeta potential analysis is used to find out the stability of dispersed nanoparticles and surface charge (Ingale and Chaudhari 2013; Khandel and Shahi 2016). DLS gives information about the particle size distribution (Ingale and Chaudhari 2013) and surface charge of nanoparticles (Khandel and Shahi 2016). The crystallinity and phase information are determined by XRD analysis. UV–visible spectroscopy identifies the functional groups of nanoparticles. It also helps in the quantification of nanoparticles as well as determines plasmon resonance (Ingale and Chaudhari 2013; Khandel and Shahi 2016). FTIR detects organic functional groups at the surface of nanoparticles. Energy dispersive spectroscopy (EDS) helps to gain facile knowledge about the elemental composition of nanomaterials (Khandel and Shahi 2016). The composition of nanoparticles and surface area is determined by X-ray photoelectron spectroscopy and BET analysis, respectively (Ealias and Saravanakumar 2017). Therefore, synthesis and characterization of nanoparticles are the processes that work synchronously in nanotechnology.

8.5 Green Synthesis of Nanoparticles

Green synthesis has arisen as a substitute for the chemical and physical method of nano-synthesis (Baker et al. 2013). It involves the use of prokaryotes (bacteria, virus) and eukaryotes (plants, algae, fungi) for the biosynthesis (Khandel and Shahi 2016; Baker et al. 2013) of silver, palladium, platinum, gold, iron, zirconium, cadmium, and metal oxides, for example, zinc oxide and titanium oxide nanoparticles (Hasan 2015). The micro and nanostructures of microbes are also considered as efficient templates for the biological synthesis of nanoparticles (Selvakumar et al. 2014). Phytochemicals present in plants and enzymes of microbes are involved in a reduction of metals to their corresponding nanoparticles (Nachiyar et al. 2015). Plant-mediated synthesis uses plant extract along with metal salts which are easily reduced to their respective nanoparticles in a short time (Mittal et al. 2013). According to the site of biosynthesis, intracellular and extracellular synthesis of

nanoparticles can be achieved by microorganisms (Velusamy et al. 2016; Baker et al. 2013). Intracellular synthesis uses cells for the reduction of metal salts into nanoparticles, whereas extracellular procedure employs the use of supernatant of the harvested microbial cell for nanoparticle synthesis (Baker et al. 2013). Many factors influence the biosynthesis of nanoparticles like pH, time, temperature, the concentration of metal salts, type of biomass, and incubation process such as static or in a shaker. Optimization of these parameters helps in the formation of nanoparticles with controlled size, morphology, and the dispersity (Khandel and Shahi 2016; Baker et al. 2013).The mechanism of the intracellular synthesis method comprises of the transportation of a specific ion inside the microbial cell. The cell wall of the microorganism plays a crucial part in the intracellular synthesis of nanoparticles. The negatively charged cell wall of microorganism interacts with the positively charged metal ions; further, the enzymes present within the cell wall catalyze the reduction of metal ions into nanoparticles which get diffused off through the cell wall. Extracellular biosynthesis mainly involves the use of enzyme NADH-dependent reductase and other secretory components for metallic nanoparticles synthesis (Mohanpuria et al. 2008; Narayanan and Sakthivel 2010). Today, green synthesis acquires a major focus because of its diverse advantages. Green synthesis of nanoparticles is a biocompatible process that occurs in ambient conditions with no toxicity, and it requires less time, money, and space. It does not need extra capping and stabilizing agents (Mukherjee and Patra 2017). Green-synthesized nanomaterials can be used for numerous healthcare applications like imaging, in therapeutics (Mukherjee and Patra 2017), and also in pharmaceutics (Baker et al. 2013). The role of different biogenic entities in nanoparticles synthesis is explained below.

8.5.1 Plant-Mediated Synthesis of Nanoparticles

Biosynthesis of nanoparticles by utilizing plant extract has gained more light because it serves many advantages over other biological sources (Ingale and Chaudhari 2013). Plant-based synthesis of nanoscale particles is a rapid (Karnani and Chowdhary 2013), one-step process which involves the use of natural capping agents (Ingale and Chaudhari 2013; Saxena et al. 2014) and also the absence of toxic chemicals (Ingale and Chaudhari 2013; Velusamy et al. 2016; Saxena et al. 2014). The use of plant extract does not require an aseptic environment, costly culture media, and tricky isolation process which are required by microorganisms; therefore, this method is more feasible and cost-effective for nano-synthesis (Velusamy et al. 2016; Ahmed et al. 2016). Plant parts like leaves, flower, root, bark, stem, and fruit can be used for green nano-synthesis (Chandra et al. 2020). But one of the major limitations of plant-mediated synthesis is that the biochemical composition of plant extracts of the same species differs geographically (Ahmed et al. 2016).

8.5.2 Bacteria-Mediated Synthesis of Nanoparticles

Bacteria have reaped the most attention in the synthesis of nanoscale materials among all microorganisms (Saxena et al. 2014) due to their unique ability to reduce metal ions into nanoparticles, easy handling, and high growth rates, and also they can be genetically manipulated (Gahlawat and Choudhury 2019). The defense mechanism to combat the toxicity of metal ions either by developing water-soluble complexes or by reduction of metal ions helps bacteria for the synthesis of nanoparticles (Sastry et al. 2003). Bacteria mainly synthesize metal nanoparticles by intracellular and extracellular mechanism (Gahlawat and Choudhury 2019). Intracellular synthesis takes place by electrostatic interaction between negatively charged microbial cell walls and positively charged metal ions, whereas enzymes within the cell wall reduce metal ions to nanoparticles (Hulkoti and Taranath 2014) that are released by ultrasound treatment or by reaction with appropriate detergents. On the other hand, extracellular synthesis involves soluble secreted enzymes or the reductive enzymes (Narayanan and Sakthivel 2010) mainly nitrate reductase (Hulkoti and Taranath 2014). The reduction of metallic ions into nanoparticles relies on various factors. The important environmental factors are temperature, medium composition, pH, and concentration of metal salts and organic functional molecules on the cell wall which encourages biomineralization (Gahlawat and Choudhury 2019). Bacterial structures like flagella and pili which play a role in mobility and communication between bacteria have been also used as a biotemplate for the synthesis of nanoparticles. Also, bacteria possess self-assembled nanostructures made up of surface layer proteins commonly known as S-layer present either attached to the cell wall or within the cell wall (Selvakumar et al. 2014). The bacterial exopolysaccharides which are secreted extracellularly are also explored as a biotemplate for the synthesis of metal nanoparticles because of its stabilizing and metallic ions reducing ability (Gahlawat and Choudhury 2019). Biofilms are the active growth mode of bacteria, which are too used for biosynthesis of nanomaterials. The attributes such as controlled electrochemical reactions, catalytic property, and highly reducing matrix make biofilms viable for the biosynthesis of nanoparticles (Gahlawat and Choudhury 2019).

8.5.3 Virus-Mediated Synthesis of Nanoparticles

Viruses are obligate intracellular parasites in both prokaryotic and eukaryotic organisms (Narasimha 2013). The virus seizes the replication machinery of the host cell and stops most of the cellular activities. The genetic material of the virus is either RNA or DNA (Velusamy et al. 2016). The outer covering of the virus is known as a capsid; it is an apt area for interaction with metal ions (Gahlawat and Choudhury 2019). Viruses can be modulated by genetic engineering for the development of novel nano assemblies. The features such as size, monodispersity, and a variety of chemical groups are available for modification to form nanodevices (Velusamy et al. 2016). The characteristics of viruses make them an ideal template

for the synthesis of nano-conjugates with noble metal nanoparticles which have application in cancer therapy and drug delivery (Gahlawat and Choudhury 2019). Bacteriophages are a class of viruses that multiply and survive inside bacteria (Ahiwale et al. 2017). Plant viruses, bacteriophages, and viral assemblies are gaining importance in nanobiotechnology due to their simple production, structural and chemical stability, absence of toxicity, and pathogenicity in animals or humans (Velusamy et al. 2016). However, virus-mediated synthesis of nanoparticles faces certain drawbacks because it is not a properly developed synthesis procedure and involves the participation of the host organism (Gahlawat and Choudhury 2019).

8.5.4 Fungi-Mediated Synthesis of Nanoparticles

Mycological synthesis of nanoparticles is reaping an enormous interest of researchers due to the great diversity and noteworthy countenances of fungi over other microorganisms (Saxena et al. 2014). Eukaryotic microorganisms, fungi, are considered as brilliant nanofactories for nanoscale materials production, because of its ability to secrete enzymes for the reduction of metal ion (Saxena et al. 2014; Gupta et al. 2012). Fungal synthesis of nanoparticles can be achieved intracellularly and extracellularly (Saxena et al. 2014) to obtain nanoparticles with different sizes, composition, superb monodispersity (Hulkoti and Taranath 2014), and stability (Gade et al. 2010). Fungi are preferred over other microbes and plants because of their ability of bioaccumulation and tolerance to toxic conditions (Selvakumar et al. 2014; Ingale and Chaudhari 2013). The mycelial mesh of fungi can tolerate high-flow pressure, agitation, and other challenging circumstances inside the bioreactors (Velusamy et al. 2016; Saxena et al. 2014) which makes their scaling up easy (Uddandarao et al. 2019). Further, fungal nanofactories are easy to handle with better growth control and manipulation feature and are cost-effective (Thakkar et al. 2010; Saxena et al. 2014). Mycosynthesis of nanoparticles gives higher yield of nanoparticles with a faster rate due to the excellent protein-secreting capability of fungi (Saxena et al. 2014). However, the simple downstream processing makes the extracellular fungi-mediated synthesis of nanoparticles more advantageous than intracellular mycosynthesis (Saxena et al. 2014; Kashyap et al. 2012). Fungal-derived nanoparticles have vast use in healthcare, textile, food industry, etc. (Saxena et al. 2014). Numerous fungi have been utilized for biosynthesis of gold, zirconium, silver, silica, platinum, titanium, and iron nanoparticles (Gade et al. 2010).

8.5.4.1 Methods for Fungi-Mediated Synthesis of Nanoparticles

The intracellular and extracellular mycosynthesis of nanoparticles (Velusamy et al. 2016) is a bottom-up approach of synthesis (Kashyap et al. 2012). In intracellular synthesis, nanoparticles are synthesized in the fungal cell, while in extracellular synthesis, fungi cell filtrate is used. In an intracellular method, fungal biomass is treated with a metal salt solution and incubated for 24 h in the dark. However, the downstream process for recovery is difficult (Yadav et al. 2015) and involves the use of various instruments (Gade et al. 2010), which makes it a costly affair (Yadav et al.

2015). The size of nanoparticles is smaller in intracellular synthesis because particles nucleate in cells (Narayanan and Sakthivel 2010). In contrast, in extracellular synthesis, the fungal filtrate is treated with a metal salt solution for nanoparticle synthesis. Extracellular synthesis is a faster, efficient, and cheaper (Yadav et al. 2015) process mostly preferred for mycosynthesis (Devi and Joshi 2015) due to its simple downstream processing (Yadav et al. 2015). The various enzymes and metabolites secreted by fungi act as reducing, capping, and stabilizing agents for nanoparticle formation (Kashyap et al. 2012; Narayanan and Sakthivel 2010). Figure 8.2a, b summarizes the intracellular and extracellular methods of the fungi-mediated synthesis of nanoparticles, respectively. Researchers in India have explored various fungi like *Aspergillus tamarii* PFL2, *Aspergillus niger* PFR6, *Penicillium ochrochloron* PFR8, *Schizophyllum commune*, *Rhizopus oryzae*, *Aspergillus niger*, *Fusarium semitectum*, *F. oxysporum*, *Verticillium* sp., *Cladosporium* sp., *Humicola* sp., *Coriolus versicolor*, etc. for synthesis of inorganic nanoparticles either extracellularly or intracellularly. In Table 8.1, the detailed comprehensive account of fungi-mediated synthesis of various nanoparticles in India is presented.

8.5.4.2 Factors Involved in Mycosynthesis of Nanoparticles

In mycosynthesis of nanoparticles, it is required to pay attention towards parameters such as temperature, pH, reaction time, biomass concentration, the existence of certain enzyme, and substrate concentration which affect the morphology, size, and monodispersity of nanoparticles (Kashyap et al. 2012; Khandel and Shahi 2016). The temperature has an important role in controlling activities of fungus as well as in ionic movement. Thus, it can be assumed that temperature employs striking influence on the growth of fungus and in metal uptake from the neighboring environment. Therefore, incubation temperature has a direct influence on the rate of synthesis of the nanoparticles. In one of the studies, the temperature for mycosynthesis of silver nanoparticles by fungus *Guignardia mangiferae* was varied from 30 to 80 °C. It has been observed that 30 °C was optimum for the synthesis of stable silver nanoparticles (Balakumaran et al. 2015). Similarly, Singh et al. (2014) studied the effect of temperature from 25 to 45 °C on silver nanoparticles synthesis by fungi *Penicillium* sp. They observed maximum absorbance at 390 nm in UV–visible spectroscopy at 25 °C, which indicates the synthesis of AgNPs. The synthesis was decreased at 40 °C (Singh et al. 2014).

The pH also plays a vital role in the fungal-based synthesis; Balakumaran et al. (2015) observed that in *G. mangiferae*-based AgNPs, the color of the solution was unchanged, but agglomeration of nanoparticles was found at acidic pH. However, at pH 5 and 6, brown color formation was initiated, and with the increase in pH, the intensity of brown color was also increased. The monodispersed as well as stable silver nanoparticles were formed at neutral pH (Balakumaran et al. 2015). In *Penicillium* sp.-mediated synthesis of silver nanoparticles, the maximum absorption in UV–visible spectrum was obtained at 425 nm at pH 7 which shows that the particle synthesized was in the array of 10–100 nm. It was concluded that at pH 7, the capping protein secreted by *Penicillium* sp. was stable, so synthesized AgNPs

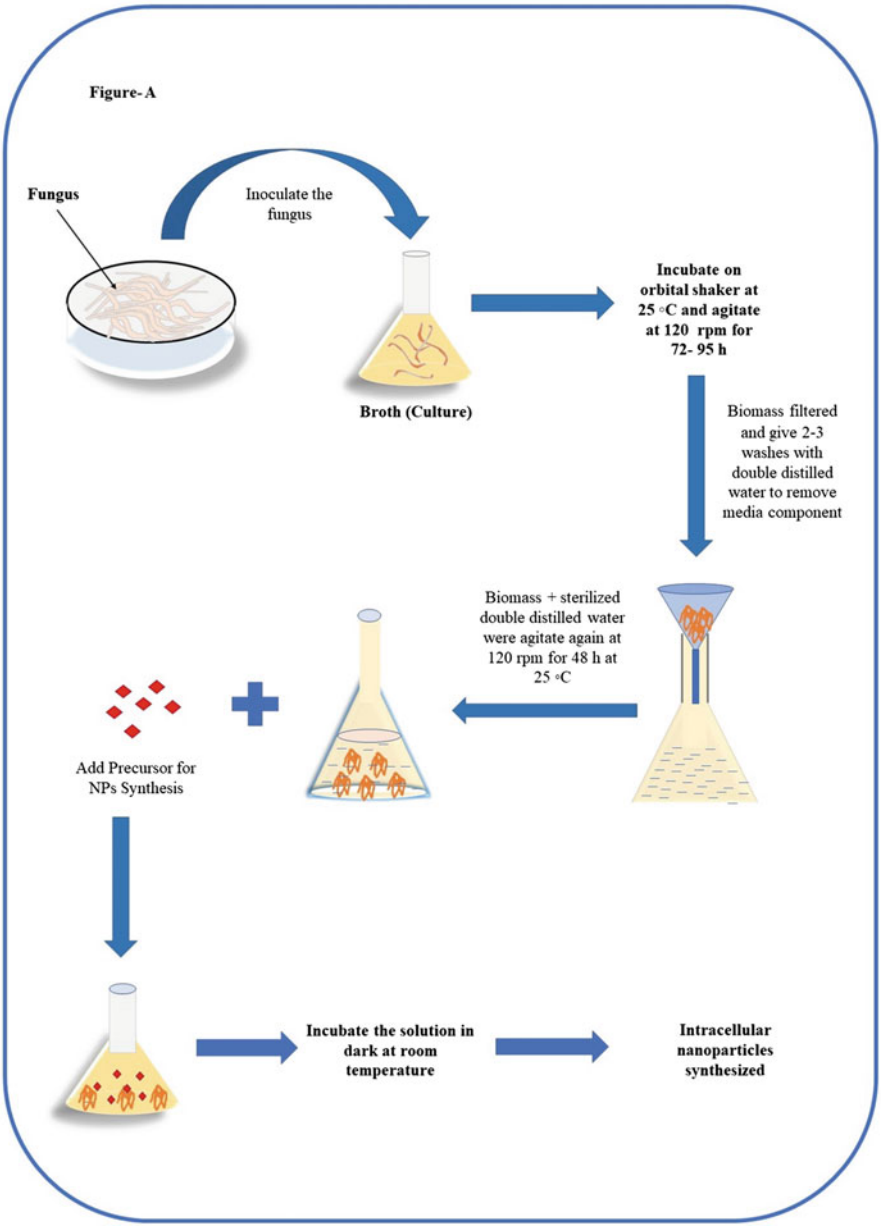

Fig. 8.2 (**a**) General representation of intracellular mycosynthesis of nanoparticles. (**b**) General representation of extracellular mycosynthesis of nanoparticles

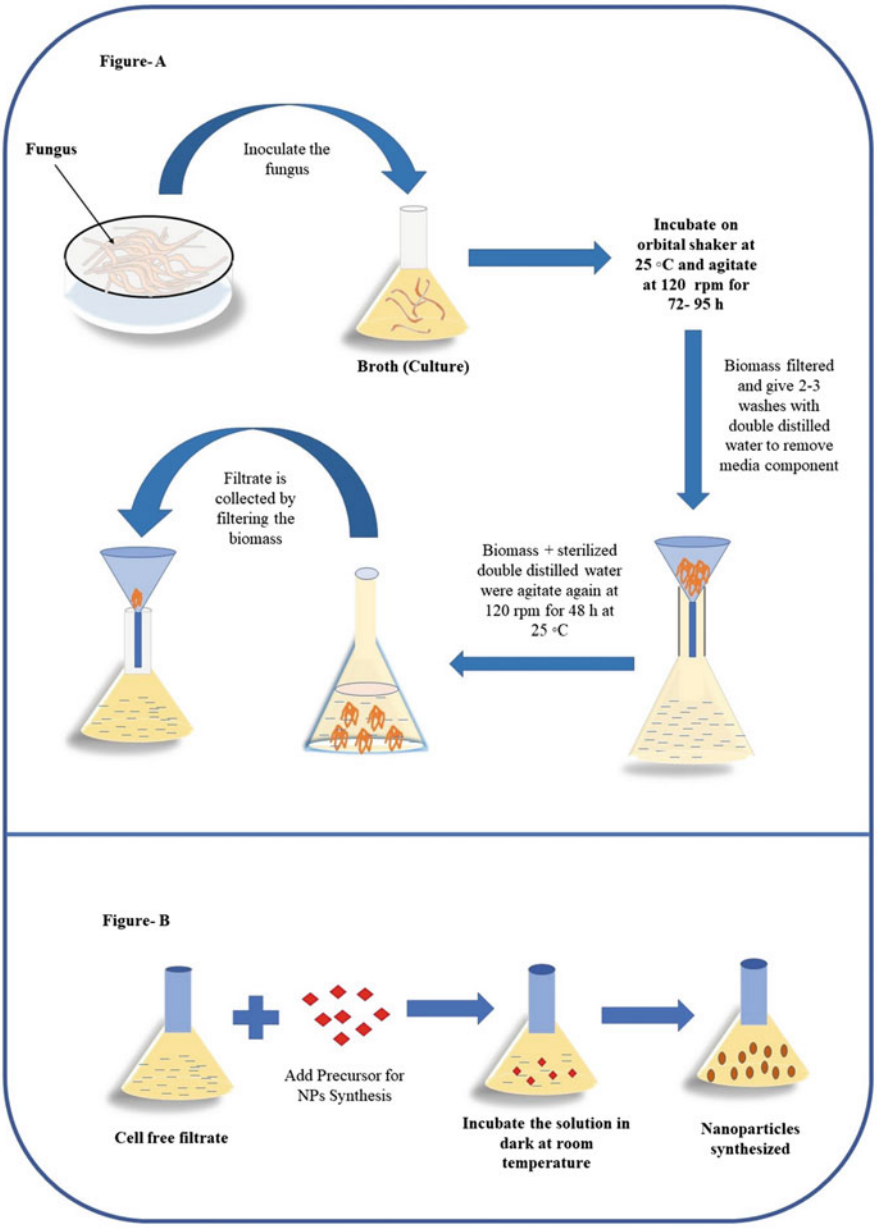

Fig. 8.2 (continued)

Table 8.1 Fungi-mediated nano-synthesis and their biomedical applications

Sr. no.	Fungi	Types of nanoparticles	Methodology of synthesis	Size of the nanoparticles	Shapes of the nanoparticles	Biomedical applications	References
1	*Aspergillus tamarii* PFL2, *Aspergillus niger* PFR6, and *Penicillium ochrochloron* PFR8	Ag	Extracellular	*A. tamarii*, 3.5 ± 3 nm; *A. niger*, 8.7 ± 6 nm; *P. ochrochloron*, 7.7 ± 4.3 nm	Spherical	NA	Devi and Joshi (2015)
2	*Trametes ljubarskyi* and *Ganoderma enigmaticum*	Ag	Extracellular	15–25 nm	Spherical	Antibacterial	Gudikandula et al. (2017)
3	*Fusarium oxysporum* sp.	Fe and Ag	Extracellular	Fe, 20–40 nm; Ag, 10–20 nm	Spherical	Antimicrobial	Abdeen et al. (2013)
4	*Cladosporium cladosporioides*	Au	Extracellular	<100 nm	–	Antimicrobial and antioxidant	Manjunath et al. (2017)
5	*Chrysosporium keratinophilum*, *Verticillium lecanii*, and *Fusarium oxysporum* f.sp. pisi	Ag	Extracellular	*C. keratinophilum*, 24–51 nm; *V. lecanii*, 20–50 nm; and *F. oxysporum* f.sp. pisi, 20–40 nm	Spherical	Adulticidal (mosquito)	Soni and Prakash (2012a)
6	*Aspergillus conicus*, *Penicillium janthinellum*, and *Phomopsis* sp.	Ag	Extracellular	*A. conicus*, 6–12 mm; *P. janthinellum*, 8–14 mm; and *Phomopsis* sp., 10–16 mm	Spherical or hexagonal particles	Antibacterial	Bharathidasan and Panneerselvam (2012)
7	*Bipolaris nodulosa*	Ag	Extracellular	10–60 nm	Symmetrical and spherical in shape, but some were hexahedral, triangular, and semi-pentagonal	Antimicrobial	Saha et al. (2010)

8	*Fusarium* sp.	Ag	Extracellular	12–20 nm	Spherical	Antibacterial	Singh et al. (2015)
9	*Verticillium* sp.	Au	Intracellular synthesis	20 ± 8 nm	Spherical	NA	Mukherjee et al. (2001)
10	*Yarrowia lipolytica* and melanin isolated from *Yarrowia lipolytica*	Ag	Intracellular and extracellular synthesis	15 nm	Spherical or hexagonal particles	Antimicrobial and antibiofilm	Apte et al. (2013)
11	*Fusarium oxysporum*	BaTi (BT)	Extracellular	4–5 nm	Irregular quasi-spherical	NA	Bansal et al. (2006)
12	*F. semitectum*	Ag	Extracellular	10–60 nm	Spherical	NA	Basavaraja et al. (2008)
13	*Penicillium* sp.	Ag	Extracellular	25–30 nm	Spherical	Antibacterial	Singh et al. (2014)
14	*Aspergillus fumigatus*	Ag	Extracellular	5–25 nm	Spherical	NA	Bhainsa and D'Souza (2006)
15	*Aspergillus niger*	Au	Extracellular	12.79 ± 5.61 nm	Variable	NA	Bhambure et al. (2009)
16	*Penicillium oxalicum*	Ag	Extracellular	–	–	Antimicrobial	Bhattacharjee et al. (2017)
17	*F. semitectum*	Au and Ag–Au	Extracellular	Au, 28 nm, and Ag–Au, 20 nm	Spherical	NA	Sawle et al. (2008)
18	*Curvularia lunata*	Ag	Extracellular	10–50 nm	Spherical	Antimicrobial	Ramalingmam et al. (2015)
19	*Penicillium* sp.	Ag	Extracellular	8.63–30.91 nm	Cubic	NA	Chandrappa et al. (2016)
20	*Aspergillus terreus*, *Paecilomyces*	Ag	Extracellular	5–50 nm	Spherical	Antimicrobial	Devi and Joshi (2012)

(continued)

Table 8.1 (continued)

Sr. no.	Fungi	Types of nanoparticles	Methodology of synthesis	Size of the nanoparticles	Shapes of the nanoparticles	Biomedical applications	References
	lilacinus, and *Fusarium* sp.						
21	*Rhizopus oryzae*	Au	Intracellular and extracellular	~15 nm	Roughly spherical	NA	Das et al. (2012)
22	*Cryptosporiopsis ericae*	Ag	Extracellular	5.5 ± 3.1 nm	Spherical	Antimicrobial	Devi and Joshi (2014)
23	*Rhizopus stolonifer*	Ag	Extracellular	5–50 nm	Spherical	Antibacterial	Afreen and Ranganath (2011)
24	*Trichoderma viride*	Ag	Extracellular	5–40 nm	Spherical	Antimicrobial	Fayaz et al. (2010)
25	*Mucor hiemalis*	Ag	Extracellular	5–15 nm	Spherical	Antibacterial and antifungal	Aziz et al. (2016)
26	*Aspergillus niger*	Ag	Extracellular	20 nm	Spherical	Antibacterial	Gade et al. (2008)
27	*Penicillium* species	Ag	Extracellular	65.92 nm	Spherical	Antibacterial and antioxidant	Govindappa et al. (2016)
28	*Cladosporium* sp.	Zn	Extracellular	50–100 nm	Ellipsoidal, rod shape, irregular	NA	Sri and Rajagopal (2016)
29	*Aspergillus niger* and *Aspergillus terreus*	Ag	Extracellular	*A. niger*, 26.5–42.3 nm, and *A. terreus*, 60–120 nm	Spherical	NA	Baskar et al. (2015)
30	*Pleurotus* sp.	Fe	Intracellular and extracellular	–	–	NA	Mazumdar and Haloi (2011)
31	*Penicillium fellutanum*	Ag	Extracellular	5–25 nm	Spherical	NA	Kathiresan et al. (2009)

32	*Orange Monascus* pigments (OMPs) and red *Monascus* pigments *(RMPs)*	Au	Extracellular	OMPs, 10–50 nm; RMPs, 10–60 nm	Spherical, triangular, and irregular shapes	NA	Koli et al. (2017)
33	*Trichoderma viride* and *Hypocrea lixii*	Au	Extracellular	*T. viride*, 20 nm; *H. lixii,* 20 nm	Spherical	Antimicrobial	Mishra et al. (2014)
34	*Trichoderma viride*	Ag	Extracellular	2–4 nm	Spherical	NA	Fayaz et al. (2009)
35	*Fusarium oxysporum*	Au	Extracellular	20–40 nm	Spherical and triangular	NA	Mukherjee et al. (2002)
36	*Helvella leucopus*	Ag	Extracellular	80–100 nm	Spherical	Antifungal	Talie et al. (2020)
37	*Aspergillus versicolor*	Ag	Extracellular	3–40 nm	Spherical	Antibacterial and antifungal	Netala et al. (2016a)
38	*B. tetramera*	Ag and Au	Extracellular	Ag, 54.78–73.49 nm; Au, 58.4 nm	Spherical	Antibacterial and antifungal	Fatima et al. (2015)
39	*Pestalotia* sp.	Ag	Extracellular	10–40 nm	Spherical	Antibacterial	Raheman et al. (2011)
40	*Chaetomium globosum*	Ag and Au	Extracellular	AgNPs, 20 nm; AuNPs, 40 nm	Spherical	Antibacterial	Singh et al. (2017b)
41	*Aspergillus clavatus*	Ag	Extracellular	10–25 nm	Spherical	Antimicrobial	Verma et al. (2010)
42	*Fusarium oxysporum*	Ag	Extracellular	19–50 nm	Spherical	Antibacterial	Vijayan et al. (2016)
43	*Alternaria* sp.	Ag	Extracellular	4–30 nm	Spherical	Antibacterial	Singh et al. (2017)
44	*Guignardia mangiferae*	Ag	Extracellular	5–30 nm	Spherical	Antibacterial, antifungal, and cytotoxic effects	Balakumaran et al. (2015)

(continued)

Table 8.1 (continued)

Sr. no.	Fungi	Types of nanoparticles	Methodology of synthesis	Size of the nanoparticles	Shapes of the nanoparticles	Biomedical applications	References
45	*Coriolus versicolor*	Cd	Extracellular	5–9 nm	Spherical	NA	Sanghi and Verma (2009a)
46	*Aspergillus nidulans*	Co	Extracellular	20.29 nm	Spherical	NA	Vijayanandan and Balakrishnan (2018)
47	*Rhizopus oryzae*	Au	Extracellular and intracellular	28–52 nm	Spherical	NA	Vala (2014)
48	*Aspergillus clavatus*	Au	Intracellular	20–35 nm	Nanotriangle	NA	Verma et al. (2011)
49	*Trichothecium* sp.	Au	Extracellular and intracellular	Extracellular, 5–200 nm; intracellular, 10–25 nm	Extracellular—mostly triangles and hexagons; intracellular, spherical	NA	Ahmad et al. (2005)
50	*Aspergillus fumigatus* and *A. flavus*	Au	Extracellular and intracellular	Extracellular, 16 and 17 nm; intracellular, 26 and 22 nm	Triangles, spherical, and hexagonal	NA	Saurabh Gupta and Bector (2013)
51	*Candida parapsilosis*	Au	Extracellular and intracellular	Extracellular, 52 nm; intracellular, 27 nm	Spherical	NA	Krishnan et al. (2016)
52	*Trichoderma harzianum*	Au	Extracellular	26–34 nm	Spherical	Sensing of Hg^{2+}	Tripathi et al. (2014)
53	*Colletotrichum* sp.	Au	Extracellular	8–40 nm	Spherical	NA	Shankar et al. (2003)
54	*Fusarium oxysporum* and *Verticillium* sp.	Fe	Extracellular	*F. oxysporum*, 20–50 nm; *Verticillium* sp., 10–40 nm	*F. oxysporum*, quasi-spherical; *Verticillium* sp., cubo-octahedral	NA	Bharde et al. (2006)

55	*Fusarium oxysporum*	Pt	Extracellular	5–30 nm	Spherical	NA	Syed and Ahmad (2012)
56	*Fusarium oxysporum*	Ag	Extracellular	5–15 nm	Spherical and few triangular	NA	Ahmad et al. (2003)
57	*Fusarium oxysporum*	Ag	Extracellular	10–25 nm	Spherical	NA	Kumar et al. (2007)
58	*Coriolus versicolor*	Ag	Extracellular and intracellular	Extracellular, 25–75 nm; intracellular, 444–491 nm	Spherical	NA	Sanghi and Verma (2009b)
59	*Penicillium nodositatum*	Ag	Extracellular	<100 nm	Ellipsoidal	Antibacterial	Manjunath et al. (2014)
60	*Aspergillus niger*	Ag	Extracellular	3–30 nm	Roughly spherical	Antibacterial and antifungal	Jaidev and Narasimha (2010)
61	*Lecanicillium lecanii*	Ag	Extracellular	45–100 nm	Spherical	Antibacterial	Namasivayam and Avimanyu (2011)
62	*Schizophyllum commune*	Ag	Extracellular and intracellular	Extracellular, 51–93 nm; intracellular, 54–99 nm	Spherical	Antibacterial, antifungal, and anticancer	Arun et al. (2014)
63	*Phoma glomerata*	Ag	Extracellular	60–80 nm	Spherical	Antibacterial	Birla et al. (2009)
64	*Alternaria solani* and *Penicillium funiculosum*	Ag	Extracellular	*A. solani*, 5–20 nm; *P. funiculosum*, 5–10 nm	Spherical	Antibacterial and antifungal	Devi et al. (2013)
65	*Aspergillus niger*	Ag	Extracellular	1.7–20 nm	Spherical	Antibacterial	Vala and Shah (2012)
66	*Fusarium acuminatum*	Ag	Extracellular	5–40 nm	Spherical	Antibacterial	Ingle et al. (2008)

(continued)

Table 8.1 (continued)

Sr. no.	Fungi	Types of nanoparticles	Methodology of synthesis	Size of the nanoparticles	Shapes of the nanoparticles	Biomedical applications	References
67	*Alternaria alternata*	Ag	Extracellular	20–60 nm	Spherical	Antifungal	Gajbhiye et al. (2009)
68	*Monascus purpureus*	Ag	Extracellular	10–40 nm	Spherical	Antibacterial and sensing of Hg^{2+}	Koli et al. (2018)
69	*Phytophthora infestans*	Ag	Extracellular	5–80 nm	Spherical and occasionally rod like	Wound healing	Thirumurugan et al. (2011)
70	*Aspergillus niger*	Ag	Extracellular	–	–	Wound healing	Sundaramoorthi et al. (2009)
71	*Rhodosporidium diobovatum*	PbS	Intracellular	2–5 nm	Spherical	NA	Seshadri et al. (2011)
72	*Saccharomyces cerevisiae*	ZnS	Intracellular	30–40 nm	Spherical	NA	Sandana Mala and Rose (2014)
73	*Humicola* sp.	Gd	Extracellular	3–8 nm	Quasi-spherical	NA	Khan et al. (2014)
74	*Pestalotiopsis microspora*	Ag	Extracellular	2–10 nm	Spherical	Anticancer and antioxidant	Netala et al. (2016b)
75	*P. citrinum*	Ag	Extracellular	2–20 nm	Roughly spherical	Anticancer, antioxidant, and larvicidal	Danagoudar et al. (2020)
76	*Piriformospora indica*	Ag	Extracellular	6–15 nm	Spherical	Anticancer and antioxidant	Aziz et al. (2019)
77	*Aspergillus flavus*	Zn and Zn: Gd	Extracellular	Zn, 12–24 nm; Zn:Gd, 10–18 nm	Spherical	Sensing of metals (Pb(II), Cd(II), Hg(II), Cu(II), and Ni (II))	Uddandarao et al. (2019)

78	*Aspergillus flavus*	ZnS	–	–	–	Sensing of Cu^{2+} and Mn^{2+}	Uddandarao and Balakrishnan (2017)
79	*Aspergillus flavus*	ZnS	Extracellular	18 nm	Spherical	ROS activity	Uddandarao and Balakrishnan (2016)
80	*Aspergillus flavus*	Ti	–	60 ± 5 nm	Spherical and hexagonal	Antimicrobial	Rajakumar et al. (2012)
81	*Beauveria bassiana*	Ag	Extracellular	36.88–60.93 nm	Spherical	Larvicidal	Banu and Balasubramanian (2014)
82	*Penicillium citrinum*	Au	Extracellular	<100 nm	–	Antioxidant	Manjunath et al. (2016)
83	*Cochliobolus lunatus*	Ag	–	3–21 nm	Spherical	Larvicidal	Salunkhe et al. (2011)
84	*Aspergillus niger*	Au	–	10–30 nm	Different shapes	Larvicidal	Soni and Prakash (2012b)

were also stable, but as the pH decreases, the proteins get denatures which results in the aggregation of AgNPs (Singh et al. 2014).

The concentration of precursor is another important consideration in the mycological synthesis of nanoparticles. A study tested concentrations of the precursor that was silver nitrate from 1 to 10 mM for the synthesis of silver nanoparticles by *Guignardia mangiferae*. Further, it has been found that at 1 mM concentration, silver nanoparticles with good monodispersity were formed (Balakumaran et al. 2015). Silver nitrate concentration was also varied from 0.5 to 2 mM by Singh et al. (2014) to study its effect on the synthesis of AgNPs by *Penicillium* sp. It was found that 1 mM was the optimum precursor concentration because of the maximum absorbance peak at 425 nm and the color of the solution, whereas at 2 mM concentration, nanoparticles were aggregated (Singh et al. 2014).

The fungal biomass amount also affects nano-synthesis. Balakumaran et al. (2015) observed better AgNPs formation at 10 g/100 ml concentration of *Guignardia mangiferae* biomass (Balakumaran et al. 2015). However, Singh et al. (2014) reported the maximum absorbance (410 nm) of silver nanoparticles at 15 and 20 g of wet biomass of fungi *Penicillium* sp. Nanoparticles were found to be well separated at this biomass concentration (Singh et al. 2014).

The variation in reaction time also varies the shape, size, and yield of nanoparticles (Khandel and Shahi 2016). Balakumaran et al. (2015) reported that fungus *G. mangiferae* had produced silver nanoparticles in 12 h by using 1 mM silver nitrate and also the particles were stable (Balakumaran et al. 2015). On the contrary, *Penicillium* sp. have produced AgNPs after 24 h of incubation with the same precursor concentration (Singh et al. 2014).

Specific fungal enzymes have an excellent function in the nano-synthesis and are also responsible for various morphologies and compositions of nanoparticles. It was reported that *F. oxysporum* was able to synthesis silver nanoparticles because of a specific NADH-dependent reductase, whereas *F. moniliforme* did not synthesize silver nanoparticles due to the absence of enzyme NADH-dependent reductase (Ahmad et al. 2003). Thus, to synthesize nanoparticles of the desired traits by using fungi, the optimization of the above mentioned parameters is needed.

8.5.4.3 Mechanism of Fungi-Mediated Nano-Synthesis

The synthesis of nanoparticles by fungi is broadly an outcome of abiotic stress (high metal ion concentration) tolerating ability of fungus (Kashyap et al. 2012). Some of the stress combating mechanisms are transport across the cell membrane, precipitation, entrapment in extracellular capsules, complexation, and oxidation–reduction reactions (Narayanan and Sakthivel 2010). These stress conditions are dealt by the secretion of various extracellular bioactive molecules by the fungi (Yadav et al. 2015). The knowledge of the precise mechanism of nano-synthesis engaging biological entities is unclear (Hulkoti and Taranath 2014; Yadav et al. 2015). Many studies suggested the role of enzymes and proteins as bioreducing and capping agents for nano-synthesis (Gahlawat and Choudhury 2019); therefore, the enzymes and proteins provide stability to nanoparticles by preventing their aggregation (Hulkoti and Taranath 2014). This was also proposed in the study of the synthesis

of cobalt oxide nanoparticles by *Aspergillus nidulans*. The FTIR analysis of cobalt oxide nanoparticles revealed the presence of bonds specific to proteins that were carboxylic and amide, and the proteins were attached to nanoparticles via their cysteine and amine groups. They further concluded that these proteins helped in the reduction of metal ion and also in the stabilization of nanoparticles by capping them. This finding was also supported by zeta potential study (Vijayanandan and Balakrishnan 2018). Krishnan et al. (2016) also reported the involvement protein in capping and formation of gold nanoparticles (Krishnan et al. 2016). Proteins were capped around ZnS:Gd nanoparticles and ZnS NPs via the thiol group of cysteine and methionine which was proved by the FTIR analysis (Uddandarao et al. 2019).

Another mechanism of fungi-mediated synthesis of nanoparticles is the Michaelis–Menten type which was proposed by Mukherjee et al. (2008) where the reaction primarily shows pseudo-zero order kinetics which means slow reaction at high concentration of silver nitrate, and as the concentration of silver nitrate decreases with reaction, it trails to higher-order kinetics reactions. The silver nanoparticles were formed due to dehydrogenation of -SH group of cysteine amino acid of protein, on its reaction with silver nitrate. The amino group was speculated as a capping agent for AgNPs (Mukherjee et al. 2008; Yadav et al. 2015). A study performed by Vala and Shah (2012) also supports the formation of silver nanoparticles by dehydrogenation of –SH bond in amino acid (Vala and Shah 2012).

Fungi can synthesize nanoparticles intracellularly as well as extracellularly (Velusamy et al. 2016; Hulkoti and Taranath 2014). The speculated intracellular synthesis mechanism involves trapping of metal ions on the fungal cell surface maybe because of electrostatic interaction between cell wall components and metal ions, followed by the reduction of metal ions to form nanoparticles by enzymes present inside the fungal cell wall. It is also possible that some metal ions may disseminate through the cell wall; such metal ions are bioreduced by the cytoplasmic enzymes (Kashyap et al. 2012; Yadav et al. 2015). However, it is assumed that the reductase enzymes; electron shuttle quinones (Kashyap et al. 2012) like ubiquinol, hydroxyquinoline, NADH, etc.; and c-type cytochromes redox proteins have a vital function in the extracellular synthesis of fungi-mediated nanoparticles (Kashyap et al. 2012; Gahlawat and Choudhury 2019). Many studies have reported the use of nitrate reductase enzyme for extracellular mycosynthesis of nanoparticles (Gahlawat and Choudhury 2019). Kumar et al. (2007) proved the role of α-NADPH-dependent nitrate reductase and phytochelatin or hydroxyquinoline as an electron shuttle for silver nanoparticles formation by using an enzyme from *Fusarium oxysporum*. The process involves the conversion of α-NADPH to α-$NADP^+$, and hydroxyquinoline probably transferred the electron formed during the reduction of nitrate for the conversion of Ag^{2+} to Ag (Kumar et al. 2007). As stated earlier, the actual mechanism of fungal synthesis of nanoscale material is not clear (Hulkoti and Taranath 2014; Yadav et al. 2015), so, Fig. 8.3a, b gives the hypothetical view of the intracellular and extracellular mechanism of mycosynthesis of nanoparticles, respectively.

In one of the studies, the extracellular reduction of Ag^+ to Ag^0 was reported due to the reductase enzymes present in the fungal extract. They also proposed the role of

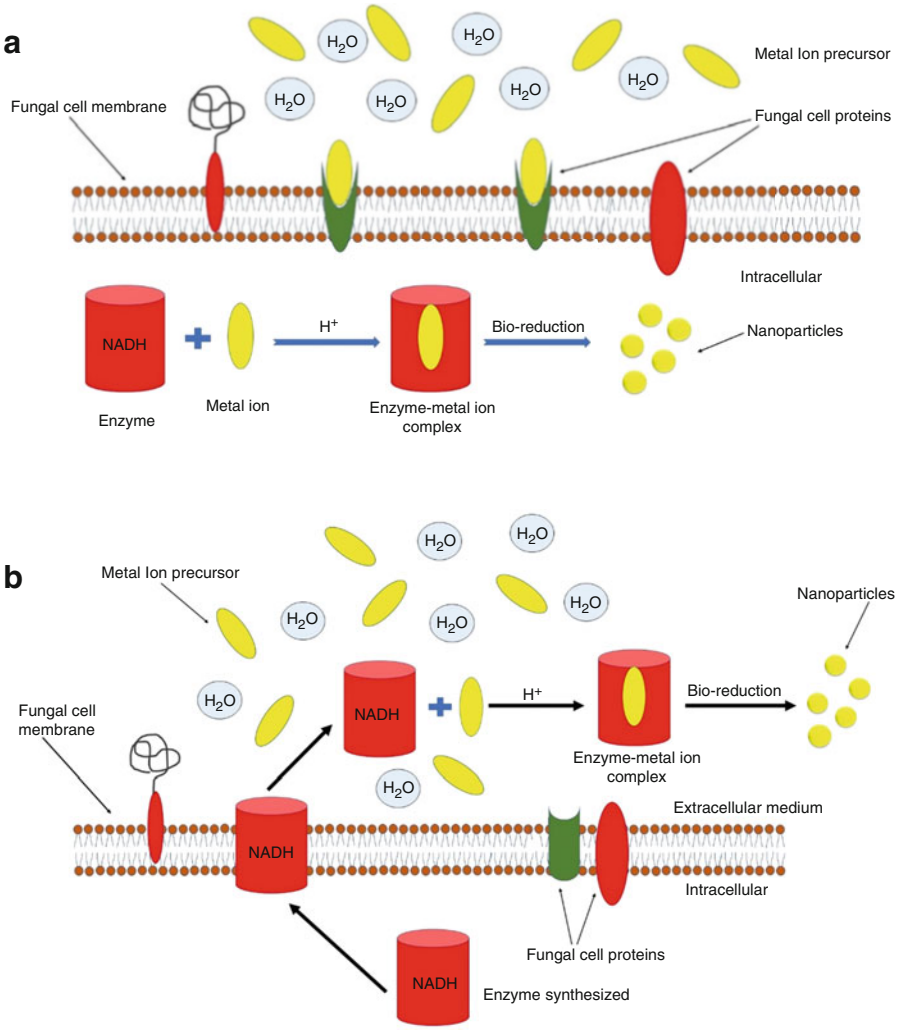

Fig. 8.3 (**a**) Speculated mechanism of intracellular fungi-based nano-synthesis. (**b**) Speculated mechanism of extracellular fungi-based nano-synthesis

protein in the capping of AgNPs by obtaining an absorbance peak around 208 nm in UV–visible spectrum (Devi and Joshi 2015). Similarly, Jaidev and Narasimha (2010) also corroborated the existence of enzyme nitrate reductase in the extract of fungus which was liable for the reduction of Ag^{+} to Ag^{0} (Jaidev and Narasimha 2010). Likewise, *Saccharomyces cerevisiae* MTCC 2918-mediated intracellular reduction of ZnS nanoparticles occurred by the enzyme mechanism of oxidoreductase (Sandana Mala and Rose 2014).

In another study, it has been shown that the reduction of Au^{3+} to Au^{0} for the synthesis of gold nanoparticles extracellularly was mediated by enzyme, whereas intracellular bioreduction of Au^{3+} for AuNPs formation in fungus *Candida parapsilosis* ATCC 7330 was found to be metabolism dependent. They found alcohol dehydrogenase (ADH) and glutathione reductase (GR) enzymes in the cell-free extract which was used for mycosynthesis of gold nanoparticles. Interestingly, by observing the formation of gold nanoparticles in heat-denatured cell extract, they proposed that the bioreduction for the synthesis of gold nanoparticles was not solely enzyme-dependent; rather, there was the involvement of multiple reductions and stabilization processes (Krishnan et al. 2016). NADH-dependent reductase enzyme was also used for the reduction of Au^{3+} to Au^{0} in extracellular mycosynthesis of gold nanoparticles by fungus *T. harzianum*. In the reaction, NADH transfers an electron to reductase and itself gets oxidized to NAD^{+} (Tripathi et al. 2014).

8.6 Applications of Nanoparticles Synthesized Using Fungi in Healthcare

Nanoparticles synthesized using fungi have a wide array of applications, due to which, myconanotechnology has gained attention from researchers (Kashyap et al. 2012). The pronounced applications of fungal-based nanoparticles in healthcare are described below. Table 8.1 also reviews the biomedical applications of different fungal-based nanoparticles.

8.6.1 Antimicrobial Activity

Infections related to microbes have been the core reason for illness and subsequent deaths in the world. Further, the advent of multidrug-resistant microorganisms paved the way for the production of novel antimicrobial agents (Patel et al. 2017). The exceptional attributes of nanoparticles make them an effective antimicrobial agent (Khandel and Shahi 2016) which can serve as a valuable substitute to antibiotic (Patel et al. 2017). In general, the antimicrobial mechanism of nanoscale materials is mainly established by electrostatic interactions between the cell wall and cell membrane of microbes with nanoparticles due to their opposite charges. Further, these interactions disrupt membrane permeability and respiration. Nanoparticles damage the cell wall and lead to a release of the cellular components like proteins, DNA, and other metabolites finally causing cell death (Gahlawat and Choudhury 2019; Ahmed et al. 2016). Also, nanoparticles react with cellular components like nucleic acid and protein by penetrating inside the cell resulting in their denaturation and loss of cell function. Besides this, the formation of reactive oxygen species (ROS) by nanoparticles too causes cell toxicity (Gahlawat and Choudhury 2019).

Biogenic silver nanoparticles of 4–30 nm were synthesized by employing an endophytic fungus have shown effective antibacterial activity against Gram-

negative bacteria (*Serratia marcescens*, MTCC 97 and *Escherichia coli*, MTCC 443) and Gram-positive bacteria (methicillin-resistant *Bacillus subtilis*, MTCC 441 and *Staphylococcus aureus*, MTCC 740) (Singh et al. 2017a). It was also observed that both antibiotics (chloramphenicol and gentamicin) and silver nanoparticles synergistically have shown a spike in antibacterial activity against pathogenic bacteria, *E. coli* MTCC 730, *S. pyogenes* MTCC 1925, and *E. faecalis* MTCC 2729 (Devi et al. 2013). Silver nanoparticles act as an efficient antimicrobial agent (Singh et al. 2017b) and also are biocompatible with human tissues (Thirumurugan et al. 2011). Because of the cell wall structure, Gram-negative bacteria are more vulnerable to silver ions than Gram-positive bacteria (Ahmed et al. 2016). A study revealed that mycosynthesized crystalline gold nanoparticles also exhibit antibacterial activity against *E. coli* MTCC 118, *Pseudomonas aeruginosa* MTCC 424, *Staphylococcus aureus* MTCC 7443, and *Bacillus subtilis* MTCC 441 (Manjunath et al. 2017). Moreover, fungal-derived metal oxides nanoparticles such as ZnO (Chauhan et al. 2015) and TiO_2 (Rajakumar et al. 2012) also showed significant antibacterial activity against pathogenic bacteria. *Pseudomonas aeruginosa* was inhibited by ZnO (Chauhan et al. 2015), whereas TiO_2 acted as an antibacterial agent against *S. aureus* (MTCC-3160), *B. subtilis* (MTCC-1427), *P. aeruginosa* (MTCC-1034), *E. coli* (MTCC-1721), and *Klebsiella pneumoniae* (MTCC-4030) at different MIC values (Rajakumar et al. 2012). Namasivayam and Avimanyu (2011) explored the antibacterial property of silver nanoparticles formed from *Lecanicillium lecanii* for the preparation of antibacterial cotton fabric. Interestingly, the cotton fabric coated with both antibiotics and silver nanoparticles has shown an increase in antibacterial capacity (Namasivayam and Avimanyu 2011).

Disruption of lipids and enzymes on the fungal cell wall and inactivation of -SH group on the same lead to cell death (Jaidev and Narasimha 2010). The antifungal activity of intracellularly and extracellularly synthesized silver nanoparticles by *Schizophyllum commune* was found to be concentration-dependent. These silver nanoparticles have averted the growth of pathogenic dermatophyte fungi—*Trichophyton mentagrophytes*, *Trichophyton simii*, and *Trichophyton rubrum* (Arun et al. 2014). Similarly, spherical nanoparticles of silver at 1 mg/mL concentration have inhibited the growth of *Colletotrichum* sp., *C. lunata*, and *Rhizoctonia solani*, which are plant pathogenic fungi (Balakumaran et al. 2015). Significant antifungal activity was also exhibited by extracellularly synthesized gold nanoparticles from *Cladosporium cladosporioides* against *Aspergillus niger* MTCC 281 (Manjunath et al. 2017). A study has shown that extracellularly mycosynthesized crystalline ZnO NPs exhibit significant antifungal activity towards pathogen *Ganoderma* sp. JAS4, *Fusarium* sp., and *Aspergillus terreus* strain JAS1 (Chauhan et al. 2015).

Silver nanoparticles synthesized using *Agaricus bisporus* were of sizes 3–10 nm and have shown antiviral activity against a bacteriophage. It was observed that viral growth was completely stopped in host bacteria at 140–160 ppm concentration of nanoparticles (Narasimha 2013).

8.6.2 Antioxidant Activity

It is a free radical scavenging activity mainly studied by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method of Brand-Williams et al. (1995) (Manjunath et al. 2016; Danagoudar et al. 2020). DPPH assay is a very accurate and basic process to determine the antioxidant activity of natural products (Danagoudar et al. 2020) and synthetic compounds (Manjunath et al. 2016). This assay uses ascorbic acid as a positive control (Danagoudar et al. 2020). Biologically synthesized, fungus *Pestalotiopsis microspora*-mediated stable silver nanoparticles were observed as an effective antioxidative agent with IC_{50} values of 76.95 ± 2.96 μg/mL and 94.95 ± 2.18 μg/mL against DPPH and H_2O_2 radicals, respectively (Netala et al. 2016b). Recently, silver nanoparticles synthesized from *P. citrinum* CGJ-C2 showed concentration-dependent antioxidant properties which mean that with the increase in the amount of AgNPs, its radical scavenging potential also increases. They also proposed that the antioxidant nature of silver nanoparticles may be due to phenolic compounds on its surface (Danagoudar et al. 2020). Biogenic gold nanoparticles synthesized by *Penicillium citrinum* also showed dose-dependent antioxidant activity (Manjunath et al. 2016).

8.6.3 Anticancer/Cytotoxic Activity

According to WHO (World Health Organization) report of 2018, cancer is among the foremost reason for mortality. There are various drawbacks of conventional cancer therapies that can be overcome by developing biocompatible drugs for cancer treatment. Green-synthesized nanoparticles especially by fungi (Danagoudar et al. 2020) have gained the attention of lots of researchers because of their specific properties and various applications in the healthcare sector (Aziz et al. 2019). Netala et al. (2016b) studied the cytotoxic activity of AgNPs (2–10 nm) synthesized by an endophytic fungus. It was found that the biogenic silver nanoparticles displayed cytotoxic activity against B16F10 (mouse melanoma), A549 (human lung adenocarcinoma), SKOV3 (human ovarian carcinoma), and PC3 (human prostate carcinoma) cell lines with IC_{50} values of 26.43 ± 3.41 μg/mL, 39.83 ± 3.74 μg/mL, 16.24 ± 2.48 μg/mL, and 27.71 ± 2.89 μg/mL, respectively, and were biocompatible towards normal CHO cells. Further, the apoptotic changes were dose-dependent in SKOV3 cells (Netala et al. 2016b). In one of the studies, the mycosynthesized silver nanoparticles have exhibited significant cytotoxic effects against MCF-7 (human breast adenocarcinoma), HepG2 (human liver hepatocellular carcinoma), and HeLa (human cervical carcinoma) cell lines than chemically synthesized AgNPs (Aziz et al. 2019). In another study, it was observed that mycologically synthesized AgNPs have apoptotic activity against MCF-7 cells, and also they revealed concentration reliant cytotoxic potential of AgNPs against cells HepG2, A431, and MCF-7 (Danagoudar et al. 2020).

8.6.4 Larvicidal Activity

The ways to inhibit vectors causing dengue, lymphatic filariasis, chikungunya fever, etc. have been developed around the world (Salunkhe et al. 2011). Due to favorable environmental situations, India is an endemic country to mosquito-borne diseases. Larvicides play a key role in controlling these disease-causing vectors. The adverse effects of insecticides have led to finding out an eco-friendly way to control mosquito larvae. Silver nanoparticles of 3–21 nm were produced by fungus *C. lunatus* which have displayed larvicidal potential against the second, third, and fourth larval stages of *Aedes aegypti* (dengue vector) and *Anopheles stephensi* (malaria vector). It was also observed that larvicidal activity was concentration-dependent (Salunkhe et al. 2011). In another study, the larvicidal potential of mycogenic AgNPs synthesized by an entomopathogenic fungus *Beauveria bassiana* against dengue vector was studied, and it was observed that first and second instar larvae of *Aedes aegypti* showed 100% mortality in 21 h. They concluded that the larvicidal rate was dependent on concentration (Banu and Balasubramanian 2014). Soni and Prakash (2012a) assessed the adulticidal potential of mycosynthesized silver nanoparticles against *C. quinquefasciatus* (filariasis vector) for the first time (Soni and Prakash 2012a). Further, the efficiency of gold nanoparticles formed by using *Aspergillus niger* against *A. stephensi*, *A. aegypti*, and *C. quinquefasciatus* was reported in a study. In 48 hrs, 100% mortality was observed in all the larval instar of *Culex quinquefasciatus* vector (Soni and Prakash 2012b). Based on all these studies, we can conclude that nanoparticles derived from fungi are the efficient mosquito vector control agents.

8.6.5 Wound-Healing Activity

The silver nanoparticles synthesized by using *Aspergillus niger* (Sundaramoorthi et al. 2009) and *Phytophthora infestans* (Thirumurugan et al. 2011) were explored for wound-healing potential in rat models. Sundaramoorthi et al. (2009) studied two models for wound healing—the excision wound model and the thermal wound model. In the excision model, wound was healed completely in 8 days with 20 mg of AgNPs, while in the thermal wound model, it took 14 days (Sundaramoorthi et al. 2009). Another study by Thirumurugan et al. (2011) also showed significant wound-healing potential of 5–30 nm biogenic AgNPs with ointment in the rat model in 8 days. They have also found that as compared to standard ointment 0.125% (w/w), silver nanoparticle ointments gave better results (Thirumurugan et al. 2011). Wound healing is a natural phenomenon. It appears that the precise use of fungi-mediated nanoparticles can certainly potentiate the wound healing.

8.6.6 Drug Delivery

The surface features and size of nanoparticles make them an efficient agent for delivering the drug. Targeted as well as multidrug delivery can be achieved by modulating nanoparticle surfaces with specific ligands and other molecules (Patel et al. 2017). Biogenic nanoparticles are covered by biological molecules which make them a superior agent for drug delivery (Birla et al. 2009). Highly stable and protein-capped gadolinium oxide (Gd_2O_3) nanoparticles were synthesized by Khan et al. (2014) with the help of fungus *Humicola* sp. (thermophilic fungus). Further, they observed the biodistribution of gadolinium oxide nanoparticles in rat models by tagging them with radiolabel Tc-99m. They found that in 45 min, the nanoparticles were cleared through urine. They also conjugated these nanoparticles with anticancer drug taxol which may serve as a potent drug delivery agent (Khan et al. 2014).

8.6.7 Sensing

Mycosynthesized gold nanoparticle-based colorimetric sensor was developed for the detection of mercury ions (Tripathi et al. 2014). Mercury is toxic to humans even at low concentrations and also leads to cause disorders of the kidney, digestive system, reproductive systems, etc. (Tripathi et al. 2014; Koli et al. 2018). It was proposed that AuNPs were able to detect Hg^{2+} ions due to the interaction of –SH group of cysteine on the surface of AuNPs with the Hg^{2+} ions. The minimum concentration that was detected is 2.6 nM (Tripathi et al. 2014). Silver nanoparticles of 10–40 nm synthesized extracellularly by fungal metabolite Monascus pigments also have the potential to detect mercury ions at the micromolar range (Koli et al. 2018). Green-synthesized nanoparticles get aggregated when their protein capping interacts with metals ions; hence, biogenic nanoparticles are used for metal sensing. Semiconductor ZnS NPs were used for detecting Cu^{2+} and Mn^{2+} ions. Copper and manganese ions were detected by the change in color of a yellow color solution of nanoparticles to blue and dark pink, respectively. The LOD of Mn^{2+} was found to be 2.14 μM and of Cu^{2+} 1.24 μM. Copper in higher amounts can cause Alzheimer disease and liver damage, while manganese can cause Parkinson's disease (Uddandarao and Balakrishnan 2017). Further, mycogenic ZnS nanoparticles derived from fungus *A. flavus* were doped with rare earth metal Gd and were used for sensing various metal ions—Pb(II), Hg(II), Cu(II), Cd(II), and Ni(II). The fluorescence property of 10–18 nm ZnS:Gd and ZnS nanoparticles was used for sensing (Uddandarao et al. 2019).

Bioinspired silver nanoparticles formed by using fungus *Cladosporium* species were explored for other healthcare use like in vitro antidiabetic and antiacetylcholinesterase activities (Popli et al. 2018). Mycosynthesized gold and silver nanoparticles conjugated with DNA of *C. albicans* were used as a nano template for rapid and sensitive detection of candidiasis in PCR (Bansod et al. 2013). This infers that fungal-derived nanoparticles can be used in molecular diagnosis. Moreover, the anti-inflammatory and tyrosine kinase inhibitory activities

of silver nanoparticles synthesized by *Penicillium* species were also reported (Govindappa et al. 2016).

8.7 Conclusions and Future Prospects

This chapter stretches the understanding of the apparent function of fungi in nanotechnology along with the applications of nanoparticles formed by using fungi in the biomedical area. Myconanotechnology offers a huge number of advantages which were very well explored by the Indian researchers. Although to plug the unattended knowledge gaps, this vast field requires more attention from the Indian researchers. It is quite evident that fungi are majorly explored for extracellular synthesis of metallic nanoparticles. Hence, there is a need to use fungi for the synthesis of other types of nanomaterials. Another bottleneck is to understand the exact mechanism of fungal nano-synthesis which will benefit in the significant and functional use of these nanoparticles in the healthcare area. Fungal-based nanoparticles can serve as a biocompatible alternative to combat different bacterial and fungal diseases over the conventional drugs owing to their significant antibacterial and antifungal potential. However, further research is necessary to explore the antiviral activity of these nanoparticles. In the future, to use fungal-derived nanoparticles for drug delivery, extensive understanding of its biodistribution, release, and clearance is to be investigated. Owing to the special fluorescent property, quantum dots of fungal origin have the potential to be utilized in diagnostic methods such as bioimaging as well as in the detection of biomolecules in extracellular fluids. Further, to commercialize nano products derived from fungi, its large-scale production is required, but there is a lack of scale-up studies by using fungi especially in India. The other void in the fungi-mediated synthesis of nanoparticles is the evaluation of extremophiles and marine fungi for nano-synthesis. There are various unique ecosystems in India, for example, the Lonar crater whose fungal diversity is needed to be explored for nano-synthesis, and these nanoscale materials can be subsequently used in unmapped biomedical applications. Therefore, by overcoming the curbs of fungi-mediated synthesis, researchers in India can establish their more persuasive contribution in the emerging field of myconanotechnology.

References

Abdeen S, Isaac RR, Geo S, Sornalekshmi S, Rose A, Praseetha PK (2013) Evaluation of antimicrobial activity of biosynthesized iron and silver nanoparticles using the fungi *Fusarium oxysporum* and Actinomycetes sp. on human pathogens. Nano Biomed Eng 5:39–45. https://doi.org/10.5101/nbe.v5i1.p39-45

Afreen RV, Ranganath E (2011) Synthesis of monodispersed silver nanoparticles by *Rhizopus stolonifer* and its antibacterial activity against MDR strains of Pseudomonas aeruginosa from burnt patients. Int J Environ Sci 1:1582–1592

Ahiwale SS, Bankar AV, Tagunde S, Kapadnis BP (2017) A bacteriophage mediated gold nanoparticles synthesis and their anti-biofilm activity. Indian J Microbiol 57:188–194. https://doi.org/10.1007/s12088-017-0640-x

Ahmad A, Mukherjee P, Senapati S, Mandal D, Khan MI, Kumar R, Sastry M (2003) Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum*. Colloids Surf B Biointerfaces 28:313–318. https://doi.org/10.1016/S0927-7765(02)00174-1

Ahmad A, Senapati S, Khan MI, Kumar R, Sastry M (2005) Extra-/intracellular biosynthesis of gold nanoparticles by an alkalotolerant fungus, *Trichothecium* sp. J Biomed Nanotechnol 1:47–53. https://doi.org/10.1166/jbn.2005.012

Ahmed S, Ahmad M, Swami BL, Ikram S (2016) A review on plants extract mediated synthesis of silver nanoparticles for antimicrobial applications: a green expertise. J Adv Res 7:17–28. https://doi.org/10.1016/j.jare.2015.02.007

Apte M, Sambre D, Gaikawad S, Joshi S, Bankar A, Kumar AR, Zinjarde S (2013) Psychrotrophic yeast Yarrowia lipolytica NCYC 789 mediates the synthesis of antimicrobial silver nanoparticles via cell-associated melanin. AMB Express 3:1–8. https://doi.org/10.1186/2191-0855-3-32

Arun G, Eyini M, Gunasekaran P (2014) Green synthesis of silver nanoparticles using the mushroom fungus *Schizophyllum commune* and its biomedical applications. Biotechnol Bioprocess Eng 19:1083–1090. https://doi.org/10.1007/s12257-014-0071-z

Asmathunisha N, Kathiresan K (2013) A review on biosynthesis of nanoparticles by marine organisms. Colloids Surf B Biointerfaces 103:283–287. https://doi.org/10.1016/j.colsurfb.2012.10.030

Aziz N, Pandey R, Barman I, Prasad R (2016) Leveraging the attributes of *Mucor hiemalis*-derived silver nanoparticles for a synergistic broad-spectrum antimicrobial platform. Front Microbiol 7:1–11. https://doi.org/10.3389/fmicb.2016.01984

Aziz N, Faraz M, Sherwani MA, Fatma T, Prasad R (2019) Illuminating the anticancerous efficacy of a new fungal chassis for silver nanoparticle synthesis. Front Chem 7:1–11. https://doi.org/10.3389/fchem.2019.00065

Baker S, Harini BP, Rakshith D, Satish S (2013) Marine microbes: invisible nanofactories. J Pharm Res 6:383–388. https://doi.org/10.1016/j.jopr.2013.03.001

Balakumaran MD, Ramachandran R, Kalaichelvan PT (2015) Exploitation of endophytic fungus, *Guignardia mangiferae* for extracellular synthesis of silver nanoparticles and their in vitro biological activities. Microbiol Res 178:9–17. https://doi.org/10.1016/j.micres.2015.05.009

Bansal V, Poddar P, Ahmad A, Sastry M (2006) Room-temperature biosynthesis of ferroelectric barium titanate nanoparticles. J Am Chem Soc 128:11958–11963. https://doi.org/10.1021/ja063011m

Bansod S, Bonde S, Tiwari V, Bawaskar M, Deshmukh S, Gaikwad S, Gade A, Rai M (2013) Bioconjugation of gold and silver nanoparticles synthesized by *Fusarium oxysporum* and their use in rapid identification of *Candida* species by using bioconjugate-nano-polymerase chain reaction. J Biomed Nanotechnol 9:1962–1971. https://doi.org/10.1166/jbn.2013.1727

Banu AN, Balasubramanian C (2014) Myco-synthesis of silver nanoparticles using *Beauveria bassiana* against dengue vector, Aedes aegypti (Diptera: Culicidae). Parasitol Res 113:2869–2877. https://doi.org/10.1007/s00436-014-3948-z

Basavaraja S, Balaji SD, Lagashetty A, Rajasab AH, Venkataraman A (2008) Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium semitectum*. Mater Res Bull 43:1164–1170. https://doi.org/10.1016/j.materresbull.2007.06.020

Baskar G, Chandhuru J, Fahad KS, Praveen AS, Bharathi R, Fyna S (2015) Mycological synthesis and characterization of silver nanoparticles by *Aspergillus* species. J Chem Pharm Res 7:300–306

Bhainsa KC, D'Souza SF (2006) Extracellular biosynthesis of silver nanoparticles using the fungus Aspergillus fumigatus. Colloids Surf B Biointerfaces 47:160–164. https://doi.org/10.1016/j.colsurfb.2005.11.026

Bhambure R, Bule M, Shaligram N, Kamat M, Singhal R (2009) Extracellular biosynthesis of gold nanoparticles using *Aspergillus niger*—its characterization and stability. Chem Eng Technol 32:1036–1041. https://doi.org/10.1002/ceat.200800647

Bharathidasan R, Panneerselvam A (2012) Biosynthesis and characterization of silver nanoparticles using endophytic fungi Aspergillus concius, *Penicillium janthinellum* and *Phomosis* sp. Int J Pharm Sci Res 3:3163–3169

Bharde A, Rautaray D, Bansal V, Ahmad A, Sarkar I, Yusuf SM, Sanyal M, Sastry M (2006) Extracellular biosynthesis of magnetite using fungi. Small 2:135–141. https://doi.org/10.1002/smll.200500180

Bhattacharjee S, Debnath G, Das AR, Saha AK, Das P (2017) Characterization of silver nanoparticles synthesized using an endophytic fungus, *Penicillium oxalicum* having potential antimicrobial activity. Adv Nat Sci Nanosci Nanotechnol 8:045008. https://doi.org/10.1088/2043-6254/aa84ec

Birla SS, Tiwari VV, Gade AK, Ingle AP, Yadav AP, Rai MK (2009) Fabrication of silver nanoparticles by Phoma glomerata and its combined effect against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Lett Appl Microbiol 48:173–179. https://doi.org/10.1111/j.1472-765X.2008.02510.x

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. LWT - Food Sci Technol 28:25–30. https://doi.org/10.1016/S0023-6438(95)80008-5

Chandra H, Kumari P, Bontempi E, Yadav S (2020) Medicinal plants: treasure trove for green synthesis of metallic nanoparticles and their biomedical applications. Biocatal Agric Biotechnol 24:101518. https://doi.org/10.1016/j.bcab.2020.101518

Chandrappa CP, Govindappa M, Chandrasekar N, Sarkar S, Ooha S, Channabasava R (2016) Endophytic synthesis of silver chloride nanoparticles from *Penicillium* sp. of Calophyllum apetalum. Adv Nat Sci Nanosci Nanotechnol 7:025016. https://doi.org/10.1088/2043-6262/7/2/025016

Chauhan R, Reddy A, Abraham J (2015) Biosynthesis of silver and zinc oxide nanoparticles using Pichia fermentans JA2 and their antimicrobial property. Appl Nanosci 5:63–71. https://doi.org/10.1007/s13204-014-0292-7

Danagoudar A, Pratap GK, Shantaram M, Chatterjee B, Ghosh K, Kanade SR, Joshi CG (2020) Cancer cell specific cytotoxic potential of the silver nanoparticles synthesized using the endophytic fungus, *Penicillium citrinum* CGJ-C2. Mater Today Commun 25:101442. https://doi.org/10.1016/j.mtcomm.2020.101442

Das SK, Liang J, Schmidt M, Laffir F, Marsili E (2012) Biomineralization mechanism of gold by zygomycete fungus *Rhizopous oryzae*. ACS Nano 6:6165–6173. https://doi.org/10.1021/nn301502s

Devi LS, Joshi SR (2012) Antimicrobial and synergistic effects of silver nanoparticles synthesized using soil fungi of high altitudes of eastern Himalaya. Mycobiology 40:27–34. https://doi.org/10.5941/MYCO.2012.40.1.027

Devi LS, Joshi SR (2014) Evaluation of the antimicrobial potency of silver nanoparticles biosynthesized by using an endophytic fungus, Cryptosporiopsis ericae PS4. J Microbiol 52:667–674. https://doi.org/10.1007/s12275-014-4113-1

Devi LS, Joshi SR (2015) Ultrastructures of silver nanoparticles biosynthesized using endophytic fungi. J Microsc Ultrastruct 3:29–37. https://doi.org/10.1016/j.jmau.2014.10.004

Devi LS, Bareh DA, Joshi SR (2013) Studies on biosynthesis of antimicrobial silver nanoparticles using endophytic fungi isolated from the ethno-medicinal plant *Gloriosa superba* L. Proc Natl Acad Sci India B Biol Sci 84:1091–1099. https://doi.org/10.1007/s40011-013-0185-7

Ealias AM, Saravanakumar MP (2017) A review on the classification, characterisation, synthesis of nanoparticles and their application. IOP Conf Series Mater Sci Eng 263:032019. https://doi.org/10.1088/1757-899X/263/3/032019

Fatima F, Bajpai P, Pathak N, Singh S, Priya S, Verma SR (2015) Antimicrobial and immunomodulatory efficacy of extracellularly synthesized silver and gold nanoparticles by a novel

phosphate solubilizing fungus Bipolaris tetramera. BMC Microbiol 15:1–10. https://doi.org/10.1186/s12866-015-0391-y

Fayaz AM, Balaji K, Kalaichelvan PT, Venkatesan R (2009) Fungal based synthesis of silver nanoparticles—an effect of temperature on the size of particles. Colloids Surf B Biointerfaces 74:123–126. https://doi.org/10.1016/j.colsurfb.2009.07.002

Fayaz AM, Balaji K, Girilal M, Yadav R, Kalaichelvan PT, Venketesan R (2010) Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. Nanomedicine 6:103–109. https://doi.org/10.1016/j.nano.2009.04.006

Gade AK, Bonde P, Ingle AP, Marcato PD, Durán N, Rai MK (2008) Exploitation of *Aspergillus niger* for synthesis of silver nanoparticles. J Biobaased Mater Bioenergy 2:243–247. https://doi.org/10.1166/jbmb.2008.401

Gade A, Ingle A, Whiteley C, Rai M (2010) Mycogenic metal nanoparticles: progress and applications. Biotechnol Lett 32:593–600. https://doi.org/10.1007/s10529-009-0197-9

Gahlawat G, Choudhury AR (2019) A review on the biosynthesis of metal and metal salt nanoparticles by microbes. RSC Adv 9:12944–12967. https://doi.org/10.1039/c8ra10483b

Gajbhiye M, Kesharwani J, Ingle A, Gade A, Rai M (2009) Fungus-mediated synthesis of silver nanoparticles and their activity against pathogenic fungi in combination with fluconazole. Nanomedicine 5:382–386. https://doi.org/10.1016/j.nano.2009.06.005

Govindappa M, Farheen H, Chandrappa CP, Channabasava R, Rai RV, Raghavendra VB (2016) Mycosynthesis of silver nanoparticles using extract of endophytic fungi, *Penicillium* species of *Glycosmis mauritiana*, and its antioxidant, antimicrobial, anti-inflammatory and tyrokinase inhibitory activity. Adv Nat Sci Nanosci Nanotechnol 7:035014. https://doi.org/10.1088/2043-6262/7/3/035014

Gudikandula K, Vadapally P, Charya MAS (2017) Biogenic synthesis of silver nanoparticles from white rot fungi: their characterization and antibacterial studies. OpenNano 2:64–78. https://doi.org/10.1016/j.onano.2017.07.002

Gupta S, Bector S (2013) Biosynthesis of extracellular and intracellular gold nanoparticles by *Aspergillus fumigatus* and A. flavus. Anton Leeuw Int J Gen Mol Microbiol 103:1113–1123. https://doi.org/10.1007/s10482-013-9892-6

Gupta S, Sharma K, Sharma R (2012) Myconanotechnology and application of nanoparticles in biology. Recent Res Sci Technol 4:36–38

Hasan S (2015) A review on nanoparticles: their synthesis and types. Res J Recent Sci 4:1–3

Hasan S, Gupta G (2012) Fungal biodiversity: evolution & distribution—a review. Int J Appl Res Stud 1:1–8

Hulkoti NI, Taranath TC (2014) Biosynthesis of nanoparticles using microbes—a review. Colloids Surf B Biointerfaces 121:474–483. https://doi.org/10.1016/j.colsurfb.2014.05.027

Ingale AG, Chaudhari AN (2013) Biogenic synthesis of nanoparticles and potential applications: an eco-friendly approach. J Nanomed Nanotechol 4:1–7. https://doi.org/10.4172/2157-7439.1000165

Ingle A, Gade A, Pierrat S, Sonnichsen C, Rai M (2008) Mycosynthesis of silver nanoparticles using the fungus *Fusarium acuminatum* and its activity against some human pathogenic bacteria. Curr Nanosci 4:141–144. https://doi.org/10.2174/157341308784340804

Jaidev LR, Narasimha G (2010) Biointerfaces fungal mediated biosynthesis of silver nanoparticles, characterization and antimicrobial activity. Colloids Surf B Biointerfaces 81:430–433. https://doi.org/10.1016/j.colsurfb.2010.07.033

Jain N, Bhargava A, Majumdar S, Tarafdar JC, Panwar J (2011) Extracellular biosynthesis and characterization of silver nanoparticles using *Aspergillus flavus* NJP08: a mechanism perspective. Nanoscale 3:635–641. https://doi.org/10.1039/c0nr00656d

Karnani RL, Chowdhary A (2013) Biosynthesis of silver nanoparticle by eco-friendly method. Indian J Nanosci 1:25–31

Kashyap PL, Kumar S, Srivastava AK, Sharma AK (2012) Myconanotechnology in agriculture: a perspective. World J Microbiol Biotechnol 29:191–207. https://doi.org/10.1007/s11274-012-1171-6

Kathiresan K, Manivannan S, Nabeel MA, Dhivya B (2009) Studies on silver nanoparticles synthesized by a marine fungus, *Penicillium fellutanum* isolated from coastal mangrove sediment. Colloids Surf B Biointerfaces 71:133–137. https://doi.org/10.1016/j.colsurfb.2009.01.016

Khan SA, Gambhir S, Ahmad A (2014) Extracellular biosynthesis of gadolinium oxide (Gd2O3) nanoparticles, their biodistribution and bioconjugation with the chemically modified anticancer drug taxol. Beilstein J Nanotechnol 5:249–257. https://doi.org/10.3762/bjnano.5.27

Khandel P, Shahi SK (2016) Microbes mediated synthesis of metal nanoparticles: current status and future prospects. Int J Nanomater Biostruct 6:1–24

Koli SH, Mohite BV, Borase HP, Patil SV (2017) *Monascus* pigments mediated rapid green synthesis and characterization of gold nanoparticles with possible mechanism. J Cluster Sci 28:2719–2732. https://doi.org/10.1007/s10876-017-1254-5

Koli SH, Mohite BV, Suryawanshi RK, Borase HP, Patil SV (2018) Extracellular red *Monascus* pigment-mediated rapid one-step synthesis of silver nanoparticles and its application in biomedical and environment. Bioprocess Biosyst Eng 41:715–727. https://doi.org/10.1007/s00449-018-1905-4

Krishnan S, Narayan S, Chadha A (2016) Whole resting cells vs. cell free extracts of *Candida parapsilosis* ATCC 7330 for the synthesis of gold nanoparticles. AMB Express 6:1–15. https://doi.org/10.1186/s13568-016-0268-y

Kumar SA, Abyaneh MK, Gosavi SW, Kulkarni SK, Pasricha R, Ahmad A, Khan MI (2007) Nitrate reductase-mediated synthesis of silver nanoparticles from AgNO3. Biotechnol Lett 29:439–445. https://doi.org/10.1007/s10529-006-9256-7

Kumar S, Kumari P, Singh R (2020) Emerging nanomaterials for Cancer therapy. In: Shukla A (ed) Nanoparticles in medicine. Springer, Singapore, pp 25–54. https://doi.org/10.1007/978-981-13-8954-2_2

Manjunath HM, Joshi CG, Raju NG (2014) Biogenic synthesis of silver nano particles using endophytic fungi Penicillium nodositatum and its antibacterial activity. J Chem Pharm Res 6:112–117

Manjunath HM, Joshi CG, Raju NG (2016) Biofabrication of gold nanoparticles using marine endophytic fungus–*Penicillium citrinum*. IET Nanobiotechnol 11:40–44. https://doi.org/10.1049/iet-nbt.2016.0065

Manjunath Hulikere M, Joshi CG, Ananda D, Poyya J, Kudva AK, Dhananjaya BL (2017) Biogenic synthesis of gold nanoparticles by marine endophytic fungus-*Cladosporium cladosporioides* isolated from seaweed and evaluation of their antioxidant and antimicrobial properties. Process Biochem 63:137–144. https://doi.org/10.1016/j.procbio.2017.09.008

Mazhar T, Shrivastava V, Tomar RS (2017) Green synthesis of bimetallic nanoparticles and its applications: a review. J Pharm Sci Res 9:102–110

Mazumdar H, Haloi N (2011) A study on biosynthesis of iron nanoparticles by Pleurotus sp. J Microbiol Biotechnol Res 1:39–49

Mishra A, Kumari M, Pandey S, Chaudhry V, Gupta KC, Nautiyal CS (2014) Biocatalytic and antimicrobial activities of gold nanoparticles synthesized by *Trichoderma* sp. Bioresour Technol 166:235–242. https://doi.org/10.1016/j.biortech.2014.04.085

Mittal AK, Chisti Y, Banerjee UC (2013) Synthesis of metallic nanoparticles using plant extracts. Biotechnol Adv 31:346–356. https://doi.org/10.1016/j.biotechadv.2013.01.003

Mohanpuria P, Rana NK, Yadav SK (2008) Biosynthesis of nanoparticles: technological concepts and future applications. J Nanoparticle Res 10:507–517. https://doi.org/10.1007/s11051-007-9275-x

Mukherjee S, Patra CR (2017) Biologically synthesized metal nanoparticles: recent advancement and future perspectives in cancer theranostics. Future Sci OA 3:FSO203

Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Ramani R, Parischa R, Ajayakumar PV, Alam M, Sastry M, Kumar R (2001) Bioreduction of AuCl4− ions by the fungus, *Verticillium* sp. and surface trapping of the gold nanoparticles formed. Angew Chem Int Ed 40:3585–3588. https://doi.org/10.1002/1521-3773(20011001)40:19<3585::AID-ANIE3585>3.0.CO;2-K

Mukherjee P, Senapati S, Mandal D, Ahmad A, Khan MI, Kumar R, Sastry M (2002) Extracellular synthesis of gold nanoparticles by the fungus *Fusarium oxysporum*. Chembiochem 3:461–463. https://doi.org/10.1002/1439-7633(20020503)3:5<461::AID-CBIC461>3.0.CO;2-X

Mukherjee P, Roy M, Mandal BP, Dey GK, Mukherjee PK, Ghatak J, Tyagi AK, Kale SP (2008) Green synthesis of highly stabilized nanocrystalline silver particles by a non-pathogenic and agriculturally important fungus *T. asperellum*. Nanotechnology 19:075103. https://doi.org/10.1088/0957-4484/19/7/075103

Mustafa S, Khan HM, Shukla I, Shujatullah F, Shahid M, AshfaqM AA (2011) Effect of ZnO nanoparticles on ESBL producing *Escherichia coli* & *Klebsiella* spp. Eastern J Med 16:253–257

Nachiyar V, Sunkar S, Prakash P, Bavanilatha (2015) Biological synthesis of gold nanoparticles using endophytic fungi. Der Pharma Chem 7:31–38

Namasivayam SKR, Avimanyu (2011) Silver nanoparticle synthesis from *Lecanicillium lecanii* and evalutionary treatment on cotton fabrics by measuring their improved antibacterial activity with antibiotics against Staphylococcus aureus (ATCC 29213) and E. coli (ATCC 25922) strains. Int J Pharm Pharm Sci 3:190–195

Narasimha G (2013) Virucidal properties of silver nanoparticles synthesized from white button mushrooms (*Agaricus bisporus*). Int J Nano Dimension 3:181–184. https://doi.org/10.7508/IJND.2012.03.002

Narayanan KB, Sakthivel N (2010) Biological synthesis of metal nanoparticles by microbes. Adv Colloid Interf Sci 156:1–13. https://doi.org/10.1016/j.cis.2010.02.001

Netala VR, Kotakadi VS, Bobbu P, Gaddam SA, Tartte V (2016a) Endophytic fungal isolate mediated biosynthesis of silver nanoparticles and their free radical scavenging activity and antimicrobial studies. 3 Biotech 6:1–9. https://doi.org/10.1007/s13205-016-0433-7

Netala VR, Bethu MS, Pushpalatha B, Baki VB, Aishwarya S, Rao JV, Tartte V (2016b) Biogenesis of silver nanoparticles using endophytic fungus *Pestalotiopsis microspora* and evaluation of their antioxidant and anticancer activities. Int J Nanomed 11:5683–5696. https://doi.org/10.2147/IJN.S112857

Omar RA, Afreen S, Talreja N, Chauhan D, Ashfaq M, Srituravanich W (2019) Impact of nanomaterials on the microbial system. In: Prasad R (ed) Microbial nanobionics. Nanotechnology in the life sciences. Springer, Cham, pp 141–158. https://doi.org/10.1007/978-3-030-16383-9_6

Patel S, Singh D, Srivastava S, Singh M, Shah K, Chauahn DN, Chauahn NS (2017) Nanoparticles as a platform for antimicrobial drug delivery. Adv Pharmacol Pharm 5:31–43. https://doi.org/10.13189/app.2017.050301

Popli D, Anil V, Subramanyam AB, Namratha MN, Ranjitha VR, Rao SN, Rai RV, Govindappa M (2018) Endophyte fungi, Cladosporium species-mediated synthesis of silver nanoparticles possessing in vitro antioxidant, anti-diabetic and anti-Alzheimer activity. Artif Cells Nanomed Biotechnol 46:676–683. https://doi.org/10.1080/21691401.2018.1434188

Raheman F, Deshmukh S, Ingle A, Gade A, Rai M (2011) Silver nanoparticles: novel antimicrobial agent synthesized from an endophytic fungus Pestalotia sp. isolated from leaves of *Syzygium cumini* (L). Nano Biomed Eng 3:174–178. https://doi.org/10.5101/nbe.v3i3.p174-178

Rajakumar G, Rahuman AA, Roopan SM, Khanna VG, Elango G, Kamaraj C, Zahir AA, Velayutham K (2012) Fungus-mediated biosynthesis and characterization of TiO_2 nanoparticles and their activity against pathogenic bacteria. Spectrochim Acta A Mol Biomol Spectrosc 91:23–29. https://doi.org/10.1016/j.saa.2012.01.011

Ramalingmam P, Muthukrishnan S, Thangaraj P (2015) Biosynthesis of silver nanoparticles using an endophytic fungus, *Curvularia lunata* and its antimicrobial potential. J Nanosci Nanoeng 1:241–247

Saha S, Sarkar J, Chattopadhyay D, Patra S, Chakraborty A, Acharya K (2010) Production of silver nanoparticles by a phytopathogenic fungus *Bipolaris nodulosa* and its antimicrobial activity. Dig J Nanomater Biostruct 5:887–895

Salunkhe RB, Patil SV, Patil CD, Salunke BK (2011) Larvicidal potential of silver nanoparticles synthesized using fungus *Cochliobolus lunatus* against Aedes aegypti (Linnaeus, 1762) and

Anopheles stephensi Liston (Diptera; Culicidae). Parasitol Res 109:823–831. https://doi.org/10.1007/s00436-011-2328-1

Sandana Mala JG, Rose C (2014) Facile production of ZnS quantum dot nanoparticles by *Saccharomyces cerevisiae* MTCC 2918. J Biotechnol 170:73–78. https://doi.org/10.1016/j.jbiotec.2013.11.017

Sanghi R, Verma P (2009a) A facile green extracellular biosynthesis of CdS nanoparticles by immobilized fungus. Chem Eng J 155:886–891. https://doi.org/10.1016/j.cej.2009.08.006

Sanghi R, Verma P (2009b) Biomimetic synthesis and characterisation of protein capped silver nanoparticles. Bioresour Technol 100:501–504. https://doi.org/10.1016/j.biortech.2008.05.048

Sastry M, Ahmad A, Khan MI, Kumar R (2003) Biosynthesis of metal nanoparticles using fungi and actinomycete. Curr Sci 85:162–170

Sawle BD, Salimath B, Deshpande R, Bedre MD, Prabhakar BK, Venkataraman A (2008) Biosynthesis and stabilization of Au and Au-Ag alloy nanoparticles by fungus, *Fusarium semitectum*. Sci Technol Adv Mater 9:035012. https://doi.org/10.1088/1468-6996/9/3/035012

Saxena J, Sharma MM, Gupta S, Singh A (2014) Emerging role of fungi in nanoparticle synthesis and their applications. World J Pharm Sci 3:1586–1613

Selvakumar R, Seethalakshmi N, Thavamani P, Naidu R, Megharaj M (2014) Recent advances in the synthesis of inorganic nano/microstructures using microbial biotemplates and their applications. RSC Adv 4:52156–52169. https://doi.org/10.1039/c4ra07903e

Seshadri S, Saranya K, Kowshik M (2011) Green synthesis of lead sulfide nanoparticles by the lead resistant marine yeast, *Rhodosporidium diobovatum*. Biotechnol Prog 27:1464–1469. https://doi.org/10.1002/btpr.651

Shankar SS, Ahmad A, Pasricha R, Sastry M (2003) Bioreduction of chloroaurate ions by geranium leaves and its endophytic fungus yields gold nanoparticles of different shapes. J Mater Chem 13:1822–1826. https://doi.org/10.1039/b303808b

Singara Charya MA (2015) Fungi: an overview. In: Bahadur B, Venkat Rajam M, Sahijram L, Krishnamurthy K (eds) Plant biology and biotechnology. Springer, New Delhi, pp 197–215. https://doi.org/10.1007/978-81-322-2286-6_7

Singh D, Rathod V, Ninganagouda S, Hiremath J, Singh AK, Mathew J (2014) Optimization and characterization of silver nanoparticle by endophytic fungi *Penicillium* sp. isolated from Curcuma longa (turmeric) and application studies against MDR E. coli and S. aureus. Bioinorg Chem Appl 2014:1–8. https://doi.org/10.1155/2014/408021

Singh AK, Rathod V, Singh D, Ninganagouda S, Kulkarni P, Mathew J, Haq MU (2015) Bioactive silver nanoparticles from endophytic fungus *Fusarium* sp. isolated from an ethanomedicinal plant Withania somnifera (Ashwagandha) and its antibacterial activity. Int J Nanomater Biostruct 5:15–19

Singh DK, Kumar J, Sharma VK, Verma SK, Singh A, Kumari P, Kharwar RN (2017a) Mycosynthesis of bactericidal silver and polymorphic gold nanoparticles: physicochemical variation effects and mechanism. Nanomedicine 13:191–207. https://doi.org/10.2217/nnm-2017-0235

Singh T, Jyoti K, Patnaik A, Singh A, Chauhan R, Chandel SS (2017b) Biosynthesis, characterization and antibacterial activity of silver nanoparticles using an endophytic fungal supernatant of *Raphanus sativus*. J Genet Eng Biotechnol 15:31–39. https://doi.org/10.1016/j.jgeb.2017.04.005

Soni N, Prakash S (2012a) Fungal-mediated nano silver: an effective adulticide against mosquito. Parasitol Res 111:2091–2098. https://doi.org/10.1007/s00436-012-3056-x

Soni N, Prakash S (2012b) Synthesis of gold nanoparticles by the fungus *Aspergillus niger* and its efficacy against mosquito larvae. Rep Parasitol 2012:1–7. https://doi.org/10.2147/rip.s29033

Sri S, Rajagopal K (2016) Isolation and identification of thermo tolerant endophytic fungi from *Melia dubia* and synthesis of zinc nano particles. Int J Nanosci Nanotechnol 7:99–112

Sundaramoorthi C, Kalaivani M, Mathews DM, Palanisamy S, Kalaiselvan V, Rajasekaran A (2009) Biosynthesis of silver nanoparticles from *Aspergillus niger* and evaluation of its wound healing activity in experimental rat model. Int J PharmTech Res 1:1523–1529

Syed A, Ahmad A (2012) Extracellular biosynthesis of platinum nanoparticles using the fungus *Fusarium oxysporum*. Colloids Surf B Biointerfaces 97:27–31. https://doi.org/10.1016/j.colsurfb.2012.03.026

Talie MD, Wani AH, Ahmad N, Bhat MY, War JM (2020) Green synthesis of silver nanoparticles (Agnps) using Helvella Leucopus Pers. and their antimycotic activity against fungi causing fungal rot of apple. Asian J Pharm Clin Res 13:161–165. https://doi.org/10.22159/ajpcr.2020.v13i4.37024

Thakkar KN, Mhatre SS, Parikh RY (2010) Biological synthesis of metallic nanoparticles. Nanomedicine 6:257–262. https://doi.org/10.1016/j.nano.2009.07.002

Thirumurugan G, Veni VS, Ramachandran S, Rao JVLNS, Dhanaraju MD (2011) Superior wound healing effect of topically delivered silver nanoparticle formulation using eco-friendly potato plant pathogenic fungus: synthesis and characterization. J Biomed Nanotechnol 7:659–666. https://doi.org/10.1166/jbn.2011.1336

Tripathi RM, Gupta RK, Singh P, Bhadwal AS, Shrivastav A, Kumar N, Shrivastav BR (2014) Ultra-sensitive detection of mercury(II) ions in water sample using gold nanoparticles synthesized by *Trichoderma harzianum* and their mechanistic approach. Sensors Actuators B Chem 204:637–646. https://doi.org/10.1016/j.snb.2014.08.015

Uddandarao P, Balakrishnan RM (2016) ZnS semiconductor quantum dots production by an endophytic fungus *Aspergillus flavus*. Mater Sci Eng B 207:26–32. https://doi.org/10.1016/j.mseb.2016.01.013

Uddandarao P, Balakrishnan RM (2017) Thermal and optical characterization of biologically synthesized ZnS nanoparticles synthesized from an endophytic fungus *Aspergillus flavus*: a colorimetric probe in metal detection. Spectrochim Acta A Mol Biomol Spectrosc 175:200–207. https://doi.org/10.1016/j.saa.2016.12.021

Uddandarao P, Balakrishnan RM, Ashok A, Swarup S, Sinha P (2019) Bioinspired ZnS:Gd nanoparticles synthesized from an endophytic fungi *Aspergillus flavus* for fluorescence-based metal detection. Biomimetics 4:1–10. https://doi.org/10.3390/biomimetics4010011

Vala AK (2014) Intra-and extracellular biosynthesis of gold nanoparticles by a marine-derived fungus *Rhizopus oryzae*. Synth React Inorg Metal Org Nano-Metal Chem 44:1243–1246. https://doi.org/10.1080/15533174.2013.799492

Vala AK, Shah S (2012) Rapid synthesis of silver nanoparticles by a marine-derived fungus *Aspergillus niger* and their antimicrobial potentials. Int J Nanosci Nanotechnol 8:197–206

Velusamy P, Kumar GV, Jeyanthi V, Das J, Pachaiappan R (2016) Bio-inspired green nanoparticles: synthesis, mechanism, and antibacterial application. Toxicol Res 32:95–102

Verma VC, Kharwar RN, Gange AC (2010) Biosynthesis of antimicrobial silver nanoparticles by the endophytic fungus *Aspergillus clavatus*. Nanomedicine 5:33–40. https://doi.org/10.2217/NNM.09.77

Verma VC, Singh SK, SolankiR PS (2011) Biofabrication of anisotropic gold nanotriangles using extract of endophytic *Aspergillus clavatus* as a dual functional reductant and stabilizer. Nanoscale Res Lett 6:1–7. https://doi.org/10.1007/s11671-010-9743-6

Vijayan S, Divya K, George TK, Jisha MS (2016) Biogenic synthesis of silver nanoparticles using endophytic fungi *Fusarium oxysporum* isolated from *Withania somnifera* (L.), its antibacterial and cytotoxic activity. J Bionanosci 10:369–376. https://doi.org/10.1166/jbns.2016.1390

Vijayanandan AS, Balakrishnan RM (2018) Biosynthesis of cobalt oxide nanoparticles using endophytic fungus *Aspergillus nidulans*. J Environ Manag 218:442–450. https://doi.org/10.1016/j.jenvman.2018.04.032

Yadav A, Kon K, Kratosova G, Duran N, Ingle AP, Rai M (2015) Fungi as an efficient mycosystem for the synthesis of metal nanoparticles: progress and key aspects of research. Biotechnol Lett 37:2099–2120. https://doi.org/10.1007/s10529-015-1901-6

Mycofabrication of Metal Nanoparticles: A Green Approach

9

Aniket Gade and Mahendra Rai

Abstract

Nanotechnology is a broad and multidisciplinary area of research, which is growing explosively worldwide. Nanoparticles are considered as basic or fundamental units of nanotechnology, arising the need to develop safer, reliable, clean, and eco-friendly methods for the fabrication of these nanoparticles. The fabrication of nanoparticles is an important aspect of the rapidly emerging field of nanoscale engineering. Nanoparticles can be fabricated either by top-down or by bottom-up approach. Broadly, methods can be classified into biological, chemical, and physical methods of nanoparticle fabrication. The use of the biological system (plants, bacteria, fungi, algae, protozoa, and animals) for the fabrication of nanoparticles is a relatively new phenomenon. Mycofabrication is the fabrication of metal nanoparticles using fungi, which is superior as compared to other biological systems used for the fabrication of metal nanoparticles. Mycofabrication process involves the use of water as a solvent and biomolecules (fungal secretions) as reducing and stabilization agents rendering it a green process. The exact mechanism of mycofabrication of metal nanoparticles needs to be unraveled to have better control on the fabrication of metal nanoparticles of particular size and shape. The in-depth study will help to elucidate the exact mechanistic aspect for the mycofabrication of metal nanoparticles, as different fungal secretions react differently during the fabrication of metal nanoparticles.

Keywords

Fungi · Green method · Mycofabrication · Myconanotechnology · Nanoparticles

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9.1 Introduction

Nanotechnology promises the fabrication of materials and products, which can be functionalized for a wide range of applications. In the coming years, we can experience a major technological changes resulting due to the fabrication of nano-scale (1–100 nm) materials by novel fabrication techniques or method. The small size of the materials can result in unique physicochemical properties like improved surface area, solubility, and reactivity. The multifunctionality of nanoparticles can open up new research avenues for researchers.

Nanotechnology can be defined as research and technology development at nano-scale, whereby creating a structure, devices, or systems possessing unique properties and having at least one dimension in the range of 1–100 nm. Every material or substance regardless of composition will exhibit different properties at nano-scale. Materials at nano-scale size show larger surface-area-to-volume ratio resulting into an increased number of molecules on the surface of the material. The more the number of molecules at the surface, the higher will be the reactivity, which results into increase surface tension, decreased gravitation, and confinement of electrons. The unusual properties of nanomaterials can be attributed to the confinement of electrons on the surface, the process known as quantum confinement (Fendler 1992; Alivisatos 1996); the transport of electrons can result from a phenomenon like Coulomb blockade (Feldheim and Keating 1998). Customization of catalytic and thermodynamic properties of structures can be achieved while designing the materials on this length scale. This can lead to exhibiting unique or novel physico-chemical properties by nano-scale material as compared to the bulk material of the similar elemental composition. This is attracting the attention of several workers to exploit the multiple functionalities of nano-scale materials (Rai et al. 2011).

The fabrication of metal nanoparticles can be classified on the basis of starting precursor material used for the fabrication of nanoparticles into either bottom-up approach or top-down approach. In the top-down approach of nanoparticle fabrication, the starting material is a bulk metal, which can be converted into nano-scale material by either cutting, carving, or molding. The nano-scale material is further used to fabricate a variety of machinery, devices, and system. The limiting factor in top-down approach is the ability to cut, carve, or mold the material at a nano-scale without much defects. Most of the physical methods like electron beam lithography, ball milling, mechanical grinding, or vapor deposition follow the top-down approach for the fabrication of nanoparticles, where the bulk metal is reduced to nano-scale material and is subsequently stabilized by the addition of capping agent or by stabilizing agents. In the case of the bottom-up approach for the fabrication of metal nanoparticles, the starting material will be simple molecules or atoms having a size smaller than those of nanoparticles and which are held together by the covalent forces that are much stronger than the forces involved in the holding of macroscale components. Chemical methods like wet chemical reduction, electrochemical method, and biological methods which generally rely on the reduction of precursor metal salt solution for the fabrication of metal nanoparticles follow the bottom-up approach for the fabrication of metal nanoparticles (Rai and Gade 2009).

All the methods of nanoparticle fabrication like physical, chemical, biological, or hybrid can be a part of either top-down or bottom-up approach of nanoparticle fabrication. Both approaches play a significant role in nanotechnology in general and in fabrication of metal nanoparticles in particular, though each approach possess certain advantages and limitations of their own. The major limitation in the case of top-down approach is the creation of nano-scale structures with greater accuracy or without any structural defect, whereas in the case of bottom-up approach, the major issue is the creation of larger size nanostructure and of sufficient quantity in a short time to be used as nanomaterial (Fendler 1998). Bottom-up approach has the advantage of obtaining nanostructure with lesser defects, monodispersed nanostructure, and greater control with respect to the fabrication of diverse nanostructures of different sizes and shapes, since the fabricated nanostructures are in thermodynamic equilibrium, which is driven by a decrease in Gibbs free energy. By top-down approach, it is advantageous to fabricate the large quantity of nanoparticles within short time. Therefore, the selection of the approach for the fabrication of nanoparticle is based on the application to be performed by the fabricated nanoparticles.

Another classification includes chemical, physical, biological, and hybrid methods for nanoparticle fabrication. The chemical methods for the fabrication of metal nanoparticles are energy-consuming, employ toxic chemicals, and often yield nanoparticles in non-polar organic solution, thereby impeding biomedical applications (Ayyub et al. 2001; Kokura et al. 2010). Similarly, the physical methods of nanoparticle fabrication are also energy-intensive, where narrow size distribution of the fabricated nanoparticles is often tough to be achieved. Moreover, physical method of metal nanoparticle fabrication uses high temperature and pressure and thus is not eco-friendly in nature. The biological method of nanoparticle fabrication is performed at ambient temperature and pressure, thus making it the viable candidate for the development of eco-friendly method for the fabrication of metal nanoparticles (Jahagirdar et al. 2020; Salem and Fouda 2020).) Among the biological system, the fungal system has been found to be the better system as compared to other biological systems. The amalgamation of mycology and nanotechnology is known as "myconanotechnology," the term proposed by Rai and Gade (2009) to point out research on mycology in the fabrication of metal nanoparticles and their contribution in the field of nanotechnology research.

Fabrication of metal nanoparticles by the biological system has been studied by several researchers due to the ease and convenience of the method. In this chapter, the focus will be on one of the most versatile biological systems used for the fabrication of nanoparticles, i.e., fungi. The chapter highlights the advantages of mycofabrication process and mycofabrication as a green process, enlists the metal nanoparticles fabricated by different fungal species and applications of mycofabricated nanoparticles, and also focuses on elucidating the mechanistic aspect of mycofabrication process.

9.2 Mycofabrication of Metal Nanoparticles

In the current bioeconomy, the fungal system plays a significant role in solving key global problems for sustainable development. Fungi enhance resource efficiency, converting waste to economically valuable ingredients, enabling crop plants stronger to survive harsh climatic conditions, and working as host organisms for the fabrication of new metabolites (Lange 2014). Also, fungi are one of the most promising organisms to search for new drug candidates including antimicrobials. Moreover, the switching from chemical to biological process can be accomplished by fungal enzymes, while the chemical processes such as sol-gel, chemical vapor deposition, borohydride reduction, citrate reduction, alcohol reduction, etc. can affect significantly the human and environment. The fabrication of metal nanoparticles by fungi is known as mycofabrication and the process mycosynthesis. The fungal system was found to be a versatile biological system with the ability to fabricate the metal nanoparticles intracellularly as well as extracellularly. The fungal system is generally preferred over other biological systems because the fungi are omnipresent in nature and play a crucial role in the fabrication of metal nanoparticles (Yadav et al. 2015).

In the past, several fungal species have been exploited for the fabrication of diverse metal nanoparticles of different shapes and sizes. *Colletotrichum* species, an endophytic fungus dwelling in the leaves of geranium, form gold nanoparticles when treated with chloroaurate ions. The fabricated particles were mainly decahedral and icosahedral in shape with a size ranging from 20 to 40 nm (Shivshankar et al. 2003). Later, Bansal et al. (2004) reported that exposure of *Fusarium oxysporum* secretions to an aqueous solution of K_2ZrF_6 leads to the formation of crystalline zirconia nanoparticles on hydrolysis of the zirconium hexafluoride ions extracellularly at room temperature by the proteins. Among the different fungal genera available for the fabrication of nanoparticles, the genus *Fusarium* was reported by many investigators. The mycofabrication of nanocrystallites in yeast has been reported by Dameron et al. (1989) in *Candida glabrata* and *Schizosaccharomyces pombe* grown in the presence of cadmium salt. Kowshik et al. (2002) claimed the fabrication of cadmium sulfide nanoparticles inside the cells by *S. pombe* strain when treated with 1 mM cadmium precursor salt solution. The fabricated CdS nanoparticles demonstrated an absorbance at 305 nm. X-ray diffraction data reveal that the nanoparticles had Wurtzite (Cd16S20)-type hexagonal lattice crystal arrangement with a size range of 1–1.5 nm.

There are numerous reports on the fabrication of metal nanoparticles by different fungi; the exhaustive list of different fungi involved with the fabrication of different metal nanoparticles is given in Table 9.1.

The mycofabricated metal nanoparticles have exhibited distinct, optical, electromagnetic, biological, and catalytic properties (Rai et al. 2008). This has generated greater interest among the researchers to functionalize the fabricated nanoparticles and used them in several applications. A schematic representation of different applications of mycofabricated metal nanoparticles is given in Fig. 9.1.

Table 9.1 List of mycofabricated metal nanoparticles

Name of fungi	Type of nanoparticle	References
Alternaria alternata	Gold, silver	Sarkar et al. (2012), Gajbhiye et al. (2009)
Alternaria sp.	Silver	Gaikwad et al. (2013b)
Aspergillus clavatus	Gold	Verma et al. (2011)
Aspergillus flavus	Silver	Vigneshwaran et al. (2007)
Aspergillus fumigatus	Zinc oxide, silver	Raliya and Tarafdar (2013), Bhainsa and D'Souza (2006)
Aspergillus niger	Silver, gold, copper	Gade et al. (2008), Bhambure et al. (2009), Noor et al. (2020)
Aspergillus oryzae	Iron, gold	Raliya (2013), Binupriya et al. (2010)
Aspergillus sydowii	Gold	Vala (2015)
Aspergillus terreus	Silver	Li et al. (2012)
Aureobasidium pullulans	Gold	Zhang et al. (2011)
Candida albicans	Gold	Ahmad et al. (2013)
Candida. glabrata	Cadmium sulfide	Krumov et al. (2007)
Cladosporium cladosporioides	Silver	Balaji et al. (2009)
Colletotrichum sp.	Gold	Shankar et al. (2003)
Coriolus versicolor	Gold, silver	Sanghi and Verma (2009, 2010)
Curvularia indicum	Silver	Gaikwad et al. (2013b)
Curvularia sp.	Silver	Gaikwad et al. (2013b)
Cylindrocladium floridanum	Gold	Narayanan and Sakthivel (2013)
Epicoccum nigrum	Gold	Govender et al. (2009)
Fusarium acuminatum	Silver, gold	Ingle et al. (2008), Tidke et al. (2014)
Fusarium culmorum	Silver	Bawaskar et al. (2010)
Fusarium equiseti	Silver	Gaikwad et al. (2013a)
Fusarium graminearum	Silver	Gaikwad et al. (2013a)
Fusarium moniliforme	Silver	Gaikwad et al. (2013a)
Fusarium oxysporum	Silver, gold, lead and cadmium carbonate, strontium carbonate, cadmium sulfide, silica and titania, barium titanate, zirconia	Birla et al. (2013), Bansod et al. (2013), Sanyal et al. (2005), Rautaray et al. (2004), Ahmad et al. (2002), Bansal et al. (2004, 2005, 2006)
Fusarium proliferatum	Silver	Gaikwad et al. (2013a)
Fusarium scirpi	Silver	Gaikwad et al. (2013a)
Fusarium semitectum	Gold and gold-silver alloy, silver	Sawle et al. (2008), Gaikwad et al. (2013a)
Fusarium solani	Silver	Ingle et al. (2009)
Fusarium tricinctum	Silver	Gaikwad et al. (2013a)

(continued)

Table 9.1 (continued)

Name of fungi	Type of nanoparticle	References
Ganoderma lucidum	Silver	Karwa et al. (2011)
Hansenula anomala	Gold	Sathish Kumar et al. (2011)
Helminthosporium solani	Gold	Kumar et al. (2009)
Helminthosporium tetramera	Silver	Shelar and Chavan (2014)
Helvella leucopus	Silver	Talie et al. (2020)
Hormoconis resinae	Gold	Mishra et al. (2010)
Lasiodiplodia theobromae	Silver	Ranjani et al. (2020)
Lentinus edodes	Silver	Adeeyo and Odiyo (2018)
Lentinus sajor-caju	Silver	Chan and Mashitah (2012)
Macrophomina phaseolina	Silver	Joshi et al. (2013)
Nemania sp.	Silver	Farsi and Farokhi (2018)
Neurospora crassa	Silver, gold, and bimetallic	Castro Longoria et al. (2011)
Nigrospora oryzae	Gold	Kar et al. (2014)
Penicillium brevicompactum	Gold	Mishra et al. (2011)
Penicillium citrinum	Silver	Honary et al. (2013)
Penicillium fellutanum	Silver	Kathiresan et al. (2009)
Penicillium glabrum	Silver	Nanda and Majeed (2014)
Penicillium nalgiovense AJ12	Silver	Maliszewska et al. (2014)
Penicillium ochrochloron	Silver	Devi and Joshi (2015)
Penicillium rugulosum	Gold	Mishra et al. (2012)
Penicillium sp.	Gold	Du et al. (2011)
Pestalotia sp.	Silver	Raheman et al. (2011)
Phanerochaete chrysosporium	Gold, cadmium sulfide	Sanghi et al. (2011), Chen et al. (2014)
Phoma arachidicola	Silver	Gade et al. (2013)
Phoma betae	Silver	Gade et al. (2013)
Phoma capsulatum	Silver	Rai et al. (2015a)
Phoma citri	Silver	Rai et al. (2015a)
Phoma destructiva	Silver	Gade et al. (2013)
Phoma exigua var. *exigua*	Silver	Shende et al. (2017)
Phoma fimeti	Silver	Gade et al. (2013)
Phoma gardeniae	Silver	Rai et al. (2015b)
Phoma glomerata	Silver, iron oxide, gold	Birla et al. (2009), Gade et al. (2014), Gudadhe et al. (2011), Kuralkar et al. (2015)
Phoma herbarum	Silver	Gade et al. (2013)

(continued)

Table 9.1 (continued)

Name of fungi	Type of nanoparticle	References
Phoma macrostoma	Silver	Gade et al. (2013)
Phoma medicaginis	Silver	Gade et al. (2013)
Phoma multirostrata	Silver	Gade et al. (2013)
Phoma pomorum	Silver	Gade et al. (2013)
Phoma putaminum	Silver	Rai et al. (2015a)
Phoma sorghina	Silver rods	Gade et al. (2011)
Phoma sp.	Silver	Gaikwad et al. (2013b)
Phoma tropica	Silver	Gade et al. (2013)
Pleurotus sp.	Silver	Gade et al. (2007)
Pythium sp.	Silver	Gade et al. (2016)
Pycnoporus sanguineus	Silver	Chan and Mashitah (2012)
Rhizopus oryzae	Gold	Das et al. (2012)
Saccharomyces cerevisiae	Gold	Sen et al. (2011)
Schizophyllum commune	Silver	Chan and Mashitah (2012)
Schizosaccharomyces pombe	Cadmium sulfide	Kowshik et al. (2002)
Sclerotium rolfsii	Gold	Narayanan and Sakthivel (2011)
Trichoderma asperellum	Silver	Mukherjee et al. (2008)
Trichoderma harzianum	Silver	Ahluwalia et al. (2014)
Trichoderma koningii	Gold	Maliszewska et al. (2009)
Trichoderma reesei	Silver	Vahabi et al. (2011)
Trichoderma viride	Silver	Fayaz et al. (2010)
Verticillium luteoalbum	Gold	Gericke and Pinches (2006)
Verticillium sp.	Gold	Mukherjee et al. (2001)
Volvariella volvacea	Silver, gold, and silver-gold	Philip (2009)
Yarrowia lipolytica	Gold	Agnihotri et al. (2009)

9.3 Why Mycofabrication?

Fabrication of metal nanoparticles to date has been reported by biological agents like bacteria, actinomycetes, cyanobacteria, fungi, yeast, algae, protozoa, animals, and plants (Yadav et al. 2015). Among all the biological systems used for the fabrication of metal nanoparticles, the fungal system scores over the other biological systems. Moreover, the fungal system was found to be the most versatile and efficient system for the fabrication of metal nanoparticles as fungi possess distinctive characters that

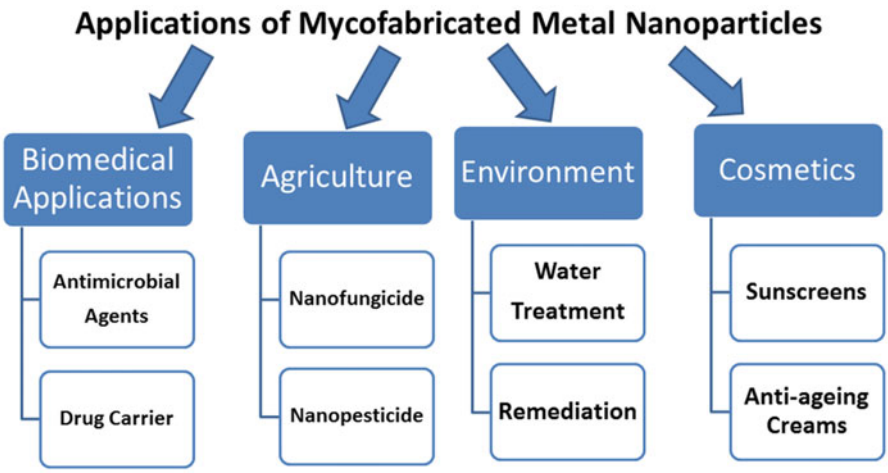

Fig. 9.1 Applications of mycofabricated metal nanoparticles

include easy to grow and culture in lab, convenient biomass handling, high cell wall binding capacity, etc. The mycofabricated nanoparticles were found to be highly monodispersed, biocompatible, and stable. As fungi are known to secrete a high amount of biomolecules, they can fabricate and produce nanoparticles in bulk quantity. The fungal secretions, which include proteins, are capable of hydrolyzing metal ions (Rai et al. 2010). Moreover, the nanoparticles are fabricated extracellularly, making their purification or downstream processing simpler and even handling of fungal biomass less complex. Fungi being eukaryote show a higher accumulation of metal ions by physicochemical and biological mechanisms. The accumulation on metal ions by the fungal biomass can be enhanced by extracellularly bound metabolites, polymers, and polypeptides (Volesky and Holan 1995). The repeated use of fungal biomass for nanoparticle fabrication has made the process more effective, and fungal biomass can be easily immobilized; it becomes easy to separate the immobilized fungal biomass from the reaction mixture and used it for another batch. The fungal secretion of enzymes makes the process extracellular with the advantage in the downstream processing (Gade et al. 2008), as compared to the bacterial system. The bacterial system generally fabricates the nanoparticles intracellularly; therefore, purification of nanoparticles involves the use of sophisticated instruments and makes the downstream processing a tedious one. Fungi are excellent secretors of protein, sugar, or metabolites as compared to bacteria and actinomycetes, enabling fungi to fabricate large quantities of nanoparticles within a short time (Sastry et al. 2003), thus making the fungi as the preferred biological system for the rapid and eco-friendly fabrication of metal nanoparticles.

9.4 Mycofabrication, a Green Approach

Mycofabrication process involves the three important reagents, viz., a solvent for the mycofabrication process, a reducing agent for the fabrication of metal ions into nanoparticles, and a capping or stabilizing agent, responsible for maintaining the size of metal nanoparticles in nano-scale or preventing the aggregation of nanoparticles. Raveendran et al. (2003) reported three crucial steps in the fabrication of nanoparticles which were evaluated on the basis of green chemistry perspective. These include the selection of non-toxic solvent medium for the fabrication of metal nanoparticles, the choice of eco-friendly reducing agent, and the selection of harmless material for the capping/stabilization of fabricated metal nanoparticles. Most of the chemical and physical methods of metal fabrication of nanoparticles rely on using organic solvents, which can be toxic. The second concern for metal nanoparticle fabrication is the selection of the reducing agent. The reported reducing agents such as hydrazine, sodium borohydride ($NaBH_4$), and dimethylformamide (DMF) are strong reducing agents and are highly reactive chemicals which can be detrimental to the environmental and living organisms. The third and final and possibly the most significant issue in the fabrication of metal nanoparticle is the selection of the capping/stabilizing agent protecting the metal nanoparticles since the behavior of the metal nanoparticles depends on the capping or stabilizing agent used. There are several concerns that need to be kept in mind before considering the capping or stabilization agent, which varies significantly based on the required size and topology of the fabricated nanoparticles.

As nanotechnology is advancing day by day, people are becoming more aware of the potential benefits of these new technologies in consumer products. The number of consumer products with metal nanoparticles in them is increasing. To document the availability of nano-based products available in the commercial marketplace leads to the creation of Nanotechnology Consumer Products Inventory (CPI) in 2005 by the scholars of Woodrow Wilson International Center. Silver nanoparticle ranks the first and was the most predominant nanomaterial present in consumer products. To support the sustainable fabrication of metal nanoparticles, the US Environmental Protection Agency (EPA) is working with the international community for the popularization of green methods for the fabrication of nanoparticles to be used in the manufacturing of nano-products, which is a more environmental-friendly process.

The fabrication of metal nanoparticles using a fungal system, i.e., mycofabrication process, generally involves water as a solvent for fabrication of metal nanoparticles; the fungal secretions which include proteins, sugars, and metabolites are accountable for the reduction of metal ions and are environmentally benign reducing agents; biomolecules like proteins also serve as a capping or stabilizing agent, which are non-toxic and even make metal nanoparticles biocompatible. Therefore, mycofabrication of metal nanoparticles is aptly referred as a green approach.

9.5 Mechanistic Aspect

The mycofabrication is either intracellular or extracellular, most of the time it is extracellular and preferred, and the exact mechanism for mycofabrication is yet unknown (Rai et al. 2011). In the case of intracellular mycofabrication, the precise role of fungal cell wall needs to be elucidated, whereas in the case of extracellular mycofabrication, the role of fungal secretions including proteins, sugars, and metabolites in the process needs to be studied in detail. In extracellular fabrication of metal nanoparticles, numerous reducing and capping agents are involved. The role of these reducing agents on the fabrication of nanoparticles with definite shape and size also needs to be elucidated. Moreover, the influence of different factors on the dispersion of metal nanoparticles also needs to be evaluated.

Unraveling the exact mechanistic aspect of the mycofabrication of metal nanoparticles and understanding the role of each component involved in the fabrication process will help us in the following ways:

1. Large-scale fabrication of nanoparticles.
2. Better control over size and shape by controlling the physicochemical conditions.
3. Enhancing the rate of reactions by adding various inducers.
4. Convenient and easy downstream processing or purification of nanoparticles.
5. Enhancing the stability of nanoparticles.
6. Biofunctionalization of nanoparticles for various applications.

Fungal cell wall components are likely to play a significant role in the reduction of metal ions. The dynamic structure of the fungal cell wall keeps altering at different stages in the life cycle of a fungus and plays a crucial role in the absorption of heavy metals (Rai et al. 2010). Mukherjee et al. (2001) proposed a step-wise mechanism for intracellular fabrication of nanoparticles using *Verticillium* sp. The authors have explained intracellular fabrication of nanoparticles by a step-wise mechanism. The mycofabrication is initiated by trapping the metal ions at the fungal cell surface; the trapping of metal ions was due to the electrostatic interaction by the positively charged groups in enzymes present on the cell wall surface. After trapping of metal ions, they were reduced by the enzymes present within the cell wall, leading to the formation of a nucleation center for the nanoparticles (Mukherjee et al. 2001).

The extracellular mycofabrication of metal nanoparticles is possible either by the action of the reductase enzyme or by the involvement of electron shuttle quinones or by both (Rai et al. 2011). Duran et al. (2005) demonstrated the nitrate reductase assay by the reaction of nitrite with 2,3-diaminophthalene. The emission spectrum showed two major peaks at 405 and 490 nm relating to the emission maximum of nitrite and 2,3-diaminonaptho-triazole (DAN), respectively. The absorbance at these two wavelengths was found to increase with the addition of a 0.1% KNO_3 solution, endorsing the presence of nitrate reductase, thus confirming the role of enzyme reductase in the reduction of Ag^+ ions into silver nanoparticles. The findings of Duran et al. (2005) were supported by Ingle et al. (2008); in the study, commercially available chromogenic substrate discs for nitrate reductase were used; the color of

the disc changes to reddish pink from white in the presence of reductase. The discs when challenged with fungal filtrate turned to reddish pink signifying the presence of nitrate reductase in the fungal filtrate. Thus, it can be inference that the enzyme NADH-dependent reductase is responsible for the reduction of Ag^{+} to Ag^{0} in the case of fungi.

A similar mechanism for mycofabrication was reported by Anilkumar et al. (2007). They reported the mycofabrication of silver nanoparticles extracellularly using *F. oxysporum* by providing the direct evidence for the involvement of nitrate reductase in the mycofabrication process. The authors used the purified nitrate reductase from *F. oxysporum* for the fabrication of silver nanoparticle in vitro, since reaction mixture comprises of only the enzyme nitrate reductase, silver nitrate, and NADPH.

The process of mycofabrication by *Coriolus versicolor* was monitored using Fourier transform infrared spectroscopy (FTIR), which demonstrated a shifting of absorbance band of aromatic group on reduction of silver ions by fungal extract. Apart from that appearance of a new band at 1735 cm^{-1} indicates the oxidation of hydroxyl groups present in fungal mycelium during silver nanoparticle fabrication (Sanghi and Verma 2009), mycofabricated silver nanoparticles demonstrating the presence of amide I and II bands clearly indicated the capping of fabricated nanoparticles with proteins. This capping or stabilization was due to the electrostatic attraction between free amine groups or cysteine residues in the proteins with that of carboxylate groups in enzymes present in the cell wall of fungus (Gole et al. 2001).

Mukherjee et al. (2008) reported a mycofabrication of nanocrystalline silver nanoparticles by *Trichoderma asperellum*. They hypothesized the possible mechanism involved in the fabrication of these silver nanoparticles on the basis of FTIR studies; the data revealed the presence of protein-capped silver nanoparticles. Further analysis of fungal extract after removal of silver nanoparticles showed a diminution in the intensity of the amine II band along with O–H, S–H, and carboxylic C=O stretching bands, demonstrating the decrease in peptide concentration after silver nanoparticle fabrication. They further concluded the involvement of fungal extract comprising proteins, in general, and S–H bond, in particular, participating in the mycofabrication, probably hinting at the cysteine amino acids involvement in the mycofabrication process.

A three-step mechanism for mycofabrication of silver nanorods using *Phoma sorghina* was reported by Gade et al. (2011). The authors reported the steps like nucleation, elongation, and termination in the fabrication of silver nanorods. Nucleation was initiated by the protein capping, leading to the formation of reaction centers to initiate the silver nanorod fabrication; these reaction centers were elongated in the next step by the photosensitized anthraquinone derivative acting as electron shuttle, and finally when this electron shuttle stops, supplying electrons to the elongated rod or providing electrons to other nucleation center for elongation will bring about the termination of silver nanorod fabrication process. In another study, the authors described three steps for the mycofabrication of spherical silver nanoparticles by *Phoma glomerata* that include as follows: the process is initiated by the photosensitization or activation of aromatic compounds in the fungal filtrate on

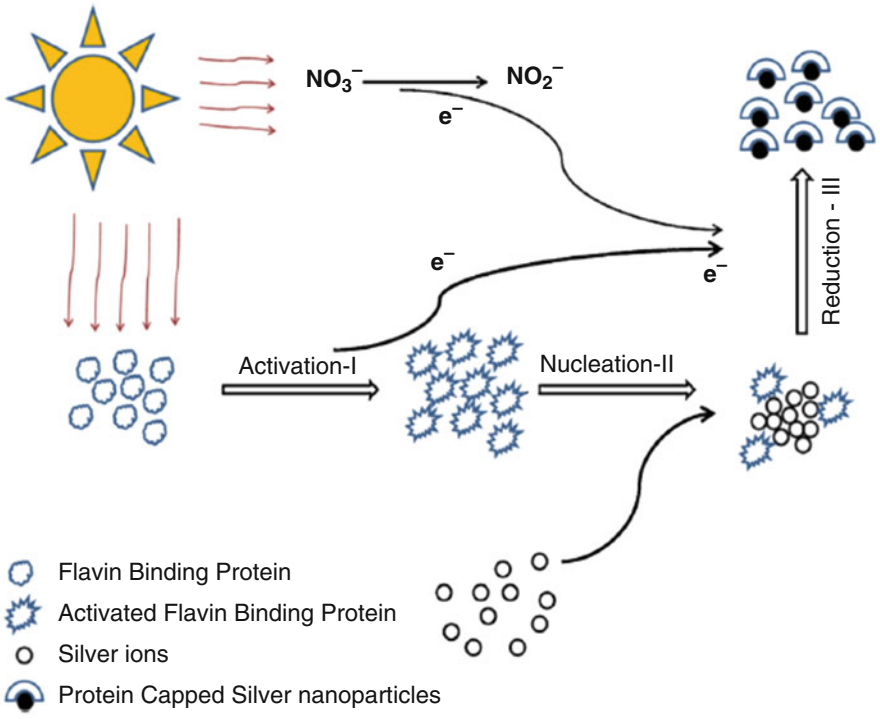

Fig. 9.2 Three-step mechanism of spherical silver nanoparticles mycofabrication by *P. glomerata*. (Reprinted from Gade et al. (2014). Green synthesis of silver nanoparticles by *Phoma glomerata*. *Micron*. 2014;59:52–59 copyright © 2014, with permission from Elsevier)

exposure to bright sunlight; followed by the formation of reaction centers, i.e., nucleation, which involves the role of protein or photosensitized aromatic compounds in capping to initiate the mycofabrication of spherical silver nanoparticles; and the third and final step comprises the actual mycofabrication by reduction of silver ions to form silver nanoparticles (Gade et al. 2014) (Fig. 9.2).

For mycofabrication of metal nanoparticles, the mechanism based on the involvement of nitrate reductase is widely accepted, but still there are some issues that need to be addressed before considering it. The proper understanding of the in-depth mechanism involved in the fabrication of silver nanoparticles and analyzing the role of different factors in the reduction of metal ions will assist in developing low-cost technology for the fabrication and purification of metal nanoparticles.

9.6 Conclusions and Perspectives

The versatility of the fungal system among the biological systems and the ability of different fungi to reduce the precursor metal salts have opened up a new exciting, rapid, cost-effective, scalable, and eco-friendly approach towards the

mycofabrication of metal nanoparticles and development of green and sustainable nanotechnology. However, still, a few issues need to be addressed from the mycofabrication point of view before addressing the mycofabrication approach as the universal approach for the fabrication of metal nanoparticles. The exact mechanism of the mycofabrication is still a mystery, and the revelation of process at the molecular level for metal ion reduction and mycofabrication is desired in order to get fine control over the fabrication of metal nanoparticles of diverse shapes and sizes. The rational use of constrained environments outside the fungal cells such as temperature, pH, light intensity, the concentration of salt, and quantity of secreted proteins to modulate metal nanoparticle size and shape is an exciting possibility. The range of chemical compositions of nanoparticles currently fabricated by the bio-based approach is extremely limited and confined to metals, metal sulfides, and iron oxide. Extension of the mycofabrication protocols to enable the reliable fabrication of nanocrystals of other oxides and nitrides, carbides, etc. could make this process a commercially viable option.

Another major concern with the fabrication of metal nanoparticles could be of its toxicity, though the mycofabricated metal nanoparticles are biocompatible since biomolecules act as capping or stabilizing agents. Still, exhaustive dose-dependent study of mycofabricated metal nanoparticles will provide more confidence not only to the nano-product manufacturer but also to the consumer. Thus, unraveling the mechanism of mycofabrication and addressing the toxicity concerns would assist in developing the mycofabrication process as an efficient process for the fabrication of metal nanoparticles.

The mycofabrication of metal nanoparticles seems to be rapid, eco-friendly, simple, safe, and scalable as compared to the fabrication of metal nanoparticles demonstrated by other biological systems. Moreover, it is a green approach, which fits into the criteria of sustainability encouraged by agencies like EPA and OECD. Before making mycofabrication as the most versatile approach, elucidation of the exact mechanism of mycofabrication and toxicity concern needs to be addressed.

References

Adeeyo AO, Odiyo JO (2018) Biogenic synthesis of silver nanoparticle from mushroom exopolysaccharides and its potentials in water purification. Open Chem J 5(1):64–75

Agnihotri M, Joshi S, Ravikumar A, Zinjarde S, Kulkarni S (2009) Biosynthesis of gold nanoparticles by the tropical marine yeast *Yarrowia lipolytica* NCIM 3589. Mater Lett 63:1231–1234

Ahluwalia V, Kumar J, Sisodia R, Shakil NA, Walia S (2014) Green synthesis of silver nanoparticles by Trichoderma harzianum and their bio-efficacy evaluation against Staphylococcus aureus and Klebsiella pneumonia. Ind Crop Prod 55:202–206

Ahmad A, Mukherjee P, Mandal D, Senapati S, Khan MI, Kumar R, Sastry M (2002) Enzyme mediated extracellular synthesis of CdS nanoparticles by the fungus, *Fusarium oxysporum*. J Am Chem Soc 124:12108–12109

Ahmad T, Wani IA, Manzoor N, Ahmed J, Asiri AM (2013) Biosynthesis, structural characterization and antimicrobial activity of gold and silver nanoparticles. Colloids Surf B Biointerfaces 107:227–234

Alivisatos AP (1996) Semiconductor clusters, nanocrystals, and quantum dots. Science 271 (5251):933–937

Anilkumar S, Abyaneh MK, Gosavi SW, Kulkarni SK, Pasricha R, Ahmad A, Khan MI (2007) Nitrate reductase mediated synthesis of silver nanoparticles from $AgNO_3$. Biotechnol Lett 29:439–445

Ayyub P, Chandra R, Taneja P, Sharma AK, Pinto R (2001) Synthesis of nanocrystalline material by sputtering and laser ablation at low temperatures. Appl Phys A Mater Sci Process 73 (1):67–73

Balaji DS, Basavaraja S, Deshpande R, Mahesh DB, Prabhakar BK, Venkataraman A (2009) Extracellular biosynthesis of functionalized silver nanoparticles by strains of *Cladosporium cladosporioides* fungus. Colloids Surf B Biointerfaces 68:88–92

Bansal V, Rautaray D, Ahmad A, Sastry M (2004) Biosynthesis of zirconia nanoparticles using the fungus *Fusarium oxysporum*. J Mater Chem 14:3303–3305

Bansal V, Rautaray D, Bharde A, Ahire K, Sanyal A, Ahmad A, Sastry M (2005) Fungus-mediated biosynthesis of silica and titania particles. J Mater Chem 15:2583–2589

Bansal V, Poddar P, Ahmad A, Sastry M (2006) Room-temperature biosynthesis of ferroelectric barium titanate nanoparticles. J Am Chem Soc 128:11958–11963

Bansod S, Bonde S, Tiwari V, Bawaskar M, Deshmukh S, Gaikwad S, Gade A, Rai M (2013) Bioconjugation of gold and silver nanoparticles synthesized by *Fusarium oxysporum* and their use in rapid identification of *Candida* species by using bioconjugate-nano-polymerase chain reaction. J Biomed Nanotechnol 9(12):1962–1971

Bawaskar M, Gaikwad S, Ingle A, Rathod D, Gade A, Duran N, Marcato P, Rai M (2010) A new report on mycosynthesis of silver nanoparticles by *Fusarium culmorum*. Curr Nanosci 6 (4):376–380

Bhainsa KC, D'Souza SF (2006) Extracellular biosynthesis of silver nanoparticles using the fungus *Aspergillus fumigatus*. Colloids Surf B Biointerfaces 47:160–164

Bhambure R, Bule M, Shaligram N, Kamat M, Singhal R (2009) Extracellular biosynthesis of gold nanoparticles using *Aspergillus niger*—its characterisation and stability. Chem Eng Technol 32:1036–1041

Binupriya AR, Sathishkumar M, Vijayaraghavan K, Yun SI (2010) Bioreduction of trivalent aurum to nanocrystalline gold particles by active and inactive cells and cell free extract of *Aspergillus oryzae* var. viridis. J Hazard Mater 177:539–545

Birla SS, Tiwari VV, Gade AK, Ingle AP, Yadav AP, Rai MK (2009) Fabrication of silver nanoparticles by *Phoma glomerata* and its combined effect against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. Lett Appl Microbiol 48:173–179

Birla SS, Gaikwad SC, Gade AK, Rai MK (2013) Rapid synthesis of silver nanoparticles from Fusarium oxysporum by optimizing physicocultural conditions. Sci World J 2013:796018

Castro Longoria E, Vilchis Nestor AR, Avalos Borja M (2011) Biosynthesis of silver, gold and bimetallic nanoparticles using the filamentous fungus *Neurospora crassa*. Colloids Surf B Biointerfaces 83:42–48

Chan YS, Mashitah MD (2012) Instantaneous biosynthesis of silver nanoparticles by selected macro-fungi. Aust J Basic Appl Sci 6:222–226

Chen G, Yi B, Zeng G, Niu Q, Yan M, Chen A, Du J, Huang J, Zhang Q (2014) Facile green extracellular biosynthesis of CdS quantum dots by white rot fungus Phanerochaete chrysosporium. Colloids Surf B Biointerfaces 117:199–205

Dameron CT, Reese RN, Mehra RK, Kortan AR, Carroll PJ, Steigerwald ML, Brus LE, Winge DR (1989) Biosynthesis of cadmium sulfide quantum semiconductor crystallites. Nature 338:596–597

Das SK, Dickinson C, Laffir F, Brougham DF, Marsili E (2012) Synthesis, characterization and catalytic activity of gold nanoparticles biosynthesized with *Rhizopus oryzae* protein extract. Green Chem 14:1322–1344

Devi LS, Joshi SR (2015) Ultrastructures of silver nanoparticles biosynthesized using endophytic fungi. J Microsc Ultrastruct 3:29–37

Du L, Xian L, Feng JX (2011) Rapid extra-/intracellular biosynthesis of gold nanoparticles by the fungus *Penicillium* sp. J Nanopart Res 13:921–930

Duran N, Marcato PD, Alves OL, D'Souza G, Esposito E (2005) Mechanistic aspects of biosynthesis of silver nanoparticles by several *Fusarium oxysporum* strains. J Nanobiotechnol 3:8–14

Farsi M, Farokhi S (2018) Biosynthesis of antibacterial silver nanoparticles by endophytic fungus *Nemania* sp. isolated from *Taxus baccata* L. (Iranian Yew). Zahedan J Res Med Sci 20(6): e57916

Fayaz M, Tiwary CS, Kalaichelvan PT, Venkatesan R (2010) Blue orange light emission from biogenic synthesized silver nanoparticles using *Trichoderma viride*. Colloids Surf B Biointerfaces 75:175–178

Feldheim DL, Keating CD (*1998*) Self-assembly of single electron transistors and related devices. Chem Soc Rev 27:1–12

Fendler JH (1992) Membrane mimetic chemistry approach to advanced materials. Springer-Verlag, Berlin

Fendler JH (1998) Nanoparticles and nanostructured films: preparation, characterization and applications. John Wiley & Sons, New York

Gade A, Rai M, Karwa A, Bonde P, Ingle A (2007) Extracellular biosynthesis of silver nanoparticles by *Pleurotus* species. In: International medical mushroom conference-4 (IMMC-4). Ljubljana (Slovenia) from 23 to 37th Sept. 2007. Int J Med Mushroom 9 (3–4):298–299

Gade AK, Bonde PP, Ingle AP, Marcato PD, Duran N, Rai MK (2008) Exploitation of *Aspergillus niger* for fabrication of silver nanoparticles. J Biobased Mater Bioenergy 2(3):243–247

Gade AK, Rai MK, Kulkarni SK (2011) *Phoma sorghina,* a phytopathogen mediated synthesis of unique silver rods. Int J Green Nanotechnol 3(3):153–159

Gade AK, Gaikwad SC, Duran N, Rai MK (2013) Screening of different species of *Phoma* for the synthesis of silver nanoparticles. Biotechnol Appl Biochem 60(5):482–493

Gade AK, Gaikwad SC, Duran N, Rai MK (2014) Green synthesis of silver nanoparticles by *Phoma glomerata*. Micron 59:52–59

Gade A, Adams J, Britt DW, Shen FA, McLean JE, Jacobson A, Kim Y-C, Anderson AJ (2016) Ag nanoparticles generated using bio-reduction and-coating cause microbial killing without cell lysis. Bio Metals 29(2):211–213

Gaikwad SC, Birla SS, Ingle AP, Gade AK, Marcato PD, Rai MK, Duran N (2013a) Screening of different *Fusarium* species to select potential species for the synthesis of silver nanoparticles. J Brazil Chem Soc 24(12):1974–1982

Gaikwad S, Ingle A, Gade A, Rai M, Falanaga A, Incoronato N, Galdiero S, Galdiero M (2013b) Antiviral activity of mycosynthesized silver nanoparticles against herpes simplex virus and human parainfluenza virus type 3. Int J Nanomedicine 8:4303–4314

Gajbhiye MB, Kesharwani JG, Ingle AP, Gade AK, Rai MK (2009) Fungus mediated synthesis of silver nanoparticles and its activity against pathogenic fungi in combination of fluconazole. J Nanomed NBM 5(4):282–286

Gericke M, Pinches A (2006) Microbial production of gold nanoparticles. Gold Bull 39:22–28

Gole A, Dash C, Ramakrishnan V, Sainkar SR, Mandale AB, Rao M, Sastry M (2001) Pepsin–gold colloid conjugates: preparation, characterization, and enzymatic. Langmuir 17:1674–1679

Govender Y, Riddin T, Gericke M, Whiteley CG (2009) Bioreduction of platinum salts into nanoparticles: a mechanistic perspective. Biotechnol Lett 31:95–100

Gudadhe JA, Bonde SR, Gaikwad SC, Gade AK, Rai MK (2011) *Phoma glomerata*: a novel agent for fabrication of iron oxide nanoparticles. J Bionanosci 5(2):138–142

Honary S, Barabadi H, Gharaei-Fatahabad E, Naghibi F (2013) Green synthesis of silver nanoparticles induced by the fungus *Penicillium citrinum*. Trop J Pharma Res 12:7–11

Ingle AP, Gade AK, Pierrat S, Sönnichsen C, Rai MK (2008) Mycosynthesis of silver nanoparticles using the fungus *Fusarium acuminatum* and its activity against some human pathogenic bacteria. Curr Nanosci 4:141–144

Ingle A, Rai M, Gade A, Bawaskar M (2009) *Fusarium solani*: a novel biological agent for the extracellular synthesis of silver nanoparticles. J Nanopart Res 11(8):2079–2085

Jahagirdar AS, Shende S, Gade A, Rai M (2020) Bioinspired synthesis of copper nanoparticles and its efficacy on seed viability and seedling growth in mung bean (*Vigna radiata* L.). Curr Nanosci 16(2):246–252

Joshi PA, Bonde SR, Gaikwad SC, Gade AK, Abd-Elsalam K, Rai MK (2013) Comparative studies on synthesis of silver nanoparticles by *Fusarium oxysporum* and *Macrophomina phaseolina* and it's efficacy against bacteria and *Malassezia furfur*. J Bionanosci 7(4):378–385

Kar P, Murmu S, Saha S et al (2014) Anthelmintic efficacy of gold nanoparticles derived from a phytopathogenic fungus, *Nigrospora oryzae*. PLoS One 9:e84693

Karwa AS, Gaikwad S, Rai MK (2011) Mycosynthesis of silver nanoparticles using Lingzhi or Reishi medicinal mushroom, Ganoderma lucidum (W. Curt.: Fr.) P. Karst. and their role as antimicrobials and antibiotic activity enhancers. Int J Med Mushrooms 13(5):483–491

Kathiresan K, Manivannan S, Nabeel M, Dhivya B (2009) Studies on silver nanoparticles synthesized by a marine fungus, *Penicillium fellutanum* isolated from coastal mangrove sediment. Colloids Surf B Biointerfaces 71:133–137

Kokura S, Handa O, Takagi T, Ishikawa T, Naito Y, Yoshikawa T (2010) Silver nanoparticles as a safe preservative for use in cosmetics. Nanomedicine 6:570–574

Kowshik M, Deshmukh N, Vogel W, Urban J, Kulkarni SK, Paknikar KM (2002) Microbial synthesis of semiconductor CdS nanoparticles, their characterization, and their use in the fabrication of an ideal diode. Biotechnol Bioeng 78:583–588

Krumov N, Oder S, PernerNochta I, Angelov A, Posten C (2007) Accumulation of CdS nanoparticles by yeasts in a fed-batch bioprocess. J Biotechnol 132:481–486

Kumar SA, Peter YA, Nadeau J (2009) Biosynthesis, separation and conjugation of gold nanoparticles to doxorubicin for cellular uptake and toxicity. In: 2009 IEEE 35th annual northeast bioengineering conference, Apr 2009, pp 1–8

Kuralkar M, Ingle A, Gaikwad S, Gade A, Rai M (2015) Gold nanoparticles: novel catalyst for the preparation of direct methanol fuel cell. IET Nanobiotechnol 9(2):66–70

Lange L (2014) The importance of fungi and mycology for addressing major global challenges. IMA Fungus 5(2):463–471

Li G, He D, Qian Y, Guan B, Gao S, Cui Y, Yokoyama K, Wang L (2012) Fungus-mediated green synthesis of silver nanoparticles using *Aspergillus terreus*. Int J Mol Sci 13:466–476

Maliszewska I, Aniszkiewicz Ł, Sadowski Z (2009) Biological synthesis of gold nanostructures using the extract of *Trichoderma koningii*. Acta Phys Pol A 116:163–165

Maliszewska I, Juraszek A, Bielska K (2014) Green synthesis and characterization of silver nanoparticles using ascomycota fungi *Penicillium nalgiovense* AJ12. J Clust Sci 25:989–1004

Mishra AN, Bhadaurla S, Singh Gaur M, Pasricha R (2010) Extracellular microbial synthesis of gold nanoparticles using fungus *Hormoconis resinae*. JOM 62:45–48

Mishra A, Tripathy SK, Wahab R, Jeong SH, Hwang I, Yang YB, Kim YS, Shin HS, Yun SII (2011) Microbial synthesis of gold nanoparticles using the fungus *Penicillium brevicompactum* and their cytotoxic effects against mouse mayo blast cancer C2C12 cells. Appl Microbiol Biotechnol 92:617–630

Mishra A, Tripathy SK, Yuna SI (2012) Fungus mediated synthesis of gold nanoparticles and their conjugation with genomic DNA isolated from *Escherichia coli* and *Staphylococcus aureus*. Process Biochem 47:701–711

Mukherjee P, Ahmad A, Mandal D, Senapati S, Sainkar SR, Khan MI, Ramani R, Parischa R, Ajayakumar PV, Alam M, Sastry M, Kumar R (2001) Bioreduction of AuCl4-ions by the fungus *Verticillium* sp and surface trapping of the gold nanoparticles formed. Angew Chem Int Ed 40(19):3585–3588

Mukherjee P, Roy M, Mandal BP, Dey GK, Mukherjee PK, Ghatak J, Tyagi AK, Kale SP (2008) Green synthesis of highly stabilized nanocrystalline silver particles by a non-pathogenic and agriculturally important fungus *T. asperellum*. Nanotechnology 19:1–7

Nanda A, Majeed S (2014) Enhanced antibacterial efficacy of biosynthesized AgNPs from *Penicillium glabrum* (MTCC1985) pooled with different drugs. Int J Pharm Tech Res 6:217–223

Narayanan KB, Sakthivel N (2011) Facile green synthesis of gold nanostructures by NADPH-dependent enzyme from the extract of *Sclerotium rolfsii*. Colloids Surf A Physicochem Eng Asp 380:156–161

Narayanan KB, Sakthivel N (2013) Mycocrystallization of gold ions by the fungus *Cylindrocladium floridanum*. World J Microbiol Biotechnol 29:2207–2211

Noor S, Shah Z, Javed A, Ali A, Hussain SB, Zafar S, Ali H, Muhammad SA (2020) A fungal based synthesis method for copper nanoparticles with the determination of anticancer, antidiabetic and antibacterial activities. J Microbiol Methods 174:105966

Philip D (2009) Biosynthesis of Au, Ag and Au-Ag nanoparticles using edible mushroom extract. Spectrochim Acta Mol Biomol Spectrosc 73:374–381

Raheman F, Deshmukh S, Ingle A, Gade A, Rai M (2011) Silver nanoparticles: novel antimicrobial agent synthesized from an endophytic fungus *Pestalotia* sp. isolated from leaves of *Syzygium cumini* (L). Nano Biomed Eng 3(3):174–178

Rai MK, Gade AK (2009) Strategic synthesis of nanoparticles by mycetes. In: Maheshwari DK (ed) Biotechnology of industrial microorganisms—an industrial approach. IK International, New Delhi, pp 322–332

Rai MK, Yadav AP, Gade AK (2008) Current trends in phytosynthesis of metal nanoparticles. Crit Rev Biotechnol 28(4):277–284

Rai M, Yadav A, Gade A (2010) Mycofabrication, mechanistic aspects and multifunctionality of metal nanoparticles-where are we? And where should we go? In: Mendez-Vilas A (ed) Current research, technology and education topics in applied microbiology and microbial biotechnology. Formatex Research Center, Badajoz, pp 1343–1354

Rai M, Gade A, Yadav A (2011) Biogenic nanoparticles: an introduction to what they, how they are synthesized and their applications. In: Rai MK, Duran N (eds) Metal nanoparticles, Microbiology. Springer-Verlag, Berlin, pp 1–16

Rai M, Ingle A, Gade A, Duran N (2015a) Synthesis of silver nanoparticles by *Phoma gardeniae* and *in vitro* evaluation of their efficacy against human disease-causing bacteria and fungi. IET Nanobiotechnol 9(2):71–75

Rai M, Ingle AP, Gade AK, Duarte MCT, Duran N (2015b) Three *Phoma* spp. synthesised novel silver nanoparticles that possess excellent antimicrobial efficacy. IET Nanobiotechnol 9 (5):280–287

Raliya R (2013) Rapid, low-cost, and ecofriendly approach her for iron nanoparticle synthesis using *Aspergillus oryzae* TFR9. J Nanopart 141274:4

Raliya R, Tarafdar JC (2013) ZnO nanoparticle biosynthesis and its effect on phosphorous-mobilizing enzyme secretion and gum contents in cluster bean (*Cyamopsis tetragonoloba* L.). Agric Res 2:48–57

Ranjani S, Shariq Ahmed M, Adnan M, Senthil Kumar N, Ruckmani K, Hemalatha S (2020) Synthesis, characterization and applications of endophytic fungal nanoparticles. Inorg Nano-Metal Chem. https://doi.org/10.1080/24701556.2020.1784231

Rautaray D, Sanyal A, Adyanthaya SD, Ahmad A, Sastry M (2004) Biological synthesis of strontium carbonate crystals using the fungus *Fusarium oxysporum*. Langmuir 20:6827–6833

Raveendran P, Fu J, Wallen SL (2003) Completely "green" synthesis and stabilization of metal nanoparticles. J Am Chem Soc 125(46):13940–13941

Salem SS, Fouda A (2020) Green synthesis of metallic nanoparticles and their prospective biotechnological applications: an overview. Biol Trace Elem Res. https://doi.org/10.1007/s12011-020-02138-3

Sanghi R, Verma P (2009) Biomimetic synthesis and characterisation of protein capped silver nanoparticles. Bioresour Technol 100:501–504

Sanghi R, Verma P (2010) pH dependant fungal proteins in the "green" synthesis of gold nanoparticles. Adv Mater Lett 1:193–199

Sanghi R, Verma P, Puri S (2011) Enzymatic formation of gold nanoparticles using *Phanerochaete chrysosporium*. Adv Chem Eng Sci 1:154–162
Sanyal A, Rautaray D, Bansal V, Ahmad A, Sastry M (2005) Heavy-metal remediation by a fungus as a means of production of lead and cadmium carbonate crystals. Langmuir 21:7220–7224
Sarkar J, Ray S, Chattopadhyay D, Laskar A, Acharya K (2012) Mycogenesis of gold nanoparticles using a phytopathogen *Alternaria alternata*. Bioprocess Biosyst Eng 35:637–643
Sastry M, Ahmad A, Khan MI, Kumar R (2003) Biosynthesis of metal nanoparticles using fungi and actinomycetes. Curr Sci 85(2):162–170
Sathish Kumar K, Amutha R, Arumugam P, Berchmans S (2011) Synthesis of gold nanoparticles: an ecofriendly approach using *Hansenula anomala*. ACS Appl Mater Interfaces 3:1418–1425
Sawle BD, Salimath B, Deshpande R, Bedre MD, Prabhakar BK, Venkataraman A (2008) Biosynthesis and stabilization of Au and Au-Ag alloy nanoparticles by fungus, *Fusarium semitectum*. Sci Technol Adv Mater 9:1–6
Sen K, Sinha P, Lahiri S (2011) Time dependent formation of gold nanoparticles in yeast cells: a comparative study. Biochem Eng J 55:1–6
Shankar SS, Ahmad A, Pasricha R, Sastry M (2003) Bioreduction of chloroaurate ions by geranium leaves and its endophytic fungus yields gold nanoparticles of different shapes. J Mater Chem 13:1822–1826
Shelar GB, Chavan AM (2014) Fungus–mediated biosynthesis of silver nanoparticles and its antibacterial activity. Arch Appl Sci Res 6:111–114
Shende S, Gade A, Rai M (2017) Large-scale synthesis and antibacterial activity of fungal-derived silver nanoparticles. Environ Chem Lett 15(3):427–434
Shivshankar S, Ahmad A, Pasricha R, Sastry M (2003) Bioreduction of chloroaurate ions by geranium leaves and its endophytic fungus yields gold nanoparticles of different shapes. J Mater Chem 13:1822–1826
Talie MD, Wani AH, Ahmad N, Bhat MY, War JM (2020) Green synthesis of silver nanoparticles (AgNPs) using Helvella Leucopus Pers. and their antimycotic activity against fungi causing fungal rot of apple. Asian J Pharm Clin Res 13(4):161–165
Tidke PR, Gupta I, Gade A, Rai M (2014) Fungus-mediated synthesis of gold nanoparticles and standardization of parameters for its biosynthesis. IEEE Trans NanoBiosci 13(4):397–402
Vahabi K, Mansoori GA, Karimi S (2011) Biosynthesis of silver nanoparticles by fungus *Trichoderma reesei* (a route for large-scale production of AgNPs). Insci J 1:65–79
Vala AK (2015) Exploration on green synthesis of gold nanoparticles by a marine-derived fungus *Aspergillus sydowii*. Environ Prog Sustain Energy 34:194–197
Verma VC, Singh SK, Solanki R, Prakash S (2011) Biofabrication of anisotropic gold nanotriangles using extract of endophytic *Aspergillus clavatus* as a dual functional reductant and stabilizer. Nanoscale Res Lett 6:16–22
Vigneshwaran N, Ashtaputre NM, Varadarajan PV, Nachane RP, Paralikar KM, Balasubramanya RH (2007) Biological synthesis of silver nanoparticles using the fungus *Aspergillus flavus*. Mater Lett 61:1413–1418
Volesky B, Holan ZR (1995) Biosorption of heavy metals. Biotechnol Prog 11:235–250
Yadav A, Kon K, Kratosova G, Duran N, Ingle AP, Rai M (2015) Fungi as an efficient mycosystem for the synthesis of metal nanoparticles: progress and key aspects of research. Biotechnol Lett 37 (11):2099–2120
Zhang X, He X, Wang K, Yang X (2011) Different active biomolecules involved in biosynthesis of gold nanoparticles by three fungus species. J Biomed Nanotechnol 7:245–254

Nanosensors for the Detection of Plant and Human Fungal Pathogens

10

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Abstract

Fungal plant and human pathogens have inflicted large losses in terms of agricultural yields (20–30%/year) and human lives (>1.6 million) worldwide. Increasing reports of fungal infections are compounded by spread of pathogenic fungi, climate change, prevailing agricultural practices, and increased usage of antifungal and immunosuppressive drugs. Rapid detection of fungal pathogens plays an important role in mitigating their threat to public health, food security, and ecosystems. Currently, the prevailing practices of symptomatic fungal disease detection are time-consuming, and the methods like PCR, ELISA, HPLC, etc. require expensive equipment and skilled personnel in laboratory settings, leading to a delayed diagnosis. Radiological imaging of human subjects and spectral imaging of field plants are being used to some extent, but these methods lack specificity. Very few on-site detection devices are available for rapid and specific detection of fungal pathogens. Nanotechnology can contribute to diagnostics by miniaturization of detection platforms and portability by incorporating the "nanoscale" aspect at the physical and material levels. Nano tools can contribute significantly by the improved sample processing, signal detection, and ease of handling portable devices such as lateral flow assays, nanosensors, nanoarrays, nanobarcodes, and nano detection kits that will expedite rapid on-site detection of plant and human fungal pathogens and their subsequent management in the agricultural and healthcare sectors.

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Fungal pathogens · Plant · Human · Nanotechnology · Detection

10.1 Introduction

Spread of fungal diseases has increased globally be it plant infections or human infections. Plant diseases caused a 13% loss associated with $2000 B losses worldwide (Casadevall 2017), while human fungal pathogens cause infections to >1 billion people that lead to >1.6 million deaths annually (Almeida et al. 2019). Human pathogens cause infections mostly among the immune-compromised patients, and the infection mortalities are upward of 50%. The rise in untimely rainfall and change in climate promote humid condition causing increased incidences of fungal pathogen infections (Garcia-Solache and Casadevall 2010). Fungal diseases cause damage to plants and crops, leading to major losses in agricultural yields and food production (Savary et al. 2012). Almost a third of all food crops are affected by pathogenic fungi (Fisher et al. 2012). Increased humidity causes increase infection by plant and human pathogens. Rapid identification of fungal pathogens in the agricultural and healthcare sectors is a challenge, and control costs are still limited. Parallelly, the human infections are also increasing with comorbidities and increasing number of immune-compromised patients.

Traditional methods for accurate fungal pathogen detection are used in laboratories, which are expensive and are mostly not applicable for on-site detection. The conventional methods for pathogen culture are tedious and time-consuming. Other methods for identification by ELISA, HPLC, or LCMS are expensive and require skilled personnel. The PCR methods are available for a few pathogens and are not optimized for a wide range of pathogens. Currently molecular diagnostic tools are available for few fungal pathogenic organisms with high degree of sensitivity and specificity. However, these procedures require expensive instruments and cannot be applied in the field for rapid on-site detection. Furthermore, the high price and short shelf-life of molecular biology reagents like enzymes, antibodies, etc. limit the application of conventional molecular methods in developing countries.

Nanotechnology can contribute to the diagnostics wherein "nanoscale" aspect can contribute to the miniaturization of the sampling and testing platforms. Nano tools can contribute to improved sample preparation, reducing the sample volumes, signal detection and amplification, ease of handling, and portability (Kumar and Arora 2020). Portable diagnostic systems such as nanosensors, nanobarcodes, and nano detection kits can contribute immensely to the quick on-site detection of fungal pathogens and to manage different fungal plant and human diseases in the agricultural and healthcare sectors. Here, we describe the concepts and current state-of-the-art nanotechnology applications in the detection of plant and human fungal pathogens.

10.2 Plant Pathogens and Current Ways of Their Detection

Timely detection of pathogens is important for crops, horticulture, and plant breeding for better fungicide efficacy in proper management and averting disasters. More than 19,000 phytopathogenic fungi cause disease in plants, and some are recorded to cause devastating effects on agricultural crops (Jain et al. 2019). The historical Irish famine during 1845–1849 led to more than a million deaths, and mass migrations were caused by potato blight fungus, *Phytophthora infestans*. Pathogenic fungi infect the plants to acquire their nutrients from host tissues. Biotrophic fungi obtain their nutrition from growing host tissues, while necrotrophic fungi necrotize or kill the host tissues to get nutrition. Hemibiotrophic fungi grow as biotrophs initially and cause necrosis in the later stages of infection. *Magnaporthe oryzae*, a hemibiotrophic pathogen, is the most economically destructive fungus that causes blast disease in rice, a staple food consumed by more than half of the world's population (Dean et al. 2012). Recently, *Magnaporthe oryzae Triticum* caused ~100% yield losses due to wheat blast disease in Bangladesh, and the areas in neighboring country (India) have banned wheat cultivation for 2 years in the nine adjoining districts in West Bengal (Islam et al. 2016). Heavy damages are caused by *Botrytis cinerea* or grey mold, a necrotrophic pathogen infecting more than 200 spp. of plants, and the three rust diseases, black (*Puccinia graminis* f. sp. *tritici*), yellow or stripe (*P. striiformis*), and brown (*P. triticina*), are obligate biotrophs that infect wheat crops globally (Dean et al. 2012). *Fusarium graminearum*, the causal agent of wilt disease, is another plant pathogen that damages several plants by reducing the grain yield and quality. Further, the grains are contaminated with mycotoxins such as deoxynivalenol and zearalenone, making grains unfit for human or animal consumption. *Blumeria graminis*, an obligate biotrophic pathogen, causes the powdery mildew of important cereal crops to affect the grain quality and yield. *Mycosphaerella graminicola*, an obligate biotrophic fungal pathogen, causes blotch disease of wheat to reduce the yields, particularly in temperate regions. *Colletotrichum* spp., a hemibiotrophic pathogen, causes anthracnose, blights of aerial plant parts, and postharvest rots of sorghum, banana, cassava, fruits, vegetables, and ornamentals. *Ustilago maydis* is a biotrophic pathogen of corn, causing corn smut, and another biotrophic pathogen, *Melampsora lini*, infects the flax plant. Other pathogenic fungi are, namely, *Phakopsora pachyrhizi*, the causal agent of Asian soybean rust; *Rhizoctonia solani*, causal agent of root rot and collar rot of several plants; and *Alternaria solani*, a necrotrophic pathogen that causes early blight of potato and tomato. In several countries that have agro-based economies, farmers experience a lot of problem in detecting and preventing these fungal diseases. These pathogens inflict severe crop losses and are usually detected by conventional methods as described below.

Plant pathogenic fungi produce typical symptoms on leaves, stem, and fruits that can be recognized visually. However, these symptoms are produced later in the infection cycle and are rarely useful for the early detection of the infections. The traditional method of visual observation for disease detection largely depends on the professional expertise. The obligate fungal pathogens *Plasmopara viticola* and *Erysiphe necator* causing grape downy and powdery mildew, respectively, and

Oidium neolycopersici causing tomato powdery mildew are difficult to detect and manage due to non-symptomatic and quiescent infections early in fruit and leaf development. Downy and powdery mildews cause ~30% loss in grapes and tomatoes (Thakur and Mathur 2002; Worrall et al. 2018). Humid weather promotes fungal infections, and wetting of foliage during irrigation makes the plants susceptible to fungal infections.

Recently, several plant studies are reported for the disease detection in grapevine using sensor techniques based on thermal, fluorescence, and optical reflectance (Agati et al. 2013; Belanger et al. 2008; Latouche et al. 2015; Oberti et al. 2014; Šebela et al. 2014). A recent study reported an automated image processing system to detect the disease on grapes (Mutha et al. 2018). Optical sensors that use the RGB (red, green, blue color) sensing were reported for detection of *Cercospora* leaf spot in sugar beet (Mahlein 2016). Spectral sensors were used for the detection of wheat head blight and yellow rust caused by *F. graminearum* and *P. striiformis*, respectively (Bravo et al. 2003; Moshou et al. 2004; Mahlein 2016). A thermal sensor was reported for downy mildew caused by *Pseudoperonospora cubensis* in cucumber, while fluorescence imaging was utilized for detection of wheat leaf rust caused by *P. triticina* (Berdugo et al. 2014; Oerke et al. 2014; Burling et al. 2011). Multispectral analysis has been carried out for the detection of fungal presence in the leaf though the specific presence is not indicated (Fahrentrapp et al. 2019; Oerke et al. 2016). Although spectral imaging differentiated necrosis and discoloration preceding downy mildew infection, the specific fungal presence is not indicated (Fahrentrapp et al. 2019; Oerke et al. 2016; Stoll et al. 2008). Alarcon et al. (2015) differentiated between cultivars resistant or susceptible to *P. viticola* by using a mass spectrometer for the detection of volatile organic compounds.

Conventionally, disease detection is based on tedious culturing and microscopic identification. Plant specimens are sourced from infected plant parts, and the spores, mycelium, and fruiting bodies are observed for disease diagnosis. Identification keys are available, and pathogens are cultivated on selective artificial media for pathogen identification. Identification relies on the key morphological characters, such as asexual or sexual spore formation, that usually occur during the later stages of the pathogen growth. *Erysiphe necator*, the causal agent of powdery mildew of grapes, represent a challenge as its growth is dependent on the host tissues. Grapevine leaves are sterilized with calcium hypochlorite solution (2%) or 30% ethanol and kept alive in water agar for the maintenance of this fungi (Corio-Costet 2015). Similarly, other obligate pathogens such as *P. graminis* var. *tritici*, *B. graminis*, *M. graminicola*, and *M. oryzae* are cultivated in a similar manner (Dean et al. 2012; Gupta et al. 2020). The tedious methods present a challenge for their easy identification.

Highly specific serological methods are available for the identification of the pathogenic fungi. Wang et al. (2017) identified PO8-VHH, a nanobody formed by single variable heavy chain bearing the antigen recognition site, from the alpaca nanobody library. A sandwich ELISA format was developed with nanobody capture and rabbit anti-*Aspergillus* polyclonal antibodies for detection with low limit of detection (1 μg/ml) for *Aspergillus flavus*. Serological detection of fungi presents a problem as the antibodies are raised towards a particular life form of the fungus in its

life cycle. This may allow the pathogen to pass undetected during other stages of its life cycle. Further serology may also detect non-viable pathogens that may lead to flawed interpretations. Serological ELISA detection platforms are developed more for viral and bacterial plant pathogens.

Nucleic acid-based methods have become popular for the detection of plant pathogens. Molecular methods such as polymerase chain reaction (PCR) and real-time quantitative PCR are specific and sensitive and can detect the pathogen during early stages of the infection (Ortega et al. 2018). *Fusarium fujikuroi*, a seed-borne pathogen causing bakanae disease of rice, was detected by PCR and real-time PCR with specific primers and probes developed towards elongation factor 1α (Carneiro et al. 2017; Amatulli et al. 2012). Real-time PCR detection of *M. oryzae* was carried out with unigene for hydrophobin (Suuidi et al. 2013). Rapid PCR and other such methods were developed to identify and study the genetic distribution of downy and powdery mildews among grapes and tomatoes (Gindro et al. 2014; Jankovics et al. 2008; Kiss et al. 2005; Montarry et al. 2009). There is a need for on-site detection of disease for real-time application in the field.

LAMP assay was developed for the detection of *F. fujikuroi* and *M. oryzae*, the seed-borne pathogens of rice. Primers were designed towards elongation factor 1α of *F. fujikuroi* and calmodulin sequence of *M. oryzae* having high specificity, sensitivity, and repeatability. The LAMP assay had a limit of detection of 100–999 fg and 10–99 pg, respectively, that could be useful in the surveillance of the disease (Ortega et al. 2018). Thiessen et al. (2016) developed a LAMP assay for the management of powdery mildew in grapes by detecting the airborne spores of *Erysiphe necator* to initiate the fungicide application. The early detection of the fungal pathogens reduced the application of fungicides in vineyard as compared to standard practices and can contribute in overall reduction of fungicide application by commercial growers.

Rapid detection of *B. cinerea*, grey mold disease, was developed by Duan et al. (2014) for tomato and strawberry using LAMP reaction with hydroxynaphthol blue for rapid visual detection. The blue color was indicative of the presence of pathogen DNA, and the method was tenfold more sensitive than conventional PCR.

Detection by microbiological methods is tedious, while PCR and qPCR are expensive and require skilled personnel (Rallos and Baudoin 2015; Falacy et al. 2007). Polymerase chain reaction (PCR) methods have advantages of sensitivity and specificity during pathogen detection, but the infrastructure, cost, and complexity of the thermal cycling equipment prevent their use in on-site detection of pathogens. Loop-mediated isothermal amplification (LAMP) is a method to amplify a target nucleic acid sequence under isothermal conditions that eliminates the need for thermal cycling equipment, allowing testing to be carried out with minimal equipment (a water bath or heated block). LAMP method overcomes many limitations of PCR-based methods and can be used in resource poor settings as a simplified method for the detection of amplification. However, these methods are not specific for fungal or bacterial detection.

The need for nanotechnological methods for plant disease management in epidemiology of plant diseases has potential to address the current lacunae in the detection (Khater et al. 2017; Khiyami et al. 2014). The detection of plant pathogens requires portable devices with on-site field application. Nanotechnology can contribute for the miniaturization of the detection platforms, increasing the rapidity, sensitivity, and overall diagnostic performance.

10.3 Human Pathogens and Current Ways of Their Detection

Opportunistic fungal pathogens like *Candida* and *Aspergillus* sp. invade the human body to cause either systemic or local infections. They cause infection among immune-compromised patients having HIV, diabetes, and cancer orthoses undergoing surgery or organ transplant and cause high mortality (Nweze et al. 2012; de Pauw 2011). **Systemic mycoses** spread throughout the body and are often caused by dimorphic fungi such as *Candida* spp., *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii*. Dimorphic fungi have the ability to reversibly change their morphology from yeast to hyphae. Hyphal form permits penetration into body tissues, while yeast form is the circulatory form. Another category of infections involves the skin mycosis at the cutaneous or subcutaneous levels. Cutaneous fungal infections are caused by dermatophytic fungi that invade the superficial layers of the skin, hair, and nail. Cutaneous mycoses are caused by three genera of dermatophytes like *Trichophyton*, *Epidermophyton*, and *Microsporum* and are termed as tineas on the body foot or groin recognized by the infection site on the body such as tinea pedis, tinea cruris, and tinea corporis.

Subcutaneous mycoses are caused by the penetration of epidermis and dermis by fungus to infect inner layer. Common subcutaneous mycoses are sporotrichosis, mycetoma, chromoblastomycosis, rhinosporidiosis, zygomycosis, phaeohyphomycosis, and lobomycosis.

Apart from these pathogenic fungi, several genera of rare yeast fungi like *Rhodotorula*, *Malassezia*, and *Geotrichum*; hyaline filamentous fungi such as *Fusarium*, *Acremonium*, *Scedosporium*, and *Paecilomyces*; dematiaceous fungi, namely, *Alternaria*, *Bipolaris*, and *Exophiala*; and zygomycetes like *Rhizopus*, *Mucor*, and *Lichtheimia* account for 1–2% of all fungemia cases (Meletiadis and Roilides 2013).

Recently, contaminated steroid preparations caused an outbreak of the rare pathogen *Exserohilum rostratum* leading to 750 infections and 61 deaths in patients due to fungal meningitis (Katragkou et al. 2014). The etiologic agent was identified only when a case of fungal meningitis was linked to an epidural injected few weeks earlier by a clinician (Larone and Walsh 2013). Similarly, in 2011, a soil fungus called *Apophysomyces trapeziformis* infected several people leading to numerous cases of necrotizing mucormycosis when a tornado hit Joplin, Missouri, and sent contaminated debris flying into the air (Fanfair et al. 2012). The fungus was only identified by a coordinated response from the Centers for Disease Control and Prevention. Such problems illustrate a common challenge faced in medical

mycology, where laboratories lack expertise and sophisticated equipment for correct identification (Steinbach et al. 2003).

The increasing reports of human fungal pathogens may be prompted by reduced immunity of hosts, increasing fungal resistance and environmental changes. Many a time, the rare fungal genera cause infections having similar clinical manifestations as the common fungal pathogens which make diagnosis difficult. Rare fungal pathogens may account for ~10% of all opportunistic fungal infections in a few hospitals and are associated with high mortality rates (60–100% mortality) despite antifungal therapy complicating the management of these infections (Walsh et al. 2004; Fleming et al. 2002).

Mycological diagnosis is carried out with clinical samples such as sputum, serum, bronchoalveolar lavage (BAL), and lung biopsy tissue specimen to detect presence of fungal pathogen, related molecular markers, and immunoreactive components. Pathologists employ the prevailing practice of symptomatic detection of fungal infections to detect the pathogens.

Traditional diagnosis for detection of fungal pathogens is carried out by culturing the fungus from clinical specimens of serum, oral lavage, skin or membrane scraping, or bronchoalveolar lavage (BAL). In case of lung infections caused by aspergillosis, blastomycosis, and histoplasmosis, diagnosis of BAL requires a tedious procedure of bronchoscopy for sample collection from the patients and displays low sensitivity (Barton 2013; Guarner and Brandt 2011; Klont et al. 2001). Detection of the organism by blood culture is slow and insensitive (Barton 2013; Brown et al. 2012; Kousha et al. 2011). Most frequently, the causative organism is isolated from sputum or serum samples.

Although culture-based techniques are cost-effective and straightforward, they are tedious and often require a minimum of 2–7 days for growth at specific temperatures on media like Sabouraud agar, potato dextrose agar or blood agar for positive confirmation (Mortensen et al. 2011). On obtaining the positive culture of the causative organism, further antifungal susceptibility testing may be initiated. Alternatively, automated BACTEC blood culture systems (Becton Dickinson, NJ, USA) may help in efficient isolation and detection of human fungal pathogens; however, these systems lack multicenter validation (Rosa et al. 2011). Culture-based diagnostic methods for human fungal pathogens are not sufficient to distinguish between invasive infections and colonization and have to be supported by radiographic and clinical tests (Horvath and Dummer 1996).

Further, the diagnosis of culture-based detection is improved by 15–20% with microscopic examination of clinical samples (Denning 1998). The detection of *Aspergillus* is highly improved with the staining of clinical specimens with fluorescent stains such as Calcofluor white and Blankophor mixed with potassium hydroxide (KOH, 10–20%). Morpho-taxonomic characteristics and taxonomic keys are used for identification of the pathogenic fungi (Guarner and Brandt 2011). Microscopy of clinical specimens is unable to distinguish between different fungal infections due to the close similarities in hyphal growth in tissues (Barton 2013; Denning 1998; Kousha et al. 2011; Meersseman et al. 2008; Paugam et al. 2010).

Histopathological examination of biopsy samples often shows angioinvasion by fungal hyphae resulting in hemorrhage or necrotic patches in the surrounding tissue (Guarner and Brandt 2011). Histological diagnosis of tissue sections is non-specific and faces the shortcomings of variations in stain quality, sampling error, and inconsistency in observations (Hayden et al. 2002). Most invasive fungal infections present similar histopathological evidences with lesions and invading hyphae, thus making specific diagnosis challenging (Merz et al. 1988). Immunohistochemistry could differentiate *Aspergillus* hyphae in biopsy tissue from other fungal pathogens by staining with specific monoclonal or polyclonal antibodies labeled with fluorescent probe or peroxidase (Fukuzawa et al. 1995; Jensen et al. 1997; Kaufman et al. 1997; Phillips and Weiner 1987; Piérard et al. 1991).

Recently, the highly sensitive and discriminative molecular detection using 5S/18S ribosomal RNA displayed accurate identification and differentiation of distinct fungal infections. The in situ hybridization with sequence-specific DNA probes for rRNA demonstrated 93% sensitivity and 100% specificity (Hayden et al. 2002).

The cell wall component 1,3-β-D-glucan (BDG) is the most abundant fungal cell wall polysaccharide that has great importance in fungal pathogenesis (Wright et al. 2011). The cell walls of medically important fungi such as *Aspergillus* spp., *Fusarium* spp., *Candida* spp., *Pneumocystis jirovecii* and *Acremonium* spp. contain BDG (Hope et al. 2005; Tran and Beal 2016).The fungi release BDG while growing which can be useful in its early detection. However, *Cryptococcus*, *Blastomyces*, and zygomycetous fungi go undetected by this test. Presently, the US FDA-approved Fungitell assay, for BDG determination, is the only non-invasive laboratory test. Fungitell assay has a limit of detection (LOD) of 1 pg/ml and is widely used clinically (Hope et al. 2005; Wright et al. 2011). Due to the non-specific nature of the test, it is usually used in conjunction with other tests for early diagnosis of fungal infections (Pazos et al. 2005; Theel and Doern 2013).

Galactomannan (GM) is the major constituent of *Aspergillus* cell wall that is released through the growing hyphal tip during early infection and contains the immunoreactive β-(1–5)-galactofuranosyl (gal*f*) moiety (Mennink-Kersten et al. 2004, 2006). Presently, the detection of GM is carried out with a commercial Platelia™ *Aspergillus* Ag sandwich ELISA kit (Bio-Rad, Marnes-la-Coquette, France) for serum, BAL, urine, and cerebrospinal fluid samples (Chong et al. 2016; Duettmann et al. 2014; Wheat 2003). The monoclonal antibody EB-A2 (IgM), included in the Platelia assay, binds specifically to the gal*f* side chains of GM and had a limit of detection of 1 ng/ml of GM in serum (Mennink-Kersten et al. 2006; Stynen et al. 1992; Verdaguer et al. 2007). Although the Platelia assay shows substantial heterogeneity in sensitivity and specificity, it is utilized for the preemptive diagnosis of invasive aspergillosis in conjunction with chest CT scans (Dixon et al. 2011; Busca et al. 2006; Maertens et al. 2001).

High-resolution computed tomography (HRCT) is an effective non-invasive diagnostic procedure for IA detection at early stages of infection (Blum et al. 1994; Caillot et al. 2001). HRCT scans of lungs in aspergillosis, coccidioidomycosis, and cryptococcosis patients show typical radiological abnormalities such as

well-circumscribed lung lesions and consolidation with or without a halo sign or a reversed halo sign, air crescent sign, cavity, or ground-glass attenuation (Caillot et al. 2001; Georgiadou et al. 2011; Jin et al. 2017; Orlowski et al. 2017; Kuhlman et al. 1985). However, radiological HRCT chest scans are non-specific and are unable to differentiate between IA and other invasive pulmonary mold infections (Chamilos and Kontoyiannis 2006; Lee et al. 2005).

Detection of nucleic acid by polymerase chain reaction (PCR) is a sensitive and specific test for fungal pathogens. Commercially available PCR kits for detection of invasive aspergillosis such as MycAssay *Aspergillus* PCR assay, *Asp*ID® multiplex PCR test, Light Cycler® Septi*Fast*, and MagicPlex™ assays have the detection limit of 1–10 fg DNA (Hope et al. 2005). The AccuProbe Coccidioides, Blastomyces, and Histoplasma culture identification tests (Hologic) employ a single-stranded DNA probe with chemiluminescent label for rapid identification. The fungi could be identified from blood culture with >98% sensitivity and specificity (Wickes and Wiederhold 2018). However, this kit showed false positives with other fungi. BioFire FilmArray (bioMérieux) detected *Candida* spp. from blood cultures on basis of nested multiplex PCR and DNA melt curve analysis with >96% sensitivity. However, this test is limited due to high reagent and equipment costs (Wickes and Wiederhold 2018). However, also, PCR is unable to discriminate between colonization and invasive infection (Hayette et al. 2001; Kousha et al. 2011). Nucleic acid detection by PCR has the potential for early diagnosis of fungal infections when combined with antigen detection. Additionally, detection of genetic markers associated with antifungal resistance may help in active treatment (Barnes and White 2016).

10.4 Nanomolecular Methods for Detection of Plant and Human Pathogenic Fungi

10.4.1 Nanoparticles for Improved Molecular Diagnostics

Nanotechnology can contribute to the detection of fungal pathogens in agriculture as well as human healthcare. Nanoparticles have potential applications in development of various colorimetric, fluorometric enzymatic, and electrochemical diagnostic assays owing to unique size- and shape-dependent physical and chemical properties (Ghormade et al. 2011). Different nanoparticles such as semiconductors, noble metals, and metal oxides can be harnessed for diverse sensing and imaging application. Development of specific detection methods by signal amplification involves the conjugation of nanoparticles as labels to biomolecular recognition elements such as antibodies, peptides, aptamers, DNA, or RNA (Fig. 10.1a). Gold nanoparticles display colorimetric change due to aggregation of the gold nanoparticles, and the associated color change from red to blue can be used as an indicator for presence of the target molecules (Fig. 10.1b, c). Several assay platforms can be developed depending on the mode of signal amplification and detection. Nanobiosensors such as lab-on-a-chip devices and array biosensors can be coupled with surface

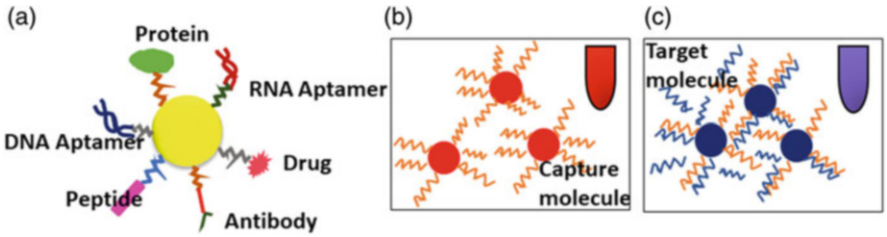

Fig. 10.1 Application of gold nanoparticles in detection (**a**) Schematic representing the functionalization by different capture molecule (DNA, Protein, Peptide, RNA). Colorimetric assay for detection change in color from (**b**) Red, due to colloidal NPs and (**c**) Purple, due to aggregation of gold nanoparticles due to binding of capture molecules with the target

plasmon resonance, fluorescence, or optical detection (Fig. 10.2). These devices can compress the entire workflow from sample preparation, signal detection, and amplification onto a single platform allowing rapid detection (Fig. 10.2). These platforms can handle detection of multiple targets for multiplexing. Lateral flow assays have the ease of visual detection and can be easily used without the need of any sophisticated instruments.

10.4.2 Lateral Flow Assays

10.4.2.1 Detection of Plant Pathogens

Lateral flow assays that operate on a simple paper-based chromatographic flow separation to give a colorimetric visual signal are based on the affinity of recognition molecule-tagged gold nanoparticle labeled to target of interest. Gold nanoparticles and quantum dots are often used in either direct or indirect formats. The lateral flow assay can be used for multiplexing and simultaneous detection of different target molecules (Fig. 10.3).

Rhizoctonia solani, an ubiquitous soil-borne pathogen, causes root rot in vegetables, grasses, horticultural plants, fruit, and forest trees leading to considerable economic losses. A LFA platform prototype was developed for detecting the fungus using the monoclonal antibody (mAb) raised against surface antigens of *R. solani*, in combination with gold NPs (Thornton et al. 2004). The *R. solani* isolate antigens gave a positive signal as did *Thanatephorus orchidicola*, *T. praticola*, and *R. fragariae* (teleomorph: *Ceratorhiza fragariae*). The test did not detect the related *R. carotae*, *R. cerealis*, *R. crocorum*, and *R. zeae* isolates as well as unrelated fungi and oomycetes. The specificity of the technique was in agreement with the identification of the fungi using PCR-based identification of ITS region of the rRNA and conventional colony counting method.

Alternaria brassicae causes dark leaf spot and pod spot in *Brassica* spp. worldwide and causes yield losses. A competitive immunochromatographic lateral flow device was developed for the detection of the fungus, and the test took 6 min. It was suggested that the air sampling of conidia could be integrated with the CFLD

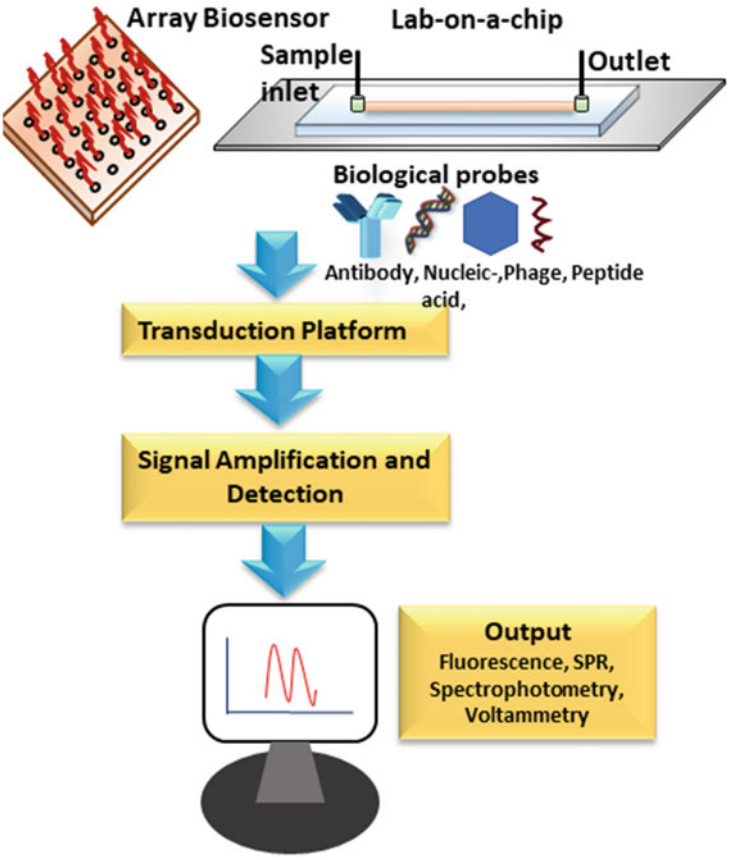

Fig. 10.2 Schematic representing the array biosensor and lab-on-a-chip devices in which the signal is transduced by different biological molecules which can be immobilized on the sensor platform, amplified, and then detected by different methods, i.e., fluorescence, SPR, spectrophotometry, and voltammetry

detection for improved disease management (Wakeham and Keane 2016). *Alternaria panax* is known to cause serious infection and economic losses in ginseng, a highly valued medicinal plant. Early infection was identified accurately with a single-tube nested PCR-lateral flow biosensor assay. The assay was highly sensitive which detected up to 0.01 pg of pathogen DNA and did not cross-react with the common fungal pathogens like *Cylindrocarpon destructans*, *B. cinerea*, *Sclerotionia schinseng*, and *Phytophthora cactorum* (Wei et al. 2018). Kang et al. (2020) developed a rapid lateral flow detection platform for the fungal pathogen *Magnaporthe oryzae* that mainly affects the two main cereal crops, rice and wheat. The CRISPR-Cas12a single-stranded DNAse activation was used with recombinase polymerase amplification (RPA) in the detection. The pathogen DNA was amplified in first RPA at 37–39 °C, 5 min, and treated with CRISPR-Cas12a for cleavage at

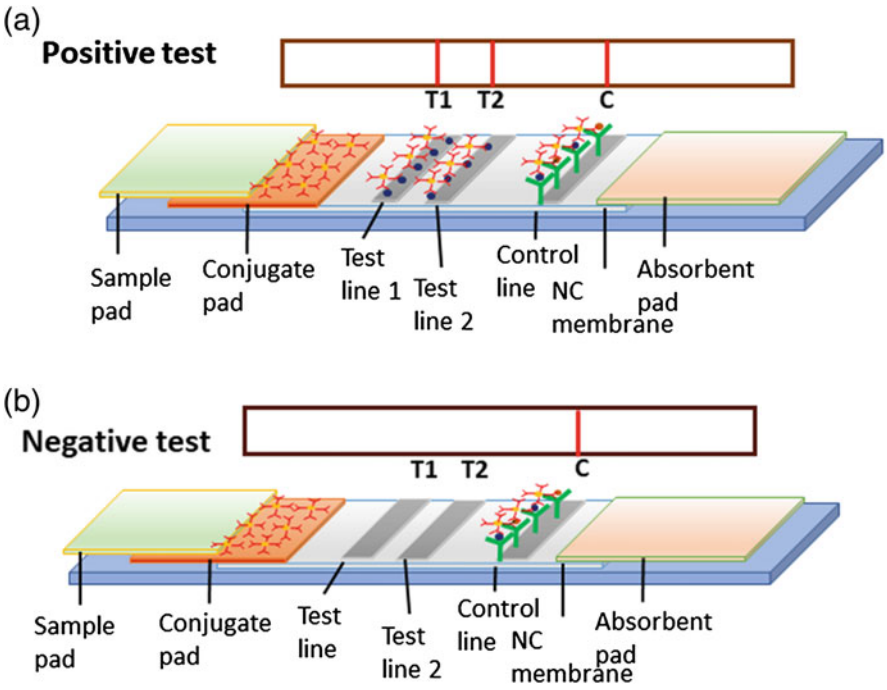

Fig. 10.3 Schematic representing the detection by multiplex lateral flow assay. (**a**) The schematic shows the negative result with control line only; (**b**) the positive result is represented by test line T1 and T2 for different targets and control line

specific sites. The resultant DNA strands were amplified with FITC/biotin primers with a second RPA at 37–39 °C, 5 min, and detected by the commercial on the lateral flow strip with the help of neutravidin-coated carbon particles. The detection was visualized by recognition of the DNA amplicons by the immobilized antibodies towards FITC/biotin and aggregation of the carbon nanoparticles. The detection platform can aid the plant pathologists, quarantine specialists, and agricultural workers to identify infected plant seed lots and alternate hosts.

10.4.2.2 Detection of Human Pathogens

Aspergillosis is caused mainly by the human fungal pathogen *Aspergillus fumigatus* and other species such as *A. flavus* and *A. terreus* in immune-compromised patients. The disease presents clinical symptoms varying from allergic reactions to systemic invasive infection depending on host immunity. Thornton (2008) developed an immunochromatographic lateral flow device (LFD) for invasive aspergillosis (IA) now marketed as "*Aspergillus* Lateral Flow Device (*Asp*LFD)" (by OLM Diagnostics, Newcastle upon Tyne, UK). The LFD comprises of a mouse monoclonal antibody (mAb JF5, IgG3) specific to a protein epitope of a cell wall-associated N-linked glycoprotein of *Aspergillus* with a limit of detection of ~35 ng/ml in serum and BAL samples (Thornton 2008; Thornton et al. 2012). The LFD performance in

immune-compromised patient groups reports moderate to high sensitivity (38–100%) and specificity (63–100%). The detection was influenced by an ongoing antifungal treatment as in case of Platelia ELISA (Prattes et al. 2014). AspLFD detected most *Aspergillus* species and a few other closely related species like *Emericella nidulans* (*A. nidulans* teleomorph), *Eurotium amstelodami* (*A. amstelodami* teleomorph), and *Neosartorya fischeri* (*A. fischeri* teleomorph). JF5 mAbs did not react with other invasive fungal pathogens including *Candida albicans*, *Fusarium solani*, *Cryptococcus neoformans*, *Trichosporon*, *Scedosporium*, *Pseudallescheria boydii*, and *Rhizopus oryzae* but were weakly cross-reactive towards *Paecilomyces variotii* antigens (Thornton 2008, 2013). However, cross-reactivity of both AspLFD and Platelia ELISA to a few *Penicillium* species remains an issue (Thornton 2010).

Recently, a lateral flow assay "sōna *Aspergillus* Galactomannan LFA" was commercialized by IMMY, Norman, OK, USA, for the detection of *Aspergillus* GM in serum and BAL samples and evaluated in hematological malignancy and non-neutropenic (Jenks et al. 2019a, 2019b) patients. The detection limit of the LFA was 1.7 and 2.25 ng/ml of GM in serum and BAL, respectively. Another LFD reported the detection of Gal*f* antigen of *Aspergillus* GM in urine, with monoclonal antibodies (MAb476, Dufresne et al. 2012). The renal excretion of circulating *Aspergillus* GM in urine was detected up to 100 ng/ml from IA patients (Bennett et al. 1985; Dupont et al. 1987; Haynes et al. 1990).

Recently a nano-immunodiagnostic assay for invasive aspergillosis was reported for rapid detection of galactomannan from serum and BAL samples. The assay employed gold nanoparticles conjugated with polyclonal antibodies for galactomannan detection and was highly sensitive with LOD of 1 pg/ml. The assay was more sensitive than the commercial Platelia ELISA for *Aspergillus* antigen having a cutoff value of 0.5 associated with 1 ng/ml. The developed nano-immunodiagnostic assay had good potential for use in rapid, specific, sensitive, on-site diagnosis of invasive aspergillosis under resource poor settings (Rawal et al. 2019).

The metalloenzyme enolase, which converts 2-phosphoglycerate to phosphoenolpyruvate. located in *Candida* cell walls, is known to elicit antibody response in infected hosts. A lateral flow immunoassay (LFIA) was developed using recombinant enolase as the antibody capture agent, and detection was performed with gold nanoparticles labeled anti-human IgG (He et al. 2016). The specificity and sensitivity were 98.2% and 84.8%, respectively. The LFIA was in agreement with the standard ELISA test when tested with sera from 38 clinically proven cases and 50 healthy control subjects. The LFIA test was proposed for serological surveillance of invasive candidiasis in resource poor settings. *Candida albicans* is the predominant cause of invasive candidiasis of nosocomial occurrence. Recently Zhao et al. (2019) employed the multiple cross displacement amplification (MCDA) method with gold nanoparticle-based lateral flow biosensor (LFB) to detect the organism sensitively. MCDA is an isothermal amplification technology similar to LAMP but with higher sensitivity and has been employed for detection of bacterial pathogens. The

highly sensitive MCDA-LFB assay was simple and specific and could detect as low as 200 pg of pathogen DNA.

Cryptococcal meningitis is caused by the breathing in of the yeast *C. neoformans* or its spore that later disseminates from the lung to the central nervous system. Cryptococcal cases account for a million cases per year globally and 13–14% mortality in immune-compromised patients (Park et al. 2009). The CrgLFA employs a cocktail of two monoclonal antibodies to detect the four capsular serotypes of cell wall antigen glucuronoxylomannan rapidly in 10 min using a small, lightweight, dipstick format. The initial sensitivity of the assay was greater than the commercially available LA and EIA assays for the CrAg of A, B, C, and D serotypes. LFA meets the ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and deliverable to end users) criteria of WHO for rapid diagnostic tests. The assay is user-friendly and recommended for use with serum or urine samples (Kozel and Bauman 2012). Vidal and Boulware (2015) employed the CrgLFA to detect asymptomatic patients requiring preemptive antifungal treatment, thus preventing symptomatic infection and contributing in better disease management.

Progressive disseminative histoplasmosis is caused by *Histoplasma capsulatum* in immune-compromised patients. Cáceres et al. (2019) evaluated the commercial MiraVista Diagnostics *Histoplasma* antigen LFA for diagnosis of PDH in HIV patients. The test displayed a high sensitivity and specificity and required minimal laboratory equipment. Some cross-reactivity was reported in case of patients with paracoccidioidomycosis.

Fungal melanonychia or nail infection is caused by the fungal pathogen *T. rubrum* or sometimes by the non-dematiaceous *Aspergillus niger*, and a gold nanoparticle-based rapid detection system was developed for rapid detection (Sojinrin et al. 2017). The aggregation of colloidal gold nanoparticles leads to color change from red to purple (Fig. 10.4). The color change correlated with the presence of the *A. niger* fungal cells that were confirmed by Raman spectroscopy (Sojinrin et al. 2017).

The application of "nano"-enabled lateral flow assays can reduce the time and dependence on expensive equipment required for the detection of plant and human pathogenic fungi (Fig. 10.4). The direct and rapid detection will permit the appropriate control of the diseases by application of appropriate antifungal treatments.

10.4.3 Rapid Nano-LAMP Assay

Loop-mediated isothermal amplification (LAMP) is a method to amplify a target nucleic acid sequence under isothermal conditions in a water bath or heated block, thereby eliminating the need for thermal cycling equipment. LAMP method overcomes many limitations of PCR-based methods and can be used in resource poor settings as a simplified method for the detection of amplification. However, these methods are not specific for fungal or bacterial detection.

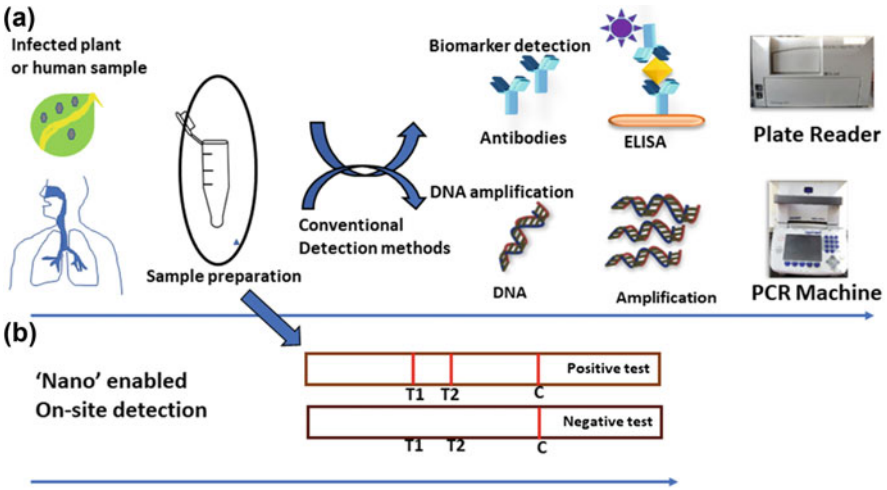

Fig. 10.4 Schematic comparing the traditional method for detection with "on-site" nano-enabled lateral flow assay. (**a**) The infected plant or human samples are processed for extraction of pathogenic protein or DNA detection and detected with ELISA reader and PCR machine. (**b**) The extracted samples can be directly detected by the LFA, thus reducing the time required for diagnosis

The accurate on-site detection of plant pathogens is important for the timely implementation of disease management and eradication strategies. Loop-mediated isothermal amplification (LAMP) is an attractive technique due to its suitability for on-site field application. The reaction can be monitored visually by precipitation of magnesium pyrophosphate that gives a white turbidity or SYBR Green I and hydroxynaphthol blue color indicators (Mori et al. 2001, 2004; Goto et al. 2009; Iwamoto et al. 2003). In an alternative approach, functionalized AuNPs were used to control aggregation/separation based on the presence/absence of Mg^{2+} in the LAMP reaction buffer providing a visible color change (Prado et al. 2016). Functionalized gold nanoparticles were used to visualize the LAMP-based DNA amplification of a bacterial pathogen, *Salmonella*, in microfluidic chip platform (Garrido-Maestu et al. 2017).

The recombinase polymerase amplification (RPA) that is a rapid isothermal amplification method having high specificity was combined with surface-enhanced Raman scattering (SERS) labeled gold nanotags to develop a rapid, highly specific and sensitive point-of-care method for multiplex detection of plant pathogens (Lau et al. 2016). Three agriculturally important plant pathogens (*B. cinerea*, *Pseudomonas syringae*, and *Fusarium oxysporum*) of *Arabidopsis thaliana* and tomato were detected with multiplex diagnostic platform. Further, the single-tube assay could identify the tomato pathogen *B. cinerea* rapidly in 40 min on the field.

An RPA assay was developed in conjunction with lateral flow dipstick assay for detecting *Phytophthora sojae*, the causal agent of soybean root and stem rot and seedling damping off (Dai et al. 2019). The 25-min assay was completed in two steps; initial amplification step within 20 min was followed by the detection of RPA

amplicons on the dipstick in 5 min. The rapid, sensitive RPA dipstick method was efficient in pathogen detection in comparison with the LAMP, conventional PCR, and bait leaf method.

10.4.4 Array Biosensors

Array biosensors are detection platforms that can detect multiple targets or organisms simultaneously and are integrated with waveguide surface for improved signal detection (Fig. 10.2). SERS imaging is a promising technique for sensor applications which relies on the enhancement of signals from biomolecules in close proximity of nanostructured surfaces based on their electromagnetic and chemical enhancement. It has been used for detection of single species of fungi.

The surface-enhanced RAMAN spectroscopy (SERS) signals from silver nanoparticle-coated FTO electrode were measured for different cell constituents like protein, nucleotides, and cell wall components like chitin, β(1,3)-glucans, and galactomannan for the identification of pathogenic organisms (Witkowska et al. 2016). Four different fungi, *Aspergillus flavus*, *Trichophyton rubrum*, *Scopulariopsis brumptii*, and *Candida krusei* were differentiated based on their specific signals rapidly, and multiple detections were possible in conjunction with the principal component analyses. *A. flavus* is the causal agent of invasive aspergillosis, and *T. rubrum* is a dermatophytic fungus. Dematiaceous fungi, like *S. brumptii*, which contains melanin in their cell walls, are increasing reported as causal agents of human phaeohyphomycosis/onychomycosis in patients with low immunity. *Candida krusei*, a budding yeast, displays inherent resistance to fluconazole, a first-in-line drug used for candidiasis treatment. Simultaneous label-free, rapid identification of the fungi was successfully carried out with the SERS-based array biosensor.

The SERS technique as a rapid tool detection was integrated with the dielectrophoretic (DEP) method for cell separation on a chip for multiplex *Candida* detection. Cheng et al. (2007) fabricated a microfluidic chip with 3D DEP gates for deflecting and separating fungal *C. albicans* and bacterial *E. coli* and *Lactobacillus* pathogen cells into channels based on their negative DEP mobility. Further identification was based on collection of the silver nanoparticle-enhanced SERS signals.

10.4.5 Lab on a Chip

Lab on a chip integrates sample preparation and detection and signal amplification on a single platform as a device for detection (Fig. 10.2). A platform integrating single-wall carbon nanotube (SWCNT) and field-effect transistors for signal transduction was used for detection of the human pathogenic fungus, *Candida*. One-dimensional single-walled carbon nanotubes are suitable for biosensing as they combine their signal transduction capability with easy functionalization by tagging with biomolecules for molecular recognition. Villamizar et al. (2009) fabricated a SWCNT-field-effect transistor by chemical vapor deposition on a silicon

chip and screen printing the source and drain electrodes. Monoclonal anti-*Candida* antibodies were immobilized on the surface of SWCNT and resulted in selective detection of the *Candida albicans* with a limit of detection (LOD) of 50 CFU/ml. The assay did not cross-react with other yeasts such as *Cryptococcus albidus* and *Saccharomyces cerevisiae*.

Microfluidic digital PCR platform is recently introduced as a robust platform for fungal detection. The droplet digital PCR (ddPCR) permits sample partitioning into thousands of water in oil droplet "nanoliter" reactors within microchannels on a disposable chip. These nanoreactors contain single molecules that are evaluated by endpoint PCR. The sophisticated liquid handling operations involve magnetic force and electrowetting to manipulate droplet merging and movement to carry out various steps essential for PCR, namely, DNA extraction, purification, heating, and cooling. Employing this technique, Schell et al. (2012) used the commercial Advanced Liquid Logic, Inc. microfluidic chip to detect *C. albicans* from blood samples of candidemia patients. DNA was extracted from the samples off-chip and applied to the biochip for detection. The ddPCR method achieved a sensitivity of 56% within 45 min as compared to the conventional PCR real-time analysis (69%, 70 min).

A lab-on-chip platform was reported by Schumacher et al. (2012) for the in vitro diagnosis of *Candida albicans* and other bacterial pathogens. A single microfluidic platform was constructed with sensitive electrochemical sensing by spotting nanoliter volumes of the captured molecules on the array chip and optically detected by total internal reflection fluorescence (TIRF). The highly integrated microfluidic LOC workflow consisted of on-chip DNA isolation from samples, DNA amplification by PCR, and fragmentation of amplicon by DNAse. Further, heating of hybridization compartment ensured binding of target DNA to the probes on the pre-spotted COP slides followed by detection by the optical sensing using TIRF.

10.5 Portable Genome Sequencer (Nanopore Sequencing System)

Nanopore technology from Oxford Technologies was pioneered by David Deamer at the University of California and George Church and Daniel Branton of Harvard University. This groundbreaking sequencing technology was designed on the basis of direct electronic analysis while DNA moves through the nanopore (Fig. 10.5). The α-hemolysis protein, belonging to the family of bacterial channel protein, functions as a nanopore to allow a single strand of DNA, ratcheted by the DNA polymerase, to move through it (Bayley 2015). The protein nanopore is held by a polymer bilayer membrane that is stretched over a microwell that contains a sensor chip to measure the ionic current during the passage of DNA bases through the nanopore. This technology has been applied for the rapid genome analysis during an outbreak to confirm the nosocomial hospital-borne infections caused by human fungal pathogen *Candida auris* (Rhodes et al. 2018). Etiological diagnosis of lower respiratory tract infections by bacterial and fungal strains, namely, *C. albicans* and *Candida humilis*, was identified with the aid of nanopore sequencing

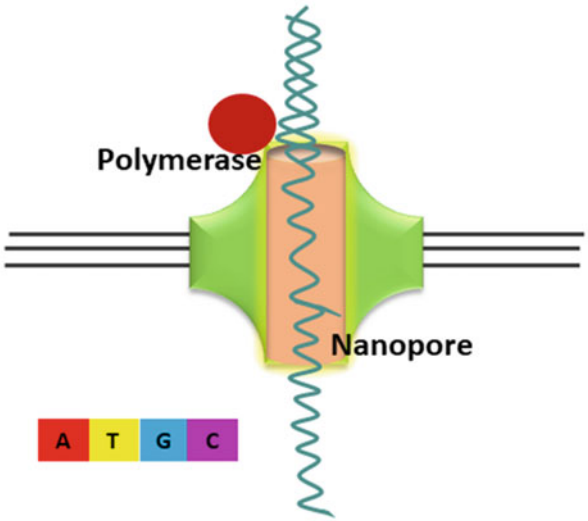

Fig. 10.5 Detection at the nanoscale in a nanopore DNA sequencer where a strand of DNA passes through a nanopore which is gated by DNA polymerase to unravel the double strand to a single strand. Further, the ionic current is measured when the molecule exits the nanopore and translated into the DNA sequence

(Chan et al. 2020). The commercial real-time DNA and RNA sequencer, MinION, is available as a handy, portable sequencer. During the recent outbreak of the resistant *Candida auris* in the UK, the MinION nanopore technology could detect the resistance alleles and the Asian origin of the strain.

Radhakrishnan et al. (2019) used the mobile nanopore sequencing platform to detect the presence of the wheat yellow rust pathogen, *P. striiformis* f. sp. *tritici (Pst)*, in wheat and assigned the strains to distinct lineages correlating with the virulence profiles and fungicide resistance. This approach was helpful for tracking the plant health.

This technology is gaining acceptance; however, the main challenges faced by nanopore sequencing are the control of the speed with which the DNA strand passes through the nanopore, as this is related with the read length and the quality of the data generated (Clarke et al. 2009). Though nanopore sequencing platform faces unique challenges, this platform is simple and straightforward and holds potential.

10.5.1 Magnetic Nanoparticles for Barcodes and NMR

Nucleic magnetic resonance (NMR) is a sensitive technique for detection of biomolecules based on characteristic release of electromagnetic radiation by nuclei in response to a magnetic field. Biological samples such as urine, blood, and serum can be analyzed without the requirement of a filtration or purification step by NMR with high sensitivity. Downsizing of NMR can be useful to target and quantify bioanalytes during pathogen detection. Neely et al. (2013) employed the sensitivity of NMR detection for detection of species-specific DNA for pathogen identification. First, pan-*Candida* PCR primers amplified the internal transcribed spacer region, and later the amplified DNA was hybridized with the superparamagnetic

nanoparticle-tagged probes. Hybridization led to clustering of the dispersed superparamagnetic nanoparticles, and the resultant T2 magnetic resonance showed a change in the relaxation time that could detect the presence of the pathogen.

The DNA bio-barcode assay employs oligonucleotide-modified magnetic nanoparticles for separation of the target DNA followed by recognition and signal amplification by oligonucleotide probe-modified gold nanoparticles (Nam et al. 2004). gold-coated magnetic nanoparticles (AuMNPs) bio-barcode test provides a highly sensitive method for rapid detection of protein and nucleic acid targets at low concentrations (Goluch et al. 2006). The concept of the bio-barcode assay is unique for the quick detection of pathogen DNA or protein.

The commercial T2X platform T2 Biosystems for *Candida* is approved by the US FDA (Mylonakis et al. 2015). The machine automatically processes serum samples to extract pathogen DNA by bead beating followed by amplification of the pathogen DNA with pan-*Candida* intertranscribed spacer primers towards RNA gene. Finally, it detected the pathogen by amplicon-induced aggregation of superparamagnetic nanoparticles and the T2MR measurements up to 1 CFU/ml of *Candida* in 3–5 h.

10.6 Conclusions

Fungi are generally harmless but can assume threatening proportions in agriculture and healthcare sectors with favorable change in climate conditions or reduction of host immunity, respectively. The detection of plant and human pathogenic fungi assumes importance for timely application of antifungal treatment for control and eradication in their proper management. One of the main limitations for rapid detection is the lack of on-site diagnosis. Nanotechnology can contribute to miniaturization of pathogen detection platforms for their on-site applicability. Lateral flow assays using gold nanoparticles are handy for rapid detection and have potential for increased scope in plant and human pathogenic fungi. Array biosensors, lab on a chip, nanopore technology, and magnetic nanoparticles are devices that can integrate detection and sample processing on a single platform. Development of robust, portable sensors for detection would allow for appropriate interventions for the management of pathogenic fungi. In case of plant pathogens, disease sensing and monitoring can be carried out on-site, permitting the farmers to make their own informed choices for timely application of pesticides or contact expert help when required. Detection of human fungal pathogens will allow timely treatment and therapy of several diseases/ailments, thus reducing hospitalization costs and mortality.

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References

Agati G, D'Onofrio C, Ducci E, Cuzzola A, Remorini D, Tuccio L et al (2013) Potential of a multiparametric optical sensor for determining in situ the maturity components of red and white *Vitis vinifera* wine grapes. J Agric Food Chem 61:12211–12218

Alarcon AA, Lazazzara V, Cappelin L, Bianchedi PL, Schuhmached R, Wohlfahrt G et al (2015) Emission of volatile sesquiterpenes and monoterpenes in grapevine genotypes following *Plasmopara viticola* inoculation *in vitro*. J Mass Spectrom 50:1013–1022

Almeida F, Rodrigues ML, Coelho C (2019) The still underestimated problem of fungal diseases worldwide. Front Microbiol 10:214

Amatulli MT, Spadaro D, Gullino ML, Garibaldi A (2012) Conventional and real time PCR for the identification of *Fusarium fujikuroi* and *Fusarium proliferatum* from diseased rice tissues and seeds. Eur J Plant Pathol 134:401–408

Barnes RA, White PL (2016) PCR technology for detection of invasive aspergillosis. J Fungi 2:1–9

Barton RC (2013) Laboratory diagnosis of invasive aspergillosis: from diagnosis to prediction of outcome. Scientifica 2013:459405, 1–29

Bayley H (2015) Nanopore sequencing: from imagination to reality. Clin Chem 61:25–31. https://doi.org/10.1373/clinchem.2014.223016

Belanger MC, Roger JM, CartolaroP VAA, Bellon-Maural V (2008) Detection of powdery mildew in grapevine using remotely sensed UV-induced fluorescence. Int J Remote Sensing 29:1707–1724

Bennett JE, Bhattacharjee AK, Glaudemans CPJ (1985) Galactofuranosyl groups are immunodominant in *Aspergillus fumigatus* galactomannan. Mol Immunol 22:251–254

Berdugo C, Zito R, Paulus S, Mahlein AK (2014) Fusion of sensor data for the detection and differentiation of plant diseases in cucumber. Plant Pathol 63:1344–1356

Blum U, Windfuhr M, Buitrago-Tellez C, Sigmund G, Herbst EW, Langer M (1994) Invasive pulmonary aspergillosis. MRI, CT, and plain radiographic findings and their contribution for early diagnosis. Chest 106:1156–1161

Bravo C, Moshou D, West J, McCartney A, Ramon H (2003) Early disease detection in wheat fields using spectral reflectance. Biosyst Eng 84:137–145

Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC (2012) Hidden killers: human fungal infections. Sci Transl Med 4:1–9

Burling K, Hunsche M, Noga G (2011) Use of blue green and chlorophyll fluorescence measurements for differentiation nitrogen deficiency and pathogen infection in wheat. J Plant Physiol 168:1641–1648

Busca A, Locatelli F, Barbui A, Limerutti G, Serra R, Libertucci D et al (2006) Usefulness of sequential *Aspergillus* galactomannan antigen detection combined with early radiologic evaluation for diagnosis of invasive pulmonary aspergillosis in patients undergoing allogeneic stem cell transplantation. Transplant Proc 38:1610–1613

Cáceres DH, Gómez BL, Tobón AM, Chiller TM, Lindsley MD (2019) Evaluation of a *Histoplasma* antigen lateral flow assay for the rapid diagnosis of progressive disseminated histoplasmosis in Colombian patients with AIDS. Mycoses 00:1–6

Caillot D, Couaillier JF, Bernard A, Casasnovas O, Denning DW, Mannone L, Lopez J, Couillault G, Piard F, Vagner O, Guy H (2001) Increasing volume and changing characteristics of invasive pulmonary aspergillosis on sequential thoracic computed tomography scans in patients with neutropenia. J Clin Oncol 19:253–259

Carneiro A, Matic G, Ortu G, Garibaldi A, Spadaro D, Gullino ML (2017) Development and validation of a Taqman real time PCR assay for the specific detection and quantitation of *Fusarium fujikuroi* in rice plants and seeds. Phytopathology 107:885–892

Casadevall A (2017) Don't forget the fungi considering global catastrophic biorisks. Health Secur 15:341–342

Chamilos G, Kontoyiannis DP (2006) Defining the diagnosis of invasive aspergillosis. Med Mycol 44:163–S173

Chan WS, Au CH, Leung SM, Ho DN, Wong EYL, To MY, Ng MK, Chan TL, Ma ESK, Tang BSF (2020) Potential utility of targeted nanopore sequencing for improving etiologic diagnosis of bacterial and fungal respiratory infection. Diagn Pathol 15:41

Cheng IF, Chang HC, Hou D, Chang HC (2007) An integrated dielectrophoretic chip for continuous bioparticle filtering, focusing, sorting, trapping, and detecting. Biomicrofluidics 1:021503

Chong GM, Maertens JA, Lagrou K, Driessen GJ, Cornelissen JJ, Rijnders BJ (2016) Diagnostic performance of galactomannan antigen testing in cerebrospinal fluid. J Clin Microbiol 54:428–431

Clarke J, Wu H-C, Jayasinghe L, Patel A, Reid S, Bayley H (2009) Continuous base identification for single-molecule nanopore DNA sequencing. Nat Nanotechnol 4:265270

Corio-Costet MF (2015) Monitoring resistance in obligate pathogens by bioassays relatin to field use: grapevine powdery and downy mildews. In: Ishii H, Holloman DW (eds) Fungicide resistance in plant pathogens. Springer, Tokyo, pp 251–279

Dai T, Yang X, Hu T, Jiao B, Xu Y, Zheng X, Shen D (2019) Comparative evaluation of a novel recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) assay, LAMP, conventional PCR, and leaf-disc baiting methods for detection of *Phytophthora sojae*. Front Microbiol 10:1884

de Pauw BE (2011) What are fungal infections? Mediterr J Hematol Infect Dis 3(1):e2011001

Dean R, Van Kan JA, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD et al (2012) The top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol 13:414–430

Denning DW (1998) Invasive aspergillosis. Clin Infect Dis 26:781–805

Dixon C, Leader MT, Bala S (2011). Biomarker qualification microbiology review, pp 1–94. https://www.fda.gov/media/90001/download

Duan YB, Ge CY, Zhang XK, Wang JX, Zhou MG (2014) Development and evaluation of a novel and rapid detection assay for Botrytis cinerea based on loop-mediated isothermal amplification. PLoS One 9(10):e111094

Duettmann W, Koidl C, Troppan K, Seeber K, Buzina W, Wölfler A, Wagner J, Krause R, Hoenigl M (2014) Serum and urine galactomannan testing for screening in patients with hematological malignancies. Med Mycol 52:647–652

Dufresne SF, Datta K, Li X, Dadachova E, Staab JF, Patterson TF, Feldmesser M, Marr KA (2012) Detection of urinary excreted fungal galactomannan-like antigens for diagnosis of invasive aspergillosis. PLoS One 7:1–10

Dupont B, Huber M, Kim SJ, Bennett JE (1987) Galactomannan antigenemia and antigenuria in aspergillosis: studies in patients and experimentally infected rabbits. J Infect Dis 155:1–11

Fahrentrapp J, Ria F, Panassiti B (2019) Detection of gray mold leaf infections prior to visual symptom appearance using a five-band multispectral sensor. Front Plant Sci 10:628

Falacy JS, Grove GG, Mahaffee WF, Galloway H, Glawe DA, Larsen RC, Vandermark GJ (2007) Detection of Erysiphe necator in air samples using the polymerase chain reaction and species-specific primers. Phytopathology 97:1290–1297

Fanfair RN, Benedict K, Bos J, Bennett SD, Lo YC, Adebanjo T, Etienne K, Deak E, Derado G, Shieh WJ, Drew C, Zaki S (2012) Necrotizing cutaneous mucormycosis after a tornado in Joplin, Missouri, in 2011. N Engl J Med 367:2214–2225

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

Fleming RV, Walsh TJ et al (2002) Emerging and less common fungal pathogens. Infect Dis Clin N Am 16(4):915–933

Fukuzawa M, Inaba H, Hayama M, Sakaguchi N, Sano K, Ito M, Hotchi M (1995) Improved detection of medically important fungi by immunoperoxidase staining with polyclonal antibodies. Virchows Arch 427:407–414

Garcia-Solache MA, Casadevall A (2010) Global warming will bring new fungal diseases for mammals. MBio 1:e00061–e00010

Garrido-Maestu A, Azinheiro S, Carvalho J, Abalde-Cela S, Carbó-Argibay E, Diéguez L, Piotrowski M, Kolenko YV, Prado M (2017) Combination of microfluidic loop-mediated

isothermal amplification with gold nanoparticles for rapid detection of salmonella spp. in food samples. Front Microbiol 8:2159

Georgiadou SP, Sipsas NV, Marom EM, Kontoyiannis DP (2011) The diagnostic value of halo and reversed halo signs for invasive mold infections in compromised hosts. Clin Infect Dis 52:1144–1155

Ghormade G, Deshpande MV, Paknikar KM (2011) Perspectives for nano-biotechnology in plant protection and nutrition. Biotechnol Adv 29:792–803

Gindro K, Lecoultre N, Molino L, de Joffery JP, Schnee S, Voinesco F, Alonso-Villaverde V, Viret O, Dubuis PH (2014) Development of rapid direct PCR assays to identify downy and powdery mildew and grey mould in *Vitis vinifera* tissues. J Int Sci Vigne Vin 48:261–268

Goluch ED, Nam JM, Georganopoulou DG, Chiesl TN, Shaikh KA, Ryu KS, Barron AE, Mirkin CA, Liu C (2006) A biobarcode assay for on-chip attomolar-sensitivity protein detection. Lab Chip 6:12931299

Goto M, Honda E, Ogura A, Nomoto A, Hanaki KI (2009) Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxynaphthol blue. BioTechniques 46:167–172

Guarner J, Brandt ME (2011) Histopathologic diagnosis of fungal infections in the 21st century. Clin Microbiol Rev 24:247–280

Gupta DR, Surovy MZ, Mahmud NU, Chakraborty M, Paul SK, Hossain MS et al (2020) Suitable methods for isolation, culture, storage and identification of wheat blast fungus *Magnaporthe oryzae Triticum* pathotype. Phytopathol Res 2:30–43

Hayden RT, Qian X, Procop GW, Roberts GD, Lloyd RV (2002) *In situ* hybridization for the identification of filamentous fungi in tissue section. Diagn Mol Pathol 11:119–126

Hayette MP, Vaira D, Susin F, Boland P, Christiaens G, Melin P, De Mol P (2001) Detection of *Aspergillus* species DNA by PCR in bronchoalveolar lavage fluid. J Clin Microbiol 39:2338–2340

Haynes KA, Latge JP, Rogers TR (1990) Detection of *Aspergillus* antigens associated with invasive infection. J Clin Microbiol 28:2040–2044

He ZX, Shi LC, Ran XY, Li W, Wang XL, Wang FK (2016) Development of a lateral flow immunoassay for the rapid diagnosis of invasive candidiasis. Front Microbiol 7:1451

Hope WW, Walsh TJ, Denning DW (2005) Laboratory diagnosis of invasive aspergillosis. Lancet Infect Dis 5:609–622

Horvath JA, Dummer S (1996) The use of respiratory-tract cultures in the diagnosis of invasive pulmonary aspergillosis. Am J Med 100:171–178

Islam MT, Croll D, Gladieux P, Soanes DM, Persoons A, Bhattacharjee P et al (2016) Emergence of wheat blast in Bangladesh was caused by a south American lineage of *Magnaporthe oryzae*. BMC Biol 14:84

Iwamoto T, Sonobe T, Hayashi K (2003) Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J Clin Microbiol 41:2616–2622

Jain A, Sarsaiya S, Wu Q, Lu Y, Shi J (2019) A review of plant leaf fungal diseases and its environment speciation. Bioengineered 10:409–424

Jankovics T, Bai Y, Kovacs GM, Bardin M, Nicot PC, Toyoda H et al (2008) *Oidium neolycopersici:* intraspecific variability inferred from amplified fragment length polymorphism analysis and relationship with closely related powdery mildew fungi infecting various plant species. Am Phytopahtol Soc 98:529

Jenks JD, Mehta SR, Taplitz R, Law N, Reed SL, Hoenigl M (2019a) Bronchoalveolar lavage *Aspergillus* galactomannan lateral flow assay versus *Aspergillus*-specific lateral flow device test for diagnosis of invasive pulmonary aspergillosis in patients with hematological malignancies. J Infect 78:249–259

Jenks JD, Mehta SR, Taplitz R, Aslam S, Reed SL, Hoenigl M (2019b) Point-of-care diagnosis of invasive aspergillosis in non-neutropenic patients: *Aspergillus* galactomannan lateral flow assay versus *Aspergillus*-specific lateral flow device test in bronchoalveolar lavage. Mycoses 62:230–236

Jensen HE, Salonen J, Ekfors TO (1997) The use of immunohistochemistry to improve sensitivity and specificity in the diagnosis of systemic mycoses in patients with haematological malignancies. J Pathol 181:100–105

Jin J, Depei W, Liu Y, Pan SS, Yan JL, Aram JA, Lou Y, Meng H, Chen X, Zhang X, Schwartz IS, Patterson TF (2017) Utility of computed tomography for the diagnosis of invasive aspergillosis in hematological malignancy. Blood 130:1–6

Kang H, Peng Y, Hua K, Deng Y, Bellizzi M, Gupta DR, Mahmud NU, Urashima AS, Paul SK, Peterson G, Zhou Y, Zhou X, Islam MT, Wang GL (2020) Rapid detection of wheat blast pathogen *Magnaporthe Oryzae Triticum* pathotype using genome specific primers and Cas12a-mediated technology. Engineering. https://doi.org/10.1016/j.eng.2020.07.016

Katragkou A, Pana ZD, Perlin DS, Kontoyiannis DP, Walsh TJ, Roilides E (2014) *Exserohilum* infections: review of 48 cases before the 2012 United States outbreak. Med Mycol 52:376–386

Kaufman L, Standard PG, Jalbert M, Kraft DE (1997) Immunohistologic identification of *Aspergillus* spp. and other hyaline fungi by using polyclonal fluorescent antibodies. J Clin Microbiol 35:2206–2209

Khater M, de la Escosura-Muñiza A, Merkoçi A (2017) Biosensors for plant pathogen detection. Biosens Bioelectron 93:72–86

Khiyami MA, Almoammar H, Awad YM, Alghuthaymi MA, Abd-ElsalamKA (2014) Plant pathogen nanodiagnostic techniques: forthcoming changes? Biotechnol Biotechnol Equip 28:775785

Kiss L, Takamasu S, Cunningtom JH (2005) Molecular identification of *Oidium neolycopersici:* as the causal agent of the recent tomato powdery mildew epidemics in North America. Plant Dis 89:491–496

Klont RR, Meis JFGM, Verweij PE (2001) Critical assessment of issues in the diagnosis of invasive aspergillosis. Clin Microbiol Infect Dis 7:32–37

Kousha M, Tadi R, Soubani AO (2011) Pulmonary aspergillosis: a clinical review. Eur Respir Rev 20:156–174

Kozel TR, Bauman SK (2012) CrAg lateral flow assay for Cryptococcosis. Expert Opin Med Diagn 6:245–251. https://doi.org/10.1517/17530059.2012.681300

Kuhlman JE, Fishman EK, Siegelman SS (1985) Invasive pulmonary aspergillosis in acute leukemia: characteristic findings on CT, the CT halo sign, and the role of CT in early diagnosis. Radiology 157:611–614

Kumar V, Arora K (2020) Trends in nano-inspired biosensors for plants. Mater Sci Energy Technol 3:255–273

Larone DH, Walsh TJ (2013) *Exserohilum rostratum*: anatomy of a national outbreak of fungal meningitis. Clin Microbiol News 35:185–193

Latouche G, Debord C, Raynal M, Milhade C, Cerovic Z (2015) First detection of the presence of naturally occurring grapevine downy mildew in the field by a fluorescence based method. Photochem Photobiol Sci 14:1807–1813

Lau HY, Wang Y, Wee EJH, Botella JR, Trau M (2016) Field demonstration of a multiplexed point-of-care diagnostic platform for plant pathogens. Anal Chem 88:8074–8081

Lee YR, Choi YW, Lee KJ, Jeon SC, Park CK, Heo JN (2005) CT halo sign: the spectrum of pulmonary diseases. Br J Radiol 78:862–865

Maertens J, Verhaegen J, Lagrou K, Van Eldere J, Boogaerts M (2001) Screening for circulating galactomannan as a noninvasive diagnostic tool for invasive aspergillosis in prolonged neutropenic patients and stem cell transplantation recipients: a prospective validation. Blood 97:1604–1610

Mahlein AK (2016) Plant disease detection by imaging sensors-parallels and specific demands for precision agriculture and plant phenotyping. Plant Dis 100:241–251

Meersseman W, Lagrou K, Maertens J, Wilmer A, Hermans G, Vanderschueren S et al (2008) Galactomannan in bronchoalveolar lavage fluid: a tool for diagnosing aspergillosis in intensive care unit patients. Am J Respir Crit Care Med 177:27–34

Meletiadis J, Roilides E (2013) Rare invasive fungal infections: epidemiology, diagnosis and management. Curr Fungal Infect Rep 7:351–360

Mennink-Kersten MA, Donnelly JP, Verweij PE (2004) Detection of circulating galactomannan for the diagnosis and management of aspergillosis. Lancet Infect Dis 4:349–357

Mennink-Kersten MA, Ruegebrink D, Wasei N, Melchers WJ, Verweij PE (2006) In vitro release by *Aspergillus fumigatus* of galactofuranose antigens, 1,3-β-D-glucan, and DNA, surrogate markers used for diagnosis of invasive aspergillosis. J Clin Microbiol 44:1711–1718

Merz WG, Karp JE, Hoagland M, Jett-Goheen M, Junkins JM, Hood AF (1988) Diagnosis and successful treatment of fusariosis in the compromised host. J Infect Dis 158:1046–1055

Montarry J, Cartolaro P, Richard-Cervera S, Delmotte F (2009) Spatio-temporal distribution of Erysiphe necator genetic groups and their relationship with disease levels in vineyards. Eur J Plant Pathol 123:61–70

Mori Y, Nagamine K, Tomita N, Notomi T (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun 289:150–154

Mori Y, Kitao M, Tomita N, Notomi T (2004) Real-time turbidimetry of LAMP reaction for quantifying template DNA. J Biochem Biophys Methods 59:145–157

Mortensen KL, Johansen HK, Fuursted K, Knudsen JD, Gahrn-Hansen B, Jensen RH, Howard SJ, Arendrup MC (2011) A prospective survey of *Aspergillus* spp. in respiratory tract samples: prevalence, clinical impact and antifungal susceptibility. Eur J Clin Microbiol Infect Dis 30:1355–1363

Moshou D, Bravo C, West J, Wahlen S, McCartney A, Ramon H (2004) Automatic detection of yellow rust in wheat using reflectance measurements and neural networks. Comput Electron Agric 44:173–188

Mutha D, Kothari M, Jain L, Sanghavi M (2018) Detection of grapes disease (downy mildew)based on IOT and image processing. In: IJREAM, pp 98–101

Mylonakis E, Clancy CJ, Ostrosky-Zeichner L, Garey KW, Alangaden GJ, Vazquez JA et al (2015) T2 magnetic resonance assay for the rapid diagnosis of candidemia in whole blood: a clinical trial. Clin Infect Dis 60:892–899

Nam JM, Stoeva SI, Mirkin CA (2004) Bio-bar-code-based DNA detection with PCR-like sensitivity. J Am Chem Soc 126:59325933

Neely LA, Audeh M, Phung NA, Min M, Suchocki A, Plourde D et al (2013) T2 magnetic resonance enables nanoparticle-mediated rapid detection of Candidemia in whole blood. Sci Transl Med 5:182–154

Nweze EI, Ghannoum A, Chandra J, Ghannoum MA, Mukherjee PK (2012) Development of a 96-well catheter-based microdilution method to test antifungal susceptibility of *Candida* biofilms. J Antimicrob Chemother 67:149–153

Oberti R, Marchi M, Tirelli P, Calcante A, Iriti M, Borghese AN (2014) Automatic detection of powdery mildew on grapevine leaves by image analysis optimal view-angle range to increase the sensitivity. Comput Electron Agric 104:1–8

Oerke EC, Mahlein AK, Steiner U (2014) Thermographic assessment of scab disease on apple leaves. Precis Agric 12:699–715

Oerke EC, Herzog K, Toepfer R (2016) Hyperspectral phenotyping of the reaction of grapevine genotypes to *Plasmopara viticola*. J Exp Bot 67:5529–5543

Orlowski HLP, McWilliams S, Mellnick VM, Bhalla S, Lubner MG, Pickhardt PJ et al (2017) Imaging spectrum of invasive fungal and fungal-like infections. Radiographics 37:1119–1134

Ortega SF, Tomlinson J, Hodgett J, Spadaro D, Gullino ML, Boonham N (2018) Development of loop mediated isothermal amplification of seedborne fungal pathogens *Fusarium fujikuroi* and *Magnaporthe oryzae* in rice seed. Plant Dis 102:1549–1558

Park BJ, Wannemuehler KA, Marston BJ (2009) Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS 23:525–530

Paugam A, Baixench MT, Lebuisson A, Dupouy-Camet J (2010) Diagnosis of invasive pulmonary aspergillosis: value of bronchoalveolar lavage galactomannan for immunocompromised patients. Pathol Biol 58:100–103

Pazos C, Pontón J, Del PA (2005) Contribution of (1→3)-β-D-glucan chromogenic assay to diagnosis and therapeutic monitoring of invasive aspergillosis in neutropenic adult patients: a comparison with serial screening for circulating galactomannan. J Clin Microbiol 43:299–305

Phillips P, Weiner MH (1987) Invasive aspergillosis diagnosed by immunohistochemistry with monoclonal and polyclonal reagents. Hum Pathol 18:1015–1024

Piérard GE, Arrese EJ, Piérard FC, Thiry A, Stynen D (1991) Immunohistochemical expression of galactomannan in the cytoplasm of phagocytic cells during invasive aspergillosis. Am J Clin Pathol 96:373–376

Prado M, Espiña B, Fernandez-Argüelles MT, Diéguez L, Fuciños P, Vial S et al (2016) Detection of foodborne pathogens using nanoparticles, advantages and trends. Antimicrob Food Packag:183–201. https://doi.org/10.1016/B978-0-12-800723-5.00014-0

Prattes J, Flick H, Prüller F, Koidl C, Raggam RB, Palfner M et al (2014) Novel tests for diagnosis of invasive aspergillosis in patients with underlying respiratory diseases. Am J Respir Crit Care Med 190:922–929

Radhakrishnan GV, Cook NM, Bueno-Sancho V, Lewis CM, Persoons A, Mitiku AD, Heaton M et al (2019) MARPLE, a point-of-care, strain-level disease diagnostics and surveillance tool for complex fungal pathogens. BMC Biol 17:65

Rallos LE, Baudoin AB (2015) Co-occurrence of two allelic variants of CYP51 in Erysiphe necator and their correlation with over-expression for DMI resistance. PLOS One 11:e0148025

Rawal KM, Ghormade V, Rajamohanan PR, Choudhary H, Rudramurthy SM, Chakrabarti A et al (2019) Development of a nano-gold immunodiagnostic assay for rapid in-site detection of invasive aspergillosis. J Med Microbiol 68:1341–1352

Rhodes J, Abdolrasouli A, Farrer RA, Cuomo CA, Aanensen DM, Armstrong-James D et al (2018) Genomic epidemiology of the UK outbreak of the emerging human fungal pathogen *Candida auris*. Emerg Microbes Infect 7:1–12

Rosa C, Araujo R, Rodrigues AG, Pinto-de-Sousa MI, Pina-Vaz C (2011) Detection of *Aspergillus* species in BACTEC blood cultures. J Med Microbiol 60:1467–1471

Savary S, Ficke A, Aubertot JN, Hollier C (2012) Crop losses due to diseases and their implications for global food production losses and food security. Food Secur 4:519–537

Schell WA, Benton JL, Smith PB, Poore M, Rouse JL, Boles DJ et al (2012) Evaluation of a digital microfluidic real-time PCR platform to detect DNA of *Candida albicans* in blood. Eur J Clin Microbiol Infect Dis 31:2237–2245

Schumacher S, Nestler J, Otto T, Wegener M, Ehrentreich-Forster E, Michel D et al (2012) Highly-integrated lab-on-chip system for point-of-care multiparameter analysis. Lab Chip 12:464–473

Šebela D, Olejnickova J, Sotolar R, Vrchtova N, Triska J (2014) Towards optical detection of Plasmopara viticola infection in the field. J Plant Pathol 96:309–320

Sojinrin T, Conde J, Liu K, Curtin J, Byrne HJ, Cui D, Tian F (2017) Plasmonic gold nanoparticles for detection of fungi and human cutaneous fungal infections. Anal Bioanal Chem 409:4647–4658

Steinbach WJ, Mitchell TG, Schell WA, Espinel-Ingroff A, Coico RF, Walsh TJ et al (2003) Status of medical mycology education. Med Mycol 41:457–467

Stoll M, Schultz HR, Baecker G, Berkelmann-Loehnertz B (2008) Early pathogen detection under different water status and the assessment of spray application on vineyards through the use of thermal imagery. Precis Agric 9:407–417

Stynen D, Sarfati J, Goris A, Prevost MC, Lesourd M, Kamphuis H et al (1992) Rat monoclonal antibodies against *Aspergillus* galactomannan. Infect Immun 60:2237–2245

Suuidi M, Kim J, Park JM, Bae SC, Kim D, Kim YH, Ahn IP (2013) Quantification of rice blasé disease progression through Taqman real time PCR. Mol Biotechnol 55:43–48

Thakur RP, Mathur K (2002) Downy mildews of India. Crop Prot 21:333–345

Theel ES, Doern CD (2013) β-D-glucan testing is important for diagnosis of invasive fungal infections. J Clin Microbiol 51:3478–3483

Thiessen LD, Keuneb JA, Neill TM, Turechek WW, Grove GG, Mahaffee WF (2016) Development of a grower-conducted inoculum detection assay for management of grape powdery mildew. Plant Pathol 65:238–249

Thornton CR (2008) Development of an immunochromatographic lateral-flow device for rapid serodiagnosis of invasive aspergillosis. Clin Vaccine Immunol 15(7):1095–1105

Thornton CR (2010) Chapter 6—Detection of invasive aspergillosis. Adv Appl Microbiol 70:187–216

Thornton CR (2013) Lateral-flow device for diagnosis of fungal infection. Curr Fungal Infect Rep 7:244–251

Thornton CR, Groenhof AC, Forrest R, Lamotte R (2004) A one-step, immunochromatographic lateral flow device specific to *Rhizoctonia solani* and certain related species, and its use to detect and quantify *R. solani* in soil. Phytopathology 94:280–288

Thornton CR, Johnson G, Agrawal S (2012) Detection of invasive pulmonary aspergillosis in haematological malignancy patients by using lateral-flow technology. J Vis Exp 61:1–5

Tran T, Beal SG (2016) Application of the 1,3-β-D-glucan (Fungitell) assay in the diagnosis of invasive fungal infections. Arch Pathol Lab Med 140:181–185

Verdaguer V, Walsh TJ, Hope W, Cortez KJ (2007) Galactomannan antigen detection in the diagnosis of invasive aspergillosis. Expert Rev Mol Diagn 7:21–32

Vidal JE, Boulware DR (2015) Lateral flow assay for cryptococcal antigen: an important advance to improve the continuum of HIV care and reduce cryptococcal meningitis-related mortality. Rev Inst Med Trop Sao Paulo 57:38–45

Villamizar RA, Maroto A, Rius FX (2009) Improved detection of Candida albicans with carbon nanotube field-effect transistors. Sensor Actuat B 136:451–457

Wakeham AJ, Keane G (2016) Field evaluation of a competitive lateral flow assay for the detection of *Alternaria brassicae* in vegetable Brassica crops. Plant Dis 100:1831–1839

Walsh TJ, Groll A, Hiemenz J, Fleming R, Roilides E, Anaissie E (2004) Infections due to emerging and uncommon medically important fungal pathogens. Clin Microbiol Infect 10:48–66

Wang T, Li P, Zhang Q, Zhang W, Zhang Z, Wang T, He T (2017) Determination of Aspergillus pathogens in agricultural products by a specific nanobody-polyclonal antibody sandwich ELISA. Sci Rep 7:4348–4359

Wei S, Sun Y, Xi G, Zhang H, Xiao M, Yin R (2018) Development of a single-tube nested PCR-lateral flow biosensor assay for rapid and accurate detection of *Alternaria panax* Whetz. PLoS One 13:e0206462

Wheat LJ (2003) Rapid diagnosis of invasive aspergillosis by antigen detection. Transpl Infect Dis 5:158–166

Wickes BL, Wiederhold NP (2018) Molecular diagnostics in medical mycology. Nat Commun 9:5135

Witkowska E, Jagielski T, Kaminska A, Kowalska A, Hryncewicz-Gwozdz A, Wa J (2016) Detection and identification of human fungal pathogens using surface-enhanced Raman spectroscopy and principal component analysis. Anal Methods 8:8427

Worrall EA, Hamid A, Mody KT, Mitter N, Pappu HR (2018) Nanotechnology for plant disease management. Agronomy 8:285

Wright WF, Overman SB, Ribes JA (2011) (1-3)-β-D-glucan assay: a review of its laboratory and clinical application. Lab Med 42:679–685

Zhao F, Niu L, Yan L, Nong J, Wang C, Wang J, Gao N, Zhu X, Wu L, Zheng F, Hu S (2019) Establishment and application of multiple cross displacement amplification coupled with lateral flow biosensor (MCDA-LFB) for visual and rapid detection of candida albicans in clinical samples. Front Cell Infect Microbiol 9:102

Part III

Plant/Human Fungal Pathogens and Their Control

Milestones in Medical Mycology in India

11

Harish C. Gugnani

Abstract

A brief account is given of the history and early progress of medical mycology in India, including the contributions of British Army physicians, surgeons, and our physicians (MDs, DM) and scientists with PhD in mycology, medical mycology, or medical microbiology. A noteworthy feature is the discovery by our eminent mycologists, Prof. S.B. Saxena and Prof. P.C. Misra, of new genera and species of fungi, namely, *Saksenaea vasiformis* and *Apophysomyces elegans*, which are now recognized as agents of human fungal infections worldwide. A mention is also made of the discovery of several new species of species of pathogenic molds and yeasts, novel pathogens, and novel lab techniques by medical mycologists. Recent progress of medical mycology in different medical institutions including the four internationally recognized centers of research on ocular fungal infections in India is covered in detail with lists of important publications (2000–2020) from these institutions. Aspects of training graduates in medicine and postgraduates in microbiology, medical microbiology, and biological sciences are adequately dealt with including the mention of self-learning resources. The need for their attending workshops and training courses offered by premier medical institutions in India is emphasized. Suggestions are given in detail for surveys by Department of Preventive and Social Medicine in medical colleges with the collaboration of state departments of health/primary health centers in communities representative of their areas to investigate the occurrence of fungal infections like ringworm, keratitis, and mycetoma to estimate their burden and chalk out preventive measures. The need for exploring antifungal therapy by herbal drugs by our mycologists and physicians is also emphasized. In the end of the chapter, the

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history of Indian Society of Medical Mycologists (ISMM) and its present status, ISMM awards, and ISMM Newsletter are mentioned.

Keywords

Historical account · Early progress · Recent progress · New species · Novel pathogens · Prevention · Herbal therapy · ISMM · Self-learning · Resources

11.1 Introduction

11.1.1 A Brief Historical Account[1]

Godfrey, a Garrison surgeon working in Bellary (Andhra Pradesh, India), first described mycetoma (as "morbus tuberculosis pedis") in 1846 in medical literature; Eyre, one of Godfrey's colleagues, described 40 cases treated between 1844 and 1848 (Eyre 1859). Carter in 1860 first suggested the fungal etiology of the disease and later in 1874 published a monograph "On Mycetoma and Fungus Diseases of India." The first isolation of the causative organism was made by Brumpt in 1906. Powell, a dermatologist in British Indian Army, reported on ringworm from Assam in 1904. The organized medical mycological research in India started in 1920 under Lt. Col. H.W. Acton (with C. McGuire, G. Panja and K.P. Banerjee) as a part of Pathology and Microbiology Department in Calcutta School of Tropical Medicine, Calcutta.

It is relevant to mention here that the work on fungal diseases of plants in India was also started by a British physician, E.J. Butler, at Pusa, Bihar, in 1903 with the publication of monograph on "Potato Diseases of India." Appointed as Imperial Plant Pathologist, Butler worked at the Indian Institute of Agricultural Research in Pusa (Bihar) from 1905 to 1921 and established a strong school of mycology and plant pathology. This institute is now located in Delhi and named as Indian Agricultural Research Institute. In 1918, Butler wrote a book on *Fungi and Disease in Plants*. Later he published an authoritative list of Indian fungi in collaboration with G.R. Bisby (Butler and Bisby 1931). This publication has been continuously updated until the last edition by Sarabhoy et al. (1993). Butler is aptly called the father of "Indian Plant Pathology." The book *Plant Pathology* (authors: E.J. Butler, S.G. Jones) published in 1949 is a classic textbook and is still a source of reference. It is noteworthy that three plant disease epidemics due to fungi, viz., *Helminthosporium*, blight of rice resulting in Bengal famine of 1943; wheat rust, *Puccinia triticina*, causing severe wheat shortage in Madhya Pradesh in 1946–1947; and the red rot of sugarcane, caused by *Colletotrichum falcatum* in several parts of northern India in 1938–1942, greatly stimulated the research on fungal diseases of plants. On the contrary, there was no such thing then to invigorate research on fungal infections in humans. However, the advent of global epidemic of AIDS and

[1]Extracted from my previous publication: s-3-eu-west-1.amazonaws.com/mycology_in_India (Training medical mycologists in developing countries 2006).

manifestation of certain opportunistic infections as indicator diseases in AIDS in the past several decades has promoted the study of human fungal infections to a great extent in developed countries and to some extent in developing countries including India.

11.1.2 Early Progress of Medical Mycology in India

It is encouraging to record here that many medical professionals (with MD, MS, DM degrees) and scientists with PhD in medical microbiology/medical mycology have undertaken the study of human pathogenic fungi and infections caused in several parts of India. The author of this chapter feels proud to have induced several MDs into the discipline of medical myology. During the past two decades, medical mycological research has progressed phenomenally; several medically qualified mycologists (MD, MD/PhDs) and biomedical scientists with PhD in microbiology/medical mycology are contributing greatly to the progress of this interdisciplinary subspecialty of medicine by working on pathogenesis, diagnosis, and epidemiology of fungal infections, frequently involving molecular techniques. As a result of this, several world-class excellent centers have emerged for training and research in medical mycology, viz., Postgraduate Institute of Medical Education & Research (PGI) Chandigarh; Vallabhbhai Patel Chest Institute (VPCI), University of Delhi, Delhi; and some AIIMS institutions and medical colleges. A special mention should be made of the Center of Advanced Research in Medical Mycology and WHO Collaborating Center for Reference and Research on Fungi of Medical Importance at PGI Chandigarh, headed by Prof. Arunaloke Chakrabarti, Head of Division of Med. Mycology. This center undertakes confirmation of species identification of the fungal isolates referred from several parts of India. Prof. Chakrabarti also shares joint responsibility with Dr. Tom Chiller of CDC, Atlanta, Georgia, USA, for Surveillance of Antimicrobial Resistance in invasive *Candida* infection under World Health Organization.

As would be seen in listed publications from different institutions, there is a continued progress with the collaboration of several globally renowned physicians and infectious disease specialists on fungal diseases in several thrust areas, namely, occurrence of candidemia in ICU settings; molecular techniques in the rapid diagnosis of fungal infections; epidemiology of chronic infections like pulmonary aspergillosis, superficial mycoses, and their management; neglected tropical diseases like histoplasmosis, mycetoma, chromoblastomycosis, and sporotrichosis; challenge of recurring and novel fungal infections; and infections in AIDS patients (e.g., talaromycosis (penicilliosis) marneffei, cryptococcosis). Work is also being done on better antifungal stewardship of all fungal diseases in the Indian setting with limited resources.

Another noteworthy feature of progress in medical mycology is that some globally recognized excellent centers of research on ocular fungal infections have been established in South India as mentioned in the later part of the chapter.

Historically, it is necessary to recall the discovery of new genus *Saksenaea* (species name *S. vasiformis*) of the order *Mucorales* by the mycologist Dr. S.-B. Saxena in 1953 from forest soils in Sagar, Madhya Pradesh, India, giving the generic name *Saksenia* in the honor of his teacher Prof. R.K. Saxena of Allahabad University. The species has been to found to be pathogenic to humans in several countries. In 2010a, Alvarez et al. demonstrated by molecular analysis that *Saksenaea vasiformis* is a complex of two species, naming these as *Saksenaea oblongispora*, characterized by oblong sporangiospores and inability to grow at 42 °C, and *Saksenaea erythrospora*, characterized by large sporangiophores and sporangia and by ellipsoid sporangiospores appearing biconcave in the later part of this view. Another mucoraceous fungus, *Apophysomyces elegans* first isolated by Misra in 1979 from soil of a mango orchard in north India and subsequently found to be pathogenic to humans, is now designated as a complex of four species, namely, *A. elegans*, *A. ossiformis*, *A. trapeziformis*, and *A. variabilis*. Chakrabarti et al. (2010a, b) described several cases of infection due to *A. elegans* (species complex) in India. Most of the isolates from India have been found to be of *A. variabilis* clade (Alvarez et al. 2010a, b).

11.1.3 Book, Manual, and Chapter in a Book

Books—Jagdish Chander's Textbook of Medical Mycology, 4th Edition (936 pages), 2018, published by Jaypee Publishers, New Delhi for Rs 2000. This book is very well illustrated and is popular among medical graduates and postgraduates in India, Pakistan, Sri Lanka, Bangladesh, Nepal, and some other countries of Southeast Asia, and recently it is being marketed in the international level. *Clinical Practice of Medical Mycology in Asia* (USD 100) at the website: customerservice@springer.nature; *Medical Mycology* by Dr. P. Vijayalakshmi (price USD 6.12) published by Sara Book Publication 303, Maharana Pratap Complex, OPP. Kapadia Guest House, B/H V.S. Hospital, Paldi, Ahmedabad (website for online purchase: sarapublication.com); and *Atlas of Clinical Fungi* (Authors: G.S. de Hoog, J. Guarro, J. Gené, S. Ahmed, A.M.S. Al-Hatmi, M.J. Figueras, and R.G. Vitale) published by Utrecht: Centraalbureau voor Schimmelcultures; Reus: UniversitatRoviraiVirgili, ©2000, Price USD 263.63.

Manuals—"https://mycology.adelaide.edu.au/docs/fungus3-book.pdf" DESCRIPTIONS OF MEDICAL FUNGI—Can be very easily searched in Google, www.researchgate.net.publication>14479267_Identification of Dematiaceous Fungi and Their Role in Human Disease. This can be very easily searched in Google. Another manual that can be easily located in the internet is "Mycotic Ulcer Treatment Trial (MUTT)" Version 10, revised on 04/23/2015. Manual of Operation and Procedures by several authors (N.V. Prajna, L. Prajna, J. Mascarenhas, R. Vijayakumar, R. Saravanan, S.R. Sumithra, S.R. Sumithra, R.D. Ravindran, T. Krishnan, R. Reddy, N. Shivananda, B. Raghunandan, K.B. Khadka, S. Patel, B.B. Thapa) from Aravind Eye Hospitals, Coimbatore, Madurai, Pondicherry, India; Bharatpur Eye Hospital, Bharatpur, Nepal; and

Lumbini Eye Institute, Bhairahawa, Nepal, and international collaborating authors from Departments of Surgery, Microbiology, and Immunology, Dartmouth Medical School, Hanover, New Hampshire, USA; Francis I. Proctor Foundation for Research in Ophthalmology, University of California, San Francisco (UCSF), USA; and Departments of Ophthalmology & Department of Epidemiology and Biostatistics, UCSF, San Francisco, California, USA.

Chapter—Gugnani HC. Ecology of dimorphic endemic pathogenic fungi in the book, *Fungi from Different Substrates.* (Eds. JK Misra, JP Tewari, SK Deshmukh and Csaba Vagvolgyi) CRC Press, USA (2011)

Other educational materials for self-learning, especially for pure mycologists sincerely interested in medical mycology, are as follows:

- Twitter Fungi SCOPE@ FungiSCOPE.
- Twitter Oliver Kurzai@Okurzai Medical microbiologist with special interest in sfg2 fungal infections.
- Twitter AberdeenFungal@AFGTweets Human fungal pathogenesis research group, fungal immunology, University of Aberdeen.
- MRC CMM@MRComm MRC Center for Medical Mycology which facilitates innovative interdisciplinary research and training to advance understanding of fungal pathogenesis and immunity.
- Gaffi—Global Action Fund for Fungal Infections. Can be very easily searched in Google.
- Asia Fungal Working Group, an ISHAM Working Group. New Diagnostic Mycology E-learning Course. This 12-module online course covers all aspects of medical mycology. **HOW TO REGISTER**: For ISHAM members, go to the Members Area to find out how to access the discount code, or non-ISHAM members, you can access the course at https://isham.scholarlms.com.
- *Medical Mycology in the United States*: *A Historical Analysis (1894–1996)*. Can be freely downloaded in Google. It also deals with history and progress of general mycology in USA.

11.1.4 Discovery of New Species of Human Pathogenic Fungi, Novel Pathogens, and Lab Techniques

Several new species of human pathogenic fungi and novel fungal pathogens have been discovered by medical mycologists in India as described in my previous publication (Amazon. s-3-eu-west-1.amazonaws.com/mycology_in_India.PDF). Subsequent to this publication, two new species of *Curvularia*, viz., *C. tamilnaduensis* and *C. coimbatorensis*, have been described as agents of keratitis by staff of Ocular Mycology Center, Coimbatore. This center also described first case report of fungal keratitis caused by *Podospora austroamericana*, *Myrothecium* sp. (Rameshkumar et al. 2019), and *Laetisaria arvalis* (Dudeja et al. 2018). Braddo et al. (2015) described rhinosinusitis caused by *Saksenaea erythrospora*; this fungus

is not previously described as a cause of this mycosis anywhere in the world record. Chowdhary et al. (2013) described *Schizophyllum commune* as a causal agent of one case each of allergic bronchopulmonary mycosis (ABPM) and pulmonary fungal ball for the first time in India. Kamalam and Thambiah (1982) described an unusual clinical manifestation, lymphedema, with subsequent elephantiasis in a case of chronic basidiobolomycosis. A new species of yeast, *Malassezia arunalokei*, was recovered from patients of seborrheic dermatitis and healthy individuals in India (Honnavar et al. 2016). Keratitis due to *Aspergillus nomius* and *Neocosmospora vasinfecta* (Manikandan et al. 2008b, 2009) and that caused by *Graphium eumorphum* (*Graphium* state of *Scedosporium apiospermum* (Palanisamy et al. 2015)) were described for the first time in India. Rudramurthy et al. (2011) described *Colletotrichum truncatum* as an unusual pathogen causing mycotic keratitis and endophthalmitis. Chowdhary et al. (2010a, b, 2020) described four novel fungal pathogens in India, viz., *Candida nivariensis* as an opportunistic pathogen, *Trichosporon mycotoxinivorans* causing blood stream infection in adults, *Candida blankii*, and *Dirkmeia churashimaensis* causing outbreaks of neonatal fungemia. First known case of keratitis due to *Aspergillus nomius* was described by Manikandan et al. (2009); Manikandan in association with other colleagues also described first two known cases of keratitis caused by *Aspergillus tubingensis* (Kredics et al. 2009). Homa et al. (2019) described another first known case of keratitis caused by *A. tamarii*. Tendolkar et al. (2014) described *Trichosporon inkin* and *Trichosporon mucoides* as unusual causes of white piedra of scalp hair. Keratitis due to *Chaetomium atrobrunneum* and *Pseudopestalotiopsis theae* was described as the first world record of ocular infection due to these fungi (Sane et al. 2019). Shankarnarayan et al. (2018) described several cases of bloodstream infections due to *Candida viswanathii*, a new species discovered by Viswanathan and Randhawa in 1959 as a cause of meningitis. Meena et al. (2019) described *Exophiala jeanselmei* and *Rhizopus oryzae* coinfection in a post renal transplant, this being a first world record of the coinfection due to these fungi. Also noteworthy is report of a case of nodular swelling caused by *Medicopsis romeroi* by Prasad et al. (2020), constituting the first record of human infection due to this fungus in India. Regarding novel lab techniques, Uma Tendolkar et al. (2003) devised a new medium, tobacco agar for pigment production of *Cryptococcus neoformans*. Nandhakumar et al. (2006) developed a new medium based on brown mustard seed for differentiation of *C. neoformans.*

11.1.5 Recent Progress of Medical Mycology in Different Institutions in India

Citations of recent important publications (2000–2020) up to 25 or at the most 27 in medical mycology from different institutions are cited in parenthesis after the write-up of each institute and are listed in a combined list under References in an alphabetical order as per the surname of first authors. Publications from some well-known medical colleges, though relatively very small in number, have been

accommodated in the list. The listed publications point out the current areas of medical mycological research in the listed institutions. The training courses offered by the Departments of Microbiology/Medical Mycology are stated for prospective students to avail of. The faculty staff in medical mycology and the available diagnostic tests are also mentioned. Due to limitations and lack of space, it is regretfully not possible to include in the list some other medical colleges and institutes, where only very little research in med. Mycology has been done recently.

Postgraduate Institute of Medical Education and Research (PGI), Division of Medical Mycology, Dept. of Microbiology, Chandigarh, India, It is recognized as the topmost medical institution in India. The division of medical mycology has the "National Reference Medical Mycology," "WHO Collaborating Center for Reference and Research on Fungi of Medical Importance," and "National Nodal Center for Antifungal Resistance Surveillance." Among WHO Collaborating Centers on fungi and related diseases, this center only has all the portfolios in the field of fungal infections. This also houses "National Culture Collection of Pathogenic Fungi" which is affiliated to the "World Federation of Culture Collections" and maintains a collection of more than 3400 standard and clinical fungal strains. The mycology center provides the service of identification of fungi isolated from clinical samples, supply of cultures, and antifungal susceptibility testing for institutes and laboratories across the country and also for some SSARC countries. It also provides regular training in the conventional and molecular techniques for the diagnosis, typing, and antifungal susceptibility testing and conducts the "External Quality Assurance Program (EQAP)" in Medical Mycology. In India, at present >140 laboratories have been enrolled in this program. Prof. Arunaloke Chakrabarti, Head of Division of Med. Mycology, shares joint responsibility with Dr. Tom Chiller of CDC, Atlanta, for Surveillance Antimicrobial Resistance in Invasive *Candida* infection under World Health Organization. The department has state-of-the-art facilities in the field of epidemiology, pathogenesis, and diagnosis of fungal infections (Biswal et al. 2017; Chakrabarti et al. 2003, 2006, 2008, 2009, 2010a, b, 2015a, b, 2019, 2020; Ghosh et al. 2015; Rastogi et al. 2016; Paul et al. 2017; Prakash et al. 2017; Rudramurthy et al. 2017b, 2018; Shankarnarayan et al. 2018, 2020; Shahi et al. 2020; Singh et al. 2011). **Faculty staff in medical mycology**: Arunaloke Chakrabarti, Shivaprakash M. Rudramurthy, Anoop Ghosh, and Harmeet Kaur; **Diagnostic tests**: Phenotypic typing and antimycotic susceptibility testing, molecular identification, DNA sequencers, MALDI-TOF, different PCRs (nested, real-time droplet), major equipment for DNA sequencing, liquid nitrogen storage, lyophilization, gel electrophoresis, etc.

Vallabhbhai Patel Chest Institute (VPCI), University of Delhi, Delhi, a leading postgraduate medical institute in India for teaching and research in chest diseases, was established by Dr. Raman Viswanathan (founder Director of the Institute), who is recognized as the father of Chest Medicine in India. He also established the Medical Mycology Department (now designated as Med. Mycology Unit, Dept. of Microbiology) in 1957 with Rajinder Singh Sandhu and Harbans Singh Randhawa as foundation staff. It was the first department of medical mycology opened in any institute in India; it progressed very fast to become a premier

center of training and research in medical mycology. The department has trained several PhDs in medical mycology, some of whom became globally recognized medical mycologists. The first six PhDs included H.S. Randhawa, Dhanwant Sandhu, Vishwanath Kurup, Harish C. Gugnani, Ziauddin Khan, and Saroj Mishra. Current Head of Med. Mycology Unit, Prof. Anuradha Chowdhary, assisted by some residents and technical staff is actively working on multidrug resistance in *Candida auris*, azole resistance in *C. glabrata* and *C. parapsilosis*, and terbinafine resistance in *Trichophyton* spp. Her team in collaboration with several scientists in other countries has generated comprehensive data on molecular types and antifungal susceptibility profiles of indigenous fungal isolates such as *Cryptococcus neoformans*, *C. gattii*, *Aspergillus* spp., *Schizophyllum commune*, and *Mucorales* (Biswal et al. 2017; Evans et al. 2000; Chowdhary et al. 2003, 2010a, b, 2013, 2016, 2017, 2020; Chowdhary and Meis 2020; Fakhim et al. 2017; Gugnani et al. 2000, 2002, 2003, 2004a, b, 2005, 2007, 2020a, b; Gugnani and Randhawa 2020; Gugnani and Sood 2020; Kathuria et al. 2014; Lalchandani et al. 2020; Randhawa and Gugnani 2018). **Faculty staff in medical mycology**: Anuradha Chowdhary assisted by residents and technical staff. H.S. Randhawa expired on11th November 2020. **Diagnostic tests**: Phenotyping and antimycotic susceptibility testing, molecular identification, MALDI-TOF, and PCR.

All India Institute of Medical Sciences (AIIMS), Department of Microbiology, New Delhi, It is one of the top ten ranking medical institutions in India and trains students for MBBS, MD in Microbiology, and DM in Infectious Diseases and other specialties and super specialties. The institute houses the oldest center of medical mycology founded by Prof. Sardari Lal Kalra (the founder Head of Microbiology Dept.) with the Mycology section, headed by Prof L.N. Mohapatra with Harish C. Gugnani working with him on an ICMR project; later Heads of the section were Uma Banerjee and Ramesh Kumar. The present head is Prof. Immaculata Xess. The Department trains students for MBBS and MD in Microbiology and Infectious Diseases (Ahuja et al. 2019; Arvind et al. 2020; Behera et al. 2010, 2011; Chakrabarti et al. 2020; Dabas et al. 2017; Duggal et al. 2015; Guha et al. 2020; Gupta et al. 2014, 2017, 2020a, b; Jain et al. 2013; Kumari et al. 2019; Meena et al. 2019; Mohanty et al. 2016; Patel et al. 2019; Seth et al. 2019; Singh et al. 2020a, b, c; Tewari et al. 2012; Rudramurthy et al. 2017a; Yoganathan et al. 2014). **Faculty staff in medical mycology**: Immaculata Xess and Gagandeep Singh; **Diagnostic tests**: Phenotypic identification, automated identification and susceptibility testing system for pathogenic fungi, MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight), conventional PCR, real-time PCR, and next-generation sequencing (NGS) **p**latform (Ion S5 next-generation systems) along with the conventional microbiological tools for diagnostic services.

AIIMS, Jodhpur (Rajasthan) Department of Microbiology is an excellent center for medical education, research, and healthcare. The Microbiology Dept. trains MDs and PhDs in microbiology and has a dynamic infection control team (Hada et al. 2018; Kombade et al. 2018; Kalita et al. 2019; Kaushal et al. 2019; Meena et al. 2020; Samaddar et al. 2019, 2020; Sharma 2019a, b; Sharma et al. 2019; 2020a, b). **Faculty staff in medical mycology**: Vijaya Lakshmi Nag, Anuradha

Sharma, and Sarika P. Kombade; **Diagnostic tests**: Direct microscopic examination of clinical specimens; phenotypical identification of pathogenic fungi, including automated culture of yeasts; and in vitro susceptibility testing;

AIIMS, Raipur (Chhattisgarh) Department of Microbiology. Faculty staff in medical mycology: Padma Das with the assistance of residents; **Diagnostic tests**: Direct microscopy of clinical specimens, phenotypic identification of pathogenic fungi, use of Vitek 2 for identification and antifungal susceptibility testing of yeasts (Das et al. 2018a, b; Patro et al. 2010; Rai et al. 2018; Sawatkar et al. 2019; Sonthalia et al. 2018; Varthya et al. 2020; Wankhade et al. 2020).

AIIMS, Bhuvneaswhar (Orrisa) Department of Microbiology (Mahapatra 2005; Mahapatra et al. 2007, 2009, 2014; Das et al. 2008; Hallur et al. 2013; Srivastva et al. 2019); **Faculty staff in medical mycology**: Ashoka Mahapatra, Vijaykumar, Hallur; **Diagnostic tests**: Direct microscopy of clinical specimens, phenotypic identification and antimycotic susceptibility testing of pathogenic fungi.

AIIMS Bhopal (MP) Departments of Microbiology and Lab Medicine, Faculty staff in medical mycology: Tadapalali Karuna, Debasis Biswas, Shashank Purwar, Robin Sharma, Samaksha Gupta, Kajal Gupta, Pradeep Kumar Gupta; **Diagnostic tests**: Direct microscopy of clinical specimens, phenotypic identification of pathogenic fungi, and PCR (Tadepalli et al. 2015; Thatoi et al. 2015; Sharma et al. 2017; Shrivastava et al. 2017; Chaurasia et al. 2020; Prasad et al. 2020).

L.V. Prasad Eye Institute, L. V. Prasad Marg, Banjara Hills, Hyderabad (LVPEI) is a comprehensive eye health facility in India, with its main campus located in Hyderabad, India. It also pursues cutting-edge research and offers training in human resources for all levels of ophthalmic personnel. LVPEI is the first major eye institute in India recognized by WHO. Microbiology Department is equipped with thermal cyclers, gel documentation units, spectrophotometer, electrophoresis equipment, dHPLC, a real-time PCR, and two automated DNA sequencers. Research laboratories offer off-campus interdisciplinary PhD programs, recognized by the Birla Institute of Technology & Science (BITS), Pilani, and the University of Hyderabad (Baine et al. 2012; Bibhudutta et al. 2011; Dave et al. 2019; Das 2020; Garg 2012; Garg et al. 2016, 2017; Gopinathan et al. 2009; Pradhan et al. 2011; Reddy et al. 2015; Sane et al. 2019; Sharma et al. 2015; Taneja et al. 2012). **Faculty staff in medical mycology**: Savitri Sharma, Usha Gopinathan, Prasant Garg, Gullapalli N. Rao, and Sreedharan Athmanathan; **Diagnostic tests**: Direct microscopic examination of clinical specimens, phenotypic identification of fungi, PCR, and molecular typing.

Arvind Eye Hospital & Post-graduate Institute of Ophthalmology (PGIOPTH) Coimbatore, Department of Microbiology & Molecular Biology, Coimbatore (Asha et al. 2014; Hassan et al. 2016; Homa et al. 2013, 2015, 2019; Kredics et al. 2015; Krizsán et al. 2015; Manikandan et al. 2008a, 2011, 2013, 2019; Mythili et al. 2014; Palanisamy et al. 2015; Selvam et al. 2014; Shobana et al. 2016); **Faculty staff in research in medical mycology**: P. Manikandan, K.P. Selvam, S.S. Shobana, Y.R.B. Singh; **Diagnostic tests**: Direct Microscopy of clinical specimens, Phenotyping identification of molds and yeasts, and PCR.

Arvind Eye Hospital & Postgraduate Institute of Ophthalmology (PGIOPTH), Madurai (Tamil Nadu) Department of Microbiology and Molecular Biology (Boomiraj et al. 2015; Chidambaram et al. 2017; Dudeja et al. 2018; Kandhavelu et al. 2017; Lalitha et al. 2006; Parthiban et al. 2019; Prajna et al. 2004; Rameshkumar et al. 2018, 2019; Shait Mohammed et al. 2020; **Faculty staff in medical mycology**: Lalitha Prajna, Rameshkumar Gunasekaran, M.S. Karpagam; **Diagnostic tests**: Direct Microscopy of clinical specimens, Phenotyping identification of molds and yeasts, and PCR.

Joseph Eye Hospital & Research Institute of Ophthalmology (RIOPTH) Department of Microbiology, Tiruchirapalli (Tamil Nadu) (Chaturvedi et al. 2018; Leck et al. 2002; Leema et al. 2011; Kalavathy et al. 2005; Geraldine and Thomas 2003; Kaliamurthy et al. 2004, 2012; Leema et al. 2010; Pinna et al. 2013a, b; Thomas 2003; Thomas et al. 2008, 2009, 2012; Ruban et al. 2013, 2015, 2018, 2019; Thomas and Kaliamurthy 2013) **Faculty staff in medical mycology**: Philip A. Thomas, J. Kaliamurthy; **Diagnostic tests**: Direct microscopic examination of clinical specimens in KOH, and various stains, phenotyping and antimycotic susceptibility testing of pathogenic fungi.

Banaras Hindu University Institute of Medical Sciences (BHUIMS), Varanasi (Uttar Pradesh), Department of Microbiology, has Bacteriology, Immunology, Parasitology, Mycology, Anaerobic Bacteriology, Mycobacteriology, and Virology sections. The Department trains MBBS, MD (Microbiology) students, and PhD scholars (Basu et al. 2015; Chowdhary et al. 2020; Gupta et al. 2015; 2018, 2019, 2020a, b; Kumar et al. 2015a, b; Mahajan et al. 2017; Pandey et al. 2019, 2020; Singh et al. 2019a, 2020a, b, c; Tilak et al. 2009). **Faculty staff engaged in medical mycology**: Ragini Tilak and Munesh Kumar Gupta; **Diagnostic tests**: Direct microscopic examination of clinical samples in KOH; gram stain; Giemsa, PAS, and GMS stains; phenotypic identification of pathogenic fungi; tests for cryptococcal antigen; and galactomannan and mannan molecular typing.

Regional Institute of Medical Sciences (RIMS), Manipur (Manipur State), Microbiology Department. This institute transferred from the North Eastern Council in 1990 in 2007 to Ministry of Health & Family Welfare, Govt. of India has excellent facilities for training students for MBBS and MD in selected specialties and super-specialties (Devi et al. 2001, 2002, 2006, 2007, 2008; Ghalige et al. 2014; Jeeten Kumar et al. 2012; Khuraijam et al. 2015; Mobing et al. 2019; Pukhramban et al. 2011; Ranjana and Singh 2016, Ranjana Devi and Singh 2018; Sreenath et al. 2014; Vijayakumar et al. 2015; **Faculty staff in medical mycology**: Khuraijam Ranjana Devi, Pratita P., Randhir Babu, Kh. Sulochana; **Diagnostic tests**. Direct microscopic examination of clinical specimens KOH and by staining, phenotypic identification and in-vitro antimycotic susceptibility testing of pathogenic fungi, PCR and Biomarkers.

Lokmanya Tilak Municipal Medical College (LTMMC) and Lokmanya Tilak Municipal General Hospital (LTMGH), Mumbai Department of Microbiology, which started in 1947, are two of the most prestigious medical colleges in India and train students for MBBS and MD in several specialties and super-specialties. LTMGH has state-of-the-art trauma center. It was the first trauma service

in India (Baradkar et al. 2011; Braddo et al. 2001, 2015; Tendolkar et al. 2003, 2014, 2016). **Faculty staff in medical mycology**: RA Braddo, Uma Tendulkar, and Dimple Kute;**Diagnostic tests**: Direct microscopic examination of clinical specimens, phenotypical identification of fungi, and antimycotic susceptibility testing

The Government Medical College Hospital (GMCH), Chandigarh, established in the year 1991 by Chandigarh Administration, trains students for students for BSc (MLT), BSc (Nursing), MBBS, and MD. In the following year, the **Department of Microbiology** came into existence (Alvarez et al. 2010b; Badali et al. 2010a, b, 2011, Bala et al. 2015; Bansal et al. 2016, 2019; Bhankhur et al. 2019; Chander et al. 2015a, b, 2017, 2018; Crous et al. 2017; Datta et al. 2018; Kaur et al. 2020; Kaushik et al. 2012; Kundu et al. 2020; Punia et al. 2019; Seidel et al. 2019; Sharma et al. 2020a, b; Singh et al. 2011, 2017b, 2019b; Singla et al. 2012, 2019; Thami et al. 2003). **Faculty staff engaged in medical mycology**: Jandish Chander, Nidhi Singla, Nelam Gulati, Sheetal Kumar and Ruby Suria; **Diagnostic tests in Medical Mycology**: Direct microscopy of clinical specimens in KOH, CFW and special stains, phenotypic identification of mycelial fungi and yeasts and their antifungal susceptibility testing.

Lady Hardinge Medical College (LHMC), New Delhi (affiliated to University of Delhi), established more than 100 years ago is the oldest medical college in Delhi and one of the oldest in India and trains students for MBBS, MD, and MCH in several specialties and super specialties. The Mycology division of Department of Microbiology headed by Prof. Ravinder Kaur currently processes approximately 2500–2600 samples annually of diagnosis for fungal infections (Arora et al. 2016; Anuradha et al. 2017; Bala et al. 2018; Jain et al. 2017, 2018; Kaur et al. 2016a, b, c, d, 2017a, b, c, d, 2018, 2019; Singh et al. 2018, 2020c, d; Thass et al. 2018; Tyagi et al. 2021; Wattal et al. 2017). **Faculty staff engaged in medical mycology**: Ravinder Kaur, Veelender Randhawa; **Diagnostic tests**: Direct microscopy of clinical specimens, brightfield, phase contrast, fluorescent microscopy, phenotypic identification of molds and their antimycotic susceptibility testing. Cryptococcus latex agglutination, galactomannan for aspergillus antigen tests, Pneumocystis antigen detection by IFAT, VITEK 2 for identification of yeasts and antifungal susceptibility testing. Real Time and Gradient PCR using Pan-fungal and species specific primers for *Candida* and *Aspergillus*.

University College of Medical Sciences (UCMS) (University of Delhi) & Guru Teg Bahadur Hospital, Delhi, Department of Microbiology is identified as a state reference laboratory offers laboratory services to patients of GTB Hospital and referred from other Hospitals of East Delhi, and is identified as state reference laboratory. It trains students for MBBS and MD and conducts MSc/MLT training courses each year (Barua et al. 2016; Chakrabarti et al. 2020; Dar et al. 2018; Das et al. 2017, 2018a, b, 2020a, b; Gupta et al. 2016; Jain et al. 2013, 2017; Kayarkatte et al. 2020; Mishra et al. 2014; Rai et al. 2018, 2019, 2020; Sharma et al. 2020a, b; Singal et al. 2019; Sonthalia et al. 2014, 2018, 2019; Tigga et al. 2018; Yadav et al. 2015a, b). **Faculty staff in medical mycology**: Shukla Das and Rupa Saha; **Diagnostic tests in mycology**: Phenotyping and antimycotic susceptibility testing of fungal isolates and PCR.

Vardhman Mahavir Medical College (VMMC) and Safdarjung Hospital, New Delhi, Department of Microbiology, trains students for MBBS and MD in several specialties and super specialties (Chakrabarti et al. 2015a, b; Capoor et al. 2005, 2008, 2010, 2011, 2014, 2015, 2017, 2019, 2020; Chopra et al. 2015; Dubey et al. 2019; Gupta et al. 2014, 2017; Kathuria et al. 2013; Kumar et al. 2019; Mohindra et al. 2017; Ramesh et al. 2010; Raj et al. 2017; Rudramurthy et al. 2017a; Sarma et al. 2009; Singh et al. 2017a, 2020a, b, c). **Faculty staff in mycology**: Malini Raj Capoor assisted by technical staff; **Diagnostic tests**: Phenotyping and antifungal susceptibility testing, galactomannan antigen ELISA, and panfungal PCR.

Christian Medical College (CMC), Vellore (Tamil Nadu), Department of Microbiology, is one of the ten top-ranking medical institutions in India and an excellent center for medical education and research and provides training for MBBS, MD, and PhD in various specialties and super specialties in medicine (Klokke and Durairaj 1967; Klokke et al. 1968; Bhagat et al. 2008; David et al. 2009; Deodhar et al. 2013; Duarte et al. 2017; Gopal et al. 2020; Sriam et al. 2007). **Faculty staff in medical mycology**: R. Samuel Promila, Jyoti Sarojini Michael, and Anish Kumar Korula; **Diagnostic tests**: Automated culture, phenotypic identification of pathogenic fungi and antifungal susceptibility testing, and PCR.

Christian Medical College (CMC), Ludhiana (Punjab), Department of Microbiology, trains students for MBBMS, MD Microbiology, BSc in Allied Medical Sciences, and Nursing. Diagnostic facilities in mycology include automated culture and in vitro antifungal susceptibility testing, phenotypic identification of pathogenic fungi, and PCR. It may not be out of place to mention that CMC, Ludhiana, is 10 years older than CMC, Vellore. Most people including medical doctors and biomedical scientists are not aware of this. It is worth mentioning that the first report of mycetoma due to *Nocardia brasiliensis* in Asia published in 1964 was by Dr. A.H. Klokke, a missionary dermatologist from the Netherlands, working in this college. It is also noteworthy that the first report of mycetoma due *Streptomyces somaliensis* from North India was also from this college (Grueber and Kumar 1970). Dr. Grueber was a missionary pathologist from the Netherlands, who worked for 10 years in this college (2 years as Head of Dept. of Pathology) and set up a museum of Pathology from autopsies performed during 1969–1971 (Alexander et al. 2013; Abraham et al. 2016, 2015; Dewan et al. 2015; Deodhar et al. 2017; Singh et al. 2014; Thomas et al. 2012). **Faculty staff engaged in mycology**: Eshani Dewan, Sangeeta Mohan, and Aroma Oberoi;**Diagnostic tests**: Phenotyping of pathogenic fungi and yeasts and in vitro antimycotic susceptibility testing.

St. John's Medical College (SJMC), Bengaluru, Department of Microbiology, trains students for MBBS, MD/MS, and (DM/MCh) in super specialties and various diploma courses—all of which are recognized by the Rajiv Gandhi University of Health Sciences (RGUHS), Bengaluru, Karnataka (Chakrabarti et al. 2015a, b, 2020; David et al. 2009; Jose and Savio 2017; Savio et al. 2006, 2011, 2016). **Faculty in medical mycology**: Jayanthi Savio, R. Muralidharan, and S. Macaden; **Diagnostic tests**: Phenotypical identification of fungi, in vitro antimycotic susceptibility tests, and PCR.

Sri Ramachandra Medical College (SRMC) Department of Microbiology, Sri Ramachandra Medical College, Porur, Chennai (Tamil Nadu), trains post-MD (Microbiology)/DNB (Microbiology), MD (Lab Medicine), and MSc (Medical Microbiology) for fellowship in medical mycology, a 6-month course (Ahmed et al. 2016; Chakrabarti et al. 2015a, b, 2019; Elavarashi et al. 2013, 2014; Graeff et al. 2017; Kindo et al. 2016; Premamalini et al. 2012; Nagarajan et al. 2015; Nucci et al. 2014; Patel et al. 2019; Ruping et al. 2010; Rajyoganandh et al. 2016; Tupaki-Sreepurna et al. 2017a, b, 2018; Vijaykumar et al. 2017; Vichitra et al. 2019). **Faculty staff in medical mycology**: Prof. Anupma Jyoti Kindo and residents; **Diagnostic tests**: Direct microscopy of clinical specimens, phenotyping of pathogenic molds and yeasts, and antimycotic susceptibility testing.

Stanley Medical College (SMC) Dept. of Dermatology, Chennai, (Tamil Nadu), Faculty staff in medical mycology: Drs. A. Kamalam and A.S. Thambiah (Chetty et al. 2009; Kamalam and Thambiah 2009a, b, c; **Diagnostic tests:** Phenotyping identification and antimycotic susceptibility testing of pathogenic fungi.

Dayanand Medical College (DMC), Ludhiana (Punjab), Department of Microbiology, is one of the reputed medical colleges in India and trains students for MBBS and MD in several specialties (Chakrabarti et al. 2014; Kucheria et al. 2016; Singh et al. 2014; Suri et al. 2014). **Faculty staff in medical myology**: Veena Gupta with the support of S.K. Gupta in Dermatology Department; **Diagnostic tests**: Phenotypic identification of pathogenic fungi and in vitro antimycotic susceptibility tests.

Sawai Man Singh Medical College (SMSMC), Jaipur Department of Microbiology, trains students for MBBS and MD (Microbiology) (Chandwani et al. 2016; Sharma and Sharma 2012; Sood et al. 2007b; Vyas et al. 2013). **Faculty staff in medical mycology**: Maheshwari R., Sharma S., A. Vyas, and S. Rishi; **Diagnostic tests in mycology**: Direct microscopy of clinical specimens, phenotyping identification of pathogenic fungi, and in vitro antimycotic susceptibility testing.

Jawaharlal Nehru Medical College (JNMC) and Associated Hospitals, Department of Microbiology, Ajmer-300501, trains students for MBBS and MD (Microbiology). **Faculty staff in medical mycology**: Rastogi and Nirwan (2007), Rastogi et al. (2013, 2016); Vijaylatha Rastogi and P.S. Nirwan with assistance of residents; **Diagnostic tests**: Phenotypic identification of pathogenic fungi and in vitro antimycotic susceptibility testing.

11.2 Training in Medical Mycology

Graduates in medicine and postgraduates in medical microbiology, microbiology, and biological sciences desiring to get training in medical mycology should contact the following centers and persons for workshops/short training courses to choose a center near their location. **An important message to pure mycologists**: If any of them are keenly interested in learning medical mycology and doing research on

fungal infections, they should get in touch any of the following centers for inquiring the dates and duration of the courses/workshop:

PGI Chandigarh: Professors Arunaloke Chakrabarti (arunaloke@hotmail.com) and Shivaprakash Rudramurhty (shivprakash@gmal.com).
AIIMS, New Delhi: Prof. Immaculata Xess (immaxess@gmail.com).
AIIMS, Jodhpur: Prof Anuradha Sharma (asharma3170@gmail.com).
VP Chest Institute, University of Delhi, Delhi (dranuradha@hitmail.com, chowdhary.anuradha@gmail.com).
Christian Medical College (CMC), Vellore: Prof Joy Sarojini Michael (joymichael@cmc.vellore.in).
St. John's Medical College, Bengaluru: Prof. Jayanthi Savio (jayanthisjmc@gmmail.com, jayanthi.s@stjohns.in).
University College of Medical Sciences, GBT Nagar, Delhi: Prof. Shukla Das (shukladas123@yahoo.com, shukladas@yahoo.com).
Vardhman Mahavir Medical College and SJ Hospital, New Delhi: Prof. Malini Raj Capoor (rajeevmalini@rediffmail.com).

It is important to mention that mycologists must learn the basic laboratory and some advanced techniques by working in medical mycology/microbiology laboratories. It is only then they would learn to identify pathogenic fungi and not commit serious errors, as many have done in the past by enthusiastically and hastily misidentifying fungal isolates from natural sources, even reporting dimorphic fungi without providing illustrations and in vitro conversion to tissue form as observed in several publications. In this way they would avoid committing diagnostic pitfalls in medical mycology. Pure mycologists should emulate their fellows in the USA and Europe, who by working in clinical and infectious disease laboratories acquired a working of knowledge of fungal diseases and their pathology and became the pioneers of several discoveries in medical mycology. Working similarly, our mycologists with their knowledge of fungal taxonomy, genetics, and physiology can discover novel fungal pathogens and can enrich the discipline of medical mycology in India, thus creating a very good image among the medical professionals in India and abroad.

A FISF (Fungal Infections Study Forum)-led 2-day training course for medically qualified professionals is organized every year by Prof. Arunaloke Chakrabarti and his team at different locations in India each year.

11.2.1 Strengthening Laboratory Facilities in Medical Mycology

India is a large country with a huge population. There is dire need to set up diagnostic medical mycology labs in those medical colleges and government hospitals, where none exists currently. These colleges and hospitals should avail of the facilities for training their staff and laboratory technicians in the centers listed earlier.

11.2.2 Community Studies in Fungal Infections and Preventive Measures

Fungal diseases are frequently caused by fungi that are common in the human environment. The Departments of Preventive and Social Medicine should carry out community services in collaboration with state departments of health/primary health centers in selected communities to investigate the occurrence of fungal infections like ringworm, keratitis, and mycetoma to estimate their burden and chalk out preventive measures. Keratitis surveys can also be done in collaboration with departments of ophthalmology. The RP Institute of Ophthalmology in AIIMS, New Delhi, has a Dept. of Community Ophthalmology, but surprisingly no survey of keratitis has been undertaken so far. There is also need for instituting preventing chemotherapy in the form of a combined antifungal/antibacterial preparation for widespread and immediate prophylactic first aid use by our farmers, carpenters, and other categories of workers, particularly in rural areas, who frequently sustain corneal injuries leading to corneal ulcers. As mentioned in CDC website (www.cdc.gov/fungal), we should have awareness campaign about fungal diseases; this is an important way to improve early recognition and reduce delays in diagnosis/treatment and chalk out preventive measures. A key clue to when a sick person may have a fungal disease is that he or she is being treated with medicine for another type of infection but does not get better. People in our country should be advised hand hygiene in their environment, home, and workplace and to avoid sharing of towels in the bathrooms in their houses and community bathing places, e.g., swimming pools. School teachers should advise their pupils not to roll on the ground while playing games in the school playground to avoid infection by soil-borne dermatophytes. Farmers need to be advised not to work in their fields barefooted, to avoid foot injury which may lead to mycetoma. Development and standardization of myco-serological/molecular techniques to detect early cases of mycetoma are need of the hour. Inhabitants near bat roosting sites and chicken pens should be educated to avoid contact with accumulations of bat guano and chicken excreta to reduce the risk of acquiring fungal infections like cryptococcosis and histoplasmosis.

11.2.3 Herbal Therapy

Most of the clinically useful antifungal compounds have some drawbacks in terms of toxicity, efficacy, and cost, and their frequent use has led to the emergence of resistant strains of pathogenic fungi. This has also led to a search for medicinal plants and compounds isolated from them for their antifungal properties. Our mycologists and physicians should explore antifungal therapy by herbal drugs. Vaijayanthimala et al. (2004) tested the antifungal activity of 23 south Indian medicinal plants against clinical isolates of *Trichophyton rubrum* and *T. mentagrophytes*; alcoholic extracts of *Allium sativum* (garlic) and *A. schoenoprasum* (chives) showed highest anti-dermatophytic activity. In another publication from south India, Balakumar et al. (2011) demonstrated significant

in vitro antifungal activity of *Ocimum sanctum* (tulsi) against clinical isolates of *T. rubrum*, *T. mentagrophytes*, *Epidermophyton floccosum*, and *Microsporum gypseum*. In Brazil, another species of *Ocimum*, *O. gratissimum*, has been shown to have antifungal activity against *Cryptococcus neoformans* (Lemos et al. 2005). *Ocimum gratissimum* (known as Ram Tulsi and Ban Tulsi in Hindi) commonly occurs in India. The clinical use of both types of Tulsi in dermatophytic and cryptococcal infections needs to be investigated. Propolis, a sticky natural substance collected by the honey bees from the resin of flowers, leaves of trees, and plants and mixed with their saliva, is shown to have significant activity against *Paracoccidioides brasiliensis*, a dimorphic pathogenic fungus (Negri et al. 2014). Our medical mycologists/biomedical scientists and physicians should evaluate propolis and antifungal products from herbs for clinical use against all dimorphic human fungal pathogens.

Indian Society of Medical Mycologists (ISMM) (until very recently called Indian Society for Human and Animal Mycologists (ISHAM)) was founded by Prof. S.M. Singh of the Dept. of Biological Sciences, Rani Durgavati University (RDU), Jabalpur (MP), where its first conference was held in 1994. Since then, conference of the society is held every 2 years. The name of the society has been changed to ISMM since 12 February 2020. The society has grown by leaps and bounds over the past two decades with a total membership of 570. The current office bearers are President-Prof. Anupma Jyoti Kindo (SRMC, Chennai), Vice-President-Prof. Immaculata Xess (AIIMS, New Delhi), General Secretary-Prof Jayanthi Savio (St. John's Medical College, Bengaluru), Joint Secretary-Dr. Bansidhar Tarai (Max Super Specialty Hospital, Delhi), and Treasure-Prof Anoop Ghosh (PGI, Chandigarh). The zonal executive members are North-Dr. Pratibha Kale (Institute of Biliary and Pancreatic Sciences, New Delhi), West-Prof. Anuradha Sharma (AIIMS, Jodhpur), South-Prof. Lalitha Prajna (Aravind Eye Institute and Hospital, Madurai), East-Dr. Vinay Kumar Hallur (AIIMS, Bhubaneswar), and Central-Dr. Anand Kumar Maurya (AIIMS, Bhopal). Email IDs for most of the office bearers are given in the later part of the chapter; that of others can be found through Google. Though the leadership of the society in initial years was provided by laboratory scientists, many clinicians who recognized the formidable challenge of emerging fungal infections in their clinical practice are taking more interest in the society. It is inspiring and encouraging to observe that the clinicians and biomedical scientists are working hand in hand to improve the outcome of patients with fungal disease in our country. In 2022, ISMM and Fungal Infections Study Forum (FISF) together will organize the 21st Congress of International Society for Human and Animal Mycology (ISHAM) at Delhi, a rare opportunity for the mycologists, physicians, and researchers to participate in this mega scientific event. All this has been possible due to the laudable efforts of the past presidents of ISHAM, viz., Arunaloke Chakrabarti and Shivaprakash M. Rudramurthy (who are also the current President and Secretary, respectively, of ISHAM), and previous presidents of ISHAM, notably K.R. Joshi, Uma Banerjee, and Hemashatiar. ISHAM has progressed to be an important internationally recognized mycological society and is affiliated to the International Society for Human and Animal Mycology (ISHAM). Several eminent

medical mycologists from England, the Netherlands, Germany, Israel, the USA, and South America have been attending the ISHAM Conferences. The 13th National Conference of ISHAM was held in Jodhpur (Rajasthan) from 12 to 15 of February 2020. In addition to members of our society, majority of the ISHAM (International Society for Human and Animal Mycology), its council members, and Fungal Infection Study Forum (FISF) members were the invited faculty of this conference. The theme of this conference "Paradigm Shift in Fungal Infections - A Global Health Challenge" was aptly chosen to discuss the challenges in the fungal infections such as emergence of *Candida auris* infection, recurrent dermatophytosis, increase in the incidence of invasive mold infections, etc. Several globally renown medical mycologists, pathologists, molecular biologists, and clinicians, viz., Malcolm Richardson (Mycology Reference Centre-ECMM, Centre of Excellence in Mycology, NHS Foundation Trust, University of Manchester, Wythenshawe Hospital, Manchester, UK), Jacques Meis (Canisius Wilhelmina Hospital, Department of Medical Microbiology and Infectious Diseases, Nijmegen, the Netherlands), John Perfect (Department of Medicine, Duke University School of Medicine, Durham NC, USA), Karl V. Clemons (Department of Medicine, Stanford University, Stanford, CA, USA), Beatriz Gomez (Corporación para Investigaciones Biológicas, Medellın, Colombia), and Oliver Cornely (Director of Translational Research, University Hospital, Cologne, Germany) excellently delivered their state-of-the-art lectures. Members of the Indian faculty, O.C. Abraham, Malini Capoor, Arunaloke Chakrabarti, Jagdish Chander, Shukla Das, Khuraijam Ranjana Devi, Anup Ghosh, Vinaykumar Hallur, Ram Gopalakrishnan, Ranganathan Iyer, KR Joshi, Pratibha Kale, Harsimrat Kaur, Anupam Kindo, Rungmei S.K. Marak, Sangeetha Mohan, Joy Sarojini Michael, Niranjan Nayak, Jaswinder Oberoi, Umabala Pamidimukkala, Atul Patel, Lalith Pranja, Rajendra Prasad, Jayanthi Savio, Nandini Sethuraman, Anuradha Sharma, Savitri Sharma, Gagandeep Singh, M.R. Shivaprakash, Rajeev Soman, Subramanian Swaminathan, Bansidhar Tarai, Karuna Tadepalli, Subhash Todi, and Immaculata Xess, also gave excellent presentations.

11.2.4 ISMM (ISHAM) Awards

At every ISMM (ISHAM) conference, papers presented as oral presentations in separate sessions are assessed for the GP Agarwal Award for young mycologists, Kamalam Glaxo Award, and Pankajlakshmi Award for best oral presentations. At some of these conferences, MJ Thirumalachar Life Time Achievement Award (funded by Dr. Arvind A. Padhye, USA) is given to outstanding medical mycologists. This award at the 13th Conference of ISMM held in Jodhpur, Rajasthan (12–15 February 2020), was endowed to Dr. Harish Chander Gugnani. It may be incidentally mentioned that Dr. A.A. Padhye collaborated as a joint author in several outstanding publications of our medical mycologists.

11.2.5 ISMM (ISHAM Newsletter)

This newsletter started in 2002 by Arunaloke Chakrabarti, initially with financial support of M/S Pfizer. India is now sponsored by MDH Pharmaceuticals PVT. Ltd., India. It is edited by Savitri Sharma with the assistance of several other members of the editorial board, viz., Malini Raj Capoor, Lalitha Pranja, Vijayalatha Rastogi, Shivaprakash M. Rudramurthy, Ruchi Mittal, P. Manikandan, Mamatha Ballal, Joveeta Joseph, and Shukla Das. The newsletter has continued to serve as a source of educational material on fungal infections and is currently a very good forum for posting the abstracts of research papers or published papers on pathogenesis, epidemiology, and laboratory diagnosis of these diseases.

This chapter is dedicated to our mycologists and medical mycologists for the discovery of new species of pathogenic fungi and to our physicians, British Army physicians and surgeons, and two missionary physicians (dermatologists and a pathologist) who worked in India, for making significant contributions to the clinicopathological aspects of fungal infections in India.

References

Abraham D, Oberoi A, Alexander V, Dewan E, Mohan S (2015) Zygomycoses - urgency in diagnosis. Int J Basic Appl Sci 4(4):3. https://doi.org/10.14419/ijbas.v4i4.5004

Abraham D, Oberoi A, Dewan E, Alexander VS (2016) *Pneumocystis jirovecii* pneumonia: prevalence in hospitalized patients, a study conducted in a tertiary center in Punjab. J Evol Med Dent Sci 5(32):1695–1698. https://doi.org/10.14260/jemds/2016/399

Ahmed SA, Khan Z, Wang X, Tarek A, Moussa A, Hassan S et al (2016) Chaetomium-like fungi causing opportunistic infections in humans: a possible role for extremotolerance. Fungal Divers 76:11. https://doi.org/10.1007/s13225-015-0338-5

Ahuja J, Soneja M, Wig N, Xess I, Biswas A, Singh G (2019) Cryptococcal antigenemia in advanced HIV infection. Open Forum Infect Dis 6(Suppl 2):S196–S196

Alexander V, Tyagi S, Oberoi A, Mohan S (2013) Changing trends: an overview of increasing burden of fungal infections from a tertiary care hospital in Punjab (North India). Int J Sci Basic Appl Res 3(1):26–29

Alvarez E, Stchigel AM, Cano J, Sutton DA, Fothergill AW, Chander J (2010a) Molecular phylogenetic diversity of the emerging mucoralean fungus *Apophysomyces*: proposal of three new species. Revista Iberoamericana Micologia 27:80–89. https://doi.org/10.1016/j.riam.2010.01.006. Epub 2010 Mar 4

Alvarez E, Garcia-Hermoso D, Sutton DA, Cano JF, Stchigel AM, Hoinard D et al (2010b) Molecular phylogeny and proposal of two new species of the emerging pathogenic fungus Saksenaea. J Clin Microbiol 48(12):4410–4416

Anuradha S, Abhaya Narayana H, Dewan R, Kaur R, Rajeshwari K (2017) Asymptomatic cryptococcal antigenemia in people living with HIV (PLHIV) with severe immunosuppression: is routine CrAg s screening indicated in India? J Assoc Physicians India 65(4):14–17

Arora S, Verma M, Gupta S, Aadithya U, Kaur R, Dhakad MS (2016) Phenotypic variability and therapeutic implications of *Candida* species in patients with oral lichen planus. Biotechnic Histochem. https://doi.org/10.3109/10520295.2015.1127425

Arvind B, Medigeshi GR, Kapil A, Xess I, Singh U, Lodha R et al (2020) Aetiological agents for pulmonary exacerbations in children with cystic fibrosis: an observational study from a tertiary care centre in northern India. Indian J Med Res 15(1):65–70

Asha P, Nisha P, Suneer K, Mythili A, Shafeeq HA, Panneer SK (2014) In-vitro activity of various potencies of homeopathic drug Thuja against molds involved in mycotic keratitis. Int J Pharm Pharm Sci 6(10):555–559

Badali H, Chander J, Bansal S, Aher A, Borkar SS, Meis JF (2010a) First autochthonous case of *Rhinocladiella mackenziei* cerebral abscess outside the Middle East. J Clin Microbiol 48:646–649

Badali H, Chander J, Gulati N, Attri A, Chopra R, Najafzadeh M et al (2010b) Subcutaneous phaeohyphomycotic cyst caused by *Pyrenochaeta romeroi*. Med Mycol 48:763–767

Badali H, Chander J, Gupta A, Rani H, Punia RS, de Hoog GS, Meis JF (2011) Fatal cerebral phaeohyphomycosis in an immunocompetent individual due to *Thielavia subthermophila*: case report and review of the literature. J Clin Microbiol 49:2336–2341

Baine PK, Nalamada S, Kodiganti M, Taneja M (2012) Fungal keratitis caused by *Chaetomium atrobrunneum*. Cornea 31(1):94–95. https://doi.org/10.1097/ICO.0b013e31821eeaed

Bala K, Chander J, Handa U, Punia RS, Attri AK (2015) A prospective study of mucormycosis in North India: experience from a tertiary care hospital. Med Mycol 53:248–257

Bala Y, Randhawa VS, Saili A, Kaur R (2018) A microbiological profile of early onset of neonatal sepsis in a tertiary care hospital in North India. Indian J Appl Microbiol 21(2):20–28

Balakumar S, Rajan S, Thirunalasundari T, Jeeva S (2011) Antifungal activity of *Ocimum sanctum* Linn. (Lamiaceae) on clinically isolated dermatophytic fungi. Asia-Pacific J Trop Med 6:54–57

Bansal Y, Chander J, Kaistha N, Singla N, Sood S, van Diepeningen AD (2016) *Fusarium sacchari*, a cause of mycotic keratitis among sugarcane farmers - a series of four cases from North India. Mycoses 59:705–709

Bansal Y, Singla N, Kaistha N, Sood S, Chander J (2019) Molecular identification of *Fusarium* species complex isolated from clinical samples and its antifungal susceptibility patterns. Curr Med Mycol 5:43–49

Baradkar VP, Tendolkar U, Baveja S, Kamath S (2011) A rare case of *Histoplasma fungemia* in an AIDS patient. Indian J Med Microbiol 29:188–191

Barua M, Shukla D, Gupta C, Saha R, Kaur IR (2016) Plasmid profile and antibiogram of *Enterococcal faecalis* isolated from tertiary care hospital in Delhi. Brief Commun Indian J Med Microbiol 34(1):109–110. https://doi.org/10.4103/0255-0857.174105

Basu S, Tilak R, Kumar A (2015) Multidrug-resistant Trichosporon: an unusual fungal sepsis in preterm neonates. Pathogens Glob Health 109(4):202–206

Behera S, Singh RI, Xess I, Mathur P, Hasan F, Misra MC (2010) *Candida rugosa*: a possible emerging cause of candidaemia in trauma patients. Infection 38(5):387–393. https://doi.org/10.1007/s15010-010-0044-x

Behera B, Singh RI, Xess I, Mathur P, Behera B, Gupta B et al (2011) Epidemiology of candidaemia in critically ill trauma patients: experiences of a level I trauma center in North India. J Med Microbiol 60(Pt 3):342–348

Bhagat SK, Kekre NS, Gopalakrishnan G, Veeraghavan B, Mathews M (2008) Changing profile of prostatic abscess. Int Braz J Urol 34(2):164–170

Bhankhur D, Singla N, Aggarwal D, Chander J (2019) Prevalence of allergic bronchopulmonary aspergillosis among patients with severe bronchial asthma in a tertiary care hospital in northern India. Indian J Pathol Microbiol 62:111–113

Bibhudutta RB, Sharma S, Kar S, Das S, Sahu SK (2011) Diagnosis and treatment outcome of mycotic keratitis at a tertiary eye care center in eastern India. BMC Ophthalmol 11:39. https://doi.org/10.1186/1471-2415-11-39

Biswal M, Rudramurthy SM, Jain N, Shamanth AS, Sharma D, Jain K, Yaddanapudi LN, Chakrabarti A (2017) Controlling a possible outbreak of *Candida auris* infection: lessons learnt from multiple interventions. J Hosp Infect 97:363–370

Boomiraj H, Mohankumar V, Lalitha P, Devarajan B (2015) Human corneal microRNA expression profile in fungal keratitis. Invest Ophthalmol Vis Sci 56(13):7939–7946

Braddo RA, Nerukar NR, Kute D, Joshi A, Tendolkar U (2001) Fungal sinusitis - a rising trend. Original/Research Articles. www.bhj.org.in/2001_4303_July 01/org_343. (Website of Dr. Reddy's Laboratories Ltd. 7.1.27. Ameerpet, Hyderabad-500016)

Braddo RA, Baveja S, Tendolkar U, Diepeningen AV, Agrawal SR, Josh AA et al (2015) Rhinosinusitis caused by *Saksenaea erythrospora* in an immunocompetent patient in India: a first report. J Med Mycol Case Rep 2(3). https://doi.org/10.1099/jmmcr.0.000044

Brumpt E (1906) Les Mycetomas. Arch Parasitol 16:489–564

Butler EJ (1903) Potato diseases of India. Agric Ledger 4:112–119

Butler EJ (1918) Fungi and disease in plants: an introduction to the diseases of field and plantation crops, especially those of India and the east. Thacker, Spink & Co., Calcutta and Simla. 547 pp

Butler EJ, Bisby GR (1931) The fungi of India, Scientific monograph 1, The Imperial Council of Agricultural Research. Central Publication Branch, The Government of India, Calcutta, India, pp i–xviii, 1–237

Butler EJ, Jones SG (1949) Plant pathology, Plant diseases. MacMillan, London. 979 pages

Capoor MR, Nair D, Deb M, Verma PK, Srivastava L, Aggarwal P (2005) Emergence of non-albicans Candida species and antifungal resistance in a tertiary care hospital. Jpn J Infect Dis 58(6):344–348

Capoor MR, Mandal P, Deb M, Aggarwal P, Banerjee U (2008) Current scenario of cryptococcosis and antifungal susceptibility pattern in India: a cause for reappraisal. Mycoses 51(3):258–265. https://doi.org/10.1111/j.1439-0507.2007.01478.x

Capoor MR, Sarabahi S, Tiwari VK, Narayanan RP (2010) Fungal infections in burns: diagnosis and management. Indian J Plastic Surg 43(Suppl):S37–S42. https://doi.org/10.4103/0970-0358.7071

Capoor MR, Ramesh V, Khanna G, Singh S (2011) Sporotrichosis in Delhi among the migrant population from Uttarakhand, India. Trop Doctor 41(1):46–48. https://doi.org/10.1258/td.2010.100276

Capoor MR, Aggarwal S, Raghvan C, Gupta DK, Jain AK, Chaudhary RA (2014) Clinical and microbiological characteristics of *Rhodotorula mucilaginosa* infections in a tertiary-care facility. Indian J Med Microbiol 32(30):304–309

Capoor MR, Gupta DK, Verma PK, Sachdeva HC (2015) Rare yeasts causing fungemia in immunocompromised and haematology patients: case series from Delhi. Indian J Med Microbiol 33(4):576–579

Capoor MR, Puri SA, Raheja H, Mohindra R, Verma PK, Gupta DK (2017) Screening of invasive fungal infections by a real time panfungal (pan-ACF) PCR assay in patients with haematological malignancy. Indian J Med Microbiol 36(1):41–47

Capoor MR, Subudhi CP, Collier A, Bal AM (2019) Antifungal stewardship with an emphasis on candidaemia. J Glob Antimicrob Resistance 19:262–268

Capoor MR, Mishra N, Kolte S, Singla G et al (2020) Disseminated *Emergomyces pasteurianum*. Mycopathologia 185:193–200. https://doi.org/10.1007/s11046-019-00387-y

Carter HV (1860) On a new and striking form of fungus disease principally affecting the foot and prevailing endemically in many parts of India. Trans Med Phys Soc Med Bombay 6:104–142

Carter HV (1874) On mycetoma and the fungus diseases of India. J & A Churchill Ltd., London

CDC (n.d.) Saving lives, protecting people. Fungal diseases. www.cdc.gov/fungal/CDC24/7

Chakrabarti A, Ghosh A, Prasad GS, David JK, Gupta S, Das A et al (2003) *Apophysomyces elegans*: an emerging zygomycete in India. J Clin Microbiol 41(2):783–788

Chakrabarti A, Das A, Mandal J, Rudramurthy SM, George VK, Tarai B et al (2006) The rising trend of invasive zygomycosis in patients with uncontrolled diabetes mellitus. Med Mycol 4:335–342

Chakrabarti A, Rudramurthy SM, Singh R, Tarai B, George VK, Fomda BA et al (2008) Fungal endophthalmitis: fourteen years' experience from a center in India. Retina 28(10):1400–1407

Chakrabarti A, Denning DW, Ferguson BJ, Ponikau J, Buzina W, Kita H et al (2009) Fungal rhinosinusitis: a categorization and definitional schema addressing current controversies. Laryngoscope 119(9):1809–1818

Chakrabarti A, Marak RSK, Rudramurthy SM, Gupta S, Garg R, Sakhuja V et al (2010a) Cavitary pulmonary zygomycosis caused by *Rhizopus homothallicus*. J Clin Microbiol 48(5):1965–1969. https://doi.org/10.1128/JCM.01272-09

Chakrabarti A, Shivaprakash MR, Curfs-Breuker I, Baghela A, Klassen CH, Meis JF (2010b) *Apophysomyces elegans*: epidemiology, amplified fragment length polymorphism, typing and *in vitro* antifungal susceptible pattern. J Clin Microbiol 48(12):4580–4585

Chakrabarti A, Sood P, Rudramurthy SP, Chen S, Kaur H, Capoor M et al (2014) Incidence, characteristics and outcome of ICU acquired candidaemia in India. Intensive Care Med. https://doi.org/10.1007/s00134-014-3603-2

Chakrabarti A, Rudramurthy SM, Panda N, Das A, Singh A (2015a) Epidemiology of chronic fungal rhinosinusitis in rural India. Mycoses 58(5):294–302

Chakrabarti A, Sood P, Rudramurthy SM, Chen S, Kaur H, Capoor M et al (2015b) Incidence, characteristics and outcome of ICU-acquired candidemia in India. Intensive Care Med 41 (2):285–295

Chakrabarti A, Kaur H, Savio J, Rudramurthy SM, Patel A, Shastri P et al (2019) Epidemiology and clinical outcomes of invasive mould infections in Indian intensive care units (FISF study). J Crit Care 51:64–70

Chakrabarti A, Sood P, Rudramurthy SM, Chen S, Jillwin J, Iyer R et al (2020) Characteristics, outcome and risk factors for mortality of paediatric patients with ICU-acquired candidemia in India: a multicentre prospective study. Mycoses. https://doi.org/10.1111/myc.13145

Chander J, Kaur M, Bhalla M, Punia RS, Singla N, Bhola K et al (2015a) Changing epidemiology of mucoralean fungi: chronic cutaneous infection caused by *Mucor irregularis*. Mycopathologia 180:181–186

Chander J, Stchigel AM, Alastruey-Izquierdo A, Jayant M, Bala K, Rani H et al (2015b) Fungal necrotizing fasciitis, an emerging infectious disease caused by *Apophysomyces* (Mucorales). Revista Iberoamericana Micologia 32:93–98

Chander J, Singla N, Kundu R, Handa U, Chowdhary A (2017) Phaeohyphomycosis caused by *Rhytidhysteron rufulum* and review of literature. Mycopathologia 182:403–407

Chander J, Kaur M, Singla N, Punia RPS, Singhal SK, Attri AK et al (2018) Mucormycosis: battle with the deadly enemy over a five-year period in India. J Fungi 4:E46

Chandwani J, Vyas N, Hooja S, Sharma B, Maheshwari R (2016) Mycological profile of sputum of HIV patients with lower respiratory tract infection and its correlation with CD4+ T lymphocyte count. J Clin Diagn Res 10(9):DC28–DC31. https://doi.org/10.7860/jcdr/2016/21601.8585

Chaturvedi V, Bouchara J-P, Hagen F, Alastruey-Izquierdo A, Badali H, Bocca AL et al (2018) Eighty years of mycopathologia: a retrospective analysis of progress made in understanding human and animal fungal pathogens. Mycopathologia. https://doi.org/10.1007/s11046-018-0306-1

Chaurasia JK, Tandon A, Tadepalli K, Naik B, Kapoor N (2020) Aspergillus encephalitis masquerading as brain tumor: diagnosis in a challenging case by intraoperative cytology. Diagn Cytopathol 49:331–334. https://doi.org/10.1002/dc.24614

Chetty GN, Kamalam A, Thambia AS (2009) Pityriasis versicolor-a study of 200 cases in a tropical skin clinic. Mycoses 22(7):234–246. https://doi.org/10.1111/j.1439-0507.1979.tb01747.x

Chidambaram JD, Prajna NV, Larke N, Macleod D, Srikanthi P, Lanjewar S et al (2017) In vivo confocal microscopy appearance of Fusarium and Aspergillus species in fungal keratitis. Br J Ophthalmol 101(8):1119–1123

Chopra S, Capoor MR, Mallik R, Gupta S, Ray A, Khanna G, Suri JC, Bhattacharya D, Raghavan S (2015) Pulmonary cryptococcosis in HIV-sero-negative patients: case series from India. Mycoses 589(5):288–293

Chowdhary A, Meis JF (2020) Antifungal resistance in clinically significant fungi. Fungal Genet Biol 144. https://doi.org/10.1016/j.fgb.2020.103369

Chowdhary A, Beckar A, Fegeler W, Gugnani HC, Kapoor L, Randhawa VS et al (2003) An outbreak of candidemia due to *Candida tropicalis* in a neonatal intensive care unit. Mycoses 46 (8):269–274

Chowdhary A, Randhawa HS, Gaur SN, Agarwal S et al (2010a) *Schizophyllum commune* as an emerging fungal pathogen: a review and report of two cases. Mycoses 56(1):1–10

Chowdhary A, Randhawa HS, Khan ZU, Ahmad S, Juneja S, Sharma B et al (2010b) First isolations in India of *Candida nivariensis*, a globally emerging opportunistic pathogen. Med Mycol 48:416–420

Chowdhary A, Sharma C, Duggal S, Agarwal K, Prakash S, Singh PK et al (2013) New clonal strain of *Candida auris*, Delhi, India. Emerg Infect Dis 19(10):1670–1673

Chowdhary A, Agarwal K, Meis JF (2016) Filamentous fungi in respiratory infections. What lies beyond aspergillosis and mucormycosis? PLoS Pathogens. https://doi.org/10.1371/journal.ppat.1005491

Chowdhary A, Sharma S, Meis JF (2017) *Candida auris*: a rapidly emerging cause of hospital-acquired multidrug-resistant fungal infections globally. PLoS Pathog 13(5):e1006290. https://doi.org/10.1371/journal.ppat.1006290

Chowdhary A, Sharada K, Singh PK, Bhagwani DK, Kumar N, de Groot T et al (2020) Outbreak of *Dirkmeia churashimaensis* fungemia in a neonatal intensive care unit, India. Emerg Infect Dis 26(4):764–768. https://doi.org/10.1016/S1473-3099(18)30609-1

Crous PW, Wingfield MJ, Burgess TI, Chander J et al (2017) *Saksenaea loutrophoriformis*. Fungal planet description sheets (622): 558–624. Persoonia 38:240–384

Dabas Y, Xess I, Kale P (2017) Molecular and antifungal susceptibility study on trichosporonemia and emergence of *Trichosporon* mycotoxinivorans as a bloodstream pathogen. Med Mycol 55 (5):518–527

Dar SA, Rai G, Ansari MA, Akhter N, Gupta N, Sharma S et al (2018) FcεR1α gene polymorphism shows association with high IgE and anti-FcεR1α in chronic rhinosinusitis with nasal polyposis. J Cell Biochem 119(5):4142–4149

Datta P, Kaur M, Gombar S, Chander J (2018) Epidemiology and antifungal susceptibility of *Candida* species isolated from urinary tract infections: a study from an intensive care unit of a tertiary care hospital. Indian J Crit Care Med 22:56–57

Das S (2020) Endophthalmitis management: stain-culture, empirical treatment, and beyond. Asia Pacific Ophthalmol (Phila) 9(1):1–3. https://doi.org/10.1097/01.APO.0000617904.11979.ae

Das P, Paty BP, Mahapatra A, Behera S, Sarangi G, Mallick B et al (2008) Mycological study of nasal polyps. Indian Med Gazette CXLII(7):253–255

Das S, Pandhi D, Rai G, Ansari MA, Gupta C, Haque S, Dar SA (2017) Challenges in culture-negative cases of *Madurella mycetomatis*: a case report re-accentuating PCR as an essential diagnostic tool. J Mycol Med 27(4):577–581

Das S, Rai G, Tigga RA, Srivastava S, Singh PK, Sharma R et al (2018a) *Candida auris* in critically ill patients: emerging threat in intensive care unit of hospitals. J Mycol Med 28(3):514–518

Das S, Tigga R, Rai G, Singh PK, Datt S, Tyagi A, Singh NP (2018b) *Candida auris* colonization in an immunocompetent patient: a new threat in medical ICU. Med Mycol Case Rep 21:54–56

Das S, Datt S, Dar SA, Bhattacharya SN, Pandhi D, Gupta C et al (2020a) Assessment of in vitro antifungal susceptibility pattern of dermatophytes isolated from patients with onychomycosis attending a tertiary care hospital of East Delhi. Indian J Dermatol Venereol Leprol 86 (3):301–314

Das S, Sharma P, Sharma R, Singh S, Kumar P, Pandhi D et al (2020b) Trichoscopy as a diagnostic tool for tinea capitis: a prospective, observational study. Int J STD AIDS 12(2):68–74

Dave TV, Dave VP, Sharma S, Koirala T, Joseph J, Pathengay A et al (2019) Infectious endophthalmitis leading to evisceration: spectrum of bacterial and fungal pathogens and antibacterial susceptibility profile. J Ophthal Inflamm Infect. https://doi.org/10.1186/s12348-019-0174-y

David VG, Korula A, Choudarie L, Michale JS, Jacob S, Jacob CK et al (2009) Cryptococcal granulomatous interstitial nephritis and dissemination in a patient with untreated lupus nephritis. Nephrol Dial Transplant 24:3243–3245. https://doi.org/10.1093/ndt/gfp293

Deodhar D, Frenzen F, Rupali P, David D, Promila M, Ramya I, Seshadri MS (2013) Disseminated histoplasmosis: a comparative study of the clinical features and outcome among immunocompromised and immunocompetent patients. Natl Med J India 26(4):214–215

Deodhar D, Koshy JM, John M, Oberoi A (2017) Clinical profile of *Pneumocystis jirovecii* infection - a comparative study. J Assoc Physicians India 66(1):28–31

Devi RK, Chakrabarti A, Kulachandra M, Lokendra K, Devendra H (2001) Sporotrichosis in Manipur: report of two cases. Indian J Dermatol Venerol Leprol 67:86–88

Devi K, Priyokumar K, Singh TJ, Gupta CHC, Sharmila L, Singh PN, Chakrabarti A (2002) Disseminated *Penicillium marneffei* infection in HIV-patients in Manipur. J Infect 45 (4):268–271

Devi K, Devi MU, Singh TN, Devi KS et al (2006) Emergence of sporotrichosis in Manipur. Indian J Med Microbiol 24(3):216–219

Devi KR, Singh RL, Rajkumari R, Usharani M, Devi SK, Singh BT (2007) *Penicillium marneffei* – an indicator in disease of AIDS: a case report. Indian J Pathol Microbiol 50(3):674–676

Devi UM, Sulochana Devi K, Lokhendro Singh H, Brajachand Singh N (2008) A study of Candida infection in relation with CD4 count and antifungal susceptibility in HIV infected patients of Manipur. J Med Soc 22(2):68–70

Dewan E, Biswas D, Barnali K, Barnali K, Verma SK, Oberoi A (2015, 2015) Epidemiological and mycological characteristics of candidemia in patients with hematological malignancies attending a tertiary-care center in India. Hematol Oncol Stem Cell Therapy 8(3). https://doi.org/10.1016/j.hemonc.2015.06.006

Duarte F, Promila MR, Michael JS, Rupal P (2017) Phaeohyphomycosis: a 10-year review (2006–2016). Open Forum Infect Dis 4(Suppl 1):S86. https://doi.org/10.1093/ofid/ofx163.038

Dubey N, Capoor MR, Hassan AS, Gupta A et al (2019) Epidemiological profile and spectrum of neglected tropical disease eumycetoma from Delhi, North India. Epidemiol Infect 147:e294. https://doi.org/10.1017/S0950268819001766

Dudeja L, Jeganathan L, Prajna NV, Prajna L (2018) Fungal keratitis caused by *Laetisaria arvalis*. Indian J Med Microbiol 36(1):140–142

Duggal SD, Jena PP, Gur R, Kumar A, Rongpharpi SR, Pandey M et al (2015) Recurring events of *Candida krusei* septicaemia: first report from an ICU. J Mycol. https://doi.org/10.1155/2015/721424

Elavarashi E, Kindo AJ, Kalyani J (2013) Optimisation of PCR-RFLP directly from the skin and nails in cases of dermatophytosis, targeting the ITS and the 18S ribosomal DNA regions. J Clin Diagn Res 7(4):646–651

Elavarashi E, Kindo AJ, Kalyani J, Sudha R (2014) Application of PCR fingerprinting using (GACA)4 primer in the rapid discrimination of dermatophytes. Indian J Med Microbiol 32 (3):236–239

Evans EGV, Bulmer GS, Aly R, Coo-Barcelona L, Chakrabarti A, De Elias-Costa MRI, Gugnani HC (2000) Training of medical mycologists in developing countries. Med Mycol 38(Suppl 1):33–40

Eyre EW (1859) An account of a peculiar disease "tubercular" of the foot. Am J Med Sci 6:513–520

Fakhim H, Chowdhary A, Prakash A, Vaezi A, Dannaoui E, Meis JF et al (2017) In vitro interactions of echinocandins with triazoles against multidrug-resistant *Candida auris*. Antimicrob Agents Chemotherapy 61(11). https://doi.org/10.1128/aac.01056-17

Garg P (2012) Fungal, mycobacterial, and Nocardia infections and the eye: an update. Eye (Lond) 26(2):245–251. https://doi.org/10.1038/eye.2011.332. Epub 2011 Dec 16

Garg P, Roy A, Roy S (2016) Update on fungal keratitis. Curr Opin Ophthalmol 4:333–339. https://doi.org/10.1097/ICU.0000000000000272

Garg P, Das S, Roy A (2017) Collagen cross-linking for microbial keratitis. Ocular Infect Update 24 (1):18–23

Ghalige HS, Sahoo B, Sharma S, Devi KR, Singh Th SC (2014) Acute abdomen due to *Penicillium marneffei*: an indicator of HIV infection in Manipur state. J Clin Diagn Res 8(9):ND05–ND06. https://doi.org/10.7860/JCDR/2014/9632.4825

Ghosh AK, Paul S, Sood P, Rudramurthy SM, Rajbanshi A, Jillwin JT, Chakrabarti A (2015) Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for the rapid identification of yeasts causing bloodstream infections. Clin Microbiol Infect 21(4):372–378

Godfrey J (1846) Disease of foot not hitherto described. Lancet 1:593–594

Gopal KA, Kalavani V, Anandna H (2020) Pulmonary infection by *Chrysosporium* species in a preexisting tuberculous cavity. Basic Med Res 10(2):62–64

Gopinathan U, Sharma S, Garg P, Rao GN (2009) Review of epidemiological features, microbiological diagnosis and treatment outcome of microbial keratitis: experience of over a decade. Indian J Ophthalmol 57(4):273–279. https://doi.org/10.4103/0301-4738.53051

Graeff LD, Seide D, Vehreschild MJGT, Hamprecht A, Kindo AJ, Racil Z (2017) Invasive infections due to *Saprochaete* and *Geotrichum* species: report of 23 cases from the FungiScope Registry. Mycoses 60(4):273–279. https://doi.org/10.1111/myc.12595. Epub 2017 Feb 2

Grueber HLE, Kumar TM (1970) Mycetoma caused by *Streptomyces somaliensis* in North India. J Med Vet Mycol 8(2):108–111. https://doi.org/10.1080/00362177085190721

Gugnani HC, Randhawa HS (2020) Laboratory-acquired fungal infections. Arch Microbiol Immunol 4:51–56

Gugnani HC, Sood N (2020) Epidemiology of talaromycosis (penicilliosis) marneffei in India, an update. Eur J Med Health Sci 2(5):1–4. https://doi.org/10.24018/ejmed.2020.2.5.478

Gugnani HC, Sood N, Singh B, Kakkar R (2000) Subcutaneous phaeohyphomycosis due to *Cladosporium cladosporioides*. Mycoses 43:85–87

Gugnani HC, Shah A, Sood N, Vijayan VK, Singh VK (2002) Subcutaneous cryptococcosis associated with rib osteomyelitis in an immunocompetent patient. J Mycol Med 12:187–189

Gugnani HC, Becker K, Fegeler W, Basu S, Chattopadhya D, Baveja U, Satyanarayana S, Kalghatgi T, Murlidhar A (2003) Oropharyngeal carriage of Candida species in HIV-infected patients in India. Mycoses 46:299–306

Gugnani HC, Vijayan VK, Tyagi P, Sharma S, Stchigel AM, Guarro J (2004a) Onychomycosis due to *Emericella quadrilineata*. J Clin Microbiol 42:914–916

Gugnani HC, Fisher MC, Paliwal-Joshi A, Vanittanakom N, Singh I, Yadav PS (2004b) Role of Cannomys badius as a natural animal host of Penicillium marneffei in India. J Clin Microbiol 42:5070–5075

Gugnani HC, Mitchell TG, Litvintseva AP, Lengeler K, Heitman J, Kumar A, Basu S, Paliwal-Joshi A (2005) Isolation of Cryptococcus gattii and C. neoformans var. grubii from the flowers and bark of Eucalyptus trees in India. Med Mycol 43:565–569

Gugnani HC, Paliwal-Joshi A, Rahman H, Padhye AA, Singh TSK, Das TK, Khanal B, Bajaj R, Rao S, Chukhani R (2007) Occurrence of pathogenic fungi in soil of burrows of rats and of other sites in bamboo plantations in India and Nepal. Mycoses 50:507–511. https://doi.org/10.1111/j.1439-0507.2007.01402.x

Gugnani HC, Mitchell TG, Paliwal-Joshi A, Rattan A (2020a) Characterization of *Cryptococcus neoformans* strains from cases of cryptococcal meningitis in India. Eur J Med Health Sci 2(4):243–249. https://doi.org/10.24018/ejmed.2020.2.4.387

Gugnani HC, Hagen F, Meis JF, Chakrabarti A (2020b) Occurrence of Cryptococcus neoformans and other yeast-like fungi in environmental sources in Bonaire (Dutch Caribbean). Germs 10(3):195–200

Guha A, Chakrabarty B, Singh G, Singh S, Kumar A, Xess I et al (2020) A rare infective cause of stroke in an immunocompetent child. Brain Dev. https://doi.org/10.1016/j.braindev.2020.07.016

Gupta A, Capoor MR, Gupta V, Kochar S, Tomar A, Gupta V (2014) Clinico-demographical profile of keratomycosis in Delhi, North India. Indian J Med Microbiol 32(3):310–314. https://doi.org/10.4103/0255-0857.136582

Gupta M, Chandra A, Prakash P, Banerjee T, Maurya OP, Tilak R (2015) Fungal keratitis in North India; spectrum and diagnosis by Calcofluor white stain. Indian J Med Microbiol 33(3):462–463

Gupta C, Das S, Ramachandran VG, Saha R, Bhattacharya SN, Dar SA et al (2016) Possible role of Trichophytin antigen in inducing impaired immunological clearance of fungus in Onychomycosis. Mycopathologia 181(3–4):247–251

Gupta A, Capoor MR, Shende T, Sharma B, Mohindra R, Suri JC et al (2017) Comparative evaluation of galactomannan test with bronchoalveolar lavage and serum for the diagnosis of invasive aspergillosis in patients with hematological malignancies. J Lab Physicians 9 (4):234–238. https://doi.org/10.4103/JLP.JLP_127_16

Gupta MK, Tilak R, Pandey N (2018) Extracellular hydrolytic enzyme activities of the different Candida spp. isolated from the blood of the intensive care unit-admitted patients. J Lab Physicians 10(4):392–396

Gupta MK, Sinha S, Pandey N, Prakash P, Ragini T (2019) Possible role of superoxidised water in the treatment of *Trichophyton* infections: an in vitro study. J Pure Appl Microbiol 13 (2):887–890

Gupta N, Kodan P, Mittal A, Singh G, George N, Ramteke P (2020a) Role of voriconazole in the management of invasive central nervous system aspergillosis: A case series from a tertiary care centre in India. J Fungi 6(3):139. https://doi.org/10.3390/jof6030139

Gupta MK, Mallick P, Pandey N, Shankar V, Chakravarty J, Tilak R (2020b) The utility of banana peel extract agar in the presumptive identification of *Cryptococcus neoformans*. J Microbiol Methods 10. https://doi.org/10.1016/j.mimet.2020.106046

Hada V, Sharma A, Nag VL (2018) Importance of mycologic evaluation in chronic non-healing ulcers. J Diagn Clin Res 12(2):DD01–DD03

Hallur V, Singh G, Rudramurthy SM, Kapoor R, Chakrabarti A (2013) Demodex mite infestation of unknown significance in a patient with rhinocerebral mucormycosis due to *Apophysomyces elegans* species complex. J Med Microbiol 62:265–266. https://doi.org/10.1099/jmm.0.029199-0G2011

Hassan AS, Al-Hatmi AMS, Shobana CS, Van Diepeningen AD, Kredics L (2016) Antifungal susceptibility and phylogeny of opportunistic members of the genus Fusarium causing human keratomycosis in South India. Med Mycol 54(3):287–294

Homa M, Shobana CS, Singh YRB, Manikandan P, Selvam KP, Kredics L (2013) *Fusarium keratitis* in South India: causative agents, their antifungal susceptibilities and a rapid identification method for the *Fusarium solani* species complex. Mycoses 56(5):501–511

Homa M, Fekete IP, Böszörményi A, Singh YRB, Selvam KP, Shobana CS (2015) Antifungal effect of essential oils against *Fusarium keratitis* isolates. Planta Med 81(14):1277–1284

Homa M, Manikandan M, Szekeres A, Kiss N, Kocsubé N, Kredics L (2019) Characterization of *Aspergillus tamarii* strains from human keratomycoses: molecular identification, antifungal susceptibility patterns and cyclopiazonic acid producing abilities. Front Microbiol. https://doi.org/10.3389/fmicb.2019.02249

Honnavar P, Prasad GS, Ghosh AK, Dogra S, Handa S, Rudramurthy SM (2016) *Malassezia arunalokei* sp. nov., a novel yeast species isolated from seborrheic dermatitis patients and healthy individuals from India. J Clin Microbiol 54:1826–1834. https://doi.org/10.1128/JCM.00683-16

Jain S, Das S, Gupta N, Malik JN (2013) Frequency of fungal isolation and antifungal susceptibility pattern of the fungal isolates from nasal polyps of chronic rhinosinusitis patients at a tertiary care centre in North India. Med Mycol 51(2):164–169

Jain C, Das S, Ramachandran VG, Saha R, Bhattacharya SN, Dar S (2017) Malassezia yeast and cytokine gene polymorphism in atopic dermatitis. J Clin Diagn Res 11(3):Dc01–DDc5

Jain C, Kaur R, Rawat D, Ansari SK (2018) Onychomycosis by *Trichosporon inkin*: a rare clinical entity. J Clin Diagn Res 12(1):DD01–DD02

Jeeten Kumar T, Babina S, Nirmal DT, Tamphasana RK, Ranjana K, Kulachandra M (2012) Clinical profile of opportunistic infections and their correlation with CD4 count in HIV infected/AIDS patients: a RIMS study. Indian Med J 106(3):202

Jose LR, Savio J (2017) Candidemia isolates, identification and antifungal susceptibility testing: a study from a tertiary care centre. Indian J Microbiol Res 4(4):464–446. https://doi.org/10.18231/2394-5478.2017.0103

Kalavathy CM, Parmar P, Kaliamurthy J, Philip VR, Ramalingam MDK, Jesudasan CAN, Thomas PA (2005) Comparison of topical itraconazole 1% with topical natamycin 5% for the treatment

of filamentous fungal keratitis. Cornea 24(4):449–452. https://doi.org/10.1097/01.ico.0000151539.92865.3e

Kaliamurthy J, Geraldine P, Thomas PA (2003) Disseminated aspergillosis due to *Aspergillus flavus* in an experimental model: efficacy of azole therapy. Mycoses 46(5–6):174–182. https://doi.org/10.1046/j.1439-0507.2003.00865.x

Kaliamurthy J, Kalavathy CM, Ramalingam MDK, Prasanth DA, Jesudasan CAN, Thomas PA (2004) Keratitis due to a coelomycetous fungus: case reports and review of the literature. Cornea 23(1):3–12. https://doi.org/10.1097/00003226-200401000-00002

Kaliamurthy J, Kalavathy CM, Nelson Jesudasan CA, Thomas PA (2012) Keratitis due to *Chaetomium* sp. Case Rep Ophthalmol Med 2011:696145

Kalita JM, Sharma A, Bharadwaj A, Nag VL (2019) Dermatophytoses and spectrum of dermatophytes in patients attending a teaching hospital in Western Rajasthan, India. J Fam Med Prim Care 8(4):141814–141821

Kamalam A, Thambiah AS (1982) Lymphedema and elephantiasis in basidiobolomycosis. Mykosen 25(9):508–511

Kamalam A, Thambiah AS (2009a) Histological study in tinea capitis. Mycoses 24(7):431–441. https://doi.org/10.1111/j.1439-0507.1981.tb01890.x

Kamalam A, Thambiah AS (2009b) Amino acids in dermatophytes under varied substrates. Mycoses 24(7):444–449. https://doi.org/10.1111/j.1439-0507.1981.tb01891.x

Kamalam A, Thambiah AS (2009c) Clotrimazole and econazole in dermatophytoses. Mycoses 23 (12):707–710. https://doi.org/10.1111/j.1439-0507.1980.tb01778.x

Kandhavelu J, Demonte NL, Namperumalsamy VP, Prajna L, Thangavel C, Jayapal JM et al (2017) *Aspergillus flavus* induced alterations in tear protein profile reveal pathogen-induced host response to fungal infection. J Proteome 152:13–21

Kathuria S, Capoor MR, Yadav S, Singh A, Ramesh V (2013) Disseminated histoplasmosis in an apparently immunocompetent individual from North India: a case report and review of literature. Med Mycol 51:774–778

Kathuria S, Singh PK, Meis JF, Chowdhary A (2014) In vitro antifungal susceptibility profile and correlation of mycelial and yeast forms of molecularly characterized *Histoplasma capsulatum* strains from India. Antimicrob Agents Chemotherapy 58(9):5613–5616. https://doi.org/10.1128/aac.02973-14

Kaur R, Kataryia P, Dhakad MS, Mehra B, Jhamb U, Dubey AP (2016a) An unusual case of cystic fibrosis associated *Pneumocystis jiroveci* pneumonia in an infant. Case Rep Infect Dis. https://doi.org/10.1155/2016/9206707

Kaur R, Dhakad MS, Goyal R, Haque A, Mukhopadhyay G (2016b) Identification and antifungal susceptibility testing of *Candida* species: a comparison of Vitek-2 system with conventional and molecular methods. J Glob Infect Dis 8(4):139–146. https://doi.org/10.4103/0974-777X.192969

Kaur R, Lavanya S, Khurana N, Gulati A, Dhakad MS (2016c) Allergic fungal rhinosinusitis: a study in a tertiary care hospital in India. J Allergy 2016:7698173. 6 pages

Kaur R, Dhakad MS, Goyal R, Kumar R (2016d) Emergence of non-*albicans Candida* species and antifungal resistance in ICU patients. Asian Pac J Trop Biomed 6(5):930–934

Kaur R, Mehra B, Dhakad MS, Goyal R, Bhalla P, Dewan R (2017a) Fungal opportunistic pneumonias in HIV/AIDS patients: an Indian tertiary care center experience. J Clin Diagn Res 11(2). https://doi.org/10.7860/JCDR/2017/24219.9277

Kaur R, Mehra B, Dhakad MS, Goyal R, Dewan R (2017b) Pulmonary Aspergillosis as an opportunistic mycoses in a cohort of HIV infected patients: report from a tertiary care hospital in North India. Int J Health Sci (Qassim) 11(2):45–50

Kaur R, Mehra B, Dhakad MS, Goyal R, Bhalla P, Dewan R (2017c) Clinico-mycological analysis and antifungal resistance pattern in human immunodeficiency virus-associated candidiasis: an Indian perspective. Indian J Sexually Transmitted Dis AIDS 39(2):111. https://doi.org/10.4103/ijstd.IJSTD8917

Kaur R, Mehra B, Dhakad MS, Goyal R, Bhalla P, Dewan R (2017d) Utility of a serum *Aspergillus* galactomannan assay in diagnosis of invasive pulmonary Aspergillosis in HIV/AIDS patients: a prospective analysis. Ann Pathol Lab Med 4(A5):515–521

Kaur R, Dhakad MS, Goyal R, Bhalla P, Dewan R (2018) Clinco-mycological analysis and antifungal resistance pattern in human immunodeficiency virus associated candidiasis: an Indian perspective. Indian J Sexually Transmitted Dis AIDS 39(2):111–119

Kaur R, Jaggi S, Dhakad MS, Rawat R (2019) An etiological and antifungal profile of candidemia in children. Int J Commun Med Public Health 6(9):3899–3904

Kaur M, Chander J, Singla N, Das A, Sood S, Guarro J (2020) Sino-orbital infection caused by *Schizophyllum commune* - rare presentation of a basidiomycetous fungus. J Mycol Med 30:100934

Kaushal D, Sharma A, Kesarwani A, Mani Kalita JM (2019) Chronic Candida osteomyelitis of hard palate and nose: a diagnostic quandary. Med Mycol Case Rep 24:1–4

Kaushik R, Chander J, Gupta S, Sharma R, Punia RS (2012) Fatal primary cutaneous zygomycosis caused by *Saksenaea vasiformis*: case report and review of literature. Surg Infect (Larchmt) 13:125–129

Kayarkatte MN, Singal A, Pandhi D, Das S, Sharma S (2020) Nail dermoscopy (onychoscopy) findings in the diagnosis of primary onychomycosis: a cross-sectional study. Indian J Dermatol Venereol Leprol 86(4):341–349

Khuraijam R, Lungran P, Yoihenba K, Laishram RS, Pukhrambam P (2015) Pancytopenia and cutaneous cryptococcosis as an indicator disease of acquired immune deficiency syndrome. J Med Microbiol 33(3):439–442

Kindo AJ, Tupaki-Sreepurna A, Yuvaraj M (2016) Banana peel culture as an indigenous medium for easy identification of late-sporulation human fungal pathogens. Indian J Med Microbiol 34:457–461

Klokke AH (1964) Mycetoma due to *Nocardia brasiliensis*. First report of *N. brasiliensis* from Asia. Trop Geogr Med 16:170–171

Klokke AH, Durairaj P (1967) The causal agents of superficial mycoses isolated in rural areas of South India. Sabouraudia 5:153–155

Klokke AH, Swamidasan G, Anguili R, Verghese A (1968) The causal agents of mycetoma in South India. Trans R Soc Trop Med Hyg 62(4):509–514

Kombade SP, Abhisehek KS, Nag VL (2018) Fungal pericarditis due to *Aspergillus nidulans*: a rare case report. J Clin Diagn Res 12(9):DDO5

Kredics L, Varga J, Kocsubé S, Rajaraman R, Raghavan A, Dóczi I, Bhaskar M, Németh TM, Antal Z, Venkatapathy N, Vágvölgyi C, Samson RA, Chockaiya M, Palanisamy M (2009) Infectious keratitis caused by Aspergillus tubingensis. Cornea 28(8):951–954

Kredics L, Narendran V, Shobana SC, Vágvölgyi C, Manikandan P (2015) Filamentous fungal infections of the cornea: a global overview of epidemiology and drug sensitivity. Mycoses 58 (4):243–260

Krizsán K, Papp T, Manikandan P, Shobana CS, Chandrasekaran M (2015) Clinical importance of the genus *Curvularia*. In: Razzaghi-Abyaneh M, Shams-Ghahfarokhi M, Rai M (eds) Medical mycology: current trends and future prospects. CRC Press, Boca Raton

Kucheria M, Gupta SK, Chinna DK, Gupta V, Hans D, Singh K (2016) Clinico-mycological profile of dermatophytic infections at a tertiary care hospital in North India. Int J Commun Health Med Res 2(2):17–22. https://doi.org/10.21276/ijchmr.2016.2.2.03

Kumar D, Banerjee T, Pratap CB, Tilak R (2015a) Itraconazole-resistant *Candida auris* with phospholipase, proteinase and hemolysin activity from a case of vulvovaginitis. J Infect Developing Countries 9(04):435–437

Kumar D, Kumar A, Singh S, Tilak R (2015b) Candidemia-induced pediatric sepsis and its association with free radicals, nitric oxide, and cytokine level in host. J Crit Care 30(2):296–303

Kumar S, Gupta R, Capoor MR, Arya S, Singh A (2019) Predictors of candidemia in pediatric patients (0–12 YEARS) admitted in a tertiary care of northern India. Indian J Child Health 6 (9):474–480. https://doi.org/10.32677/IJCH.2019.v06.i09.003

Kumari J, Jat KR, Lodha R, Jana M, Xess I, Kabra SK (2019) Prevalence and risk factors of allergic bronchopulmonary aspergillosis and *Aspergillus* sensitization in children with poorly controlled asthma. J Trop Pediatr 66(3). https://doi.org/10.1093/tropej/fmz066

Kundu R, Handa U, Punia RS, Singla N, Chander J, Attri AK (2020) Phaeohyphomycosis: cytomorphologic evaluation in eleven cases. Acta Cytol 64:406–412

Lalchandani R, Salvi BV, D'souza P, Gugnani HC (2020) Successful limb salvage in a case of advanced long-standing Eumycetoma of the foot using adjunctive local amphotericin B delivery through bioabsorbable beads. J Indian Orthopaed 50:224–227

Lalitha P, Prajna NV, Kabra A, Mahadevan K, Srinivasan M (2006) Risk factors for treatment outcome in fungal keratitis. Ophthalmology 113(4):526–530

Leck AK, Thomas PA, Hagan M, Kalamurthy J, Akaku A, John M (2002) Aetiology of suppurative corneal ulcers in Ghana and South India, and epidemiology of fungal keratitis. Br J Ophthalmol 86(11):211–215. https://doi.org/10.1136/bjo.86.11.1211

Leema G, Kaliamurthy J, Geraldine P, Thomas PA (2010) Keratitis due to *Aspergillus flavus*: clinical profile, molecular identification of fungal strains and detection of aflatoxin production. Mol Vision 16:843–854

Leema G, Chou D-S, Nelson Jesudasan CA, Geraldine P, Thomas PA (2011) Expression of genes of the aflatoxin biosynthetic pathway in *Aspergillus flavus* isolates from keratitis. Mol Vision 17:2889–2897

Lemos JA, Passos XS, Fernandes OFL, de Paula R, Ferri PH, Souza LKH (2005) Antifungal activity from Ocimum gratissimum L. towards Cryptococcus neoformans. Memorias du Instituto Oswaldo Cruz Rio de Janeiro 100(1):55–58. https://doi.org/10.1590/S0074-02762005000100011

Mahajan S, Tilak R, Kaushal SK, Mishra RN, Pandey SS (2017) Clinico-mycological study of dermatophytic infections and their sensitivity to antifungal drugs in a tertiary care center. Indian J Dermatol Venereol Leprol 83(4):436–440. https://doi.org/10.4103/ijdvl.IJDVL_519_16

Mahapatra A (2005) Coinfection of Cryptosporidium and Geotrichum in a case of AIDS. Indian J Pathol 48(1):25–27

Mahapatra A, Patnaik B, Mahapatra S, Mahapatra A (2007) Comparative evaluation of direct microscopy & culture for diagnosis of mycotic keratitis. Indian Med Gazett 104(6):239–241

Mahapatra S, Sethy SM, Mahapatra A, Bhuyan P, Mahaptra S, Mohanty P (2009) Disseminated cryptococcal lymphadenitis & meningitis in AIDS. Antiseptic 106(5):217–218

Mahapatra A, Das TC, Parida B, Chayani N (2014) Chromogenic tube test for presumptive identification and susceptibility test of Candida spp. Indian Med Gazett 60:215–217

Manikandan P, Selvam KP, Shobana CS, Ravikumar K, Rajaduraipandi K (2008a) Corneal ulcer of bacterial and fungal etiology from diabetic patients at a tertiary care eye hospital, Coimbatore, South India. Facta Universitatis-series Med Biol 15(2):59–63

Manikandan P, Vismer HF, Kredics L, Dóczi I, Marasas WFO, Bhaskar M, Anita R, Revathi R, Narendran V (2008b) Corneal ulcer due to in an immunocompetent patient. Med Mycol 46 (3):279–284

Manikandan P, Varga J, Koksube S, Samson RA, Anita R, Revathi R et al (2009) Mycotic Keratitis due to *Aspergillus nomius*. J Clin Microbiol 47(10):3382–3385. https://doi.org/10.1128/JCM.01051-09

Manikandan P, Galgóczy LN, Selvam KP, Shobana CS, Kocsubé S, Liu D (eds) (2011) Molecular detection of human fungal pathogens, Facta Universitatis-series: medicine and biology. CRC Press, Boca Raton, pp 409–425

Manikandan P, Varga J, Kocsubé S, Anita R, Revathi R, Németh TM (2013) Epidemiology of *Aspergillus keratitis* at a tertiary care eye hospital in South India and antifungal susceptibilities of the causative agents. Mycoses 56(1):26–33

Manikandan P, Abdel-Hadi AY, Singh RB, Revathi R, Anita R (2019) Fungal keratitis: epidemiology, rapid detection, and antifungal susceptibilities of *Fusarium* and *Aspergillus* isolates from corneal scrapings. Biomed Res Int. https://doi.org/10.1155/2019/6395840

Meena S, SInGH G, PanDeY M, XeSS I (2019) Exophiala jeanselmei and Rhizopus oryzae co-infection post renal transplant. J Clin Diagn Res 13(6)

Meena DS, Kumar D, Bohra GK, Garg MK, Yadav P et al (2020) Pulmonary nocardiosis with aspergillosis in a patient with COPD: a rare co-infection. IDCases 2. https://doi.org/10.1016/j.idcr.2020.e00766

Mishra K, Das S, Goyal S, Gupta C, Rai G, Ansari MA et al (2014) Subcutaneous mycoses caused by *Rhytidhysteron* species in an immunocompetent patient. Med Mycol Case Rep 5:32–34

Misra PC, Srivastava KJ, Lata K (1979) Apophysomyces, a new genus of the Mucorales. Mycotaxon 8:377–382

Mobing H, Singh JK, Devi B, Singh Ksh B, Kh Devi R, Maharajan D et al (2019) Prevalence of Cryptococcal antigenemia and its correlation with clinico-immunological status in HIV infected patients. IOSR J Dent Med Sci 18(5):6–14

Mohanty S, Xess I, Hasan F, Kapil A, Mittal S, Tolosa JE (2016) Prevalence & susceptibility to fluconazole of *Candida* species causing vulvovaginitis. Indian J Med Res 126(3):216–219

Mohindra R, Capoor MR, Puri S, Raheja H, Gupta DK, Gupta B, Chowdhury R (2017) Evaluation of serum galactomannan enzyme immunoassay at two different cut-offs for the diagnosis of invasive aspergillosis in patients with febrile neutropenia. Indian J Med Microbiol 35 (2):237–242

Mythili A, Singh YRB, Priya P, Hassan AS, Manikandan P (2014) In vitro and comparative study on the extracellular enzyme activity of molds isolated from keratomycosis and soil. Int J Ophthalmol 7(5):778–784

Nagarajan C, Premamalini T, Rajyoganandh S, Kindo AJ, Samuel J (2015) Rhinosinusitis caused by unusual fungus *Fonsecaea pedrosoi* - a rare case report from South India. Am J Med Case Rep 3(9):297–300

Nandhakumar B, Girish Kumar CP, Praabu D, Menon T (2006) Mustard seed agar, a new medium for differentiation Cryptococcus neoformans. J Clin Microbiol 44(2):674

Negri M, Salci TP, Shinobu-Mesquita CS, Capoci IRG, Svidzinski TLE, Kioshima ES (2014) Early state research on antifungal natural products. Molecules 19:2925–2956. https://doi.org/10.3390/molecules19032925

Nucci M, Marr KA, Vehreschild MJGT, de Souza CA, Velasco E, Appellano P et al (2014) Improvement in the outcome of invasive fusariosis in the last decade. Clin Microbiol Infect 20(6):580–586

Palanisamy P, Venkatapathy N, Rajendran V, Shobana CS (2015) Keratomycosis caused by *Graphium eumorphum* (*Graphium* state of *Scedosporium apiospermum*). J Clin Diagn Res 9 (4):DD03

Pandey N, Gupta MK, Paul P, Tilak R (2019) Necessity to identify *Candida* species accurately with minimum inhibitory concentration determination in each case of bloodstream infections. J Infect Public Health 13(5):753–758

Pandey N, Tripathy M, Gupta M, Tilak R (2020) Overexpression of efflux pump transporter genes and mutations in ERG11 pave the way to fluconazole resistance in *Candida tropicalis*: a study from North India region. J Glob Antimicrob Resist 22. https://doi.org/10.1016/j.jgar.2020.02.010

Parthiban N, Sampath NL, JeyaMaheshwari J, Prajna NV, Lalitha P, Dharmalingam K (2019) Quantitative profiling of tear proteome reveals down regulation of zinc alpha-2 glycoprotein in *Aspergillus flavus* keratitis patients. Exp Eye Res 186:107700

Patel A, Kaur HS, Xess I, Michael JS, Savio S, Rudramurthy SM et al (2019) A multicenter observational study on the epidemiology, risk factors, management and outcomes of mucormycosis in India. Clin Microbiol Infect 26(7). https://doi.org/10.1016/j.cmi.2019.11.0212

Patro P, Das P, Sachdev D, Borkar N, Ganguly S, Hussain N (2010) An instance of excellent response of subcutaneous zygomycosis to itraconazole monotherapy. Med Mycol Case Rep 24:13–17

Paul S, Singh P, Rudramurthy SM, Chakrabarti A, Ghosh AK (2017) Matrix-assisted laser desorption/ionization–time of flight mass spectrometry: protocol standardization and database

expansion for rapid identification of clinically important molds. Future Microbiol 12:1457–1466

Pinna A, Usai D, Zanetti S, Thomas PA, Kaliamurthy J (2013a) In vitro efficacy of contact lens solutions against various corneal fungal isolates. J Ocular Pharmacol Therapeutics 29 (7):694–699. https://doi.org/10.1089/jop.2012.0052

Pinna A, Usai D, Zanetti S, Thomas PA, Kaliamurthy J (2013b) Susceptibility of various corneal fungal isolates and *Pseudomonas aeruginosa* to contact lens disinfecting solutions. J Infect Dev Ctries 7(3):261–268. https://doi.org/10.3855/jidc.2756

Pradhan L, Sharma S, Nalamada S, Sahu SK, Das S, Garg P (2011) Natamycin in the treatment of keratomycosis: correlation of treatment outcome and *in vitro* susceptibility of fungal isolates. Indian J Ophthalmol 59(6):512–514

Prajna NV, Nirmalan PK, Mahalakshmi R, Lalitha P, Srinivasan M (2004) Concurrent use of 5% natamycin and 2% econazole for the management of fungal keratitis. Cornea 23(8):793–796

Prakash H, Rudramurthy SM, Gandham PS, Ghosh AK, Kumar MM, Badapanda C, Chakrabarti A (2017) Apophysomyces variabilis: draft genome sequence and comparison of predictive virulence determinants with other medically important Mucorales. BMC Genomics 18(1)

Prasad S, Khurana U, Tadepalli K, Brahamchari S, Sinha JK, Tandon A (2020) Fine needle aspiration of nodular cystic swelling showing a rare melanized fungus *Medicopsis romeroi*: a case report. Diagn Cytopathol. https://doi.org/10.1002/dc.24383

Premamalini T, Ambujavalli BT, Vijaykumar R, Rajyoganand SV, Kalpana S, Kindo AJ (2012) Fungal keratitis caused by *Macrophomina phaseolina*. Med Mycol Case Rep 1(1):123–126. https://doi.org/10.1016/j.mmcr.2012.10.007

Pukhramban PD, Devi RK, Singh NB (2011) Pattern of onychomycosis study–a RIMS study. J Commun Disord 43(2):105–112

Punia RS, Singhal SK, Kundu R, Das A, Chander J (2019) Fungal suppurative otitis media (histopathology) among patients in North India. Head Neck Pathol 13:149–153

Rai G, Das S, Ansari MA, Singh PK, Gupta N, Sharma S et al (2018) Phenotypic and functional profile of Th17 and Treg cells in allergic fungal sinusitis. Int Immunopharmacol 57:55–61

Rai G, Roy P, Gupta N, Sharma S, Dar SA, Ansari MA et al (2019) Computed tomography score an excellent marker: differentiates eosinophilic and non-eosinophilic variants of chronic rhinosinusitis with nasal polyp. Indian J Otolaryngol Head Neck Surg 71(Suppl 3):1787–1792. https://doi.org/10.1007/s12070-017-1154-x

Rai G, Das S, Ansari MA, Singh PK, Pandhi D, Tigga RA et al (2020) The interplay among Th17 and T regulatory cells in the immune dysregulation of chronic dermatophytic infection. Microb Pathog 139:103921

Raj S, Sharma D, Capoor MR, Mate P, Bhowmik KT (2017) A study of changes in the oral fungal flora of patients on radiotherapy for head and neck malignancies and their correlation with funguria and fungemia. Indian J Cancer 54(1):39–42. https://doi.org/10.4103/ijc.IJC_155_17

Rajyoganandh S, Ramaraj V, Kindo AJ (2016) Direct colony polymerase chain reaction for rapid identification of yeasts isolated from blood specimen. J Acad Clin Microbiologists 18(2):91–94

Ramesh V, Ramam M, Capoor MR, Sugandhan S, Dhawan J, Khanna G (2010) Subcutaneous zygomycosis: report of 10 cases from two institutions in North India. J Eur J Acad Dermatol Venereol 24(10):1220–1225. https://doi.org/10.1111/j.1468-3083.2010.03606.x

Rameshkumar G, Ponlakshmi M, Selvapandiyan A, Ramsudharsan R, Krishnan RS, Lalitha P (2018) Fungal keratitis caused by *Podospora austroamericana*: a first case report. Indian J Med Microbiol 36(4):594–596

Rameshkumar G, Sikha M, Ponlakshmi M, Selva Pandiyan A, Lalitha P (2019) A rare case of *Myrothecium* species causing mycotic keratitis: diagnosis and management. Med Mycol Case Rep 25:53–55

Randhawa HC, Gugnani HC (2018) Occurrence of histoplasmosis in the Indian sub-continent : an overview and update. J Med Res Pract 7(3):71–83

Ranjana Devi K, Singh RB (2018) A comparative study on antifungal susceptibility of *Penicillium marneffei* (*Talaromyces marneffei*) and non-marneffei *Penicillium* species. J Med Sci 32 (1):22–26

Ranjana DK, Singh RBY (2016) *In-vitro* extra cellular enzyme activity of clinical and environmental *Penicillium* spp. Indian J Appl Microbiol 19(1):1–8

Rastogi V, Nirwan PS (2007) Invasive trichosporonosis due to *Trichosporon asahii* in a non-, host: a rare case report. Indian J Med Microbiol 25(1):59–61. https://doi.org/10.4103/0255-0857.31065

Rastogi V, Rudhramurthty SM, Maheswari M, Nirwan PS, Chakrabarti A, Batra S (2013) Non-healing ulcer due to *Trichosporon loubieri* in an immunocompetent host and review of published reports. Mycopathologia 176(1–2):107–111. Epub 2013 Apr 1

Rastogi V, Honnavar P, Shivaprakash MR, Pamidi U, Ghosh A, Chakrabarti A (2016) Molecular characterisation and antifungal susceptibility of clinical Trichosporon isolates in India. Mycoses 59(8):528–534

Reddy C, Murthy R, Reddy AK, Garg P (2015) Risk factors and clinical outcomes of bacterial and fungal scleritis at a tertiary eye care hospital. Middle East Afr J Ophthalmol 22(2):203–211. https://doi.org/10.4103/0974-9233.150634

Ruban VV, Kaliamurthy J, Dineshkumar M, Jesudasan CAN, Geraldine P, Thomas PA (2013) Keratitis due to the wood saprobic ascomycete, *Auerswaldia lignicola* (family Botryosphaeriaceae), in a carpenter in India. Mycopathologia 176(5–6):463–466. https://doi.org/10.1007/s11046-013-9713-5

Ruban VV, Geraldine P, Kaliamurthy J, Jesudasan CAN, Thomas PA (2015) Keratitis due to *Fusarium langsethiae*: clinical profile, molecular identification, and susceptibility to antifungals. Mycopathologia 179(5–6):453–458. https://doi.org/10.1007/s11046-015-9866-5

Ruban VV, Archana PT, Sundararajan M, Geraldine P, Thomas PA (2018) Inflammation and oxidative stress in corneal tissue in experimental keratitis due to *Fusarium solani*: amelioration following topical therapy with voriconazole and epigallocatechin gallate. Mycoses 61 (3):159–171. https://doi.org/10.1111/myc.12718

Ruban VV, Anbukkarasi M, Anand T, Thomas PA, Geraldine P (2019) Oxidative stress in corneal tissue in experimental keratitis due to *Aspergillus flavus*: effect of topical voriconazole therapy. Biocatal Agric Biotechnol 21:101323. https://doi.org/10.1016/j.bcab.2019.101323

Rudramurthy SM, Appannanavar SB, Dhaliwal M, Gupta A, Gupta S, Gupta A et al (2011) *Colletotrichum truncatum*: an unusual pathogen causing mycotic keratitis and endophthalmitis. J Clin Microbiol 49(8):2894–2898. https://doi.org/10.1128/JCM.00151-11

Rudramurthy SM, Chakrabarti A, Paul RA, Sood P, Kaur H, Capoor MR et al (2017a) *Candida auris* candidaemia in Indian ICUs: analysis of risk factors. J Antimicrob Agents Chemotherapy

Rudramurthy SM, Syedmousavi S, Dhaliwal M, Chakrabarti A, Meis JF, Mouton JW (2017b) Pharmacodynamics of voriconazole against wild-type and azole-resistant Aspergillus flavus isolates in a nonneutopenic murine model of disseminated aspergillosis. Antimicrob Agents Chemother 61:e01481–e01416

Rudramurthy SM, Shankarnaryan SA, Shaw D, Mushtaq K, Paul RA et al (2018) Mutation in the squalene epoxidase gene of Trichophyton interdigitale and Trichophyton rubrum associated with allylamine resistance. Antimicrob Agents Chemother 62:e0149–e0116

Ruping MJT, Heinz WJ, Kindo AJ, Rickerts V, Lass-Florl C (2010) Forty one recent cases of invasive zygomycosis from a global clinical registry. J Antimicrob Chemother 65(2):296–302

Samaddar A, Sharma A, Kumar APH, Srivastava S et al (2019) Disseminated histoplasmosis in immunocompetent patients from an arid zone in Western India: a case series. Med Mycol Case Rep 25:49–52

Samaddar A, Sharma A, Shrimali T (2020) Pulmonary infection due to *Acrophialophora fusispora* in a patient with underlying mixed connective tissue disease and chronic pulmonary aspergillosis. J Mycol Med 30(2):100932. https://doi.org/10.1016/j.mycmed.2020.100932

Sane S, Sharma S, Konduri R, Fernandes M (2019) Emerging corneal pathogens: first report of *Pseudopestalotiopsis theae* keratitis. Indian J Ophthalmol 67(1):150–152. https://doi.org/10.4103/ijo.IJO_791_18

Sarabhoy AK, Varshney JL, Agarwal DK (1993) Fungi of India (1982–92). CBS Publishers, Delhi. 350 pp

Sarma S, Capoor MR, Deb M, Ramesh V, Aggarwal P (2009) Epidemiologic and clinicomycologic profile of onychomycosis from North India. Int J Dermatol 47(6):584–587. https://doi.org/10.1111/j.1365-4632.2008.03674.x

Savio J, Murlidhran S, MacAden RS, D'Souza G, Mysore S, Ramachandran P (2006) Blastomycosis in a South Indian patient after visiting an endemic area in USA. Med Mycol 44(6):522–529. https://doi.org/10.1080/13693780500406865

Savio J, Srinivasa S, Mathew A, Chakrabarti A, McNamara DR, Hemashettar BM et al (2011) Coccidioidomycosis in India: report of a third imported case. J Mycol Med 21(1):33–36. https://doi.org/10.1016/j.mycmed.2010.11.004

Savio J, Nikhilesh R, Menon NR, Sudharma AR, Vinutha JV, Mathew J (2016) Galactomannan assay and invasive pulmonary aspergillosis. Comparison of the test performance at an in-house and the Kit Cut-off. J Clin Diagn Res 10(8):DC01–DC04. https://doi.org/10.7860/JCDR/2016/19175.8310

Sawatkar GU, Wankhade VH, Supekar BB, Rajesh Pratap RP, Dharitri M, Bhat DM, Tankhiwale SS (2019) Mycetoma: a common yet unrecognized health burden in Central India. Indian Dermatol Online J 10(3):256–261

Saxena SB (1953) A new genus of mucorales. Mycologia 45:426–436

Seidel D, Meißner A, Lackner M, Chander J et al (2019) Prognostic factors in 264 adults with invasive *Scedosporium* spp. and *Lomentospora prolificans* infection reported in the literature and FungiScope(®). Crit Rev Microbiol 45:1–21

Selvam KP, Singh YRB, Shobana CS, Karunakaran NK, Vágvölgyi C, Al-Baradie RS et al (2014) Extracellular enzymes and mycotoxins as virulence factors in *Fusarium* and *Aspergillus keratitis*. Biosci Biotechnol Res Asia 11:479–490

Seth R, Xess I, Jana M (2019) Diagnosis of invasive fungal infection in children. Indian Pediatr 56:229–236

Shahi S, Kumar S, Rudramurthy SM, Chakrabarty A, Gaur S et al (2020) A detailed study of human pathogenic fungi *Candida auris*. FEMS Yeast Res 20(6). https://doi.org/10.1093/femsyr/foaa045

Shait Mohammed MR, Krishnan S, Amrathlal RS, Jayapal JM, Namperumalsamy VP, Prajna L et al (2020) Local activation of the alternative pathway of complement system in mycotic keratitis patient tear. Front Cell Infect Microbiol 10:205

Shankarnarayan AS, Rudramurthy SM, Chakrabarti A, Shaw D, Paul S, Sethuraman N et al (2018) Molecular typing and antifungal susceptibility of *Candida viswanathii*, India. Emerg Infect Dis 24(10):1956–1958

Shankarnarayan AS, Shaw D, Sharma A, Chakrabarti A, Dogra S, Kumaran MS et al (2020) Rapid detection of terbinafine resistance in *Trichophyton* species by amplified refractory mutation system-polymerase chain reaction. Sci Rep 10:1297. https://doi.org/10.1038/s41598-020-58187-0

Sharma A (2019a) Establishing a mycology laboratory. Infect Cont Trends Newsl 2I(1):7–10

Sharma A (2019b) Prevention of nosocomial fungal infections. Infect Cont Trends Newsl I (1):16–17

Sharma M, Sharma R (2012) Profile of dermatophytic and other fungal infections in Jaipur. Indian J Microbiol 52(2):70–274. https://doi.org/10.1007/s12088-011-0217-z

Sharma S, Balne PK, Motukupally SR, Das S, Garg P, Srikant K et al (2015) *Pythium insidiosum* keratitis: clinical profile and role of DNA sequencing and zoospore formation in diagnosis. Cornea 34(4):438–442. https://doi.org/10.1097/ICO.0000000000000349

Sharma R, Gupta S, Asati DP, Karuna T, Purwar SA, Biswas D (2017) Pilot study for the evaluation of PCR as a diagnostic tool in patients with suspected dermatophytoses. Indian Dermatol Online J 8(3):176–180

Sharma A, Saxena R, Sinha A, Singh S, Yadav T (2019) Disseminated gastrointestinal basidiobolomycosis (GIB) in an infant from Western India. Med Mycol Case Rep 26:38–41. https://doi.org/10.1016/j.mmcr.2019.10.001

Sharma P, Bhalla M, Thami GP, Chander J (2020a) Evaluation of efficacy and safety of oral terbinafine and itraconazole combination therapy in the management of dermatophytosis. J Dermatol Treat 31(7):749–753

Sharma A, Bisht D, Das S, Rai G, Dutt S, Arora VK (2020b) Molecular detection of *Aspergillus* in sputum of patients with lower respiratory tract infections. Int J Appl Basic Med Res 10(2):86–90

Shobana CS, Mythili A, Homa M, Galgóczy L, Priya R, Singh YRB (2016) In-vitro susceptibility of filamentous fungi from mycotic keratitis to azole drugs. J Mycol Med 25(1):44–49

Shrivastava A, Tadepalli K, Goel G, Gupta K, Gupta PK (2017) Melanized fungus as an epidural abscess: a diagnostic and therapeutic challenge. Med Mycol Case Rep 16:20. https://doi.org/10.1016/j.mmcr.2017.04.001

Singal A, Jakhar D, Kaur I, Pandhi D, Das S (2019) *Tinea pseudoimbricata* as a unique manifestation of steroid abuse: a Clinico-mycological and dermoscopic study from a tertiary care hospital. Indian Dermatol Online J 10(4):422–425

Singh R, Rudramurthy SM, Chakrabarti A (2011) Biofilm formation by zygomycetes: quantification, structure and matrix composition. Microbiology 157(Pt 9):2611–2618

Singh T, Kashyap AK, Ahluwalia G, Chinna D, Sidhu SS (2014) Epidemiology of fungal infections in critical care setting of a tertiary care teaching hospital in North India: a prospective surveillance study. J Clin Sci Res 3:14–25

Singh GK, Capoor MR, Nair D, Bhowmik TK (2017a) Spectrum of fungal infection in head and neck cancer patients on chemoradiotherapy. J Egypt Natl Canc Inst 29(1):33–37. https://doi.org/10.1016/j.jnci.2017.01.006

Singh A, Singh PK, Kumar A, Chander J, Khanna G, Roy P (2017b) Molecular and matrix-assisted laser desorption ionization - time of flight mass spectrometry-based characterization of clinically significant melanized fungi in India. J Clin Microbiol 55:1090–1103

Singh GB, Solo M, Kaur R, Arora R, Kumar S (2018) Mycology of chronic suppurative otitis media-cholesteatoma disease: an evaluative study. Am J Otolaryngol 39(2):157–161

Singh T, Arya SK, Handa U, Chander J (2019a) Usability of donor corneas harvested from the deceased having septicaemia or malignancy. Q J Med 112:681–683

Singh SK, Patwa DK, Tilak R, Das A, Singh TB (2019b) In vitro susceptibility of dermatophytes to oral antifungal drugs and amphotericin B in Uttar Pradesh, India. Indian J Dermatol Venereol Leprol 85(4):388–392. https://doi.org/10.4103/ijdvl.IJDVL_319_18

Singh SK, Subba N, Tilak R (2019c) Effect of topical corticosteroid on yield of fungal culture and response to treatment: a prospective cohort study. J Dermatol Treat 11:1. https://doi.org/10.1080/09546634.2019.1687829

Singh S, Capoor MR, Varshney S, Gupta DK, Verma PK, Ramesh R (2020a) Epidemiology and antifungal susceptibility of infections caused by *Trichosporon* species: an emerging non-*Candida* and non-*Cryptococcus* yeast worldwide. Indian J Med Microbiol 37:536–541. https://doi.org/10.4103/ijmm.IJMM_19_146

Singh A, Sharma B, Mahto KK, Meis JF, Chowdhary A (2020b) High-frequency direct detection of triazole resistance in *Aspergillus fumigatus* from patients with chronic pulmonary fungal diseases in India. J Fungi (Basel) 6(2):67. https://doi.org/10.3390/jof6020067

Singh SK, Subba N, Tilak R (2020c) Efficacy of terbinafine and itraconazole in different doses and in combination in the treatment of tinea infection: a randomized controlled parallel group open labeled trial with clinico-mycological correlation. Indian J Dermatol 65(4):284–289. https://doi.org/10.4103/ijd.IJD_548_19\

Singh GB, Solo M, Kaur R (2020d) A rare case of candida mastoiditis in an immunocompetent patient with cholesteatoma ear disease. Ear Nose Throat J. https://doi.org/10.1177/0145561320957757

Singla N, Gulati N, Kaistha N, Chander J (2012) *Candida* colonization in urine samples of ICU patients: determination of etiology, antifungal susceptibility testing and evaluation of associated risk factors. Mycopathologia 174:149–155

Singla N, Gulati N, Chander J (2019) Biofilms: fungal perspective. Indian J Pathol Microbiol 62:514–515

Sonthalia S, Singal A, Das S (2014) Tinea cruris and Tinea corporis masquerading as *Tinea indecisiva*: case report and review of the literature. J Cutan Med Surg 18(5):1–6

Sonthalia S, Agrawal M, Goldust M, Das S, Bhattacharya SN (2018) Antifungal therapeutic failures in India: an important issue being overlooked. Lancet Infect Dis 18(11):1181–1182

Sonthalia S, Bhattacharya SN, Agrawal M, Das S (2019) High-magnification universal serial bus dermoscopy: a convenient alternative to direct microscopic examination. J Am Acad Dermatol. https://doi.org/10.1016/j.jaad.2019.08.083

Sood N, Gugnani HC (2007) Penicillium marneffei infection in a Myanmar refugee settled in Mizoram State in Northeast India. Indian J Pathol Microbiol 53:361–363. https://www.ijpmonline.org/article.asp?issn=0377

Sood N, Gugnani HC, Rashmi B, Ramesh V, Padhye AA (2007a) Mucocutaneous nasal histoplasmosis in an immunocompetent young adult. Indian J Dermatol Venerol Leprol 73:182–184

Sood S, Sharma R, Gupta S, Pathak D, Rishi S (2007b) Neuroaspergillosis in an immunocompetent patient. Indian J Med Microbiol 25(1):67–69

Sreenath K, Sanjeeb S, Singh THG, Gojen KH, Singh K, Devi R (2014) Localised cryptococcal scalp osteomyelitis in an immunocompetent man – a case report. Int J Anat Radiol Surg 3 (4):10–12

Sriam KP, Mathews PS, Gopalakrishnan G (2007) Paecilomyces pyelonephritis in a patient with urolithiasis. Indian J Urol 23(2):195–197. https://doi.org/10.1590/S1677-55382008000200006

Srivastva A, Mohapatra M, Mahapatra A (2019) Maxillary fungal osteomyelitis: a review of literature and report of a rare case. Ann Maxillofacial Surg 90(1):168. https://doi.org/10.4103/ams.ams_218_18

Suri P, Chhina DK, Kaushal V, Kaushal RK, Singh J (2014) Cerebral phaeohyphomycosis due to *Cladophialophora bantiana* - a Case report and review of literature from India. J Clin Diagn Res 8(4):DD01–DD05. https://doi.org/10.7860/jcdr/2014/7444.4216

Tadepalli K, Asati DP, Biswas D, Purwar S (2015) Subcutaneous entomophthoromycoses. Indian Dermatol Online J 6(6):410–412

Taneja M, Ashar JN, Mathur A, Nalamada S, Garg P (2012) Microbial keratitis following vegetable matter injury. Int J Ophthalmol 32(2). https://doi.org/10.1007/s10792-012-9643-0

Tendolkar U, Tainwala S, Jog S, Mathur M (2003) Use of a new medium - tobacco agar, for pigment production of *Cryptococcus neoformans*. Indian J Med Microbiol 21(4):227–229

Tendolkar U, Shinde A, Baveja S, Dhurat R, Phiske M (2014) *Trichosporon inkin and Trichosporon mucoides* as unusual causes of white piedra of scalp hair. Indian J Dermatol Venerol Leprol 80(4):324–327

Tendolkar U, Shet B, Baveja S, Mehta N, Samadhar A, Banshelkkar S et al (2016) Unusual presentation of *Madurella mycetomatis* mycetoma in a paediatric patient in India. MOJ Clin Med Case Rep 4(5):00103

Tewari A, Behera B, Mathur P, Xess I (2012) Comparative analysis of the Vitek 2 antifungal susceptibility system and E-test with CLSI M27-A3 broth microdilution method for susceptibility testing of Indian clinical isolates of *Cryptococcus neoformans*. Mycopathologia 173:427–433

Thami GP, Kaur S, Bawa AS, Chander J, Mohan H, Bedi MS (2003) Post-surgical zygomycotic necrotizing subcutaneous infection caused by Absidia corymbifera. Clin Exp Dermatol 28:251–253. https://doi.org/10.1046/j.1365-2230.2003.01244.x

Thass N, Kumar M, Kaur R (2018) *Myroides* spp. causing urinary tract infection in an immunocompromised patient: a case report. Indian Curr Microbiol Appl Sci 7(4):997–1001

Thatoi PK, Khadanga S, Khurana U, Dugar D, Satapathy SP, Tadepalli K (2015) Ileo-cecal mucormycosis in an immune competent adult: treating successfully a rare disease with high mortality. J Gastrointest Infect 5:50–53

Thomas PA (2003) Current perspectives on ophthalmic mycoses. Clin Microbiol Rev 16 (4):730–797. https://doi.org/10.1128/cmr.16.4.730-797

Thomas PA, Kaliamurthy J (2013) Mycotic keratitis: epidemiology, diagnosis and management. Clin Microbiol Infect 19(3). https://doi.org/10.1111/1469-0691.12126

Thomas PA, Kaliamurthy J, Jesudasan CAN, Geraldine P (2008) Use of Chlorazol black E mounts of corneal scrapes for diagnosis of filamentous fungal keratitis. Am J Ophthalmol 145 (6):971–976.e2. https://doi.org/10.1016/j.ajo.2008.01.020

Thomas P, Abraham DJ, Kalavathy CM, Rajasekaran J (2009) Oral Itraconazole therapy for mycotic keratitis. Mycoses 31(5):271–279

Thomas PA, Teresa P, Theodore J, Geraldine P (2012) PCR for the molecular diagnosis of mycotic keratitis. Expert Rev Mol Diagn 12(7):703–718. https://doi.org/10.1586/erm.12.65

Tigga RA, Das S, Bhattacharya SN, Saha R, Pandhi D, Datt S et al (2018) Burden of chronic dermatophytosis in a tertiary care hospital: interaction of fungal virulence and host immunity. Mycopathologia 183(6):951–959

Tilak R, Raina P, Gupta SK, Tilak V, Praksh P, Gulati AK (2009) Cutaneous zygomycosis: a possible postoperative complication in immunocompetent individuals. Indian J Dermatol Venereol Leprol 75(6):596–599

Training medical mycologists in developing countries - Presidential Oration: 6th Conference of Society for Indian Human and Animal Mycologists (ISHAM) at Hyderabad (19–22 January 2006). s-3-eu-west-1.amazonaws.com/mycology_in_India

Tupaki-Sreepurna A, Abdullah MS, Al-Hatmi KAJ, Murugan S, Hoog GS (2017a) Multidrug resistant fusarium in keratitis: a clinical mycological study of keratitis infections in Chennai, India. Mycoses 60(4):230–233. https://doi.org/10.1111/myc.12578. Epub 2016 Oct 21

Tupaki-Sreepurna A, Jishnu BT, Thanneru V, Sharma S, Gopi A, Sundaram M et al (2017b) An assessment of in vitro antifungal activities of efinaconazole and itraconazole against common non-dermatophyte fungi causing onychomycosis. J Fungi 3(2):20. https://doi.org/10.3390/jof3020020. www.mdpi.com/journal/jof

Tupaki-Sreepurna A, Sharma SNS, Sundaram, Kindo AJ (2018) Phylogenetic diversity and in vitro susceptibility profiles of human pathogenic members of the *Fusarium fujikuroi* species complex isolated from South India. Mycopathologia 183(3):529–540. https://doi.org/10.1007/s11046-0180248-7

Tyagi S, Kaur R, Rawat D (2021) Mycological profile and prevalence of superficial mycoses agents: a study from North India. Int J Med Dent Sci 10(1). https://doi.org/10.18311/ijmds/2021/25828,January

Vaijayanthimala J, Prasad NR, Anandi C, Pugalendi KV (2004) Anti-dermatophytic activity of some Indian medicinal plants. J Nat Remedies 4:26–31

Varthya SB, Thangaraju P, Venketesan S (2020) Curcumin and fungal infection – commonly available herbs for common female infection. J Fam Med Prim Care 9(2). https://doi.org/10.4103/jfmpc.jfmpc_1218_19

Vichitra K, Gopal L, Govindarao B, Kindo AJ (2019) Fungal infection in a post-renal transplant patient with Diaporthe. Indian J Med Microbiol 37(3):454–456

Vijayakumar S, Sahoo B, Singh S, Devi PP, Lilavati N, Roel T (2015) Fungaemia in an immunocompetent infant due to *Candida pelliculosa*. Int J Sci Res 4(1):1718–1719

Vijaykumar R, Rajyoganandh SV, Sudha ER, Kindo AJ (2017) Molecular strain typing of clinical isolates, *Trichophyton rubrum* using non-transcribed spacer (NTS) region as a molecular marker. J Clin Diagn Res 11(5):DCO4–DCO9. https://doi.org/10.7860/jcdr/2017/21994.9843

Viswanathan R, Randhawa HS (1959) *Candida viswanathii* sp. nov. isolated from a case of meningitis. Sci Cult 25:86–87

Vyas A, Pathan N, Sharma R, Vyas L (2013) A clinicomycological study of cutaneous mycoses in Sawai Man Singh hospital of Jaipur, North India. Ann Med Health Sci Res 3(4):593–597. https://doi.org/10.4103/2141-9248.122125

Wankhade AB, RD PPA, Nagarkar NM (2020) Nasofacial rhinoentomophthoramycosis: a report of two cases from Central India. J Oral Maxillofacial Pathol 24(4):124–127

Wattal C, Chakrabarti A, Oberoi J, Peter D, Barnes RA, Sherwal B et al (2017) Issues in antifungal stewardship: an opportunity that should not be lost. J Antimicrob Chemother 72(4):969–974

Yadav P, Singal A, Pandhi D, Das S (2015a) Comparative efficacy of continuous and pulse dose terbinafine regimes in toenail dermatophytosis: a randomized double-blind trial. Indian J Dermatol Venereol Leprol 81(4):363–369

Yadav P, Singal A, Pandhi D, Das S (2015b) Clinico-mycological study of dermatophyte toenail onychomycosis in New Delhi, India. Indian J Dermatol 60(2):153–158

Yoganathan S, Chakrabarty B, Gulati S, Kumar A, Singh M, Xess I (2014) *Candida tropicalis* brain abscess in a neonate: an emerging nosocomial menace. Ann Indian Acad Neurol 17(4):448–450

Fungal Enzymes in Biocontrol of Phytopathogens

12

Manvika Sahgal

Abstract

Soil-dwelling fungi have been excellent biocontrol agents (BCAs). Among all known mechanisms of biocontrol in fungi, mycoparasitism is identified as an effective way to control the growth of phytopathogens in the rhizosphere. Mycoparasitism involves the production of an array of cell wall degrading enzymes by a biocontrol agent. Generally, fungal BCAs produce proteases, glucanases, and chitinases. In recent years, metabolic pathways involved in the production of CWDEs have been elucidated. This helps to understand why some strains control a given pathogen more efficiently than others and why strains stimulate plant defense reactions to varying levels. The present chapter presents the details of the lytic apparatus of fungal BCAs and their role in the biocontrol of soil-borne fungal diseases. *Trichoderma*, *Clonostachys*, *Coniothyrium*, *Verticillium*, *Pythium*, and *Aspergillus* are examples of the most successful fungal biocontrol agents.

Keywords

Soil-borne fungi · Fungal biocontrol agents · Mycoparasitism · Lytic enzymes · *Trichoderma* · Signaling pathways

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12.1 Introduction

Globally phytopathogens, both fungi and bacteria inflict huge losses to crop production and thus represent a major threat to agriculture (Pimentel et al. 2001). The extent of crop loss, type, and intensity of diseases vary according to crop, type of pathogen, prevailing environmental conditions, and control methods. Among various control methods, biological control is an ecologically friendly approach to plant disease protection. Biological control is the use of specific microorganisms that interfere with plant pathogens and pests. Lately, biocontrol agents (BCAs) are becoming popular and have replaced synthetic pesticides (Glare et al. 2012). BCAs suppress plant pathogens through both direct and indirect mechanisms (Fig. 12.1). The direct mechanisms include the production of antibiotics and lytic enzymes, whereas tolerance to stress, solubilization or acquisition of inorganic nutrients, and induction of defense responses through elicitation of induced systemic resistance (ISR) and systemic acquired resistance (SAR) are indirect mechanisms of biological control. SAR is an immune response elicited in plants after primary pathogen infection while ISR upon challenge with fungi and non-pathogenic bacteria (van Loon et al. 1998; Park and Kloepper 2000; Yedidia et al. 1999). During SAR, pathogenesis-related (PR) proteins, for example, chitinases and β-1,3-glucanases, are activated. In recent years, besides chitinases and glucanases, other enzymes, implicated in biological control, have been identified. Moreover, genetic and biochemical bases of biocontrol are not yet fully understood. In this chapter, we detail the lytic apparatus of fungal BCAs and their role in the biocontrol of soil-borne fungal diseases.

12.2 Fungi as Biocontrol Agents

The first commercially available biocontrol agents contained a few species of rhizosphere-competent bacteria and more than ten species of fungi (Chernin and Chet 2001). Both rhizospheric and endophytic fungi, along with mycoparasitic fungi, show the biocontrol potential against fungal phytopathogens. To date, the most successful fungal biocontrol agents include strains of the genera *Clonostachys rosea*, *Coniothyrium minitans*, *Pythium oligandrum* (an oomycete), *Trichoderma*, and *Verticillium biguttatum* (Daguerre et al. 2017). These inhibit the plant pathogens in various ways. *Pythium oligandrum* attacks hyphae, while *Coniothyrium minitans* attacks sclerotia. Sometimes multiple strains attack a single fungal pathogen. For example, *Acremonium alternatum*, *Acrodontium crateriforme*, *Ampelomyces quisqualis*, *Cladosporium oxysporum*, and *Gliocladium virens* parasitize powdery mildew-causing pathogens (Kiss 2003). Similarly, three endophytic fungi *Colletotrichum gloeosporioides*, *Clonostachys rosea*, and *Botryosphaeria ribis* act as biocontrol agents against *Phytophthora* spp. and *Moniliophthora roreri* (frosty pod rot) in *Theobroma cacao* L. (Mejía et al. 2008). Similarly, various mycoparasitic fungi possess biocontrol potential against plant diseases caused by *Fusarium oxysporum* (Thangavelu et al. 2004), *Phytophthora* sp. (Krauss et al. 2001), and *Colletotrichum* sp. (Bankole and Adebanjo 1996). *Talaromyces variabilis*

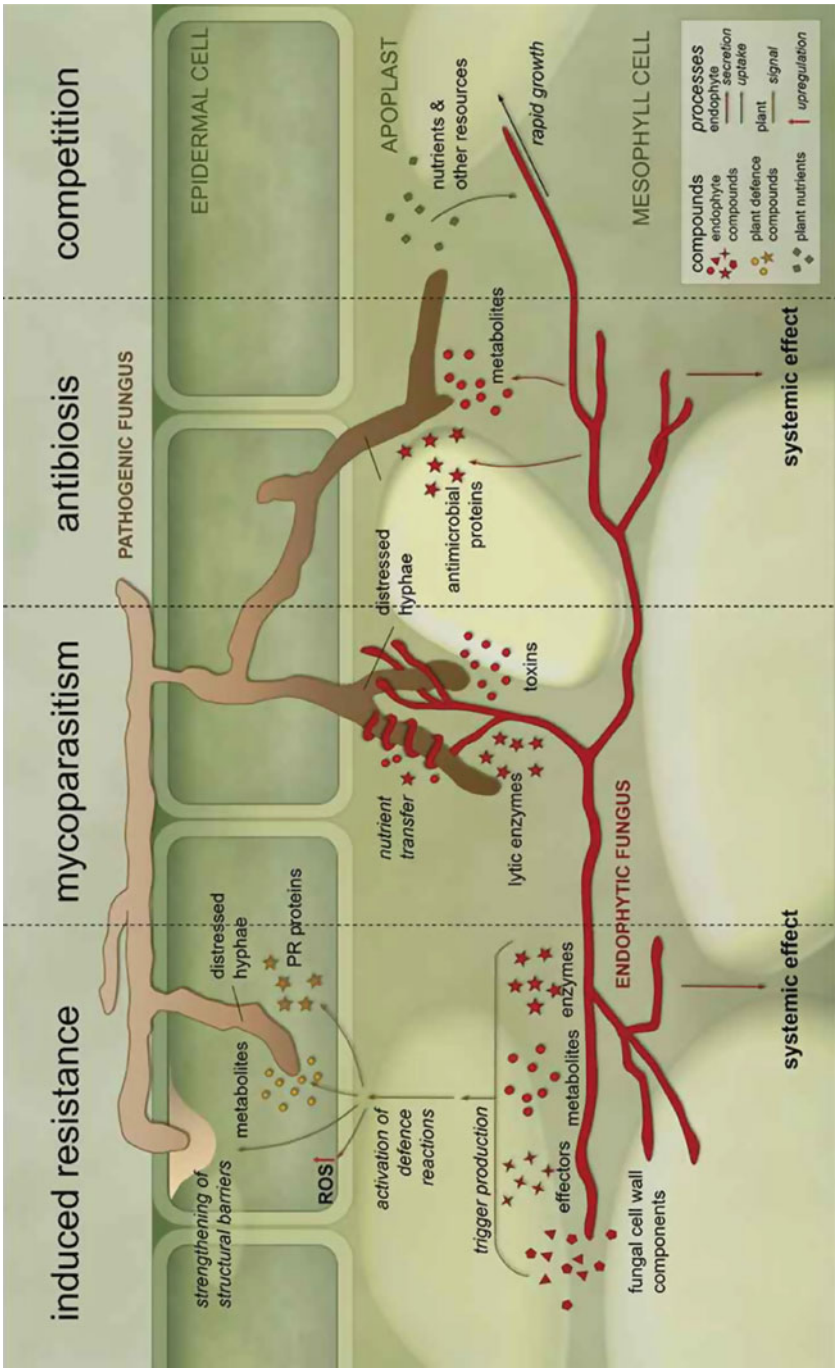

Fig. 12.1 Fungal mechanisms in biological control of phytopathogens. (Adopted from Latz et al. 2018 with permission)

suppresses *Pythium*-induced damping-off of cucumbers and tomatoes through inhibition of mycelial growth and oospore production in *Pythium aphanidermatum*. *T. variabilis* is known to produce glucanase, cellulase, and siderophore (Jain et al. 2018). Hence, the biocontrol efficacy of *Talaromyces* isolates against damping-off disease may be attributed to cellulase production by the isolates. The biocontrol ability of *Clonostachys rosea* f. *catenulate* was attributed to the production of chitinase and β-1,3-glucanase (Chatterton and Punja 2009). The biocontrol potential of *Trichoderma* (de Souza et al. 2018) and *Aspergillus* (Mohapatra et al. 2018; Zhao et al. 2018) is attributed to the production of cellulase. It is the most studied fungal biocontrol agent. The biocontrol activity of mycoparasitic fungi is also due largely to the production of chitinase and chitobiose (Leger et al. 1991; Valadares-Inglis and Peberdy 1997). The well-known examples of entomopathogenic fungal biocontrol agents are *Beauveria* spp., *Metarhizium anisopliae*, and *Verticillium lecanii* (Strasser et al. 2000; Tsujibo et al. 2003).

12.2.1 *Trichoderma* as Biocontrol Agent

The genus *Trichoderma* includes common filamentous fungi. The fungus shows pleomorphism and exists in two stages, sexual and asexual. Both the stages are morphologically and physiologically different. The sexual (or teleomorphic) stage is known as *Hypocrea*, and the asexual (or anamorphic) stage is called *Trichoderma*. The fungus interacts with other fungi and higher organisms (animals and plants) in varied lifestyles. The species of *Hypocrea/Trichoderma* growing within sclerotia of various plant pathogenic fungi such as *Macrophomina phaseolina*, *Sclerotinia* spp., *Typhula* spp., and *Verticillium dahliae* are used as plant disease biocontrol agents as they can antagonize plant pathogenic fungi and stimulate plant growth and defense responses. *Trichoderma* species mainly *T. harzianum*, *T. viride*, *T. asperellum*, and *T. asperelloides* are excellent biocontrol agents (Leger et al. 1991; Kang et al. 1999). The *Trichoderma* strains possess multiple mechanisms of biocontrol that include antibiosis, competition for rhizosphere colonization, and nutrients in the rhizosphere, necrotrophic hyperparasitism and mycoparasitism, induction of systemic resistance in plants, and secretion of toxigenic secondary metabolites and peptides with fungicidal action (Harman et al. 2004; Chet and Inbar 1994; Gruber et al. 2012; Ruiz et al. 2007; Dang et al. 2010; López-Mondéjar et al. 2011*). Trichoderma* is also known to inactivate the pathogenicity-related proteins in pathogens (Saravanakumar et al. 2016). The biocontrol potential of *Trichoderma* was first identified in 1932 when *Trichoderma lignorum* (present name *T. viride*) was observed to parasitize the hyphae of *Rhizoctonia solani* (Weindling 1932). Since then biocontrol potential of *Trichoderma* has been reported/validated in several studies. Plant root colonization of biocontrol agent is the key to effective biocontrol of phytopathogens. The protein elicitor *TVHYDII2* is reportedly involved in plant root colonization of *Trichoderma viride* and increased its antagonistic activity against the pathogen (Guzmán-Guzmán et al. 2017). The inoculation with *Trichoderma* spores reduced the damping-off in citrus seedlings

(Lo 1997). *Trichoderma asperellum* is reported as an effective biocontrol agent of *Phytophthora megakarya*, *P. capsici*, *P. citrophthora*, and *P. palmivora* (Tondje et al. 2007). The mycoparasitism and ISR are major mechanisms of biocontrol by *Trichoderma* strains and elicited through the production of lytic enzymes. The major hydrolytic enzymes produced by *Trichoderma* sp. are chitinase and β-1,3-glucanases (Saravanakumar et al. 2016b). For example, the biocontrol activity of *T. harzianum* strain CCTCC-RW0024 against *Fusarium graminearum*, the causal agent of maize stalk rot, depended on the combined action of CWDEs. This was evident by a positive correlation between the antagonistic activity and chitinase (19%) and β-1,3-glucanase activities (50%), in *Trichoderma* strains (Saravanakumar et al. 2017). The microscopic examination of *Trichoderma* spp. displaying antagonism against *F. oxysporum* revealed the formation of appressoria structures and their coiling around the pathogen hyphae, thereby confirming mycoparasitism as the biocontrol mechanism (Ghanbarzadeh et al. 2014). Similarly, *T. harzianum* parasitized hyphae of *Phytophthora capsici* and *Rhizoctonia solani* first by coiling around and then degrading the cell wall through secretion of CWDEs chitinase, glucanase, and protease (Alamri et al. 2012). Similarly, the principal mechanism of antagonism in *Trichoderma asperellum* is also mycoparasitism (Samuels et al. 2010). *T. asperellum* is antagonistic against *Pythium aphanidermatum* (Kipngeno et al. 2015), *F. oxysporum* f. sp. *lycopersici*, and *A. alternata*, and the mechanism of action is mycoparasitism (Galarza et al. 2015). Besides, *T. asperellum* is also antagonistic against *Rhizoctonia solani* causing damping-off of beans, *Sclerotinia sclerotiorum* causing white mold disease in soybean plants (Sumida et al. 2018), and *Sclerotium rolfsii* (Sacc.) causing stem rot in groundnut (Doley et al. 2014). *T. asperellum* reduced the incidence of the *F. oxysporum* disease by more than 85% both in in vitro and in vivo conditions (Patel and Saraf 2017). It is also reported that *Trichoderma* sp. in combination with other bacterial biocontrol agents reduce the various fungal plant diseases. For example, co-inoculation of *T. asperelloides* and *Bacillus paralicheniformis* effectively reduced the infection of *Alternaria alternata* and *Fusarium oxysporum* in tomato plants. The principal mechanism of biocontrol, in this case, was mycoparasitism (Ramírez-Carinõ et al. 2020). The enzymes, endochitinases, and chitobiosidases from *T. harzianum* have been reported to show antifungal activity against various fungal pathogens including *Botrytis cinerea*, *Fusarium solani*, *Fusarium graminearum*, *Pythium ultimum*, *Saccharomyces cerevisiae*, *Ustilago avenae*, and *Uncinula necator* (Lorito et al. 1993). Besides, *Trichoderma* strains *Aspergillus niger* is reported to be antagonistic against plant pathogenic strains of *Fusarium solani*, *F. culmorum*, and *R. solani* with chitinolytic ability as the major mechanism of biological control (Brzezinska and Jankiewicz 2012).

12.3 Mycoparasitism

In mycoparasitism, fungi acting as BCA produce cell wall degrading enzymes (CWDEs) such as chitinases, glucanases, and proteases to inhibit/kill pathogenic fungi. So far, among antifungal CWDEs, chitinases and β-1,3-glucanases are the most studied for biocontrol action (Selitrennikoff 2001). This is because chitin and β-1,3-glucans are the predominant structural components of fungal cell walls (Latgé 2007), whereas β-1,6-glucan is a comparatively minor component and cross-links cell wall proteins to the β-1,3-glucan/chitin layer. However, β-1,6-glucan is essential for the construction of rigid fungal cell walls in *Candida albicans*, *Cryptococcus neoformans*, and *Colletotrichum graminicola* (Umeyama et al. 2006; Gilbert et al. 2010; Oliveira-Garcia and Deising 2016). Accordingly, β-1,6-glucanases may contribute to the efficient disorganization and further degradation of fungal cell walls during predation. The antifungal properties of β-1,6-glucanases were confirmed through a study where knockout of a gene, Tvbgn3 which encodes for β-1,6-glucanase, decreased biocontrol efficiency of *Trichoderma virens* (Djonović et al. 2006).

12.4 Major Fungal Enzymes in Biocontrol

Soil-dwelling fungi destroy or inhibit phytopathogens by secreting lytic enzymes. The lytic enzymes are generally hydrolases which can degrade cell walls of plant pathogens and generally known as CWDEs. Several studies confirm the biocontrol activity of CWDEs against various plant pathogenic fungi and bacteria (Table 12.1). Investigation of some fungal enzymes revealed that besides degrading cell wall of plant pathogens, they can inhibit or modify cell wall synthesis and perforate cell membrane (Roberti et al. 2002; Mota et al. 2017; Mishra et al. 2020). The following section details the role and mechanisms of fungal enzymes in biocontrol.

12.4.1 Proteases

Proteases are also known as peptidases or proteinases. They cleave the peptide bond present between the amino acid residues in a polypeptide chain. The proteases can be classified into two major types—(1) endopeptidases and (2) exopeptidases—based on catalytic reaction, chemical nature of the catalytic site, and evolutionary relationships. The endopeptidases cleave amino acids internally, whereas exopeptidases cleave amino acids either from the amino-terminal or carboxy-terminal of the protein. Exopeptidases that remove amino acids from amino-terminal are known as aminopeptidases and those removing amino acids from carboxy-terminal as carboxypeptidases. Based on the architecture of a catalytic site, the following proteases are categorized: aspartic proteases (EC 3.4.23), cysteine proteases (EC 3.4.22), metalloproteases (EC 3.4.24), serine proteases (EC 3.4.21), and threonine proteases (EC 3.4.25) (Garcia-Carreon 1997; Clark and Pazdernik

Table 12.1 Fungal enzymes with biocontrol potential against various phytopathogens (modified from Daguerre et al. 2017)

S. no.	Name of the enzyme(s)	Producing microbes	Phytopathogen	Reference
1	Chitinase	*Trichoderma asperellum*	*Rigidoporus microporus*	Sakpetch et al. (2018)
2	Chitinase	*Trichoderma harzianum Rifai T24*	*S. rolfsii*	El-Katatny et al. (2001)
3	Cellulase	*Trichoderma longibrachiatum*	*Pythium ultimum*	Migheli et al. (1998)
4	Cellulase	*Trichoderma harzianum*	*P. ultimum*	Thrane et al. (1997)
5	α-1,3-Glucanases	*T. harzianum*	*Aspergillus niger*, *Botrytis cinerea*, *C. acutatum*, *F. oxysporum*, *Penicillium aurantiogriseum*, and *R. solani*	Ait-Lahsen et al. (2001)
6	α-1,3-Glucanases	*T. harzianum*	*P. ultimum*	Thrane et al. (1997)
7	Aspartic protease	*T. harzianum*	*Aspergillus fumigatus*, *Aspergillus flavus*, *B. cinerea*, *Candida albicans*, *Mucor circinelloides*, and *R. solani*	Deng et al. (2018)
8	Protease	*T. harzianum*	*M. phaseolina* and *R. solani*	El-Bendary et al. (2016)
9	Chitinase, glucanase, and protease	*T. harzianum*	*Phytophthora cactorum* and *Rhizoctonia solani*	Schirmböck et al. (1994)

2016). Among these, serine proteases are enzymes that cleave peptide bonds in proteins with serine at the (enzyme's) active site. They are low molecular weight (18–35 kDa) proteins usually active at neutral to alkaline pH, with optima between pH 7.0 and 11.0 (Gupta et al. 2002), and found ubiquitously in eukaryotes and prokaryotes.

According to the MEROPS peptidase database, serine proteases are classified into 13 clans and 40 families. Of these, clan SB (subtilases) plays a significant role in biocontrol agents. Within the clan, SB two families, subtilisin-like proteases (S8) and serine–carboxyl proteases (S53), were identified (Rawlings et al. 2016). The S8 proteases possess an Asp-His-Ser catalytic triad where the Ser residue is essential for activity (Ekici et al. 2008). The S8 family is further grouped into two subfamilies, namely, S8A and S8B. The majority of the characterized subtilisin-like proteases are placed in the S8A subfamily (Muszewska et al. 2011). For example, proteinase K produced by the fungus, *the Engyodontium album*, is placed within the S8A subfamily (Gunkel and Gassen 1989).

Several reports highlight the role of proteases in biocontrol of phytopathogenic fungi. Subtilisin-like serine proteases have been reported in entomopathogenic, mycoparasitic, and nematophagous fungi. The examples of entomopathogenic fungi which produce subtilisin-like proteases are *Beauveria bassiana* (Xiao et al. 2012), *Cordyceps militaris* (Zheng et al. 2011), and *Metarhizium* spp. (Gao et al. 2011). The nematode-parasitizing fungi showing production of subtilisin-like proteases are *Arthrobotrys oligospora* (Yang et al. 2011a, b), *Drechslerella stenobrocha* (Liu et al. 2014), *Hirsutella minnesotensis* (Lai et al. 2014), *Monacrosporium haptotylum* (Meerupati et al. 2013), *Pochonia chlamydosporia* (Larriba et al. 2014), and *Purpureocillium lilacinum* (Prasad et al. 2015). Similarly a few examples of mycoparasitic fungi showing production of subtilisin-like proteases are *Trichoderma atroviride*, *T. longibrachiatum*, *T. reesei* (Xie et al. 2014), and *T. virens* (Kubicek et al. 2011).

Serine proteases exhibit biological control of different phytopathogens through various mechanisms. The serine proteases SprT from *Trichoderma longibrachiatum* (Chen et al. 2009) and PRA1 from *T.* cf. *harzianum* reduced hatching of eggs in *Meloidogyne incognita* (Suarez et al. 2004), while ThSS45, a serine protease from *T.* cf. *harzianum*, inhibited the growth of *Alternaria alternata* (Fan et al. 2014). There are reports that overexpression of serine protease Prb1 in *T. confer* (cf.) *harzianum* (Flores et al. 1997) and Tvsp1 in *T. virens* (Pozo et al. 2004) improved the biocontrol ability of respective *Trichoderma* strains against the *Rhizoctonia solani infection in cotton seedlings*. Serine proteases from nematode-parasitizing fungi, like *A. oligospora*, *Dactylella shizishanna*, *H. rhossiliensis*, *Lecanicillium psalliotae*, and *P. lilacinus*, play a significant role in controlling nematode infections (Li et al. 2010; Minglian et al. 2004). Similarly proteases of entomopathogenic fungi control insect population (Leger et al. 1992). The extracellular proteases of entomopathogenic and nematode-parasitizing fungi are widely used as potential bioagents for preventing crop loss due to insect attack as they hydrolyze proteinaceous insect cuticle. Proteases with nematicidal activity have been reported in some strains of *Trichoderma*, *Monacrosporium microscaphoides*, and *Arthrobotrys oligospora* (Tunlid et al. 1992; Suárez et al. 2005; Wang et al. 2006). Transcriptome studies revealed that during infection of nematodes, PrC, a serine protease, was upregulated (Zou et al. 2010). The biocontrol action of *Clonostachys rosea* against *Helminthosporium solani*, the silver scurf pathogen of potato, was attributed to the production of serine proteases. During the parasitism of *Helminthosporium solani*, several serine protease genes from *C. rosea* are upregulated (Lysøe et al. 2017). The serine protease belonging to families S8A, S9X, and S33 is important for mycoparasitic lifestyle. They are expressed in aggressive mycoparasitic species like *T. virens* or *T. atroviride*. The antagonistic action of *Trichoderma* strains against *Fusarium* sp., *Colletotrichum* sp., *Gloeocercospora* sp., and *Botrytis* sp. (Elad and Kapat 1999; Schirmböck et al. 1994; Jayalakshmi et al. 2009; Sharma et al. 2016) has been attributed to production of extracellular proteases. The alkaline and serine proteases from *T. harzianum* and *T. virens* have been found to be effective against phytopathogen *R. solani* (Benítez et al. 1998; Pozo et al. 2004). In last few years, recombinant proteases with enhanced antifungal activity have also been investigated

against *Penicillium expansum*, *B. cinerea*, *Monilinia fructicola*, and *A. alternata* (Banani et al. 2014; Fan et al. 2014).

12.4.2 Glucanases

Glucanases are enzymes that break down a glucan by hydrolysis of glycosidic bonds and, hence, are also known as hydrolases. They are widespread in bacteria, fungi, and higher plants (Simmons 1994). Since glucan is a major constituent of fungal cell wall, glucanases degrade their cell wall (Zhongcun et al. 2004) and penetrate into the host mycelium (Fridlender et al. 1993).

12.4.2.1 β-Glucanases

The fungi produce both endo- and exo-β-glucanases. However, exocellular non-cellulolytic β-(1,3)-, β-(1,6)-, and β-(1,4)-glucanases are prominently produced. A few fungi also produce β-(1,2)-glucanases. The non-cellulolytic exocellular β-(1,3)- and β-(1,6)-glucanases degrade β-(1,3)- and β-(1,6)-glucans, respectively. The β-1,3-glucanases are of two types: (1) exo-β-1,3-glucanases which act randomly inside a glucan chain and (2) endo-β-1,3-glucanases which release glucose residues from the non-reducing end. β-1,3-Glucanases hydrolyze the O-glycosidic linkages of β-glucan chains by two mechanisms: (1) hydrolysis of the substrate by sequentially cleaving glucose residues from the non-reducing end and (2) cleaving linkages at random sites along the polysaccharide chain, releasing smaller oligosaccharides (Fig. 12.2) (Noronha and Ulhoa 1996). Exo-β-1,3-glucanases (EC 3.2.1.58) hydrolyze a substrate by first mechanism, whereas endo-β-1,3-glucanases (EC 3.2.1.39) cleave the substrate by second mechanism. In most of the fungal species, multiple β-glucanases rather than a single enzyme have been identified (Simmons 1994; Stahmann et al. 1993).

Fungi accomplish the degradation of glucan by the synergistic action of both endo- and exo-β-glucanases (Pitson et al. 1993; Simmons 1994). Various studies have elucidated the biocontrol of phytopathogens through the action of glucanases. *T. harzianum* produces four β-1,3-glucanases distinguishable on the basis of differences in molecular weight and isoelectric point. However, only one gene (*bgn13.1*) has been cloned from *T. harzianum*. Expression of this gene might be induced by fungal cell walls and mycelia (de la Cruz et al. 1995a, b). Several research studies have highlighted that secretion of β-1,3-glucanase by *Trichoderma* species is also one of the several mechanisms of biocontrol. The β-1,3-glucanases of *Trichoderma* can degrade glucan chain in the cell wall of various fungal pathogens such as *B. cinerea*, *Fusarium* sp., and *R. solani* (Monteiro and Ulhoa 2006). The production of β-1,3-glucanase by *T. harzianum* was implicated in the biocontrol of *S. rolfsii* (El-Katatny et al. 2001). However, antagonistic action of *T. asperellum* against *F. graminearum* maize stalk rot pathogen was due to combined action of glucanases, chitinases, and proteases produced by *T. asperellum* (Li et al. 2016). Similarly, effective management of *F. oxysporum* by *T. cerinum* was attributed to the lytic enzymes β-1,3-glucanases and chitinase along with peptaibols (Khare et al.

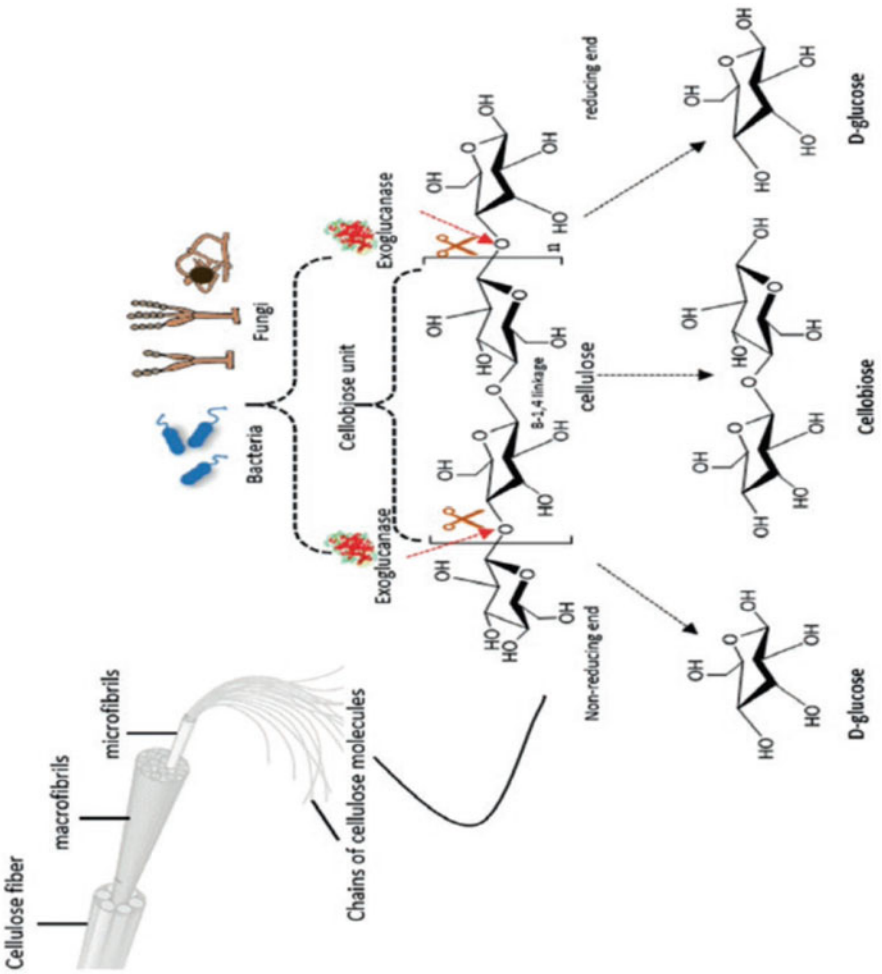

Fig. 12.2 Mechanisms of glucanases that lead to biological control during mycoparasitism. (Source: Mishra et al. 2020)

2018). In another study, β-1,3-glucanases and chitinase of *T. spirale* were involved in the biocontrol of *Corynespora cassiicola* or *Curvularia aeria*, a causative agent of leaf spot disease in lettuce (Baiyee et al. 2019).

12.4.2.2 Biocontrol Action of Glucanase

Glucan is the cell wall polysaccharide, made predominantly of β-1,3-linked backbone linked via β-1,6-linkages, and is an important component of fungal and yeast cell wall. Glucanase hydrolyzes the glucan in the cell wall of yeasts and fungi. Glucanases have exo- and endo-hydrolase activity as well as glucosyltransferase

activities. The exo-hydrolases hydrolyze the β-glucan chain by sequential cleavage of glucose residues from the non-reducing end and releasing glucose as the sole product of hydrolysis. Sometimes, gentiobiose is also generated if the internal β-(1,3)-glucosidic linkages adjacent to the β-(1,6)-glucosyl residues in branched β-(1,3), β-(1,6) glucans are cleaved, for example, in *Acremonium persicinum*. Endo-hydrolases randomly cleave β-linkages all along the polysaccharide chain, releasing smaller oligosaccharides. Endo-hydrolases are of two types depending on the type of products generated. One group generates oligosaccharides, and the other produces glucose and disaccharides (Khalikova et al. 2005). Substrate specificity data and inhibition studies suggest that the exocellular β-glucanases from yeasts are better considered as β-glucosidase. Glucanases may attack by multichain single attack strategy, as with the wall-associated exo-β-(1,3)-glucanases in *Aspergillus fumigatus*, where another glucan chain is attacked soon after the initial glucan chain is cleaved. Alternatively, these enzymes may cleave a single glucan chain several times before being freed from their substrates for further attack.

12.4.3 Chitinases

Chitin is the second most abundant organic compound next to cellulose. Chemically it is a poly-β-1,4-*N*-acetylglucosamine (GlcNAc). Chitin is the major constituent of exoskeletons, tendons, and linings of respiratory, excretory, and digestive systems of arthropods and cell walls of most fungi (Clark and Smith 1936). Chitinases are enzymes that hydrolyze chitin. The enzymes, chitinases, have been divided into two types, endochitinases and exochitinases, based on their mode of action. Endochitinases (EC 3.2.1.14) randomly cleave internal points over the entire length of chitin polymer and produce dimer (diacetylchitobiose) and multimeric compounds such as chitotriose and chitotetraose (Fig. 12.3). Exochitinases are further subclassified into (1) chitobiosidases (EC 3.2.1.29) which cleave non-reducing ends of chitin and produce diacetylchitobiose and (2) β-l,4-glucosaminidases (EC 3.2.1.30) which cleave oligomers obtained through action of endochitinases into monomers of *N*-acetylglucosamine (Sahai and Manocha 1993). Among chitinases, endochitinases specifically show the antifungal activity (EC 3.2.1.14). Endochitinases are expressed in all organisms including higher plants, animals, and microbes for different purposes such as nutrition, morphogenesis, and defense against chitin-containing pathogens (Hamid et al. 2013; Bhattacharya et al. 2007; Henrissat and Davies 1997). In plants, chitinases are produced as defense mechanism against invading pathogens. In plants, chitinases act synergistically with β(1→3)-glucanases. Chitinases have been discovered recently in bacteria (viz., *Streptomycetes* and *Bacilli*) and fungi (Singh et al. 2009). Bacterial chitinases assist in the breakdown and assimilation of fungal cell walls, whereas fungal chitinases participate in morphogenesis of fungal cell wall. Extracellular chitinases along with β-glucanases attack and degrade hyphae in mycoparasitic fungi, such as *Trichoderma harzianum*, *Aphanocladium album*, and *Gliocladium virens*.

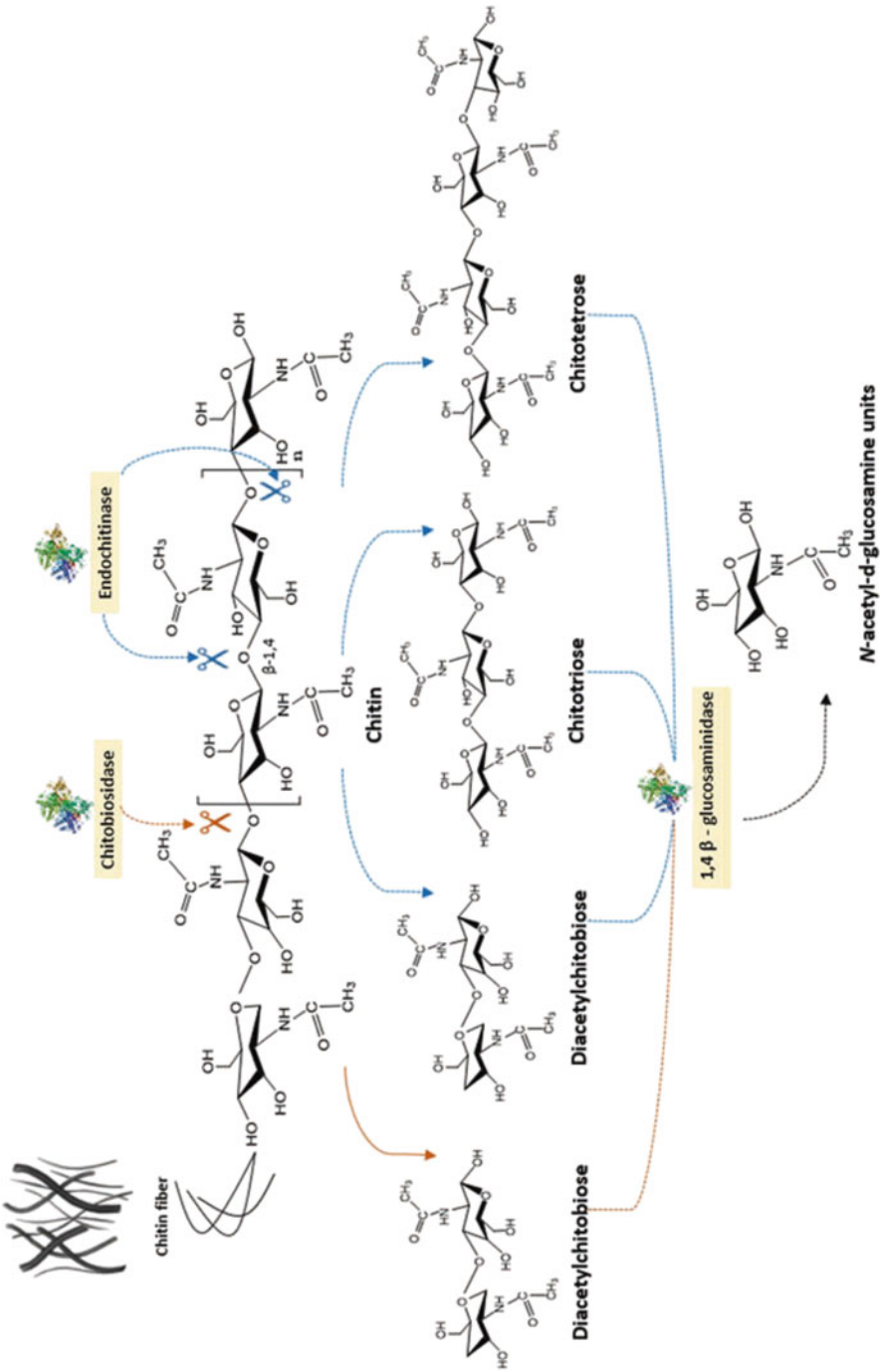

Fig. 12.3 Mechanism of action of exo- and endo-chitinases during biological control of phytopathogens. (Source: Mishra et al. 2020)

The chitinolytic enzymes are placed into glycosyl hydrolase (GH) families numbering 18, 19, and 20 based on sequence similarity of constituent amino acids (Henrissat 1991). In addition, the chitinases have further been grouped into six different classes on the basis of various characteristics. The characteristics considered for grouping of enzymes are N-terminal sequence, enzyme localization, isoelectric pH, signal peptides, and inducers (Iseli et al. 1996). Family 18 contains class III and V chitinases, whereas family 19 includes class I, II, and IV chitinases. Family 19 primarily comprises of plant chitinases. Family 20 consists of chitinolytic enzymes *N*-acetylglucosaminidase (EC 3.2.1.30) from *Vibrio harveyi* and *N*-acetylhexosaminidases (EC 3.2.1.52) from *Dictyostelium discoideum* (Patil et al. 2000). The plant, yeast, and bacterial and fungal chitinases differ in their molecular structure. Chitinases from plant, yeast, fungi, and bacteria consist of one, four, five, and three domains, respectively. Plant chitinases possess a single catalytic domain, whereas chitinase from yeast contains four domains, namely, a signal sequence, a catalytic domain, a serine/threonine rich region, and a C-terminal chitin-binding domain. The fungal chitinases are comprised of five different domains: (a) N-terminal signal peptide region, (b) catalytic domain, (c) chitin-binding domain, (d) serine/threonine-rich region, and (e) C-terminal extension region. In contrast, bacterial chitinases consist of three domains: a catalytic domain, a chitin-binding domain, and a fibronectin III domain. All the chitinases possess specific affinity towards polymer chitin. Hence, degradation of chitin by chitinases is highly specific.

12.5 Regulation of Mycoparasitism

The mycoparasitism is outcome of an antagonistic interaction between a biocontrol fungus and a target phytopathogen. This interaction involves two steps: (1) recognition of target pathogen by antagonistic fungus and (2) signaling pathways leading to gene expression and destruction of the pathogenic fungus, although the recognition of host by a fungi biocontrol agent and subsequent signaling cascade that results in an effective antagonistic response is not fully understood as yet. However, a few studies using *Trichoderma* species as a model fungal BCA have been undertaken (Table 12.2).

12.5.1 Host Recognition and Signaling Pathways

In *Trichoderma* species, adenylate cyclase G proteins and G protein-coupled receptors are important for secretion and production of CWDEs, as well as formation of infection structures. For example, silencing of the gene Gpr1, encoding seven transmembrane receptors, prevented the adhesion of *Trichoderma* hyphae to the surface of *Rhizoctonia solani* and also the induction of protease (prb1) and chitinase genes (nag1 and ech42) (Omann et al. 2012). Similarly, Tga1 and Tga3, the two G protein α subunits, are identified as critical to mycoparasitic activity of *T. atroviride*. Δtga1 mutants of *T. atroviride* lost mycoparasitic activity against *Botrytis cinerea*,

Table 12.2 Fungal enzymes and genes associated with mycoparasitism (modified from Daguerre et al. 2017)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
Endochitinases (GH 18)				
Chitinase	*chit32*, *chit41*	*Talaromyces flavus*	*Alternaria alternata*, *Fusarium moniliforme*, *Magnaporthe grisea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*	Duo-Chuan et al. (2005)
Chitinase	*chi1*	*Aphanocladium album*	*Rust fungi*	Kunz et al. (1992)
Chitinase	*chit42*	*Trichoderma atroviride*	*Alternaria brassicicola*, *Botrytis cinerea*, *Fusarium graminearum*, *F. oxysporum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Verticillium dahliae*	Kowsari et al. (2014)
	chiA5, *chiA6*	*Clonostachys rosea*	*Botrytis cinerea*, *Rhizoctonia solani*	Tzelepis et al. (2015)
Endochitinase	*ech30*	*Trichoderma atroviride*	*Botrytis cinerea*	Klemsdal et al. (2004)
Endochitinases	*chit33 (ech33)*	*Trichoderma atroviride*, *Trichoderma harzianum*	*Rhizoctonia solani*, *Fusarium solani*, *Sclerotinia sclerotiorum*	Dana et al. (2001), de la Cruz et al. (1992), Matroudi et al. (2008), Troian et al. (2014)
	Tv-cht1, *Tv-cht2*	*Trichoderma virens*	*Rhizoctonia solani*	Kim et al. (2002)
Endochitinases	*chit36*	*Trichoderma harzianum*	*Botrytis cinerea*, *Fusarium oxysporum* f. sp. *melonis*, *Sclerotium rolfsii*	Viterbo et al. (2001)

(continued)

Table 12.2 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
	chit36Y	*Trichoderma asperellum*	*Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *melonis*, *Rhizoctonia solani*	Viterbo et al. (2002)
Endochitinases	*cr-ech37*, *cr-ech42*	*Clonostachys rosea*	*Alternaria radicina*, *Botrytis cinerea*, *Fusarium culmorum*	Mamarabadi et al. (2008a, b)
Endochitinases	*chit42*	*Trichoderma atroviride*, *Trichoderma hamatum*	*Botrytis cinerea*, *Penicillium digitatum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*	Deng et al. (2007), Kullnig et al. (2000), Mach et al. (1999), Pérez-Martínez et al. (2007), Steyaert et al. (2004)
	chit42 (ech42)	*Trichoderma harzianum*	*Botrytis cinerea*, *Gibberella fujikuroi*, *Fusarium solani*, *Rhizoctonia solani*	Vieira et al. (2013)
	echi42	*Trichoderma asperellum*	*Rhizoctonia solani*, *Sclerotinia sclerotiorum*	Liu et al. (2010)
	Tv-ech1, *Tv-ech2*	*Trichoderma virens*	*Rhizoctonia solani*	Kim et al. (2002), Baek et al. (1999)
Endochitinase	*sechi44*	*Stachybotrys elegans*	*Rhizoctonia solani*	Morissette et al. (2003)
Endochitinase	*chi46*	*Chaetomium cupreum*, *C. globosum*, *Trichoderma asperellum*, *T. reesei*	*Fusarium oxysporum*, *Phytophthora sojae*, *Sclerotium rolfsii*, *S. sclerotiorum*, *S. tritici*, *Rhizoctonia solani*, *Valsa sordida*	Zhang et al. (2009)

(continued)

Table 12.2 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
Endochitinase	*cr-ech58*	*Clonostachys rosea*	*Alternaria radicina*, *Fusarium culmorum*	Mamarabadi et al. (2008a)
Endochitinases	*crchi1*	*Clonostachys rosea*, *Trichoderma harzianum*	*Botrytis cinerea*, *Rhizoctonia solani*	Gan et al. (2007), Limón et al. (1995)
	trchi1	*Trichothecium roseum*	*Alternaria alternata*, *Cercospora nicotianae*	Xian et al. (2012)
Glucosaminidases (GH 20)				
N-acetyl-β-D-glucosaminidases	*cr-nag1*	*Clonostachys rosea* (*G. roseum*)	*Botrytis cinerea*, *Fusarium culmorum*	Mamarabadi (2007)
	eng18B	*Trichoderma atroviride*	*Botrytis cinerea*, *Rhizoctonia solani*	Dubey et al. (2012)
	exc1Y	*Trichoderma asperellum*	*Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *melonis*, *Rhizoctonia solani*	Viterbo et al. (2002)
	nag1	*Trichoderma atroviride*, *Trichoderma harzianum*	*Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*	Brunner et al. (2003)
	nag68	*Stachybotrys elegans*	*Rhizoctonia solani*	Taylor et al. (2002)
	Tvnag1, *Tvnag2*	*Trichoderma virens*	*Rhizoctonia solani*	Kim et al. (2002)
Glucanases				
α-1,3-Glucanase	*agn13.1*	*Trichoderma harzianum*	*Aspergillus niger*, *Botrytis cinerea*, *Colletotrichum acutatum*, *Fusarium oxysporum*, *Penicillium aurantiogriseum*	Ait-Lahsen et al. (2001)

(continued)

Table 12.2 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
	agn13.2	*Trichoderma asperellum*	*Botrytis cinerea*	Sanz et al. (2005)
	a13gluc	*Trichoderma harzianum*	*Sclerotinia sclerotiorum*	Troian et al. (2014)
β-1,3-Glucanases	*cmg1*	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	Giczey et al. (2001)
	exgA	*Ampelomyces quisqualis*	*Sphaerotheca fusca*	Rotem et al. (1999)
	glu1	*Clonostachys rosea* f. *catenulata*	*Fusarium oxysporum* f. sp. *radicis-cucumerinum*	Chatterton and Punja (2009)
	gluc78	*Trichoderma atroviride*	*Phytophthora* sp., *Pythium* sp.	Donzelli et al. (2001)
	lam1.3	*Trichoderma harzianum*	*Rhizoctonia solani*, *Sclerotium rolfsii*	Cohen-Kupiec et al. (1999)
	tag83	*Trichoderma asperellum*	*Rhizoctonia solani*	Bara et al. (2003), Marcello et al. (2010)
78 kDa β-1,3-glucanase	*bgn13.1*	*Trichoderma harzianum*	*Botrytis cinerea*, *Gibberella fujikuroi*, *Phytophthora citrophthora*, *Rhizoctonia solani*	de la Cruz et al. (1995a, b)
β-1,3-Glucanase	*Tvbgn1*, *Tvbgn2*	*Trichoderma virens*	*Pythium oligandrum*, *Rhizoctonia oryzae*, *R. solani*	Kim et al. (2002)
Endoglucanases	*cel12B*, *cel12D*	*Clonostachys rosea*	*Botrytis cinerea*	Mamarabadi et al. (2008a)
Exo-β-1,3-Glucanase	*PAEXG1*, *PAEXG2*	*Pichia anomala*	*Botrytis cinerea*	Friel et al. (2007)
β-1,6-glucanase	*bgn16.2*	*Trichoderma harzianum*	*Botrytis cinerea*, *Rhizoctonia solani*	de la Cruz et al. (1995a, b)
	b16gluc	*Trichoderma harzianum*	*Sclerotinia sclerotiorum*	Troian et al. (2014)
	Tvbgn3	*Trichoderma virens*	*Pythium oligandrum*, *Rhizoctonia oryzae*, *R. solani*	Djonović et al. (2007)

(continued)

Table 12.2 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
Proteases				
Aspartic proteases	*P6281*	*Trichoderma harzianum*	*Botrytis cinerea*, *Pythium ultimum*, *Rhizoctonia solani*	Suárez et al. (2005)
	Sa76	*Trichoderma harzianum*	*Fusarium oxysporum*, *Phytophthora sojae*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Valsa sordida*	Liu and Yang (2007)
	TaAsp	*Trichoderma asperellum*	*Alternaria alternata*, *Cytospora chrysosperma*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*	Yang et al. (2013)
	ASP55	*Trichoderma asperellum*	*Alternaria alternata*	Dou et al. (2014)
	PAPA	*Trichoderma asperellum*	*Rhizoctonia solani*	Viterbo et al. (2004)
Serine proteases	*prb1*	*Trichoderma harzianum*	*Rhizoctonia solani*, *Sclerotium rolfsii*, *Sclerotinia sclerotiorum*	Steyaert et al. (2004)
	Spm1	*Trichoderma asperellum*	*Rhizoctonia solani*, *Sclerotinia sclerotiorum*	Liu et al. (2010)
	SL41	*Trichoderma harzianum*	*Fusarium oxysporum*, *Phytophthora sojae*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Valsa sordida*	Liu and Yang (2013)

(continued)

Table 12.2 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
	SS10	*Trichoderma harzianum*	*Alternaria alternata*, *Cytospora chrysosperma*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*	Yan and Qian (2009)
	ThSS45	*Trichoderma harzianum*	*Alternaria alternata*	Fan et al. (2014)
	tvsp1	*Trichoderma virens*	*Rhizoctonia solani*	Pozo et al. (2004)
Trypsin-like protease	*pra1*	*Trichoderma harzianum*	*Sclerotinia sclerotiorum*	Troian et al. (2014)

Rhizoctonia solani, and *Sclerotinia sclerotiorum* (Rocha-Ramírez et al. 2002), and Δtga3 mutant was unable to mycoparasitize *Rhizoctonia solani* and *Botrytis cinerea* (Zeilinger et al. 2005). The signaling pathways are host-specific. For example, Δtga4 mutant of *T. virens* exhibited reduced ability to antagonize *Sclerotium rolfsii* whereas showed normal antagonism against *Rhizoctonia solani* (Mukherjee et al. 2004).

Besides G proteins and coupled receptors, several mitogen-activated protein kinases (MAPK) implicated in fungal mycoparasitism have been identified in *Trichoderma* species. The Δtvk1 mutants of *T. virens* displayed increased lytic enzyme secretion and were considerably more effective in disease control than wild-type strains (Mendoza-Mendoza et al. 2003). Similarly ΔtmkA mutant of *T. virens* completely antagonized *Rhizoctonia solani*, however partially antagonized or failed to parasitize *Sclerotium rolfsii* (Mukherjee et al. 2003). In contrast, *T. atroviride* Δtmk1 mutants exhibited reduced mycoparasitic activity against *R. solani* and *B. cinerea*. The overexpression of hog1 strongly affected the antagonistic activity of *Trichoderma harzianum* against *Phoma betae* and *Colletotrichum*. Hence it is evident that MAPKs and G proteins play crucial role in fungus–fungus interaction. However, their overlapping roles and host specificities are obvious. Therefore, further characterization is required to fully understand the complexity of the signaling pathway related to biocontrol of specific fungal pathogen. Adenylate cyclase is associated with the synthesis of cAMP from ATP. The α subunit of heterotrimeric G protein regulates the activity of adenylate cyclase in fungi. In *Trichoderma atroviride*, α subunits tga3 and Gna3 positively stimulated the activity of adenylate cyclase and consequently mycoparasitism (Zeilinger et al. 2005; Silva et al. 2009; Schmoll et al. 2009). Moreover, deletion of tac1, an adenylate cyclase

gene, abolished the biocontrol activity of *T. virens* against *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Pythium* sp.

12.5.2 Transcription Factors in Biocontrol

The transcription factors (TFs) that regulate gene transcription during antagonism are not fully elucidated at the cellular level. TFs such as AreA/Nit2, Msn2/Msn4, and AceI, involved in nitrogen repression, stress response, and regulation of CWDEs in plants, respectively, bind to specific motifs in the promoter region of biocontrol genes in *Trichoderma* spp. The carbon catabolite repressor protein, Cre1, is involved in mycoparasitic interactions. During the interaction of *T. harzianum* with *Botrytis cinerea*, Cre1 binds to the promoter sequences of endochitinase-encoding gene ech4 (Lorito et al. 1996). The xylanase transcription regulator Xyr1 from *T. atroviride* was implicated in mycoparasitism and induction of plant defense responses (Reithner et al. 2014). Vel1 and Lae1 may be master regulators of antagonistic properties of *Trichoderma* spp. *T. atroviride* Lae1 is essential for antagonism towards *Alternaria alternata*, *A. solani*, and *Botrytis cinerea* (Karimi-Aghcheh et al. 2013), and Vel1 is involved in antagonism along with conidium and chlamydospore formation. PacC and heat shock factor 1 are reported to be associated with antagonistic property in *Coniothyrium minitans.*

12.6 Conclusion and Future Perspectives

In recent years, several studies on the biocontrol of different phytopathogens have highlighted mycoparasitism as a prominent mechanism in fungi. It manifests via the secretion of lytic enzymes such as chitinases, glucanases, and proteases. Although the genes responsible for CWDE secretion and genetics of regulation and signaling mechanism leading to biocontrol are known, the interaction of the biocontrol genes with environmental stimuli needs to be investigated. During the past decade, various "omics" techniques including genomics, transcriptomics, proteomics, and metabolomics data have been available to elucidate pathways affected by environmental stimulus and how. Furthermore, analyzing the secretomes during a dynamic interaction between biocontrol fungi–phytopathogen would be a direct and idealistic approach to obtain a comprehensive snapshot of its control mechanism. Hence, research related to the identification of effector-like proteins from biocontrol fungi holds promise. Besides, the direct manipulation of mycoparasitism-related genes could be exploited to develop better biocontrol agents. For example, *T. virens* deletion mutant for tvk1 gene (*that* encodes MAP kinases regulated by external signals) is a more aggressive parasite and, consequently, a better biocontrol agent than wild type. Hence, mapping the behavior of mutant strains in other phytopathogenic fungi could be a step forward in the development of better biocontrol agents.

In conclusion, there is a need to investigate how environmental factors affect the molecular switches that regulate the expression of genes encoding CWDEs. Such

studies will help define the range of environments in which fungal biocontrol agents function to the best and provide an opportunity to create formulations, which maximize biocontrol activity. For use in integrated disease management, the compatibility of fungal BCAs with chemical fungicides and fertilizers needs to be understood. Moreover, designing microarrays based on the presence or absence of proteins/metabolites could be used in pathogen control and help in developing a suitable cultivation scheme for high-value crops.

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References

Ait-Lahsen H, Soler A, Rey M, de la Cruz J, Monte E, Llobell A (2001) An antifungal exo-α-1,3-glucanase (AGN13.1) from the biocontrol fungus *Trichoderma harzianum*. Appl Environ Microbiol 67:5833–5839

Alamri S, Hashem M, Mostafa SY (2012) In vitro and in vivo biocontrol of soil-borne phytopathogenic fungi by certain bioagents and their possible mode of action. Biocontrol Sci 17:155–167. https://doi.org/10.4265/bio.17.155

Baek JM, Howell CR, Kenerley CM (1999) The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani*. Curr Genet 35:41–50

Baiyee B, Pornsuriya C, Ito S-i, Sunpapao A (2019) *Trichoderma spirale* T76-1 displays biocontrol activity against leaf spot on lettuce (*Lactuca sativa* L.) caused by *Corynespora cassiicola* or *Curvularia aeria*. Biol Control 129:195–200

Banani H, Spadaro D, Zhang D, Matic S, Garibaldi A, Gullino ML (2014) Biocontrol activity of an alkaline serine protease from *Aureobasidium pullulans* expressed in *Pichia pastoris* against four postharvest pathogens on apple. Int J Food Microbiol 182:1–8

Bankole SA, Adebanjo A (1996) Biocontrol of brown blotch of cowpea caused by *Colletotrichum truncatum* with *Trichoderma viride*. Crop Protect 15:633–636

Bara MT, Lima AL, Ulhoa CJ (2003) Purification and characterization of an exo-beta-1,3-glucanase produced by *Trichoderma asperellum*. FEMS Microbiol Lett 219:81–85

Benítez T, Delgado-Jarana J, Rincon A, Rey M, Limón C (1998) Biofungicides: *Trichoderma* as a biocontrol agent against phytopathogenic fungi. Recent Res Dev Microbiol 2:129–150

Bhattacharya D, Nagpure A, Gupta RK (2007) Bacterial chitinases: properties and potential. Crit Rev Biotechnol 27:21–28

Brunner K, Peterbauer CK, Mach RL, Lorito M, Zeilinger S, Kubicek CP (2003) The Nag1 N-acetylglucosaminidase of *Trichoderma atroviride* is essential for chitinase induction by chitin and of major relevance to biocontrol. Curr Genet 43:289–295

Brzezinska MS, Jankiewicz U (2012) Production of antifungal chitinase by *Aspergillus niger* LOCK 62 and its potential role in the biological control. Curr Microbiol 65:666–672

Chatterton S, Punja ZK (2009) Chitinase and β-1,3-glucanase enzyme production by the mycoparasite Clonostachys rosea f. catenulata against fungal plant pathogens. Can J Microbiol 55:356–367

Chen LL, Liu LJ, Shi M, Song XY, Zheng CY, Chen XL et al (2009) Characterization and gene cloning of a novel serine protease with nematicidal activity from *Trichoderma pseudokoningii* SMF2. FEMS Microbiol Lett 299:135–142

Chernin L, Chet I (2001) Microbial enzymes in biocontrol of plant pathogens and pests. In: Burns R, Dicks R (eds) Enzymes in the environment: activity, ecology and applications. Marcel Dekker, Inc., New York, pp 171–226

Chet I, Inbar J (1994) Biological control of fungal pathogens. Appl Biochem Biotechnol 48:37–43. https://doi.org/10.1007/BF02825358

Clark DP, Pazdernik NJ (2016) Chapter 9: Proteomics. In: Clark DP, Pazdernik NJ (eds) Biotechnology, 2nd edn. Academic Cell, Boston, MA, pp 295–333

Clark GL, Smith AF (1936) X-ray diffraction studies of chitin, chitosan, and derivatives. J Phys Chem 40:863–879

Cohen-Kupiec R, Broglie KE, Friesem D, Broglie RM, Chet I (1999) Molecular characterization of a novel beta-1,3-exoglucanase related to mycoparasitism of *Trichoderma harzianum*. Gene 226:147–154

Daguerre Y, Edel-Harmann V, Steinberg C (2017) Fungal genes and metabolites associated with the biocontrol of soil-borne plant pathogenic fungi. In: Mérillon J-M, Ramawat KG (eds) Fungal metabolites, Reference series in phytochemistry. Springer International, Cham. https://doi.org/10.1007/978-3-319-25001-4_27

Dana MD, Limón MC, Mejías R, Mach R, Benítez T, Pintor-Toro J, Kubicek CP (2001) Regulation of chitinase 33 (chit33) gene expression in *Trichoderma harzianum*. Curr Genet 38:335–342

Dang L et al (2010) Chemical constituents from the endophytic fungus *Trichoderma ovalisporum* isolated from *Panax notoginseng*. Ann Microbiol 60:317–320. https://doi.org/10.1007/s13213-010-0043-2

de la Cruz J, Hidalgo-Gallego A, Lora JM, Benitez T, Pintor-Toro JA, Llobell A (1992) Isolation and characterization of three chitinases from *Trichoderma harzianum*. Eur J Biochem 206:859–867

de la Cruz J, Pintor-Toro JA, Benítez T, Llobell A (1995a) Purification and characterization of an endo-beta-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. J Bacteriol 177:1864–1871

de la Cruz J, Pintor-Toro JA, Benitez T, Llobell A, Romero LC (1995b) A novel endo-β-1,3-glucanase, BGN13.1, involved in the mycoparasitism of *Trichoderma harzianum*. J Bacteriol 177:6937–6945

de Souza MF, da Silva ASA, Bon EP (2018) A novel *Trichoderma harzianum* strain from the Amazon Forest with high cellulolytic capacity. Biocatal Agric Biotechnol 14:183–188

Deng S, Lorito M, Penttila M, Harman GE (2007) Overexpression of an endochitinase gene (ThEn-42) in *Trichoderma atroviride* for increased production of antifungal enzymes and enhanced antagonist action against pathogenic fungi. Appl Biochem Biotechnol 142:81–94

Deng J-J, Huang W-Q, Li Z-W, Lu D-L, Zhang Y, Luo X-c (2018) Biocontrol activity of recombinant aspartic protease from *Trichoderma harzianum* against pathogenic fungi. Enzym Microb Technol 112:35–42

Djonović S, Pozo MJ, Kenerley CM (2006) Tvbgn3, a β-1,6-glucanase from the biocontrol fungus *Trichoderma virens*, is involved in mycoparasitism and control of *Pythium ultimum*. Appl Environ Microbiol 72:7661–7670

Djonović S, Vittone G, Mendoza-Herrera A, Kenerley CM (2007) Enhanced biocontrol activity of *Trichoderma virens* transformants constitutively coexpressing beta-1,3- and beta1,6-glucanase genes. Mol Plant Pathol 8:469–480

Doley K, Dudhane M, Borde M, Jite PK (2014) Effects of *Glomus fasciculatum* and *Trichoderma asperelloides* in roots of groundnut (cv. western-51) against pathogen *Sclerotium rolfsii*. Int J Phytopathol 3:89–100. https://doi.org/10.1016/j.ijcard.2012.06.064

Donzelli BG, Lorito M, Scala F, Harman GE (2001) Cloning, sequence and structure of a gene encoding an antifungal glucan 1,3-beta-glucosidase from *Trichoderma atroviride* (*T. harzianum*). Gene 277:199–208

Dou K, Wang ZY, Zhang RS, Wang N, Fan HJ, Diao GP, Liu ZH (2014) Cloning and characteristic analysis of a novel aspartic protease gene Asp55 from *Trichoderma asperellum* ACCC30536. Microbiol Res 169:915–923

Dubey MK, Ubhayasekera W, Sandgren M, Jensen DF, Karlsson M (2012) Disruption of the Eng18B ENGase gene in the fungal biocontrol agent *Trichoderma atroviride* affects growth, conidiation and antagonistic ability. PLoS One 7:e36152

Duo-Chuan LI, Chen S, Jing LU (2005) Purification and partial characterization of two chitinases from the mycoparasitic fungus *Talaromyces flavus*. Mycopathologia 159:223–229

Ekici ÖD, Paetzel M, Dalbey RE (2008) Unconventional serine proteases: variations on the catalytic Ser/his/asp triad configuration. Protein Sci 17:2023–2037

Elad Y, Kapat A (1999) The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. Eur J Plant Pathol 105(2):177–189

El-Bendary MA, Hamed HA, Moharam ME (2016) Potential of *Bacillus* isolates as bio-control agents against some fungal phytopathogens. Biocatal Agric Biotechnol 5:173–178

El-Katatny M, Gudelj M, Robra K-H, Elnaghy M, Gübitz G (2001) Characterization of a chitinase and an endo-β-1,3-glucanase from *Trichoderma harzianum* Rifai T24 involved in control of the phytopathogen *Sclerotium rolfsii*. Appl Microbiol Biotechnol 56:137–143

Fan HJ, Liu ZH, Zhang RS, Wang N, Dou K, Mijiti G, Diao GP, Wang ZY (2014) Functional analysis of a subtilisin-like serine protease gene from biocontrol fungus *Trichoderma harzianum*. J Microbiol 52:129–138

Flores A, Chet I, Herrera-Estrella A (1997) Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene prb1. Curr Genet 31:30–37

Fridlender M, Inbar J, Chet I (1993) Biological control of soil borne plant pathogens by a β-1,3-glucanase producing *Pseudomonas cepacia*. Soil Biol Biochem 25:1211–1221

Friel D, Pessoa NMG, Vandenbol M, Jijakli MH (2007) Separate and combined disruptions of two exo-beta-1,3-glucanase genes decrease the efficiency of *Pichia anomala* (strain K) biocontrol against Botrytis cinerea on apple. Mol Plant Microbe Interact 20:371–379

Galarza L, Akagi Y, Takao K, Kim CS, Maekawa N, Itai A, Peralta E, Santos E, Kodama M (2015) Characterization of *Trichoderma* species isolated in Ecuador and their antagonistic activities against phytopathogenic fungi from Ecuador and Japan. J Gen Plant Pathol 81:201–210. https://doi.org/10.1007/s10327-015-0587-x

Gan Z, Yang J, Tao N, Yu Z, Zhang K-Q (2007) Cloning and expression analysis of a chitinase gene Crchi1 from the mycoparasitic fungus *Clonostachys rosea* (syn. Gliocladium roseum). J Microbiol 45:422–430

Gao Q, Jin K, Ying SH, Zhang Y, Xiao G, Shang Y et al (2011) Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genet 7:e1001264

Garcia-Carreon FL (1997) Classification of proteases without tears. Biochem Educ 25:161–167

Ghanbarzadeh B, Safaie N, Goltapeh EM (2014) Antagonistic activity and hyphal interactions of *Trichoderma* spp. against *Fusarium proliferatum* and *F. oxysporum in vitro*. Arch Phytopathol Plant Prot 47:1979–1987. https://doi.org/10.1080/03235408.2013.864506

Giczey G, Kerényi Z, Fülöp L, Hornok L (2001) Expression of cmg1, an exo-beta1,3-glucanase gene from *Coniothyrium minitans*, increases during sclerotial parasitism. Appl Environ Microbiol 67:865–871

Gilbert NM, Donlin MJ, Gerik KJ, Specht CA, Djordjevic JT, Wilson CF et al (2010) KRE genes are required for β-1,6-glucan synthesis, maintenance of capsule architecture and cell wall protein anchoring in *Cryptococcus neoformans*. Mol Microbiol 76:517–534

Glare T, Caradus J, Gelernter W, Jackson T, Keyhani N, Köhl J et al (2012) Have biopesticides come of age? Trends Biotechnol 30:250–258

Gruber S, Omann M, Rodrìguez CE, Radebner T, Zeilinger S (2012) Generation of *Trichoderma atroviride* mutants with constitutively activated G protein signaling by using genetic in resistance as selection marker. BMC Res Notes 5:641. https://doi.org/10.1186/1756-0500-5-641

Gunkel FA, Gassen HG (1989) Proteinase K from *Tritirachium album* limber. Eur J Biochem 179:185–194

Gupta R, Beg QK, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15–32

Guzmán-Guzmán P, Alemán-Duarte MI, Delaye L, Herrera-Estrella A, Olmedo-Monfil V (2017) Identification of effector-like proteins in *Trichoderma* spp. and role of a hydrophobin in the plant-fungus interaction and mycoparasitism. BMC Genet 18:16. https://doi.org/10.1186/s12863-017-0481-y

Hamid R, Khan MA, Ahmad M, Ahmad MM, Abdin MZ, Musarrat J, Javed S (2013) Chitinases: an update. J Pharm Bioallied Sci 5:21–29

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) *Trichoderma* species-opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2:43–56. https://doi.org/10.1038/nrmicro797

Henrissat B (1991) A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 280:309–316

Henrissat B, Davies G (1997) Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol 1997(7):637–644

Iseli B, Boller T, Neuhaus J-M (1996) Functional analysis of the domains of a plant PR-3 chitinase. In: Muzzarelli RAA (ed) Chitin enzymology, vol 2. Atec Edizioni, Grottammare, Italy, pp 136–142

Jain L, Kurmi AK, Agrawal D (2018) Conclusive selection of optimal parameters for cellulase production by Talaromyces verruculosus IIPC 324 under SSF via saccharification of acid-pretreated sugarcane bagasse. Biofuels:1–9

Jayalakshmi SK, Raju S, Rani SU, Benagi VI, Sreeramulu K (2009) *Trichoderma harzianum* L1 as a potential source for lytic enzymes and elicitor of defense responses in chickpea (*Cicer arietinum* L.) against wilt disease caused by *Fusarium oxysporum* f. sp. ciceri. Aust J Crop Sci 3(1):44

Kang SC, Park S, Lee DG (1999) Purification and characterization of a novel chitinase from the entomopathogenic fungus, *Metarhizium anisopliae*. J Invertebr Pathol 73:276–281

Karimi-Aghcheh R, Druzhinina IS, Kubicek CP (2013) The putative protein methyltransferase LAE1 of *Trichoderma atroviride* is a key regulator of asexual development and mycoparasitism. PLoS One 8:e67144

Khalikova E, Susi P, Korpela T (2005) Microbial dextran-hydrolyzing enzymes: fundamentals and applications. Microbiol Mol Biol Rev 69(2):306–325. https://doi.org/10.1128/MMBR.69.2.306-325

Khare E, Kumar S, Kim K (2018) Role of peptaibols and lytic enzymes of *Trichoderma cerinum* Gur1 in biocontrol of *Fusarium oxysporum* and chickpea wilt. Environ Sust 1:39–47

Kim D-J, Baek J-M, Uribe P, Kenerley CM, Cook DR (2002) Cloning and characterization of multiple glycosyl hydrolase genes from *Trichoderma virens*. Curr Genet 40:374–384

Kipngeno P, Losenge T, Maina N, Kahangi E, Juma P (2015) Efficacy of *Bacillus subtilis* and *Trichoderma asperellum* against *Pythium aphanidermatum* in tomatoes. Biol Control 90:92–95. https://doi.org/10.1016/j.biocontrol.2015.05.017

Kiss L (2003) A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. Pest Manag Sci 59:475–483

Klemsdal SS, Clarke JL, Elen O (2004) Chitinase gene from *Trichoderma atroviride* confers Fusarium resistance to GM-barley. In: McIntyre M, Nielsen J, Arnau J, van der Brink H, Hansen K, Madrid S (eds) Proceedings of the 7th European conference on fungal genetics. DTU, Copenhagen, p 68

Kowsari M, Motallebi M, Zamani M (2014) Protein engineering of Chit42 towards improvement of chitinase and antifungal activities. Curr Microbiol 68:495–502

Krauss U, Matthews P, Bidwell R, Hocart M, Anthony F (2001) Strain discrimination by fungal antagonists of *Colletotrichum musae:* implications for biocontrol of crown rot of banana. Mycol Res 105:67–76

Kubicek CP, Herrera-Estrella A, Seidl-Seiboth V, Martinez DA, Druzhinina IS, Thon M et al (2011) Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma*. Genome Biol 12:R40

Kullnig C, Mach RL, Lorito M, Kubicek CP (2000) Enzyme diffusion from Trichoderma atroviride (=T. harzianum P1) to *Rhizoctonia solani* is a prerequisite for triggering of *Trichoderma* ech42 gene expression before mycoparasitic contact. Appl Environ Microbiol 66:2232–2234

Kunz C, Ludwig A, Bertheau Y, Boller T (1992) Evaluation of the antifungal activity of the purified chitinase 1 from the filamentous fungus *Aphanocladium album*. FEMS Microbiol Lett 90:105–109

Lai Y, Liu K, Zhang X, Zhang X, Li K, Wang N et al (2014) Comparative genomics and transcriptomics analyses reveal divergent lifestyle features of nematode endoparasitic fungus *Hirsutella minnesotensis*. Genome Biol Evol 6:3077–3093

Larriba E, Jaime MD, Carbonell-Caballero J, Conesa A, Dopazo J, Nislow C et al (2014) Sequencing and functional analysis of the genome of a nematode egg parasitic fungus, *Pochonia chlamydosporia*. Fungal Genet Biol 65:69–80

Latgé JP (2007) The cell wall: a carbohydrate armour for the fungal cell. Mol Microbiol 66:279–290

Latz MAC, Jensen B, Collinge DB, Jørgensen HJL (2018) Endophytic fungi as biocontrol agents: elucidating mechanisms in disease suppression. Plant Ecol Divers 11(5–6):555–567. https://doi.org/10.1080/17550874.2018.1534146

Leger RS, Cooper R, Charnley A (1991) Characterization of chitinase and chitobiase produced by the entomopathogenic fungus *Metarhizium anisopliae*. J Invertebr Pathol 58:415–426

Leger RJ, Staples RC, Roberts DW (1992) Cloning and regulatory analysis of starvation-stress gene, ssgA, encoding a hydrophobin-like protein from the entomopathogenic fungus, *Metarhizium anisopliae*. Gene 120(1):119–124

Li J, Yu L, Yang J, Dong L, Tian B, Yu Z et al (2010) New insights into the evolution of subtilisin-like serine protease genes in Pezizomycotina. BMC Evol Biol 10:68

Li Y, Sun R, Yu J, Saravanakumar K, Chen J (2016) Antagonistic and biocontrol potential of *Trichoderma asperellum* ZJSX5003 against the maize stalk rot pathogen *Fusarium graminearum*. Indian J Microbiol 56(3):318–327. https://doi.org/10.1007/s12088-016-0581-9

Limón MC, Lora JM, García I, de la Cruz J, Llobell A, Benítez T, Pintor-Toro JA (1995) Primary structure and expression pattern of the 33-kDa chitinase gene from the mycoparasitic fungus *Trichoderma harzianum*. Curr Genet 28:478–483

Liu Y, Yang Q (2007) Cloning and heterologous expression of aspartic protease SA76 related to biocontrol in *Trichoderma harzianum*. FEMS Microbiol Lett 277:173–181

Liu Y, Yang Q (2013) Cloning and heterologous expression of serine protease SL41 related to biocontrol in *Trichoderma harzianum*. J Mol Microbiol Biotechnol 23:431–439

Liu Z, Yang X, Sun D, Song J, Chen G, Juba O, Yang Q (2010) Expressed sequence tags-based identification of genes in a biocontrol strain *Trichoderma asperellum*. Mol Biol Rep 37:3673–3681

Liu K, Zhang W, Lai Y, Xiang M, Wang X, Zhang X et al (2014) *Drechslerella stenobrocha* genome illustrates the mechanism of constricting rings and the origin of nematode predation in fungi. BMC Genomics 15:114

Lo CT (1997) Biological control of turf grass diseases using *Trichoderma harzianum*. Plant Protect Bull 39:207–225

López-Mondéjar R, Ros M, Pascual JA (2011) Mycoparasitism-related genes expression of *Trichoderma harzianum* isolates to evaluate their efficacy as biological control agent. Biol Control 56:59–66. https://doi.org/10.1016/j.biocontrol.2010.10.003

Lorito M, Harman G, Hayes C, Broadway R, Tronsmo A, Woo SL, Di Pietro A (1993) Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. Phytopathology 83:302–307

Lorito M, Mach RL, Sposato P, Strauss J, Peterbauer CK, Kubicek CP (1996) Mycoparasitic interaction relieves binding of the Cre1 carbon catabolite repressor protein to promoter sequences of the ech42 (endochitinase-encoding) gene in *Trichoderma harzianum*. Proc Natl Acad Sci U S A 93:14868–14872

Lysøe E, Dees MW, Brurberg MB (2017) A three-way transcriptomic interaction study of a biocontrol agent (*Clonostachys rosea*), a fungal pathogen (*Helminthosporium solani*), and a potato host (*Solanum tuberosum*). Mol Plant Microbe Interact 30:646–655

Mach RL, Peterbauer CK, Payer K, Jaksits S, Woo SL, Zeilinger S, Kullnig CM, Lorito M, Kubicek CP (1999) Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. Appl Environ Microbiol 65:1858–1863

Mamarabadi M (2007) Characterization of genes encoding chitinolytic enzymes and their expression pattern in the biocontrol strain Clonostachys rosea IK726. PhD thesis, Faculty of Life Sciences, University of Copenhagen, Denmark

Mamarabadi M, Jensen B, Jensen DF, Lübeck M (2008a) Real-time RT-PCR expression analysis of chitinase and endoglucanase genes in the three-way interaction between the biocontrol strain *Clonostachys rosea* IK726, *Botrytis cinerea* and strawberry. FEMS Microbiol Lett 285:101–110

Mamarabadi M, Jensen B, Lübeck M (2008b) Three endochitinase-encoding genes identified in the biocontrol fungus *Clonostachys rosea* are differentially expressed. Curr Genet 54:57–70

Marcello CM, Steindorff AS, da Silva SP, Silva Rdo N, Mendes Bataus LA, Ulhoa CJ (2010) Expression analysis of the exo-beta-1,3-glucanase from the mycoparasitic fungus *Trichoderma asperellum*. Microbiol Res 165:75–81

Matroudi S, Zamani MR, Motallebi M (2008) Molecular cloning of chitinase 33 (chit33) gene from *Trichoderma atroviride*. Braz J Microbiol 39:433–437

Meerupati T, Andersson KM, Friman E, Kumar D, Tunlid A, Ahrén D (2013) Genomic mechanisms accounting for the adaptation to parasitism in nematode trapping fungi. PLoS Genet 9:e1003909

Mejía LC et al (2008) Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biol Control 46:4–14

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

Migheli Q, González-Candelas L, Dealessi L, Camponogara A, Ramón-Vidal D (1998) Transformants of *Trichoderma longibrachiatum* overexpressing the β-1,4-endoglucanase gene egl1 show enhanced biocontrol of *Pythium ultimum* on cucumber. Phytopathology 88:673–677

Minglian Z, Minghe M, Keqin Z (2004) Characterization of a neutral serine protease and its full-length cDNA from the nematode-trapping fungus *Arthrobotrys oligospora*. Mycologia 96:16–22

Mishra P, Mishra J, Dwivedi SK, Arora NK (2020) Microbial enzymes in biocontrol of phytopathogens. In: Arora NK et al (eds) Microbial enzymes: roles and applications in industries, Microorganisms for sustainability, vol 11. Springer Nature, Singapore, pp 259–285. https://doi.org/10.1007/978-981-15-1710-5_10

Mohapatra S, Padhy S, Mohapatra PKD, Thatoi H (2018) Enhanced reducing sugar production by saccharification of lignocellulosic biomass, *Pennisetum* species through cellulase from a newly isolated *Aspergillus fumigatus*. Bioresour Technol 253:262–272. https://doi.org/10.1016/j.biortech.2018.01.023

Monteiro VN, Ulhoa CJ (2006) Biochemical characterization of a β-1,3-glucanase from *Trichoderma koningii* induced by cell wall of *Rhizoctonia solani*. Curr Microbiol 52(2):92–96

Morissette DC, Driscoll BT, Jabaji-Hare S (2003) Molecular cloning, characterization, and expression of a cDNA encoding an endochitinase gene from the mycoparasite *Stachybotrys elegans*. Fungal Genet Biol 39:276–285

Mota MS, Gomes CB, Souza Júnior IT, Moura AB (2017) Bacterial selection for biological control of plant disease: criterion determination and validation. Braz J Microbiol 48(1):62–70

Mukherjee PK, Latha J, Hadar R, Horwitz BA (2003) TmkA, a mitogen-activated protein kinase of *Trichoderma virens*, is involved in biocontrol properties and repression of conidiation in the dark. Eukaryot Cell 2:446–455

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

Muszewska A, Taylor JW, Szczesny P, Grynberg M (2011) Independent subtilases expansions in fungi associated with animals. Mol Biol Evol 28:3395–3404

Noronha EF, Ulhoa CJ (1996) Purification and characterization of an endo-β-1,3-glucanase from *Trichoderma harzianum*. Can J Microbiol 42(1):1039–1044

Oliveira-Garcia E, Deising HB (2016) Attenuation of PAMP-triggered immunity in maize requires down-regulation of the key β-1,6-glucan synthesis genes KRE5 and KRE6 in biotrophic hyphae of *Colletotrichum graminicola*. Plant J 87:355–375

Omann MR, Lehner S, Escobar Rodríguez C, Brunner K, Zeilinger S (2012) The seven transmembrane receptor Gpr1 governs processes relevant for the antagonistic interaction of *Trichoderma atroviride* with its host. Microbiology 158:107–118

Park KS, Kloepper JW (2000) Activation of PR1a promoter by rhizobacteria that induce systemic resistance in tobacco against *Pseudomonas syringae* pv. *tabaci*. Biol Control 18:2–9

Patel S, Saraf M (2017) Biocontrol efficacy of *Trichoderma asperellum* MSST against tomato wilting by *Fusarium oxysporum* f. sp. *lycopersici*. Arch Phytopathol Plant Protect 50:228–238. https://doi.org/10.1080/03235408.2017.1287236

Patil R, Ghormade V, Deshpande M (2000) Chitinolytic enzymes: an exploration. Enzyme Microb Technol 26:473–483. https://doi.org/10.1016/S0141-0229(00)00134-4

Pérez-Martínez AS, De León-Rodríguez A, Harris LJ, Herrera-Estrella A, Barba de la Rosa AP (2007) Overexpression, purification and characterization of the *Trichoderma atroviride* endochitinase, Ech42, in *Pichia pastoris*. Protein Expr Purif 55:183–188

Pimentel D, McNair S, Janecka J, Wightman J, Simmonds C, O'Connell C et al (2001) Economic and environmental threats of alien plant, animal, and microbe invasions. Agric Ecosyst Environ 84:1–20

Pitson SM, Seviour RJ, McDougall BM (1993) Noncellulolytic fungal β-glucanases: their physiology and regulation. Enzym Microb Technol 15:178–192

Pozo MJ, Baek JM, Garcıa JM, Kenerley CM (2004) Functional analysis of tvsp1, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal Genet Biol 41:336–348

Prasad P, Varshney D, Adholeya A (2015) Whole genome annotation and comparative genomic analyses of bio-control fungus *Purpureocillium lilacinum*. BMC Genomics 16:1004

Ramírez-Carinõ HF, Guadarrama-Mendoza PC, Sánchez-López V, Cuervo-Parra JA, Ramírez-Reyes T, Dunlap CA, Valadez-Blanco R (2020) Biocontrol of *Alternaria alternata* and *Fusarium oxysporum* by *Trichoderma asperelloides* and *Bacillus paralicheniformis* in tomato plants. Antonie Van Leeuwenhoek 113:1247–1261. https://doi.org/10.1007/s10482-020-01433-2

Rawlings ND, Barrett AJ, Finn R (2016) Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res 44:D343–D350

Reithner B, Mach-Aigner AR, Herrera-Estrella A, Mach RL (2014) The transcriptional regulator Xyr1 of *Trichoderma atroviride* supports the induction of systemic resistance in plants. Appl Environ Microbiol 80:5274–5281

Roberti R, Zakrisson E, Flamigni F, De Vero L, Cesari A (2002) Antagonistic fungi producing hydrolytic enzymes, active in degrading the cell wall of some foot rot pathogens (*Fusarium* spp.) of wheat. J Plant Dis Protect 109(1):101–108

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

Rotem Y, Yarden O, Sztejnberg A (1999) The mycoparasite *Ampelomyces quisqualis* expresses exgA encoding an exo-beta-1,3-glucanase in culture and during mycoparasitism. Phytopathology 89:631–638

Ruiz N et al (2007) New Trichobrachins, 11-residue peptaibols from a marine strain of *Trichoderma longibrachiatum*. Peptides 28:1351–1358. https://doi.org/10.1016/j.peptides.2007.05.012

Sahai AS, Manocha MS (1993) Chitinases of fungi and plants: their involvment in morphogenesis and host-parasite interaction. FEMS Microbiol Rev 11:317–338

Sakpetch P, Aran H, Kuwahara Y, Komeda H, Asano Y (2018) Isolation of indigenous antagonistic microorganism to inhibit *Rigidoporus microporus* and other plant pathogens and analysis of the bioactive compounds. Biol Control 124:53–60

Samuels GJJ, Ismaiel A, Bon M-CC, De Respinis S, Petrini O (2010) *Trichoderma asperellum* sensu lato consists of two cryptic species. Mycologia 102:944–966. https://doi.org/10.3852/09-243

Sanz L, Montero M, Redondo J, Llobell A, Monte E (2005) Expression of an alpha-1,3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. FEBS J 272:493–499

Saravanakumar K et al (2016) Synergistic effect of *Trichoderma*-derived antifungal metabolites and cell wall degrading enzymes on enhanced biocontrol of *Fusarium oxysporum* f. sp. *cucumerinum*. Biol Control 94:37–46. https://doi.org/10.1016/j.biocontrol.2015.12.001

Saravanakumar K et al (2016b) Cellulase from *Trichoderma harzianum* interacts with roots and triggers induced systemic resistance to foliar disease in maize. Sci Rep 6:35543. https://doi.org/10.1038/srep35543

Saravanakumar K, Li Y, Yu C, Wang Q-q, Wang M, Sun J, Gao J-X, Chen J (2017) Effect of *Trichoderma harzianum* on maize rhizosphere microbiome and biocontrol of *Fusarium* stalk rot. Sci Rep 7:1771. https://doi.org/10.1038/s41598-017-01680-w

Schirmböck M et al (1994) Parallel formation and synergism of hydrolytic enzymes and peptaibol antibiotics, molecular mechanisms involved in the antagonistic action of *Trichoderma harzianum* against phytopathogenic fungi. Appl Environ Microbiol 60:4364–4370

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

Selitrennikoff C (2001) Antifungal proteins. Appl Environ Microbiol 67:2883–2894. https://doi.org/10.1128/AEM.67.7.2883-2894.2001

Sharma V, Salwan R, Sharma PN (2016) Differential response of extracellular proteases of *Trichoderma harzianum* against fungal phytopathogens. Curr Microbiol 73:419–425

Silva RD, Steindorff AS, Ulhoa CJ, Félix CR (2009) Involvement of G-alpha protein GNA3 in production of cell wall-degrading enzymes by *Trichoderma reesei* (*Hypocrea jecorina*) during mycoparasitism against Pythium ultimum. Biotechnol Lett 31:531–536

Simmons CR (1994) The physiology and molecular biology of plant 1–3-β-D-glucanase and 1,3;1–4-β-D-glucanase. Crit Rev Plant Sci 13:325–387

Singh R, Kumar R, Bishnoi K, Bishnoi NR (2009) Optimization of synergistic parameters for thermostable cellulase activity of *Aspergillus heteromorphus* using response surface methodology. Biochem Eng J 48:28–35

Stahmann KP, Schimz KI, Sahm H (1993) Purification and characterization of four extracellular 1,3-b-glucanases of *Botrytis cinerea*. J Gen Microbiol 139:2833–2840

Steyaert JM, Stewart A, Jaspers MV, Carpenter M, Ridgway HJ (2004) Co-expression of two genes, a chitinase (chit42) and proteinase (prb1), implicated in mycoparasitism by *Trichoderma hamatum*. Mycologia 96:1245–1252

Strasser H, Vey A, Butt TM (2000) Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium*, *Tolypocladium* and *Beauveria* species? Biocontrol Sci Tech 10:717–735

Suarez B, Rey M, Castillo P, Monte E, Llobell A (2004) Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. Appl Microbiol Biotechnol 65:46–55

Suárez MB, Sanz L, Chamorro MI, Rey M, González FJ, Llobell A, Monte E (2005) Proteomic analysis of secreted proteins from *Trichoderma harzianum*: identification of a fungal cell wall-induced aspartic protease. Fungal Genet Biol 42:924–934

Sumida CH, Daniel JFS, Araujod APCS, Peitl DC, Abreu LM, Dekker RFH, Canteri MG (2018) *Trichoderma asperelloides* antagonism to nine *Sclerotinia sclerotiorum* strains and biological

control of white mold disease in soybean plants. Biocontrol Sci Tech 28:142–156. https://doi.org/. https://doi.org/10.1080/09583157.2018.1430743

Taylor G, Jabaji-Hare S, Charest PM, Khan W (2002) Purification and characterization of an extracellular exochitinase, beta-N-acetylhexosaminidase, from the fungal mycoparasite *Stachybotrys elegans*. Can J Microbiol 48:311–319

Thangavelu R, Palaniswami A, Velazhahan R (2004) Mass production of *Trichoderma harzianum* for managing fusarium wilt of banana. Agric Ecosyst Environ 103:259–263

Thrane C, Tronsmo A, Jensen DF (1997) Endo-1,3-β-glucanase and cellulase from *Trichoderma harzianum*: purification and partial characterization, induction of and biological activity against plant pathogenic Pythium spp. Eur J Plant Pathol 103:331–344

Tondje PR, Roberts DP, Bon MC, Widmer T, Samuels GJ, Ismaiel A, Begoude AD et al (2007) Isolation and identification of mycoparasitic isolates of *Trichoderma asperellum* with potential for suppression of black pod disease of cacao in Cameroon. Biol Control 43:202–212. https://doi.org/10.1016/j.biocontrol.2007.08.004

Troian RF, Steindorff AS, Ramada MHS, Arruda W, Ulhoa CJ (2014) Mycoparasitism studies of *Trichoderma harzianum* against *Sclerotinia sclerotiorum*: evaluation of antagonism and expression of cell wall-degrading enzymes genes. Biotechnol Lett 36:2095–2101

Tsujibo H et al (2003) Characterization of chitinase genes from an alkaliphilic actinomycete, *Nocardiopsis prasina* OPC-131. Appl Environ Microbiol 69(2):894–900

Tunlid A, Jansson H-B, Nordbring-Hertz B (1992) Fungal attachment to nematodes. Mycol Res 96 (6):401–412

Tzelepis G, Dubey M, Jensen DF, Karlsson M (2015) Identifying glycoside hydrolase family 18 genes in the mycoparasitic fungal species *Clonostachys rosea*. Microbiology 161:1407–1419

Umeyama T, Kaneko A, Watanabe H, Hirai A, Uehara Y, Niimi M et al (2006) Deletion of the CaBIG1 gene reduces β-1,6-glucan synthesis, filamentation, adhesion, and virulence in *Candida albicans*. Infect Immun 74:2373–2381

Valadares-Inglis MC, Peberdy JF (1997) Location of chitinolytic enzymes in protoplasts and whole cells of the entomopathogenic fungus *Metarhizium anisopliae*. Mycol Res 101:1393–1396

van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36:453–483

Vieira PM, Coelho ASG, Steindorff AS, de Siqueira SJL, Silva RDN, Ulhoa CJ (2013) Identification of differentially expressed genes from *Trichoderma harzianum* during growth on cell wall of *Fusarium solani* as a tool for biotechnological application. BMC Genomics 14:177

Viterbo A, Haran S, Friesem D, Ramot O, Chet I (2001) Antifungal activity of a novel endochitinase gene (chit36) from *Trichoderma harzianum* Rifai TM. FEMS Microbiol Lett 200:169–174

Viterbo A, Montero M, Ramot O, Friesem D, Monte E, Llobell A, Chet I (2002) Expression regulation of the endochitinase chit36 from *Trichoderma asperellum* (*T. harzianum* T-203). Curr Genet 42:114–122

Viterbo A, Harel M, Chet I (2004) Isolation of two aspartyl proteases from *Trichoderma asperellum* expressed during colonization of cucumber roots. FEMS Microbiol Lett 238:151–158

Wang RB, Yang JK, Lin C, Zhang Y, Zhang KQ (2006) Purification and characterization of an extracellular serine protease from the nematode trapping fungus *Dactylella shizishanna*. Lett Appl Microbiol 42:589–594

Weindling R (1932) Studies on lethal principle effective in the parasitic action of Trichoderma hamatum on Rhisoctonia solani and other soil fungi. Phytopathology 22:837–845

Xian H, Li J, Zhang L, Li D (2012) Cloning and functional analysis of a novel chitinase gene Trchi1 from *Trichothecium roseum*. Biotechnol Lett 34:1921–1928

Xiao G, Ying SH, Zheng P, Wang ZL, Zhang S, Xie XQ et al (2012) Genomic perspectives on the evolution of fungal entomopathogenicity in *Beauveria bassiana*. Sci Rep 2:483

Xie BB, Qin QL, Shi M, Chen LL, Shu YL, Luo Y et al (2014) Comparative genomics provide insights into evolution of *Trichoderma* nutrition style. Genome Biol Evol 6:379–390

Yan L, Qian Y (2009) Cloning and heterologous expression of SS10, a subtilisin-like protease displaying antifungal activity from *Trichoderma harzianum*. FEMS Microbiol Lett 290:54–61

Yang J, Wang L, Ji X, Feng Y, Li X, Zou C et al (2011a) Genomic and proteomic analyses of the fungus *Arthrobotrys oligospora* provide insights into nematode-trap formation. PLoS Pathog 7: e1002179

Yang J, Zhao X, Liang L, Xia Z, Lei L, Niu X, Zou C, Zhang K-Q (2011b) Overexpression of a cuticle-degrading protease Ver112 increases the nematicidal activity of *Paecilomyces lilacinus*. Appl Microbiol Biotechnol 89:1895–1903

Yang X, Cong H, Song J, Zhang J (2013) Heterologous expression of an aspartic protease gene from biocontrol fungus *Trichoderma asperellum* in *Pichia pastoris*. World J Microbiol Biotechnol 29:2087–2094

Yedidia II, Benhamou N, Chet I (1999) Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Appl Environ Microbiol 65:1061–1070

Zeilinger S, Reithner B, Scala V, Peissl I, Lorito M, Mach RL (2005) Signal transduction by Tga3, a novel g protein subunit alpha of *Trichoderma atroviride*. Appl Environ Microbiol 71 (3):1591–1597

Zhang HY, Yang Q, Wang G, Shang FD (2009) Analysis of expressed sequence tags from *Chaetomium cupreum* grown under conditions associated with mycoparasitism. Lett Appl Microbiol 48:275–280

Zhao C, Deng L, Fang H (2018) Mixed culture of recombinant *Trichoderma reesei* and *Aspergillus niger* for cellulase production to increase the cellulose degrading capability. Biomass Bioenergy 112:93–98

Zheng P, Xia Y, Xiao G, Xiong C, Hu X, Zhang S et al (2011) Genome sequence of the insect pathogenic fungus *Cordyceps militaris*, a valued traditional Chinese medicine. Genome Biol 12: R1

Zhongcun P, Kodo O, Yasunori S et al (2004) Purification and characterization of an endo-1,3-β-glucanase from *Arthrobacter* sp. J Biol Macromol 4(2):57–66

Zou CG, Tao N, Liu WJ, Yang JK, Huang XW, Liu XY et al (2010) Regulation of subtilisin-like protease prC expression by nematode cuticle in the nematophagous fungus *Clonostachys rosea*. Environ Microbiol 12:3243–3252

Candida: A Model Fungus to Study Differentiation, Pathogenesis, and Bioprospecting

13

Ejaj K. Pathan and Mukund V. Deshpande

Abstract

Candida albicans is one of the most dreadful human fungal pathogens. Nowadays, non-*albicans Candida* (NAC) species like *C. glabrata*, *C. krusei*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis* too account for a substantial part of clinical isolates collected worldwide. These observations highlight the need to look beyond *C. albicans* to combat fungal infections. Therefore, in the present chapter, NAC species have been discussed concerning their emergence, biology, pathogenesis, and evolutionary relatedness. Yeast-hypha morphogenesis is one of the most discussed and essential phenomena associated with the virulence of *C. albicans* and few NAC species. Therefore, the biochemical and molecular basis of morphogenesis in *Candida* species has also been discussed. The knowledge of the molecular basis of multidrug resistance in emerging *Candida* pathogens is necessary to design the antifungal drugs. Nevertheless, beyond the pathogenesis and treatment of *Candida* infections, the significant biotechnological contributions of *Candida* species are also documented.

Keywords

Bioprospecting · *Candida albicans* · *Candida glabrata* · Morphogenesis · Multidrug resistance · Non-*albicans Candida* species

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13.1 Introduction

Candida genus has more than 150 species and nearly 20 species are known to cause human infections. *C. albicans*, normal commensal of humans, is the most frequent causative agent of candidiasis. Under immunosuppressive conditions caused due to immunosuppressive drugs, or chemotherapeutic agents, or to the patients in surgical intensive care units for prolonged time, the commensal *C. albicans* yeast cells turn into pathogenic cells implicated in life-threatening invasive candidiasis. Moreover, multidrug resistance in *C. albicans* strains is a major concern in treating systemic candidiasis (Reyna-Beltrán et al. 2019). Interestingly, non-*albicans Candida* (NAC) species like *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* now account for a substantial part of clinical isolates collected worldwide (Papon et al. 2013). These observations highlight the need to look beyond *C. albicans* to combat the candidiasis. Therefore, in the subsequent section, non-*albicans Candida* species have been discussed with respect to their emergence, biology, pathogenesis, and evolutionary relatedness. The yeast-hypha morphogenesis is one the most discussed and essential phenomenon associated with virulence of *C. albicans* and few non-*albicans Candida* species (Jacobsen et al. 2012). Therefore, the account of biochemical and molecular basis of morphogenesis in *Candida* species has also been taken. Further, the continued use of antifungals leads to development of resistance in *Candida* species is well documented, and clinical isolates are therefore more prone to develop a resistance against routinely used antifungals (Prasad et al. 2015; Walker et al. 2013). In view of this, molecular basis of multidrug resistance in emerging *Candida* pathogens is discussed. Furthermore, in an effort to look beyond their pathogenic characteristics, various biotechnological applications of *Candida* species are also documented in the following sections.

13.2 Human Fungal Pathogens

Fungi cause various diseases in humans, ranging from superficial skin and mucosal infections to invasive infections of internal organs (Kim 2016). However, past more than 150 years of Louis Pasteur's discovery that fungi cause disease in animals, the fungal infections and their impact on human health are still underestimated (Janbon et al. 2019). On the contrary, ~1.5 billion of the world population suffer from various fungal infections, and associated mortality is around 1.5–2 million/year (Brown et al. 2012). According to Denning and Bromley (2015), the mortality rate due to fungal infections is higher than that caused by malaria or tuberculosis. Interestingly, around 1000 species among the estimated 5.1 million fungal species can cause disease in humans (O'Brien et al. 2005; Köhler et al. 2014). Further, only four pathogens among them, viz., *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, and *Pneumocystis jirovecii* account for >90% of deaths (Brown et al. 2012). *C. albicans* alone contribute to ~40% of these deaths and therefore considered as one of the most dreadful human fungal pathogens (Pukkila-Worley et al. 2009). Other important fungal pathogens are *Blastomyces dermatitidis*, *Coccidioides*

immitis, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Penicillium marneffei*, *Rhizopus oryzae*, and non-*albicans Candida* species, viz., *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, and *C. krusei* (Brown et al. 2012; Kam and Xu 2002). These fungal pathogens are acquired either environmentally (e.g., *Aspergillus* and *Cryptococcus*) or endogenously (e.g., *Candida*). For instance, *A. fumigatus* is a saprophyte that grows in environmental niches (e.g., compost and soil), and inhalation of the spores by the patients leads to infection (Reedy et al. 2007). On the contrary, *C. albicans* is a harmless member of the human microbiota and becomes pathogenic by disrupting the balanced interaction with the host cells (Höfs et al. 2016). In short, the pathogenic potential of different human fungal pathogens has evolved independently, either in environmental niches or as a member of the human microbiota. However, fungal infections are no longer associated only with the immunocompromised patients; fungi like *Basidiobolus* sp., *Conidiobolus* sp., *B. dermatitidis*, *H. capsulatum*, *P. brasiliensis*, and *C. immitis* can cause pathogenic lesions in healthy individuals too (Köhler et al. 2014; Kim et al. 2006).

13.3 Human Pathogenic *Candida* Species

The genus *Candida* contains a diverse group of organisms, and ~20 *Candida* spp. were reported as human pathogens (Kämmer et al. 2020; Papon et al. 2013). These pathogenic *Candida* species fulfill all four features needed for a fungus to become human pathogen, viz., they grow very well at and above human body temperature; they produce multiple adhesion molecules to bind to host surface and are able to penetrate host tissue by forming hyphae; their hyphae produce range of lytic enzymes to facilitate tissue invasion and also to digest human macromolecules; lastly, hyphae constitutively produce the yeast cells, important for disseminating to distant sites to cause pathogenesis (Köhler et al. 2014). Indeed, *C. albicans* remains the most frequent cause of candidiasis and, along with *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*, accounts for about 90% of all *Candida* bloodstream infections (Kämmer et al. 2020). Another important non-*albicans Candida* (NAC) clinical isolate collected worldwide in hospitals is *C. krusei* (Pfaller et al. 2010a; Papon et al. 2013) and represents the most common causes of invasive fungal infections of humans. Further, the less-prominent species, viz., *C. guilliermondii*, *C. lusitaniae*, *C. kefyr*, *C. famata*, *C. inconspicua*, *C. rugosa*, *C. dubliniensis*, and *C. norvegensis*, were also reported (Pfaller et al. 2010b; Papon et al. 2013; Turner and Butler 2014). The variation in virulence potential of these pathogenic *Candida* species is reported to be emerged several times independently during evolution (Papon et al. 2013). The characteristics of some important pathogens (established as well as emerging) of *Candida* genus are described in the following sections.

13.3.1 *C. albicans*

This is the most dreadful pathogen among the *Candida* species causing invasive candidiasis, accounting for the mortality rate of ~50% (Pfaller and Diekema 2007; Hameed and Fatima 2013). *C. albicans* is normally associated with the humans as a part of commensal microbiota and becomes pathogenic in immunocompromised hosts (Brown and Netea 2012). It is a polymorphic fungus that shows different morphological forms: yeast (Y), pseudohypha, true hypha (H), and also the chlamydospores, under distinct growth conditions (Citiulo et al. 2009). Yeast cells are usually 10–12μm in diameter and switch to hypha and *vice a versa* for survival and proliferation in the host (Gow et al. 2012; Yang et al. 2015). The ability to switch between Y and H morphologies in response to environmental conditions is the most discussed and main virulence attribute of *C. albicans* (Jacobsen et al. 2012; Lu et al. 2014). Indeed, both the morphologies are important for pathogenesis events, viz., adhesion (Y and H), invasion (H), dissemination (Y), biofilm formation (Y and H), and secretion of hydrolytic enzymes by hyphae (H) to digest host macromolecules (Pathan et al. 2017; Rizzetto et al. 2014).

13.3.2 *C. glabrata*

C. glabrata is second most pathogenic species after *C. albicans* causing invasive candidiasis (Kämmer et al. 2020). It is haploid, non-dimorphic yeast (2–3μm in diameter) and normally reproduces by budding, with limited capacity to produce pseudohyphae under nitrogen starvation condition (Csank and Haynes 2000; Sasani et al. 2016). Recently, Pathan et al. (2019) showed the germ tube formation in *C. glabrata* heterologously expressing hyphal form specific *BpNADPGDH II* of dimorphic fungus *Benjaminiella poitrasii*. Similar to *C. albicans*, *C. glabrata* is also a part of normal human microbiota and becomes pathogenic causing superficial infections to life-threatening systemic infections in immunocompromised patients (Fidel Jr et al. 1999; Kämmer et al. 2020). Further, it spreads very fast and difficult to treat because of resistance to many existing antifungal drugs. The mortality due to *C. glabrata*-induced candidemia is therefore more than 50% in cancer patients and immunocompromised patients undergoing organ transplantation and renal deficiency in hospitals (Fidel Jr et al. 1999; Gupta et al. 2015; Yang and Rao 2018).

13.3.3 *C. tropicalis*

Another emerging NAC species, *C. tropicalis*, is a more frequent cause of candidiasis in leukemia and bone marrow transplantation patients worldwide (Chakrabarti et al. 2015a, b; Kothavade et al. 2010). According to Chakrabarti et al. (2015a, b) in the Indian subcontinent, *C. tropicalis* is the most common non-*albicans Candida* species reported responsible for life-threatening bloodstream infections. *C. tropicalis* is non-dimorphic diploid yeast (2–10μm in diameter). It can form biofilm on medical

devices (De Brucker et al. 2013). *C. tropicalis* is an osmotolerant species that can withstand high salt concentrations, contributing to virulence and drug resistance.

13.3.4 *C. auris*

C. auris has recently attracted the clinical world's attention due to its rapid and widespread occurrence as a highly drug-tolerant NAC species. It is challenging to identify the *C. auris* from clinical samples due to its capacity to spread rapidly, the mechanism of which is still to be understood (Jeffery-Smith et al. 2017). *C. auris* grows as yeast cells that can form a biofilm. It forms pseudohyphae when grown at high salt condition, but not otherwise (Kumar et al. 2017; Satoh et al. 2009; Wang et al. 2018). The virulence factors are biofilm, transporter genes, and protein kinases. The ICU (intensive care unit) patients are more prone to candidiasis caused by *C. auris* (Vallabhaneni et al. 2017). Although the full genome sequence of *C. auris* is available, the molecular basis of pathogenicity and virulence is still poorly understood (Chatterjee et al. 2015).

13.3.5 *C. parapsilosis*

C. parapsilosis is a typical commensal of human skin that has rapidly increased its prevalence as a pathogen in the last few decades (Yang and Rao 2018). It grows as a diploid yeast and can form pseudohyphae in the presence of citrulline (Kim et al. 2006). The virulence factors include adhesion, secretion of hydrolytic enzymes, and biofilm formation (Trofa et al. 2008). *C. parapsilosis* causes nosocomial infections, accounting for ~35% cases of candidemia in newborn babies (Pammi et al. 2014; Trofa et al. 2008). Further, it also causes systemic infections and candidemia in cancer patients (Sun et al. 2019; van Asbeck et al. 2009).

The non-*albicans Candida* species previously considered as non-pathogenic, viz., *C. kefyr*, *C. krusei*, *C. rugosa*, *C. guilliermondii*, *C. dubliniensis*, and *C. lusitaniae*, are also emerging as pathogens, and it clearly indicates the need to look beyond *C. albicans* and other established pathogens to combat the fungal infections (Yang and Rao 2018).

13.4 Evolutionary Relatedness in *Candida* Species

Initially, the genus name *Candida* was attributed to yeast species able to form hyphae or pseudohyphae but not producing sexual spores. Nevertheless, recent phylogenetic analysis has clarified that *Candida* species represent a polyphyletic group within the *Saccharomycotina* (Fitzpatrick et al. 2006). More precisely, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, *C. lusitaniae*, *C. famata*, *C. rugosa*, and *C. dubliniensis* form the part of *Candida* CTG clade (Fig. 13.1) and translate CTG codons as serine instead of leucine (Papon et al. 2013). On the

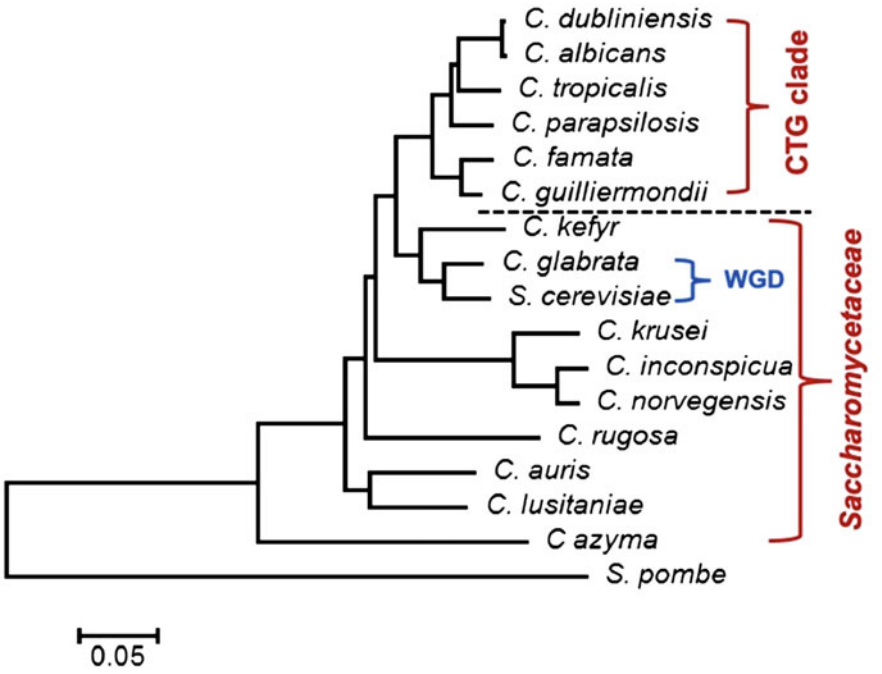

Fig. 13.1 Evolutionary relationship between the *Candida* species. The evolutionary history of *Candida* species based on 18S rDNA sequences was inferred using the minimum evolution (ME) method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The neighbor-joining algorithm was used to generate the initial tree. The analysis involved 17 nucleotide sequences of 18S rDNA. The tree is rooted with 18S rDNA sequence of fission yeast *Schizosaccharomyces pombe* as an out-group. Evolutionary analyses were conducted in MEGA6. The CTG clade represents *Candida* species that translate CTG codons as serine instead of leucine. WGD stands for whole-genome duplication

other hand, species like *C. glabrata* and *C. kefyr* belong to the group *Saccharomycetaceae*. *C. glabrata* is closely related to baker's yeast *S. cerevisiae*, and both share the whole-genome duplication (WGD) clade in phylogeny (Fig. 13.1). Further, other *Candida* species, viz., *C. krusei*, *C. inconspicua*, and *C. norvegensis*, were also found to be originated from the *Saccharomycetaceae* clade (Fig. 13.1). Interestingly, among the four most dreadful *Candida* sp. (*C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*), except *C. glabrata*, the remaining were found in the CTG clade, suggesting the correlation between their pathogenicity and evolution. Further, *C. tropicalis* is more closely related to *C. albicans* than the other *Candida* species in phenotypic and biochemical characteristics (Zuza-Alves et al. 2017). However, the non-pathogenic *Candida* species were interspersed within the pathogens (Papon et al. 2013; Silva et al. 2012), indicating their independent evolutionary origins. The comparative genomic analyses indicate the involvement of the host environment in developing

pathogenicity in *Candida* species. However, it would be interesting to see whether these species show a similar level of infection and survival in the host due to their evolutionary relatedness. One should also look at the emergence of multidrug resistance in *Candida* species through evolutionary prospect. In view of this, detailed analysis of available whole-genome sequences of CTG clade genomes, including *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. guilliermondii*, and *C. lusitaniae*, and their comparison with *C. glabrata* genome can provide new insights into gene family evolution within *Candida* species.

13.5 Morphological Switching and Virulence in *Candida*

Most *Candida* sp. is opportunistic pathogens and exhibit morphological change to facilitate infection in the host cell. Seven NAC species are of significant medical importance, in that *C. tropicalis* and *C. glabrata* are most frequently observed in the clinical specimens. Other species are *C. parapsilosis*, *C. stellatoidea*, *C. guilliermondii*, *C. krusei*, and *C. pseudotropicalis* (Scully et al. 1994). Several *Candida* species may develop pseudohyphae, but very few can form true hyphae, including *C. albicans* and *C. tropicalis* (Zuza-Alves et al. 2017). The role of morphogenesis in *C. albicans* infection is extensively studied, and hyphae are considered essential for tissue invasion (Jacobsen et al. 2012). Lo et al. (1997) reported that mutants unable to form hyphae were avirulent. Though hyphae are often seen in tissue invasion areas in patients with candidiasis and thought to be involved in disseminating disease (Saville et al. 2003), yeast cells of *C. albicans* and *C. glabrata* were shown to traverse the gut wall of infant mice (Pope and Cole 1982). Many reports from the literature suggest that yeast forms are also involved in disseminating the fungus through the bloodstream and establishing infection at distant sites. The yeast form produces many aspartyl proteases involved in virulence (Hube and Naglik 2001). Earlier Ray and Payne (1988) demonstrated that proteinase production by *C. albicans* yeast cells formed pits in the mouse skin surface ex vivo.

The effect of environmental conditions, transcription regulators, and target genes on Y-H morphogenesis is well-established for *C. albicans*. For instance, the yeast and hyphal forms were triggered by change in incubation temperature combined with other factors such as blood glucose and serum (Shepherd et al. 1980). Brown and Gow (1999) reported that nitrogen deprivation stimulated filamentation in *C. albicans*. The zinc also affected germ tube formation in *C. albicans* (Sabie and Gadd 1992). Further, the pH of the medium also affects the morphological outcome in *C. albicans*. At pH range 6–8, cytoplasmic alkalinization accompanied the germ tube formation, whereas yeast growth was prevalent in an acidic medium (Stewart et al. 1988). The animal serum (horse, cattle, or human) was reported to induce hyphal growth in *C. albicans*. When incubated with serum at 37 °C and neutral pH, *C. albicans* undergoes a morphological change to form hyphae. However, at high serum concentration in the medium, even at acidic pH, hyphae are induced, indicating an overriding effect of serum over pH (Odds 1988).

Microarray analysis showed that 742 genes (out of 6500) were differentially expressed during Y-H transition in *C. albicans* (Nantel et al. 2002). It was further suggested that transcriptional factors *Efg1p* and *Cph1p* were important for germ tube formation in *C. albicans* (Nantel et al. 2002). Genes encoding for cellular regulators like transcriptional activators (*TUP1*, *EFG1*) were reported to play an essential role in the morphological transition of *C. albicans* (Magee 1997). Further, the expression of genes coding for cellular building blocks also changed during yeast to hypha transition (Gow 1995). For instance, the genes *ECE1* (gene expressed in relation to the extent of cell elongation) and *CHS2* (chitin synthase gene) were expressed in the hyphal form. The genes such as *HYR1* are expressed only in the hyphal form of *C. albicans* (Bailey et al. 1996). Further, genes such as *EFG1* coding for enhanced filamentous growth (Stoldt et al. 1997), *HST7* coding for mitogen-activated protein kinase components (Gow 1995), *RBF1* coding for RPG box binding factor1 (Magee 1997), *SAP1-10* coding for aspartyl proteinases (Hube et al. 1994; Naglik et al. 2003), and *NADPGDH* coding for NADP-dependent glutamate dehydrogenase (Han et al. 2019) were also reported to show correlation with Y-H morphogenesis in *C. albicans*. Similarly, in nonpathogenic, dimorphic model fungus *Benjaminiella poitrasii*, the cause-effect relationship between *BpNADPGDH* genes and morphological outcome was reported (Pathan et al. 2019). Interestingly, expression of H-form specific *BpNADPGDH II* induced the germ tube formation in human pathogenic, non-dimorphic yeast *C. glabrata* (Pathan et al. 2019).

Involvement of various signal transduction pathways for the control of Y-H transition in *C. albicans* was also established by studying different mutants. Based on the information from *S. cerevisiae* mitogen-activated protein kinase (MAPK) pathway conserved element *ste12p*, homologous *cph1Δ/cph1Δ C. albicans* mutants were constructed and found to be defective in hyphal development on solid media. These MAPK pathway mutants were able to form hyphae in liquid culture in response to serum (Liu et al. 1994). Another *Ste12p*-independent pathway with *EFG1* gene was identified, and its reduced expression suppressed the formation of true hyphae (Stoldt et al. 1997); however, pseudohyphae were formed in response to serum. When double mutants for both the pathways, i.e., *cph1Δ/cph1Δ efg1Δ/efg1Δ*, were constructed, they remained locked in yeast form and unable to form hyphae or pseudohyphae in response to any stimuli, including serum or macrophages. These mutants were avirulent when tested in a mouse model (Lo et al. 1997). The *EFG1* is a part of the cyclic AMP-mediated signaling pathway. Another transcription factor, *Flo8p* from this pathway, controls hyphae formation in response to various environmental stimuli. Further, *flo8Δ/flo8Δ* mutants showed defects in hyphae formation with attenuated virulence in animal models of candidiasis (Cao et al. 2006). Similar results with the above three individual mutants and *cph1Δ/cph1Δ efg1Δ/efg1Δ* double mutant in the *Caenorhabditis elegans* infection model confirmed the importance of hypha formation in the pathogenesis of *C. albicans* (Pukkila-Worley et al. 2009).

13.6 Multidrug Resistance in *Candida*

Number of the *Candida* species show multidrug (MD) resistance against the polyene, azole, and echinocandin class of antifungal molecules that are neither structurally nor functionally similar (Papon et al. 2013; Prasad and Goffeau 2012). Although *C. albicans* isolated from the clinical samples remained the primary source of MDR strains, other non-*albicans Candida* species, viz., *C. glabrata*, *C. krusei*, *C. inconspicua*, *C. rugosa*, *C. norvegensis*, *C. parapsilosis*, and *C. guilliermondii*, exhibiting resistance against azoles and echinocandins have also become a significant concern in past few years (Papon et al. 2013; Walker et al. 2013). Several factors contribute to drug resistance, and the molecular mechanism underlying the MDR remains either of the (a) alteration of the drug target, (b) overexpression of the drug target, (c) inability to uptake the drug, or (d) drug efflux, alone or in combination (Healey et al. 2016).

The molecular studies of azole resistance *Candida* strain attributed this phenomenon to either mutation or overexpression of its target *ERG11*, coding for lanosterol 14 α-demethylase, an essential enzyme in ergosterol biosynthesis (Perea et al. 2001; Xiang et al. 2013). Interestingly, most of the clinical isolates of *Candida* species showed either R467K or G464S single point mutations in *ERG11* (Lamb et al. 2000). These isolates showed reduced susceptibility and improved resistance against fluconazole (Xiang et al. 2013; You et al. 2017). Few other point mutations in *ERG11* were also reported but did not show any alteration in drug response (Flowers et al. 2015). Further, overexpression of *ERG11* due to mutation in transcription factor *CaUpc2* also led to drug resistance in clinical isolates of *C. albicans* (Flowers et al. 2012; Heilmann et al. 2010). The mutation in *ERG3*, coding for ergosterol biosynthetic enzyme, sterol-Δ-(5,6)-desaturase, is another mechanism for azole resistance in *Candida* species (Morio et al. 2012; Whaley et al. 2017). The echinocandin resistant *Candida* species primarily showed the mutation in its target, *FKS1*, coding for β-1-3-glucan synthase, an important enzyme of cell wall biosynthesis (Dannaoui et al. 2012). These mutations enhanced the echinocandin tolerance in *Candida* species by more than a 1000-folds. Amphotericin B (AmB) is the only antifungal molecule against which no resistance was reported in the last 25 years, and therefore it remained the most potent option against invasive fungal infections (Mesa-Arango et al. 2012). However, recent reports suggested the emergence of amphotericin B resistance in *C. auris* and *C. haemulonii*; however, the mechanism is not understood yet (Shin et al. 2012; Wu et al. 2020).

The inhibition of drug uptake is another mechanism by which *Candida* species show high drug resistance. It is based on the permeability constraint imposed by the fungal cell wall and the membrane, thereby controlling the intracellular drug concentration. The antifungal molecules are mostly hydrophobic, and the plasma membrane plays a vital role in determining the rate of their passive diffusion across the membrane (Krishnamurthy 1999; Mukhopadhyay et al. 2002). Recently, Dawaliby et al. (2016) showed that imbalances in lipid homeostasis of plasma membrane also affect the diffusion of fluconazole in *Candida* species.

The efflux of the drug outside the cells using protein pumps contributes to developing the multidrug resistance in *Candida* species (Prasad and Goffeau 2012; Prasad et al. 2015). The MDR *Candida* species overexpress the efflux proteins leading to rapid drug extrusion resulting in decreased susceptibility toward antifungals. In *C. albicans*, two major drug efflux systems, viz., ATP-binding cassette (ABC) superfamily and the major facilitator superfamily (MFS), have been characterized (Prasad and Goffeau 2012). The ABC transporters are ATP-dependent, whereas MFS proteins are drug/H^+ antiporters that use proton motive force. These membrane transporters recognize a wide array of drugs/molecules; thus, their activation remains the predominant drug resistance mechanism in *Candida* species (Healey et al. 2016; Prasad et al. 2015).

Other than healthcare concern, a number of *Candida* species exhibit beneficial activities for the mankind.

13.7 Bioprospecting of *Candida* Species

Zuza-Alves et al. (2017) extensively reviewed the biology, clinical, and applied aspects of *C. tropicalis*, a second most studied species next to *C. albicans.* This species is a strong biofilm producer as compared to *C. albicans*, a producer of wide range of other virulence factors and also exhibits resistance toward different antifungal agents. In spite of this, *C. tropicalis* is one of the well-studied *Candida* species for biotechnological applications. Unlike most other *Candida* species, *C. tropicalis* can grow on a large variety of carbon sources, which include mon-, di-, and polysaccharides, phenols, alkanes, alkane derivatives, and fatty acids (Jamai et al. 2001).

13.7.1 *Candida* in Ethanol Production

The efforts are being made to use *C. tropicalis* to produce ethanol from soluble starch, cellulosic and hemicellulosic biomass. Jamai et al. (2001) compared ethanol production from glucose fermentation by *S. cerevisiae* and *C. tropicalis* using free and calcium alginate-immobilized cells. However, immobilized *C. tropicalis* cells showed morphological alteration with reduced fermentation capacity as compared to *S. cerevisiae.* Nevertheless because of its thermotolerance, *C. tropicalis* is suitable for coupled saccharification fermentation (CSF) of hemicellulose that can be achieved at a temperature above 40 °C. On the other hand, *S. cerevisiae* is not able to carry out fermentation at >30 °C. Shariq and Sohail (2019) identified a strain of *C. tropicalis* which showed ability to produce xylanase using lignocellulosic waste and to produce ethanol. Additionally, *C. tropicalis* has the ability to ferment C5 sugars from hemicellulose. Similarly, Tanimura et al. (2012) reported isolation of *C. shehatae* for ethanol production at elevated temperature from glucose and xylose. Sugarcane bagasse and rice straw are potent renewable sources for ethanol production as they contain significant quantities of glucose and xylose. A co-cultivation of *S. cerevisiae* and xylose utilizing yeasts such as *Pichia stipitis*, *C. shehatae*, and

C. utilis was reported earlier. To improve xylose fermentation for ethanol production, Meethit et al. (2016) further proposed to use co-immobilization of *S. cerevisiae* and *C. shehatae* in Ca-alginate beads or on delignified cellulose. The entrapment of cells in Ca-alginate beads was reported to be better than immobilization on delignified cellulose.

13.7.2 *Candida* in Wine Fermentation

While studying natural yeast flora of wine grape varieties, Chavan et al. (2009) for the first time reported *C. azyma* and *C. quercitrusa* from the berries of Bangalore blue and Cabernet varieties. *C. quercitrusa* usually is associated with insects, while *C. azyma* is found in sugarcane field. The unusual presence of *C. azyma* on grapes could be attributed to the change in cropping pattern from sugarcane to grapes by the local farming community (Chavan et al. 2009). Earlier Heard and Fleet (1985) reported the significant growth of *C. stellata*, *C. colliculosa*, and *C. pulcherrima* during four different Australian wine fermentations. Bagheri et al. (2015) observed that the agriculture practices influence the natural flora. For instance, the integrated pest management with chemicals usually affected the yeast flora. A number of yeasts species including *C. azyma* were more in percentage in the farm managed with an integrated approach, while in biodynamic (self-sustaining) ecosystem, *C. glabrata* and *C. parapsilosis* were more. The conventional (using some chemicals) practice showed presence of *C. apicola* (Bagheri et al. 2015). Different *Candida* species were reported to be associated with distinctive wine characteristics, for instance, *C. vanderwaltii* and *C. amapae* with regional wines of Spain while *C. albidus* with Slovene wine (Chavan 2014). *C. zemplinina* is low-temperature-tolerant and osmo (high sugar)-tolerant fructophilic yeast useful in sweet wine, such as "Passito wines," production. In fermentation using dried grapes (with high sugar contents), in response to osmotic stress, *S. cerevisiae* produces more acetic acid due to upregulation of aldehyde dehydrogenase gene(s). However, in a mixed fermentation with *C. zemplinina*, acetic acid contents were reduced (Rantsiou et al. 2012). It was suggested that *C. zemplinina* consumed sugars at the beginning thus alleviating the problem of osmotic stress to *S. cerevisiae*. It was observed that in sequential inoculation (*C. zemplinina* and then after 48 h *S. cerevisiae*), the acetic acid contents were low but also ethanol and glycerol contents. While in co-inoculation with both the species in a specific proportion, the ethanol and glycerol concentrations were maintained as desired, and acetic acid contents were drastically reduced (Rantsiou et al. 2012).

It has been suggested that yeast strain and species diversity in a fermentation increases sensory complexity and chemical aroma compound diversity in the final fermented product (Carrau et al. 2015). In yeast, these compounds have role in survival, dispersion and defense strategies, and "quorum sensing" mechanisms for cell communication. While in wine fermentation, these compounds contribute immensely for aroma and flavor. Carrau et al. (2015) extensively reviewed the yeast diversity and flavor compounds. Similar to other non-*Saccharomyces* yeasts,

Candida species also has β-glycosidase activity, which is involved in flavor production. Different volatile sesquiterpenes were reported as cell-cell signaling molecules in *Candida* species: farnesol for *C. albicans* and nerolidol for *C. parapsilosis*. The terpenoids and benzyl alcohol are produced by number of yeasts including *C. stellata.* All these compounds have significant contribution in aroma and flavor.

13.7.3 *Candida* in Sugar Alcohol Production

Biodiesel, a mixture of fatty acid alkyl esters, is a product of chemical reaction between vegetable oil and animal fat with either methanol or ethanol. As a result, biodiesel industry has a glycerol as a main by-product. Yoshikawa et al. (2014a) screened different yeast species for the effective utilization of raw glycerol for mannitol production. *C. azyma* was reported to be an efficient mannitol (0.3 g/g glycerol) producer from 25% (w/v) glycerol. While *Candida quercitrusa* was reported to be the best D-arabitol producer from glycerol (Yoshikawa et al. 2014b.)

13.7.4 *Candida* in Long-Chain Dicarboxylic Acid Production

Dodecanedioic acid (*α*, *ω*-dodecanedioic acid) is one of the important precursors to produce the polyamide nylon-6,12, which is used to make heat- and chemical-resistant sheaths. This is also used in antiseptics, painting materials and coatings, corrosion inhibitors, etc. *C. tropicalis* converts petrochemical-based *n*-dodecanes to the corresponding dicarboxylic acids by targeted functionalization. Funk et al. (2017) reported transesterification of coconut oil to dodecanoic acid methyl ester by *C. tropicalis* which can further be used to produce dodecanedioic acid. Similarly Akmalina et al. (2018) reported use of *C. tropicalis* natural isolate from ambarella fruit for conversion of lauryl (dodecanoic acid) methyl ester to dodecanedioic acid. Cao et al. (2017) reported production of *α*, *ω*-dodecanedioic acid (DC_{12}) using *Candida viswanathii* strain by co-utilization of wheat straw and *n*-dodecane. The organism efficiently could utilize xylose and glucose simultaneously. Earlier Picataggio et al. (1992) engineered *C. tropicalis*, for the efficient production of long-chain dicarboxylic acids. The sequential disruption of the four genes encoding isozymes of the acyl-CoA oxidase, cytochrome P450 monooxygenase, and NADPH-cytochrome reductase was reported. The modified strain demonstrated increased omega-hydroxylase activity. It was suggested that this strain would be commercially used to produce a wide range of saturated and unsaturated dicarboxylic acids with a high degree of purity. Recently, Ibrahim et al. (2020) using CRISPR-Cas9 system genetically manipulated a lipolytic yeast *C. aseri*, which was isolated from the compost of oil palm empty fruit bunches for the production of bio-based chemicals. The acyl-CoA oxidase (AOX) mutant in which β-oxidation was blocked showed accumulation of dodecanedioic acid from dodecane.

13.7.5 Oleaginous Yeast: *Candida phangngensis*

The yeast which accumulates lipids, known as oleaginous yeast species, usually non-*Saccharomyces*, up to 20–65% of the dry weight. Lipid accumulation is triggered by nitrogen limitation along with an excess of carbon. Other than nitrogen limitation, phosphorus and sulfur-limited conditions also induce lipid accumulation. Oleaginous yeasts identified so far are *Cryptococcus curvatus*, *Lipomyces lipofer*, *Rhodotorula glutinis*, *Rhodosporidium toruloides*, *Trichosporon pullulan*, *Yarrowia lipolytica*, and a number of *Candida* species including *Candida phangngensis* too (Lamers et al. 2016; Quarterman et al. 2018). Lamers et al. (2016) screened 24 species from different genera including *Candida* species such as *C. glabrata*, *C. tropicalis*, *C. lusitaniae*, *C. bombicola*, and *C. intermedia*. However, according to them, *Schwanniomyces occidentalis* was the strain which has commercial potential to produce high amounts of lipids. While Quarterman et al. (2018) genetically engineered *C. phangngensis*, a species phylogenetically related to *Y. lipolytica* to improve lipid production using cellulose as a carbon source. To enhance in situ detoxification of aldehyde fermentation inhibitors, such as furfural generated during biomass treatment, the overexpression of *S. cerevisiae ADH6* in *C. phangngensis* was reported, while the expression of *Y. lipolytica DGA1* increased lipid accumulation in *C. phangngensis* almost 32% more than the parent strain.

13.7.6 Use of *Candida* to Develop Recombinant Strains

S. cerevisiae is one of the important organisms for ethanol production using glucose. However, it cannot use *N*-acetylglucosamine (GlcNAc, a monomner of chitin) as a sole carbon source because of absence of genes for chitin catabolism. Wendland et al. (2009) generated *S. cerevisiae* strains that could use GlcNAc as a carbon source by expressing four *C. albicans* genes encoding a GlcNAc permease, a GlcNAc kinase, a GlcNAc-6-phosphate deacetylase, and a glucosamine-6-phosphate deaminase. They further tested recombinant strains for the utilization of a GlcNAc catabolic pathway for bioethanol production. According to the investigators, this could be the first step toward the use of GlcNAc for biofuel production.

13.8 *Candida* Research: An Indian Prospective

Datta and co-workers have done pioneering work on *Candida* species in India. *C. albicans* as a model system to understand the biochemical and molecular basis of fungal morphogenesis and its correlation with the pathogenesis was the main focus initially for the research (Biswas et al. 2007). They studied the effect of various molecules, viz., serum, amino acids, amino sugars, Ca^{2+}, etc., on Y-H morphogenesis of *C. albicans*. Further, the receptors, ligands, and signaling pathways involved in sensing environmental signals were identified, and thereby it was demonstrated that these pathways play significant role in morphogenesis and pathogenesis of

C. albicans (Biswas et al. 2007). It was the basis for further studies on designing and development of new antifungal therapies to combat candidiasis. The extensive work on the *C. albicans* membrane to understand the molecular basis of drug resistance was carried out by Prasad and co-workers (Prasad et al. 2015; Prasad and Goffeau 2012). They have demonstrated the role of membrane transporter proteins belonging to the ABC (ATP-binding cassette) superfamily as a prime factor responsible for multidrug resistance in *C. albicans*. The cause-effect relationship between the ABC transporter proteins, viz., Cdr1p and Cdr2p, and efflux of antifungal drugs from inside to outside of the cells leading to MDR in *C. albicans* was also reported. Further, the role of each protein of the ABC superfamily was studied to uncover the novel mechanism of MDR in *C. albicans*. At the same time, Banerjee and co-workers studied the role of *TAC1*, a transcription activator of CDR genes, to understand the regulation of ABC transporters in *Candida* species (Jain et al. 2018). The role of membrane receptor protein Rta3 in biofilm formation by *C. albicans* and the possibility of exploring it as a target for antifungal therapy have been extensively studied by Panwar and co-workers (Srivastava et al. 2017). The focus of further research was on the role of transcription factors, mitochondria, and iron homeostasis in *C. albicans* pathogenesis (Thomas et al. 2013). The role of DNA polymerase eta (*Polh/Rad30*) in genome stability, Y-H morphogenesis, and drug sensitivity of *C. albicans* has been studied by Acharya and co-workers (Manohar et al. 2018). The role of DNA polymerase eta in serum-induced Y-H dimorphism of *C. albicans* has also been demonstrated. Further, they showed that *C. albicans* strain with catalytically inactive DNA polymerase eta was defective in hyphae formation and more sensitive to antifungal drugs. Their finding gave a new paradigm to explore new antifungal therapy. The molecular basis of glycosylphosphatidylinositol (GPI) anchor biosynthesis and Ras signaling, and its correlation with morphogenesis and pathogenesis of *C. albicans* was studied by Komath and co-workers (Yadav et al. 2014). The role of multifunctional SAGA chromatin-modifying complex in stress response of *C. albicans* is extensively studied by Natrajan and co-workers (Sinha et al. 2017). The analysis of different mutants was useful to demonstrate that the SAGA complex works in a module-specific manner that induces stress response and hyphae formation in *C. albicans*. Ganesan and co-workers dissected the molecular mechanism of amphotericin B (AmpB) resistance in *Candida* species. They found the role of *FEN1* and *SUR4* genes (coding for fatty acid elongases involved in sphingolipid biosynthetic), in AmpB resistance of *Candida* species (Bari et al. 2015). The hybrid histidine kinase 3 (HHK3), a new target for antifungal therapy, has been proposed by Mondal and co-workers (Kaur et al. 2014). The sudden rise in the incidence of non-*albicans Candida* infections posed difficulty in diagnosing and managing candidemia. In this regard, Shivaprakash and co-workers studied the epidemiology of candidiasis caused by different *Candida* species. Also, they proposed techniques such as PCR-based assays, T2 magnetic resonance, DNA microarrays, and analysis of IL-17 as a marker for their rapid diagnosis (Chakrabarti et al. 2015a, b). The *Candida* species as a model to understand the gene regulation in eukaryotes was also ventured in India. For instance, Sanyal and co-workers characterized the centromeric elements in *C. albicans* to understand the epigenetic

regulations (Thakur and Sanyal 2013). The studies were also carried out to understand the role of centromeres in the evolution of non-*albicans Candida* species (Chatterjee et al. 2016). While most Indian researchers studied various aspects of *C. albicans*, Kaur and co-workers focused on non-*albicans* pathogenic yeast *C. glabrata* (Rai et al. 2015). They elucidated the various strategies employed by *C. glabrata* to evade the host immune system. The DNA damage response in this yeast upon macrophage internalization has also been studied, and the role of histone H4 levels in this process was proposed.

13.9 Epilogue

Indeed, opportunistic fungi like *Candida* species, which are commensal to humans, have become a significant concern in human health because of the increase in immunocompromised patients for various reasons. Despite the variety of physiological distinctions, the existence of *C. albicans* and non-*albicans Candida* species has become a threat to mankind. Nevertheless, it is necessary to explore the biotechnological potential of the number of *Candida* species at a commercial level, with immediate applications like biofuel production, developing avirulent strains, or manipulating the incubation conditions that negatively affect their virulent nature.

References

Akmalina R, Purwadi R, Sitompul J (2018) Bioconversion studies of methyl laurate to dodecanedioic acid using a wild-type of *Candida tropicalis*. MATEC Web Conf 156:01001

Bagheri B, Bauer FF, Setati ME (2015) The diversity and dynamics of indigenous yeast communities in grape must from vineyards employing different agronomic practices and their influence on wine fermentation. S Afr J Enol Viticult 36:243–251

Bailey DA, Feldmann JFP, Bovey M, Gow NAR, Brown APJ (1996) The *Candida albicans HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. J Bacteriol 178:5353–5360

Bari VK, Sharma S, Alfatah M, Mondal AK, Ganesan K (2015) Plasma membrane proteolipid 3 protein modulates Amphotericin B resistance through sphingolipid biosynthetic pathway. Sci Rep 5:9685

Brown AJP, Gow NAR (1999) Regulatory networks controlling *Candida albicans* morphogenesis. Trends Microbiol 7:333–338

Brown GD, Netea MG (2012) Exciting developments in the immunology of fungal infections. Cell Host Microbe 11:422–424

Brown GD, Denning DW, Gow NAR, Levits SM, Netea MG, White TC (2012) Hidden killers: human fungal infections. Sci Transl Med 4:1–9

Biswas S, Van Dijck P, Datta A (2007) Environmental sensing and signal transduction pathways regulating morpho-pathogenic determinants of *Candida albicans*. Microbiol Mol Biol Rev 71: 348–376

Cao F, Lane S, Raniga PP, Lu Y, Zhou Z, Ramon K, Chen J, Liu H (2006) The *Flo8* transcription factor is essential for hyphal development and virulence in *Candida albicans*. Mol Biol Cell 17:295–307

Cao W, Liu B, Luo J, Yin J, Wan Y (2017) α, ω-Dodecanedioic acid production by *Candida viswanathii* ipe-1 with co-utilization of wheat straw hydrolysates and *n*-dodecane. Bioresour Technol 243:179–187

Carrau F, Boido E, Dellacassa E (2015) Yeast diversity and flavor compounds. In: Mérillon JM, Ramawat KG (eds) Fungal metabolites. Springer International Publishing, Switzerland, pp 1–29

Chakrabarti A, Sood P, Rudramurthy SM, Chen S, Kaur H, Capoor M, Chhina D, Rao R, Eshwara VK, Xess I, Kindo AJ, Umabala P, Savio J, Patel A, Ray U, Mohan S, Iyer R, Chander J, Arora A, Sardana R, Roy I, Appalaraju B, Sharma A, Shetty A, Khanna N, Marak R, Biswas S, Das S, Harish BN, Joshi S, Mendiratta D (2015a) Incidence, characteristics, and outcome of ICU-acquired candidemia in India. Intensive Care Med 41:285–295

Chakrabarti A, Sood P, Rudramurthy SM et al (2015b) Incidence, characteristics and outcome of ICU-acquired candidemia in India. Intensive Care Med 41:285–295

Chatterjee S, Alampalli SV, Nageshan RK, Chettiar ST, Joshi S, Tatu US (2015) Draft genome of a commonly misdiagnosed multidrug resistant pathogen *Candida auris*. BMC Genomics 16:686

Chatterjee G, Sankaranarayanan SR, Guin K, Thattikota Y, Padmanabhan S, Siddharthan R, Sanyal K (2016) Repeat-associated fission yeast-like regional centromeres in the ascomycetous budding yeast *Candida tropicalis*. PLoS Genet 12:e1005839

Chavan P (2014) Biochemical and microbial profiling of yeasts to identify their role in wine making. PhD thesis, Savitribai Phule Pune University, Pune, India

Chavan P, Mane S, Kulkarni G, Shaikh S, Ghormade V, Nerkar DP, Shouche YS, Deshpande MV (2009) Natural yeast flora of different varieties of grapes used for wine making in India. Food Microbiol 26:801–808

Citiulo F, Moran GP, Coleman DC, Sullivan DJ (2009) Purification and germination of *Candida albicans* and *Candida dubliniensis* chlamydospores cultured in liquid media. FEMS Yeast Res 9:1051–1060

Csank C, Haynes K (2000) *Candida glabrata* displays pseudohyphal growth. FEMS Microbiol Lett 189:115–120

Dannaoui E, Desnos-Ollivier M, Garcia-Hermoso D, Grenouillet F, Cassaing S, Baixench MT, Bretagne S, Dromer F, Lortholary O, French Mycoses Study Group (2012) *Candida* spp. with acquired echinocandin resistance, France, 2004–2010. Emerg Infect Dis 18:86–90

Dawaliby R, Trubbia C, Delporte C, Noyon C, Ruysschaert JM, Van Antwerpen P, Govaerts C (2016) Phosphatidylethanolamine is a key regulator of membrane fluidity in eukaryotic cells. J Biol Chem 291:3658–3667

De Brucker K, Bink A, Meert E, Cammue BP, Thevissen K (2013) Potentiation of antibiofilm activity of Amphotericin B by superoxide dismutase inhibition. Oxidative Med Cell Longev 2013:704654

Denning DW, Bromley MJ (2015) How to bolster the antifungal pipeline. Science 347:1414–1416

Fidel PL Jr, Vazquez JA, Sobel JD (1999) *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. Clin Microbiol Rev 12:80–96

Fitzpatrick DA, Logue ME, Stajich JE, Butler G (2006) A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. BMC Evol Biol 6:99

Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, Morschhäuser J, Rogers PD (2012) Gain-of-function mutations in UPC2 are a frequent cause of *ERG11* upregulation in azole-resistant clinical isolates of *Candida albicans*. Eukaryot Cell 11:1289–1299

Flowers SA, Colón B, Whaley SG, Schuler MA, Rogers PD (2015) Contribution of clinically derived mutations in *ERG11* to azole resistance in *Candida albicans*. Antimicrob Agents Chemother 59:450–460

Funk I, Rimmel N, Schorsch C et al (2017) Production of dodecanedioic acid via biotransformation of low cost plant-oil derivatives using *Candida tropicalis*. J Ind Microbiol Biotechnol 44:1491–1502

Gow NAR (1995) Yeast-hyphal dimorphism. In: Gow NAR, Gadd GM (eds) The growing fungus. Chapman and Hall, London, pp 403–422

Gow NAR, van de Veerdonk FL, Brown APJ, Netea MG (2012) *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. Nat Rev Microbiol 10:112–122

Gupta A, Gupta A, Varma A (2015) *Candida glabrata* candidemia: an emerging threat in critically ill patients. Indian J Crit Care Med 19:151–154

Hameed S, Fatima Z (2013) Novel regulatory mechanisms of pathogenicity and virulence to combat MDR in *Candida albicans*. Int J Microbiol 2013:240209

Han TL, Cannon RD, Gallo SM, Villas-Bôas SG (2019) A metabolomic study of the effect of *Candida albicans* glutamate dehydrogenase deletion on growth and morphogenesis. NPJ Biofilms Microbiomes 5:13

Healey KR, Jimenez Ortigosa C, Shor E, Perlin DS (2016) Genetic drivers of multidrug resistance in *Candida glabrata*. Front Microbiol 7:1995

Heard GM, Fleet GH (1985) Growth of natural yeast flora during the fermentation of inoculated wines. Appl Environ Microbiol 50:727–728

Heilmann CJ, Schneider S, Barker KS, Rogers PD, Morschhäuser J (2010) An A643T mutation in the transcription factor *Upc2p* causes constitutive *ERG11* upregulation and increased fluconazole resistance in *Candida albicans*. Antimicrob Agents Chemother 54:353–359

Höfs S, Mogavero S, Hube B (2016) Interaction of *Candida albicans* with host cells: virulence factors, host defence, escape strategies, and the microbiota. J Microbiol 54:149–169

Hube B, Naglik J (2001) *Candida albicans* proteinases: resolving the mystery of a gene family. Microbiology 147:1997–2005

Hube B, Monod M, Schofield DA, Brown APJ, Gow NAR (1994) Expression of seven members of the gene family encoding secretory aspartyl proteinase in *Candida albicans*. Mol Microbiol 14:87–99

Ibrahim ZH, Bae JH, Lee SH, Sung BH, Rashid AH, Sohn JH (2020) Genetic manipulation of a lipolytic yeast *Candida aseri* SH14 using CRISPR-Cas9 system. Microorganisms 8:526

Jacobsen ID, Wilson D, Wächtler B, Brunke S, Naglik JR, Hube B (2012) *Candida albicans* dimorphism as a therapeutic target. Expert Rev Anti-Infect Ther 10:85–93

Jain T, Muktapuram PR, Sharma K, Ravi O, Pant G, Mitra K, Bathula SR, Banerjee D (2018) Biofilm inhibition and anti-*Candida* activity of a cationic lipo-benzamide molecule with twin-nonyl chain. Bioorg Med Chem Lett 28:1776–1780

Jamai L, Sendide K, Ettayebi K, Errachidi F, Hamdouni-Alami O, Tahri-Juti MA, McDermott T, Ettayebi M (2001) Physiological difference during ethanol fermentation between calcium alginate-immobilized *Candida tropicalis* and *Saccharomyces cerevisiae*. FEMS Microbiol Lett 204:375–379

Janbon G, Quintin J, Lanternier F et al (2019) Studying fungal pathogens of humans and fungal infections: fungal diversity and diversity of approaches. Genes Immun 20:403–414

Jeffery-Smith A, Taori SK, Schelenz S, Jeffery K, Johnson EM, Borman A, Candida auris Incident Management Team, Manuel R, Brown CS (2017) *Candida auris*: a review of the literature. Clin Microbiol Rev 31:e00029–e00017

Kam AP, Xu J (2002) Diversity of commensal yeasts within and among healthy hosts. Diagn Microbiol Infect Dis 43:19–28

Kämmer P, McNamara S, Wolf T, Conrad T, Allert S, Gerwien F, Hünniger K, Kurzai O, Guthke R, Hube B, Linde J, Brunke S (2020) Survival strategies of pathogenic *Candida* species in human blood show independent and specific adaptations. mBio 11:e02435–e02420

Kaur H, Singh S, Rathore YS, Sharma A, Furukawa K, Hohmann S, Ashish, Mondal AK (2014) Differential role of HAMP-like linkers in regulating the functionality of the group III histidine kinase *DhNik1p*. J Biol Chem 289:20245–20258

Kim JY (2016) Human fungal pathogens: why should we learn? J Microbiol 54:145–148

Kim S-K, El Bissati K, Ben Mamoun C (2006) Amino acids mediate colony and cell differentiation in the fungal pathogen *Candida parapsilosis*. Microbiology 152:2885–2894

Köhler JR, Casadevall AC, Perfect J (2014) The spectrum of fungi that infects human. Cold Spring Harb Perspect Med 5:a019723

Kothavade RJ, Kura MM, Valand AG, Panthaki MH (2010) *Candida tropicalis*: its prevalence, pathogenicity and increasing resistance to fluconazole. J Med Microbiol 59:873–880

Krishnamurthy S (1999) Membrane fluidity affects functions of *Cdr1p*, a multidrug ABC transporter of *Candida albicans*. FEMS Microbiol Lett 173:475–481

Kumar A, Sachu A, Mohan K et al (2017) Simple low-cost differentiation of *Candida auris* from *Candida haemulonii* complex using CHROMagar *Candida* medium supplemented with Pal's medium. Rev Iberoam Micol 34:109–111

Lamb DC, Kelly DE, White TC, Kelly SL (2000) The R467K amino acid substitution in *Candida albicans* sterol 14alpha-demethylase causes drug resistance through reduced affinity. Antimicrob Agents Chemother 44:63–67

Lamers D, van Biezen N, Martens D, Peters L, van de Zilver E, Jacobs-van Dreumel N, Wijffels RH, Lokman C (2016) Selection of oleaginous yeasts for fatty acid production. BMC Biotechnol 16:45

Liu H, Kohler J, Fink GR (1994) Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. Science 266:1723–1726

Lo HJ, Köhler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR (1997) Non-filamentous *C. albicans* mutants are avirulent. Cell 90:939–949

Lu Y, Su C, Liu H (2014) *Candida albicans* hyphal initiation and elongation. Trends Microbiol 22:707–714

Magee PT (1997) Which came first, the hypha or the yeast? Science 277:52–53

Manohar K, Peroumal D, Acharya N (2018) TLS dependent and independent functions of DNA polymerase eta (Polη/Rad30) from pathogenic yeast *Candida albicans*. Mol Microbiol 110:707–727

Meethit P, Ratanaprasit P, Sakdaronarong C (2016) *Candida shehatae* and *Saccharomyces cerevisiae* work synergistically to improve ethanol fermentation from sugarcane bagasse and rice straw hydrolysate in immobilized cell bioreactor. Eng Life Sci 16:706–719

Mesa-Arango AC, Scorzoni L, Zaragoza O (2012) It only takes one to do many jobs: Amphotericin B as antifungal and immunomodulatory drug. Front Microbiol 3:286

Morio F, Pagniez F, Lacroix C, Miegeville M, Le Pape P (2012) Amino acid substitutions in the *Candida albicans* sterol Δ5,6-desaturase (*Erg3p*) confer azole resistance: characterization of two novel mutants with impaired virulence. J Antimicrob Chemother 67:2131–2138

Mukhopadhyay K, Kohli A, Prasad R (2002) Drug susceptibilities of yeast cells are affected by membrane lipid composition. Antimicrob Agents Chemother 46:3695–3705

Naglik JR, Challacombe SJ, Hube B (2003) *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 67:400–428

Nantel A, Dignard D, Bachewich C, Harcus D, Marcil A, Bouin A, Sensen CW, Hogues H, Hoog M, Gordon P, Rigby T, Benoit F, Tessier DC, Thomas DY, Whiteway M (2002) Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. Mol Biol Cell 13:3452–3465

O'Brien HE, Parrent JL, Jackson JA, Moncalvo JM, Vilgalys R (2005) Fungal community analysis by large-scale sequencing of environmental samples. Appl Environ Microbiol 71:5544–5550

Odds FC (1988) *Candida* and *candidosis*. Balliere Tindall, London

Pammi M, Holland L, Butler G, Gacser A (2014) *Candida parapsilosis* is a significant neonatal pathogen: a systematic review and meta-analysis. Pediatr Infect Dis J 32:1–23

Papon N, Courdavault V, Clastre M, Bennett RJ (2013) Emerging and emerged pathogenic *Candida* species: beyond the *Candida albicans* paradigm. PLoS Pathog 9:e1003550

Pathan EK, Tupe SG, Deshpande MV (2017) Fungal differentiation: a model phenomenon to screen antifungal drugs. In: Satyanarayana T, Deshmukh S, Johri B (eds) Developments in fungal biology and applied mycology. Springer, Singapore, pp 227–246

Pathan EK, Ghormade V, Panwar SL, Prasad R, Deshpande MV (2019) Molecular studies of NAD- and NADP-glutamate dehydrogenases decipher the conundrum of yeast-hypha dimorphism in zygomycete *Benjaminiella poitrasii*. FEMS Yeast Res 19:foz074

Perea S, López-Ribot JL, Kirkpatrick WR, McAtee RK, Santillán RA, Martínez M, Calabrese D, Sanglard D, Patterson TF (2001) Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob Agents Chemother 45:2676–2684

Pfaller MA, Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 20:133–163

Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Ellis D et al (2010a) Results from the ARTEMIS DISK global antifungal surveillance study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. J Clin Microbiol 48:1366–1377

Pfaller MA, Moet GJ, Messer SA, Jones RN, Castanheira M (2010b) *Candida* bloodstream infections: comparison of species distributions and antifungal resistance patterns in community-onset and nosocomial isolates in the SENTRY antimicrobial surveillance program, 2008–2009. Antimicrob Agents Chemother 55:561–566

Picataggio S, Rohrer T, Deanda K, Lanning D, Reynolds R, Mielenz J, Eirich LD (1992) Metabolic engineering of *Candida tropicalis* for the production of long-chain dicarboxylic acids. Nat Biotechnol 10:894–898

Pope LM, Cole GT (1982) Comparative studies of gastrointestinal colonization and systemic spread by *Candida albicans* and non-lethal yeast in the infant mouse. Scanning Microsc 4:1667–1676

Prasad R, Goffeau A (2012) Yeast ATP-binding cassette transporters conferring multidrug resistance. Annu Rev Microbiol 66:39–63

Prasad R, Banerjee A, Khandelwal NK, Dhamgaye S (2015) The ABCs of *Candida albicans* multidrug transporter *Cdr1*. Eukaryot Cell 14:1154–1164

Pukkila-Worley R, Peleg AY, Tampakakis E, Mylonakis E (2009) *Candida albicans* hyphal formation and virulence assessed using a *Caenorhabditis elegans* infection model. Eukaryot Cell 8:1750–1758

Quarterman JC, Slininger PJ, Hector RE, Dien BS (2018) Engineering *Candida phangngensis*: an oleaginous yeast from the *Yarrowia* clade for enhanced detoxification of lignocellulose derived inhibitors and lipid overproduction. FEMS Yeast Res 18:foy102

Rai MN, Sharma V, Balusu S, Kaur R (2015) An essential role for phosphatidylinositol 3-kinase in the inhibition of phagosomal maturation, intracellular survival and virulence in *Candida glabrata*. Cell Microbiol 17:269–287

Rantsiou K, Dolci P, Giacosa S, Torchio F, Tofalo R, Torriani S, Suzzi G, Rolle L, Cocolina L (2012) *Candida zemplinina* can reduce acetic acid produced by *Saccharomyces cerevisiae* in sweet wine fermentations. Appl Environ Microbiol 78:1987–1994

Ray TL, Payne CD (1988) Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase from protein-supplemented cultures. Infect Immun 56:1942–1949

Reedy JL, Bastidas RJ, Heitman J (2007) The virulence of human pathogenic fungi: notes from the south of France. Cell Host Microbe 2:77–83

Reyna-Beltrán E, Bazán Méndez CI, Iranzo M, Mormeneo S, Luna-Arias JP (2019) The cell wall of *Candida albicans*: a proteomics view. In: Sandai D (ed) *Candida albicans*. IntechOpen, Rijeka, Croatia. https://doi.org/10.5772/intechopen.82348

Rizzetto L, De Filippo C, Cavalieri D (2014) Richness and diversity of mammalian fungal communities shape innate and adaptive immunity in health and disease. Eur J Immunol 44:3166–3181

Sabie FT, Gadd GM (1992) Effect of nucleosides and nucleotides and the relationship between cellular adenosine 3′,5′-cyclic monophosphate (cyclic AMP) and germ tube formation in *Candida albicans*. Mycopathologia 119:147–156

Sasani E, Khodavaisy S, Agha KAS et al (2016) Pseudohyphae formation in *Candida glabrata* due to CO_2 exposure. Curr Med Mycol 2:49–52

Satoh K, Makimura K, Hasumi Y, Nishiyama Y, Uchida K, Yamaguchi H (2009) *Candida auris* sp. nov., a novel ascomycetous yeast isolated from the external ear canal of an inpatient in a Japanese hospital. Microbiol Immunol 53:41–44

Saville SP, Lazzell AL, Monteagudo C, Lopez-Ribot JL (2003) Engineered control of cell morphology *in vivo* reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. Eukaryot Cell 2:1053–1060

Scully C, el-Kabir M, Samaranayake LP (1994) *Candida* and oral candidosis: a review. Crit Rev Oral Biol Med 5:125–157

Shariq M, Sohail M (2019) Application of *Candida tropicalis* MK-160 for the production of xylanase and ethanol. J King Saud Univ Sci 31:1189–1194

Shepherd MG, Yin YC, Ram SP, Sullivan PA (1980) Germ tube induction in *Candida albicans*. Can J Microbiol 26:21–26

Shin JH, Kim MN, Jang SJ, Ju MY, Kim SH, Shin MG, Suh SP, Ryang DW (2012) Detection of Amphotericin B resistance in *Candida haemulonii* and closely related species by use of the Etest, Vitek-2 yeast susceptibility system, and CLSI and EUCAST broth microdilution methods. J Clin Microbiol 50:1852–1855

Silva S, Negri M, Henriques M, Oliveira R, Williams DW, Azeredo J (2012) *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: biology, epidemiology, pathogenicity and antifungal resistance. FEMS Microbiol Rev 36:288–305

Sinha I, Kumar S, Poonia P, Sawhney S, Natarajan K (2017) Functional specialization of two paralogous *TAF12* variants by their selective association with SAGA and TFIID transcriptional regulatory complexes. J Biol Chem 292:6047–6055

Srivastava A, Sircaik S, Husain F, Thomas E, Ror S, Rastogi S, Alim D, Bapat P, Andes DR, Nobile CJ, Panwar SL (2017) Distinct roles of the 7-transmembrane receptor protein Rta3 in regulating the asymmetric distribution of phosphatidylcholine across the plasma membrane and biofilm formation in *Candida albicans*. Cell Microbiol 19:10

Stewart E, Gow NAR, Bowen DV (1988) Cytoplasmic alkalinization during germ tube formation in *Candida albicans*. J Gen Microbiol 134:1079–1087

Stoldt VR, Sonneborn A, Leuker CE, Ernst JF (1997) *Efg1p*, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J 16:1982–1991

Sun M, Chen C, Xiao W, Chang Y, Liu C, Xu Q (2019) Increase in *Candida Parapsilosis* candidemia in cancer patients. Mediterr J Hematol Infect Dis 11:e2019012

Tanimura A, Nakamura T, Watanabe I, Ogawa J, Shima J (2012) Isolation of a novel strain of *Candida shehatae* for ethanol production at elevated temperature. SpringerPlus 1:27

Thakur J, Sanyal K (2013) Efficient neocentromere formation is suppressed by gene conversion to maintain centromere function at native physical chromosomal loci in *Candida albicans*. Genome Res 23:638–652

Thomas E, Roman E, Claypool S, Manzoor N, Pla J, Panwar SL (2013) Mitochondria influence CDR1 efflux pump activity, Hog1-mediated oxidative stress pathway, iron homeostasis, and ergosterol levels in *Candida albicans*. Antimicrob Agents Chemother 57:5580–5599

Trofa D, Gacser A, Nosanchuk JD (2008) *Candida parapsilosis*, an emerging fungal pathogen. Clin Microbiol Rev 21:606–625

Turner SA, Butler G (2014) The *Candida* pathogenic species complex. Cold Spring Harb Perspect Med 4:a019778

Vallabhaneni S, Kallen A, Tsay S et al (2017) Investigation of the first seven reported cases of *Candida auris*, a globally emerging invasive, multidrug-resistant fungus-United States, May 2013–August 2016. Am J Transplant 17:296–299

van Asbeck EC, Clemons KV, Stevens DA (2009) *Candida parapsilosis*: a review of its epidemiology, pathogenesis, clinical aspects, typing and antimicrobial susceptibility. Crit Rev Microbiol 35:283–309

Walker LA, Gow NA, Munro CA (2013) Elevated chitin content reduces the susceptibility of *Candida* species to caspofungin. Antimicrob Agents Chemother 57:146–154

Wang X, Bing J, Zheng Q, Zhang F, Liu J, Yue H, Tao L, Du H, Wang Y, Wang H, Huang G (2018) The first isolate of *Candida auris* in China: clinical and biological aspects. Emerg Microbes Infect 7:93

Wendland J, Schaub Y, Walther A (2009) *N*-acetylglucosamine utilization by *Saccharomyces cerevisiae* based on expression of *Candida albicans NAG* genes. Appl Environ Microbiol 75:5840–5845

Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD (2017) Azole antifungal resistance in *Candida albicans* and emerging non-*albicans Candida* species. Front Microbiol 7:2173

Wu Y, Totten M, Memon W, Ying C, Zhang SX (2020) *In vitro* antifungal susceptibility of the emerging multidrug-resistant pathogen *Candida auris* to Miltefosine alone and in combination with Amphotericin B. Antimicrob Agents Chemother 64:e02063–e02019

Xiang MJ, Liu JY, Ni PH et al (2013) *Erg11* mutations associated with azole resistance in clinical isolates of *Candida albicans*. FEMS Yeast Res 13:386–393

Yadav A, Singh SL, Yadav B, Komath SS (2014) *Saccharomyces cerevisiae Gpi2*, an accessory subunit of the enzyme catalyzing the first step of glycosylphosphatidylinositol (GPI) anchor biosynthesis, selectively complements some of the functions of its homolog in *Candida albicans*. Glycoconj J 31:497–507

Yang B, Rao R (2018) Emerging pathogens of the *Candida* species. In: Sandai D (ed) *Candida albicans*. IntechOpen, Rijeka, Croatia. https://doi.org/10.5772/intechopen.80378

Yang L, Weil T, Cavalieri D (2015) Systems level dissection of *Candida* recognition by dectins: a matter of fungal morphology and site of infection. Pathogens 4:639–661

Yoshikawa J, Habe H, Morita T, Fukuoka T, Imura T, Iwabuchi H, Uemura S, Tamura T, Kitamoto D (2014a) Production of mannitol from raw glycerol by *Candida azyma*. J Biosci Bioeng 117:725–729

Yoshikawa J, Habe H, Morita T, Fukuoka T, Imura T, Iwabuchi H, Shingo U, Tkamitsu T, Dai K (2014b) Production of D-arabitol from raw glycerol by *Candida quercitrusa*. Appl Microbiol Biotechnol 98:2947–2953

You L, Qian W, Yang Q, Mao L, Zhu L, Huang X, Jin J, Meng H (2017) *ERG11* gene mutations and *MDR1* upregulation confer pan-azole resistance in *Candida tropicalis* causing disseminated candidiasis in an acute lymphoblastic leukemia patient on posaconazole prophylaxis. Antimicrob Agents Chemother 61:e02496–e02416

Zuza-Alves DL, Silva-Rocha WP, Chaves GM (2017) An update on *Candida tropicalis* based on basic and clinical approaches. Front Microbiol 8:1927

Research Contributions from India on Membrane-Modifying Peptides: Motivations from Fungal Peptaibiotics

14

Varatharajan Sabareesh and Gurunath Ramanathan

Abstract

Peptaibiotics are non-ribosomally synthesized linear peptides that are secondary metabolites of mainly fungal origin. They contain unusual non-protein amino acids, e.g. α-aminoisobutyric acid (*Aib*) besides possessing modified amino and carboxyl termini. These peptides are capable of altering cell membrane permeability by forming transmembrane voltage-gated ion channels. In India, much of this research was done in Prof. P. Balaram's group at the Indian Institute of Science, Bengaluru. Initially, peptides based on *alamethicin*'s sequence were chemically synthesized and their biophysical functions were studied. Further, using fluorescence spectroscopy, aggregation behaviour of synthetic *emerimicin* and *alamethicin* fragments in solution was investigated. Subsequently, solution-phase and solid-state conformations of synthetic fragments of *suzukacillin*, *zervamicin* and *trichogin* were probed by circular dichroism, nuclear magnetic resonance spectroscopy and X-ray crystallography. Notably, full-length natural [*Leu*-1]*zervamicin* and *antiamoebin I* were isolated, and their three-dimensional molecular structures were determined by X-ray crystallography. In the latter half of the 1990s, mass spectrometry (MS) was employed to delineate the mechanism of *efrapeptin* biosynthesis. Tandem MS was applied to obtain insights into the microheterogeneous *trichotoxin* sequences. A strategy based on tandem MS was devised to distinguish isobaric residues, leucine and isoleucine in *zervamicin* and

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antiamoebin. Recently, other groups from India have discovered some new fungal metabolites that belong to this class of peptides.

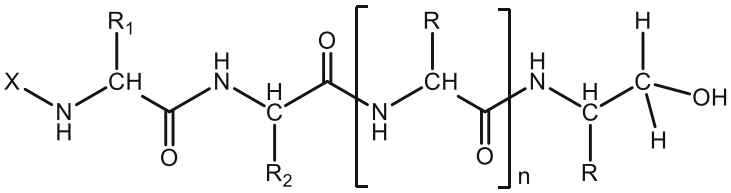
Keywords

Fungal peptides · Channel-forming peptides · Peptaibiotics · Peptaibols · *Zervamicin* · *Antiamoebin* · *Trichogin A IV* · *Efrapeptins*

14.1 Introduction

14.1.1 Molecular Properties

Linear peptides mainly of fungal origin that are secreted as secondary metabolites often have a high content of a non-proteinogenic amino acids such as alpha-aminoisobutyric acid (*Aib*). They exhibit antibacterial and antifungal activity. These molecules are called as *peptaibiotics* (Toniolo and Brückner 2007). Several of these peptides were found to possess membrane-modifying activities (Mueller and Rudin 1968; Sansom 1993a, b). These peptides also possess modified amino (*N*-) and carboxyl (*C*-) termini. A major class of *peptaibiotics*, which have *N*-terminus 'acetyl' group modification and *C*-terminus alcohol (2-amino alcohol), is classified as *peptaibols* (Krause et al. 2006). Another class of *peptaibiotics* comprises of *lipopeptaibols*, which possess *N*-terminus acyl modification, e.g. *N*-terminus contains an octanoyl group, etc. (Auvin-Guette et al. 1992, 1993; Rowley et al. 2003; Singh et al. 2018). A representation of the general molecular structure of *peptaibols* is illustrated in Fig. 14.1, where 'X' denotes *N*-terminus acyl modification, e.g. acetyl, octanoyl, etc. Other than the *C*-terminus alcohol group, several *peptaibiotics* also possess *C*-terminus amine or amide functional groups, for instance, *leucinostatins*, *efrapeptins* and *trichopolyns* (Momose et al. 2019; Stoppacher et al. 2013; Degenkolb et al. 2003). This chapter will describe about different *peptaibol*-like peptides, such as synthetic peptide esters possessing protected *N*-termini and some natural *peptaibols*/*peptaibiotics* that have been studied in India (Table 14.1). In addition to *Aib*, another α,α-dialkyl α-amino acid commonly found in several *peptaibols*/*peptaibiotics* is isovaline (ethylalanine). This chiral amino acid has been found to occur in both its *L*- and *D*-stereochemistry, though the occurrence of *D-Iva* predominates (Degenkolb et al. 2003; Raap et al. 2005). Figure 14.2 shows the structures of three different α,α-dialkyl α-amino acids and their respective residues, wherein ethylnorvaline is an unusual case, which was discovered in *lipopeptaibols* isolated from a fungus *Tolypocladium geodes* (Tsantrizos et al. 1996). Some of the widely found *C*-terminal alcohol groups in many *peptaibols*/*peptaibiotics* are valinol, leucinol and phenylalaninol, whose structures are shown in Fig. 14.3. Proteinogenic amino acids that frequently occur in numerous *peptaibols*/*peptaibiotics* are shown in Fig. 14.4. Asparagine, glutamine, glutamic acid, threonine and hydroxyproline are some polar proteinogenic amino acids present in several *peptaibols*. Table 14.2 shows the sequences of the *peptaibol-*



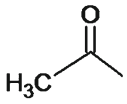
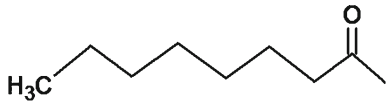
Structure of 'X'	Name of the N-terminus modifying group
	**Acetyl** (43.018 Da)
	**n-Octanoyl** (127.112 Da)

Fig. 14.1 General molecular structure of *peptaibol*. 'X' represents 'acyl' or 'fatty acyl' modification. 'R' represents the amino acid side chain. The stereochemistry is omitted for better clarity. The value of n can be $1 \leq n \leq 18$. A few examples of 'X' are also shown. The values in the parenthesis are monoisotopic masses expressed in Daltons (Da) rounded off to three decimal places

like peptides and natural *peptaibols*/*peptaibiotics*, which have been investigated and identified in India.

Peptaibols are capable of modifying the membrane properties of cells by inserting into the lipid bilayer structure forming transmembrane ion channels or pores (Nagaraj and Balaram 1981b; Balaram et al. 1992). Within the lipid bilayer environment of the cell membrane, a few *peptaibol* monomers (e.g. ca. 6–8 molecules) aggregate leading to the formation of a pore-like structure, through which cation translocation is facilitated (Nagaraj and Balaram 1981b; Mathew et al. 1982; Sansom 1993b). Such channels/pores are known to get activated in the presence of electric fields, and hence they are referred to as voltage-dependent ion channels (Sansom 1993b).

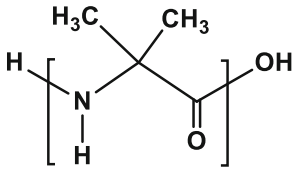
Thus far, more than 700 different *peptaibols* have been identified, and many of those were discovered from the fungus belonging to the genus *Trichoderma* (Stoppacher et al. 2013). The lengths of the *peptaibols* vary (4–21 residues) including the *C*-terminus alcohol residue. Majority of the *peptaibols* are 11 residues long. Twenty residues long *peptaibols* constitute the second-populous category, and there is a significant number of *peptaibols* that contain 18, 19 and 14 residues as well (Stoppacher et al. 2013).

Biosynthesis of *peptaibols* is primarily mediated by non-ribosomal peptide synthetases (NRPSs), which are large multidomain/multi-modular enzymes (Kleinkauf and Von Döhren 1996; Marahiel et al. 1997). A characteristic feature of *peptaibols* (and several other related classes of *peptaibiotics*) is that they are

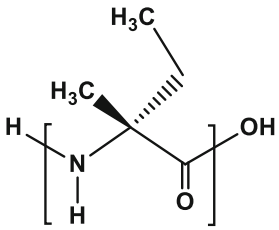
Table 14.1 Different types of investigations carried out on *peptaibol*-like peptides and natural *peptaibols*/*peptaibiotics* in India: an overview

S. no.	Name of peptaibol/ peptaibiotic[a]	Nature of studies	References
1.	***Emerimicin*** Synthetic fragments	Aggregation studies and mitochondrial uncoupling activity	Nagaraj and Balaram (1979), Raj et al. (1988)
2.	***Alamethicin*** Synthetic fragments	Divalent cation permeability activity and ion channel-forming property	Nagaraj et al. (1980), Mathew et al. (1981, 1982), Nagaraj and Balaram (1981a)
3.	***Suzukacillin*** Synthetic fragments	Structural characterization[b]	Iqbal and Balaram (1981a, b), Iqbal and Balaram (1982), Francis et al. (1982a, b, 1983)
4.	***Antiamoebin*** Natural full form and its micro heterogeneous components	Structural characterization[b], mitochondrial uncoupling and Ca^{2+} transport activities	Das et al. (1986, 1988), Karle et al. (1998), Gupta et al. (2012)
5a.	***Zervamicin IIA*** (1) Synthetic fragment analogue (ten residues) (2) Full-length synthetic analogue (16 residues)	Structural characterization[b], ion channel-forming activity of 16 residues synthetic analogue	Karle et al. (1986, 1987), Balaram et al. (1992)
5b.	***Zervamicins IIB and IC*** Natural full-length forms	Ion channel-forming activity and tandem MS studies	Agarwalla et al. (1992), Balaram et al. (1992), Gupta et al. (2012)
5c.	***[Leu^1]zervamicin*** or ***zervamicin Z-L*** Natural full length	Structural characterization[b] and ion channel formation activity	Karle et al. (1991, 1994), Agarwalla et al. (1992), Balaram et al. (1992)
6.	***Trichogin A IV*** and **its synthetic fragments**	Structural characterization[b]	Gurunath and Balaram (1995)
7.	***Efrapeptins***[a] Natural full length	Biosynthetic mechanistic studies and antimalarial activity	Uma et al. (2001), Nagaraj et al. (2001)
8.	***Trichotoxins*** Natural full length	Tandem MS studies	Sabareesh and Balaram (2006)
9.	***Lipovelutibols*** Natural full length	Identification from fungus in Himalayan regions and cytotoxic activity in cancer cell lines	Singh et al. (2018)
10.	***Velutibols*** Natural full length	Identification from fungus in Himalayan regions and cytotoxic activity in cancer cell lines	Singh et al. (2020)

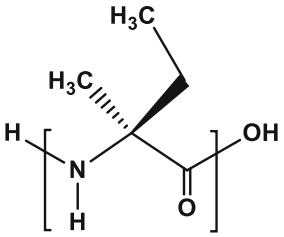
[a]*Peptaibiotics*
[b]X-ray crystallography and/or NMR spectroscopy



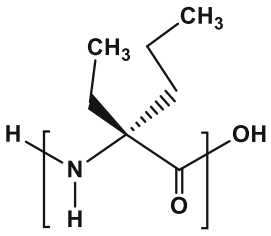
α-aminoisobutyric acid (Aib*) : α
(85.053 Da)



R-Isovaline (*D*-Iva) : ɪ†
(99.068 Da)



S-Isovaline (*L*-Iva) : ɪ†
(99.068 Da)



R-Ethylnorvaline : Z
(127.100 Da)

Fig. 14.2 Non-proteinogenic α,α-dialkyl α-amino acids in *peptaibols/peptaibiotics*. (Portion of structure within the square bracket is called 'residue or amino acid residue'). *α is used as the single letter code to denote *Aib* all through this chapter, as the letter U has been denoted for selenocysteine by IUPAC-IUB. We suggest the use of Greek letters β for beta alanine, γ for gamma amino butyric acid (GABA) and ɪ for isovaline, which are some amino acids found in nature, but not in proteins. †Prevalence of *D*-Iva is greater than *L*-Iva (Degenkolb et al. 2003; Raap et al. 2005). Note: Residue mass = (amino acid molecular mass − 18.011 Da) is shown in parenthesis below each structure. These are monoisotopic masses rounded off to three decimal places

almost always produced as a mixture of isoforms; thus, they are often called as 'microheterogeneous sequences'. It has been hypothesized that lack of selectivity or decreased selectivity of one module or a few modules in the NRPSs might be the reason for the observation of 'microheterogeneity' in various *peptaibol* sequences and a few experiments seem to provide support for this hypothesis (Raap et al. 2005). Nevertheless, further research into NRPS catalysed synthesis is required to delineate the underlying mechanisms that are responsible for the formation of microheterogeneous sequences.

14.1.2 Analytical Techniques

Various kinds of analytical techniques have been applied to study diverse aspects of *peptaibols*. The ability of *peptaibols* to form transmembrane ion channels kindled interests to determine their three-dimensional (3D) molecular structure, particularly in a membrane or micellar environments, to correlate their 3D structure to the

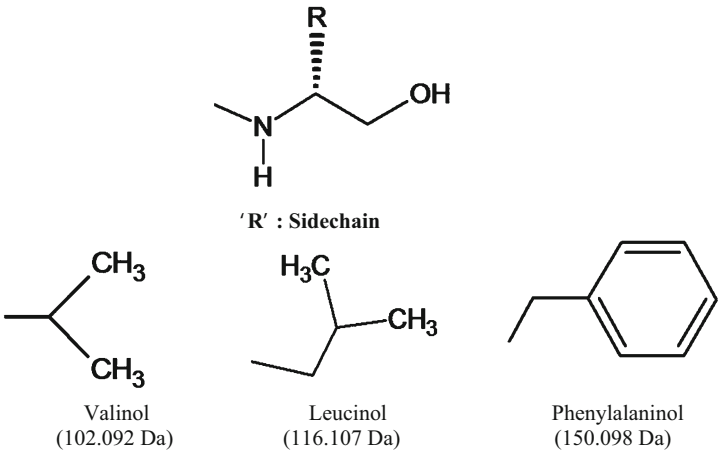
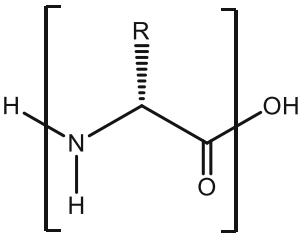


Fig. 14.3 Commonly found '*C*-terminus amino alcohol residues' in *peptaibols*. Monoisotopic 'residue' masses rounded off to three decimal places are shown in parenthesis

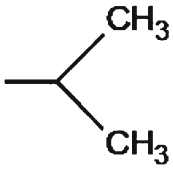
property of ionic conductance across the membranes. Elucidation of the mechanism (s) of ion transport across the membrane by the *peptaibols* was the focus of this field. So, X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy were employed extensively to determine 3D molecular structures of *peptaibols* in different environments (Fox and Richards 1982; Karle et al. 1998; Anders et al. 2000; Du et al. 2017). Circular dichroism (CD) spectroscopy also played a crucial role in determining the secondary structures adopted by the *peptaibols* in various solvent environments (Gurunath and Balaram 1995). High-performance liquid chromatography (HPLC), principally reverse phase (RP)-HPLC, has been applied extensively to isolate and purify natural *peptaibols* from the fungal cultures (Brückner and Przybylski 1984; Krishna et al. 1990) because the *peptaibols* are mostly hydrophobic (Figs. 14.2, 14.3, and 14.4). However, RP-HPLC fractionation might not be useful to separate some components, since the polar or non-polar character of microheterogeneous *peptaibols* may not be drastically different. But, if there are differences in the molecular masses of such microheterogeneous *peptaibols*, then they can be distinguished by mass spectrometry (MS). Mass spectrometric methods can provide evidence for the presence of microheterogeneous sequences (Brückner and Przybylski 1984; Przybylski et al. 1984). In particular, mass spectral fragmentation patterns can be useful to find those specific regions of the sequences that are microheterogeneous (Pandey et al. 1977a, b, c; Brückner et al. 1979; Rinehart Jr. et al. 1981; Jaworski and Brückner 1999; Sabareesh and Balaram 2006). Thus, in recent years, mass spectrometric experiments have been directly conducted on crude extracts or on whole cells, with limited or no chromatographic purification. In this context, HPLC particularly RP-HPLC, coupled to electrospray ionization mass spectrometry, viz. LC-ESI-MS, has been applied to identify and 'analytically' characterize several *peptaibols* from diverse fungal species (Poirier et al. 2007; Stoppacher et al. 2007). With regard to the direct mass spectral analysis of fungal



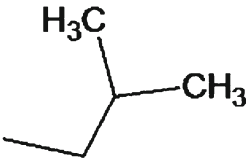
'R' : Sidechain

Glycine: Gly (G)
(57.021 Da)

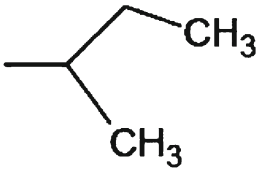
L-Alanine: Ala (A)
(71.037 Da)



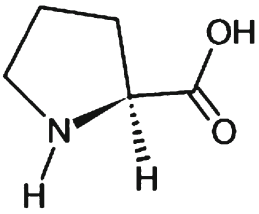
L-Valine: Val (V)
(99.068 Da)



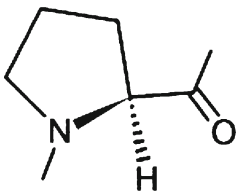
L-Leucine: Leu (L)
(113.084 Da)



L-Isoleucine: Ile (I)
(113.084 Da)



L-Proline : Pro (P)
(115.063 Da)



L-Proline Residue (P)
(97.053 Da)

Fig. 14.4 Proteinogenic *L*-amino acids commonly found in *peptaibols*/*peptaibiotics*. (Portion of structure within the square bracket is called 'residue or amino acid residue'). Below each structure, monoisotopic residue mass is shown in parenthesis (refer footnote of Fig. 14.2). Since proline is an imino acid, both its complete structure as well as the structure of its residue are shown

Table 14.2 Sequences of *Peptaibol*-like peptides and natural *peptaibols/peptaibiotics* studied in India

S. No.	Name of Peptaibol / Peptaibiotic[a]	Name of the Fungi	Sequences
1.	***Emerimicin***[b] Synthetic fragments	*Emericellopsis microspora* (*E. microspora*)	1 3 5 9 Dansyl - F α α α V G L α α – OMe
2.	***Alamethicin***[c] Synthetic fragments	*Trichoderma viride* (*T. viride*)	1 6 10 13 17 Dansyl - α P α A α A Q α V α G L α P V α α - OBz
3.	***Suzukacillin***[d] Synthetic fragments	*T. viride*	Boc - α P V α V A α A α α - OMe (1 - 10) Boc - Q α L α G L α P V α α - OMe (11 - 21) Boc - A α α Q α L α G L α P V α α - OMe (8 - 21) Boc - A α A α α Q α L α G L α P V α α - OMe (6 - 21)
4.	***Antiamoebin I*** Natural full form & microheterogenous components	*E. poonensis* Thirum., *E. synnematicola* Mathur and Thirum., *Cephalosporium pimprina* Thirum.	Ac - F α α α ι G L α α O Q ι O α P F - CH_2OH
5a.	***Zervamicin IIA***[e] Synthetic fragment Analogues	*E. salmosynnemata*	1 10 16 Boc - W I A α I V α L α P A α P α P F – OMe ↑ ↑ ↑
5b.	***Zervamicins IIB & IC*** Natural Full Length forms	*E. salmosynnemata*	3 **IIB:** Ac - W I **Q** ι I T α L α O Q α O α P F - CH_2OH **IC:** **E**
5c.	***[Leu¹]Zervamicin***[f] Natural Full Length	*E. salmosynnemata*	1 Ac - **L** I Q ι I T α L α O Q α O α P F - CH_2OH

Table 14.2 (continued)

S. No.	Name of Peptaibol / Peptaibiotic[a]	Name of the Fungi	Sequences
6.	***Trichogin A IV*** Synthetic fragments	*T. longibrachiatum*	Boc - G G L α G I L - OMe
7.	***Efrapeptins***[a] Natural Full Length	*Tolypocladium niveum*	**C:** Ac – Pip α Pip **α** α L β G α α Pip α **G** L **α** – DBN **D:** **ι** **E:** **ι** **ι** **F:** **A** **ι** **G:** **ι** **A** **ι**
8.	***Trichotoxins*** Natural Full Length	*T. viride*	**A_50_I:** Ac – α **G** α L α **Q** α α α A α α P L α **ι** **Q** V - CH_2OH **A_40_Va:** **A** **α** **E** **New:** **A** **E** **α** **Q**
9.	***Lipovelutibols*** Natural Full Length	*T. velutinum*	**A:** Oct – G A L **α** **S** I L - CH_2OH **B:** **ι** **C:** **A** **D:** **ι** **A**
10.	***Velutibols*** Natural Full Length	*T. velutinum*	**A:** Ac – **α** Q **L** α P V **L** α P α α α P L - CH_2OH **B:** **I** **C:** **I** **D:** **V** **Lxx** **Lxx**

[a]Peptaibiotics
[b]Synthetic fragments: 1–3 and 1–5
[c]Synthetic fragments: 1–6, 1–10, 1–13 and 1–17
[d]Synthetic fragments: 1–5, 6–10 and 16–20
[e]Gln-3, Thr-6 and Hyp-10 (marked with ↑) of native *zervamicin IIA*, replaced with Ala-3, Val-6 and Pro-10, respectively; synthetic fragment analogue, 1–10 and synthetic analogue, 1–16
[f]*Zervamicin Z-L*
Boc, tertiary-butyloxycarbonyl (*N*-terminus); Ac, acetyl (*N*-terminus); OBz, benzyl ester (*C*-terminus); OMe, methyl ester (*C*-terminus); –CH_2OH, *C*-terminus alcohol; F, phenylalanine; O, hydroxyproline (Hyp); W, tryptophan (Trp); Q, glutamine (Gln); E, glutamic acid (Glu); T, threonine (Thr); Thirum., Thirumalachar; Pip, pipecolic acid; DBN, 1,5-diazabicyclo [4:3:0] nonene; –CH2OH, *C*-terminus alcohol; Oct, octanoyl (N-terminus); Lxx, Leu or Ile

cells, matrix-assisted laser desorption/ionization (MALDI) has been quite fruitful, which is referred as intact cell mass spectrometry (ICMS) or IC-MALDI-MS (Fenselau and Demirev 2001; Neuhof et al. 2007; Shishupala 2009; Sharma et al. 2016; Patel 2019; Katoch et al. 2019).

14.1.3 *Peptaibol*/*Peptaibiotic* Research in India

Peptaibol research in India was ignited by the discovery of *peptaibols* in the laboratory of Dr. M. J. Thirumalachar at the Hindustan Antibiotics Ltd (Thirumalachar 1968). It was subsequently continued by Prof. P. Balaram at the Indian Institute of Science (IISc), Bengaluru. The initial investigations were focused on membrane perturbation studies by *peptaibols*, to understand the molecular structural determinants that a *peptaibol* needed to possess to perform ionophoric activity within the cellular membrane. This study began by choosing '*Alamethicin*' (20 residue long *peptaibol*) as a model since electrical conductance properties of alamethicin in black lipid membranes were already reported (Mueller and Rudin 1968; Boheim and Kolb 1978). Subsequently, Balaram's group began to carry out studies on other *peptaibols* such as *suzukacillin*, *antiamoebins*, *zervamicins*, *trichogin A IV*, *efrapeptins* and *trichotoxins*. Correspondingly, the research objectives of his group also expanded and diversified, for which a variety of analytical and experimental approaches encompassing chemical synthesis, HPLC, different spectroscopic techniques (infrared (IR), CD and NMR), X-ray crystallography and mass spectrometry have been applied thoroughly. Recently, fungi existing in the cold habitats of Himalayan region have been explored in search of *peptaibols*, by a group of scientists led by Dr. Ram A. Vishwakarma and Dr. Deepika Singh, at the CSIR-Indian Institute of Integrative Medicine (CSIR-IIIM), Jammu. Their efforts have resulted in the identification of a few novel *lipopeptaibols* as well as *peptaibols*, which elicit cytotoxic activities. The ensuing sections of this review will discuss various details on *peptaibol*-like peptides and conventional *peptaibols*/*peptaibiotics*, mainly focusing on the contributions from India.

14.2 Chemical Synthesis and Molecular Structural Characterization

14.2.1 *Peptaibol* Synthesis

Peptaibol sequences contain a liberal spread of non-protein amino acids like *Aib*, *Iva* with the 'normal' protein amino acids with the *C*-terminus ending as an alcohol group. This poses unusual problems in peptide synthesis. While the *C*-terminal functionality can often be obtained by reduction of a protecting ester group, the coupling of various residues itself is fraught with some difficulties—some of which can be overcome by choosing a careful synthetic strategy.

In India, mostly solution-phase strategies have been used to achieve the synthesis of *peptaibols* and their analogues. The solution-phase synthesis has primarily used ^{t}Boc/ Fmoc protection at the *N*-terminus and methyl ester functionality at the *C*-terminus. The final protected peptide is suitably deprotected and reduced to get to the final product. The first solution-phase attempt to synthesize *Alamethicin* was way back in the 1980s when Nagaraj and Balaram got 350 mg from a fragment condensation approach (Nagaraj and Balaram 1981a). Even single crystals were obtained of several fragments and that of the final synthesized peptide in this report.

The synthesis by solution phase of these peptides has several associated problems that are listed below. The strategies employed by various research groups to circumvent them are also discussed.

(a) Lower yields in couplings involving hindered Aib/Iva residues: $C^{\alpha,\alpha}$-dialkylated amino acids are notorious in giving abysmal yields often due to steric hindrance due to the presence of (Leplawy et al. 1960; Jones et al. 1965; Goodman and McGahren 1967) geminal dimethyl groups. A second contributing factor to low yields is often the secondary structures imposed by these hindered residues (Venkatraman et al. 2001). These not only cause aggregation of peptides in organic solvents but also have low solubility in polar solvents. These can be overcome by using soluble carbodiimide couplings in the presence of 1-hydroxybenzotriazole (HOBt) instead of the traditional dicyclohexylcarbodiimide (DCC/HOBt). Subsequent synthesis even used anhydride coupling methods (Gurunath Ramanathan 1994).
(b) Racemization at chiral residues: To overcome racemization at chiral residues, which resulted from long reaction times (sometimes extending between 5 and 7 days) and to obtain greater than 75% yields in coupling—one often employed racemization minimizing synthetic strategies. The *Aib* or *Iva* residue was almost always kept as the C-terminal residue in these couplings. These gave satisfactory yields in synthesis (Gurunath Ramanathan 1994; Gurunath and Balaram 1995).
(c) Longer coupling times involving hindered residues like *Aib*/*Iva*.
(d) The final step of obtaining the *C*-terminal alcohol with modification of the N-terminus with an acyl (or acetyl) group as warranted.

For instance, the solution-phase synthesis of the *lipopeptaibol* fragment of *Trichogin A IV* was attempted using the strategy outlined in Fig. 14.5 (Gurunath Ramanathan 1994).

Some researchers in India focused only on methodology development for the synthesis of *peptaibols*. For instance, the group of Dr. Suresh Babu at Central College in Bangalore University developed methods of making peptide alcohols (Babu et al. 2006; Prabhu et al. 2015). The group of H. N. Gopi at IISER Pune used sodium borohydride reduction of *N*-hydroxy succinamide esters to obtain high yields of racemization free products (Jadhav et al. 2011). However, these groups have as yet not reported a total synthesis of a natural *peptaibol* (personal communication) (Fig. 14.6).

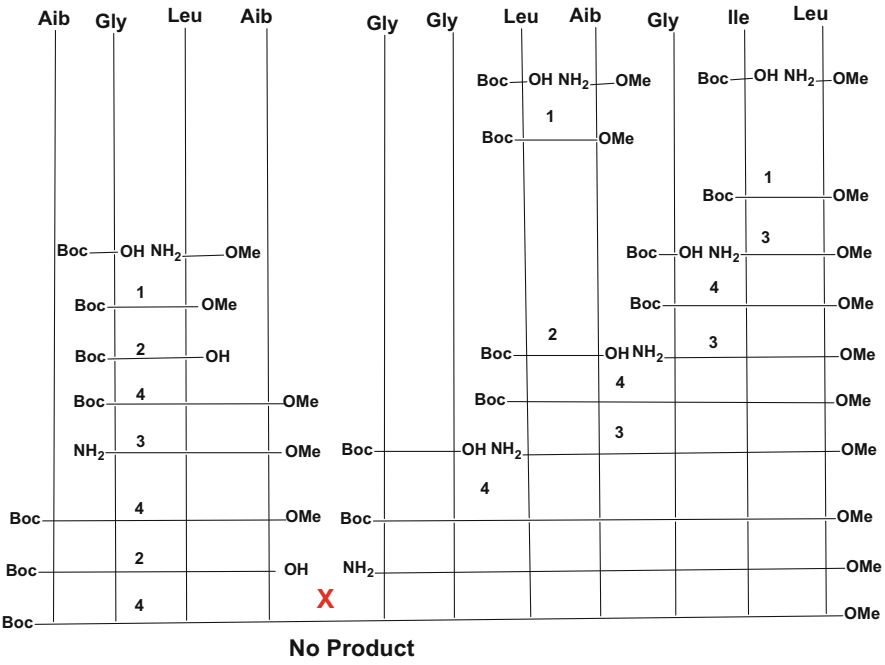


Fig. 14.5 Strategy followed for solution-phase synthesis of *Trichogin A IV*. The final coupling attempt was not successful (see text for details)

The first solid-phase peptide synthesis of a *peptaibol* was attempted in India by one of the authors in the laboratory of Prof. P. Balaram at IISc, Bangalore, as a graduate student (Gurunath Ramanathan 1994). The synthesis of *Trichogin A IV*, an 11 residue *lipopeptaibol* was attempted by solution-phase method as well as by solid-phase method. But, the *N*-terminal tetrapeptide fragment could not yield the 11 mer on DCC coupling (Fig. 14.5). The synthetic strategy was quickly changed to solid-phase on an LKB-Biolynx peptide synthesizer using the Fmoc strategy on a polyamide resin using a continuous flow protocol. The coupling of *Aib* residues was done as an anhydride using fourfold excess to achieve efficient couplings. The coupling reactions were monitored by Kaiser test, and the octonoylation was done on the resin using the anhydride method. A negative Kaiser test confirmed acylation. The product was completely characterized by MS, ^{1}H and ^{13}C NMR spectroscopy. Reduction of the peptide-resin ester linkage using known protocols at that time did not yield the final *peptaibol*. Subsequently, the peptide acid was isolated and converted into a methyl ester by reaction with diazomethane. The methyl ester product was finally reduced in cold lithium borohydride to yield the *peptaibol* antibiotic on a 2 mg scale. However, one could only obtain sufficient amount to characterize the product by HPLC, MS and NMR. As a result, conformational studies were restricted to the peptide acid.

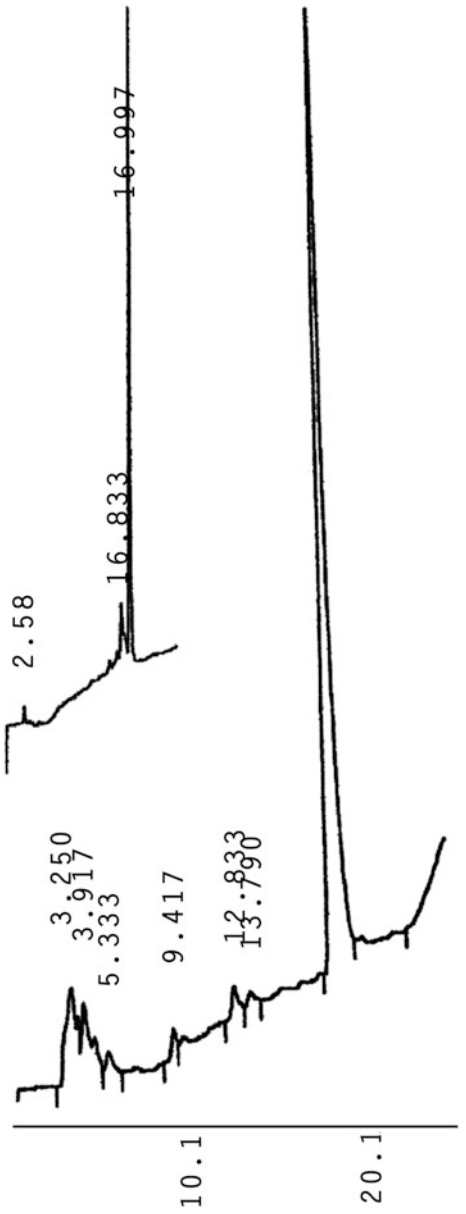


Fig. 14.6 HPLC trace of purified *Trichogin A IV* synthesized by the solid-phase peptide synthesis protocol; inset: HPLC trace of the Trichogin A IV acid, cleaved from the resin. The HPLC was run on a LiChrosorb C-18 analytical column (5μm, 4 × 250 mm) on a Shimadzu HPLC, following a linear gradient of 80–95% methanol-water in 15 min, followed by holding the composition of mobile phase at 95% for 10 min, when the peptide eluted out

14.2.2 Nuclear Magnetic Resonance (NMR) Studies

Peptaibol research in India mostly involved synthesis, purification and spectroscopic characterization utilizing either NMR or circular dichroic spectroscopy

(Venkatraman et al. 2001). NMR spectroscopy was also used for characterizing synthesized peptides and studying its conformation in the solution state (Iqbal and Balaram 1982; Gurunath Ramanathan 1994).

In this section, we will discuss the spectroscopic techniques used and the way these investigations evolved vis-à-vis in *peptaibol* research. NMR spectroscopy was usually used at every stage of the *peptaibol* synthesis to characterize the products. Initially, only 60 or 80 MHz instruments were available in India, and hence mostly either solvent titration techniques or variable temperature NMR along with one-dimensional nuclear Overhauser effect (NOE) experiments were performed.

With the availability of modern NMR spectrometers in the late 1980s and early 1990s, the one-dimensional experiments gradually evolved to more modern two-/multidimensional experiments. Indeed, these became so popular that later thesis from the Balaram group rarely recorded 1D NOE experiments. Eventually, data from these two-dimensional experiments were used to augment the data obtained from other solution spectroscopic methods. The literature survey only reveals model building with NMR constraints. The creative use of one-dimensional experiments to ascertain 3_{10}- or α-helix type secondary structures were also correlated to helix transitions and fraying in the study of peptide fragments (Gurunath Ramanathan 1994; Venkatraman et al. 2001).

Initially, Balaram and co-workers worked on synthetic fragments of *alamethicin* (Nagaraj and Balaram 1981a; Rao et al. 1981), *emerimicin* (Raj et al. 1988) and *suzukacillin* (Balaram 1985). They were quite successful in delineating the effect of *Aib* in these peptides using solution NMR experiments. Subsequently, Das et al. (1986) worked on a natural *peptaibol*, *antiamoebin*. Using just a 270 MHz NMR spectrometer and performing a set of simple ^{1}H-^{1}H COSY experiments in dimethyl sulfoxide, the authors could assign all resonance signals. Using additional sets of one-dimensional experiments, such as temperature-dependent chemical shift and difference NOE spectra, the authors could infer the hydrogen-bonding scheme in *antiamoebin* that was consistent with all NMR data. The results suggested that the peptide was highly intramolecularly hydrogen-bonded and at least two distinct folded structures could be ascertained. These computer-generated structures were different from each other. One was a curved helix, and the other was a frayed helix. CD spectra of *antiamoebin* were found to be solvent dependent. This fact was correlated with their finding of multiple conformations in *antiamoebin*. During this period of mid-1980s, Balaram's group also investigated the structures of *zervamicins*, *efrapeptins* and other *peptaibols* in solution, successfully.

The next successful NMR structure derived was for *Trichogin A IV* fragment (Gurunath and Balaram 1995) and its synthesized peptide (Gurunath Ramanathan 1994). By this time a 400 MHz spectrometer had become available, and the authors could use reliable COSY and ROESY experiments (viz. two-dimensional NMR spectroscopy) to infer the structure in solution. From the circular dichroic spectrum, it was concluded that this full-length *peptaibol* adopted helical conformation in methanol as well as in 2,2,2-trifluoroethanol (TFE), but the seven-residue long fragment showed a multiple overlapping β-turns. This result was hardly surprising

considering that the peptide contained a large number of glycine and α-aminoisobutyric acid residues are mainly present at the *N*-terminus.

Later, Balaram's group began to investigate *efrapeptins* (Uma et al. 2001). *Efrapeptins* are strictly not *peptaibols* as they have a *C*-terminal 1,5-diazabicyclo [4:3:0]nonene, and hence they are regarded as *peptaibiotics*. The biosynthetic pathway of *efrapeptins* from *elvapeptins* was successfully worked out with the help of ESI-MS (refer Sect. 14.3.2).

The other group that had applied NMR spectroscopy for *peptaibol* research was the group of Ram Vishwakarma (Singh et al. 2018). This group from Jammu reported sequences of four short *lipopeptaibols* from *Trichoderma velutinum*, which was found in the cold habitat of the Himalayan region. These four are six-residue *lipopeptaibols*, all of them possessing *N*-terminus octanoyl group. Two of these lack *Aib* residues but contain *R*-Iva instead, whereas two other *peptaibols* contain *Aib*. Albeit, in these studies, NMR spectroscopy was not used for conformational analysis; rather modern NMR methods were applied for molecular structure determination.

14.2.3 Crystal Structures

In this section, we will discuss the progress made in the solid-state structure determination of those *peptaibols* and their fragments that could be crystallized. Initially, the synthesized fragments of *suzukacillin* and *alamethicin* were crystallized by slow evaporation from methanol-water mixtures. Initially, only the column purified peptide fragments were successfully crystallized. Eventually, HPLC availability in the early eighties ensured that crystallization attempts were generally more successful, if the peptide was pure and the amount was 3–5 mg/mL of solvent. Several of these were solved with inter-/intra-departmental collaboration (Francis et al. 1982a, b, 1983; Rao et al. 1981) or by the expertise available in-house itself (Rao and Balaram 1982).

Most of the structures solved were protected peptides with an *N*-terminal *tert*. *Boc* group and a *C*-terminal methyl ester. These structure determinations yielded a wealth of information on *peptaibol* conformations in the solid state. For instance, in the classic case of *suzukacillin* fragments, the conformation of the individual fragments was successfully correlated to their conformation in solution determined by solvent titration and temperature experiments using NMR spectroscopy. These results were a nice 'icing on the cake'. Most of the peptide fragments in the solid-state had φ, ψ angles that were ($\pm 30^{\circ}$) close to ideal 3_{10}-/α-helical conformations (Ramakrishnan and Soman 1982). The pitch of the helix determined through crystallography was also quite close to ideal values reported for these types of helices in the literature. The hydrogen-bonding pattern often revealed mixed 5→1 and 4→1 type of bonding. The *C*-terminal methyl ester group was often treated as equivalent to an amide in these reports for determining the Ramachandran angles from the molecular structure for the last residue in these peptides. The reader is referred to an excellent review on this by Balaram himself (Balaram 1983).

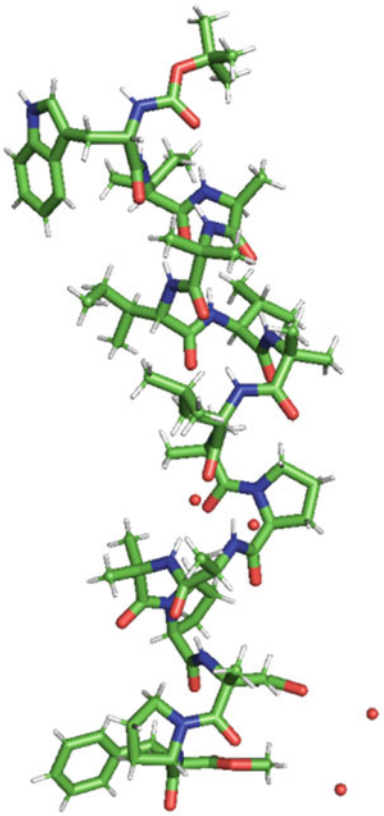


Fig. 14.7 A stick model of synthetic ***Zervamicin IIA* analogue** that only differs in the capping residues. A tertiary-butyloxycarbonyl group instead of the acetyl group at the *N*-terminus and a methyl ester instead of the traditional *C*-terminal alcohol are the only differences in this ***Zervamicin IIA* analogue**. The small red spheres indicate water molecules found in the crystal structure. The structure is redrawn based on the deposited coordinates by the authors (CCDC deposition no. 1167455). The *N*-terminus of the peptide is at the top in this orientation

The first major success in determining the crystal structure of a large synthetic *peptaibol* fragments ester of significant length is the case of *zervamicin* (Karle et al. 1986, 1987). The sequences of these two synthetic peptide fragments were based on the native 16-residue *zervamicin* from *Emericellopsis salmosynnemata*. This peptide crystallized with one molecule in a triclinic space group, with a parallel aggregation of the helical columns. The packing of these columns was considered to be 'rather inefficient'. The structure of synthetic 16-residue analogue of the *peptaibol zervamicin* was reported in 1987 (Karle et al. 1987). This structure revealed a unique 3_{10}-/α-helical mix that terminated with three successive β-bends (Fig. 14.7) each of which had an *L*-proline residue at its corners. These helices stacked in a parallel fashion in crystals. The presence of proline residues that lacked amide hydrogen ensured the formation of a curved helix, in which several water molecules were inserted through hydrogen bonds in *peptaibol* analogues for the first time.

Subsequently, the structure of a complete *peptaibol [Leu^1]zervamicin* was determined which was a major success in the early 1990s (Karle et al. 1991). The structure of *[Leu^1]zervamicin* was a profound contribution that resulted in several intriguing results. In this seminal manuscript, atomic resolution structures of naturally isolated

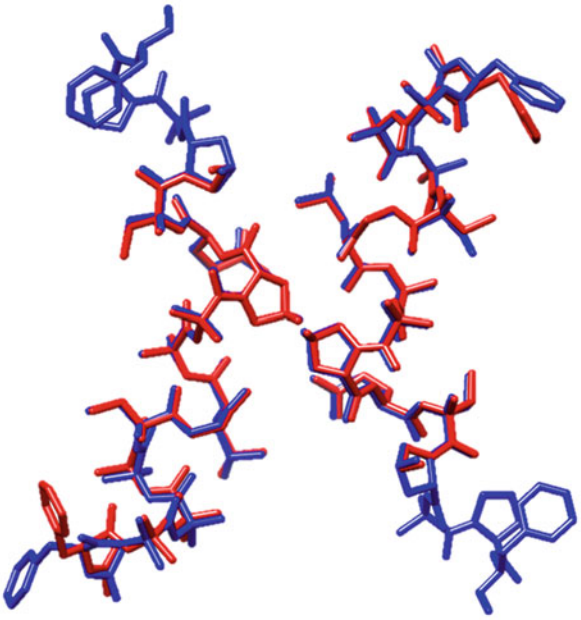


Fig. 14.8 Superposition of the crystal structures of the ***Antiamoebin*** solved by the groups of Karle-Balaram (Red) and Bonnie Wallace (1JOH, blue). The RMS deviation of the structure obtained by direct methods to molecule A (left) and B (right) in the unit cell of the Bonnie Wallace structure determination were 0.296 Å and 0.283 Å, respectively. (The authors acknowledge the help received from Prof. Devapriya Chowdhury and Dr. Kiran Lata for this figure)

[Leu^1]zervamicin that differed from the earlier reported synthetic analogue only at the *N*-terminus were solved in four different crystal forms. Two different types of crystal morphologies were obtained from the same batch of methanol-water solution. Another form was obtained from ethylene glycol-ethanol solution. A fourth polymorph was obtained by soaking the crystals obtained from ethylene glycol-ethanol mixtures in aqueous potassium chloride. Two different space groups ($P2_1$ and $P2_12_12_1$) were found in these four polymorphs. The structure of *[Leu^1]zervamicin* revealed a curved amphipathic helix that had polar residues towards the outer curve and hydrophobic residues towards the concave side of the helix curve. The side chains of Glutamine 11 were folded back on to the peptide backbone. There were also several water molecules lining the unit cell. The structure prompted the authors to propose a mechanism for voltage gating, invoking the movement of the Glutamine 11 side chain (Karle et al. 1991). In later work, the same authors expanded on their mechanism, wherein glutamine 3 was invoked as a residue critical to control access to the channel mouth (Agarwalla et al. 1992; Karle et al. 1994). The structures in these polymorphs were distinct only in the conformation of the glutamine 11 side chain. These were interpreted to illustrate open and closed forms of the channel gating. These models were expanded with more experimental results by other workers (Woolley and Wallace 1992).

The crystal structure of *antiamoebin* was reported almost within months of each other by British group of Bonnie Wallace-Tom Blundell on one side and the other from the groups of Balaram and Isabella Karle; see Fig. 14.8 (Snook et al. 1998; Karle et al. 1998). While the group in India crystallized the natural *peptaibol* from a methanol-octanol mixture over several weeks to afford diffraction quality crystals,

which were solved by direct methods in the United States, the group in the United Kingdom crystallized the same from methanol. The group from the United Kingdom had the data from these crystals in the P_1 space group for several years but had considerable difficulty in refining and solving the same (Snook et al. 1998; Snook and Wallace 1999). Eventually, the UK group got around this problem by using molecular replacement with a structural model of *[Leu^1]zervamicin* reported earlier. Using the search model as a peptide fragment 6–16 of the solved *[Leu^1]zervamicin* with all nonequivalent residues reduced to alanine side chains, this group managed to obtain an initial *R*-factor of 48% that could be eventually be refined to 15.6%. The final resolution was 1.4 Å. This still gave a fairly decent idea about the backbone conformation.

The Karle-Balaram's groups used direct methods for structure determination. They were thus able to solve the structure to a resolution of 1.0 Å in what was a remarkable feat at that time. This was the second *peptaibol* antibiotic whose structure was being reported at atomic resolution (the first being *[Leu^1]zervamicin*). The structure yielded several octanol molecules some of which were structurally quite ordered—almost as if they were mimicking the membrane environment. Superposition of the *[Leu^1]zervamicin* structure on the *antiamoebin* structure revealed that the two backbones were remarkably similar. Fifteen of the C^{α} carbon atoms when superposed had an RMS deviation of 0.5 Å. The molecules formed curved helices that gave the shape of a classical hourglass to the proposed ionic channel. The polar residues were lining the outer curved surface, and the hydrophobic residues were lining the outside with principally hydrophobic interactions with the octanol.

These results for the first time threw light on the subtle differences that exist between *peptaibol* length, conformation and helix length. The four helices found in the unit cell had identical conformation. While the structure at 1.4 Å gave a helix of length of approximately 27 Å, direct methods gave helices of around 29 Å. This 2 Å difference can be attributed to crystal packing forces and resolution at which the structures of these have been determined. Both structures had an hourglass curvature on their helices. Thus the 2 Å difference in length could be significant for *antiamoebin I*'s function as an ion channel.

A discussion of Indian contribution to *peptaibol* research will be incomplete without mention of H. N. Gopi's work from IISER, Pune (Jadhav et al. 2011). This group originally investigated techniques to make *C*-terminal peptide alcohols. Some of their products crystallized readily and their structures revealed interesting folds. In the case of the tripeptide alcohol Boc-Aib-*L*-Ala-*L*-Leu-ol, there were four molecules in the asymmetric unit, wherein pairs of molecules were intermolecularly hydrogen-bonded. A classical type III β-turn was held together in these short *peptaibol* sequences similar to what had been reported earlier by Toniolo's group (Toniolo et al. 1983).

14.3 Mass Spectrometric Characterization

Since *peptaibols* possess an acyl residue at the *N*-terminus, the traditional *N*-terminal sequencing protocols, viz. Edman's sequencing method, cannot be applied for deducing their primary structure. Also, classical sequencing techniques often failed in the case of peptaibiotics because these peptides have *C*-terminus alcohol, an *N*-terminal acyl group and several sterically hindered amino acid residues (Sect. 14.1). Moreover, enzymatic techniques to cleave certain specific peptide bonds in such peptides were not very useful. Thus, NMR spectroscopy and MS-based methods have been quite successful for sequence elucidation of the *peptaibols*. The success of mass spectrometry in determining the primary structure of these peptides ensured that the technique could be applied for protein sequencing as well. The first attempt to sequence the *peptaibols* successfully was by the group of Rinehart (Pandey et al. 1977a, b, c).

14.3.1 Applications of MS to Sequence *Peptaibols*

14.3.1.1 Use of Different Ionization Modes

Amino acid composition and sequence of a few *peptaibol* classes (*antiamoebin*, *emerimicins*, *alamethicins*) were initially determined using gas chromatography (GC)-MS, which involved electron ionization (EI), viz. GC-EI-MS, as well as field desorption (FD) MS (Pandey et al. 1977a, b, c; Brückner et al. 1985). GC-EI-MS and FD-MS were conducted on chemically derivatized hydrolysis products (Brückner et al. 1985). However, other *peptaibols*, such as *zervamicins*, *emerimicins*, *trichotoxins*, *tricholongins*, *trichogin*, *trichoningins* and *trichokindins*, could be sequenced, with the arrival of fast atom bombardment (FAB) ionization method (Rinehart Jr. et al. 1981; Brückner and Przybylski 1984; Przybylski et al. 1984; Rebuffat et al. 1991; Auvin-Guette et al. 1992, 1993; Iida et al. 1994). Chemical derivatization was rather not preferred or not required for FAB-MS analysis (Barber et al. 1981a, b). FAB could generate ions of polar molecules of masses up to 6000 Da (e.g. human insulin), and molecular fragmentation did not occur to a great extent (de Hoffmann and Stroobant 2007). So, it was possible to determine 'intact molecular mass' of both polar and non-polar *peptaibols* using FAB-MS, as the molecular masses of most of the *peptaibols* are typically less than 3000 Da. Liquid secondary ion mass spectrometry (LSIMS), an extended version of FAB-MS, has also proven to be useful in a few studies for identifying *peptaibols* (Pócsfalvi et al. 1997; Leclerc et al. 2001; Wiest et al. 2002). In LSIMS, caesium ions (Cs^+) are allowed to bombard on 'liquid' sample for generating molecular ions in the gas phase.

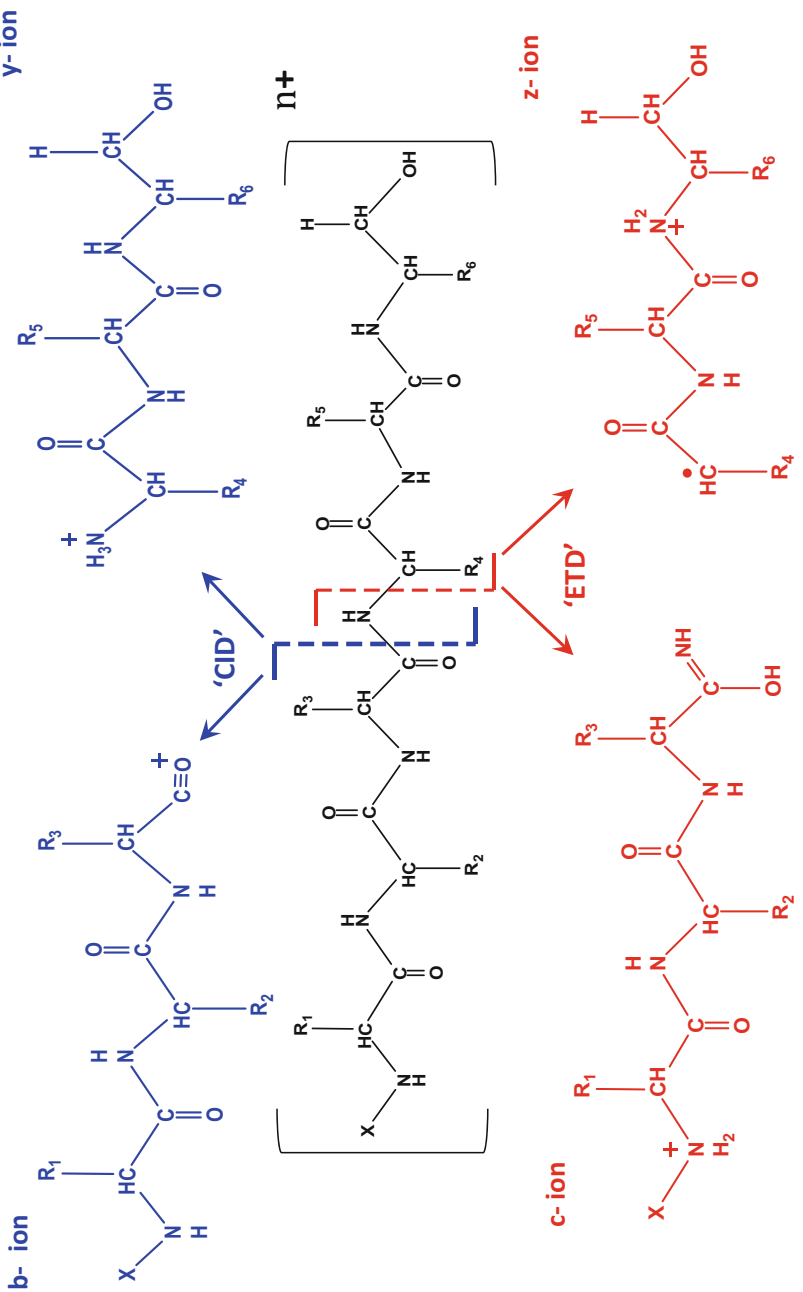
However, significant advancements followed with the arrival of two novel soft ionization modes: (1) electrospray ionization (ESI) and (2) matrix-assisted laser desorption/ionization (MALDI), during the late 1980s and early 1990s (Fenn et al. 1989; Karas and Hillenkamp 1988; Tanaka et al. 1988; Fenn 2003; Tanaka 2003). The soft nature of ionization processes ensures the feasibility of determining 'intact molecular mass' of very large-sized and highly polar molecules, such as proteins and

oligonucleotides (de Hoffmann and Stroobant 2007; Kinter and Sherman 2000). Consequently, these two ionization modes were applied for investigating *peptaibols* also from diverse natural sources. *Peptaibol* analysis by MALDI and ESI has been predominantly carried out in positive ion mode, wherein both protonated $[M + H]^+$ (where 'M' is the *peptaibol* molecule) as well as metal-cationized species, especially sodium-adduct ions $[M + Na]^+$, have been observed. The $[M + Na]^+$ ions have also been found to be useful in some studies to probe the sequences of various *peptaibols* (Pócsfalvi et al. 1997; Sabareesh and Balaram 2006; Ruiz et al. 2007). Even the doubly charged $[M + 2Na]^{2+}$ precursor ion was fruitful in characterizing and deriving the *peptaibol* sequences (Poirier et al. 2007). Intriguingly, the negative ESI mode too (in conjunction with positive ESI mode) is fruitful for deducing the sequence of *peptaibols* by LC-MS mode (Jaworski and Brückner 1999). Survey of the literature available, thus far, shows more applications of ESI than MALDI for sequencing and characterizing *peptaibols*.

14.3.1.2 Role of Mass Analysers and Tandem Mass Spectrometry

Another essential component of a mass spectrometer is the 'mass analyser'. The purpose of the mass analyser is to 'separate ions based on their respective *m/z* (mass-to-charge) values', where this process of separation takes place in the vacuum. Quadrupole and ion trap are mass analysers that usually offer medium resolution, whereas ToF and FT-ICR are high-resolution mass analysers. In a typical mass spectrometer, a particular ionization mode would be coupled to a mass analyser. However, there are also instruments, where two or more different analysers are combined together in various configurations to perform experiments of 'tandem mass spectrometry', which in short is denoted as 'MS/MS' (Kinter and Sherman 2000; de Hoffmann and Stroobant 2007). Q-ToF, ToF/ToF and ion trap-ToF are a few examples of tandem mass spectrometers, in which MS/MS experiments can be carried out. Such tandem mass analysers can be coupled to either ESI or MALDI, e.g. MALDI-ToF/ToF, ESI-Q-ToF and MALDI-ion trap/ToF. Both MALDI and ESI can be used with MS/MS for deducing the sequences of several *peptaibols*, though relatively more applications of ESI MS/MS are known, thus far.

MS/MS is a process involving dissociation of the selected intact molecular ion, also known as parent ion or 'precursor ion' and recording the mass spectrum of the resulting fragment ions. The *m/z* values of the fragment ions can be useful to elucidate the sequence of the peptide/*peptaibol*. Different methods of dissociation have been developed for effecting MS/MS. Collision-induced dissociation (CID) is a widely followed MS/MS method, which has proven to be successful in various studies, including for *peptaibols'* characterization. CID MS/MS can be performed on either singly charged or on multiply charged precursor ions. CID of peptides usually causes fragmentation of backbone peptide units (C′-N) (Roepstorff and Fohlman 1984; Biemann 1990; Kinter and Sherman 2000). Electron transfer dissociation (ETD) is another MS/MS method, which leads to fragmentation of backbone N-Cα bonds in multiply protonated peptide precursor ions (Syka et al. 2004). Scheme 14.1 shows the chemical structures of major fragment ions that can be anticipated due to CID and ETD of *peptaibols*. It is important to note that the



Scheme 14.1 Schematic illustration of MS/MS fragmentation of *peptaibol* due to collision-induced dissociation (CID) and electron transfer dissociation (ETD). Structures of **b-** and **y-**type ions resulting from CID and structures of **c-** and **z-**type ions resulting from ETD are shown. For ETD, the charge state of the *peptaibol*, **n** must be $\geq +2$

***Elvapeptin C*: 1626 Da**

Ac-Pip-*Aib*-Pip-*Aib*-*Aib*-Leu-β-Ala-Gly-*Aib*-*Aib*-Pip-*Aib*-Gly-Leu-*Aib*-**spermidine**

Chemically driven Oxidative Cyclization ↓ CuCl/Pyridine (48 hours); rt ΔM = 20 Da

***Efrapeptin C*: 1606 Da**

Ac-Pip-*Aib*-Pip-*Aib*-*Aib*-Leu-β-Ala-Gly-*Aib*-*Aib*-Pip-*Aib*-Gly-Leu-*Aib*-**DBN**$^{+}$

Scheme 14.2 Decrease in the molecular mass of *Elvapeptin C* by 20 Da due to chemical oxidation, resulting in the formation of *Efrapeptin C*. Note: Pip is 'pipecolic acid residue', refer Table 14.2

chemical structures shown in Scheme 14.1 are drawn based on the CID and ETD data of peptides (Kinter and Sherman 2000; Syka et al. 2004; Good et al. 2007).

14.3.2 In vitro Chemical Conversion of *Elvapeptins* to *Efrapeptins* Probed by ESI-MS

The fungus *Tolypocladium niveum* produces a group of microheterogeneous peptaibiotics called *efrapeptins*, whose *N*-termini are acetylated and have an unusual *C*-terminus modifying group 1,5-diazabicyclo [4:3:0] nonene (DBN) (Gupta et al. 1991, 1992). An ESI-MS (single quadrupole) study was carried out in order to investigate the hypothesis that the *C*-terminus spermidine moiety in *elvapeptins* is the biosynthetic precursor of DBN in *efrapeptins* (Uma et al. 2001). ESI-MS confirmed the optimized production of *elvapeptins*, which were obtained by appropriate control of the fermentation conditions. These *elvapeptins* were then subjected to acetylation reaction using acetic anhydride and pyridine. Characterization of all these reaction products by ESI-MS confirmed that each of these *elvapeptins* had the spermidine moiety. Subsequently, the *elvapeptins* were treated with cuprous chloride (CuCl) and pyridine at room temperature (rt), which led to the conversion of the amine group of the spermidine moiety to aldehyde, thereby yielding 1,5-diazabicyclo [4:3:0] nonane. The use of CuCl/pyridine also provided an oxidative environment that could trigger hydrogen abstraction from the 1,5-diazabicyclo [4:3:0] nonane, thereby forming DBN. Therefore, treating *elvapeptins* with CuCl/pyridine eventually decreased their respective molecular mass by 20 Da, which was finally confirmed by ESI-MS. Thus, by proving this hypothesis successfully, a new understanding was obtained about the biosynthetic relationship between the *elvapeptins* and *efrapeptins*, especially with respect to its *C*-terminus modifying group. Summary of this work is illustrated in Scheme 14.2 below for *elvapeptin C* as a representative example:

14.3.3 ESI-Ion Trap MS/MS Studies on *Peptaibols*

14.3.3.1 ESI-CID MS/MS Reveals Microheterogeneous *Trichotoxin* Sequences

A study from Balaram's group was aimed to identify novel *peptaibol* in a HPLC fraction that was processed from the soil fungus *Trichoderma*, and this was addressed by ESI-CID MS/MS experiments (Sabareesh and Balaram 2006). The normal ESI mass spectrum recorded for this HPLC fraction (containing methanol) in an ion trap mass spectrometer (Esquire 3000 Plus, Bruker Daltonics) showed two intense peaks: *m/z* 1717 and *m/z* 1740 (Fig. 14.9). The difference of 22 units between these two *m/z* values suggested the first peak was due to singly protonated (H^+) adduct (viz. $[M + H]^+$) and the peak at *m/z* 1740 was due to singly charged Na^+ adduct (viz. $[M + Na]^+$). Considering these observed *m/z* values as intact molecular ions (viz. not any fragment ion), the *peptaibol* database: http://www.cryst.bbk.ac.uk/peptaibols, was searched to find the sequence(s) of those *peptaibol*s, whose molecular mass(es) is/are about 1717 Da. Some sequences within a class called *trichotoxins* were available in this database, whose molecular masses were ~1717 Da (see Table 14.3). Two different *trichotoxin* sequences had the same monoisotopic molecular mass, 1717.051 Da, and monoisotopic molecular masses of three different *trichotoxin* sequences were 1718.035 Da. The sequences of these *trichotoxins* are actually not very different. The sequences are very similar, but microheterogeneous

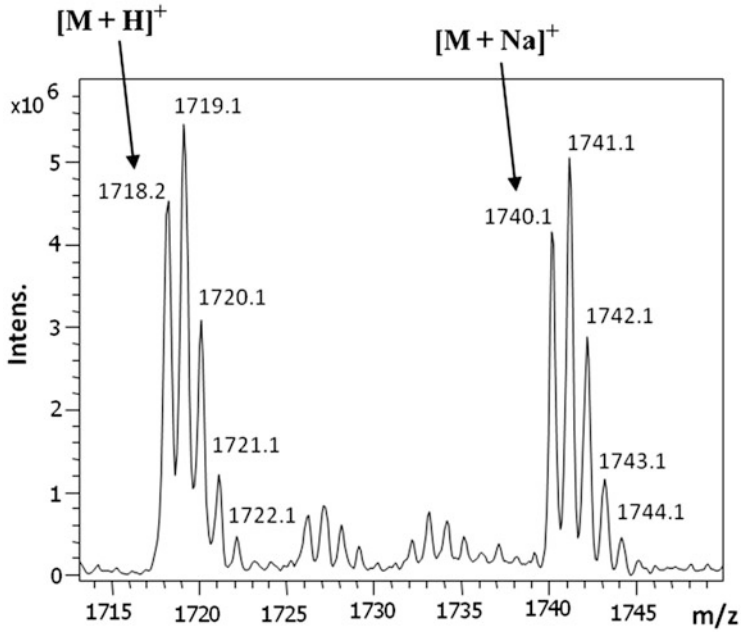


Fig. 14.9 Conventional ESI mass spectrum acquired by directly infusing the HPLC fraction into the mass spectrometer. (Esquire 3000 Plus, Bruker Daltonics)

Table 14.3 Sequences of *Trichotoxins*, whose molecular masses are ~1717 Da/1718 Da, taken from *peptaibol* database[a]

S. No.	Name of Trichotoxin	Sequence																				Monoisotopic molecular mass
	Neutral peptides			1	↓				5					10	↓				15			18		
1.	**Trichotoxin_A-50_H**	Ac	α	A	α	L	α	Q	α	α	α	A	A	α	P	L	α	ι	Q	V	CH_2OH	**1717.05065 Da**
2.	**Trichotoxin_A-50_I**	Ac	α	G	α	L	α	Q	α	α	α	A	α	α	P	L	α	ι	Q	V	CH_2OH	**1717.05065 Da**
	Acidic peptides			1	↓			5	↓				10					15	↓	↓	18		
3.	**Trichotoxin_A-40_V**	Ac	α	G	α	L	α	Q	α	α	α	A	α	α	P	L	α	ι	E	V	CH_2OH	**1718.03466 Da**
4.	**Trichotoxin_A-40**	Ac	α	G	α	L	α	E	α	α	α	A	α	α	P	L	α	ι	Q	V	CH_2OH	**1718.03466 Da**
5.	**Trichotoxin_A-40_Va**	Ac	α	A	α	L	α	Q	α	α	α	A	α	α	P	L	α	α	E	V	CH_2OH	**1718.03466 Da**

Ac, Acetyl (Fig. 14.1). α: alpha-aminoisobutyric acid (Aib, Fig. 14.2). ι: isovaline (Iva, Fig. 14.2). Single letter codes A, G, L and P; see Fig. 14.4. Q: glutamine (Gln). E: glutamic acid (Glu). V CH_2OH: valinol (Fig. 14.3)
Variable positions in the sequences are 'boxed' and are indicated by ↓
[a]http://www.cryst.bbk.ac.uk/peptaibols

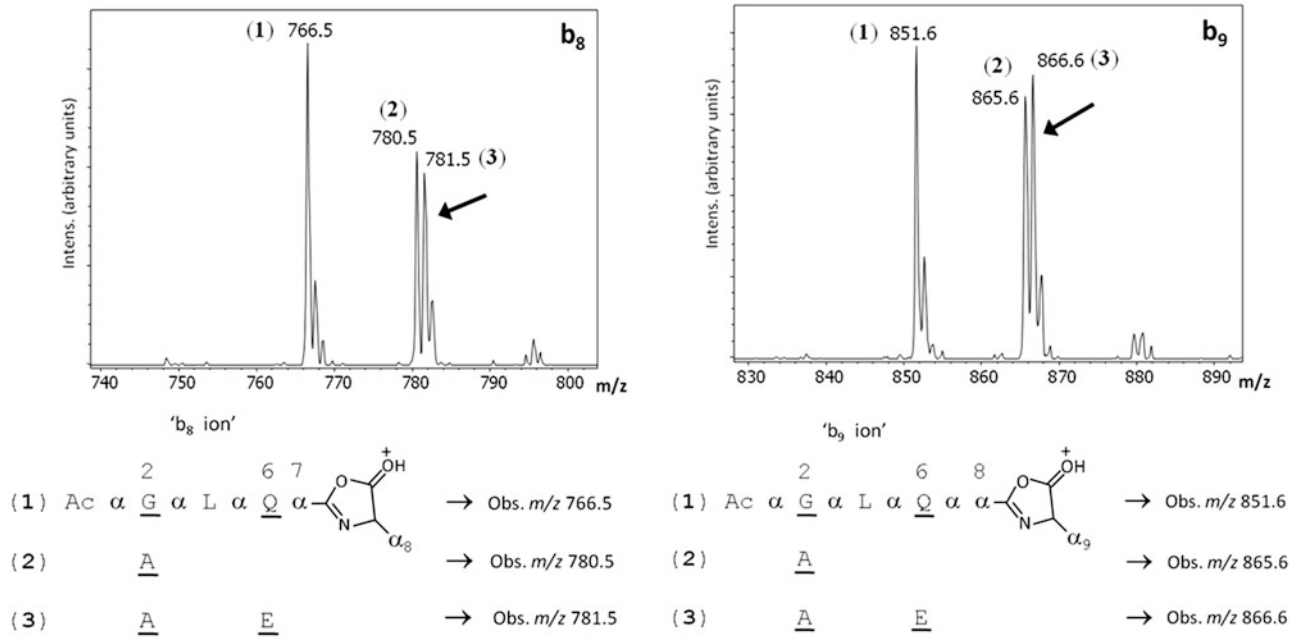
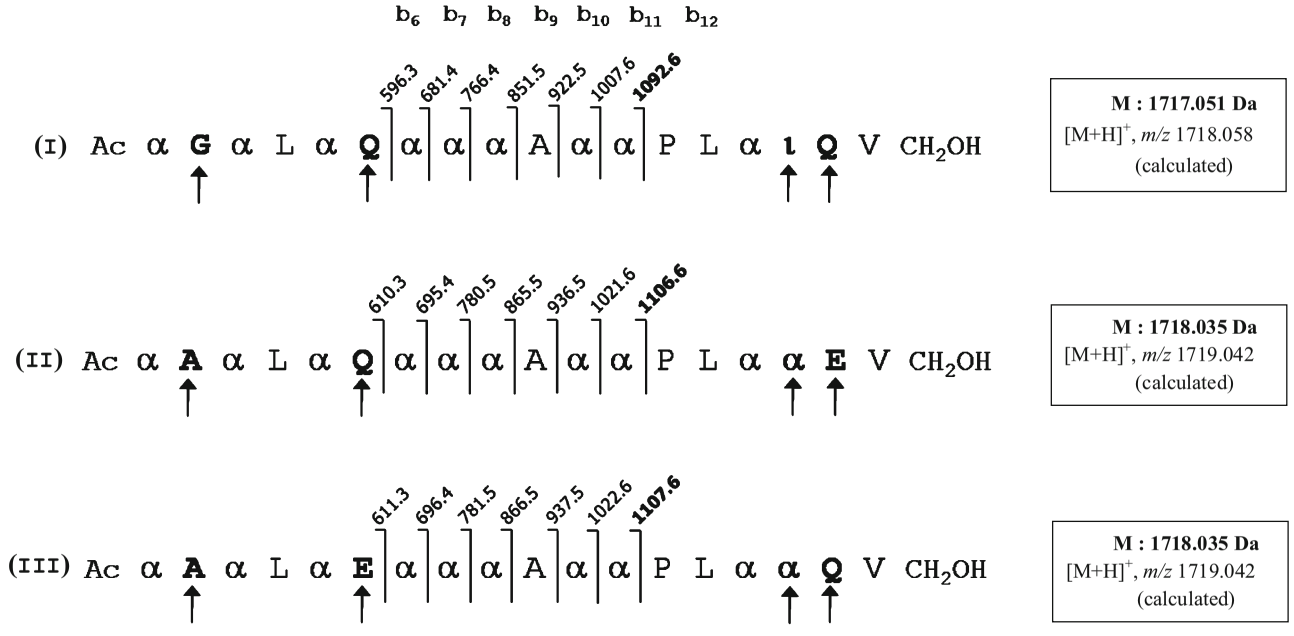
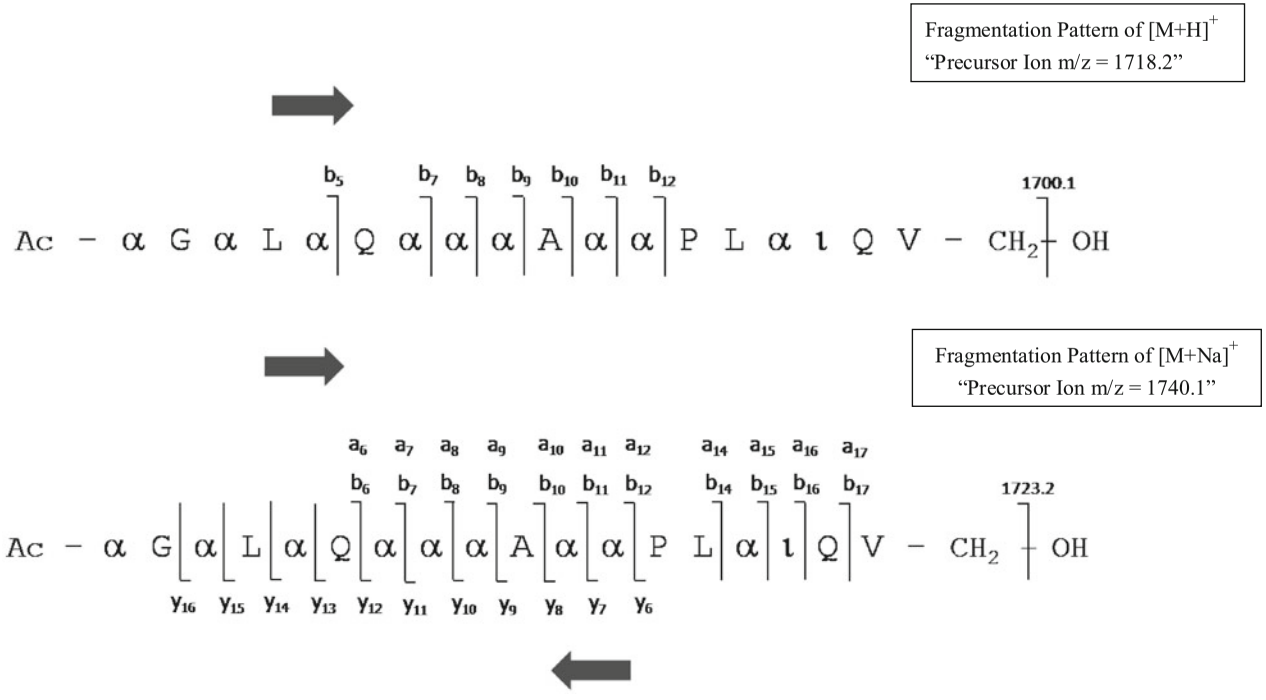


Fig. 14.10 MS/MS spectrum of $[M + H]^+$ precursor ion centred at *m/z* 1718.1: two different mass ranges, *m/z* 740–800 and *m/z* 830–890 edited from this spectrum are zoomed in and shown here. Also shown are the schematic representations of sequences corresponding to b_8 and b_9 ions in their oxazolone form. Note that the peaks at *m/z* 781.5 and *m/z* 866.6 do not appear to be the isotope peaks of *m/z* 780.5 and *m/z* 865.6, respectively, and this suggests the presence of third peptidic component in the HPLC fraction



Scheme 14.3 Fragmentation pattern as observed in CID MS/MS spectrum of $[M + H]^+$ precursor ion *m/z* 1718.1 (see Figs. 14.9 and 14.10). Calculated monoisotopic molecular masses and the *m/z* values corresponding to H^+ adduct of each peptidic component are also shown



Scheme 14.4 Comparative summary of CID fragmentation patterns of H^+ and Na^+ adducts of the neutral *peptaibol*, ***Trichotoxin_A-50_I***, observed in ESI-ion trap mass spectrometer (Esquire 3000 plus, Bruker Daltonics). In the fragmentation pattern of $[M + Na]^+$, the **a-**, **b-** and **y-**ions carry Na^+, viz. **sodiated a-**, **b-** and **y-**ions

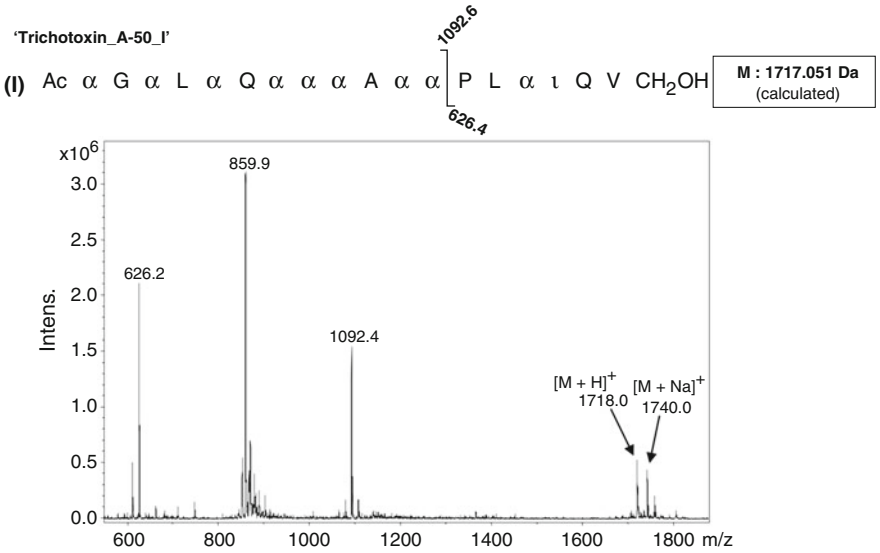


Fig. 14.11 Conventional ESI mass spectrum of an apparently homogeneous HPLC fraction obtained from soil fungus *Trichoderma*. The peaks at *m/z* 1092.4 and *m/z* 626.2 correspond to N-terminal fragment ion and C-terminal fragment ion, respectively, which result from the 'in-source fragmentation' of *Aib-Pro* peptide bond in ***Trichotoxin_A-50_I***, (whose sequence is shown), without resorting to MS/MS fragmentation

in that the variations were observed only at a few positions in their sequences, as remarked previously.

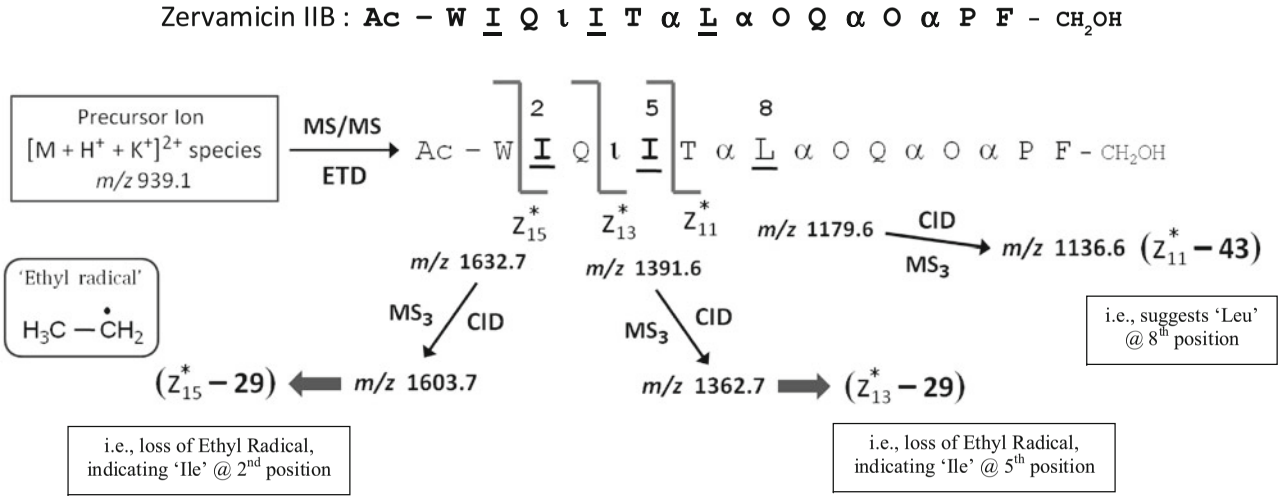
The microheterogeneity was due to exchanges or replacements such as Gly ↔ Ala (G ↔ A), Ala ↔ Aib (A ↔ α), Aib ↔ ^{D}Iva (α ↔ ι) and Gln ↔ Glu (Q ↔ E), which leads to differences of 1 Da or 14 Da or 15 Da between their molecular masses (Table 14.3). Thus, the task was to identify the sequence of the *peptaibol* present in this HPLC fraction. Consequently, both H^+ and Na^+ adducts were chosen as precursors to perform CID in the same ion trap (Esquire 3000 Plus, Bruker Daltonics). The MS/MS spectrum of $[M + H]^+$ (precursor ion *m/z* 1718.2) contained interesting features, in that the intensities of the isotope (neighbouring) peaks of the b-type fragment ions clearly indicated the presence of three different sequences of *peptaibols*, which could be attributed to the 'microheterogeneous' sequences (see Fig. 14.10). Note that the b-type ions, b_8 and b_9, are depicted in the **oxazolone** form in Fig. 14.10, instead of acylium form, which is shown in Scheme 14.1. The formation of oxazolone structure in b-type ions has been proposed mechanistically, and it has been experimentally detected in certain studies as well (Polfer et al. 2005; Schlosser and Lehmann 2000).

Scheme 14.3 shows those three sequences of *trichotoxins*, which were interpreted by correlating the ESI-CID MS/MS spectrum of H^+ adduct, with the sequences taken from the *peptaibol* database (Table 14.3).

The sequence **III** in the Scheme 14.3 was actually interpreted by swapping the positions of Gln 6 (Q6) and Glu 17 (E17) in the sequence **II**, so as to fit to the observed *m/z* values of the b-type ions in the MS/MS spectrum of H^+ adduct. Interestingly, the sequence **III** was not available in the *peptaibol* database, and, hence, the sequence **III** eventually turned out to be a *new trichotoxin*. The pattern of fragment ions obtained from the MS/MS of $[M + Na]^+$ precursor were not only drastically different from the fragmentation pattern observed in the MS/MS spectrum of $[M + H]^+$ precursor (see Scheme 14.4), but the MS/MS spectrum of $[M + Na]^+$ also proved useful to confirm the sequence of the 'neutral' *peptaibol*, sequence **I**, which lacked the acidic amino acid (glutamic acid, Glu: 'E') (Scheme 14.4). Altogether, MS/MS experiments carried out on both H^+ and Na^+ adducts established the identity of three microheterogeneous *trichotoxin peptaibols* in the HPLC fraction isolated from a soil fungus *Trichoderma*.

With respect to precursor-ion isolation step, the ion trap mass analyser used in this investigation has, in fact, selected more than one *peptaibol* molecular (precursor) ion, whose *m/z* values differ by 1 unit, i.e. 1718.1 and 1719.1 (Fig. 14.9). Consequently, in the MS/MS spectrum of H^+ adduct, it was possible to observe fragment ions (mainly b-type ions) that resulted from the CID of more than one *peptaibol*, which not only aided in knowing that this HPLC fraction contained microheterogeneous *trichotoxins*, but it also proved to be useful to precisely identify the sites of microheterogeneity, based on the observed *m/z* values of the b-type fragment ions. Further, in the 'normal' ESI mass spectrum of this HPLC fraction, in addition to the peaks due to H^+ and Na^+ adducts, it was intriguing to find two more peaks that resulted from the dissociation of *Aib-Pro* peptide unit (Fig. 14.11), whereby one peak (*m/z* 1092.4) could be interpreted due to the fragment ion possessing the *N*-terminus and another peak (*m/z* 626.2) was assigned as the (complementary) *C*-terminal fragment ion (Sabareesh and Balaram 2006). This observation was interesting since this dissociation has occurred without MS/MS experiment, viz. without 'precursor-ion selection' step.

Such a process is referred as 'in-source fragmentation', whereby the intact molecular ion undergoes fragmentation during the ionization process. Although, ESI is established as a soft ionization mode in many studies, the process of in-source fragmentation can be attributed to the fragile nature of certain chemical bonds, and in this case, the peptide or the tertiary amide bond between *Aib* and Pro seems to be relatively weaker, in comparison to other peptide bonds in the *peptaibol*. In-source fragmentation can be controlled and minimized, by suitably altering and optimizing certain parameters that are specific to the ionization source, which are rather proprietary, depending on the instrument. Notably, the in-source fragmentation of *Aib-Pro* peptide bond has been observed in other *peptaibols* also (Huang et al. 1995; Mohamed-Benkada et al. 2006).



Scheme 14.5 Distinguishing 'Leu' from 'Ile' in ***Zervamicin IIB***, by combining ETD MS/MS and CID MS_3. The precursor ion for ETD MS/MS was a doubly charged species of the *peptaibol* bound to a H^+ and a K^+. CID MS_3 was performed on three specific z-ions, z^*_{11}, z^*_{13} and z^*_{15}, where each of these three are potassiated z-ions, viz. each z-fragment ion is bound to a K^+. CID MS_3 of potassiated z-ions led to dissociation of Cβ-Cγ bonds, which corresponds to loss of isopropyl radical from the 'Leu side chain' (z^*_{11} ion–43) and loss of ethyl radical from the 'Ile side chains' (z^*_{13}–29 and z^*_{15}–29)

14.3.3.2 ESI-ETD MS/MS in Conjunction with CID for Distinguishing Leu from Ile in *Peptaibol* Sequences: Application of MS_3 on *Zervamicin IIB* and *Antiamoebin*

Electron transfer dissociation (ETD) is another MS/MS method, which is relatively new, when compared to CID (Syka et al. 2004). As already mentioned earlier (vide supra), ETD MS/MS requires multiply charged peptide precursor cations, and it causes fragmentation of backbone N-C^{α} bonds, giving rise to c- and z-ions (Scheme 14.1). It is important to note that the z-type ions are radical ions.

In India, ETD MS/MS was first used by Balaram's group (IISc, Bengaluru), to characterize *peptaibols*, whereby ETD method was applied in combination with CID MS/MS, for the purpose of distinguishing 'leucine (Leu)' from 'isoleucine (Ile)', which have the same residue masses (see Fig. 14.4) (Gupta et al. 2012). Since, Leu and Ile are isobaric residues (same molecular mass), applying only CID or only ETD cannot be fruitful or sufficient. Therefore, experiments were done by combining these two MS/MS methods, and such a combination is possible in ion trap mass analyser. In order to distinguish Leu from Ile by MS_3, it is important to choose only those peptide 'fragment ion(s)' (obtained in the first MS/MS stage), which contain Leu and/or Ile. Hence, the peptide fragment ions that do not contain Leu and/or Ile need not be selected for MS_3 stage of dissociation. The MS_3 experimental design of Balaram's group entailed ETD in the first MS/MS stage and then CID in the MS_3 level. This means that CID was carried out on c- and z-type fragment ions (refer Scheme 14.1). Two different *peptaibols* of already known sequences: *antiamoebin* and *zervamicin IIB*, were chosen to examine this experimental design. These experiments were carried out in an ESI ion trap mass spectrometer, HCT-Ultra ETD II (Bruker Daltonics). The ETD MS/MS experiments were performed on doubly charged species of *peptaibols*, one being H^+ and another charge was potassium ion K^+; so, the precursor ion subjected to ETD in the first MS/MS stage was $[M + H^+ + K^+]^{2+}$ species. Fluoranthene radical anion was used as the electron transfer reagent, where the radical anions are created by chemical ionization of fluoranthene. z-type ions carrying K^+ ion, which was obtained from ETD MS/MS, were chosen for the CID MS_3 experiments.

The sequence of *antiamoebin* contains one 'Leu' (Leu-7). *Zervamicin IIB* has two 'Ile' (Ile-2 and Ile-5) and a 'Leu-8' (see Scheme 14.5). Thus, according to the position of the Leu/Ile residues in the sequence, appropriate z-type ion was selected for CID MS_3 experiments (Scheme 14.5). A peak arising due to neutral loss of 43 mass units upon CID of a particular z-type ion would suggest the presence of 'Leu' in that z-ion. The neutral loss of 43 mass units can be ascribed to the loss of isopropyl radical because of cleavage of Cβ-Cγ bond in the side chain of Leu. If a peak was detected due to neutral loss of 29 mass units, due to CID of a particular z-type ion, then it would indicate the presence of 'Ile' in that z-ion, whereby 29 mass units can be attributed to the 'ethyl radical', which is released due to the dissociation of Cβ-Cγ bond, from the side chain of Ile.

Overall, this particular study from Balaram's group highlighted the significance of MS_3 experiments for distinguishing 'Leu' from 'Ile' not only in *peptaibols* but also in a few other peptide natural products. Another important aspect is the

hydroxyproline (Hyp), whose residue mass is identical to that of Leu and Ile; however, the dissociation of N-Cα bond in Hyp is not feasible upon ETD, and, therefore, no c- and z-type ions would arise at the site of Hyp. Consequently, 'absence of c- and z-type ions' can provide a clue regarding the presence of Hyp in the sequence. Nevertheless, Hyp can be identified in a sequence from CID MS/MS data. Upon CID, the peptide bond preceding Hyp, i.e. 'Xxx-Hyp peptide bond' in a sequence, can get cleaved, giving rise to appropriate b-/y-ions, where the y-ion would possess the Hyp residue. Hence, CID MS/MS can prove fruitful to infer the presence of Hyp in a peptide sequence.

14.3.4 ESI-Q-TOF MS/MS of *Lipopeptaibols* and *Peptaibols* from the Himalayan Cold Habitat Fungus

Four novel *lipopeptaibols* (*lipovelutibols*) and four peptaibols (*velutibols*) were discovered from a fungus *Trichoderma velutinum*, isolated from the soils of Himalayan cold habitats (see Sect. 14.2). ESI-Q-TOF MS/MS in combination with NMR spectroscopy was useful to elucidate the sequences of these peptides. High-resolution MS/MS data acquired on $[M + H]^+$ precursor ions yielded predominantly b-type ions, which enabled to derive the sequences of all these new peptides.

In the case of *velutibols*, MS_3 experiments also were performed on certain specific b-type ions: b_{12} and b_8, for determining the full sequence, wherein these b-ions resulted from the fragmentation of *Aib-Pro* bonds at two different sites of the peptaibol (Singh et al. 2020). Although, Na^+ adducts of three *lipovelutibols* (A, C and D) and one *velutibol* (A) were detected, these cationized adducts were not shown to be chosen for MS/MS experiments, to deduce their respective sequences, in these studies (Singh et al. 2018, 2020).

14.3.5 Intact Cell Mass Spectrometry (ICMS) by MALDI-TOF MS of *Peptaibols*

In this technique, the intact or the whole cells are directly studied by mass spectrometry, mostly by MALDI-MS, without cell lysis. A typical procedure for sample preparation prior to mass spectral data acquisition is briefly described here. The fungal mycelia from the agar petri plates are carefully scrapped and suspended in a suitable solvent system. The fungal mycelia/conidia suspension solution is usually prepared using acetonitrile/water mixture or acetonitrile/methanol/water mixture. Such a fungal mycelia suspension is then thoroughly mixed (vortexed) with matrix solution, e.g. α-cyano-4-hydroxycinnamic acid, which is prepared in acetonitrile/water, typically in the proportion 70:30 (v/v). Subsequently, a few microlitre of this sample is allowed to dry on the sample target plate of the MALDI mass spectrometer, for data acquisitions. Because the samples are not subjected to any extraction procedures involving different types of solvents and since chromatographic fractionation steps are also not applied, this technique offers expeditious snapshots of

cellular metabolite profiles, in terms of their molecular masses. Further, it is possible to elucidate and verify molecular structures as well, since MS/MS experiments also can be performed. When such experimentally observed mass profiles are appropriately interrogated with certain suitable databases or libraries that contain various classes/families of metabolites (including *peptaibols*/*peptaibiotics*), it would be possible to narrow down the search space for identification of unknown compounds, or even the known compounds can be identified quite rapidly (Smedsgaard and Frisvad 1996). Thus, ICMS data can be useful for rapid screening of prospective metabolites/compounds, based on which further experiments involving extraction, isolation and purification can be planned.

ICMS technique has been attempted to explore the production of *peptaibols* by three different species of *Trichoderma*, found in India. Whole-cell profiling of the fungus *Trichoderma harzianum* has been carried out by MALDI-TOF-MS, which showed two sets of peaks in the *m/z* range 1750–1810 and 1900–1950, in the mass spectrum, indicating presence of two different classes of *peptaibols* produced by this fungal species (Shishupala 2009). Non-ribosomal peptides containing *Aib* residues were discovered for the first time in a fungus *Trichoderma velutinum* ACR-P1 of Himalayan origin, with the help of IC-MALDI-TOF MS, whereby the mass spectral data indicated that these peptides belong to two different subfamilies, SF1 and SF4 (Sharma et al. 2016). The presence of *Aib* residues in the peptides belonging to both the families was known from the MS/MS data, which were also acquired directly from the whole cells. Further, IC-MALDI-TOF MS has been applied to explore peptides/*peptaibols* in another fungus collected from Himalayan region, *Trichoderma lixii* (IIIM-B4), which is actually an endophyte of *Bacopa monnieri* L. plant, commonly known as Brahmi in Ayurvedic medicine (Katoch et al. 2019).

14.4 Biosynthesis

The biosynthesis of *peptaibols* is primarily accomplished employing non-ribosomal peptide synthetases (NRPSs) (Kleinkauf and Von Döhren 1996; Marahiel et al. 1997). NRPSs are multifunctional enzymes, possessing multi-modular structures, whereby each module in an NRPS performs a particular catalytic function. In other words, they are large multienzyme complexes or multidomain enzymes. By identifying the genes that code for different modules in an NRPS and by suitably engineering these gene sequences, it is possible to carry out 'directed biosynthesis' of *peptaibols* viz., through genetic engineering of NRPSs, novel functional *peptaibols* can be produced (Wiest et al. 2002). The process of engineering gene sequences of NRPS might involve the replacement of one module with another or re-combining the modules in a different possible manner or by deleting certain specific modules (knock-out); these are, in fact typical molecular cloning strategies. These approaches would be similar to the techniques used by Chaitan Khosla to engineer polyketide synthases (Khosla et al. 2014).

The gene (*tex1*) encoding a peptaibol synthetase was first identified and characterized by Kenerly and co-workers from the fungus *Trichoderma virens*, strain

TV29-8 (Wiest et al. 2002). *tex1* was found to code for an 18-module NRPS, which can synthesize 18-residue long *peptaibol*, and its size was estimated to be ~2.3 MDa. By creating disruption mutants of *tex1*, which failed to produce *peptaibols*, it was known that *tex1* codes for peptaibol synthetase. Mukherjee et al. identified another gene, *tex2*, which was shown to code for a single 14-module NRPS that could synthesize both the 11- and 14-residues long *peptaibols* in the strain Gv 29–8 of *T. virens* (Mukherjee et al. 2011).

14.5 Biophysical and Biological Functions

Alamethicin was the first *peptaibol* to be investigated in voltage-dependent ion channel studies (Mueller and Rudin 1968). In an attempt to understand and rationalize the observed electrical conductance behaviour, a model was proposed, which suggested the formation of pore-like structures consisting of central aqueous core spanning the lipid bilayer, due to aggregation or oligomerization of monomeric *alamethicin* molecules (Boheim and Kolb 1978). The 3D molecular structure of *alamethicin* derived from X-ray diffraction by Fox and Richards (1982) also revealed the formation of pore-like structure upon association of six monomeric molecules in the crystal, which was in support of the proposed model. The interest to investigate the mechanism of ion channel formation by *peptaibols* stemmed from the receptor ion-gated channels, which are transmembrane proteins. Because these proteins are quite large-sized and adopt complex 3D structures, high-resolution X-ray crystallography and/or electron microscopic imaging would be more suitable, so that a clearer picture of the ion channelling mechanism can be obtained. However, during that period (1980s and 1990s), because of limited access to these two techniques (that were rather very sophisticated), studying simpler model systems utilizing channel-forming peptides (CFPs), which can mimic the functions of ion-channelling proteins, was more preferred (Balaram et al. 1992). Since CFPs were chosen to be investigated, electrochemical or electrophysiological devices and fluorescence spectroscopy were used extensively to understand the mechanism of their ionophoric activity. The understanding derived from the behaviour of CFPs was extrapolated to comprehend the mechanism of ion-channelling activity carried out by transmembrane receptor proteins. Most *peptaibols* are indeed CFPs that contain an unusually high percentage of helix-promoting amino acid residue, *Aib*.

14.5.1 Channel-Forming Ionophores and Uncouplers of Mitochondrial Oxidative Phosphorylation

***Alamethicin*:** In India, membrane perturbation studies by *peptaibols* were first started by Balaram's research group in the late 1970s. Drawing inspiration from the report by Mueller and Rudin, the investigations were focused on the effect of peptide's chain length (size) and charge on ion conductance (Mueller and Rudin 1968). Several *alamethicin* fragments of different sizes were chemically

synthesized (Nagaraj et al. 1980; Mathew et al. 1981). Fluorescence spectroscopic studies revealed that two synthetic *alamethicin* fragment esters of 13- and 17-residues long were able to translocate divalent cations in unilamellar liposomes. These *alamethicin* fragments could also uncouple the activity of oxidative phosphorylation in rat liver mitochondria, suggesting that these two peptide esters formed functional channels (Mathew et al. 1982). The corresponding peptide acids also were active. Further, it was noticed that the longer peptides, but not the shorter ones, aggregated at lower concentrations and the channels' activity improved with the better formation of aggregates, viz. the functional channels composed of longer peptides elicited better activity (Mathew et al. 1982). Thus, the investigations performed on fragments aided in understanding the molecular structural requirements for optimal ionophoric activity and for uncoupling mitochondrial oxidative phosphorylation.

Zervamicins: *Zervamicins* are a family of 16-residue *peptaibols* identified in the fungus *Emericellopsis salmosynnemata*, and they contain a larger proportion of hydrophilic amino acids, such as glutamine, threonine and hydroxyproline (Rinehart Jr. et al. 1981). Therefore, it was surmised that they might possess better amphipathic character and, hence, may be better models than *alamethicin*, for investigating the mechanism of transmembrane ionophoric function (Balaram et al. 1992). The inferences drawn from the experiments carried out on three natural *zervamicins* and one chemically synthesized peptide, which is an analogue of *zervamicin*, are summarized (Agarwalla et al. 1992; Balaram et al. 1992). From the voltage-dependent and concentration-dependent macroscopic conductance measurements carried out on *zervamicin-IIB* (***Zrv-IIB***) within the lipid bilayer (diphytanoyl phosphatidylcholine), the number of ***Zrv-IIB*** molecules involved in the formation of ion channel was estimated to be ~13. So, it was inferred that 'this' pore-like structure created within the lipid bilayer might not have a circular cross-section but perhaps a torpedo or an elliptical-shaped cross-section. One of the interpretations proposed to understand such an unusual aggregation or oligomerization behaviour was that there might be a mismatch between the peptide's length and the thickness of the lipid bilayer. Energetics of helix packing and dipole-dipole repulsions can also influence the oligomerization process of pore formation by ***Zrv-IIB***. In the case of ***zervamicin-IC***, the channels were activated by use of cis-negative potentials and not by positive potentials. The behaviour of *[Leu-1]zervamicin* was found to be qualitatively very similar to that of ***Zrv-IIB***. The results obtained from the synthetic analogue of *zervamicin*, ***Zrv-A1-16***, were ambiguous to decide, whether it formed ion-channel or not (Balaram et al. 1992).

Antiamoebins: ***Antiamoebin I***, a 16-residue long *peptaibol* could perturb the mitochondrial membrane of rat liver, whereby it uncoupled (but did not inhibit) oxidative phosphorylation in a medium containing phosphate in the concentration range: 2.5–100 mM (Das et al. 1986). Further, four HPLC fractions consisting of microheterogeneous components of ***antiamoebin*** were found to induce Ca^{2+} flux across lipid bilayer membranes, and these four fractions were also noted to be uncouplers of mitochondrial oxidative phosphorylation (Das et al. 1988).

14.5.2 Antimalarial Activity

Hemalatha Balaram's research group at Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, has investigated the antimalarial potential of three different classes of Peptaibiotics. ***Zervamicins***, ***antiamoebin I*** and ***efrapeptins*** were all found to kill *Plasmodium falciparum* in the culture with 50% inhibitory concentrations in the micromolar range (Nagaraj et al. 2001).

14.5.3 Cytotoxic Activity

The cytotoxic potential of four lipovelutibols (*lipopeptaibols*) and a *peptaibol* from the Himalayan cold habitat fungus *Trichoderma velutinum* was tested on four different cancer cell lines: HL-60 (human myeloid leukaemia), LS180 (human colon cancer), MDA-MB-231 (human breast cancer) and A549 (human lung cancer), by a team of researchers guided by Deepika Singh and Ram A. Vishwakarma, at CSIR-IIIM, Jammu (Singh et al. 2018, 2020). One lipovelutibol showed cytotoxic activity with 50% inhibitory concentration (IC_{50}) of 2μM and 4μM, against HL-60 and MDA-MB-231, respectively (Singh et al. 2018). Another lipovelutibol elicited cytotoxicity with IC_{50} in the range of ~4–7μM, against all the four cancer cell lines (Singh et al. 2018). In the case of the 14-residue long velutibol A, better cytotoxicity was observed against HL-60 (IC_{50} ~ 4μM) and MDA-MB-231 (IC_{50} ~ 7μM), than against other two cancer cell lines (Singh et al. 2020).

14.6 Future Perspectives

One of the mechanisms, through which the *peptaibols* elicit antibiotic or cytotoxic activity against various targets such as bacterial, fungal and mammalian cells, is by forming ion channels or pores by inserting into the cell membranes (lipid bilayer) thereby enabling translocation of ions across the membrane. Some *peptaibols* also form voltage-dependent ion channels within this cellular membrane environment (Nagaraj and Balaram 1981b; Mathew et al. 1982; Sansom 1993b). Despite that many *peptaibols* have been demonstrated to have antimicrobial properties, only a few studies are available showing their activity on mammalian cells, especially the cytotoxic activity (Du et al. 2017; Singh et al. 2018, 2020). Also, not many *peptaibol*-based drug compounds are known to have reached the pharmaceutical market. This may be attributed to their toxic properties or lower pharmacological potencies (viz. higher 50% inhibitory concentrations (IC_{50}), which may be in the range of mM or μM), due to which they might have been ruled out in different stages of preclinical and/or clinical trials. Nevertheless, it may be possible to engineer the natural *peptaibols* to yield greater potency with less toxicity than the respective natural *peptaibol's* biological/pharmacological activity. In this regard, the semi-synthetic modified analogues of *gichigamins* (identified in a *Tolypocladium* species derived from the deep-water sediment sample collected in Lake Superior, Michigan)

were found to display far better cytotoxic activity towards pancreatic tumour cell lines than the native *gichigamin* and its natural analogues (Du et al. 2017). Thus, the study on *gichigamins* offers encouragement to continue further research on *peptaibols*/*peptaibiotics*, whereby several different semi-synthetic analogues can be prepared and screened for identifying those candidates that elicit better biological or pharmacological activity. Despite lacking therapeutic potential, *peptaibols*, particularly from the genus *Trichoderma*, have been found to be major components possessing potential as biocontrol agents against plant pathogens in agriculture (Degenkolb et al. 2015). So, perhaps widespread agricultural applications of *peptaibols*/*peptaibiotics* seem imminent.

With regard to *peptaibol* biosynthesis, it seems that not many studies have been undertaken either for understanding their biosynthetic pathways or to engineer the genetic elements that code for NRPS for directed biosynthesis of different *peptaibol* sequences, barring a few (Wiest et al. 2002; Raap et al. 2005; Wei et al. 2005; Mukherjee et al. 2011). Therefore, ample scope is left for directed or customized biosynthesis of *peptaibols*, whereby the *peptaibol* sequences may be pertinently tailored by designing and/or manipulating those genes that correspond to appropriate modules in the NRPS and, thus, produce *peptaibols* of desirable biological or pharmacological functions, which may be suitable to be taken up for biotechnological (industrial), agricultural as well as for biomedical applications. Thus, biosynthesis of *peptaibols* through genetic engineering may have bright prospects in future.

Thus, for all future investigations, mass spectrometry may be an indispensable analytical technique, as it can rapidly provide details about the size and sequence of the newly produced *peptaibols*.

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References

Agarwalla S, Mellor IR, Sansom MSP, Karle IL, Flippen-Anderson JL, Uma K, Krishna K, Sukumar M, Balaram P (1992) Zervamicins, a structurally characterised peptide model for membrane ion channels. Biochem Biophys Res Commun 186:8–15

Anders R, Ohlenschläger O, Soskic V, Wenschuh H, Heise B, Brown LR (2000) The NMR solution structure of the ion channel peptaibol chrysospermin C bound to dodecylphosphocholine micelles. Eur J Biochem 267:1784–1794. https://doi.org/10.1046/j.1432-1327.2000.01177.x

Auvin-Guette C, Rebuffat S, Prigent Y, Bodo B (1992) Trichogin A IV, an 11-residue lipopeptaibol from *Trichoderma longibrachiatum*. J Am Chem Soc 114:2170–2174. https://doi.org/10.1021/ja00032a035

Auvin-Guette C, Rebuffat S, Vuidepot I, Massias M, Bodo B (1993) Structural elucidation of Trikoningins KA and KB, peptaibols from *Trichoderma koningii*. J Chem Soc Perkin Trans 1:249–255

Babu VVS, Kantharaju, Sudarshan NS (2006) Synthesis of Fmoc-protected β-amino alcohols and peptidyl alcohols from Fmoc-amino acid/peptide acid azides. Indian J Chem Sect B Org Chem Incl Med Chem 45B:1880–1886

Balaram P (1983) Membrane active peptides containing α-aminoisobutyric acid. Pierce Chem. Co., Dallas, TX, pp 477–486

Balaram P (1985) Proton NMR studies of peptide conformations. Proc Indian Acad Sci Chem Sci 95:21–38

Balaram P, Krishna K, Sukumar M, Mellor IR, Sansom MSP (1992) The properties of ion channels formed by zervamicins. Eur Biophys J 21:117–128. https://doi.org/10.1007/BF00185426

Barber M, Bordoli RS, Sedgwick RD, Tyler AN (1981a) Fast atom bombardment of solids as an ion source in mass spectrometry. Nature 293:270–275

Barber M, Bordoli RS, Sedgwick RD, Tyler AN (1981b) Fast atom bombardment of solids (F.A. B.): a new ion source for mass spectrometry. J Chem Soc Chem Commun:325–327

Biemann K (1990) Appendix 5. Nomenclature for peptide fragment ions (positive ions). Methods Enzymol 193:886–887

Boheim G, Kolb HA (1978) Analysis of the multi-pore system of alamethicin in a lipid membrane. J Membr Biol 38:99–150

Brückner H, Konig WA, Greiner M, Jung G (1979) The sequences of the membrane-modifying peptide antibiotic Trichotoxin A-40. Angew Chem Int Ed 18:476–477

Brückner H, Przybylski M (1984) Isolation and structural characterization of polypeptide-antibiotics of the peptaibol class by high-performance liquid chromatography with field desorption and fast atom bombardment mass spectrometry. J Chromatogr 296:263–275

Brückner H, König WA, Aydin M, Jung G (1985) Trichotoxin A40. Purification by counter-current distribution and sequencing of isolated fragments. Biochim Biophys Acta (BBA)/Protein Struct Mol 827:51–62. https://doi.org/10.1016/0167-4838(85)90100-1

Das MK, Raghothama S, Balaram P (1986) Membrane channel forming polypeptides. Molecular conformation and mitochondrial uncoupling activity of antiamoebin, an alpha-aminoisobutyric acid containing peptide. Biochemistry 25:7110–7117

Das MK, Krishna K, Balaram P (1988) Membrane modifying activity of four peptide components of antiamoebin, a microheterogeneous fungal antibiotic. Indian J Biochem Biophys 25:560–565

de Hoffmann E, Stroobant V (2007) Mass spectrometry: principles and applications. 3rd Edition, Wiley, Hoboken, NJ

Degenkolb T, Berg A, Gams W, Schlegel B, Gräfe U (2003) The occurrence of peptaibols and structurally related peptaibiotics in fungi and their mass spectrometric identification via diagnostic fragment ions. J Pept Sci 9:666–678. https://doi.org/10.1002/psc.497

Degenkolb T, Nielsen KF, Dieckmann R, Branco-Rocha F, Chaverri P, Samuels GJ, Thrane U, von Döhren H, Vilcinskas A, Brückner H (2015) Peptaibol, secondary-metabolite, and hydrophobin pattern of commercial biocontrol agents formulated with species of the *Trichoderma harzianum* complex. Chem Biodivers 12:662–684

Du L, Risinger AL, Mitchell CA, You J, Stamps BW, Pan N, King JB, Bopassa JC, Judge SIV, Yang Z, Stevenson BS, Cichewicz RH (2017) Unique amalgamation of primary and secondary structural elements transform peptaibols into potent bioactive cell-penetrating peptides. Proc Natl Acad Sci U S A 114:E8957–E8966. https://doi.org/10.1073/PNAS.1707565114

Fenn JB (2003) Electrospray wings for molecular elephants (Nobel Lecture). Angew Chem Int Ed 42:3871–3894

Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM (1989) Electrospray ionization for mass spectrometry of large biomolecules. Science 246:64–71

Fenselau C, Demirev PA (2001) Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev 20:157–171

Fox RO, Richards FM (1982) A voltage-gated ion channel model inferred from the crystal structure of alamethicin at 1.5-Å resolution. Nature 300:325–330. https://doi.org/10.1038/300325a0

Francis AK, Iqbal M, Balaram P, Vijayan M (1982a) The crystal structure of the amino-terminal pentapeptide of suzukacillin. Occurrence of a four-fold peptide helix. J Chem Soc Perkin Trans Phys Org Chem 2:1235–1239

Francis AK, Rao CP, Iqbal M, Nagaraj R, Vijayan M, Balaram P (1982b) Helical conformations of three crystalline pentapeptide fragments of suzukacillin, a membrane channel forming polypeptide. Biochem Biophys Res Commun 106:1240–1247. https://doi.org/10.1016/0006-291x(82)91245-1

Francis AK, Iqbal M, Balaram P, Vijayan M (1983) Crystal structure of Boc-Ala-Aib-Ala-Aib-Aib-methyl ester, a pentapeptide fragment of the channel-forming ionophore suzukacillin. Biopolymers 22:1499–1505. https://doi.org/10.1002/bip.360220606

Good DM, Wirtala M, McAlister GC, Coon JJ (2007) Performance characteristics of electron transfer dissociation mass spectrometry. Mol Cell Proteomics 6:1942–1951

Goodman M, McGahren WJ (1967) Mechanistic studies of peptide oxazolone racemization. Tetrahedron 23:2031–2050

Gupta S, Krasnoff SB, Roberts DW, Renwick JAA, Brinen LS, Clardy J (1991) Structures of the efrapeptins: potent inhibitors of mitochondrial ATPase from the fungus *Tolypocladium niveum*. J Am Chem Soc 113:707–709. https://doi.org/10.1021/ja00002a068

Gupta S, Krasnoff SB, Roberts DW, Renwick JAA, Brinen LS, Clardy J (1992) Structure of efrapeptins from the fungus *Tolypocladium niveum*: peptide inhibitors of mitochondrial ATPase. J Org Chem 57:2306–2313

Gupta K, Kumar M, Chandrashekara K, Krishnan KS, Balaram P (2012) Combined electron transfer dissociation-collision-induced dissociation fragmentation in the mass spectrometric distinction of leucine, isoleucine, and hydroxyproline residues in peptide natural products. J Proteome Res 11:515–522. https://doi.org/10.1021/pr200091v

Gurunath R, Balaram P (1995) A nonhelical, multiple beta-turn conformation in a glycine-rich heptapeptide fragment of Trichogin A IV containing a single central alpha-aminoisobutyric acid residue. Biopolymers 35:21–29. https://doi.org/10.1002/bip.360350104

Huang Q, Tezuka Y, Kikuchi T, Nishi A, Tubaki K, Tanaka K (1995) Studies on metabolites of mycoparasitic fungi. II Metabolites of *Trichoderma koningii*. Chem Pharm Bull 43:223–229

Iida A, Sanekata M, Fujita T, Tanaka H, Enoki A, Fuse G, Kanai M, Rudewicz PJ, Tachikawa E (1994) Fungal metabolites XVI. Structures of new peptaibols, Trichokindins I–VII, from the fungus, *Trichoderma harzianum*. Chem Pharm Bull 42:1070–1075

Iqbal M, Balaram P (1981a) Membrane channel forming polypeptides. 270-MHz Hydrogen-1 Nuclear Magnetic Resonance studies on the conformation of the 11–21 fragment of suzukacillin. Biochemistry 20:4866–4871

Iqbal M, Balaram P (1981b) The 3_{10} helical conformation of the amino terminal decapeptide of suzukacillin. 270 MHz ^{1}H NMR evidence for eight intramolecular hydrogen bonds. J Am Chem Soc 103:5548–5552

Iqbal M, Balaram P (1982) The helical conformations of 14- and 16-residue fragments of suzukacillin, a membrane channel-forming polypeptide. Biochim Biophys Acta Protein Struct Mol Enzymol 706:179–187. https://doi.org/10.1016/0167-4838(82)90485-x

Jadhav SV, Bandyopadhyay A, Benke SN, Mali SM, Gopi HN (2011) A facile synthesis and crystallographic analysis of N-protected β-amino alcohols and short peptaibols. Org Biomol Chem 9:4182–4187. https://doi.org/10.1039/c0ob01226b

Jaworski A, Brückner H (1999) Detection of new sequences of peptaibol antibiotics trichotoxins A-40 by on-line liquid chromatography-electrospray ionization mass spectrometry. J Chromatogr A 862:179–189. https://doi.org/10.1016/S0021-9673(99)00931-0

Jones DS, Kenner GW, Preston J, Sheppard RC (1965) Peptides. XVII. Synthesis of peptides and polymers of some sterically hindered amino acids via oxazolone intermediates. J Chem Soc:6227–6239. https://doi.org/10.1039/jr9650006227

Karas M, Hillenkamp F (1988) Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem 60:2299–2301

Karle IL, Sukumar M, Balaram P (1986) Parallel packing of alpha-helices in crystals of the zervamicin IIA analog Boc-Trp-Ile-Ala-Aib-Ile-Val-Aib-Leu-Aib-Pro-OMe.2H2O. Proc Natl Acad Sci U S A 83:9284–9288

Karle IL, Flippen-Anderson J, Sukumar M, Balaram P (1987) Conformation of a 16-residue zervamicin IIA analog peptide containing three different structural features: 3(10)-helix, alpha-helix, and beta-bend ribbon. Proc Natl Acad Sci U S A 84:5087–5091

Karle IL, Flippen-Anderson JL, Agarwalla S, Balaram P (1991) Crystal structure of [Leu1] zervamicin, a membrane ion-channel peptide: implications for gating mechanisms. Proc Natl Acad Sci U S A 88:5307–5311

Karle IL, Flippen-Anderson JL, Agarwalla S, Balaram P (1994) Conformation of the flexible bent helix of Leu1-zervamicin in crystal C and a possible gating action for ion passage. Biopolymers 34:721–735

Karle IL, Perozzo MA, Mishra VK, Balaram P (1998) Crystal structure of the channel-forming polypeptide antiamoebin in a membrane-mimetic environment. Proc Natl Acad Sci U S A 95:5501–5504. https://doi.org/10.1073/pnas.95.10.5501

Katoch M, Singh D, Kapoor KK, Vishwakarma RA (2019) *Trichoderma lixii* (IIIM-B4), an endophyte of *Bacopa monnieri* L. producing peptaibols. BMC Microbiol 19:1–10. https://doi.org/10.1186/s12866-019-1477-8

Khosla C, Herschlag D, Cane DE, Walsh CT (2014) Assembly line polyketide synthases: mechanistic insights and unsolved problems. Biochemistry 53:2875–2883

Kinter M, Sherman NE (2000) Protein sequencing and identification using tandem mass spectrometry. Wiley, Hoboken, NJ

Kleinkauf H, Von Döhren H (1996) A nonribosomal system of peptide biosynthesis. Eur J Biochem 236:335–351. https://doi.org/10.1111/j.1432-1033.1996.00335.x

Krause C, Kirschbaum J, Brückner H (2006) Peptaibiomics: an advanced, rapid and selective analysis of peptaibiotics/peptaibols by SPE/LC-ES-MS. Amino Acids 30:435–443. https://doi.org/10.1007/s00726-005-0275-9

Krishna K, Sukumar M, Balaram P (1990) Structural chemistry and membrane modifying activity of the fungal polypeptides zervamicins, antiamoebins and efrapeptins. Pure Appl Chem 62:1417–1420. https://doi.org/10.1351/pac199062071417

Leclerc G, Goulard C, Prigent Y, Bodo B, Wróblewski HRS (2001) Sequences and antimycoplasmic properties of longibrachins LGB II and LGB III, two novel 20-residue peptaibols from *Trichoderma longibrachiatum*. J Nat Prod 64:164–170

Leplawy MT, Jones DS, Kenner GW, Sheppard RC (1960) Peptides. XI. Synthesis of peptides derived from α-methylalanine. Tetrahedron 11:39–51. https://doi.org/10.1016/0040-4020(60)89006-0

Marahiel MA, Stachelhaus T, Mootz HD (1997) Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem Rev 97:2651–2673. https://doi.org/10.1021/cr960029e

Mathew MK, Nagaraj R, Balaram P (1981) Alamethicin and synthetic peptide fragments as uncouplers of mitochondrial oxidative phosphorylation. Effect of chain length and charge. Biochem Biophys Res Commun 98:548–555

Mathew MK, Nagaraj R, Balaram P (1982) Membrane channel-forming polypeptides. J Biol Chem 257:2170–2176

Mohamed-Benkada M, Montagu M, Biard J-F, Mondeguer F, Verite P, Dalgalarrondo M, Bissett J, Pouchus YF (2006) New short peptaibols from a marine *Trichoderma* strain. Rapid Commun Mass Spectrom 20:1176–1180. https://doi.org/10.1002/rcm.2430

Momose I, Onodera T, Doi H, Adachi H, Lijima M, Yamazaki Y, Sawa R, Kubota Y, Igarashi M, Kawada M (2019) Leucinostatin Y: a peptaibiotic produced by the entomoparasitic fungus *Purpureocillium lilacinum* 40-H-28. J Nat Prod 82:1120–1127. https://doi.org/10.1021/acs.jnatprod.8b00839

Mueller P, Rudin DO (1968) Action potentials induced in biomolecular lipid membranes. Nature 217:713–719

Mukherjee PK, Wiest A, Ruiz N, Keightley A, Moran-Diez ME, McCluskey K, Pouchus YF, Kenerley CM (2011) Two classes of new peptaibols are synthesized by a single non-ribosomal peptide synthetase of *Trichoderma virens*. J Biol Chem 286:4544–4554. https://doi.org/10.1074/jbc.M110.159723

Nagaraj R, Balaram P (1979) Fluorescent hydrophobic peptide fragments of emerimicin. Models for the study of peptide aggregation and interactions with lipids and proteins. Biochem Biophys Res Commun 89:1041–1049

Nagaraj R, Balaram P (1981a) Solution phase synthesis of alamethicin I. Tetrahedron 37:1263–1270. https://doi.org/10.1016/S0040-4020(01)92061-7

Nagaraj R, Balaram P (1981b) Alamethicin, a transmembrane channel. Acc Chem Res 14:356–362. https://doi.org/10.1021/ar00071a005

Nagaraj R, Mathew MK, Balaram P (1980) Cation translocating effects of alamethicin and its synthetic fragments in lipid membranes. FEBS Lett 121:365–368

Nagaraj G, Uma MV, Shivayogi MS, Balaram H (2001) Antimalarial activities of peptide antibiotics isolated from fungi. Antimicrob Agents Chemother 45:145–149. https://doi.org/10.1128/AAC.45.1.145-149.2001

Neuhof T, Dieckmann R, Druzhinina IS, Kubicek CP, von Döhren H (2007) Intact-cell MALDI-TOF mass spectrometry analysis of peptaibol formation by the genus *Trichoderma/Hypocrea*: can molecular phylogeny of species predict peptaibol structures? Microbiology 153:3417–3437. https://doi.org/10.1099/mic.0.2007/006692-0

Pandey RC, Cook JC Jr, Rinehart KL Jr (1977a) High resolution and field desorption mass spectrometry studies and revised structures of alamethicins I and II. J Am Chem Soc 99:8469–8483

Pandey RC, Cook JC Jr, Rinehart KL Jr (1977b) Reptaibophol antibiotics. 2. Structures of the peptide antibiotics emerimicins III and IV. J Am Chem Soc 99:5205–5206

Pandey RC, Meng H, Cook JC Jr, Rinehart KL Jr (1977c) Structure of antiamoebin I from high resolution field desorption and gas chromatographic mass spectrometry studies. J Am Chem Soc 99:5203–5205

Patel R (2019) A moldy application of MALDI: MALDI-ToF mass spectrometry for fungal identification. J Fungi 5:4. https://doi.org/10.3390/jof5010004

Pócsfalvi G, Ritieni A, Ferranti P, Randazzo G, Vékey K, Malorni A (1997) Microheterogeneity characterization of a paracelsin mixture from *Trichoderma reesei* using high-energy collision-induced dissociation tandem mass spectrometry. Rapid Commun Mass Spectrom 11:922–930

Poirier L, Amiard JC, Mondeguer F, Quiniou F, Ruiz N, Pouchus YF, Montagu M (2007) Determination of peptaibol trace amounts in marine sediments by liquid chromatography/electrospray ionization-ion trap-mass spectrometry. J Chromatogr A 1160:106–113. https://doi.org/10.1016/j.chroma.2007.04.006

Polfer NC, Oomens J, Suhai S, Paizs B (2005) Spectroscopic and theoretical evidence for oxazolone ring formation in collision-induced dissociation of peptides. J Am Chem Soc 127:17154–17155. https://doi.org/10.1021/ja056553x

Prabhu G, Narendra N, Basavaprabhu, Panduranga V, Sureshbabu VV (2015) Amino acid fluorides: viable tools for synthesis of peptides, peptidomimetics and enantiopure heterocycles. RSC Adv 5:48331–48362. https://doi.org/10.1039/C4RA16142D

Przybylski M, Dietrich I, Manz I, Brückner H (1984) Elucidation of structure and microheterogeneity of the polypeptide antibiotics paracelsin and trichotoxin A-50 by fast atom bombardment mass spectrometry in combination with selective in situ hydrolysis. Biomed Mass Spectrom 11:569–582

Raap J, Erkelens K, Ogrel A, Skladnev DA, Brückner H (2005) Fungal biosynthesis of non-ribosomal peptide antibiotics and α, α-dialkylated amino acid constituents. J Pept Sci 11:331–338. https://doi.org/10.1002/psc.621

Raj PA, Das MK, Balaram P (1988) Conformations and mitochondrial uncoupling activity of synthetic emerimicin fragments. Biopolymers 27:683–701

Ramakrishnan C, Soman KV (1982) Identification of secondary structures in globular proteins - a new algorithm. Int J Pept Protein Res 20:218–237. https://doi.org/10.1111/j.1399-3011.1982.tb03052.x

Ramanathan G (1994) Non protein amino acids in de novo design: an evaluation. Indian Institute of Science, Bangalore

Rao CP, Balaram P (1982) Molecular structure of t-butyloxycarbonyl-Leu-Aib-Pro-Val-Aib-methyl ester, a fragment of alamethicin and suzukacillin: a 310-helical pentapeptide. Biopolymers 21:2461–2472. https://doi.org/10.1002/bip.360211210

Rao CP, Shamala N, Nagaraj R, Rao CNR, Balaram P (1981) Hydrophobic channels in crystals of an α-aminoisobutyric acid pentapeptide. Biochem Biophys Res Commun 103:898–904. https://doi.org/10.1016/0006-291x(81)90895-0

Rebuffat S, Prigent Y, Auvin-Guette C, Bodo B (1991) Tricholongins BI and BII, 19-residue peptaibols from *Trichoderma longibrachiatum*. Solution structure from two-dimensional NMR spectroscopy. Eur J Biochem 201:661–674

Rinehart KL Jr, Gaudioso LA, Moore ML, Pandey RC, Cook JC Jr, Barber M, Sedgwick RD, Bordoli RS, Tyler AN, Green BN (1981) Structures of eleven zervamicin and two emerimicin peptide antibiotics studied by fast atom bombardment mass spectrometry. J Am Chem Soc 103:6517–6520

Roepstorff P, Fohlman J (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biomed Mass Spectrom 11:601

Rowley DC, Kelly S, Kauffman CA, Jensen PR, Fenical W (2003) Halovirs A–E, new antiviral agents from a marine-derived fungus of the genus *Scytalidium*. Bioorg Med Chem 11:4263–4274. https://doi.org/10.1016/S0968-0896(03)00395-X

Ruiz N, Wielgosz-Collin G, Poirier L, Grovel O, Petit KE, Mohamed-Benkada M, Robiou du Pont T, Biddett J, Vérité P, Barnathan G, Pouchus YF (2007) New Trichobrachins, 11-residue peptaibols from a marine strain of *Trichoderma longibrachiatum*. Peptides 28:1351–1358. https://doi.org/10.1016/j.peptides.2007.05.012

Sabareesh V, Balaram P (2006) Tandem electrospray mass spectrometric studies of proton and sodium ion adducts of neutral peptides with modified N- and C-termini: synthetic model peptides and microheterogeneous peptaibol antibiotics. Rapid Commun Mass Spectrom 20:618–628. https://doi.org/10.1002/rcm.2349

Sansom MSP (1993a) Alamethicin and related peptaibols - model ion channels. Eur Biophys J 22:105–124. https://doi.org/10.1007/bf00196915

Sansom MSP (1993b) Structure and function of channel-forming peptaibols. Q Rev Biophys 26:365–421

Schlosser A, Lehmann WD (2000) Five-membered ring formation in unimolecular reactions of peptides: a key structural element controlling low-energy collision-induced dissociation of peptides. J Mass Spectrom 35:1382–1390. https://doi.org/10.1002/1096-9888(200012)35:12<1382::AID-JMS84>3.0.CO;2-6

Sharma R, Singh VP, Singh D, Yusuf F, Kumar A, Vishwakarma RA, Chaubey A (2016) Optimization of nonribosomal peptides production by a psychrotrophic fungus: *Trichoderma velutinum* ACR-P1. Appl Microbiol Biotechnol 100:9091–9102. https://doi.org/10.1007/s00253-016-7622-5

Shishupala S (2009) Bioactive peptaibol antibiotics from *Trichoderma*. In: Sridhar KR (ed) Front fungal ecology, diversity and metabolites. I.K. International Publishing House Pvt. Ltd., New Delhi, pp 300–320

Singh VP, Yedukondalu N, Sharma V, Kushwaha M, Sharma R, Chaubey A, Kumar A, Singh D, Vishwakarma RA (2018) Lipovelutibols A–D: cytotoxic lipopeptaibols from the Himalayan cold habitat fungus *Trichoderma velutinum*. J Nat Prod 81:219–226. https://doi.org/10.1021/acs.jnatprod.6b00873

Singh VP, Pathania AS, Kushwaha M, Singh S, Sharma V, Malik FA, Khan IA, Kumar A, Singh D, Vishwakarma RA (2020) 14-Residue peptaibol velutibol A from *Trichoderma velutinum*: its

structural and cytotoxic evaluation. RSC Adv 10:31233–31242. https://doi.org/10.1039/d0ra05780k

Smedsgaard J, Frisvad JC (1996) Using direct electrospray mass spectrometry in taxonomy and secondary metabolite profiling of crude fungal extracts. J Microbiol Methods 25:5–17. https://doi.org/10.1016/0167-7012(95)00073-9

Snook CF, Wallace BA (1999) The molecular-replacement solution of an intermediate-sized helical polypeptide, antiamoebin I. Acta Crystallogr Sect D Biol Crystallogr 55:1539–1545. https://doi.org/10.1107/S0907444999007921

Snook CF, Woolley GA, Oliva G, Pattabhi V, Wood SP, Blundell TL, Wallace BA (1998) The structure and function of antiamoebin I, a proline-rich membrane-active polypeptide. Structure 6:783–792. https://doi.org/10.1016/S0969-2126(98)00079-3

Stoppacher N, Reithner B, Omann M, Zeilinger S, Krska R, Schuhmacher R (2007) Profiling of trichorzianines in culture samples of *Trichoderma atroviride* by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 21:3963–3970

Stoppacher N, Neumann NKN, Burgstaller L, Zeilinger S, Degenkolb T, Brückner H, Schuhmacher R (2013) The comprehensive peptaibiotics database. Chem Biodivers 10:734–743. https://doi.org/10.1002/cbdv.201200427

Syka JEP, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. Proc Natl Acad Sci U S A 101:9528–9533

Tanaka K (2003) The origin of macromolecule ionization by laser irradiation (Nobel Lecture). Angew Chem Int Ed 42:3860–3870

Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T, Matsuo T (1988) Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 2:151–153

Thirumalachar MJ (1968) Antiamoebin, a new antiprotozoal-anthelmintic antibiotic. I. Production and biological studies. Hindustan Antibiot Bull 10:287–289

Toniolo C, Brückner H (2007) Peptaibiotics. Chem Biodivers 4:1021–1022

Toniolo C, Bonora GM, Bavoso A, Beneditti E, Di Blasio B, Pavone V, Pedone C (1983) Preferred conformations of peptides containing α,α-disubstituted α-amino acids. Biopolymers 22:205–215. https://doi.org/10.1002/bip.360220129

Tsantrizos YS, Pischos S, Sauriol F, Widden P (1996) Peptaibol metabolites of *Tolypocladium geodes*. Can J Chem 74:165–172. https://doi.org/10.1139/v96-020

Uma MV, Sudha R, Balaram P (2001) Spermidine as a potential biosynthetic precursor to the 1,5-diazabicyclo[4:3:0]nonene residue in the efrapeptins. J Pept Res 58:375–379. https://doi.org/10.1034/j.1399-3011.2001.00915.x

Venkatraman J, Shankaramma SC, Balaram P (2001) Design of folded peptides. Chem Rev 101:3131–3152. https://doi.org/10.1021/cr000053z

Wei X, Yang F, Straney DC (2005) Multiple non ribosomal peptide synthetase genes determine peptaibol synthesis in *Trichoderma virens*. Can J Microbiol 51:423–429

Wiest A, Grzegorski D, Xu BW, Goulard C, Rebuffat S, Ebbole DJ, Bodo B, Kenerley C (2002) Identification of peptaibols from *Trichoderma virens* and cloning of a peptaibol synthetase. J Biol Chem 277:20862–20868. https://doi.org/10.1074/jbc.M201654200

Woolley GA, Wallace BA (1992) Model ion channels: gramicidin and alamethicin. J Membr Biol 129:109–136. https://doi.org/10.1007/bf00219508

Development of Mycotoxicology in India

15

Anjali Sharma and Geeta Sumbali

Abstract

Mycotoxigenic fungi grow on a wide range of agricultural crops and their products and have the ability to produce one or more, low-molecular-weight toxic secondary metabolites known as mycotoxins. This term was coined after a veterinary outbreak in England (UK) in 1962 where lakhs of poultry birds died after ingesting aflatoxin-contaminated peanut meal. Since then, mycotoxins have assumed global importance. From India also, a number of acute and chronic diseases and in some cases fatal consequences have been reported among humans and animals after ingestion of food/feed contaminated with mycotoxins. Various environmental parameters like temperature, water activity and pH along with the nutritional status of the substrate are the key determinants of fungal colonization and biosynthesis of mycotoxins. The most important mycotoxigenic species belong to the fungal genera *Aspergillus*, *Penicillium* and *Fusarium*, which produce mycotoxins like aflatoxins, ochratoxins, fumonisins, patulin, citrinin, zearalenone, deoxynivalenol and many more. In view of being natural unavoidable contaminants of foods and feeds, mycotoxins impose health risks to the consumers as they are heat stable and have diverse toxic effects and synergistic properties. This review summarizes data on the natural occurrence of mycotoxins with acute toxicological characteristics found associated with cereals, animal feeds, dried medicinal herbs, fresh and sun-dried fruits and vegetables, dry nuts, spices and condiments, oil seeds, oilseed cakes, vegetable oils and many other consumables of importance grown and consumed in India.

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15.1 Introduction

Mycotoxicology is the branch of mycology that focuses on analysing and studying the toxins produced by filamentous fungi. These mould toxins known as mycotoxins are a group of structurally diverse, low-molecular-weight secondary metabolites, which evoke a toxic response in higher vertebrates and other animals when introduced by a natural route in low concentration (Bennett 1987). According to him, mycotoxins exclude mushroom and yeast poisons. So far, approximately 350 mould species are known to produce around 300 mycotoxins and related metabolites (Singh et al. 1991). The mycotoxic contaminants of importance are produced chiefly by the species of *Aspergillus*, *Penicillium* and *Fusarium*, and they include aflatoxins, ochratoxins, cyclopiazonic acid, patulin, citrinin, fumonisins, deoxynivalenol, nivalenol and zearalenone (Reddy et al. 2009a, b).

Mycotoxins have been implicated as causative agents in a number of disease syndromes known as mycotoxicoses. Symptoms of mycotoxicoses depend on the type of mycotoxin ingested, duration of exposure, species, age, sex, nutritional status and presence of other infectious diseases (Pohland 1993). According to Pier et al. (1980), mycotoxicoses in humans and animals can be divided into three general forms:

1. Acute primary mycotoxicoses, which develop after consumption of moderate to high amounts of mycotoxins. Specific symptoms of the toxic effect of the mycotoxins can be observed in the consumers.
2. Chronic primary mycotoxicoses, which result by the intake of mycotoxins varying from moderate to low levels. Often non-specific effects, such as reduced weight gain and interference with reproductive efficiency, occur.
3. Secondary mycotoxic diseases, which result from low levels of mycotoxin intake and do not cause overt mycotoxicoses. However, such individuals get predisposed to infectious diseases through impairment of immune system and native mechanism of resistance.

Toxigenic moulds are ubiquitous in nature as they regularly infest susceptible agricultural commodities. They belong to two distinct groups, the first group commonly called as the field fungi includes those which invade and produce toxins before harvest, and the second group, the storage fungi that are problematic during post-harvest phase. In both the cases, field is usually implicated as the original source of inoculum (Miller 1995). Species of *Fusarium* often affect the growing crops, such as corn, wheat, rice and barley in the field and propagate inside the plant, whereas species of *Aspergillus* and *Penicillium* grow on the foods and feeds under storage

conditions (Tanaka et al. 1988; Bennett and Klich 2003). Presently, more than 300 mycotoxins have been reported and identified, but only a few are known to contaminate food and feed stuffs. These include aflatoxins, ochratoxins, patulin, citrinin, fumonisins, zearalenone and trichothecenes including deoxynivalenol and T-2 toxin (De Boevre et al. 2012; Pereira et al. 2014).

Factors that determine fungal infestation and the type and amount of mycotoxin produced include right combination of the toxigenic strains of the fungus, substrate composition and the prevailing environmental conditions (Tola and Kebede 2016). Preharvest fungal invasion is dependent on the host-parasite interaction and the presence of other biological forms such as insects. In addition, heavy rainfall or droughts, poor soil fertility, weed competition, application of fertilizers and fungicides and other agronomic practices favour accumulation of more mycotoxins in the plants by influencing the metabolism of toxigenic fungal species (Miller 1994). On the other hand, post-harvest mycotoxin contamination of plant products usually occur when there is delay in sun-drying due to humid weather conditions, inappropriate storage structures and conditions and prolonged storage period.

15.2 Historical Records of Mycotoxicoses

Mycotoxins have caused major epidemics in man and animals since the beginning of organized crop production. The first historical record of human and livestock mycotoxicoses is that of ergotism or St. Anthony's fire, a disease that was recognized centuries ago and finally associated with *Claviceps purpurea* (ergot fungus) in 1711. Ergotism resulted from the consumption of rye and other cereals that were contaminated with the long, hard and dark sclerotia of *Claviceps purpurea*. Early symptoms of poisoning include nausea, vomiting, muscle pain, weakness, numbness, which later progress to convulsions, hallucinations, gangrene of the extremities, unconsciousness and death. Ergotism killed hundreds of thousands of people in Europe in the last millennium (Smith and Moss 1985).

Another case of mycotoxicoses recognized to have seriously affected human populations occurred in Russia during World War II, where thousands of people in certain regions of erstwhile Soviet Union died as a consequence of consuming baked products made from millet that had spent the winter under the snowdrifts. The incident occurred in the 1940s, but information about it was not commonly known until early 1960s. Later, investigations revealed that the millet had supported growth of moulds, principally the genus *Fusarium*, which had produced toxic substances, probably trichothecenes that caused the illness named alimentary toxic aleukia (ATA) or septic angina. Patients with ATA typically exhibited dermonecrosis, GI irritation, diarrhoea, excessive salivation and a burning sensation in the oral cavity and oesophagus followed by leucopenia, anaemia and jaundice, the severity of which correlated with increasing trichothecene exposure (Joffe 1974).

The event that caused concern about toxigenic moulds occurred in England (UK) in 1960 and was associated with illness in turkeys, ducklings and baby chicks. Thousands of these animals died, which resulted in a serious economic loss for

farmers. The disease was given the name 'Turkey X disease', and later investigations revealed that it occurred due to consumption of mouldy peanut meal imported from Brazil, which was contaminated with ubiquitous mould, *Aspergillus flavus*, that produced various aflatoxins. Again in 1960, widespread occurrence of trout hepatoma was detected in many hatcheries in the USA, and in due course this outbreak was also attributed to aflatoxins present in the cottonseed meal diet (Sinnhuber et al. 1965).

Another major outbreak of mycotoxicoses known as acute cardiac beriberi occurred in Japan in the second half of the nineteenth century, which caused heart distress, nausea, vomiting, pain and anguish and, in extreme cases, respiratory failure and death. This disease was associated with fungal growth in rice—the yellow rice syndrome—due to the toxins from *Penicillium citrinum* and *P. islandicum* (Uraguchi 1969). Similarly, in 1971, cases of acute encephalopathy or Reye's syndrome occurred in children aged 1–6 years, who lived in the suburban or rural areas of Bangkok, Thailand. It is now widely accepted that aflatoxin B_1 was involved in part with this disease syndrome, which resulted in a 60–80% death rate (Sunakorn and Kalayanarooj 1990). Some evidences have even proved that Balkan endemic nephropathy (BEN), a kidney disease first identified in the 1920s among several discrete communities living along the Danube River in Europe, is also due to human exposure to ochratoxin A (Hult et al. 1982; Stoev 1998). There is also evidence that facial eczema (pithomycotoxicosis) of sheep and cattle in New Zealand known for over 100 years is due to a toxin (sporidesmin) produced by the saprophytic fungus, *Pithomyces chartarum*, which grows on the litter at the base of pastures and sporulates; it produces the toxin sporidesmin, which when eaten by sheep, cattle, goats and deer also causes liver injury with inflammation and blockage of bile ducts (Di Menna et al. 2009). Similarly, in Denmark, mycotoxic nephropathy in pigs has been frequently identified and has been associated with the ingestion of ochratoxin A contaminated barley grains (Elling and Moller 1973).

Another outbreak of mycotoxicoses occurred in Wisconsin during winter months of 1970–1971, where 20% of the lactating cows in a dairy farm died after consuming a diet containing corn infested with *Fusarium tricinctum* and T-2 toxin (Hsu et al. 1972). During 1977–1978, Ethiopia recorded major outbreak of gangrenous ergotism affecting nearly 140 people of whom 34% died (King 1979). This outbreak was due to a long wet season that favoured infection of *C. purpurea* in wild oats. In another outbreak that occurred in 13 provinces of northern China during 1972–1988, at least 884 persons got affected due to food poisoning known as mouldy sugarcane poisoning (MSP) caused by *Arthrinium* species that produce a secondary toxic metabolite, 3-nitropropionic acid. At least 88 fatalities were recorded during this outbreak (Liu et al. 1992).

These cases of early mycotoxicoses formed the basis for mycotoxicological researches all over globe and stimulated analysis of other mycotoxins, especially those produced by species of *Aspergillus*, *Penicillium* and *Fusarium*, which may occur in cereals and other plant products that are destined for human and animal consumption.

15.3 Outbreaks of Mycotoxicoses in India

In India, the history of mycotoxin research began with the newspaper report (The Indian Express, New Delhi, Edition, March 25, 1975) about an outbreak of hepatitis that affected 397 people in Banswada (Rajasthan) and Panchmahals (Gujarat) during 1974–1975, of whom 106 died. It was reported that hepatitis was due to aflatoxins and the outbreak of aflatoxins lasted for about 2 months and was confined to tribal population. The outbreak was traced to the consumption of maize, which was heavily contaminated with *Aspergillus flavus* that produced aflatoxin concentration of 6.25–15.6 ppm (Krishnamachari et al. 1975a, b). Childhood cirrhosis in India also seems to be due to the consumption of parboiled rice and crude peanut oil containing up to 0.1 mg aflatoxin B_1/kg food (Robinson 1967; Amla et al. 1970, 1974).

In several outbreaks that occurred in Sikar and Jaipur districts of Rajasthan during 1956–1957 and 1975, hundreds of people were affected, and the poisoning is attributed to the consumption of pearl millet infected with *Claviceps* (Patel et al. 1958; Krishnamachari and Bhat 1976). From Tamil Nadu, toxicosis of chicks fed on *Aspergillus terreus* contaminated poultry feed was reported by Sekhar and Shanmugasundram (1979) and, from Andhra Pradesh, acute aflatoxicosis in pigs was recorded by Rao et al. (1980).

Later, an outbreak of gastrointestinal disorder occurred in Kashmir Valley (J&K) in 1987 where the main cause of toxicosis was consumption of locally prepared wheat bread contaminated with *Fusarium* species and fusarial toxins—deoxynivalenol, nivalenol, T-2 toxin and 3-acetyldeoxynivalenol (Bhat et al. 1989). Similarly, the outbreak that occurred during 1996 in the rural regions of Deccan Plateau affecting 1424 people was due to the consumption of sorghum and maize that was contaminated by the mycotoxin fumonisin B1. This investigation also revealed that sorghum has the potential for the occurrence of high levels of fumonisin that could result in human diseases (Bhat et al. 1997a, b). Later, mycotoxicoses had been reported in poultry from Deccan Plateau region which was again due to the fumonisin contaminated feed containing rain damaged maize (Vasanthi and Bhat 1998).

15.4 Mycotoxins, Types of Toxicity and Risk Assessment

Toxigenic strains of *Aspergillus*, *Penicillium* and *Fusarium* species regularly infest the susceptible agricultural commodities and produce some important mycotoxins like aflatoxins, cyclopiazonic acid, ochratoxin A, fumonisins, patulin, citrinin, deoxynivalenol, zearalenone and many more. These mycotoxins present acute food safety challenges as they affect the health of consumers by causing hepatotoxicity (Li et al. 2018), nephrotoxicity (Schulz et al. 2018), carcinogenesis (Ostry et al. 2017), mutagenesis (Kim et al. 2016), cytotoxicity (Malekinejad et al. 2015), genotoxicity (Domijan et al. 2015), immunotoxicity (Hueza et al. 2014), neurotoxicity (Malekinejad et al. 2015) and other estrogenic affects (Fink-Grernmels 1999;

Table 15.1 Important mycotoxins, types of toxicity and their major producers

Mycotoxins	Major types of mycotoxicity	Producer fungal species
Aflatoxins	Hepatotoxic, hepatocarcinogenic, immunosuppressive, mutagenic, teratogenic, carcinogenic	*Aspergillus flavus* *A. parasiticus* *A. nomius*
Sterigmatocystin	Carcinogenic	*Aspergillus nidulans* *A. versicolor* *A. sydowii* *Emericella* species
Ochratoxin A	Nephrotoxic, genotoxic, immunosuppressive, carcinogenic, teratogenic	*Aspergillus ochraceus* *A. carbonarious* *A. glaucus* *A. melleus* *Penicillium verrucosum*
Citrinin	Nephrotoxic, genotoxic	*Penicillium citrinum* *Aspergillus terreus*
Patulin	Carcinogenic, teratogenic, mutagenic, neurotoxic, genotoxic, immunosuppressive, gastrointestinal affects	*Penicillium expansum* *P. patulum* (*P. griseofulvum*) *Byssochlamys nivea* *Aspergillus terreus*
Cyclopiazonic acid	Affects muscle tissues, neurotoxic	*Aspergillus flavus* *Penicillium commune*
Zearalenone (F-2 toxin)	Estrogenic effects, disruption of sex steroid hormone functions, abortions in various animals	*Fusarium roseum* (*F. graminearum*) *F. culmorum* *F. crookwellense*
Fumonisins	Oesophagal cancer, immunosuppressive	*F. verticillioides* (*F. moniliforme*) *F. proliferatum* *F. fujikuroi*
Deoxynivalenol (vomitoxin)	Nausea, vomiting, diarrhoea, inhibition of reproductive performance and immune function, contact dermatitis	*F. graminearum* *F. culmorum*
Nivalenol	Bone marrow toxicity, toxicity of lymphoid organs	*F. graminearum* *F. crookwellense*

Vejdovszky et al. 2017). A summary of known types of mycotoxins, their producers and major health implications due to their ingestion are presented in Table 15.1.

The World Health Organization (WHO) and Food and Agricultural Organization (FAO) are responsible for assessing the risk of mycotoxins to humans through food contamination and also for recommending adequate protection. It is done by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and used by the Codex Alimentarius Commission and governments to establish maximum permissible levels of mycotoxins in food or provide other risk management advice to prevent contamination. Codex standards are the international reference for national food trade and supplies, so that people from any place can be confident that the food they purchase meets the agreed standards for quality and safety, no matter where it was produced. Outcome of such risk assessments can either be a maximum tolerable intake level or the guidance to indicate the level of health concern and also includes advice on risk management to prevent contamination.

Among the noted mycotoxins, aflatoxin B_1 is the most abundant and potent natural carcinogen whose tolerance limit is 20 ppb (WHO 1979). Indian government under 57 A of prevention of Food Adulteration Rules has imposed maximum permissible limit of 30 ppb for aflatoxin B_1 in all the foods prone to aflatoxin contamination (Sinha 1995). In the USA, 20 μg/kg is the maximum aflatoxin residue limit allowed in food for human consumption. However, according to European Union, 4 μg/kg is the maximum acceptable limit in food for human consumption, the strictest in standard worldwide (EC 2006). However, depending on the country involved, human foods are allowed 4–30 ppb aflatoxins (FDA 2004). International Agency for Research on Cancer (1993) has classified aflatoxin B_1 and naturally occurring mixture of aflatoxins as group 1 carcinogen.

Another mycotoxin, sterigmatocystin is closely related to aflatoxins and is a precursor of aflatoxin biosynthesis. It has been classified as class 2B carcinogen by International Agency for Research on Cancer (Engelhart et al. 2002). However, the acute and chronic toxicities of this toxin are considerably lower than aflatoxins. Similarly, the mycotoxin cyclopiazonic acid (CPA) is also not as acutely toxic as aflatoxin B_1, yet it is unique as it is the only mycotoxin that affects muscle tissue in animals (Norred 1990). In the case of ochratoxin A (OTA), International Agency for Research on Cancer has classified it in group 2B because of its toxicity to humans (IARC 1993). Committee on Toxicity (COT) of chemicals in food, consumer products and environment considers OTA as a genotoxic carcinogen and proposed that its level in consumables be reduced to the lowest level that can be technologically attained (COT 1997). The FAO/WHO Joint Expert Committee on Food Additives (JECFA) have established a provisional tolerable weekly intake (PTWI) of OTA at 100μg/kg body weight (Benford et al. 2001). The scientific committee on food (SCF) of the European Union proposed that the maximum daily intake of OTA should not exceed 5 μg/kg body weight. However, few countries have legislative limits ranging from 5 to 50 μg/kg (Webley et al. 1997).

In the case of patulin, US FDA (2000) believes that human beings may be at risk of exposure to it as its thermal processing results in just moderate reduction in its level and it is also able to survive the pasteurization process (IARC 1986). World

Health Organization (WHO) and several European Countries have prescribed limit for patulin in food at 50 μg/L or 50 μg/kg (Welke et al. 2009). Another *Penicillium* toxin, citrinin, is mostly formed in association with other mycotoxins like patulin and OTA, and it is reported that toxicity of citrinin increases in combination with other mycotoxins (Frisvad and Filtenborg 1983). However, so far, no specific legislation for citrinin has been fixed.

Among the fusarial toxins, International Agency for Research on Cancer has evaluated the carcinogenicity of zearalenone and found it to be a possible human carcinogen (IARC 1993). A limit between 60 and 200 μg/kg of zearalenone in raw and finished products has been set in several countries (FAO 1997). In the case of deoxynivalenol (DON), which is another fusarial toxin, guidelines on the permissible concentration of DON in cereal products exist in several countries including Canada and the USA (Van Egmond 1989; Kuiper-Goodman 1994). The US Food and Drug Administration has designated 2 μg of DON per gram as 'level of concern' for wheat products intended for human consumption (Ueno 1987), but for the grains destined for animal feed, the US guideline for DON is 10 mg/kg (Webley et al. 1997). Similarly, in the case of nivalenol (NIV), the effects are in the same range as that of DON.

Another group of fusarial toxins are the fumonisins. According to WHO (2001), the maximum tolerable daily intake of fumonisins for humans is 2 μg/kg body weight. The European Commission (2006, 2007) also established a maximal fumonisin level of 1000 μg/kg in maize and maize-based foods for humans, 800 μg/kg in maize-based breakfast cereals and snacks and 200 μg/kg in maize-based infant food.

15.5 Natural Occurrence of Mycotoxins in Indian Foods and Feeds

Mycotoxins have attracted the attention of many researchers in India because of their acute toxicological characteristics and the fact that they are unpredictable and unavoidable contaminants found in a variety of foods and feeds. The natural occurrence of mycotoxins in agricultural commodities is influenced by warm and humid climatic conditions existing in most part of our country along with the improper and unscientific storage facilities, which usually lead to the proliferation of toxigenic fungi both in the field and storage. Although problems associated with mycotoxin contamination of agricultural commodities are universal, yet in a developing country, like India, they have more serious consequences as they affect agricultural economics, reduce annual production and exports and above all affect the health of the consumers. Many times, the good-quality products are exported, whereas the inferior and contaminated lots that are rejected find way to the domestic markets. In India, scientists and researchers of many institutes like National Institute of Nutrition (NIN), Hyderabad; Central Food Technological Research Institute (CFTRI), Mysore; Indian Grain Storage Institute (IGSI), Hapur; Central Drug Research Institute (CDRI), Lucknow; Industrial Toxicology Research Centre

(ITRC), Lucknow; Vallabhbhai Patel Chest Institute, New Delhi; ICAR; and its centres and many Indian Universities have been working on various mycotoxin contaminants of Indian crops and ways to mitigate it.

15.5.1 Mycotoxin Contamination in Cereal Grains

Cereals are the most important sources of food and feed, which are affected by different mycotoxigenic fungi and their toxins. In India, occurrence of mycotoxins in cereal grains is well documented (Reddy et al. 1984; Singh et al. 1984; Balasubramanian 1985; Bhat et al. 1997a, b). Among the various cereals, maize is cultivated throughout the year in most of the Indian states, and the large part of the produce is consumed as human food. Production of aflatoxin B_1 in maize during cultivation, harvesting and storage is a big problem because of high temperature, high humidity and unseasonal rains and floods in different regions of the country (Bilgrami 1984). The first report of aflatoxin contamination was from maize consumed by the tribals of Gujarat and Rajasthan (Krishnamachari et al. 1975a, b). Later, more work on mycotoxin contamination of cereals was done by the mycotoxicologists of Bhagalpur University, Bihar, and Kakatiya University, Warangal. Sinha (1990) surveyed maize-growing areas of Bihar for 3 consecutive years (1984–1986) and found that maize samples of the kharif crop had a greater incidence of aflatoxins (47%) than the samples of rabi crop (17%), whereas stored maize grains also had a high incidence of aflatoxins (43%). In India, incidence of aflatoxins in maize is more due to the non-availability of proper storage facilities (Sinha and Punam Kumari 1992). Later, in a collective study from different geographical regions of India, Bhat et al. (1997a, b) found that 26% of the maize samples were having aflatoxin B_1 contamination, which exceeded the level of 30 ppb set by Indian government under rule STA of Prevention of Food Adulteration Rules, 1995. Vasanthi et al. (1997) assessed aflatoxin level of maize-based diets in rural population in Southern India and found that the contamination level before cooking was higher by 36% in comparison to cooked maize.

Occurrence of high levels of aflatoxin B_1 in maize had also been reported by Dutta and Das (2001). Similarly in 2002, a survey was carried out on the occurrence of aflatoxins in maize, and 43.16% samples were found to be contaminated with aflatoxins (Reddy et al. 2002). Waliyar et al. (2003) reported 43% of maize samples contaminated with aflatoxins with the highest contamination level of aflatoxin B_1 (806 g/kg). In 2009, Karthikeyan and his coworkers reported variable range of aflatoxin producing potential of *Aspergillus flavus* isolates from maize samples. Similarly, Vijayasamundeeswari et al. (2009) reported aflatoxin B_1 in 61.3% of maize kernels, and 26% of them had more than 20 μg/kg AFB_1. Another study was conducted on the occurrence of aflatoxin contamination in maize kernels from 16 districts of Tamil Nadu. Results indicated that 40.22% of the maize samples were contaminated with aflatoxins and aflatoxin B_1 was detected in 22.97% of pre-harvest, and 53.93% of post-harvest maize samples and 12.05% of the total samples were found to have more than 20 μg/kg of AFB_1 (Karthikeyan et al. 2013).

Mudili et al. (2014) conducted a study on freshly harvested maize collected from three Indian states (Karnataka, Tamil Nadu and Andhra Pradesh) during winter seasons of 2010–2012 and found the samples positive for variable range of mycotoxin contamination like aflatoxin B_1 (48–58 μg/kg), ochratoxin A (<5 μg/kg), fumonisin B1 (76–123 μg/kg), deoxynivalenol (72–94 μg/kg) and T-2 toxins (38–58 μg/kg).

In India, wheat had also been shown to be naturally contaminated with different levels of aflatoxin contamination during storage (Sinha and Sinha 1988, 1990). Further, differential behaviour of wheat varieties towards aflatoxin production by the same toxigenic strain of *Aspergillus flavus* has also been observed (Sinha and Sinha 1993). Toteja et al. (2006) conducted a survey on the prevalence of aflatoxin B_1 contamination in wheat grain samples collected from urban and rural areas of ten Indian states representing different geographical regions and found that 40.3% of the samples were contaminated. From other cereals, Sashidhar et al. (1991) reported aflatoxin B_1 contamination from 150 samples of sorghum, and the range varied from 16 to 40 μg/kg. Mishra and Daradhiyar (1991) reported that 66.3% stored samples and 40.5% cooked samples of pearl millet contained aflatoxin B_1 in the range of 14–2110 μg/kg and 18–549 μg/kg, respectively. Studies conducted in several parts of Tamil Nadu showed that the fungal contamination of paddy rice was more than that of the milled rice (Palaniswami et al. 1989). In another study conducted by Elangovan et al. (1999) from some districts of Andhra Pradesh, Karnataka and Tamil Nadu, nearly 62% samples of rice bran were found to have low levels of aflatoxin B_1. Reddy et al. (2004) reported various species of *Aspergillus* as the major mycotoxigenic fungi in rice. Later, Reddy (2008) explored the prevalence of *Aspergillus* species in about 1200 rice samples consisting of milled rice and paddy collected from 43 locations in 20 rice growing states across India.

Among the fusarial toxins, deoxynivalenol (DON), fumonisin B1 and zearalenone are the most prevalent mycotoxins that occur in cereal grains. Contamination of wheat flour with *Fusarium* species, DON (0.346–8.38 μg/g), nivalenol, T-2 toxin and 3-acetyl deoxynivalenol was reported from Kashmir Valley (J&K) by Bhat et al. (1989). Later, Bhat et al. (1997a, b) reported fumonisin contamination of sorghum and maize from Deccan Plateau. Similarly, a number of fusarial toxins like fumonisins, moniliformin, fusaric acid, fusarins and fusaproliferin have been reported from sorghum (Thakur et al. 2006). Earlier in 1999, Janardhana and coworkers analysed maize samples collected from different agroclimatic regions of Karnataka for the presence of various moulds and mycotoxins like aflatoxin B_1, zearalenone, T-2 toxin, ochratoxin A, citrinin and deoxynivalenol (DON).

Ochratoxin A, one of the most important mycotoxins produced by *Aspergillus ochraceous*, *A. carbonarius*, *A. glaucus*, *A. melleus* and *Penicillium verrucosum* is also predominant. Among cereals, wheat and its products are important group of food commodities where OTA has great impact, as wheat is used as a staple food for majority of world population. Kumar et al. (2012) conducted a study on the detection of OTA in wheat samples from different regions of India. This study revealed that 85% of wheat samples from Bihar, 42% samples from Punjab, 20% samples from Delhi and 12% from Uttar Pradesh were found to exceed the European Union

(EU) limit of 5 ppb, whereas, none of the Haryana wheat samples exceeded the European Union (EU) limit. In addition, there are few more mycotoxins that have been reported from Indian cereals and millets. Rao and Husain (1985) reported presence of cyclopiazonic acid (CPA) in kodo millet (*Paspalum scrobiculatum*) causing 'kodua poisoning' in man. Ansari and Shrivastava (1990) reported natural occurrence of *Alternaria* toxins in sorghum and ragi from North Bihar. Bilgrami et al. (1995) also reported widespread occurrence of toxigenic *Alternaria* in cereals.

15.5.2 Mycotoxin Contamination in Animal Feeds

Feedborne fungi have gained considerable importance due to the production of toxic metabolites. At times the processing of feedstuffs may kill the moulds without destruction of the mycotoxins, and, conversely, presence of moulds does not necessarily imply the presence of toxins. Usually the poorest-quality grains are used for making animal feed and the ingredients include peanuts, oil cakes, coconut and cottonseed cakes, which often contain mycotoxins (Jelinek et al. 1989). Mycotoxins present in animal feeds have a negative effect on the health and fertility of animals. In addition, when animals ingest such contaminated feedstuffs, it poses a threat to food safety due to the possible carryover of mycotoxins to animal-derived products like eggs, meat and milk, which may lead to mycotoxin intake by humans (Richard 2007). However, in comparison to agricultural commodities, the level of these toxins is much lower in the secondary sources of food (Adeyeye 2016).

From India, Reddy and Reddy (1994) screened the sesame and groundnut presscakes used as animal feed in Andhra Pradesh and observed some *Fusarium* species and their toxins like zearalenone, vomitoxin, nivalenol, diacetoxyscirpenol, T-2 toxin, neosolaniol and solaniol. Later, Thirumala-Devi et al. (2002) conducted a study on the occurrence of aflatoxins and ochratoxin A in Indian poultry feeds and found much higher level of mycotoxins than the permissible level. Waliyar et al. (2003) collected feed samples containing groundnut presscake from the peri-urban areas and found more than 75% of the tested samples to contain 4100 μg/kg or more aflatoxin content. In order to understand mycotoxin prevalence in animal feed ingredients, a study was done for 10 months in which a total of 540 raw ingredients of the samples across India were collected and analysed for aflatoxins, ochratoxins, fumonisins and T-2 trichothecene (Benison media 2018). Recently, a study has been done on the seasonal variations, and prevalence of aflatoxins in cattle feed from Bihar in which maximum level of aflatoxin B_1 contamination was detected during wet seasons (Choudhary et al. 2020).

15.5.3 Mycotoxin Contamination in Dried Medicinal Plants

The quality of crude and finished medicinal plants is adversely affected by fungal contamination since most of them are collected from the wild sources, sun-dried and piled in heaps under warm and humid storage conditions before sale in the herbal

markets. In India, marked fluctuation of temperature and relative humidity in different seasons favours the growth of storage fungi and mycotoxin formation (Roy and Chourasia 1990a, b; Chourasia 1995). The World Health Organization (1991) showed serious concern for mycotoxin contamination in herbal drugs and developed guidelines for their assessment based on toxicological studies. These fungal contaminants have been reported to thrive on herbal drug plants by utilizing some active components like phenols, alkaloids and proteins (Roy 2003).

From India, perhaps for the first time, Reddy and Reddy (1983) investigated the toxigenic potential of fungi associated with the seed samples of medicinally important *Sesamum indicum* and detected a wide range of mycotoxins including aflatoxins. Later, more information on mycotoxin contamination of crude medicinal plants and finished drugs became known due to the active work of ethnomycologists from Bhagalpur University, Bihar, and mycotoxicologists from University of Jammu, J&K. Roy and Chourasia (1989) gave elaborate account of aflatoxin contamination in some seeds of medicinal value. In the same year, Kumari and coworkers studied at least 20 dried medicinal plants from storage centres located in Bihar and detected toxigenic *Aspergillus* strains producing aflatoxins B_1 and B_2 in variable amounts. Subsequently, Roy and Chourasia (1990a, 1991, 2001) gave an elaborate account of various mycotoxins including aflatoxins, ochratoxin A and citrinin that were associated with some traditional herbal drug plants and seeds of India. Roy and Kumari (1991) also studied medicinal seed samples kept under storage for mycotoxin contamination and found them to be positive for variable amounts of aflatoxins and citrinin. Screening of herbal drugs of Indian origin for OTA was also attempted by Roy and Kumar (1993) who found high concentration of OTA in them. Kumar and Roy (1994) found aflatoxins and citrinin in some liver and kidney curative herbal drugs collected from different places of Bihar. Similarly, Chourasia (1995) investigated the mould and toxin profile of some crude and finished drugs of Indian pharmaceutical industries and found that 43% samples were positive for aflatoxins and 6% were positive for ochratoxin A. Singh (2003) investigated mycotoxin spectrum of triphala and detected aflatoxins and citrinin in variable amounts. Koul and Sumbali (2008a, b) detected aflatoxin B_1 and B_2 in very high concentration (0.07–2.01 μg/g) from some medicinally important dried rhizomes of *Acorus calamus*, *Bergenia ciliata*, *Curcuma longa* and *Zingiber officinale* collected from different districts of J&K. Later, Koul and Sumbali (2009) recorded aflatoxins B_1, B_2 and ochratoxin A as contaminants from dried root tubers of Indian kudzu, *Pueraria tuberosa*, a plant with multi-medicinal value procured from different markets of J&K. Gautam and Bhadauria (2010) investigated some medicinally important stored fruits for mycotoxin contamination and detected aflatoxins B_1, B_2, G_1 and G_2 in 12.88%, 34.43%, 9.22% and 11.10% samples respectively. In addition, they found 3.07% samples contaminated with citrinin also. Gupta et al. (2013) detected natural aflatoxin contamination in some traditional medicinal plants from Jammu (J&K). Bala et al. (2016) reported high range of aflatoxins B_1 and B_2 contamination from medicinally important seeds of quinces collected from Jammu markets.

During the last decade, few more aspergilli and penicilli toxins detected from medicinal plants include patulin, citrinin, ochratoxins and cyclopiazonic acid (CPA). Koul (2007) detected contamination of CPA in 18.51% and 15.38% rhizome samples of *Zingiber officinale* and *Acorus calamus*, respectively, but the level of CPA was quite high varying between 1.20 and 2.36 μg/g in *Z. officinale* samples and between 0.50 and 1.76 μg/g in *A. calamus* samples. Zakir (2009) analysed mycotoxin contamination from medicinally important wild pomegranate seeds (anardana) sampled from different districts of Jammu province and found mycotoxic contaminants like aflatoxins B_1, B_2, ochratoxin A and patulin. Koul and Sumbali (2010) assessed market samples of medicinally important dried rhizomes and root tubers collected from wholesale and retail shops of Jammu, Kashmir and Leh and observed 32.30% of the tested samples to be positive for citrinin, whereas 9.23% were positive for patulin contamination. While studying mycobial contamination and mycotoxinogenesis of dried stem portions of *Tinospora cordifolia*, an important medicinal plant of India, Sharma et al. (2013a) detected contamination of aflatoxin B_1 (0.11–1.27 mg/kg), aflatoxin B_2 (0.27–0.77 mg/kg), ochratoxin A (0.45–0.54 mg/kg), patulin (3.75 mg/kg) and citrinin (0.25–0.28 mg/kg), but none of the fusarial toxins were detected. In the same year, Sharma et al. (2013b) also analysed dried leaves of medicinally important *Azadirachta indica* and *Justicia adhatoda* for mycotoxin contamination and found them having very high concentration of aflatoxins B_1 and B_2. They also found contamination of patulin associated with some leaf samples of *Azadirachta indica* and zearalenone contamination in some leaf samples of *Justicia adhatoda*. However, other mycotoxins like OTA, citrinin, zearalenone and deoxynivalenol were not detected from these samples, which were collected from eight districts of erstwhile Jammu and Kashmir states. Rawat et al. (2014) detected toxigenic fungi and mycotoxins from some stored medicinal plant samples procured from different markets of Agra and nearby regions during the year 2009–2010. Jeswal and Kumar (2015) reported natural occurrence of citrinin in dry ginger rhizomes. Recently, mycotoxin contamination and induced biochemical changes have also been investigated in some medicinal plants by Singh (2017) and Chandra et al. (2019).

Among the fusarial toxins, zearalenone has been detected in variable amounts from a diverse range of dried medicinal plants by various Indian ethnomycologists (Kumar and Roy 1994; Chourasia 1995; Roy and Chourasia 1989, 1990a, b, 2001; Sharma 2005; Koul 2007). High levels of zearalenone (0.64–14.51 μg/g) were detected by Koul and Sumbali (2008b) from some medicinally important rhizomes and root tuber samples procured from Jammu and Kashmir. They also detected zearalenol (ZOL) contamination (1.28 μg/g) from one root tuber sample of *Pueraria tuberosa* and deoxynivalenol (DON) contamination (1.19–14.45 μg/g) from few samples of the investigated rhizomes and root tubers. Earlier, Sharma (2005) detected contamination of ZOL ranging between 0.26 and 1.60 mg/kg from dried leaves of *Justicia adhatoda* and dried fruits of *Phyllanthus emblica* and *Terminalia chebula*. Sharma (2005) also detected contamination of deoxynivalenol (DON) ranging between 1.15 and 6.43 mg/kg from dried fruit samples of *Phyllanthus emblica*.

15.5.4 Mycotoxin Contamination in Fresh Fruits and Vegetables

India is bestowed by variable agroclimatic conditions, which allow the cultivation of almost every kind of fruit and vegetable. However, during the post-harvest phase, fresh fruits and vegetables get infected by the opportunistic rot causing fungal pathogens, which not only cause quantitative loss but also produce mycotoxins that may affect the health of the consumers. From India, some of the earliest reports of natural occurrence of mycotoxins in some fruit rots caused by fungal species are by Sinha and Singh (1982), Singh and Sinha (1982, 1983), Madhukar and Reddy (1992) and Agarwal and Roy (1994). Later, Singh and Sumbali (2000) observed that *Aspergillus flavus* infection not only resulted in maximum loss of ascorbic acid from the ripe fruits of jujube (ber) but also induced aflatoxin production during pathogenesis. They further noted that approximately 85.7% of the *A. flavus* isolates associated with jujube fruit rot were aflatoxigenic. Singh (2002) while studying fruit rot of apple caused by *Penicillium expansum* found that the mycotoxin patulin is produced during pathogenesis, which migrated to the surrounding unaffected tissues making them also unfit for human consumption. Singh and Sumbali (2004) recorded 83% of the tested *Alternaria alternata* isolates as producers of tenuazonic acid (TeA) in apples. However, analysis of the apple fruit tissue immediately surrounding the rot showed that it was free from this specific toxin. Bamba and Sumbali (2005) demonstrated for the first time that sour lime are a favourable substrate for aflatoxin B_1 and cyclopiazonic acid (CPA) production by *A. flavus* isolates. They also detected TeA as the main mycotoxin produced by *A. alternata* isolates in citrus fruits like mandarin orange, kinnow mandarin and sour lime (Bamba and Sumbali 2006). Singh and Sumbali (2008a) conducted investigations to elucidate differential toxigenic behaviour of *P. expansum* strains towards five cultivar varieties of pear. They found that the toxigenic strains produced variable amount of patulin (14.75–40.08 mg/kg) and citrinin (0.10–8.04 mg/kg) in the infected fruits of apple cultivars, whereas the levels produced by toxigenic strains in pear cultivars were comparatively low. In the same year, they detected that *P. expansum* commonly caused post-harvest rot of crab apples cv. scarlet Siberian (*Docynia indica* Dcne) and produced patulin and citrinin up to 36.00 mg/kg and 6.04 mg/kg, respectively, in the rotten tissue (Singh and Sumbali 2008b). Nallathambi and Umamaheshwari (2009) detected aflatoxins from *Aspergillus*-infected pomegranate arils. Later, Singh and Sumbali (2011) detected aflatoxin B_1 contamination in commercial varieties of apple and pear fruits infected with *Aspergillus flavus*. Bagwan (2011) also detected aflatoxin B_1 contamination in papaya fruits during post-harvest pathogenesis.

Very few Indian workers have assessed fresh vegetables for mycotoxin contamination. Gupta et al. (2009) assessed mycotoxin-producing potential of some pathogens causing vegetable rots. Samyal and Sumbali (2011) detected that *Alternaria alternata*, a common pathogen causing tomato fruit rot in the field, during harvesting, transportation and even in refrigerated storage produced an array of *Alternaria* toxins. Of the tested isolates, they found that 45% produced tenuazonic acid (TeA), 35% produced altertoxin-I (ATX-I) and 20% produced alternariol (AOH) in the tomatoes during pathogenesis. Later, they detected aflatoxin B_1

ranging from 141.6 to 1481.3 μg/kg and aflatoxin B_2 ranging from 49.0 to 2497.6 μg/kg in the fruits of tomato cv. marglobe infected with *Aspergillus flavus* and encountered during post-harvest survey (Samyal and Sumbali 2012). These values are quite high and much beyond the regulatory limit of 20 μg/kg fixed by WHO (1979), thus representing a potential hazard, considering that these toxins may be transferred into processed food products.

15.5.5 Mycotoxin Contamination in Sun-Dried Fruits, Vegetables and Nuts

In India, most of the fruits and vegetables are seasonal, and when they are in plenty, dehydration is one of the most ancient and widely practiced methods of their preservation. However, if proper care is not taken during storage and marketing, even sun-dried fruits, vegetables and nuts may become infested with moulds and their toxic metabolites, which are hazardous to the health of consumers and may impede their export. One of the earliest records of mycotoxins is that of Singh (1983) who analysed 116 samples of various dry fruits and found 26 samples contaminated with aflatoxins and other mycotoxins. Bilgrami (1984) investigated mould contaminated samples of almond, walnut, foxnut (makhana) and coconut and detected 20–2840 ppb of aflatoxin B_1 contamination. Kulkarni et al. (1985) reported aflatoxins from dried dates. Later, Krishnakumari and Nusrath (1987a, b) found natural occurrence of citrinin and ochratoxin A in coconut products. Roy (1990) detected natural contamination of aflatoxins from imported dried figs. Sharma and Sumbali (1999a, b) assessed dry fruit slices of quince (*Cydonia oblonga* Mill.), an important rosaceous pome of Jammu and Kashmir, which is valued both for its religious and commercial significance. They observed natural occurrence of aflatoxin B_1, ochratoxin A and patulin in the dried slices of quinces collected from different markets of Jammu and Kashmir. Saxena and Mehrotra (1990) studied mycotoxin contamination in some dried fruits sampled from retail markets of Nainital. High contamination of aflatoxins B_1 and B_2 has also been detected from the Jammu market samples of chilgoza pine nuts (Sharma et al. 2013) and apricot kernels (Bala et al. 2014). Later, Sharma et al. (2014) detected co-occurrence of aflatoxins, ochratoxin A and patulin in chilgoza pine nuts marketed in Jammu. In the same year, Sharma and Sumbali (2014) detected natural occurrence of aspergilli and penicilli and co-contamination of aflatoxins B_1, B_2 and sterigmatocystin in some market samples of dried walnut kernels from Jammu and Kashmir. Similarly, in dried apricot samples collected from Jammu, Kashmir and Ladakh, co-occurrence of aflatoxins, ochratoxin A and patulin was observed by Gupta et al. (2015). Some fusarial toxins, viz. zearalenone (ZEN), zearalenol (ZOL) and deoxynivalenol (DON) and their co-occurrence were also detected from the in-shell walnuts sampled from Jammu and Kashmir. However, a large proportion of these walnut samples had fairly low levels of individual mycotoxins. In an attempt to analyse dried date plum persimmons (*Diospyros lotus* L.) of Jammu province for mycotoxin contamination,

Gupta et al. (2017) detected aflatoxins, ochratoxin A and patulin in 69.23%, 47.69% and 32.30% market samples, respectively.

From India, very few investigations have been carried with respect to sun-dried vegetables and mycotoxin contamination. Kour and Sumbali (2006) investigated domestic and market samples of dried slices of brinjal, tomato, bottle gourd, turnip, cauliflower and bitter gourd for patulin and citrinin contamination. They detected patulin as an important fungal contaminant ranging from 2.95 to 10.25 mg/kg in all the dried vegetables excepting cauliflower samples, whereas citrinin was found in only four dried vegetables, viz. brinjal, tomato, turnip and cauliflower, and the range of contamination was comparatively low (0.04–2.01 mg/kg). Later, Kour and Sumbali (2012) assessed the sun-dried vegetables for fusarial mycotoxins and reported contamination of ZEN, ZOL and DON from some of them.

15.5.6 Mycotoxin Contamination in Spices and Condiments

India is the largest producer and exporter of spices in the world (Power 2013). Common spices and condiments used in Indian cuisine are red chillies, turmeric, fennel, dry ginger, cumin, black pepper, coriander and caraway. In India, spices are chiefly cultivated in Tamil Nadu, Kerala, Andhra Pradesh, Karnataka, Rajasthan, Gujarat, Madhya Pradesh, Uttar Pradesh, Punjab and Jammu and Kashmir based on different agroclimatic conditions (Divakara and Sharma 2001). Many of these spices are even used for their effective medicinal properties. However, spices like any other agricultural commodity face fungal and mycotoxin contamination, which is one of the most common problems that hamper their trade.

One of the earliest study on the mycoflora and mycotoxins of spices is by Madhyastha and Bhat (1984) who analysed black and white pepper for their substrate efficiency for *Aspergillus flavus* growth and aflatoxin B_1 production. They found that black pepper is a better substrate for *A. flavus* growth and aflatoxin B_1 production than white pepper. Later, Madhyastha and Bhat (1985) analysed red pepper, black pepper, cardamom, turmeric and dry ginger for their substrate efficacy for aflatoxin B_1 production. Their results indicated that ginger and red pepper were better substrates for the production of aflatoxin B_1, whereas cardamom did not support the fungal growth and aflatoxin B_1 production. Misra and Batra (1987) studied mycotoxins associated with fennel seeds. Saxena and Mehrotra (1989) screened samples of 15 spices collected from various Indian markets for natural occurrence of mycotoxins like aflatoxins, rubratoxin, ochratoxin, citrinin, sterigmatocystin and zearalenone. They found maximum samples of coriander and fennel to be contaminated with aflatoxins, and their levels were higher than the prescribed limits for consumption. Rani and Singh (1990) reported high contamination of aflatoxin B_1 from samples of cumin, coriander and fennel. However, Roy et al. (1988) and Chourasia (1995) detected <20 μg/kg contamination of aflatoxin B_1 in samples of cumin, cardamom and long pepper. Chourasia (1995) also reported low levels of citrinin contamination from *Zingiber officinale*.

Thirumala Devi and coworkers (2000) collected 100 chilli samples from the Khammam and Guntur districts of Andhra Pradesh and detected ochratoxin A (OTA) contamination ranging from 10 to 120 μg/kg in all the samples. Later, they reported OTA contamination from coriander, black pepper, turmeric and dry ginger samples also (Thirumala-Devi et al. 2001). In another study from Andhra Pradesh, Reddy et al. (2001) collected three grades of chilli pods and chilli powder samples from the cold storage facilities and markets of the major chilli growing areas of the state and detected aflatoxin B_1 >30 μg/kg of the samples by using ELISA. Keshri and Basu (2003) undertook a study during 1997–2000 and detected aflatoxins B_1, B_2, G_1 and G_2 contamination in seed spices obtained from various retail markets of Uttar Pradesh. Koul and Sumbali (2008a, b) collected dry ginger rhizome samples from nine districts of Jammu, Kashmir and Leh and found citrinin in 33.33%, patulin in 11.11%, DON in 14.81%, ZEN in 7.40%, aflatoxin B_1 in 11.1% and aflatoxin B_2 in 14.8% samples. Similarly, among the market samples of dried turmeric rhizomes, 25.92% samples were detected positive for citrinin contamination, 18.51% were positive for ZEN, 3.7% were positive for DON, 37.0% were positive for aflatoxin B_1, and 22.2% were positive for aflatoxin B_2, whereas none was positive for patulin (Koul and Sumbali 2008a, b, 2010).

Moorthy et al. (2011) collected 16 types of spices from three different districts (Namakkal, Karur and Erode) of Tamil Nadu and found aflatoxin B_1 contamination to be highest in black pepper. Samyal and Sumbali (2013) collected market samples of loose red chilli powder from Jammu and Kashmir and detected aflatoxin B_1 and B_2 contamination in 19% and 29% samples, respectively. Later, they investigated loose and packed chilli powder samples for fusarial toxins and found them to be contaminated with high concentration of ZEN, ZOL and DON (Samyal and Sumbali 2014). Ramesh and Jayagoudar (2014) investigated OTA contamination from selected spices of Dharwad, Karnataka, by HPLC method and found that OTA concentration of all the samples was within the EU consumption limit. Jeswal and Kumar (2015) analysed nine different Indian spices (red chillies, coriander, dry ginger, turmeric, black pepper, fenugreek, cumin, caraway and fennel) for natural occurrence of toxigenic mycoflora and mycotoxins. They detected red chilli samples to be highly contaminated with aflatoxins, followed in decreasing order by dry ginger, whereas OTA was detected from all the spices except cumin, and citrinin was detected from dry ginger and black pepper. Aiko and Mehta (2015) conducted a study on the prevalence of toxigenic fungi and mycotoxins in common spices of India. Their analysis revealed presence of toxigenic fungi on the investigated samples of Indian spices, but mycotoxins were not detected, thereby showing that spices possess intrinsic properties that may inhibit mycotoxin contamination. Recently, Samyal and Sumbali (2020) observed quality deterioration of dried whole red chillies by several toxigenic fungal species and mycotoxic contaminants like aflatoxin B_1 (0.24–2.14 μg/g), aflatoxin B_2 (2.08–2.14 μg/g), cyclopiazonic acid (1.65–144.78 μg/g) and sterigmatocystin (5.10–24.03 μg/g). In addition, they also observed co-occurrence of mycotoxins from chilli pericarp samples, which may generate additive or synergistic effect in consumers.

15.5.7 Mycotoxin Contamination in Oil Seeds, Cakes and Oils

India is well-known for growing a number of oil seed crops like groundnut (peanut), sunflower, mustard, coconut, etc. Among these, groundnut is being produced annually in thousands of tons for both domestic and export purposes. Since groundnut pods develop underneath the soil, they get contaminated with soil mycoflora, which may lead to both pre- and post-harvest infection and mycotoxin elaboration. In addition, adverse climatic conditions and improper storage are some of the predisposing factors for post-harvest mycotoxin contamination of groundnuts. In 1964, a survey of freshly harvested groundnuts from coastal districts of Andhra Pradesh was done, which showed some samples to be contaminated with aflatoxins (Rao et al. 1965). Sreenivasamurthy et al. (1965) found that only few *A. flavus* isolates from peanuts formed aflatoxin B_1. Dwarakanath et al. (1969) detected low values of aflatoxins from Indian peanut oil extracted from freshly harvested peanuts. In 1970, Wagle surveyed 500 samples of groundnuts from west coast of India and found 50% of them to contain aflatoxins in the range of 0.10–0.50 ppm. Subrahmanyam and Rao (1974a) recovered 72 toxigenic strains of *A. flavus* from groundnut samples collected from seven Indian states, which produced aflatoxins B_1 and G_1. They also reported occurrence of citrinin in some peanut samples (Subrahmanyam and Rao 1974b). In 1977, Tulpule and coworkers studied variation in aflatoxin production due to fungal isolates and crop genotypes and also their scope in prevention of aflatoxin production. Similarly, Mehan et al. (1982) studied seed colonization and aflatoxin production in genotypes of groundnut inoculated with different *A. flavus* strains.

In India, major groundnut producing regions have this crop growing in the rainy season, which encourages *A. flavus* infection and aflatoxin contamination (Ghewande et al. 1987; Bhat and Rao 1990; Ghewande 1997). Sahay and Ranjan (1990) investigated distribution of aflatoxin B_1 from the groundnut seeds to their oil and cakes and found maximum concentration in the groundnut seeds (316.22 μg/kg), whereas the extracted oil contained lesser concentration of aflatoxin B_1 (159.31 μg/kg) and the residual cakes contained even more less amount (148.05 μg/kg). Sharma et al. (1994) estimated aflatoxin B_1 content in the de-oiled groundnut and groundnut oil cakes collected periodically during different seasons. Kolhe et al. (1994) assessed the oil seed cakes from Jalgaon district, Maharashtra, for aflatoxin contamination and found that more than 50% of the peanut cake samples were contaminated with aflatoxin B_1 having maximum concentration of 515.86 ppb. A study by Indian Council of Medical Research (ICMR) revealed that 21% of the samples were contaminated with aflatoxin B_1 above 30 ppb (Verma et al. 1995). In the same year, Bhat et al. (1996) in their study showed that 28% samples were contaminated below the permissible limit and 8% samples were contaminated above the permissible limit. Kumar et al. (2001) estimated prevalence of aflatoxin contamination in groundnut samples from Tumkur, Karnataka, and found that levels of many of the positive samples were below 20 ppb. Kishore et al. (2002) collected groundnut samples from the fields of Andhra Pradesh and detected 20.3% and 16.5% of them to be contaminated with aflatoxins in the year 1999 and 2000, respectively.

Reddy et al. (2003) conducted study in four rainy seasons and found that 90% of the groundnut samples were contaminated with aflatoxins. Waliyar et al. (2003) studied aflatoxin contamination in the insect damaged groundnut samples collected from farmers of Andhra Pradesh and found more than 500 μg/kg aflatoxin levels from such samples. Navya et al. (2013) surveyed groundnut samples across different agroclimatic regions of India. Rajarajan et al. (2013) isolated and quantified aflatoxins from *A. flavus* infected peanuts stored in godown in Madurai (Tamil Nadu). Similarly, Ommi et al. (2019) reported aflatoxin contamination in groundnut samples collected from different locations in Rayalaseema area (Andhra).

Another important oil seed crop of India is sunflower, which is cultivated in different states particularly Karnataka, Andhra Pradesh, Maharashtra, Tamil Nadu, Haryana, Punjab and Uttar Pradesh. Suryanarayanan and Suryanarayanan (1990) tested *A. flavus* isolates from sunflower seeds for their aflatoxin production. Dawar and Ghaffar (1991) observed that *A. flavus* and *A. parasiticus* can infect sunflower and cause accumulation of aflatoxins in the seeds and cakes. Banu and Muthumary (2010a) analysed raw, refined and filtered sunflower oil obtained from a refinery in Karnataka and found aflatoxin B_1 contamination in 44% samples of raw and filtered oil, whereas no contamination was detected from refined oil. They also recovered *A. flavus* isolates from samples of sunflower seeds, oil cake, de-oiled cake, raw oil and filtered oil from oil refineries of Tamil Nadu and found that the majority of the isolates were aflatoxigenic and produced only aflatoxin B_1 (Banu and Muthumary 2010b).

Aflatoxin contamination has also been reported from dried coconut, an important source of vegetable oil that is used for cooking and industrial applications. The ill effects of consuming aflatoxin-contaminated coconut oil feed stuffs in poultry and milch cattles have been reported by Bhat et al. (1978). Kumari and Nusrath (1987a, b) collected 384 market samples of dry copra, coconut oil and copra meal during different seasons for evaluation of natural mycotoxic contaminants and found only 3 samples of dry copra and 1 sample of copra meal to be contaminated with citrinin up to a level of 60 μg/kg and 10 μg/kg, respectively. In addition to citrinin, they found two samples of dry copra to have co-occurrence with either ochratoxin A or aflatoxin B_1. Srinivasulu et al. (2003) also reported that several fungi attack copra and result in aflatoxin contamination.

In India, some work has also been done on the incidence of mycotoxin-producing fungi on mustard seeds, oil and cakes. Sahay and Prasad (1990) studied mycotoxin contamination in mustard and mustard products and found aflatoxin at concentrations of 750, 870 and 1420 μg/kg in 44%, 33% and 80% samples each of mustard seeds, oil and cakes respectively. Sahay and Ranjan (1990) investigated distribution of aflatoxins from the mustard seeds to oil and cake. They found that the concentration of aflatoxin B_1 was maximum (273.59 μg/kg) in the seeds, lesser in residual cake (257.29 μg/kg) and least in the oil (96.33 μg/kg). Kolhe et al. (1994) while investigating various oil cakes found least contamination of aflatoxin B_1 (79.72 ppb) in mustard cakes.

15.5.8 Mycotoxin Contamination in Other Consumables

The xerophilic fungi are the most important spoilage-causing and mycotoxin-producing organisms of the dry consumables because they are capable of growing at a water activity below 0.85 (Pitt 1975). However, the level of fungal and mycotoxin contamination is affected by the interrelationship of several factors including climatic conditions, geographical location, chemical nature of the substrate, mode of commodity handling, type of dehydration process, type of storage containers used, spore load and microbial interaction. Some Indian researchers have even attempted analysis of mycotoxins from consumables that are utilized by only few. For example, Neelkantan et al. (1982) reported contamination of patulin from scented supari. Later, Raisuddin and Misra (1991) conducted a study on the aflatoxin contamination of areca nuts and found 37% of the tested samples to be positive for aflatoxin B_1 contamination (94 μg/kg), whereas total aflatoxin content ($B_1 + B_2 + G_1 + G_2$) was up to 137 μg/kg. Rai and Chaturvedi (1993) detected aflatoxin B_1 from tobacco (chewing type). Similarly, Verma et al. (1995) analysed samples of betel nuts, tobacco and pan masala from Western India for the incidence of aflatoxigenic fungi and aflatoxin contamination. Few other food and feed commodities of India, which have been investigated for mycotoxin contamination, include pulses and their products for human use (Ahmed 1993; Rani and Singh 1994), bakery bread (Sinha and Prasad 1994), groundnut fodder (Surekha and Reddy 1987), cotton seed (Vedanayagam et al. 1986), safflower seeds (Ghosal et al. 1977), gram flour (Ramakrishna et al. 1990), forest tree seeds (Khan and Singh 2000), dairy products (Mor and Singh 2000), dried fishes (Prasad et al. 1987; Sam et al. 2015) and dried morels (Bala et al. 2017).

15.6 Detection of Mycotoxins

Presence of mycotoxins in food products is a major global issue of food safety (Van Emon 2010; Moretti et al. 2017). Its detection in food products is usually achieved by certain methods including sampling, homogenization, extraction of mycotoxins, extract clean-up and finally the detection and quantification by some techniques (Pereira et al. 2014; Shephard 2016).

Sampling is the most important step for mycotoxin analysis of food commodities as it significantly contributes to the reliability of the results and also decides the compliance or non-compliance of an entire food batch (Richard et al. 1993; Turner et al. 2015). Excepting liquid food samples such as milk or some other highly processed foods like peanut butter, the traditional methods of sampling for food products are not suitable for mycotoxin analysis. The presence of mycotoxins in food is not homogenous (Whitaker 2006; Ridgway and Scientific 2012; Shephard 2016). In view of their uneven distribution, it is difficult to get a representative sample of the bulk (Scudamore 2008; Ridgway and Scientific 2012). Therefore, a good sampling plan is required to ensure that the tested sample is representative of the whole bulk, which assures the accuracy of the results (Shephard 2016).

Considering the problems associated with the sampling, various sampling plans have been developed based on statistical parameters (Whitaker and Johansson 2005; Shephard 2016). EU under the Commission Regulation (EC) No. 401/2006 has described sampling methods (Krska et al. 2008). In addition, different governmental regulatory agencies worldwide are also governing to improve the sampling plans for the analysis of mycotoxins in food and feed in order to reduce the variability of the analytical results.

Extraction of mycotoxins from solid food samples into liquid phase is the initial step in sample preparation. Extraction of mycotoxins requires organic solvents like chloroform, methanol, acetonitrile, acetone, etc., and it is followed by clean-up process to enhance the specificity and sensitivity of a given detection method. The extraction and clean-up of mycotoxins in food samples are usually governed by three major factors (1) chemical properties of the mycotoxin, (2) nature of food matrix (3) and the method of detection. Generally, the liquid food samples like milk, apple juice, wine, etc. are initially subjected to liquid-liquid extraction method to separate the mycotoxins. However, for grains, cereal food and other solid food materials, solid-liquid extraction methods can be used.

Mostly mycotoxins are highly soluble in organic solvents, but least soluble in water (Rahmani et al. 2009; Turner et al. 2009). However, fumonisins are soluble in water due to the presence of four carboxyl groups, and one amino group and FB1-type fumonisin is highly stable in a mixture of acetonitrile and water (Bennett and Klich 2003). These days, various instrumental-automated solvent extraction methods are being used for analysing mycotoxins including accelerated solvent extraction (ASE), supercritical fluid extraction (SFE) and microwave-assisted extraction (MAE) (Maragos and Busman 2010; Pereira et al. 2014). As compared to conventional methods, the latest methods may be costly, but they accelerate the process of extraction, require less amount of chemical solvents, thus making it eco-friendly with better efficiency (Kralj Cigic and Prosen 2009). Following extraction, filtration and centrifugation are important steps to eliminate any interfering particles before performing further clean-ups. Cleaning up of the extract improves the accuracy and precision, which is enhanced by its specificity and sensitivity. Various clean-up methods that are being implemented include liquid-liquid partitioning (LLP), solid phase extraction (SPE), immune affinity columns (IAC), ion exchange columns, column chromatography and multifunctional clean-up columns (e.g. Mycosep™) (Rahmani et al. 2009; Pereira et al. 2014). Commonly used clean-up methods are SPE and IAC, due to their rapidity, efficiency, reproducibility, safety and selectivity. The quick, easy, cheap, effective, rugged and safe (QuEChERS) sample preparation method has also been applied for extraction and clean-up of mycotoxins (Koesukwiwat et al. 2014).

As the mycotoxins are of low-molecular-weight, their analysis requires a well-equipped instrumentation laboratory and technical personnel with sufficient experience in handling of samples and the highly toxic and pure reference standards. Mycotoxins are commonly detected and quantified by using chromatography techniques, absorption and emission spectrometric techniques and by using antibody-based assays. The most common analytical methods for mycotoxin

analysis in food and feed are the chromatographic techniques. Among them, thin layer chromatography (TLC) is the earliest method used as chromatographic technique. These days it is used as a rapid screening method for some mycotoxins by instrumental densitometry and visual assessment (Shephard 2016). High-performance liquid chromatography (HPLC) represents the technique of choice for the analysis of mycotoxins as it is sensitive, reproducible and accurate and has a high degree of automation. On the other hand, gas chromatography (GC) is used less frequently since most of the mycotoxins are not sufficiently volatile and therefore, must be derivatized, which increases the time and cost of analysis. HPLC and GC have detection limit of 0.05 ppm for many mycotoxins but there is requirement of expensive equipment and technical support. Recently, ultra-high-performance liquid chromatography combined with time-of-flight-mass spectrometry (UHPLC-ToF-MS) method has been developed for determination of nine mycotoxins, namely, (AFB1, AFB2, AFG1 and AFG2), OTA, ZEA, T-2 and fumonisins (FB1 and FB2) in maize by Sanches-Silva et al. (2019).

The most widely used absorption and emission spectrometric techniques are ultraviolet (UV) and fluorescence detection (FLD), the latter being preferred when mycotoxins exhibit natural fluorescence. Since the discovery of mycotoxins, various methods have been validated and used to examine the presence of mycotoxins in food and feed such as HPLC coupled with FLD, DAD, UV and MS detection; TLC, GC coupled with FID, ECD or MS detection; ultra-performance liquid chromatography; and rapid strip screening tests (Krska et al. 2008; Turner et al. 2015). Despite tremendous progress in this field, there are still major challenges and drawbacks to these methods which need to be addressed (Yazdanpanah 2011).

Different types of immunochemical methods for mycotoxin detection have been developed, among which the most important ones are the ELISA test (enzyme-linked immunosorbent assay) and RIA (radioimmunoassay). Most of these methods are very sensitive, specific and easy to use, which have given rise to new dimensions in terms of methodology for mycotoxin analysis. ELISA is one of the most affordable methods for mycotoxin detection, but the limit of detection for many mycotoxins often exceeds 0.2 ppm. Availability of immunochemical methods has led to the development of various rapid and sensitive methods for monitoring and quantifying AFB_1 in contaminated foods and feeds (Liu et al. 2013). According to Zhang et al. (2011), ELISA is the most convenient screening tool, which can provide a great saving in cost resources, efforts and toxic solvents. This test method has proven to be simple, portable and reliable for screening a large number of samples (Li et al. 2009; Guan et al. 2011) and has become the most common and rapid method for the detection of mycotoxins in food and feedstuffs (Zheng et al. 2005; Wang et al. 2011). According to Zhang et al. (2011), the detection limits of ELISA can be comparable with or even lower than those obtained by instrumental methods. There are several commercial ELISA kits available for aflatoxins, deoxynivalenol, fumonisins, ochratoxins and zearalenone. However, commercial ELISA kits differ in the detection of AFB_1, and some manufacturers launch one ELISA product for the detection of AFB_1 in all the substances that may reduce the performance of ELISA kits because different interfering substances co-extract from different food and feed

matrixes (Li et al. 2009). Furthermore, factors like antibodies, co-extracted compounds, power hydrogen of the extract composition of the extraction solvent, etc. can result in under or overestimation of AFB_1 contamination. As a result, the concentration of AFB_1 in the same sample might be discrepant by kits from different manufacturers, even different batches of the same manufacturers. A study was conducted to evaluate the quality of five commercial enzyme-linked immunosorbent assay (ELISA) kits procured from different suppliers for detecting AFB_1, and the results revealed that the qualities of five test ELISA kits were significantly different (Sun et al. 2015).

15.7 Strategies for the Management of Mycotoxins

In India, risk of mycotoxin contamination is on the rise due to various environmental, agronomical and socio-economical factors. Therefore, management of mycotoxin contamination is of dire importance for the purpose of public health maintenance and economic improvement in the country. A number of strategies for reduction and control of mycotoxins have been considered in different countries including India. The two main strategies are the preharvest and post-harvest strategies.

15.7.1 Preharvest Strategies

They remain to be first priority and mean prevention of fungal growth and mycotoxin production on crops in the field. Preharvest prevention strategies include good agricultural practices like implementation of cultural and biological practices; use of registered fungicides, herbicides and insecticides to control fungal infection; seed and seed bed treatment; soil analysis; and enhancing the genetic synthesis to suppress mycotoxin production (Alberts et al. 2017; Adebiyi et al. 2019). Although most of the field fungi get destroyed during the storage period, yet the mycotoxins produced by them remain in the raw material even after processing due to their heat stability and may get redistributed and transferred from the crops to foods and feeds. In India, most of the preharvest strategies have been evolved for groundnut. Mehan et al. (1982, 1983) screened several groundnut cultivars for seed resistance to *Aspergillus flavus* and aflatoxin production at ICRISAT. Upadhyaya et al. (2002) described genetic enhancement for resistance to aflatoxin contamination in groundnut. Sharma et al. (2018) achieved a high level of resistance in peanut by overexpressing antifungal plant defensins MsDef1 and MtDef4.2 and host-induced gene silencing (HIGS) of aflM and aflP genes from aflatoxin biosynthetic pathway.

15.7.2 Post-harvest Strategies

Post-harvest prevention measures such as adequate storage, detection, disposal and continuous monitoring of potential contamination during processing and marketing of agricultural commodities have proved to be indispensible in ensuring food and feed safety. Studies have shown that mycotoxins can be eliminated by physical, chemical and biological methods. Of these, physical and chemical detoxification methods have many limitations like nutrient loss and are time-consuming, ineffective and expensive. In comparison to physical and chemical methods, biological methods have proved to be highly effective, specialized and eco-friendly.

Physical methods of detoxification/decontamination like grading, sorting, milling, roasting, boiling, irradiation, microwaving, heating and peeling are commonly used and have been attempted with success by a number of Indian researchers. Shantha and Murthy (1981) used sunlight to partially detoxify groundnut cake flour and casein contaminated with aflatoxin B_1. Samarajeewa et al. (1985) also detoxified aflatoxin B_1-contaminated coconut oil by solar radiation and found that 75% of the toxin was degraded. Later, Samarajeewa et al. (1990) observed that inactivation of microorganisms depends on the dosage of gamma radiation as low dosage (0.1 MRad) stimulated aflatoxin production in bread and other foods, whereas high dosage (0.3–0.4 MRad) suppressed mould growth as well as aflatoxin production. Bhat et al. (2007) reported significant reduction of aflatoxin B_1 at 10 kGy irradiation dose in seeds of *Mucuna pruriens*. Garg et al. (2013) investigated optimization of conditions for reducing aflatoxin contamination in peanuts using UV radiations.

Storage conditions and packaging practices also play a major role in controlling mycotoxins since they affect the overall fungal growth. Roy and Chourasia (2001) reported that dry medicinal plants stored in gunny bags had significantly higher incidence of mycoflora and mycotoxins as compared to other storage containers. Sharma (2005) and Koul (2007) used vacuum packing immediately after sun-drying for storing medicinal plants and found that number of fungal species detected was very low, whereas mycotoxins were completely absent in such samples at the end of 12 months storage. This may be due to the closed oxygen-free atmosphere of the vacuum packs that gradually becomes richer in carbon dioxide content due to the respiration of existing microbes. Samyal (2013) also recommended the use of polythene packs but preferably vacuum packs immediately after sun-drying for minimizing mould growth and threat of mycotoxin contamination in red chillies.

Chemical methods of detoxification include oxidation, reduction, hydrolysis, alcoholysis and adsorption. In 1967, Sreenivasamurthy and coworkers found that the aflatoxin molecule contains lactone ring, which opens in an alkaline medium and is susceptible to oxidation. Dwarakanath et al. (1968) investigated the aflatoxin reduction in cottonseed and peanut meal by ozonation. In this study, aflatoxin B_1 and aflatoxin G_1 were readily destroyed by ozonation, but aflatoxin B_2 was relatively resistant. Similarly, detoxification of coconut products such as coconut flour, coconut milk, desiccated coconut, coconut cake and coconut oil, which are prone to aflatoxin, have been conducted by many researchers using different methods like

extraction with NaCl (Shantha and Sreenivasamurthy 1975), filtration (Basappa and Sreenivasamurthy 1979), photolysis and ammoniation (Shantha and Sreenivasamurthy 1977). However, none of the procedures/methods could detoxify the commodities completely. Basappa and Sreenivasamurthy (1979) decontaminated aflatoxins from groundnut oil by adsorption-cum-filtration method. Samarajeewa et al. (1977) worked on the degradation of aflatoxins in coconut oil and copra meal. Shantha et al. (1986) detoxified 85% of aflatoxins from groundnut seeds using urea and sunlight. Chaturvedi et al. (2002) investigated the adsorption capacity of some indigenous aflatoxin adsorbents such as shelkhari, multani and bentonite and compared them with a commercial adsorbent. They observed that commercial adsorbent and hydrated sodium bentonite showed highest aflatoxin adsorption capacity.

In the last two decades, many research groups made great achievements in the search for biological agents for mycotoxin detoxification. In comparison to chemical treatments, the use of spices, herbs and essential oils is usually preferred for the removal of toxigenic fungi and mycotoxins because they are considered safe to humans and are environmentally friendly. Singh et al. (2008) demonstrated efficacy of employing essential oil of *Cinnamomum camphora* (L.) leaves. Their study showed that the oil completely inhibited aflatoxin B_1 production. Zakir (2009) worked on the efficacy of aqueous leaf extracts of some medicinal plants such as *Artemisia maritima*, *Azadirachta indica*, *Mentha piperita*, *Coriandrum sativum* and *Ocimum sanctum* in the management of mycoflora and mycotoxin contamination in wild pomegranate (anardana). In the same year, Reddy et al. (2009a, b) used few plant extracts and biocontrol agents for growth inhibition of *A. flavus* and AFB_1 production in stored rice. Their results revealed that extract of *Syzygium aromaticum* (5 g/kg) showed complete growth inhibition of *A. flavus* and AFB_1 production, whereas *Allium sativum*, *Curcuma longa* and *Ocimum sanctum* effectively inhibited growth of *A. flavus* (65–78%) and AFB_1 production (72.2–85.7%). Among the biocontrol agents used, culture filtrates of *Rhodococcus erythropolis* showed complete inhibition of AFB_1 production at 25 ml/kg concentration, while *Bacillus subtilis*, *Pseudomonas fluorescens* and *Trichoderma virens* showed 68%, 93% and 80% reduction of *A. flavus* growth and 58%, 83.7% and 72.2% reduction of AFB_1 at 200 ml/kg, respectively. Sudhakar et al. (2009) studied effect of methyleugenol in inhibiting *A. flavus* colonization and aflatoxin production on peanut pods and kernels. They observed 0.5% methyleugenol spray on pods and kernels checked *A. flavus* colonization and aflatoxin synthesis. Prakash et al. (2011) found essential oil from *Ocimum gratissimum* as a safe plant-based antimicrobial effective against fungal and aflatoxin B_1 contamination of spices. Panda and Mehta (2013) detoxified aflatoxins at room temperature by using extracts of *Ocimum tenuiflorum*. Aiko and Mehta (2013) observed inhibitory effect of clove on the growth of *Penicillium citrinum* and citrinin production. Similarly, Vijayanandraj et al. (2014) used aqueous extracts of *Adhatoda vasica* leaves, which completely degraded AFB_1 after 24 h at 37 °C. An investigation has also been done to assess the efficacy of aqueous extracts of *Mentha arvensis* (mint) and *Piper betle* (betel) on the mycelial growth and citrinin production of *P. citrinum* (Panda et al. 2014). Results revealed that mint extract

inhibited production of citrinin up to 73% without inhibiting mycelia growth, and, on the contrary, betel extract resulted in stimulatory effect on citrinin production and mycelia growth.

15.8 Conclusions and Future Perspectives

The extensive research efforts of the last few decades on mycotoxin contamination clearly indicate that many fungal species are capable of producing different mycotoxins, which are hazardous to human and animal health. As the levels of mycotoxin contamination vary from season to season, place to place and year to year depending on the susceptibility of the plant variety, prevalence of toxigenic fungal strains and optimum environmental conditions, continuous surveillance programs are required to be conducted by the government and government-designated agencies so that withdrawal of the contaminated commodity is exercised and contamination remains at the lowest level in various ecological systems. Although it may not be possible to produce a supply of food and feed completely free of mycotoxins, yet improvement in handling, drying, better storage and marketing facilities can minimize fungal growth and, therefore, reduce the risk of mycotoxin contamination.

The consumers also need to be advised to remove visibly infected and damaged foods before eating and storing. In addition, they have to make sure that the foods are stored in a dry and cool place and kept free of insects. Every consumer has also to ensure that his/her diet is diverse as this not only helps to mitigate mycotoxin exposure but also improves nutrition and health. Therefore, it is very important to introduce awareness programs about the impact and sources of mycotoxins to both the producers and the consumers.

In India, there is also an urgent need to form national guidelines or regulations for the prevention and control of mycotoxin contamination in different agricultural commodities as it exists for many other countries. In fact there is a dire need to achieve universal standardization of regulatory limits for at least the mycotoxins of concern. This is very much required as the developed countries have adopted stringent laws with respect to mycotoxin contaminants and the export rejected consignments of various agricultural commodities are usually sold in the domestic markets, which is a matter of great concern.

At the state/national level, there is also need of removing mycotoxins from the feeds and foods by using some mycotoxin binding agents, which may effectively adsorb the mycotoxins or by any other process of deactivation of mycotoxins or by using the modern storage practices, which can decrease the mycobial load and simultaneously decrease mycotoxin contamination. This needs to be implemented aggressively by the Food Safety and Standards Authority of India (FSSAI) before marketing of the products.

References

Adebiyi JA, Kayitesi E et al (2019) Food fermentation and mycotoxin detoxification: an African perspective. Food Control 106:106731

Adeyeye SAO (2016) Fungal mycotoxins in foods: a review. Cogent Food Agric 2:1–11

Agarwal S, Roy A (1994) Screening analysis and quantitation of mycotoxins screened by different fungal pathogens causing fruit rots. J Indian Bot Soc 73:167–168

Ahmed KS (1993) Mycoflora changes and aflatoxin production in stored black gram seed. J Stored Food Res 29:33–36

Aiko V, Mehta A (2013) Inhibitory effect of clove (*Syzygium aromaticum*) on the growth of *Penicillium citrinum* and citrinin production. J Food Saf 33:440–444

Aiko V, Mehta A (2015) Occurrence, detection and detoxification of mycotoxins. J Biosci 40:943–954

Alberts JF, Lilly M et al (2017) Technological and community-based methods to reduce mycotoxin exposure. Food Control 73:101–109

Amla I, Kumari S, Murthy VS, Jayaraj P, Parpia HAB (1970) Role of aflatoxin in Indian childhood cirrhosis. A preliminary communication. Indian Pediatr 7:262–270

Amla I, Sreenivasamurthy V, Jayaraj A, Parpia HAB (1974) Aflatoxin in Indian childhood cirrhosis (ICC)-a review. J Trop Pediatr Environ Child Health 20:28–33

Ansari AA, Shrivastava AK (1990) Natural occurrence of *Alternaria* mycotoxins in sorghum and ragi from North Bihar, India. Food Addit Contam 7:815–820

Bagwan NB (2011) Aflatoxin B_1 contamination in papaya fruits (*Carica papaya* L.) during post-harvest pathogenesis. Indian Phytopathol 64:48–50

Bala P, Gupta D, Sharma YP (2014) Mycobiota and aflatoxin (B_1 and B_2) contamination of apricot kernels *Prunus armeniaca* L. from Jammu and Kashmir, India. Environ Conserv 15:69–76

Bala P, Gupta D, Sharma YP (2016) Mycoflora and natural aflatoxin contamination in dried quince seeds from Jammu, India. J Environ Biol 37:101–106

Bala P, Gupta D, Sharma Y (2017) Mycotoxin research and mycoflora in some dried edible morels marketed in Jammu and Kashmir, India. J Plant Dev Sci 9:771–778

Balasubramanian T (1985) Incidence of aflatoxin B_1 in animal feeds. Indian Vet J 62:982–988

Bamba R, Sumbali G (2005) Co-occurrence of aflatoxin B_1 and cyclopiazonic acid in sour lime (*Citrus aurantifolia* Swingle) during post-harvest pathogenesis by *Aspergillus flavus*. Mycopathologia 159:407–411

Bamba R, Sumbali G (2006) Differential toxigenic behaviour of *Alternaria alternata* isolates from citrus fruits. Indian Phytopathol 59:194–198

Banu N, Muthumary JP (2010a) Aflatoxin B1 contamination in sunflower oil collected from sunflower oil refinery situated in Karnataka. Health 2:973–987

Banu N, Muthumary JP (2010b) Screening of aflatoxigenic property of some *Aspergillus flavus* isolated from sunflower seeds and its products at sunflower oil refineries. Res J Sci Technol 2:102–107

Basappa SC, Sreenivasamurthy V (1979) Decontamination of groundnut oil from aflatoxin by adsorption-cum-filtration. Indian J Technol 17:440–441

Benford D, Boyle C et al (2001) Ochratoxin A safety evaluation of certain mycotoxins in food. WHO Food Additives Series 47. FAO Food and Nutrition Paper, vol 74. WHO, Geneva, Switzerland, pp 281–415

Benison Media (2018) A comprehensive survey of mycotoxin prevalence in India (2017–18)

Bennett JW (1987) Mycotoxins, mycotoxicoses, mycotoxicology and mycopathologia. Mycopathologia 100:3–5

Bennett JW, Klich M (2003) Mycotoxins. Clin Microbiol Rev 16:497–516

Bhat RV, Rao RN (1990) Practical guide for prevention and control of aflatoxins in groundnuts. National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India. 16pp

Bhat RV, Nagarajan V, Tulpule PG (1978) Health hazards of mycotoxins in India. Indian Council of Medical Research, New Delhi

Bhat RV, Ramakrishna Y, Shashidhar RB (1989) Outbreak of mycotoxicosis in Kashmir Valley. Nutr News 11

Bhat RV, Vasanthi S et al (1996) Aflatoxin B_1 contamination in groundnut samples collected from different geographical regions of India: a multi-centric study. Food Addit Contam 13:325–331

Bhat RV, Prathapkumar H et al (1997a) A foodborne disease outbreak due to the consumption of moldy sorghum and maize containing fumonisin mycotoxins. J Toxicol Clin 35:249–255

Bhat RV, Shetty HPK, Amruth RP, Sudershan RV (1997b) A foodborne disease outbreak due to consumption of moldy sorghum and maize containing fumonisin mycotoxins. J Toxicol Clin Toxicol 35:249–255

Bhat R, Sridhar K, Velmourougane K (2007) Microbial quality evaluation of velvet bean seeds (*Mucuna pruriens* L. DC.) exposed to ionizing radiation. Trop Subtrop Agroecosyst 7:29–40

Bilgrami KS (1984) Mycotoxin in food. Indian Bot Soc 63:109–120

Bilgrami KS, Sinha KK, Ansari AA, Rahman MF (1995) Widespread occurrence of toxigenic *Alternaria* in cereals and oilseeds. Indian Phytopathol 48:150–153

Chandra H, Kumari P, Yadav S (2019) Evaluation of aflatoxin contamination in crude medicinal plants used for the preparation of herbal medicine. Orient Pharm Exp Med 19:137–143

Chaturvedi VB, Singh KS, Agnihotri AK (2002) *In vitro* aflatoxin adsorption capacity of some indigenous aflatoxin adsorbents. Indian J Anim Sci 72:257–260

Choudhary AK, Tudu S, Kumari P, Ranjan A (2020) Present status, prevalence and seasonal variations of aflaxtoxin in cattle feed, Bihar, India. Indian J Sci Technol 13:1738–1745

Chourasia HK (1995) Mycobiota and mycotoxins in herbal drugs of Indian pharmaceutical industries. Mycol Res 99:697–703

Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) (1997) Statement on ochratoxin A in dried wine fruits. Department of Health, London, pp 1–4

Dawar S, Ghaffar A (1991) Detection of seed-borne mycoflora of sunflower. Pak J Bot 23:173–178

De Boevre M, Di Mavungu JD et al (2012) Natural occurrence of mycotoxins and their masked forms in food and feed products. World Mycotoxin J 5:207–219

Di Menna M, Smith B, Miles C (2009) A history of facial eczema (pithomycotoxicosis) research. N Z J Agric Res 52:345–376

Divakara SEV, Sharma RK (2001) In: Agrawal S, Divakara Sastry EV, Sharma RK (eds) Seed spices, production, quality and export. Pointer Pub., Jaipur, India, p 19

Domijan A, Novak Jovanovic I et al (2015) *In vitro* genotoxicity of mycotoxins ochratoxin A and fumonisin B_1 could be prevented by sodium copper chlorophyllin–implication to their genotoxic mechanism. Food Chem 170:455–462

Dutta TK, Das P (2001) Isolation of aflatoxigenic strains of *Aspergillus* and detection of aflatoxin B_1 from feeds in India. Mycopathologia 151:29–33

Dwarakanath CT, Rayner ET et al (1968) Reduction of aflatoxin levels in cottonseed and peanut meals by ozonization. J Am Oil Chem Soc 45:93–95

Dwarakanath CT, Sreenivusamurthy V, Parpia AB (1969) Aflatoxin in Indian peanut oil. J Food Sci Technol 6:107–109

Elangovan TVP, Indira K, Kalyanasundaram I (1999) Prevalence of AFB_1 in rice bran and some associated factors. Indian Phytopathol 52:129–133

Elling F, Moller T (1973) Mycotoxic nephropathy in pigs. Bull World Health Organ 49:411–418

Engelhart S, Annette L et al (2002) Occurrence of toxigenic *Aspergillus versicolor* isolates and sterigmatocystin in carpet dust from damp indoor environments. Appl Environ Microbiol 68:3886–3890

European Commission (2007) European Commission Regulation (EC) No. 1126/2007 of 28 September 2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products. Off J Eur Union 255:14–17

European Commission (EC) (2006) Commission Regulation EC No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off J Eur Union L 364:5–24

Fink-Grernmels J (1999) Mycotoxins: their implications for human and animal health. Vet Q 21:115–120
Food and Agriculture Organization (1997) Worldwide regulations for mycotoxins 1995. A compendium, FAO food and nutrition paper 64. FAO, Rome, Italy
Food and Drug Administration (FDA) (2004) Compliance guidance manual. http://www.cfsan.fda.gov
Frisvad JC, Filtenborg O (1983) Classification of terverticillate penicillia based on profiles of mycotoxin and other secondary metabolites. Appl Environ Microbiol 46:1301–1310
Garg N, Aggarwal M, Javed S, Khandal RK (2013) Studies for optimization of conditions for reducing aflatoxin contamination in peanuts using ultraviolet radiations. Int J Drug Dev Res 5:408–424
Gautam AK, Bhadauria R (2010) Fungal and mycotoxin contamination of some common stored herbal fruit samples. J Indain Bot Soc 89:74–79
Ghewande MP (1997) Aflatoxin contamination of groundnut and its management in India. Aflatoxin contamination problem in groundnut in Asia. In: Mehan VK, Gowda CLL (eds) Proceedings of the first Asia working group meeting, 27–29 May 1996; Ministry of Agricultural and Rural Development Hanoi Vietnam. International Crops Research Institute for Semi-Arid Tropics, Patancheru, India
Ghewande MP, Nagaraj G, Reddy PS (1987) Aflatoxins in ground-nut: technologies for better crops. Technical Bulletin no. 33. National Research Centre for Groundnut, Junagadh, Gujarat, p 10
Ghosal S, Chakrabarti DK, Basuchaudhary KC (1977) The occurrence of 12,13-epoxytrichothecenes in seeds of safflower infected with *Fusarium oxysporum* f. sp. *carthami*. Experientia 33:547–545
Guan S, Yin Y et al (2011) Microbial strategies to control aflatoxins in food and feed. World Mycotoxin J 4:413–424
Gupta S, Aggarwal R, Ahluwalia P (2009) Assessing the mycotoxin producing potential of pathogens causing vegetable rots. Indian Phytopathol 62:1–7
Gupta D, Sharma S, Sharma Y (2013) Mycoflora and natural aflatoxin contamination in some traditional medicinal plants from Jammu, India. J Mycol Plant Pathol 43:360–368
Gupta D, Bala P, Sharma YP (2015) Evaluation of fungal flora and mycotoxin contamination in whole dried apricots (*Prunus armeniaca* L.) from J&K, India. Proc Natl Acad Sci India Sect B Biol Sci 87:81–87
Gupta D, Bala P, Sharma YP (2017) Survey of mycoflora and mycotoxin contamination in dried date plum persimmons (*Diospyros lotus* L.) from markets of Jammu province. J Plant Dev Sci 9:983–989
Hsu IC, Smalley EB, Strong FM, Ribelin WE (1972) Identification of T-2 toxin in moldy corn associated with a lethal toxicosis in dairy cattle. Appl Microbiol 24:684–690
Hueza IM, Raspantini PCF et al (2014) Zearalenone, an estrogenic mycotoxin, is an immunotoxic compound. Toxins 6:1080–1095
Hult K, Plestina R et al (1982) Ochratoxin A in human blood and Balkan endemic nephropathy. Arch Toxicol 51:313–321
IARC (1986) Patulin. In: IARC monographs on the evaluation of carcinogenic risks to humans, vol 40. IARC, Lyon, France, pp 83–98
International Agency for Research on Cancer (1993) Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. In: IARC monographs on the evaluation of carcinogenic risks to humans. International Agency for Research on Cancer, Geneva, pp 489–521
Janardhana GR, Raveesha KA, Shetty HS (1999) Mycotoxin contamination of maize grains grown in Karnataka (India). Food Chem Toxicol 37:863–868
Jelinek CPA, Pohland E, Wood GE (1989) Worldwide occurrence of mycotoxins in food and feeds, an update. J Assoc Off Anal Chem 72:223–247

Jeswal P, Kumar D (2015) Mycobiota and natural incidence of aflatoxins, ochratoxin A, and citrinin in Indian spices confirmed by LC-MS/MS. Int J Microbiol 2015:1–8

Joffe AZ (1974) Toxicity of *Fusarium pone* and *F. sporotrichioides* and its relation to alimentary toxic aleukia. In: Purchase IEH (ed) Mycotoxins. Elsevier, Amsterdam, pp 229–262

Karthikeyan M, Sandosskumar R et al (2009) Genetic variability and aflatoxigenic potential of *Aspergillus flavus* isolates from maize. Arch Phytopathol Plant Protect 42:83–91

Karthikeyan M, Karthikeyan A et al (2013) Occurrence of aflatoxin contamination in maize kernels and molecular characterization of the producing organism, *Aspergillus*. Afr J Biotechnol 12:5839–5844

Keshri S, Basu M (2003) Evaluation of aflatoxin contamination in different spices. Indian Phytopathol 56:457–459

Khan SN, Singh PK (2000) Mycotoxin producing potential of seed mycoflora of some forest trees. Indian Forester 126:1231–1233

Kim J, Park SH et al (2016) Interference with mutagenic aflatoxin B_1-induced checkpoints through antagonistic action of ochratoxin A in intestinal cancer cells: a molecular explanation on potential risk of crosstalk between carcinogens. Oncotarget 7:39627–39639

King B (1979) Outbreak of ergotism in Wollo, Ethiopia. Lancet 1:1411

Kishore G, Krishna S et al (2002) Occurrence of mycotoxins and toxigenic fungi in groundnut (*Arachis hypogaea* L.) seeds in Andhra Pradesh, India. Plant Pathol J 18:204–209

Koesukwiwat U, Sanguankaew K, Leepipatpiboon N (2014) Evaluation of a modified QuEChERS method for analysis of mycotoxins in rice. Food Chem 153:44–51

Kolhe A, Verma RJ, Dube HC (1994) Aflatoxin contamination in oil cakes. Indian Phytopathol 47:270–272

Koul A (2007) Studies on the surface mycoflora and associated mycotoxins produced in dried underground parts of some medicinal plants during storage. PhD thesis, Department of Botany, University of Jammu, Jammu

Koul A, Sumbali G (2008a) Detection of aflatoxin B_1 and B_2 from medicinally important dried rhizomes. J Mycol Plant Pathol 38:531–533

Koul A, Sumbali G (2008b) Detection of zearalenone, zearalenol and deoxynivalenol from medicinally important dried rhizomes and root tubers. Afr J Biotechnol 7:4136–4139

Koul A, Sumbali G (2009) Incidence of aflatoxins and ochratoxin A in dried root tubers of Indian kudzu, *Pueraria tuberosa* (Roxb. ex Willd.) DC. Proc Natl Acad Sci India Sect B Biol Sci 79:61–64

Koul A, Sumbali G (2010) Contamination of medicinally important dried rhizomes and root tubers with fungi and their toxins. Indian Phytopathol 63:55–58

Kour H, Sumbali G (2006) Detection of penicilli, aspergilli and their toxins from market and domestic samples of dehydrated vegetables. Indian Phytopathol 59:460–466

Kour H, Sumbali G (2012) Occurrence of zearalenone, zearalenol and deoxynivalenol in some market samples of dried vegetables in J&K State. Proc Natl Acad Sci India Sect B Biol Sci 82:531–535

Kralj Cigic I, Prosen H (2009) An overview of conventional and emerging analytical methods for the determination of mycotoxins. Int J Mol Sci 10:62–115

Krishnamachari KA, Bhat RV (1976) Poisoning of ergoty bajra (pearl millet) in man. Indian J Med Res 64:1624–1628

Krishnamachari KA, Bhat RV, Nagarajan V, Tilak TBG (1975a) Hepatitis due to aflatoxicosis: an outbreak in Western India. Lancet 1:1061–1063

Krishnamachari KA, Bhat RV, Nagarajan V, Tilak TB (1975b) Investigations into an outbreak of hepatitis in parts of Western India. Indian J Med Res 63:1036–1049

Krska R, Schubert-Ullrich P et al (2008) Mycotoxin analysis: an update. Food Addit Contam Part A 25:152–163

Kuiper-Goodman T (1994) Prevention of human mycotoxicoses through risk assessment. In: Miller JD, Trenholm HL (eds) Mycotoxins in grain. Compounds other than aflatoxin. Eagan Press, St. Paul, MN, pp 439–469

Kulkarni JR, Padule DN, Shinde PA, Salunkhe DK (1985) Aflatoxins in dried dates. Curr Res Reptr Mahatma Phule Agric Univ 2:26–31
Kumar S, Roy AK (1994) Mycotoxin contamination of some kidney curative herbal drugs. J Indian Bot Soc 73:341–342
Kumar VKK, Thakur RP, Desai S (2001) Prevalence of aflatoxin contamination in groundnut in Tumkur district of Karnataka, India. Int Arachis Newsl 21:37–39
Kumar R, Ansari K et al (2012) Detection of ochratoxin A in wheat samples in different regions of India. Food Control 26:63–67
Kumari CK, Nusrath M (1987a) Natural occurrence of citrinin and ochratoxin A in coconut products. Natl Acad Sci Lett (India) 10:303–305
Kumari CK, Nusrath M (1987b) Natural occurrence of citrinin and ochratoxin A in coconut products. Natl Acad Sci Lett 10:303–305
Li P, Zhang Q, Zhang W (2009) Immunoassays for aflatoxins. Trends Anal Chem 28:1115–1126
Li Y, Wang TQ, Wu J, Zhang XL, Xu YY, Quian YZ (2018) Multi-parameter analysis of combined hepatotoxicity induced by mycotoxin mixtures in HepG2 cells. Mycotoxins J 11:225–235
Liu X, Luo X, Hu W (1992) Studies on the epidemiology and etiology of moldy sugarcane poisoning in China. Biomed Environ Sci 5:161–177
Liu BH, Hsu YT, Lu CC, Yu FY (2013) Detecting aflatoxin B_1 in foods and feeds by using sensitive rapid enzyme-linked immunosorbent assay and gold nanoparticle immuno-chromatographic strip. Food Control 30:184–189
Madhukar J, Reddy SM (1992) Incidence of mycotoxin elaboration in guava fruits. Geobios New Rep 11:188–189
Madhyastha MS, Bhat RV (1984) *Aspergillus parasiticus* growth and aflatoxin production on black and white pepper and the inhibitory action of their chemical constituents. Appl Environ Microbiol 48:376–379
Madhyastha MS, Bhat RV (1985) Evaluation of substrate potentiality and inhibitory effects to identify high risk spices for aflatoxin contamination. J Food Sci 50:376–378
Malekinejad H, Aghazadeh-Attari J et al (2015) Neurotoxicity of mycotoxins produced in vitro by *Penicillium roqueforti* isolated from maize and grass silage. Hum Exp Toxicol 34:997–1005
Maragos CM, Busman M (2010) Rapid and advanced tools for mycotoxin analysis: a review. Food Addit Contam Part A 27:688–700
Mehan VK, McDonald D, Gibbons RW (1982) Seed colonization and aflatoxin production in groundnut genotypes inoculated with different strains of *Aspergillus flavus*. Oleagineux 37:185–191
Mehan VK, McDonald D, Lalitha B (1983) Effect of season, location and field-drying treatment on in vitro seed colonization of groundnut genotypes by *Aspergillus flavus*. Oleagineux 38:553
Miller JD (1994) Epidemiology of *Fusarium graminearum* diseases of wheat and corn. In: Miller JD, Trenholm HL (eds) Mycotoxins in grain: compounds other than aflatoxin. Egan Press, St. Paul, MN, pp 19–36
Miller JD (1995) Fungi and mycotoxins in grain: implications for stored product research. J Stored Prod Res 31:1–6
Mishra NK, Daradhiyar SK (1991) Mold flora and aflatoxin contamination of stored and cooked samples of pearl millet in the Paharia tribal belt of Santhal paragana, Bihar, India. Appl Environ Microbiol 57:1223–1226
Misra N, Batra S (1987) Mycotoxins in fennel seeds. J Food Saf 8:157–160
Moorthy K, Prasanna I et al (2011) Determination of aflatoxin contamination in various spices. Biosci Biotechnol Res Asia 8:893–900
Mor S, Singh K (2000) Incidence of toxigenic molds in dairy foods and animal feeds. Indian J Anim Sci 70:766–768
Moretti A, Logrieco AF, Susca A (2017) Mycotoxins: an underhand food problem. Methods Mol Biol 1542:3–12
Mudili V, Siddaih C et al (2014) Mould incidence and mycotoxin contamination in freshly harvested maize kernels originated from India. J Sci Food Agric 94:2674–2683

Nallathambi P, Umamaheshwari C (2009) Detection of aflatoxins in pomegranate arils infected by *Aspergillus* species. Indian Phytopathol 62:178–182
Navya HM, Hariprasad P et al (2013) Natural occurrence of aflatoxin, aflatoxigenic and non-aflatoxigenic *Aspergillus flavus* in groundnut seeds across India. Afr J Biotechnol 12:2587–2597
Neelkantan S, Balasaraswati R, Balasubramanian T, Indrajasmin G (1982) Incidence of patulin in scented supari. Madras Agric J 69:326–328
Norred WP (1990) Cyclopiazonic acid: toxicity and tissue distribution. Vet Hum Toxicol 32:20–26
Ommi S, Medikonda M, Gudela K (2019) Identification and HPLC quantification of aflatoxins in groundnut (*Arachis hypogaea* L.) samples collected in Rayalaseema area, Andhra Pradesh. Int J Pharm Sci Res 10:1312–1318
Ostry V, Malir F, Toman J, Grosse Y (2017) Mycotoxins as human carcinogens-the IARC Monographs classification. Mycotoxin Res 33:65–73
Palaniswami A, Manickam A, Neelakantan S (1989) Surveillance of toxigenic fungi and aflatoxins in rice and paddy grains. Madras Agric J 76:481–489
Panda P, Mehta A (2013) Aflatoxin detoxification potential of *Ocimum tenuiflorum*. J Food Saf 33:265–272
Panda P, Aiko V, Mehta A (2014) Effect of aqueous extracts of *Mentha arvensis* (mint) and *Piper betle* (betel) on growth and citrinin production from toxigenic *Penicillium citrinum*. J Food Sci Technol 52:3466–3474
Patel TB, Boman TJ, Dalal UC (1958) An epidemic of ergot poisoning through ingestion of infected bajra in southern parts of Bombay state. Indian J Med Sci 12:257–261
Pereira VL, Fernandes JO, Cunha SC (2014) Mycotoxins in cereals and related foodstuffs: a review on occurrence and recent methods of analysis. Trends Food Sci Technol 36:96–136
Pier AC, Richard JL, Cysewski SJ (1980) Implications of mycotoxins in animal disease. J Am Vet Med Assoc 176:719–724
Pitt J (1975) Xerophilic fungi and the spoilage of foods of plant origin. In: Duckworth RB (ed) Water relations of foods. Academic, London, pp 273–307. https://doi.org/10.1016/B978-0-12-223150-6.50021-3
Pohland AE (1993) Mycotoxins in review. Food Addit Contam 10:17–28
Power SR (2013) Present scenario of Indian spice industry and its trend in production and export. Asian J Manag Cases 4:197–204
Prakash B, Shukla R et al (2011) Efficacy of chemically characterized *Ocimum gratissimum* L. essential oil as an antioxidant and a safe plant based antimicrobial against fungal and aflatoxin B_1 contamination of spices. Food Res Int 44:385–390
Prasad T, Sinha RK, Jeswal P (1987) Seed mycoflora of cereals and aflatoxin contamination under storage systems. J Indian Bot Soc 66:156–160
Rahmani A, Jinap S, Soleimany F (2009) Qualitative and quantitative analysis of mycotoxins. Compr Rev Food Sci Food Saf 8:202–251
Rai S, Chaturvedi SN (1993) Aflatoxin contamination in chewing tobacco (*Nicotiana tabacum* L). J Indian Bot Soc 72:189–190
Raisuddin S, Misra JK (1991) Aflatoxin in betel nut and its control by use of food preservatives. Food Addit Contam 8:707–712
Rajarajan PN, Rajasekaran KM, Devi NK (2013) Isolation and quantification of aflatoxin from *Aspergillus flavus* infected stored peanuts. Indian J Pharm Sci 1:76–80
Ramakrishna Y, Bhat RV, Vasanthi S (1990) Natural occurrence of mycotoxins in staple foods in India. J Agric Food Chem 38:1857–1859
Ramesh C, Jayagoudar S (2014) Determination of ochratoxin A in spices from Dharwad by high performance liquid chromatography. Asian J Plant Sci 4:42–52
Rani N, Singh S (1990) Aflatoxin contamination of some umbelliferous spices of human use. In: International symposium and workshop on Food cont. mycotoxins and phycotoxins, 4–-15 November, 1990, Cairo, Egypt, Abstract book, pp 79–80

Rani N, Singh S (1994) Natural occurrence of mycoflora and mycotoxins in some pulse products of human use. J Indain Bot Soc 75:2893–2895
Rao LB, Husain A (1985) Presence of cyclopiazonic acid in kodo millet (*Paspalum scrobiculatum*) causing 'kodua poisoning' in man and its production by associated fungi. Mycopathologia 89:177–180
Rao KS, Madhavan TV, Tulpule PG (1965) Incidence of toxigenic strains of *Aspergillus flavus* affecting groundnut crop in certain coastal districts of India. Indian J Med Res 53:1196–1202
Rao PR, Murthy AS, Naidu NR, Sriram PK (1980) Notes on acute aflatoxicosis in pigs. Indian J Anim Sci 51:800–802
Rawat A, Mahajan S et al (2014) Detection of toxigenic fungi and mycotoxins in some stored medicinal plant samples. Int J Appl Sci Biotechnol 2:211–216
Reddy KRN (2008) Estimation and prevention of aflatoxin contamination in rice. PhD thesis, Osmania University, Hyderabad, India
Reddy AS, Reddy SM (1983) Elaboration of mycotoxins by fungi associated with til. (*Sesamum indicum* L.). Curr Sci 52:613–614
Reddy GL, Reddy SM (1994) Incidence of trichothecene mycotoxin producing fungi in oil seed cakes. Indian J Anim Nutr 11:101–106
Reddy PS, Reddy CV, Reddy VR, Rao PV (1984) Occurrence of aflatoxin in some feed ingredients in three geographical regions of Andhra Pradesh. Indian J Anim Sci 54:235–238
Reddy SV, Mayi DK et al (2001) Aflatoxin B_1 in different grades of chillies (*Capsicum annuum* L.) in India as determined by indirect competitive ELISA. Food Addit Contam 18:553–558
Reddy DVR, Thirumala-Devi K et al (2002) Estimation of aflatoxin level in selected food and feeds in India. In: Hanak E, Boutrif E, Fabre P, Pineiro M (eds) Food safety management in developing countries. Proceeding of the International workshop, CIRAD-FAO, 11–-13 December 2000, Montpellier, France
Reddy TY, Sulochanamma BN et al (2003) Influence of weather, dryspells and management practices on aflatoxin contamination in groundnut. Indian Phytopathol 56:262–265
Reddy CS, Reddy KRN et al (2004) Exploration of aflatoxin contamination and its management in rice. J Mycol Plant Pathol 34:816–820
Reddy KRN, Hamed A, Abel C, Shier W, Oliveira C, Raghavender C (2009a) Mycotoxin contamination of commercially important agricultural commodities. Toxin Rev 28:154–168
Reddy KRN, Reddy CS, Muralidharan K (2009b) Potential of botanical and biocontrol agents on growth and aflatoxin production by *Aspergillus flavus* infecting rice grains. Food Control 20:173–178
Richard JL (2007) Some major mycotoxins and their mycotoxicoses-an overview. Int J Food Microbiol 119:3–10
Richard JL, Bennett GA et al (1993) Analysis of naturally occurring mycotoxins in feedstuffs and food. J Anim Sci 71:2563–2574
Ridgway K, Scientific R (2012) Sample preparation for food contaminant analysis. LC GC Eur 25:1–8
Robinson P (1967) Infantile cirrhosis of the liver in India: with special reference to probable aflatoxin etiology. Clin Pediatr 6:57–62
Roy R (1990) Aflatoxins in imported figs. Acta Hortic 269:521–526
Roy AK (2003) Mycological problems of crude herbal drugs - overview and challenges. Indian Phytopathol 56:1–13
Roy AK, Chourasia HK (1989) Aflatoxin problems in some medicinal plants under storage. Int J Crude Drug Res 27:156–160
Roy AK, Chourasia HK (1990a) Mycotoxin incidence in root drugs. Int J Crude Drug Res 28:157–160
Roy AK, Chourasia HK (1990b) Mycoflora, mycotoxin producibility and mycotoxins in traditional herbal drugs from India. J Gen Appl Microbiol 36:295–302
Roy AK, Chourasia HK (1991) Aflatoxin occurrence in crude drugs of plant origin. Ann Phytopathol Soc Jpn 57:256–258

Roy AK, Chourasia HK (2001) Mycotoxin contamination in herbal seed samples under storage and their prevention. In: Seed technology and seed pathology. Pointer Publishers, Jaipur, India, pp 131–144

Roy AK, Kumar S (1993) Occurrence of ochratoxin A in herbal drugs of Indian origin - a report. Mycotoxin Res 9:94–98

Roy AK, Kumari V (1991) Aflatoxin and citrinin in seeds of some medicinal plants under storage. Int J Pharmacogn 29:62–65

Roy AK, Sinha KK, Chourasia HK (1988) Aflatoxin contamination of some common drug plants. Appl Environ Microbiol 54:842–843

Sahay SS, Prasad T (1990) The occurrence of aflatoxins in mustard and mustard products. Food Addit Contam 7:509–513

Sahay SS, Ranjan KS (1990) Distribution of aflatoxin in oil seeds to their oil and cakes. Geobios 17:47–48

Sam F, Patterson Edward JK, Devadason S (2015) Aflatoxins investigation on dried fishes of Tuticorin, South East Coast of India. J Foodborne Zoonotic Dis 3:49–62

Samarajeewa U, Arseculeratne SN, Bandunatha CHSR (1977) Degradation of aflatoxin in coconut oil and copra meal. J Natl Sci Found Sri Lanka 5:1–12

Samarajeewa U, Jayatilaka CLV et al (1985) A pilot plant for detoxification of aflatoxin B1-contaminated coconut oil by solar irradiation. World J Microbiol Biotechnol 1:333–343

Samarajeewa U, Sen AC, Cohen MD, Wei CI (1990) Detoxification of aflatoxins in foods and feeds by physical and chemical methods. J Food Prot 53:489–501

Samyal S (2013) Studies on the mycotoxic contaminants of dried red chillies (*Capsicum annuum* Linn.) from Jammu and Kashmir. PhD thesis, Department of Botany, University of Jammu, Jammu

Samyal S, Sumbali G (2011) Toxins of *Alternaria alternata* from decaying tomato fruits during marketing. Plant Pathol 41:259–262

Samyal S, Sumbali G (2012) Detection of aflatoxin B_1 and B_2 in tomato (*Lycopersicon esculentum*) c.v. marglobe infected with *Aspergillus flavus*. J Mycol Plant Pathol 42:459–462

Samyal S, Sumbali S (2013) Aflatoxin B_1 and B_2 contamination in red chilli powder sold loose in the markets of Jammu and Kashmir. J Biosphere 2:10–12

Samyal S, Sumbali G (2014) Detection of fusarial toxins from loose and packed red chilli powder marketed in Jammu and Kashmir state, India. Int J Adv Res 2:1397–1404

Samyal S, Sumbali G (2020) Toxigenic mycoflora and natural co-occurrence of toxins in red chillies from Jammu and Kashmir. Kavaka 54:89–95

Sanches-Silva A, Brites C et al (2019) UHPLC-ToF-MS method for determination of multi-mycotoxins in maize: development and validation. Curr Res Food Sci 1:1–7

Sashidhar RB, Ramakrishna Y, Ramnath T, Bhat RV (1991) Lack of relationship between sorghum consumption mycotoxin contamination and pellagra in a traditionally sorghum eating population. Trop Geogr Med 43:165–170

Saxena J, Mehrotra BS (1989) Screening of spices commonly marketed in India for natural occurrence of mycotoxins. J Food Compos Anal 2:286–292

Saxena J, Mehrotra BS (1990) The occurrence of mycotoxins in some dry fruits retail marketed in Nainital district of India. Acta Alimentaria 19:221–224

Schulz M, Schumann L et al (2018) Synergistic action of the nephrotoxic mycotoxins ochratoxin A and citrinin at nanomolar concentrations in human proximal tubule-derived cells. Toxicol Lett 291:149–157

Scudamore KA (2008) In: Gilbert J, Enyuva HZ (eds) Bioactive compounds in foods. Blackwell Publishing Ltd., Oxford, UK, pp 134–172

Sekhar V, Shanmugasundram ERB (1979) Toxicosis due to *Aspergillus terreus* in chicks. Indian J Exp Biol 14:521

Shantha T, Murthy VS (1981) Use of sunlight to partially detoxify groundnut (peanut) cake flour and casein contaminated with aflatoxin B_1. J Assoc Off Anal Chem 64:291–293

Shantha T, Sreenivasamurthy V (1975) Detoxification of groundnut oil. J Food Sci Technol 12:20–22
Shantha T, Sreenivasamurthy V (1977) Photodestruction of aflatoxin in groundnut oil. Indian J Technol 15:453–454
Shantha T, Sreenivasamurthy V, Rati ER, Prema V (1986) Detoxification of groundnut seeds by urea and sunlight. J Food Saf 7:225–231
Sharma S (2005) Studies on the mycobial contamination and mycotoxicity of some dried medicinal plants. PhD thesis, Department of Botany, University of Jammu, Jammu
Sharma Y, Sumbali G (1999a) Incidence of aflatoxin producing strains and aflatoxin contamination in dry fruit slices of quinces (*Cydonia oblonga* Mill.) from the Indian State of Jammu and Kashmir. Mycopathologia 148:103–107
Sharma YP, Sumbali G (1999b) Natural occurrence of ochratoxin A and toxigenic *Aspergillus ochraceus* strains in dry fruit slices of quinces from Jammu and Kashmir. Indian Phytopathol 52:148–150
Sharma R, Sumbali G (2014) Fungal diversity associated with the commercial grades of walnut kernels sold in the markets of Jammu and Kashmir State (India). Int J Pharm Sci Invent 3:50–57
Sharma RS, Trivedi KR et al (1994) Aflatoxin B_1 content in deoiled/oil cakes, cattle feeds and damaged grains during different seasons in India. J Food Sci Technol 31:244–246
Sharma S, Gupta D, Sharma YP (2013) Aflatoxin contamination in chilgoza pine nuts (*Pinus gerardiana* Wall.) commercially available in retail markets of Jammu, India. Int J Pharma Bio Sci 4:751–759
Sharma S, Sumbali G, Sharma V (2013a) Mycobial contamination and mycotoxinogenesis of *Tinospora cordifolia* - an important medicinal plant of India. Int J Agric Res Innov Technol 3:16–21
Sharma S, Sumbali G, Sharma V (2013b) Investigations on the mycoflora and mycotoxin contamination of dried medicinal leaves of *Azadirachta indica* A. Juss. and *Justicia adhatoda* Linn. from Jammu and Kashmir state (India). Indian J Adv Res 1:131–138
Sharma S, Gupta D, Sharma YP (2014) Natural incidence of aflatoxins, ochratoxin A, patulin and their co-occurrence in chilgoza pine nuts marketed in Jammu, India. Proc Natl Acad Sci India Sect B Biol Sci 85:45–50
Sharma KK, Pothana A et al (2018) Peanuts that keep aflatoxin at bay: a threshold that matters. Plant Biotechnol J 16:1024–1033
Shephard GS (2016) Current status of mycotoxin analysis: a critical review. J AOAC Int 99:842–848
Singh A (1983) Mycotoxin contamination in dry fruits and spices. In: Proc symp mycotoxin in food and feed, Bhagalpur, pp 55–68
Singh YP (2002) Studies on the market diseases of some pome fruits and their management. PhD thesis, Department of Botany, University of Jammu, Jammu
Singh PK (2003) Mycotoxin elaboration in Triphala and its constituents. Indian Phytopathol 56:380–383
Singh PK (2017) Mycotoxin contamination and induced biochemical changes associated with selected medicinal plants. Microbiol Res J Int 20:1–7
Singh A, Sinha KK (1982) Aflatoxin production on some fruits by *Aspergillus flavus* Link. ex Fries and *Aspergillus parasiticus* Spear. Curr Sci 51:282
Singh A, Sinha KK (1983) Biochemical changes and aflatoxin production in guava fruits by *Aspergillus flavus* and *A. parasiticus*. Indian Phytopathol 36:365–366
Singh YP, Sumbali G (2000) Natural incidence of toxigenic *Aspergillus flavus* strains on the surface of pre-harvest jujube fruits. Indian Phytopathol 53:404–406
Singh YP, Sumbali G (2004) Occurrence of tenuazonic acid-producing strains of *Alternaria alternata* in natural rots of apple. Indian Phytopathol 57:68–69
Singh YP, Sumbali G (2008a) Patulin and citrinin production in apple and pear fruits by *Penicillium expansum* strains collected from Jammu markets. Indian Phytopathol 61:471–477

Singh YP, Sumbali G (2008b) Mycopathogens and mycotoxins associated with post-harvest fruit rot of crab apple cv. scarlet siberian (*Docynia indica* Dcne.). Geobios 35:61

Singh YP, Sumbali G (2011) Aflatoxin B_1 contamination in commercial varieties of apple and pear fruits with *Aspergillus flavus* Link ex Fries. Indian Phytopathol 64:100–101

Singh T, Tyagi RPS, Ram B (1984) Occurrence of aflatoxin B_1 in animal and poultry feeds. Indian J Dairy Sci 37:167–169

Singh K, Frisvd JC, Thrane U, Mathur SB (1991) An illustrated manual on identification of some seed-borne Aspergilli, Fusaria, Penicillia and their mycotoxins. Danish Government Institute of Seed Pathology for Developing Countries, Hellerup, Denmark

Singh P, Srivastava B, Kumar A, Dubey N (2008) Fungal contamination of raw materials of some herbal drugs and recommendation of *Cinnamomum camphora* oil as herbal fungitoxicant. Microb Ecol 56:555–560

Sinha KK (1990) Incidence of mycotoxins in maize grains in Bihar State, India. Food Addit Contam 7:55–61

Sinha AK (1995) Mycotoxin - health hazard. Yojana 39:15–16

Sinha AK, Prasad G (1994) Monitoring and identification of mycotoxins in marketed bakery bread during monsoon. Indian Phytopathol 47:164–167

Sinha KK, Punam Kumari (1992) Incidence of aflatoxins in maize and its impact on physiological processes of seed. In: Bilgrami KS, Sinha KK (eds) Mycotoxin incidence and effects. CBS Publishing, New Delhi, India, pp 73–81

Sinha KK, Singh A (1982) Aflatoxin producing potentials of *Aspergillus flavus* and natural occurrence of aflatoxins in some fruits and vegetables. Natl Acad Sci Lett 5:213–214

Sinha KK, Sinha AK (1988) Occurrence of aflatoxin and *Aspergillus flavus* in marketed wheat. Indian Phytopathol 41:44–47

Sinha AK, Sinha KK (1990) Insect pests, *Aspergillus flavus* and aflatoxin contamination in wheat: a survey at North Bihar, India. J Stored Prod Res 26:223–226

Sinha KK, Sinha AK (1993) Effect of aflatoxin B1 on germination index and seedling growth in wheat varieties. Mycopathologia 123:165–169

Sinnhuber R, Wales JH et al (1965) Aflatoxins in cottonseed meal and hepatoma in Rainbow Trout. Fed Proc 24:627

Smith JE, Moss MO (1985) Mycotoxins. Formation, analyses and significance. Wiley, Chichester, UK, pp 1–148

Sreenivasamurthy V, Jayaraman A, Parpia HAB (1965) Aflatoxin in Indian peanuts: analysis and extraction. In: Wogan GN (ed) Mycotoxins in foodstuffs. Mass. Inst. of Technol. Press, Cambridge, MA, pp 251–260

Sreenivasamurthy V, Parpia HAB, Srikanta S, Shankar Murti A (1967) Detoxification of aflatoxin in peanut meal by hydrogen peroxide. J Assoc Off Anal Chem 50:350–354

Srinivasulu B, Aruna K et al (2003) Investigations of post-harvest aflatoxin contamination of copra. Indian Coconut J 34:8–9

Stoev SD (1998) The role of ochratoxin A as a possible cause of Balkan endemic nephropathy and its risk evaluation. Vet Hum Toxicol 40:352–360

Subrahmanyam P, Rao AS (1974a) A note on aflatoxin production by some Indian strains of *Aspergillus flavus*. Indian J Microbiol 14:31–34

Subrahmanyam P, Rao AS (1974b) Occurrence of aflatoxin and citrinin in groundnut (*Arachis hypogaea* L.) at harvest in relation to pod condition and kernel moisture content. Curr Sci 43:707–710

Sudhakar P, Latha P et al (2009) Inhibition of *Aspergillus flavus* colonization and aflatoxin (AFB_1) in peanut by methyleugenol. Indian J Exp Biol 47:63–67

Sun DD, Gu X et al (2015) Quality evaluation of five commercial enzyme linked immunosorbent assay kits for detecting aflatoxin B_1 in feedstuffs. Asian-Australas J Anim Sci 28:691–696

Sunakorn P, Kalayanarooj S (1990) Reye's syndrome in Thailand. Acta Paediatr Jpn 32:391–398

Surekha M, Reddy SM (1987) Mycoflora and mycotoxin production in groundnut fodder. Ind J Mycol Plant Pathol 17:337–338

Suryanarayanan TS, Suryanarayanan CS (1990) Fungi associated with stored sunflower seeds. J Econ Taxon Bot 14:174–176
Tanaka T, Hasegawa A et al (1988) Natural occurrence of aflatoxins in traditional herbal drugs from Indonesia. Proc Japanese Assoc Mycotoxicol 28:33–35
Thakur RP, Reddy BVS et al (2006) Sorghum grain mold. Information Bulletin No. 72. International Crops Research Institute for the Semi Arid Tropics, Patancheru, Andhra Pradesh, India
Thirumala-Devi K, Reddy G, Reddy SV, Delfosse P, Reddy D (2000) Production of polyclonal antibodies against ochratoxin A and its detection in chilies by ELISA. J Agric Food Chem 48:5079–5082
Thirumala-Devi K, Mayo MA et al (2001) Occurrence of ochratoxin A in black pepper, coriander, ginger and turmeric in India. Food Addit Contam 18:830–835
Thirumala-Devi K, Reddy G, Reddy D (2002) Occurrence of aflatoxins and ochratoxin A in Indian poultry feeds. J Food Prot 65:1338–1340
Tola M, Kebede B (2016) Occurrence, importance and control of mycotoxins: a review. Cogent Food Agric 2:1191103
Toteja GS, Mukherjee A et al (2006) Aflatoxin B1 contamination of parboiled rice samples collected from different states of India: a multi-centre study. Food Addit Contam 23:411–414
Tulpule PG, Bhat RV, Nagarajan V (1977) Variations in aflatoxin production due to fungal isolates and crop genotypes and their scope in prevention of aflatoxin production. Arch Inst Pasteur Tunis 54:487–493
Turner NW, Subrahmanyam S, Piletsky S (2009) Analytical methods for determination of mycotoxins: a review. Anal Chim Acta 632:168–180
Turner NW, Bramhmbhatt H et al (2015) Analytical methods for determination of mycotoxins: an update (2009–2014). Anal Chim Acta 901:12–33
Ueno Y (1987) Trichothecenes in food. In: Krogh P (ed) Mycotoxins in food, food science and technology. Academic, London
Upadhyaya HD, Nigam SN, Thakur RP (2002) Genetic enhancement for resistance to aflatoxin contamination in groundnut. In: Proceedings of the seventh ICRISAT regional groundnut meeting for Western and Central Africa, 6–8 Dec. 2000, Cotonou, Benin. International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh, pp 29–36
Uraguchi K (1969) Mycotoxic origin of cardiac beriberi. J Stored Prod Res 5:227–236
US Food and Drug Administration (2000) Patulin in apple juice, apple juice concentrates and apple juice products. US Food and Drug Administration Center for Food Safety and Applied Nutrition Office of Plant and Dairy Foods and Beverages
Van Egmond HP (1989) Aflatoxin M_1: occurrence, toxicity, regulation. In: Van Egmond HP (ed) Mycotoxins in dairy products. Elsevier Applied Science, London, pp 11–55
Van Emon JM (2010) Bioanalytical methods for food contaminant analysis. J AOAC Int 93:1681–1691
Vasanthi S, Bhat RV (1998) Mycotoxins in food: occurrence, health and economic significance and food control measures. Indian J Med Res 108:212–222
Vasanthi S, Bhat RV, Subbulakshmi G (1997) Aflatoxin intake from maize-based diets in a rural population in Southern India. J Sci Food Agric 73:226–230
Vedanayagam HS, Indulkar AS, Rao SR (1986) The occurrence of *Aspergillus flavus* and aflatoxins in Indian cotton seed: a survey. J Food Sci Technol 23:73–76
Vejdovszky K, Hahn K et al (2017) Synergistic estrogenic effects of *Fusarium* and *Alternaria* mycotoxins *in vitro*. Arch Toxicol 91:1447–1460
Verma RJ, Kolhe A, Dube HC (1995) Aflatoxin contamination in chewing products. Proc Natl Acad Sci India 65:167–170
Vijayanandraj S, Brinda R et al (2014) Detoxification of aflatoxin B_1 by an aqueous extract from leaves of *Adhatoda vasica* Nees. Microbiol Res 169:294–300
Vijayasamundeeswari A, Mohankumar M et al (2009) Prevalence of aflatoxin B1 contamination in pre- and post-harvest maize kernels, food products, poultry and livestock feeds in Tamil Nadu, India. J Plant Protect Res 49:221–224

Wagle NG (1970) Detection and estimation of aflatoxin in groundnuts and groundnut products. Indian Standards Inst Bull 22:299–301
Waliyar F, Reddy SV et al (2003) Importance of mycotoxins in food and feed in India. Aspects Appl Biol 68:147–154
Wang YC, Wang J et al (2011) Preparation of monoclonal antibodies and development of an indirect competitive ELISA for fumonisin B_1 detection. J Shanghai Jiao Tong Univ 29:69–74
Webley DJ, Jackson KL, Mullins JD (1997) Mycotoxins in food: a review of recent analyses. Food Aust 49:375–379
Welke JE, Hoeltz M, Dottori HA, Noll IB (2009) Effect of processing stages of apple juice concentrate on patulin levels. Food Control 20:48–52
Whitaker TB (2006) Sampling foods for mycotoxins. Food Addit Contam 23:50–61
Whitaker TB, Johansson AS (2005) Sampling uncertainties for the detection of chemical agents in complex food matrices. J Food Prot 68:1306–1313
WHO (1979) Environmental health criteria II. Mycotoxins. World Health Organization, Geneva
WHO (1991) Documents No. A 44/20, 22 March 1991 (Progress Report by the Director General). World Health Organization, Geneva
World Health Organization (WHO) (2001) Safety evaluation of certain mycotoxins in food. Fumonisins (WHO Food Additives Series 47), FAO food and nutrition paper, vol 74. WHO, Geneva
Yazdanpanah H (2011) Mycotoxins: analytical challenges. Iran J Pharm Res 10:653–654
Zakir S (2009) Studies on post-harvest spoilage of dried seeds of pomegranate (*Punica granatum* L.) from Jammu province. PhD thesis, Department of Botany, University of Jammu, Jammu
Zhang H, Wang S, Fang G (2011) Applications and recent developments of multi-analyte simultaneous analysis by enzyme-linked immunosorbent assays. J Immunol Methods 368:1–23
Zheng Z, Humphrey CW et al (2005) Validation of an ELISA test kit for the detection of total aflatoxins in grain and grain products by comparison with HPLC. Mycopathologia 159:255–263

Fungi a Potential Source of Bioactive Metabolites an Indian Prospective

16

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Abstract

Fungi are multifaceted microbes and are one of the largest groups among living organisms and have established themselves as an important source of the diverse bioactive metabolites. These metabolites are of different attributes such as antibacterial, antifungal, immunosuppressant, cholesterol-lowering, anticancer, anti-inflammatory, antidiabetic, etc. This group of microbes has been explored for producing bioactive metabolites for their pharmaceutical applications after the discovery of penicillin. Interestingly, some fungal metabolites or their derivatives have been brought to the market in the form of drugs under different categories such as penicillin G, cephalosporin C, fusidic acid, retapamulin, valnemulin, tiamulin (antibacterial), griseofulvin, micafungin, anidulafungin, caspofungin (antifungal), lovastatin (anti-hypercholesterolemic), and cyclosporine A (immunosuppressant). In earlier work in India, the bioactive metabolites were isolated from soil fungi by Hindustan Antibiotics Ltd. at Pune and Rishikesh and Hoechst Pharmaceuticals, Mumbai. Later, the emphasis was shifted to marine and endophytic fungi as interest shown by prime institutes like Hoechst Pharmaceuticals, Mumbai; Piramal Enterprises Ltd., Mumbai; Indian Institute of Integrated Medicine, Jammu; Banaras Hindu University, Varanasi; University of Jammu, Jammu; and University of Agricultural Sciences, Bangalore, to name few. This chapter highlights the bioactive metabolites from fungi isolated from various sources (soil, plants, marine, etc.) with some examples.

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Keywords

Soil fungi · Endophytic fungi · Bioactive metabolites · Antibacterial · Antifungal · Anticancer

16.1 Introduction

Fungi are now generally accepted as the largest group of organisms on earth after the insects. The 1.5 million species hypothesized by Hawksworth (1991) is a commonly used figure. An updated estimate of fungal diversity showed that the fungal species ranged from 2.2 to 3.8 million worldwide (Hawksworth and Luecking 2017). Based on the recently generated data from culture-dependent and culture-independent survey on same samples, the fungal species on the earth were estimated to be 12 (11.7–13.2) million (Wu et al. 2019). The figures of fungi identified, described, and documented are less than 10% worldwide, and rest of the fungi still await their exploration. Fungi are used for various biotechnological applications such as in the pharmaceutical and agrochemical industries, in bioremediation, and in biological control, as natural scavengers, for recycling of elements and dyes, etc. Fungi are known to mankind since civilization and have been used by people for various purposes through different ways. Beautifully coloured umbrella-shaped mushrooms and toad stools growing on soils forming 'fairy rings' attracted men from the very beginning. After the discovery of penicillin (**1**) (Fig. 16.1) by Sir Alexander Fleming in 1928, the role of fungi as a source of antibiotics was established for the first time. Later, the work of Brotzu (1948) who isolated cephalosporin C (**2**) (Fig. 16.1) has strengthened the potential of fungi. These discoveries were milestones in the discovery of antibacterials from fungi. The discovery of griseofulvin (**3**) (Fig. 16.1) gave the first antifungal agent of fungal origin. Compounds like echinocandin B (**4**), deoxymulundocandin (**5**), papulacandin B (**6**), pneumocandin B_0 (**7**), caspofungin (**8**), micafungin (**9**) (Fig. 16.1), and anidulafungin (**10**) isolated from fungi have been developed as antifungal agents against humans fungal infections. Cyclosporine A (**11**) (Fig. 16.2) with immunosuppression activity was extracted from *Tolypocladium inflatum* isolated from a soil sample in Norway by H.P. Frey in 1970 (Stahelin 1996). Fingolimod (FTY720) (**12**) (Fig. 16.2) orally active immunomodulatory drug was used for the treatment of multiple sclerosis (MS). This drug was derived from myriocin (**13**) (Fig. 16.2) isolated from a fungus *Isaria sinclairii* (ATCC24400) and mycelia sterilia (Fujita et al. 1994), and subsequently clinical trial was approved by FDA in 2010 and is currently being marketed under the trade name Gilenya by Novartis for the treatment of multiple sclerosis. Mevinolin (**14**) (Fig. 16.2), a statin isolated in 1979 from a fungus *Aspergillus terreus*, later known as lovastatin, was also reported to be produced by *Monascus ruber* (Alberts et al. 1980), and it was finally approved by FDA in August 1987 as the first anti-hypercholesterolemic drug (Tolbert 2003). In the recent years, several classes of chemically distinct metabolites have been reported from fungi which have a wide range of activities against different

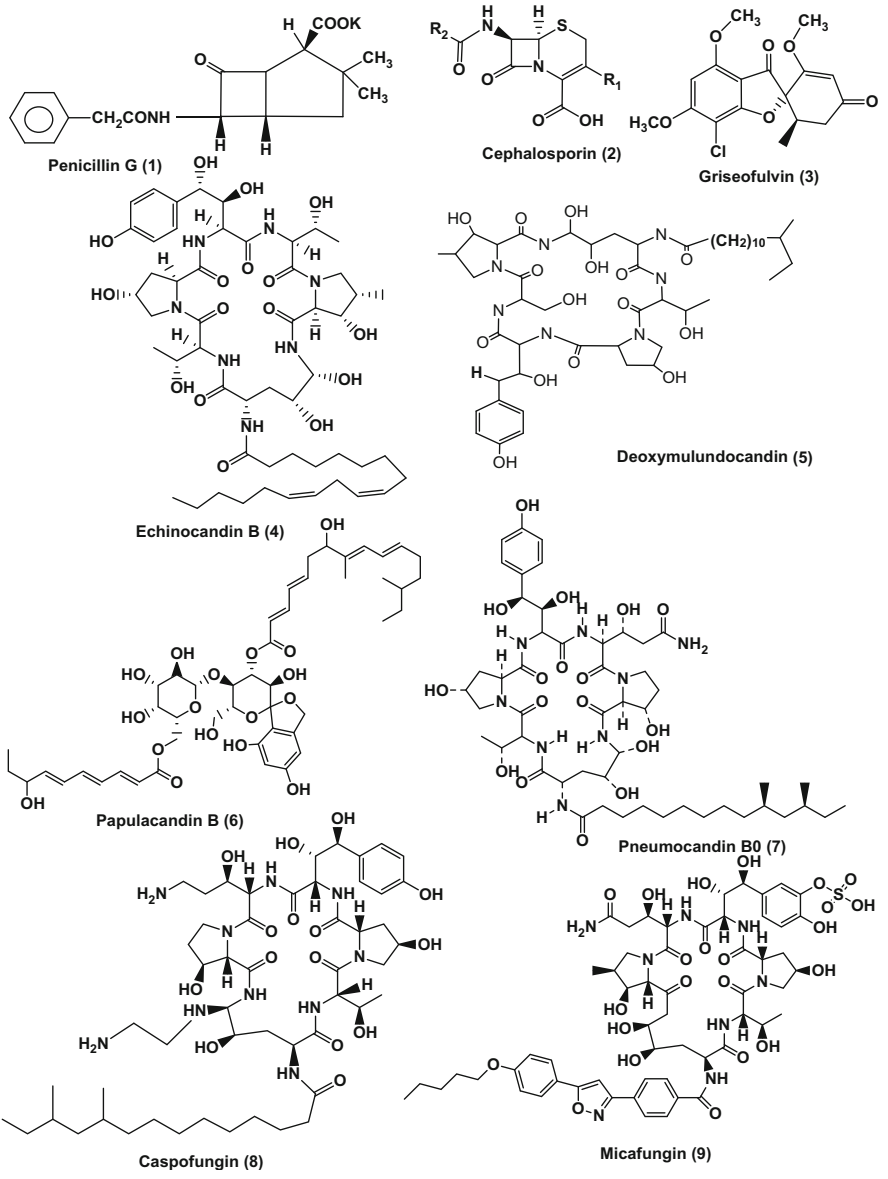

Fig. 16.1 Secondary metabolites obtained from fungi (**1**–**9**)

targets (Kharwar et al. 2011; Deshmukh et al. 2018a, b, 2019a, 2021; Agrawala et al. 2020).

In India, the major work on bioactives was done in Hoechst Pharmaceuticals, Mumbai, and started way back in 1969 and discovered the antifungal compound deoxymulundocandin which is at various stages of clinical trials. The other schools

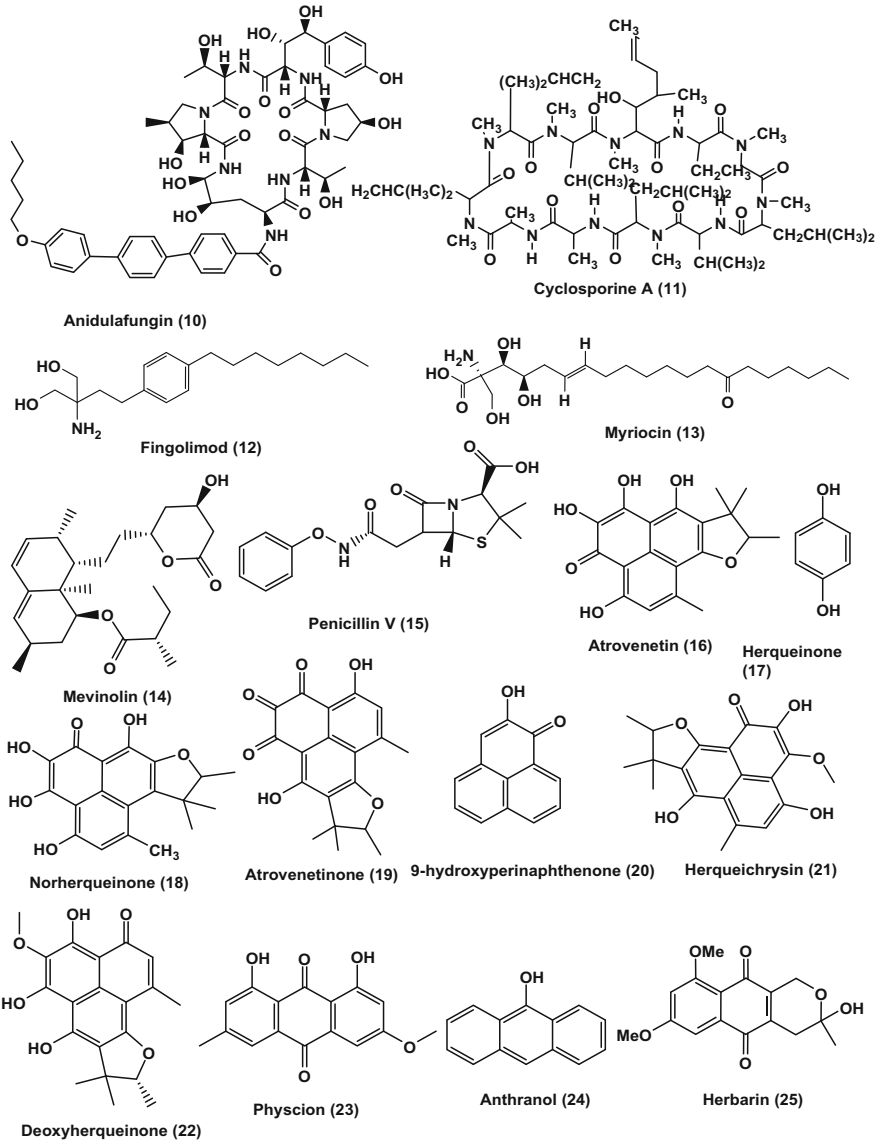

Fig. 16.2 Secondary metabolites obtained from fungi (**10**–**25**)

including Piramal Enterprises Ltd., Mumbai; Indian Institute of Integrated Medicine, Jammu; Banaras Hindu University, Varanasi; University of Jammu, Jammu; University of Agricultural Sciences, Bangalore; National Chemical Laboratory, Pune; and NIO, Goa, to name a few are involved in search of novel bioactive compounds. Primarily, the source of fungi was soil, but later orientation of emphasis has been moved towards endophytic fungi, marine fungi, mushrooms, coprophilous fungi,

and endolichenic fungi. These fungi primarily screened for antibacterial and antifungal activities followed by anticancer, anti-inflammatory, and antidiabetic activities. After the discovery of taxol, the attempts were also made to isolate compounds originally produced by plants (host memetic) from endophytic fungi. This review highlights the discovery of bioactive compounds in Indian Laboratories keeping in mind to emphasizing the exploitation of Indian bioresources by the Indians.

16.2 Preliminary Work

The initial work on bioactive compounds was started at Hindustan Antibiotics Ltd., Pimpri, Pune, under the guidance of Krishnamurthi Ganapathi in the year 1953, where initial work was done on the production of penicillin. He successfully replaced imported raw materials with indigenous ones. Penicillin V (**15**) (Fig. 16.2), the 'oral penicillin' was produced using phenoxyacetic acid as the precursor in place of phenyl acetic acid. Crude potassium penicillin thus was obtained was very white. A mechanism of the biosynthesis of penicillin by the mould *Penicillium chrysogenum* was suggested as a diversion of protein synthesis from the available amino acids as the growth phase of the mould is stopped (Ganapathi 1957; Ganapathi and Deshpande 1957). Penicillin synthesis was stimulated by a variety of carbohydrates, inositol, glycerol, fatty acids, fatty oils, and many amino acids; the Kreb's cycle intermediates did not cause any stimulation (Ganapathi and Deshpande 1958; Ganapathi and Irani 1958a, b, 1959). The biosynthesis was inhibited by dinitrophenol, cyanide, and arsenite, implicating the electron transport system in the biosynthesis. It was postulated that the compounds that caused stimulation were serving as sources of continuous production of ATP molecules required to form the peptide bonds and the removal of two hydrogen atoms; dehydrogenation was postulated to be mediated by the cytochromes in the respiratory chain.

Penicillium chrysogenum utilizes a variety of carbohydrates and converts them into glucose which seemed to polymerize into glucan. There was a difference between the 'seed mycelium' which does not produce penicillin and the 'mature mycelium' which does so. The mature mycelium contained about 10–20% of free reducing sugars in the cold-water extract, called the 'free sugar pool'; this was depleted in the seed mycelium. In the mature mycelium, irrespective of the sugar used for growth promotion, the free sugar pool contained mostly glucose, with more sugars detected by paper chromatography; free ribose was present. The seed mycelium in the aqueous extract contained fructose and ribose with small amount of glucose. Hydrolysate of the extract yielded galactose. Thus, it appeared that numerous pathways involving the carbohydrate function in the mould explaining the vast adaptabilities of the moulds to varying environmental conditions (Ganapathi and Irani 1960a, b).

Techniques were developed to work with 25–50 μg of the mycelia to identify various pentoses and hexoses by paper chromatography and resorcinol-sulphuric

reactions which give characteristic spectra for each sugar. They were able to differentiate glucose from galactose, so very closely related. Taking advantage of the differences found between riboses and deoxyriboses, a method for differential assays of DNA and RNA was also worked out (Ganapathi and Boyce 1965). A review on the chemical pathways in carbohydrate metabolism (Ganapathi and Irani 1960c) and another on regulatory mechanisms in microorganisms resolve these issues involving the feedback inhibitions (Ganapathi 1961). Penicillin production was observed in vegetative and spore inoculum and waste mycelium of *P. chrysogenum* for evaluating the impact of nitrogen (Bhuyan et al. 1961; Ghosh and Ganguli 1961).

Later an antibiotic atrovenetin (**16**) (Fig. 16.2) was isolated from a strain of *Penicillium herquei* along with herquinone and norherquinone (Narasimhachari et al. 1963). At the same time, several minor pigments from the cultures of *P. herquei* including herqueinone (**17**), norherqueinone (1**8**), atrovenetin (**16**) atrovenetinone (**19**), and 9-hydroxyperinaphthenone (**20**) (Fig. 16.2) were reported from *P. heryuei* (Narasimhachari and Vining 1963). Herqueichrysin (**21**), a new phenalenone antibiotic along with atrovenetin (**16**), deoxyherqueinone (**22**), physcion (**23**), and anthranol (**24**) were isolated from *P. herque* (Narasimhachari and Vining 1972). *Torula herbarum*, associated with dry leaves and twigs of *Felia microphylla* was the prime source of pigments herbarin (**25**) (Fig. 16.2) and dehydroherbarin (**26**) (Fig. 16.3), with weak antimicrobial activity (Kadkol et al. 1971; Nagarajan et al. 1971). Deshmukh and Vaidya in 1968 reported a biologically active polypeptide, antiamoebin (L-2-amino-3-phenyl-1-propanol (L-phenylalaninol)) (**27**) (Fig. 16.3) from mycelium of *Emericellopsis poonensis* (Deshmukh and Vaidya 1968).Volucrisporin (**28**) (Fig. 16.3) was isolated from *Volucrispora aurantiaca* and identified as 2,5-(*m*-hydroxyphenyl)-l,4-benzoquinone, the pigment as red rhombic plates (Divekar et al. 1959a, b).

Later in the year 1964, Ganapathi joined as Director at the Regional Research Laboratory Jammu and bioactives like botryodiplodin (**29**) (Fig. 16.3), with antibacterial activity which was reported from *Botryodiplodia theobromae* at RRL Jammu (San Gupta et al. 1966). Other compounds reported from RRL Jammu, now Indian Institute of Integrative Medicine (**IIIM**), will be discussed later.

16.3 Bioactives from Soil Fungi

3-Methoxy-2,5-toluquinone (**30**) (Fig. 16.3), isolated from *Aspergillus* sp. HPL Y-30,212 was a soil fungus collected near Jodhpur, India, with moderate antibacterial and antifungal activities, and penicillic acid and neoaspergillic acids were coproduced (Sood et al. 1982).

Mulundocandin (**31**) (Fig. 16.3) and deoxymulundocandin (**5**) (Fig. 16.1) are lipohexapeptides and potent antifungal antibiotics of the echinocandin class produced by *Aspergillus sydowii* var. *mulundensis* (Y-30462 = DSMZ 5745), isolated at Hoechst India Ltd., Mulund, Mumbai, from a soil sample collected from Bangladesh (Roy et al. 1987; Mukhopadhyay et al. 1992). Later on, Bills et al.

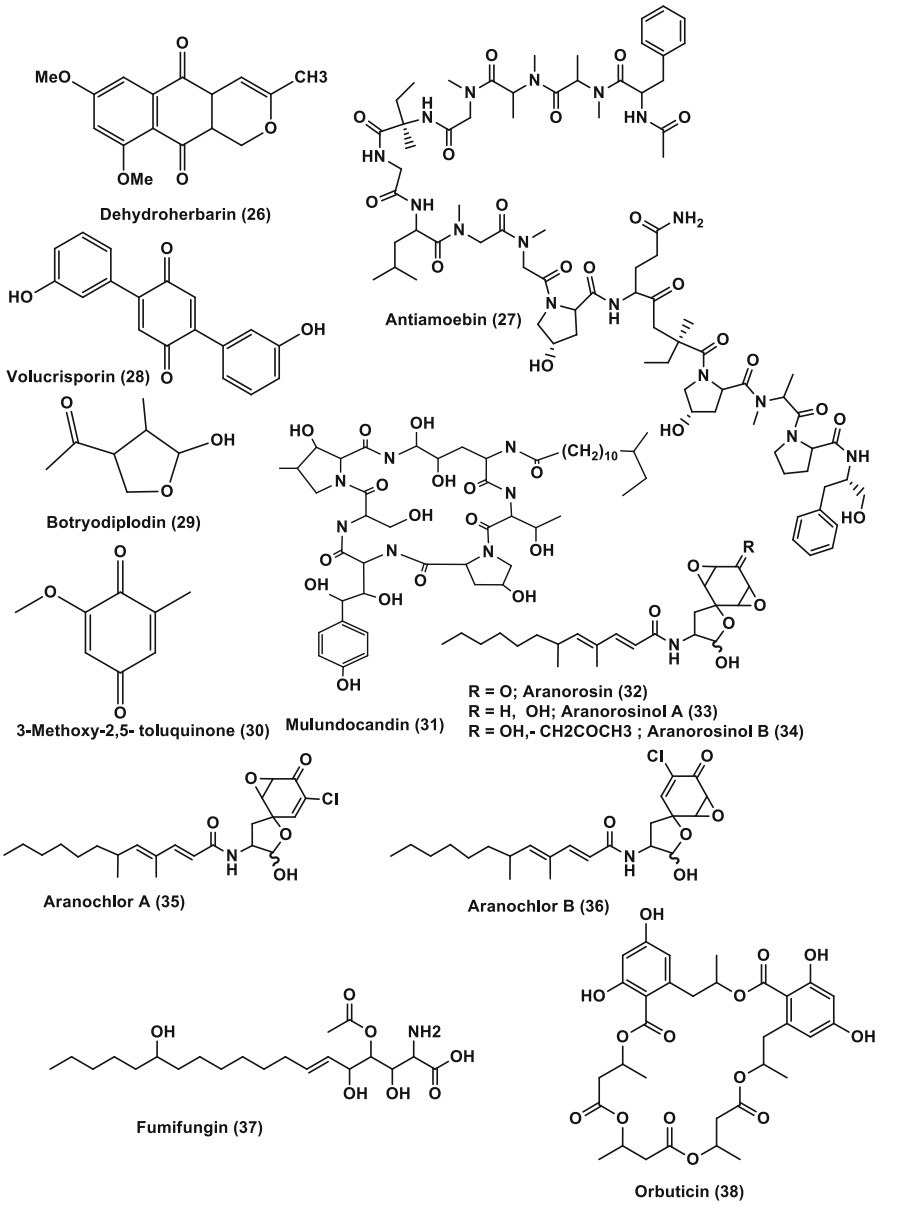

Fig. 16.3 Secondary metabolites obtained from fungi (**26**–**38**)

(2016) had reported *Aspergillus mulundensis* as a novel species of *Aspergillus* section *Nidulantes*, based on its morphological, molecular, phylogenetic, and metabolic data. However, among the echinocandins described to date from aspergilli, mulundocandin is unique because of its combination of a 12-methymyristoyl side

and serine in the fifth position of the core peptide. Aminocandin, a semi-synthetic derivative of deoxymulundocandin, displayed a good potency against fluconazole-resistant *C. albicans* (Ghannoum et al. 2007) and itraconazole-resistant *A. fumigatus* strains (Warn et al. 2010).

Aranorosin (**32**), aranorosinol A (**33**), aranorosinol B (**34**), aranochlor A (**35**) and aranochlor B (**36**) (Fig. 16.3) were purified from *Pseudoarachniotus roseus* Kuehn (No. Y-30,499), isolated from a soil sample collected near Wai, Maharashtra, India (Roy et al. 1988, 1992; Mukhopadhyay et al. 1998). Aranorosin displayed an impressive antimicrobial activity against *Staphylococcus aureus* FDA 209P, *Bacillus subtilis*, *Micrococcus luteus*, *Salmonella typhimurium*, *Klebsiella aerogenes* 1082E, *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Penicillium italicum*, *Cercospora beticola*, *Botrytis cinerea*, and *Microsporum gypseum* with MIC of 1.50, 1.50, 1.50, 50.00, 30.00, 500.00, 7.50, 30.00, 3.00, 30.00, and 3.00 μg/ml, respectively (Roy et al. 1988).

A novel antifungal fumifungin (**37**) (Fig. 16.3) was reported from *Aspergillus fumigatus* (Y-83, 0405) isolated from Himalayan soil samples. Fumifungin exhibited antifungal activity against *C. albicans*, *Saccharomyces cerevisiae*, *A. niger*, *Penicillium digitatum*, *Trichophyton mentagrophytes*, *B. cinerea*, *Fusarium culmorum*, *Alternaria solani*, *C. beticola*, *Cladosporium resinae*, *Pyricularia oryzae* with MIC of 62.5, 62.5, 7.8, 7.8, 15.6, 15.0, 25.0, 31.2, 0.9, 0.9, and 125 μg/ml, respectively (Mukhopadhyay et al. 1987).

Orbuticin (**38**) (Fig. 16.3), a cell wall active compound, produced by *Acremonium butyric* (HIL Y-87,1745) was isolated from a soil sample collected at Kashet ghat, Alibag, and Maharashtra, India. Orbuticin gave turbid zone and an unidentified orange pigment at 37 °C after 18–36 h incubation of *Neurospora crassa* SGF 18 strain. Microscopic observation revealed an enlarged and ballooned hyphae and sphaeroplast-/protoplast-like bodies in addition to normal hyphae around the turbid zone. Orbuticin displayed a poor antifungal activity in vivo against *Phytophthora infestans*, *Pyricularia oryzae*, and *Erysiphe graminis* f. sp. *hordei* (Roy et al. 1996).

The psychrotrophic fungus *Trichoderma velutinum* isolated from the soil collected during extreme winters from Pancheri, Udhampur, Jammu, and Kashmir was the source of four new lipovelutibols A (**39**), B (**40**), C (**41**), and D (**42**) (Fig. 16.4). Lipovelutibol D (**42**) displayed cytotoxic activity against HL-60, LS180, MDA-MD-231, and A-549 cell lines with IC_{50} values of 4.0, 7.0, 5.0, and 4.0 μM, while lipovelutibol B (**40**) exhibited an impressive activity with IC_{50} values of 2.0 and 4.0 μM against HL-60 and MDA-MD-231, respectively. Paclitaxel a positive control displayed cytotoxicity against HL-60, LS180, MDA-MD-231, and A-549 cell lines with IC_{50} values of 2.0, 2.8, 3.0, and 6.8 nM, respectively (Singh et al. 2018).

Two new antifungals, L970843 (**43**) and L970844 (**44**) (Fig. 16.4), were obtained from an unidentified fungal species HIL Y-903146, isolated from a soil sample collected at Chandigarh, India. The compound L970843 (**43**) showed antimicrobial activity against *P. digitatum*, *F. culmorum* 100, *Alternaria mali* P37, *Botrytis cinerea* A06, *B. cinerea* D01, *B. cinerea* E02, *Pyricularia grisea*, *Leptosphaeria nodorum* JO2, *P. oryzae* 154, *Pseudocercosporella herpotrichoides* 008, *P. infestans* J08, *Neurospora crassa* SGF 18, *C. albicans*, *S. aureus* 209P,

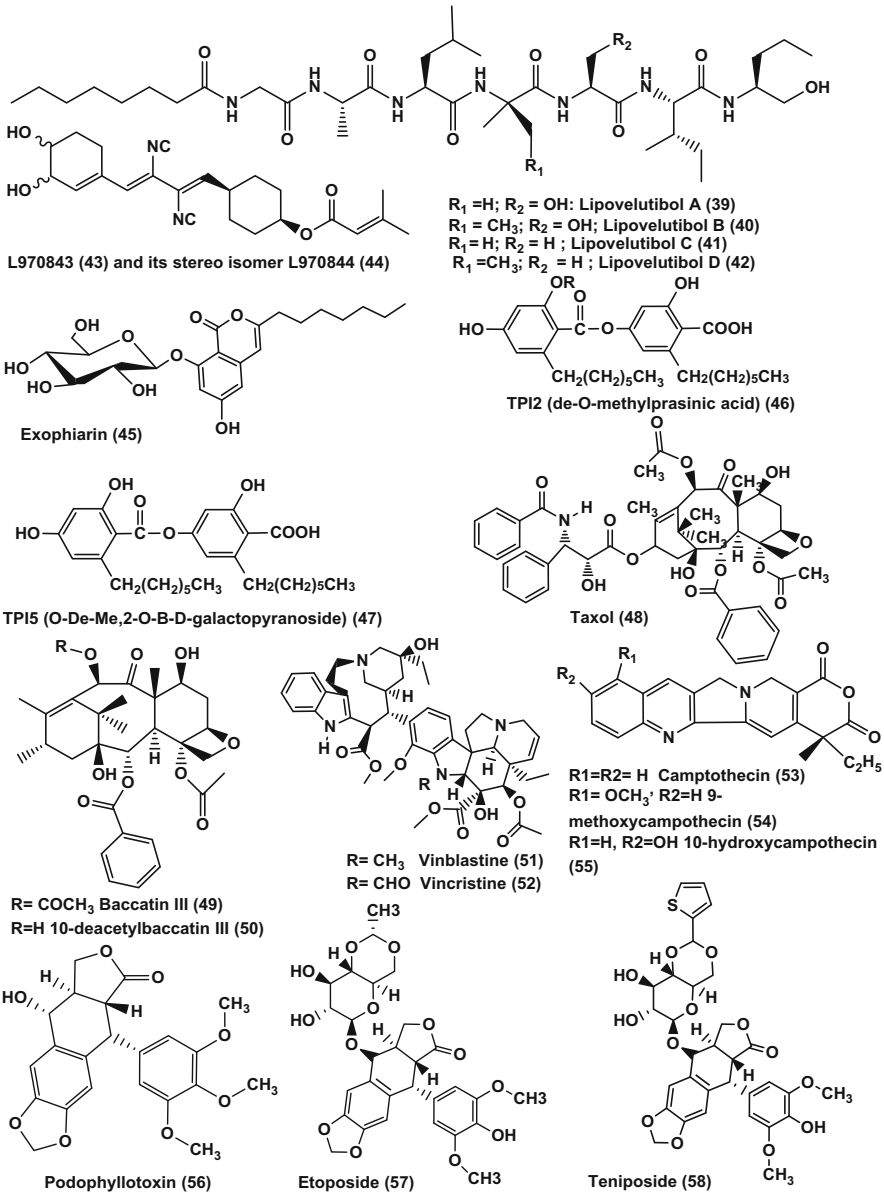

Fig. 16.4 Secondary metabolites obtained from fungi (**39**–**58**)

Escherichia coli 9632, and *Pseudomonas aeruginosa* with zones of inhibition of 24, 18, 23, 23, 17, 20, 23, 28, 23, 28, 20, 27, 22, 23/26 h, 17, 17 mm at the concentration of 1 mg/ml in methanol applied on filter paper disc. Similarly, compound L970844 (**44**) also displayed antimicrobial activity against the same

sets (mentioned above) of phytopathogens with zones of inhibition 25, 19, 27, 27, 18, 23, 27, 27, 30, 30, 21, 27, 18/27 h, 26, 17, and 22 mm, at the concentration of 1 mg/ml in methanol applied on filter paper disc (Vijayakumar et al. 2001). Under greenhouse conditions at 2500 ppm, compound (**43**) showed 90%, 40%, and 100% fungicidal efficiency against *P. infestans* of LILY, *Erysiphe graminis* f. sp. *hordei* of *Hordeum vulgare*, and *Sclerotinia fuckeliana* St.E02 of *Cucumis sativus*, while compound (**44**) exhibited fungicidal efficiency of 75%, 65%, and 42% against *P. infestans* of LILY, *P. oryzae* of *Oryza sativa* and *Sclerotinia fuckeliana* St.E02 of *C. sativus*, respectively (Vijayakumar et al. 2001).

A new isocoumarin, exophiarin (**45**) along with known compound TPI-2 (O-De-Me,2-O-B-D-galactopyranoside) (**46**) and TPI-5 (de-*O*-methylprasinic acid) (**47**) (Fig. 16.4) was isolated and purified from *Exophiala* sp., inhabitant of soil from Kaziranga National Park in Assam. In rat skeletal muscle cell line L6, compounds (**45**, **46**, and **47**) revealed mild improvement in glucose uptake activity in comparison with rosiglitazone (Gohil et al. 2019).

Apart from these, many known compounds isolated from soil fungi at Hoechst Research Centre and Piramal Enterprises Limited, Mumbai, with antimicrobial and anticancer activities are presented in Tables 16.1 and 16.2.

16.4 Compounds Produced by Endophytic Fungi

16.4.1 Taxol from Endophytic Fungi

Endophytic fungi are prolific producers of chemically diverse compounds of agricultural, industrial, and pharmaceutical interest. Endophytic microbes are known to produce considerable numbers of antimicrobial compounds, and their efficacy and probable uses have been elegantly described in some recent reviews (Verma et al. 2009; Mishra et al. 2012; Deshmukh et al. 2015, 2018c, 2020). Surprisingly, more than 100 anticancer compounds have been reported from fugal endophytes during the period of 1990–2010 (Kharwar et al. 2011).

Paclitaxel (taxol) (**48**) (Fig. 16.4), a highly lipophilic diterpenoid compound, was first purified from *Taxus brevifolia* (pacific yew tree) (Wani et al. 1971). It was reported in very low quantity in the needles, bark, and roots of yews (*Taxus* sp.). It displayed potent anticancer activity against ovarian and breast cancer with a wide spectrum of carcinomas of the lung, colon, prostate, and brain (Rowinsky 1997). Paclitaxel enhances polymerization of microtubules and can block cell mitosis, which is responsible for the antitumour activities of the drug (Vedantham et al. 2010). After the discovery of paclitaxel from *Taxomyces andreanae* an endophytic fungus from *T. brevifolia* by Stierle et al. (1993), endophytic fungi were explored a relatively less explored group of microbes for the production of taxol and other active metabolites. After the discovery of taxol, a large number of publications from India on taxol-producing fungi from yews (Shrestha et al. 2001; Kumaran et al. 2010; Kumaran and Hur 2009) and other plant species had appeared (Gangadevi and Muthumary 2009a, b; Gohar et al. 2015; Doss et al. 2016). Twenty one (21) genera

Table 16.1 Antimicrobial compounds isolated from soil fungi

Sr. no.	Culture no.	Compounds isolated	Fungus
1	Y-30,213	Terreic acid	*Aspergillus terreus*
2	Y-30,459	Neoaspergillic acid	*Aspergillus* sp.
3	Y-30,459	Neohydroxyaspergillic acid	*Aspergillus* sp.
4	Y-30,462	Mulundocandin[a]	*Aspergillus mulundensis*
5	Y-30,462	Deoxymulundocandin[a]	*Aspergillus mulundensis*
6	Y-30,499	Aranorosin[a]	*Pseudoarachniotus roseus*
7	Y-30,499	Aranorosinol A[a]	*P. roseus*
8	Y-30,499	Aranorosinol B[a]	*P. roseus*
9	Y-30,499	Aranochlor A[a]	*P. roseus*
10	Y-30,499	Aranochlor B[a]	*P. roseus*
11	Y-820016	Patulin	*Byssochlamys* sp.
12	Y-820102	Roridin-A	*Myrothecium* sp.
13	Y-820102	Verrucarin-A	*Myrothecium* sp.
14	Y-820112	Floccosin	*Gymnascella hyalinospora*
15	Y-820119	Monorden	*Humicola fuscoatra*
16	Y-820286	Wortmannin	*Penicillium wortmanni*
17	Y-830313	Mycophenolic acid	*Penicillium* sp.
18	Y-830394	Soyasapogenol E	*Aspergillus* sp.
19	Y-830405	Fumifungin[a]	*Aspergillus fumigatus*
20	Y-830405	Viriditoxin	*Aspergillus fumigatus*
21	Y-840700	Isobongkrekic acid	*Eubacterium* sp.
22	Y-840816	Trichoviridin	*Trichoderma* sp.
23	Y-850886	Xanthocillin x. monomethyl ether	Unidentified fungus
24	Y-850886	Xanthocillin x. dimethyl ether	Unidentified fungus
25	Y-850901	Chloroflavonin	*Aspergillus* sp.
26	Y-850901	Dechloroflavonin	*Aspergillus* sp.
27	Y-850926	Cyclosporin A	*Fusarium* sp.
28	Y-851002	Trichodermin	*Trichoderma* sp.
29	Y-851270	Crotocin	*Mycogone* sp.
30	Y-851270	Trichothecin	*Mycogone* sp.
31	Y-861636	Trichodermol (roridin-C)	Unidentified fungus
32	Y-871745	Orbuticin[a]	*Acremonium butyri*
33	Y-913416	Citrinin	*Penicillium* sp.
34	Y87-2002	Cheatoglobosin A	*Chaetomium globosum*
35	Y-872100	2,4 dihydroxy-2-vinyl-4-cyclopentenone[a]	*Trichoderma koningii*
36	Y-872164	Echinocandin B	*Aspergillus* sp.
37	Y-872164	Aspertetronin	*Aspergillus* sp.
38	Y-872164	Aspertetronin CH_3 ether	*Aspergillus* sp.
39	Y-882380	LL-Z-1271-gama	*Spiromyces* sp.
40	Y-882380	LL-Z-1271-alpha	*Spiromyces* sp.
41	Y-882491	Roridin-D	*Myrothecium* sp.

(continued)

Table 16.1 (continued)

Sr. no.	Culture no.	Compounds isolated	Fungus
42	Y-882491	Roridin-A	*Myrothecium* sp.
43	Y-882633	Myriocin	Unidentified fungus
44	Y-892769	Terreic acid	*Aspergillus terreus*
45	Y-892769	Terremutin	*Aspergillus terreus*
46	Y-892874	Variotin	*Aspergillus* sp.
47	Y-903022	Arthrichitin[a]	*Arthrinium phaeospermum*
48	Y90-3037	Chloroflavonin	*Aspergillus* sp.
49	Y-903146	L970843[a]	Unidentified fungus
50	Y-903146	L970844[a]	Unidentified fungus
51	Y-903170	Brefeldin A	*Aspergillus* sp.
52	Y-903274	Paracelsin B	*Trichoderma saturnisporum*
53	Y-903274	Paracelsin H	*Trichoderma saturnisporum*
54	Y903333	Illicicolin A	Unidentified fungus
55	Y903333	Illicicolin D	Unidentified fungus
56	Y903333	Illicicolin E	Unidentified fungus
57	Y903333	Illicicolin F	Unidentified fungus
58	Y903333	Dechloro-illicicolin D	Unidentified fungus
59	Y-913377	Griseofulvin	*Penicillium* sp.
60	Y-9201077	CAF.0603	Unidentified fungus
65	Y93 3158	Gliotoxin	*Aspergillus* sp.
66	Y9404602	Enniatin B1	*Fusarium* sp.
67	Y9404602	Enniatin A1	*Fusarium* sp.
68	Y9404602	Enniatin A	*Fusarium* sp.
69	Y9404602	Enniatin B	*Fusarium* sp.
70	Y-30,042 L	Fusidic acid	*Microsporum gypseum*
71	Y-30,011	Penicillic acid	*Penicillium* sp.
72	Y-30,212	Neoaspergillic acid	*Aspergillus* sp.
73	Y-30,212	Aspergillic acid	*Aspergillus* sp.
74	Y-30,212	Neohydroxyaspergillic acid	*Aspergillus* sp.
75	Y-30,212	3-Methoxy toluquinone	*Aspergillus* sp.
76	Y-30,489	Helvolic acid	*Cephalosporium* sp.
77	Y-30,489	Cephalosporin C	*Cephalosporium* sp.
78	Y-30,489	Desacetylcephalosporin C	*Cephalosporium* sp.
79	Y-30,489	Ergosterol	*Cephalosporium* sp.
80	Y-30,309	Bostrycin	*Nigrospora* sp.
81	M-30,000	Agrocybin	*Agrocybe* sp.
82	PM0407754	Papulacandin	*Papularia* sp.
83	PM0306084	Aspirochlorin	*Aspergillus* sp.

[a]New compounds isolated

Table 16.2 Compounds isolated from soil fungi with an anticancer activity

Sr. no.	Culture no.	Compounds isolated	Fungus
1	PM0204024	Flavipin	*Aspergillus* sp.
2	PM0408976	Bostrycin	*Nigrospora sphaerica*
3	PM0509340	Cladosporol	*Cladosporium* sp.
4	PM0307260	A52688 and its analogues	*Mycoleptodiscus terrestris*
5	PM0408982	Nectripyrone	*Diaporthe phaseolorum*
6	PM0409281	Cytochalasin D	*Xylaria* sp.
7	PM0509399	Curvularin	*Curvularia* sp.
8	PM0509512	Rumbrin	*Auxarthron conjugatum*
9	PM0509671	Aranorosin	*Gymnoascella* sp.
10	PM0509945	Stachybotryal	*Stachybotrys chartarum*
11	PM0509971	6-*O*-methyl norrubrofusarin	*Fusarium* sp.
12	PM0550004	Gliotoxin and acetyl derivative	Unidentified fungus
13	PM0550399	Chaetomin	*Chaetomium globosum*
14	PM0550454	Harzianin A	*Trichoderma* sp.
15	PM0550466	Microperfuranone	*Emericella* sp.
16	PM0550563	Eritadenine methyl ester	*Phoma* sp.
17	PM0550563	Napiradiomycin B-2	*Phoma putaminum*
18	PM0651670	Trichurusin A and B	Unidentified fungus
19	PM0651717	Diketopiperazine/maculocin	*Embellisia* sp.
20	PM0854391	Radicicol	Unidentified fungus
21	PM0954782	Hypericin, emodinbianthrone (meso), emodinbianthrone (racemic)	Unidentified fungus
22	PM0752445	Deacetylsclerotiorin	*Cunninghamella* sp.
23	PM0752531	Malformin A1, A2, A3, epi-neovasinin, neovasinin	*Aspergillus aculeatus*
24	PM0753236	Brefeldin A	*Humicola fuscoatra*
25	PM0752609	Dichlorodiportin	Unidentified fungus
26	PM0753339	Alterperylenol	*Alternaria* sp.
27	PM0853779	PR-toxin	Fungal endophyte
28	PM0853873	Hamigerone, radicinol	*Bipolaris* sp.
29	PM0854143	Trapoxin A, trapoxin A analogue, spirolaxine	*Hypoxylon haematostroma*

(continued)

Table 16.2 (continued)

Sr. no.	Culture no.	Compounds isolated	Fungus
30	PM0954825	Periconicin A, B	*Massaria inquinans*
31	PM1055084	Mycophenolic acid	*Penicillium* sp.
32	PM1055088	Patulin	*Aspergillus giganteus*
33	PM1055227	Triticone A	Sterile mycelium
34	PM1055233	Dehydrocurvularin	*Curvularia* sp.
35	PM1055475	Radicinin	Unidentified fungus
36	PM0651780	Theilocin analogue	*Chaunopicnis alba*
37	PM0306549	[a]Compactin	*Aspergillus* sp.

[a]HMG-CoA reductase inhibitor

of endophytic fungi, viz. *Aspergillus*, *Bartalinia*, *Botryodiplodia*, *Chaetomella*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Epicoccum*, *Fusarium*, *Gliocladium*, *Grammothele*, *Lasiodiplodia*, *Paraconiothyrium*, *Penicillium*, *Periconia*, *Phoma*, *Phomopsis*, *Pestalotiopsis*, *Phyllosticta*, *Sporormia*, *Trichothecium*, and an unidentified dimorphic fungus, have been described till date to produce taxol and its analogues baccatin III (**49**) and 10-deacetylbaccatin III (**50**) (Fig. 16.4), from Indian subcontinent (Table 16.3). Kumar et al. (2019) screened endophytic fungi isolated from *Taxus* sp. from Shimla, Himachal Pradesh (India), using dbat gene essential for the taxol biosynthetic pathway for taxol production and obtained from *Aspergillus fumigatus* KU-837249 strain based on the PCR amplification data.

The hosts of paclitaxel-producing fungi mainly include the *Taxus* species (i.e., *Taxus baccata*, *T. celebica*, *T. cuspidata*, *T. media*, and *T. wallichiana*) of the family Taxaceae; however, some non-*Taxus* hosts were also reported to harbour the fungi producing the paclitaxel, viz. *Aegle marmelos* (Rutaceae), *Aloe vera* (Liliaceae), *Calotropis gigantean* (Asclepiadaceae), *Capsicum annuum* (Solanaceae), *Cardiospermum halicacabum* (Sapindaceae), *Citrus medica* (Rutaceae), *Corchorus olitorius* (Malvaceae), *Corylus avellana* (Betulaceae), *Cupressus* sp. (Cupressaceae), *Ficus infectoria* (Moraceae), *Ginkgo biloba* (Ginkgoaceae), *Hibiscus rosa-sinensis* (Malvaceae), *Justicia gendarussa* (Acanthaceae), *Larix leptolepis* (Pinaceae), *Morinda citrifolia* (Rubiaceae), *Michelia champaca* (Magnoliaceae), *Moringa oleifera* (Moringaceae), *Mesua ferrea* (Calophyllaceae), *Melochia corchorifolia* (Malvaceae), *Plumeria acutifolia* (Apocynaceae), *Torreya grandifolia* (Taxaceae), *Taxodium mucronatum* (Cupressaceae), *Tabebuia pentaphylla* (Bignoniaceae), *Terminalia arjuna* (Combretaceae), etc. This indicates that there is a broad biological diversity in both taxol-producing fungi and their hosts (Deshmukh and Verekar 2014; Deshmukh et al. 2019b; Gond et al. 2014).

Table 16.3 List of taxol, baccatin III, and 10-deacetyl-baccatin III producing endophytic fungi and their host plants

Sr. no.	Fungus	Plant source	Compound	Reference
1	*Aspergillus fumigatus*	*Taxus* sp.	Taxol	Kumar et al. (2019)
2	*Bartalinia robillardoides* AMB-9	*Aegle marmelos*	Taxol	Gangadevi and Muthumary (2008a)
3	*Botryodiplodia theobromae* BT115	*Taxus baccata*	Taxol	Venkatachalam et al. (2008)
4	*Lasiodiplodia theobromae* Pat.	*Morinda citrifolia*	Taxol	Pandi et al. (2011)
5	*Chaetomella raphiger* TAC-15	*Terminalia arjuna*	Taxol	Gangadevi and Muthumary (2009a)
6	*Chaetomium* sp.	*Michelia champaca* L.	Taxol	Rebecca et al. (2012)
7	*Cladosporium cladosporioides* UH-10	*Taxus* sp.	Taxol	Gohar et al. (2015)
8	*Cladosporium oxysporum*	*Moringa oleifera*	Taxol	Gokul et al. (2015)
9	*Cladosporium* sp.	*Taxus baccata*	Taxol	Kasaei et al. (2017)
10	*Colletotrichum capsici*	*Capsicum annuum*	Taxol	Kumaran et al. (2011)
11	*Colletotrichum gloeosporioides*	*Justicia gendarussa*	Taxol	Gangadevi and Muthumary (2008b)
12	*Colletotrichum gloeosporioides*	*Plumeria acutifolia*	Taxol	Nithya and Muthumary (2009)
13	*Epicoccum nigrum*	*Taxus baccata*	Taxol	Somjaipeng et al. (2016)
14	*Fusarium redolens*	*Taxus baccata* L. subsp. *wallichiana*	Taxol	Garyali et al. (2013, 2014a, b)
15	*Fusarium solani*	*Taxus celebica*	Taxol, baccatin III	Chakravarthi et al. (2013)
16	*Fusarium solani*	*Taxus celebica*	Taxol	Chakravarthi et al. (2008)
17	*Gliocladium* sp.	*Taxus baccata*	Taxol, 10-deacetyl baccatin III	Sreekanth et al. (2009)
18	*Gliocladium* sp.	*Taxus baccata*	Taxol	Sreekanth et al. (2011)
19	*Grammothele lineata*	*Corchorus olitorius* acc. 2015	Taxol	Das et al. (2017)
20	*Lasiodiplodia theobromae*	*Morinda citrifolia*	Taxol	Pandi et al. (2011)

(continued)

Table 16.3 (continued)

Sr. no.	Fungus	Plant source	Compound	Reference
21	*Paraconiothyrium* sp.	*Taxus media*	Taxol	Soliman et al. (2011)
22	*Paraconiothyrium* SSM001	*Taxus media*	Taxol	Soliman et al. (2017)
23	*Paraconiothyrium* SSM001	*Taxus baccata*	Taxol	Soliman et al. (2015)
24	*Paraconiothyrium variabile*	*Taxus baccata*	Taxol	Somjaipeng et al. (2016)
25	*Penicillium aurantiogriseum*	*Corylus avellana*	Taxol	Liu et al. (2016)
26	*Penicillium aurantiogriseum* NRRL 62431	*Corylus avellana*	Taxol	Yang et al. (2014)
27	*Periconia* sp. No. 202	*Torreya grandifolia*	Taxol	Li et al. (1998)
28	*Pestalotiopsis microspora*	*Taxodium mucronatum*	7-Epi-10-deacetyltaxol	Subban et al. (2017)
29	*Pestalotiopsis pauciseta* CHP-11	*Cardiospermum halicacabum*	Taxol	Gangadevi et al. (2008)
30	*Pestalotiopsis pauciseta* VM1	*Tabebuia pentaphylla*	Taxol	Vennila et al. (2010, 2012)
31	*Pestalotiopsis stellata*	*Ficus infectoria*	Taxol	Doss et al. (2016)
32	*Pestalotiopsis terminaliae*	*Terminalia arjuna*	Taxol	Gangadevi and Muthumary (2009b)
33	*Pestalotiopsis versicolor*	*Taxus cuspidata*	Taxol	Kumaran et al. (2010)
34	*Phoma betae*	*Ginkgo biloba*	Taxol	Kumaran et al. (2012)
35	*Phoma* sp.	*Calotropis gigantea*	Taxol	Hemamalini et al. (2015)
36	*Phoma* sp.	*Aloe vera*	Taxol	Rebecca et al. (2011)
37	*Phomopsis* sp. BKH 27	*Taxus cuspidata*	Taxol	Kumaran and Hur (2009)
38	*Phomopsis* sp. BKH 30	*Gingko biloba*	Taxol	Kumaran and Hur (2009)
39	*Phomopsis* sp. BKH 35	*Larix leptolepis*	Taxol	Kumaran and Hur (2009)
40	*Phomopsis longicolla*	*Mesua ferrea*	Taxol	Jayanthi et al. (2015)
41	*Phyllosticta* sp.	*Ocimum basilicum*	Taxol	Gangadevi and Muthumary (2007)
42	*Phyllosticta citricarpa* No. 598	*Citrus medica*	Taxol	Kumaran et al. (2008a)

(continued)

Table 16.3 (continued)

Sr. no.	Fungus	Plant source	Compound	Reference
43	*Phyllosticta dioscoreae* No. 605	*Hibiscus rosa-sinensis*	Taxol	Kumaran et al. (2009)
44	*Phyllosticta melochiae* Yates	*Melochia corchorifolia*	Taxol	Kumaran et al. (2008b)
45	*Phyllosticta spinarum* No. 625	*Cupressus* sp.	Taxol	Kumaran et al. (2008c)
46	*Sporormia minima*	*Taxus wallichiana*	Taxol	Shrestha et al. (2001)
47	*Trichothecium* sp.	*Taxus wallichiana*	Taxol	Shrestha et al. (2001)
48	Unidentified dimorphic fungus	*Taxus wallichiana*	Taxol	Shrestha et al. (2001)

16.4.2 Compounds Other Than Taxol from Endophytic Fungi

Other than taxol, a large number of compounds are reported from endophytic fungi in the following sections, and the details of these compounds are given in Table 16.4.

Vinblastine (**51**) and vincristine (**52**) (Fig. 16.4), are dimeric alkaloids isolated from the Madagascar periwinkle plant (*Catharanthus roseus*), with antineoplastic activity. The antineoplastic activity of vinca alkaloids is due to their capacity to arrest cell division in metaphase by binding to the microtubule protein, tubulin that forms the mitotic spindle (Kwok et al. 2016).These alkoloids were also extracted from endophytic fungi of *C. roseus*. In India, vinblastine (**51**) and vincristine (**52**) were reported from an endophytic fungus *Fusarium oxysporum* isolated from *Catharanthus roseus* (Kumar and Ahmad 2013). Kumar et al. (2013a) also reported fungal transformation of vinblastine to vincristine by the same fungus isolated from the same host. Vinblastine dissolved in sterile water, when incubated with the fungal mycelial mass, was transformed into vincristine at room temperature.

The topoisomerase I-DNA inhibitor alkaloid camptothecin (**53**) (Fig. 16.4) was purified from the bark of *Camptotheca acuminata* and *Nothapodytes foetida* (Wall et al. 1966; Fulzele et al. 2001). Later, it was extracted from several endophytic fungi such as *Entrophospora infrequens* and *Nodulisporium* sp. associated with *N. foetida* (Puri et al. 2005; Rehman et al. 2009a, b), *Neurospora crassa* from seeds of *C. acuminata* (Rehman et al. 2008), *Botryosphaeria parva* from *N. nimmoniana* (Gurudatt et al. 2010), and *Fusarium solani* from *Apodytes dimidiata* (Shweta et al. 2010). Camptothecin binds to the topoisomerase I and DNA complex resulting in a ternary complex, stabilizing it and preventing DNA religation, therefore, causes DNA damage which results in apoptosis (Cragg and Newman 2004). 9-Methoxycamptothecin (**54**) and 10-hydroxycamptothecin (**55**) (Fig. 16.4), the analogues of camptothecin, were also purified from an endophyte *F. solani* of *C. acuminata* (Kusari et al. 2009a). An endophyte *Fusarium solani* obtained from

Table 16.4 Bioactive metabolites isolated from endophytic fungi

Sr. no.	Endophytic fungal strain	Host plant(s)	Plant part or tissue/locality of host plants	Isolated metabolite	Tested systems	Activity response	References
	Anticancer metabolites						
1.	*Phomopsis glabrae*	*Pongamia pinnata*	Leaf, Karnala Bird Sanctuary, Raigad (MS), India	PM 181110 (**69**)	40 human cancer cell lines 24 human tumour xenografts	Mean IC_{50} value 0.089 μM Mean IC_{50} value 0.245 μM	Verekar et al. (2014)
2.	*Phomopsis* sp.	*Nyctanthes arbor-tristis*	Leaf, Mumbai, India	Altersolanol A (**70**)	34 human cell lines	Mean IC_{50} value 0.005 μM	Mishra et al. (2015)
3.	*Cephalotheca faveolata*	*Eugenia jambolana*	Leaf, Mumbai, India	Sclerotiorin (**71**)	ACHN, Panc-1, Calu-1, HCT-116, and H460 cell lines, MCF 10A cell lines	IC_{50} values 1.2, 1.6, 2.1, 0.63, 1.6, and >10 μM	Giridharan et al. (2012)
4.	Unidentified fungus	*Mimusops elengi*		Ergoflavin (**72**)	Inhibited TNF-α and IL-6 ACHN, H460, Panc-1, HCT16, Calu-1 cell lines	IC_{50} values 1.9, 1.2 μM IC_{50} values 1.2, 4.0, 2.4, 8.0, 1.5 μM	Deshmukh et al. (2009)
5.	*Aspergillus aculeatus*	Marine sponge *Cinachyra cavernosa*	Mandapam, Tamil Nadu, India	Secalonic acid D (**73**)	Panc-1, H460, ACHN, Calu-1, HCT-116, and WI-38 cell lines	IC_{50} values 0.2, 0.2, 0.19, 0.2, 0.2, and 4.9 μg/ml	Deshmukh (2018)
6.	*Trichoderma* sp.	*Azadirachta indica*		Heptelidic acid (**74**)	T47D, SKOV-3, KM-12, NAMALWA, MDAMB-231, NCI-H460, HOP-62, Colo-	IC_{50} values 0.21, 0.34, 0.41, 0.45, 0.45, 0.49, 0.51, 0.63, 0.66, 0.69, 0.76, 0.76,	Rahier et al. (2015)

					205, TK10, OVCAR-3, BXPC3, HL-60, WM-266-4, DU145, HCT-116, A-549 cell lines	0.84, 0.89, 0.93, and 0.99 μM	
7.	*Bipolaris setariae*	*Parthenium hysterophorus*	Leaf, Mumbai, India	Ophiobolin A (**75**)	A2780, PC-3, MDAMB-231, MCF-7, MM1R, RPMI8226, U266B168, and Jurkat cell lines	Mean IC_{50} value 0.4–4.3 μM	Bhatia et al. (2016)
8.	*Diaporthe* sp.	*Taxus baccata*	Bhaderwah, Doda district, India	Trichalasin E (**76**), F (**77**), and H (**78**)	MCF-7 and HeLa cancer cell lines	IC_{50} values of 1058 and 1257 μg/ml	Vasundhara et al. (2017)
9.	*Talaromyces* sp.	Twigs of *Cedrus deodara*	Lolab Valley in the Western Himalayas of Kashmir, India	(3*S*,4a*R*,7*S*)-7,8-dihydroxy-3-methyl-3,4,10,5,6,7-hexahydro-1*H*-isochromen-1-one (**79**)	A-549, HEP-1, THP-1, PC-3, and HCT-116 cells	35%, 3%, 40%, 34%, and 35% cytotoxicity at 50 μM	Kumar et al. (2013a, b)
				(1*S*,3*R*,5*R*)-3-methyl 2-oxabicyclo[3.3.1] nonan-7-one (**80**)	A-549, HEP-1, PC-3, and HCT-116 cells	71%, 26%, 23%, and 59% cytotoxicity at 50 μM	
				(−)-Ramulosin (**81**)	A-549, HEP-1, THP-1, PC-3, and HCT-116 cells	15%, 23%, 54%, 23%, and 44% cytotoxicity at 50 μM	
				(−)-Epoformin (**82**)	A-549, HEP-1, THP-1, PC-3, and HCT-116 cells	98%, 100%, 50%, 22%, and 56%	

(continued)

Table 16.4 (continued)

Sr. no.	Endophytic fungal strain	Host plant(s)	Plant part or tissue/locality of host plants	Isolated metabolite	Tested systems	Activity response	References
						cytotoxicity at 50 μM	
				Paclitaxel A-549	THP-1, HCT-116 cells	82%, 71%, and 72% cytotoxicity at 1 μM concentration	
				Fluorouracil A-549	THP-1, and HCT-116 cells	22%, 84%, and 55% cytotoxicity at 20 μM concentration	
				Compounds (**79**–**82**)	HL-60 cells	Induce apoptosis and microtubule inhibition	
10.	*Humicola fuscoatra*	*Mangifera indica*	Mulund, Mumbai, India	Radicicol (**83**)	ACHN, Panc-1, Calu-1, H460, HCT-116, MCF 10A cell line	IC_{50} values of 0.29, 0.45, 0.41, 0.27, 0.29, and 2.7 μM, respectively	Deshmukh (2018)
11.	*Phoma macrostoma*	*Glycyrrhiza glabra*	Inner tissue of collected from Jammu	Macrophin (**84**)	MDA-MB-231, T47D, MCF-7, and MIAPaCa-2 cell lines, respectively	IC_{50} values of 14.8, 8.12, 13.0, and 0.9 μM	Nalli et al. (2019)
12.				Macrophin (**84**)	MiaPaca-2 cell lines	Induces apoptosis through S-phase arrest in at the concentration of 600 nM	

				Rosellisin (**85**), 2-(2-hydroxy-5-6-methoxy-3-methylene-1,4-benzodioxin-2(3*H*)-one) (**86**), methoxyphenoxyacrylic acid (**87**)			
13.	*Cryptosporiopsis* sp.	*Clidemia hirta*	Hawaii, USA	*4-epi*-ethisolide (**94**)	HL-60, PC-3, A-549, Colo-205, and MDA-MB-231 cell lines	IC_{50} values of 11.0, 30.0, 75.0, 48.0, 90.0 μM	Kumar et al. (2017)
				4-Ethyl-3a,4,4′,5′-tetrahydro-2*H*-spiro[furo[3,4-b]furan3,3′-pyrazole]-2,6(6a*H*)-dione (**93**)	*E. coli*, *B. cereus*, and *C. albicans*	IC_{50} values of 90.0, 65.0, and 70.0 μg/ml	
				4-Ethyl-3-methyldihydrofuro[3,4-b]furan-2,6(3*H*,6a*H*)-dione (**90**), (*S*)-3-hydroxy-5-methyl-4-propyl-2*H*-pyran-2,6(3*H*)-dione (**91**), (*E*)-oxacyclododec-3-ene-2,12-dione (**92**), Orsellinic acid (**95**)			
14.	*Cladosporium tenuissimum*	*Pinus wallichiana*	Lolab Valley, Kashmir, India	(3*S*, 5*S*, 11*S*)-trihydroxydodecanoic acid (**96**)	MCF-7 and T47D cell lines	IC_{50} values of 15.0 and 100 μM	Naseer et al. (2017)
				Scytalone (**97**)	MCF-7 and T47D cell lines	IC_{50}, 68.0, and 75.0 μM	

(continued)

Table 16.4 (continued)

Sr. no.	Endophytic fungal strain	Host plant(s)	Plant part or tissue/locality of host plants	Isolated metabolite	Tested systems	Activity response	References
				Ortho-hydroxyphenyl acetone (**98**)	MCF-7 and T47D cell lines	IC_{50}, 42.0, and 82.0 μM	
				Positive control doxorubicin	MCF-7 and T47D cell lines	IC_{50}, 0.22, and 0.41 μM	
				Compounds (**96–98**)	*Bacillus cereus* IIIM 25	IC_{50}, 63.6, 68.5, and 23.3 μg/ml	
				Compound (**98**)	*Staphylococcus aureus* ATCC 29978 and *Escherichia coli* ATCC 25922	IC_{50} values of 89.8 and 43.7 μg/ml	
15.	*Diaporthe terebinthifolii* GG3F6	Rhizome of *Glycyrrhiza glabra*	Jammu, J&K, India	Diapolic acid A–B (**99**, **100**) and known molecule xylarolide (**101**) and phomolide G (**102**)	MCF-7, MIA-Pa-Ca-2, NCI-H226, HepG2, and DU145 cells		Yedukondalu et al. (2017)
				Xylarolide (**101**)	T47D cell lines	IC_{50} value of 7.0 μM	
				Positive control paclitaxel		IC_{50}, 0.011 μM	
				Xylarolide (**101**)	*Candida albicans*	IC_{50}, 78.8 μM	
				Positive control nystatin		IC_{50}, 21.5 μM	
				Compounds (**99–102**)	*Yersinia enterocolitica*	IC_{50},78.4, 73.4, 72.1, and 69.2 μM	
				Positive control Ciprofloxacin		IC_{50}, 3.5 μM	

16.	*Diaporthe* sp.	*Datura inoxia*	Jammu region of Jammu and Kashmir, India	Xylarolide A (**103**), diportharine A (**104**), xylarolide B (**105**), xylarolide (**106**)			
				Xylarolide A (**103**), xylarolide (**106**)	MIAPaCa-2 cell	IC_{50}, 20 and 32 μM	Sharma et al. (2018a, b)
					PC-3	IC_{50}, 14 and 18 μM	
				Xylarolide A (**103**)	DPPH assay	EC_{50}, 10.3 μM	
17.	*Aspergillus fumigatus* (GA-L7)	Leaves of *Grewia asiatica*	Shiwalik region, Jammu, India	Pseurotin A (**107**), pseurotin D (**108**), pseurotin F2 (**109**), fumagillin (**110**), tryprostatin C (**111**), gliotoxin (**112**), bis (methylthio)gliotoxin (**113**), fumiquinazoline C (**114**)			Magotra et al. (2017)
18.	*Xylaria psidii*	Leaf sample of *Aegle marmelos*	Yamuna Nagar district of Haryana, India	Xylarione A (**115**), (−) 5-methylmellein (**116**)	MCF-7, MIA-Pa-Ca-2, NCI-H226, HepG2, and DU145 cancer cell	IC_{50} in the range of 16–37 μM	Arora et al. (2016a, b)
					MIA-Pa-Ca-2	Cell cycle arrest at the sub-G1 phase, induced apoptosis and displayed substantial decrease in membrane potential of mitochondria	

(continued)

Table 16.4 (continued)

Sr. no.	Endophytic fungal strain	Host plant(s)	Plant part or tissue/locality of host plants	Isolated metabolite	Tested systems	Activity response	References
19.	*Penicillium pinophilum* MRCJ-326	*Allium schoenoprasum*	Nathatop, J&K, India	Oxyskyrin (**117**)	HCT-116 cells	IC_{50}, 48.00 μg/ml	
				Skyrin (**118**)	A-549, MIA-PaCa-2, HCT-116, and PC-3, cells	IC_{50}, 38.00, 27.00, 32.00, and 47.00 μg/ml	
				Dicatenarin (**119**)	A-549, MIA-PaCa-2, HCT-116, T47D, PC-3, and OVCAR-3 cells	IC_{50}, 23.00, 12.00, 17.00, 29.00, 35.00, and 26.00 μg/ml	
				Skyrin (**118**), Dicatenarin (**119**)	In MIA PaCa-2 cells	Induction of caspase-3 apoptotic proteins	
				1,6,8-Trihydroxy-3-hydroxy methylanthraquinone (**120**)			
20.	*Rosellinia sanctae-cruciana*	*Albizia lebbeck*	Jammu region of Jammu and Kashmir, India	Jammosporin A (**121**), 19,20-epoxycytochalasin D (**122**), cytochalasin D (**123**), 19,20-epoxycytochalasin C (**124**), cytochalasin C (**125**)	MOLT-4 cell line	IC_{50} values of 20.0, 10.0, 25.0, 8.0, and 6.0 μM	Sharma et al. (2018a, b)

21.	*Alternaria alternata* FC-46	*Capsicum annum*		Capsaicin (**126**), 2,4-di-*tert*-butyl phenol (**127**)			Devari et al. (2014)
				Alternariol-10-methyl ether (**128**)	HL-60 and A431 cell lines	IC_{50}, 85 and 95 μM	
					HL-60 cells	Induces apoptosis, loss of mitochondrial-membrane	
22.	*Alternaria alternata* KT380662	*Passiflora incarnata*	Tiruchirappalli, Tamil Nadu, India	Chrysin (**129**)	MCF-7 cell lines	IC_{50} value of 37.97 μg/ml	Seetharaman et al. (2017)
					HepG2 cells	Loses their viability in a time- and dose-dependent manner	
					HepG2 cells	Formation of condensed nuclei, membrane, blebbing, and apoptotic bodies	
23.	*Cryptosporiopsis* sp.	*Clidemia hirta*		Compounds (*R*)-5-hydroxy-2-methylchroman-4-one (**130**)	HL-60 cell line	IC_{50} of 4 μg/ml	Zilla et al. (2013)
				1-(2,6-dihydroxyphenyl) pentan-1-one (**131**)	*B. cereus*, *E. coli*, *L. monocytogenes*, *P. fluorescens*, *S. flexneri*, *S. aureus* ATCC 29978, *S. aureus* P1571 with	IC_{50}, 20.0, 30.0, 60.0, 47.0, 92.0, 18.0, 15.0 μg/ml, respectively	

(continued)

Table 16.4 (continued)

Sr. no.	Endophytic fungal strain	Host plant(s)	Plant part or tissue/locality of host plants	Isolated metabolite	Tested systems	Activity response	References
				(*Z*)-1-(2-(2- butyryl-3-hydroxyphenoxy)-6-hydroxyphenyl)-3-hydroxybut-2-en-1-one (**132**)	*B. cereus*, *E. coli*, *L. monocytogenes*, *P. fluorescens*, *S. aureus* ATCC 29978, and *S. aureus* P1571, *S. pyogenes*	IC_{50}, 20.0, 50.0, 75.0, 6.0, 51.0, 24.0, and 62.0 μg/ml	
				Positive control ciprofloxacin	*B. cereus*, *E. coli*, *L. monocytogenes*, *P. fluorescens*, *S. flexneri*, *S. aureus* ATCC 29978, and *S. aureus* P1571, *S. pyogenes*	IC_{50}, 0.04, 0.10, 0.005, 0.03, 0.005, 0.09, 0.60, and 0.005 IC_{50} (μg/ml)	
24.	*Trichothecium* sp.	*Phyllanthus amarus*	Pune, India	Trichothecinol A (**133**)	HeLa and B16F10 cells	Causes 50% cell death in at the concentration of 500 nM	Taware et al. (2014)
					MDA-MB-231 cells	Checked migration of wound by 50% at 500 nM of suggesting its antimetastatic property	

25.	*Aspergillus* sp.	seeds of *Gloriosa superba*	Tirupati, India	6-methyl-1,2,3-trihydroxy-7,8-cyclohepta-9,12-diene11-one-5,6,7,8-tetralene-7-acetamide (KL-4) (**134**)	A-549, HEP-2, MCF-7, CV-1, and OVCAR-5 cell lines	Displayed 23%, 70%, 35%, 43%, and 80% growth inhibition at 100 μg/ml	Budhiraja et al. (2013)
26.	*Lepidosphaeria sp.*	Unidentified plant	Rajkot, India	Mutolide (**135**)	TNF-α and IL-6	IC_{50} 1.27 and 1.07 μM	Shah et al. (2015)
27.	*Dendryphion nanum*	*Ficus religiosa*	Leaf, India	Herbarin (**25**)	TNF-α and IL-6 GUA	IC_{50} 0.60 and 0.60 μM IC_{50}, 0.3 μg/ml Toxicity >10 μg/ml	Mishra et al. (2013)
28.	*Alternaria longissima*	*Sphaeranthus* sp.		Ustilaginoidin (**137**)	GUA	IC_{50}, 0.1 μM Toxicity >1 μM	Deshmukh (2018)
29.	*Arthrinium phaeospermum*	Unidentified grass	Not reported, India	Arthrichitin (**138**)	Fungicidal efficiency against *Pyricularia oryzae* infection of rice and *B. cinerea* infection of cucumber at 5000 ppm	75% and 85%	Vijayakumar et al. (1996)
30.	*Phialophora mustea*	*Crocus sativus*		Phialomustin C (**141–142**)	*C. albicans*	IC_{50} values of 14.3 and 73.6 mM	Nalli et al. (2015)
				Phialomustin B(**140**)	T47D cell line	IC_{50} of 1 mM	
31.	*Aspergillus terreus* JAS-2	*Achyranthes aspera*	Varanasi, India	Terrein (**143**)	*Enterococcus faecalis*	IC_{50} value of about 20 μg/ml	Goutam et al. (2017, 2020)
					S. aureus and *Aeromonas hydrophila*	Greater than 20 μg/ml	

(continued)

Table 16.4 (continued)

Sr. no.	Endophytic fungal strain	Host plant(s)	Plant part or tissue/locality of host plants	Isolated metabolite	Tested systems	Activity response	References
					Bipolaris sorokiniana, *Aspergillus flavus*, and *Alternaria alternata*	Displayed 57.14%, 52.5%, and 91.25%, inhibition	
					DPPH assay	IC_{50} value 112 μg/ml	
					A-549 cell line	IC_{50} 121.9 μg/ml	
32.	*Alternaria alternata*	*Grewia asiatica*		Alternariol (1) (**145**)	*S. aureus* (ATCC 29213), VRE, and MRSA	MIC (MBC) of 32 (>256), 32 (>256), and 8 (>256) μg/ml	Deshidi et al. (2017)
				Alternariol 9-methyl ether (2) (**146**)	*S. aureus* (ATCC 29213), VRE, and MRSA	MIC (MBC) of 128 (>256), 128 (>256) and 64 (>256) μg/ml	
				Ciprofloxacin	*S. aureus* (ATCC 29213), VRE, and MRSA	MIC of 0.125, 32, and 8 μg/ml	
33.	*Chloridium* sp.	*Azadirachta indica*	Varanasi, India	Javanicin (**147**)	*P. fluorescens* and *P. aeruginosa*	MIC value of 2 μg/ml	Kharwar et al. (2009)
	Talaromyces funiculosus	*Diorygma hieroglyphicum*	India	Funiculosone (**148**), mangrovamide J (**149**), ravenelin (**150**)	*Escherichia coli* and *Staphylococcus aureus*	IC_{50} range 23–104 pg/ml	Padhi et al. (2019)
				Funiculosone (**148**)	*Candida albicans*	IC_{50} 35 μg/ml	

the plant *Apodytes dimidiata* was the source of both the analogues from Western Ghats (Shweta et al. 2010).

Podophyllotoxin (**56**) (Fig. 16.4), an aryltetralin lignin, isolated from the resin of *Podophyllum emodi* was used for the treatment of genital warts (Leiter et al. 1950), a precursor for etoposide (**57**), teniposide (**58**) (Fig. 16.4), etoposide phosphate (**59**) (Fig. 16.5), the topoisomerase I inhibitors, and anticancer target (Eyberger et al. 2006; Puri et al. 2006). Podophyllotoxin had also been isolated from *Trametes hirsuta* associated with the rhizomes of *Podophyllum hexandrum* collected from the Himalayan region of India (Puri et al. 2006) and from *F. oxysporum* from *Juniperus recurva* (Kour et al. 2008). An anticancer prodrug deoxypodophyllotoxin (**60**) was extracted from *A. fumigatus*, an endophyte from *Juniperus communis* (Kusari et al. 2009b).

Hypericin (**61**) (Fig. 16.5), a naphthodianthrone derivative, was extracted from *Hypericum perforatum*. It commonly used for 'the blues' or depression and symptoms that sometimes go along with mood such as nervousness, tiredness, poor appetite, and trouble in sleeping (Brockmann et al. 1939; Nahrstedt and Butterweck 1997). Anticancer activity of hypericin is due to photodynamic activity which was suggested by several in vitro studies (Hadjur et al. 1996; Delaey et al. 2001; Kamuhabwa et al. 2001). Hypericin (**61**), along with emodin (**62**) (Fig. 16.5), were isolated from *Thielavia subthermophila*, an endophyte of *H. perforatum*, collected from Jammu and Kashmir (Kusari et al. 2008). Hypericin (**61**) and emodin (**61**) displayed the photodynamic cytotoxicity against the human acute monocytic leukaemia cell line (THP-1), at 92.7% vs 4.9%, and 91.1% vs 1.0% viability by resazurin and ATPlite assays, in the light and dark, respectively (Kusari et al. 2009c).

Rohitukine (**63**) (Fig. 16.5), a chromane alkaloid, was purified from *Amoora rohituka* and *Dysoxylum binectariferum* both belonging to family Meliaceae (Harmon et al. 1979; Naik et al. 1988). Rohitukine displayed anti-inflammatory, immunomodulatory, and anticancer properties (Naik et al. 1988). Rohitukine displayed anticancer activity against SKOV-3, T47D, MDAMB 273, NCI/ADR-RES, and MCF-7 cells (IC_{50} values of 20, 50, 3, 2.8, and 15 μg/ml, respectively) (Kumara et al. 2010). It was also purified from *Fusarium proliferatum* a resident of the inner bark of *D. binectariferum* (Kumara et al. 2012).

Piperine (**64**) (Fig. 16.5), an alkaloid was extracted from *Piper longum* and *Piper nigrum* plants belonging to family Piperaceae. Piperine is also extracted from *Periconia* sp., an endophyte of *P. longum*, *Mycosphaerella* sp., and *Colletotrichum gloeosporioides* endophytes of *P. nigrum* (Chithra et al. 2014a, b; Verma et al. 2011). Piperine possesses a wide range of biological activities, viz. antimicrobial, antimycobacterial, antitumour, antidepressant, anti-inflammatory, antioxidative, immunoregulatory, and antihyperlipidemic (Sunila and Kuttan 2004; Maneesai et al. 2012; Huan et al. 2015).

Withanolides are a group of C28 steroids built on an ergostane scaffold functionalized at carbons 1, 22, and 26, commonly known as the withanolide skeleton (Chen et al. 2011). These withanolides were found to be safe and active against inflammation-mediated chronic diseases, e.g. arthritis, autoimmune, cancer, neurodegenerative, and neurobehavioural. Withanolides exhibit unusual biologic

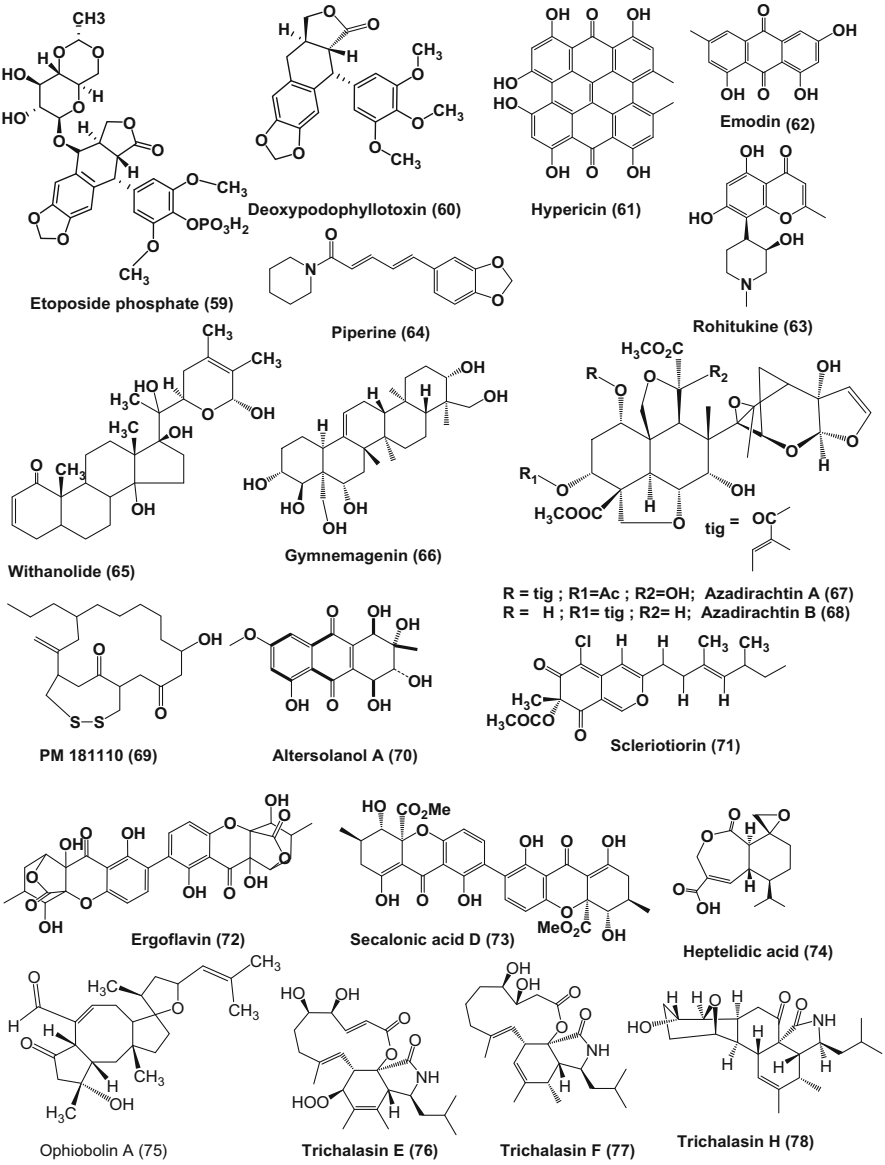

Fig. 16.5 Secondary metabolites produced by fungi (**59**–**78**)

activity across these complex disease processes while showing minimal adverse effects (Chen et al. 2011; White et al. 2016). Cardiovascular and Alzheimer's diseases can be treated with withanolides, isolated from the root and leaf of *W. somnifera* (Sabir et al. 2013; Sangwan et al. 2014). Withanolide (**65**) (Fig. 16.5), has also been isolated from *Talaromyces pinophilus*, an endophyte

residing in the leaves of *Withania somnifera*. The fungus produces a higher amount of withanolides compared to leaf and root of *W. somnifera* (Sathiyabama and Parthasarathy 2018).

Gymnemagenin (**66**) (Fig. 16.5), a triterpenoid, was purified from plant *Gymnema sylvestre* and possesses antidiabetic, anti-obesity, and antiviral activities (Liu et al. 1992; Kanetkar et al. 2007; Rao and Sinsheimer 1971). It had also been isolated from *Penicillium oxalicum*, an endophyte residing in the leaves of *Gymnema sylvestre* (Parthasarathy and Sathiyabama 2014) and can help in curing diabetes and obesity (Pothuraju et al. 2014; Rathore et al. 2016).

Azadirachtin is a highly oxygenated tetranortriterpenoid, isolated from the seed kernels of *Azadirachta indica* (Meliaceae) (Butterworth 1968). Azadirachtin A (**67**) and B (**68**) (Fig. 16.5), were extracted from *Eupenicillium parvum*, an endophyte of *A. indica* (Kusari et al. 2012). Azadirachtin exhibits deleterious effects on phytophagous insects. It disrupts insect moulting by antagonizing the effects of ecdysteroids. The desert locust *Schistocerca gregaria* and many species of *Lepidoptera* are among the most sensitive, being deterred by as little as 0.007 ppm (in diets), whereas the *Hemiptera* and *Coleoptera* are much less sensitive with EC_{50} values of around 100 ppm or more. Surprisingly, *Schistocerca gregaria* prefers to starve to death rather than ingest azadirachtin; the primary antifeedant effect on aphids of azadirachtin applied systemically in plants occurs at levels far higher than those causing IGR and sterility effects (Nisbet 2000; Morgan 2009).

A novel depsipeptide PM181110 (**69**) (Fig. 16.5) was extracted and characterized from *Phomopsis glabrae* (PM0509732), an endophyte residing in the leaves of *Pongamia pinnata* collected from Raigarh district of Maharashtra. The compound PM181110 exhibited cytotoxic activity against a set of 40 human cancer cell lines with the mean IC_{50} value of 0.089 μM and displayed the mean $IC_{50} = 0.245$ μM in ex vivo assay towards 24 human tumour xenografts (Verekar et al. 2014).

Altersolanol A (**70**) (Fig. 16.5), an anthraquinone derivative, was reported from an endophytic fungus *Phomopsis* sp. associated with plant *Nyctanthes arbor-tristis* collected in Mumbai. Compound altersolanol A exhibited noticeable toxicity with mean IC_{50} (IC_{70}) values of 0.005 μg/ml (0.024 μg/ml) against 34 human cancer lines (Mishra et al. 2015). The cellular activity of altersolanol A has been studied in detail, which inhibits kinase that follows caspase-dependent pathways for inducing apoptosis. Altersolanol A inhibited a variety of kinases which suggested that the kinase inhibition might be the mode behind cytotoxic activity (Debbab et al. 2009) and blocks NF-B transcriptional activity (Teiten et al. 2013).

Cephalotheca faveolata, an endophyte residing inside the petiole of *Eugenia jambolana* collected from Goregaon, Mumbai, was the source of sclerotiorin (**71**) (Fig. 16.5). Sclerotiorin (**71**) showed the cytotoxicity against HCT-116, H460, ACHN, Panc-1, Calu-1, and MCF 10A cell lines with the IC_{50} values of 0.63, 1.6, 1.2, 1.6, 2.1, and >10 μM, respectively. It also induced apoptosis in HCT-116 cells via the triggering of BAX and downregulation of Bcl-2 that result in stimulation of cleaved caspase-3 thereby causing the death of cancerous cells (Giridharan et al. 2012).

Ergoflavin (**72**) (Fig. 16.5) belonging to a class ergochromes was purified from the fungus PM0651480 residing inside the leaf of *Mimusops elengi* collected from Sagar, Madhya Pradesh, India. Ergoflavin exhibited cytotoxic activity against ACHN, H460, Panc-1, HCT16, and Calu-1 cancer cell lines with the IC_{50} values of 1.2, 4.0, 2.4, 8.0, and 1.5 μM, respectively. It also displayed anti-inflammatory activity against TNF-α and IL-6, with IC_{50} values of 1.9 and 1.2 μM, respectively (Deshmukh et al. 2009).

Secalonic acid D (SAD) (**73**) (Fig. 16.5) was reported from *Aspergillus aculeatus*, isolated from a marine sponge *Cinachyra cavernosa* collected at Mandapam, Tamil Nadu. It displayed cytotoxic activity with IC_{50} values of 0.2, 0.2, 0.19, 0.2, 0.2, and 4.9 μg/ml against Panc-1, H460, ACHN, Calu-1, HCT-116, and WI-38 cell lines, respectively (Deshmukh 2018). SAD was also purified from mangrove endophyte *Paecilomyces* sp. (tree 17) and exhibited cytotoxicity with an $IC_{50} < 1$ μg/ml against KB cell lines and inhibited topoisomerase I with an IC_{50} of 0.16 μM (Guo et al. 2007).

Heptelidic acid (**74**) (Fig. 16.5) also known as koningic acid, a sesquiterpene lactone, is purified from *Trichoderma* sp. residing inside the leaf of *A. indica* collected at Goregaon, Mumbai. Heptelidic acid (**74**) displayed modest cytotoxic activity with $IC_{50} < 1$ μg/ml against T47D, SKOV-3, KM-12, NAMALWA, MDAMB-231, NCI-H460, HOP-62, Colo-205, TK10, OVCAR-3, BXPC3, HL-60, WM-266-4, DU145, HCT-116, and A-549 cell lines (Rahier et al. 2015). Heptelidic acid is a potent inhibitor of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which binds to the cysteine residue at the active site (Kato et al. 1992).

Ophiobolin A (**75**) (Fig. 16.5) was purified from *Bipolaris setariae* associated with the leaf of *Parthenium hysterophorus*. Ophiobolin A displayed phosphorylation of S6, ERK, and RB with IC_{50} values of 1.9, 0.28, and 1.42 μM, respectively. Sorafenib PI103 and staurosporine were used as standard for pERK, pS6, and pRB assays, with IC_{50} values of 0.06, 0.19, and 0.1 μM, respectively. Ophiobolin A displayed cytotoxicity against solid cancer cell lines A2780 PC-3 MDAMB-231 and MCF-7 with IC_{50} values of 1.2, 2.5, 1.9, and 4.3 μM, respectively. It also exhibited cytotoxicity against haematological cancer cell lines MM1R, RPMI8226, U266B168 and Jurkat with IC_{50} values of 0.7, 0.4, 0.7, and 1.03 μM, respectively, while it exhibited cytotoxicity against normal cells hPBMC with IC_{50} value of 20.9 μM. Ophiobolin A checked the progress of cell cycle and induced cell death in MDAMB-231 cancer cell line with simultaneous inhibition of pS6, pAKT, pERK, pRB, and cyclin D1 proteins and simultaneous blockage of different cancer regulatory pathways like PI3K/mTOR, Ras/Raf/ERK, and CDK/RB (Bhatia et al. 2016).

Compounds trichalasin E (**76**), F (**77**), and H (**78**) (Fig. 16.5) were isolated from *Diaporthe* sp. T1, an endophyte associated with *Taxus baccata* collected from Bhaderwah, Doda district, India. The crude extract of *Diaporthe* sp.T1 displayed cytotoxic activity with IC_{50} values of 1058 and 1257 μg/ml against MCF-7 and HeLa cell lines, respectively (Vasundhara et al. 2017).

Two new compounds (3*S*,4a*R*,7*S*)-7,8-dihydroxy-3-methyl-3,4,10,5,6,7-hexahydro1*H*-isochromen-1-one (**79**) and (1*S*,3*R*,5*R*)-3-methyl-2-oxabicyclo [3.3.1]nonan-7-one (**80**), along with known compounds (−)-ramulosin (**81**) and

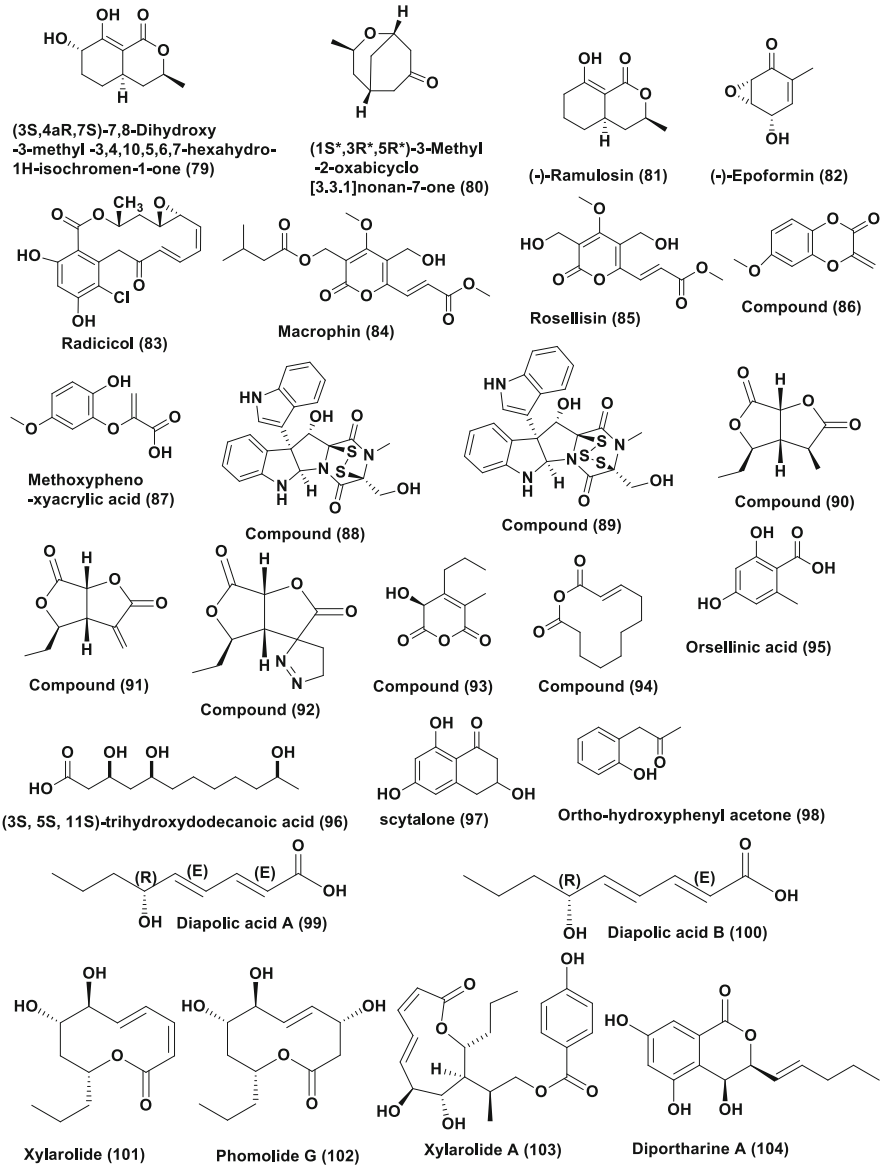

Fig. 16.6 Secondary metabolites obtained from fungi (**79**–**104**)

(−)-epoformin (**82**) (Fig. 16.6), were reported from an endophytic fungus *Talaromyces* sp., which resides inside the plant *Cedrus deodara* collected from Lolab Valley in Western Himalayas of Kashmir. Compound (**82**) displayed cytotoxic activity with 98%, 100%, 50%, 22%, and 56% against A-549, HEP-1, THP-1, PC-3, and HCT-116 cell lines, respectively, at 50 μM concentration. Compound (**80**)

exhibited cytotoxic activity with 71%, 26%, 23%, and 59% inhibition against A-549, HEP-1, PC-3, and HCT-116 cells, respectively, at the same concentration. Compound (**81**) also displayed cytotoxicity with 15%, 23%, 54%, 23%, and 44% against A-549, HEP-1, THP-1, PC-3, and HCT-116 cells at 50 μM concentration. Compound (**79**) exhibited poor cytotoxicity with 35%, 3%, 40%, 34%, and 35% against A-549, HEP-1, THP-1, PC-3, and HCT-116 cell lines at 50 μM concentration. Paclitaxel, the positive control, displayed cytotoxicity with 82%, 71%, and 72% inhibition at 1 μM concentration against A-549, THP-1, and HCT-116 cell lines. Compounds (**79–82**) induced apoptosis in HL-60 cells, fluorescence and SEM studies reveal the microtubule inhibition in HL-60 cells (Kumar et al. 2013b).

Radicicol (**83**) (Fig. 16.6) was purified from an endophytic fungus *Humicola fuscoatra* associated with *Mangifera indica* collected from Mumbai, India. Radicicol displayed cytotoxic activity against ACHN, Panc-1, Calu-1, H460, HCT-116, MCF 10A cell lines with IC_{50} values of 0.29, 0.45, 0.41, 0.27, 0.29, and 2.7 μM, respectively. Based on the efficacy, radicicol was further evaluated for molecular signature in Panc-1 cells using high content screening tools. Radicicol remarkably upregulates p21 and p53 and showed noteworthy downregulation of NFkB and STAT-3 protein levels in Panc-1 cells. In addition, the levels of $pAKT^{S473}$, pRB^{S780} were notably downregulated in Panc-1 cells. Radicicol also expressed upregulation of caspase-3 compared to untreated cells (Deshmukh 2018). It was also observed that radicicol specifically targets aggregated/misfolded α-synuclein's toxicity (Derf et al. 2019).

Four known metabolites, macrophin (**84**), rosellisin (**85**), 2-(2-hydroxy-5-6-methoxy-3-methylene-1,4-benzodioxin-2(3*H*)-one) (**86**), and methoxyphenoxyacrylic acid (**87**) (Fig. 16.6), were reported from *Phoma macrostoma* an endophytic fungus of *Glycyrrhiza glabra*, collected from Jammu. Macrophin (**84**) displayed an impressive cytotoxic activity with IC_{50} values of 14.8, 8.12, 13.0, and 0.9 μM, against the MDA-MB-231, T47D, MCF-7, and MIAPaCa-2 cell lines, respectively. Macrophin (**84**) induces apoptosis through S-phase arrest in MiaPaca-2 cell lines at the concentration of 600 nM analysed by flow cytometer (Nalli et al. 2019).

Two known thiodiketopiperazine derivatives (**88**) and (**89**) (Fig. 16.6) were purified from *Phoma cucurbitacearum*, an endophyte associated with the plant *Glycyrrhiza glabra* collected from Jammu (J&K). Both the compounds exhibited antibacterial activity with IC_{50} values of <10 μM against the battery of bacterial pathogens including *S. aureus* and *Streptococcus pyogenes*. Both the compounds potentially inhibited biofilm formation in *S. aureus* and *S. pyogenes* and acted synergistically with streptomycin and inhibited transcription/translation. It was also observed that the *sea* (Staphylococcal enterotoxin A) gene was expressed several fold on treatment with compound (**88**), while its expression was not affected significantly with compound (**89**). The expression of agrA gene was also not affected significantly in *S. aureus* with the treatment of either of the compounds (Arora et al. 2016a).

Three new compounds 4-ethyl-3-methyldihydrofuro[3,4-b]furan-2,6(3*H*,6a*H*)-dione (**90**), (*S*)-3-hydroxy-5-methyl-4-propyl-2*H*-pyran-2,6(3*H*)-dione (**91**), (*E*)-oxacyclododec-3-ene-2,12-dione (**92**) and one semi-synthetic 4-ethyl-

3a,4,4′,5′-tetrahydro-2*H*-spiro[furo[3,4-b]furan3,3′-pyrazole]-2,6(6a*H*)-dione (**93**), together with known molecules *4-epi*-ethisolide (**94**), orsellinic acid (**95**) (Fig. 16.6), were isolated from *Cryptosporiopsis* sp. an endophyte of *Clidemia hirta* obtained from Hawaii, USA. Compound (**94**) displayed an average activity against HL-60, PC-3, A-549, Colo-205, and MDA-MB-231 cell lines (IC_{50}, 11.0, 30.0, 75.0, 48.0, 90.0 μM). The compound (**93**) exhibited mild activity against *E. coli*, *B. cereus*, and *C. albicans* with (IC_{50}, 90.0, 65.0, and 70.0 μg/ml) (Kumar et al. 2017).

Three bioactive molecules, viz. (3*S*, 5*S*, 11*S*)-trihydroxydodecanoic acid (**96**), scytalone (**97**), and *ortho*-hydroxyphenyl acetone (**98**) (Fig. 16.6) were purified from *Cladosporium tenuissimum*, an endophyte of *Pinus wallichiana* collected from Lolab Valley (Kashmir India). Compounds (**96**) were active against MCF-7 and T47D cell lines with IC_{50} values of 15.0 and 100 μM, respectively. Compounds (**97**) were found active against MCF-7 and T47D cell lines with IC_{50} values of 68.0 and 75.0 μM, respectively. Compounds (**98**) were found active against MCF-7 and T47D cell lines with IC_{50} values of 42.0 and 82.0 μM, respectively. Positive control doxorubicin was found highly active against MCF-7 and T47D cell lines with IC_{50} values of 0.22 and 0.41 μM, respectively. Compounds (**96–98**) also exhibited antibacterial activity against *Bacillus cereus* IIIM 25 with IC_{50} values of 63.6, 68.5, and 23.3 μg/ml, respectively. Compound (**98**) also exhibited an antibacterial activity against *S. aureus* ATCC 29978 and *E. coli* ATCC 25922 (IC_{50}, 89.8 and 43.7 μg/ml, respectively) (Naseer et al. 2017).

Two new hydroxylated unsaturated fatty acids, namely, diapolic acid A–B (**99**, **100**) and known molecules xylarolide (**101**) and phomolide G (**102**) (Fig. 16.6), were purified from *Diaporthe terebinthifolii* GG3F6, an endophyte associated with the rhizome of *Glycyrrhiza glabra* collected from Jammu, J&K, India. The compound xylarolide (**101**) displayed a potent cytotoxic activity against T47D cell lines with an IC_{50} of 7.0 μM and paclitaxel, the positive control exhibited the cytotoxic activity with IC_{50} value of 0.011 μM, and the cell death induced was of apoptotic nature. Xylarolide (**101**) displayed strong antifungal activity against *C. albicans*, with IC_{50} value of 78.8 μM, while the positive control nystatin displayed antifungal activity with an IC_{50} value of 21.5 μM. Compounds (**99–102**) found active against *Yersinia enterocolitica* with an IC_{50} value of 78.4, 73.4, 72.1, and 69.2 μM, respectively, while positive control ciprofloxacin displayed antibacterial activity with an IC_{50} value of 3.5 μM (Yedukondalu et al. 2017).

Xylarolide A (**103**), diportharine A (**104**) (Fig. 16.6), and xylarolide B (**105**) along with one known compound xylarolide (**106**) (Fig. 16.7) were purified from *Diaporthe* sp. an endophyte associated with the plant *Datura inoxia* collected from the Jammu region of Jammu and Kashmir, India, was treated with histone deacetylases inhibitor, valproic acid. Compounds (**103** and **106**) exhibited anticancer activity with an IC_{50} of 20 and 32 μM against MaPaCa-2 cell lines, respectively. Both the compounds also displayed anticancer activity against PC-3 with an IC_{50} of 14 and 18 μM, respectively. Xylarolide A (**103**) exhibited good antioxidant activity with EC_{50} of 10.3 μM in DPPH assay, and ascorbic acid was used as the positive control (Sharma et al. 2018a).

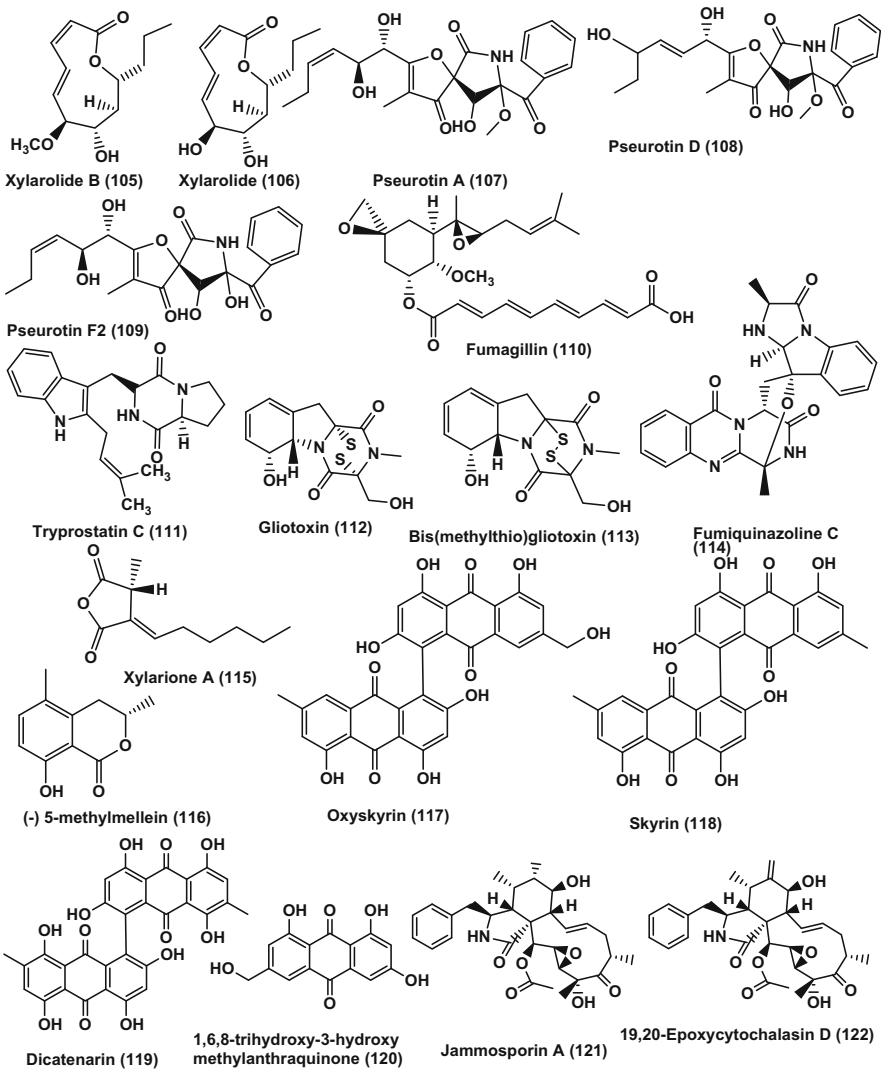

Fig. 16.7 Secondary metabolites obtained from fungi (**105**–**122**)

Compounds pseurotin A (**107**), pseurotin D (**108**), pseurotin F2 (**109**), fumagillin (**110**), tryprostatin C (**111**), gliotoxin (**112**), and bis(methylthio)gliotoxin (**113**) (Fig. 16.7) were isolated from an endophytic fungus *Aspergillus fumigatus* (GA-L7), a resident of *Grewia asiatica* collected from the Shiwalik region, Jammu, India. When *A. fumigatus* (GA-L7) treated with valproic acid, an HDAC inhibitor, there was tenfold enhancement in the production of fumiquinazoline C, which was earlier present in very low quantities. By the addition of valproic acid, the genes (Afua_6g 12040, Afua_6g 12050, Afua_6g 12060, Afua_6g 12070, and

Afua_6g 12080) involved in the biosynthesis of fumiquinazoline C (**114**) were over expressed 7.5-, 8.8-, 3.4-, 5.6-, and 2.1-fold, respectively, which led to overall tenfold enhancement of fumiquinazoline C (Magotra et al. 2017).

A new compound xylarione A (**115**) and known molecule (−)5-methylmellein (**116**) (Fig. 16.7) were purified from *Xylaria psidii*, an endophyte of *Aegle marmelos* collected from Yamuna Nagar district of Haryana, India. Compounds (**115** and **116**) displayed an impressive activity against MIA-Pa-Ca-2 cancer cell line with IC_{50} values of 16.0 and 19.0 μM, respectively. Both the compounds displayed activity against normal cells (fR2), with IC_{50} values which were 79.0 and 76.0 μM, respectively. Compounds (**115** and **116**) also displayed activity against MCF-7, MIA-Pa-Ca-2, NCI-H226, HepG2, and DU145 cancer cell line with IC_{50} in the range of 16–37 μM. The cell cycle distribution in MIA-Pa-Ca-2 cells confirmed a cell cycle arrest at the sub-G1 phase. Compounds (**115** and **116**) induced apoptosis and displayed substantial decrease in membrane potential of mitochondria in a concentration-dependent manner (Arora et al. 2016b).

The anthraquinones, oxyskyrin (**117**), skyrin (**118**), dicatenarin (**119**), and 1,6,8-trihydroxy-3-hydroxy methylanthraquinone (**120**) (Fig. 16.7) were isolated from *Penicillium pinophilum* MRCJ-326, an endophyte associated with the plant *Allium schoenoprasum* collected from Nathatop, J&K, India. Dicatenarin (**119**) exhibited anticancer activity against A-549, MIA-PaCa-2, HCT-116, T47D, PC-3, and OVCAR-3 cell lines with IC_{50} values of 23.00, 12.00, 17.00, 29.00, 35.00, and 26.00 μg/ml, respectively. Skyrin (**118**) exhibited anticancer activity against A-549, MIA-PaCa-2, HCT-116, and PC-3, cell lines with IC_{50} values of 38.00, 27.00, 32.00, and 47.00 μg/ml. Oxyskyrin (**117**) exhibited anticancer activity against HCT-116 cell lines with IC_{50} value of 48.00 μg/ml. Compounds, (**119** and **118**), significantly induced apoptosis and transmit the apoptotic signal via intracellular ROS generation, thereby inducing a change in the mitochondrial transmembrane potential and induction of the mitochondrial-mediated apoptotic pathway. It has been found that compounds (**119** and **118**) induce ROS-mediated mitochondrial permeability transition and resulted in an increased induction of caspase-3 apoptotic proteins in MIA PaCa-2 cell lines (Koul et al. 2016).

Jammosporin A (**121**), a novel cytochalasin and its analogues 19,20-epoxycytochalasin D (**122**) (Fig. 16.7), cytochalasin D (**123**), 19,20-epoxycytochalasin C (**124**), and cytochalasin C (**125**) (Fig. 16.8) were purified from *Rosellinia sanctae-cruciana*, an endophyte of *Albizia lebbeck* collected from the Jammu region of Jammu and Kashmir, India. Compounds (**121–125**) exhibited cytotoxicity against MOLT-4 cell line with IC_{50} values of 20.0, 10.0, 25.0, 8.0, and 6.0 μM, respectively (Sharma et al. 2018b).

Capsaicin (**126**), 2,4-di-*tert*-butyl phenol (**127**), and alternariol-10-methyl ether (**128**) (Fig. 16.8) were purified from *Alternaria alternata* FC-46, an endophyte associated with *Capsicum annum*. Compound (**128**) showed an antiproliferative activity against HL-60 and A431 cell lines with IC_{50} values of 85 and 95 μM and induced apoptosis verified by Hoechst staining and loss of mitochondrial membrane potential in HL-60 cells (Devari et al. 2014).

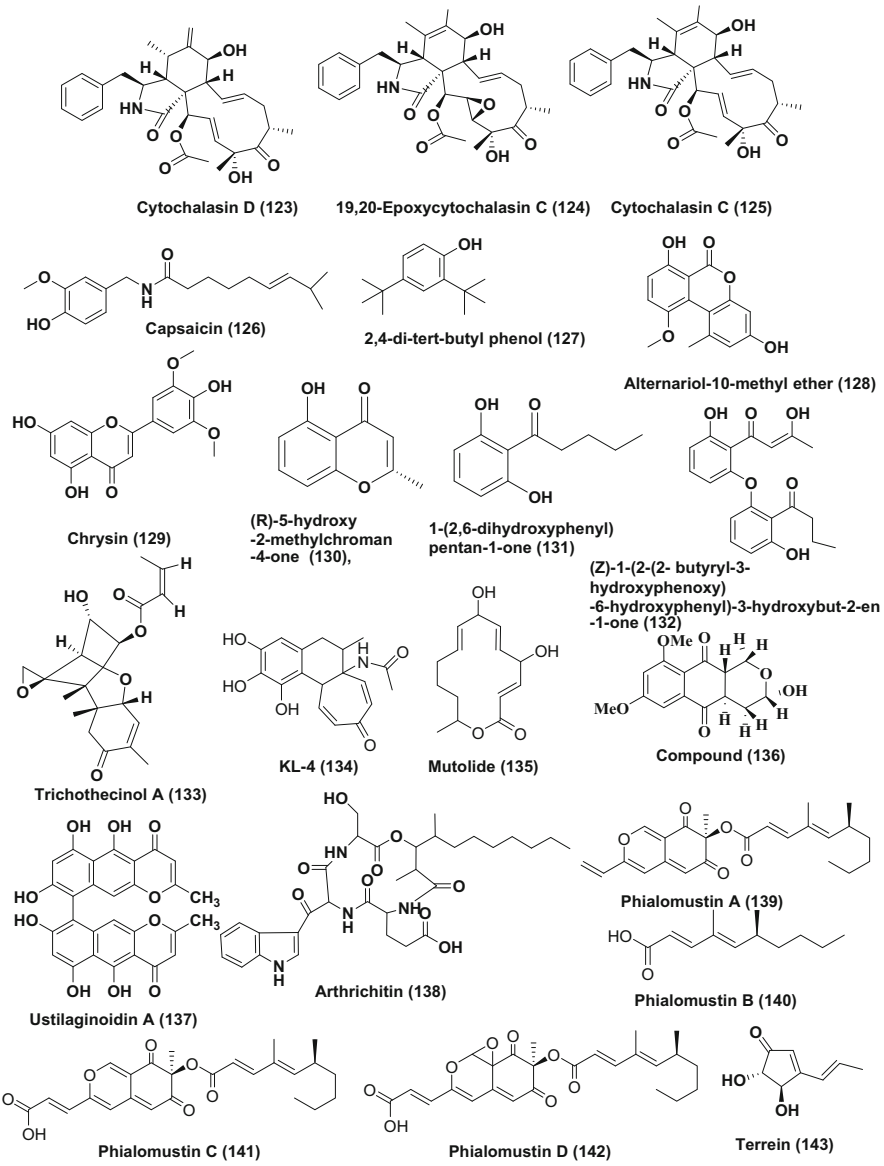

Fig. 16.8 Secondary metabolites obtained from fungi (**123**–**143**)

Chrysin (ChR) (**129**) (Fig. 16.8) was purified from *Alternaria alternata* KT380662, an endophyte associated with the plant *Passiflora incarnata.* The site of collection was Tiruchirappalli, Tamil Nadu, India. Fungal chrysin displayed an antiproliferative activity with an IC_{50} value of 37.97 μg/ml against MCF-7 cell line. HepG2 cells lose their viability in a time- and dose-dependent manner when treated

with chrysin. Formation of condensed nuclear membrane, blebbing, and apoptotic bodies clearly indicate that ChR triggers immediate cellular responses and apoptotic cell death against HepG2 cells (Seetharaman et al. 2017).

Compounds (*R*)-5-hydroxy-2-methylchroman-4-one (**130**), 1-(2,6-dihydroxyphenyl) pentan-1-one (**131**), and (*Z*)-1-(2-(2-butyryl-3-hydroxyphenoxy)-6-hydroxyphenyl)-3-hydroxybut-2-en-1-one (**132**) (Fig. 16.8) were isolated from *Cryptosporiopsis* sp., an endophytic resident of *Clidemia hirta* obtained from Hawaii, USA. Compound (**130**) did not exhibit any antimicrobial activity up to a concentration of 100 μg/ml. Compound (**131**) displayed antibacterial activity against *Bacillus cereus*, *E. coli*, *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Shigella flexneri*, *S. aureus* ATCC 29978, and *S. aureus* P1571 with IC_{50} values of 20.0, 30.0, 60.0, 47.0, 92.0, 18.0, and 15.0 μg/ml, respectively. Compound (**132**) showed antibacterial activity against *B. cereus*, *E. coli*, *L. monocytogenes*, *P. fluorescens*, *S. aureus* ATCC 29978, and *S. aureus* P1571, *S. pyogenes*, with IC_{50} values of 20.0, 50.0, 75.0, 6.0, 51.0, 24.0, and 62.0 μg/ml, respectively. The positive control ciprofloxacin showed acute activity against *B. cereus*, *E. coli*, *L. monocytogenes*, *P. fluorescens*, *S. flexneri*, *S. aureus* ATCC 29978, and *S. aureus* P1571, *S. pyogenes*, with IC_{50} values of 0.04, 0.10, 0.005, 0.03, 0.005, 0.09, 0.60, and 0.005 IC_{50} (μg/ml). Compound (**130**) exhibited a potent cytotoxic activity against, HL-60 cell line with an IC_{50} of 4.0 μg/ml (Zilla et al. 2013). Compound (**130**) arrests cells at G2/M phase and altered the expression of key proteins involve in cell cycle regulation. It induced caspase-dependent and caspase-independent apoptosis in cells and targets STAT-3 signaling cascade. HMC-mediated pSTAT-3 inhibition involves ubiquitin dependent pathway in HL-60 cells (Pathania et al. 2015).

Trichothecium sp., an endophyte associated with *Phyllanthus amarus* collected from Pune was the source of trichothecinol A (**133**) (Fig. 16.8). Trichothecinol A (**133**) causes 50% cell death in HeLa and B16F10 cells at 500 nM and developed apoptosis later and also checked migration of wound by 50% at 500 nM of MDA-MB-231 cells suggesting its antimetastatic property (Taware et al. 2014).

Compounds 6-methyl-1,2,3-trihydroxy-7,8-cyclohepta-9,12-diene11-one-5,6,7,8-tetralene-7-acetamide (KL-4) (**134**) (Fig. 16.8) were purified from an endophyte *Aspergillus* sp., isolated from the seeds of *Gloriosa superba* collected from Tirupati. Compound (**134**) displayed 23%, 70%, 35%, 43%, and 80% growth inhibition at 100 μg/ml of A-549, HEP-2, MCF-7, CV-1, and OVCAR-5 cell lines, respectively (Budhiraja et al. 2013).

16.4.3 Metabolites with Anti-inflammatory Activity

The compound mutolide (**135**) (Fig. 16.8) with an anti-inflammatory potential was purified from *Lepidosphaeria* sp. isolated from the unidentified plant collected from Rajkot, India. It exhibited anti-inflammatory activity against TNF-α and IL-6, with IC_{50} values of 1.27 and 1.07 μM, respectively. It also abrogated IL-17 secretion from anti-CD3/CD28 and stimulated hPBMCs. NF-κB, the major transcription factor involved in the secretion of pro-inflammatory cytokines including IL-17. Mutolide blocks TNF-α-induced NF-κB expression in a CEM-κB cell line transfected with the

κB element. Mutolide also inhibits TNF-α-induced translocation of NF-κB from cytoplasm into the nucleus but has no significant effect on IκB activation and mutolide does not inhibit p38 MAPK enzyme activity. The results indicated that anti-inflammatory effect of mutolide is via NF-κB inhibition. Oral administration of mutolide at 50 and 100 mg/kg inhibited LPS-induced production of TNF-α in Balb/c mice in an acute model of inflammation (Shah et al. 2015).

16.4.4 Metabolites with Antidiabetic Activity

A naphthoquinone, herbarin (**25**), and its analogue (**136**) (Fig. 16.8) were purified from *Dendryphion nanum* an endophyte of *Ficus religiosa* collected from Goregaon, Mumbai. Herbarin (**25**) inhibits cytokines TNF-α and IL-6 with IC_{50}values of 0.60 and 0.60 μM, respectively, while the positive control dexamethasone blocks cytokine production with IC_{50} values of 0.06 and 0.01 μM for TNF-α and IL-6, respectively. Herbarin, also displayed antidiabetic activity with EC_{50} value of 0.8 μM in GUA assay, while positive control rosiglitazone exhibited antidiabetic activity with EC_{50} value of 3.0 μM (Mishra et al. 2013).

Ustilaginoidin A (**137**) (Fig. 16.8) is another antidiabetic metabolite, purified from *Alternaria longissima*, an endophyte recovered from the leaf of *Sphaeranthus* sp., collected at Tungareshwar, (Thane, district), which displayed antidiabetic activity with IC_{50}, GUA 0.1 μM; toxicity >1 μM (Deshmukh 2018). It was also purified from *Claviceps virens* with a weak cytotoxic activity against human epidermoid carcinoma (Koyama and Natori 1988; Koyama et al. 1998).

16.4.5 Metabolites with Antimicrobial Activity

Arthrichitin (**138**) (Fig. 16.8), a cyclic depsipeptide purified from *Arthrinium phaeospermum* associated with an unidentified grass, collected at Mumbai (Vijayakumar et al. 1996) and also from the marine derived fungus *Hypoxylon oceanicum* as LL156256g (Schlingmann et al. 1998). Arthrichitin (**138**) was found active against *Candida* sp., *Trichophyton* sp., and a battery of plant pathogens. It displayed poor in vitro activity to be used in the clinic, but further derivatization can improve the activity (Vijayakumar et al. 1996). Arthrichitin (**138**) also exhibited morphological abnormalities for in vitro *Botrytis cinerea.* It also displayed fungicidal efficiency with 75% and 85% against infection of *P. oryzae* to rice and *Botrytis cinerea* to cucumber at 5000 ppm under greenhouse conditions, respectively (Vijayakumar et al. 1996).

Phialomustin A–D (**139–142**) (Fig. 16.8) were purified from *Phialophora mustea* an endophyte associated with the corms of *Crocus sativus*. Compounds (**141**) and (**142**) exhibited antifungal activity against *C. albicans* with IC_{50} values of 14.3 and 73.6 mM, whereas compound (**140**) displayed potent antiproliferative activity against T47D cell line with an IC_{50} of 1 mM (Nalli et al. 2015).

The compound terrein (**143**) (Fig. 16.8), a polyketide, was purified from *Aspergillus terreus* JAS-2 associated with the plant *Achyranthes aspera*. The site of collection was Varanasi, India. Terrein (**143**) exhibited antibacterial activity with an IC_{50} value of 20 μg/ml against *Enterococcus faecalis* and greater than 20 μg/ml against *S. aureus* and *Aeromonas hydrophila*. And the compound showed only 38.3% and 48% inhibition. It also exhibited antifungal activity against *Bipolaris sorokiniana*, *Aspergillus flavus*, and *Alternaria alternata* with 57.14%, 52.5%, and 91.25%, respectively. Terrein (**143**) showed free radical scavenging activity with IC_{50} value 112 μg/ml for DPPH assay; it also displayed the cytotoxic activity against A-549 cell line with an IC_{50} value 121.9 μg/ml. At 150 μg/ml of the compound, the maximum cells were found arrested in sub G1 phase indicating apoptotic dead cells (Goutam et al. 2017, 2020). Previously it was reported to have an anticancer activity against MCF-7, PANC-1, and HepG2 cell lines with IC_{50} values of 1.1, 9.8, and 66.8 μM, respectively (Liao et al. 2012).

A new structural isomer alternariol (**144**) of alternariol (**145**), along with alternariol 9-methyl ether (**146**) (Fig. 16.9), was purified from *Alternaria alternata* from the leaves of *Grewia asiatica*. The collection site was Jammu, J&K, India. The compound (**145**) displayed activity against *S. aureus* (ATCC 29213), VRE, and MRSA with MIC (MBC) of 32 (>256), 32 (>256), and 8 (>256) mg/ml, respectively. The compound (**146**) displayed activity against *S. aureus* (ATCC 29213), VRE, and MRSA with MIC (MBC) of 128 (>256), 128 (>256), and 64 (>256) mg/ml, respectively, while the positive control ciprofloxacin exhibited an activity against *S. aureus* (ATCC 29213), VRE, and MRSA with MIC of 0.125, 32, and 8 mg/ml, respectively (Deshidi et al. 2017).

A naphthoquinone, javanicin (**147**) (Fig. 16.9), was reported from *Chloridium* sp., an endophyte residing inside the plant *A. indica* collected from Varanasi, India. Javanicin (**147**) displayed an impressive antibacterial activity against *P. fluorescens* and *P. aeruginosa* with MIC value of 2.0 μg/ml (Kharwar et al. 2009).

A new compound funiculosone (**148**) and its two analogues mangrovamide J (**149**) and ravenelin (**150**) (Fig. 16.9) were purified from *Talaromyces funiculosus*, an endolichenic fungus isolated from lichen thallus of *Diorygma hieroglyphicum* (*Graphidaceae*), in India. All the compounds displayed antibacterial activity with an IC_{50} in the range of 23–104 μg/ml when assayed against *Escherichia coli* and *Staphylococcus aureus*. Funiculosone also showed anticandidal activity against *Candida albicans* with an IC_{50} 35 μg/ml (Padhi et al. 2019).

16.5 Useful Strategies to be Adopted in Cultivation of Fungi

The genomic approach applied for getting bioactives indicates the significant difference between the number of secondary metabolite biosynthetic gene or gene clusters (BGCs) and the actual number of secondary metabolites obtained from microorganisms. Cryptic/or silent BGCs are not expressed normally in the laboratory conditions mainly due to the lack of stimuli required for activation of secondary metabolite BGCs (Romano et al. 2018). This is one of the major limitations in

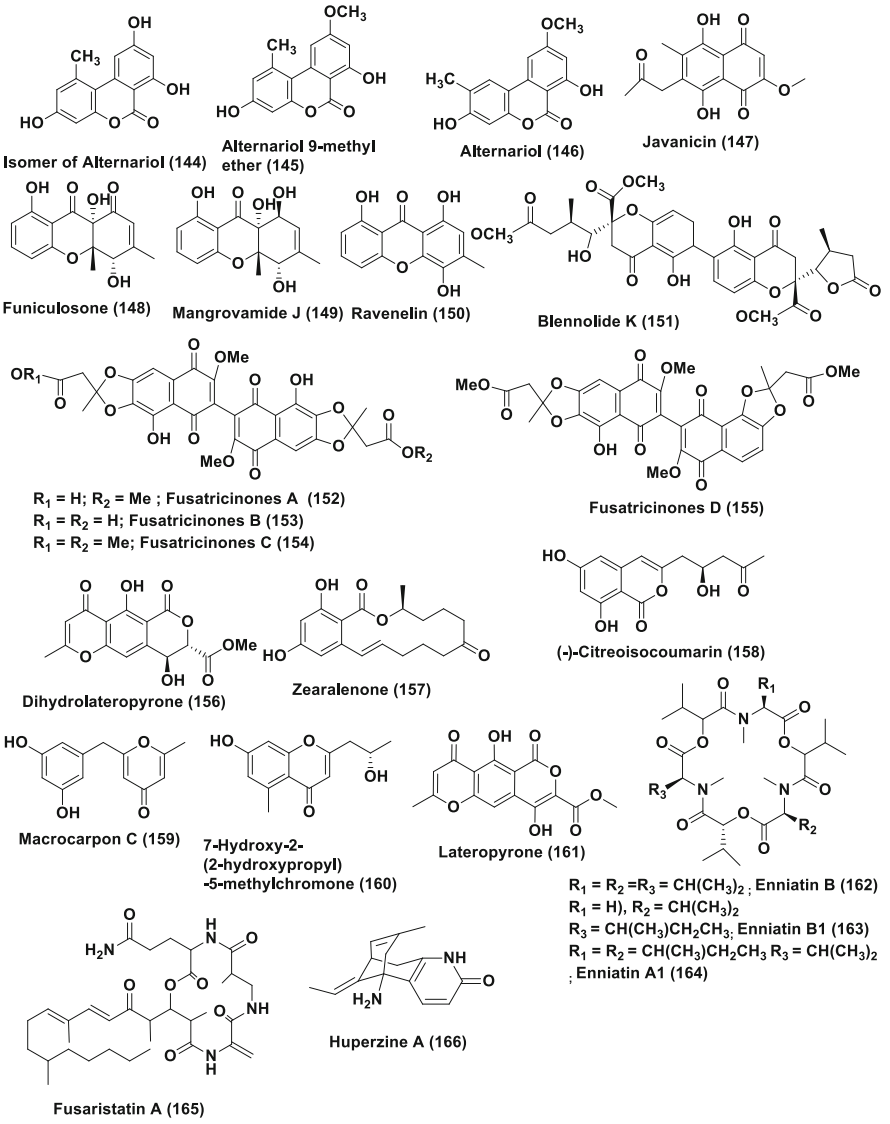

Fig. 16.9 Secondary metabolites obtained from fungi (**144**–**165**)

in vitro fermentation under laboratory conditions. One strain, many compounds (OSMAC) or co-cultivation-based strategies to evoke the expression of silent BGCs to produce new compounds may be adopted (Reen et al. 2015; Sharma et al. 2017). Diverse approaches were developed in order to optimize the production of undiscovered bioactives. Some of the strategies to be used for producing diverse bioactive metabolites are described briefly here.

16.5.1 One Strain, Many Compounds (OSMAC)

One of the methods varying the composition of the culture medium, pH, temperature, aeration, and luminosity besides the incorporation of precursors or inhibitors of metabolite biosynthesis (Bode et al. 2002; Rateb and Ebel 2011). This approach was developed based on the hypothesis that even a small amendment in the culture medium modulates secondary metabolite biosynthesis, and the term one strain, many compounds (OSMAC) is generally used in this approach (Elias et al. 2006; Bills et al. 2008). The success of this approach depends upon selection of suitable culture media, optimal growth conditions that favour fungal growth, and mycelium formation, and determining the capacity of secondary metabolite biosynthesis, optimization of culture conditions may greatly help.

This method is accomplished in generating the intracellular signals that enhance the capability of enzymatic potential of a fungal species (Paranagama et al. 2007), which in turn results in the production of metabolites, not detected otherwise, and is considered unusual in natural biosynthesis.

16.5.2 Metabolite Expression Modulated by Epigenetic Modifiers

Genomics has revealed that many microorganisms have far greater potential to produce specialized metabolites than was thought from the classic bioactivity screening; realizing this potential has been hampered by the fact that many specialized metabolite biosynthetic gene(s) clusters (BGCs) are not expressed in the laboratory cultures. To stimulate the expression of inactive biosynthetic gene clusters and secondary metabolite biosynthesis, epigenetic modifiers can used in fungal culture media (de la Cruz et al. 2012). The use of epigenetic modifiers such as histone deacetylase (HDACs) inhibitors and DNA methyltransferase (DNMT) inhibitors has been explored increasingly as a technique to induce the production of additional microbial secondary metabolites (Vander Molen et al. 2014; Sharma et al. 2017; Jasim et al. 2019) or to improve the yield of bioactive metabolites by activation of silent biosynthetic gene(s) (Magotra et al. 2017). For an example, valproic acid, an HDAC inhibitor, induced the biosynthesis of three novel compounds, xylarolide A (**103**), diportharine A (**104**), and xylarolide B (**105**) along with known compound xylarolide (**105**) from *Diaporthe* sp. that exhibits potent anticancer activity (Sharma et al. 2018a). These results clearly indicate that the expression of cryptic gene or gene clusters in fungi using epigenetic modifier would lead to the discovery of new bioactive metabolites (Brakhage and Schroeckh 2011; Chiang et al. 2011; Lyu et al. 2020).

16.5.3 Co-culture of Different Strains

The co-culture strategy is one of the encouraging promising approaches to obtain chemically undiscovered compounds, mainly because the microbes live in an

exceptionally biodiverse community in their natural habitats. These microorganisms share the same niche but vary in their morphology, growth, adaptation, and development patterns; this may also include changes in secondary metabolite production as a result of chemical interactions among the organisms (Kusari et al. 2014; Pamphile et al. 2017). For example, a new polyketide blennolide K (**151**) (Fig. 16.9) was obtained from the co-culture of *Setophoma terrestris* and *Bacillus amyloliquefaciens* (Arora et al. 2018).

When *Fusarium tricinctum*, an endophyte, was co-cultivated with *Streptomyces lividans*, four novel naphthoquinone dimers were formed: fusatricinones A–D (**152–155**), a new lateropyrone derivative, dihydrolateropyrone (**156**), along with four known compounds zearalenone (**157**), (−)-citreoisocoumarin (**158**), macrocarpon C (**159**), and 7-hydroxy-2-(2-hydroxypropyl)-5-methylchromone (**160**) (Fig. 16.9), which were not detected in the control. Increased production of the lateropyrone (**161**); the depsipeptides enniatins B (**162**), B1 (**163**), and A1 (**164**); and the lipopeptide fusaristatin A (**165**) (Fig. 16.9) were found in co-culture extracts as compared to the fungal culture alone (Moussa et al. 2019). More recently huperzine A (**166**) (Fig. 16.9) was isolated from *Alternaria brassicae* AGF041 residing inside *Huperzia serrata* from Meihua Mountain in Fujian Province, P.R. China. Hyperproduction of huperzine A was optimizes via response surface methodology. When statistically optimized compound was irradiated (0.70 kGy of γ treatment followed by 42.49 min of UV exposure sequentially), it led to a 53.1% enhancement in production (Zaki et al. 2020).

16.6 Conclusions and Future Perspectives

We have reviewed 165 compounds reported from the soil and endophytic fungi indicating their immense potential from Indian subcontinent for producing diverse metabolites; there is, however, a need to explore the functional and metabolic potential of fungi. In India, there are two biodiversity hot spots, Eastern Himalayas and the Western Ghats, and six wetlands, more than 8000 km of coastline with endemic mangroves, included in nine phytogeographic zones, and 35% plants are endemic. Indian subcontinent has different geographical regions and climatic zones ranging from tropical to alpine (Himalayas) and possesses cold and hot deserts which make India a hot spot for finding novel fungi and bioactive metabolites.

From India, 2080 fungal genera have been reported, among which 205 are new with approximately 27,000 species (Manoharachary et al. 2005). Using modified improved techniques, there is, however, a plenty of scope of getting a large number of yet to be described fungi which can be explored for bioprospection. High-throughput techniques developed for natural product isolation and identification include automated coupling of HPLC and MS spectrometry, NMR cryoprobes, LC-DAD-TOFMS (liquid chromatography with UV/VIS diode array detection and ESI+/ESI– time-of-flight MS for the assignment of molecular entities), HPLC bioactivity profiling/microtiter plate technique in conjunction with capillary probe NMR instrumentation, requiring mg quantities of extracts, and use of ‘biosynthetic

enzymes', and combinatorial biosynthesis, application of genetic approach for the development of improved strains, systematic chemical mutagenesis, and synthetic mimetics will help in understanding chemical diversity. There is an urgent need to study the whole genome sequences of Indian fungi which may certainly help in the prediction of genes/gene clusters responsible for synthesis of novel classes of bioactive compounds with diverse chemical scaffolds.

Cold and hot desserts are unexplored or less explored for the diversity of fungi that needs more attention. Though marine fungi are screened for bioactives, there is still a plenty of scope for their bioprospecting. Another specific group which needs to be explored is entomopathogenic fungi because of their diverse chemical attributes. There are limited reports of peptaiboles; a group of small peptides with a high aminoisobutyric acid (Aib), have been reported from India, such as an antiamoebin (L-2-Amino-3-phenyl-1-propanol (L-phenylalaninol)) produced by *Emericellopsis poonensis* (Deshmukh and Vaidya 1968). Peptaiboles are economically important because of their antimicrobial and anticancer properties and their ability to induce systemic resistance in plants against microbial invasion.

The compound deoxymulundocandin isolated at Hoechst India Ltd., Mumbai, its semi-synthetic derivative aminocandin, has displayed a potent antifungal activity against fluconazole-resistant *C. albicans* and itraconazole-resistant *Aspergillus fumigatus* strains. These compounds displayed chemical diversity along with their in vitro activity. In most of the cases, their efficacy is not tested in vivo which needs to be done. The fate and production of various bioactive metabolites are affected by various external and internal environmental factors. Deep understanding of the system biology is one of the holistic approaches that can be applied as a valuable tool for identifying important metabolites. There is a need to develop culture collections at national and regional levels with adequate facilities.

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References

Agrawala S, Deshmukh SK, Reddy MS, Prasad R, Goel M (2020) Endolichenic fungi: a hidden source of bioactive metabolites. South Afr J Bot. https://doi.org/10.1016/j.sajb.2019.12.008

Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, Rothrock J, Lopez M, Joshua H, Harris E, Patchett A (1980) Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proc Natl Acad Sci U S A 77(7):3957–3961

Arora P, Wani ZA, Nalli Y, Ali A, Riyaz-Ul-Hassan S (2016a) Antimicrobial potential of thiodiketopiperazine derivatives produced by *Phoma* sp., an endophyte of *Glycyrrhiza glabra* Linn. Microb Ecol 72(4):802–812

Arora D, Sharma N, Singamaneni V, Sharma V, Kushwaha M, Abrol V, Guru S, Sharma S, Gupta AP, Bhushan S et al (2016b) Isolation and characterization of bioactive metabolites from *Xylaria psidii*, an endophytic fungus of the medicinal plant *Aegle marmelos* and their role in

mitochondrial dependent apoptosis against pancreatic cancer cells. Phytomedicine 23 (12):1312–1320

Arora D, Chashoo G, Singamaneni V, Sharma N, Gupta P, Jaglan S (2018) *Bacillus amyloliquefaciens* induces production of a novel blennolide K in coculture of *Setophoma terrestris*. J Appl Microbiol 124(3):730–739

Bhatia DR, Dhar P, Mutalik V, Deshmukh SK, Verekar SA, Desai DC, Kshirsagar R, Agarwal V, Thiagarajan P (2016) Isolation, structure elucidation and novel anticancer activity of ophiobolin A, isolated from fungus *Bipolaris setariae*. Nat Prod Res 30(12):1455–1458

Bhuyan BK, Ganguli BN, Ghosh D (1961) Comparative study of penicillin production with vegetative and spore inoculum of *Penicillium chrysogenum*. Appl Microbiol 9(1):85–90

Bills GF, Platas G, Fillola A, Jiménez MR, Collado J, Vicente F, Martín J, González A, Bur-Zimmermann J, Tormo JR, Peláez F (2008) Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. J Appl Microbiol 104:1644–1658

Bills GF, Yue Q, Chen L, Li Y, An Z, Frisvad JC (2016) *Aspergillus mulundensis* sp. nov., a new species for the fungus producing the antifungal echinocandin lipopeptides, mulundocandins. J Antibiot Res 69(3):141–148

Bode HB, Bethe B, Höfs R, Zeeck A (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. ChemBioChem 3:619–627

Brakhage AA, Schroeckh V (2011) Fungal secondary metabolites—strategies to activate silent gene clusters. Fungal Genet Biol 48(1):15–22

Brockmann H, Haschad MN, Maier K, Pohl F (1939) Hypericin, the photodynamically active pigment from *Hypericum perforatum*. Nebr Med J 32:550–555

Brotzu G (1948) Ricerche su di un nuovo antibiotico. Lavori dell'Istituto d'Igiene di Cagliari 4–18

Budhiraja A, Nepali K, Sapra S, Gupta S, Kumar S, Dhar KL (2013) Bioactive metabolites from an endophytic fungus of Aspergillus species isolated from seeds of Gloriosa superba Linn. Med Chem Res 22(1):323–329

Butterworth JH (1968) Isolation of a substance that suppresses feeding in locusts. Chem Commun:23–24

Chakravarthi BV, Das P, Surendranath K, Karande AA, Jayabaskaran C (2008) Production of paclitaxel by *Fusarium solani* isolated from *Taxus celebica*. J Biosci 33(2):259–267

Chakravarthi BV, Sujay R, Kuriakose GC, Karande AA, Jayabaskaran C (2013) Inhibition of cancer cell proliferation and apoptosis-inducing activity of fungal taxol and its precursor baccatin III purified from endophytic *Fusarium solani*. Cancer Cell Int 13:105

Chen LX, He H, Qiu F (2011) Natural withanolides: an overview. Nat Prod Rep 28(4):705–740

Chiang YM, Chang SL, Oakley BR, Wang CC (2011) Recent advances in awakening silent biosynthetic gene clusters and linking orphan clusters to natural products in microorganisms. Curr Opin Chem Biol 15(1):137–143

Chithra S, Jasim B, Anisha C, Mathew J, Radhakrishnan EK (2014a) LC-MS/MS based identification of piperine production by endophytic *Mycosphaerella* sp. PF13 from *Piper nigrum*. Appl Biochem Biotechnol 173:30–35

Chithra S, Jasim B, Jyothis M, Sachidanandan P, Radhakrishnan EK (2014b) Piperine production by endophytic fungus *Colletotrichum gloeosporioides* isolated from *Piper nigrum*. Phytomedicine 21(4):534–540

Cragg GM, Newman DJ (2004) A tale of two tumor targets: topoisomerase I and tubulin. The Wall and Wani contribution to cancer chemotherapy. J Nat Prod 67(2):232–244

Das A, Rahman MI, Ferdous AS, Al-Amin R, Mahbubur M, Nahar N (2017) An endophytic Basidiomycete, *Grammothele lineata*, isolated from *Corchorus olitorius*, produces paclitaxel that shows cytotoxicity. PLoS One 12(6):e0178612/1–e0178612/17

de la Cruz M, Martín J, González-Menéndez V, Pérez-Victoria I, Moreno C, Tormo JR, El Aouad N, Guarro J, Vicente F, Reyes F, Bills GF (2012) Chemical and physical modulation of antibiotic activity in *Emericella* species. Chem Biodivers 9(6):1095–1113

Debbab A, Aly AH, Proksch P, Edrada-Ebel R, Wray V, Müller WEG, Totzke F, Zirrgiebel U, Schächtele C, Kubbutat MHG, Wen HL, Mosaddak M, Hakiki A, Ebel R (2009) Bioactive metabolites from the endophytic fungus *Stemphylium globuliferum* isolated from Mentha pulegium. J Nat Prod 72(4):626–631

Delaey EM, Obermueller R, Zupko I, De Vos D, Falk H, de Witte PA (2001) *In vitro* study of the photocytotoxicity of some hypericin analogs on different cell lines. Photochem Photobiol 74:164–171

Derf A, Verekar SA, Jain S, Deshmukh SK, Bharate SK, Chaudhuri B (2019) Radicicol rescues yeast cell death triggered by expression of human α-Synuclein and its A53T mutant, but not by human βA4 peptide and proapoptotic protein bax. Bioorg Chem 85:152–158

Deshidi R, Devari S, Kushwaha M, Gupta AP, Sharma R, Chib R, Khan IA, Jaglan S, Shah BA (2017) Isolation and quantification of alternariols from endophytic fungus, *Alternaria alternata*: LC-ESI-MS/MS analysis. Chem Select 2(1):364–368

Deshmukh SK (2018) Translating endophytic fungal research towards pharmaceutical applications. Kavaka 50:1–13

Deshmukh PV, Vaidya MG (1968) L-2-Amino-3-phenyl-l-propanol (L-Phenylalaninol) as a constituent of a fungal metabolite. Nature 217(5131):849–849

Deshmukh SK, Verekar SA (2014) Fungal endophytes: an amazing and hidden source of cytotoxic compounds anticancer compounds from endophytic fungi. In: Kharwar RN, Upadhyay RS, Dubey NK, Raghubanshi R (eds) Microbial diversity and biotechnology in food security. Springer, New York, pp 59–89

Deshmukh SK, Mishra PD, Kulkarni-Almeida A, Verekar SA, Sahoo MR, Periyasamy G, Goswami H, Khanna A, Balakrishnan A, Vishwakarma R (2009) Anti-inflammatory and anticancer activity of Ergoflavin isolated from an endophytic fungus. Chem Biodivers 6:784–789

Deshmukh SK, Verekar SA, Bhave S (2015) Endophytic fungi: an untapped source for antibacterials. Front Microbiol. https://doi.org/10.3389/fmicb.2014.00715

Deshmukh SK, Gupta MK, Prakash V, Saxena S (2018a) Endophytic fungi: a source of potential antifungal compounds. J Fungi 4:77. https://doi.org/10.3390/jof4030077

Deshmukh SK, Prakash V, Ranjan N (2018b) Marine fungi: a potential source of cytotoxic compounds. Front Microbiol 8:2536. https://doi.org/10.3389/fmicb.2017.02536

Deshmukh SK, Gupta MK, Prakash V, Reddy MS (2018c) Mangrove-associated fungi a novel source of potential anticancer compounds. J Fungi 4:101. https://doi.org/10.3390/jof4030101

Deshmukh SK, Gupta MK, Prakash V, Reddy MS (2019a) Fungal endophytes - a novel source of cytotoxic compounds. In: Jha S (ed) Endophytes and secondary metabolites, Reference series in phytochemistry. Springer, Cham, pp 1–62

Deshmukh SK, Gupta MK, Lenka SK (2019b) Endophytic fungi are the alternate source of high value plant secondary metabolites. In: Satyanarayana T, Das SK, Johri BN (eds) Microbial diversity in ecosystem sustainability and biotechnological applications, Soil and agroecosystems, vol 2. Spinger, Cham, pp 493–532

Deshmukh SK, Agrawala S, Prakash V, Reddy MS, Gupta MK (2020) Anti-infectives from mangrove endophytic fungi. South Afr J Bot 134:237–263

Deshmukh SK, Agrawala S, Gupta MK, Patidar RK, Ranjan N (2021) Fungi: a novel source of antiviral compounds. Curr Pharm Biotechnol. https://doi.org/10.2174/1389201022666210615120720

Devari S, Jaglan S, Kumar M, Deshidi R, Guru S, Bhushan S, Kushwaha M, Gupta AP, Gandhi SG, Sharma JP, Taneja SC (2014) Capsaicin production by *Alternaria alternata*, an endophytic fungus from Capsicum annum; LC–ESI–MS/MS analysis. Phytochemistry 98:183–189

Divekar PV, Read G, Vining LC, Haskins RH (1959a) Volucrisporin: isolation, structure, and synthesis of the methyl ether. Can J Chem 37(12):1970–1976

Divekar PV, Read G, Vining LC (1959b) Volucrisporin: a novel fungal pigment. Chem Ind 24:731–732

Doss V, Govindharajan K, Ravichandran D (2016) Screening of Taxol, an anticancer drug produced from *Pestalotiopsis stellata* isolated from *Ficus infectoria*. Life Sci J 13(10):13–21

Elias BC, Said S, Albuquerque S, Pupo MT (2006) The influence of culture conditions on the biosynthesis of secondary metabolites by *Penicillium verrucosum* Dierck. Microbiol Res 161:273–280

Eyberger AL, Dondapati R, Porter JR (2006) Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. J Nat Prod 69(8):1121–1124

Fujita T, Inoue K, Yamamoto S, Ikumoto T, Sasaki S, Toyama R, Chiba K, Hoshino Y, Okumoto T (1994) Fungal metabolites. Part II. A potent immunosuppressive activity of *Isaria sinclairii* metabolites. J Antibiot 47:208–215

Fulzele DP, Satdive RK, Pol BB (2001) Growth and production of camptothecin by cell suspension cultures of *Nothapodytes foetida*. Planta Medica 67:150–152

Ganapathi K (1957) The biosynthesis of penicillin. Experietia 13:172–176

Ganapathi K (1961) Regulatory mechanisms in microorganisms their molecular basis. J Sci lnd Res 20A:569–576

Ganapathi K, Boyce RS (1965) Use of resorcinol-sulphuric acid reagents for the identification and estimation of some sugars and nucleic acids. Indian J Biochem 2:53

Ganapathi K, Deshpande VN (1957) Biosynthesis of benzylpenicillin by mycelial suspensions of *Penicillium chrysogenum*. Experientia 13:475

Ganapathi K, Deshpande VN (1958) Biosynthesis of benzylpenicillin by resting cells of *Penicillium chrysogenum*. Part I - Effect of various carbohydrates & carbon sources. J Sci Ind Res 17C:59–66

Ganapathi K, Irani RJ (1958a) The effect of glycerol on the biosynthesis of benzylpenicillin by the washed cells of *Penicillium chrysogenum*. Experientia 14:329

Ganapathi K, Irani RJ (1958b) Carbohydrate constituents of the mycelium of *Penicillium chrysogenum* grown in media with different sources of carbon. Nature 183:758–760

Ganapathi K, Irani RJ (1959) Myo-inositol in the biosynthesis of benzylpenicillin by the mycelial suspensions of *Penicillium chrysogenum*. Experientia 15:22

Ganapathi K, Irani RJ (1960a) Carbohydrate constituents of the mycelium of *Penicillium chrysogenum* grown in media with different sources of carbon. J Sci Ind Res 19C:207–216

Ganapathi K, Irani RJ (1960b) Effect of carbohydrates & some carbon sources on biosynthesis of benzylpenicillin by washed cells of *Penicillium chrysogenum*. J Sci Ind Res 19C:216–222

Ganapathi K, Irani RJ (1960c) Chemical pathways in carbohydrate metabolism. J Sci Ind Res 19A:9–18

Gangadevi V, Muthumary J (2007) Endophytic fungal diversity from young, mature and senescent leaves of *Ocimum basilicum* L. with special reference to taxol production. Indain J Sci Technol 1(1):1–12

Gangadevi V, Muthumary J (2008a) Taxol, an anticancer drug produced by an endophytic fungus *Bartalinia robillardoides* Tassi, isolated from a medicinal plant, *Aegle marmelos* Correa ex Roxb. World J Microbiol Biotechnol 24(5):717–724

Gangadevi V, Muthumary J (2008b) Isolation of *Colletotrichum gloeosporioides*, a novel endophytic taxol-producing fungus from the leaves of a medicinal plant *Justicia gendarussa*. Mycol Balcanica 5(1):1–4

Gangadevi V, Muthumary J (2009a) A novel endophytic Taxol-producing fungus *Chaetomella raphigera* isolated from a medicinal plant *Terminalia arjuna*. Appl Biochem Biotechnol 158 (3):675–684

Gangadevi V, Muthumary J (2009b) Taxol production by *Pestalotiopsis terminaliae*, an endophytic fungus of *Terminalia arjuna* (arjun tree). Biotechnol Appl Biochem 52:9–15

Gangadevi V, Murugan M, Muthumary J (2008) Taxol determination from *Pestalotiopsis pauciseta*, a fungal endophyte of a medicinal plant. Chin J Biotechnol 24:1433–1438

Garyali S, Kumar A, Reddy MS (2013) Taxol production by an endophytic fungus, *Fusarium redolens*, isolated from Himalayan yew. J Microbiol Biotechnol 23(10):1372–1380

Garyali S, Kumar A, Reddy MS (2014a) Diversity and antimitotic activity of taxol-producing endophytic fungi isolated from Himalayan yew. Ann Microbiol 64(3):1413–1422

Garyali S, Kumar A, Reddy MS (2014b) Enhancement of taxol production from endophytic fungus *Fusarium redolens*. Biotechnol Bioprocess Eng 19(5):908–915

Ghannoum MA, Kim HG, Long L (2007) Efficacy of aminocandin in the treatment of immunocompetent mice with haematogenously disseminated fluconazole-resistant candidiasis. J Antimicrob Chemother 59:556–559

Ghosh D, Ganguli BN (1961) Production of penicillin with waste mycelium of *Penicillium chrysogenum* as the sole source of nitrogen. Appl Microbiol 9:252–255

Giridharan P, Verekar SA, Khanna A, Mishra PD, Deshmukh SK (2012) Anticancer activity of sclerotiorin, isolated from an endophytic fungus *Cephalotheca faveolata* Yaguchi, Nishim. & Udagawa. Indian J Exp Biol 50:464–468

Gohar UF, Mukhtar H, Ul Haq I (2015) Isolation and screening of endophytic fungi for the reduction of taxol. Pak J Bot 47(Spec.Issue):355–358

Gohil A, Deshmukh SK, Bhattacharya V, Verekar SA, Lavhale R, Kate AS (2019) A novel isocoumarin from fungus *Exophiala spinifera* with anti-diabetic activity. Nat Prod Res. https://doi.org/10.1080/14786419.2019.1624957

Gokul RK, Manikandan R, Arulvasu C, Pandi M (2015) Antiproliferative effect of fungal taxol extracted from *Cladosporium oxysporum* against human pathogenic bacteria and human colon cancer cell line HCT 15. Spectrochim Acta A Mol Biomol Spectrosc 138:667–674

Gond SK, Kharwar RN, White JF (2014) Will fungi be the new source of the blockbuster drug taxol? Fungal Biol Rev 28:77–84

Goutam J, Sharma G, Tiwari VK, Mishra A, Kharwar RN, Ramaraj V, Koch B (2017) Isolation and characterization of "Terrein" an antimicrobial and antitumor compound from endophytic fungus *Aspergillus terreus* (JAS-2) associated from *Achyranthes aspera* Varanasi, India. Front Microbiol 8:1334. https://doi.org/10.3389/fmicb.2017.01334

Goutam J, Kharwar RN, Tiwari VK, Singh R, Sharma D (2020) Efficient production of the potent antimicrobial metabolite terrein from the fungus *Aspergillus terreus*. Nat Prod Commun 15 (3):1–5

Guo Z, She Z, Shao C, Wen L, Liu F, Zheng Z, Lin Y (2007) 1H and 13C NMR signal assignments of paecilin A and B, two new chromone derivatives from mangrove endophytic fungus *Paecilomyces* sp. (tree 1–7). Magn Reson Chem 45(9):777–780

Gurudatt PS, Priti V, Shweta S, Ramesha BT, Ravikanth G, Vasudeva R, Amna T, Deepika S, Ganeshaiah KN, Shaanker RU, Puri S, Qazi GN (2010) Attenuation of camptothecin production and negative relation between hyphal biomass and camptothecin content in endophytic fungal strains isolated from *Nothapodytes nimmoniana* Grahm (Icacinaceae). Curr Sci 98 (8):1006–1010

Hadjur C, Richard MJ, Parat MO, Jardon P, Favier A (1996) Photodynamic effect of Hypericin on lipid peroxidation and antioxidant status in melanoma cells. J Photochem Photobiol 64:375–381

Harmon AD, Weiss U, Silverton JV (1979) The structure of rohitukine, the main alkaloid of *Amoora rohituka* (syn. *Aphanamixis polystachya*) (Meliaceae). Tetrahedron Lett 8:721–724

Hawksworth DL (1991) The fungal dimension of biodiversity: magnitude, significance, and conservation. Mycol Res 95:641–655

Hawksworth DL, Luecking R (2017) Fungal diversity revisited: 2.2 to 3.8 million species. Microbiol Spectr 5(4):FUNK-0052-2016

Hemamalini V, Kumar DJ, Mukesh RA, Immaculate N, Srimathi S, Muthumary J, Kalaichelvan PT (2015) Isolation and characterization of taxol producing endophytic *Phoma* sp. from *Calotropis gigantea* and its anti-proliferative studies. JAIR 3(12):645–649

Huan QU, Min LV, Hui XU (2015) Piperine: bioactivities and structural modifications. Mini Rev Med Chem 15:145–156

Jasim B, Sahadevan N, Chithra S, Mathew J, Radhakrishnan EK (2019) Epigenetic modifier based enhancement of piperine production in endophytic *Diaporthe* sp. PF20. Proc Natl Acad Sci India Sect B Biol Sci 89:671–677

Jayanthi G, Karthikeyan K, Muthumary J (2015) Isolation and characterization of anticancer compound, taxol from an endophytic fungus *Phomopsis longicolla*. Int J Curr Res 7 (2):12727–12734

Kadkol MV, Gopalkrishnan KS, Narasimhachari N (1971) Isolation and characterization of naphthaquinone pigments from *Torula herbarum* (Pers.). Herbarin and dehydroherbarin. J Antibiot 24(4):245–248

Kamuhabwa AR, Agostinis PM, D'Hallewin MA, Baert L, de Witte PA (2001) Cellular photo destruction induced by hypericin in AY-27 rat bladder carcinoma cells. Photochem Photobiol 74 (2):126–132

Kanetkar PV, Singhal RS, Kamat MY (2007) *Gymnema sylvestre*: a memoir. Recent advances in Indian herbal drug research. J Clin Biochem Nutr 41:77–81

Kasaei A, Mobini-Dehkordi M, Mahjoubi F, Saffar B (2017) Isolation of taxol-producing endophytic fungi from Iranian yew through novel molecular approach and their effects on human breast cancer cell line. Curr Microbiol 74(6):702–709

Kato M, Sakai K, Endo A (1992) Koningic acid (heptelidic acid) inhibition of glyceraldehyde-3-phosphate dehydrogenases from various sources. Biochim Biophys Acta 1120:113–116

Kharwar RN, Verma VC, Kumar A, Gond SK, Harper JK, Hess WM, Lobkovosky E, Ma C, Ren Y, Strobel GA (2009) Javanicin, an antibacterial naphthaquinone from an endophytic fungus of neem, *Chloridium* sp. Curr Microbiol 58(3):233–238

Kharwar RN, Mishra A, Gond SK, Stierle A, Stierle D (2011) Anticancer compounds derived from fungal endophytes: their importance and future challenges. Nat Prod Rep 28:1208–1228

Koul M, Meena S, Kumar A, Sharma PR, Singamaneni V, Riyaz-Ul-Hassan S, Hamid A, Chaubey A, Prabhakar A, Gupta P, Singh S (2016) Secondary metabolites from endophytic fungus *Penicillium pinophilum* induce ROS-Mediated apoptosis through mitochondrial pathway in pancreatic cancer cells. Planta Medica 82(4):344–355

Kour A, Shawl AS, Rehman S, Sultan P, Qazi PH, Suden P, Khajuria RK, Verma V (2008) Isolation and identification of an endophytic strain of *Fusarium oxysporum* producing podophyllotoxin from *Juniperus recurva*. World J Microbiol Biotechnol 24(7):1115–1121

Koyama K, Natori S (1988) Further characterization of seven bis(naphtho-γ-pyrone) congeners of ustilaginoidins, coloring matters of *Claviceps virens* (*Ustilaginoidea virens*). Chem Pharm Bull 36:146–152

Koyama K, Ominato K, Natori S, Tashiro T, Tsuruo T (1998) Cytotoxicity and antitumor activities of fungal bis(naphtho-gamma-pyrone) derivatives. J Pharmacobiodyn 11:630–635

Kumar A, Ahmad A (2013) Biotransformation of vinblastine to vincristine by the endophytic fungus *Fusarium oxysporum* isolated from *Catharanthus roseus*. Biocatal Biotransform 31 (2):89–93

Kumar A, Patil D, Rajamohanan PR, Ahmad A (2013a) Isolation, purification and characterization of vinblastine and vincristine from endophytic fungus *Fusarium oxysporum* isolated from *Catharanthus roseus*. PLoS One 8(9):e71805

Kumar M, Qadri M, Sharma PR, Kumar A, Andotra SS, Kaur T, Kapoor K, Gupta VK, Kant R, Hamid A, Johri S (2013b) Tubulin inhibitors from an endophytic fungus isolated from *Cedrus deodara*. J Nat Prod 76(2):194–199

Kumar S, Nalli Y, Qadri M, Riyaz-Ul-Hassan S, Satti NK, Gupta V, Bhushan S, Ali A (2017) Isolation of three new metabolites and intervention of diazomethane led to separation of compound 1 & 2 from an endophytic fungus, *Cryptosporiopsis* sp. depicting cytotoxic activity. Med Chem Res 26(11):2900–2908

Kumar P, Singh B, Thakur V, Thakur A, Thakur N, Pandey D, Chand D (2019) Hyper-production of taxol from *Aspergillus fumigatus*, an endophytic fungus isolated from *Taxus* sp. of the Northern Himalayan region. Biotechnol Rep 24:e00395

Kumara MP, Sreejayan N, Priti V, Ramesha BT, Ravikanth G, Ganeshaiah KN, Vasudeva R, Mohan J, Santhoshkumar TR, Mishra PD, Viswakarma R, Shaanker RU (2010) Dysoxylum binectariferum Hook. f (Meliaceae), a rich source of rohitukine. Fitoterapia 81(2):145–148

Kumara MP, Zuehlke S, Priti V, Ramesha BT, Shweta S, Ravikanth G, Vasudeva R, Santhoshkumar TR, Spiteller M, Shaanker RU (2012) *Fusarium proliferatum*, an endophytic fungus from *Dysoxylum binectariferum* Hook. f, produces rohitukine, a chromane alkaloid possessing anticancer activity. Antonie Van Leeuwenhoek 101(2):323–329

Kumaran RS, Hur BK (2009) Screening of species of the endophytic fungus *Phomopsis* for the production of the anticancer drug taxol. Biotechnol Appl Biochem 54(1):21–30

Kumaran RS, Muthumary J, Hur BK (2008a) Taxol from *Phyllosticta citricarpa*, a leaf spot fungus of the angiosperm *Citrus medica*. J Biosci Bioeng 106:103–106

Kumaran RS, Muthumary J, Hur BK (2008b) Isolation and identification of taxol, an anticancer drug from *Phyllosticta melochiae* Yates, an endophytic fungus of *Melochia corchorifolia* L. Food Sci Biotechnol 17(6):1246–1253

Kumaran RS, Muthumary J, Hur BK (2008c) Production of taxol from *Phyllosticta spinarum*, an endophytic fungus of *Cupressus* sp. Eng Life Sci 8:438–446

Kumaran RS, Muthumary J, Kim EK, Hur BK (2009) Production of taxol from *Phyllosticta dioscoreae*, a leaf spot fungus isolated from *Hibiscus rosa-sinensis*. Biotechnol Bioproc Eng 14:76–83

Kumaran RS, Kim HJ, Hur BK (2010) Taxol promising fungal endophyte, *Pestalotiopsis* species isolated from *Taxus cuspidata*. J Biosci Bioeng 110(5):541–546

Kumaran RS, Jung H, Kim HJ (2011) *In vitro* screening of taxol, an anticancer drug produced by the fungus, *Colletotrichum capsici*. Eng Life Sci 11:264–271

Kumaran RS, Choi YK, Lee S, Jeon HJ, Jung H, Kim HJ (2012) Isolation of taxol, an anticancer drug produced by the endophytic fungus, *Phoma betae*. Afr J Biotechnol 11(4):950–960

Kusari S, Lamshoeft M, Zuehlke S, Spiteller M (2008) An endophytic fungus from *Hypericum perforatum* that produces hypericin. J Nat Prod 71(2):159–162

Kusari S, Zuhlke S, Spiteller M (2009a) An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogs. J Nat Prod 72(1):2–7

Kusari S, Lamshoeft M, Spiteller M (2009b) *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. J Appl Microbiol 107(3):1019–1030

Kusari S, Zuhlke S, Kosuth J, Cellarova E, Spiteller M (2009c) Light-independent metabolomics of endophytic *Thielavia subthermophila* provides insight into microbial hypericin biosynthesis. J Nat Prod 72(10):1825–1835

Kusari S, Verma VC, Lamshoeft M, Spiteller M (2012) An endophytic fungus from *Azadirachta indica* A. Juss.that produces azadirachtin. World J Microbiol Biotechnol 28:1287–1294

Kusari S, Singh S, Jayabaskaran C (2014) Rethinking production of Taxol®(paclitaxel) using endophyte biotechnology. Trends Biotechnol 32(6):304–311

Kwok KK, Vincent EC, Gibson JN (2016) Antineoplastic drugs in pharmacology and therapeutics for dentistry, 7th edn, Chapter 036, pp 530–562

Leiter J, Downing V, Hartwell JL, Shear MJ (1950) Damage induced in sarcoma 37 with podophyllin, podophyllotoxin alpha-peltatin, beta-peltatin, and quercetin. J Natl Cancer Inst 10 (6):1273–1293

Li J, Sidhu R, Ford E et al (1998) The induction of taxol production in the endophytic fungus—*Periconia* sp from *Torreya grandifolia*. J Ind Microbiol Biotechnol 20:259–264

Liao WY, Shen CN, Lin LH, Yang YL, Han HY, Chen JW, Kuo SC, Wu SH, Liaw CC (2012) Asperjinone, a nor-neolignan, and terrein, a suppressor of ABCG2-expressing breast cancer cells, from thermophilic *Aspergillus terreus*. J Nat Prod 75(4):630–635

Liu HM, Kiuchi F, Tsuda Y (1992) Isolation and structure elucidation of gymnemic acids, anti-sweet principles of *Gymnema sylvestre*. Chem Pharm Bull 40:1366–1375

Liu HW, Yang YF, Li YY, Wang S, Qiu DY (2016) Cloning and expression of GGPP synthase gene from paclitaxel-producing endophytic fungi (*Penicillium aurantiogriseum*) in *Corylus avellana*. Guangxi Zhiwu 36(4):456–461

Lyu HN, Liu HW, Keller NP, Yin WB (2020) Harnessing diverse transcriptional regulators for natural product discovery in fungi. Nat Prod Rep 37(1):6–16

Magotra A, Kumar M, Kushwaha M, Awasthi P, Raina C, Gupta AP, Shah BA, Gandhi SG, Chaubey A (2017) Epigenetic modifier induced enhancement of fumiquinazoline C production in *Aspergillus fumigatus* (GA-L7): an endophytic fungus from Grewia asiatica L. AMB Express 7(1):1–10

Maneesai P, Norman S, Krongkarn C (2012) Piperine is anti-hyperlipidemic and improves endothelium-dependent vasorelaxation in rats on a high cholesterol diet. J Physiol Biomed Sci 25:27–30

Manoharachary C, Sridhar K, Singh R, Adholeya A, Suryanarayanan TS, Rawat S, Johri BN (2005) Fungal biodiversity: distribution, conservation and prospecting of fungi from India. Curr Sci 89:58–71

Mishra A, Gond SK, Kumar A, Sharma VK, Verma SK, Kharwar RN, Sieber TN (2012) Season and tissue type affect fungal endophyte communities of the Indian medicinal plant *Tinospora cordifolia* more strongly than geographic location and their antimicrobial potential. Microb Ecol 64:388–398

Mishra PD, Deshmukh SK, Kulkarni-Almeida A, Roy S, Jain S, Verekar SA, Balakrishnan A, Vishwakarma R (2013) Anti-inflammatory and anti-diabetic naphthoquinones from an endophytic fungus. Indian J Chem Sect B 52:555–557

Mishra PD, Verekar SA, Deshmukh SK, Joshi KS, Fiebig HH, Kelter G (2015) Altersolanol A: a selective cytotoxic anthraquinone from a *Phomopsis* sp. Lett Appl Microbiol 60(4):387–391

Morgan ED (2009) Azadirachtin, a scientific gold mine. Bioorg Med Chem 17(12):4096–4105

Moussa M, Ebrahim W, Bonus M, Gohlke H, Mándi A, Kurtán T, Hartmann R, Kalscheuer R, Lin W, Liu Z, Proksch P (2019) Co-culture of the fungus *Fusarium tricinctum* with *Streptomyces lividans* induces production of cryptic naphthoquinone dimers. RSC Adv 9(3):1491–1500

Mukhopadhyay T, Roy K, Coutinho L, Rupp RH, Ganguli BN, Fehlhaber HW (1987) Fumifungin, a new antifungal antibiotic from *Aspergillus fumigatus* Fresenius 1863. J Antibiot 40 (7):1050–1052

Mukhopadhyay T, Roy K, Bhat RG, Sawant SN, Blumbach J, Ganguli BN, Fehlhaber HW, Kogler H (1992) Deoxymulundocandin—a new echinocandin type antifungal antibiotic. J Antibiot 45 (5):618–623

Mukhopadhyay T, Bhat RG, Roy K, Vijayakumar EKS, Ganguli BN (1998) Aranochlor A and Aranochlor B, two new metabolites from *Pseudoarachniotus roseus*. J Antibiot 51(4):439–441

Nagarajan R, Narasimhachari N, Kadkol MV, Gopalkrishnan KS (1971) Structure of herbarin. J Antibiot 24(4):249–252

Nahrstedt A, Butterweck V (1997) Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. Pharmacopsychiatry 30(Suppl 2):129–134

Naik RG, Kattige SL, Bhat SV, Alreja B, de Souza NJ, Rupp RH (1988) An anti-inflammatory cum immunomodulatory piperidinylbenzopyranone from *Dysoxylum binectariferum*: isolation, structure and total synthesis. Tetrahedron 44:2081–2086

Nalli Y, Mirza DN, Wani ZA, Wadhwa B, Mallik FA, Raina C, Chaubey A, Riyaz-Ul-Hassan S, Ali A (2015) Phialomustin A-D, new antimicrobial and cytotoxic metabolites from an endophytic fungus, Phialophora mustea. RSC Adv 5:95307–95312

Nalli Y, Arora P, Khan S, Malik F, Riyaz-Ul-Hassan S, Gupta V, Ali A (2019) Isolation, structural modification of macrophin from endophytic fungus *Phoma macrostoma* and their cytotoxic potential. Med Chem Res 28(3):260–266

Narasimhachari N, Vining LC (1963) Studies on the pigments of *Penicillium herquei*. Can J Chem 41:641–648

Narasimhachari N, Vining LC (1972) Herqueichrysin, a new phenalenone antibiotic from *Penicillium herquei*. J Antibiot 25(3):155–162

Narasimhachari N, Gopalkrishnan KS, Haskins RH, Vining LC (1963) Production of the antibiotic atrovenetin by a strain of *Penicillium herquei* Bainier and Sartory. Can J Microbiol 9:134–136

Naseer S, Bhat KA, Qadri M, Riyaz-Ul-Hassan S, Malik FA, Khuroo MA (2017) Bioactivity-guided isolation, antimicrobial and cytotoxic evaluation of secondary metabolites from *Cladosporium tenuissimum* associated with *Pinus wallichiana*. Chem Select 2(3):1311–1314

Nisbet AJ (2000) Azadirachtin from the neem tree *Azadirachta indica*: its action against insects. An Soc Entomol Brasil 29(4):615–632

Nithya K, Muthumary J (2009) Growth studies of *Colletotrichum gloeosporioides* (Penz.) Sacc.-a taxol producing endophytic fungus from *Plumeria acutifolia*. Ind J Sci Technol 6:14–19

Padhi S, Masi M, Cimmino A, Tuzi A, Jena S, Tayung K, Evidente A (2019) Funiculosone, a substituted dihydroxanthene-1, 9-dione with two of its analogues produced by an endolichenic fungus *Talaromyces funiculosus* and their antimicrobial activity. Phytochemistry 157:175–183

Pamphile JA, dos Santos Ribeiro MA, Polonio JC (2017) Secondary metabolites of endophyte fungi: techniques and biotechnological approaches. In: Diversity and benefits of microorganisms from the tropics. Springer, Cham, pp 185–206

Pandi M, Kumaran RS, Choi YK, Kim HJ, Muthumary J (2011) Isolation and detection of taxol, an anticancer drug produced from *Lasiodiplodia theobromae*, an endophytic fungus of the medicinal plant *Morinda citrifolia*. Afr J Biotechnol 10(8):1428–1435

Paranagama PA, Wijeratne EMK, Burns AM, Marron MT, Gunatilaka MK, Arnold AE, Gunatilaka AAL (2007) Heptaketides from *Corynespora* sp. inhabiting the cavern beard lichen, *Usnea cavernosa*: first report of metabolites of an endolichenic fungus. J Nat Prod 70:1700–1705

Parthasarathy R, Sathiyabama M (2014) Gymnemagenin-producing endophytic fungus isolated from a medicinal plant *Gymnema sylvestre* R.Br. Appl Biochem Biotechnol 172(6):3141–3152

Pathania AS, Guru SK, Ul Ashraf N, Riyaz-Ul-Hassan S, Ali A, Tasduq SA, Malik F, Bhushan S (2015) A novel stereo bioactive metabolite isolated from an endophytic fungus induces caspase dependent apoptosis and STAT-3 inhibition in human leukemia cells. Eur J Pharmacol 765:75–85

Pothuraju R, Sharma RK, Chagalamarri J, Jangra S, Kumar Kavadi P (2014) A systematic review of *Gymnema sylvestre* in obesity and diabetes management. J Sci Food Agric 94(5):834–840

Puri SC, Verma V, Amna T, Qazi GN, Spiteller M (2005) An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. J Nat Prod 68:1717–1719

Puri SC, Nazir A, Chawla R, Arora R, Riyaz-ul-Hasan S, Amna T, Ahmed B, Verma V, Singh S, Sagar R, Sharma A, Kumar R, Sharma RK, Qazi GN (2006) The endophytic fungus *Trametes hirsuta* as a novel alternative source of Podophyllotoxin and related aryltetralin lignans. J Biotechnol 122(4):494–510

Rahier NJ, Molinier N, Long C, Deshmukh SK, Kate AS, Ranadive P, Verekar SA, Jiotode M, Lavhale RR, Tokdar P, Balakrishnan A, Meignan S, Robichon C, Gomes B, Aussagues Y, Samson A, Sautel F, Bailly C (2015) Anticancer activity of koningic acid and semisynthetic derivatives. Bioorg Med Chem 23:3712–3721

Rao GS, Sinsheimer JE (1971) Constituents *Gymnema sylvestre* leaves VIII: isolation, chemistry, and derivatives of gymnemagenin and gymnestrogenin. J Pharm Sci 60(2):190–1933

Rateb ME, Ebel R (2011) Secondary metabolites of fungi from marine habitats. Nat Prod Rep 28 (2):290–344

Rathore PK, Arathy V, Attimarad VS, Kumar P, Roy S (2016) *In-silico* analysis of gymnemagenin from *Gymnema sylvestre* (Retz.) R.Br. with targets related to diabetes. J Theor Biol 391:95–101

Rebecca AIN, Kumar DJM, Srimathi S, Muthumary J, Kalaichelvan PT (2011) Isolation of *Phoma* species from *Aloe vera*: an endophyte and screening the fungus for Taxol production. World J Sci Technol 1(11):23–31

Rebecca AIN, Hemamalini V, Kumar DJM, Srimathi S, Muthumary J, Kalaichelvan PT (2012) Endophytic *Chaetomium* sp. from *Michelia champaca* L. and its taxol production. J Acad Indus Res 1(2):68–72

Reen FJ, Romano S, Dobson AD, O'Gara F (2015) The sound of silence: activating silent biosynthetic gene clusters in marine microorganisms. Mar Drugs 13:4754–4783

Rehman S, Shawl AS, Kour A, Andrabi R, Sudan P, Sultan P, Verma V, Qazi GN (2008) An endophytic *Neurospora* sp. from *Nothapodytes foetida* producing camptothecin. Appl Biochem Microbiol 44:203–209

Rehman S, Shawl AS, Kour A, Sultan P, Ahmad K, Khajuria R, Qazi GN (2009a) Comparative studies and identification of camptothecin produced by an endophyte at shake flask and bioreactor. Nat Prod Res 23(11):1050–1057

Rehman S, Shawl AS, Sultana S, Kour A, Riyaz-ul-Hassan S, Qazi GN (2009b) *In vitro* cytotoxicity of an endophytic fungus isolated from *Nothapodytes foetida*. Ann Microbiol 59(1):157–161

Romano S, Jackson S, Patry S, Dobson A (2018) Extending the "One Strain Many Compounds" (OSMAC) principle to marine microorganisms. Mar Drugs 16:244

Rowinsky EK (1997) The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu Rev Med 48(1):353–374

Roy K, Mukhopadhyay T, Reddy GCS, Desikan KR, Ganguli BN (1987) Mulundocandin, a new lipopeptide antibiotic. I. Taxonomy, fermentation, isolation and characterization. J Antibiot 40:275–280

Roy K, Mukhopadhyay T, Reddy GCS, Desikan KR, Rupp RH, Ganguli BN (1988) Aranorosin, a novel antibiotic from *Pseudoarachniotus roseus*. J Antibiot 41(12):1780–1784

Roy K, Vijayakumar EKS, Mukhopadhyay T, Chatterjee S, Bhat RG, Blumbach J, Ganguli BN (1992) Aranorosinol A and aranorosinol B, two new metabolites from *Pseudoarachniotus roseus*: production, isolation, structure elucidation and biological properties. J Antibiot 45 (10):1592–1598

Roy K, Chatterjee S, Deshmukh SK, Vijayakumar EKS, Ganguli BN, Fehlhaber HW (1996) Orbuticin, a new secondary metabolite from *Acremonium butyri*. J Antibiot 49(11):1186–1187

Sabir F, Mishra S, Sangwan RS, Jadaun JS, Sangwan NS (2013) Qualitative and quantitative variations in withanolides and expression of some pathway genes during different stages of morphogenesis in *Withania somnifera* Dunal. Protoplasma 250:539–549

San Gupta R, Chandran RR, Divekar PV (1966) Botryodiplodin, a new antibiotic from *Botryodiplodia theobromae* II. Production, isolation and biological properties. Indian J Exp Biol 4(3):152–153

Sangwan NS, Sabir F, Mishra S, Bansal S, Sangwan RS (2014) Withanolides from *Withania somnifera* Dunal: development of cellular technology and their production. Recent Pat Biotechnol 8:25–35

Sathiyabama M, Parthasarathy R (2018) Withanolide production by fungal endophyte isolated from *Withania somnifera*. Nat Prod Res 32(13):1573–1577

Schlingmann G, Milne L, Williams DR, Carter GT (1998) Cell wall active antifungal compounds produced by the marine fungus Hypoxylon oceanicum LL-15G256 II. Isolation and structure determination. J Antibiot 51(3):303–316

Seetharaman P, Gnanasekar S, Chandrasekaran R, Chandrakasan G, Kadarkarai M, Sivaperumal S (2017) Isolation and characterization of anticancer flavone chrysin (5,7-dihydroxy flavone)-producing endophytic fungi from *Passiflora incarnata* L. leaves. Ann Microbiol 67(4):321–331

Shah M, Deshmukh SK, Verekar SA, Kate AS, Rekha V, Kulkarni-Almeida A (2015) Anti-inflammatory properties of mutolide, a major constituent of the fungus. PM0651419. SpringerPlus 4:706. https://doi.org/10.1186/s40064-015-1493-6

Sharma VK, Kumar J, Singh DK, Mishra A, Verma SK, Gond SK, Kumar A, Singh N, Kharwar RN (2017) Induction of cryptic and bioactive metabolites through natural dietary components in an endophytic fungus *Colletotrichum gloeosporioides*. Front Microbiol 8:1126

Sharma V, Sharma N, Arora D, Singamaneni V, Kumar A, Kushwaha M, Bhushan S, Jaglan S, Gupta P (2018a) Valproic acid induces three novel cytotoxic secondary metabolites in *Diaporthe* sp., an endophytic fungus from *Datura inoxia* Mill. Bioorg Med Chem Lett 28 (12):2217–2221

Sharma N, Kushwaha M, Arora D, Jain S, Singamaneni V, Sharma S, Shankar R, Bhushan S, Gupta P, Jaglan S (2018b) New cytochalasin from *Rosellinia sanctae-cruciana*, an endophytic fungus of *Albizia lebbeck*. J Appl Microbiol 125(1):111–120

Shrestha K, Strobel GA, Shrivastava SP, Gewali MB (2001) Evidence for paclitaxel from three new endophytic fungi of Himalayan yew of Nepal. Planta Medica 67(04):374–376

Shweta S, Zuehlke S, Ramesha BT, Priti V, Kumar PM, Ravikanth G, Spiteller M, Vasudeva R, Shaanker RU (2010) Endophytic fungal strains of *Fusarium solani*, from *Apodytes dimidiate* E. Mey.ex Arn (Icacinaceae) produce camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin. Phytochemistry 71:117–122

Singh VP, Yedukondalu N, Sharma V, Kushwaha M, Sharma R, Chaubey A, Kumar A, Singh D, Vishwakarma RA (2018) Lipovelutibols A–D: cytotoxic lipopeptaibols from the himalayan cold habitat fungus *Trichoderma velutinum*. J Nat Prod 81(2):219–226

Soliman SSM, Tsao R, Raizada MN (2011) Chemical inhibitors suggest endophytic fungal paclitaxel is derived from both mevalonate and non-mevalonate-like pathways. J Nat Prod 74 (12):2497–2504

Soliman SSM, Greenwood JS, Bombarely A, Mueller LA, Tsao R, Mosser DD, Raizada MN (2015) An endophyte constructs fungicide-containing extracellular barriers for its host plant. Curr Biol 25(19):2570–2576

Soliman SSM, Mosa KA, El-Keblawy AA, Husseiny MI (2017) Exogenous and endogenous increase in fungal GGPP increased fungal taxol production. Appl Microbiol Biotechnol 101 (20):7523–7533

Somjaipeng S, Medina A, Magan N (2016) Environmental stress and elicitors enhance taxol production by endophytic strains of *Paraconiothyrium variabile* and *Epicoccum nigrum*. Enzyme Microb Technol 90:69–75

Sood RS, Roy K, Reddy GC, Reden J, Ganguli BN (1982) 3-Methoxy-2,5-toluquinone from *Aspergillus* sp. HPL Y-30,212. Fermentation, isolation, characterization and biological properties. J Antibiot 35(8):985–987

Sreekanth D, Syed A, Sarkar S, Sarkar D, Santhakumari B, Ahmad A, Khan MI (2009) Production, purification, and characterization of taxol and 10-DABIII from a new endophytic fungus *Gliocladium* sp. isolated from the Indian yew tree, *Taxus baccata*. J Microbiol Biotechnol 19 (11):1342–1347

Sreekanth D, Sushim GK, Syed A, Khan BM, Ahmad A (2011) Molecular and morphological characterization of a taxol-producing endophytic fungus *Gliocladium* sp., from *Taxus baccata*. Mycobiology 39(3):151–157

Stahelin HF (1996) The history of Cyclosporin A (Sandimmune) revisited: another point of view. Experientia 52:5–13

Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. Science 260(5105):214–217

Subban K, Singh S, Chelliah J, Subban K, Johnpaul M, Subramani R (2017) Fungal 7-epi-10-Deacetyltaxol produced by an endophytic *Pestalotiopsis microspora* induces apoptosis in human hepatocellular carcinoma cell line (HepG2). BMC Complement Altern Med 17(1):504

Sunila ES, Kuttan G (2004) Immunomodulatory and antitumor activity of *Piper longum* Linn.and piperine. J Ethnopharmacol 90:339–346

Taware R, Abnave P, Patil D, Rajamohananan PR, Raja R, Soundararajan G, Kundu GC, Ahmad A (2014) Isolation, purification and characterization of Trichothecinol-A produced by endophytic fungus *Trichothecium* sp. and its antifungal, anticancer and antimetastatic activities. Sustain Chem Process 2:1–9

Teiten MH, Mack F, Debbab A, Aly AH, Dicato M, Proksch P, Diederich M (2013) Anticancer effect of altersolanol A, a metabolite produced by the endophytic fungus *Stemphylium globuliferum*, mediated by its proapoptotic and anti-invasive potential via the inhibition of NF-jB activity. Bioorg Med Chem 21:3850–3858

Tolbert JA (2003) Lovastatin and beyond: the history of HMG-CoA reductase inhibitors. Nat Rev Drug Discov 2:517–526

Vander Molen KM, Darveaux BA, Chen WL, Swanson SM, Pearce CJ, Oberlies NH (2014) Epigenetic manipulation of a filamentous fungus by the proteasome-inhibitor bortezomib induces the production of an additional secondary metabolite. RSC Adv 4(35):18329–18335

Vasundhara M, Baranwal M, Sivaramaiah N, Kumar A (2017) Isolation and characterization of trichalasin-producing endophytic fungus from *Taxus baccata*. Ann Microbiol 67(3):255–261

Vedantham K, Chaterji S, Kitsongsermthon J, Park K, Garner J (2010) Future outlook for drug eluting stents. In: Drug-device combination products. Woodhead Publishing, Cambridge, UK, pp 117–153

Venkatachalam R, Subban K, Paul MJ (2008) Taxol from *Botryodiplodia theobromae* (BT 115)-an endophytic fungus of *Taxus baccata*. J Biotechnol 136:S189–S190

Vennila R, Thirunavukkarasu SV, Muthumarya J (2010) *In-vivo* studies on anticancer activity of taxol isolated from an endophytic fungus *Pestalotiopsis pauciseta* Sacc. VM1. Asian J Pharm Clin Res 3(4):30–34

Vennila R, Kamalraj S, Muthumary J (2012) *In vitro* studies on anticancer activity of fungal taxol against human breast cancer cell line MCF-7 cells. Biomed Aging Pathol 2(1):16–18

Verekar SA, Mishra PD, Sreekumar ES, Deshmukh SK, Fiebig HH, Kelter G, Maier A (2014) Anticancer activity of a new depsipeptide compound isolated from an endophytic fungus. J Antibiot 67:697–701

Verma VC, Kharwar RN, Strobel GA (2009) Chemical and functional diversity of natural products from plant associated endophytic fungi. Nat Prod Commun 4(11):1511–1532

Verma VC, Lobkovsky E, Gange AC, Singh SK, Prakash S (2011) Piperine production by endophytic fungus *Periconia* sp. isolated from *Piper longum* L. J Antibiot 64:427–431

Vijayakumar EKS, Roy K, Chatterjee S, Deshmukh SK, Ganguli BN, Kogler H, Fehlhaber HW (1996) Arthrichitin a new cell wall active metabolite from *Arthrinium phaeospermum*. J Org Chem 61:6591–6593

Vijayakumar EKS, Roy K, Hiramath CP, Deshmukh SK, Mukhopadhyay T, Kogler H (2001) L970843 and L970844, two new antifungal metabolites from an unidentified fungal species HIL Y-903146. J Antibiot 54:973–976

Wall ME, Wani MC, Cook CE et al (1966) Plant antitumor agents I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *C. acuminata*. J Am Chem Soc 88(16):3888–3890

Wani MC, Taylor HL, Wall ME, Coggon P, McPhail AT (1971) Plant antitumor agents VI: the isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. J Am Chem Soc 93:2325–2327

Warn PA, Sharp A, Morrissey G, Denning DW (2010) Activity of aminocandin (IP960; HMR3270) compared with amphotericin B, itraconazole, caspofungin and micafungin in neutropenic murine models of disseminated infection caused by itraconazole-susceptible and -resistant strains of *Aspergillus fumigatus*. Int J Antimicrob Agents 35:146–151

White PT, Subramanian C, Motiwala HF, Cohen MS (2016) Natural withanolides in the treatment of chronic diseases. Adv Exp Med Biol 928:329–373

Wu B, Hussain M, Zhang W, Stadler M, Liu X, Xiang M (2019) Current insights into fungal species diversity and perspective on naming the environmental DNA sequences of fungi. Mycology 10 (3):127–140

Yang Y, Zhao H, Barrero RA, Zhang B, Sun G, Wilson IW, Xie F, Walker KD, Parks JW, Bruce R et al (2014) Genome sequencing and analysis of the paclitaxel-producing endophytic fungus *Penicillium aurantiogriseum* NRRL 62431. BMC Genomics 15:69/1–69/14. 14 pp

Yedukondalu N, Arora P, Wadhwa B, Malik FA, Vishwakarma RA, Gupta VK, Riyaz-Ul-Hassan S, Ali A (2017) Diapolic acid A-B from an endophytic fungus, *Diaporthe terebinthifolii* depicting antimicrobial and cytotoxic activity. J Antibiot 70(2):212–215

Zaki AG, El-Shatoury EH, Ahmed AS, Al-Hagar OE (2020) Response surface methodology-mediated improvement of the irradiated endophytic fungal strain, *Alternaria brassicae* AGF041 for Huperzine A-hyperproduction. Lett Appl Microbiol. https://doi.org/10.1111/lam.13435

Zilla MK, Qadri M, Pathania AS, Strobel GA, Nalli Y, Kumar S, Guru SK, Bhushan S, Singh SK, Vishwakarma RA, Riyaz-Ul-Hassan S (2013) Bioactive metabolites from an endophytic *Cryptosporiopsis* sp. inhabiting *Clidemia hirta*. Phytochemistry 95:291–297

Part IV

Bioprospecting of Fungi

Fungal Pigment Research in India: An Overview

17

Sunil K. Deshmukh, Ajay Lagashetti, Sanjay K. Singh, Hina F. Badgujar, and Umesh Kumar

Abstract

Color is an integral part of human life predominantly used for food, textile, craft, painting, etc. Plants, lichens, and insects have been sources of natural colorants during the early part of civilization. Over the years, focus was shifted toward synthetic colors, mainly due to high demand, low production cost, and issues related to stability. But it is well recognized that artificial dyes cause serious side effects like hyperactivity, allergies, and even cancer. Some countries have banned or heavily regulated these additives. Due to such serious side effects, colors obtained from natural hosts like plants, insects, and other microorganisms have emerged as alternative option. Due to certain ecological limitations in the use of plants and insects as natural sources, microorganisms, however, are considered as better choice for pigment production. Among the microbes, fungi are one of the sources of pigments used in the food industry. Fungi like *Aspergillus*, *Fusarium*, *Penicillium*, *Monascus*, *Trichoderma*, *Laetiporus*, and others are reported to produce various pigments, viz., red, purple, yellow, brown, orange, and green. These pigments belong to the chemical class of carotenoids, melanins, azaphilones, flavins, phenazines, quinones, polyketides etc. In this review, bio-active pigments produced by Indian fungi and the challenges faced during

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the production of fungal pigments and how to deal with these challenges using advanced techniques and nanotechnology are reviewed.

Keywords

Red pigment · Fungal metabolites · Azaphilone pigments · Metabolic engineering · Nano-emulsions

17.1 Introduction

Color plays an important role in human life and has been used in food, clothes, furniture, etc. (Lagashetti et al. 2019; Ramesh et al. 2019). Coloring of food has been known since centuries. During the early days, food colorants were of natural sources like turmeric, indigo, saffron, various flowers, paprika (*Capsicum annuum*), and berries (Gulrajani 2001; Aberoumand 2011).

History dates back to the 1800s where there was a shift toward the development of synthetic colors for enhancing chemical stability, low production cost, and a larger range of hue and shades. In 1856, the English chemist Sir William Henry Perkin discovered the first synthetic organic dye called mauveine (Rao et al. 2017) which promoted the discovery of several other synthetic dyes manufactured by chemical reactions. These were used in the food and pharmaceutical industries. Commonly used synthetic dyes include tartrazine, sunset yellow, amaranth, allura red, quinolone yellow, brilliant blue, and indigo carmine. Later on, it was realized that synthetic colors have many drawbacks and were found to be hazardous to health and environment. Some of them are found be allergic, toxic, and carcinogenic (Ratna and Padhi 2012; Arora 2014). Because of these adverse effects, public demand for natural pigments over the synthetic pigments has been increasing globally. Moreover, increasing consumer awareness as well as strict environmental rules and regulation has drastically shifted global interest toward the use of natural colors (Caro et al. 2017).

Natural colors are dyes or pigments which have their origin from natural sources, like plants, animals, microbes, or mineral sources. Natural colors have no toxic accumulation after they are consumed by consumers and are safe for use in all age groups. Currently, many studies have shown that microbes (bacteria, fungi, and algae) are an excellent alternative source of natural pigments. Due to their rapid growth, ease of processing, and simple cultural techniques involved, microorganisms are becoming popular in large-scale production of pigments. Natural pigments from fungi have been well recognized worldwide. Pigments of diverse chemical classes such as carotenoids, melanins, azaphilones, flavins, phenazines, quinones, monascin, violacein, indigo, etc. have been discovered from various fungal species (Caro et al. 2017; Lagashetti et al. 2019; Ramesh et al. 2019). In addition to coloring property, most of these fungal pigments possess beneficial attributes such as antioxidant, antimicrobial, antiviral, and anticancer activities. These fungal pigments have been reported to be used in extensive applications in

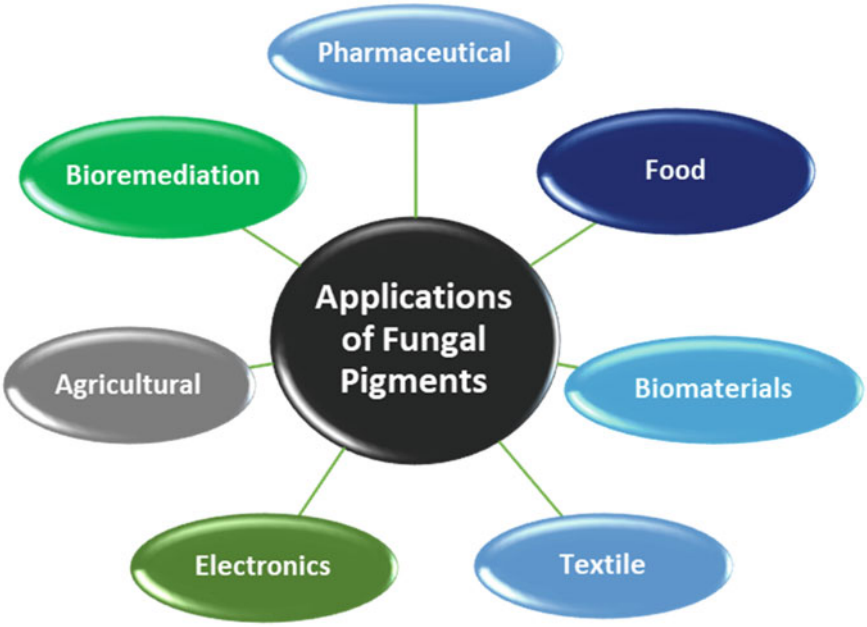

Fig. 17.1 Application of fungal pigments in various industries

different industries including pharmaceutical, food, textile, cosmetic, paint, food, and beverage industries (Lagashetti et al. 2019; Ramesh et al. 2019) (Fig. 17.1).

17.2 Investigations on Fungal Pigments in India

India is a country with rich biodiversity. Reviews reveal that pigments of wide range of chemical classes have been discovered from different groups of fungi in India (Mukherjee et al. 2017; Ramesh et al. 2019). In addition to their coloring property, some of these pigments have exhibited different biological properties such as antibacterial, antifungal, antioxidant, anticancer, antiviral, antimalarial, and insecticidal activities (Rao et al. 2017; Lagashetti et al. 2019; Sen et al. 2019). On the other hand, numerous fungi have been reported from India showing very good pigment production potential, but very less attention has been paid toward the application of these pigments in different industries.

Some institutions including industries in India are actively involved in isolation and screening of fungi for bioactive pigments such as Piramal Enterprises Ltd. Mumbai, Banaras Hindu University, IIM Jammu, Agharkar Research Institute, Pune, and others. Some of the reported pigment producing fungal isolates are depicted in Fig. 17.2.

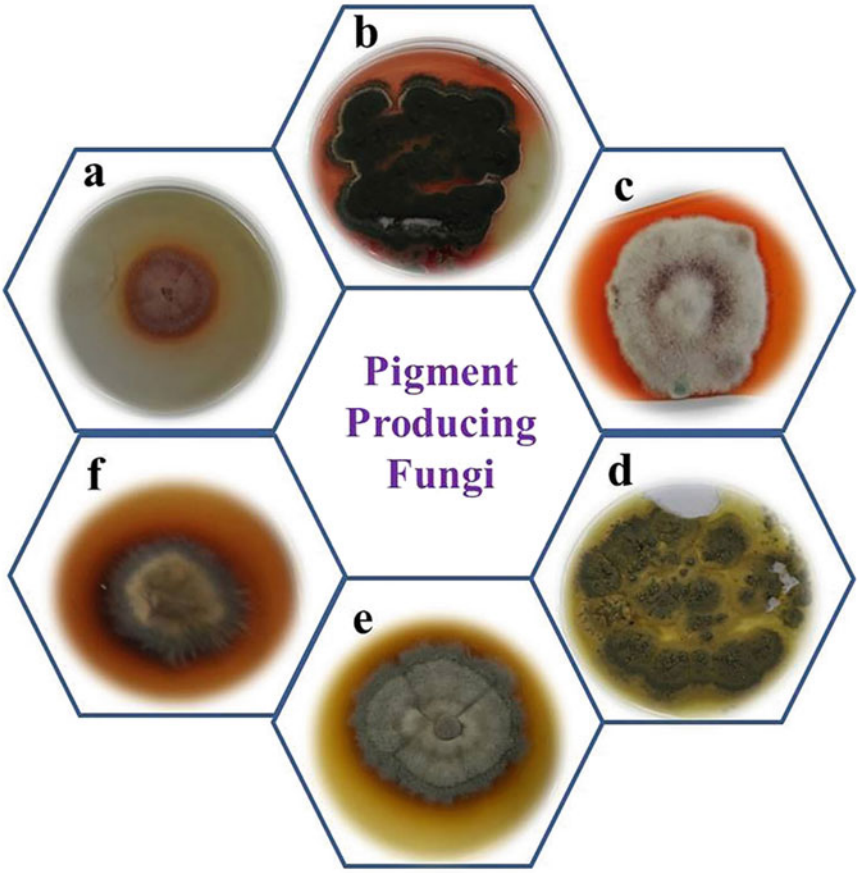

Fig. 17.2 Some pigment-producing fungi: (a) *Monascus* sp. (b) *Talaromyces purpureogenus* (c) *Fusarium* sp. (d) *Emericella versicolor* (e) *Alternaria* sp. (f) unidentified fungus

17.3 Pigment-Producing Fungi from India

Numerous fungal species belonging to different taxonomic genera like *Alternaria*, *Chlorociboria*, *Curvularia*, *Fusarium*, *Ganoderma*, *Monascus*, *Penicillium*, *Scytalidium*, *Talaromyces*, *Trichoderma*, etc. producing colorful pigments of wide range of chemical classes have been discovered from India (Table 17.1 and Fig. 17.2).

Melanin, a black pigment from an endophyte *Phyllosticta capitalensis*, was characterized by chemical tests and UV-visible, IR, and ESR spectral analysis (Suryanarayanan et al. 2004). *Talaromyces marneffei* (syn. *Penicillium marneffei*) has been reported to produce a brick red pigment. The structural ellucidataion of this pigment was done by UV-VIS, fluorescence, atomic absorption (AAS), infra red

Table 17.1 Pigment-producing fungi isolated from India

Sr. no.	Pigment-producing fungi	Pigment	Color	Bioactivity	References
1	*Alternaria alternata*	Uncharacterized	Brown	–	Devi and Karuppan (2014)
2	*Alternaria alternata*	Uncharacterized	Brilliant olive	–	Sharma et al. (2012)
3	*Alternaria longissima*	Ustilaginoidin A	Yellow	Antidiabetic	Deshmukh (2018)
4	*Aspergillus* sp.	Uncharacterized	Yellow	–	Narendrababu and Shishupala (2017)
5	*Aspergillus* sp.	Uncharacterized	Yellow	–	Narendrababu and Shishupala (2017)
6	*Aspergillus* sp.	Uncharacterized	Yellow	–	Saravanan and Radhakrishnan (2016)
7	*Aspergillus* sp.	Uncharacterized	Brown	–	Saravanan and Radhakrishnan (2016)
8	*Aspergillus* sp.	Uncharacterized	Black	Antibacterial activity	Saravanan and Radhakrishnan (2016)
9	*Aspergillus terreus*	Uncharacterized	Yellow	–	Akilandeswari and Pradeep (2017)
10	*Cephalotheca faveolata*	Sclerotiorin	Orange-yellow	Cytotoxic	Giridharan et al. (2012)
11	*Chloridium* sp.	Javanicin	Red–orange	Antibacterial	Kharwar et al. (2009)
12	*Cochliobolus kusanoi*	Oosporein	Red	Antibacterial, antifungal, antioxidant, cytotoxic	Alurappa et al. (2014), Ramesha et al. (2015)
13	*Curvularia lunata*	Uncharacterized	Brown	–	Sharma et al. (2012)
14	*Dendryphion nanum*	Herbarin	Bright yellow	Anti-inflammatory and antidiabetic	Mishra et al. (2013)

(continued)

Table 17.1 (continued)

Sr. no.	Pigment-producing fungi	Pigment	Color	Bioactivity	References
15	*Dendryphion nanum*	Herbarin's analog	Bright yellow	Anti-inflammatory and antidiabetic	Mishra et al. (2013)
16	*Fusarium chlamydosporum*	Long-chain hydrocarbons with poly-unsaturated groups	Red	Cytotoxic activity	Soumya et al. (2018)
17	*Fusarium fujikuroi* (formerly known as *Fusarium moniliforme*)	2-(4-((3*E*,5*E*)-14-aminotetr adeca-3,5-dienyloxy)butyl)-1,2,3,4-tetrahydroisoquinol in-4-ol (ATDBTHIQN)	Pink	Larvicidal activity	Pradeep et al. (2015)
18	*Fusarium fujikuroi*	Uncharacterized	Pinkish violet	–	Pradeep and Pradeep (2013)
19	*Fusarium* sp.	Uncharacterized	Red	–	Saravanan and Radhakrishnan (2016)
20	*Ganoderma applanatum*	Uncharacterized	Orange	–	Karuppan et al. (2014)
21	*Ganoderma lucidum*	Uncharacterized	–	–	Karuppan et al. (2014)
22	*Monascus purpureus*	9-(1-hydroxyhexyl)-3-(2-hydroxypropyl)-6a-methyl-9,9a-dihydrofuro[2,3-h]isoquinoline-6,8(2*H*,6a*H*)-dione	Red	–	Mukherjee and Singh (2011)
23	*Monascus purpureus*	Uncharacterized	Red	Antibacterial activity	Chatterjee et al. (2009)
24	*Monascus purpureus*	Uncharacterized	Red	–	Kaur et al. (2008)
25	*Monascus* sp.	Monascorubrin	Orange	Antibacterial activity	Akilandeswari and Pradeep (2016)
26	*Nigrospora* sp.	Bostrycin	Red	Cytotoxic	Deshmukh (2018)
27	*Penicillium pinophilum*	Oxyskyrin	Orange-red	Cytotoxic	Koul et al. (2016)
28	*Penicillium pinophilum*	Skyrin	Orange-red	Cytotoxic	Koul et al. (2016)

29	*Penicillium pinophilum*	Dicatenarin	Orange	Cytotoxic	Koul et al. (2016)
30	*Penicillium pinophilum*	1,6,8-trihydroxy-3-hydroxy methylanthraquinone	Yellow-orange	Cytotoxic	Koul et al. (2016)
31	*Penicillium purpurogenum*	Uncharacterized	Red	–	Sethi et al. (2016)
32	*Penicillium purpurogenum*	Uncharacterized	Red	Antimicrobial activity	Patil et al. (2015)
33	*Penicillium* sp.	Uncharacterized	Orange-red	–	Narendrababu and Shishupala (2017)
34	*Penicillium* sp.	Uncharacterized	Orange-red	–	Narendrababu and Shishupala (2017)
35	*Penicillium* sp.	Uncharacterized	Orange-red	–	Narendrababu and Shishupala (2017)
36	*Penicillium* sp.	Citrinin	Lemon-yellow	Antimicrobial	Deshmukh et al. (2005)
37	*Penicillium* sp.	Uncharacterized	Green	Antibacterial activity	Saravanan and Radhakrishnan (2016)
38	*Penicillium* sp.	Uncharacterized	Yellow	–	Saravanan and Radhakrishnan (2016)
39	*Phomopsis vexans*	Mevinolin (lovastatin)	Red		Parthasarathy and Sathiyabama (2015)
40	*Phomopsis* sp.	Altersolanol A	Yellow to red	Cytotoxic	Mishra et al. (2015)
41	*Phyllosticta capitalensis*	Melanin	Black	–	Suryanarayanan et al. (2004)
42	*Pleurotus cystidiosus*	Melanin	Black		Selvakumar et al. (2008)
43	*Pycnoporus* sp.	Uncharacterized	Orange		Subramanian et al. (2014)

(continued)

Table 17.1 (continued)

Sr. no.	Pigment-producing fungi	Pigment	Color	Bioactivity	References
44	*Schizophyllum commune*	Melanin	Black	Antimicrobial, antioxidant, and anticancer activity	Arun et al. (2015)
45	*Talaromyces marneffei*	Herqueinone like	Brick red	–	Bhardwaj et al. (2007)
46	*Thelebolus microsporus*	β-Carotene	Orange	–	Singh et al. (2014)
47	*Thermomyces* sp.	Uncharacterized	Yellow	Antibacterial activity	Poorniammal et al. (2013)
48	*Thermomyces* sp.	Uncharacterized	Yellow	Antibacterial activity	Prathiban et al. (2016)
49	*Trichoderma* sp.	Uncharacterized	Yellow	–	Gupta et al. (2013)
50	*Trichoderma virens*	Uncharacterized	Yellow	–	Sharma et al. (2012)
51	Unidentified fungus	Ergoflavin	Cherry red	Anti-inflammatory, cytotoxic	Deshmukh et al. (2009)
52	Unidentified fungus	Hypericin	Dark red	Cytotoxic	Deshmukh et al. Unpublished data
53	Unidentified fungus	Emodin	Yellow	Cytotoxic	Deshmukh et al. Unpublished data
54	*Aspergillus* sp.	Viomellein	Reddish-brown	Antibacterial activity	Deshmukh et al. Unpublished data
55	*Aspergillus* sp.	Xanthomegnin	Orange	Antibacterial activity	Deshmukh et al. Unpublished data
56	*Aspergillus* sp.	Flavipin	Brown	Cytotoxic	Deshmukh et al. Unpblshed data
57	*Aspergillus* sp. (Y-30,459)	Physcion	Yellow	Cytotoxic	Deshmukh et al. Unpublished data
58	*Epicoccum nigrum*	Epicorazine B	Amber	Antimicrobial/anti-inflammatory	Deshmukh et al. Unpublished data

(IR), tandem mass spectrometry (MS-MS), and nuclear magnetic resonance (NMR) spectroscopy. The pigment has shown some structural resemblance with herquinone (copper-colored pigment) (Bhardwaj et al. 2007). Similarly, a black pigment was isolated from the edible mushroom *Pleurotus cystidiosus* var. *formosensis* and finally confirmed as melanin based on chemical tests and UV, IR, and EPR spectral analysis (Selvakumar et al. 2008). Production of an extracellular red pigment has been reported from *Monascus purpureus* MTCC 410 in solid-state fermentation on cooked autoclaved rice as well as on MEA containing ammonium nitrate. This purified red pigment was found thermolabile over 70 °C and less light sensitive compared to pigment produced by submerged fermentation. This study has also shown that pH and temperature stability of the pigment can be enhanced by altering substrate and nitrogen source (Kaur et al. 2008).

Hypericin, a naphthodianthrone derivative, along with emodin, an anthroquinone, has been extracted from an unidentified fungus (PM0954782), isolated from soil samples collected from Kaziranga National Park, Assam, India. Both compounds displayed photodynamic cytotoxicity against the human acute monocytic leukemia cell line (THP-1), at 92.7% vs. 4.9%, and 91.1% vs. 1.0% viability by resazurin and ATPlite assays, in light and in the dark, respectively (Kusari et al. 2009). Ergoflavin belonging to class ergochromes has been purified from the fungus PM0651480 residing inside the leaf of *Mimusops elengi* collected from Sagar, Madhya Pradesh, India. Ergoflavin exhibited cytotoxic activity against ACHN, H460, Panc1, HCT16, and Calu1 cancer cell lines with the IC_{50} value of 1.2, 4.0, 2.4, 8.0, and 1.5μM, respectively. It also displayed anti-inflammatory activity against TNF-α and IL-6, with IC_{50} values of 1.9 and 1.2μM, respectively (Deshmukh et al. 2009).

Chatterjee et al. (2009) reported maximum production of red pigment from *Monascus purpureus* in medium (pH 6.0) supplemented with 20 g/L glucose and 0.3% MSG and incubated at 30 °C in complete darkness. Purified pigment has shown antibacterial activity only against Gram-positive bacteria. Similarly, a red-orange naphthoquinone, javanicin, has been reported from an endophyte *Chloridium* sp., residing inside the plant *Azadirachta indica* collected from Varanasi, India. Javanicin displayed an impressive antibacterial activity against *P. fluorescens* and *P. aeruginosa* with MIC values of 2μg/mL (Kharwar et al. 2009). A novel red pigment 9-(1-hydroxyhexyl)-3-(2-hydroxypropyl)-6a-methyl-9,9a-dihydrofuro[2,3-h]isoquinoline-6,8(2*H*,6a*H*)-dione has been isolated from *Monascus purpureus* and chemically characterized by UV-VIS, IR, GC-MS, and NMR analyses. Pigment has shown close similarities with rubropunctamine and monascorubramine and displayed activity against Gram-positive bacteria (Mukherjee and Singh 2011).

An orange-yellow pigment sclerotiorin has been purified from *Cephalotheca faveolata*, an endophyte associated with the *Eugenia jambolana* collected from Goregaon Mumbai. Sclerotiorin showed the cytotoxicity against HCT-116, H460, ACHN, Panc-1, Calu-1, and MCF10A cell lines with the IC_{50} value of 0.63, 1.6, 1.2, 1.6, 2.1, and >10 μM, respectively. It also induced apoptosis in HCT116 cells via the triggering of BAX and downregulation of Bcl-2 that result in the stimulation of cleaved caspase-3 thereby causing the death of cancerous cells (Giridharan et al.

2012). A bright yellow naphthoquinone, herbarin, and its analog were purified from *Dendryphion nanum*, associated with the plant *Ficus religiosa* collected from Goregaon, Mumbai. Herbarin was found to inhibit cytokines TNF-α and IL-6 with IC_{50} values of 0.60 and 0.60μM, respectively, while the positive control dexamethasone blocks cytokine production with IC_{50} values of 0.06 and 0.01μM for TNF-α and IL-6, respectively. It has also displayed antidiabetic activity with an EC_{50} value of 0.8μM in GUA assay, while positive control rosiglitazone exhibited antidiabetic activity with an EC_{50} value of 3.0μM (Mishra et al. 2013). *Curvularia lunata*, *Alternaria alternata*, and *Trichoderma virens* produce brown, brilliant olive, and yellow pigments, respectively, which can be used for dyeing textile fabrics (wool and silk) (Sharma et al. 2012). A study by Pradeep and Pradeep in the year 2013 reported maximum biomass and pigment production from *Fusarium fujikuroi* in potato dextrose broth containing 2% glucose as carbon source, peptone 1% as nitrogen source, 0.5% methionine, 0.5% KH_2PO_4, and pH 5.5 when incubated at 28 °C for 8 days. In 2013, Poorniammal and colleagues had reported yellow pigment from *Thermomyces* sp., and purified pigment was assessed for dyeing cotton, silk, and wool fabrics. Similarly, a study has reported the pigments from the *Trichoderma* sp., and extracted pigments have been tested for dyeing silk and wool fabrics (Gupta et al. 2013).

Devi and Karuppan (2014) investigated different natural and synthetic media for maximum mycelial growth and brown pigment production from *Alternaria alternata* and found that *Sorghum* extract medium shows maximum mycelial growth and brown pigment production at 28 °C ± 2 °C temperature and pH 6 under stationary condition. *Ganoderma applanatum* and *G. lucidum* have also been mentioned to produce pigments and have been tested for dyeing cotton and silk yarns (Karuppan et al. 2014). Subramanian et al. (2014) isolated an orange dye from *Pycnoporus* sp. and reported the orange pigment for dyeing cotton and silk yarns and fabrics. An orange pigment β-carotene has been reported from a cold-tolerant fungus *Thelebolus microsporus* in addition to fatty acids like myristic acid, palmitic acid, stearic acid, heptadecanoic acid, linolenic acid, and linoleic acid (Singh et al. 2014).

Oosporein, a red pigment, has been isolated from *Cochliobolus kusanoi*, an endophytic fungus associated with the plant *Nerium oleander*. It displayed antimicrobial activity against *S. aureus*, *B. cereus*, *E. coli*, *S. typhimurium*, *P. aeruginosa*, and *C. albicans*, with an IC_{50} value of 13, 46, 40, 48, 64, and 35 mM, respectively. Oosporein also exhibited antioxidant activity with an IC_{50} value of 0.194 mM, which is very close to the reference standard (ascorbic acid) at 0.189 mM. Oosporine also displayed cytotoxicity with an IC_{50} value of 21 mM against the A549 cell line (Alurappa et al. 2014; Ramesha et al. 2015). An extracellular melanin pigment has been isolated from the mushroom *Schizophyllum commune*. It was characterized by biochemical tests, and its melanin nature was confirmed by analysis of UV, IR, EPR, NMR, and MALDI-TOF mass spectra. It has shown significant antimicrobial activity against *E. coli*, *K. pneumoniae*, *B. subtilis*, *P. fluorescens*, *Trichophyton simii*, and *T. rubrum* at 100μg/mL concentration. Melanin (50μg/mL) has also shown the antioxidant activity when tested with DPPH free radical scavenging assay. In addition to this, it has also shown the anticancer activity against Human Epidermoid

Larynx Carcinoma (HEP-2) cell line (Arun et al. 2015). Altersolanol A, an anthraquinone with a yellow to red hue, was purified from *Phomopsis* sp. isolated from plant *Nyctanthes arbor-tristis* collected at Mumbai. Compound altersolanol A exhibited potent cytotoxicity with mean IC_{50} (IC_{70}) values of 0.005μg/mL (0.024μg/mL) against 34 human cancer lines (Mishra et al. 2015). A novel pigment 2-(4-((3*E*,5*E*)-14-aminotetradeca-3,5-dienyloxy)butyl)-1,2,3,4-tetrahydroisoquinolin-4-ol (ATDBTHIQN) has been reported from *Fusarium fujikuroi* (formerly known as *Fusarium moniliforme*) KUMBF1201 isolated from paddy field soil. It has shown promising larvicidal activity against third and fourth instar larvae of *Aedes aegypti* with LC_{50} value of 237.0 and 276.4 ppm, respectively, and against *Anopheles stephensi* with LC_{50} value of 335.6 and 258.1 ppm, respectively (Pradeep et al. 2015). Another study has described production of red exopigment from *Penicillium purpurogenum* isolated from spoilt onion and reported maximum pigment production in media supplemented with 2% xylose and 2% peptone, at pH 5.0 and 27 °C temperature. Red pigment has shown significant antibacterial activity against both Gram-positive and Gram-negative bacteria (Patil et al. 2015). A red pigment lovastatin (potent drug for lowering blood cholesterol) has been reported from an endophytic fungus *Phomopsis vexans* isolated from healthy leaves of the medicinal plant *Solanum xanthocarpum* (Parthasarathy and Sathiyabama 2015).

The anthraquinones oxyskyrin (orange-red), skyrin (orange-red), dicatenarin (orange), and 1,6,8-trihydroxy-3-hydroxy methylanthraquinone (yellow-orange) were isolated from an endophytic fungus *Penicillium pinophilum* MRCJ-326, isolated from the plant *Allium schoenoprasum*, collected from Nattha Top, J and K, India. Dicatenarin exhibited anticancer activity against A549, MIA-PaCa-2, HCT-116, T47D, PC-3, and OVCAR-3 cell lines with IC_{50} values of 23.00, 12.00, 17.00, 29.00, 35.00, and 26.00μg/mL, respectively. Skyrin exhibited anticancer activity against A549, MIA-PaCa-2, HCT-116, and PC-3 cell lines with IC_{50} values of 38.00, 27.00, 32.00, and 47.00μg/mL, respectively, whereas oxyskyrin exhibited anticancer activity against HCT-116 cell lines with IC_{50} values of 48.00μg/mL. Compounds dicatenarin and skyrin significantly induced apoptosis and transmitted the apoptotic signal via intracellular ROS generation, thereby inducing a change in the mitochondrial transmembrane potential and induction of the mitochondrial-mediated apoptotic pathway. It has been found that dicatenarin and skyrin induce ROS-mediated mitochondrial permeability transition and resulted in an increased induction of caspase-3 apoptotic proteins in MIA PaCa-2 cell lines (Koul et al. 2016). Researchers have assessed pigments of *Thermomyces* sp. for the development of antibacterial silk sutures for healthcare application. Silk sutures dyed with fungal pigment have significantly retarded the growth of *E. coli* and *S. aureus* showing its potential in healthcare applications (Prathiban et al. 2016). Saravanan and Radhakrishnan (2016) in their study screened 15 fungal isolates, and among them 6 strains have been found to produce pigments which include *Aspergillus* sp. MF2 (Black), *Aspergillus* sp. MF15 (Brown), *Aspergillus* sp. MF11 (yellow), *Penicillium* sp. MF5 (green), *Penicillium* sp. MF7 (yellow), and *Fusarium* sp. MF10 (red). Among them, *Penicillium* sp. MF5 has shown good antibacterial activity against

tested pathogen, and MIC of the purified green pigment was found to be 12.5μg/mL for *Bacillus subtilis*. In 2016, Sethi and co-workers screened 15 fungal isolates for their pigment production potential, and among them, only one *Penicillium* sp. BKS 9 has exhibited excellent red pigment production potential on Sabouraud dextrose agar (SDA) medium as well as in broth medium (SD broth). Maximum red pigment (0.79 Abs/mL) and biomass (0.877 g/50 mL) production was achieved at pH 6.0 and 30 °C temperature upon 18 days of incubation. Moreover, pigment has shown no significant negative effect on *Cicer arietinum* seed germination (Sethi et al. 2016).

Narendrababu and Shishupala (2017) detected and confirmed the yellow pigments of *Aspergillus* species (DUMB 13 and DUMB 14) and orange-red pigments of *Penicillium* species (DUMB 11, DUMB 12, and DUMB 15) by UV-spectroscopy and suggested their possible potential application in the industry. Ustilaginoidin A belonging to bis-naphtho-γ-pyrones has been purified from *Alternaria longissima*, an endophyte associated with the plant *Sphaeranthus* sp. collected from Tungareshwar (Thane district), and exhibited antidiabetic activity (IC_{50}, GUA 0.1μM) and toxicity >1μM (Deshmukh 2018). A novel tetrahydroanthraquinone red pigment bostrycin was isolated from *Nigrospora* sp., an endophyte of *Avicennia marina* collected from Thane creek which displayed cytotoxicity with IC_{50} value in the range of 1.2–3.5μg/mL against ACHN, Panc1, Calu 1, H460, HCT 116, and MCF 10A cell lines (Deshmukh 2018). Soumya et al. (2018) extracted the pigment from *Fusarium chlamydosporum*. FTIR, ESI-MS, LC-MS, and NMR (1HNMR, 13C NMR) analysis revealed structure of red pigment as "long-chain hydrocarbons with poly unsaturated groups." It has shown selective cytotoxicity against human breast adenocarcinoma (MCF-7) cell lines, suggesting its possible application in cosmetics and as an anticancer drug.

Orange compound xanthomegnin and reddish-brown compound viomellein possessing antibacterial activity were obtained from *Aspergillus* sp. (PM0100024). Brown pigment flavipin and yellow pigment physcion extracted from *Aspergillus* sp. (PM0204024) and *Aspergillus* sp. (Y-30,459) have shown very good cytotoxic activity. Amber-colored compound epicorazine B was extracted from *Epicoccum nigrum* isolated from marine wood collected from Goa (QIL-27548). Citrinin, a polyketide-derived mycotoxin which forms lemon-yellow crystals, has been isolated from *Penicillium* sp. Y-871987 possessing antimicrobial activity (Deshmukh et al. 2005).

17.4 Optimization of Pigment Production

Various methods, mode of isolation, cultivation, and techniques and strategies have been assessed for the characterization and enhancement of pigment production from fungi (Fig. 17.3). Different factors like medium composition, pH of the media, supply of carbon, and nitrogen sources have been found to influence fungal pigment production. Similarly, fermentation parameters such as temperature, agitation rate, and supply of oxygen, water activity, and light availability have also been found to affect the pigment production of fungi.

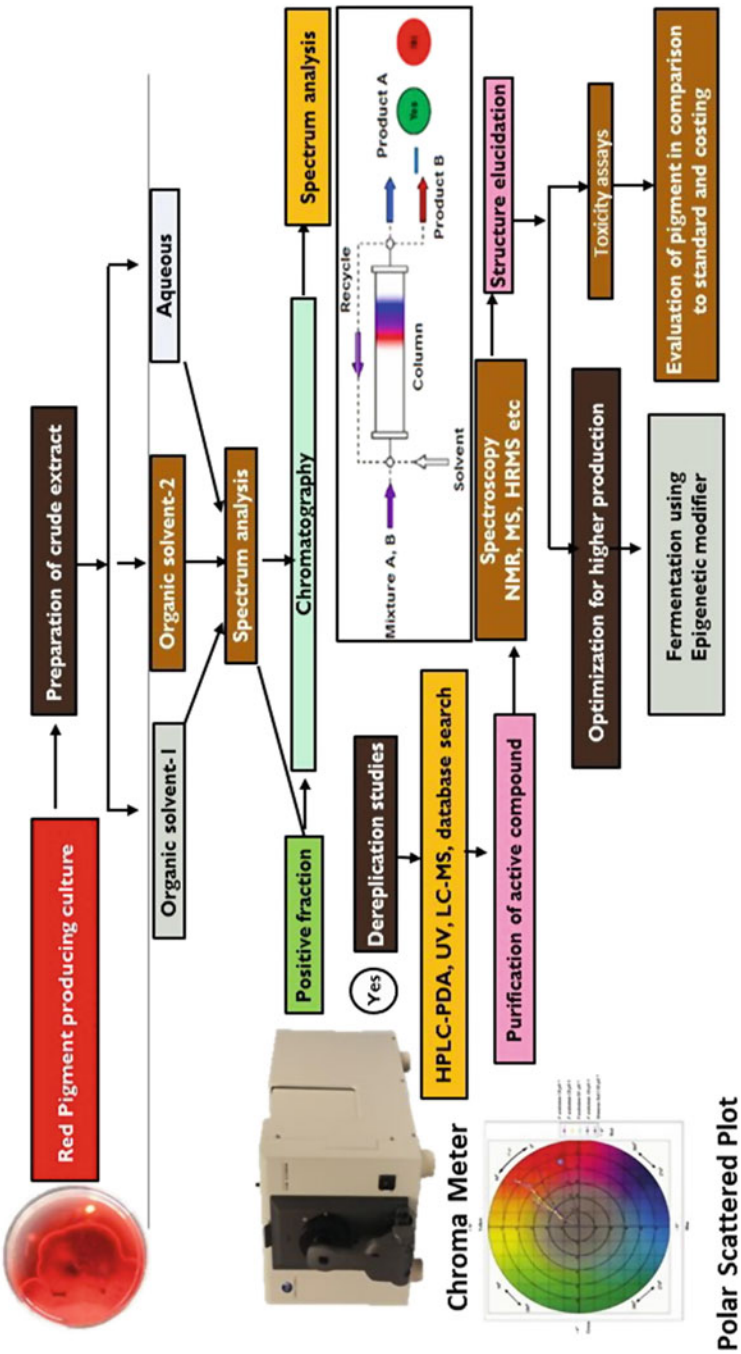

Fig. 17.3 Isolation and characterization of natural pigments

17.5 Technologies for Enhancing Pigment Production

Fungal pigments are the secondary metabolites belonging to polyketides or carotenoids. Various factors include pH, substrate, oxygen, temperature, water activity, and light availability influence the production of these secondary metabolites. Medium composition, aeration rate, agitation rate, supply of carbon and nitrogen sources, and micronutrient affect production of secondary metabolites. Here, we summarize the details of the optimization process for the production of pigments by filamentous fungi.

17.5.1 Media Optimization

Media components such as different carbon sources (glucose, fructose, sucrose, lactose, and maltose) and nitrogen sources (peptone, sodium nitrate, ammonium, tryptophan, 6-furturylaminopurine, glutamic acid, and monosodium glutamate) have also been found to influence the fungal pigment production. *Monascus purpureus* and *M. ruber* have shown increased pigment production in the presence of fructose and lactose, respectively, as a carbon source (Tseng et al. 2000; Costa and Vendruscolo 2017). In addition to yield of pigment, color characteristics and light stability of the *Monascus* pigments were also altered with respect to type of nitrogen sources and type of amino acids added in the media (Carels and Shepherd 1978; Chen and Johns 1993; Pastrana et al. 1995; Tseng et al. 2000; Jung et al. 2003; Zhang et al. 2013).

The pH of the medium plays a very important role in fungal pigment production. Chen and Johns (1993) studied the effect of pH and nitrogen source on pigment production by *Monascus purpureus* 192F using glucose as the carbon and energy source. The pigment reported from this fungus includes yellow pigments monascin and ankaflavin, the orange rubropunctatin, and the red pigment monascorubramine. The highest red pigment production was obtained using a glucose-peptone medium at pH 6.5. Fungal growth and ankaflavin synthesis were favored at low pH (pH 4.0), whereas production of the other pigments was relatively independent of pH. Ammonium and peptone as nitrogen sources gave superior growth and pigment concentrations compared to nitrate, and ankaflavin was not detected in nitrate cultures. Likewise, a study by Kang et al. in 2013 have shown that the pH of the medium strongly affects red pigment production in *M. purpureus* since red pigments (extracellular and water-soluble) are produced by the chemical modification of orange pigments under relatively higher pH values in the presence of a suitable nitrogen source. At pH 5.5, the maximum production of red pigment was observed in *M. purpureus* CCT3802 (Orozco and Kilikian 2008). Most of the studies have reported that enhanced pigment production was observed at low pH (Carels and Shepherd 1978; Tudor et al. 2013; Li et al. 2019). A recent study has also shown the reduction in pigment production by *M. purpureus* upon increasing or decreasing the pH values of medium (Silbir and Goksungur 2019).

17.5.2 Optimization of Fermentation Parameters

Pigment production by fungi was also affected by different fermentation parameters, such as temperature, pH, agitation speed, color, and intensity of light. Several studies have shown that fungal pigment production varies with temperature (Mendez et al. 2011; Huang et al. 2017; Stange et al. 2019). Variation in fungal pigment production was also observed with respect to the color and intensity of the light. *Alternaria alternata* has shown enhancement in pigment production and inhibition of mycotoxin production upon exposure to blue light (Haggblom and Unestam 1979). Similarly, *M. ruber* has shown enhanced pigment production upon exposure to red light, whereas *F. oxysporum* reported maximum pigment production upon exposure to blue and green light (Buhler et al. 2015; Palacio-Barrera et al. 2019). Enhanced biomass and pigment production were also observed in many fungi, when incubated in total darkness (Velmurugan et al. 2010; Buhler et al. 2015; Stange et al. 2019). Besides these, researchers have found that agitation speeds as well supply of oxygen also have major impact on pigment production by fungi (Stange et al. 2019; Zhou et al. 2019).

17.5.3 Co-culturing

Microbial co-culture or mixed fermentation proved to be an efficient strategy to expand chemical diversity by the induction of cryptic biosynthetic pathways and in many cases led to the production of new antimicrobial agents. In co-culture, *Penicillium* sp. HSD07B and *C. tropicalis* produced a stable and apparently non-toxic red pigment, and after culture optimization, a pigment yield of 2.75 and 7.7 g/L was obtained in a shake flask and a 15 L bioreactor, respectively. These observations suggest that the production of a natural red pigment by the co-culture is feasible (Hailei et al. 2011). Similarly, studies have shown that co-culturing of fungi with bacteria or yeast results in the enhancement of pigment production. Increase in pigment production was observed in *Monascus* and *A. chevalieri*, when co-cultured with *S. cerevisiae* or *A. oryzae* (Shin et al. 1998; Palacio-Barrera et al. 2019). *C. neoformans* has also shown melanin production upon co-culturing with *Klebsiella aerogenes* (Frases et al. 2006).

17.5.4 Strategies/Modes of Cultivation and Extraction

Different modes of cultivation such as use of various surfactants including Tween 80, Triton X-100, Span 20, and polyethylene glycerol polymer 8000 were assessed for the enhancement of pigment production (Carvalho et al. 2007). Moreover, for the rapid extraction and improved recovery of pigments from *Chlorociboria aeruginosa* and *Scytalidium cuboideum*, different solvents like methanol, chloroform, acetone, hexane, chloramphenicol, acetonitrile, dimethyl sulfoxide cyclohexane, dichloromethane, isooctane, methyl sulfoxide, pyridine, tetrahydrofuran, and water

were tested and compared and finally confirmed dichloromethane as an ideal solvent for extraction (Robinson et al. 2014).

17.5.5 Technologies for Enhancing Pigment Production

Global demand for natural pigments such as anthocyanins, carotenoids, betalains, and chlorophylls is increasing because of their many advantages. However, these pigments are less stable compared to synthetic colorants. Various stabilizing methods and techniques have been developed for enhancing the stability of these natural pigments which include additions of co-pigment compounds (polymers, metals, and phenolic compounds), encapsulation, etc. (Cortez et al. 2017).

17.5.6 Cost-Effective Downstream Processing and Metabolic Engineering

Cost-effective recovery and separation techniques for fungal pigment are important aspects in the pigment industry. The convential method used for industrial scale is not cost-effective. Use of various solvents for extraction purpose is complicated and time-consuming, and substantial amounts of organic solvents are exhausted. Other limitations include the low yield of high-purity product. The use of solvents other than ethanol and water can defeat the purpose of obtaining a natural pigment for regulatory purposes, since most organic solvents are not natural. Therefore, it is necessary to develop a simple but efficient and environmentally friendly technique to extract and purify pharmacologically active natural products. Adsorption and desorption onto non-ionic macroporous resins have proved to be an efficient technique in this field due to its advantages, such as high adsorption capacity, low operational expense, and easy regeneration of the adsorbent (Li and Chase 2010). The advantages of this method includes low operational cost, lowered solvent consumption, and low amounts of residual chemicals in the final product, and it can be used for separation of nucleic acids, organic acids, peptides, and others (Sen et al. 2019). Wang et al. (2004) used non-ionic resins directly into the culture broth, for separation and purification of prodigiosin, thereby eliminating the cell separation step, yielding a concentrated and semi-purified product, which was found to be an efficient method.

Another method can be an aqueous two-phase system (ATPS), the methods also used for the extraction of pigmented compounds from the fermented broth (Iqbal et al. 2016). This approach uses liquid-liquid fractionation and is based on the use of green ionic liquids for extraction of pigmented molecules. Separation of different hydrophilic solutes into two immiscible aqueous phases is mainly based upon their differential selectivity toward different polymer-polymer, polymer-salt, or salt-salt and solute combinations formed in these two phases. Aqueous two-phase systems offer several advantages for downstream control of biomolecules: Both phases (1) are composed of water compared to organic solvents in conventional extraction

thus providing favorable environment to molecules and supporting the stability of their structure and bioactivity, (2) are an environmental friendly process, and (3) are economically favorable (McQueen and Lai 2019).

Tan et al. (2012) have successfully isolated aloe anthraquinones using microemulsion of ionic liquids (IL). Recently, similar techniques coupling IL with ultrasound, microemulsion, or ATPS were performed with good results on biomass or culture broth of filamentous fungi. Ventura et al. (2013) succeeded the isolation of anthraquinones from 14-day-old fermentation broth of *Penicillium purpurogenum*. Similarly, Shen et al. (2014) obtained good results during hydrophobic IL microemulsion extraction of red *Monascus* pigments from 7-day-old fermentation broth.

17.5.7 Metabolic Engineering Using the CRISPR-Cas9 System

In recent years, the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) genome editing system has been frequently used for genome editing. This technique can be used in manipulating bioactive compound production, virus resistance, disease resistance, physical and chemical resistance, crop production enhancement, etc. CRISPR genome editing system consists of only two elements, a Cas9 nuclease and a single-guide RNA (sgRNA) composed of two small RNAs, a target-recognizing CRISPR RNA (crRNA) and auxiliary non-coding trans-activating crRNA (tracrRNA). The synthetic sgRNA binds to Cas9, and the resulting complex can catalyze a double-strand break (DSB) in the target DNA comprising a 20-bp sequence matching the protospacer of the sgRNA and a downstream protospacer adjacent motif (PAM) sequence. CRISPR-Cas9 system can be very well used for metabolic engineering in fungi to make cellular factories for cost-efficient production of natural food colors.

Literature survey reveals that the first reports on CRISPR-Cas9-mediated genome editing in filamentous fungi were published in 2015 (Arazoe et al. 2015; Fuller et al. 2015; Liu et al. 2015; Matsu-Ura et al. 2015; Nødvig et al. 2015). Since then, the technology has conquered the field and has established 41 different species of fungi and oomycetes including several species where genome manipulation had not been possible before (Nielsen et al. 2017). This technique can be used to create genetically engineered microorganisms to produce natural colors, by inserting the color-producing gene into its genome using the CRISPR-Cas9 system (Lian et al. 2018).

Monascus red, produced by *Monascus purpureus*, is used as a food colorant associated with the co-production of the nephrotoxic compound citrinin responsible for disease and death in both humans and animals. The application of genetic engineering techniques is difficult in a polykaryotic fungus such as *M. purpureus* industrial strain KL-001. Using CRISPR-Cas9 system, 15-kb citrinin biosynthetic gene cluster was deleted, overcoming the long-standing problem in the food industry. It has also helped to increase monascus red pigment production by 2–5% (Liu et al. 2020). There is limited research done in the field of genetic or metabolic engineering of fungi for enhanced pigment production that requires more attention.

There is a great need of applying CRISPR/Cas system for yield improvement and to delete the gene responsible for the production of unwanted metabolites including mycotoxins.

17.6 Microencapsulation, Nano-emulsions, and Nano-formulations

A plethora of fungal resources are known to produce a wide array of pigments which includes metabolites from different classes of compounds such as melanins, anthraquinones, hydroxyanthraquinones, azaphilones, carotenoids, oxopolyene, quinones, and naphthoquinone (Mukherjee et al. 2017; Deshmukh et al. 2018, 2020). The microencapsulation method is broadly used in numerous fields, especially in the food industry. This includes internal phase core material which is surrounded by external coating material, also called encapsulating agents or wall material. The size and shape of the microcapsule inevitably depend on the selection of the materials and methods used to prepare them. Some of them are maltodextrins, starch, protein, inulin, gum arabic, ascorbic acid, carrageenan, silicon dioxide, sodium caseinate, soy lecithin, and sodium alginate typically considered for wall material during drying process (Chen et al. 2017; Kalra et al. 2020).Various forms of microencapsulation systems can be prepared through spray drying, freeze draying, coacervation, and emulsion formation to preserve the integrity of microbial pigments. Maltodextrins are commonly used as encapsulating agents, and spray drying is the most suitable drying method for encapsulation.

Microemulsions are isotropic systems consisting water-in-oil formulations stabilized by the addition of small amounts of surfactants. They are thermodynamically stable, fluid, and isotropically clear and may have droplets in the range of 5–100 nm (Morales et al. 2015). Nano-emulsions are classically shaped in small spherical droplets, less than 100 nm, using oil and water system with the addition of emulsifier agent. Nano-emulsions are inspired by the understanding of microemulsions, in which the fundamental key components are mostly considered similar, except the concept of formulation stability. Notably, nano-emulsions offer several advantages, such as the ability to penetrate extracellular and intracellular areas that may be inaccessible to other delivery systems due to their small size (Bermúdez et al. 2017). The structure of the nano-emulsion play a vital role when introduced to an emulsifier, establishing significant stability and giving effectual protection from various external hindrance (Rao and McClements 2011). The emulsifiers used are mostly surfactants, but proteins and lipids are also used. These surfactants noticeably differ when applied for breaking up the macroscopic phase into smaller, nano-scale droplets using high-energy devices such as high-shear stirrers, ultrasonic homogenizers, and colloid mills (Morales et al. 2015). Thus, the development of desired nano-encapsulation design provides advanced protection to microbial pigment by yielding a uniform population of droplets in nanometer ranges and enhancing stability. Some of the major pigments found in fungi such as canthaxanthin, cycloprodigiosin, naphthoquinone, riboflavin, beta-carotene,

monascorubramin, azaphilones, xanthomonadin, and lycopene are potential sources of food colorants. The isolated fungal pigments are encapsulated for the development of nano-formulation that can be designed to deliver nutrients through effective encapsulation that would not negatively impact the flavor or stability of the nutrient or bio-active.

The food pigment or the incorporated bioactive molecules in emulsions experiences a complex series of physiological processes when it passes in human body enabling eating pleasure, food intake, and nutrient absorption. Therefore, the design of micro- and nano-emulsions is being developed as a delivery system for enhancing the health benefits of food products, and the bioactivity of such molecules depends on the behavior of the droplet diameter within GIT, for example, rate, extent, and location of digestion and absorption. Solid lipid nanoparticles (SLN) are composed of lipids particularly useful for nano-formulation of hydrophobic bioactive compounds. Nik et al. observed that SLN dispersions of β-carotene stabilized with the non-ionic surfactants Poloxamer 188 and Tween-20 were stable when they were exposed under gastric conditions; they were more stable compared to a canola oil-in-water emulsion. In contrast, the drastic increase in particle size instigated when emulsions were stabilized with milk proteins like whey protein isolates/β-lactoglobulin due to proteolysis of the protein interfacial layers along the surface charge of the emulsion was lost (Nik et al. 2012). Carotenoids and anthocyanins have been focused on for their color, strength, and bioavailability which can be achieved by making carrier matrix used to develop the nano-emulsion system. The smallest particles had good stability at GIT track and higher bio-accessibility. Many studies reported to produce encapsulated carotenoids using different encapsulating agents through spray and freeze drying (Loksuwan 2007). Nano- and microemulsions techniques have been developed for the delivery of a wide range of compounds to the skin for dermatological and cosmetic/cosmeceutical application owing to their nano-diameters which are advantageous in improving the penetration of bioactive compounds into skins and the controlled release of nano-formulation. Nastiti et al. investigated the targeted follicular delivery of hydrophilic caffeine from nano-emulsion composed of the skin penetration enhancer oleic acid and eucalyptol, while aqueous solution as control. The nano-emulsions significantly enhanced the permeation of caffeine by 23-fold for oleic acid and 43-fold for eucalyptol compared to control. They have collected data from two different topical formulations using oleic acid and eucalyptol nano-emulsions for caffeine as a penetration enhancer of hydrophilic compounds (Nastiti et al. 2017). A similar nano-formulation containing resveratrol, retinoids, lipids, antioxidants, and humectants has been established to improve skin smoothness and moisture and develop skin barrier function (Clares et al. 2014; Liu et al. 2007). Nano-emulsions are valued because of their sensorial and biophysical properties, with studies indicating their stability for a few years, which is an advantageous property for carriers in cosmetic products (Liu et al. 2007). However, in this field, more extensive studies regarding in vivo system of encapsulated carotenoids, stilbenes, or anthocyanins are necessary for a view on their potential application in the field of the food industry.

17.7 Addressing Toxicity Issue

Although numerous fungal pigments have been reported, every pigment can't be used as food color, because microbial pigments have to satisfy several criteria concerning its toxicity, stability, regulatory approval, and economical investment (Dufossé et al. 2014). To counteract the toxicity issue, nonpathogenic fungal strains are selected. Otherwise, toxin production can be controlled by analyzing their metabolic biosynthesis pathway or by selection of a particular growth media containing variable carbon, nitrogen, and metal ion sources. Besides toxicity problems, fungal pigments are also selected based on their stability at extreme temperature and pH and cost-effectiveness of the whole production process (Padmapriya and Murugesan 2016; Dufossé 2017, 2018).

17.8 Conclusions and Future Perspectives

The pigments produced by a large spectrum of fungi are an important part of their broad range of biological activities. In different organism, variable amounts of pigments are produced. The chemical properties of these pigments may have extremely variable roles in their metabolic pathways. The in-depth investigation of pigments produced by fungi has incredible use in medical, textile coloring, food, and cosmetics industries. Fungi occupy countless diverse niches and thus are able to survive against a plethora of environmental factors which could immensely impact on the function of fungal pigment. Melanins are located outside the plasma membrane and seem to be important structural components of the cell wall of fungal species. Melanins and carotenoids play miscellaneous, vigorously protective functions against a large range of ecological stressors; it may protect the fungal cell from damage caused by excessive visible light, UV radiation, and oxidative stress. Thus, understanding the role that pigment plays in fungal pathobiology is vital to exploring new antimicrobial drugs. Despite steady advancements in fungal pigments, a shorter shelf life due to instability of biomolecule in diverse conditions may minimize its application. Micro- and nano-encapsulation approach can be employed to enhance the physical stability and solubility issues associated with natural pigments. Pigment formulation has functional advances when encapsulated in micro and nano-formulation and shows elusive stability to ambient conditions such as pH, light, moisture, and temperature. Recently, Matsuo et al. (2018) have prepared two different nano-encapsulation systems using *Monascus* pigments by using hydroxypropyl cellulose (HPC) and poly(lactic-co-glycolic) acid (PLGA) copolymer. The PLGA-based formulation showed effective sustained release and photobleaching of pigment which is limited to pharmaceutical applications. Because of the pharmaceutical use, it can be explored for several advantages over other topical products. On the other hand, *Monascus* pigments-encapsulated HPC nanoparticles can be used as food additives, though it shows slight improvement in photostability compared to PLGA formulation. These novel formulations are the miscellaneous example of the *Monascus*-based pigment for the researchers to

develop effective and stable formulations which incorporate fungal pigments. Co-production of mycotoxin is one of the problems associated with the fungal pigments which can be solved by gene editing techniques like CRISPR/Cas9. The stability-related issues of fungal colorants could be overcome by adopting nanotechnological approaches.

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References

Aberoumand A (2011) A review article on edible pigments properties and sources as natural biocolorants in foodstuff and food industry. World J Dairy Food Sci 6(1):71–78

Akilandeswari P, Pradeep BV (2016) Exploration of industrially important pigments from soil fungi. Appl Microbiol Biotechnol 100:1631–1643

Akilandeswari P, Pradeep BV (2017) *Aspergillus terreus* KMBF1501 a potential pigment producer under submerged fermentation. Int J Pharm Pharm Sci 9:38–43

Alurappa R, Bojegowda MRM, Kumar V, Mallesh NK, Chowdappa S (2014) Characterisation and bioactivity of oosporein produced by endophytic fungus *Cochliobolus kusanoi* isolated from *Nerium oleander* L. Nat Prod Res 28(23):2217–2220

Arazoe T, Miyoshi K, Yamato T, Ogawa T, Ohsato S, Arie T, Kuwata S (2015) Tailor-made CRISPR/Cas system for highly efficient targeted gene replacement in the rice blast fungus. Biotechnol Bioeng 112(12):2543–2549

Arora S (2014) Textile dyes: its impact on the environment and its treatment. J Bioremed Biodegr 5:1

Arun G, Eyini M, Gunasekaran P (2015) Characterization and biological activities of extracellular melanin produced by *Schizophyllum commune* (Fries). Indian J Exp Biol 53:380–387

Bermúdez JM, Cid AG, Romero AI, Villegas M, Villegas NA, Palma SD (2017) New trends in the antimicrobial agents delivery using nanoparticles. In: Antimicrobial nanoarchitectonics: from synthesis to applications. Elsevier, Amsterdam. https://doi.org/10.1016/B978-0-323-52733-0.00001-X

Bhardwaj S, Shukla A, Mukherjee S, Sharma S, Guptasarma P, Chakraborti AK, Chakrabarti A (2007) Putative structure and characteristics of a red water-soluble pigment secreted by *Penicillium marneffei*. Sabouraudia 45(5):419–427

Buhler RMM, Muller BL, Moritz DE, Vendruscolo F, Oliveira D, Ninow JL (2015) Influence of light intensity on growth and pigment production by *Monascus ruber* in submerged fermentation. Appl Biochem Biotechnol 176:1277–1289

Carels M, Shepherd D (1978) The effect of pH and amino acids on conidiation and pigment production of *Monascus major* ATCC 16362 and *Monascus rubiginosus* ATCC 16367 in submerged shaken culture. Can J Microbiol 24:1346–1357

Caro Y, Venkatachalam M, Lebeau J, Fouillaud M, Dufossé L (2017) Pigments and colorants from filamentous fungi. In: Merillon JM, Ramawat KG (eds) Fungal metabolites. Springer International Publishing, Cham, Switzerland, pp 499–568

Carvalho JC, Oishi BO, Woiciechowski AL, Pandey A, Babitha S, Soccol CR (2007) Effect of substrates on the production of *Monascus* biopigments by solid-state fermentation and pigment extraction using different solvents. Indian J Biotechnol 6:194–199

Chatterjee S, Maity S, Chattopadhyay P, Sarkar A, Laskar S, Sen SK (2009) Characterization of red pigment from *Monascus* in submerged cultured pigment from *Monascus purpureus*. J Appl Sci Res 5:2102–2108

Chen MH, Johns MR (1993) Effect of pH and nitrogen source on pigment production by *Monascus purpureus*. Appl Microbiol Biotechnol 40(1):132–138
Chen J, Li F, Li Z, McClements DJ, Xiao H (2017) Encapsulation of carotenoids in emulsion-based delivery systems: enhancement of β-carotene water-dispersibility and chemical stability. Food Hydrocoll 69:49–55
Clares B, Calpena AC, Parra A, Abrego G, Alvarado H, Fangueiro JF, Souto EB (2014) Nanoemulsions (NEs), liposomes (LPs) and solid lipid nanoparticles (slns) for retinyl palmitate: effect on skin permeation. Int J Pharm 473(1–2):591–598
Cortez R, Luna-Vital DA, Margulis D, Gonzalez de Mejia E (2017) Natural pigments: stabilization methods of anthocyanins for food applications. Compr Rev Food Sci Food Saf 16(1):180–198
Costa JPV, Vendruscolo F (2017) Production of red pigments by *Monascus ruber* CCT 3802 using lactose as a substrate. Biocatal Agric Biotechnol 11:50–55
Deshmukh SK (2018) Translating endophytic fungal research towards pharmaceutical applications. Kavaka 50:1–13
Deshmukh SK, Roy K, Mukhopadhyay T, Vijayakumar EKS, Chatterjee S, Ganguli BN (2005) Antifungal agents from fungi: an industrial experience. In: Goutam SP, Sharma A, Sandhu SS, Pandey AK (eds) Microbial diversity: opportunities and challenges, Prof. Rajak Festschrift. Shree Publishers and Distributors, New Delhi, pp 361–372
Deshmukh SK, Mishra PD, Kulkarni-Almeida A, Verekar SA, Sahoo MR, Periyasamy G, Goswami H, Khanna A, Balakrishnan A, Vishwakarma R (2009) Anti-inflammatory and anti-cancer activity of Ergoflavin isolated from an endophytic fungus. Chem Biodivers 6:784–789
Deshmukh SK, Gupta MK, Prakash V, Saxena S (2018) Endophytic fungi: a source of potential antifungal compounds. J Fungi 4:77
Deshmukh SK, Agrawala S, Prakash V, Reddy MS, Gupta MK (2020) Anti-infectives from mangrove endophytic fungi. S Afr J Bot 134:237–263
Devi S, Karuppan P (2014) Influence of culture condition and pH on growth and production of brown pigment from *Alternaria alternata*. Int J Sci Res 3:458–461
Dufossé L (2017) Current carotenoid production using microorganisms. In: Singh OV (ed) Bio-pigmentation and biotechnological implementations, 1st edn. Hoboken, NJ, Wiley, pp 87–106
Dufossé L (2018) Microbial pigments from bacteria, yeasts, fungi, and microalgae for the food and feed industries. In: Grumezescu AM, Holban AM (eds) Natural and artificial flavoring agents and food dyes, Handbook of food bioengineering, vol 7. Elsevier, Amsterdam, pp 113–132
Dufossé L, Fouillaud M, Caro Y, Mapari SAS, Sutthiwong N (2014) Filamentous fungi are large-scale producers of pigments and colorants for the food industry. Curr Opin Biotechnol 26:56–61
Frases S, Chaskes S, Dadachova E, Casadevall A (2006) Induction by *Klebsiella aerogenes* of a melanin-like pigment in *Cryptococcus neoformans*. Appl Environ Microbiol 72:1542–1550
Fuller KK, Chen S, Loros JJ, Dunlap JC (2015) Development of the CRISPR/Cas9 system for targeted gene disruption in *Aspergillus fumigatus*. Eukaryot Cell 14(11):1073–1080
Giridharan P, Verekar SA, Khanna A, Mishra PD, Deshmukh SK (2012) Anticancer activity of sclerotiorin, isolated from an endophytic fungus *Cephalotheca faveolata* Yaguchi, Nishim. and Udagawa. Indian J Exp Biol 50:464–468
Gulrajani ML (2001) Present status of natural dyes. IJFTR 26:191–201
Gupta C, Sharma D, Aggarwal S, Nagpal N (2013) Pigment production from *Trichoderma* spp. for dyeing of silk and wool. Int J Sci Nat 4:351–355
Haggblom P, Unestam T (1979) Blue light inhibits mycotoxin production and increases total lipids and pigmentation in *Alternaria alternata*. Appl Environ Microbiol 38:1074–1077
Hailei W, Zhifang R, Ping L, Yanchang G, Guosheng L, Jianming Y (2011) Improvement of the production of a red pigment in *Penicillium* sp. HSD07B synthesized during co-culture with *Candida tropicalis*. Bioresour Technol 102:6082–6087
Huang T, Tan H, Chen G, Wang L, Wu Z (2017) Rising temperature stimulates the biosynthesis of water-soluble fluorescent yellow pigments and gene expression in *Monascus ruber* CGMCC10910. AMB Express 7:134

Iqbal M, Tao Y, Xie S et al (2016) Aqueous two-phase system (ATPS): an overview and advances in its applications. Biol Proced Online 18:18

Jung H, Kim C, Kim K, Shin CS (2003) Color characteristics of *Monascus* pigments derived by fermentation with various amino acids. J Agric Food Chem 51:1302–1306

Kalra R, Conlan XA, Goel M (2020) Fungi as a potential source of pigments: harnessing filamentous fungi. Front Chem 8:1–23

Kang B, Zhang X, Wu Z, Qi H, Wang Z (2013) Effect of pH and nonionic surfactant on profile of intracellular and extracellular *Monascus* pigments. Process Biochem 48:759–767

Karuppan P, Subramanian CS, Velusamy K, Sadasivam M, Mahalingam P et al (2014) Prospective aspects of myco-chrome as promising future textiles. In: Proceedings of 8th International conference on mushroom biology and mushroom products, New Delhi, 19–22 November 2014, vol I and II. ICAR-Directorate of Mushroom Research, New Delhi, pp 412–416

Kaur B, Chakraborty D, Kaur H (2008) Production and evaluation of physicochemical properties of red pigment from *Monascus purpureus* MTCC 410. Internet J Microbiol 7:1–6

Kharwar RN, Verma VC, Kumar A, Gond SK, Harper JK, Hess WM, Lobkovosky E, Ma C, Ren Y, Strobel GA (2009) Javanicin, an antibacterial naphthaquinone from an endophytic fungus of neem, *Chloridium* sp. Curr Microbiol 58(3):233–238

Koul M, Meena S, Kumar A, Sharma PR, Singamaneni V, Riyaz-Ul-Hassan S, Hamid A, Chaubey A, Prabhakar A, Gupta P, Singh S (2016) Secondary metabolites from endophytic fungus *Penicillium pinophilum* induce ROS-Mediated apoptosis through mitochondrial pathway in pancreatic cancer cells. Planta Med 82(4):344–355

Kusari S, Zühlke S, Kosuth J, Cellarova E, Spiteller M (2009) Light-independent metabolomics of endophytic *Thielavia subthermophila* provides insight into microbial hypericin biosynthesis. J Nat Prod *72*(10):1825–1835

Lagashetti AC, Dufossé L, Singh SK, Singh PN (2019) Fungal pigments and their prospects in different industries. Microorganisms 7(12):604

Li J, Chase HA (2010) Development of adsorptive (non-ionic) macroporous resins and their uses in the purification of pharmacologically-active natural products from plant sources. Nat Prod Rep 27(10):1493–1510

Li L, Chen S, Gao M, Ding B, Zhang J, Zhou Y, Liu Y, Yang H, Wu Q, Chen F (2019) Acidic conditions induce the accumulation of orange *Monascus* pigments during liquid-state fermentation of *Monascus ruber* M7. Appl Microbiol Biotechnol 103:8393–8402

Lian J, HamediRad M, Zhao H (2018) Advancing metabolic engineering of *Saccharomyces cerevisiae* using the CRISPR/Cas system. Biotechnol J 1:700601. https://doi.org/10.1002/biot.201700601

Liu J, Hu W, Chen H, Ni Q, Xu H, Yang X (2007) Isotretinoin-loaded solid lipid nanoparticles with skin targeting for topical delivery. Int J Pharm *328*(2):191–195

Liu R, Chen L, Jiang Y, Zhou Z, Zou G (2015) Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. Cell Discov 1(1):1–11

Liu W, An C, Shu X, Meng X, Yao Y, Zhang J, Chen F, Xiang H, Yang S, Gao X, Gao SS (2020) A dual-plasmid CRISPR/Cas system for mycotoxin elimination in polykaryotic industrial fungi. ACS Synth Biol 9(8):2087–2095

Loksuwan J (2007) Characteristics of microencapsulated β-carotene formed by spray drying with modified tapioca starch, native tapioca starch and maltodextrin. Food Hydrocoll 21 (5–6):928–935

Matsu-ura T, Baek M, Kwon J, Hong C (2015) Efficient gene editing in *Neurospora crassa* with CRISPR technology. Fungal Biol Biotechnol 2(1):4

Matsuo T, Hara T, Ikemoto H, Jacques K, Kasai S, Kinjo A, Ogata M, Fukuda K, Tachibana S (2018) Improvement of photobleaching of Monascus pigments using novel nanoencapsulation technology. Sci Bull Facul Agric Univ Ryukyus 65:83–89

McQueen L, Lai D (2019) Ionic liquid aqueous two-phase systems from a pharmaceutical perspective. Front Chem 7:135

Mendez A, Perez C, Montanez JC, Martinez G, Aguilar CN (2011) Red pigment production by *Penicillium purpurogenum* GH2 is influenced by pH and temperature. J Zhejiang Univ Sci B (Biomed Biotechnol) 12:961–968

Mishra PD, Deshmukh SK, Kulkarni-Almeida A, Roy S, Jain S, Verekar SA, Balakrishnan A, Vishwakarma R (2013) Anti-inflammatory and anti-diabetic naphthoquinones from an endophytic fungus. Indian J Chem Sect B 52:555–557

Mishra PD, Verekar SA, Deshmukh SK, Joshi KS, Fiebig HH, Kelter G (2015) Altersolanol A: a selective cytotoxic anthraquinone from a *Phomopsis* sp. Lett Appl Microbiol 60(4):387–391

Morales JO, Valdés K, Morales J, Oyarzun-Ampuero F (2015) Lipid nanoparticles for the topical delivery of retinoids and derivatives. Nanomedicine 10(2):253–269

Mukherjee G, Singh SK (2011) Purification and characterization of a new red pigment from *Monascus purpureus* in submerged fermentation. Process Biochem 46:188–192

Mukherjee G, Mishra T, Deshmukh SK (2017) Fungal pigments: an overview. In: Satyanarayana T, Deshmukh SK, Johri BN (eds) Developments in fungal biology and applied mycology. Springer Nature, Singapore, pp 525–541

Narendrababu BN, Shishupala S (2017) Spectrophotometric detection of pigments from *Aspergillus* and *Penicillium* isolates. J Appl Biol Biotechnol 5:53–58

Nastiti CMRR, Ponto T, Abd E, Grice JE, Benson HAE, Roberts MS (2017) Topical nano and microemulsions for skin delivery. Pharmaceutics 9(4):1–25

Nielsen ML, Isbrandt T, Rasmussen KB, Thrane U, Hoof JB, Larsen TO, Mortensen UH (2017) Genes linked to production of secondary metabolites in *Talaromyces atroroseus* revealed using CRISPR-Cas9. PLoS One 12(1):e0169712

Nik AM, Langmaid S, Wright AJ (2012) Digestibility and β-carotene release from lipid nanodispersions depend on dispersed phase crystallinity and interfacial properties. Food Funct 3(3):234–245

Nødvig CS, Nielsen JB, Kogle ME, Mortensen UH (2015) A CRISPR-Cas9 system for genetic engineering of filamentous fungi. PLoS One 10(7):e0133085

Orozco SFB, Kilikian BV (2008) Effect of pH on citrinin and red pigments production by *Monascus purpureus* CCT3802. World J Microbiol Biotechnol 24:263–268

Padmapriya C, Murugesan R (2016) Characterization of methanolic extract of red pigment from *Penicillium purpurogenum* and its antioxidant activity. J Pure Appl Microbiol 10:1505–1511

Palacio-Barrera AM, Areiza D, Zapata P, Atehortúa L, Correa C, Peñuela-Vásquez M (2019) Induction of pigment production through media composition, abiotic and biotic factors in two filamentous fungi. Biotechnol Rep 21:e00308

Parthasarathy R, Sathiyabama M (2015) Lovastatin-producing endophytic fungus isolated from a medicinal plant *Solanum xanthocarpum*. Nat Prod Res 29(24):2282–2286

Pastrana L, Blanc PJ, Santerre AL, Loret MO, Goma G (1995) Production of red pigments by *Monascus ruber* in synthetic media with a strictly controlled nitrogen source. Process Biochem 30:333–341

Patil SA, Sivanandhan G, Thakare DB (2015) Effect of physical and chemical parameters on the production of red exopigment from *Penicillium purpurogenum* isolated from spoilt onion and study of its antimicrobial activity. Int J Curr Microbiol Appl Sci 4:599–609

Poorniammal R, Parthiban M, Gunasekaran S, Murugesan R, Thilagavathi G (2013) Natural dye production from *Thermomyces* sp. fungi for textile application. Indian J Fibre Text Res 38:276–279

Pradeep FS, Pradeep BV (2013) Optimization of pigment and biomass production from *Fusarium moniliforme* under submerged fermentation conditions. Int J Pharm Pharm Sci 5:526–535

Pradeep FS, Palaniswamya M, Ravib S, Thangamanib A, Pradeep BV (2015) Larvicidal activity of a novel isoquinoline type pigment from *Fusarium moniliforme* KUMBF1201 against *Aedes aegypti* and *Anopheles stephensi*. Process Biochem 50:1479–1486

Prathiban M, Thilagavathi G, Viju S (2016) Development of antibacterial silk sutures using the natural fungal extract for healthcare applications. J Text Sci Eng 6:249

Ramesh C, Vinithkumar NV, Kirubagaran R, Venil CK, Dufossé L (2019) Multifaceted applications of microbial pigments: current knowledge, challenges and future directions for public health implications. Microorganisms 7(7):186

Ramesha A, Venkataramana M, Nirmaladevi D, Gupta VK, Chandranayaka S, Srinivas C (2015) Cytotoxic effects of oosporein isolated from endophytic fungus *Cochliobolus kusanoi*. Front Microbiol 6:870

Rao J, McClements DJ (2011) Formation of flavor oil microemulsions, nanoemulsions and emulsions: influence of composition and preparation method. J Agric Food Chem 59 (9):5026–5035

Rao MPN, Xiao M, Li WJ (2017) Fungal and bacterial pigments: secondary metabolites with wide application. Front Microbiol 8:1113

Ratna, Padhi BS (2012) Pollution due to synthetic dyes toxicity and carcinogenicity studies and remediation. Int J Environ Sci 3(3):940–955

Robinson SC, Hinsc E, Weber GL, Freitas S (2014) Method of extraction and resolubilisation of pigments from *Chlorociboria aeruginosa* and *Scytalidium cuboideum*, two prolific spalting fungi. Coloration Technol 130:221–225

Saravanan D, Radhakrishnan M (2016) Antimicrobial activity of pigments produced by fungi from the Western Ghats. J Chem Pharm Res 8:634–638

Selvakumar P, Rajasekar S, Periasamy K, Raaman N (2008) Isolation and characterization of melanin pigment from *Pleurotus cystidiosus* (telomorph of *Antromycopsis macrocarpa*). World J Microbiol Biotechnol 24(10):2125–2131

Sen T, Barrow CJ, Deshmukh SK (2019) Microbial pigments in the food industry—challenges and the way forward. Front Nutr 6:7

Sethi BK, Parida P, Sahoo SL, Dikshit B, Pradhan C, Sena S, Behera BC (2016) Extracellular production and characterization of red pigment from *Penicillium purpurogenum* BKS9. Alger J Nat Prod 4:379–392

Sharma D, Gupta C, Aggarwal S, Nagpal N (2012) Pigment extraction from fungus for textile dyeing. Indian J Fibre Text Res 37:68–73

Shen L, Zhang X, Liu M, Wang Z (2014) Transferring of red *Monascus* pigments from nonionic surfactant to hydrophobic ionic liquid by novel microemulsion extraction. Sep Purif Technol 138:34–40

Shin CS, Kim HJ, Kim MJ, Ju JY (1998) Morphological change and enhanced pigment production of *Monascus* when co-cultured with *Saccharomyces cerevisiae* or *Aspergillus oryzae*. Biotechnol Bioeng 59:576–581

Silbir S, Goksungur Y (2019) Natural red pigment production by *Monascus purpureus* in submerged fermentation systems using a food industry waste: Brewer's spent grain. Foods 8 (5):161

Singh SM, Singh PN, Singh SK, Sharma PK (2014) Pigment, fatty acid and extracellular enzyme analysis of a fungal strain *Thelebolus microsporus* from Larsemann Hills, Antarctica. Polar Record 50:31–36

Soumya K, Narasimha Murthy K, Sreelatha GL, Tirumale S (2018) Characterization of a red pigment from *Fusarium chlamydosporum* exhibiting selective cytotoxicity against human breast cancer MCF-7 cell lines. J Appl Microbiol 125:148–158

Stange S, Steudler S, Delenk H, Werner A, Walther T, Wagenführ A (2019) Influence of the nutrients on the biomass and pigment production of *Chlorociboria aeruginascens*. J Fungi 5:40

Subramanian CS, Velusamy K, Karuppan P (2014) Orange dye from *Pycnoporus* sp. for textile industries. In: Proceedings of 8th international conference on mushroom biology and mushroom products, New Delhi, 19–22 November 2014, vol I and II. ICAR-Directorate of Mushroom Research, New Delhi, pp 456–460

Suryanarayanan TS, Ravishankar JP, Venkatesan G, Murali TS (2004) Characterization of the melanin pigment of a cosmopolitan fungal endophyte. Mycol Res 108:974–978

Tan Z, Li F, Xu X (2012) Isolation and purification of aloe anthraquinones based on an ionic liquid/salt aqueous two-phase system. Sep Purif Technol 98:150–157

Tseng YY, Chen MT, Lin CF (2000) Growth, pigment production and protease activity of *Monascus purpureus* as affected by salt, sodium nitrite, polyphosphate, and various sugars. J Appl Microbiol 88:3–37

Tudor D, Robinson SC, Cooper PA (2013) The influence of pH on pigment formation by lignicolous fungi. Int Biodeter Biodegr 80:22–28

Velmurugan P, Lee YH, Venil CK, Lakshmanaperumalsamy P, Chae JC, Oh BT (2010) Effect of light on growth, intracellular and extracellular pigment production by five pigment-producing filamentous fungi in synthetic medium. J Biosci Bioeng 109:346–350

Ventura SP, Santos-Ebinuma VC, Pereira JF, Teixeira MF, Pessoa A, Coutinho JA (2013) Isolation of natural red colorants from fermented broth using ionic liquid-based aqueous two-phase systems. J Ind Microbiol Biotechnol 40(5):507–516

Wang X, Tao J, Wei D, Shen Y, Tong W (2004) Development of an adsorption procedure for the direct separation and purification of prodigiosin from culture broth. Biotechnol Appl Biochem 40:277–280

Zhang X, Wang J, Chen M, Wang C (2013) Effect of nitrogen sources on production and photostability of *Monascus* pigments in liquid fermentation. IERI Proc 5:344–350

Zhou B, Tian Y, Zhong H (2019) Application of a two-stage agitation speed control strategy to enhance yellow pigments production by *Monascus anka* mutant. J Microbiol Biotechnol Food Sci 8:1260–1264

Bioprospecting of Marine Fungi

18

Prabha Devi and Narsinh Thakur

Abstract

Marine fungi are eukaryotic organisms occurring in water and sediments as parasitic and/or symbiotic on marine flora and fauna. The Indian subcontinent has a rich marine habitat, and fungi have high diversity and distribution colonizing marine flora and fauna as endophytes. Since marine fungi have to compete with a diverse array of organisms, accordingly, they develop several mechanisms for survival and growth. These unique attributes of fungi seem to herald great promise in the production of valuable secondary metabolites which mainly comprise of alkaloids, terpenoids, steroids, peptides, polyketides, and others. All of these have excellent bioprospecting potentials such as antitumor, anticancer, antimicrobial, and enzymatic activities, bioremediation, as well as in the production of several therapeutically bioactive agents. It is believed that exploring fungi from new and extreme habitats especially those endophytic with other marine flora and fauna may lead to the isolation of novel fungal metabolites with immense bioactivities. This review summarizes the biologically active secondary metabolites produced by marine-derived fungi. It also briefs some of the products isolated and identified currently in our laboratory. We further discuss that marine fungi can be made to induce production of novel metabolites by using modern and sophisticated metabolic engineering techniques like mutagenomics, recombinant DNA techniques, and improved molecular microbiology techniques. Several such bioprospecting strategies may be utilized in order to develop competent fungal endophytes for the discovery of novel metabolites.

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Anticancer · Antitumor · Antimicrobial · Bioprospecting · Marine-derived fungi

18.1 Introduction

Marine endophytic fungi are those which infect living tissues of marine organisms, while marine-derived fungi live and survive in marine or estuarine environments. Both these groups constitute a very interesting and commercially viable group of fungi (Raghukumar 2012; Thirunavukkarasu et al. 2011). The endophytic fungi live in a robust, stressful, and competitive environment with significant variation in biotic and abiotic factors including dissolved oxygen, nutrients, and space. The endophytic fungi have evolved physiological and chemical capabilities to withstand harsh conditions brought about by changes in pressure, salinity, pH, temperature, pollution, and climate in the environment. Studies have shown that endophytic fungi are not host specific (Cohen 2006). In spite of their abundances and widespread distribution, surprisingly, marine fungi have not received adequate attention when compared to their terrestrial counterparts. This is mainly because only a small fraction (5%) of the marine fungi has been cultured in the laboratory and hence a very small percentage is exploited (Demain 2006; Shin 2020). Their adaptations have provided them with the ability to produce chemically diverse secondary metabolite possessing unique biotechnological applications (Bhadury et al. 2006; Saleema et al. 2007; Devi et al. 2013). If we can overcome the difficulties involved in their isolation and culturing, several more new and novel compounds would be discovered (Rappe and Giovannoni 2003; Stevenson et al. 2004). Moreover, advancement in newer technologies and engineering offering scuba diving, snorkeling, and manned submersible and remotely operated vehicles (ROVs) would be an added advantage for scientific exploration of invertebrates and their associates.

18.2 Natural Products from Marine Fungi

Marine fungi have been studied as early as 1848 when the first record of the species *Sphaeria posidoniae* from the rhizome of seagrass, *Posidonia oceanic*, was reported (Montagne 1846). Moricca and Ragazzi (2008) have shown that the type of interaction between the endophyte and the host is controlled by the genes of both the organisms and modulated by the environment. The diversity of marine fungi is not adequately represented, and most of the published work on their secondary metabolites is only centered around few common genera like *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichoderma*. Hence, research for discovering novel bioactive secondary metabolites from several other marine fungi growing in adverse habitats is progressively improving.

Marine fungal metabolites, viz., pyrones, macrolides, aromatic compounds, cytochalasins, naphthalenones, anthracenones, terpenes, amino acids, butanolides,

and several more, have been identified (Turner and Aldridge 1983; Cole et al. 2003). Several useful hits have also been obtained from the discovery of drug perspective (Molinski et al. 2009; Butler et al. 2014). These compounds isolated possess an array of bioactivities from antibacterial, antiviral, antifungal, anticancer, antiplasmodial, anti-inflammatory, antifeeding, or antioxidant among several others (Schlingmann et al. 2002; Bugni and Ireland 2004; Bhadury et al. 2006; Ebel 2006). After celebrating 90 years of discovery of penicillin from a fungal culture by Alexander Fleming, which began the era of antibiotic chemotherapy, the next to follow was cephalosporin C which was the first bioactive compound to be reported by Brotzu in 1945 from a marine fungus, *Acremonium chrysogenum* (Newton and Abraham 1955; Godzeski et al. 1968; Bugni and Ireland 2004; Bhadury et al. 2006; Sithranga and Kathiresan 2011). Ever since, the discovery of compounds and biological activities from marine fungi has been increasing several folds (Wiese et al. 2011; Imhoff 2016). Out of more than 22,000 bioactive metabolites, 38% are of fungal origin. Compounds like aphidicolin and phenylahistin which are promising anticancer compounds were isolated from the culture broth of *Nigrospora sphaerica* (Starratt and Loschiavo 1974) and *Aspergillus utus* (Kanoh et al. 1997), respectively. The new alkaloid sorbicillactone A isolated from *Penicillium chrysogenum* (sponge-derived fungus) showed significant antileukemic activity (Bringmann et al. 2007). Other significant fungal metabolites include phomactins, peribysins, tryprostatins, prialt, yondelis, and several others (Table 18.1). Despite significant progress in drug discovery, newer drugs are still required to combat global resistance to new infections like SARS, Zika viruses, corona viruses, etc.

Marine fungi have been a huge reservoir for biologically active compounds with a potential for drug development (Gomes et al. 2015; Wu et al. 2015, 2016; Deshmukh et al. 2017). Some of the marine-based fungi have been associated with sea sediments and invertebrates (sponges, corals, mollusks, bryozoans, algae, mangroves, etc.). Those derived from sponges called sponge-derived fungi like *Penicillium chrysogenum* produced sorbicillactone A, showing promising antileukemic activity (Ebel 2006; Bringmann et al. 2007). *Engyodontium album*, isolated from marine sponge *Cacospinga scalaris*, produces polyketides engyodontochones A–F showing antibiotic properties (Wu et al. 2016). Fungi belonging to family *Lindgomycetaceae* were reported to produce lindgomycin (Wu et al. 2015) and also derivative of ascosetin (Ondeyka et al. 2014) that possessed significant antibiotic properties. Furthermore, the pyridine trichodin A produced by marine-derived *Trichoderma* sp. inhibited MRSA and *Candida albicans* (Wu et al. 2014). The genus *Aspergillus* from the marine environment is a well-known producer of several novel compounds. Some of the most significant reports of fungi isolated from different ecological niches like those associated with marine sponge (Bringmann et al. 2007; Liu et al. 2010; Bhatnagar and Se-Kwon 2010; Ingavat et al. 2011; Cohen et al. 2011; Tsukada et al. 2011; Sun et al. 2012; Pinheiro et al. 2012; Fukuda et al. 2014; Elissawy et al. 2015), gorgonian (Wei et al. 2005; Trisuwan et al. 2011), soft coral (Afiyatullov et al. 2012; Li et al. 2012), algae (Cui et al. 2010; Elsebai et al. 2010, 2013; Ishino et al. 2010, 2012; Gao et al. 2011; Debbab et al. 2012; Sun et al. 2012; Zhuravleva et al. 2012; Liu et al. 2010; Wu et al. 2013; Fang et al. 2014;

Table 18.1 Some of the significant compounds isolated from marine fungi between 2000 and 2020 with activities

Secondary metabolites	Fungal name	Activity	Reference
Halamide	*Aspergillus* sp.	Chemotherapeutic	Fenical et al. (2000)
Enfumafungin	*Hormonema* sp.	Antifungal	Peláez et al. (2000)
Fusapyrone and deoxyfusapyrone	*Fusarium semitectum*	Antifungal	Altomare et al. (2000)
Dictyonamides	*Ceratodictyon spongiosum*	Anticancer	Komatsu et al. (2001)
5-Hydroxyramulosin and 7-methoxycoumarin	*Phoma* sp.	Anticancer, anti HIV, antifungal	Konig et al. (2002)
Kojic acid	*Aspergillus wentii*	Antibiotic	Devi et al. (2002)
Stachyflin (terpenoid) and acetylstachyflin	*Stachybotrys*	Antiviral	Minagawa et al. (2002)
Aigialomycin A–E	*Aigialusparvus*	Antimalarial	Isaka et al. (2002)
Trichodermamide B (dipeptide)	*Trichoderma* sp.	Cytotoxic	Garo et al. (2003)
Isocyclocitrinol and 22-acetylisocyclocitrinol A	*P. citrinum*	Antibiotic	Amagata et al. (2003)
Halovirs A–E lipopeptides	*Scytalidium* sp.	Antiviral	Rowley et al. (2003)
Keisslone	*Keissleriella* sp.	Antifungal	Liu et al. (2003)
Griseofulvin	*Penicillium waksmanii*	Antifungal and immunosuppressive	Petit et al. (2004)
Verrucarin A	*Myrothecium roridum*	Antitumor	Oda et al. (2005)
Leptosins	*Leptosphaeria* sp.	Cytotoxic	Yanagihara et al. (2005a, b)
Shimalactones A	*Emericella variecolor*	Cytotoxic	Wei et al. (2005)
Siccayne and deacetoxyyanuthone	*Aspergillus* sp.	Antibacterial	Li et al. (2005)
Clonostachysins A–B	*Clonostachys rogersoniana*	Antialgal	Adachi et al. (2005)
Leptosins	*Leptosphaeria* sp.	Anticancer	Yanagihara et al. (2005a, b)
Hirsutellic acid A	*Hirsutella* sp.	Antimalarial	Thongtan et al. (2006)
Guisinol	*Emericella unguis*	Antibacterial	Punyasloke et al. (2006)
Lunatin	*Curvularia lunata*	Antibacterial	Punyasloke et al. (2006)
Microsphaeropsin	*Microsphaeropsis* sp.	Antifungal	Punyasloke et al. (2006)
Naphthoquinoneimine and nigerasperone A–C	*A. niger*	Antifungal	Zhang et al. (2007)

(continued)

Table 18.1 (continued)

Secondary metabolites	Fungal name	Activity	Reference
Zygosporamide	*Zygosporium masonii*	Cytotoxic	Oh et al. (2007)
Cephalimysin A	*A. fumigatus*	Cytotoxic	Yamada et al. (2007)
Cephalimysin A	*A. fumigatus*	Cytotoxicity	Yamada et al. (2007)
Sorbicillactone A	*P. chrysogenum*	Antileukemic	Bringmann et al. (2007)
Phomolides A and B	*Phomopsis* sp.	Antimicrobial	Du et al. (2008)
Chlorohydroaspyrones A and B	*Exophiala* sp.	Antibacterial	Zhang et al. (2008)
Nigrospoxydons A–C	*Nigrospora* sp.	Antibiotic	Trisuwan et al. (2008)
Balticolid	*Ascomycetes*	Antiviral	Mi-Hee et al. (2008)
Fumigaclavine	*P. viridicatum*	Antibiotic	Mi-Hee et al. (2008)
Trichodermaketone A	*T. koningii*	Antifungal	Mi-Hee et al. (2008)
Chaetochromin A	*Hypocreavinosa*	Cytotoxic	Mi-Hee et al. (2008)
Averatin	*A. versicolor*	Cytotoxic/ antibacterial	Mi-Hee et al. (2008)
Fusaquinon A, B, C	*Fusarium* sp.	Cytotoxic	Mi-Hee et al. (2008)
Circundatin A, B, E, H	*A. ostianus*	Antibacterial	Mi-Hee et al. (2008)
Hirsutanol A	*Chondrostereum* sp.	Cytotoxic	Mi-Hee et al. (2008)
Balticolid	Ascomycetes	Antiviral	Mi-Hee et al. (2008)
Fumigaclavine	*P. viridicatum*	Antibiotic	Mi-Hee et al. (2008)
Neomangicol B	*Fusarium* sp.	Antibacterial	Zhang et al. (2009)
Cottoquinazoine A	*A. versicolor*	Cytotoxic	Fremlin et al. (2009)
Conidiogenone C	*P. chrysogenum*	Cytotoxicity	Du et al. (2009)
Verticinols A	*Verticillium tenerum*	Antifungal	Almeida et al. (2010)
Aspergiolide A	*Aspergillus glaucus*	Antitumor	Xueqian et al. (2010)
Protulactones A and B	*Aspergillus* sp.	Antibiotic	Jae and Hyuncheol (2010)
Nigrosporanenes A and B	*Nigrospora* sp.	Cytotoxic	Rukachaisirikul et al. (2010)
Paeciloxocins A, B	*Paecilomyces* sp.	Cytotoxic	Wen et al. (2010)
Expansols A and B	*P. expansum*	Cytotoxic	Lu et al. (2010)
Clodepsipeptide	*Clonostachys* sp.	Antibacterial	Samuel and Prince (2011)
Emerimidine A and B	*Emericella* sp.	Antiviral	Zhang et al. (2011)
Austin and Austinol	*Emericella* sp.	Antiviral	Zhang et al. (2011)
Balticolid	*Pleosporales* sp.	Antiviral	Shushni et al. (2011)
Xanthocillin, chrysogine, meleagrin	*Penicillium commune*	Cytotoxic	Shang et al. (2012a, b), Zhao et al. (2012)

(continued)

Table 18.1 (continued)

Secondary metabolites	Fungal name	Activity	Reference
Xanthocillin X	*Penicillium commune*	Cytotoxic	Shang et al. (2012a, b)
Diaporthin B and Pimarane diterpenes	*Epicoccum* sp.	Antibacterial	Xia et al. (2012)
Bisabolane (sesquiterpenoid) aspergiterpenoid	*Aspergillus* spp.		Li et al. (2012)
Lajollamide A (pentapeptide)	*Asteromyces cruciatus*	Antimicrobial	Gulder et al. (2012)
Citrinin	*Penicillium chrysogenum*	Antibacterial	Devi et al. (2012)
Sterolic acid	*Penicillium* sp.	Antitumor	Li et al. (2012)
Breviones A, I	*Penicillium* sp.	Cytotoxic	Li et al. (2012)
Neochinulin A	*Microsporium* sp.	Cytotoxic	Wijesekara et al. (2013)
Neochinulin A	*Penicillium* sp.	Antibacterial	Devi et al. (2012)
Asperolides A, B and wentilactones, and Kojic acid	*A. wentii*	Cytotoxic Antibacterial	Sun et al. (2012)
Neomangicol B	*Fusarium* sp.	Antibacterial	Swathi et al. (2013)
Penicinoline	*Penicillium* sp.	Cytotoxic	Bladt et al. (2013)
6-n-pentyl α-pyrone Cyclonerodiol	*Trichoderma harzianum*	Antifungal	Devi et al. (2013)
Fumagillin	*Aspergillus fumigatus*	Anticancer	Bladt et al. (2013); Eble and Hanson (1951)
Aogacillins A and B	*Simplicillium* sp.	Antibiotic	Keiko Takata et al. (2013)
Plinabulin	*Aspergillus* sp.	Cytotoxic	Bladt et al. (2013)
Wentilactone G	*Aspergillus wentii*	Cytotoxic	Zhang et al. (2013)
Wentilactone B	*A. dimorphicus*	Anticancer	Zhang et al. (2013)
Phenylspirodrimanes	*Stachybotrys chartarum*	Antiviral	Ma et al. (2013)
Aspinotriol A, B, aspilactonols A–F, chaetoquadrin F	*Aspergillus* sp.	Cytotoxic	Chen et al. (2014)
Cinnamolide	*A. ochraceus*	Cytotoxic	Fang et al. (2014)
Physcion	*Microsporum* sp.	Apoptosis	Wijesekara et al. (2014)
Psychrophilins and versicotide C	*A. versicolor*	Hypolipidimic	Peng et al. (2014)
Psychrophilin E (cyclic tripeptide)	*Aspergillus* sp.	Cytotoxic	Ebada et al. (2014)
Preussin B (pyrrolidine alkaloid)	*Simplicillium lanosoniveum*	Antimicrobial	Fukuda et al. (2014)

(continued)

Table 18.1 (continued)

Secondary metabolites	Fungal name	Activity	Reference
Aphidicolin	*Cephalosporium aphidicola*	Anticancer	Baranovskiy et al. (2014)
Fellutamide C	*A. versicolor*	Cytotoxic	Evidente et al. (2014)
Ascomycotin A	*Ascomycota* sp.	Antibiotic	Tian et al. (2014)
Asperelines A–F	*Trichoderma asperellum*	Antibiotic	Brito et al. (2014)
Similanamide and isocoumarin	*A. similanensis*	Cytotoxic	Prompanya et al. (2015)
Anguidine	*Fusarium* sp.	Anticancer	Kornienko et al. (2015)
Spartinols A–D	*Phaeosphaeria spartinae*	Cytotoxic	Elsebai et al. (2015)
Terrelumamides (lumazine peptides)	*A. terreus*	Improves insulin sensitivity	You et al. (2015)
Penicitrinine A	*P. citrinum*	Antitumor	Liu et al. (2015)
Circumdatin and westerdijikin A	*A. westerdijkiae*	Antiproliferative	Fredimoses et al. (2015)
Chrodrimanins I–J	*Penicillium funiculosum*	Antiviral	Zhou et al. (2015)
Asteltoxins	*Aspergillus* sp.	Antiviral	Tian et al. (2016)
Simplicilliumtides A–H	*Simplicillium obclavatum*	Immunostimulant	Liang et al. (2016)
Acaromycin and cryptosporin	*Acaromyces ingoldii*	Antitumor	Gao et al. (2016a)
Asperolide E	*Aspergillus wentii*	Cytotoxic	Li et al. (2016)
Chlorofolipastatin and unguinol	*A. ungui*	Anti-atherosclerotic	Uchida et al. (2016)
Varioloid A, B	*Paecilomyces variotii*	Cytotoxic	Zhang et al. (2016)
Spirobrocazines C and brocazine G	*Penicillium brocae*	Cytotoxic	Meng et al. (2016)
Rhizovarins A, B, E Penitrems A, C, F	*Mucor irregularis*	Cytotoxic	Gao et al. (2016b)
Preussomerins Chloropreussomerins A and B Spreussomerin K, H, G, F	*Lasiodiplodia threobromae*	Cytotoxic	Chen et al. (2016)
Campyridones D Ilicicolin H	*Campylocarpon* sp.	Cytotoxic	Zhu et al. (2016)
Calocerins A–D 9-Oxostrobilurin	*Favolaschia calocera*	Cytotoxic, antibiotic	Chepkirui et al. (2016)
Dihydroaltersolanol D	*Stemphylium globuliferum*	Cytotoxic	Liu et al. (2015)
Altersolanols A, B, N Alterporriol C	*Stemphylium globuliferum*	Antimicrobial	Debbab et al. (2012)
Cephalosporin C	*C. acremonium*	Antibacterial	Khan (2017)

(continued)

Table 18.1 (continued)

Secondary metabolites	Fungal name	Activity	Reference
Lovastatin	*Aspergillus terreus*	Cholesterol lowering	Boruta and Bizukojc (2017)
Conidiogenone C	*Penicillium* sp.	Cytotoxic	Liu et al. (2017)
Aspergilol G–I	*Aspergillus versicolor*	Antioxidant	Huang et al. (2017)
Neoechinulin A	*Microsporium* sp.	Cytotoxic	Deshmukh et al. (2017)
Enniatin B	*Fusarium* sp.	Antibacterial	Prosperini et al. (2017)
Indole derivative	*Paecilomyces variotii*	Cytotoxic	Deshmukh et al. (2017)
Aspersymmetide A (cyclohexapeptide)	*A. versicolor*	Cytotoxic	Hou et al. (2017)
Aspergillipeptides D–G	*Aspergillus* sp.	Antiviral	Ma et al. (2017)
Penicillixanthone A	*A. fumigates*	Anti-HIV	Tan et al. (2017)
Trichodermanones A–D and sorbicillinoid	*Trichoderma citrinoviride*	Cytotoxic	Marra et al. (2018)
Cyclosporin A	*Tolypocladium inflatum*	Antifungal	Yang et al. (2018)
Fusaperazine	*Penicillium crustosum*	Cytotoxic	Liu et al. (2018a, b)
Cyclic tetrapeptide	*A. violaceofuscus*	Anti-inflammatory	Liu et al. (2018a, b)
Auxarthrols D–H	*Sporendonema casei*	Cytotoxic activity	Ge et al. (2019)
Aspergillamides (tripeptides)	*A. terreus*	Cytotoxic	Luo et al. (2019)
Asperterrestide (tetrapeptide)	*A. terreus*	Cytotoxic, antiviral	Youssef et al. (2019)
Tenellone H	*Phomopsis lithocarpus*	Cytotoxic activity	Shin (2020)
Pestalotionol	*Penicillium* sp.	Antibiotic	Arifeen et al. (2020)

Elsebai et al. 2015), mangroves ecosystem (Lin et al. 2001; Huang et al. 2006, 2011, 2013; Lu et al. 2009, 2010; Gao et al. 2010; Chokpaiboon et al. 2011; Li et al. 2011; Zhou et al. 2011; Bhimba et al. 2012; Mei et al. 2012; Song et al. 2012; Sun et al. 2012; Hemberg et al. 2013; Xiao et al. 2013; Zeng et al. 2014), and sediments (Du et al. 2009; Zhang et al. 2011; Sun et al. 2012; Arai et al. 2013; Wu et al. 2013; Liao et al. 2014; Xia et al. 2014) for secondary metabolites are worth mentioning.

A detailed list of compounds from marine fungi is presented in Table 18.1. Antibacterial drugs that exist in the market like mycophenolic acid, penicillins, cephalosporin, pleuromutilin, retapamulin, fusidic acid, griseofulvin, etc. are of fungal origin (Grove et al. 1954; Newton and Abraham 1955; Godtfredsen et al. 1962; Novak and Shlaes 2010). Fungal metabolites like enfumafungin,

pneumocandin, favolon, and sporothriolode possess antifungal activities against human pathogens (Peláez et al. 2000; Surup et al. 2014; Chepkirui et al. 2016). Significant anticancer compounds were also reported from marine fungi like irofulven, illudin, aphidicolin, and leptosin C & F (Bucknall et al. 1973; Starratta and Loschiavo 1974; Yanagihara et al. 2005a, b; Chin et al. 2006; Pejin et al. 2013). Some fungal metabolites like hinnuliquinone, stachybosin-D, integrastatin-A, stachyflin, vanitaracin A, 4-hydroxypleurogrisein, cytosporaquinone B, and rhodatin showed significant antiviral activities (Minagawa et al. 2002; Singh et al. 2002, 2004; Ma et al. 2013; Kaneko et al. 2015; Sandargo et al. 2018, 2019; Narmani et al. 2019). Immunosuppressive and immunomodulatory agents like cyclosporin A and mycophenolate mofetil have fungal origin (Dreyfuss et al. 1996; Allison and Eugui 2000).

However, in comparison to other organisms, the number of products from marine fungi is still quite small. Till 2015, there were reports of 1000 and odd number of potent bioactive molecules showing varied activities targeted from marine-derived fungi (Gomes et al. 2015). In addition to the above, fungi also represent a remarkable source of enzymes possessing significant application as neutraceuticals and industrial, cosmetics, and biotechnological aspects (Farha and Hatha 2019).

18.3 Economically Important Compounds Isolated and Identified from India

Indian coastline extends over 8000 km with rich marine habitats which are not just restricted to the sea but also to the intertidal rocky, sandy, and muddy shores, mangroves, algae, and coral reefs. Due to the wide range of climatic and topological conditions, India offers a robust marine life harboring associated fungal cultures. Recently, alga-associated fungal endophytes of Konkan coast reportedly a rich and novel source of pharmaceutically active compounds have been reported by Kamat et al. (2020).

During the course of our ongoing research programs at the bioorganic laboratory at CSIR-NIO, Goa (India), we isolated several marine-derived fungi from different marine sources for their chemical characterization (Fig. 18.1). These epiphytic fungi were isolated from sponges, soft corals, ascidians, mangroves, seaweeds/algae, and sea grasses using latest techniques in order to get many culturable fungal isolates. They were grown in the laboratory using batch fermentation for producing significant amounts of crude extracts for further chemical characterization of bioactive compounds. For example, two poly-substituted aromatic compounds tetrahydroauroglaucin and isodihydroauroglaucin were identified from a marine-derived fungus *Eurotium* sp., isolated from the sea grass, *Porteresia coarctata* (Roxb) (Gawas et al. 2002). From *Porteresia coarctata*, *Fusarium nivale* was isolated that produced cyclosporine. Cyclosporine is an immunosuppressant drug given to patients immediately after organ transplant to prevent organ rejection. Five compounds, viz., two alpha pyrone derivatives, a sesquiterpene, an unsaturated hydroxy-fatty acid methyl ester, and a phenolic compound possessing significant

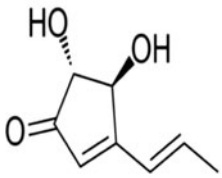
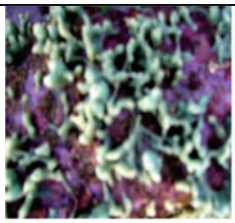
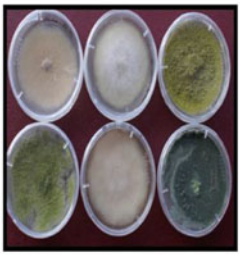
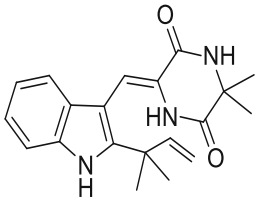
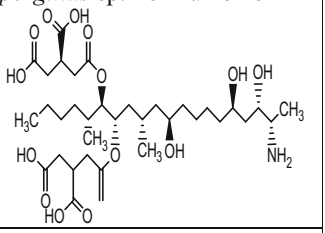
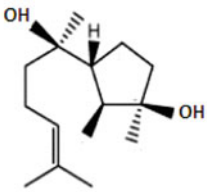
Marine organisms	Associated Fungal cultures	Mass Cultured
SPONGE  *Lissodendoryx sinensis*		*Aspergillusflavus* for terrein 
 *Haliclonatenuijamosa* (Burton)Blue sponge		*P.chrysogenum* for Neochinulin A 
 *Toxadociatoxius* (Topsent)Pink sponge		*Aspergillus* sp. for Fumonisin B 
ASCIDIAN 		*T.harzianum* for Cyclonerodiol 

Fig. 18.1 Fungal endophytes isolated from different sources for chemical characterization

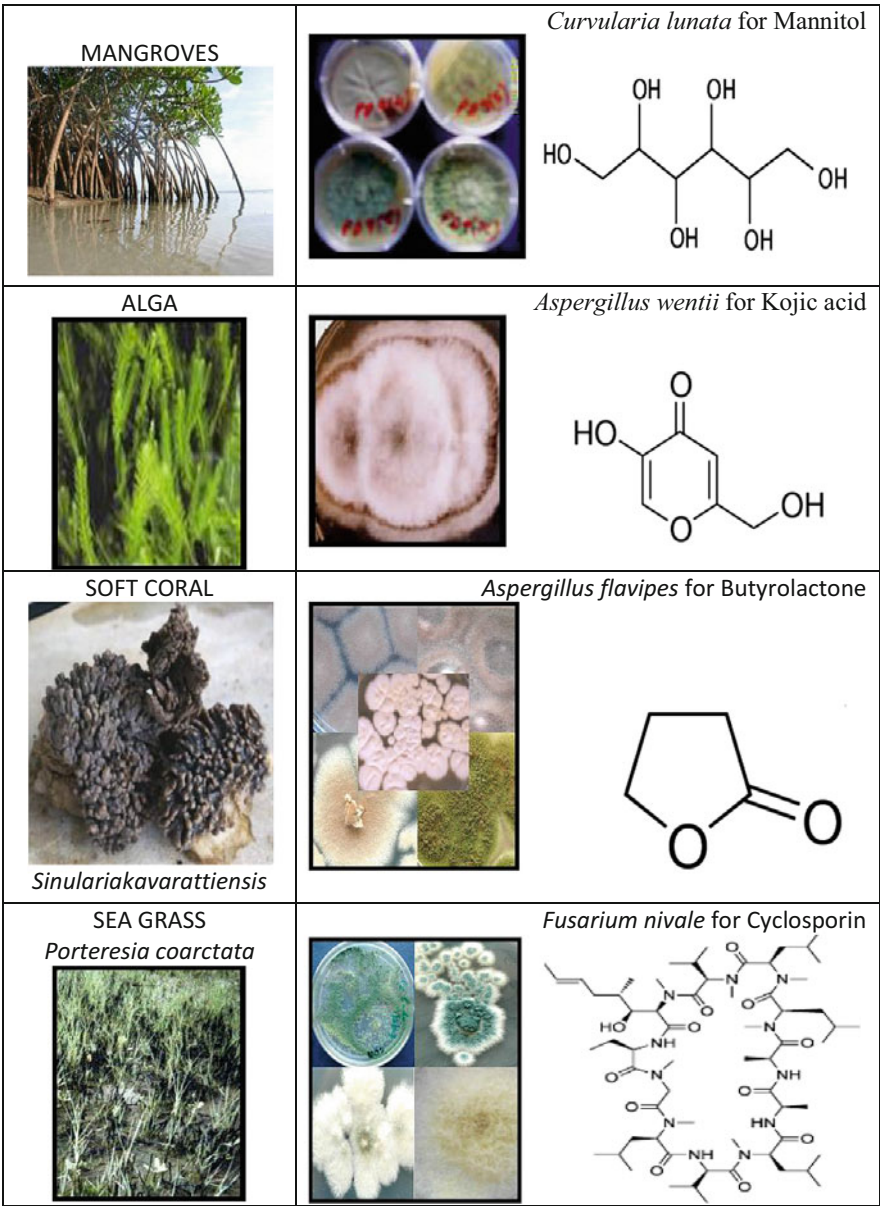


Fig. 18.1 (continued)

antifungal properties, were identified from *Trichoderma harzianum*, which was isolated from marine ascidian (Devi et al. 2013). *Aspergillus flavipes* from soft coral yielded butyrolactone, and *A. wentii* from the surface of green alga produced

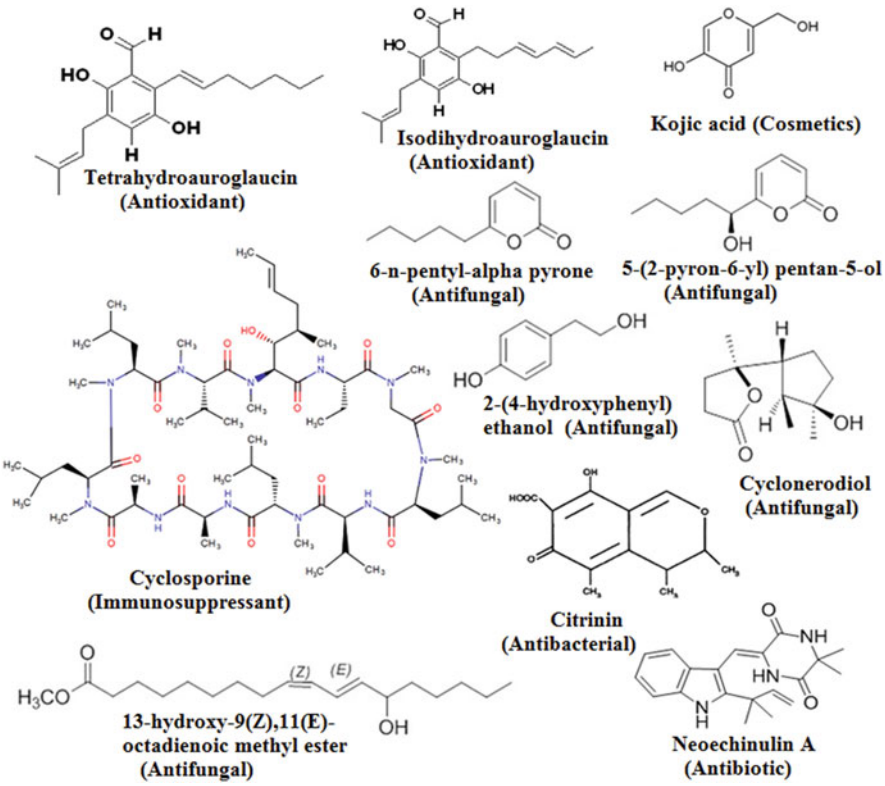


Fig. 18.2 Significant secondary metabolites identified in-house from marine fungi

Kojic acid which is widely used in cosmetics as well as an antibiotic compound (CSIR-Patent WO2002111203). *Curvularia lunata* isolated from a mangrove plant yields high quantities of mannitol (CSIR-Patent WO2004111204; EP1639094; US2004259217). Another *Aspergillus* sp. isolated from sponge *Toxodocia toxius* produced fumonisin B, and from *A. flavus* isolated from another sponge *Lissodendoryx sinensis* yield significant quantities of antiseptic compound terrain. *Penicillium chrysogenum* isolated from a mangrove produced neochinulin A (Devi et al. 2012) which was highly active against bacterial pathogen *Vibrio cholerae*. Citrinin was also produced from the same fungus *P. chrysogenum* (Fig. 18.2).

18.4 Present Status of Fungal Metabolites

Several life-saving bioactive secondary metabolites have been discovered from marine fungi possessing interesting bioactivities. Some of them are listed in Table 18.1. Beta-lactam antibiotics, followed by lipid-lowering agents atorvastatin

and simvastatin, immunosuppressive agent cyclosporine A, and antifungal agent griseofulvin, are among some important drugs from marine fungi (Aly et al. 2011; Kück et al. 2014). Although it is known that fungal metabolites have immense applications, there exist many reasons to explain as to why development in this area is still slow. Firstly, the foremost reason could be the difficulty in the isolation of culturable endophytic fungi from marine organisms. Secondly is the difficulty in obtaining sufficient quantities of the harvested material, permitting a detailed investigation related to its chemical structure and biological activity.

A large number of compounds reported to be produced by the host organisms is also produced by the endophytic fungi. Therefore, it becomes convenient to obtain similar compounds from the fungal culture rather than targeting the already extinct organisms in the sea. Marine fungi produce novel antibiotic, anti-inflammatory, anticancer, and several such beneficial compounds (Kupka et al. 1981; Davidson 1995; Fenical 1997; Pietra 1997; Liberra et al. 1998; Daferner et al. 2002; Isaka et al. 2002; Son et al. 2002; Rowley et al. 2003; Tziveleka et al. 2003; Liu et al. 2003; Abdel-Lateff et al. 2003; Gautschi et al. 2004), and it has been observed that several of the bioactives are found to be analogues to those reported earlier from fungi of terrestrial origin (Davidson 1995). Compounds like halimide, sargassamide, and avrainvillamide showed selective inhibition toward cancer cell lines. These drugs are currently reported to be in preclinical development. Pestalone, which is a chlorinated benzophenone, was isolated from *Pestalotia* sp., a marine-derived fungus, and several others like modiolide A and B, guisinol, corollosporine, xestodecalactone B, ascochital, speradine A, keisslone, seragikinone A, trichodermamide B, and zopfiellamide A showed potent antibiotic activity (Cueto et al. 2001; Daferner et al. 2002; Garo et al. 2003; Liu et al. 2003). Some of the significant antiviral compounds showing promising results are integric acid, equisetin, phomasetin, halovirs A–E, sansalvamide A, stachyflin, and many more (Rowley et al. 2003). Hence, few of the above mentioned metabolites with significant clinical importance could be produced in bulk from natural resources thereby reducing the cost of production and increasing the yield. Despite the fact that several metabolites of fungal origin had promising activity, only cephalosporin C from *Acremonium chrysogenum* was listed as a drug from marine origin (Abraham 1979; Gomes et al. 2015). Over the years, there were many metabolites known to display anticancer properties (Evidente et al. 2014). A list of drugs of fungal origin currently in the market as well as those in the final stages of clinical trial is presented in Fig. 18.3.

18.5 Potential Applications and Future Perspectives

The success stories on marine fungi and its activities have led to improving techniques for isolation and identification coupled with screening for biological activities. In addition to commercial applications, they are also considered important from the view point of biotechnological applications. According to Zilinskas et al. (1995), marine biotechnology can be defined as the direct or indirect application of

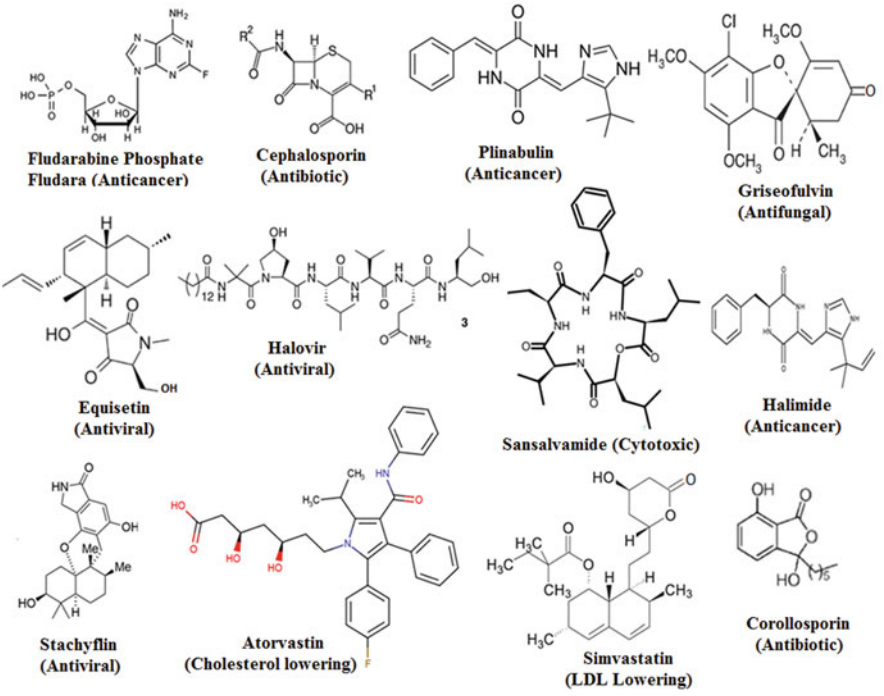


Fig. 18.3 Fungal-derived compounds currently in the market as drugs or in the final stages of clinical trials

scientific and engineering principles to the biological agents to provide required results by way of goods or products. The ocean comprises more than 70% of the earth's surface, and the marine-associated fungi are now designated as a treasured resource for the development of newer compounds. Compounds like those useful in cosmetics possess biotechnological applications, in addition to agricultural, nutritional, and pharmaceutical potentials for human well-being. A number of fungal metabolites are known to possess anticancer activities which have reached various stages of clinical trials and are also regarded as a strong and probable source of important natural products for other activities (Schiehser et al. 1986; Kobayashi and Ishibashi 1993; Toske et al. 1998; Altomare et al. 2000; Holler et al. 2000; Namikoshi et al. 2000; Wegner et al. 2000; Amagata et al. 2003; Capon et al. 2003; Lee et al. 2003; Liu et al. 2003; Tan et al. 2003; Tziveleka et al. 2003; Klemke et al. 2004; Luo et al. 2004). This is attributed to the fact that when grown under extreme conditions, they are capable of modifying the existing analogs, making them more superior in activity than the original compounds. Attempt is now being made to check the new novel compounds for antiviral activity with special reference to anti-corona virus. Such studies are still in its infancy, and it is important to try and screen as many compounds as possible from marine sources

against COVID-19. The main limitation for such studies is the absence of appropriate antiviral testing systems; this can be overcome by collaborating with labs having facilities for the same.

Marine-derived fungi thus possess this unique capability of producing compounds reportedly similar to that produced by the hosts. Due to which, there is an increase in interest in the recent decade on the bioactivities produced by them. This could be further perceived by exploiting combinatorial genetic alongside metabolic engineering to optimize the yield of target metabolites from the selected marine fungi (Moore and Piel 2000). Improving metabolite production of potential candidates by applying recombinant DNA methods to restructure metabolic network could be the best strategy for future research. The discovery and development of an array of novel bioactive compounds through metagenomic cloning and expression has proven that this technology is an excellent alternative for exploiting unculturable microbes for natural product drug discovery (Thakur et al. 2008 and references cited therein). We can use this approach to explore potential marine fungi, which are difficult to culture.

The use of high-throughput genome scanning for analyzing gene clusters involved in natural-product biosynthesis has been highlighted earlier. Polyketide synthases (PKSs) are a class of enzymes that are involved in the biosynthesis of secondary metabolites such as erythromycin, rapamycin, tetracycline, lovastatin, and resveratrol (Gokhale and Tuteja 2001). Several researchers have successfully isolated polyketide biosynthetic genes from marine bacteria and fungi, which have been cloned, sequenced, and expressed in heterologous hosts. One could follow this reverse engineering approach for detecting biosynthesis gene clusters responsible for the production of active metabolites in marine fungi. Once we detect such gene clusters, we can trigger those clusters by providing a conducive culture environment for marine fungi and get production of the desired active metabolites.

In addition to the above, new culturing techniques can also increase the rate of discovering novel compounds from marine fungi. These techniques can help in activating the dormant biogenetic gene clusters enabling the production of significantly different metabolites which were probably unexpressed when the same culture was grown under normal laboratory conditions. Furthermore, manipulating growth regulators can help to increase production of targeted secondary metabolites of interest. Cultivating microbes in mixed fermentation is yet another approach to exploit secondary metabolite synthesis, wherein the growth of one microbe in the presence of another may induce desired products. Hence, when mixed cultures were fermented, it could result in either increase in the yield of a desired compound or increase in the activity of a new compound. There are some compounds like bryostatin which are produced through aquaculture, whereas corollosporine, ziconotide, halichondrin B, dolastatin, etc. are produced synthetically. Although there are many compounds which are now produced synthetically, there are several others that are not economically viable. Hence, metabolic engineering can help in increasing the yield of selective compounds.

18.6 Conclusions

It is clear beyond doubt that the marine environment can largely influence the production of anti-infective drugs and marine-derived fungi will be a gold mine for natural bioactive compounds. Hence, we ascertain that sincere efforts may be required in terms of bulk production and processing using metabolic engineering and genomic technology resulting in better understanding and manipulating microorganism resources to make it more beneficial to mankind. Therefore, the first most important step will be to search for potential marine-derived fungal resources from the marine environment. Later through mutation, protoplast fusion, gene manipulation, and other DNA recombination techniques, highly productive candidates suitable for industrial fermentation should be established. When compared to higher marine organisms, the fungal culture medium is simple and inexpensive. Moreover, the fermentation period is also short and can be manipulated by optimizing its growth according to the time of metabolite production. Hence, in summary, marine-derived fungi can act as an important microbial resource for producing bioactive compounds. Marine flora and fauna from which these endophytic fungi are isolated also play a significant role and, hence, both in combination need to be preserved and protected. The present review thus highlights the potential of marine-derived fungi as a renowned source of bioactive compounds.

References

Abdel-Lateff A, Klemke C, Konig GM, Wright AD (2003) Two new xanthone derivatives from the algicolous marine fungus *Wardomyces anomalus*. J Nat Prod 66:706–708

Abraham EP (1979) A glimpse of the early history of the cephalosporins. Rev Infect Dis 1:99–105. https://doi.org/10.1093/clinids/1.1.99

Adachi K, Kanoh K, Wisespongp P, Nishijima M, Shizuri Y (2005) Clonostachysins A and B, new anti-dinoflagellate cyclic peptides from a marine-derived fungus. J Antibiot 58:145. https://doi.org/10.1038/ja.2005.17

Afiyatullov SS, Zhuravleva OI, Antonov AS, Kalinovsky AI, Pivkin MV, Menchinskaya ES, Aminin DL (2012) New metabolites from the marine-derived fungus *Aspergillus fumigatus*. Nat Prod Commun 7:497–500

Allison AC, Eugui EM (2000) Mycophenolate mofetil and its mechanisms of action. Immunopharmacology 47:85–118

Almeida C, Elsaedi S, Kehraus S, Konig GM (2010) Novel bisabolane sesquiterpenes from the marine-derived fungus *Verticillium tenerum*. Nat Prod Commun 5(4):507–510

Altomare C, Perrone G, Zonno MC, Evidente A, Pengue R, Fanti F, Polonelli L (2000) Biological characterization of fusapyrone and deoxyfusapyrone, two bioactive secondary metabolites of *Fusarium semitectum*. J Nat Prod 63:1131–1135

Aly AH, Debbab A, Proksch P (2011) Fifty years of drug discovery from fungi. Fungal Divers 50:3–19. https://doi.org/10.1007/s13225-011-0116-y

Amagata T, Amagata A, Tenney K, Valeriote FA, Lobkovsky E, Clardy J, Crews P (2003) Unusual C25 steroids produced by a sponge-derived *Penicillium citrinum*. Org Lett 5:4393–4396

Arai M, Niikawa H, Kobayashi M (2013) Marine-derived fungal sesterterpenes, ophiobolins, inhibit biofilm formation of *Mycobacterium* species. J Nat Med 67:271–275

Arifeen MZ, Ma YN, Xue YR, Liu CH (2020) Deep sea fungi could be the new arsenal for bioactive molecules. Mar Drugs 18(1):9–28

Baranovskiy AG, Babayeva ND, Suwa Y, Gu J, Pavlov YI, Tahirov TH (2014) Structural basis for inhibition of DNA replication by aphidicolin. Nucleic Acids Res 42(22):14013–14021. https://doi.org/10.1093/nar/gku1209

Bhadury P, Mohammad BT, Wright PC (2006) The current status of natural products from marine fungi and their potential as anti-infective agents. J Ind Microbiol Biotechnol 33:325–337

Bhatnagar I, Se-Kwon K (2010) Immense essence of excellence: marine microbial bioactive compounds. Mar Drugs 8:2673–2701

Bhimba B, Franco AD, Mathew JM, Jose GM, Joel EL, Thanagraj M (2012) Anticancer and antimicrobial activity of mangrove derived fungi *Hypocrea lixii* VB1. Chin J Nat Med 10:77–80. https://doi.org/10.1016/S1875-5364(12)60017-X

Bladt TT, Frisvad JC, Knudsen PB, Larsen TO (2013) Anticancer and antifungal compounds from *Aspergillus*, *Penicillium* and other filamentous fungi. Molecules 18:11338–11376. https://doi.org/10.3390/molecules180911338

Boruta T, Bizukojc M (2017) Production of lovastatin and itaconic acid by *Aspergillus terreus* a comparative perspective. World J Microbiol Biotechnol 33(2):34–34

Bringmann G, Gulder TA, Lang G, Schmitt S, Stohr R, Wiesc J, Magel K, Imhoff JF (2007) Large scale biotechnological production of the antileukemic marine natural product sorbicillactone A. Mar Drugs 5(2):23–30

Brito JPC, Ramada MHS, Magalhaes MTQ, de Silva LP, Ulhoa CJ (2014) Peptaibols from *Trichoderma asperellum* TR356 strain isolated from Brazilian soil. Springerplus 3:600–610

Bucknall RA, Moores H, Simms R, Hesp B (1973) Antiviral effects of aphidicolin, a new antibiotic produced by *Cephalosporium aphidicola*. Antimicrob Agents Chemother 4:294–298

Bugni TS, Ireland CM (2004) Marine derived fungi: a chemically and biologically diverse group of microorganisms. Nat Prod Rep 21:143–163

Butler MS, Robertson AA, Cooper MA (2014) Natural product and natural product derived drugs in clinical trials. Nat Prod Rep 31:1612–1661. https://doi.org/10.1039/C4NP00064A

Capon RJ, Skene C, Stewart M, Ford J, O'Hair RA, Williams L, Lacey E, Gill JH, Heiland K, Friedel T (2003) Aspergillicins A–E: five novel depsipeptides from the marine-derived fungus *Aspergillus carneus*. Org Biomol Chem 1:1856–1862

Chen XW, Li CW, Cui CB, Hua W, Zhu TJ, Gu QQ (2014) Nine new and five known polyketides derived from a deep sea-sourced *Aspergillus* sp. 16-02-1. Mar Drugs 12:3116–3137

Chen S, Chen D, Cai R, Cui H, Long Y, Lu Y, Li C, She Z (2016) Cytotoxic and antibacterial preussomerins from the mangrove endophytic fungus *Lasiodiplodia theobromae* ZJ-HQ1. J Nat Prod 79:2397–2402. https://doi.org/10.1021/acs.jnatprod.6b00639

Chepkirui C, Richter C, Matasyoh JC, Stadler M (2016) Monochlorinated calocerins A–D and 9-oxostrobilurin derivatives from the basidiomycete *Favolaschia calocera*. Phytochemistry 132:95–101

Chin YW, Balunas MJ, Chai HB, Kinghorn AD (2006) Drug discovery from natural sources. AAPS J 8:239–253

Chokpaiboon S, Sommit D, Bunyapaiboonsri T, Matsubara K, Pudhom K (2011) Antiangiogenic effect of chamigrane endoperoxides from a Thai mangrove-derived fungus. J Nat Prod 74:2290–2294

Cohen SD (2006) Host selectivity and genetic variation of *Discula umbrinella* isolates from two oak species: analyses of intergenic spacer region sequences of ribosomal DNA. Microb Ecol 52:463–469

Cohen E, Koch L, Thu KM, Rahamim Aluma Y, Ilan M, Yarden O, Carmeli S (2011) Novel terpenoids of the fungus *Aspergillus insuetus* isolated from the Mediterranean sponge *Psammaocinia* sp. collected along the coast of Israel. Bioorg Med Chem 19:6587–6593

Cole RJ, Jarvis BB, Schweikert MA (2003) Handbook of secondary fungal metabolites, vol III. Academic, Amsterdam

Cueto M, Jensen PR, Kauffman C, Fenical W, Lobkovsky E, Clardy J (2001) Pestalone, a new antibiotic produced by a marine fungus in response to bacterial challenge. J Nat Prod 64:1444–1446

Cui CM, Li XM, Meng L, Li CS, Huang CG, Wang BG (2010) 7-Nor-ergosterolide, a pentalactone-containing norsteroid and related steroids from the marine-derived endophytic *Aspergillus ochraceus* EN-31. J Nat Prod 73:1780–1784

Daferner M, Anke T, Sterner O (2002) Zopfiellamides A and B, antimicrobial pyrrolidinone derivatives from the marine fungus *Zopfiella latipes*. Tetrahedron 58:7781–7784

Davidson BS (1995) New dimensions in natural products research: cultured marine microorganisms. Curr Opin Biotechnol 6:284–291

Debbab A, Aly AH, Edrada-Ebel R, Wray V, Pretsch A, Pescitelli G, Kurtan T, Proksch P (2012) New anthracene derivatives—structure elucidation and antimicrobial activity. Eur J Org Chem 2012(7):1351–1359. https://doi.org/10.1002/ejoc.201101442

Demain AL (2006) From natural products discovery to commercialization: a success story. J Ind Microbiol Biotechnol 33:486–495

Deshmukh SK, Prakash V, Ranjan N (2017) Marine fungi: a source of potential anticancer compounds. Front Microbiol 8:2536–2560

Devi P, Wahidullah S, D'Souza L (2002) Kojic acid production from Fungal culture MTCC 5374. Patent filed under Council of Scientific and Industrial Research (CSIR)

Devi P, Rodrigues C, Naik CG, D'Souza L (2012) Isolation and characterization of antibacterial compound from a mangrove-endophytic fungus, *Penicillium chrysogenum* MTCC 5108. Ind J Microbiol 52(4):617–623

Devi P, Ciavatta ML, Wahidullah S, Vuppala S, D'Souza L (2013) Fungicidal compounds from a marine Ascidian-associated fungus *Trichoderma harzianum*. Discov Biotechnol 4(10):12–17

Dreyfuss DH, Kelleher CA, Jones JF, Gelfand EW (1996) Epstein-Barr virus infection of t cells: implications for altered t-lymphocyte activation, repertoire development and autoimmunity. Immunol Rev 152(89–110):314–322

Du X, Lu C, Li Y, Zheng Z, Su W, Shen Y (2008) Three new antimicrobial metabolites of *Phomopsis* sp. J Antibiot 61:250–253

Du L, Li D, Zhu T, Cai S, Wang F, Xiao X, Gu Q (2009) New alkaloids and diterpenes from a deep ocean sediment derived fungus *Penicillium* sp. Tetrahedron 65:1033–1039

Ebada SS, Fischer T, Hamacher A, Du FY, Roth YO, Kassack MU, Wang BG, Roth EH (2014) Psychrophilin E, a new cyclotripeptide, from co-fermentation of two marine alga-derived fungi of the genus *Apsergillus*. Nat Prod Res 28(11):776–781. https://doi.org/10.1080/14786419.2014.880911

Ebel R (2006) Secondary metabolites from the marine-derived fungi. In: Proksch P, Muller WEG (eds) Frontiers in marine biotechnology. Horizon Bioscience, Norwich, England, pp 73–143

Eble TE, Hanson FR (1951) Fumagillin, an antibiotic from *Aspergillus fumigatus* H-3. Antibiot Chemother 1:54–58

Elissawy AM, El-Shazly M, Ebada SS, Singab AB, Proksch P (2015) Bioactive terpenes from marine-derived fungi. Mar Drugs 13:1966–1992

Elsebai MF, Kehraus S, Gütschow M, König GM (2010) Spartinoxide, a new enantiomer of A82775C with inhibitory activity toward HLE from the marine-derived fungus *Phaeosphaeria spatinae*. Nat Prod Commun 5:1071–1076

Elsebai MF, Kehraus S, König GM (2013) Caught between triterpene- and steroid-metabolism: 4α-carboxylixpregnane-derivative from the marine alga-derived fungus *Phaeosphaeria spatinae*.Steroids 78:880-883

Elsebai M, Maddah FE, Kehraus S, Konig GM (2015) New bicyclo-spartinols from the marine-derived fungus *Phaeosphaeria spartinae*. Nat Prod Commun 10(4):637–639

Evidente A, Kornienko A, Cimmino A, Andolfi A, Lefranc F, Mathieu V, Kiss R (2014) Fungal metabolites with anticancer activity. Nat Prod Rev 31:617–627. https://doi.org/10.1039/c3np70078j

Fang W, Lin X, Zhou X, Wan J, Lu X, Yang B, Ai W, Lin J, Zhang T, Tu Z (2014) Cytotoxic and antiviral nitrobenzoyl sesquiterpenoids from the marine-derived fungus *Aspergillus ochraceus* Jcma1F17. Med Chem Commun 5:701–705

Farha AK, Hatha AM (2019) Bioprospecting potential and secondary metabolite profiling of a novel sediment-derived fungus *Penicillium* sp. ArCSPf from continental slope of Eastern Arabian Sea. Mycology 10(2):109–117

Fenical W (1997) New pharmaceuticals from marine organisms. Trends Biotechnol 15:339–341

Fenical W, Jensen PR, Cheng XC (2000) Halimide, a cytotoxic marine natural product, and derivatives thereof. U.S. Patent US6358957B1, 30 May 2000

Fredimoses M, Zhou X, Ai W, Tian X, Yang B, Lin X, Xian JY, Liu Y (2015) Westerdijkin A, a new hydroxyphenylacetic acid derivative from deep sea fungus *Aspergillus westerdijkiae* SCSIO 05233. Nat Prod Res 29:158–162. https://doi.org/10.1080/14786419.2014.968154

Fremlin LJ, Piggott AM, Lacey E, Capon RJ (2009) Cottoquinazoline A and cotteslosins A and B, metabolites from an Australian marine-derived strain of *Aspergillus versicolor*. J Nat Prod 72 (4):666–670

Fukuda T, Sudoh Y, Tsuchiya Y, Okuda T, Igarashi Y (2014) Isolation and biosynthesis of Preussin B, a pyrrolidine alkaloid from *Simplicillium lanosoniveum*. J Nat Prod 77(4):813–817

Gao H, Hong K, Chen GD, Wang CX, Tang JS, Yu Y, Jiang MM, Li MM, Wang NL, Yao XS (2010) New oxidized sterols from *Aspergillus awamori* and the *endo*-boat conformation adopted by the cyclohexene oxide system. Magn Reson Chem 48:38–43

Gao SS, Li XM, Li CS, Proksch P, Wang BG (2011) Penicisteroids A and B, antifungal and cytotoxic polyoxygenated steroids from the marine alga-derived endophytic fungus *Penicillium chrysogenum* QEN-24S. Bioorg Med Chem Lett 21:2894–2897

Gao XW, Liu HX, Sun ZH, Chen YC, Tan YZ, Zhang WM (2016a) Secondary metabolites from the deep sea derived fungus *Acromyces ingoldii* JS121. Molecules 21(4):371–378

Gao SS, Li XM, Williams K, Proksch P, Ji NY, Wang BG (2016b) Rhizovarins A-F, indole-diterpenes from the mangrove-derived endophytic fungus *Mucor irregularis* QEN-189. J Nat Prod 79:2066–2074. https://doi.org/10.1021/acs.jnatprod.6b00403

Garo E, Starks CM, Jensen PR, Fenical W, Lobkovsky E, Clardy J (2003) Trichodermamides A and B, cytotoxic modified dipeptides from the marine derived fungus *Trichoderma virens*. J Nat Prod 66:423–426

Gautschi JT, Amagata T, Amagata A, Valeriote FA, Mooberry SL, Crews P (2004) Expanding the strategies in natural product studies of marine-derived fungi: a chemical investigation of *Penicillium* obtained from deep water sediment. J Nat Prod 67:362–367

Gawas D, Devi P, Tilvi S, Naik CG, Parameswaran PS (2002) Fungal metabolites: tetrahydroauroglaucin and isodihydroauroglaucin from the marine fungus, *Eurotium* sp. In: Proceedings of the national conference on utilization of Bioresources (NATCUB-2002). Bhubaneswar, India, pp 453–457

Ge X, Sun C, Feng Y, Wang L, Peng J, Che Q, Gu Q, Zhu T, Li D, Zhang G (2019) Anthraquinone derivatives from a marine-derived fungus Sporendonema casei HDN16-802. Mar Drugs 17:334

Godtfredsen WO, Von Daehne W, Tybring L, Vangedal S (1962) Fusidic acid derivatives. Relationships between structure and antibacterial activity. J Med Chem 9:15–22

Godzeski CWJ, Kobayashi J, Ishibashi M (1968) Bioactive metabolites of symbiotic marine microorganisms. Chem Rev 93:1753–1769

Gokhale RS, Tuteja D (2001) Biochemistry of polyketide synthases. Biotechnology 10:341–372

Gomes NG, Lefranc F, Kijjoa A, Kiss R (2015) Can some marine derived fungal metabolites become actual anticancer agents? Mar Drugs 13:3950–3991

Grove JF, MacMillan J, Mulholland TPC, Rogers MAT (1954) Griseofulvin, Part XIV. Isolation of the Bromo-analogue from *Penicillium griseofulvum* and *Penicillium nigricans*. J Chem Soc:2885

Gulder TAM, Hong H, Correa J, Egereva E, Wiese J, Imhoff JF, Gross H (2012) Isolation, structure elucidation and total synthesis of Lajollamide A from the marine fungus *Asteromyces cruciatus*. Mar Drugs 10(12):2912–2935

Hemberg Y, Xu J, Wray V, Proksch P, Wu J, Bringmann G (2013) Pestaliopens A and B: stereochemically challenging flexible sesquiterpene-cyclopaldic acid hybrids from *Pestalotiopsis* sp. Chem A Eur J 19:15556–15564

Holler U, Wright AD, Matthee GF, Konig GM, Draeger S, Aust HJ, Schulz BJ (2000) Fungi from marine sponges: diversity, biological activity and secondary metabolites. Mycol Res 104 (11):1354–1365

Hou XM, Zhang YH, Hai Y, Zheng JY, Gu YC, Wang CY, Shao CL (2017) Aspersymmetide A, a new centrosymmetric cyclohexapeptide from the marine-derived fungus *Aspergillus versicolor*. Mar Drugs 15(11):363–371

Huang HR, Xia XK, She ZG, Lin YC, Vrijmoed LLP, Jones EBG (2006) A new chloromonoterpene from the mangrove endophytic fungus *Tryblidiopycnis* sp. (4275). J Asian Nat Prod Res 8:609–612

Huang H, Feng X, Xiao Z, Liu L, Li H, Ma L, Lu Y, Ju J, She Z, Lin Y (2011) Azaphilones and *p*-terphenyls from the mangrove endophytic fungus *Penicillium chermesinum* (Zh4-E2) isolated from the South China Sea. J Nat Prod 74:997–1002

Huang X, Huang H, Li H, Sun X, Huang H, Lu Y, Lin Y, Long Y, She Z (2013) Asperterpenoid A, a new sesterterpenoid as an inhibitor of *Mycobacterium tuberculosis* protein tyrosine phosphatase B from the culture of *Aspergillus* sp. 16-5c. Org Lett 15:721–723

Huang Z, Nong X, Ren Z, Wang J, Zhang X, Qi S (2017) Anti-HSV-1, antioxidant and antifouling phenolic compounds from the deep-sea-derived fungus *Aspergillus versicolor* SCSIO 41502. Bioorg Med Chem Lett 27:787–791

Imhoff JF (2016) Natural products from marine fungi—still an under represented resource. Mar Drugs 14:1–19

Ingavat N, Mahidol C, Ruchirawat S, Kittakoop P (2011) Asperaculin A, a sesquiterpenoid from a marine-derived fungus, *Aspergillus aculeatus*. J Nat Prod 74:1650–1652

Isaka M, Suyarnsestakorn C, Tanticharoen M, Kongsaeree P, Thebtaranonth Y (2002) Aigialomycins A–E, new resorcylic macrolides from the marine mangrove fungus *Aigialus parvus*. J Org Chem 67:1561–1566

Ishino M, Kiyomichi N, Takatori K, Sugita T, Shiro M, Kinoshita K, Takahashi K, Koyama K (2010) Phomactin I, 13-*epi*-phomactin I, and phomactin J, three novel diterpenes from a marine-derived fungus. Tetrahedron 66:2594–2597

Ishino M, Kinoshita K, Takahashi K, Sugita T, Shiro M, Hasegawa K, Koyama K (2012) Phomactins K–M, three novel phomactin-type diterpenes from a marine-derived fungus. Tetrahedron 68:8572–8576

Jae HS, Hyuncheol O (2010) Protulactones A and B: two new polyketides from the marine-derived fungus *Aspergillus* sp. SF-5044. Bull Korean Chem Soc 31:6

Kamat S, Kumari M, Taritla S, Jayabaskaran C (2020) Rich source of bioactive material. Front Mar Sci 7(31):1–16

Kaneko M, Watashi K, Kamisuki S, Matsunaga HL (2015) A novel tricyclic polyketide, vanitaracin A, specifically inhibits the entry of hepatitis B and D viruses by targeting sodium taurocholate co-transporting polypeptide. J Virol 89:11945–11953

Kanoh K, Konho S, Asari T, Harada T, Katada J, Muramatsu M, Kawashima H, Sekiya H, Uno I (1997) Phenylahistin: a new mammalian cell cycle inhibitor produced by *Aspergillus ustus*. Bioorg Med Chem Lett 7:2847–2852

Khan NT (2017) Cephalosporin C production from *Acremonium chrysogenum*. Enz Eng 6(1):1–4

Klemke C, Kehraus S, Wright AD, Konig GM (2004) New secondary metabolites from the marine endophytic fungus *Apiospora montagnei*. J Nat Prod 67:1058–1063

Kobayashi J, Ishibashi M (1993) Bioactive metabolites of symbiotic marine microorganisms. Chem Rev 93:1753–1769

Komatsu K, Shigemori H, Kobayashi JI (2001) Dictyonamides A and B, new peptides from marine-derived fungus. J Org Chem 66:6189–6192. https://doi.org/10.1021/jo0156767

Konig GM, Jones PG, Wright AD (2002) 5-Hydroxyramulosin, a new natural product produced by phomatropica, a marine-derived fungus isolated from the alga *Fucus spiralis*. Planta Med 68 (11):1052–1054

Kornienko A, Evidente A, Vurro M, Mathieu V, Cimmino A, Evidente M, Van Otterlo WAL, Dasari R, Lefranc F, Kiss R (2015) Towards a cancer drug of fungal origin. Med Res Rev 35 (5):937–967

Kück U, Bloemendal S, Teichert I (2014) Putting fungi to work: harvesting a cornucopia of drugs, toxins, and antibiotics. PLoS Pathog 10:e1003950. https://doi.org/10.1371/journal.ppat.1003950

Kupka J, Anke T, Steglich W, Zechlin L (1981) Antibiotics from basidiomycetes. XI. The biological activity of siccayne, isolated from the marine fungus *Halocyphina villosa* J. & E. Kohlmeyer. J Antibiot (Tokyo) 34:298–304

Lee GT, Lee SY, Jeong JH, Jo BK, Li XF, Son BW (2003) PP-35 Screening of tyrosinase inhibiting activity from the marine derived fungus. Pigment Cell Res 16:604

Li Y, Li X, Son BW (2005) Antibacterial and radical scavenging epoxycyclohexenones and aromatic polyols from a marine isolate of the fungus *Aspergillus*. Nat Prod Sci 11:136–138

Li H, Huang H, Shao C, Huang H, Jiang J, Zhu X, Liu Y, Liu L, Lu Y, Li M (2011) Cytotoxic norsesquiterpene peroxides from the endophytic fungus *Talaromyces flavus* isolated from the mangrove plant *Sonneratia apetala*. J Nat Prod 74:1230–1235

Li Y, Ye D, Shao Z, Cui C, Che Y (2012) A sterol and spiroditerpenoids from a *Penicillium* sp. isolated from a deep sea sediment sample. Mar Drugs 10:497–508

Li XD, Li X, Li X, Xu GM, Zhang P, Meng LH, Wang BG (2016) Tetranorlabdane diterpenoids from the deep sea sediment-derived fungus *Aspergillus wentii* SD-310. Planta Med 82:877–881

Liang X, Zhang XY, Nong XH, Wang J, Huang ZH, Qi SH (2016) Eight linear peptide from the deep sea fungus *Simplicillium obclavatum* EIODSF 020. Tetrahedron 72(22):3092–3097

Liao L, Lee JH, You M, Choi TJ, Park W, Lee SK, Oh DC, Oh KB, Shin J (2014) Penicillipyrones A and B, meroterpenoids from a marine-derived *Penicillium* sp. fungus. J Nat Prod 77:406–410

Liberra K, Jansen R, Lindequist U (1998) Corollosporine, a new phthalide derivative from the marine fungus *Corollospora maritima* Werderm 1069. Pharmazie 53:578–581

Lin Y, Wu X, Feng S, Jiang G, Luo J, Zhou S, Vrijmoed LLP, Jones EBG, Krohn K, Steongrover K, Zsila F (2001) Five unique compounds: xyloketales from mangrove fungus *Xylaria* sp. from the South China Sea coast. J Org Chem 66:6252–6256

Liu CH, Liu JY, Huang LL, Zou WX, Tan RX (2003) Absolute configuration of keisslone, a new antimicrobial metabolite from *Keissleriella* sp. YS4108, a marine filamentous fungus. Planta Med 69:481–483

Liu WC, Li CQ, Zhu P, Yang JL, Cheng KD (2010) Phylogenetic diversity of culturable fungi associated with two marine sponges: Haliclona simulans and Gelliodes carnosa, collected from the Hainan Island coastal waters of the South China Sea. Fungal Div 42:1–15

Liu QY, Zhou T, Zhao YY, Chen L, Gong MW, Xia QW, Ying MG, Zheng QH, Zhang QQ (2015) Antitumor effects and related mechanisms of penicitrinine A, a novel alkaloid with a unique spiro skeleton from the marine fungus *Penicillium citrinum*. Mar Drugs 13:4733–4753

Liu S, Su M, Song SJ, Jung JH (2017) Marine derived *Penicillium* species as producer of cytotoxic metabolites. Mar Drugs 15(10):329–373

Liu CC, Zhang ZZ, Feng YY, Gu QQ, Li DH, Zhu TJ (2018a) Secondary metabolites from Antarctic marine-derived fungus *Penicillium crustosum* HDN153086. Nat Prod Res 33:414–419

Liu J, Gu B, Yang L, Yang F, Lin H (2018b) New anti-inflammatory cyclopeptides from a sponge derived fungus *Aspergillus violaceofuscus*. Front Chem 6:226–234

Lu Z, Wang Y, Miao C, Liu P, Hong K, Zhu W (2009) Sesquiterpenoids and benzofuranoids from the marine-derived fungus *Aspergillus ustus* 094102. J Nat Prod 72:1761–1767

Lu Z, Zhu H, Fu P, Wang Y, Zhang Z, Lin H, Liu P, Zhuang Y, Hong K, Zhu W (2010) Cytotoxic polyphenols from the marine-derived fungus *Penicillium expansum*. J Nat Prod 73:911–914

Luo J, Yang Y, Lin Y, Chen Z, Jiang G (2004) Antioxidative activities of two metabolites of cultured marine fungus, *Halorosellinia oceanicum* 323 in vitro. Zhong Yao Cai 27:188–192

Luo XW, Yun L, Liu YJ, Zhou XF, Liu YH (2019) Peptides and polyketides isolated from the marine sponge-derived fungus *Aspergillus terreus* SCSIO 41008. Chin J Nat Med 17:149–154

Ma X, Li L, Zhu T, Ba M, Li G, Gu Q, Guo Y, Li D (2013) Phenylspirodrimanes with anti-HIV activity from the sponge-derived fungus *Stachybotrys chartarum* MXH-X73. J Nat Prod 76 (12):2298–2306

Ma X, Nong XH, Zhe R, Wang J, Liang X, Wang L, Qi SH (2017) Antiviral peptides from marine-derived fungus *Aspergillus* sp. SCSIO 41501. Tetrahedron Lett 58(12):1151–1155

Marra R, Nicoletti R, Pagano E, Della Greca M, Salvatore MM, Borrelli F, Lombardi N, Vinale F, Woo SL, Andolfi A (2018) Inhibitory effect of trichodermanone C, a sorbicillinoid produced by *Trichoderma citrinoviride* associated to the green alga *Cladophora* sp., on nitrite production in LPS-stimulated macrophages. Nat Prod Res 33(23):1478–6427

Mei WL, Zheng B, Zhao YX, Zhong HM, Chen XLW, Zeng YB, Dong WH, Huang JL, Proksch P, Dai HF (2012) Meroterpenes from endophytic fungus A1 of mangrove plant *Scyphiphora hydrophyllacea*. Mar Drugs 10:1993–2001

Meng LH, Wang CY, Mandi A, Li XM, Hu XY, Kassack MU, Kurtan T, Wang BG (2016) Three diketopiperazine alkaloids with spirocyclic skeletons and one bisthiodiketopiperazine derivative from the mangrove-derived endophytic fungus *Penicillium brocae* MA-231. Org Lett 18:5304–5307

Mi-Hee C, Hyeon-Jin KK, Yeop J, Seong CH (2008) The isolation and characterisation of *Pseudozyma* sp. JCC207 a novel producer of squalene. Appl Microbiol Biotechnol 78:963–972

Minagawa K, Kouzuki S, Yoshimoto J, Kawamura Y, Tani H, Iwata T, Terui Y, Nakai H, Yagi S, Hattori N, Fujiwara T, Kamigauchi T (2002) Stachyflin and acetylstachyflin, novel anti-influenza A virus substances, produced by *Stachybotrys* sp. RF-7260. I. Isolation, structure elucidation and biological activities. J Antibiot (Tokyo) 55(2):155–164

Molinski TF, Dalisay DS, Lievens SL, Saludes JP (2009) Drug development from marine natural products. Nat Rev Drug Discov 8:69. https://doi.org/10.1038/nrd2487

Montagne C (1846) In: Durieu De Maisonneuve MC (ed) Exploration scientifique de l'Algérie pendant les années 1840, 1841, 1842. Imprimerie Royale, Paris, France, pp 1–197

Moore BS, Piel J (2000) Engineering biodiversity with type II polyketide synthase genes. Antonie Van Leeuwenhoek 78:391–398

Moricca S, Ragazzi A (2008) Fungal endophytes in Mediterranean oak forests: a lesson from *Discula quercina*. Phytopathology 98(3):380–386

Namikoshi M, Kobayashi H, Yoshimoto T, Meguro S, Akano K (2000) Isolation and characterization of bioactive metabolites from marine-derived filamentous fungi collected from tropical and sub-tropical coral reefs. Chem Pharm Bull (Tokyo) 48:1452–1457

Narmani A, Teponno RB, Arzanlou M, Surup F (2019) Cytotoxic, antimicrobial and antiviral secondary metabolites produced by the plant pathogenic fungus *Cytospora* sp. CCTU A309. Fitoterapia 134:314–322

Newton GG, Abraham EP (1955) Cephalosporin *C*, a new antibiotic containing sulphur and D-α-aminoadipic acid. Nature 175:548

Novak R, Shlaes DM (2010) The pleuromutilin antibiotics: a new class for human use. Curr Opin Investig Drugs 11(2):182–191

Oda T, Namikoshi M, Akano K, Kobayashi H, Honma Y, Kasahara T (2005) Verrucarin a inhibition of map kinase activation in a pma-stimulated promyelocytic leukemia cell line. Mar Drugs 3:64–73

Oh DC, Jensen PR, Fenical W (2007) Zygosporamide, a cytotoxic cyclic depsipeptide from the marine-derived fungus *Zygosporium masonii*. Tetrahedron Lett 47(48):8625–8628

Ondeyka JG, Smith SK, Zink DL, Vicente F, Basilio A, Bills GF, Polishook JD, Garlisi C, Mcguinness D, Smith E, Qiu H, Gill CJ, Donald RGK, Phillips JW, Goetz MA, Singh SB (2014) Isolation, structure elucidation and antibacterial activity of a new tetramic acid, ascosetin. J Antibiot 67:527–531

Pejin BK, Jovanovic K, Mojovic MG, Savic A (2013) New and highly potent antitumor natural products from marine-derived fungi: covering the period from 2003 to 2012. Curr Top Med Chem 13:2745–2766

Peláez F, Cabello A, Platas G, Diez MT, del Val AG, Basilio A, Martan I, Vicente F, Bills GF, Giacobbe RA, Schwartz RE, Onishi JC, Meinz MS, Abruzzo GK, Flattery AM, Kong L, Kurtz MB (2000) The discovery of enfumafungin, a novel antifungal compound produced by an endophytic *Hormonema* species. Biological activity and taxonomy of the producing organism. Syst Appl Microbiol 23:333–343

Peng J, Gao H, Zhang X, Wang S, Wu C, Gu Q, Guo P, Zhu T, Li D (2014) Psychrophilins E–H and versicotide C, cyclic peptides from the marine derived fungus *Aspergillus versicolor* ZLN-60. J Nat Prod 77(10):2218–2223

Petit K, Mondeguer F, Roquebert MF, Biard JF, Pouchus YF (2004) Detection of griseofulvin in a marine strain of *Penicillium waksmanii* by ion trap mass spectrometry. J Mircobiol Methods 58 (1):59–65

Pietra F (1997) Secondary metabolites from marine microorganisms: bacteria, protozoa, algae and fungi. Achievements and prospects. Nat Prod Rep 14:453–464

Pinheiro A, Dethoup T, Bessa J, Silva AMS, Kijjoa AA (2012) A new bicyclic sesquiterpene from the marine sponge associated fungus *Emericellopsis minima*. Phytochem Lett 5:68–70

Prompanya C, Fernandes C, Cravo S, Pinto MM, Dethoup T, Silva AM, Kijjoa A (2015) A new cyclic hexapeptide and a new isocoumarin derivative from the marine sponge-associated fungus *Aspergillus similanensis* KUFA 0013. Mar Drugs 13(3):1432–1450

Prosperini A, Berrada H, Ruiz MJ, Caloni F, Coccini T, Spicer LJ, Perego MC, Lafranconi A (2017) A review of the mycotoxin enniatin B. Front Public Health 5:304–315

Punyasloke B, Balsam TM, Phillip C (2006) The current status of natural products from marine fungi and their potential as anti-infective agents. J Ind Microbiol Biotechnol 33:325–337

Raghukumar C (ed) (2012) Biology of marine fungi, Progress in molecular and subcellular biology, vol 53. Springer, Berlin

Rappe MS, Giovannoni SJ (2003) The uncultured microbial majority. Annu Rev Microbiol 57:369–394

Rowley DC, Kelly S, Kauffman CA, Jensen PR, Fenical W (2003) Halovirs A–E, new antiviral agents from a marine-derived fungus of the genus *Scytalidium*. Bioorg Med Chem 11:4263–4274

Rukachaisirikul V, Khamthong N, Sukpondma Y, Phongpaichit S, Hutadilok-Towatana N, Graidist P, Sakayaroj J, Kirtikara K (2010) Cyclohexene, diketopiperazine, lactone and phenol derivatives from the sea fan-derived fungi *Nigrospora* sp. PSU-F11 and PSU-F12. Arch Pharm Res 33:375–380

Saleema M, Ali MS, Hussain S, Jabbar A, Ashraf M, Lee YS (2007) Marine natural products of fungal origin. Nat Prod Rep 24:1142–1152

Samuel P, Prince LP (2011) Antibacterial activity of marine derived fungi collected from south east coast of Tamilnadu, India. J Microbiol Biotechnol Res 1(4):86–94

Sandargo B, Thongbai B, Padutya D, Steinmann E (2018) Antiviral 4-hydroxypleurogrisein and antimicrobial pleurotin derivatives from cultures of nematophagous basidiomycete *Hohenbuehelia grisea*. Molecules 23:2697

Sandargo B, Michehl M, Praditya D, Steinmann E, Stadler M, Surup F (2019) Antiviral Meroterpenoid Rhodatin and Sesquiterpenoids Rhodocoranes A–E from the Wrinkled Peach Mushroom. Org Lett 21(9):3286–3289

Schiehser GA, White JD, Matsumoto G, Pezzanite JO, Clardy J (1986) The structure of leptosphaerin. Tetrahedron Lett 27:5587–5590

Schlingmann G, Milne L, Carter G (2002) Isolation and identification of antifungal polyesters from the marine fungus *Hypoxylon oceanicum* LL-15G256. Tetrahedron 58:6825–6835

Shang Z, Li X, Meng L, Li C, Gao S, Huang C, Wang B (2012a) Chemical profile of the secondary metabolites produced by a deep-sea sediment-derived fungus *Penicillium commune* SD-118. Chinese J Oceanol Limnol 30:305–314

Shang Z, Li X, Meng L, Li C, Gao S, Huang C, Wang B (2012b) Chemical profile of the secondary metabolites produced by a deep-sea sediment-derived fungus *Penicillium commune* SD-118. Chin J Oceanol Limnol 30(2):305–314

Shin HJ (2020) Natural products from marine fungi. Mar Drugs 18:230. https://doi.org/10.3390/md18050230

Shushni MAM, Singh R, Mentel R, Lindequist U (2011) Balticolid: a new 12-membered macrolide with antiviral activity from an ascomycetous fungus of marine origin. Mar Drugs 9:844–851

Singh SB, Zink DL, Bills GF, Pelaez F (2002) Discovery, structure and HIV-1 integrase inhibitory activities of integracins, novel dimeric alkyl aromatics from *Cytonaema* sp. Tetrahedron Lett 43:1617–1620

Singh SB, Ondeyka JG, Tsipouras N, Ruby C (2004) Hinnuliquinone, a C2-symmetric dimeric non-peptide fungal metabolite inhibitor of HIV-1 protease. Biochem Biophys Res Commun 324:108–113

Sithranga NB, Kathiresan K (2011) Anticancer drugs from marine flora: an overview. J Oncol 2010:18. https://doi.org/10.1155/2010/214186

Son BW, Choi JS, Kim JC, Nam KW, Kim DS, Chung HY, Kang JS, Choi HD (2002) Parasitenone, a new poxycyclohexenone related to gabosine from the marine-derived fungus *Aspergillus parasiticus*. J Nat Prod 65:794–795

Song Y, Wang J, Huang H, Ma L, Wang J, Gu Y, Liu L, Lin Y (2012) Four eremophilane sesquiterpenes from the mangrove endophytic fungus *Xylaria* sp. BL321. Mar Drugs 10:340–348

Starratt AN, Loschiavo SR (1974) The production of aphidicolin by *Nigrospora sphaerica*. Can J Microbiol 20:416–417

Starratta N, Loschiavo SR (1974) The production of aphidicolin by *Nigrospora sphaerica*. Can J Microbiol 20:416–417

Stevenson BS, Eichorst SA, Wertz JT, Schmidt TM, Breznak JA (2004) New strategies for cultivation and detection of previously uncultured microbes. Appl Environ Microbiol 70:4748–4755

Sun LL, Shao CL, Chen JF, Guo ZY, Fu XM, Chen M, Chen YY, Li R, de Voogd NJ, She ZG (2012) New bisabolane sesquiterpenoids from a marine-derived fungus *Aspergillus* sp. isolated from the sponge *Xestospongia testudinaria*. Bioorg Med Chem Lett 22:1326–1329

Surup F, Kuhnert E, Lehmann E, Heitkamper S, Hyde KD, Fournier J, Stadler M (2014) Sporothriolide derivatives as chemotaxonomic markers for hypoxylon monticulosum. Mycology 5(3):110–119

Swathi J, Sowjanya KM, Narendra K, Krishna Satya A (2013) Bioactivity assay of an isolated marine *Fusarium* spp. Int J Biosci Biotechnol 5(5):179–186

Takata K, Iwatsuki M, Yamamoto T, Shirahata T, Nonaka K, Masuma R, Hayakawa Y, Hanaki H, Kobayashi Y, Petersson GA, Omura S, Shiomi K (2013) Aogacillins A and B produced by *Simplicillium* sp. FKI-5985: new circumventors of Arbekacin resistance in MRSA. Org Lett 15 (18):4678–4681

Tan LT, Cheng XC, Jensen PR, Fenical W (2003) Scytalidamides A and B, new cytotoxic cyclic heptapeptides from a marine fungus of the genus *Scytalidium*. J Org Chem 68:8767–8773

Tan S, Yang B, Liu J, Xun T (2017) Penicillixanthone A, a marine-derived dual-coreceptor antagonist as anti-HIV-1 agent. Nat Prod Res 19:1–5

Thakur NL, Jain R, Natalio F, Thakur AN, Hammer B, Müller WEG (2008) Marine molecular biology: an emerging field of biological sciences. Biotechnol Adv 26:233–245

Thirunavukkarasu N, Suryanarayanan TS, Murali TS, Ravishankar JP, Gummadi SN (2011) L-Asparaginase from marine derived fungal endophytes of seaweeds. Mycosphere 2 (2):147–155

Thongtan J, Saenboonrueng J, Rachtawee P, Isaka M (2006) An antimalarial tetrapeptide from the Entomopathogenic fungus *Hirsutella* sp. BCC 1528. J Nat Prod 69(4):713–714

Tian YQ, Lin XP, Liu J, Kaliyaperumal K, Ai W, Ju ZR, Yang B, Wang J, Yang XW, Liu Y (2014) Ascomycotin A, a new citromycetin analogue produced by *Ascomycota* sp. Ind19F07 isolated from deep sea sediment. Nat Prod Res 29(9):820–826

Tian Y, Lin X, Wang Z, Zhou X (2016) Asteltoxins with antiviral activities from the marine sponge-derived fungus *Aspergillus* sp. SCSIO XWS02F40. Molecules 21:34/1–34/10

Toske SG, Jensen PR, Kauffman CA, Fenical W (1998) Aspergillamides A and B: modified cytotoxic tripeptides produced by a marine fungus of the genus *Aspergillus*. Tetrahedron 54:13459–13466

Trisuwan K, Rukachaisirikul V, Sukpondma Y, Preedanon S, Phongpaichit S, Rungjindamai N, Sakayaroj J (2008) Epoxydons and a pyrone from the marine-derived fungus *Nigrospora* sp. PSU-F5. J Nat Prod 71(8):1323–1326

Trisuwan K, Rukachaisirikul V, Kaewpet M, Phongpaichit S, Hutadilok-Towatana N, Preedanon S, Sakayaroj J (2011) Sesquiterpene and xanthone derivatives from the sea fan-derived fungus *Aspergillus sydowii* PSU-F154. J Nat Prod 74:1663–1667

Tsukada M, Fukai M, Miki K, Shiraishi T, Suzuki T, Nishio K, Sugita T, Ishino M, Kinoshita K, Takahashi K (2011) Chemical constituents of a marine fungus *Arthrinium sacchari*. J Nat Prod 74:1645–1649

Turner WB, Aldridge DC (1983) Fungal metabolites II. Academic, London

Tziveleka LA, Vagias C, Roussis V (2003) Natural products with anti-HIV activity from marine organisms. Curr Top Med Chem 3:1512–1535

Uchida R, Nakajyo K, Kobayashi K, Ohshiro T, Terahara T, Imada C, Tomoda H (2016) 7-Chlorofolipastatin, an inhibitor of sterol O-acyltransferase, produced by marine-derived *Aspergillus ungui* NKH-007. J Antibiot 69:647

Wegner C, Schwibbe M, Konig GM, Wright AD (2000) HPLC-DAD and HPLC-MS investigations of marine and terrestrial *Phoma* species. Phytochem Anal 11:288–294

Wei H, Ito T, Kinoshita M, Kotoku N, Aoki S, Kobayashi M (2005) Shimalactone A, a novel polyketide from marine-derived fungus *Emericella* variecolor GF10. Tetrahedron 61 (33):8054–8058

Wen L, Chen G, She Z, Yan C, Cai J, Mu L (2010) Two new paeciloxocins from a mangrove endophytic fungus *Paecilomyces* sp. Russian Chem Bull 59:1656–1659

Wiese J, Ohlendorf B, Blumel M, Schmaljohann R, Imhoff JF (2011) Phylogenetic identification of fungi isolated from the marine sponge *Tethya aurantium* and identification of their secondary metabolites. Mar Drugs 9:561–585

Wijesekara I, Li YX, Vo TS, Van Ta Q, Ngo DH, Kim SK (2013) Induction of apoptosis in human cervical carcinoma HeLa cells by neoechinulin A from marine-derived fungus *Microsporum* sp. Process Biochem 48:68–72

Wijesekara I, Hang C, Van TQ, Vo TS, Li YX, Kim SK (2014) Physcion from marine-derived fungus *Microsporum* sp. induces apoptosis in human cervical carcinoma HeLa cells. Microbiol Res 169:255–261. https://doi.org/10.1016/j.micres.2013.09.001

Wu B, Wu X, Sun M, Li M (2013) Two novel tyrosinase inhibitory sesquiterpenes induced by $CuCl_2$ from a marine-derived fungus *Pestalotiopsis* sp. Z233. Mar Drugs 11:2713–2721

Wu B, Oesker V, Wiese J, Malien S, Schmaljohann R, Imhoff JF (2014) Spirocyclic drimanes from the marine fungus *Stachybotrys* sp. strain MF347. Mar Drugs 12:1924–1938

Wu B, Wiese J, Labes A, Kramer A, Schmaljohann R, Imhoff JF (2015) Lindgomycin, an unusual antibiotic polyketide from a marine fungus of the *Lindgomycetaceae*. Mar Drugs 13:4617–4632

Wu B, Wiese J, Wenzel-Storjohann A, Malien S, Schmaljohann R, Imhoff JF (2016) Engyodontochones, new antibiotics from a marine fungus *Engyodontium* album strain LF069. Chemistry 22:7452–7462

Xia X, Zhang J, Zhang Y, Wei F, Liu X, Jia A, Liu C, Li W, She Z, Lin Y (2012) Pimarane diterpenes from the fungus *Epicoccum* sp. HS-1 associated with *Apostichopus japonicas*. Bioorg Med Chem Lett 22(8):3017–3019

Xia MW, Cui CB, Li CW, Wu CJ (2014) Three new and eleven known unusual C25 steroids: activated production of silent metabolites in a marine-derived fungus by chemical mutagenesis strategy using diethyl sulfate. Mar Drugs 12:1545–1568

Xiao Z, Huang H, Shao C, Xia X, Lin M, Huang X, Lu Y, Lin Y, Long Y, She Z (2013) Asperterpenols A and B, new sesterterpenoids isolated from a mangrove endophytic fungus *Aspergillus* sp. 085242. Org Lett 15:2522–2525

Xueqian S, Xiangshan Z, Menghao C, Jiushun Z, Yuanxing Z (2010) Significant stimulation of o-phthalic acid in biosynthesis of aspergiolide A by a marine fungus *Aspergillus glaucus*. Bioresour Technol 101:3609–3616

Yamada T, Imai E, Nakatuji K, Numata A, Tanaka R (2007) Cephalimysin A, a potent cytotoxic metabolite from an *Aspergillus* species separated from a marine fish. Tetrahedron Lett 48 (36):6294–6296

Yanagihara M, Sasaki-Takahashi N, Sugahara T, Yamamoto S, Shinomi M, Yamashita I, Hayashida M, Yamanoha B, Numata A, Yamori T, Andoh T (2005a) Leptosins isolated from marine fungus *Leptosphaeria* species inhibit DNA topoisomerases I and/or II and induce apoptosis by inactivation of Akt/protein kinase B. Cancer Sci 96(11):816–824

Yanagihara M, Sasaki-Takahashi N, Sugahara T, Yamamoto S (2005b) Leptosins isolated from marine fungus *Leptoshaeria* species inhibit DNA topoisomerases I and/or II and induce apoptosis by inactivation of Akt/protein kinase B. Cancer Sci J 96:816–824

Yang X, Feng P, Yin Y, Bushley KE, Spatafora JW, Wang C (2018) Cyclosporine biosynthesis in *Tolypocladium inflatum* benefits fungal adaptation to the environment. Mol Biol Physiol 9 (5):1211–1218

You M, Liao L, Hong SH, Park W, Kwon DI, Lee J, Noh M, Oh DC, Oh KB, Shin J (2015) Lumazine peptides from the marine-derived fungus *Aspergillus terreus*. Mar Drugs 13 (3):1290–1303

Youssef FS, Ashour ML, Singab ANB, Wink M (2019) A comprehensive review of bioactive peptides from marine fungi and their biological significance. Mar Drugs 17(10):559–583

Zeng YB, Gu HG, Zuo WJ, Zhang LL, Bai HJ, Guo ZK, Proksch P, Mei W, Dai HF (2014) Two new sesquiterpenoids from endophytic fungus J3 isolated from mangrove plant *Ceriops tagal*. Arch Pharm Res. https://doi.org/10.1007/s12272-014-0448-8

Zhang Y, Wang S, Li XM, Cui CM, Feng C, Wang BG (2007) New sphingolipids with a previously unreported 9-methyl-C20-sphingosine moiety from a marine algous endophytic fungus *Aspergillus niger* EN-13. Lipids 42(8):759–764

Zhang D, Yang X, Kang JS, Choi HD, Son BW (2008) Chlorohydroaspyrones A and B, antibacterial aspyrone derivates from the marine-derived fungus *Exophiala* sp. J Nat Prod 71 (8):1458–1460

Zhang Y, Mu J, Feng Y, Kang Y, Zhang J, Gu PJ, Wang Y, Ma LF, Zhu YH (2009) Broad-spectrum antimicrobial epiphytic and endophytic fungi from marine organisms: isolation, bioassay and taxonomy. Mar Drugs 7(2):97–112

Zhang G, Sun S, Zhu T, Lin Z, Gu J, Li D, Gu Q (2011) Antiviral isoindolone derivatives from an endophytic fungus *Emericella* sp. associated with *Aegiceras corniculatum*. Phytochemistry 72 (11):1436–1442

Zhang Z, Miao L, Lv C, Sun H, Wei S, Wang B, Huang C, Jiao B (2013) Wentilactone B induces G2/M phase arrest and apoptosis via the Ras/Raf/MAPK signaling pathway in human hepatoma SMMC-7721 cells. Cell Death Dis 4:657–669. https://doi.org/10.1038/cddis.2013.182

Zhang P, Li XM, Mao XX, Mandi A, Kurtan T, Wang BG (2016) Varioloid A, a new indolyl-6,10b-dihydro-5aH-[1]benzofuro[2,3-b]indole derivative from the marine alga-derived endophytic fungus *Paecilomyces variotii* EN-291. Beilstein J Org Chem 12:2012–2018

Zhao Y, Chen H, Shang Z, Jiao B, Yuan B, Sun W, Wang B, Miao M, Huang C (2012) SD118-xanthocillin X (1), a novel marine agent extracted from *Penicillium commune*, induces autophagy through the inhibition of the MEK/ERK pathway. Mar Drugs 10:1345–1359. https://doi.org/10.3390/md10061345

Zhou H, Zhu T, Cai S, Gu Q, Li D (2011) Drimane sesquiterpenoids from the mangrove-derived fungus *Aspergillus ustus*. Chem Pharm Bull 59:762–766

Zhou H, Li L, Wang W, Che Q (2015) Chrodrimanins I and J from the Antarctic moss-derived fungus *Penicillium funiculosum* GWT2-24. J Nat Prod 78:1442–1445

Zhu M, Zhang X, Feng H, Che Q, Zhu T, Gu Q, Li D (2016) Campyridones A–D, pyridone alkaloids from a mangrove endophytic fungus *Campylocarpon* sp. HDN13-307. Tetrahedron 72:5679–5683

Zhuravleva OI, Afiyatullov SS, Denisenko VA, Ermakova SP, Slinkina NN, Dmitrenok PS, Kim NY (2012) Secondary metabolites from a marine-derived fungus *Aspergillus carneus* Blochwitz. Phytochemistry 80:123–131

Zilinskas RA, Colwell RR, Lipton DW, Hill RT (1995) The global challenge of marine biotechnology: a status report on the United States, Japan, Australia and Norway (College Park, MD), 372

Recent Developments and Future Prospects of Fungal Sophorolipids

19

Ngangom Bidyarani, Jyoti Jaiswal, Priyanka Shinde, and Umesh Kumar

Abstract

Sophorolipids (SPLs) are glycolipid biosurfactants and are amphiphilic molecules. Structurally SPLs consist of sugar sophorose head group (2-*O*-β-D-glucopyranosyl-D-glucopyranose) attached to a long chain of C18 or C16, hydroxyl fatty acid tail group by a glycosidic linkage between the anomeric C atom of the sugar and the hydroxyl group of the fatty acid. SPLs are synthesized by a variety of microorganisms as a mixture of related molecules with differences in the fatty acid part (chain length, saturation and position of hydroxylation) and the lactonization and acetylation pattern. SLPs are one of the most promising biosurfactants that belong to the glycolipid group and are synthesized extracellularly. SLPs are secreted as secondary metabolites in the stationary phase during nitrogen-limiting conditions. SLPs have been widely studied for their potential application in various fields and are more attractive for commercial purposes. In this chapter, we have discussed the various fermentation parameters essential for optimum production of SPLs and their applications in agriculture, cosmetics, nanotechnology, bioremediation, antimicrobial, anticancer, immunomodulation, drug delivery and others.

Keywords

Sophorolipids (SPLs) · Biosurfactant · Nanotechnology · Cosmetics · Agriculture

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19.1 Introduction

The global production of surfactants has reached around ten million tons per year to fulfil the demands for household and industrial applications (Van Bogaert et al. 2007). The petrochemical-based production has been remarkably replaced by the development of economical and sustainable methods through bioactivity (Ma et al. 2020). Surfactants are amphiphilic, surface active molecules containing both hydrophilic and hydrophobic moieties, and those of microbial origin from bacteria, yeasts and fungi that are known as biosurfactants. Biosurfactants are produced by renewable primary products or agro-industrial wastes having better prospects than chemical surfactants, which possess low-toxicity, biodegradable and environment-friendly characteristics and better adjustable biological activities (Makkar et al. 2011; Marchant and Banat 2012; Saharan et al. 2011; Vatsa et al. 2010). The demand for biosurfactants in the coming years will increase enormously due to their value in many industries including cosmetics, laundry detergents, textile, pharmaceutics, food and agriculture (Sekhon Randhawa and Rahman 2014). Biosurfactants possess unique chemical structures ensuring better interfacial properties which make them good emulsifying agents with a lower value of critical micellar concentration (CMC) as compared to synthetic surfactants, thus widening their applications (Jahan et al. 2020). Biosurfactants have come across long journeys since the first biosurfactant called "surfactin" which was purified and characterized by Arima (1968). Though many researchers have satisfactorily studied biosurfactants, some features are still left to be understood. At present, biosurfactants are replacing chemical surfactants as potential alternatives in many industries because of the environmental concerns of the latter (Banat et al. 2010; Marchant and Banat 2012).

Biosurfactants are categorized into low molecular weight (LMW) [e.g. glycolipids and lipopeptides] and high molecular weight (HMW) (e.g. polysaccharides, proteins and lipoproteins or lipopolysaccharides) (Banat et al. 2010; Ron and Rosenberg 2001; Rosenberg and Ron 1999). Biosurfactants have promising applications in agricultural, pharmaceutical, food, cosmetics, and detergent industries. So far, there are more than 250 patents granted for biodegradable molecules (Rahman and Gakpe 2008; Shete et al. 2006). Some studies reported that microbial biosurfactants are advantageous over plant-based biosurfactants due to their scale-up capacity, rapid production and versatile properties. Various plant-based biosurfactants like saponins and lecithins and soy proteins have significant emulsification properties but are costly at industrial scale and thus have other disputable factors such as solubility and hydrophobicity (Xu et al. 2011). Among different categories of biosurfactants (sophorolipids, rhamnolipids, trehalose lipids, cellobiose lipids, mannosylerythritol lipids, surfactin and emulsan) are glycolipid biosurfactants except for surfactin and emulsan. Glycolipid biosurfactants belong to the non-ionic class which are composed of a carbohydrate head and a lipid tail. Since glycolipid biosurfactants offer promising properties as compared to other traditional and chemical surfactants, the first generation of glycolipids was produced from renewable resources by chemical synthesis [e.g. alkyl polyglycosides (APGs)]; the second generation was achieved through the biotechnological procedure, and thus at

present, glycolipids are produced through fermentation, particularly rhamnolipids and sophorolipids (Van Bogaert et al. 2007). Sophorolipids are produced mainly from non-pathogenic yeasts; in contrast to rhamnolipids which are mainly obtained from the bacterial species *Pseudomonas aeruginosa*, sophorolipids make them more attractive for commercial purposes.

19.1.1 Sophorolipid-Producing Strains

A century back, Gorin et al. (1961) were the first to describe the extracellular glycolipid obtained from the yeast *Torulopsis magnolia*, and later the authors in 1968 corrected and identified the same species as *Torulopsis apicola* (Hajsig), which is currently known as *Candida apicola*.

The structure of the hydroxy-fatty acid sophoroside mixture was elucidated as a partially acetylated 2-*O*-β-D-glucopyranosyl-D-glucopyranose unit attached β-glycosidically to 17-L-hydroxyoctadecanoic or 17-L-hydroxy-Δ9-octadecenoic acid (Tulloch et al. 1962, 1968b). Also, Tulloch et al. (1968a) discovered a new sophorolipid from *Candida bogoriensis* (now known as *Rhodotorula bogoriensis*). The overall structure is similar to the sophorolipids of *Candida apicola* but differs in its hydroxy-fatty acid moiety: the sophorose unit is linked to 13-hydroxydocosanoic acid. A third sophorolipid-secreting yeast strain was identified by the same researchers as *Candida bombicola* (named initially as *Torulopsis bombicola*). The glycolipids and production characteristics of this species are nearly identical to those of *Candida apicola* (Spencer et al. 1970). Rosa and Lachance (1998) described the novel yeast species *Starmerella bombicola* and introduced it as the teleomorph of *Candida bombicola* based on the high 18S rDNA identity between both strains (more than 98%) and their ability to mate with each other to form ascospores. Chen et al. (2006a) also proved sophorolipid synthesis in a new strain of *Wickerhamiella domericqiae*. They observed more than six glycolipids and identified one of the three main products as 17-L-(-oxy)-octadecanoic acid 1,4″-lactone 6′,6″-diacetate, which is identical to the major component of the sophorolipids of *C. apicola* and *C. bombicola*. SPLs are produced not only by a single yeast species but also by other related microorganisms which belong to the *Wickerhamiella*, *Starmerella* and *Rhodotorula* species which is capable of producing similar kind of SPL molecules (Van Bogaert et al. 2011, 2007).

19.1.2 Sophorolipid Structure

SPLs are glycolipid biosurfactants and are amphiphilic molecules. They structurally consist of a sugar sophorose head group (2-*O*-β-D-glucopyranosyl-D-glucopyranose) attached to a long chain of C18 or C16, hydroxyl fatty acid tail group by a glycosidic linkage between the anomeric C atom of the sugar and the hydroxyl group of the fatty acid (Baccile et al. 2017). The head group, sophorose, is a disaccharide having a β-1,2 bond and acetylated on the 6′- and/or 6″-positions (Fig. 19.1). The acetylation

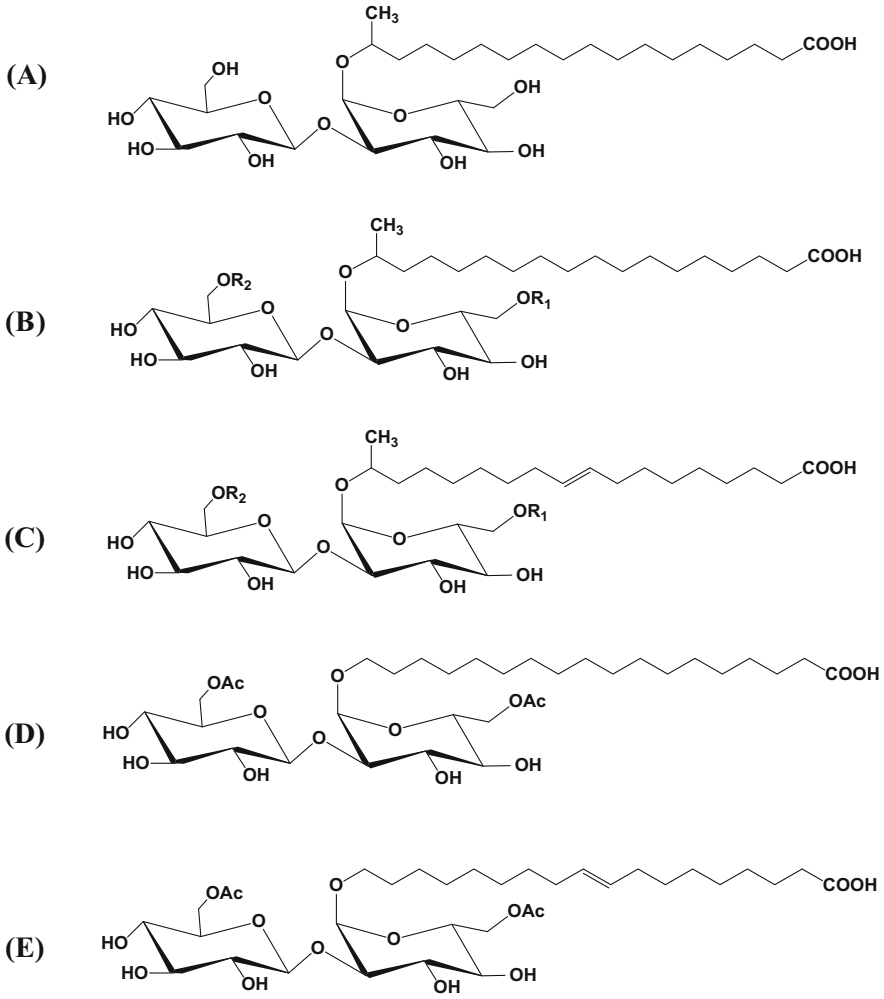

Fig. 19.1 Structures of sophorolipids: (**a**) Deacetylated sophorolipids, (**b**, **c**) major sophorolipids of *Starmerella bombicola* and (**d**, **e**) major sophorolipids of *Candida batistae*

is on the 6- and/or 6′-positions of sophorose residue. One terminal or subterminal hydroxylated fatty acid is β-glycosidically linked to the sophorose molecule. The hydroxy-fatty acid residue can have one or more unsaturated bonds (Fig. 19.1). The carboxylic group of fatty acids is either free (acidic or open form) or internally esterified (lactonic form) (Fig. 19.1). Sophorolipids can exist in the form of lactones both in monomeric and in dimeric forms (Nuñez et al. 2004). The carboxylic end of this fatty acid is either free (acidic or open form) or internally esterified at the 4″ or in some rare cases at the 6′- or 6″-position (lactonic form). The hydroxy-fatty acid itself counts in general 16 or 18 carbon atoms and can have one more unsaturated bond (Asmer et al. 1988; Davila et al. 1993). As such, the SPLs synthesized by

C. bombicola are a mixture of related molecules with differences in the fatty acid part (chain length, saturation and position of hydroxylation) and the lactonization and acetylation pattern. Asmer et al. (1988) were the first to shed light on this structural variation. However, differences in fatty acid length and hydroxylation patterns were not taken into account. Davila et al. (1993) separated the SPLs mixture by a gradient elution high-performance liquid chromatography (HPLC) method and used an evaporative light scattering for the detection of the individual SPLs. The group analysed the fatty acid chain and identified over 20 components.

19.2 Biosynthesis of Sophorolipids

Sophorolipids (SLPs) are one of the most promising biosurfactants that belong to the glycolipid group and synthesized extracellularly. SLPs are produced by the non-pathogenic yeast strain *Candida bombicola* as a mixture of different molecules. Generally found in two groups, acidic and lactonic, they hence show variation in physicochemical and biological properties (Daverey and Pakshirajan 2009). SLPs are secreted as secondary metabolites in the stationary phase during nitrogen-limiting conditions. Secretion can be induced in the presence of both lipophilic and hydrophilic carbon source, agitation-aeration, and growing the cells under stress condition (Desai and Banat 1997). The various factors are discussed below.

19.2.1 Carbon Source

The carbon source plays an important role in the production of SLPs. When both hydrophobic and hydrophilic carbon sources are applied to the medium, then SLPs production is optimal. SLPs biosynthesis is observed with two main inputs, glucose and fatty acids (FA), where the process begins with hydroxylation of FA. In the absence of a hydrophobic substrate, FA is formed by the de novo pathway from acetyl-CoA (Inoue and Ito 1982). Glucose is added to the synthesis pathway after the conversion of FA to hydroxy-FA. Glucose is further glycosidically converted to ω-1 hydroxyl group of FA by a specific transferase enzyme. A second glucose molecule is added to C2′ position of first glucose by transferase II (Esders and Light 1972). Acidic non-acetylated SLPs are obtained after this second glycosylation. The mixture of SLPs is obtained by further modifications caused by acetylation or lactonization of the sophorose unit. When either glucose or vegetable oil was used for the production of biosurfactant, a low yield was obtained by *Torulopsis*. An increased yield of SLPs was produced by the same organism when both the carbon sources were used in optimum quantity (Kim et al. 1997). Also, a higher yield of SLPs was obtained by *Candida bombicola* within 8 days by using sugar and oil as a carbon source (Casas et al. 1997).

19.2.2 Nitrogen Source

Nitrogen is the second most important component for the production of SL. Mainly when the yeast cell enters the stationary phase, SLP synthesis begins and is triggered in nitrogen starvation conditions (Davila et al. 1992). Normally, in fermentation processes, a higher C/N ratio that has lower nitrogen levels limits cell growth leading to the synthesis of secondary metabolites. It is assumed that *C. bombicola* prefers the same mechanism for the production of SLPs which is synthesized as extracellular storage material. Later, it was discovered that SLPs were not metabolized at a higher C/N ratio; indeed when added, carbon source is depleted (Garcia-Ochoa and Casas 1997). Hence, monitoring both the source is important for obtaining a high yield of SL. Initially, yeast extract and urea were used as a nitrogen source to increase SLP production by *Candida* and *Torulopsis*. The alternative for costly glucose and nitrogen source was first found by Solaiman and colleagues. They made the use of low-cost soy molasses with oleic acid for SLP production (Solaiman et al. 2007). Further using different yeast strains and by the combination of various fermentation parameters, Makoto et al. showed optimum SLP production by use of only sugarcane molasses and water as the most economic process (Takahashi et al. 2011).

19.3 Types of Biosurfactant Produced by Yeast/Fungi

Biosurfactants are amphiphilic molecules having a definite structure. The hydrophobic portion contains the hydrocarbon tail of long-chain fatty acid linked to hydrophilic moieties like alcohol, carbohydrate, amino acid or phosphate. Most biosurfactants are neutral or anionic, whereas few with amine groups are cationic. Synthetic surfactants are classified according to their polar group, but biosurfactants are categorized by chemical composition and microbial origin. Some of the most important types of biosurfactants are described in Table 19.1.

19.4 Advantages of Biosurfactants Over Normal Surfactants

Surfactants are widely used in almost every sector of industrial chemicals. The industrial demand of surfactants as well as household consumption is also growing faster accounting for larger production of surfactants to meet the increasing demand. Presently available commercial surfactants are synthesized mostly from petrochemicals (Farn 2008). Two major concerns related to the use of petrochemicals are an economic burden and an increase in environmental pollution. With the concern of global environment protection, there is a transformation in the use of chemical products according to environmental regulation, and demand for green alternative products has increased. The industries now propose the use of biological materials or methods in wide areas such as waste management, energy conservation, product modification and more. Thus advances in biological science have opened a way for the replacement of synthetic surfactant from petroleum

Table 19.1 Classes of biosurfactants and producing organisms

Biosurfactant			
Group	Class	Microorganism	References
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa*	Nitschke et al. (2011)
	Sophorolipids	*Candida*, *Torulopsis*	de Jesus Cortes-Sanchez et al. (2013)
	Trehalolipids	*Rhodococcus*, *Mycobacterium*, *Arthrobacter*	Lang and Philp (1998)
		F. fujikuroi	dos Reis et al. (2018)
Phospholipids and fatty acids		*Acinetobacter*	Gautam and Tyagi (2006)
		Candida sp. strain SY16	Kim et al. (2006)
		Candida antarctica	Kim et al. (2002)
Lipopeptides	Surfactin	*Bacillus* sp.	Fox and Bala (2000)
	Lichenysin	*Bacillus licheniformis*	Joshi et al. (2016)
Polymeric surfactant	Liposan	*Candida lipolytica*	Campos et al. (2013)
	Emulsan	*Acinetobacter calcoaceticus*	Gakpe et al. (2007)
		Candida lipolytica	Sarubbo et al. (2007)

feedstock to possible alternative biosurfactant (De et al. 2015). Being derived from natural products and organism sources, biosurfactants are eco-friendly. They possess various advantages over synthetic surfactants in terms of low-cost production, low toxicity, availability and sustainability. Also, they are considered a safe alternative for food, pharma and cosmetic industries (Bhadoriya et al. 2013). Biosurfactants have specific activity even at higher pH, temperature or other extreme conditions and are highly selective compared to synthetic surfactants. Few more advantages of biosurfactants are described below.

19.5 Availability of Raw Materials

In recent years, the disposal of industrial residues has been a major cause of pollution. The most efficient way of consuming this residual waste is by utilizing it as a substrate for the production of commercial compounds. These materials contain a large number of carbohydrates, oils and proteins which can serve as ideal raw material for the fermentation process. Biosurfactants are mostly produced extracellularly or as cell membrane part by various microorganisms. This bacteria, yeast or fungus uses sugars, oils, residues and waste materials as raw materials for their growth and synthesis of surfactant. Thus, biosurfactants can be produced from various oil refinery wastes, potato effluent, cassava waste, bagasse, etc. which are cheap and available in large quantities (Muthusamy et al. 2008).

19.6 Diversity

Surfactants produced by microorganisms are available in a wide range depending upon the environment and growth condition they are synthesized (Bodour et al. 2003). The different industrial processes demand a variety of surfactant to satisfy the commercial application. By changing the growth parameters and optimizing other fermentation conditions, different variants of surfactants can be generated from the same organism. This results in the formation of a surfactant mixture, and thus, even a small difference in structure can have a profound effect on its function (Symmank et al. 2002). This is of particular interest in the production of biosurfactant and is also economically favourable.

19.7 Selectivity and Specificity

Biomolecules are often found to be complex with specific functional groups. Microbial surfactants also possess particular functional moiety that shows specificity in action as compared to synthetic surfactants (Wick et al. 2002).

19.8 Low Toxicity

Biosurfactants are commonly considered as low toxic and cause no serious damage to the biotic ecosystem. Due to the low degree of toxicity, they are used in food, cosmetics and pharmaceutical industries. Biosurfactants do not have any harmful effects on the lung, heart, kidney or circulatory system. They hold lower chronic and acute toxicity compared to synthetic surfactants. It has been reported that synthetic anionic surfactant possesses ten times lower LC50 (lethal concentration) on test species compared to rhamnolipid. Flasz and colleagues perform an assay of toxicity and mutagenic properties of synthetic surfactant (Marlon) and biosurfactant derived from *Pseudomonas aeruginosa*. They found that synthetic surfactants showed higher toxicity and mutagenic effect (Flasz et al. 1998).

19.9 Biodegradability

Biodegradability of surfactants is the most important issue when evaluating environmental pollution (Berna et al. 2007). Biodegradation of surface-active agent occurs due to breaking down the molecule by a natural processor with the help of microorganisms. Biosurfactants can be easily degraded in nature by microorganisms into basic components. Microorganisms use BS as a carbon and energy source by transforming the hydrocarbon chain into CO_2, water and minerals (Garcia et al. 2006). However, there is very little literature available on the biodegradability of biosurfactants. However, it is shown recently that lipid biosurfactants are degraded under aerobic, sulphate-reducing, nitrate-reducing and fermentation conditions.

Also, biosurfactants surfactin, iturin and fengycin show degradation potential by soil microorganism as well as in liquid media, thus reducing the risk of environmental accumulation.

19.10 Applications

19.10.1 Antibacterial Activity

Sophorolipids (SLPs) have been widely studied for their potential antibacterial applications. Many researchers have used various methods for determining the antibacterial activity of sophorolipids which include serial dilution, microtitration and agar diffusion to determine the minimal inhibitory concentrations (MIC) or minimal lethal doses (50% lethal dose) of SLPs against various bacterial strains. In addition to SLPs acting as an antibacterial agent, they also act as anti-algal, antifungal, anticancer and antiviral agents. The antimicrobial activity of SLPs depends on its chemical structure and microbial cell wall structure.

Dengle-Pulate et al. (2014) produced SLPs by *Candida bombicola* with hydrophobic moiety derived from lauryl alcohol (SLPs-LA) having an effective antimicrobial activity against gram-negative bacteria, gram-positive bacteria and the pathogenic yeast. SLPs-LA shows a complete inhibition against gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* along with gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. Gram-positive bacteria when treated with SLPs-LA results in the rupture of cells (lysis), while gram-negative bacteria showed shrinking of the cells rather than rupture.

Further, Gaur et al. (2019) isolated SLPs from the yeast strains *Candida albicans* SC5314 and *Candida glabrata* CBS138 which showed antibacterial properties against pathogenic bacteria and also generated reactive oxygen species in *Bacillus subtilis* and *Escherichia coli*. Some previous studies have reported that ROS generation results in the killing of pathogenic strains. SLPs exhibit bactericidal activities of antimicrobial agents which contribute to the generation of free hydroxyl radicals resulting in the killing of bacteria. The antibacterial properties of sophorolipids against pathogenic bacteria strongly suggest that they are likely to be used in food emulsions that protect against pathogenic bacteria.

Recent studies by Ceresa et al. (2020) showed that SLPs exhibit properties like anti-adhesive and antibiofilm, which can be exploited for the surface coating to prevent and treat infections in humans and animals. This study demonstrated the antimicrobial effect of sophorolipids on medical-grade silicone material surfaces using microbial strains: *Candida albicans*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. Hence, SLPs help to reduce the cell attachment of microbial strain suggesting its effective role as coating agents on medical-grade silicone devices for the preventions of gram-positive bacteria and yeast infections.

19.10.2 Anticancer Activity

SLPs exhibit anticancer activity against many types of tumour cells and may have potential use in cancer treatment. Cell death is usually achieved by necrosis or apoptosis. Necrotic cell death is the process by which cells are destroyed resulting in lysis, while apoptosis involves programmed cell death through either intrinsic or extrinsic stimulation (Roelants et al. 2019).

The very first report of sophorolipids production by a new yeast strain of *Wickerhamiella domercqiae* showed anticancer effect and exhibited cytotoxic effects on different cancer cell lines, e.g., human liver cancer H7402, lung cancer A549, HL60 and K562. These results indicate a dose-dependent response on cell viability according to the drug concentration $\leq$62.5μg/ml (Chen et al. 2006b).

Further, Nawale et al. (2017) showed the apoptotic response of sophorolipids against HeLa cells through mitochondrial membrane depolymerization and by increasing the intracellular calcium levels which activate caspase-3, caspase-8 and caspase-9, playing essential roles in programmed cell death.

Besides this, Li et al. (2017) demonstrated SLPs to be effective against human cervical cancer cells. In that work, they synthesized SLPs by fermentation of *Stamerella bombicola* and reveal the anti-proliferative activity on HeLa and CaSki cells. The cytotoxic response of the SLPs molecule was proved to be influenced by the carbon chain length of sophorolipids. A direct correlation was observed between the length of the carbon chain and the cytotoxic response of SLPs. To increase the anticancer activity of SLPs, its structure can be modified with the enzymatic method. Thus, their work suggested a potential use of SLPs as anticancer medicine for cervical cancer treatment.

In this work, they have demonstrated SLPs to be effective against human pancreatic cancer cells. SLPs were synthesized by fermentation of *Candida bombicola* and showed the cytotoxic effect of the natural mixture or their derivatives (ethyl ester, methyl ester, ethyl ester monoacetate, ethyl ester diacetate, acidic sophorolipid, lactonic sophorolipid diacetate) against human pancreatic cancer cell lines by LDH release which often involves necrosis as a mechanism of action (Fu et al. 2008).

19.10.3 Antifungal activity

SLPs also exhibit antifungal activity against yeast including the various strain of *Candida*, *Pichia*, *Debaryomyces*, *Saccharomycopsis* and *Lodderomyces*.

Haque et al. (2019) demonstrated that the treatment of *Candida albicans* cells with SLPs increases the production of reactive oxygen species and upregulates the expression of SOD1 and CAT1, indicating high levels of oxidative stress and activation of stress response mechanism. Increased intracellular ROS level causes ER stress and the release of Ca^{2+} in the cytoplasm and changes in mitochondrial membrane potential (MMP). This study helps to know the mechanism of cell death initiation by glycolipids and indicated that further modification of these molecules

can lead to the development of a new therapeutic agent against fungal infection like *C. albicans*.

Sanada et al. (2014) reported the use of polyhexamethylene biguanide (PHMB) along with SLPs to prevent *Tinea pedis*. PHMB possesses antifungal activity and is used for disinfectant swimming pools, contact lenses and antimicrobial wound dressings. Non-woven textiles with PHMB are effective against *Trichophyton rubrum* and *Trichophyton mentagrophytes*. Non-woven textiles containing PHMB with SLPs increase PHMB access into the cuticle extensively reducing colony-forming units of *Trichophyton rubrum* and *Trichophyton mentagrophytes* suggesting that PHMB and SLPs are effective for *Tinea pedis* prevention.

19.10.4 Drug Delivery System

The use of biosurfactants as drug delivery agents offers attractive applications such as passive immunization, particularly where drug treatment options are limited.

Lactonic sophorolipid was formulated to develop solid lipid nanoparticle (SLPs) by the solvent injection method to encapsulate the antileprosy drug such as rifampicin and dapsone. For rifampicin, the EE (%) was 98.6 ± 0.2 and 98.8 ± 0.2, for SLN-3 and SLN-7 formulations, and for dapsone, it was 96.8 ± 0.2, and 96.9 ± 0.2, for SLN-3 and SLN-7 formulations, respectively. The kinetic model showed that the transport mechanism of rifampicin-releasing drugs is non-Fickian, and for dapsone, it is a Fickian-driven process. Due to easy preparation, biocompatibility, high entrapment efficiency, sustained release, increased bioavailability, etc., they have become a viable option for further research (Kanwar et al. 2018).

Darne et al. (2016) discussed the limiting factors for curcumin because of its low aqueous solubility, low retention time and poor bioavailability. SLPs were synthesized by non-pathogenic yeast such as *Candida bombicola* (ATCC 22214) and formulated curcumin-sophorolipids nano-conjugates (CurSL) to enhance the bioavailability. They have used gold salts, which act as potent-reducing and capping agents, resulting in synthesizing monodispersed, spherical gold nanoparticles (CurSL-GNPs) with sizes of 8–10 nm. Thus, Cur-SLPs-based nano-gold formulation was used as a good drug delivery carrier.

Another work done to enhance the bioavailability of the hydrophobic drug was studied (Yuan et al. 2019). In their study, they have fabricated the lutein-loaded zein nanoparticles with sophorolipid (ZSLNPs). These nanoparticles show good dispersibility and enhance water solubility of lutein with about 80 times higher than that of lutein alone.

19.10.5 Cosmetics

SLPs biosurfactants have been produced and commercially applied as an active ingredient in cosmetics products for body and skin applications.

Sophorolipids have antibacterial properties and are particularly active against gram-positive bacteria such as *Propionibacterium acnes* and *Corynebacterium xerosis*, the causal agents of acne and dandruff. Ashby et al. (2011) demonstrated different biopolymer matrices used to produce SLPs composite films with multiple antimicrobial effects against *P. acnes*. Pectin and alginate improve the transparency character of SLP composite films and also act as successful carriers of SLPs to *P. acnes*. SLPs in the lactone form have the best antimicrobial effect and work synergistically with all types of pectin or alginate biopolymers. It is a reproducible and eco-friendly measure against acne.

SLPs exhibited lower cytotoxicity than surfactin, which is a commercialized cosmetic ingredient (Hirata et al. 2009b).

19.10.6 Bioremediation

Bioremediation is a process that uses microorganisms to speed up the degradation of environmental contaminants. Biosurfactants produced by bacteria, fungi and yeast increase the surface area, solubility and bioavailability of hydrophobic water-insoluble substrates, stimulating the growth of oil-degrading microorganisms and improving their ability to utilize hydrocarbons.

The major problem facing today is the pollution of soils contaminated with poorly soluble polycyclic aromatic hydrocarbons (PAHs) like anthracene, fluorene, phenanthrene and pyrene. Phenanthrene is a model substrate for biodegradation research. SLPs increase the solubility and availability of phenanthrene stimulating the microbial biodegradation by *Sphingomonas yanoikuyae*. The maximum biodegradation achieved by *Sphingomonas yanoikuyae* is 1.3 mg/L h in the presence of SLPs compared to 0.8 mg/L h without SLPs. Two main problems can be solved by the use of surfactant—longer process time and residual pollutants (Schippers et al. 2000).

Oil pollution has caused a huge environmental problem for terrestrial and marine ecosystems. The components of petroleum have low aqueous solubility and strong binding and absorption in water and solid particles. The common method of remediation is based on the extraction of organic solvents or surfactants. SLPs improve the bioremediation of sites contaminated with hydrocarbons thereby increasing the bioavailability of microbial consortium for biodegradation. Adding SLPs to the site can improve the biodegradability of model compounds: 2-methylnaphthalene (95% degradation in 2 days), hexadecane (97%, 6 days) and pristane (85%, 6 days). SLPs show effective biodegradation of crude oil in soil (80% biodegradation of saturates and 72% aromatics hydrocarbon within in 8 weeks) (Kang et al. 2010).

19.10.7 Immunomodulatory Activity

Septic shock is a common cause of death in hospitals. In patients with sepsis caused by gram-negative bacteria, bacterial components including DNA, endotoxin and cell

wall lipopolysaccharide (LPS) are thought to be responsible for septic shock by inducing a cytokine cascade. Septic shock can lead to activation of the coagulation cascade and apoptosis, causing further organ damage and diffuse intravascular coagulation.

Hagler et al. (2007) demonstrated that SLPs decrease IgE production in U266 cells (IgE-producing myeloma cell line), by affecting the activity of plasma cells. This suggests that SLPs act as an anti-inflammatory agent and potential therapy in diseases with altered IgE regulation.

Sources of SPL	Application	Advantages	References
Candida bombicola	Antiviral and spermicidal activity	• It shows virucidal activity against HIV and sperm-immobilizing activity against human semen which is similar to nonoxynol-9 • Shows less cytotoxicity and higher activity	Shah et al. (2005)
Candida bombicola ATCC 22214	Bioremediation of lubricating oil-contaminated soils	• It increases the solubility, surface area and bioavailability of aqueous insoluble compounds • It also helps stimulate the growth of microorganisms that breakdown hydrocarbon and enhance their ability to use these hydrocarbons	Minucelli et al. (2017)
Candida bombicola ATCC 22214	Enhanced oil recovery	• Reduction of ST and IFT; it has a high %E24 for various hydrocarbons (including light and heavy crude oils) and also shows high stability under extreme conditions of salinity, pH and temperature	Elshafie et al. (2015)
Candida tropicalis	Bioremediation	• Good degrading agent of diesel oil • Higher emulsifying activity reduce surface tension	Chandran and Das (2012)
Candida kuoi NRRL Y-27208	Agriculture	• Natural surfactants/emulsifiers for post-emergence herbicides • SLPs can replace synthetic surfactants like polyethoxylated tallow amines (POEA)	Vaughn et al. (2014)
Candida bombicola	Cleaning agent	• Low cytotoxicity to human keratinocytes and fibroblasts • Biodegradable low-foaming surfactants with high detergency and hardness tolerance	Hirata et al. (2009a)
Torulopsis apicola and *Torulopsis bombicola*	Laundry detergent	• The detergent compositions show enhanced oily soil detergency in fabric washing	Flasz et al. (1998)

(continued)

Candida bombicola	Food	• Suitable for cleaning fruits, vegetables, skin and hair • This composition is sufficient to kill 100% of *E. coli*, *Salmonella typhi* and *Shigella dysenteriae* in 30 s	Data et al. (2001)
Chemically modified SLPs such as sophorolipid alkyl esters	Food	• Enhance the characteristics of prepared food products (bakery and oily emulsions)	Allingham (1971)
Candida species	Antibacterial activity	• Exhibited potential antibacterial activity towards pathogenic gram-negative and gram-positive bacteria	Archana et al. (2019)
Starmerella bombicola MTCC1910	Antifungal	• Inhibit *C. albicans* biofilm formation • It also shows inhibitory effect on hyphae formation	Haque et al. (2016)

19.11 Conclusions and Future Perspectives

Based on the origin, production and physicochemical properties of light-molecular-weight (LMW) and high-molecular-weight (HMW) microbial biosurfactants need to be explored and studied thoroughly to find their possible applications in nanotechnology. Most of the previous literature advocate the application of biosurfactant in synthesizing or stabilizing metal/metal oxide nanoparticles, but very recently (Bidyarani et al. 2020; Shinde et al. 2020), biosurfactants have been exploited for stabilizing protein nanoparticles for their application in drug delivery and agriculture. Due to their wide availability, low cost, biodegradability and environment-friendly nature, sophorolipids can be used in various industrial applications including food processing.

Sophorolipids are one of the most promising biosurfactants that belong to the glycolipid group and are synthesized extracellularly. These are produced mainly from non-pathogenic yeasts, in contrast to rhamnolipids which are mainly obtained from bacterial species *Pseudomonas aeruginosa*, making them more attractive for commercial purposes. Applications of sophorolipids have spiked over the last few decades, and their utilization has enhanced currently in the pursuit of natural ingredients. Various fermentation parameters essential for their optimal production and their various applications in agriculture, cosmetics, nanotechnology, bioremediation, antimicrobial, anticancer, immunomodulation, drug delivery, etc. have led to the replacement of synthetic surfactants.

References

Allingham RP (1971) United States Patent 72

Archana K, Reddy KS, Parameshwar J, Bee H (2019) Isolation and characterization of sophorolipid producing yeast from fruit waste for application as antibacterial agent. Environ Sustain 2:107–115

Arima K (1968) Surfactin, acrystalline peptidelipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochem Biophys Res Commun 31:488–494

Ashby RD, Zerkowski JA, Solaiman DKY, Liu LS (2011) Biopolymer scaffolds for use in delivering antimicrobial sophorolipids to the acne-causing bacterium Propionibacterium acnes. New Biotechnol 28:24–30. https://doi.org/10.1016/j.nbt.2010.08.001

Asmer H-J, Lang S, Wagner F, Wray V (1988) Microbial production, structure elucidation and bioconversion of sophorose lipids. J Am Oil Chem Soc 65:1460–1466

Baccile N, Babonneau F, Banat IM, Ciesielska K, Cuvier AS, Devreese B, Everaert B, Lydon H, Marchant R, Mitchell CA, Roelants S (2017) Development of a cradle-to-grave approach for acetylated acidic sophorolipid biosurfactants. ACS Sustain Chem Eng 5(1):1186–1198

Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R (2010) Microbial biosurfactants production, applications and future potential. Appl Microbiol Biotechnol 87:427–444

Berna JL, Cassani G, Hager C-D, Rehman N, López I, Schowanek D, Steber J, Taeger K, Wind T (2007) Anaerobic biodegradation of surfactants–scientific review. Tenside Surfact Deterg 44:312–347

Bhadoriya SS, Madoriya N, Shukla K, Parihar MS (2013) Biosurfactants: a new pharmaceutical additive for solubility enhancement and pharmaceutical development. Biochem Pharmacol Open Access 2:113

Bidyarani N, Srivastav AK, Gupta SK, Kumar U (2020) Synthesis and physicochemical characterization of rhamnolipid-stabilized carvacrol-loaded zein nanoparticles for antimicrobial application supported by molecular docking. J Nanopart Res 22(10):1–3

Bodour AA, Drees KP, Maier RM (2003) Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. Appl Environ Microbiol 69:3280–3287

Campos JM, Montenegro Stamford TL, Sarubbo LA, de Luna JM, Rufino RD, Banat IM (2013) Microbial biosurfactants as additives for food industries. Biotechnol Prog 29:1097–1108

Casas JA, de Lara SG, Garcia-Ochoa F (1997) Optimization of a synthetic medium for Candida bombicola growth using factorial design of experiments. Enzym Microb Technol 21:221–229

Ceresa C, Fracchia L, Williams M, Banat IM, De Rienzo MD (2020) The effect of sophorolipids against microbial biofilms on medical-grade silicone. J Biotechnol 309:34–43

Chandran P, Das N (2012) Role of sophorolipid biosurfactant in degradation of diesel oil by Candida tropicalis. Biorem J 16:19–30. https://doi.org/10.1080/10889868.2011.628351

Chen J, Song X, Zhang H, Qu Y (2006a) Production, structure elucidation and anticancer properties of sophorolipid from Wickerhamiella domercqiae. Enzym Microb Technol 39:501–506

Chen J, Song X, Zhang H, Qu Y, Miao J (2006b) Sophorolipid produced from the new yeast strain *Wickerhamiella domercqiae* induces apoptosis in H7402 human liver cancer cells. Appl Microbiol Biotechnol 72:52–59

Darne PA, Mehta MR, Agawane SB, Prabhune AA (2016) Bioavailability studies of curcumin–sophorolipid nano-conjugates in the aqueous phase: role in the synthesis of uniform gold nanoparticles. RSC Adv 6:68504–68514

Data RUSA, Examiner P, Dees JG, Leigh C (2001) (12) United States Patent 1

Daverey A, Pakshirajan K (2009) Production, characterization, and properties of sophorolipids from the yeast Candida bombicola using a low-cost fermentative medium. Appl Biochem Biotechnol 158:663–674

Davila A-M, Marchal R, Vandecasteele J-P (1992) Kinetics and balance of a fermentation free from product inhibition: sophorose lipid production by *Candida bombicola*. Appl Microbiol Biotechnol 38:6–11

Davila AM, Marchal R, Monin N, Vandecasteele JP (1993) Identification and determination of individual sophorolipids in fermentation products by gradient elution high-performance liquid chromatography with evaporative light-scattering detection. J Chromatogr A 648:139–149

de Jesus Cortes-Sanchez A, Hernandez-Sanchez H, Jaramillo-Flores ME (2013) Biological activity of glycolipids produced by microorganisms: new trends and possible therapeutic alternatives. Microbiol Res 168:22–32

De S, Malik S, Ghosh A, Saha R, Saha B (2015) A review on natural surfactants. RSC Adv 5:65757–65767

Dengle-Pulate V, Chandorkar P, Bhagwat S, Prabhune AA (2014) Antimicrobial and SEM studies of sophorolipids synthesized using lauryl alcohol. J Surfactant Deterg 17:543–552

Desai JD, Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev 61:47–64

dos Reis CBL, Morandini LMB, Bevilacqua CB, Bublitz F, Ugalde G, Mazutti MA, Jacques RJS (2018) First report of the production of a potent biosurfactant with α, β-trehalose by Fusarium fujikuroi under optimized conditions of submerged fermentation. Brazilian J Microbiol 49:185–192

Elshafie AE, Joshi SJ, Al-Wahaibi YM, Al-Bemani AS, Al-Bahry SN, Al-Maqbali D, Banat IM (2015) Sophorolipids production by *Candida bombicola* ATCC 22214 and its potential application in microbial enhanced oil recovery. Front Microbiol 6:1–11. https://doi.org/10.3389/fmicb.2015.01324

Esders TW, Light RJ (1972) Glucosyl- and acetyltransferases involved in the biosynthesis of glycolipids from *Candida bogoriensis*. J Biol Chem 247:1375–1386

Farn RJ (2008) Chemistry and technology of surfactants. Wiley, Hoboken, NJ

Flasz A, Rocha CA, Mosquera B, Sajo C (1998) A comparative study of the toxicity of a synthetic surfactant and one produced by *Pseudomonas aeruginosa* ATCC 55925. Med Sci Res 26:181–185

Fox SL, Bala GA (2000) Production of surfactant from *Bacillus subtilis* ATCC 21332 using potato substrates. Bioresour Technol 75:235–240

Fu SL, Wallner SR, Bowne WB, Hagler MD, Zenilman ME, Gross R, Bluth MH (2008) Sophorolipids and their derivatives are lethal against human pancreatic cancer cells. J Surg Res 148:77–82

Gakpe EE, Rahman PP, Hatha AAMM (2007) Microbial biosurfactants—review. J Mar Atmos Res 3:1–17

Garcia MT, Campos E, Dalmau M, Illan P, Sanchez-Leal J (2006) Inhibition of biogas production by alkyl benzene sulfonates (LAS) in a screening test for anaerobic biodegradability. Biodegradation 17:39–46

Garcia-Ochoa F, Casas JA (1997) Process for the production of sophorose by *Candida bombicola*. Spanish Pat 2103688

Gaur VK, Regar RK, Dhiman N, Gautam K, Srivastava JK, Patnaik S, Kamthan M, Manickam N (2019) Biosynthesis and characterization of sophorolipid biosurfactant by *Candida* spp.: application as food emulsifier and antibacterial agent. Bioresour Technol 285:121314

Gautam KK, Tyagi VK (2006) Microbial surfactants: a review. J Oleo Sci 55:155–166

Gorin PAJ, Spencer JFT, Tulloch AP (1961) Hydroxy fatty acid glycosides of sophorose from *Torulopsis magnoliae*. Can J Chem 39:846–855

Hagler M, Smith-Norowitz TA, Chice S, Wallner SR, Viterbo D, Mueller CM, Gross R, Nowakowski M, Schulze R, Zenilman ME, Bluth MH (2007) Sophorolipids decrease IgE production in U266 cells by downregulation of BSAP (Pax5), TLR-2, STAT3 and IL-6. J Allergy Clin Immunol 119:S263. https://doi.org/10.1016/j.jaci.2006.12.399

Haque F, Alfatah M, Ganesan K, Bhattacharyya MS (2016) Inhibitory effect of sophorolipid on *Candida albicans* biofilm formation and hyphal growth. Sci Rep 6:23575

Haque F, Verma NK, Alfatah M, Bijlani S, Bhattacharyya MS (2019) Sophorolipid exhibits antifungal activity by ROS mediated endoplasmic reticulum stress and mitochondrial dysfunction pathways in *Candida albicans*. RSC Adv 9:41639–41648

Hirata Y, Ryu M, Igarashi K, Nagatsuka A, Furuta T, Kanaya S, Sugiura M (2009a) Natural synergism of acid and lactone type mixed sophorolipids in interfacial activities and cytotoxicities. J Oleo Sci 58:565–572. https://doi.org/10.5650/jos.58.565

Hirata Y, Ryu M, Oda Y, Igarashi K, Nagatsuka A, Furuta T, Sugiura M (2009b) Novel characteristics of sophorolipids, yeast glycolipid biosurfactants, as biodegradable low-foaming surfactants. J Biosci Bioeng 108:142–146. https://doi.org/10.1016/j.jbiosc.2009.03.012

Inoue S, Ito S (1982) Sophorolipids from *Torulopsis bombicola* as microbial surfactants in alkane fermentations. Biotechnol Lett 4:3–8

Jahan R, Bodratti AM, Tsianou M, Alexandridis P (2020) Biosurfactants, natural alternatives to synthetic surfactants: physicochemical properties and applications. Adv Colloid Interf Sci 275:102061

Joshi SJ, Al-Wahaibi YM, Al-Bahry SN, Elshafie AE, Al-Bemani AS, Al-Bahri A, Al-Mandhari MS (2016) Production, characterization, and application of Bacillus licheniformis W16 biosurfactant in enhancing oil recovery. Front Microbiol 7:1853

Kang SW, Kim YB, Shin JD, Kim EK (2010) Enhanced biodegradation of hydrocarbons in soil by microbial biosurfactant, sophorolipid. Appl Biochem Biotechnol 160:780–790. https://doi.org/10.1007/s12010-009-8580-5

Kanwar R, Gradzielski M, Mehta SK (2018) Biomimetic solid lipid nanoparticles of sophorolipids designed for antileprosy drugs. J Phys Chem B 122:6837–6845

Kim SY, Oh DK, Lee KH, Kim JH (1997) Effect of soybean oil and glucose on sophorose lipid fermentation by *Torulopsis bombicola* in continuous culture. Appl Microbiol Biotechnol 48:23–26

Kim H-S, Jeon J-W, Kim S-B, Oh H-M, Kwon T-J, Yoon B-D (2002) Surface and physicochemical properties of a glycolipid biosurfactant, mannosylerythritol lipid, from Candida antarctica. Biotechnol Lett 24:1637–1641

Kim H-S, Jeon J-W, Kim B-H, Ahn C-Y, Oh H-M, Yoon B-D (2006) Extracellular production of a glycolipid biosurfactant, mannosylerythritol lipid, by *Candida* sp. SY16 using fed-batch fermentation. Appl Microbiol Biotechnol 70:391–396

Lang S, Philp JC (1998) Surface-active lipids in rhodococci. Antonie Van Leeuwenhoek 74:59–70

Li H, Guo W, Ma X, Li J, Song X (2017) In vitro and in vivo anticancer activity of sophorolipids to human cervical cancer. Appl Biochem Biotechnol 181:1372–1387

Ma X, Meng L, Zhang H, Zhou L, Yue J, Zhu H, Yao R (2020) Sophorolipid biosynthesis and production from diverse hydrophilic and hydrophobic carbon substrates. Appl Microbiol Biotechnol 104:77–100

Makkar RS, Cameotra SS, Banat IM (2011) Advances in utilization of renewable substrates for biosurfactant production. AMB Express 1:5

Marchant R, Banat IM (2012) Microbial biosurfactants: challenges and opportunities for future exploitation. Trends Biotechnol 30:558–565

Minucelli T, Ribeiro-Viana RM, Borsato D, Andrade G, Cely MVT, de Oliveira MR, Baldo C, Celligoi MAPC (2017) Sophorolipids production by *Candida bombicola* ATCC 22214 and its potential application in soil bioremediation. Waste Biomass Valorization 8:743–753. https://doi.org/10.1007/s12649-016-9592-3

Muthusamy K, Gopalakrishnan S, Ravi TK, Sivachidambaram P (2008) Biosurfactants: properties, commercial production and application. Curr Sci 94:736–747

Nawale L, Dubey P, Chaudhari B, Sarkar D, Prabhune A (2017) Anti-proliferative effect of novel primary cetyl alcohol derived sophorolipids against human cervical cancer cells HeLa. PLoS One 12(4):e0174241

Nitschke M, Costa SG, Contiero J (2011) Rhamnolipids and PHAs: recent reports on *Pseudomonas*-derived molecules of increasing industrial interest. Process Biochem 46:621–630

Nuñez A, Ashby R, Foglia TA, Solaiman DKY (2004) LC/MS analysis and lipase modification of the sophorolipids produced by Rhodotorula bogoriensis. Biotechnol Lett 26:1087–1093

Rahman PKSM, Gakpe E (2008) Production, characterisation and applications of biosurfactants—review. Biotechnology 7(2):360–370

Roelants S, Solaiman DKY, Ashby RD, Lodens S, Van Renterghem L, Soetaert W (2019) Production and applications of sophorolipids. In: Biobased surfactants. Elsevier, Amsterdam, pp 65–119

Ron EZ, Rosenberg E (2001) Natural roles of biosurfactants: mini review. Environ Microbiol 3:229–236

Rosa CA, Lachance M-A (1998) The yeast genus *Starmerella* gen. nov. and *Starmerella bombicola* sp. nov., the teleomorph of *Candida bombicola* (Spencer, Gorin & Tullock) Meyer & Yarrow. Int J Syst Evol Microbiol 48:1413–1417

Rosenberg E, Ron EZ (1999) High-and low-molecular-mass microbial surfactants. Appl Microbiol Biotechnol 52:154–162

Saharan BS, Sahu RK, Sharma D (2011) A review on biosurfactants: fermentation, current developments and perspectives. Genet Eng Biotechnol J 2011:1–14

Sanada H, Nakagami G, Takehara K, Goto T, Ishii N, Yoshida S, Ryu M, Tsunemi Y (2014) Antifungal effect of non-woven textiles containing polyhexamethylene biguanide with sophorolipid: a potential method for Tinea pedis prevention. Healthcare 2:183–191. https://doi.org/10.3390/healthcare2020183

Sarubbo LA, Farias CBB, Campos-Takaki GM (2007) Co-utilization of canola oil and glucose on the production of a surfactant by *Candida lipolytica*. Curr Microbiol 54:68–73

Schippers C, Geßner K, Müller T, Scheper T (2000) Microbial degradation of phenanthrene by addition of a sophorolipid mixture. J Biotechnol 83:189–198. https://doi.org/10.1016/S0168-1656(00)00304-7

Sekhon Randhawa KK, Rahman PKSM (2014) Rhamnolipid biosurfactants—past, present, and future scenario of global market. Front Microbiol 5:454

Shah V, Doncel GF, Seyoum T, Eaton KM, Zalenskaya I, Hagver R, Azim A, Gross R (2005) Sophorolipids, microbial glycolipids with anti-human immunodeficiency virus and sperm-immobilizing activities. Antimicrob Agents Chemother 49:4093–4100. https://doi.org/10.1128/AAC.49.10.4093-4100.2005

Shete AM, Wadhawa G, Banat IM, Chopade BA (2006) Mapping of patents on bioemulsifier and biosurfactant: a review. J Sci Ind Res 65:91–115

Shinde P, Agraval H, Srivastav AK, Yadav UC, Kumar U (2020) Physico-chemical characterization of carvacrol loaded zein nanoparticles for enhanced anticancer activity and investigation of molecular interactions between them by molecular docking. Int J Pharm 588:119795

Solaiman DKY, Ashby RD, Zerkowski JA, Foglia TA (2007) Simplified soy molasses-based medium for reduced-cost production of sophorolipids by *Candida bombicola*. Biotechnol Lett 29:1341–1347

Spencer JFT, Gorin PAJ, Tulloch AP (1970) *Torulopsis bombicola* sp. n. Antonie Van Leeuwenhoek 36:129–133

Symmank H, Franke P, Saenger W, Bernhard F (2002) Modification of biologically active peptides: production of a novel lipohexapeptide after engineering of *Bacillus subtilis* surfactin synthetase. Protein Eng 15:913–921

Takahashi M, Morita T, Wada K, Hirose N, Fukuoka T, Imura T, Kitamoto D (2011) Production of sophorolipid glycolipid biosurfactants from sugarcane molasses using Starmerella bombicola NBRC 10243. J Oleo Sci 60:267–273

Tulloch AP, Spencer JFT, Gorin PAJ (1962) The fermentation of long-chain compounds by Torulopsis magnoliae: I. Structures of the hydroxy fatty acids obtained by the fermentation of fatty acids and hydrocarbons. Can J Chem 40:1326–1338

Tulloch AP, Hill A, Spencer JFT (1968a) Structure and reactions of lactonic and acidic sophorosides of 17-hydroxyoctadecanoic acid. Can J Chem 46:3337–3351

Tulloch AP, Spencer JFT, Deinema MH (1968b) A new hydroxy fatty acid sophoroside from *Candida bogoriensis*. Can J Chem 46:345–348

Van Bogaert INA, Saerens K, De Muynck C, Develter D, Soetaert W, Vandamme EJ (2007) Microbial production and application of sophorolipids. Appl Microbiol Biotechnol 76:23–34. https://doi.org/10.1007/s00253-007-0988-7

Van Bogaert INA, Zhang J, Soetaert W (2011) Microbial synthesis of sophorolipids. Process Biochem 46:821–833. https://doi.org/10.1016/j.procbio.2011.01.010

Vatsa P, Sanchez L, Clement C, Baillieul F, Dorey S (2010) Rhamnolipid biosurfactants as new players in animal and plant defense against microbes. Int J Mol Sci 11:5095–5108

Vaughn SF, Behle RW, Skory CD, Kurtzman CP, Price NPJ (2014) Utilization of sophorolipids as biosurfactants for postemergence herbicides. Crop Prot 59:29–34. https://doi.org/10.1016/j.cropro.2014.01.014

Wick L, De Munain A, Springael D, Harms H (2002) Responses of *Mycobacterium* sp. LB501T to the low bioavailability of solid anthracene. Appl Microbiol Biotechnol 58:378–385

Xu Q, Nakajima M, Liu Z, Shiina T (2011) Biosurfactants for microbubble preparation and application. Int J Mol Sci 12:462–475

Yuan Y, Li H, Liu C, Zhang S, Xu Y, Wang D (2019) Fabrication and characterization of lutein-loaded nanoparticles based on zein and sophorolipid: enhancement of water solubility, stability, and bioaccessibility. J Agric Food Chem 67:11977–11985

Fungi: A Sustainable and Versatile Tool for Transformation, Detoxification, and Degradation of Environmental Pollutants

20

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Abstract

Environmental pollution is one of the major concerns for all living beings. The eco-friendly management for the disposal of waste is the basic problem faced worldwide. The attention of the public toward the environment is a very essential element for the survival of both humans and biotic organisms. Considering the energy cost and severe environmental hazards, bioremediation is one of the practical solutions. Mycoremediation is a cleanup technique within the larger field of remediation. It is a process of degrading or remediation of toxicant from the environment by using fungi. Fungi are a very diverse potential group of organism for the elimination of pollutants present in different niches. Fungi with their array of enzyme system can effectively neutralize the most persistent chemical compounds. Fungal biomass as well as metabolites aids in the removal of toxicants by the process of adaptation. This chapter describes recent advances in the field of mycoremediation. An attempt has been made to compile noteworthy findings regarding detoxification, mineralization, and transformation of polycyclic aromatic hydrocarbons (PAHs), dyes, heavy metals, endocrine-disrupting chemicals (EDCs), halogenated compounds, and others. Various fungi with potential of bioremediation have been discussed in the chapter. Furthermore, this study will also lay a good foundation and interest of scientists to look for indigenous fungi and their exploitation using genetic engineering in the future.

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Keywords

Mycoremediation · Dye degradation · Pesticides · Polycyclic aromatic hydrocarbons · EDCs · White rot fungi · Genomics and proteomics

20.1 Introduction

The quality of life on earth is linked inexorably to the overall quality of the environment. The advancement made in science and technology and industrialization has improved the quality of life, but at the same time, it has posed a serious threat to the ecosystem. The problem on pollution is of concern globally, and poor management of waste disposal from household, industries, and agriculture is deteriorating the environment. According to the reports by the World Health Organization, approximately, 2.2 billion people are unable to access safe water services, and nearly 144 million people consume contaminated water. It is anticipated that 50% of the population will be living in water-deficient regions by 2025 (WHO factsheet report, 2019). Owing to the reason cited above especially population explosion and industrialization, management of chemical and solid waste has become a major cause of concern today as environment is being constantly loaded with large quantities of organic and inorganic waste. In some case, the release of contaminants is deliberate, accidental, and sometimes well regulated. Many of the contaminants are toxic and persistent in aquatic as well as terrestrial environment. The most common organic pollutants are pesticides, dyes, and polycyclic aromatic hydrocarbons (PAHs). Pesticides are utilized in agriculture to control pests and some other parasites. Among South Asian countries, India is by far the largest consumer of pesticides (Abhilash and Singh 2009). India being one of the biggest producers as well as consumer of textile products releases large amounts of various types of dyes like azo dyes, anthraquinone dyes, etc. Wastes generated in textile industries are very difficult to treat; thus, it is directly released into water bodies after employing only preliminary treatments. Human activities like burning of fossil fuels, wood, oil drilling, and coal mining have led to generation of recalcitrant pollutants such as PAHs (Gupta et al. 2017; Verdin et al. 2004). These pollutants persist in the environment for a very long time and may generate more hazardous compounds if left untreated.

Generally, such pollutants are directly dumped for land-filling or flushed into water bodies. Such disposal methods have been proven to be ineffective due to economic and health concerns (Singh et al. 2020; Karigar and Rao 2011). Apart from land-filling, incineration is one of the common methods for remedy of environmental contaminants. The conventional techniques used for disposal of such pollutants are by digging and filling it with waste material and/or by its release in water bodies. Other methods such as chemical decomposition (base-catalyzed dechlorination, UV oxidation) have evolved. Although they can be effective at reducing a wide range of contaminants, these methods are complex, uneconomical, and unacceptable to the public. They demand high energy and manpower input too. Furthermore, these

methods have drawback of releasing pollutant gases like hydrogen cyanide, methane, carbon monoxide, etc. It has been reported that these pollutants have reached up to groundwater levels and even found their way back to humans by contaminating food crops as well as water, which led to increased health-related issues such as heavy metal toxicity, birth and fertility defects, and cancer (Tokar et al. 2013). In order to minimize such problems, recently, researchers are getting attracted toward alternate remediation strategies; bioremediation is one of those which have been found very effective (Agnihotri 2020).

20.2 Bioremediation

Bioremediation is an ecologically sound and state-of-the-art technique which employs natural biological process to potentially hazardous pollutants which are either degraded or transformed into a non-hazardous form with the use of microorganisms, fungi, green plants, or their enzymatic machinery. The process of bioremediation can be traced to the ancient period dating back to 600 BC when Romans used it for treatment of wastewater (Agnihotri 2020). Bioremediation of pollutants can be carried out by the application of any of the processes such as natural attenuation, bio-stimulation, and bio-augmentation, or combination thereof. The process of bioremediation mainly depends upon microorganisms and their enzyme systems (Azubuike et al. 2016). Characteristics like pervasiveness, vast diversity, high growth rate and biomass production, ability to survive and operate under extreme conditions, as well as catalytic capabilities make microbes as potent agents for bioremediation (Gupta et al. 2017; Megharaj et al. 2011). Furthermore, microbes interact with each other either via chemotaxis or via some physical means, which in turn leads to co-metabolism of pollutants. Co-metabolism can result in transformations of complex pollutants, and it may even result in complete degradation. These microbes are capable of removing, detoxifying, or immobilizing pollutants like heavy metals, pesticides, and others. In order to either detoxify metals or to degrade other pollutants, microbes may employ a variety of chemical reactions such as redox reactions, methylation, bioemulsification, and others which involve metal chelating compounds such as siderophores. The kind of process and mechanism to be applied depends greatly on the type of pollutants, degree of complexity, and environmental factors.

Generally, there are two approaches for conducting bioremediation: in situ and ex situ. In situ bioremediation method involves treatment of contaminant on the same site where pollutants exist. On the other hand, ex situ bioremediation includes treatment of contaminants on the other site after physically removing pollutants from the contaminated area (Kumar et al. 2011).

Bioremediation strategies are chosen as per requirement, cost, and efficacy in a particular environment. Although both strategies have their own pros and cons, as compared to ex situ, in situ bioremediation methods are relatively efficient, more promising, economic, and environment-friendly (Vidali 2001). Currently available bioremediation techniques are summarized in Fig. 20.1.

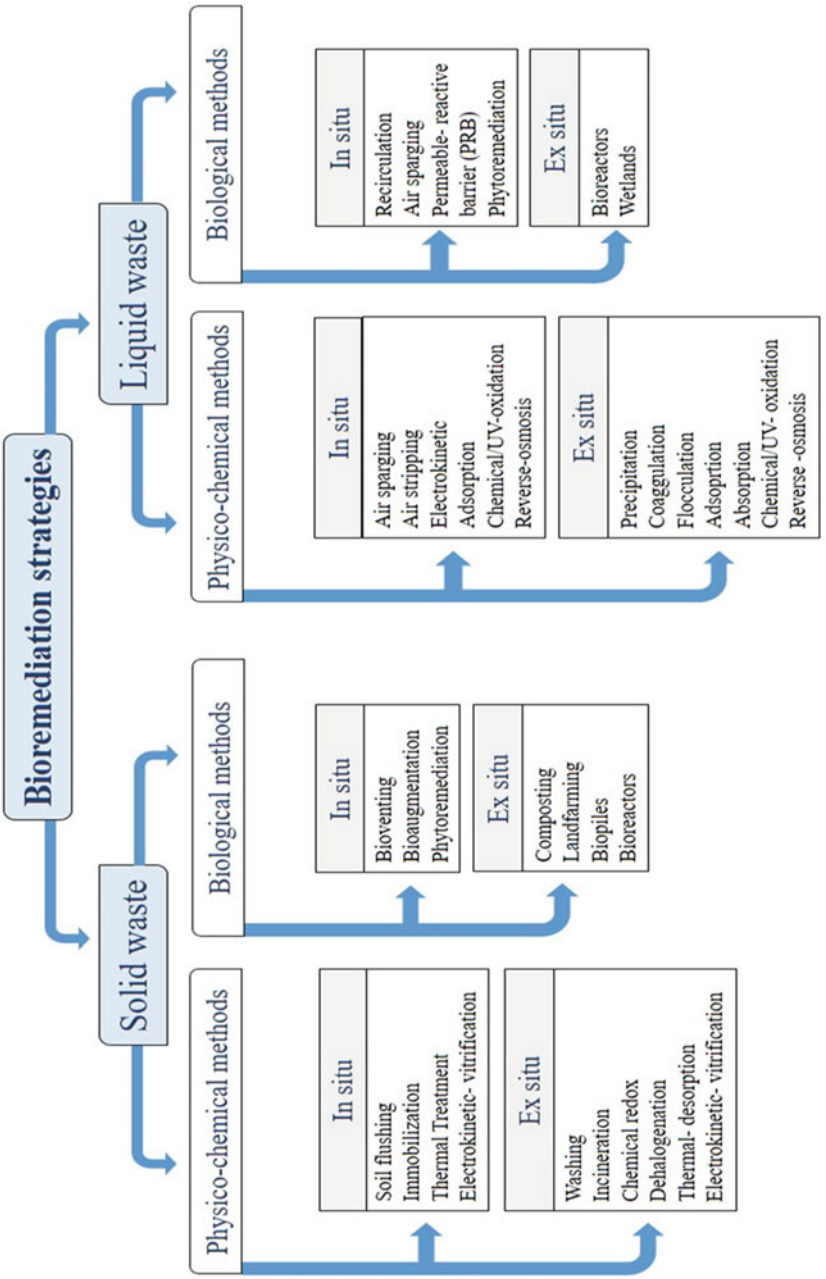

Fig. 20.1 Overview of current bioremediation technologies

20.3 Mycoremediation

Fungi are one of the earliest organisms to evolve on land, beginning over 1430 million years ago. Over time, fungi have created intricate relationships with plants and bacteria to break down organic materials and build a symbiotic web. Fungi are saprophytic microorganisms and serve many purposes. They rely on dead or decaying organic matters for energy. Fungi are found in aqueous, desert, forest, and even marine ecosystem. They are capable of surviving under very harsh environmental conditions and proven to be very efficient in degrading complex organic matter (Niehaus et al. 1999). The kingdom *Fungi* has been broadly classified into seven divisions (phyla): *Microsporidia*, *Chytridiomycota*, *Blastocladiomycota*, *Neocallimastigomycota*, *Glomeromycota*, *Ascomycota*, and *Basidiomycota* (Hibbett et al. 2007). Among all divisions, fungi from *Ascomycota* and *Basidiomycota* divisions have greater potential for remediation. Some fungi which have been utilized for bioremediation purposes are enlisted in Table 20.1.

Fungi can be considered as ideal organisms for bioremediation purposes because they are known to habitat in different ecosystems and thus capable of remediating pollutants present in diverse environmental zones. Many fungi have been reported from terrestrial as well as marine environment and from extremophilic zones too (Rampelotto 2013). Fungi having remediation potential from terrestrial ecosystem include many members of *Ascomycota* and several mushroom species. Members of *Ascomycota* division are usually fast-growing fungi and excellent decomposers of complex carbohydrates such as cellulose, hemicellulose, pectin, and starch. Apart from this, they are also reported to degrade complex materials such as hydrocarbons, chitin, fat, as well as keratin (Gupta et al. 2017). Among mushrooms, white rot fungi

Table 20.1 Bioremediation-capable fungal species

Division	Fungi	References
Ascomycota	*Pseudallescheria boydii*, *Aspergillus niger*, *Aspergillus foetidus*, *Aspergillus jegita*, *Penicillium* sp., *Trichoderma* sp., *Geotrichum candidum*, *Doratomyces nanus*, *Myceliophthora thermophila*, *Curvularia* sp., *Chrysosporium keratinophilum*, *Exophiala xenobiotica*, *Acremonium* sp., *Thielavia* sp. HJ22, *Fusarium incarnatum* UC-14	April et al. (1998), Mouhamadou et al. (2013), Jebapriya and Gnanadoss (2013), Mtibaà et al. (2020), Chhaya and Gupte (2013), Raina et al. (2020)
Basidiomycota	*Pleurotus ostreatus*, *Pleurotus eryngii*, *Tricholoma giganteum*, *Bjerkandera adusta*, *Schizophyllum commune* AGMJ-1, *Trametes versicolor*, *Phanerochaete sordida*, *Ceriporia metamorphosa*, *Phanerochaete chrysosporium*, *Ganoderma cupreum* AG-1	Joshi et al. (2013), Rosales et al. (2013), Gahlout et al. (2013), Safferman et al. (1995), Dhiman et al. (2020)

are the most exploited for remediation purposes as they possess excellent capabilities for the degradation of lignocellulosic biomass wastes. Furthermore, their fruiting bodies as well as mycelia have an advantage of absorbing hazardous pollutants such as heavy metals (Harms et al. 2011). Due to their preference of habitat rich in organic materials, white rot fungi have been known to produce a wide variety of enzymes which are very effective in transforming many organic and inorganic pollutants such as pesticides, PAHs, dyes, and many more (Verma et al. 2020). Marine fungi have an advantage over terrestrial ones of being tolerant to high salinity, pH, and high atmospheric pressure. They have been particularly utilized for remediation of hydrocarbon and heavy metal pollution (Raghukumar et al. 2008). Extremophilic microorganisms are the most sought-after ones because they possess unique characteristics of displaying optimum activity at extreme pH, temperature, and stress. Extremophilic fungi have been keenly investigated by many researchers as they have a robust biocatalytic system which is advantageous under environmental conditions which normally restrict typical bioconversion processes (Peeples 2014). Fungi don't have photosynthetic abilities, so they cannot generate energy on their own, and they rely on sources which are rich in organic matter. These limitations have led fungi to predominantly utilize biocatalysis as a main strategy to digest complex organic materials.

20.4 Fungal Enzymes in Bioremediation

Many researchers have reported that enzymes from fungal origin can serve as sustainable alternatives for the remediation of contaminated sites. The use of enzyme is desirable to perform the specific function under ambient conditions. Enzymes (biocatalysts) have the ability to enhance the rate of biochemical reactions by lowering activation energy. Enzymes are proteinaceous in nature, produced by almost all living beings. Most of the enzymes involved in bioremediation are extracellular (Verma et al. 2020). Enzyme technologies have advantages of being quicker, reusable, eco-friendly, as well as economic. Since enzymes replace chemical reactions, no hazardous chemicals are employed, and less amount of undesirable pollutant by-products is produced, so they are also known as "Green Catalysts," and this technology has been often referred to as "Green Technology." Fungi produce vast variety of enzymes, and many of those have been proven to possess good bioremediation capabilities. Some of the important enzymes employed in remediation include laccases, peroxidases, and catalases (Marco et al. 2013). Among these fungal enzymes, laccases, lignin peroxidases (LiP), and manganese peroxidases (MnP) are the most exploited ones.

Laccase is a multi-copper oxidase which catalyzes the four-electron reduction of molecular oxygen to water. It only interacts with the phenolic compounds that results in Cα oxidation, Cα-Cβ cleavage, and aryl-alkyl bond cleavage (Chaurasia et al. 2013). Lignin peroxidases (LiP) catalyze hydrogen peroxide-dependent oxidative degradation of lignin. Additionally, they also oxidize β-*O*-4 linkage-type aryl-glycerol-aryl ethers which are non-phenolic lignin compounds. LiP carries out

oxidation by the formation of radical cation through one-electron oxidation which leads to side-chain cleavage, demethylation, intramolecular addition, and rearrangements (Falade et al. 2017). Similarly, manganese peroxidase (MnP) carries out the oxidation of Mn^{2+} to Mn^{3+} in the presence of hydrogen peroxide. The oxidation of phenolic lignin structures to phenoxy radicals or aryl cation radicals is performed by these oxidized Mn^{3+} ions which act like low-molecular-weight diffusible redox mediator (Hildén et al. 2013). Apart from these three enzymes, other enzymes such as monooxygenases, tyrosinases, heme-peroxigenases, nitro-reductases, and dehalogenases carry out transformation of xenobiotic compounds by either O_2/H_2O_2-assisted oxidation or NADPH-dependent reduction reactions (Singh et al. 2020). Such enzymes can be found from various sources having wide range of applications. The sources and applications of some of the most important fungal enzymes for bioremediation are presented in Table 20.2.

20.5 Fungi-Assisted Bioremediation of Various Organic as Well as Inorganic Pollutants.

For a long time, fungi have already been known to humankind and mostly being utilized as one of the food resources. In the twentieth century, exploration of fungi has unlocked a new paradigm of novel applications for remediating pollutants which are otherwise very much difficult to degrade by other means and may lead to health crisis (Treu and Falandysz 2017). Initially, fungi were mostly employed for degradation of lignocellulosic residues, but now their capabilities are being tested and utilized for transformation, detoxification, elimination, as well as degradation of various complex organic and inorganic compounds such as dyes, pigments, pesticides, poly-aromatic hydrocarbons, aliphatic hydrocarbons, heavy metals, endocrine-disrupting chemicals, and halogenated compounds (Rudakiya et al. 2019). Fungi transform or detoxify such hazardous chemicals by means of enzymatic actions that basically involve oxidation, reduction, and transferase reactions too. Apart from these, fungal biomass itself has the ability to adsorb as well as absorb some of these compounds like metal ions. The mechanism to be employed for remediation of aforementioned pollutants depends largely on the type and complexity of the chemicals. Some of the bioremediation strategies are depicted in Fig. 20.2.

20.5.1 Bioremediation of Dyes

Rise in population and improved quality of life have propelled growth of textile industries in India. Textile industries of India are very diverse in terms of raw materials as well as product range, and it is dependent on multiple industries such as cotton and yarn, dye industries, etc. Among these, dye industries are one of the important sectors, and it has many applications in paper and pulp, textile, plastic, and paint industries. Dyes are organic compounds or mixture that chemically bind and impart color to substrates like cloth, paper, plastic, or leather. Although dyes have

Table 20.2 Role of various fungal enzymes in bioremediation

Enzymes	Name of fungi and applications	References
Laccase	*Fusarium incarnatum* UC-14 • Remediation of bisphenol A	Chhaya and Gupte (2013)
	Trametes versicolor • Bioremediation of agro waste	Pinheiro et al. (2020)
	Phanerochaete chrysosporium • Transformation of graphene	Yang et al. (2019)
	Ganoderma cupreum AG-1 • Decolorization of azo dye	Gahlout et al. (2013)
	Flavodon flavus • Decolorization of anthraquinone dye	Soares et al. (2001)
Lignin peroxidase	*Aspergillus flavus* • Degradation of tri- and tetrameric lignin-related compounds	Betts and Dart (1989)
	Ganoderma lucidum IBL-05 • Dye decolorization	Shaheen et al. (2017)
	Phanerochaete chrysosporium • Degradation of lignin	Vandana et al. (2019)
Manganese peroxidase	*Phanerochaete chrysosporium* • Transformation of fluorene	Bogan et al. (1996)
	Cerrena unicolor BBP6 • Dye decolorization and denim bleaching	Zhang et al. (2018)
	Lentinus arcularius • Decolorization and detoxification of textile dyes	Bayburt et al. (2020)
Tyrosinase	*Penicillium simplicissimum* • Biodegradation of triphenylmethane dyes	Chen et al. (2019)
	Amylomyces rousii • Degradation of pentachlorophenol	Montiel et al. (2004)
Heme-thiolate peroxigenases and monooxygenases	*Coprinopsis (Coprinus) cinerea* • Peroxigenation of heterocylic, aromatic, and aliphatic compounds	Hofrichter et al. (2010)
	Aspergillus niger SK 9317 • Pyrene degradation	Wunder et al. (1994)
Nitro-reductase	*Aspergillus niger* • Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine	Bhushan et al. (2002)
Reductive dehalogenase	*Gloeophyllum trabeum* • Removal of chlorinated compounds	Jensen et al. (2001)

been naturally extracted and used traditionally by ancient civilizations of Greece, India, and some countries from the Middle East, in the modern time, synthetic dyes have replaced the use of natural dyes. The textile industries utilize large amounts of water and chemical compounds for wet processing of textiles (Jebapriya and Gnanadoss 2013). The discharges of wastewater are the main cause of the harmful

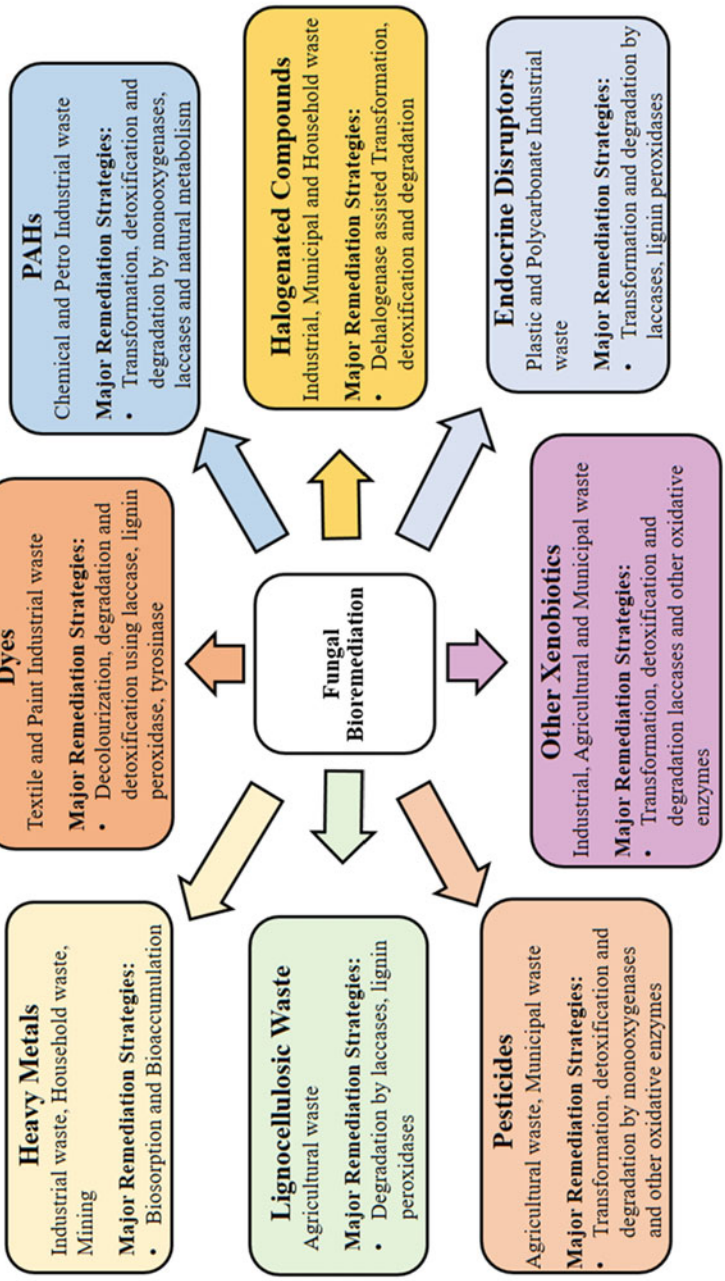

Fig. 20.2 Fungal bioremediation strategies for various types of pollutants

environmental impact of the textile industries. It is estimated that almost 10–15% of textile dyes are released into waterways as effluent which contain high chloride, sodium, sulfate, and hardness and organic carcinogenic compounds (Robinson et al. 2001).

In order to reduce the impact of synthetic dyes on environmental pollution, various methods are being employed which can be categorized as physical, chemical, electrochemical, and biological. Although the physicochemical treatment methods are effective and rapid, some drawbacks such as superfluous usage of chemicals, subsequent disposal problems, and high installation, energy, and operational costs have limited their implementation as one of the dye-removal strategies (Sarioglu et al. 2007). For the first time, it was reported that biological methods can be used to degrade dyes occurring in industrial effluents as microorganisms like fungi, bacteria, yeasts, and algae are able to accumulate in the effluents (Banat et al. 1996). Thus, biological processes have been gaining recognition because of their low cost and eco-friendly nature (Van Der Zee and Villaverde 2005).

Many fungal species from ascomycete as well as basidiomycete classes are reported for degradation and detoxification of synthetic dyes. The key feature for dye degradation is ligninolytic enzyme system which mainly includes laccase, lignin peroxidase, and manganese peroxidase enzymes. These enzymes are directly involved in degradation of dyes as dyes possess a phenolic ring which is structurally similar to lignin. Three major processes involved in decolorization or biodegradation of dyes mainly are (1) modification in additional organic molecules without changing the core structure of the dye molecules, (2) breakdown of the complex structural organic molecules in to simpler forms, and (3) complete mineralization of the complex molecules (Gulzar et al. 2020). The general mechanism for decolorization of the azo dyes involves cleavage of the azo bond (–N=N–) in two steps. In each step, two electrons are transferred to the azo dye, where the dye molecule acts as the final electron acceptor (Guo et al. 2010). Figure 20.3 illustrates the mechanism of azo dye (Acid orange 7) decolorization by means of azo reductase enzyme.

A number of white rot fungi have been employed for degradation and decolorization of a range of textile dyes. Fungal cultures, fermentation broths, and crude as well as purified enzymes produced by fungi were used to degrade various textile as well as polymeric dyes. Various fungal species such as *Trametes versicolor* (Erkurt et al. 2007), *Pleurotus ostreatus* (Faraco et al. 2009), *T. trogii*, and *T. villosa* (Levin et al. 2010) were identified to degrade and decolorize various synthetic dyes. The cultures of *Phanerochaete chrysosporium* played an important role in the decolorization of reactive dyes such as Reactive Black 15, Reactive Red C-4 BL, and Lemon Yellow C-4 GL dyes (Kiran et al. 2019). A novel fungus *Lasiodiplodia* sp. was recently reported to carry out the decolorization and degradation of the potent carcinogenic and mutagenic dye Malachite Green, through the laccase-mediated oxidation, biosorption, as well as mineralization processes (Arunprasath et al. 2019). The azo dyes Acid Blue 161 and Procion Red MX-5B were biologically absorbed by the cultures of *Aspergillus niger* CCT 1435, *Aspergillus terreus* CCT 2679, and *Rhizopus oligosporus* CCT 3762. Furthermore, their degradation using *Aspergillus terreus* CCT 2679 imparted intense molecular level changes in the

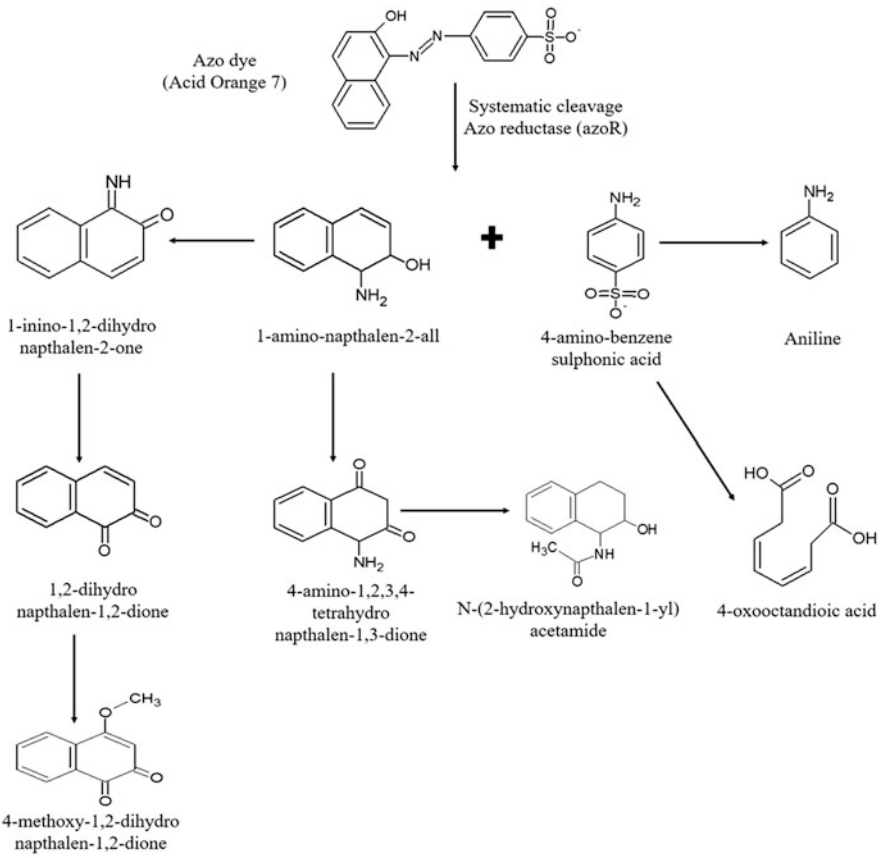

Fig. 20.3 Pathway for transformation and decolonization of Acid Orange 7 azo dye. (Adapted from Zhuang et al. 2020)

structures of these azo dyes (Almeida and Corso 2019). An antraquinone dye Remazol Brilliant Blue R was degraded by laccase producing an immobilized fungal strain, *Pleurotus ostreatus* URM 4809 (Simões et al. 2019).

20.5.2 Bioremediation of Poly-Aromatic Hydrocarbons (PAHs)

Polycyclic Aromatic Hydrocarbons (PAHs) are uncharged, nonpolar, as well as lipophilic organic compounds which are naturally found in petroleum crude, coal, and tar deposits. PAHs are also generated upon incineration of oils, tobacco, wood, gas, and coal. Apart from these, PAHs are also mass produced in industries by thermal decomposition and chemical reactions. PAH compounds may find their way into the environment through partial combustion of organic materials arising from natural phenomenon such as forest fires and volcanic eruptions, but its major source

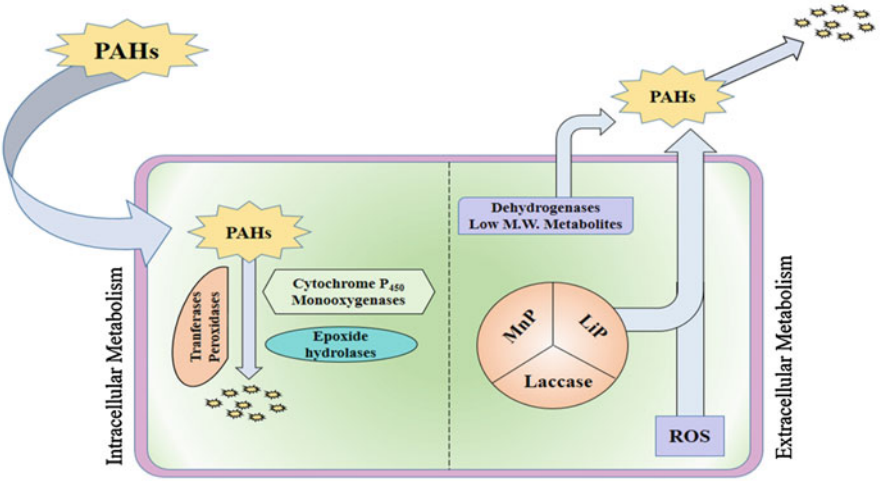

Fig. 20.4 Strategies adopted for metabolism of PAHs

is human activities such as industrial release of pollutants in air and water, transportation, gasification, and plastic ware incinerations. PAH compounds persist in the environment for longer period and may lead to health hazards (Fawell and Hunt 1988). PAH compounds may also get absorbed to soil sediments rich in organic matter, which sometime reaches aquatic ecosystem and later accumulates in aquatic organism followed by transfer to humans via consumption of seafood (Meador et al. 1995). Due to these concerns, it has become essential to find ways to remove PAHs from the environment; microbe-assisted bioremediation has shown great potential. Since mycelia of filamentous fungi can easily proliferate throughout the inner layers of the soil matrix, they can effectively degrade PAHs which are generally inaccessible to unicellular organisms (Cerniglia 1997). Both ligninolytic and non-ligninolytic fungi have capabilities to degrade PAHs, but they adopt different strategies for the same (Fig. 20.4). Ligninolytic fungi transform PAHs through the extracellular pathway with the help of ligninolytic enzyme system as well as intracellular metabolism, whereas non-ligninolytic fungi perform it via only intracellular metabolism and convert them into harmless simple organic compounds (Morelli et al. 2013).

Recent genomic analysis of PAHs degrading microbial species has revealed metabolic and genetic mechanism of detoxification and mineralization of PAH compounds. It was observed that several catabolic genes such as *nah*, *phn*, *nid*, etc. encode many PAH-degrading enzymes like mono-oxygenases, PAH ring-hydroxylating dioxygenase, catechol 1,2-dioxygenase, and lipases. These genes are reported to be actively involved in the transformation of PAHs like phenanthrene, napthalene, pyrene, anthracene, and fluorine (Haritash and Sakshi 2020). The biotransformation pathway of phenanthrene and naphthalene is depicted in Fig. 20.5a, b.

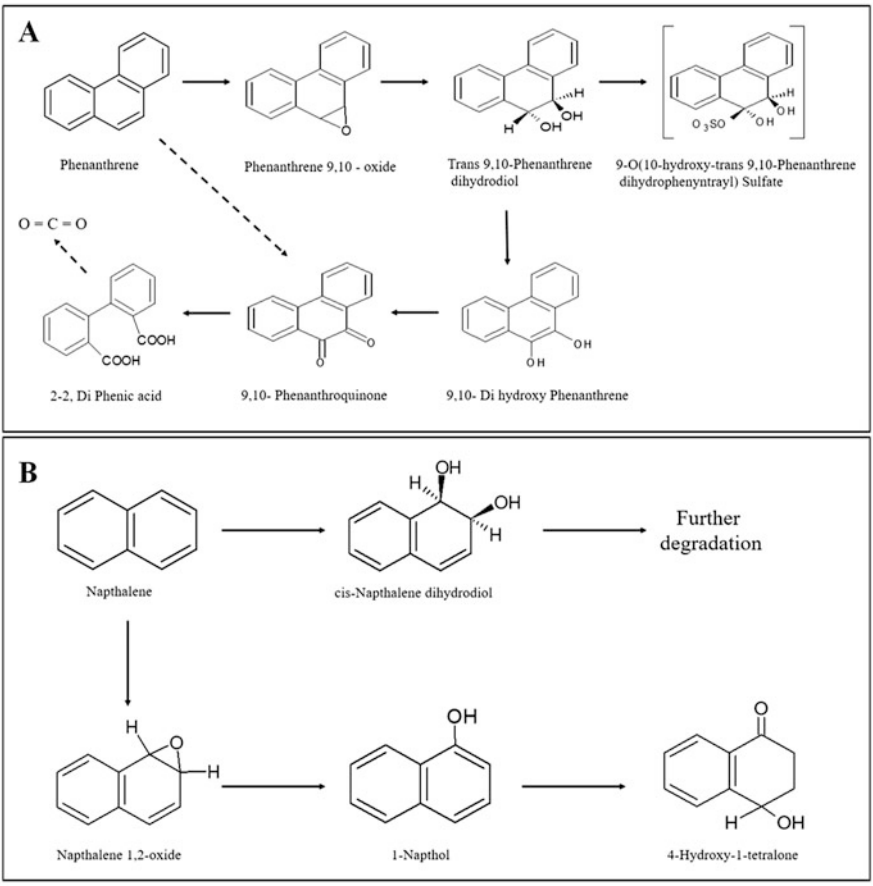

Fig. 20.5 Biotransformation pathway of phenanthrene (**a**) and napthalene (**b**). (Adapted from Ghosal et al. 2016)

The degradation of phenanthrene and naphthalene proceeds via incremental oxidation by PAH-degrading enzymes. After oxidation, the ring structure opens up, and further metabolism continues to convert them into simpler compounds (Ghosal et al. 2016). Recently, many fungi have been discovered for their excellent capabilities to transform and degrade PAHs. A novel fungal species belonging to *Agaricomycetes* had been reported to degrade a range of PAH compounds present in contaminated soils, such as phenanthrene, pyrene, fluoranthene, and fluorine; maximum of 78.53%, 85.05%, 20.5%, and 70.49% degradation of these compounds was achieved, respectively. It was also reported that after degradation, these compounds were converted into simpler forms (Pandey and Gupte 2018). A unique fungus *Dentipellis* sp. KUC8613 was isolated and reported to degrade about 90% of 100 ppm PAH within 10 days by means of non-ligninolytic PAH-transforming enzyme system including FAD-dependent monooxygenases, epoxide hydrolases,

dehydrogenases, dioxygenases, and glutathione transferases enzymes (Park et al. 2019). Recently, researchers have reported the use of halophilic fungi like *Aspergillus sydowii* and *A. destruens* for elimination of PAHs for the first time in history. Both fungi successfully removed about 90% of benzo-α-pyrene and phenanthrene in saline conditions. However, *Aspergillus sydowii* followed biodegradation mechanism, whereas *Aspergillus destruens* used bioadsorption strategy. Interestingly, both fungi were also successful in removing 100% of PAH present in saline wastewater of biorefinery (González-Abradelo et al. 2019). A filamentous soil fungus *Trichoderma* sp. F03 was reported to degrade 78% of pyrene with the aid of biosurfactant and converted into three various metabolites: acetic acid, 3-hydroxybenzoic acid, and benzoic acid (Al Farraj et al. 2020).

20.5.3 Bioremediation of Heavy Metals

Heavy metals are generally defined as metal elements with high density or atomic mass. Heavy metals are nonbiodegradable and are bioaccumulated in tissues and biomagnified in the food chain. Volcanic eruptions and melting of rocks can discharge heavy metals into the surrounding environment. Heavy metals are normally occurring elements that are found throughout the earth's crust. Heavy metal pollution occurs as a result of natural and anthropomorphic activities like mining, smelting, and industrial production. These sources have been reported by many researchers (Ahluwalia and Goyal 2007; Kalinowska et al. 2020; Zhang and Wang 2020). Heavy metals are regarded as significant environmental pollutants due to high density and high toxicity even at lower concentrations. According to US EPA, eight heavy metals, namely, lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni), are listed to be the most widespread heavy metals in the environment. Heavy metals and their chemical derivatives are toxic, teratogenic, carcinogenic, as well as mutagenic in nature (Rudakiya and Pawar 2013). Exposure to heavy metals has led to the occurrence of diseases like infertility, anemia, birth defects, metal poisoning, organ failures, Hunter-Russell syndrome, Wilson's disease, and Minamata disease (Bhatt and Flora 2009; Krishnamoorthy et al. 2019). Due to these life-threatening problems, many researchers are paying more attention toward remediation of heavy metals.

Elimination of such heavy metals is possible either through physicochemical methods or via biological means. Physicochemical methods include oxidation-reduction, lime coagulation, chemical precipitation, solvent precipitation, reverse osmosis, incineration, electrodialysis, etc. The application of such methods has several drawbacks such as high energy cost and low economic viability. Furthermore, it also leads to environmental pollution due to disposal of by-products and waste generated (Ahluwalia and Goyal 2007; Pandey et al. 2013). On the other hand, bioremediation approach has better economic as well as environment-friendly aspects. Biosorbents for the heavy metals include bacteria, fungi, algae, and agricultural and industrial wastes (Gadd 2010); fungi-assisted bioremediation has remained the most attractive option for the researchers (Lacina et al. 2003). Fungal system

offers some advantages over other biological agents like faster growth, higher biomass production, tolerance to environmental stress, as well as low specific nutrient requirements. Moreover, some accessories such as functional groups like amine, phosphate, carboxyl, and hydroxyl groups present in fungal cell walls help in metal chelation (Baldrian 2003). Additionally, it was also noted that fungal extracellular polymeric substances (EPS) assisted in reduction of heavy metals from environment via bioabsorption or bioadsorption (Pal and Paul 2008).

Several unique fungal genera such as *Tetracladium*, *Coprinellus*, *Coprinus*, *Pilidium*, *Dendroclathra*, *Rosellinia*, and *Hypomyces* have been reported for their excellent biosorption and bioleaching capabilities (Taleski et al. 2020). In one of the studies, out of three isolated metallotolerant fungal strains—*Aspergillus niger* M1DGR, *Aspergillus fumigatus* M3Ai, and *Penicillium rubens* M2Aii—*A. niger* was reported to have maximum bioaccumulation efficiency of 98% and 43% for Cd and Cr, respectively. The other fungus, *A. fumigatus*, efficiently leached out about 79% of Cd and 69% of Cr (Khan et al. 2019). An entomopathogenic fungus *Beauveria bassiana* was employed for multiple heavy metal [Zn^{2+}, Cu^{2+}, Cd^{2+}, Cr^{6+} and Ni^{2+}] removal from contaminated water. It was observed that fungi displayed higher capabilities to remove multiple-metal (84%) as compared to individual-metal (61–75%) removal (Gola et al. 2016). Recently, the multi-metal-tolerant earthworm gut fungus *Trichoderma brevicompactum* QYCD-6 was reported to eliminate metals individually as well as in combination. This fungus had tolerance toward heavy metals [Cu^{2+}, Cr^{6+}, Cd^{2+}, and Zn^{2+}] at 150–200 ppm on composite medium, and surprisingly for Pb^{2+}, it was highest with 1600 ppm on potato dextrose (PD) medium. Noticeably, it had bioaccumulation as high as 80% and effectively removed up to 97.5% Pb^{2+} with maximum biomass generation of 6.13 g/L in PD medium (Zhang et al. 2020). Similarly, mineralization and biological elimination of Pb^{2+} (200 ppm) were achieved using mycelia of *Phanerochaete chrysosporium* from polluted water (Zhao et al. 2020). Mycofiltration is also a similar process which uses fungal mycelia to filter toxic waste and microorganisms from water in soil through stimulation of microbial and enzymatic activity. Fungi such as *A. niger*, *Aureobasidium pullulans*, *Ganoderma lucidum*, *Penicillium* sp., *Trametes versicolor*, and *Rhizopus* are efficient and capable of recovering heavy metals from polluted sites (Ahluwalia and Goyal 2007).

20.5.4 Bioremediation of Pesticides

Pesticides are organic chemical compounds or mixture of the same which are utilized for preventing, controlling, or destroying pest as well as weed. Depending upon the target organism, pesticides can be classified as herbicides (plants), insecticides (insects), fungicides (fungi), bactericides (bacteria), algicides (algae), avicides (birds), molluscicides (snails), rodenticides (rodents), nematicides (nematodes), as well as miticides (mites) (Gilden et al. 2010). Chemically, most of the pesticides are organophosphates, organochlorines, and carbamates (Kamrin 1997). Evidently, humankind has been utilizing pesticides for thousands of years. Till the discovery

of organochlorine pesticide dichlorodiphenyltrichloroethane (DDT) in 1948 by Paul Müller (Dash et al. 2007), arsenic-based pesticides have been used predominantly. By 2019, global consumption of pesticides reached up to approximately 2 million tons annually, and it is also expected to rise up to 3.5 million tons (Sharma et al. 2019). In the decades of the 1960s and 1970s, India had used pesticides in huge quantities as a part of "Green Revolution." It helped in increasing agricultural output as well as improving quality of the crops; however, at that time, the environmental impact of the pesticides was unknown. Later on, it was revealed that pesticides are not degraded easily and keep accumulating in soil which also disturbed terrestrial ecosystem. It even seeps into water bodies, thus affecting the aquatic ecosystem too (Sharma and Singhvi 2017). In the late 1960s, for the first time, it was investigated that DDT was found to be hampering reproducibility of many fish-eating birds, which was a serious threat to biodiversity (Fry 1995). It is now very well-known that pesticides cause many kinds of cancer, chronic and acute damage to the nervous system, ailments of the respiratory system, damage to organs of the reproductive system, birth defects, and disruption of the immune and endocrine systems (Thuy 2015).

The most common pesticides which are known to pollute the environment are chlorpyrifos, profenofos, thiophanate-methyl, Turbacil, Benlate, Dalapon, Paraquat, mancozeb, tordon, Dacthal, Malathion, Sevin, methoxychlor, methomyl, diazonine, Treflan, Zineb, dieldrin, Captan, PMAS, Calo, chlordane, DDT, aldrin, simazine, Cd compounds, etc. (Vargas 1975; Nyakundi et al. 2011; Rudakiya et al. 2020). Some of the aforementioned pest-controlling compounds are degradable, and the others are nondegradable. Many bacterial and fungal species have been exploited for their pesticide degradation abilities; major focus has always been on fungi-assisted remediation. Fungi have superior pesticide degradation capabilities due to the presence of diverse metabolic and enzymatic systems. Fungi commonly use co-metabolism as a mode of pesticide degradation, and for that they employ a variety of chemical reactions. The most commonly reported biochemical reactions by which fungi co-metabolize the pesticides are oxidation, reduction, alkylation, ether cleavage, amide or ester hydrolysis, dealkylation, dehydrogenation, dehalogenation, hydroxylation, ring cleavage, conjugate formation, and condensation (Bollag 1974). *Aspergillus niger* YAT-mediated degradation of cypermethrin is illustrated in Fig. 20.6. Here, aliphatic chain of cypermethrin is initially separated by esterases, and further cleavage of aromatic ring structure is performed with the help of dehydrogenases, hydrolases, and dioxygenases which yield catechol and simple chain of olefin acid which are further mineralized by means of fungal metabolism (Huang et al. 2018).

Laccase from *Tricholoma giganteum* AGDR-1 was capable of removing 29% of chlorpyrifos, 7% of profenofos, and 72% of thiophanate-methyl within 15 h. Furthermore, through molecular-docking studies, it was revealed that these pesticides had higher binding efficacy with amino acids H83, H320, A95, V384, and P366 which were adjacent to catalytic site of laccase (Rudakiya et al. 2020). In another investigation, three white rot fungi: *Coriolus versicolor*, *Hypholoma fasciculare*, and *Stereum hirsutum* were reported to degrade various pesticides. The cultures

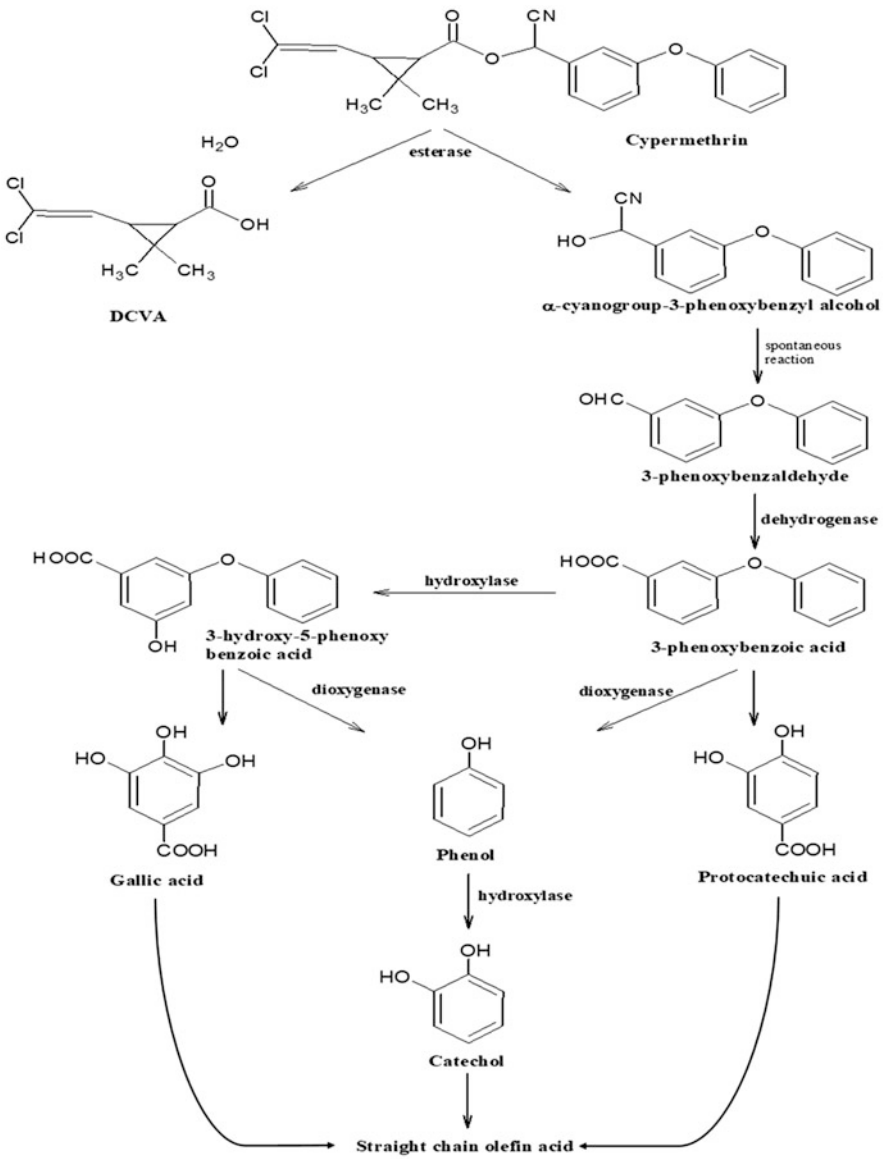

Fig. 20.6 Biodegradation of cypermethrin by *Aspergillus niger* YAT. (Adapted from Huang et al. 2018)

degraded more than 86% of diuron, atrazine, and terbuthylazine pesticides after 42 days (Bending et al. 2002).

In another study, five white rot fungal isolates labeled as WR1, WR2, WR4, WR9, and WR15 were tested for their capabilities to degrade methomyl and

diazinon pesticides individually as well as by co-culturing. It was observed that a mixture of fungal cultures was able to degrade diazinon pesticide faster (within 50 days) than that of individual ones (about 60–100 days), whereas methomyl was degraded in 22–25 days (Nyakundi et al. 2011). *Aspergillus niger*, *Ganoderma austral*, *Trichosporon* spp., and *Verticillium dahliae* were examined for degradation of various pesticides. Among the four fungi, *Aspergillus niger* successfully degraded 59% of endosulfan and 29% of lindane. The culture of *Ganoderma austral* was effective in degrading 61% of lindane, whereas it was not found to be much effective for degradation of chlorpyrifos (16%). On the other hand, *Trichosporon* sp. has shown highest degradation capabilities for chlorpyrifos (55%) and much lower for endosulfan (10%) and lindane (7%). About 64% of chlorpyrifos, 8% of endosulfan, and 10% of both Malathion and lindane were successfully degraded by *Verticillium dahliae* (Hussaini et al. 2013). Recently, researchers were able to isolate 20 fungi which were highly tolerant to endosulfan and chlorpyrifos. Out of the 20 fungi, *P. chrysosporium* was reported to be the most tolerant toward endosulfan. Similarly, *P. decumbens* was more tolerant to chlorpyrifos. In the same study, other fungal species such as *I. lacteus*, *S. commune*, *Trametes hirsuta*, *C. cladosporioides*, *P. decumbens*, *P. frequentans*, *P. fimeti*, *T. harzianum*, *T. virens*, and *T. viride* have also been reported to show good tolerance toward endosulfan and chlorpyrifos. These ten fungi were also tested for degradation of both α- and β-endosulfan. Among these, *T. hirsute* displayed 100% degradation of both α- and β-endosulfan after 15 days followed by *C. cladosporioides* which showed 96.87% degradation (Bisht et al. 2019).

20.5.5 Bioremediation of Miscellaneous Pollutants

Aforementioned pollutants have the highest impact on the environment, but many others such as halogenated compounds, ligninogenic waste, endocrine-disrupting chemicals, as well as dioxins have also been a matter of serious concern. Despite being highly hazardous, these miscellaneous pollutants are often ignored by the researchers. Thus, it has now become mandatory to remediate such pollutants.

Abundant quantity of lignocellulosic waste is present on earth, and most of it is directly disposed that persists in the environment for a longer time. When such waste is being treated in order to take out cellulosic and hemicellulosic components, remaining lignin and its derivatives are directly discarded which in turn pollute the environment. Lignin being highly complex in nature, it is very much difficult to be degraded naturally, and chemical treatments results in generation of highly toxic compounds. Thus, researchers have come up with biological strategies to remediate lignin. Biological system for remediation of lignin generally comprises of lignin-degrading enzymes especially laccases, lignin peroxidases, and manganese peroxidases. Using solid-state fermentation technology, *Tricholoma giganteum* AGHP that produces laccase was shown to successfully degrade wheat straw (Patel and Gupte 2016). Similarly, a mutant of *Fusarium incarnatum* LD-3 strain was also able to degrade lignin containing substrates like rice bran and wheat straw

(Chhaya and Gupte 2019). Interestingly, 31% delignification of *Eucalyptus globulus* wood was achieved through the secretome of *Marasmiellus palmivorus* in the absence of any laccase mediators. On the contrary, laccase containing secretome was able to detoxify 70% of eucalyptus biomass (Schneider et al. 2020).

Dioxins and furans are toxic by-products of pesticides, paper and pulp, as well as textile industries. Additionally, such compounds are also found as impurities of chlorophenols like trichlorophenol, tetrachlorophenol, and pentachlorophenol (PCP) (Anasonye et al. 2014). Dioxins are known to cause damage to the reproductive system, immune system, as well as endocrine system (White and Birnbaum 2009). Several fungi are reported to have adequate potential to remediate dioxins and their derivatives. Among eight fungal strains, *Trichosporon mucoides* was shown to convert six major metabolites by transforming dibenzofuran (Hammer et al. 1998). In one study, it was reported that *Phanerochaete chrysosporium* was able to metabolize mono- and dichloro-dibenzo-*p*-dioxins by virtue of its cytochromes P_{450} enzyme system (Kasai et al. 2010). Recently, an isolate FMD21, a *Rigidoporus* sp., was identified to degrade 73% of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin after 28 days (Dao et al. 2019). In one of the most recent studies, *Pleurotus pulmonarius* had been used for enhanced bioremediation of highly PCDD/F-contaminated field by solid-state fermentation, where 96% removal was recorded in 72 days (Kaewlaoyoong et al. 2020).

Halogenated compounds are another class of organic compounds which are very notorious pollutants for their resistance toward natural degradation. Chemically, they have a high electronegativity which not only gives them chemical stability but also makes them recalcitrant. As a result, they get accumulated in the environment and may enter the food chain as well. Additionally, what makes them more problematic is their capability to bestow or elevate toxicity as well as mutagenicity of other organic compounds (Mohn 2004). Polychlorinated biphenyls (PCBs) are one of the most common halo-organic pollutants. Laccase-mediated degradation of 7100 ppm PCBs was achieved by using *Pleurotus ostreatus* (Gayosso-Canales et al. 2012). Recently, two fungal species, *Penicillium simplicissimum* and *Trichoderma harzianum*, were studied to record their responses toward hexachlorocyclohexane (HCH) and to evaluate their potential for biotransformation of HCH-contaminated sites (Russo et al. 2019). In one of the recent studies, researchers isolated 27 fungi from marine invertebrates and studied their biodegradation potential for 2,4-dichlorophenol (2,4-DCP). Out of these 27, 4 fungal strains, namely, *Cladosporium* sp. ML6-S1, *Aspergillus* sp. ML147-S2, *Penicillium chrysogenum* ML156-S8, and *Tritirachium* sp. ML197-S3, achieved 64.0%, 55.1%, 59.5%, and 66.3% bioconversion, respectively (Nikolaivits et al. 2020).

Endocrine-disrupting chemicals (EDCs) are a recently identified class of pollutants which specifically affect functioning of hormones. Endocrine disrupters are organic compounds which are analogues of hormones and thus can bind with hormone receptors and modify normal functioning of the endocrine system. Xenoestrogens, bisphenol S (BPS), bisphenol A (BPA), alkylphenols, and phthalates are some examples of EDCs. These chemicals are common ingredients and by-products of plastic, epoxy resin, and polycarbonate industries. Plastic products

are an unavoidable necessity of modern human life. Their application varies from household products such as toys, water bottles, to industrial products like packaging materials, and such plastic products are a main source of endocrine disruptive compounds. This puts millions of human lives at risk of their potential exposure to EDC. In order to remediate EDC present in the environment, researchers have come up with novel bioremediation strategies. Mycoremediation has been proven the most promising technology for EDC remediation. Fungi can transform or degrade EDC by either metabolizing them or with assistance of enzymes like laccases and Mn peroxidases. Laccase from *Fusarium incarnatum* UC-14 showed excellent oxidative degradation of bisphenol A. Furthermore, efficiency of the process was enhanced by using reverse micelle technology (Chhaya and Gupte 2013). The abilities of *Trametes versicolor*, *Pleurotus ostreatus*, and *Phanerochaete chrysosporium* for degradation of five different EDCs [bisphenol A (BPA), nonylphenol (NP), methylparaben (MTPRB), butylparaben (BTPRB), and dimethylphthalate (DMPTL)] were assessed. The mixture of all five EDCs was efficiently removed by *T. versicolor* which had the ability to eliminate all five EDCs (Pezzella et al. 2017). The removal of 17α-ethinylestradiol (EE2) using cultures of *Lentinula edodes* enhanced by laccase enzymes was reported for the first time. This fungal culture was able to break down 57% of EE2 within a week's time (Eldridge et al. 2017). Researchers have also reported the use of laccase as an immobilized biocatalyst from *Coriolopsis polyzona* for elimination of nonylphenol (NP), bisphenol A (BPA), and triclosan (TCS) just within 200 min. The amount of laccase used was 1.88 units for NP and 3.75 units for BPA and TCS, and the capability of this immobilized laccase system to eliminate all three EDCs was maintained for five consecutive treatment cycles (Cabana et al. 2009).

20.6 Fungal Genomics and Proteomics in Bioremediation

Fungi have the ability to transform and metabolize hazardous pollutants which persist in the environment for a longer period of time. They have been utilized for remediation in diverse environmental conditions, but the advantage of their true potential has not been achieved due to the lack of genomic and proteomic data which can provide information on biochemical activities as well as metabolic pathways (Deshmukh et al. 2016).

The genomic study of fungi involves rDNA technology, DNA sequencing methods, and use of bioinformatics tools to sequence, assemble, and analyze the function and structure of genomes. One of the most common techniques to improve bioremediation capabilities is the use of genetically modified organisms (GMOs) for achieving improvements in the rate of degradation of pollutants and their transformation into less toxic to nontoxic products at laboratory level (Sayler and Ripp 2000; Kumar et al. 2020). rRNA sequencing, next-generation sequencing (NGS), and DNA microarrays have been proven to be beneficial in identifying genes involved in remediation of particular compounds and helped in predicting biochemical pathways. Stable-isotope probing (SIP) is a relatively new technology in which

labeled isotopes are used to isolate fractions of genomic DNA specifically involved in remediation (Radajewski et al. 2000). Haldar and Nazareth (2018) were able to identify fungal phyla having the most potential for remediation by using metagenomics approach by means of paired-end Illumina sequencing technique in samples from a polluted mangrove sediment site in Zuari, Goa.

Proteomics can be simply defined as a large-scale study of structure and function of cellular proteins. Commonly used techniques for proteomics are gel electrophoresis, mass spectroscopy, CD spectroscopy, etc. Proteomics studies help in identifying phenotypes of microorganism that may not be observed in the genome sequence (Fulekar and Sharma 2008; Kumavath and Deverapalli 2013). Proteomics have been employed in bioremediation, and membrane proteins are considered to be of great interest. By employing proteomics approach, the physicochemical changes occurring in microorganism during remediation can provide further information regarding bioremediation-rerated genes and their regulation (Hivrale et al. 2015). The role of ABC transporter, peroxidases, and stress-responsive genes is well-known for remediation, especially in case of dyes. Furthermore, engineering and manipulating fungal enzymes can help increase their activity and achieve bioremediation (Park et al. 2019).

20.7 Conclusions and Future Prospects

Bioremediation is an emerging technology which can be used simultaneously with other physical and chemical treatment methods for the complete and effective management of diverse groups of environmental pollutants. Though there have been reports of the use of fungi for bioremediation purposes, in-depth assessment on the multifaceted role of fungi to degrade various recalcitrant, persistent, and toxic pollutants like PAH, pesticide, EDCs, dioxins, etc. is lacking. In this review, we have tried to collate various aspects describing diverse and novel metabolic capacities of fungi and their role in bioremediation potential on a common platform. Most of the studies have shown the role of various extracellular enzymes like laccases, peroxidases, oxygenases along with some stress response proteins like ABC transporter play active role in fungi to degrade many toxic pollutants, and there is a need of exploring the genes.

Genomics and proteomics studies could be an area that needs to be explored to understand the mechanism of mycoremediation. Recent studies in these areas will provide a proper insight toward the genes and proteins involved. The whole genome studies can help us understand and explore the biodegradative pathways. The information observed can further be used to genetically modify the fungi of different groups to facilitate the pollutant removal from the environment. The study on indigenous fungi growing in polluted sites should be continued as they are adapted to harsh environmental conditions. In addition, efficient biomarkers for bioremediation can emerge from gene expression studies in fungi which can aid in bioremediation studies employing fungal systems. Thus, there are enough evidences that

support safe utilization of mycoremediation for the remediation of environmental pollutants and support green technology.

References

Abhilash PC, Singh N (2009) Pesticide use and application: an Indian scenario. J Hazard Mater 165 (1–3):1–12

Agnihotri V (2020) Advancement in bioremediation of pharmaceutical and personal care products. In: Waste management: concepts, methodologies, tools, and applications. IGI Global, Hershey, PA, pp 1150–1168

Ahluwalia SS, Goyal D (2007) Microbial and plant derived biomass for removal of heavy metals from wastewater. Bioresour Technol 98(12):2243–2257

Al Farraj DA, Hadibarata T et al (2020) Biotransformation and degradation pathway of pyrene by filamentous soil fungus *Trichoderma* sp. F03. Water Air Soil Pollut 231:1–9

Almeida EJR, Corso CR (2019) Decolorization and removal of toxicity of textile azo dyes using fungal biomass pelletized. Int J Environ Sci Technol 16(3):1319–1328

Anasonye F, Winquist E et al (2014) Fungal enzyme production and biodegradation of polychlorinated dibenzo-p-dioxins and dibenzofurans in contaminated sawmill soil. Chemosphere 110:85–90

April TM, Abbott SP et al (1998) Degradation of hydrocarbons in crude oil by the ascomycete *Pseudallescheria boydii* (Microascaceae). Can J Microbiol 44(3):270–278

Arunprasath T, Sudalai S et al (2019) Biodegradation of triphenylmethane dye malachite green by a newly isolated fungus strain. Biocatal Agric Biotechnol 17:672–679

Azubuike CC, Chikere CB, Okpokwasili GC (2016) Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. World J Microbiol Biotechnol 32(11):180

Baldrian P (2003) Interactions of heavy metals with white-rot fungi. Enzym Microb Technol 32 (1):78–91

Banat IM, Nigam P et al (1996) Microbial decolorization of textile-dyecontaining effluents: a review. Bioresour Technol 58(3):217–227

Bayburt C, Karaduman AB et al (2020) Decolourization and detoxification of textile dyes by Lentinus arcularius in immersion bioreactor scale. Int J Environ Sci Technol 17(2):945–958

Bending GD, Friloux M, Walker A (2002) Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. FEMS Microbiol Lett 212(1):59–63

Betts WB, Dart RK (1989) Initial reactions in degradation of tri- and tetrameric lignin-related compounds by *Aspergillus flavus*. Mycol Res 92(2):177–181

Bhatt K, Flora SJS (2009) Oral co-administration of α-lipoic acid, quercetin and captopril prevents gallium arsenide toxicity in rats. Environ Toxicol Pharmacol 28(1):140–146

Bhushan B, Halasz A et al (2002) Biotransformation of hexahydro-1,3,5-trinitro-1,3,5-triazine catalyzed by a NAD(P)H: nitrate oxidoreductase from *Aspergillus niger*. Environ Sci Technol 36(14):3104–3108

Bisht J, Harsh NSK et al (2019) Biodegradation of chlorinated organic pesticides endosulfan and chlorpyrifos in soil extract broth using fungi. Remediat J 29(3):63–77

Bogan BW, Schoenike B et al (1996) Manganese peroxidase mRNA and enzyme activity levels during bioremediation of polycyclic aromatic hydrocarbon-contaminated soil with *Phanerochaete chrysosporium*. Appl Environ Microbiol 62(7):2381–2386

Bollag JM (1974) Microbial transformation of pesticides. In: Advances in applied microbiology, vol 18. Academic, New York, pp 75–130

Cabana H, Alexandre C et al (2009) Immobilization of laccase from the white rot fungus Coriolopsis polyzona and use of the immobilized biocatalyst for the continuous elimination of endocrine disrupting chemicals. Bioresour Technol 100(14):3447–3458

Cerniglia CE (1997) Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. J Ind Microbiol Biotechnol 19:324–333
Chaurasia PK, Yadav RSS, Yadava S (2013) A review on mechanism of laccase action. Res Rev Biosci 7(2):66–71
Chen SH, Cheow YL et al (2019) Mechanisms for metal removal established via electron microscopy and spectroscopy: a case study on metal tolerant fungi *Penicillium simplicissimum*. J Hazard Mater 362:394–402
Chhaya U, Gupte A (2013) Possible role of laccase from *Fusarium incarnatum* UC-14 in bioremediation of bisphenol A using reverse micelles system. J Hazard Mater 254:149–156
Chhaya U, Gupte A (2019) Studies on a better laccase-producing mutant of *Fusarium incarnatum* LD-3 under solid substrate tray fermentation. 3 Biotech 9(3):100
Dao AT, Vonck J et al (2019) Screening white-rot fungi for bioremediation potential of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Ind Crop Prod 128:153–161
Dash AP, Raghavendra K, Pillai MKK (2007) Resurrection of DDT: a critical appraisal. Indian J Med Res 126(1):1–4
Deshmukh R, Khardenavis AA, Purohit HJ (2016) Diverse metabolic capacities of fungi for bioremediation. Indian J Microbiol 56(3):247–264
Dhiman N, Jasrotia T et al (2020) Immobilization interaction between xenobiotic and Bjerkandera adusta for the biodegradation of atrazine. Chemosphere 257:127060
Eldridge HC, Milliken A et al (2017) Efficient remediation of 17α-ethinylestradiol by *Lentinula edodes* (shiitake) laccase. Biocatal Agric Biotechnol 10:64–68
Erkurt EA, Ünyayar A, Kumbur H (2007) Decolorization of synthetic dyes by white rot fungi, involving laccase enzyme in the process. Process Biochem 42(10):1429–1435
Falade AO, Nwodo UU et al (2017) Lignin peroxidase functionalities and prospective applications. Microbiol Open 6(1):1–14
Faraco V, Pezzella C et al (2009) Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes. Biodegradation 20(2):209–220
Fawell JK, Hunt S (1988) The polycyclic aromatic hydrocarbons. Environmental toxicology: organic pollutants. Ellis Horwood, West Susex, pp 241–269
Fry DM (1995) Reproductive effects in birds exposed to pesticides and industrial chemicals. Environ Health Perspect 103(7):165–171
Fulekar MH, Sharma J (2008) Bioinformatics applied in bioremediation. Innov Romanian Food Biotechnol 3:28–36
Gadd GM (2010) Metals, minerals and microbes: geomicrobiology and bioremediation. Microbiology 156(3):609–643
Gahlout M, Gupte S, Gupte A (2013) Optimization of culture condition for enhanced decolorization and degradation of azo dye reactive violet 1 with concomitant production of ligninolytic enzymes by *Ganoderma cupreum* AG-1. 3 Biotech 3(2):143–152
Gayosso-Canales M, Rodríguez-Vázquez R et al (2012) PCBs stimulate laccase production and activity in Pleurotus ostreatus thus promoting their removal. Folia Microbiol 57(2):149–158
Ghosal D, Ghosh S et al (2016) Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): a review. Front Microbiol 7:1–27
Gilden RC, Huffling K, Sattler B (2010) Pesticides and health risks. J Obstet Gynecol Neonatal Nurs 39(1):103–110
Gola D, Dey P et al (2016) Multiple heavy metal removal using an entomopathogenic fungi *Beauveria bassiana*. Bioresour Technol 218:388–396
González-Abradelo D, Pérez-Llano Y et al (2019) First demonstration that ascomycetous halophilic fungi (*Aspergillus sydowii* and *Aspergillus destruens*) are useful in xenobiotic mycoremediation under high salinity conditions. Bioresour Technol 279:287–296
Gulzar ABM, Vandana UK et al (2020) The role of mushrooms in biodegradation and decolorization of dyes. In: An introduction to mushroom. IntechOpen, Rijeka

Guo J, Kang L et al (2010) Decolorization and degradation of azo dyes by redox mediator system with bacteria. In: Biodegradation of azo dyes. Springer, Berlin, pp 85–100
Gupta S, Wali A et al (2017) Fungi: an effective tool for bioremediation. In: Plant-microbe interactions in agro-ecological perspectives. Springer, Singapore, pp 593–606
Haldar S, Nazareth SW (2018) Taxonomic diversity of bacteria from mangrove sediments of Goa: metagenomic and functional analysis. 3 Biotech 8(10):436
Hammer E, Krowas D et al (1998) Isolation and characterization of a dibenzofuran-degrading yeast: identification of oxidation and ring cleavage products. Appl Environ Microbiol 64 (6):2215–2219
Haritash AK, Sakshi (2020) A comprehensive review of metabolic and genomic aspects of PAH-degradation. Arch Microbiol 202(8):2033–2058
Harms H, Schlosser D, Wick LY (2011) Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. Nat Rev Microbiol 9(3):177–192
Hibbett DS, Binder M et al (2007) A higher-level phylogenetic classification of the Fungi. Mycol Res 111(5):509–547
Hildén K, Mäkelä MR et al (2013) *Agaricus bisporus* and related *Agaricus* species on lignocellulose: production of manganese peroxidase and multicopper oxidases. Fungal Genet Biol 55:32–41
Hivrale UA et al (2015) Application of genomics and proteomics in bioremediation. In: Toxicity and waste management using bioremediation. IGI Global, Hershey, USA, pp 97–112
Hofrichter M, Ullrich R et al (2010) New and classic families of secreted fungal heme peroxidases. Appl Microbiol Biotechnol 87(3):871–897
Huang Y, Xiao L et al (2018) Microbial degradation of pesticide residues and an emphasis on the degradation of cypermethrin and 3-phenoxy benzoic acid: a review. Molecules 23(9):2313
Hussaini SZ, Shaker M, Iqbal MA (2013) Isolation of fungal isolates for degradation of selected pesticides. Bull Environ Pharmacol Life Sci 2(4):50–53
Jebapriya GR, Gnanadoss JJ (2013) Bioremediation of textile dye using white rot fungi: a review. Int J Curr Res Rev 5(3):1–13
Jensen KA, Houtman CJ et al (2001) Pathways for extracellular Fenton chemistry in the brown rot basidiomycete Gloeophyllum trabeum. Appl Environ Microbiol 67(6):2705–2711
Joshi M, Patel H et al (2013) Nutrient improvement for simultaneous production of exopolysaccharide and mycelial biomass by submerged cultivation of *Schizophyllum commune* AGMJ-1 using statistical optimization. 3 Biotech 3(4):307–318
Kaewlaoyoong A, Cheng CY et al (2020) White rot fungus Pleurotus pulmonarius enhanced bioremediation of highly PCDD/F-contaminated field soil via solid state fermentation. Sci Total Environ 738:139670
Kalinowska A, Szopińska M et al (2020) Heavy metals in a high arctic fiord and their introduction with the wastewater: A case study of Adventfjorden-Longyearbyen System, Svalbard. Water 12 (3):794
Kamrin MA (1997) Pesticide profiles: toxicity, environmental impact, and fate. CRC press, Boca Raton, FL
Karigar CS, Rao SS (2011) Role of microbial enzymes in the bioremediation of pollutants: a review. Enzyme Res 2011:805187
Kasai N, Ikushiro SI et al (2010) Metabolism of mono- and dichloro-dibenzo-p-dioxins by *Phanerochaete chrysosporium* cytochromes P450. Appl Microbiol Biotechnol 86(2):773–780
Khan I, Aftab M et al (2019) Mycoremediation of heavy metal (Cd and Cr)-polluted soil through indigenous metallotolerant fungal isolates. Environ Monit Assess 191(9):585–595
Kiran S, Huma T et al (2019) Lignin degrading system of *Phanerochaete chrysosporium* and its exploitation for degradation of synthetic dyes wastewater. Pol J Environ Stud 28(3):1749–1757
Krishnamoorthy R, Venkatramanan V et al (2019) Management of heavy metal polluted soils: perspective of arbuscular mycorrhizal fungi. In: Sustainable green technologies for environmental management. Springer, Singapore, pp 67–85

Kumar A, Bisht BS et al (2011) Review on bioremediation of polluted environment: a management tool. Int J Environ Sci 1(6):1079–1093
Kumar RS, Singh D et al (2020) Biodegradation of environmental pollutant through pathways engineering and genetically modified organisms approaches. In: Microorganisms for sustainable environment and health. Elsevier, Amsterdam, pp 137–165
Kumavath RN, Deverapalli P (2013) Scientific swift in bioremediation: an overview. Int J Pharm Pharm Sci 4:375–388
Lacina C, Germain G, Spiros AN (2003) Utilization of fungi for biotreatment of raw wastewaters. Afr J Biotechnol 2(12):620–630
Levin L, Melignani E, Ramos AM (2010) Effect of nitrogen sources and vitamins on ligninolytic enzyme production by some white-rot fungi. Dye decolorization by selected culture filtrates. Bioresour Technol 101(12):4554–4563
Marco E, Font X et al (2013) Co-composting as a management strategy to reuse the white-rot fungus *Trametes versicolor* after its use in a biotechnological process. Int J Environ Waste Manag 11(1):100–108
Meador JP, Stein JE et al (1995) Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. In: Reviews of environmental contamination and toxicology. Springer, New York, NY, pp 79–165
Megharaj M, Ramakrishnan B et al (2011) Bioremediation approaches for organic pollutants: a critical perspective. Environ Int 37(8):1362–1375
Mohn WW (2004) Biodegradation and bioremediation of halogenated organic compounds. In: Biodegradation and bioremediation. Springer, Berlin, pp 125–148
Montiel AM, Fernández FJ et al (2004) A fungal phenoloxidase (tyrosinase) involved in pentachlorophenol degradation. Biotechnol Lett 26(17):1353–1357
Morelli IS, Saparrat MCN et al (2013) Bioremediation of PAH-contaminated soil by fungi. In: Fungi as bioremediators. Springer, Berlin, pp 159–179
Mouhamadou B, Faure M et al (2013) Potential of autochthonous fungal strains isolated from contaminated soils for degradation of polychlorinated biphenyls. Fungal Biol 117(4):268–274
Mtibaà R, Ezzanad A et al (2020) Biodegradation and toxicity reduction of nonylphenol, 4-tert-octylphenol and 2,4-dichlorophenol by the ascomycetous fungus *Thielavia* sp HJ22: identification of fungal metabolites and proposal of a putative pathway. Sci Total Environ 708:135129
Niehaus F, Bertoldo C et al (1999) Extremophiles as a source of novel enzymes for industrial application. Appl Microbiol Biotechnol 51(6):711–729
Nikolaivits E, Agrafiotis A et al (2020) Degradation mechanism of 2,4-dichlorophenol by fungi isolated from marine invertebrates. Int J Mol Sci 21(9):3317
Nyakundi WO, Magoma G et al (2011) Biodegradation of diazinon and methomyl pesticides by white rot fungi from selected horticultural farms in rift valley and Central Kenya. In: Scientific conference proceedings, pp 639–654
Pal A, Paul AK (2008) Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. Indian J Microbiol 48(1):49–64
Pandey A, Gupte A (2018) Polycyclic aromatic hydrocarbons (PAHs) metabolism by white rot fungi Agaricomycetes sp AGAT and its microcosm study. Int J Environ Bioremed Biodegrad 6 (2):36–56
Pandey AK, Jamalludin A, Pandey AK (2013) Biosorption characteristics of Aspergillus flavusin removal of nickel from an aqueous solution. Int J Green Herb Chem 2:47–51
Park H, Min B et al (2019) Comprehensive genomic and transcriptomic analysis of polycyclic aromatic hydrocarbon degradation by a mycoremediation fungus, Dentipellis sp. KUC8613. Appl Microbiol Biotechnol 103(19):8145–8155
Patel H, Gupte A (2016) Optimization of different culture conditions for enhanced laccase production and its purification from *Tricholoma giganteum* AGHP. Bioresour Bioprocess 3(11):1–10
Peeples TL (2014) Bioremediation using extremophiles. In: Microbial biodegradation and bioremediation. Elsevier, Amsterdam, pp 251–268

Pezzella C, Macellaro G et al (2017) Exploitation of *Trametes versicolor* for bioremediation of endocrine disrupting chemicals in bioreactors. PLoS One 12(6):1–12
Pinheiro VE, Michelin M et al (2020) *Trametes versicolor* laccase production using agricultural wastes: a comparative study in Erlenmeyer flasks, bioreactor and tray. Bioprocess Biosyst Eng 43(3):507–514
Radajewski S, Ineson P et al (2000) Stable-isotope probing as a tool in microbial ecology. Nature 403(6770):646–649
Raghukumar C, D'Souza-Ticlo D, Verma A (2008) Treatment of colored effluents with lignin-degrading enzymes: an emerging role of marine-derived fungi. Crit Rev Microbiol 34 (3–4):189–206
Raina SA, Yahmed NB et al (2020) Mycoremediation: a sustainable tool for abating environmental pollution. In: Bioremediation and biotechnology. Springer, Cham, pp 269–291
Rampelotto PH (2013) Extremophiles and extreme environments. Life 3(3):482–485
Robinson T, McMullan G et al (2001) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. Bioresour Technol 77(3):247–255
Rosales E, Pazos M, Ángeles Sanromán M (2013) Feasibility of solid-state fermentation using spent fungi-substrate in the biodegradation of PAHs. CLEAN Soil Air Water 41(6):610–615
Rudakiya DM, Pawar K (2013) Evaluation of remediation in heavy metal tolerance and removal by *Comamonas acidovorans* MTCC 3364. IOSR J Environ Sci Toxicol Food Technol 5(5):26–32
Rudakiya DM, Tripathi A et al (2019) Fungal bioremediation: a step towards cleaner environment. In: Advancing frontiers in mycology & mycotechnology. Springer, Singapore, pp 229–249
Rudakiya DM, Patel DH, Gupte A (2020) Exploiting the potential of metal and solvent tolerant laccase from *Tricholoma giganteum* AGDR1 for the removal of pesticides. Int J Biol Macromol 144:586–595
Russo F, Ceci A et al (2019) Understanding fungal potential in the mitigation of contaminated areas in the Czech Republic: tolerance, biotransformation of hexachlorocyclohexane (HCH) and oxidative stress analysis. Environ Sci Pollut Res 26(24):24445–24461
Safferman SI, Lamar RT et al (1995) Treatability study using *Phanerochaete sordida* for the bioremediation of DDT contaminated soil. Toxicol Environ Chem 50(1–4):237–251
Sarioglu M, Bali U, Bisgin T (2007) The removal of CI Basic Red 46 in a mixed methanogenic anaerobic culture. Dyes Pigments 74(1):223–229
Sayler GS, Ripp S (2000) Field applications of genetically engineered microorganisms for bioremediation processes. Curr Opin Biotechnol 11(3):286–289
Schneider WDH, Fontana RC et al (2020) Lignin degradation and detoxification of eucalyptus wastes by on-site manufacturing fungal enzymes to enhance second-generation ethanol yield. Appl Energy 262(114493):1–12
Shaheen R, Asgher M et al (2017) Immobilized lignin peroxidase from *Ganoderma lucidum* IBL-05 with improved dye decolorization and cytotoxicity reduction properties. Int J Biol Macromol 103:57–64
Sharma N, Singhvi R (2017) Effects of chemical fertilizers and pesticides on human health and environment: a review. Int J Agric Environ Biotechnol 10(6):675–680
Sharma A, Kumar V et al (2019) Worldwide pesticide usage and its impacts on ecosystem. SN Appl Sci 1(11):1446–1461
Simões MF, Maiorano AE et al (2019) Microbial fuel cell-induced production of fungal laccase to degrade the anthraquinone dye Remazol Brilliant Blue R. Environ Chem Lett 17(3):1413–1420
Singh RK, Tripathi R et al (2020) Fungi as potential candidates for bioremediation. In: Abatement of environmental pollutants. Elsevier, Amsterdam, pp 177–191
Soares GM, de Amorim MP, Costa-Ferreira M (2001) Use of laccase together with redox mediators to decolourize Remazol Brilliant Blue R. J Biotechnol 89(2–3):123–129
Taleski V, Dimkić I et al (2020) Bacterial and fungal diversity in the lorandite (TlAsS2) mine 'Allchar' in the Republic of North Macedonia. FEMS Microbiol Ecol 96(9):fiaa155
Thuy TT (2015) Effects of ddt on environment and human health. J Educ Soc Sci 2:108–114

Tokar EJ, Boyd WA et al (2013) Toxic effects of metals. In: Klaassen CD (ed) Casarett and Doull's toxicology: the basic science of poisons, 8th edn. McGraw-Hill Medical, New York. ISBN 978-0-07-176923-5

Treu R, Falandysz J (2017) Mycoremediation of hydrocarbons with basidiomycetes—a review. J Environ Sci Health B 52(3):148–155

Van der Zee FP, Villaverde S (2005) Combined anaerobic–aerobic treatment of azo dyes—a short review of bioreactor studies. Water Res 39(8):1425–1440

Vandana T, Kumar SA et al (2019) Purification, characterization, and biodelignification potential of lignin peroxidase from immobilized Phanerochaete chrysosporium. Bioresources 14 (3):5380–5399

Vargas JM (1975) Pesticide degradation. J Arboric 1(12):232–233

Verdin A, Sahraoui ALH, Durand R (2004) Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. Int Biodeterior Biodegradation 53(2):65–70

Verma ML, Thakur M et al (2020) Biotechnological applications of fungal enzymes with special reference to bioremediation. In: Environmental biotechnology, vol 2. Springer, Cham, pp 221–247

Vidali M (2001) Bioremediation. An overview. Pure Appl Chem 73(7):1163–1172

White SS, Birnbaum LS (2009) An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. J Environ Sci Health C 27(4):197–211

Wunder T, Kremer S et al (1994) Metabolism of the polycyclic aromatic hydrocarbon pyrene by *Aspergillus niger* SK 9317. Appl Microbiol Biotechnol 42(4):636–641

Yang H, Wu X et al (2019) Fungal transformation of graphene by white rot fungus *Phanerochaete chrysosporium*. Chemosphere 216:9–18

Zhang Q, Wang C (2020) Natural and human factors affect the distribution of soil heavy metal pollution: a review. Water Air Soil Pollut 231(350):1–13

Zhang J, Sun L et al (2018) A novel homodimer laccase from Cerrena unicolor BBP6: purification, characterization, and potential in dye decolorization and denim bleaching. PLoS One 13 (8):1–20

Zhang D, Yin C et al (2020) Multiple heavy metal tolerance and removal by an earthworm gut fungus Trichoderma brevicompactum QYCD-6. Sci Rep 10(1):1–9

Zhao WW, Zhu G et al (2020) Removal and biomineralization of Pb^{2+} in water by fungus *Phanerochaete chrysosporium*. J Clean Prod 2020:120980

Zhuang M, Sanganyado E et al (2020) Azo dye degrading bacteria tolerant to extreme conditions inhabit nearshore ecosystems: optimization and degradation pathways. J Environ Manag 261:110222

Heterologous Protein Expression in Yeast and Molds

21

Shilpa Mohanty, Babbal, and Yogender Pal Khasa

Abstract

The high-level and cost-effective production of biopharmaceuticals and other compounds of commercial interest is possible because of advancements in molecular biology and fermentation techniques. Yeast represents an attractive and popular expression host choice among all the available heterologous expression platforms, mainly due to their GRAS status and post-translational modification mechanisms. *Saccharomyces cerevisiae* and other non-conventional yeast such as *Pichia pastoris* and *Hansenula polymorpha* are successfully employed for biopharmaceutical's commercial production. A series of yeast-based expression vectors comprising promoters, secretion signals, affinity tags, and selection markers have been constructed to drive efficient intracellular or secretory recombinant protein production. The yeast cell surface display technology has been successfully employed for many biotechnological applications such as antibody engineering, bioremediation, and whole-cell biocatalysis. Genome shuffling, directed evolution, metabolic engineering, and synthetic biology approaches have improved product yields, catalytic activity, and substrate specificity of various enzymes. Optimization of fermentation conditions and bioprocess optimization strategies resulted in the cost-effective production of numerous commercially useful products.

Keywords

Yeast expression platforms · *Saccharomyces cerevisiae* · Yeast cell surface display · Metabolic engineering · Bioprocess optimization · Recombinant DNA technology

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21.1 Introduction

Recombinant DNA technology (RDT) has resulted in overpowering the inadequacies of earlier extraction processes for several commercially important microbial products and therapeutic biomolecules from their natural reservoirs. It has laid a platform for the successful and efficient production of these compounds in heterologous expression systems. Herbert Boyer, the founder of Genentech, is credited for successfully producing human insulin in the prokaryotic host strain, *Escherichia coli*. Partnering with Eli Lilly, the first commercially available biosynthetic insulin of human origin was synthesized. It was sold under the brand name Humulin™. In addition to human insulin, there were nine more products, including cytokines, hormones, and antibody molecules, which were permitted for therapeutic use in the 1980s. The application of RDT, especially in drug discovery, has come a long way, which is evident from its current market size. According to a study carried out by MarketsandMarkets, it was proclaimed that the protein expression market had a worth of $1645 million in the year 2017, and the numbers are expected to shoot up to $2850.5 million in the year 2022 (Vieira Gomes et al. 2018). The commercial availability of pharmaceutical products is considerably dependent on achieving large-scale and cost-effective production of recombinant proteins. The generation of a stable and superior expression platform with high expression yields and its amiability to scalable and optimized fermentation conditions will enable the efficient production of the target protein. A suitable and efficient purification strategy will make the entire production process economically viable (Porro et al. 2011; Gupta and Shukla 2016; Sanchez-Garcia et al. 2016; Vieira Gomes et al. 2018).

Several microbial strains and cell lines from higher organisms like mammals, plants, and insects are now well established to function as heterologous protein expression hosts. The microbial strains are considered beneficial due to their high growth rate and amenability to genetic manipulation. *E. coli* is a widely studied bacterial host that is regarded as an expression workhorse of heterologous proteins (Porro and Mattanovich 2004; Porro et al. 2011). However, the bacterial expression platforms also suffer from numerous drawbacks, like their inability to fold the recombinant proteins accurately, leading to protein aggregates or inclusion bodies (IBs). The IB formation presents a considerable hindrance in the biologically active and soluble protein expression. Despite this drawback, about 30% of biopharmaceutical products are produced in industries using this expression platform (Porro and Mattanovich 2004; Singhvi et al. 2020).

Yeast represents a group of expression hosts that amalgamate the eukaryotic system's benefits with simple growth requirements and ease of genetic manipulation with Generally Regarded As Safe (GRAS) status. On the other hand, they possess eukaryotic post-translational and protein processing mechanisms such as protein folding, assembly, and modification, giving them an upper hand in protein expression systems. Further, the recombinant products expressed using the yeast-based expression systems are devoid of endotoxins and oncogenic or viral DNA. Since the 1980s, numerous proteins have been produced using *Saccharomyces cerevisiae* for therapeutic and industrial applications. The in-depth knowledge about its genetic

makeup, physiology, biochemical characteristics, and fermentation strategies makes this organism the choice of host for recombinant protein production. Despite several advantages, *S. cerevisiae* is also overwhelmed by several disadvantages, such as hyperglycosylation and retention of proteins in periplasmic space. The retention of the proteins results in the partial degradation of the product, thereby making product separation difficult. Hence, keeping in view of the microbial world's high diversity, novel expression systems are being developed using alternate yeast strains such as *Yarrowia lipolytica*, *Zygosaccharomyces rouxii*, *Hansenula polymorpha*, and *Pichia pastoris* (Porro and Mattanovich 2004; Radecka et al. 2015; Rebello et al. 2018). The use of filamentous fungi has gained immense attraction due to their inherent capacity to secrete hydrolytic enzymes into the culture medium. The presence of eukaryotic post-translational mechanisms is an added advantage that enables fungi to be used as expression hosts (Nevalainen et al. 2005). In the following sections, various characteristic features of these expression platforms regarding their biotechnological applications are systematically discussed.

21.2 Different Expression Platforms

21.2.1 Introduction

Recombinant protein expression can be achieved using different expression platforms such as bacteria, yeast, molds, insects, and mammalian cells. The bacterial system is often the first choice to produce recombinant proteins. Bacterial hosts have various advantages like cultivation on readily available inexpensive medium, rapid cell growth, flexibility in genetic manipulation, and ability to achieve higher cellular biomass using fermentation strategies (Choi et al. 2006; Demain and Vaishnav 2009; Gopal and Kumar 2013; Tripathi 2016). However, the misfolding of proteins, poor protein stability, protein degradation, aggregation as inclusion bodies, and endotoxin production are some of the limitations. The recombinant protein production using a gram-negative *Escherichia coli* system is well established, while among gram-positive bacterium *Bacillus subtilis* is extensively employed (Drejer et al. 2018). Further, the expression of eukaryotic proteins requiring post-translational modifications for biological activity can be achieved using higher organisms such as yeast and molds.

Yeast holds the highest potential to produce recombinant proteins for commercial applications. The most widely used yeast system for heterologous expression of target genes includes *Saccharomyces cerevisiae*, *Pichia pastoris*, *Schizosaccharomyces pombe*, and *Hansenula polymorpha* (Balamurugan et al. 2007; Gomes et al. 2016). The filamentous molds such as *Aspergillus oryzae*, *A. niger*, and *Trichoderma* species are used to produce industrially important enzymes (Gomes et al. 2016; Landowski et al. 2016). Furthermore, the baculovirus system has been well exploited for the heterologous production of vaccines, therapeutic and diagnostic antibodies for human use. The insect cells can be easily grown in a serum-free medium and amenable to scale-up studies at bioreactors

(Balamurugan et al. 2007; Gomes et al. 2016). The mammalian cell lines also serve as an excellent expression host to produce biologically active recombinant proteins (Picanço-Castro et al. 2014; Gomes et al. 2016). One of the emerging platforms for recombinant protein production is the single-celled slime mold known as *Dictyostelium discoideum*. The production of therapeutic or biological proteins synthesized using *D. discoideum* as the expression host still requires FDA approval (Arya et al. 2008).

21.2.2 Heterologous Protein Expression in Yeast

Yeast are majorly exploited compared to all the available systems since they offer easy protein production strategies due to their unicellular cell structure, post-translational modifications, and low nutritional requirements compared to mammalian and insect cell lines. Hormones such as insulin and glucagon have been produced at an industrial scale in *S. cerevisiae*, indicating their immense potential in the biopharmaceutical industry. The eukaryotic host *S. cerevisiae* remains the excellent expression platform due to fast growth in protein-free media and secretion of proteins into the culture supernatant. *Pichia pastoris* is a methylotrophic yeast that has been used for the industrial-scale and commercial recombinant protein expression. The *P. pastoris* expression system was introduced decades ago by Phillips Petroleum, resulting in the commercial-scale synthesis of single-cell protein (SCP) as an additive for animal feeds. The expression of heterologous proteins can be carried out with the help of a strong, tightly regulated, and inducible alcohol oxidase (*AOX1*) promoter. It possesses cellular machinery that can enable the production of intracellular and extracellular recombinant proteins with excellent efficacy. Its capacity to attain high cell densities in cheap and inexpensive media makes it a choice of host for cost-effective protein production and product development (Porro et al. 2011; Gupta and Shukla 2017; Vieira Gomes et al. 2018).

Although *S. cerevisiae* presents itself as the best expression platform for recombinant protein production, it is also associated with several drawbacks. It cannot grow on a wide range of substrates such as xylose, arabinose, and glycerol. The non-conventional yeast strains, such as *Kluyveromyces lactis*, *Hansenula polymorpha*, *Yarrowia lipolytica*, and *Pichia pastoris*, are widely studied and explored to develop protein expression systems and other biotechnological applications (Table 21.1). The physiology of these yeast strains, metabolic pathways, and their regulation have several benefits compared to the conventional *S. cerevisiae* strains. They possess a mechanism that aids in the survival and growth of these yeast cells in extreme environmental conditions. Their divergent evolution from *S. cerevisiae* led to the development of specific pathways and machinery that permits them to thrive under different stress conditions. Furthermore, the non-conventional hosts such as *K. lactis* and *P. pastoris* utilize a wide range of substrates and have higher protein titers than *S. cerevisiae* (Madhavan et al. 2017a).

Table 21.1 Heterologous expression of target proteins using yeast as the expression host

Expression host	Target protein	Yield/product titers/ enzyme activity	Reference
S. cerevisiae	Simvastatin	55 mg/L	Bond and Tang (2019)
S. cerevisiae	Violaxanthin	7.3 mg/gDCW	Cataldo et al. (2020)
S. cerevisiae	*Rahnella aquatilis* levansucrase	50 U/mL	Ko et al. (2019)
S. cerevisiae	*Trametes versicolor* laccase	45 U/L	Iimura et al. (2018)
S. cerevisiae	Levopimaric acid	400.31 mg/L	Liu et al. (2018)
S. cerevisiae	Taxadiene	129 ± 15 mg/L	Nowrouzi et al. (2020)
S. cerevisiae	*Thermoascus aurantiacus* β-glucosidase	7.25–7.83 g/L (ethanol yield)	Smekenov et al. (2020)
Pichia pastoris	Human serum albumin	8.86 g/L	Zhu et al. (2018)
P. pastoris	*Beauveria bassiana* chitosanase	5.48 U/mg	Liu et al. (2020)
P. pastoris	Hyaluronidase of scorpion venom	0.266 mg/mL	Amorim et al. (2018)
P. pastoris	Human DNA topoisomerase I	0.33 mg/mL	Chan et al. (2018)
P. pastoris	Dengue virus non-structural protein (NS1)	0.3 mg/mL	Allonso et al. (2019)
P. pastoris	Hispidalin	98.6 μg/mL	Meng et al. (2019)
P. pastoris	Tachyplesin I	27.24–29.53 mg/L	Li et al. (2019)
P. pastoris	Transglutaminase	37,640 U/L	Özçelik et al. (2019)
Hansenula polymorpha	Human papilloma virus type 16 (HPV-16) L1–L2 proteins	132.10 mg/L	Bredell et al. (2018)
H. polymorpha	Streptavidin	~751 mg/L	Wetzel et al. (2016)
H. polymorpha	Rotavirus VP6 protein	3350.717 mg/L	Bredell et al. (2016)
Y. lipolytica	α-Farnesene	259.98 ± 2.15 mg/L	Yang et al. (2016)
Y. lipolytica	(+)-Nootkatone	978.2 μg/L	Guo et al. (2018)
Y. lipolytica	Itaconic acid	4.6 g/L	Blazeck et al. (2015)
Y. lipolytica	Oleanolic acid	540.7 mg/L	Li et al. (2020)

21.3 Components of a Yeast Expression System

The design of a suitable expression system for yeast involves several steps. The first critical component is the host strain that will enable the efficient synthesis of recombinant proteins. The cloning procedure requires the design of an appropriate expression cassette comprising an episomal or integrative vector, a strong constitutive/inducible or repressible promoter, and a selection marker. The desired protein gene can be codon-optimized for efficient translation and fused with a tag for affinity

purification and detection. Depending on whether the heterologous protein production is intracellular or extracellular, an appropriate signal sequence must be used for targeting the protein to the culture supernatant. Strategies must be developed to prevent the recombinant protein's proteolytic cleavage due to the secretion of proteases by the host strain. Optimization of cultivation conditions such as temperature, pH, dissolved oxygen (DO), and media components and determination of fermentation conditions like suitable carbon and nitrogen sources are other parameters that need to be standardized (Celik and Calik 2012).

21.3.1 Host Strains

There are several *S. cerevisiae* wild-type and mutant strains that are being used for heterologous protein production. The yeast strain *S. cerevisiae* BJ5464 was used to express the fungal polyketide synthase after the deletion of *PEP4* and *PRB1* genes responsible for the expression of aspartyl protease and proteinase B, respectively (Bond et al. 2016). There are techniques available for engineering the N-glycosylation pathway that allow for the successful production of glycoproteins in yeast with a glycan structure similar to the human type. The GlycodExpress™ and YAC-express innovative strategies have been developed for *S. cerevisiae* to promote recombinant glycoprotein production via selective glycosylation (Arico et al. 2013).

The *P. pastoris* strains have been derived from the NRRL-Y 11430 strain (NRRL, Peoria, IL). Several strains possess a modification in the *HIS4* gene that encodes for histidinol dehydrogenase (*HIS4*). This permits the selection of recombinant host cells harboring *Pichia* vectors carrying the *HIS4* selection marker after transformation on minimal media. The functional *HIS4* gene now enables the growth of recombinant host cells on minimal media. The other selection markers employed include *S. cerevisiae* ARG4 and the kanamycin resistance gene against G418, which is of bacterial origin. The *Sh ble* gene native to *Streptoalloteichus hindustanus* has also been known to confer resistance to Zeocin, which is a bleomycin-related antibiotic. There are three different *P. pastoris* strains available, depending on their capability to utilize methanol. The Mut^+ phenotype shows a normal cell growth rate on methanol. The minus phenotype (Mut^-) and the methanol slow utilization phenotype (Mut^s) carry deletion on both or one of the *AOX* genes, respectively. The advantage of using these strains is that they require a low amount of methanol for induction and perform better than wild-type hosts. Some of the commonly used *P. pastoris* strains include GS115 (*his4*), which is a wild-type strain having *AOX1* and *AOX2* genes. In the KM71 host, the deleted *AOX1* gene is substituted by the *ARG4*, whereas the MC100-3 strain cannot survive on methanol owing to the omission of both of the *AOX* genes. The protease-deficient strains of *Pichia pastoris* can successfully control the proteolytic degradation of heterologous proteins by vacuolar proteases (Li et al. 2007). In *Yarrowia lipolytica*, the wild-type W29 strain has been modified to Po1d, Po1e, Po1f, Po1g, and YLP21 strains (Nicaud et al. 2002).

21.3.2 Vectors and Promoters

21.3.2.1 *Saccharomyces cerevisiae*

The yeast expression vector comprises a transcriptional unit of eukaryotic origin. The integrative plasmids are responsible for merging within the yeast genome via homologous recombination. It possesses a DNA sequence that is homologous to the target locus. The genomic integration approach is beneficial since it eliminates the selection pressure requirement during the recombinant protein production process. Several shuttle vectors are now available that propagate in *E. coli* and help gene insertion via genomic integration to yeast cells. An additional component of the shuttle vectors is *E. coli* origin of replication and its specific antibiotic resistance marker. The expression vectors also include a secretion signal present in-frame to the MCS. In addition, sequences encoding for fusion tags such as 6× Histidine tag, FLAG, HA, and c-myc are also present within the expression vectors that aid in affinity chromatography and allow for easy purification and detection of proteins (Celik and Calik 2012). The examples of shuttle vectors of *S. cerevisiae* are the YXplac and the pRS series, where the former is based on the pUC19 plasmid. The pRS plasmid series is a type of hybrid vectors constructed by ligating the pBluescript or pBluescriptII polylinker/multiple cloning site (MCS) with the origin of replication of the pBluescribe. These vectors also allow for blue-white screening. Different selection markers used in these vectors include *S. cerevisiae* biosynthetic genes such as *TRP1*, *URA3*, and *LEU2* compatible with most of the laboratory strains auxotrophic for tryptophan, uracil, and leucine, respectively (Chee and Haase 2012).

In *S. cerevisiae*, the native promoters can be classified into two groups, the constitutive and inducible promoters. The constitutive promoters enable stable transcription and do not depend on extracellular or intracellular stimuli. The most commonly used constitutive promoters include P_{TEF1}, P_{TDH3}, P_{PGK1}, P_{TPI1}, P_{CCW12}, and P_{ENO2}. On the other hand, the inducible promoters are highly influenced by the presence or absence of stimuli or an inducer. In *S. cerevisiae*, these inducers can be galactose, maltose, glucose, sucrose, glycerol, acetate, or ethanol. Apart from these, certain environmental parameters such as pH, temperature, and light and chemicals like hormones, metabolites, amino acids, and metal ions can also act as inducer molecules. The strong inducible promoters are P_{GAL1}, P_{GAL2}, P_{GAL7}, and P_{GAL10}, which are galactose-based and have been used to drive high-level expression of desired proteins (Tang et al. 2020).

21.3.2.2 *Pichia pastoris*

The standard design of a *P. pastoris* vector involves a bifunctional structure and auxotrophic selection markers such as *HIS4*, *ARG4*, *URA3*, and *URA5* or antibiotic resistance genes against Zeocin™, geneticin (G418), and blasticidin S. The *Pichia* expression vector's general characteristic features include a promoter (commonly *AOX1*), followed by a single or multiple cloning site and a terminator sequence that is derived from *AOX1*. Some vectors were developed for the extracellular secretion of target proteins and hence contained the secretion signals (Damasceno et al. 2012; Ahmad et al. 2014). Several alternate promoters include the inducible *FLD1*

promoter obtained from the *P. pastoris FLD1* gene that uses methanol or methylamine as the induction molecule. The *FLD1* promoter can direct high levels of protein expression in the presence of methylamine, which is an inexpensive source of nitrogen. The constitutive *GAP* promoter is another example of an alternate promoter native to *P. pastoris*. The foremost benefit conferred by this promoter is the lack of methanol requirement for the induction step. Moreover, it is not essential to shift the yeast cells to a medium containing another carbon source, thereby making the entire process convenient and straightforward under constitutive promoters. The moderate-level protein production can be targeted by the *PEX8* and *YPT7* promoters, where the former gene encodes for a peroxisomal protein matrix required for peroxisome biogenesis (Li et al. 2007). There are several other inducible promoters for protein expression in *P. pastoris* such as *DAS*, *ADH3*, *ICL1*, *LRA3*, *LRA4*, and *CUP1* and constitutive ones like *TEF1* and *PGK1* (Turkanoglu Ozcelik et al. 2019). BioGrammatics (Carlsbad, CA, USA) provides the GlycoSwitch® vectors that aid in producing proteins with a humanized glycosylation pattern. The PichiaPink™ expression system based on the *AOX1* promoter contains eight different secretion signals and is supplied by Life Technologies (Ahmad et al. 2014).

21.3.2.3 *Yarrowia lipolytica*

Yarrowia lipolytica also utilizes two types of shuttle vectors based on their maintenance mode. Although natural episomal vectors haven't been observed in this organism, replicative plasmids can be developed. The genomic integration of foreign DNA occurs via homologous recombination. It is undertaken by linearizing the plasmid DNA at the homologous site and transforming it, which dramatically enhances the frequency of transformation (10^6 transformants/μg of DNA). The utilization of auxotrophic strains of *Y. lipolytica* is considered a best choice for the selection marker. The extensively used markers in *Y. lipolytica* are *LEU2* and *URA3* (Madzak et al. 2004). In addition, several promoters are being used to drive protein expression in *Y. lipolytica*. A common example of constitutive and inducible promoter is p*TEF1* and p*POX2*, respectively. Other promoters that have been used in this strain include p*LEU2*, p*XPR2*, p*POT1*, and p*RPS7* (Madzak 2015).

21.3.2.4 Promoters Used in Other Fungi and Yeast

The basis of the widely used promoter in the fission yeast *Schizosaccharomyces pombe* is the strong *nmt1* (no message in thiamine) promoter and its two different kinds that possess mutations (*nmt41* and *nmt81*) in the TATA box. Another inducible promoter is the *urg1* that gets strongly induced by uracil under nitrogen deprivation. A series of stable integrative vectors (SIV) are constructed that has been expanded to incorporate antibiotic resistance genes, promoters, fluorescent tags, and terminator sequences (Vjestica et al. 2020). A small number of promoters have been identified to express heterologous proteins in *Aspergillus*. Some of these include the constitutive promoters *PgpdA* and *Ptef1* and the inducible *PalcA* and *PglaA* (Rendsvig et al. 2019). Although the number of promoters for filamentous fungi is less as compared to *S. cerevisiae*, promoter tools for fungi such as

Trichoderma reesei, *A. niger*, *Penicillium chrysogenum*, and *Ustilago maydis* have been developed (Umemura et al. 2020).

21.3.3 Selection Marker

The introduction of new characteristics into the yeast genome, deletion of native genes, and transformation events involving centromeric or episomal plasmids require selection markers. In non-integrative plasmids, the maintenance of selection pressure is necessary to avoid plasmid loss. The most commonly used markers are the prototrophic markers derived from either amino acid or nucleotide base from biosynthesis pathways. Markers such as *LEU2*, *TRP1*, *URA3*, and *ADE2* require the auxotrophic strain that carries a deleted or nonfunctional gene. The *URA3* of *Kluyveromyces lactis* and the *his5*$^{+}$ gene of *S. pombe* can complement auxotrophic markers. Genes that enable the organism's growth on specific carbon or nitrogen sources can also be employed as selection markers. The *FCYI* and *GAP1* genes in *S. cerevisiae* encode cytosine deaminase and general amino acid permease. Thus, the selection of cells expressing these genes takes place on a cultivation media supplemented with cytosine and L-citrulline, respectively. In the auto-selection system, the marker's presence is vital for the cells to survive under most growth conditions. Hence, the application of selection pressure can be attained even in a complex cultivation medium. In the absence of appropriate auxotrophic or auto-selection markers, other semi-dominant markers are generally employed. These markers are majorly responsible for conferring resistance to either toxic or growth-inhibitory compounds. The *CUP1*, *ble*, *cat*, and *kan* resistance genes confer resistance to copper and cadmium, phleomycin, chloramphenicol, and G418, respectively (Siewers 2014).

21.3.4 Secretion Signals

The budding yeast *S. cerevisiae* and *P. pastoris* are commonly used to express desirable proteins for therapeutic and research use. Most of the proteins enter the secretory system, necessitating an N-terminal signal sequence to translocate the protein into the endoplasmic reticulum (ER). A secretion signal's characteristic structure involves a zone of hydrophobic amino acid residues followed by a recognition site for the signal peptidase in the ER's lumen. Several signal sequences have been used to drive the extracellular production of foreign proteins. The most widely accepted one is the *S. cerevisiae* pre-pro-α-factor that acts as a precursory molecule to a peptide mating pheromone. The pre-pro-α-factor consisting of 19 amino acids gets terminated due to the cleavage by signal peptidase. After the signal sequence, a 66-amino-acid-long pro-region gets removed by the Kex2 endoprotease in the late Golgi. The downstream region of the dibasic Kex2 cleavage site contains the EAEA tetrapeptide that is further cleaved by Ste13, a dipeptidyl aminopeptidase (Fitzgerald and Glick 2014).

The *S. cerevisiae* α-mating factor with 89-amino acid residues has been widely used in *P. pastoris* to efficiently translocate the protein to the extracellular medium. Other secretion signals successfully used in *Pichia* include α-amylase signal sequence from *Aspergillus niger*, glucoamylase of *Saccharomyces diastaticus*, *Kluyveromyces marxianus* inulinase, and *S. cerevisiae SUC2*. The signal sequence of *S. cerevisiae* killer toxin, chicken lysozyme, and the human serum albumin have also been used. The enzyme levanase native to *Bacillus subtilis* has been successfully secreted from *P. pastoris* CBS7435 using its native α-factor signal sequence. Its efficiency is comparable to that of the *S. cerevisiae* α-factor (Daly and Hearn 2005; Juturu and Wu 2018). Several alternate signal sequences are being explored in *P. pastoris* to direct recombinant proteins to the culture supernatant. The secretion signals such as the bovine β-casein, E-CALB, and P23 have been utilized in *P. pastoris* for extracellular production of recombinant xylanase, lipase, and human growth hormone, respectively (Kang et al. 2017). A comparative study on signal sequences in *P. pastoris* was carried out to secrete human interferon-alpha 2b (IFN-α2b) in extracellular broth (Ghosalkar et al. 2008). The secretory expression platform for recombinant streptokinase molecule was developed in *P. pastoris* using its native *P*rotein with *I*nternal *R*epeats (PIR1) signal peptide (Adivitiya et al. 2019).

21.3.5 Recovery of Intracellular Proteins

The scale-up of recombinant proteins in yeast is subsequently followed by collecting the cells via filtration or centrifugation for product recovery. The strategies required for cell disruption are divided into two groups: the mechanical methods that involve applying shear force and non-mechanical methods involving chemical or enzymatic treatment. Usually, the non-mechanical techniques are employed at the laboratory scale. In contrast, mechanical procedures are suitable for cell disruption events at a larger scale since they are inexpensive and easily scaled-up. The mechanical means include bead mills, homogenizers, and jet streams. The use of lyticase or zymolase enzymes and physical means such as decompression, sonication, thermolysis, and osmotic shock constitute the non-mechanical strategies of cell lysis. The glass bead lysis method is suitable for small samples at a preparatory scale. However, this method has the disadvantage of producing heat, and hence the samples need to be cooled on ice in between the process. Ultrasonication is a widely used cell disruption technique that utilizes high-frequency sound waves of magnitude greater than 16 kHz, resulting in high shear force generation (Liu et al. 2013). The cell wall disintegration by ultrasound occurs due to the cavitation phenomenon that involves the creation, development, and breakdown of the gas and vapor bubbles. The breakdown of the cavitation bubbles leads to the conversion of sonic energy into mechanical energy in intense elastic shock waves where the pressure can reach as high as thousands of atmospheres (Jamshad and Darby 2012; Liu et al. 2013).

The large-scale cell disintegration in the biopharmaceutical manufacturing units is carried out using high-pressure homogenization. Factors such as turbulence, cavitation, inertial forces, and the valve's design play a substantial role in this cell

disruption process (Shepard et al. 2002). The French Press is another device that is employed for cell disintegration. It is powered by a motor that is responsible for disrupting the cells using variable pressure. Although numerous methods can be used to disrupt cells, the separation of the cellular debris, membrane components, and soluble proteins can be undertaken in two phases. The first phase constitutes low-speed centrifugation that will settle down the cell debris, whereas the second step involves high-speed centrifugation of the culture supernatant. The cell membrane fractionation is accomplished by utilizing standard sucrose gradient techniques (Jamshad and Darby 2012).

21.3.6 Affinity Tags and Protein Purification

The addition of a fusion tag to a target protein is a very reliable approach to enhance its expression yields, facilitate purification, and accelerate the process of characterizing the protein's structural and functional aspects. Many solubility-enhancer tags, genetically modified epitopes, and endoproteases have been introduced to improve protein detection and purification strategies. The *Schistosoma japonicum* glutathione-*S*-transferase (GST) and yeast small ubiquitin-related modifier (SUMO) are among the popular fusion tags used to improve solubility. The GST is a 26 kDa N- or C-terminal fusion tag that is utilized to target single-step purification of desirable proteins. The *Smt3* gene of *S. cerevisiae* is responsible for encoding the SUMO protein that is used as an N-terminal solubility enhancer tag. This aids in enhancing protein stability in terms of its folding and structure. One of the advantages of using SUMO as a fusion tag is the presence of a SUMO protease that cleaves SUMO explicitly at the Gly-Gly motif. Several other affinity tags are developed to facilitate the easy purification of the recombinant proteins. The c-myc, hemagglutinin antigen (HA), and FLAG epitopes are examples of such tags that are primarily used in protein detection techniques. The c-myc tag is an epitope of the human c-myc proto-oncogene product that can be specifically detected by the anti-c-myc antibody. The fusion tags are widely compatible with protein engineering techniques such as Western blot, flow cytometry, IP, and co-IP in yeast and bacterial systems. The HA tag is another widely used protein tag that is obtained from the human influenza hemagglutinin protein. Another fusion tag that is a popular choice for antibody-based purification is the FLAG tag. It is an octapeptide that inherently contains an enterokinase cleavage site that is especially beneficial for therapeutic proteins, where tag removal is an essential step. The Strep-tag II is a small inert peptide and has been used in various hosts, including yeast and bacteria (Young et al. 2012). The hexahistidine or 6× His tag is one of the most commonly used tags in high-throughput protein purification techniques. It possesses the ability to bind to the immobilized transition metals, and hence the immobilized metal affinity chromatography (IMAC) strategy is employed. The most common matrix used for polyhistidine-tagged protein purification is the Ni(II)-nitrilotriacetic acid (Ni-NTA) that demonstrates an enhanced affinity for the histidine residues. There are several advantages of using polyhistidine residues as an affinity tag as it is smaller in size

and uses the relatively inexpensive Ni-NTA column for binding and the matrix exhibits a high binding capacity. Further, the protein can be eluted under mild conditions with the aid of 100–250 mM imidazole. Another benefit of using this versatile purification technique is its utilization under denaturing conditions (Waugh 2005).

21.4 Yeast Expression Platforms

21.4.1 *Saccharomyces cerevisiae* as an Expression Host

The budding yeast *Saccharomyces cerevisiae* is an extensively studied eukaryotic organism. This yeast's ability to produce ethanol and carbon dioxide makes it a desired microbial host for bakers and brewers. It has been awarded the "GRAS" status. *S. cerevisiae* is widely accepted to produce several functional molecules such as antibody fragments, G-protein-coupled receptor, ABC transporters, and proteins involved in drug resistance. The *Saccharomyces* Genome Database (SGD) containing information regarding yeast molecular biology and genetic makeup is available in the public domain. The database includes the phenotypic information on the mutant strains of *Saccharomyces* available for protein production (Darby et al. 2012). Majorly, the transformation event in *S. cerevisiae* depends on the plasmids belonging to the YEp type. These plasmids contain the elements of the 2μ plasmid with a high copy number of approximately 30. They exist independently of the chromosomal DNA. The other type of vectors is of the YIp type that lacks ARS sequences, is stable, and integrates within the genome in low copy numbers. There is an extensive repertoire of molecules of pharmaceutical importance that have been produced in *S. cerevisiae*. Biomolecules such as insulin, hepatitis B vaccines, glucagon, and hirudin are few examples that are developed using this organism (Boer et al. 2007).

The first therapeutic protein that was expressed in this budding yeast was human alpha-interferon, after which numerous other proteins of therapeutic importance were successfully expressed. Hormones such as glucagon, insulin, and its analogs, tissue plasminogen activator, granulocyte-macrophage colony-stimulating factor, albumin, human platelet-derived growth factor, and human papillomavirus (HPV) vaccine Gardasil™ are a few examples of successfully commercialized products from this host (Zhang and An 2010; Baeshen et al. 2014; Wang et al. 2017). Insulin aspart and insulin detemir, the fast-acting and long-acting analogs of insulin, were developed by Novo Nordisk and approved by the US FDA and the European authorities, respectively (Baeshen et al. 2014).

Several groups of metabolites that are of prime importance include flavonoids, isoprenoids such as terpene and terpenoids, polyketides, and vitamin C. The isoprenoids constitute the largest group of plant metabolites where their members, such as Taxol, artemisinin, and vinblastine, are recognized as promising pharmaceutical agents. *S. cerevisiae* was employed to produce three variants of plant sesquiterpenes, i.e., valencene, cubebol, and patchoulol. Some of the examples of

the terpenoids that have been expressed in *S. cerevisiae* are artemisinic acid, geraniol, and amorpha-4,11-diene. Vitamin C or L-ascorbic acid is a water-soluble antioxidant molecule that acts as a strong antioxidant agent. The yeast cells are not the natural producers of this metabolite, but the biosynthesis of ascorbic acid in *S. cerevisiae* was demonstrated using a plant pathway (Huang et al. 2008).

S. cerevisiae does not possess the natural ability to utilize xylose found in lignocellulosic materials. Still, it can undertake xylulose assimilation via native xylulokinase (*XKS1*) and pentose phosphate pathway. Therefore, metabolic engineering techniques are employed to insert pathways involved in xylose metabolism into *S. cerevisiae* to generate xylose-fermenting yeast strains for biofuels, fatty alcohol, and lactic acid production (Kwak and Jin 2017). The *S. cerevisiae*-based yeast cell surface display approach has been widely accepted as an alternate strategy to display cellulolytic enzymes. The developed whole-cell biocatalyst acts as a platform to carry out saccharification and fermentation simultaneously (Hasunuma and Kondo 2012).

21.4.2 *Pichia pastoris* as an Expression Host

Pichia pastoris is a methylotrophic yeast that has gained immense popularity in the field of biotechnology. One of its engineered strains, GS115, has been widely used in the industrial and pharmaceutical sectors. The eukaryotic yeast contains two genes *AOX1* and *AOX2*, encoding for the alcohol oxidase enzyme involved in the methanol utilization pathway. When methanol is present in the medium, activation of both these genes results in an upregulated enzyme expression. Out of both the *AOX1* and *AOX2* genes, *AOX1* is majorly responsible for synthesizing alcohol oxidase. Several advantages seem to be associated with *P. pastoris* while utilizing it to produce heterologous proteins. *P. pastoris* exclusively produced membrane proteins such as histamine H1 receptor, calcium, and potassium channels (Byrne 2015). It efficiently secretes desired recombinant proteins into the extracellular medium without secretion of its native proteins. It glycosylates proteins efficiently to maintain their folding, stability, solubility, and biological activity. It has been reported that proteins undergoing glycosylation in *P. pastoris* may not be hyperglycosylated. The presence of terminal α-1,3 glycan linkages in the core oligosaccharides is not observed in *P. pastoris*. Hence, it does not add the immunogenic mannose residues that are detected in *S. cerevisiae*. However, despite these benefits, some shortcomings are also associated with this expression platform. Unlike the bacterial expression system, the *P. pastoris* transformation process requires high amounts of recombinant plasmid DNA (~μg). The inducible expression system is based on methanol, whose high accumulation in the induction phase negatively affects protein synthesis machinery and cellular viability due to its toxic nature. Moreover, methanol is a fire hazard and hence is unsuitable for the synthesis of food items. The alternate glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter supports the high-level constitutive expression, but it cannot be utilized for toxic protein production. The *P. pastoris* system lacks promoters, which can drive moderate-level expression. The

AOX1, *FLD1*, and *GAP* promoters cause high-level expression of the desirable proteins, which in some instances may be toxic to the cells. The resultant overburdening of the cellular machinery leads to misfolding of the target protein. Further, the release of proteases may lead to the degradation of the anticipated product (Balamurugan et al. 2007; Karbalaei et al. 2020). Numerous parameters determine the recombinant protein expression in *P. pastoris*. These factors include gene dosage or copy number, codon usage, the signal sequence for extracellular secretion, promoter strength, physiological characteristics of the host strain, folding events in the endoplasmic reticulum, and the optimization studies at the bioreactor level. Among all these parameters, the copy number of the target gene and optimization studies are two essential factors that limit the final cost of the production process (Dagar and Khasa 2018). *P. pastoris* is viewed and developed as a promising platform for the synthesis of several proteins at the bioreactor level due to its ability to attain high cell densities. A vast collection of proteins and molecules of commercial relevance has been efficiently produced in this expression host (Ahmad et al. 2014). The biotechnological applications of this expression platform are discussed in the latter sections of this chapter.

21.4.3 *Hansenula polymorpha* as an Expression Host

H. polymorpha is a non-conventional methylotrophic yeast that is being explored due to its unique properties. It is a thermotolerant microorganism that can withstand and grow in a temperature range of 30–50 °C. It glycosylates protein to a certain extent and tends to avoid hyperglycosylation as compared to other yeast. Apart from methanol as the sole carbon source, it can utilize glycerol and sugars such as glucose, xylose, and cellobiose. Currently, three different strains of *H. polymorpha* are used as expression hosts. The DL-1 strain, CBS4732 strain, and NCYC495 strain have been isolated from soil samples, irrigated soil in Brazil, and orange juice in Florida, respectively. Currently, *H. polymorpha* expression platform-based vaccines for hepatitis B, i.e., Hepavax-Gene®, GeneVac-B®, and Biovac-B®, are produced on a commercial scale and are available in markets. Besides, other commercially available therapeutic molecules expressed using this yeast are hirudin, insulin, and IFNα-2a (Kulkarni et al. 2006; Manfrao-Netto et al. 2019). A detailed list of recombinant therapeutic products commercially synthesized in yeast including *S. cerevisiae*, *P. pastoris*, and *H. polymorpha* has been prepared by Walsh (2018).

21.4.4 *Kluyveromyces* sp. as an Expression Host

Kluyveromyces sp. displays a respiring rather than a fermentative mode of metabolism, which is desirable for the recombinant protein expression platform. They can survive at high temperatures, have a broad substrate range, and give high product and secretion yields. *K. marxianus* can grow solely on monosaccharides such as glucose, fructose, galactose, and disaccharides like lactose and sucrose. The

occurrence of high temperatures during the fermentation process is considered advantageous for specific reactions. It minimizes bacterial contamination at the industrial scale as *K. marxianus* can grow at a temperature as high as 45 °C. Moreover, it is the fastest-growing eukaryote that possesses a short doubling time of only 52 min, which might reduce the time required to carry out genetic engineering and strain improvement (Gombert et al. 2016). *K. marxianus* is known to produce several products like short-chain alcohols, carotenoids, and polyketides. It has also been used to synthesize aromatic compounds such as phenylalanine. The aromatic amino acids phenylalanine, tyrosine, and tryptophan are required to produce valuable secondary metabolites that are members of the flavonoid, stilbenoid, and alkaloid groups. Hence, strategies have been aimed to metabolically engineer *K. marxianus* to overproduce phenylalanine (Rajkumar and Morrissey 2020). It has also been used for the generation of vaccines against porcine circovirus 2 (PCV2), responsible for the postweaning multisystemic wasting syndrome (PMWS) and porcine circovirus diseases (PCVDs) that causes substantial damage to the swine industry. Therefore, to target the low-cost and efficient production of this vaccine, the Cap protein of PCV2 virus was expressed in *K. marxianus*, to form virus-like particles. These PCV2-VLPs successfully induced antibody response and decreased the viral titers in challenged mice (Duan et al. 2019).

The other member of the genus *Kluyveromyces* is *K. lactis* that has gained popularity in the biotechnological field due to its GRAS status. It is a heterothallic yeast with a predominant haploid life cycle whose foremost variance from *S. cerevisiae* is its primary metabolism. *S. cerevisiae* majorly depends on glycolysis, while *K. lactis* employs the pentose-phosphate pathway for hexose utilization. It naturally secretes fewer proteins into the culture supernatant and efficiently produces large proteins such as the pGKL1 killer toxin's large subunit. It can attain high cell densities, and hence it is used to produce recombinant proteins for food and feed industries. Some of these products include inulinase, chitinase, phospholipase B, xylanase, and therapeutic molecules such as recombinant interferon-α, macrophage colony-stimulating growth factor (M-CSF), and β-lactoglobulin (Spohner et al. 2016). The β-galactosidase or the enzyme lactase catalyzes lactose's breakdown into glucose and galactose. It has immense applications in the field of medicine, food technology, and the environment. The members of the yeast *Kluyveromyces* genus widely contribute to the production of β-galactosidase. Its member *K. lactis* serves as the major commercial source of this enzyme. The structural gene encoding for β-galactosidase is the *LAC4* gene in *K. lactis*. Improved secretion strategies of *K. lactis* β-galactosidase have been developed that will aid in addressing issues such as poor stability and intracellular nature (Becerra et al. 2001; Kim et al. 2003).

21.4.5 *Yarrowia lipolytica* as an Expression Host

Yarrowia lipolytica is a dimorphic fungus that is known to degrade *n*-paraffins and oils. It is naturally found in oil-contaminated regions and food products such as cheese, yogurt, poultry, and meat products. Although the organism was initially

recognized to be distantly related to *S. cerevisiae*, its genetic mechanisms seem to differ. It possesses an inherent capacity to secrete out proteins and has a high secretion efficiency. It also follows a typical hyperglycosylation pattern, provides a good yield, and shows reproducibility. Several commercially viable enzymes have been successfully expressed in this host. One such example is of laccase that is native to the white-rot fungus *Trametes versicolor*. A recombinant β-glucanase derived from *Aspergillus fumigatus* was overexpressed in *Y. lipolytica* with a FLAG tag that assisted in easy purification. *Y. lipolytica* has also been genetically manipulated to display desirable proteins over its cell surface. Numerous proteins such as *A. pullulans* alkaline protease, EGFP, and hemolysin from *Vibrio harveyi* and alginate lyase derived from *Vibrio* sp. have been targeted for yeast immobilization. These recombinant yeast cells carry a vast potential for bioremediation, bioconversions, and the development of biocatalysts and live vaccines (Bankar et al. 2009).

A laccase enzyme isolated from a basidiomycete *Pycnoporus cinnabarinus* was cloned and expressed in *Y. lipolytica* (Madzak et al. 2005). Several enzymes, antibody molecules, and proteins of mammalian, fungal, viral, plant, and bacterial origin are being successfully produced in *Y. lipolytica* (Rao et al. 2011). A comparative study has also been done in which the recombinant protein production by the methylotrophic yeast *P. pastoris* and *Y. lipolytica* at bioreactor levels was carried out. The *Candida antarctica* lipase B (CALB) was efficiently expressed in both the hosts at the bioreactor level, where *Y. lipolytica* showed a five-fold higher ability to secrete extracellular lipase as compared to *P. pastoris*. Moreover, *Y. lipolytica* required half the cultivation time to reach its maximum production level than *P. pastoris* (Theron et al. 2020).

21.4.6 *Zygosaccharomyces* sp. as an Expression Host

Among the non-conventional yeast employed for recombinant protein production, two members belonging to the genus *Zygosaccharomyces* are currently under investigation. *Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii* are known for high salt tolerance and high resistance to sugars, respectively. Further, *Z. bailii* also tolerates acidic conditions and high temperatures and can thrive in chemical preservatives. It is a non-methylotrophic and Crabtree positive yeast capable of handling high osmotic pressure. These features help in preventing unwanted bacterial contamination in bioreactors. With a high specific growth rate, *Z. bailii* presents itself as a potential candidate for biotechnological studies (Branduardi et al. 2004).

The other important species of *Zygosaccharomyces* is the osmotolerant *Z. rouxii*, which is closely associated with *S. cerevisiae*. Since it resembles the model organism *S. cerevisiae*, therefore, its genes for osmotolerance are of beneficial interest. However, the availability of molecular tools to manipulate and explore *Z. rouxii* is still limited. *Z. rouxii* has been in use for several years to manufacture traditional Japanese fermented food products such as soy sauce and soybean paste. Its economic

significance is also crucial as it is associated with the spoilage of food items (Pribylova et al. 2007; Watanabe et al. 2010). DNA manipulations, transformation procedures, and selection mechanisms for this organism have been attempted to develop it as an expression platform. Since certain *S. cerevisiae* centromeric plasmids are not stably maintained in *Z. rouxii*, therefore, *Z. rouxii*-specific plasmids have been generated (Pribylova et al. 2007). The lithium acetate and polyethylene glycol method used for facilitating transformation in *S. cerevisiae* cannot be used with pronounced efficacy for *Z. rouxii*. Instead, the spheroplast-polyethylene glycol method was developed by Ushio and co-workers in 1988, but it is time-intensive and involves cell wall regeneration steps. The alternate electroporation method is a quicker and efficient method developed by Pribylova and Sychrova (Pribylova and Sychrova 2003; Watanabe et al. 2010). Certain modifications to the transformation protocol were incorporated where glycerol was supplemented to the electroporation buffer, and the cells were maintained at −80 °C. These changes significantly improved the transformation efficiency of *Z. rouxii* and will facilitate research at the genetic and molecular levels (Watanabe et al. 2010). Soy sauce is one of the most widely used dressings in Japan. Its umami flavor enhancement is brought by the conversion of L-glutamine to L-glutamic acid by glutaminases. Most of these hydrolases are secreted by *A. sojae* or *A. niger* whose activity decreases in the saltwater present during soy sauce preparation. Therefore, to circumvent this problem, a recombinant *Z. rouxii* strain was developed, which expressed these key hydrolases during the soy sauce fermentation. Since it is a salt-tolerant organism, it can quickly grow in miso and *moromi*, which comprises 10–18% (w/v) of sodium chloride (Yuzuki et al. 2015).

21.4.7 *Candida boidinii* as an Expression Host

C. boidinii is a methylotrophic yeast with the capacity to utilize methanol efficiently. Other methylotrophs like *P. pastoris*, *P. methanolica*, and *H. polymorpha* have been investigated for strong and inducible methanol-based promoters. The alcohol oxidase (*AOD1*) promoter shows an enhanced gene transcription when methanol or a combination of methanol and glycerol is used as a carbon source. The presence of glycerol in the growth medium showed an intermediate level of expression. The low-level expression of proteins is observed in media supplemented with glucose or ethanol. The supplementation of both methanol and glycerol in the growth medium, therefore, supports high-cell density. Such a strategy cannot be adopted in *P. pastoris* as glycerol represses the *AOX1* promoter (Yurimoto and Sakai 2009).

A novel *C. boidinii*-based expression platform has also been developed for toxic protein production where the accumulation of these proteins can occur within the membrane-bound peroxisomes. Several proteins, such as *S. cerevisiae* adenylate kinase and human α_1-antitrypsin, have been expressed in the cytosolic fraction of *C. boidinii*. Heterologous proteins such as *R. oryzae* glucoamylase, *Schwanniomyces occidentalis* phytase, bovine cathepsin C, and transglutaminase from *Streptomyces mobaraensis* have been successfully secreted into the culture supernatant.

Peroxisomal protein expression was also observed in *Penicillium janthinellum* FAOD protein (Yurimoto and Sakai 2009). The *AOD* promoter was used to drive the expression of a D-amino acid oxidase (*DAO*) gene native to the *C. boidinii* in the native host. It was a monomeric enzyme expressed in the peroxisomal fraction and was found to be biologically active toward several substrates such as D-Ala, D-Met, and D-Ser. The expression of recombinant products was improved by employing an alcohol oxidase disrupted strain rather than the wild-type organism (Yurimoto et al. 2001).

21.4.8 *Debaryomyces* sp. as an Expression Host

The members belonging to the genus *Debaryomyces* are explored for biotechnological applications due to their extremophilic nature. These organisms are known to be osmotolerant as they can thrive with a NaCl concentration of as high as 4 M. One of its members, *D. hansenii*, is naturally found in low water activity regions such as seawater, cheese, soil, beer, meat, fruit, and wine. It is also found in products containing high sugar content. In addition, *D. hansenii* is an oleaginous yeast that has the ability to accumulate lipids. This organism's high-level ability to synthesize and store lipids makes it an attractive choice for the production of several valuable products (Breuer and Harms 2006).

One of the unconventional yeast is *Debaryomyces occidentalis* (syn. *Schwanniomyces occidentalis*), which was earlier recognized as a "super yeast" due to its ability to utilize whole starch to release glucose without any prior hydrolysis reaction. The utilization of *Debaryomyces* is especially beneficial for carrying out cost-effective fermentation processes as they have the natural ability to tolerate extreme environments (Kregiel 2019).

21.4.9 *Arxula adeninivorans* as an Expression Host

Arxula adeninivorans, also known as *Blastobotrys adeninivorans*, is a temperature-tolerant yeast that is fully characterized, and its genome has been completely sequenced. It can survive a temperature of up to 48 °C, and its LS3 wild-type strain displays three different morphologies based on cultivation temperature. When grown at a temperature of up to 42 °C, it shows budding; following which, at 42 °C, it forms pseudomycelia, and at temperatures above 42 °C, it forms mycelia. *A. adeninivorans* is also a halotolerant yeast that can grow in the presence of 20% NaCl. It has been employed to successfully produce proteins and molecules such as human interleukin-6, human interferon α2a (IFNα2a), and *Kluyveromyces lactis* β-galactosidase. Presently, there are about ten commercially relevant products available in the market based on *A. adeninivorans*. Products such as tannase and three different *A. adeninivorans* cutinases have been developed by ASA Spezialenzyme GmbH (Germany). An A-YES kit has been commercialized by "QuoData" GmbH (Germany) that acts as an estrogen biosensor. There are several kits available that are

commercially viable and can be used as detection kits for dioxin and estrogen in ultrapure, potable, and saline waters, as well as for estrogen and progesterone. These kits utilize transgenic *A. adeninivorans* as the detection component (Malak et al. 2016). The heterologous expression of the enzyme YlLip11 native to *Y. lipolytica* was targeted in *A. adeninivorans*, *H. polymorpha*, and *S. cerevisiae*, where the maximum expression of the lipase enzyme was observed to be 2654 U/L in the host *A. adeninivorans*. The productivity in the case of *A. adeninivorans* was higher than *H. polymorpha* and *S. cerevisiae* by 2.3- and 1.6-fold, respectively (Kumari et al. 2015).

21.4.10 *Aspergillus* sp. as an Expression Host

Several members of the kingdom Fungi are utilized widely for recombinant protein production since they have the inherent capability to secrete large quantities of protein to the culture supernatant. Recombinant protein expression in filamentous fungi is quite popular due to their natural capacity to express industrially important hydrolytic enzymes. The filamentous fungi, including *Aspergillus niger*, *A. oryzae*, *A. nidulans*, and *Trichoderma reesei*, have been used as cell factories for recombinant products. Members of the *Aspergillus* genus are generally related to biomass degradation and synthesize large varieties of hydrolase enzymes. One of its species, *Aspergillus nidulans*, is a model organism used in bioengineering research and can be manipulated via molecular methods. Its physiological, morphological, and growth characteristics are well studied, and it has been used for the expression of lipase, xylanase, aryl alcohol oxidase, and xyloglucanase. *A. nidulans* A773 host strain has been used to successfully produce xylanase enzyme that subsequently helped in the release of xylooligosaccharide from rice husk. *Penicillium funiculosum* endoxylanase and *Aspergillus niger* arabinofuranosidase were expressed in *A. nidulans*. The medium containing pyridoxine and 2% maltose as an inducer was used for the cultivation of the engineered *A. nidulans* strain for the hypersecretion of endoxylanase (301.2 U/mg) and arabinofuranosidase (115.55 U/mg) (Kumar 2020).

Aspergillus niger is another industrially important strain that is capable of secreting several commercially viable enzymes. The selection of the transformants is made based on dominant and nutritional markers. *A. niger* is a known hyperproducer of citrate, and its fermentation capabilities under acidogenic conditions are well established. It follows a saprophytic mode of nutrition that allows the utilization of several raw substrates. The host can grow at a pH of 3 or less. The overall benefits of *A. niger* and its GRAS status make it a suitable expression platform for the synthesis of citrate and gluconate. It has been engineered to express oxalate, succinate, and itaconate using genetic engineering strategies. The mouse lactate dehydrogenase (*mldhA*) gene was expressed in *A. niger* using a strong constitutive citrate synthase promoter from *A. niger*. The expression host is not a natural producer of L-lactate. However, using molecular techniques, the transformants

could aerobically synthesize L-lactate on minimal media (Dave et al. 2012; Dave and Punekar 2015).

21.4.11 Other Fungi as Hosts for Recombinant Protein Production

Most of the filamentous fungi can be transformed with vectors that can be integrated within the fungal genome to ensure longer stability. Apart from the yeast such as *Pichia pastoris*, there are filamentous fungi that have been tested for their ability to produce homologous as well as heterologous proteins. Although *Aspergillus* has emerged as one of the most useful hosts for protein production, alternate organisms such as *Trichoderma reesei*, *Chrysosporium lucknowense*, and *Mortierella alpina* also have enormous potential and need to be explored. One such expression system is *Penicillium chrysogenum* developed using *xylanase xylA* and *glutamate dehydrogenase gdhA* as inducible and constitutive promoters, respectively (Wang et al. 2005; Sonderegger et al. 2016). The model fungus *Ustilago maydis* is recently developed as a protein expression platform (Sarkari et al. 2014). *Neurospora crassa* is another fungal system that has been designed for targeting recombinant protein production. Its genetic and biochemical features are well studied, and the genome has been fully sequenced. It has been tested for its ability to generate subunit vaccines. The initial vaccine candidate that was chosen is against the seasonal variant of the influenza virus, H1N1 (Allgaier et al. 2009).

21.5 Metabolic Engineering and Systems Biology for Strain Improvement

21.5.1 Introduction

The strategies of strain improvement have led to the development of microbes with improved characteristics, including higher production, fewer by-products, amenability to bioreactor environment, higher productivity, and utilization of complex raw material as a growth substrate. Microbes that produce commercially pertinent compounds when acquired from their natural habitat are not suitable for industrial use as they lack higher product yields. To improve the yield of the desired products, the genes encoding them can be manipulated. During strain improvement, the regulatory steps and checkpoints limiting higher product yields could be avoided. The alterations of desired pathway genes allow a shift in metabolic machinery toward producing the anticipated product, thereby improving the product yield. In some instances, it has been observed that changes at the DNA level could enhance the characteristic properties of an enzyme, such as substrate specificity, improved catalytic activity, and the ability to tolerate different inhibitors. On the other hand, alteration in the regulatory regions such as promoters can cause gene regulation loss. One example of such modification includes the overproduction of amylase without

the need of an inducer (starch) due to the generation of a constitutive mutant (Parekh 2009).

The procedures for hyper-producer's screening are developed based on the information regarding the rate-limiting steps in product formation, enzyme activity, and environmental factors. To improve the yield of any specific enzyme, the gene dosage strategy can be applied to enhance the gene copy number, which in turn translates to higher product concentrations. The strain improvement via alteration at the genetic level has been a prerequisite for secondary metabolites and antibiotics. Further, the strains that can thrive on cheaper raw materials such as corn syrup, molasses, and lignocellulosic hydrolysate will significantly reduce the fermentation cost. Additionally, the genetically modified or metabolically engineered strains can produce various biologically relevant molecules that do not have a microbial origin, such as viral vaccines, insulin, human growth factor, interferon, and interleukins. All strain improvement techniques involve the changes at the DNA level via mutation and genetic engineering (Parekh 2009).

21.5.2 Introduction of Genetic Variability in Yeast

Yeast are recognized as a diverse group of organisms where strains classified under the same species exhibit significant variability at the genetic level (Steensels et al. 2014). The occurrence of natural diversity or genetic variation has been confirmed using high-throughput techniques such as next-generation sequencing. Apart from naturally occurring variation, yeast can be artificially modified to produce the desired metabolite. The development of artificial yeast can be achieved via mutagenesis, sexual reproduction, insertion or deletions of DNA fragments, changes in ploidy, genetic recombination, and transposons and even by the horizontal gene transfer method. The sexual reproduction approach was employed to introduce genetic variability in *S. pastorainus* via mating between *S. cerevisiae* and *S. eubayanus*. The resulting interspecific hybrid was able to perform better during fermentation at low temperatures as compared to *S. cerevisiae* (Dunn and Sherlock 2008). The sexual hybridization strategy is an effortless way of introducing artificial diversity in yeast. The commonly used approaches for producing superior yeast strains via sexual hybridization, such as direct mating, rare mating, and genome shuffling, are described below in brief.

21.5.2.1 Genome Shuffling

The engineering of microbial strains for industrially important metabolites or products is an essential aspect of biotechnological research. The genome shuffling method provides an alternative to the traditionally used mutagenesis approach to design an efficient microbial strain with desirable phenotypes for food, chemical, biofuel, and pharmaceutical industries. It permits the recombination between a genetically diverse population and accelerates the directed evolution process without the complete knowledge of genetic background for microbial strain improvement. Further, the genome shuffling is a sexual process where the entire generation gets

evolved. This strategy significantly contributes to the development of the combinatorial engineering approach. The genome shuffling procedure does not require the whole of the microbial genome for recombination, as it employs the usage of DNA fragments. The cellular phenotype depends on metabolic activity, nutritional availability, global gene expression, and cellular stresses (Patnaik et al. 2002; Biot-Pelletier and Martin 2014; Magocha et al. 2018).

Stemmer (1994) first described the method of genome shuffling. It has revolutionized metabolic engineering and strain improvement technology due to its cost-effectiveness and ease of handling. It has proven to be an efficient technique in carrying out whole-cell engineering, thereby allowing quick advancement in industrially relevant phenotype improvement. The enhanced production of tylosin, a polyketide antibiotic (from *Streptomyces fradiae*), was reported (Zhang et al. 2002). In another example, the *Lactobacillus* strains capable of tolerating high acid (low pH) during fermentation were obtained in only a few shuffling rounds (Patnaik et al. 2002).

It has been more efficient and successful in achieving improved phenotype than rational and classical strain improvement methods. Further, the genome shuffling has reduced the time required for gaining strains with desirable properties. It involves repetitive rounds of protoplast fusion between multiple parental strains, which results in more hybrid generation than other methods. The protoplast fusion method is the backbone of this latest technology. This method involves the fusion of two cells, which differ in their genetic traits, thus resulting in the generation of recombinants with both the parental cells' features. In protoplast fusion, only two parental cells per generation can be used for fusion. In genome shuffling, multiple parents can be recombined per generation and involve numerous rounds of shuffling. The shuffled strains are not considered genetically modified and can be safely used in the food industry.

Bajwa et al. (2010) used the genome shuffling method for strain improvement of *Pichia stipitis*. The mating between haploid strains permitted the accumulation of beneficial characteristics exhibited by both the strains via genetic recombination. The resulting strain showed enhanced ability to tolerate the inhibitors present in hardwood spent sulfite liquor (HW SSL) and can be used for bioethanol production from other lignocellulosic hydrolysates (Bajwa et al. 2010).

21.5.2.2 Cytoduction

There are certain industrially relevant phenotypes whose genes are not nuclear but lie in the mitochondrial DNA or are present in the cytoplasm. Cytoduction is a method to transfer such cytoplasmic traits from parent to recipient strain. During the cytoduction experiment, the donor strain (harboring cytoplasmically transferable element) carries a nonfunctional *KAR1* gene (Georgieva and Rothstein 2002). The *kar1* mutants cannot undergo karyogamy after the hybridization step. Hence, after protoplast fusion or mating, a transient heterokaryon (like a zygote) is formed. The heterokaryon undergoes further meiotic events, and the resulting heteroplasmons contain a nucleus from one parent and mixed cytoplasmic factors from both the parental strains (Conde and Fink 1976; Steensels et al. 2014). The hybrids thus

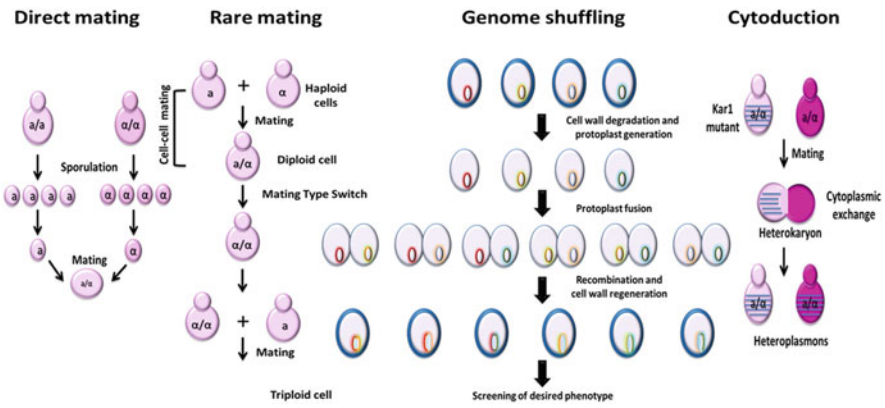

Fig. 21.1 Strain improvement techniques using sexual hybridization (direct mating, rare mating, genome shuffling) and asexual hybridization (cytoduction) methods

generated are also called cytoductants. This technique is often employed to create yeast strains exhibiting killer-toxin-positive phenotype. It can be used to transfer yeast artificial chromosomes and flocculation characteristics (Barre et al. 1993; Spencer et al. 1994; Steensels et al. 2014) (Fig. 21.1).

21.5.2.3 Directed Evolution

Directed evolution combines the random mutagenesis and sexual hybridization approach to achieve the desired phenotypic variants or to fine-tune the existing phenotype. The molecular evolution method provides a cost-effective way of rapidly identifying and screening the superior variants of strains/enzymes. It has been used majorly for enzymes involved in the rate-limiting reaction. Further, screening process is done via high-throughput methods. The culture is grown under selective conditions such as using a batch system for continuous passaging of cells and a continuous system like turbidostat and chemostat. The selection of strain is performed by mimicking conditions present during industrial level cultivation like high ethanol level, nutrient deprivation, and osmotic stress to identify and isolate multi-stress-tolerant strains (Gresham et al. 2008; Steensels et al. 2014). It can provide additional features to strain generated by metabolic engineering or genome shuffling. The xylose utilizing *S. cerevisiae* strain was developed using a directed evolution approach where xylose metabolism enzymes were transferred. Further rounds of alteration were introduced using the mutagenesis and genome shuffling approach. The strains thus produced were made to undergo evolutionary adaptation via a serial transfer of strains in hydrolysate containing xylose for cell growth (Demeke et al. 2013; Steensels et al. 2014).

21.5.2.4 Metabolic Engineering

In 1991, Bailey was the pioneer scientist who first demonstrated the metabolic engineering method via rewiring pathways to improve product yields (Bailey

1991). Metabolic engineering involves improving the product yield by altering the existing pathway reactions or introducing new reaction steps with recombinant DNA technology. It requires the knowledge of metabolic fluxes, quantification of fluxes using analytical methods, and a molecular approach to enhance the desired product yields. Further, the metabolic flux is governed by several steps of the pathway and not solely dependent on the rate-limiting steps. The commonly employed strategies to produce metabolite include altering regulatory elements, structural genes, and pathway engineering. Manipulation of regulatory genes is achieved by introducing the multiple copies of positive regulatory genes and also by disrupting the gene whose activity negatively impacts the product formation (Antoniewicz et al. 2007; Woolston et al. 2013). Similarly, pathway engineering requires the alteration in the pathway steps, such as the rate-limiting steps. For example, overexpression of the rate-limiting enzyme has been shown to enhance tylosin and penicillin production (Cox et al. 1987; Demain and Adrio 2008).

Cohen and Boyer were the first to introduce the foreign gene in a bacterial cell (Cohen et al. 1973). The bacterial system and other microbial cells were then employed as factories for targeting the overproduction of genes of interest (such as pharmaceuticals, chemicals, and therapeutics). Overexpression of insulin, amylase, and lipase had been obtained by cloning the target gene under strong promoters present in multi-copy plasmids. However, the production of bioethanol requires the engineering of pathways comprising of multiple genes and enzymes. Thus, metabolic engineering involves understanding of integrated metabolic pathways and regulating networks rather than focusing on only one gene or enzyme. For yeast, both the inducible and constitutive promoters are available for optimizing the production of the target gene (Curran et al. 2013). The COMPACTER method, i.e., combinatorial transcriptional engineering, has been demonstrated in customized optimization of metabolic pathways. This approach was used in fine-tuning the genes required for xylose metabolism (Du et al. 2012).

The overexpression of pyruvate carboxylase (*pyc*) and aspartokinase (*ask*) genes has been shown to improve lysine productivity up to 150% without any deleterious effect on cellular health. The production of cephalosporin C is often limited by the intermediate penicillin N (Koffas et al. 2003). Thus, elimination of this pathway intermediate resulted in a 15% enhancement in cephalosporin production. The accumulation of DAC (deacetylcephalosporin C) as an intermediate also limited cephalosporin production in *A. chrysogenum*. Therefore, cloning of *CefG* gene coding DAC acetyltransferase under strong promoter increased cephalosporin C levels by two- to three-fold with lower DAC accumulation (Mathison et al. 1993; Demain and Adrio 2008). The genetic engineering of *S. cerevisiae* was also done via the transfer of xylose metabolism pathways from *Pichia stipitis* such that simultaneous hydrolysis of both pentose and hexose sugar present in lignocellulosic hydrolysate could take place leading to higher yields of bioethanol. Recently, the CRISPR (clustered regularly interspaced short palindromic repeat)-Cas method was utilized in *S. cerevisiae* to improve its xylose utilization (Bao et al. 2015; Jansen et al. 2017).

21.5.2.5 Inverse Metabolic Engineering

The strategy of metabolic engineering requires the knowledge of genetic factors involved in the pathway. Lack of this knowledge impedes pathway engineering. To overcome this drawback, the concept of "inverse metabolic engineering," also known as "reverse engineering," was described (Bailey et al. 2002; Oud et al. 2012). The first step in this approach involves determining genetics behind the phenotypic trait exhibited by a strain under certain conditions. The genetic information can be obtained by gene mapping, using omics methods to unravel the difference at gene, mRNA, and protein levels under different conditions in different strains, followed by linking these genetic parameters to the phenotype. The complete information thus obtained is then used to modify the production strain genetically. The exo-metabolome and transcriptome study of wine-producing yeast during fermentation showed the *BAT1*, *ACS1*, *AAD10*, and *AAD14* gene expression was responsible for different aromas (Rossouw et al. 2008).

21.5.2.6 Synthetic Biology

The synthetic biology approach is another powerful tool for creating industrially relevant novel yeast strains. The primary goal of synthetic biology is to construct biological systems ranging from single genes to the whole organism or to redesign and generate existing natural systems. Using "biobricks" (individual genetic elements), complex systems such as metabolic pathways can be generated. Synthetic biology thus involves the creation of artificial life by mimicking natural molecules. The use of this approach for enhancing protein production requires the synthesis of codon-optimized gene sequences, synthetic promoters, expression cassettes, and artificial transcription factors (Kim et al. 2015). Using this methodology, the Synthetic Yeast (2.0) project has been conceptualized to redesign and construct the *S. cerevisiae* genome. Further, via synthetic biology, the artemisinic acid (antimicrobial drug) was produced in *S. cerevisiae*. Genes from *Artemisia annua* such as cytochrome P450 monooxygenase and amorphadiene synthase were introduced and expressed in baker's yeast, which resulted in artemisinic production at a level of 100 mg/L (Ro et al. 2006).

21.6 Yeast Cell Surface Display Technology

21.6.1 Introduction

The yeast surface display technology (YSD) has gained immense popularity due to its applicability in antibody engineering, bioadsorption, peptide library screening, and many commercial bioconversion processes (Tanaka and Kondo 2015). The targeting of proteins on the microbial cell surface has aided in dealing with problems associated with conventional protein immobilization strategies. The covalent attachment of proteins during immobilization causes changes in target proteins' structure and features due to harsh treatment. In the case of ionic interactions, the immobilized enzymes are not stable for multiple cycles. In YSD, the target protein expression is

directed to the cell surface, which helps in dealing with the disadvantages of conventional immobilization techniques (Ueda and Tanaka 2000; Fukuda et al. 2009; Kuroda and Ueda 2011b).

The passenger protein is generally the target that needs to be immobilized, and its choice depends on the application for which it is being immobilized on the cell surface. The anchor protein should possess certain characteristic features like a suitable secretion signal, strong anchoring capacity, compatibility with the heterologous protein, and must not lose its efficiency or stability. The anchoring protein should also be resistant to proteases present in the extracellular medium or in the periplasmic space. The success of the surface display technology also depends on the choice of appropriate host strain. It must not secrete host proteases in the culture supernatant (Lee et al. 2003).

The YSD technology is advantageous over bacterial or phage-based systems as they allow the surface expression of large-molecular-weight proteins. Amongst the yeast, *Saccharomyces cerevisiae* is a popular host that possesses quality control mechanisms (Kuroda and Ueda 2011a). However, apart from *S. cerevisiae*, methylotrophic yeast like *Pichia pastoris* and *Hansenula polymorpha* have also been used as a protein displaying platform. These yeast cells can be grown to high cell densities on low-cost carbon sources, thus making them suitable for large-scale fermentation processes such as the development of whole-cell biocatalysts (Pepper et al. 2008). The cell surface display technology is also extended to include other yeast such as *Yarrowia lipolytica* and *Kluyveromyces*. The inherent ability of *Y. lipolytica* to secrete high quantities of protein into the culture supernatant has enabled the researchers to exploit its potential as a display host. The "GRAS" status of *Kluyveromyces* and its thermotolerance properties make it an excellent host for food fermentation processes. Although members of this genus, *K. marxianus* and *K. lactis*, are well-known for carrying out industrial processes, however, their acceptability for surface display is still very limited (Andreu and Del Olmo 2018).

The yeast cell wall proteins majorly comprise of the GPI (glycosylphosphatidylinositol)-modified cell wall proteins. The covalently linked GPI modified proteins can be dissociated from the yeast cell walls via the action of β-1,3-glucanases and β-1,6-glucanases as they are indirectly connected to the β-1,3-glucans via short β-1,6-glucan bridges. The GPI anchored proteins are crucial for maintaining cell viability and morphology and are found across all eukaryotic organisms. The GPI-linked proteins function as hydrolytic enzymes, receptors, and adhesion molecules. They also participate in flocculation, mat and biofilm formation, and mating. The general characteristic of these proteins includes a signal sequence at N-terminus and a C-terminal domain having GPI attachment site or the ω site. They also contain a Ser/Thr-rich region and lack a transmembrane region (Ecker et al. 2006; Klis et al. 2006; Zhang et al. 2013). The other group contains proteins that are linked to β-1,3-glucans of the cell wall via an alkali-sensitive linkage. The PIR group proteins (protein with internal repeats) are covalently associated with the β-1,3-glucans via an alkali-labile ester linkage and possess repetitive amino acid blocks. They lack a GPI attachment signal, and their linkage to the yeast cell wall makes them susceptible to mild alkali; hence they are released by treating the cell

wall with 30 mM NaOH at 4 °C. Since they are associated with the β-1,3-glucans directly, therefore, they can be extracted by the enzymatic action of β-1,3-glucanases but not β-1,6-glucanases (Ecker et al. 2006; Klis et al. 2006).

The *S. cerevisiae* α-agglutinin and **a**-agglutinin are two different GPI-linked cell wall proteins frequently used as anchors in cell surface display strategy. These two proteins undertake the phenomenon of sexual adhesion during mating. The presence of a secretion signal at the N-terminal region enables their transport via the action of phosphatidylinositol-specific phospholipase C (PI-PLC) on the cell surface. The **a**-agglutinin display system utilizes a different strategy as it comprises two protein subunits, Aga1p and Aga2p. The formation of two disulfide bonds between them helps associate the secreted Aga2p protein to the Aga1p, which are then linked to the yeast cell wall. The Aga1p–Aga2p display platform allows for the dual strategy to connect the target protein to the N-terminus or the Aga2p protein's C-terminus (Cherf and Cochran 2015; Ueda 2016). The flocculin protein Flo1p is another established cell wall protein in which the truncated form of Flo1p lacking its GPI attachment site is utilized. The adhesive features of this protein's functional domain are exploited where the N-terminal region of the target is fused with the truncated Flo1p protein. The other strategy involving the Flo1p protein employs its GPI attachment signal, where the C-terminal region of the passenger protein is linked with the Flo1p protein. Variable anchor lengths of Flo1p have been used depending on repeat sequences and the GPI attachment site. Several other GPI-modified proteins, such as Sed1p, Tir1p, Cwp1p, Cwp2p, and Tip1p, have been tested for their anchoring properties (Kondo and Ueda 2004; Ueda 2016).

The PIR proteins are a group of cell wall proteins having repetitive sequences consisting of highly conserved amino acid residues including serine, glutamine, aspartic acid, glycine, alanine, and threonine (S, Q, D, G, Q, Q, A, T) and a C-terminal domain comprising four cysteine residues. They possess an N-terminal signal peptide and a site recognized for cleavage by the Kex2 protease. They do not use the GPI anchoring apparatus and instead have two different mechanisms by which these proteins bind to the cell wall. The first interaction involves an alkali-sensitive ester linkage. The second is based on disulfide linkages involving at least three out of the four cysteine residues with the cell wall. The PIR proteins are versatile as they help in the introduction of three different approaches to yeast cell surface display. The N-terminal fusion strategy is undertaken when the C-terminal cysteine residues ensure attachment to the cell wall via the disulfide bonds. The C-terminal fusion strategy is used when the repetitive blocks within the PIR sequence result in the formation of ester linkages with the cell wall. The third strategy involves the insertion of target protein in between the PIR protein, with or without replacing its sequence. This strategy has been employed for the Pir4 protein, where the elements responsible for the cell wall attachment must be preserved. The changes in regions responsible for cell wall attachment led to protein secretion in the extracellular medium (Andreu and Del Olmo 2018). PIR proteins play a role in the response against heat shock, maintenance of the cell wall structure, and conferring resistance to osmotin, a plant antifungal agent. In *S. cerevisiae*, five different PIR family members possessing a variable number of repetitive units have been

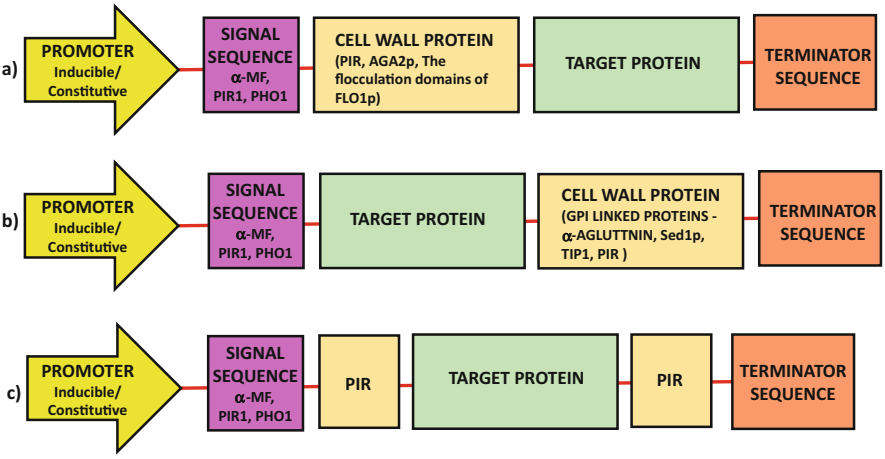

Fig. 21.2 The different strategies employed for yeast cell surface display. (**a**) The C-terminal fusion strategy: The C-terminus of the yeast cell wall protein is fused to the N-terminus of the target protein. (**b**) The N-terminal fusion strategy: The N-terminus of the yeast cell wall protein is fused with the C-terminal region of the target protein. (**c**) The internal fusion strategy: The target protein is sandwiched between the parts of the anchor protein. This strategy has been undertaken for the Pir protein where the components involved in the cell wall attachment are retained

reported. The presence of PIR proteins in the cell walls of other yeasts such as *Candida albicans*, *Yarrowia lipolytica*, *Pichia pastoris*, *Zygosaccharomyces rouxii*, and *Kluyveromyces lactis* has been reported (Kandasamy et al. 2000; Jaafar et al. 2003; Khasa et al. 2011) (Fig. 21.2).

21.6.2 Applications of Yeast Cell Surface Display

The construction of whole-cell biocatalysts provides an added advantage at an industrial scale since the expressed enzymes demonstrate higher stability and reusability. Several amylolytic and cellulolytic enzymes have been surface expressed to hydrolyze cellulose and raw starch to glucose for ethanol production (Apiwatanapiwat et al. 2011; Baek et al. 2012). *Rhizopus oryzae* glucoamylase-displaying recombinant *S. cerevisiae* cells successfully hydrolyzed starch to glucose and subsequently to bioethanol. With a similar approach, the Flo1p flocculation protein was also used to display glucoamylase. Cellulolytic enzymes such as *Aspergillus aculeatus* endoglucanase and β-glucosidase and *Trichoderma reesei* endoglucanase II have been expressed on yeast cells' surface, possessing the capacity to thrive only on β-glycan. The conversion of cellulose to ethanol after a pretreatment step with phosphoric acid was catalyzed by armed yeast cells co-displaying three cellulose-degrading enzymes, endoglucanase II from *T. reesei*, cellobiohydrolase II, and *A. aculeatus* β-glucosidase (Padkina and Sambuk 2018). The co-expression of β-mannanase and β-mannosidase was successfully

demonstrated on *S. cerevisiae*, where recombinant cells showed efficient utilization of linear mannans to produce bioethanol (Ishii et al. 2016).

Lipases are commercially important enzymes responsible for catalyzing transesterification reactions and esters synthesis. The recombinant arming yeast cells displaying lipase are used in biofuel production, synthesis of food, pharmaceutical agents, and chemicals. Several anchor proteins have been used to display lipases from different sources. The Flo1p and α-agglutinin proteins have been used to display *Rhizopus oryzae* lipase (ROL) on *S. cerevisiae*'s surface (Matsumoto et al. 2002; Shiraga et al. 2005). The same anchor proteins have been tested to display *Rhizomucor miehei* lipase (RML) on *P. pastoris* and *S. cerevisiae* cells. Several other lipases from *Geotrichum candidum*, *Candida antarctica*, *Rhizopus stolonifer*, *Aspergillus niger*, *Pseudomonas fluorescens* and *Bacillus subtilis* have been immobilized using different anchor proteins. Using Aga2 subunit of **a**-agglutinin, Flo1p and Cwp2, three lipases from *Y. lipolytica* were successfully expressed on *S. cerevisiae* and *P. pastoris* cell surface (Liu et al. 2014).

The concept of bioremediation using microbial surface display has gained immense interest and investigation. Bioadsorption of metal ions found in polluted water bodies can be carried out by surface expression of metal-binding proteins. Proteins such as metallothionein, six residues of histidine, mercury-responsive metalloregulatory proteins, as well as histidine and cysteine-rich peptides are used (Shibasaki and Ueda 2014). A hexa-his displaying armed *S. cerevisiae* strain was engineered, which had acquired the self-accumulation ability in response to heavy metals. The bioremediation of xenobiotic compound was also studied by surface expression of organophosphorus hydrolase (OPH) of *Flavobacterium* sp. on *S. cerevisiae* (Saleem et al. 2008).

The yeast display technology has been comprehensively used for the affinity maturation technique for antibodies. A high affinity is a desirable characteristic property for therapeutic and diagnostic biomolecules. The process of affinity maturation is initiated by constructing a yeast library consisting of protein mutants generated by random mutagenesis. Further, the molecular display technique is used for protein engineering to increase their thermal stability and catalytic efficiency (Cherf and Cochran 2015).

The yeast display approach has gained enormous popularity in the field of biomedical science. The mapping of protein epitopes, vaccine development, and surface expression of antibacterial agents such as pediocin are some of its significant potential applications. The surface display of immunogenic molecules is a novel approach to develop oral vaccines. The oral vaccine against candidiasis was developed using the *S. cerevisiae* display system. A recombinant budding yeast was genetically modified to express PCV2b (Porcine circovirus type 2b) Cap protein and fed in a freeze-dried form to pigs that helped to confer protection against PCV2b (Kumar and Kumar 2019). The envelope protein from HIV was also displayed using the yeast cell surface display system (Mathew et al. 2018). The α-agglutinin cell wall anchoring protein was successfully employed to target pediocin to the cell wall, followed by a demonstration of its inhibitory activity against *Shigella boydii* and *Shigella flexneri* (Nguyen et al. 2020).

21.7 Yeast Expression Systems: Contribution by Indian Scientists and Authors

The heterologous expression of recombinant proteins has been undertaken in a variety of yeast expression platforms. Further, cell surface engineering's strategy enables the display of target proteins on the yeast cell surface with great efficacy leading to the development of whole-cell biocatalysts. Several researchers from India have successfully expressed different target proteins in recombinant yeast cells that find several biotechnological applications. Indian scientists have also documented several reviews on the research work carried out in this field. The following section highlights the experimental studies and reviews authored by Indian researchers and the biotechnological applications associated with different yeast expression platforms.

S. cerevisiae is a standard host that is employed for the production of antigens required for vaccine development. The recombinant proteins can be expressed on yeast cells' surface by carrying out fusions with cell wall proteins such as the Aga2p protein of the **a**-agglutinin system. To study antigen-specific antibody response in mice, the *S. cerevisiae* cells were used to express the envelope protein of Japanese encephalitis virus (JEV), a neurotropic virus. Strong antibody response was detected in the mice following immunization; however, a neutralizing activity of the antibody molecules was not observed (Upadhyaya and Manjunath 2009). The data regarding the specific antibody binding sites or epitopes of an antigen is crucial during vaccine development against many pathogens. A simple, dependable, and rapid method for mapping the conformational epitopes, determining immuno-dominant regions of antigen and protein-ligand interaction sites, was developed using the yeast cell surface display approach. A range of cysteine mutants were targeted to the yeast cell surface, and the epitopes were identified by determining the loss of antibody binding ability via flow cytometry analysis (Najar et al. 2017).

Chymosin is a primary component of the rennet that is frequently used in the cheese industry as a milk clotting enzyme. A buffalo prochymosin was produced using the *P. pastoris* expression system, where its applicability was tested for the manufacturing of Mozzarella cheese (Tyagi et al. 2017). A recombinant goat prochymosin was also successfully expressed using a modified methanol feeding strategy in *P. pastoris* Mut^+ strains (Tyagi et al. 2016). The nitrile hydratase (NHase) enzyme isolated from *Rhodococcus rhodochrous* PA-34 mutant 4D was heterologously expressed in the *P. pastoris* KM-71 strain. The enzyme catalyzed the successful conversion of 3-cyanopyridine to nicotinamide (Pratush et al. 2017). The high demands for global energy have increased focus on the breakdown of lignocellulosic biomass into reducing sugars that can be utilized for biofuel production. Endoglucanases are a group of enzymes involved in the breakdown of cellulose's internal chains. Therefore, thermostable endoglucanase from *Thermoascus aurantiacus* RCKK was cloned in *P. pastoris* X-33 strain, and its high-level expression was optimized. The enzyme was characterized based on its temperature and pH stability and resistance toward ionic liquid, highlighting its usage in the deinking of recycled paper pulp (Jain et al. 2018). The recombinant

expression of a chimeric α-amylase enzyme was targeted in *P. pastoris*, where multicopy integrants were achieved using multiple transformations and post-translational vector amplification (Parashar and Satyanarayana 2017). β-glucosidase is another enzyme with hydrolytic activity and is useful in synthesizing several compounds such as oligosaccharides and alkyl- and aryl-glucosides. *Pichia etchellsii* is an oenological yeast that acts as a good source of thermostable β-glucosidases. Hence, overexpression of this enzyme was targeted in *P. pastoris*. The one-factor at a time (OFAT) approach was undertaken to optimize the effects of antifoam and casamino acid addition along with sorbitol and methanol levels. The predicted model's efficacy was also tested at the bioreactor level (Batra et al. 2014). A novel α-galactosidase (AG) native to *Aspergillus neoniger* and having a high transglycosylation activity was selected and subsequently cloned in *P. pastoris* to improve its productivity (Kumar and Mutturi 2020).

Another enzyme of economic importance, i.e., phytase (PPHY) from *Pichia anomala*, was successfully expressed using the *P. pastoris* expression system. It is an acid phosphatase responsible for removing at least a single phosphate group from phytate. Phosphatase is an attractive candidate as a poultry and fish feed additive and can be employed to deproteinize soymilk. *P. pastoris* was selected as an alternate expression host to target high-level enzyme titers (Joshi and Satyanarayana 2014).

The *P. pastoris* expression system has achieved immense popularity due to its versatility and utility in producing molecules as well as therapeutic and diagnostic compounds. *S*-Adenosylmethionine (SAM) is one such molecule that is a product of methionine metabolism. It is exclusively used as a therapeutic agent for osteoarthritis, liver disease, and depression. Therefore, to achieve a high-level production of recombinant SAM, the *SAM2* gene native to *S. cerevisiae* was used to transform *P. pastoris* cells and grown in the presence of a methionine-rich medium. Fermentation studies were carried out using a 14-L bioreactor, where the heterologous SAM was detected and quantified using the HPLC and LC-MS/MS methods (Kamarthapu et al. 2013). The human growth hormone (rhGH) is another therapeutically relevant molecule that is crucial for treating several diseases like fractures, skin burns, dwarfism, renal malfunction, and growth hormone deficiency. Hence, to target its high-level extracellular expression, the *rhGH* gene was cloned in *P. pastoris* expression vector pPIC9K under the methanol inducible *AOX1* promoter's control (Apte-Deshpande et al. 2009). Galectins are a group of S-type lectins known to play a significant role in inflammation, cancer, and fibrosis. Galectin3 has multiple effects on different physiological processes, such as cell adhesion, activation, chemoattraction, apoptosis, and motility. Hence, the *P. pastoris* expression platform was employed to clone, express, and purify the human galectin3 protein. The recombinant human galectin3 was purified via affinity chromatography, and hemagglutinin inhibition assay was performed to test its biological activity (Kumar Vemuri and Veeravalli 2014). The human interferon-alpha 2b is one of the 13 variants of the IFNα reported for its antiviral, antiproliferative, and immunomodulatory functions. Human interferon-alpha 2b was successfully expressed in a glycoengineered strain of *P. pastoris* (SuperMan5), with human-type N-glycan moieties. The biological activity was further demonstrated by determining its antiproliferative capacity and

growth inhibition using breast cancer cell lines (Katla et al. 2019). Human GM-CSF is a hematopoietic growth factor frequently used to treat neutropenia, aplastic anemia, and myeloid leukemia. However, its recombinant production is hampered because of its toxicity toward expression hosts. To achieve its maximum productivity, its constitutive expression was studied in continuous fermentation mode where a high volumetric productivity of 16.4 mg/L/h was reported in *P. pastoris* under *GAP* promoter in complex media (Khasa et al. 2007).

One of the significant medical interventions for treating cardiovascular diseases is the intravenous administration of thrombolytics. Streptokinase is a thrombolytic agent that aids in the dissolution of fibrin blood clots. *P. pastoris* was chosen as the host for its expression, where a large-scale secretory system for the extracellular production of streptokinase was developed (Adivitiya et al. 2016). A comparative analysis of the secretion efficiency of the α-factor of *S. cerevisiae* and the PIR1 signal sequence of *P. pastoris* was also carried out where both the signal sequences were successful in targeting the protein to the culture supernatant with comparable efficiency. The approach of cell recycling countered the problem of proteolytic degradation in the case of streptokinase. The gene-dosage optimization strategy and cell-retention technique helped to achieve 2.4-fold higher volumetric product yield (Adivitiya et al. 2019).

The human interleukin-3 (hIL-3) is a multipotent hematopoietic growth factor that plays a therapeutic role in treating blood cancer, immunodeficiency conditions, bone marrow transplantation, cytopenia, and different forms of cancers. Due to its low expression in bacterial systems such as *E. coli*, *Bacillus subtilis*, and *Streptomyces lividans*, as well as baculovirus and mammalian cell lines, an alternate expression host, *P. pastoris*, was chosen for its high-level production. Using the α-mating factor secretion signal, the constitutive and inducible expression of hIL-3 was targeted in *P. pastoris* cells (Dagar et al. 2016).

The *P. pastoris*-based expression system is broadly engaged for vaccine development and manufacturing. Several antigenic peptides have been expressed in *P. pastoris* for targeting efficient vaccine generation. A high yield of 1.5–2.0 g/L was observed when the Mb86 antigen of *Boophilus microplus* tick affecting cattle was expressed in *P. pastoris*. The recombinant protein could assemble to form immunogenic particles that could effectively protect against ticks. Several other antigenic peptides like *Bordetella pertussis* pertactin (P69) and Human papillomavirus (HPV) type 6 L1 protein were efficaciously produced in this heterologous system (Balamurugan et al. 2007).

Rabies is a fatal condition that is lethal in 100% of cases if a proper treatment line is not available. Hence, efforts were made to develop vaccines based on rabies virus glycoprotein that is both antigenic and immunogenic. The CVS rabies glycoprotein was produced with success in *P. pastoris* at a level of 200 mg/L (Nagesha et al. 2010). The 2009 H1N1 pandemic created a need for vaccines that were produced in chicken eggs. However, the production system had severe complications such as allergic reactions and limited availability of the vaccines. Hence, *Pichia*-based HA production was established, where purified product elicited the immune response and produced neutralizing antibodies in BALB/c mice and rabbits. The expression of

the HA protein's biologically active trimeric form was successfully targeted for extracellular production using the *P. pastoris* system (Athmaram et al. 2011, 2012). The generation of virus-like particles (VLPs) is a viable approach to prevent viral transmission and infection. The VLPs are nano-sized non-infectious particles that hold the viral structural proteins. However, due to the several drawbacks associated with the other expression systems, the *P. pastoris* expression platform was employed to generate VLPs of the Chikungunya virus (CHIKV). The yeast-derived VLPs were morphologically similar to the CHIKV and successfully elicited a humoral and cell-mediated immune response (Saraswat et al. 2016). Like the CHIKV VLPs, the virus-like particles for the hepatitis E virus were expressed in *P. pastoris* to confirm the yeast system's better efficiency for the VLPs production than the baculovirus system. The purified protein showed enhanced immune response in the BALB/c mice, further elucidating that the yeast system is a better alternative than the existing baculovirus expression system (Gupta et al. 2020).

The yeast *H. polymorpha* has also emerged as one of the platforms for the development of vaccines. Hepatitis B is a major human disease of world's concern where the hepatitis B virus (HBV) significantly affects more than two billion people. A vaccine for HBV, Engerix-B, was developed by the pharmaceutical giant GlaxoSmithKline (Rixensart, Belgium). The GeneVac-B is another HBV vaccine manufactured by the Serum Institute of India Ltd., Pune, India. A comparative analysis of these vaccines has been carried out where the immunogenicity, efficiency, and safety of the low-cost Indian recombinant vaccine have been compared with other commercially available vaccines. In the case of Shanvac B® (Shantha Biotechniques Ltd., Hyderabad, India) and Engerix-B (GlaxoSmithKline, Rixensart, Belgium), no significant deviation in therapeutic efficacy was obtained (Shivananda et al. 2006; Velu et al. 2007).

The industrially important phytase and lipase enzyme production was successfully obtained in *H. polymorpha*. *Pichia anomala* phytase enzyme was cloned and expressed in different eukaryotic expression hosts, *S. cerevisiae*, *Arxula adeninivorans*, and *H. polymorpha*, where *H. polymorpha* was observed to be the preferable host for enzyme production (Kaur et al. 2010). In another work, the *Yarrowia lipolytica* lipase enzyme, YlLIP11, was expressed along with a signal sequence in the three hosts, *S. cerevisiae*, *Arxula adeninivorans*, and *H. polymorpha*, by utilizing the *Arxula*-derived *TEF1* promoter. Recombinant lipase showed low expression yields in *H. polymorpha* as compared to others but displayed a higher extent of glycosylation and better thermostability (Kumari et al. 2015).

Therapeutic proteins such as human serum albumin have been successfully expressed using the *K. marxianus* expression system. An efficient human interferon-beta extracellular expression was targeted using a codon-optimized signal sequence of glucoamylase native to *Aspergillus niger*. The recombinant protein was successfully purified in its biologically active form (Madhavan and Sukumaran 2016). A group of researchers isolated the tyrosinase gene from *Aspergillus oryzae* and expressed it in *Y. lipolytica* strain Polg. The recombinant strain expressing tyrosinase with a high specific activity of 10.94 U/mg was screened for its L-tyrosine

to L-DOPA transformational capacity. The final drug product is used to treat Parkinson's disease (Rao et al. 2011).

One major issue reported in filamentous fungi is their peculiar growth characteristics during cultivation at a large scale. Under submerged fermentation conditions, they tend to form mycelium clumps that form cotton-like large balls. These structures tend to stick to the fermenter surface and pose a barrier to oxygen and mass transfer. However, the pelleted growth displayed by some fungi is considered to be beneficial since it allows for enhanced biomass and product formation. A novel filamentous fungus, *Aspergillus unguis* NII 08123, was isolated that demonstrated excellent properties while growing under submerged fermentation conditions. It grew in the form of pelleted culture in an even suspension but did not aggregate to form large balls, thereby permitting better oxygen transfer. The organism has also shown good growth properties on lignocellulosic biomass. The production of EGFP protein was reported in *A. unguis*, where its expression was controlled by the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) promoter highlighting its potential as an expression host (Madhavan et al. 2017b). Heterologous production of enzymes such as aminopeptidase, β-glucanase, and mannanase has been done in *Trichoderma reesei*, whereas *T. longibrachiatum* has been successfully used for pullulanase production from *Hormoconis* sp. (Sharma et al. 2009).

21.8 Recombinant Gene Expression and Bioprocess Optimization Using Yeast Expression System

21.8.1 Introduction

The optimization studies at bioreactor is a critical aspect of the cost-effective production of many proteins of therapeutic and industrial use. Hence, the process designed at the laboratory scale for 0.5–10 L fermentors is further advanced to manufacturing-scale bioreactors of thousands of liters. Scaling-up studies at the industrial level are carried out in two different phases. The initial step comprises the pilot-scale fermentation work in 100–100,000 L bioreactors with an integrated downstream processing scheme. The second phase of the scale-up process is the demonstration plant, where 10,000–100,000 L fermenters are operated. The demo plant's role is to further analyze the risks involved in the manufacturing process as it confirms and validates the process, raw materials, and market needs. Additionally, the fermentation quality also impacts its downstream process, the quantity, and the nature of the wastes generated. The fermentation run is considered an intricate process as several of the affecting parameters tend to deviate during the scale-up process. Hence, it is imperative that these factors need to be well understood to ensure good performance of the fermentation run. An optimized fermentation process must perform well at the manufacturing scale to ease product recovery (Crater and Lievense 2018).

There are several parameters that a yeast host strain requires to express the heterologous protein with high efficacy. The development of a robust, quick, and reproducible fermentation process with an economical downstream processing is a prerequisite for the synthesis of foreign proteins using batch and fed-batch fermentation design. The important group of Crabtree-negative yeast is the methylotrophs. In most methylotrophic yeasts, two types of phenotypes are observed, i.e., Mut^+ and the Mut^-. However, *P. pastoris* stands as a unique case since it carries two alcohol oxidase genes (*AOXI* and *AOX2*). The *AOX1* hyper-expression is majorly responsible for methanol utilization, whereas *AOX2* expression was observed at the basal level. The *AOXI* mutated strain shows slower growth on methanol, thereby allowing cellular growth in the presence of controlled methanol concentration at lower growth rates (Porro and Mattanovich 2004).

21.8.2 Effect of Cultivation Media

The design of an industrial fermentation process majorly depends on the cultivation media. The media composition affects the final product yields, volumetric product concentration, as well as productivity. The medium cost significantly impacts the process economics, especially in the case of commercial products. Optimization of the cultivation medium is generally commenced with a "one-at-a-time" approach. However, this method has certain disadvantages since it is time-intensive and does not give an insight into the system's behavior. Several statistical-based experimental methods have been used to produce numerous microbial enzymes and products (Gigras et al. 2002).

The rich medium supports vigorous cellular growth compared to mineral media since it contains biosynthetic precursor molecules that can be directly utilized in the anabolic pathways. However, in mineral media, the biosynthetic precursors need to be synthesized by microorganisms by utilizing metabolic energy that significantly impacts the growth and final product yields. A threefold increase in laccase production was recorded when *Yarrowia lipolytica* was switched from Yeast Nitrogen Base (YNB) to a complex medium. The nitrogen source in fermentation cultivation media plays a crucial role in regulating cultivation pH by providing a buffering capacity. The industrial-scale bioreactors can utilize the protein components of cheese whey, which has effectively improved recombinant protein production compared to the mineral medium. However, other contradictory reports state that cheese whey usage results in decreased production levels and slower growth than mineral medium containing lactose. The nitrogen components present in the cultivation media also aid in protecting the recombinant proteins from proteolysis.

The proteolytic degradation of heterologous proteins usually results from carbon starvation conditions or glucose depletion. Hence, complex nitrogen sources also help limit degradation as observed in many yeast. The supplementation of casamino acids, skim milk, bovine serum albumin, amino acids, and peptides helps in protecting the recombinant proteins either by reducing the production of extracellular proteases or acting as a competitive substrate for proteases. The media

components enormously impact the fermentation process's economics. In some instances, it accounts for 30% of the total price. The large-scale production of low-priced products such as fuels, chemicals, and materials requires inexpensive raw materials. Hence, their industrial-scale processes generally utilize by-products of agricultural, forest, or chemical sources such as sugar beet, sugar cane molasses, spent sulfite liquor, and cheese whey as the carbon source. The spent yeast biomass replaces the expensive yeast extract as the nitrogen source. Another widely used industrial-scale nitrogen source is corn-steep liquor that is obtained as a by-product of the starch industry (Hahn-Hagerdal et al. 2005).

21.8.3 Physical Parameters: pH, Temperature, and DO Control

The temperature of the fermentation process plays a crucial role in regulating protein expression. To target the intracellular protein production, lower cultivation temperature has been shown to promote soluble expression due to enhanced chaperon activity. The protease-mediated degradation of heterologous proteins during large-scale fermentation is a major concern. Process parameters such as temperature, pH, and induction time have been shown to affect the degradation process. Hence, optimizing these factors may disrupt protease activity, thus imparting high stability to the desired protein. Cultivation temperature has a pronounced effect on cellular physiology, product stability, activity, and yields. For yeast such as *P. pastoris*, the optimal growth temperature has shown to be 30 °C, and cultivation above 32 °C drastically affects cellular growth and inhibits recombinant protein production (Cos et al. 2006). Li et al. 2001 have demonstrated that a decrease in temperature to 23 °C from 30 °C resulted in a threefold increase in yeast protein production (Li et al. 2001). Expression at 15 °C in *P. pastoris* resulted in enhanced production of ScFv; however, the total fermentation time increased.

Dissolved oxygen is a critical growth parameter that governs the microorganism's physiology and cellular health in a bioreactor environment. The higher metabolic activity during the exponential growth phase is associated with enhanced oxygen requirement and faster substrate consumption. Therefore, determining the dissolved oxygen concentration during the fermentation process is an absolute requirement. An increase in the DO concentration beyond the saturation level indicates substrate exhaustion, whereas an extremely low DO level points toward stressful conditions. Hence, maintenance of an optimized DO concentration translates into high yield and productivity (Vandermies and Fickers 2019). The optimal levels of oxygen are maintained using agitation, aeration, and supplementation of pure oxygen. In most of the fermentation processes, the DO value is set at 20% or above. In fermentation employing *Pichia* as host, the DO value must be sustained at 30–35% (Li et al. 2007).

The fermentation medium pH is another crucial factor for microbial growth and product formation. The alteration in the pH from optimal value can adversely affect cellular physiology and may cause protease release (Li et al. 2007). The deviation of pH above or below from the optimal pH of 6 releases an alkali (AEP) and acid (AXP)

class of extracellular proteases that could lead to product degradation (Young et al. 1996; Vandermies and Fickers 2019). During fermentation, a decrease in the pH from the optimal set value may indicate cell lysis and protease release. Moreover, pH also affects the recombinant protein stability and activity, and hence maintenance of optimal pH value is essential for designing scale-up processes. The optimal pH value for *P. pastoris* is in the range of 5–6. An increase in pH beyond this value causes salt precipitation and protein degradation by proteases (Cregg et al. 1993; Zhang et al. 2000; Cos et al. 2006). The cultivation of *Pichia* cells at pH 3.0 prevented the proteolysis of insulin-like growth factor (IGF-1).

21.8.4 Product Recovery and Downstream Processing

Downstream processing (DSP) includes all the steps that are performed after the completion of the fermentation process. The DSP consists of stages ranging from product collection to its final drying or crystallization. The intended use of targeted protein governs the product recovery steps, and for certain proteins, it constitutes more than 80% of the overall process cost (Lowe et al. 2001; Antecka et al. 2019). Thus, the primary objective of any recovery process is to produce a high-quality product using cost-effective measures. The fermentation parameters, such as antifoam agents, media impurities, and microorganisms, also affect the recovery steps. Downstream processing is generally divided into three key steps: solid-liquid separation, product enrichment, and purification (Hatti-Kaul 2010).

(a) *Solid-liquid separation*: This step involves the separation of cells and impurities from the fermentation broth. It can be achieved by the use of centrifugation and filtration method. The filtration methods available for solid-liquid separation are plate and frame filters, filter press, membrane filter press, and vacuum filters (Krijgsman 1992; Doran 1995; Shuler and Kargi 2002; Hatti-Kaul 2010).
(b) *Release of intracellular components*: The recombinant proteins are often expressed intracellularly as inclusion bodies. Recovery of these intracellular products requires efficient and economical methods for cell disruption without compromising the yield. Cell disruption can be achieved via mechanical and non-mechanical procedures. The mechanical process of cell disruption involves the usage of French press (at laboratory scale), Manton and Gaulin homogenizer (for pilot scale), ultrasonication, cell grinding using beads (aluminum, silica, glass beads), and use of bead mills at high speed. Further, the non-mechanical approach of cell disruption can be achieved using heat shock, repetitive freeze-thaw, osmotic shock, and autolysis (Harrison et al. 1991; Harrison 1991; Hatti-Kaul and Mattiasson 2003; Hatti-Kaul 2010).
(c) *Product recovery*: After the cellular disruption, the intracellular proteins present in the soluble fractions are recovered using centrifugation. For inclusion bodies (IBs), an extra step of protein solubilization via chaotropic agents (urea) is applied. The solubilized proteins are then treated similarly to that of extracellular produced protein (Stephanopoulos 1993; Hatti-Kaul 2010). The product can

be recovered or concentrated using membrane filtration (microfiltration and ultrafiltration), precipitation (use of inorganic salts such as ammonium sulfate), aqueous two-phase separation (used for recovery of antibiotics, vitamins), chromatography technique (adsorption, gel filtration, affinity, and ion exchange), and dialysis and distillation (for volatile product recovery) (Hatti-Kaul 2001, 2010; Khayet and Matsuura 2004; El-Bourawi et al. 2006).

(d) *Finishing steps*: After obtaining the product with high purity, it can be further subjected to drying and crystallization (Schmidt et al. 2005; Hatti-Kaul 2010).

21.8.5 Solid-State Fermentation (SSF) and Submerged Fermentation (SMF)

In SSF, the organisms are allowed to grow on a solid substrate with minimal free water presence, thus mimicking the natural environment in which the selected organism, such as fungi, grows. The moisture content is maintained between 12% and 70%. The typical example of SSF includes rice fermentation by *Aspergillus oryzae* for the Koji process and the cheese production by *Penicillium roqueforti* (Pandey 1994, 2003; Couto and Sanromán 2006; Chen 2013; Singhania et al. 2015). The foremost advantage of the SSF method is its ability to use a wide range of substrates for growth, including agricultural waste (such as lignocellulosic), thereby allowing sustainable and economical product development (Watanabe et al. 2002; Behera and Ray 2016; Manan and Webb 2017). It eliminates the need for managing process parameters and has been shown to produce higher product yield and stability. The disadvantages of the SSF process are uneven distribution of temperature, aeration, moisture, pH, and difficulty in measuring cell biomass and growth kinetics parameters (Prior et al. 1992; Pandey 2003; Manan and Webb 2017). The most suitable organisms for SSF are filamentous fungi as they can quickly grow on low moisture substrate. However, bacteria need high water activity and thus are not much suited for SSF. The dominant hosts for the SSF process are *Aspergillus*, *Trichoderma*, *Mucor*, *Rhizopus*, *Penicillium*, *Fusarium*, *Monilia*, and *Alternaria*. The filamentous fungi secrete large quantities of extracellular enzymes, and the fungal mycelia can easily penetrate the solid substrate. The SSF process has been successfully used in the production of pharmaceuticals (antibiotics), industrial enzymes (lipase, amylase, cellulase, glucoamylase, xylanases), fermented food products, biopesticides, organic acids (Kojic acid, ethanol), aroma substances, and polymers (xanthan gum) (Manpreet et al. 2005; Wang et al. 2010; Lizardi-Jiménez and Hernández-Martínez 2017).

Submerged fermentation (SMF) is the most commonly used and popularized system for microbial fermentation. SMF allows homogeneous nutrient distribution and ease of controlling process parameters such as pH, temperature, and agitation, thus permitting optimal growth. The disadvantages of SMF include overall expensive procedure due to the high cost of raw material, equipment, and high energy requirements. The production of foam is another major hurdle during process scale-up. The recovery of the product is complicated as compared to SSF and generates a

lot of wastewater. However, the problem of mass and heat transfer in SSF limits its use at large-scale fermentation. In contrast, SMF allows a homogeneous environment where physical parameters can be easily monitored and controlled. Hence, the SMF process is commonly employed in large-scale fermentation to produce various industrially relevant enzymes, therapeutics, antibiotics, and organic acids (Manpreet et al. 2005; Wang and Yang 2007; nee'Nigam and Pandey 2009; Patro and Gupta 2012; Doriya et al. 2016; Manan and Webb 2017). Mrudula and Murugammal (2011) demonstrated the cellulase enzyme production by *Aspergillus niger* culture under both SMF and SSF conditions, where coir waste was used as a substrate for fungal growth. Under optimized conditions, SSF resulted in 14.6-fold higher cellulase productivity compared to SMF (Mrudula and Murugammal 2011).

21.8.6 CSTR Operations with Yeast

Continuous stirred tank reactor (CSTR) involves a constant fresh medium supply throughout the fermentation process with simultaneous removal of spent medium and culture. The advantages of CSTR operation include maximal protein production under optimized conditions and high volumetric productivity. The dilution factor is an essential parameter of the CSTR run. The fermentation process at different dilution rates results in varied product yields, productivities, and quality (Bayrock and Ingledew 2005; Huang and Tang 2007). The use of a continuous process prevents substrate or by-product inhibition as the product is continuously removed. The culture is also maintained under an exponential phase for a prolonged period, resulting in high product yield. The CSTR process is susceptible to contamination due to longer operational time. Moreover, complex equipment, process designing, and high downstream processing cost are a few more drawbacks than fed-batch operations (Ingledew 2003).

The continuous culturing can be accomplished as single-stage or multistage operations. The use of multistage CSTR is more advantageous compared to single stage due to efficient substrate utilization. Further, the residence time for product formation such as ethanol was reduced from 42 to 18 h, when fermentation was carried out in a series of two fermenters having equal volume (Ghose and Tyagi 1979; Bayrock and Ingledew 2005). During the continuous run, one major problem is the maintenance of process and biological system stability for an extended period. The correlation between dilution rate and plasmid stability has been demonstrated during continuous cultivation. In the chemostat system, the dilution rate correlates with the growth rate and can determine the relationship between growth rate and plasmid stability (Khasa et al. 2007). In the case of *S. cerevisiae*, an increase in plasmid stability was observed with an increase in growth rate when grown on a selective medium (Parker and DiBiasio 1987; Zhang 1997). In contrast, under non-selective medium conditions, inverse relation was seen where increased growth rate resulted in a decrease in plasmid stability. This could be due to the plasmid carrying cells' enhanced specific growth rate and an increase in the dilution rate in selective medium (Zhang 1997).

For increasing the biomass and product yield, the concept of cell recycling has been put forward. This strategy successfully enhanced recombinant streptokinase yields in *Pichia pastoris* (Adivitiya et al. 2019). Further, immobilized cells are also used to improve the product yield in a continuous process. Immobilization allows ease of cell recovery, controls cell washout at a high dilution rate, and prevents cell damage. A fibrous bed bioreactor has also been used for a continuous process using immobilized cells (Huang et al. 1998; Zhu et al. 2002; Huang and Tang 2007).

Continuous culture run at optimal dilution rate has been employed for improving recombinant product formation in *P. pastoris*. Production of puroindoline a at an optimized dilution rate of 0.025 h^{-1} in continuous culture resulted in tenfold increase in the expression levels (Issaly et al. 2001). Bayrock and Ingledew (2005) demonstrated bioethanol production in batch, single-stage, and multistage continuous reactors. The multistage CSTR had the same operating volume as that of a single stage. The cultivation of *S. cerevisiae* in a medium containing 260 g/L of glucose resulted in maximum ethanol productivity under multistage CSTR operation. The multistage CSTR yielded 12.7 g/L/h of ethanol productivity (Bayrock and Ingledew 2005).

21.9 Conclusions

Different microbial hosts such as bacteria, yeast, molds, and mammalian system are used to produce vaccines, enzymes, proteins, and secondary metabolites. The yeast expression system, specifically, the *S. cerevisiae* and *P. pastoris*, holds an upper hand compared to others to produce authentic target proteins, especially of eukaryotic origin. Various strain improvement methods involving sexual and asexual hybridization are employed to achieve higher metabolite production. Yeast strains have been engineered using metabolic engineering approach to achieve higher production of desired products, where pathways encoding for the target protein are modified using molecular genetics. Further, the surface display technique allows whole-cell immobilization and cost-effective production of various industrially relevant value-added products. The optimization of scale-up strategies at the bioreactor level leads to gram levels of product formation.

References

Adivitiya, Dagar VK et al (2016) High level production of active streptokinase in *Pichia pastoris* fed-batch culture. Int J Biol Macromol 83:50–60

Adivitiya, Babbal et al (2019) Development of a streptokinase expression platform using the native signal sequence of the protein with internal repeats 1 (PIR1) in *P. pastoris*: gene dosage optimization and cell retention strategies. Process Biochem 83:64–76

Ahmad M, Hirz M et al (2014) Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol 98:5301–5317

Allgaier S, Taylor RD et al (2009) Vaccine production in *Neurospora crassa*. Biologicals 37:128–132

Allonso D, Pereira IB et al (2019) Expression of soluble, glycosylated and correctly folded dengue virus NS1 protein in *Pichia pastoris*. Protein Expr Purif 162:9–17

Amorim FG, Boldrini-França J et al (2018) Heterologous expression of rTsHyal-1: the first recombinant hyaluronidase of scorpion venom produced in *Pichia pastoris* system. Appl Microbiol Biotechnol 102:3145–3158

Andreu C, Del Olmo ML (2018) Yeast arming systems: pros and cons of different protein anchors and other elements required for display. Appl Microbiol Biotechnol 102:2543–2561

Antecka A, Blatkiewicz M et al (2019) Comparison of downstream processing methods in purification of highly active laccase. Bioprocess Biosyst Eng 42:1635–1645

Antoniewicz MR, Kraynie DF et al (2007) Metabolic flux analysis in a nonstationary system: fed-batch fermentation of a high yielding strain of *E. coli* producing 1,3-propanediol. Metab Eng 9:277–292

Apiwatanapiwat W, Murata Y et al (2011) Direct ethanol production from cassava pulp using a surface-engineered yeast strain co-displaying two amylases, two cellulases, and beta-glucosidase. Appl Microbiol Biotechnol 90:377–384

Apte-Deshpande A, Rewanwar S et al (2009) Efficient expression and secretion of recombinant human growth hormone in the methylotrophic yeast *Pichia pastoris*: potential applications for other proteins. Biotechnol Appl Biochem 54:197–205

Arico C, Bonnet C, Javaud C (2013) N-Glycosylation humanization for production of therapeutic recombinant glycoproteins in *Saccharomyces cerevisiae*. Methods Mol Biol 988:45–57

Arya R, Bhattacharya A, Saini KS (2008) *Dictyostelium discoideum*—a promising expression system for the production of eukaryotic proteins. FASEB J 22:4055–4066

Athmaram T, Saraswat S et al (2011) Yeast expressed recombinant Hemagglutinin protein of novel H1N1 elicits neutralising antibodies in rabbits and mice. Virol J 8:1–13

Athmaram T, Saraswat S et al (2012) Influence of copy number on the expression levels of pandemic influenza hemagglutinin recombinant protein in methylotrophic yeast *Pichia pastoris*. Virus Genes 45:440–451

Baek SH, Kim S et al (2012) Cellulosic ethanol production by combination of cellulase-displaying yeast cells. Enzyme Microb Technol 51:366–372

Baeshen NA, Baeshen MN et al (2014) Cell factories for insulin production. Microb Cell Fact 13:141

Bailey JE (1991) Toward a science of metabolic engineering. Science 252:1668–1675

Bailey JE, Sburlati A et al (2002) Inverse metabolic engineering: a strategy for directed genetic engineering of useful phenotypes. Biotechnol Bioeng 79:568–579

Bajwa PK, Pinel D et al (2010) Strain improvement of the pentose-fermenting yeast *Pichia stipitis* by genome shuffling. J Microbiol Methods 81:179–186

Balamurugan V, Reddy G, Suryanarayana V (2007) *Pichia pastoris*: a notable heterologous expression system for the production of foreign proteins—vaccines. Indian J Biotechnol 6:175–186

Bankar AV, Kumar AR, Zinjarde SS (2009) Environmental and industrial applications of *Yarrowia lipolytica*. Appl Microbiol Biotechnol 84:847–865

Bao Z, Xiao H et al (2015) Homology-integrated CRISPR–Cas (HI-CRISPR) system for one-step multigene disruption in *Saccharomyces cerevisiae*. ACS Synth Biol 4:585–594

Barre P, Vezinhet F et al (1993) Genetic improvement of wine yeasts. In: Fleet GH (ed) Wine microbiology and biotechnology. Taylor and Francis, New York, pp 265–287

Batra J, Beri D, Mishra S (2014) Response surface methodology based optimization of beta-glucosidase production from *Pichia pastoris*. Appl Biochem Biotechnol 172:380–393

Bayrock DP, Ingledew W (2005) Ethanol production in multistage continuous, single stage continuous, *Lactobacillus*-contaminated continuous, and batch fermentations. World J Microbiol Biotechnol 21:83–88

Becerra M, Prado SD et al (2001) New secretory strategies for *Kluyveromyces lactis* beta-galactosidase. Protein Eng 14:379–386

Behera SS, Ray RC (2016) Solid state fermentation for production of microbial cellulases: recent advances and improvement strategies. Int J Biol Macromol 86:656–669
Biot-Pelletier D, Martin VJ (2014) Evolutionary engineering by genome shuffling. Appl Microbiol Biotechnol 98:3877–3887
Blazeck J, Hill A et al (2015) Metabolic engineering of *Yarrowia lipolytica* for itaconic acid production. Metab Eng 32:66–73
Boer E, Steinborn G et al (2007) Yeast expression platforms. Appl Microbiol Biotechnol 77:513–523
Bond CM, Tang Y (2019) Engineering *Saccharomyces cerevisiae* for production of simvastatin. Metab Eng 51:1–8
Bond C, Tang Y, Li L (2016) *Saccharomyces cerevisiae* as a tool for mining, studying and engineering fungal polyketide synthases. Fungal Genet Biol 89:52–61
Branduardi P, Valli M et al (2004) The yeast *Zygosaccharomyces bailii*: a new host for heterologous protein production, secretion and for metabolic engineering applications. FEMS Yeast Res 4:493–504
Bredell H, Smith JJ et al (2016) Expression of rotavirus VP6 protein: a comparison amongst *Escherichia coli*, *Pichia pastoris* and *Hansenula polymorpha*. FEMS Yeast Res 16:fow001
Bredell H, Smith JJ et al (2018) Expression of unique chimeric human papilloma virus type 16 (HPV-16) L1–L2 proteins in *Pichia pastoris* and *Hansenula polymorpha*. Yeast 35:519–529
Breuer U, Harms H (2006) *Debaryomyces hansenii*—an extremophilic yeast with biotechnological potential. Yeast 23:415–437
Byrne B (2015) *Pichia pastoris* as an expression host for membrane protein structural biology. Curr Opin Struct Biol 32:9–17
Cataldo VF, Arenas N et al (2020) Heterologous production of the epoxycarotenoid violaxanthin in *Saccharomyces cerevisiae*. Metab Eng 59:53–63
Celik E, Calik P (2012) Production of recombinant proteins by yeast cells. Biotechnol Adv 30:1108–1118
Chan MK, Lim SK et al (2018) Expression of stable and active human DNA topoisomerase I in *Pichia pastoris*. Protein Expr Purif 141:52–62
Chee MK, Haase SB (2012) New and redesigned pRS plasmid shuttle vectors for genetic manipulation of *Saccharomyces cerevisiae*. G3 (Bethesda) 2:515–526
Chen H (2013) Modern solid state fermentation: theory and practice. Springer, Dordrecht, pp 1–324
Cherf GM, Cochran JR (2015) Applications of yeast surface display for protein engineering. Methods Mol Biol 1319:155–175
Choi JH, Keum KC, Lee SY (2006) Production of recombinant proteins by high cell density culture of *Escherichia coli*. Chem Eng Sci 61:876–885
Cohen SN, Chang AC et al (1973) Construction of biologically functional bacterial plasmids in vitro. Proc Natl Acad Sci U S A 70:3240–3244
Conde J, Fink GR (1976) A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. Proc Natl Acad Sci U S A 73:3651–3655
Cos O, Ramon R et al (2006) Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. Microb Cell Fact 5:17
Couto SR, Sanromán MA (2006) Application of solid-state fermentation to food industry—a review. J Food Eng 76:291–302
Cox K, Fishman S et al (1987) Cloning and characterization of genes involved in tylosin biosynthesis. In: Alacevic M, Hranueli D, Toman Z (eds) Genetics of industrial microorganisms, Part B. Ognjen Prica Printing Works, Karlovac, pp 337–346
Crater JS, Lievense JC (2018) Scale-up of industrial microbial processes. FEMS Microbiol Lett 365:fny138
Cregg JM, Vedvick TS, Raschke WC (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. Biotechnology 11:905–910

Curran KA, Karim AS et al (2013) Use of expression-enhancing terminators in *Saccharomyces cerevisiae* to increase mRNA half-life and improve gene expression control for metabolic engineering applications. Metab Eng 19:88–97

Dagar VK, Khasa YP (2018) Combined effect of gene dosage and process optimization strategies on high-level production of recombinant human interleukin-3 (hIL-3) in *Pichia pastoris* fed-batch culture. Int J Biol Macromol 108:999–1009

Dagar VK, Adivitiya et al (2016) Bioprocess development for extracellular production of recombinant human interleukin-3 (hIL-3) in *Pichia pastoris*. J Ind Microbiol Biotechnol 43:1373–1386

Daly R, Hearn MT (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J Mol Recognit 18:119–138

Damasceno LM, Huang CJ, Batt CA (2012) Protein secretion in *Pichia pastoris* and advances in protein production. Appl Microbiol Biotechnol 93:31–39

Darby RA, Cartwright SP et al (2012) Which yeast species shall I choose? *Saccharomyces cerevisiae* versus *Pichia pastoris* (review). Methods Mol Biol 866:11–23

Dave KK, Punekar NS (2015) Expression of lactate dehydrogenase in *Aspergillus niger* for L-lactic acid production. PLoS One 10:e0145459

Dave K, Ahuja M et al (2012) A novel selectable marker based on *Aspergillus niger* arginase expression. Enzyme Microb Technol 51:53–58

Demain AL, Adrio JL (2008) Strain improvement for production of pharmaceuticals and other microbial metabolites by fermentation. In: Petersen F, Amstutz R (eds) Natural compounds as drugs Volume I, Progress in drug research, vol 65. Birkhäuser, Basel, Switzerland, pp 251–289

Demain AL, Vaishnav P (2009) Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv 27:297–306

Demeke MM, Dietz H et al (2013) Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. Biotechnol Biofuels 6:1–24

Doran PM (1995) Bioprocess engineering principles. Academic, London, p 439

Doriya K, Jose N et al (2016) Solid-state fermentation vs submerged fermentation for the production of l-asparaginase. In: Advances in food and nutrition research. Elsevier, Burlington, pp 115–135

Drejer EB, Hakvåg S et al (2018) Genetic tools and techniques for recombinant expression in thermophilic Bacillaceae. Microorganisms 6:42

Du J, Yuan Y et al (2012) Customized optimization of metabolic pathways by combinatorial transcriptional engineering. Nucleic Acids Res 40:e142–e142

Duan J, Yang D et al (2019) Efficient production of porcine circovirus virus-like particles using the nonconventional yeast *Kluyveromyces marxianus*. Appl Microbiol Biotechnol 103:833–842

Dunn B, Sherlock G (2008) Reconstruction of the genome origins and evolution of the hybrid lager yeast *Saccharomyces pastorianus*. Genome Res 18:1610–1623

Ecker M, Deutzmann R et al (2006) Pir proteins of *Saccharomyces cerevisiae* are attached to beta-1,3-glucan by a new protein-carbohydrate linkage. J Biol Chem 281:11523–11529

El-Bourawi M, Ding Z et al (2006) A framework for better understanding membrane distillation separation process. J Membr Sci 285:4–29

Fitzgerald I, Glick BS (2014) Secretion of a foreign protein from budding yeasts is enhanced by cotranslational translocation and by suppression of vacuolar targeting. Microb Cell Fact 13:125

Fukuda H, Kondo A, Tamalampudi S (2009) Bioenergy: sustainable fuels from biomass by yeast and fungal whole-cell biocatalysts. Biochem Eng J 44:2–12

Georgieva B, Rothstein R (2002) Kar-mediated plasmid transfer between yeast strains: alternative to traditional transformation methods. Methods Enzymol 350:278–289

Ghosalkar A, Sahai V, Srivastava A (2008) Secretory expression of interferon-alpha 2b in recombinant *Pichia pastoris* using three different secretion signals. Protein Expr Purif 60:103–109

Ghose T, Tyagi R (1979) Rapid ethanol fermentation of cellulose hydrolysate. II. Product and substrate inhibition and optimization of fermentor design. Biotechnol Bioeng 21:1401–1420

Gigras P, Sahai V, Gupta R (2002) Statistical media optimization and production of ITS alpha-amylase from *Aspergillus oryzae* in a bioreactor. Curr Microbiol 45:203–208
Gombert AK, Madeira JV Jr et al (2016) *Kluyveromyces marxianus* as a host for heterologous protein synthesis. Appl Microbiol Biotechnol 100:6193–6208
Gomes AR, Byregowda SM et al (2016) An overview of heterologous expression host systems for the production of recombinant proteins. Adv Anim Vet Sci 4:346–356
Gopal GJ, Kumar A (2013) Strategies for the production of recombinant protein in *Escherichia coli*. Protein J 32:419–425
Gresham D, Desai MM et al (2008) The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. PLoS Genet 4:e1000303
Guo X, Sun J et al (2018) Heterologous biosynthesis of (+)-nootkatone in unconventional yeast *Yarrowia lipolytica*. Biochem Eng J 137:125–131
Gupta SK, Shukla P (2016) Advanced technologies for improved expression of recombinant proteins in bacteria: perspectives and applications. Crit Rev Biotechnol 36:1089–1098
Gupta SK, Shukla P (2017) Sophisticated cloning, fermentation, and purification technologies for an enhanced therapeutic protein production: a review. Front Pharmacol 8:419
Gupta J, Kaul S et al (2020) Expression, purification and characterization of the hepatitis E virus like-particles in the *Pichia pastoris*. Front Microbiol 11:141
Hahn-Hagerdal B, Karhumaa K et al (2005) Role of cultivation media in the development of yeast strains for large scale industrial use. Microb Cell Fact 4:31
Harrison ST (1991) Bacterial cell disruption: a key unit operation in the recovery of intracellular products. Biotechnol Adv 9:217–240
Harrison S, Dennis J, Chase H (1991) Combined chemical and mechanical processes for the disruption of bacteria. Bioseparation 2:95–105
Hasunuma T, Kondo A (2012) Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering. Biotechnol Adv 30:1207–1218
Hatti-Kaul R (2001) Aqueous two-phase systems. Mol Biotechnol 19:269–277
Hatti-Kaul R (2010) Downstream processing in industrial biotechnology. In: Industrial biotechnology: sustainable growth and economic success, vol 1. Wiley, Weinheim, pp 279–321
Hatti-Kaul R, Mattiasson B (2003) Release of protein from biological host. In: Isolation and purification of proteins. CRC Press, Boca Raton, FL, pp 1–28
Huang W-C, Tang I-C (2007) Bacterial and yeast cultures–process characteristics, products, and applications. In: Bioprocessing for value-added products from renewable resources. Elsevier, Dublin, pp 185–223
Huang YL, Mann K et al (1998) Acetic acid production from fructose by *Clostridium formicoaceticum* immobilized in a Fibrous-Bed bioreactor. Biotechnol Prog 14:800–806
Huang B, Guo J et al (2008) Heterologous production of secondary metabolites as pharmaceuticals in *Saccharomyces cerevisiae*. Biotechnol Lett 30:1121–1137
Iimura Y, Sonoki T, Habe H (2018) Heterologous expression of *Trametes versicolor* laccase in *Saccharomyces cerevisiae*. Protein Expr Purif 141:39–43
Ingledew W (2003) Continuous fermentation in the fuel alcohol industry: how does the technology affect yeast? In: Jacques KA, Lyons TP, Kelsall DR (eds) The alcohol textbook: a reference for the beverage, fuel and industrial alcohol industries, 4th edn. Nottingham University Press, Nottingham, pp 135–143
Ishii J, Okazaki F et al (2016) From mannan to bioethanol: cell surface co-display of beta-mannanase and beta-mannosidase on yeast *Saccharomyces cerevisiae*. Biotechnol Biofuels 9:188
Issaly N, Solsona O et al (2001) Optimization of the wheat puroindoline-a production in *Pichia pastoris*. J Appl Microbiol 90:397–406
Jaafar L, Moukadiri I, Zueco J (2003) Characterization of a disulphide-bound Pir-cell wall protein (Pir-CWP) of *Yarrowia lipolytica*. Yeast 20:417–426
Jain KK, Kumar S et al (2018) Functional expression of a thermostable endoglucanase from *Thermoascus aurantiacus* RCKK in *Pichia pastoris* X-33 and its characterization. Mol Biotechnol 60:736–748

Jamshad M, Darby RA (2012) Disruption of yeast cells to isolate recombinant proteins. Methods Mol Biol 866:237–246
Jansen ML, Bracher JM et al (2017) *Saccharomyces cerevisiae* strains for second-generation ethanol production: from academic exploration to industrial implementation. FEMS Yeast Res 17:fox044
Joshi S, Satyanarayana T (2014) Optimization of heterologous expression of the phytase (PPHY) of *Pichia anomala* in *P. pastoris* and its applicability in fractionating allergenic glycinin from soy protein. J Ind Microbiol Biotechnol 41:977–987
Juturu V, Wu JC (2018) Heterologous protein expression in *Pichia pastoris*: latest research progress and applications. ChemBioChem 19:7–21
Kamarthapu V, Ragampeta S et al (2013) Engineered *Pichia pastoris* for enhanced production of S-adenosylmethionine. AMB Express 3:40
Kandasamy R, Vediyappan G, Chaffin WL (2000) Evidence for the presence of pir-like proteins in *Candida albicans*. FEMS Microbiol Lett 186:239–243
Kang Z, Huang H et al (2017) Recent advances of molecular toolbox construction expand *Pichia pastoris* in synthetic biology applications. World J Microbiol Biotechnol 33:19
Karbalaei M, Rezaee SA, Farsiani H (2020) *Pichia pastoris*: a highly successful expression system for optimal synthesis of heterologous proteins. J Cell Physiol 235:5867–5881
Katla S, Karmakar B et al (2019) High level extracellular production of recombinant human interferon alpha 2b in glycoengineered *Pichia pastoris*: culture medium optimization, high cell density cultivation and biological characterization. J Appl Microbiol 126:1438–1453
Kaur P, Singh B et al (2010) Pphy—a cell-bound phytase from the yeast *Pichia anomala*: molecular cloning of the gene PPHY and characterization of the recombinant enzyme. J Biotechnol 149:8–15
Khasa YP, Khushoo A et al (2007) Kinetic studies of constitutive human granulocyte-macrophage colony stimulating factor (hGM-CSF) expression in continuous culture of *Pichia pastoris*. Biotechnol Lett 29:1903–1908
Khasa YP, Conrad S et al (2011) Isolation of *Pichia pastoris* PIR genes and their utilization for cell surface display and recombinant protein secretion. Yeast 28:213–226
Khayet M, Matsuura T (2004) Pervaporation and vacuum membrane distillation processes: modeling and experiments. AIChE J 50:1697–1712
Kim CS, Ji ES, Oh DK (2003) Expression and characterization of *Kluyveromyces lactis* beta-galactosidase in *Escherichia coli*. Biotechnol Lett 25:1769–1774
Kim H, Yoo SJ, Kang HA (2015) Yeast synthetic biology for the production of recombinant therapeutic proteins. FEMS Yeast Res 15:1–16
Klis FM, Boorsma A, De Groot PW (2006) Cell wall construction in *Saccharomyces cerevisiae*. Yeast 23:185–202
Ko H, Bae J-H et al (2019) Efficient production of levan using a recombinant yeast *Saccharomyces cerevisiae* hypersecreting a bacterial levansucrase. J Ind Microbiol Biotechnol 46:1611–1620
Koffas MA, Jung GY, Stephanopoulos G (2003) Engineering metabolism and product formation in *Corynebacterium glutamicum* by coordinated gene overexpression. Metab Eng 5:32–41
Kondo A, Ueda M (2004) Yeast cell-surface display—applications of molecular display. Appl Microbiol Biotechnol 64:28–40
Kregiel D (2019) Amylolytic activity of kluyver-positive *Debaryomyces occidentalis* cells immobilized in foamed alginate gel. J Microbiol Biotechnol Food Sci 5:311–313
Krijgsman BHJ (1992) In: Jenkins RO (ed) Product recovery in bioprocess technology, BIOTOL Series. Butterworth Heinemann Ltd, Oxford, p 172
Kulkarni PS, Raut SK et al (2006) Immunogenicity of a new, low-cost recombinant hepatitis B vaccine derived from *Hansenula polymorpha* in adults. Vaccine 24:3457–3460
Kumar A (2020) *Aspergillus nidulans*: a potential resource of the production of the native and heterologous enzymes for industrial applications. Int J Microbiol 2020:8894215
Kumar R, Kumar P (2019) Yeast-based vaccines: new perspective in vaccine development and application. FEMS Yeast Res 19:foz007

Kumar S, Mutturi S (2020) Expression of a novel alpha-glucosidase from *Aspergillus neoniger* in *Pichia pastoris* and its efficient recovery for synthesis of isomaltooligosaccharides. Enzyme Microb Technol 141:109653

Kumar Vemuri P, Veeravalli S (2014) Expression, purification and characterization of human recombinant galectin 3 in *Pichia pastoris*. Iran J Biotechnol 12:3–8

Kumari A, Baronian K et al (2015) Extracellular expression of *YlLip11* with a native signal peptide from *Yarrowia lipolytica* MSR80 in three different yeast hosts. Protein Expr Purif 110:138–144

Kuroda K, Ueda M (2011a) Cell surface engineering of yeast for applications in white biotechnology. Biotechnol Lett 33:1–9

Kuroda K, Ueda M (2011b) Molecular design of the microbial cell surface toward the recovery of metal ions. Curr Opin Biotechnol 22:427–433

Kwak S, Jin YS (2017) Production of fuels and chemicals from xylose by engineered *Saccharomyces cerevisiae*: a review and perspective. Microb Cell Fact 16:82

Landowski CP, Mustalahti E et al (2016) Enabling low cost biopharmaceuticals: high level interferon alpha-2b production in *Trichoderma reesei*. Microb Cell Fact 15:104

Lee SY, Choi JH, Xu Z (2003) Microbial cell-surface display. Trends Biotechnol 21:45–52

Li Z, Xiong F et al (2001) Low-temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris*. Protein Expr Purif 21:438–445

Li P, Anumanthan A et al (2007) Expression of recombinant proteins in *Pichia pastoris*. Appl Biochem Biotechnol 142:105–124

Li H, Ali Z et al (2019) Expression of recombinant tachyplesin I in *Pichia pastoris*. Protein Expr Purif 157:50–56

Li D, Wu Y et al (2020) Metabolic engineering of *Yarrowia lipolytica* for heterologous oleanolic acid production. Chem Eng Sci 218:115529

Liu D, Zeng X-A et al (2013) Disruption and protein release by ultrasonication of yeast cells. Innov Food Sci Emerg Technol 18:132–137

Liu Y, Zhang R et al (2014) Yeast cell surface display for lipase whole cell catalyst and its applications. J Mol Catal 106:17–25

Liu T, Zhang C, Lu W (2018) Heterologous production of levopimaric acid in *Saccharomyces cerevisiae*. Microb Cell Fact 17:114

Liu Y, Li Y et al (2020) Expression of a *Beauveria bassiana* chitosanase (BbCSN-1) in *Pichia pastoris* and enzymatic analysis of the recombinant protein. Protein Expr Purif 166:105519

Lizardi-Jiménez M, Hernández-Martínez R (2017) Solid state fermentation (SSF): diversity of applications to valorize waste and biomass. 3 Biotech 7:44

Lowe CR, Lowe AR, Gupta G (2001) New developments in affinity chromatography with potential application in the production of biopharmaceuticals. J Biochem Biophys Methods 49:561–574

Madhavan A, Sukumaran RK (2016) Secreted expression of an active human interferon-beta (HuIFNβ) in *Kluyveromyces lactis*. Eng Life Sci 16:379–385

Madhavan A, Jose AA et al (2017a) Synthetic biology and metabolic engineering approaches and its impact on non-conventional yeast and biofuel production. Front Energy Res 5:8

Madhavan A, Pandey A, Sukumaran RK (2017b) Expression system for heterologous protein expression in the filamentous fungus *Aspergillus unguis*. Bioresour Technol 245:1334–1342

Madzak C (2015) *Yarrowia lipolytica*: recent achievements in heterologous protein expression and pathway engineering. Appl Microbiol Biotechnol 99:4559–4577

Madzak C, Gaillardin C, Beckerich JM (2004) Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. J Biotechnol 109:63–81

Madzak C, Otterbein L et al (2005) Heterologous production of a laccase from the basidiomycete *Pycnoporus cinnabarinus* in the dimorphic yeast *Yarrowia lipolytica*. FEMS Yeast Res 5:635–646

Magocha TA, Zabed H et al (2018) Improvement of industrially important microbial strains by genome shuffling: current status and future prospects. Bioresour Technol 257:281–289

Malak A, Baronian K, Kunze G (2016) *Blastobotrys (Arxula) adeninivorans*: a promising alternative yeast for biotechnology and basic research. Yeast 33:535–547

Manan M, Webb C (2017) Design aspects of solid state fermentation as applied to microbial bioprocessing. J Appl Biotechnol Bioeng 4:91
Manfrao-Netto JHC, Gomes AMV, Parachin NS (2019) Advances in using *Hansenula polymorpha* as chassis for recombinant protein production. Front Bioeng Biotechnol 7:94
Manpreet S, Sawraj S et al (2005) Influence of process parameters on the production of metabolites in solid-state fermentation. Malays J Microbiol 2:1–9
Mathew E, Zhu H et al (2018) Display of the HIV envelope protein at the yeast cell surface for immunogen development. PLoS One 13:e0205756
Mathison L, Soliday C et al (1993) Cloning, characterization, and use in strain improvement of the *Cephalosporium acremonium* gene cefG encoding acetyl transferase. Curr Genet 23:33–41
Matsumoto T, Fukuda H et al (2002) Construction of yeast strains with high cell surface lipase activity by using novel display systems based on the Flo1p flocculation functional domain. Appl Environ Microbiol 68:4517–4522
Meng D-M, Li W-J et al (2019) Expression, purification and characterization of a recombinant antimicrobial peptide Hispidalin in *Pichia pastoris*. Protein Expr Purif 160:19–27
Mrudula S, Murugammal R (2011) Production of cellulase by *Aspergillus niger* under submerged and solid state fermentation using coir waste as a substrate. Braz J Microbiol 42:1119–1127
Nagesha S, Gowda PR et al (2010) Heterologous expression of CVS rabies virus glycoprotein gene in *Pichia pastoris*. IJGEB 1:101–110
Najar TA, Khare S et al (2017) Mapping protein binding sites and conformational epitopes using cysteine labeling and yeast surface display. Structure 25:395–406
nee'Nigam PS, Pandey A (2009) Solid-state fermentation technology for bioconversion of biomass and agricultural residues. In: Biotechnology for agro-industrial residues utilisation. Springer, Dordrecht, pp 197–221
Nevalainen KM, Te'o VS, Bergquist PL (2005) Heterologous protein expression in filamentous fungi. Trends Biotechnol 23:468–474
Nguyen TPA, Nguyen TTM et al (2020) Application of yeast surface display system in expression of recombinant pediocin PA-1 in *Saccharomyces cerevisiae*. Folia Microbiol (Praha) 65 (6):955–961
Nicaud JM, Madzak C et al (2002) Protein expression and secretion in the yeast *Yarrowia lipolytica*. FEMS Yeast Res 2:371–379
Nowrouzi B, Li RA et al (2020) Enhanced production of taxadiene in *Saccharomyces cerevisiae*. Microb Cell Fact 19:1–12
Oud B, van Maris AJ et al (2012) Genome-wide analytical approaches for reverse metabolic engineering of industrially relevant phenotypes in yeast. FEMS Yeast Res 12:183–196
Özçelik AT, Ersöz F, İnan M (2019) Extracellular production of the recombinant bacterial transglutaminase in *Pichia pastoris*. Protein Expr Purif 159:83–90
Padkina M, Sambuk E (2018) Prospects for the application of yeast display in biotechnology and cell biology. Appl Biochem Microbiol 54:337–351
Pandey A (1994) Solid state fermentation-an overview. In: Pandey A (ed) Solid-state fermentation. Wiley Eastern Limited, New Delhi, India, pp 3–10
Pandey A (2003) Solid-state fermentation. Biochem Eng J 13:81–84
Parashar D, Satyanarayana T (2017) Production of chimeric acidic alpha-amylase by the recombinant *Pichia pastoris* and its applications. Front Microbiol 8:493
Parekh S (2009) Strain improvement. In: Encyclopedia of microbiology. Academic, Indianapolis, pp 286–298
Parker C, DiBiasio D (1987) Effect of growth rate and expression level on plasmid stability in *Saccharomyces cerevisiae*. Biotechnol Bioeng 29:215–221
Patnaik R, Louie S et al (2002) Genome shuffling of *Lactobacillus* for improved acid tolerance. Nat Biotechnol 20:707–712
Patro KR, Gupta N (2012) Extraction, purification and characterization of L-asparaginase from *Penicillium* sp. by submerged fermentation. Int J Biotechnol 3:30–34
Pepper LR, Cho YK et al (2008) A decade of yeast surface display technology: where are we now? Comb Chem High Throughput Screen 11:127–134

Picanço-Castro V, Cristina Correa de Freitas M et al (2014) Patents in therapeutic recombinant protein production using mammalian cells. Recent Pat Biotechnol 8:165–171
Porro D, Mattanovich D (2004) Recombinant protein production in yeasts. Methods Mol Biol 267:241–258
Porro D, Gasser B et al (2011) Production of recombinant proteins and metabolites in yeasts: when are these systems better than bacterial production systems? Appl Microbiol Biotechnol 89:939–948
Pratush A, Seth A, Bhalla TC (2017) Expression of nitrile hydratase gene of mutant 4D strain of *Rhodococcus rhodochrous* PA 34 in *Pichia pastoris*. Biocatal Biotransform 35:19–26
Pribylova L, Sychrova H (2003) Efficient transformation of the osmotolerant yeast *Zygosaccharomyces rouxii* by electroporation. J Microbiol Methods 55:481–484
Pribylova L, de Montigny J, Sychrova H (2007) Tools for the genetic manipulation of *Zygosaccharomyces rouxii*. FEMS Yeast Res 7:1285–1294
Prior B, Du Preez J, Rein P (1992) Environmental parameters. In: Doelle H, Mitchelle D, Rolz C (eds) Solid substrate cultivation. Elsevier Applied Science, London, pp 65–85
Radecka D, Mukherjee V et al (2015) Looking beyond *Saccharomyces*: the potential of non-conventional yeast species for desirable traits in bioethanol fermentation. FEMS Yeast Res 15:fov053
Rajkumar AS, Morrissey JP (2020) Rational engineering of *Kluyveromyces marxianus* to create a chassis for the production of aromatic products. Microb Cell Fact 19:207
Rao A, Pimprikar P et al (2011) Cloning and expression of a tyrosinase from *Aspergillus oryzae* in *Yarrowia lipolytica*: application in L-DOPA biotransformation. Appl Microbiol Biotechnol 92:951–959
Rebello S, Abraham A et al (2018) Non-conventional yeast cell factories for sustainable bioprocesses. FEMS Microbiol Lett 365
Rendsvig JKH, Workman CT, Hoof JB (2019) Bidirectional histone-gene promoters in *Aspergillus*: characterization and application for multi-gene expression. Fungal Biol Biotechnol 6:24
Ro DK, Paradise EM et al (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440:940–943
Rossouw D, Næs T, Bauer FF (2008) Linking gene regulation and the exo-metabolome: a comparative transcriptomics approach to identify genes that impact on the production of volatile aroma compounds in yeast. BMC Genomics 9:530
Saleem M, Brim H et al (2008) Perspectives on microbial cell surface display in bioremediation. Biotechnol Adv 26:151–161
Sanchez-Garcia L, Martin L et al (2016) Recombinant pharmaceuticals from microbial cells: a 2015 update. Microb Cell Fact 15:33
Saraswat S, Athmaram TN et al (2016) Expression and characterization of yeast derived chikungunya virus like particles (CHIK-VLPs) and its evaluation as a potential vaccine candidate. PLoS Negl Trop Dis 10:e0004782
Sarkari P, Reindl M et al (2014) Improved expression of single-chain antibodies in *Ustilago maydis*. J Biotechnol 191:165–175
Schmidt S, Havekost D et al (2005) Crystallization for the downstream processing of proteins. Eng Life Sci 5:273–276
Sharma R, Katoch M et al (2009) Approaches for refining heterologous protein production in filamentous fungi. World J Microbiol Biotechnol 25:2083–2094
Shepard SR, Stone C et al (2002) Recovery of intracellular recombinant proteins from the yeast *Pichia pastoris* by cell permeabilization. J Biotechnol 99:149–160
Shibasaki S, Ueda M (2014) Bioadsorption strategies with yeast molecular display technology. Biocontrol Sci 19:157–164
Shiraga S, Kawakami M et al (2005) Enhanced reactivity of *Rhizopus oryzae* lipase displayed on yeast cell surfaces in organic solvents: potential as a whole-cell biocatalyst in organic solvents. Appl Environ Microbiol 71:4335–4338

Shivananda SV et al (2006) Comparison of two hepatitis B vaccines (GeneVac-B and Engerix-B) in healthy infants in India. Clin Vaccine Immunol 13:661–664
Shuler M, Kargi F (2002) Selection, scale-up, operation, and control of bioreactors. In: Bioprocess engineering basic concept, 2nd edn. Prentice Hall, Hoboken, NJ, pp 285–328
Siewers V (2014) An overview on selection marker genes for transformation of *Saccharomyces cerevisiae*. Methods Mol Biol 1152:3–15
Singhania RR, Saini R et al (2015) An integrative process for bio-ethanol production employing SSF produced cellulase without extraction. Biochem Eng J 102:45–48
Singhvi P, Saneja A et al (2020) Bacterial inclusion bodies: a treasure trove of bioactive proteins. Trends Biotechnol 38:474–486
Smekenov I, Bakhtambayeva M et al (2020) Heterologous secretory expression of β glucosidase from *Thermoascus aurantiacus* in industrial *Saccharomyces cerevisiae* strains. Braz J Microbiol 51:107–123
Sonderegger C, Galgoczy L et al (2016) A *Penicillium chrysogenum*-based expression system for the production of small, cysteine-rich antifungal proteins for structural and functional analyses. Microb Cell Fact 15:192
Spencer F, Hugerat Y et al (1994) Yeast kar1 mutants provide an effective method for YAC transfer to new hosts. Genomics 22:118–126
Spohner SC, Schaum V et al (2016) *Kluyveromyces lactis*: an emerging tool in biotechnology. J Biotechnol 222:104–116
Steensels J, Snoek T et al (2014) Improving industrial yeast strains: exploiting natural and artificial diversity. FEMS Microbiol Rev 38:947–995
Stemmer WP (1994) Rapid evolution of a protein in vitro by DNA shuffling. Nature 370:389–391
Stephanopoulos G (1993) Bioprocessing, Biotechnology, vol 3. (Rehm HJ, Reed G, Pühler A, Stadler P, series eds.). Weley VCH, Verlag, GmbH, Weinheim
Tanaka T, Kondo A (2015) Cell-surface display of enzymes by the yeast *Saccharomyces cerevisiae* for synthetic biology. FEMS Yeast Res 15:1–9
Tang H, Wu Y et al (2020) Promoter architecture and promoter engineering in *Saccharomyces cerevisiae*. Metabolites 10:320
Theron CW, Vandermies M et al (2020) Comprehensive comparison of *Yarrowia lipolytica* and *Pichia pastoris* for production of *Candida antarctica* lipase B. Sci Rep 10:1741
Tripathi NK (2016) Production and purification of recombinant proteins from *Escherichia coli*. ChemBioEng Rev 3:116–133
Turkanoglu Ozcelik A, Yilmaz S, Inan M (2019) *Pichia pastoris* promoters. Methods Mol Biol 1923:97–112
Tyagi A, Kumar A et al (2016) Functional expression of recombinant goat chymosin in *Pichia pastoris* bioreactor cultures: a commercially viable alternate. LWT Food Sci Technol 69:217–224
Tyagi A, Kumar A et al (2017) Expression of buffalo chymosin in *Pichia pastoris* for application in mozzarella cheese. LWT Food Sci Technol 84:733–739
Ueda M (2016) Establishment of cell surface engineering and its development. Biosci Biotechnol Biochem 80:1243–1253
Ueda M, Tanaka A (2000) Genetic immobilization of proteins on the yeast cell surface. Biotechnol Adv 18:121–140
Umemura M, Kuriiwa K et al (2020) Promoter tools for further development of *Aspergillus oryzae* as a platform for fungal secondary metabolite production. Fungal Biol Biotechnol 7:3
Upadhyaya B, Manjunath R (2009) Baker's yeast expressing the Japanese encephalitis virus envelope protein on its cell surface: induction of an antigen-specific but non-neutralizing antibody response. Yeast 26:383–397
Vandermies M, Fickers P (2019) Bioreactor-scale strategies for the production of recombinant protein in the yeast *Yarrowia lipolytica*. Microorganisms 7:40
Velu V, Nandakumar S et al (2007) Comparison of three different recombinant hepatitis B vaccines: GeneVac-B, Engerix B and Shanvac B in high risk infants born to HBsAg positive mothers in India. World J Gastroenterol 13:3084–3089

Vieira Gomes AM, Souza Carmo T et al (2018) Comparison of yeasts as hosts for recombinant protein production. Microorganisms 6:38
Vjestica A, Marek M et al (2020) A toolbox of stable integration vectors in the fission yeast *Schizosaccharomyces pombe*. J Cell Sci 133:jcs240754
Walsh G (2018) Biopharmaceutical benchmarks 2018. Nat Biotechnol 36:1136–1145
Wang L, Yang ST (2007) Solid state fermentation and its applications. In: Yang ST (ed) Bioprocessing for value-added products from renewable resources. Elsevier, UK, pp 465–489
Wang L, Ridgway D et al (2005) Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. Biotechnol Adv 23:115–129
Wang EQ, Li SZ et al (2010) Modeling of rotating drum bioreactor for anaerobic solid-state fermentation. Appl Energy 87:2839–2845
Wang G, Huang M, Nielsen J (2017) Exploring the potential of *Saccharomyces cerevisiae* for biopharmaceutical protein production. Curr Opin Biotechnol 48:77–84
Watanabe I, Miyata N et al (2002) Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Biochem Eng J 93:90–95
Watanabe J, Uehara K et al (2010) Improved transformation of the halo-tolerant yeast *Zygosaccharomyces rouxii* by electroporation. Biosci Biotechnol Biochem 74:1092–1094
Waugh DS (2005) Making the most of affinity tags. Trends Biotechnol 23:316–320
Wetzel D, Müller JM et al (2016) Fed-batch production and secretion of streptavidin by *Hansenula polymorpha*: evaluation of genetic factors and bioprocess development. J Biotechnol 225:3–9
Woolston BM, Edgar S, Stephanopoulos G (2013) Metabolic engineering: past and future. Annu Rev Chem 4:259–288
Yang X, Nambou K et al (2016) Heterologous production of α-farnesene in metabolically engineered strains of *Yarrowia lipolytica*. Bioresour Technol 216:1040–1048
Young TW, Wadeson A et al (1996) The extracellular acid protease gene of *Yarrowia lipolytica*: sequence and pH-regulated transcription. Microbiology 142:2913–2921
Young CL, Britton ZT, Robinson AS (2012) Recombinant protein expression and purification: a comprehensive review of affinity tags and microbial applications. Biotechnol J 7:620–634
Yurimoto H, Sakai Y (2009) Methanol-inducible gene expression and heterologous protein production in the methylotrophic yeast *Candida boidinii*. Biotechnol Appl Biochem 53:85–92
Yurimoto H, Hasegawa T et al (2001) Characterization and high-level production of D-amino acid oxidase in *Candida boidinii*. Biosci Biotechnol Biochem 65:627–633
Yuzuki M, Matsushima K, Koyama Y (2015) Expression of key hydrolases for soy sauce fermentation in *Zygosaccharomyces rouxii*. J Biosci Bioeng 119:92–94
Zhang Z (1997) Bioreactor studies of heterologous protein production by recombinant yeast. UWSpace. http://hdl.handle.net/10012/133
Zhang N, An Z (2010) Heterologous protein expression in yeasts and filamentous fungi. In: Manual of industrial microbiology and biotechnology, 3rd edn. ASM Press, Washington, DC, pp 145–156
Zhang W, Inan M, Meagher MM (2000) Fermentation strategies for recombinant protein expression in the methylotrophic yeast *Pichia pastoris*. Biotechnol Bioprocess Eng 5:275–287
Zhang YX, Perry K et al (2002) Genome shuffling leads to rapid phenotypic improvement in bacteria. Nature 415:644–646
Zhang L, Liang S et al (2013) Screening for glycosylphosphatidylinositol-modified cell wall proteins in *Pichia pastoris* and their recombinant expression on the cell surface. Appl Environ Microbiol 79:5519–5526
Zhu Y, Wu Z, Yang ST (2002) Butyric acid production from acid hydrolysate of corn fibre by *Clostridium tyrobutyricum* in a fibrous-bed bioreactor. Process Biochem 38:657–666
Zhu W, Gong G et al (2018) High level expression and purification of recombinant human serum albumin in *Pichia pastoris*. Protein Expr Purif 147:61–68

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