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Fungal Metabolites

Reference Series in Phytochemistry

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This reference works series provides a platform for all information on plant metabolites and phytochemicals, their chemistry, properties, applications, and methods. By the strictest definition, phytochemicals are chemicals derived from plants. However, the term is often used to describe the large number of secondary metabolic compounds found in and derived from plants. These metabolites exhibit a number of nutritional and protective functions for human welfare such as colorants, fragrances and flavorings, amino acids, pharmaceuticals, hormones, vitamins and agrochemicals. Besides food, fibers, fuel, cloth and shelter, a vast number of wild plants can hence provide important sources for medicines, especially in developing countries for their traditional health systems. Natural products have inspired and provided the foundation to the bulk of FDA-approved compounds and there is tremendous increase in natural products and natural products derived compounds that have been registered against many prevailing diseases. Natural product industry has shown tremendous growth and is expected to continue to do so in the near future. The present series compiles reference information on various topics and aspects about phytochemicals, including their potential as natural medicine, their role as chemo-preventers, in plant defense, their ecological role, their role in plants as well as for pathogen adaptation, and disease resistance. Volumes in the series also contain information on methods such as metabolomics, genetic engineering of pathways, molecular farming, and obtaining metabolites from lower organisms and marine organisms besides higher plants. The books in the series are hence of relevance in various fields, from chemistry, biology, biotechnology, to pharmacognosy, pharmacology, botany, or medicine. Each volume is edited by leading experts and contains authoritative contributions by renowned authors.

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Fungal Metabolites

With 164 Figures and 66 Tables

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Preface

We are pleased to present the first book entitled 'Fungal metabolites' under the new book series of Springer "Reference series in Phytochemistry". This book series provides the platform with the aim of attracting researchers involved in all aspects of natural metabolites useful for human welfare.

Fungi are present all over the planet earth in different habitat from aquatic to terrestrial ecosystems. We know since long back, fungi as harmful entity spoiling our foods and food grains, cultivated plants and causing health hazards till we did not discover antibiotics, which saved thousands of life. Other beneficial effects of fungi known since ancient times includes bread, cheese and fermentation products but colorant, drugs, enzymes, metabolic inhibitors are of relatively recent origin. This book is a timely compilation of state of information about this rapidly developing field composed by highly renowned scientists of the field.

The book aims to present comprehensive, up-to-date and well established information about complex and astonishing structure, properties and biotechnological applications of fungal metabolites and their upcoming industrial applications. The book comprises of 30 chapters and is divided in to three sections viz: Part I – General Biology, Part II – Methods and Biotechnology, and Part III – Biological activity; besides an introductory chapter describing gamut of fungal metabolites including a brief history of their developments. The book will be a valuable source on fungal metabolites to those working in the field of human wellness, industrial production and biotechnology development.

This comprehensive and thoroughly up-to-date reference book presents the sources, biology of pathogens, methods of analysis, biosynthesis, biotechnology and applications of fungal metabolites. Fungal metabolites have received much attention recently because of the emergence of different biological activities, such as anticancer, anti-oxidative, cholesterol inhibitor, in biological control of nematodes and other ones in human health. New industrial applications in pharmaceutical and medical sciences are being developed. Due to these properties and benefits, a vast body of data is being generated.

The book is intended to serve the needs of graduate students, scholars, researchers in the field of botany, agriculture, pharmacy, biotechnology and phytochemistry, industrial scientists and those involved in marketing phytochemicals and their extracts.

This work could not be completed without active support of Springer team who took pains in streamlining the production process. We are particularly indebt to Drs. Tobias Wassermann, Elizabeth Hawkins, Sylvia Blago and Sylvia Jakuscheit for their continuous professional support throughout the project.

August 2016

Jean-Michel Mérillon
Kishan Gopal Ramawat
Editors

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About the Editors

Prof. Dr. Jean-Michel Mérillon received his Pharm.D. (1979), Ph.D. (1984) and HDR (1992) from the University of Tours in France. He joined this same University as assistant professor in 1981, became associate professor in 1987. In 1993 he moved to the faculty of Pharmacy, University of Bordeaux, France, accepting a position as full professor. He is currently leading the "study group on biologically active plant substances" at the Institute of Vine and Wine Sciences, which comprises 25 scientists and research students. The group has been working on phenolic compounds from vine and wine for many years, mainly complex stilbenes and their involvement in health. Prof. Mérillon has supervised the doctoral theses of 20 students. He is involved in developing teaching on plant biology, natural bioactive compounds and biotechnology.

Prof. Mérillon has published more than 150 research papers in internationally recognized journals, resulting in an H index of 38 (documents published between 1996 and 2016). He has co-edited books and reference works on secondary metabolites and biotechnology.

Throughout his career, Prof. Mérillon has traveled widely as a senior professor. Scientists from several countries have been and are working in his laboratory, and his research is supported by funding from the Aquitaine Regional Government, the Ministry of Higher Education and Research, and various private companies. In 2004, he founded the technology transfer unit "Polyphenols Biotech", providing support for R&D programs for SMEs and major groups from the cosmetic, pharmaceutical, agricultural and health-nutrition sectors. Faculty of Pharmacy, Institut des Sciences de la Vigne et du Vin – CS 50008, University of Bordeaux, Villenave d'Ornon, France.

Prof. Dr. Kishan Gopal Ramawat is Former Professor and Head of the Botany Department, M.L. Sukhadia University, Udaipur, India, and can look back on longstanding research experience. He received his Ph.D. in Plant Biotechnology in 1978 from the University of Jodhpur, India and afterwards joined the university as a faculty member. In 1991 he moved to the M.L. Sukhadia University in Udaipur as Associate Professor and became Professor in 2001. He served as the Head of the Department of Botany (2001–2004, 2010–2012), was in charge of the Department of Biotechnology (2003–2004), was a member of the task force on medicinal and aromatic plants of the Department of Biotechnology, Government of India, New Delhi (2002–2005) and coordinated UGC-DRS and DST-FIST programmes (2002–2012).

Prof. Ramawat had done his postdoctoral studies at the University of Tours, France from 1983 to 1985, and later returned to Tours as visiting professor (1991). He also visited the University of Bordeaux 2, France several times as visiting professor (1995, 1999, 2003, 2006, 2010), and in 2005 Poland in an academic exchange programme (2005). Through these visits in France, Prof. Ramawat and Prof. Mérillon established a strong connection, which has resulted in productive collaborations and several book and reference work publications.

Prof. Ramawat has published more than 170 well cited peer reviewed papers and articles, and edited several books and reference works on topics such as the biotechnology of medicinal plants, secondary metabolites, bioactive molecules, herbal drugs, and many other topics. His research was funded by several funding agencies.

In his research group, Prof. Ramawat has supervised doctoral thesis of 25 students. He is an active member of several academic bodies, associations and editorial boards of journals. Botany Department, M.L.Sukhadia University, Udaipur, India.

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Different Shades of Fungal Metabolites: An Overview

1

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Abstract

Fungi can be found in almost all types of habitats. Its several thousand species are very diverse in morphological characters with plethora of secondary metabolites. These secondary metabolites make some of the fungi our friend as well as foe. Many of these secondary metabolites exhibit harmful effect being mycotoxins. Fungi are notoriously known as food spoiler, causing damage to cooked food and grains, and as plant pathogen, causing various severe diseases. However, fungi are beneficial to mankind as producer of antibiotics, food colorant, enzymes, and as a nutritious food. Today many industries are based on fungi or fungal products. Fungi are believed to be the future microbial cell factories for the production of food grade pigments, enzymes, and pharmaceuticals. Owing to the increasing demand of these products, the large-scale production can be achieved by using modern tools of biotechnology and appropriate use of fermentation physiology. Heterologous expression of secondary metabolite production or even manipulation of physical and chemical growth factors can enhance the desired product yield with improved functionality. But still, there is a vast scope for improved production and search for novel fungal metabolites which will render our safe future against resistance-developing bacteria and other dreaded diseases. In this brief review, we present a global scenario of fungal metabolites.

Keywords

Fungal classification • Antibiotics • Mycotoxins • Fungal bioactive molecules • Enzymes • Fungal pigments

1 Introduction

Today we all are familiar with the importance of secondary metabolites in pharmaceuticals, agrochemicals, food additives, and as ingredients in cosmetics. Secondary metabolites are believed to have no function in the life cycle of producer cells, unlike primary metabolites. They are chemically heterogeneous group with molecular weight less than 3000 Da [Dalton is the standard unit that is used for indicating mass on an atomic or molecular scale (atomic mass)]. In the search of new bioactive secondary metabolites, most of the scientists' intense interest surrounds the plants. But the fact is that until 2014 about 170,000 natural products have been described [1, 2], out of which more than 22,500 bioactive metabolites are produced by microorganisms, with about 45 % as products of actinomycetes fermentation and about 38 % of fungal origin [3, 4].

Fungi can be found in almost all types of habitats. In their need to live and reproduce, they compete with other organisms. They grow fast on the surface of plant/nutrition to prevent other competitors from reaching their nutrient source. Some live in symbiosis with the host and some off the dead organisms. In order to survive, fungi have developed a number of strategies for protection and

communication, one of which is production of different types of secondary metabolites. These fungal or fungal-induced compounds in plants increase the ability of plant to resist invasion of predators, parasites, and diseases and may be used for competition between species or to facilitate reproductive processes [5].

Fungi are notoriously known as food spoiler, causing damage to cooked food and grains, as plant pathogen, causing various severe diseases such as Potato late blight, caused by *Phytophthora infestans* responsible for potato famine of Ireland (1845–1849), and rice blast, which leads to infamous Bengal famine of 1943; however, fungi are beneficial to humankind as producer of antibiotics (penicillin, cephalosporin, cyclosporine etc) and statins. Poisoning of cattle by ergot (a fungal body formed by mycelium of *Claviceps* species, especially *C. purpurea*) and mycotoxins [produced by several species of *Aspergillus* in stored grains (aflatoxins, ochratoxin A)] is common in several countries. Beneficial effects of fungi are of relatively of recent origin (except applications in cheese making) such as producer of several enzymes (cellulase, lipase, ligninolytic enzymes), alkaloids (ergot alkaloids from *Claviceps*), pigments (anthraquinone, betalains), aroma and flavors, and in biological control of nematodes, health benefits by edible fungi (source of selenium, potassium, riboflavin, niacin, vitamin D, proteins) and in prevention of or treatment for Parkinson, Alzheimer, hypertension, cancers, and high risk of stroke [6–11]. All these drastically different activities of fungi (Fig. 1) are attributed to the plethora of metabolites they have. These metabolites represent a large source of compounds endowed with ingenious structures and potent biological activities. They comprises of aliphatic and aromatic hydrocarbons, organic acids, esters, ketones, aldehydes, alcohols, and mono-, sesqui-, and diterpenes [12]. The present article provides an

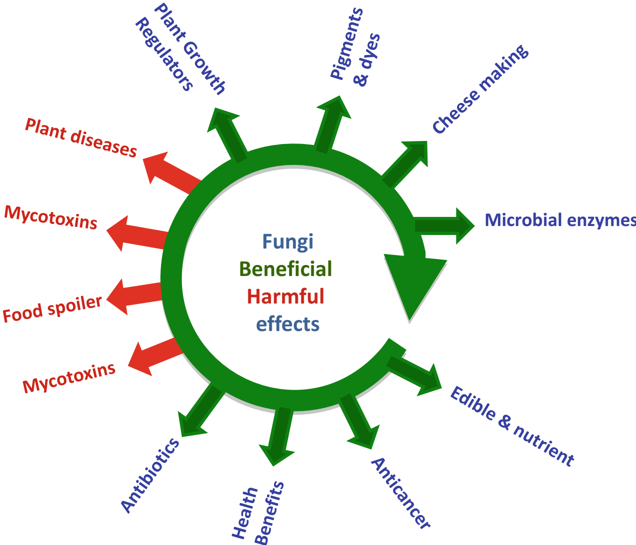

Fig. 1 Beneficial and harmful effects of fungi

overview of the current knowledge on fungal metabolites and gives prospects for the future developments.

2 Brief Classification

About 1.5 million species of the fungi have been raised to a separate kingdom of organisms, as complex and diverse as plants and animals, of which only about 10 % have been named and described. Originally there were only two kingdoms – plants and animals. If an organism did not move, it was usually placed in the plant kingdom. In 1969, a scientist named Robert Whittaker published the first major revision to Linnaeus's proposed two kingdom classification – animals and plants (which included fungi). In the revised version, Whittaker suggested that fungi should be classified as a separate kingdom, and this has been accepted by scientists [13]. Fungi are placed in a separate kingdom because they have many characteristics which are different from plants and animals. Their cell walls are made of chitin and mode of nutrition is absorption, contrasting to plants which have cell walls made of cellulose and use photosynthesis to synthesize carbohydrates. Thus, bacteria including actinobacteria (previously called as actinomyces), fungi, plants, protozoa, and animals are recognized as separate kingdoms. Major characteristic and examples of various subdivisions of fungal kingdom are given in Table 1. A comprehensive phylogenetic classification of the kingdom Fungi has been developed in light of recent molecular phylogenetic analyses and with input from diverse groups of the fungal taxonomic. The readers primarily interested in classification can consult reviews on this aspect [14, 15].

Table 1 Characteristics of various subdivisions of fungal kingdom

Sub-division	Characteristics and remarks
Basidiomycota	This division contains the mushrooms and toadstools. Divided into 3 subphyla: Agaricomycotina, Ustilaginomycotina, Pucciniomycotina such as puffballs and stinkhorns, rusts and smuts, and gilled and pored fungi. Spores are produced on a characteristic cell called a basidium (plural basidia)
Ascomycota	Three subphyla : Taphrinomycotina, Saccharomycotina, Pezizomycotina. The largest number of species occurs in this group such as the cup fungi and flask fungi. Spores are produced in a sack like structure called an ascus (plural asci)
Zygomycota	Mostly microscopic species, the pin molds with coenocytic hyphae
Oomycota	The Oomycota includes the water molds and some important pathogens such as potato blight. Many produce motile spores during their life cycle which can swim. This group is now classified along with brown algae
Deuteromycota= Fungi imperfecti	Species for which sexual reproduction is not known such as molds (*Alternaria, Aspergillus*, *Penicillium).* Mainly basidiomycotina or ascomycotina anamorph
Microsporidiomycota	Spore forming unicellular parasites

3 Fungal Metabolites Biosynthesis

Fungi produce diversified extent of metabolites ranging from antibiotics to mycotoxins. The biosynthetic pathways involve in the synthesis of these molecules are also diverse. However, it is surprising that these complex structures are synthesized by relatively few building blocks. The pathways are usually named after enzymes or intermediates involved and are also commonly used to classify secondary metabolites. Three most common pathways studied are (1) the mevalonic acid pathway (synthesize terpenoids, steroids, etc), (2) the shikimic acid pathway (synthesize aromatic amino acids, alkaloids, etc), and (3) the acetate pathway (synthesize polyketides, fatty acids, etc). The enzymes associated with these pathways are nonribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), terpene cyclases (TCs), dimethylallyl tryptophan synthetases (DMATs), and geranylgeranyl diphosphate synthases (GGPPs), etc. These enzymes utilize building blocks like acetyl-coA, amino acids, mevalonate, and their different counterparts for the production of different fungal metabolites as shown in Fig. 2. In case of terpenes, steroids, gibberellins, the actual fundamental building block is dimethylallyl diphosphate (DMAPP). DMAPP are biosynthesized via the mevalonic acid (MVA) which is formed by combining three acetyl-coenzyme A (acetyl-CoA) [16, 17]. In microorganisms, an alternative pathway for terpenes biosynthesis was described where glyceraldehyde 3-phosphate

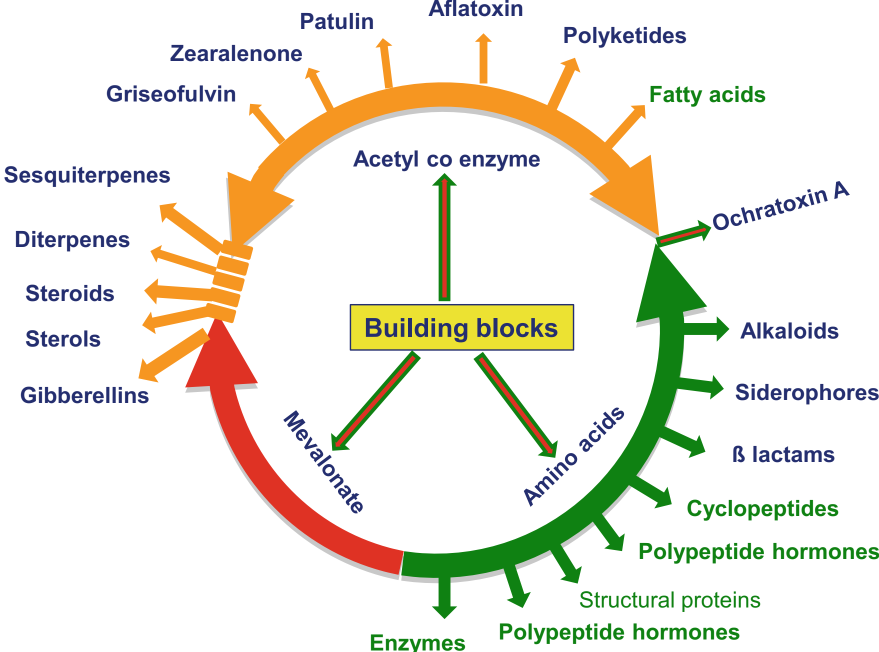

Fig. 2 Biosynthesis of different metabolites by fungi using building blocks. Building blocks are photosynthates; *green fonts*-primary metabolites, *blue fonts*-secondary metabolites

(or ultimately glyceraldehyde) and a C_2 unit derived from pyruvate decarboxylation were the only precursors of the C_5 skeleton of isoprenic units in this nonmevalonate pathway for isoprenoid biosynthesis [18]. Thus, many hybrid secondary metabolites are derived from the tandem action of these enzymes, building blocks, and cofactors.

4 Metabolic Engineering

Metabolic engineering is an emerging field where using the modern biotechnological tools and techniques, cell factories are generated. Now-a-days many genetically well-characterized fungi are modified for the production of industrially relevant enzymes, pharmaceuticals, and biofuels and are widely used by the research community to produce proteins that cannot be actively expressed in *Escherichia coli* or require glycosylation for proper folding and biological activity. Most of the heterologous expressions are mainly performed in baker's yeast *Saccharomyces cerevisiae* [19]. There are over hundred proof-of-concept chemicals that have been made in yeast [20]. One of the most important biopharmaceuticals produced by yeast is insulin and insulin analogs. It is a continuous growing market as the global insulin sale is expected to grow from USD 12 billion in 2011 to more than USD 32 billion by 2018. Other important biopharmaceuticals produced by yeast are human serum albumin, hepatitis vaccines, and human papilloma virus vaccine [21]. Besides yeast, filamentous fungi that dominate the heterologous expression of useful metabolites are *Aspergillus niger*, *A. oryzae*, *Trichoderma reesei*, and *A. nidulans*. Another fungus with great potential is *Chrysosporium lucknowense* [22]. So far, the genome has only been sequenced for a few species of filamentous fungi, and there is no detailed metabolic reconstruction of any filamentous fungi.

5 Fungi as Functional Food and Health

Fungi, particularly mushrooms such as *Cordyceps sinensis* (Fig. 3), *Ganoderma lucidum, Hericium erinaceus, Lentinus edodes* (Fig. 4), *Sclerotinia sclerotiorum, Tremella mesenterica, Trametes versicolor*, are increasingly consumed as food for their health benefits as nutraceuticals and functional foods [9, 23–27]. Besides nutritional benefits, many fungi are associated with medicinal properties and myths. These mushrooms are rich in some physiologically important components, especially β-glucan polysaccharides, which are responsible for anticancer, immunomodulating, hypolipidemic, antioxidant, and neuro-protective activities. Shiitake mushrooms have been used medicinally by the Chinese for more than 6,000 years. Another fungus *Ganoderma lucidum* is a potent immune system regulator, promising anticancer agent, and stress reducer. This mushroom is frequently used in traditional Chinese medicine [28]. All Cordyceps species (about 400 species known) are endoparasitoids, parasitic mainly on insects and other arthropods.

Fig. 3 *Cordyceps sinensis* – The caterpillar fungi emerging from larvae

Fig. 4 Cultivation of Shiitake mushroom (*Lentinus edodes*), Courtesy of Sophie Mérillon (Loches, France)

Gordon Wasson believed that the Soma plant used in religious ceremonies, over 4000 years ago by Aryans, was a mushroom (the other plants considered are Amanita, *Ephedra sinica, Cannabis sativa,* etc). The Vedic juice called "soma rasa" is said to bestow divine qualities on the soul of the consumer, even immortality [24, 25]. The fungus *Cordyceps sinensis* is also found in Tibetan medicine. Traditional healers in Sikkim recommend the fungus/mushroom *Cordyceps sinensis* to improve energy, appetite, stamina, libido, endurance, and sleeping patterns [25]. Main constituent of the extract derived from this fungus comprises a novel biometabolite called as Cordycepin (3'deoxyadenosine), which has a very potent anticancer, antioxidant, and anti-inflammatory activities [26] (see ▶ Chap. 23, "Cordycepin: A Cordyceps Metabolite with Promising Therapeutic Potential" in this book).

6 Fungi in Pharmaceuticals

The word antibiotic was used for the first time by Selman Waksman in 1941 to describe any small molecule made by a microbe that antagonizes the growth of other microbes [29]. On the morning of September 3, 1928, when Professor Alexander Fleming was in process of cleaning up plates loaded with Staphyloccocus, he observed a clean zone around the mold (*Penicillium notatum*) grown as contamination. Fleming concluded that the bacteria on the plate around the mold had been killed off by some substance that had come from the mold. It was 10 years later that Howard Florey and Ernst Chain, working at Oxford University, isolated the bacteria-killing substance found in the mold – penicillin. (http://www.historylearningsite.co.uk/a-history-of-medicine/antibiotics/).

This discovery and medicinal use of antibiotics in the 1950s revolutionized the treatment and suffering and increased the life span. Antibiotics are useful in the treatment of bacterial, fungal, and protozoal infections and some physiological diseases (e.g., lowering cholesterol) [30]. Landmark discoveries of fungal metabolites are shown in Fig. 5. With the increasing use of antibiotics, resistance to antibiotics started developing, which leads to search for new antibiotics. The number of new antibiotics, mainly analogs, increased almost exponentially. To eliminate antibiotic resistance, the pharmaceutical industry developed thousands of new semi-synthetic antibiotics, opening up a new area of antibiotic discovery [4].

More than 90 % of the studies performed by large pharmaceutical companies between 1980 and 2003 resulted in decreasing profits due to increased research expenses, the small number of new leads, and regulatory obstacles [31–33]. It is difficult to assess the number of metabolites produced by fungi but currently total entries for fungal metabolites on Google scholar is 466,000. Some of the selected recently discovered (2013–2015) bioactive molecules with medicinal properties are presented in Table 2. Some of these molecules can potentially be future medicines. Antibiotics are major contribution of fungi towards human health and alleviating suffering, for example, genus *Streptomyces* (in historical perspectives) being principal producer (~80 % of total known) of large number of antibiotics and secondary

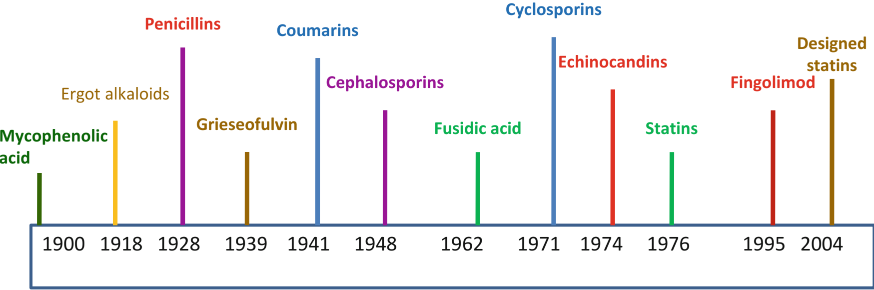

Fig. 5 Historical developments showing discovery of fungal metabolites

Table 2 Recent examples of bioactive molecules of fungi and their biological activities

Metabolites	Source	Bioactivity	References
Ascomycone B and 6-deoxyfusarubin	*Biatriospora* sp. CCF 4378	Cytotoxicity	[37]
Asperterpenoid A; asperlones A and B, mitorubrin	*Aspergillus* sp. 16-5c	Inhibitor of Mycobacterium tuberculosis protein tyrosine phosphatase B	[38, 39]
Aspiketolactonol, aspyronol, epiaspinonediol	*Aspergillus* sp. 6-02-1	Cytotoxic: human cancer cell lines K562, HL-60, HeLa, and BGC-823	[40]
Apicidin F	*Fusarium fujikuroi*	Antimalarial	[41]
Beauvericin	*Fusarium* sp.	Trypanocidal activity	[42]
Citrinin	Sponge associated *Penicillium* sp.	Antibacterial and cytotoxic	[43]
Cladosin C	*Cladosporium sphaerospermum* 2005-01-E3	Antiviral activity: influenza A H1N1 virus	[44]
Cercosporenes F	*Cercospora* sp.	Cytotoxic: human cancer cell lines HeLa, A549, MCF-7, HCT116, T24 and induces autophagy in HCT116 cells	[45]
1-(2,6-dihydroxyphenyl) pentan-1-one	*Cryptosporiopsis* sp.	Antibacterial	[46]
6,8-di-O-methylaverufin	*Aspergillus versicolor*	Antibacterial	[47]
Dihydronaphthalenone 2	*Nodulisporium* sp.	Antimycobacterial activity	[48]
Dinapinone AB2	*Talaromyces pinophilus* FKI-3864	Inhibition of triacylglycerol synthesis in mammalian cells	[49]
Fumiquinazoline Q and Protuboxepin E	*Penicillium expansum* Y32	Mitigative effect on bradycardia and vasculogenetic activity	[50]
Gliotoxin	*Aspergillus* sp. YL-06	Cytotoxic: human cancer cell lines HeLa	[51]
Ganoleucoins A and C	*Ganoderma leucocontextum*	Inhibitory activity against HMG-CoA reductase	[52]
4-Hydroxymellein	*Phoma sp.*	Inhibitory activity against P388 murine leukemia cells	[53]
Herqueidiketal	*Penicillium* sp.	Significant activity against *Staphylococcus aureus* sortase A.	[54]
Hispidin	*Phaeolus schweinitzii*	Antioxidant activity	[55]
Isosclerone	*Aspergillus fumigatus*	Antiproliferative:MCF-7 human breast cancer cells	[56]
Nodulisporiviridin G	*Nodulisporium* sp. (No. 65-17-2-1)	Amyloid β42 aggregation inhibitory activities	[57]
Neoechinulin A	*Eurotium* sp. SF-5989	Anti-inflammatory effect	[58]
Pestalotiopsone A	*Pestalotiopsis* sp.	Antibacterial	[59]

(*continued*)

Table 2 (continued)

Metabolites	Source	Bioactivity	References
Polyporusterone B	*Polyporus umbellatus*	Antitumor activity: HepG2 cells	[60]
Phenylpyropenes E and F	*Penicillium concentricum* ZLQ-69	Cytotoxic: MGC-803 cell line	[61]
Pinazaphilones B and (±)-penifupyrone	*Penicillium* sp. HN29-3B	Inhibits α-glucosidase	[62]
Reduced gliotoxin , 6-acetylbis(methylthio) gliotoxin	*Neosartorya pseudofischeri*	Cytotoxic and antibacterial	[63]
Solaninaphthoquione	*Fusarium solani* PSU-RSPG227	Cytotoxic: MCF-7 human breast cancer cells	[64]
Sorbicatechols A and B	*Penicillium chrysogenum* PJX-17	Antiviral:influenza virus A (H1N1)	[65]
Stemphyperylenol	*Botryosphaeria dothidea* KJ-1	Antifungal and cytotoxicity against HCT116 cancer cell line	[66]
Verrucosidin	*Penicillium* sp. TPU1271	Antimycobacterial activity.	[67]

metabolites [34–36]. Besides antibiotics, cholesterol synthesis inhibitor statin, ergot alkaloids, and lipase are important pharmaceutical products of fungal origin.

7 Fungal Mycotoxins

Mycotoxins are low molecular weight (MW ~ 700) toxic secondary metabolites of certain fungi. They are often very stable molecules and produced by several genera, in particular *Penicillium*, *Aspergillus*, and *Fusarium* spp. [68, 69]. Besides these, other genera exhibiting mycotoxin production includes *Alternaria, Chaetomium, Cladosporium, Claviceps, Diplodia, Myrothecium, Monascus, Phoma, Phomopsis, Pithomyces, Trichoderma*, and *Stachybotrys* spp. [70]. Mycotoxin contamination is a global problem and occurs both in temperate and tropical regions of the world, based on the species of fungi. Major food crops infected by fungi are cereals, cocoa, coffee, oil seeds, spices, nuts, dried fruit, dried peas, beans, and fruits. The cereals are usually invaded by fungi both in the field and after harvest, and as a consequence they carry multitoxins. Generally, crops that are stored for more than a few days become vulnerable to mold growth and mycotoxin formation. The production of mycotoxins is often inevitable and depends on environmental conditions during the plant growth and the subsequent food storage.

These toxins have caused major epidemics in humans and animals during ancient times. Ergotism [71] and Alimentary Toxic Alexia (ATA) [72] were some of the dreaded mycotoxicosis instances which killed hundreds and thousands of people in Europe and Russia, respectively. Mycotoxins cause four basic kinds

of toxicity: acute, chronic, mutagenic, and teratogenic. Different mycotoxins have different modes of action in the body, while some deteriorate the liver or kidney function to the extreme that may cause death or interfere with protein synthesis which produces series of other problems including extreme immunodeficiency, some are neurotoxic responsible for brain damage or death in higher doses. Due to the variety of structures of these toxins, it is impossible to use one standard technique for analysis and/or detection. The most studied mycotoxins are aflatoxins (AF), citrinin, trichothecenes such as deoxynivalenol (DON), patulin, ochratoxin A (OTA), fumonisins (FB), and zearalenone (ZEA) and some major toxins of endophytic fungi (ergot toxins and ergotamine) (Fig. 6).

7.1 Aflatoxins

Aflatoxins (AF) are mycotoxins that are produced by fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Among them, *A. flavus* has been reported to be a common contaminant of agricultural produce. However, *A. bombycis, A. ochraceoroseus, A. nomius,* and *A. pseudotamari* are also less common, AF-producing species [73–75]. Aflatoxins were discovered because of their devastating effect on turkey poults (Turkey-X-disease) and other chicks in 1960 in England, when more than 100,000 turkey poults died [76]. Aflatoxin contamination is most common in African, Asian, and South American countries with warm and humid climates, but also occurs in temperate areas of North America and Europe. The habitat of *Aspergillus* is in soil, decaying vegetation, hay, and grains, which are undergoing microbiological deterioration. It can invade all types of organic substrates with inadequate drying or improper storage conditions. They have a particular affinity for nuts and oil seeds. Peanuts, maize, and cotton seed are the three most important crops affected by *Aspergillus*.

AF are difuranocoumarin derivatives produced by a polyketide pathway. The naturally occurring aflatoxins are AFB1, AFB2, AFG1, and AFG2, here “B” and “G” refer to the blue and green fluorescent colors produced by these compounds under UV light and relative chromatographic mobility during thin-layer chromatography (TLC) [75] and the subscript numbers 1 and 2 indicate major and minor compounds, respectively. Among these, AFB1 is the most abundant, toxic, and carcinogenic [77]. Other aflatoxins AFM1 and AFM2 are the hydroxylation products of AFB1 and AFB2, respectively, and are found in milk and milk products [78]. After entering the body, aflatoxins may be metabolized by the liver to a reactive epoxide intermediate or hydroxylated to become AFM1 and AFM2. AFM1 and AFM2 are in lower toxicity than the parent molecules, but significant because of the widespread consumption of cows’ milk by infants.

Aflatoxins are hepatocarcinogenic in humans, particularly in conjunction with chronic hepatitis B virus infection; thus, the probability of people developing cancer of the liver is much higher in areas where both aflatoxins and hepatitis B are prevalent [79]. Aflatoxins are not only hepatocarcinogen, but they are also

Fig. 6 Structures of selected mycotoxins

genotoxins and immunotoxins, which can suppress both cellular and humoral responses and also be responsible for growth retardation in animals [80, 81].

7.2 Ochratoxin

Ochratoxin A (OTA) was first discovered as a fungal metabolite that showed toxic behavior towards animals, from a strain of *Aspergillus ochraceus* in 1965 [82].

Ochratoxin A is mainly produced by *Aspergillus ochraceus, Aspergillus carbonarius*, and *Penicillium verrucosum* [83]. The natural occurrence of these fungi is widespread, since all these species grow in a wide range of conditions (substrate, pH, moisture, and temperature). *A. ochraceus* dominates in tropical regions, while the *P. verrucosum* predominates in temperate regions such as eastern and north eastern Europe, Canada, and parts of South America [84]. OTA commonly contaminates grains such as corn, barley, oats, rye, and wheat and has also been reported in other plant products including coffee beans, spices, nuts, olives, grapes, beans, and figs [85, 86]. Mostly dried vine fruits, wines, and probably coffee are contaminated with *A. carbonarius* [87] which can survive sun drying. Biosynthetically, it is a pentaketide derived from the dihydrocoumarins family coupled with β-phenylalanine. The biochemistry and molecular biology of ochratoxin A biosynthesis have been comprehensively reviewed [88].OTA is a potential risk to human health not only as a result of the intake of contaminated foods of vegetable origin, but also through foods of animal origin. It is important to know that ochratoxin A is fat soluble and stable molecule and is not readily excreted, which means that the intake of OTA leads to its buildup in the circulatory system, liver, and other tissues of the animals. This is due to the feeding of mold-contaminated fodder by animals.

7.3 Fumonisins

Fumonisins are a group of polyketide mycotoxins that are produced by *Fusarium verticillioides* and *Fusarium proliferatum*, main pathogens of maize and sorghum [89]. Fumonisins were discovered in the late 1980s as the result of many years of study of the disease known as equine leucoencephalomalacia (LEM). Fumonisin B1 and fumonisin B2 were isolated for the first time by Gelderblom and coworkers [90] from cultures of *F. verticillioides* MRC 826 by means of a bioassay based on the cancer-promoting activity in rat liver. Fumonisins consist of a 20-carbon aliphatic chain with two ester-linked hydrophilic side chains, resembling sphingosine, an essential phospholipid in cell membranes. The toxic action of fumonisins appears to be a result from competition with sphingosine in sphingolipid metabolism [91]. It is also important to mention that the main concern for human health in regard to aflatoxins, ochratoxin A [92], and fumonisins in developed countries appeared to be their carcinogenic or genotoxic and teratogenic effects rather than their acute effects [93].

7.4 Zearalenone

Zearalenone (ZEA) is produced by different species of *Fusarium* fungi, for example, *F. graminearum, F. culmorum, F. cerealis, F. equiseti, F. crookwellense*, and *F. semitectum*. These common soil fungi are pathogenic to cereal crops worldwide [75]. Zearalenone is heat-stable and is found in a number of cereal crops, such as maize, barley, wheat, oats, rice, and sorghum [94]. *Fusarium* spp. infect cereals in

the field. Toxin production mainly takes place before harvesting, but may also occur during or post harvest if the crop is not handled and dried properly [95]. Zearalenone is a nonsteroidal mycotoxin biosynthesized through a polyketide pathway. ZEA is a resorcylic acid lactone. Historically, Christensen and coworkers [96] isolated this metabolite and called it F-2. Its isolation resulted from the observation of the disease syndrome in two swine herds in Minnesota. Prior to the discovery and implementation of modern milling practices, *Fusarium* species have been implicated in several human outbreaks of mycotoxicoses [97]. Unlike Ochratoxin, ZEA is rapidly biotransformed and excreted after consumption; therefore, the dietary intake of ZEA from meat is probably of less significance [98]. However, ZEA can be excreted into milk after lactating cows are fed it in high doses. ZEA and its metabolites are often referred to as a mycoestrogen because of its potent estrogenic activity. ZEA is implicated in reproductive disorders of farm animals and occasionally in hypoestrogenic syndromes in humans [99]. Studies have demonstrated the potential for ZEA to stimulate growth of human breast cancer cells containing estrogen response receptors [100]. Another recent study also established a potential role of α-ZEA in the risk of developing breast cancer [101].

7.5 Patulin

Patulin (PAT) is a toxic metabolite produced by several species of *Penicillium, Aspergillus,* and *Byssochlamys* [102, 103]. It is the most common mycotoxin in apples and apple-derived products including juice, compotes, cider, and baby foods. It also contaminates other fruits such as grapes, oranges, pears, and peaches [102, 104]. Patulin (4-hydroxy-4H-furo [3,2c] pyran-2[6H]-one) is a water-soluble lactone isolated in 1940s. Initially PAT was isolated as a broad spectrum antifungal antibiotic. Due to co-discovery of the compound by various groups, it has historically been known by names such as clavacin [105], expansine [106], claviformin [107], clavatin [108], gigantic acid [109]. Patulin poses several biological activities and induces toxicity in vitro and in vivo. It is considered as genotoxic, immunotoxic, neurotoxic to rodents, and teratogenic to chicken [110]. Cellular effects of patulin include the formation of reactive oxygen species (ROS), cell cycle arrest, cytochrome C release from mitochondria, caspase-3 activation, PARP cleavage, ATF3 expression, and subsequent apoptosis. Patulin causes DNA damage and is mutagenic, carcinogenic, and teratogenic [102, 104, 111, 112]. On the other hand, a recent study suggests that PAT induces cytotoxicity through a ROS-dependent mechanism involving endoplasmic reticulum stress and activation of mitochondrial apoptotic pathway in human intestinal and kidney cells [113].

7.6 Trichothecene Toxin: Deoxynivalenol

Mycotoxin deoxynivalenol (DON; also known as vomitoxin) is an epoxy-sesquiterpenoid produced by fungi, *Fusarium graminearum* and *F. culmorum*.

They are the most potent eukaryotic protein-synthesis inhibitors known [114]. Vomitoxin has been naturally occurring in cereal grains throughout the world. It occurs mostly in grains such as wheat, barley, oats, rye, and maize, and less often in rice, sorghum, and triticale [115]. Its capacity to induce vomiting episodes in various species including humans explains its commonly used nickname "vomitoxin."

8 Fungi as Producer of Colorants

There is an increasing demand for pigments from natural sources which can be used in foodstuff, cosmetics, and pharmaceuticals. Currently, the major sources of natural colorants are of either plant or animal origin. The production of many currently authorized natural food colorants has numerous disadvantages, like nonavailability throughout the year, variations in pigment extraction, instability against light, heat or adverse pH, and low water solubility. Even the present commercial natural food colorants have several drawbacks, for instance the violet and purple colors of anthocyanins (flavonoids) are sensitive to oxidation, bleaching by sulfur dioxide, and vary with pH, limiting their application to acidic foods and beverages. Betanins, carotenoids, and chlorophyll pigments are easily decolorized by oxidation, making them sensitive to light, heat, and oxygen. Furthermore, there are also some weird source of natural colorants like red colorant carmine; to produce 100 g carmine colorant, approximately 14,000 female cochineal insects are required [116]. To overcome these problems, alternative ways to produce natural colorants are highly desired. In this regard, fungi have attracted special attention. Fungi-derived food colorants are natural, which shows high chemical and light stability [117, 118], high yield, and variety of colors. The authorization of fungal food colorant has expedited research to explore the fungi for the biotechnological production of pigments. However, these studies require a comprehensive knowledge of already recognized fungal metabolites, pathogenic strains, and toxin producers.

Filamentous fungi produce an extraordinary range of pigments that include several chemical classes such as carotenoids, melanins, flavins, phenazines, quinones, and more specifically monascins, violacein, or indigo. These pigments show diversity of chemical structures and a spectrum of colors (Figs. 7 and 8). In nature, these pigments help fungi to serve different ecological functions, for example, melanins protect them against environmental stress, caroteniods against lethal photo-oxidations, and flavins act as cofactors in enzyme catalysis [119, 120].

Fungi are looked upon as future microbial cell factories for the production of food grade pigments. However, there are discrepancies and controversial views over the safety of these pigments. This is due to the co-production of mycotoxin along with the target compound. For instance, *Monascus* spp. are often used for rice fermentation to produce red yeast rice, a special product used either for food coloring or as a food supplement in the South East Asia for more than thousands of years [121] (see ► Chaps. 16, "Pigments and Colorants from Filamentous Fungi," ► 26, "Monascus Secondary Metabolites," and ► 8, "Melanin Pigments of Fungi" in this book).

Fig. 7 Chemical structures of a few important fungal colorants

The colored appearance (red, orange, or yellow) of *Monascus* fermented substrates is produced by a mixture of oligoketide pigments that are synthesized by a combination of polyketide and fatty acid synthases. The major pigments consist of pairs of yellow (ankaflavin and monascin), orange (rubropunctatin and monascorubrin) [122], and red (rubropunctamine and monascorubramine) compounds. Although these polyketide pigments from the *Monascus* sp. have been commercially produced and legally used as food colorants in the form of pigment extracts, European Union (EU) and USA have not approved them as food colorant due to the risk of the possible contamination by the nephrotoxic and hepatotoxic metabolite citrinin and a group of monacolin substances [123]. Other examples of mycotoxin co-production are fungal hydroxyanthraquinoid (HAQN) pigments like emodin (yellow), physcion (yellow), questin (yellow to orange-brown), erythroglaucin (red), catenarin (red), and rubrocristin (red), produced from some strains of *Aspergillus* sp. (*A. glaucus*, *A. cristatus*, and *A. repens*) [124] and are contaminated with secalonic acid D, oxaline, citrinin, tanzawaic acid A, cyclochlorotine, islanditoxin, luteoskyrin, erythroskyrin rugulosin, or aspergiolide A. All these fungal secondary metabolites, the yellow and the red HAQN pigments that show substitution on both aromatic

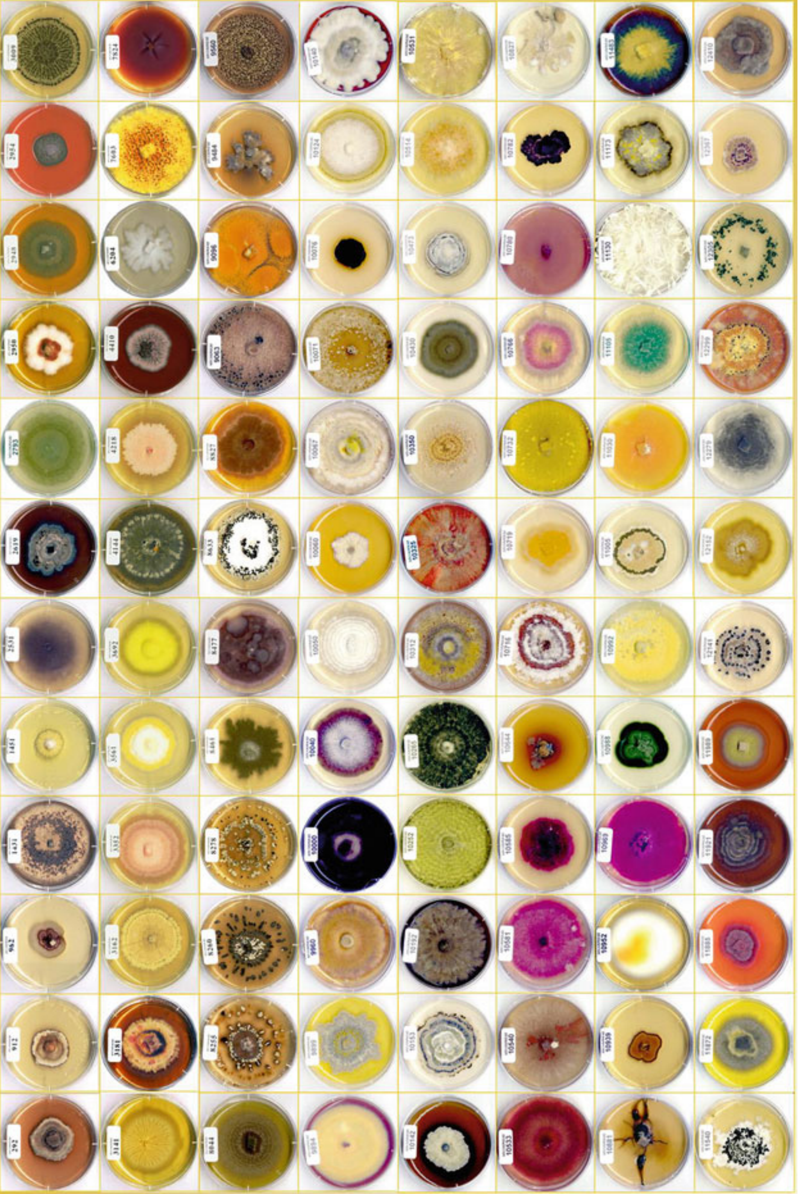

Fig. 8 A representative photo of an array of pigments produced by fungi (Courtesy of Olivier Laurence, Mycosphere, Fumel, France)

rings and the naphthoquinone type mycotoxins, are biosynthetically synthesized by polyketide pathway. Likewise, species of *Eurotium* co-produce the mycotoxin echinulin and two benzaldehyde coloring compounds: flavoglaucin (yellow) and auroglaucin (red) along with the yellow pigment physcion and the red pigment erythroglaucin [125]. This infers that these fungal strains are not safe, and there is an urge to continue to explore new strains of fungi which do not produce mycotoxins. Many of such strains are now known; for example, a strain of *Dermocybe sanguinea* (*Cortinarius sanguineus*) produces red HAQN glycoside dermocybin-1-b-D-glycopyranoside, together with the pigments emodin and physcion without co-production of mycotoxins [126]. Another polyketide pigment orevactaene is shown to be produced by nonmycotoxigenic fungi *Epicoccum nigrum* [127]. Another useful discovery is the production of *Monascus* like polyketide azaphilone (MPA) pigments without co-production of citrinin or any other known mycotoxins, by some strains of *Talaromyces* species (formerly *Penicillium sp.*) viz. *Talaromyces aculeatus, T. funiculosus, T. pinophilus*, and *T. purpurogenus* [127, 128]. Moreover, even a patent has been granted for a submerged cultivation method, for some of the nonmycotoxigenic strains of *Talaromyces* sp. [129].

Owing to the increasing demand of food colorant, an alternative route for the large-scale production of natural food colorants can be achieved by using modern tools of biotechnology and appropriate use of fermentation physiology. Heterologous expression of secondary metabolite production or even manipulation of culture medium or precursor/inhibitor feeding can enhance the desired product yield with improved functionality [130, 131]. Presently, we can find some fermentative food grade pigments in the market. Arpink red™ (now Natural Red™) manufactured by the Czech company (Ascolor Biotech followed by Natural Red) has been claimed to be produced by fermentation and bioprocess engineering using the fungal strain *Penicillium oxalicum*. Furthermore, riboflavin from *Ashbya gossypii*, lycopene and b-carotene from *Blakeslea trispora*, and bikaverin from *Fusarium* sp. are obtained from fermentation process [132–134].

9 Fungal Enzymes

Enzymes are biological macromolecules (primary metabolites) used to catalyze the chemical reactions. Human beings are using the enzymes since long back for the processes like cheese making, brewing, baking, and the production of antibiotics. In nature, fungi play a main role in degradation of plant biomass. They secrete a wide range of active enzymes and then absorb these “predigested” foodstuffs back into their cells. This natural phenomenon of fungi has lead researchers to identify and utilize various enzymes and proteases that fungi produce for industrial use [135]. Historically, Taka-diastase was the first enzyme preparation to be patented for industrial use by Dr. Jokichi Takamine in 1884. It was produced by the filamentous fungus *Aspergillus oryzae*. Since then, there are continuous advancements in exploration and extraction technology of fungal enzymes. Fungal enzymes are always preferred over plant and

Table 3 Functions and applications of fungal enzymes in diverse fields

Enzymes	Functions	Applications
Cellulases Cellulases, Beta-glucosidase	Improve paper quality and smooth fibers Ethanol production	Paper production Biofuels
Laccases	Soften paper and improving bleaching as biotransformers to remove nonionic surfactants	Paper Bioremediation
Lactase Pectinases	Part of β-glucosidase family of enzymes and can break down lactose to glucose and galactose in the manufacture of yogurt	Dairy industry
Lignocellulolytic enzymes	Breakdown of agrowaste in to ethanol	Biofuels
Lipase	Fat removal, esterification, hydrolysis	Dairy, detergent, pulp, pharmaceuticals, leather
Pectinases, cellulases –	To clarify fruit juices and form jams	Fruit and jam manufacturing
Peroxidases	Removal of pollutants by precipitation	Waste water treatment
Proteases	Protein to amino acids	Baking, brewing, detergent, leather
Yeast enzymes	Beer production and malting	Brewing industry
Tyrosinases	Convert monophenols into diphenols	Food additives, pharmaceutical drugs

animal enzymes owing to their wide varieties and exceptional properties. They are active under mild conditions related to temperature and pH. There production is cheaper and faster and the yield is higher. Moreover, fungal systems are easily susceptible to genetic manipulation. According to a report, till now about 200 fungal enzymes have been purified from fungal cultures with biochemical and catalytic properties characterized [136, 137]. These enzymes have significant uses in pharmaceutical, agricultural, food, paper, detergent, and textile-based industries (Table 3). Some of the main enzymes that dominate the industrial enzyme market are amylase, cellulase, chitinase, invertase, laccase, lipase, protease, tyrosinase, and xylase.

Amylases are possibly the most important enzymes in present day biotechnology because of their wide ranging application in food, fermentation, textiles, and paper industries. These enzymes have even successfully replaced the chemical hydrolysis of starch in starch-processing industries. Research on cellulases and related polysaccharidases actively began in the early 1950s. These enzymes convert lignocellulose to glucose and soluble sugars [138, 139]. Currently, cellulases are widely used in pharmaceutical, baking, detergent, wine, and textile industries [140]. Another enzyme of varied importance is chitinases. It hydrolyzes the linear polymer chitin. Though bacteria are one of the major sources of chitinase, filamentous fungi too have many different chitinases belonging to GH family 18 [141]. Over the past few years, chitin and chitin derivatives have been used in cosmetics, food, nutrition, and biotechnology. Chitinase gene from mycoparasitic

Trichoderma spp. when expressed in several agriculturally important plants, e.g., lemon, cotton, apple, and carrot, rendered in them defense response against various fungal pathogens [142, 143]. Unlike chitinase production, fungi are the best producers of industrial invertases. Yeast *Saccharomyces cerevisiae* is most extensively used for the purpose. This enzyme is widely used in the processed food and confectionery industry. Other uses of the enzyme include lactic acid production, fermentation of cane sugar molasses, etc [144]. Another enzyme of immense industrial potential is laccases. Laccases are copper-containing enzymes that catalyze the oxidation of a wide variety of organic and inorganic substrates including mono-, di-, and polyphenols, amino phenols, methoxy phenols, aromatic amines, and ascorbate [145]. Laccases have been found in Ascomycetes, Deuteromycetes, and Basidiomycetes; being particularly abundant in many white-rot fungi such as *Coriolus versicolor* and *Pycnoporus sanguineus* [146]. Most potential industrial applications of laccase are the delignification and pulp bleaching and the bioremediation of contaminating environmental pollutants [147, 148]. Laccase production is an expensive process. In order to find the affordable and higher yielding sources of laccase, cloning of laccase gene followed by heterologous expression is gaining impetus [149]. Similarly, lipase, an essential catalyst that digests water-insoluble lipids, also has costly purification procedures. In addition, it also exhibits extracellular instability. Therefore, in order to avoid enzyme purification step, direct use of cells or biomass with endoactivity of lipases within a porous biomass support could represent a very attractive process for lipase production and applied to several processes as in case of bulk biodiesel production. The fungi *Penicillium* sp. F2 and *Rhizomucor* sp. F18 showed great potential for extra and intracellular lipase production, aiming at its future use in processes of hydrolysis and transesterification of residual oils and greases of environmental sanitation [150]. Lipases are also beneficial in different industries such as food, pharmaceuticals, oleo chemicals, cosmetics, fuel, and detergents [151].

Among all these enzymes, one of the most important enzymes is proteases. It is estimated that proteases account for about 60 % of the total global industrial enzyme sales (>3 billion USD) [152]. Proteases hydrolyse the peptide bond (CO-NH) in a protein molecule. Proteolytic enzymes occur naturally in all living organisms. However, fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity [153, 154]. Filamentous fungal strains such as *Aspergillus* have been widely used for industrial production of protease. Industrially they have applications in food, leather, detergent, pharmaceutical, diagnostics, and waste management. Tyrosinase and xylanases are enzymes gaining importance due to their industrial and environmental applications. Tyrosinase can convert monophenols into diphenols and thus helps in production of antioxidant ortho-diphenols with beneficial properties as food additives or pharmaceutical drugs [155], while xylanases have many commercial uses, such as in paper manufacturing, animal feed, bread-making, juice and wine industries, xylitol production. Among xylanases sources, filamentous fungi are especially preferred due to their ability to secret enzyme into the medium and high xylanases production [156, 157].

10 Conclusions

The enormous biodiversity within the Mycota and the eminent role played by the fungi in the production of pharmaceuticals, chemicals, biofuels, enzymes, and food colorants is fascinating more and more people to the fungal research. Nonetheless, their studies also help in preventing the food and feed spoilage and pathogenicity caused by mycotoxins in plants, animals, and humans of different levels and intensity. Fungi as a group of microorganisms cause the most economical damage on crops [158]. Besides these notorious activities, some fungi are the great source of healthy food such as basidiomycetes. They are packed with useful vitamins, minerals, and secondary metabolites that have been found to inhibit the growth of different kinds of tumors [9, 159] and have many other beneficial effects.

Today people are more intended towards natural sources. In this regard, various fungal usage and high yields have always attracted the attention. For example, in case of food colorant, in many countries still plants are the main source of natural food colorant and fungi are feared of their co-mycotoxin production along with desired product. Thus, more imperative measures are required to carry out the necessary toxicology testing. In these ways, fungal pigments could be accepted by the current consumer round the world. Similarly, another thing in high demand is renewable sources of energy like biofuels. This can be met from agriculture waste or underutilized agriculture products if we can breakdown the cell wall polysaccharide, cellulose, hemicelluloses, and lignin into simple monomer molecules like glucose/cellobiose. However, these polysaccharides are hard nut to crack, and microbial/fungal enzymes are used to break the complex molecules in to glucose/cellobiose for biofuel generation (see chapter on Lignocellulose Degrading Enzymes from Fungi and Fungal Metabolism in this book). Production of biofuels/chemicals by microbial fermentation can have several advantages like lower costs of production than through traditional routes, use of renewable feed stocks, and production of chemicals with properties that allow for synthesis of new advanced polymers. In the past few years, rapid developments have occurred in the enzyme supply market. Present evolution in protein engineering and heterologous expression has revolutionized enzyme production and commercialization by extending the list of enzymes now available. This increasing demand and production of enzymes also increased the incidence of occupational exposure to high-molecular-weight allergens. Workers that are in direct contact with fungal enzymes are at a great risk of IgE-mediated disease and occupational asthma. Some of the majors which can be helpful in alleviation of enzyme exposure are like use of safety equipments; protein encapsulation and setting the threshold limit values [160].

Today fungi are one of the major parts of pharmaceutical industries, but still there is a need to continue to explore new bioactive molecules from fungi. As we know that now-a-days development of resistance in microbes and tumor cells has become a major problem. This resistance increasingly limits the effectiveness of current antibiotics. According to a report by Katz [161] in 2004, more than 70 % of pathogenic bacteria were estimated to be resistant to at least one of the currently available antibiotics. We believe that novel antibiotics and other bioactive secondary

metabolites can still be discovered from microbial sources knowing the fact that in fully sequenced fungi, numbers of genes and gene clusters that potentially may lead to production of secondary metabolites are very higher in accordance with the number of secondary metabolites known [130, 162]. Fungi are easy to cultivate and scale up as compared to plant cells and are grown at relatively very high volumes. Genome, transcriptome, proteome, and metabolome analyses help us in understanding fungal science as a whole. Now tools with advanced version for metabolome analysis, such as mass spectroscopy, single crystal x-ray diffraction, and nuclear magnetic resonance spectrometry with increasingly sophisticated methods of chromatography, have made possible continuing discovery of novel fungal metabolites and future wonder drugs.

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Part I

General Biology

Fungal Genes and Metabolites Associated with the Biocontrol of Soil-borne Plant Pathogenic Fungi

2

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Abstract

The biocontrol of plant pathogenic fungi includes two complementary approaches depending on whether the aim is to control soil-borne or air-borne pathogenic fungi. In the first case, natural biotic interactions within the indigenous microflora should be stimulated to regulate inoculum density and the infectious activity of pathogen populations. This strategy can be enhanced by inoculating one or more previously selected biocontrol agents. In the second case, one or more previously selected biocontrol agents can be sprayed on plant foliage to interfere with the development of the targeted pathogen through different mechanisms involving particular enzymes or metabolites. Selecting the most effective biological control agents implies (i) knowing the mechanisms of their interactions with the pathogens and (ii) checking that the environment in which the biocontrol agent is introduced will permit the expression of these mechanisms. The common thread of this chapter is the impressive diversity of metabolites and proteins produced by fungi and involved in interactions between pathogenic and nonpathogenic fungi. Many metabolites and proteins were discovered empirically or by chance a few decades ago, and what we knew about them was they inhibited the growth of pathogenic models on agar medium. Fungi producing these metabolites were not well-known fungal species and were not used as biocontrol agents. However, the demonstration of their intense metabolic activity paved the way for more investigations in this area and led to deciphering the mechanisms of interactions between fungal strains. Thus, in recent years a large number of enzymes, signal molecules, secondary metabolites, large-size proteins, as well as new metabolic pathways have been revealed by genomics, and it is now possible to understand why some strains can control a given pathogen more than others or stimulate plant defense reactions. To date, the most studied fungi include many strains of the genus *Trichoderma* but also the species *Chlonostachys rosea, Coniothyrium minitans, Verticillium biguttatum,* and the oomycete *Pythium oligandrum*. All of them are successfully used as biocontrol agents. This chapter does not aim to provide a comprehensive catalog, but rather to associate these metabolites and proteins to the modes of action involved in pathogen control. The state of the art presented in this review suggests promising prospects for rational, appropriate, and effective use of the biocontrol potential offered by the huge diversity of fungal metabolites and proteins.

Keywords
Antagonism • Antibiosis • Competition • Mycoparasitism • Host recognition • Plant defense

List of Abbreviations

6PAP	6-pentyl-alpha-pyrone
ABC	ATP-binding cassette
cAMP	Cyclic adenosine monophosphate
CBD	Carbohydrate-binding module
CWDE	Cell-wall-degrading enzyme
GH	Glycosyl hydrolase
ISR	Induced systemic resistance
MAPK	Mitogen-activated protein kinase
NAGase	*N*-acetyl-β-glucosaminidase
NRPS	Non-ribosomal peptide synthetase
PGPF	Plant-growth-promoting fungi
PGPR	Plant-growth-promoting rhizobacteria
PKS	Polyketide synthase
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
TF	Transcription factor

1 Introduction

Soil-borne diseases are a permanent acute issue in agriculture because means for pest and disease control in complex environments are limited. The traditional approach of soil-borne disease control consists in trying to eradicate the pathogens from the soil. This has led to the use of very dangerous biocides whose side effects often result in increased phytosanitary risks related to the acquisition of resistance by pathogens or the emergence of new pest populations. These biocides also affect the entire soil-borne biota and destroy the natural regulation mechanisms among populations, as in disease-suppressive soils [1–3]. Microorganisms, and also the soil microfauna, directly or indirectly interact through parasitism or antibiosis, amensalism or competition for the exploitation of common resources. They also interact via plants by priming plant defense reactions and via rhizodeposits that in turn may select microbial populations in the rhizosphere [2, 4]. The soil interferes with the relationships between and among microorganisms, pathogens, and plants in several ways, and it can modify the interactions among microorganisms themselves [5, 6]. Different situations in which such biotic and abiotic interactions may play a role in plant health have been described all around the world. They correspond to soils in which disease caused by a type of pathogen to a host plant type is weakly or not at all expressed, as if disease suppression was a constitutive feature of the biota in these soils. In other situations, disease suppression is acquired gradually thanks to the use

of farming practices including organic amendments and specific green fertilizers, appropriate crop rotation schemes, or on the contrary extended monocultures. However, the outcomes of disease suppression management are frequently variable and not sustainable yet. Disease suppression has been demonstrated for a wide range of soil-borne plant pathogens including bacteria, nematodes, oomycetes, and fungi [7–11].

All these situations fall within the concept of soil suppressiveness proposed by Cook and Baker [12], namely, a suppressive soil is a soil in which the pathogen does not establish, or establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease becomes less severe although the pathogen may persist in the soil. However, even if all these situations pertain to the same concept, each of them is very specific and involves different mechanisms, with (i) different unevenly distributed soil microorganisms, or (ii) different functions encoded by genes of various taxonomic origins but expressed according to environmental conditions, or (iii) both. Apart from some cases of acquired soil suppressiveness, such as take-all disease for which 2,4-diacetylphloroglucinol produced by *Pseudomonas* populations is involved in the suppression of *Gaeumannomyces graminis* var. *tritici* [9], so far attempts at deciphering the mechanisms involved in soil suppressiveness have revealed a limited number of genes or proteins, enzymes or secondary metabolites [13].

Conversely, studies of the modes of action of biocontrol agents and research efforts devoted to plant–pathogen interactions at the cellular and molecular levels have helped to highlight enzymes, secondary metabolites, and signal molecules that may act alone or in interaction in pathogen suppression [14]. A large number of metabolites belonging to different biochemical families have been described for their ability to inhibit the growth of plant pathogenic fungi. Much work on the genetics of biological control using fungal antagonists has been done, mainly with the genus *Trichoderma* [15–18]. Previous reviews had focused on this genus [19, 20], but they also described expressed genes involved in the modes of action of many other biocontrol fungi such as *Chlonostachys rosea*, *Coniothyrium minitans, Pseudozyma* sp., *Stachybotrys elegans*, *Verticillium biguttatum,* and the oomycete *Pythium oligandrum* [21–26]. All these studies make up a complex database that should be used to better understand the successes but also the failures encountered in biological control and in integrated pest management. The presence or absence of a given set of these metabolites in a complex environment could indeed be an indicator of biological activity capable of controlling or not the infectious activity of targeted or nontargeted pathogenic agents. For example, this could be a way to assess the risk of growing a given crop sensitive to the pathogen(s) or to evaluate the phytosanitary impact of innovative agricultural practices. However, all these data are particularly scattered in the literature, which is very abundant but should be used cautiously. Some metabolites were described more than 30 years ago but have never been exploited since. Others have been detected by chemists mainly interested in their physico-chemical structure, not by plant pathologists interested in the biological control of pathogens. Therefore, the biological significance of many of these metabolites remains to be validated because in most cases only in vitro activity has been

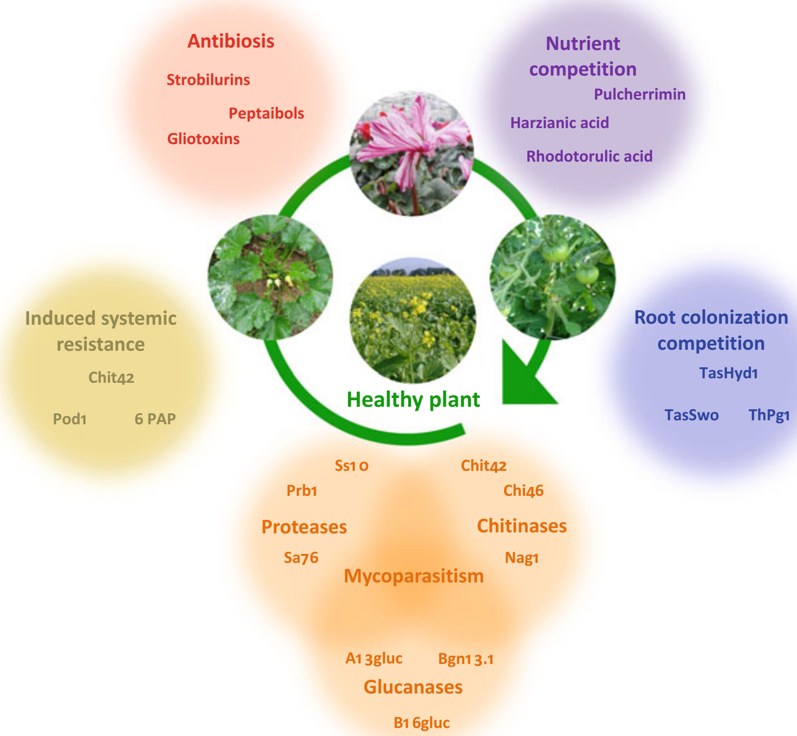

Fig. 1 Schematic representation of the five main modes of action by which biocontrol agents antagonize pathogenic fungi and promote the growth of healthy plants. *Chit42* Chitinase 42KDa, *Chi46* Chitinase 46KDa. *Nag1* *N*-acetyl-beta-d-glucosaminidase, *Ss10* subtilisin-like serine protease 10, *Prb1* basic proteinase 1, *Sa76* Secreted aspartic protease 76, *Pod1* Pythium oligandrum D-type cell wall protein 1, *6PAP* 6-pentyl-α-pyrone, *ThPg1* Trichoderma harzianum Endopolygalacturonase 1, *TasHyd1* Trichoderma asperellum Hydrophobin 1, *TasSwo* Trichoderma asperellum Swollenin

demonstrated. We do not claim to be exhaustive in this chapter. However, we went through an abundant literature and tried to list metabolites, enzymes, and other proteins described to have a potential role in the control of soil-borne fungal pathogens or post-harvest pathogens. Depending on studies, metabolites/proteins and/or their encoding genes have been identified. Biochemical characterization of proteins usually makes it possible to determine the genes encoding them, and on the other hand, knowing the gene leads to the protein. We do not aim to provide a descriptive catalog, but rather to associate these metabolites to the modes of action involved in the control of pathogens. These modes of action are antibiosis, microbial competition for nutrients and root colonization, mycoparasitism, host recognition, and induction of plant defense reactions (Fig. 1). They are successively discussed below. For each of them, we rank the various metabolites and proteins according to

their biochemical families. They are presented in Tables. In parallel, we explain the mechanisms whereby some of these metabolites directly or indirectly affect the metabolism of pathogens by picking a few examples from the many cases presented in the Tables.

2 Antibiosis

Fungal antibiosis is related to the production of secondary metabolites by fungi (e.g., *Trichoderma* spp., *Gliocladium* spp.). It results in microbial antagonism. Many different molecules involved in the suppression of several soil-borne plant pathogens have been described (Table 1, Fig. 2). They include pyrones, polyketides, peptaibols, gliotoxins, fatty acids, and glycolipids or strobilurins. They are toxic to pathogenic fungi at concentrations that depend on the compound and on the target [2]. Many fungi are known to produce secondary metabolites but these molecules are not always tested against pathogenic fungi. Table 1 groups the main fungal metabolites for which deleterious effects on pathogenic fungi or oomycetes have been demonstrated. Growth inhibition is generally observed, but the molecules are rarely tested in vivo for decreased symptoms on plants. In addition, the related mechanism is not always well understood. Different proteins and genes involved in the synthesis of secondary metabolites are also presented in Table 2.

2.1 Pyrones

One of the major pyrones produced by *Trichoderma* spp. is 6-pentyl-alpha-pyrone (6PAP) (Table 1). It was first isolated from *Trichoderma viride* [135], and it is produced by several *Trichoderma* species, among which *Trichoderma harzianum* and *Trichoderma atroviride* [30, 136]. 6PAP displays strong antifungal activity against several phytopathogenic fungi such as *Rhizoctonia solani*, *Botrytis cinerea,* and *Fusarium* species [27–30]. Although 6PAP is the best studied pyrone in *Trichoderma* spp., pyrone-like metabolites may explain why different *Trichoderma harzianum* strains more or less successfully antagonize *Gaeumannomyces graminis* var. *tritici* [137]. 6PAP is derived from the oxidation of the fatty acid linoleic acid. Although the main steps of 6PAP synthesis in *Trichoderma harzianum* have been elucidated, the enzymes involved in its biosynthesis pathway still need to be identified. A lipoxygenase gene found in *Trichoderma atroviride* may be involved, but no functional characterization has yet been performed [104] (Table 2). 6PAP is weakly toxic, biodegradable, and used as a food additive, so it is a good candidate for the development of new agricultural fungicides. To date, the major limitation to its field use is related to the cost of its synthesis. Only nontargeted pre- or post-harvest applications are economically conceivable. That is why several cheaper analogs have been synthesized and tested in vitro and/or in vivo for their antifungal activity [138, 139]. For example, (R)-5,6-dihydro-6-pentyl-2H-pyran-2-one displayed promising antifungal activity in vitro against *Penicillium* species, whereas 6-butyl-,

Table 1 Main families or groups of secondary metabolites known to be deleterious to soilborne pathogenic fungi or oomycetes

Family or group of molecules	Metabolite	Source fungus	Target pathogens[a]	References
Pyrones	6-pentyl-α-pyrone	*Trichoderma harzianum*	*Botrytis cinerea, Fusarium moniliforme, Fusarium oxysporum* f. sp. *lycopersici, F. oxysporum* f. sp. *cucumerinum, Rhizoctonia solani*	[27–30]
	6-pentyl-α-pyrone	*Trichoderma viride*	*Botrytis cinerea*	[28]
	Viridepyronone	*Trichoderma viride*	*Sclerotinia rolfsii*	[31]
	Solanapyrone C	*Nigrospora* sp. YB-141	*Aspergillus niger, Botrytis cinerea, Penicillium islandicum*	[32]
	Solanapyrones N, O	*Nigrospora* sp. YB-141	*Botrytis cinerea, Penicillium islandicum*	[32]
	Pycnophorin	*Botryosphaeria dothidea* KJ-1	*Alternaria solani*	[33]
Polyketides and derivatives	Alveolarin	*Polyporus alveolaris*	*Botrytis cinerea, Fusarium oxysporum, Mycosphaerella arachidicola, Physalospora piricola*	[34]
	Chaetochromone A	*Chaetomium indicum* CBS 860.68	*Poria placenta*	[35]
	Chaetoviridin A	*Chaetomium globosum* F0142	*Alternaria mali, Botrytis cinerea, Colletotrichum gloeosporioides, Fusarium oxysporum, Magnaporthe grisea*[a], *Phytophthora capsici, P. infestans, Pythium ultimum, P. recondita*[a]	[36]
	Chaetoviridin B	*Chaetomium globosum* F0142	*Magnaporthe grisea*[a], *Pythium ultimum, P. recondita*[a]	[36]
	Chaetoviridins A, B	*Chaetomium globosum*	*Rhizoctonia solani*	[37]
	Dechlorogriseofulvin	*Nigrospora* sp. LLGLM003	*Botrytis cinerea, Colletotrichum orbiculare, Rhizoctonia solani, Sclerotinia sclerotiorum*	[38]
	Decipinin A	*Podospora decipiens*	*Fusarium verticillioides*	[39]

(*continued*)

Table 1 (continued)

Family or group of molecules	Metabolite	Source fungus	Target pathogens[a]	References
	Griseofulvin	*Nigrospora* sp. LLGLM003	*Botrytis cinerea, Colletotrichum orbiculare, Fusarium oxysporum* f.sp. *cucumerinum, F. oxysporum* f. sp. *melonis, Pestalotia diospyri, Pythium ultimum, Rhizoctonia solani, Sclerotinia sclerotium*	[38]
	Griseofulvin	*Xylaria* sp. F0010	*Alternaria mali, Blumeria graminis f. sp. hordei*[a], *Botrytis cinerea*[a], *Colletotrichum gloeosporioides, Corticium sasaki*[a], *Fusarium oxysporum, Magnaporthe grisea*[a], *Puccinia recondita*[a]	[40]
	Hypothemycin	*Paecilomyces* sp. SC0924	*Peronophythora Litchii*[a]	[41]
	Macropsphelide A	*Coniothyrium minitans* IMI 134523	*Sclerotinia sclerotiorum, S. cepivorum*	[42]
	Phomalevone C	*Phoma* sp.	*Aspergillus flavus, Fusarium verticillioides*	[43]
	Pyrrocidines A, B	*Acremonium zeae*	*Aspergillus flavus, Fusarium verticillioides*	[44]
	Sporminarins A, B	*Sporormiella minimoides*	*Aspergillus flavus*	[45]
	Chaetoglobosins A, C, D, E, G, R	*Chaetomium globosum* No.04	*Coniothyrium diplodiella, Rhizopus stolonifer*	[46]
	Chaetoglobosin C	*Botryosphaeria dothidea*	*Alternaria solani*	[33]
	Chaetoglobosin G	*Chaetomium globosum* NM0066	*Alternaria alternata, Alternaria solani, Fusarium graminearum*	[47]
	Chaetoglobosin V	*Chaetomium globosum* NM0066	*Alternaria solani*	[47]
	Chaetoglobosin X	*Chaetomium globosum* L18	*Curvularia lunata, Exserohilum turcicum, Fusarium graminearum, Fusarium moniliforme, Fusarium oxysporum* f. sp. *cucumeris*	[48]
	Cytochalasin D	*Xylaria* sp.	*Cladosporium cladosporioides, Cladosporium sphaerospermum*	[49]

	Koningiopisin C	*Trichoderma koningiopsis* YIM PH 30002	*Plectosphaerella cucumerina*	[50]
	Aspinolide C	*Trichoderma arundinaceum*	*Botrytis cinerea*	[51]
	Monorden, monorden analog	*Humicola fuscoatra*	*Aspergillus flavus*	[52]
Peptaibols	Atroviridins A, B, C and neoatroviridins A, B, C, D	*Trichoderma atroviride* F80317	*Aspergillus niger, Cladosporium sp., Collectotrichum dematium, Curavularia inaqualis, Fusarium oxysporum, Phytophtora infestans, Verticillium dahliae*	[53]
	Chrysospermins A, B, C, D	*Apiocrea chrysosperma* Ap101	*Phoma destructiva, Sporobolomyces salmonicolor*	[54]
	Trichogin	*Tricholoma giganteum*	*Fusarium oxysporum, Mycosphaerella arachidicola, Physalospora piricola*	[55]
	Trichokonins VI, VII, VIII	*Trichoderma koningii* SMF2	*Bipolaris sorokiniana, Botrytis cinerea, Colletotrichum lagenarium, Curvularia lunata, Fusarium oxysporum f. sp. niveum, F. oxysporum* f. sp. *phaseoli, F. oxysporum* f. sp. *vasinfectum, Rhizoctonia solani*	[56]
	Trichokonins VI	*Trichoderma pseudokoningii* SMF2	*Fusarium oxysporum*	[57]
	Trichorzins HA, MA	*Trichoderma harzianum*	*Sclerotium cepivorum*	[58]
	Trichorzianin TA	*Trichoderma harzianum*	*Botrytis cinerea*	[59]
Gliotoxins	Gliotoxin	*Trichoderma virens*	*Pythium ultimum*[a], *Sclerotinia sclerotiorum*	[60]
	Gliotoxin	*Acremonium* sp.	*Pythium myriotylum*	[61]
Fatty acids and glycolipids	Flocculosin	*Pseudozyma flocculosa*	*Botrytis cinerea, Phomopsis* sp., *Phytophthora infestans, Pythium aphanidermatum*	[62]
	6-methyl-9-heptadecenoic acid	*Pseudozyma flocculosa*	*Cladosporium cucumerinum*	[63]
	4-Methyl-7,11-heptadecadienal	*Pseudozyma flocculosa, Pseudozyma rugulosa*	*Fusarium oxysporum* f. sp. *lycospersici*	[64]
	4-methyl-7,11-heptadecadienoic acid	*Pseudozyma flocculosa, Pseudozyma rugulosa*	*Fusarium oxysporum* f. sp. *lycospersici*	[64]

(*continued*)

Table 1 (continued)

Family or group of molecules	Metabolite	Source fungus	Target pathogens[a]	References
	cis-9-Heptadecenoic acid	*Pseudozyma flocculosa*	*Botrytis cinerea, Cladosporium cucumerinum, Idriella bolleyi, Phytophthora infestans, Pythium aphanidermatum*	[63, 65]
	16-(tetra-*O*-acetyl-β-cellobiosyloxy)-2-hydroxyhexadecanoic acid and 16-(tetra-*O*-acetyl-β-cellobiosyloxy)-2,15-dihydrohexadecanoic acid	*Cryptococcus humicola* 9-6	*Sclerotinia sclerotiorum*	[66]
	Ustilagic acid	*Pseudozyma fusiformata*	*Mucor mucedo, Phomopsis helianthi, Sclerotinia sclerotiorum*	[66, 67]
Terpenes	Harzianum A	*Trichoderma arundinaceum*	*Botrytis cinerea*	[68]
	Harziandione	*Trichoderma viride*	*Sclerotium rolfsii*	[69]
	α-humulene	*Fusarium oxysporum* MSA 35	*Fusarium oxysporum* f. sp. *lactucae*	[70]
	Trichodermin	*Stachybotrys cylindrospora*	*Ophiostoma crassivaginatum*	[71]
	Trichodermol	*Stachybotrys cylindrospora*	*Ophiostoma crassivaginatum*	[71]
	Trichodermol	*Stachybotrys elegans* ATCC18825	*Rhizoctonia solani*	[72]
	Trichothecin	*Stachybotrys elegans* ATCC18825	*Rhizoctonia solani*	[72]
	Trichothecin	*Trichothecium roseum*	*Botrytis allii, Fusarium graminearum, Mucor erectus, Paecilomyces varioti, Penicillium digitatum, Saccharomyces carsbergensis*	[73]
	(+)-spongiporic acid A	*Spongiporus leucomallellus* SP2	*Paecilomyces variotii*	[74]
	Viridin	*Trichoderma viride*	*Botrytis allii, Cephalosporium spp., Fusarium* spp., *Trichothecium roseum, Verticillum dahliae*	[75, 76]

Strobilurins/ oudemansins	Strobilurin A, B	*Strobilurus tenacellus*	*Botrytis cinerea, Rhizoctonia solani*	[77]
	Strobilurin C	*Xerula* sp.	*Alternaria porri, Ascochyta pisi, Ceratocystis retusi, Cladosporium cladosporioides, Nematospora coryli, Phytophthora infestans, Pleospora herbarum, Pythium debaryanum, Ustilago nuda*	[78]
	Strobilurin E	*Crepidotus fulvotomentosus*	*Alternaria porri, Botrytis cinerea, Cladosporium cladosporioides, Curvularia lunata, Nematospora coryli, Phoma clematidina, Phytophthora infestans*	[79]
	Strobilurin F	*Bolinea lutea*	*Botrytis cinerea, Cephalosporium acremonium, Cercospora arachidicola, Pythium debaryanum*	[80]
	Strobilurin G, H	*Bolinea lutea*	*Botrytis cinerea, Cephalosporium acremonium, Cercospora arachidicola, Piricularia oryzae, Pythium debaryanum*	[80]
	Strobilurin M	Mycena species	*Absidia glauca, Alternaria porri, Ascochyta pisi, Aspergillus ochraceus, Botrytis cinerea, Cladosporium cladosporioides, Fusarium fujikuroi, Nematospora coryli, Penicillium islandicum, Phoma clematidina*	[81]
	Mucidin (Strobilurin A)	*Oudemansiella mucida*	*Aspergillus flavus, A. niger, Botrytis cinerea, Fusarium oxysporum, Mucor mucedo, M. racemosus, Penicillium italicum, Rhizopus nigricans, R. oryzae*	[82]
	Noroudemansin A	*Pterula* sp. 82168	*Aspergillus ochraceus, Curvularia lunata, Fusarium fujikuroi, Phoma clematidina,*	[83]
	Oudemansin B	*Xerula* sp.	*Alternaria porri, Ascochyta pisi, Ceratocystis retusi, Cladosporium cladosporioides, Nematospora coryli, Phytophthora infestans, Pleospora herbarum, Pythium debaryanum, Ustilago nuda*	[78]
	Oudemansin X	*Oudemansiella radicata*	*Alternaria porri, Ascochyta pisi, Botrytis cinerea, Curvularia lunata, Fusarium fujikuroi, F. oxysporum, Nematospora coryli, Penicillium islandicum, Pythium debaryanum, Ustilago nuda*	[84]
	Oudemansin	*Oudemansiella mucida*	*Aspergillus panamensis*	[85]

(*continued*)

Table 1 (continued)

Family or group of molecules	Metabolite	Source fungus	Target pathogens[a]	References
Azaphilones, butenolides, nitrogen heterocyclic compounds, other lactones and derivatives	Mellein	*Pezicula* sp. zjwcf069	*Botrytis cinerea, Fulvia fulva*	[86]
	Mellein	*Nigrospora* sp. LLGLM003	*Botrytis cinerea*	[38]
	Nigrosporalactone	*Nigrospora* sp. YB-141	*Aspergillus niger, Botrytis cinerea, Penicillium islandicum*	[32]
	Cerinolactone	*Trichoderma cerinum*	*Botrytis cinerea, Pythium ultimum, Rhizoctonia solani*	[87]
	Phomalactone	*Nigrospora* sp. YB-141	*Aspergillus niger, Botrytis cinerea, Ophiostoma minus*	[32]
	Monocillin IV	*Humicola fuscoatra*	*Aspergillus flavus*	[52]
	T22azaphilone	*Trichoderma hazianum T22*	*Gaeumannomyces graminis* var. *tritici, Pythium ultimum, Rhizoctonia solani*	[88]
	Harzianolide	*Trichoderma harzianum*	*Gaeumannomyces graminis* var. *tritici*	[88]
	T39butenolide	*Trichoderma harzianum T39*	*Gaeumannomyces graminis* var. *tritici, Rhizoctonia solani*	[88]
	Harzianopyridone	*Trichoderma harzianum*	*Botrytis cinerea, Gaeumannomyces graminis* var. *tritici, Pythium ultimum, Rhizoctonia solani*	[88, 89]
Other volatile organic compounds (VOC)	1-butanol, 3-methyl-, acetate; other esters; alcohols; ketones	*Muscodor albus*	*Cercospora beticola, Fusarium solani, Pythium ultimum, Rhizoctonia solani, Sclerotinia sclerotiorum, Tapesia yallundae*	[90]
	Ethyl acetate, Ethyl propionate, Isobutyl acetate, Butyl acetate, Propyl acetate, Propyl propionate, Isopentyl acetate, Isobutyl propionate, Isoamyl alcohol, 2-Phenyl ethyl acetate, Isobutyl alcohol, Phenyl ethyl alcohol, Butanoic acid octyl ester	*Hanseniaspora uvarum, Pichia anomala, Pichia kluyveri*	*Aspergillus ochraceus*	[91]

	1,3,5,7-cyclooctatetraene; 3-methyl-1-butanol; 2-nonanone; pentanoic acid, 4-methyl-, ethyl ester; 3-methyl-1-butanol, acetate; acetic acid, pentyl ester; hexanoic acid, ethyl ester	*Candida intermedia* C410	*Botrytis cinerea*[a]	[92]
	2-phenylethanol	*Pichia anomala*	*Aspergillus flavus*	[93]
	2-phenylethanol	*Kloeckera apiculata 34–9*	*Penicillium digitatum, Penicillium italicum*	[94]
Small antifungal proteins	Killer toxins PMKT	*Pichia membranifaciens* CYC 1106	*Botrytis cinerea*[a]	[95, 96]
	Killer toxins PMKT2	*Pichia membranifaciens* CYC 1086	*Brettanomyces bruxellensis*	[97]
	Small, basic and cysteine-rich peptide PAF	*Penicillium chrysogenum*	*Aspergillus niger, Blumeria graminis* f. sp. *hordei, Botrytis cinerea, Cochliobolus carbonum, Fusarium oxysporum, Puccinia recondita* f. sp. *tritici*	[98, 99]
	Small, basic and cysteine-rich peptide AFP	*Aspergillus giganteus*	*Erysiphe graminis, Fusarium moniliforme, Fusarium oxysporum f. sp. lycopersici*[a], *F. oxysporum, Magnaporthe grisea, Phytopthora infestans*	[100, 101]
	Hydrophobin Hytlo1	*Trichoderma longibrachiatum*	*Alternaria alternata, Botrytis cinerea*	[102]
	Perilipin Per3	*Clonostachys rosea* f. *catenulata*	*Sclerotinia sclerotiorum*	[103]

[a]Pathogens for which the metabolite was tested in vivo on the host plant

6-pentyl-, 6-hexyl-, and 6-heptyl-substituted 4-methyl pyrones were active against *Macrophomina phaseolina, Pythium aphanidermatum, Pythium debaryanum, Rhizoctonia bataticola, Rhizoctonia solani,* and *Sclerotium rolfsii*. Among them, only 4-methyl-6-hexyl-alpha-pyrone has been tested in vivo. The percentage of healthy tomato plants was higher in treated *Sclerotium rolfsii*-infected soil than in the untreated control [139].

The biological effects of 6PAP are contrasted. Despite classical decreased fungal growth, it may reduce the production of fusaric acid and deoxynivalenol by *Fusarium moniliforme* and *Fusarium graminearum,* respectively [140, 141]. Moreover, Vinale et al. [142] reported auxin-like activity of 6PAP on pea, tomato, and canola, with a plant-growth-promoting effect at low concentrations (10^{-6} M) and a plant-growth-inhibiting effect at higher concentrations.

Pyrones with antifungal activity were also identified from the endophytic fungi *Nigrospora* sp. YB-141 and *Botryosphaeria dothidea* KJ-1 [32, 33] (Table 1). Solanopyrones C, N, and O are active against *Botrytis cinerea* and *Penicillium islandicum,* whereas pycnophorin is active against *Alternaria solani*.

2.2 Polyketides

Polyketides represent a highly diverse group of molecules that have carbon skeletons, including polyphenols, macrolides, polyenes, enediynes, and polyethers. Although they are structurally and functionally diverse, their synthesis results from the controlled assembly of acetate and propionate. Numerous polyketides have an antagonistic effect on various pathogens (Table 1), but little is known about their role in antagonism. Studies on mammal cells suggest that hypothemycin and monorden inhibit a subset of protein kinases and the Hsp90 molecular chaperone, respectively [143, 144]. However, full demonstrations in targeted fungi are still lacking. According to the studies conducted on mammal cells and fungal human pathogens, griseofulvin inhibits mitosis by interfering with microtubule dynamics [145]. Among polyketides, chaetoviridin and chaetoglobosin from *Chaetomium globosum* are particularly well represented. In vivo assays showed that both chaetoviridin A and B can reduce rice blast severity by at least 88 % at 62.5 μg/mL [36]. Similarly, griseofulvin from *Xylaria* sp. decreased rice sheath blight, wheat leaf rust, and barley powdery mildew symptoms by at least 87 % at 50 μg/mL [40].

Polyketide synthases (PKSs) are poorly studied among biocontrol agents (Table 2). The functional characterization of the biosynthetic pathway of chaetoviridin A in *Chaetomium globosum* is recent [36]. Among others, a gene cluster encodes a highly reducing PKS (HR-PKS) and a nonreducing PKS (NR-PKS). HR-PKS partners with NR-PKS (in sequential and convergent fashions) to produce structurally distinct fragments incorporated into chaetoviridin A [107]. Genomes of *Trichoderma* spp. are rich in polyketide synthase genes, suggesting a predominant role of polyketides in the biology of the fungus. The genomes of *Trichoderma virens* and *Trichoderma atroviride* encode 18 PKSs, and the genome of *Trichoderma reesei* encodes 11 [146]. Two *Trichoderma atroviride*

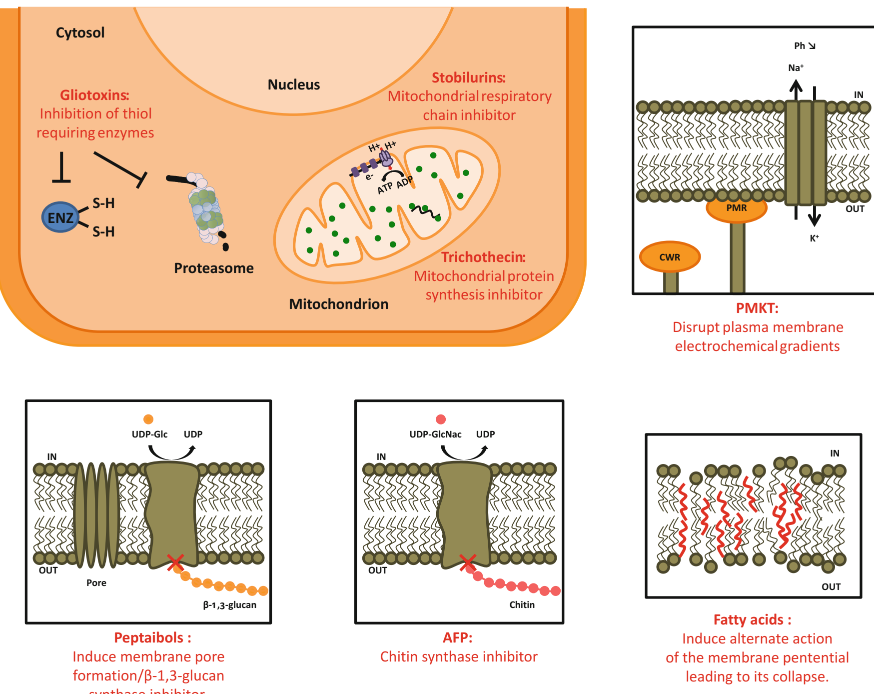

Fig. 2 Putative role of some secondary metabolites and proteins during fungal antibiosis. *ENZ* enzyme, *SH* fonction thiol, *e−* electron, *H+* proton, *ATP* adenosine triphosphate, *ADP* adenosine diphosphate, *PMKT* Pichia membranifaciens killer toxin, *Na+* Sodium, *K+* potassium, *CWR* cell wall receptor, *PMR* plasma membrane receptor, *AFP* antifungal protein, *UDP-Gluc* uridine diphosphate glucose, *UDP-GlucNac* uridine diphosphate *N*-acetylglucosamine, *UDP* uridine diphosphate

PKS genes were expressed under *Rhizoctonia solani* challenge, indicating a possible role in mycoparasitism [15, 147]. Moreover, deletion of the *Trichoderma reesei* pks4 gene reduced antagonistic activity and conidial cell wall stability and influenced the regulation of other PKS-encoding genes [105].

2.3 Peptaibols

Peptaibols are short-chain linear amphipathic polypeptides containing a high proportion of non-proteinogenic amino acids such as alpha-aminoisobutyrate (Aib) and isovaline (Iva). They are classified into three groups depending on their chain lengths: 6- to 10-residue lipopeptaibols, 11- to 16-residue peptaibols, and 18- to 20-residue peptaibols. The N-terminal end of the peptide is usually acetylated or acylated according to the group, whereas the C-terminal end is an amino alcohol [148]. Peptaibols are produced by the genera *Trichoderma* and *Gliocladium,* as well as *Acremonium, Emericellopsis,* and *Paecilomyces*. They are usually secreted as a

Table 2 Fungal and oomycete proteins associated with antagonism and involved in the synthesis of secondary metabolites deleterious to soil-borne pathogens

Protein	Encoding gene	Source fungus or oomycete	Target pathogen	References
Pyrone biosynthesis pathway				
Lipoxygenase	N.A.	*Trichoderma atroviride*	*Rhizoctonia solani*	[104]
Polyketide biosynthesis pathway				
Polyketide synthases (PKS)	*pks4*	*Trichoderma atroviride, T. reesei, T. virens*	*Alternaria alternata, R. solani, Sclerotinia sclerotiorum*	[105]
	N.A.	*Chaetomium cupreum*	*R. solani*	[106]
Highly reducing PKS	N.A.	*Chaetomium globosum*	N.A.	[107]
Nonreducing PKS	N.A.	*Chaetomium globosum*	N.A.	[107]
Peptaibol biosynthesis pathway				
Non-ribosomal peptide synthetases (NRPS)	*tex1*	*Trichoderma virens*	N.A.	[108, 109]
	tex2, tex3	*Trichoderma virens*	N.A.	[110]
	pes	*Trichoderma asperellum*	N.A.	[111]
	salps2	*Trichoderma harzianum*	N.A.	[112]
Gliotoxin and gliovirin biosynthesis pathway				
Aminotransferase	*gliI*	*Coniothyrium minitans, Trichoderma virens*	*Pythium oligandrum, Rhizoctonia solani, Sclerotinia sclerotiorum*	[25, 113, 114]
GliC Cytochrome P450	*gliC*	*Coniothyrium minitans, Trichoderma virens*	*Pythium oligandrum, Rhizoctonia solani, Sclerotinia sclerotiorum*	[25, 113, 114]
	gliF	*Trichoderma virens*	*Pythium oligandrum, Rhizoctonia solani*	[113, 114]
ɣ-glutamyl cyclotransferase-like protein	*gliK*	*Coniothyrium minitans, Trichoderma virens*	*Pythium oligandrum, Rhizoctonia solani, Sclerotinia sclerotiorum*	[25, 113, 114]
Glutathione S-transferase	*gliG*	*Coniothyrium minitans, Trichoderma virens*	*Pythium oligandrum, Rhizoctonia solani, Sclerotinia sclerotiorum*	[25, 113, 114]
Membrane dipeptidase	*gliJ*	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]

(*continued*)

Table 2 (continued)

Protein	Encoding gene	Source fungus or oomycete	Target pathogen	References
Methyltransferase	*gliN*	*Coniothyrium minitans, Trichoderma virens*	*Pythium oligandrum, Rhizoctonia solani, Sclerotinia sclerotiorum*	[25, 113, 114]
NRPS modules	*gliP*	*Coniothyrium minitans, Trichoderma virens*	*Pythium oligandrum, Rhizoctonia solani, Sclerotinia sclerotiorum*	[25, 113, 114]
O-methyltransferase	*gliM*	*Trichoderma virens*	*Pythium oligandrum, Rhizoctonia solani*	[113, 114]
Flocculosin biosynthesis pathway				
Acetyl-transferases	*fat2, fat3*	*Pseudozyma flocculosa*	Powdery mildew pathogens	[26]
ABC multidrug transporter	*atr1*	*Pseudozyma flocculosa*	Powdery mildew pathogens	[26]
BAHD family acyltransferase	*fat1*	*Pseudozyma flocculosa*	Powdery mildew pathogens	[26]
C2H2 zinc finger protein	*rfl1*	*Pseudozyma flocculosa*	Powdery mildew pathogens	[26]
Cytochrome P450 monooxygenases	*cyp1, cyp2*	*Pseudozyma flocculosa*	Powdery mildew pathogens	[26]
Fatty acid synthase	*fas2*	*Pseudozyma flocculosa*	Powdery mildew pathogens	[26]
Hydrolase	*fhd1*	*Pseudozyma flocculosa*	Powdery mildew pathogens	[26]
Hypothetical protein	*orf1*	*Pseudozyma flocculosa*	Powdery mildew pathogens	[26]
UDP-glycosyltransferase	*fgt1*	*Pseudozyma flocculosa*	Powdery mildew pathogens	[26]
Terpenoid/steroid synthesis pathway				
Adh oxidoreductase short chain dehydrogenase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
C-8 sterol isomerase	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani*	[106, 115]
Cytochrome P450 monooxygenases	*tri4*	*Trichoderma arundinaceum*	*Botrytis cinerea, Rhizoctonia solani*	[68]
	tri11	*Trichoderma arundinaceum*	*Kluyveromyces marxianus*	[116]
Delta (24)-sterol C-methyltransferase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Hydroxy-methylglutaryl-CoA reductase	*hmgR*	*Trichoderma harzianum*	*Fusarium oxysporum, Rhizoctonia solani*	[117]

(*continued*)

Table 2 (continued)

Protein	Encoding gene	Source fungus or oomycete	Target pathogen	References
Major facilitator superfamily transporter	*Thmfs1*	*Trichoderma harzianum*	*Aspergillus niger, Botrytis cinerea, Fusarium oxysporium, Gibberella saubinetii, Rhizoctonia solani*	[118]
O-methylsterigmatocystin oxidoreductase, cytochrome P450	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Oxidosqualene lanosterol-cyclase	*erg7*	*Trichoderma harzianum*	N.A.	[117]
Oxysterol-binding protein, ergosterol synthesis	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Regulatory proteins	*tri6, tri10*	*Trichoderma arundinaceum*	*Kluyveromyces marxianus*	[116]
Squalene epoxidase	*erg1*	*Trichoderma harzianum*	N.A.	[119]
Sterol-C5-desaturase	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani*	[106, 115]
Sterol C-22 desaturase	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani, Sclerotinia sclerotiorum*	[25, 106, 115]
Trichothecene 15-*O*-acetyltransferase	*tri3*	*Trichoderma arundinaceum*	*Kluyveromyces marxianus*	[116]
Trichothecene efflux pump	*tri12*	*Trichoderma arundinaceum*	*Kluyveromyces marxianus*	[116]
TRI14 protein	*tri14*	*Trichoderma arundinaceum*	*Kluyveromyces marxianus*	[116]
Trichodiene synthase	*tri5*	*Trichoderma arundinaceum*	*Botrytis cinerea, Rhizoctonia solani*	[120]
	Thtri5	*Trichoderma harzianum*	N.A.	[121]
Oxidases				
Glucose oxidase	*Gox*	*Talaromyces flavus*	*Verticillium dahliae*	[122]
	N.A.	*Aspergillus tubingensis*	*Fusarium solani*	[123]
L-amino acid oxidase	*Th-LAAO*	*Trichoderma harzianum*	*Rhizoctonia solani*	[400]
NADPH oxidase	*nox1*	*Trichoderma harzianum*	*Pythium ultimum*	[124]
Other proteins involved in secondary metabolites biosynthesis				
Gluconate dehydrogenase	N.A.	*Trichoderma harzianum*	*Sclerotinia sclerotiorum*	

(*continued*)

Table 2 (continued)

Protein	Encoding gene	Source fungus or oomycete	Target pathogen	References
Monooxygenases	*G3*	*Trichoderma harzianum*	*Sclerotium cepivorum, Sclerotinia minor, Sclerotinia sclerotiorum*	[125]
Oxaloacetate acetylhydrolase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Peroxisome biogenesis factor 6	*CmPEX6*	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[126]
4-phosphopantetheinyl transferase	*ppt1*	*Trichoderma virens*	*Alternaria solani, Botrytis cinerea, Fusarium oxysporum, Fusarium* spp.*, Phytophthora capsici, Rhizoctonia solani, Sclerotium cepivorum, S. rolfsii*	[127]
Protection of antagonistic fungus against toxins				
Transporters				
ABC transporters	*CrabcG5*	*Clonostachys rosea*	*Fusarium graminearum*	[128]
	Taabc2	*Trichoderma atroviride*	*Beauveria bassiana, Botrytis cinerea, Fusarium* spp.*, Pythium ultimum, Rhizoctonia solani*	[129]
	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani*	[106, 115]
ABC-type multidrug transport system	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Leptomycin B resistance protein, ABC transporter	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Multidrug resistance protein	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Na + −transporting ATPase ENA-1	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Inhibitors				
Cystatin-like protease inhibitor	N.A.	*Pythium oligandrum*	*Pythium infestans*	[21]
Four domain protease inhibitor	N.A.	*Pythium oligandrum*	*Pythium infestans*	[21]
Protease inhibitor, agrin-like protein	N.A.	*Pythium oligandrum*	*Pythium infestans*	[21]
Protease inhibitor, mini-agrin	N.A.	*Pythium oligandrum*	*Pythium infestans*	[21]

(*continued*)

Table 2 (continued)

Protein	Encoding gene	Source fungus or oomycete	Target pathogen	References
Detoxification				
Beta-lactamases	N.A.	*Coniothyrium minitans*, *Pythium oligandrum*	*Pythium infestans*, *Sclerotinia sclerotiorum*	[21, 25]
Copper amine oxidase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Flavin-containing amine oxidase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Hydroperoxide glutathione peroxidase	N.A.	*Pythium oligandrum*	*Pythium infestans*	[21]
Oxalate decarboxylase	*Cmoxdc1*	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[130]
Pisatin demethylase, Cytochrome P450	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Pyridine nucleotide-disulphide oxidoreductase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Thioredoxin peroxidase	N.A.	*Pythium oligandrum*	*Pythium infestans*	[21]
Zearalenone lactonohydrolases	*zhd*	*Chlonostachys catenulatum*, *C. rosea*, *Trichoderma aggressivum*	*Fusarium culmorum*, *F. graminearum*	[131]
	zhd101	*Chlonostachys rosea*	*Fusarium graminearum*	[132, 133]
DNA repair				
8-oxoguanine DNA glycosylase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Parp-like, Poly (ADP-ribose) polymerase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
PIF1 DNA helicase	*PIF1*	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[134]

N.A. not available

mixture of isoforms, and more than 300 sequences have already been identified [20]. Their antifungal activity is mainly studied in *Trichoderma* and has been demonstrated for atroviridins and neoatroviridins (*Trichoderma atroviride*), trichorzins and trichorzianin TA (*Trichoderma harzianum*), trichokonins (*Trichoderma pseudokoningii*) [53, 58, 149] (Table 1). The biological activity of peptaibols generally derives from their membrane-modifying properties, their ability

to form pores in lipid membranes [150, 151], and their ability to induce systemic resistance in plants against microbial invasion [110]. However, in 1996, Lorito and collaborators demonstrated that the peptaibol trichorzianin TA produced by *Trichoderma harzianum* inhibited *Botrytis cinerea* beta-1,3-glucan synthase activity in vitro [59]. In addition, this inhibition seemed to be synergistic with the action of beta-1,3-glucanase from *Trichoderma harzianum* on *Botrytis cinerea* cell walls. The authors suggest that membrane leakage is a nonspecific effect of trichorzianin TA at high concentrations, whereas beta-1,3-glucan synthase inhibition is a specific effect at low concentrations. Whether other fungal cell wall synthesis enzymes are affected and other peptaibols have similar inhibitory activity still remains to be determined. Peptaibols are synthesized by non-ribosomal peptide synthetases (NRPSs), large multifunctional enzyme domains that assemble different compounds from a vast range of precursors, e.g., non-proteinogenic amino acids and hydroxy or carboxyl acids [110, 152]. Several NRPSs involved in the production of peptaibols in *Trichoderma* spp. have been identified (Table 2). However, characterization of NRPSs from other biological control agents is still lacking.

2.4 Gliotoxins

Gliotoxins are sulfur-containing mycotoxins produced by several fungal species, e. g., *Gliocladium fimbriatum* (hence their name), *Trichoderma virens* (*Gliocladium virens*), *Aspergillus fumigatus, Penicillium obscurum*, and *Acremonium* sp. [61, 75, 153–155]. They have antifungal, antimicrobial, antiviral, and immunomodulating properties [151]. In particular, their antifungal activity has been demonstrated in *Trichoderma virens* and *Acremonium* sp. (Table 1). It is synergistically enhanced by the cell-wall-degrading enzymes of the biocontrol agents *Trichoderma harzianum* and *Trichoderma virens* [156]. Little is known regarding the modes of action of gliotoxins in fungal cells during antagonistic interactions. However, studies on mammal cells suggest that the dilsulfide bridge of gliotoxins reacts with the thiol groups of a number of enzymes, resulting in the inhibition of several activities including the activity of the proteasome and apoptosis induction [157]. The genes involved in gliotoxin biosynthesis were identified in both *Trichoderma reesei* and *Trichoderma virens* [25, 60, 113, 114] (Table 2). They encode a major facilitator-type transporter (gliA), a glutathione S-transferase (gliG), a hypothetical protein (gliK), a 1-aminocyclopropane-1-carboxylic acid synthase (gliI), a dipeptidase (gliJ), a two-module non-ribosomal peptide synthetase (gliP), two cytochrome P450 monooxygenases (gliC and gliF), two methyl transferases (gliM and gliN), a thioredoxin reductase (gliT), and a zinc finger transcription factor (GliZ). Some of them, in particular gliK, gliN, gliC, and gliP, are upregulated during mycoparasitism of *Rhizoctonia solani* by *Trichoderma virens* [114]. The organization and expression of the gliotoxin biosynthesis genes during sclerotial mycoparasitism by *Coniothyrium minitans* has also been studied. Despite a limited

degree of synteny, a number of these genes are upregulated during antagonistic interactions too [25].

2.5 Fatty acids and Glycolipids

The biocontrol fungus *Pseudozyma flocculosa* is well known for its ability to secrete fatty acids and glycolipids with antifungal activity. Several fatty acids like 6-methyl-9-heptadecenoic acid [63], 4-methyl-7,11-heptadecadienal and 4-methyl-7,11-heptadecadienoic acid [64], or cis-9-heptadecenoic acid [65, 158] have been purified and tested against several plant pathogens such as *Botrytis cinerea, Cladosporium cucumerinum*, *Fusarium oxysporum* f. sp. *lycopersici,* and *Phytophthora infestans* (Table 1). The current model suggests that antifungal fatty acids are uniformly distributed within target fungal membranes, which increases membrane fluidity and alters membrane integrity [65, 158]. High fatty acid concentrations may result in changes in membrane permeability and cytoplasmic disintegration [159, 160]. Fungi with high sterol contents are less impacted than fungi with low sterol contents because sterols stabilize the fatty acyl chain of phospholipids in the presence of antifungal fatty acids and thus maintain membrane integrity [65]. The glycolipid flocculosin produced by *Pseudozyma flocculosa* is active against *Botrytis cinerea, Phomopsis* sp., *Phytophthora infestans,* and *Pythium aphanidermatum* [62]. It causes rapid leakage of intracellular potassium and inhibits acidification of the medium by plasma membrane ATPases, which disrupts the surface of the fungal pathogen membrane [161]. The identification of the flocculosin biosynthesis pathway in *Pseudozyma flocculosa* is recent [26] (Table 2). A gene cluster encodes two cytochrome P450 monooxygenases (cyp1 and cyp2), a single-chain fatty acid synthase (fas2), a glycosyl transferase (fgt1), a hydroxylase (fhd1), an acyltransferase (fat1), two putative acetyl-transferases (fat2 and fat3), and orf1 that exhibits no homology to known proteins and is probably involved in glycolipid synthesis. Genes encoding the ATP-binding cassette (ABC) transporter (atr1) and the C2H2 zinc finger transcription factor (rfl1) may be involved in flocculosin export and gene cluster regulation, respectively. To date, only two other *Pseudozyma* species, i.e., *Pseudozyma fusiformata* [67] and *Pseudozyma graminicola* [162], along with *Sympodiomycopsis paphiopedili* [163], *Cryptococcus humicola* [66], and the plant pathogen *Ustilago maydis* [164] are known to produce similar antibiotic glycolipids. For example, ustilagic acid produced by *Pseudozyma fusiformata* is active against *Sclerotinia sclerotiorum* and *Phomopsis helianthi* [66, 67] (Table 1).

2.6 Terpenes

Terpenes include a wide range of molecules that consist of multiples of the formula C_5H_8. Depending on the number of carbon atoms, they are classified as hemiterpenes

(C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterpenes (C_{25}), triterpenes (C_{30}), tetraterpenes (C_{40}), or polyterpenes. Each class includes linear and cyclic molecules; cyclization is generated by terpene cyclases. Some terpenes have antifungal activity, such as the three trichothecenes (sesquiterpenes) trichodermin from *Stachybotrys cylindrospora*, trichotecin from *Trichothecium roseum* and *Stachybotrys elegans*, and harzianum A from *Trichoderma arundinaceum*, as well as the triterpene viridin from *Trichoderma viride*. Others are essential for cell membrane fluidity, like the triterpene ergosterol (Table 1).

The mode of action of trichothecenes is generally associated with inhibition of cytosolic protein synthesis in yeast and mammalian cells [165–167]. However, multiple other detrimental effects, including inhibition of DNA synthesis, RNA synthesis, cell division, and disruption of membrane structure and integrity and mitochondrial functions have been recorded in eukaryotic cells [168]. Therefore it is hard to tell whether they are primary or secondary consequences of translation arrest in the cytosol. In addition, McLaughlin and colleagues [169] showed that trichothecin inhibited mitochondrial translation in a dose-dependent manner and altered mitochondrial membrane morphology in *Saccharomyces cerevisiae*. Translation arrest in mitochondria is not due to the inhibition of cytosolic protein synthesis or alteration of mitochondrial membranes [170]. In addition, higher concentrations of trichothecin have an effect on both the inhibition and alteration above mentioned, whereas 1 μM trichothecin already impacts translation in mitochondria. All these data suggest that in yeast, trichothecin first targets mitochondrial translation before cytosolic translation. Whether this mechanism can be generalized to other fungi remains to be proved.

In *Trichoderma*, biosynthesis of terpene compounds depends primarily on hmgR, a gene encoding a hydroxy-methylglutaryl-Coenzyme A reductase (HMGR) that converts hydroxy-methylglutaryl-CoA into mevalonate (Table 2). Hemiterpenes, monoterpenes, diterpenes, triterpenes, sesquiterpenes, and trichothecenes are subsequently synthesized depending on different cluster genes and terpene cyclases. In *Trichoderma harzianum*, partial silencing of hmgR resulted in lower antifungal activity against *Fusarium oxysporum* and *Rhizoctonia solani* [117], suggesting that terpenoid compounds play a crucial role in antagonism. Baker et al. [146] inventoried 3 terpene cyclases in *Trichoderma atroviride*, 3 in *Trichoderma virens,* and 6 in *Trichoderma reesei*. Little is presently known about which terpene cyclase is involved in the biosynthesis of which compound. In 2006, Mukherjee et al. [171] identified a gene cluster in *Trichoderma virens* named vir, whose gene expression was null in a mutant that lacked viridin production. Therefore they hypothesized a possible role in viridin biosynthesis. However, subsequent experiments showed that it is rather involved in the synthesis of three categories of volatile compounds: 24 sesquiterpenes, 5 monoterpenes, and 5 C8 alkanes [172]. The role of most of them remains to be determined. They have no effect on the ability of *Trichoderma virens* to colonize maize roots, but they slightly promote fungal growth. Antifungal activity cannot be ruled out, but experimental evidence is lacking.

Harzianum A is a non-phytotoxic trichothecene that antagonizes fungal plant pathogens and induces genes involved in plant defense (Table 1). The tri gene cluster involved in harzianum A synthesis was recently characterized in *Trichoderma arundinaceum* [68, 116, 120] (Table 2). Disruption of the *tri4* and *tri5* genes stopped harzianum A production and resulted in drastically reduced biocontrol activity of the transformants against *Rhizoctonia solani* and *Botrytis cinerea*. Moreover, the *tri4* null mutant displayed reduced ability to induce the expression of tomato plant defense-related genes against *Botrytis cinerea*. The triterpene biosynthesis pathway is initiated by enzymes encoded by the *erg1*, *erg7,* and *erg9* genes that are also involved in the synthesis of viridin, a well-known antifungal molecule. Overexpression of *erg1* in *Trichoderma harzianum* increased its antifungal activity against *Botrytis cinerea* and reduced lesion size. Nevertheless, the ability of *Trichoderma harzianum* to induce salicylate-related plant defense genes and to colonize roots is low [173]. Hence antifungal activity and plant-*Trichoderma* interactions are highly regulated by the triterpene biosynthesis pathway and depend on intermediate or final metabolites of the pathway.

2.7 Strobilurins/Oudemansins

Strobilurins and the structurally close oudemansins were purified for the first time in 1969 and 1979 from the basidiomycete fungus *Oudemansiella mucida* [85, 174]. Since then, they have been discovered in other genera such as *Strobilurus, Bolinea, Crepidotus, Mycena,* and *Xerula* [77–81]. Strobilurins exhibit antifungal activity against a wide variety of ascomycete, basidiomycete, and oomycete plant pathogens, including *Alternaria porri, Botrytis cinerea, Fusarium fujikuroi, Rhizoctonia solani,* and *Pythium debaryanum* (Table 1). Their mode of action is particularly well known [175, 176]. They inhibit the electron transfer at the quinol oxidation site in the cytochrome bc1 complex of the mitochondrial respiratory chain. Thereby they prevent ATP synthesis and in turn energy production. They belong to the Quinone outside Inhibitors (QoI) family of fungicides. Since their discovery, natural strobilurins have been modified to identify analogous compounds with improved antifungal activity, stability, and dissemination in field. They are considered as low-risk molecules for human health and the environment [177]. The chemical industries Syngenta and BASF commercialized strobilurin fungicides for the first time in 1996, followed by Shionogi and Bayer [177]. Nevertheless, pathogens such as *Alternaria solani, Blumeria graminis, Magnaporthe grisea,* or *Plasmopara viticola* quickly revealed existing resistance [178–182]. The chemical industry is then constantly developing new molecules and formulas to improve the control of plant pathogens. Chemical synthesis of strobilurin and analogs is well described in the literature [183–185]. However, little is known about the strobilurin biosynthesis pathway. Some studies suggest that the aromatic part of strobilurin A is derived from the shikimate pathway, whereas the aliphatic portion could originate from the polyketide pathway [186, 187].

2.8 Small Antifungal Proteins

Some yeasts are able to secrete killer toxins (proteins or glycoproteins) with deleterious effects on sensitive yeasts and fungi [95, 188]. Although they were first discovered in *Saccharomyces cerevisiae* [189], these are not restricted to *Saccharomyces* but found in other yeast genera such as *Candida, Cryptococcus, Hansenula, Kluyveromyces*, or *Pichia*. Interestingly, the producers of these toxins are able to kill one another but are not sensitive to killer toxins they produce. Killer toxins are thought to kill sensitive cells in a two-step manner. First, they bind to a receptor site on the target cell wall. Then, they are supposed to interact with receptors on the cell membrane and induce cell death via different mechanisms. Antifungal activity on plant pathogens has been demonstrated for some of them (Table 1). A good example is *Pichia membranifaciens*. Strains CYC 1106 and CYC 1086 can antagonize *Botrytis cinerea* and *Brettanomyces bruxellensis* by secreting killer toxins PMKT and PMKT2, respectively. These toxins have different physicochemical properties and modes of action in sensitive yeasts [95, 190]. PMKT, an 18-kDa protein, first interacts with β-1,6-D-glucans [191], and then with the GPI-anchored protein CWP2p [192]. Toxins mediate cell death by forming ion-permeable channels that disrupt the plasma membrane electrochemical gradient. This is characterized by a potassium efflux, a sodium influx, and acidification of intracellular pH [95]. PMTK2, a 30-kDa protein, first interacts with mannoproteins, but the second cell membrane receptor is still unknown [97]. Unlike PMTK, PMTK2 cannot form ion-permeable channels in liposome membranes. High doses of PMTK2 result in cell cycle arrest followed by death, whereas low doses induce programmed cell death [190]. The molecular mechanism affected by PMTK2 during the cell cycle is still under investigation.

Similarly, *Aspergillus giganteus* and *Penicillium chrysogenum* secrete AFP and PAF, respectively, two small basic cysteine-rich antifungal proteins. Antifungal (or anti-oomycete) activity of AFP has been demonstrated against *Erysiphe graminis, Fusarium moniliforme, Fusarium oxysporum, Magnaporthe grisea,* and *Phytophthora infestans* [100], whereas antifungal activity of PAF has been shown against *Aspergillus niger, Blumeria graminis* f. sp. *hordei, Botrytis cinerea, Cochliobolus carbonum*, *Fusarium oxysporum,* and *Puccinia recondita* f. sp. *tritici* [98, 99]. AFP specifically localizes to the cell wall compartment of the target pathogen and inhibits chitin synthase activity [101, 193]. In vivo, AFP treatment protects tomato plants from infection by *Fusarium oxysporum* f. sp. *lycopersici* [101]. As for PAF, it localizes to the cytoplasm of sensitive pathogens and causes plasma membrane hyperpolarization, ion channel activation, an increase in reactive oxygen species in the cell, and finally programmed cell death [194, 195].

2.9 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly oxidant molecules that contain oxygen. In the absence of stress, local bursts of ROS play key roles as second messengers in

fungal cell signaling, cell differentiation, or virulence [196–198]. However, ROS production is also the first event following pathogenic interactions in eukaryotic cells, as a defense reaction [199, 200]. High concentrations of ROS react nonspecifically and rapidly with macromolecules and cause molecular damage such as DNA mutation, lipid peroxidation, and protein oxidation, which can result in cell death [201]. Some antagonistic fungi use this property to kill target pathogens. For example, *Talaromyces flavus* secretes a glucose oxidase to antagonize *Verticillium dahliae* [122, 202] (Table 2). Its antifungal properties are probably due to the production of hydrogen peroxide during the catalytic oxidation of glucose by the enzyme. Microsclerotium germination and subsequent hyphal growth of *Verticillium dahliae* are inhibited in the presence of glucose [122]. A glucose oxidase from *Aspergillus tubingensis* was recently isolated [123]. It displayed antifungal activity against *Fusarium solani* in vitro and in vivo. In in vivo assays, preventive application of glucose oxidase on tomato plants decreased symptom severity by 55 % as compared to the control, whereas curative applications totally inhibited the incidence of the disease.

NADPH oxidases (Nox) are also well-known producers of ROS during pathogenic interactions. Montero-Barrientos and collaborators [124] studied the *nox1* gene of *Trichoderma harzianum* and tested its role in antagonism against plant pathogens using overexpression mutants. They showed that *nox1* is slightly upregulated during direct challenge by *Pythium ultimum*. In addition, overexpression of the *nox1* gene was accompanied by increased ROS production in the presence of *Pythium ultimum*. Transformants exhibited higher hydrolytic patterns of protease, cellulase, and chitinase activity than the wild type.

2.10 Proteins Protecting Antagonistic Fungi Against Toxins

The efficiency of fungal antagonism towards soil-borne pathogens relies not only on the production and secretion of antimicrobial compounds but also on the capacity of antagonistic fungi to protect themselves against toxins. Several genes that encode ABC transporters and detoxify enzymes are expressed by biocontrol agents to protect them against toxins produced by pathogens or by themselves (Table 2). For example, *Trichoderma atroviride Taabc2* deletion mutants displayed reduced tolerance to fungal inhibitory compounds, including their own, and were impaired in their ability to protect tomato plants from *Pythium ultimum* and *Rhizoctonia solani* attacks [129]. Similarly, null mutation of *Clonostachys rosea CrabcG5* resulted in reduced antagonism towards *Fusarium graminearum* and failed to protect barley seedlings from foot rot diseases [128].

Cmoxdc1, an oxalate decarboxylase gene from *Coniothyrium minitans*, was recently cloned and found involved in sclerotia mycoparasitism and antibiosis [130]. The enzyme degrades oxalic acid, a multifunctional virulence factor of *Sclerotinia sclerotiorum*, toxic to plants and to *Coniothyrium minitans* [203–207]. In the presence of oxalic acid, a deletion mutant of *Cmoxdc1* displayed reduced ability to infect *Sclerotinia sclerotiorum*. Transcript levels of the

mycoparasitism-related genes *Cmch1* and *Cmg1* were lower, and protease secretion was abolished. Nevertheless, the antifungal activity of culture filtrates increased, probably because of the acidic pH condition generated by oxalic acid in the absence of its degradation by oxalate decarboxylase. DNA repair is also essential during sclerotial mycoparasitism by *Coniothyrium minitans*. Disruption of the *Coniothyrium minitans PIF1* DNA helicase gene altered morphology, reduced growth rates, and cut down the ability to mycoparasitize sclerotia of *Sclerotinia sclerotiorum* [134]. The authors suggest that *PIF1* may ensure mitochondrial stability in the presence of endogenous or exogenous reactive oxygen species produced during the antagonistic interaction.

Zearalenone (ZEA) is one of the most dangerous mycotoxins produced by *Fusarium* species, notably *Fusarium graminearum* and *Fusarium culmorum*. ZEA degradation is a useful self-protecting strategy. Genes encoding zearalenone lactonohydrolase have been isolated from the two biocontrol fungi *Clonostachys rosea* and *Trichoderma aggressivum* (Table 2). In *Clonostachys rosea*, *zhd101* is directly linked to the antagonistic activity of the fungus. A *zhd101*-deletion mutant was unable to detoxify ZEA; it displayed lower inhibition of a ZEA-producing *Fusarium graminearum* strain and failed to protect wheat seedlings against foot rot [132]. Transcriptomic studies performed on *Coniothyrium minitans* and *Pythium oligandrum* during the antagonistic interaction also suggest that ROS may be detoxified by hydroperoxide glutathione peroxidases and thioredoxin peroxidases, and proteases may be inhibited by protein inhibitors [21, 25].

3 Microbial Competition

3.1 Competition for Nutrients

In soils, trophic competition takes place for nutrients and for colonization of the plant tissues. When resources are limited, competition for nutrients regulates the population dynamics of microorganisms that share the same ecological niche and have the same physiological requirements [208]. Competition for nutrients, especially for carbon, is an important mode of action of some biological control agents, such as *Trichoderma* spp. [209]. Competition for carbon between pathogenic and nonpathogenic strains of *Fusarium oxysporum* is one of the main mechanisms resulting in the suppression of *Fusarium* wilt [210].

Biomass components, including cellulose, hemicelluloses, and lignins, are considered as important determinants of the antagonistic capacity of biocontrol fungi. They are supposed to be inherent in the saprophytic lifestyle and in competition with plant pathogens [211, 212]. Many of the presently identified genes encoding biomass-degrading enzymes are genes from the fungal decomposers of forest litter. But many are also and surprisingly from species of the *Trichoderma* genus, probably the most studied fungal biocontrol agent [213]. These saprophytic fungi secrete various hydrolytic enzymes such as proteases, amylases, cellulases, and hemicellulases that degrade biological substrates. The resulting nutrients can then

be the target of intense competition among microorganisms, especially between plant pathogenic fungi and potential biological control agents. For instance, the competitive ability of *Trichoderma* isolates and pathogenic *Rhizoctonia solani* was assessed for cellulose exploitation on wheat straw [211]. Cellulolytic activity levels were estimated as a possible mechanism involved in the competition to ensure straw possession, since the major components of wheat straw are cellulose and hemicelluloses. The rapid assimilation of carbon and nitrogen compounds, whether released or naturally present in the soil, actually plays a determining role in the competition between two fungi. Transcriptomic analyses revealed that genes encoding sugar and amino acid transporters involved in the assimilation of nutrients released from the degradation of plant cell walls were overexpressed, leading to direct competition between pathogenic and nonpathogenic fungi for soil nutrients [214, 215]. The uptake of these low-molecular-weight organics occurs via more or less specialized transporters. Some of them, such as *Botrytis cinerea* BcFRT*,* transport one sugar, while others like *Colletotrichum graminicola* MTBas can transport several carbohydrates [212, 216]. Despite the thousands of transmembrane molecular transport systems listed in the Transporter Classification Database (TCDB; http://www.tcdb.org; [217]), the sugar and amino acid transportome of soil fungal species is still poorly known. This is particularly true in the case of mycoparasitic interactions, although genes encoding sugar and amino acid transporters are overexpressed during mycoparasitism [46, 218]. Gtt1, a high-affinity glucose transporter of the mycoparasitic fungus *Trichoderma harzianum,* has been characterized [219] (Table 3). The authors showed that *gtt1* mRNA levels increased under *Rhizoctonia solani* challenge. Similarly, a di/tri-peptide transporter (PTR2) is involved in the mycoparasitic process of *Trichoderma harzianum* against *Botrytis cinerea* [220].

The best-known example of competition for micronutrients is competition for iron, which is necessary for the growth and pathogenicity of fungal pathogens. The different siderophores produced by *Trichoderma harzianum, Metschnikowia pulcherrima,* and *Rhodotorula glutinis* can inhibit the growth of many plant pathogenic fungi and oomycetes (Table 3). For example, the biocontrol yeast *Rhodotorula glutinis* produces a siderophore called rhodotorulic acid to compete with the postharvest pathogen *Penicillium expansum* [225]. Siderophores are low-molecular-weight, ferric ion-specific chelating agents produced under iron-limiting conditions. In vitro, rhodotorulic acid production is higher, and the fungal pathogen is controlled more effectively when the iron concentration is low. Moreover, in vivo assays on apple wounds showed that a combination of the biocontol agent and the siderophore was more effective than the biocontrol agent alone. Similar results were observed against a *Botrytis cinerea* strain resistant to the conventional fungicide iprodione [226]. Another biocontrol yeast, *Metschnikowia pulcherrima,* which antagonizes *Botrytis cinerea*, *Penicillium expansum,* and *Monilia* sp. among others, produces a pigment called pulcherrimin [224]. Pulcherrimin is a large complex formed nonenzymatically from pulcherriminic acid and ferric ions. Unlike diffusible water-soluble siderophores whose function is to solubilize iron, pulcherrimin is water-insoluble and cannot diffuse in agar medium [223]. In addition, it is produced

constitutively at both low and high ferric ion concentrations. Therefore pulcherrimin may play a role in the immobilization of iron inhibiting fungal pathogen growth. Competition for iron is also determining in the control of *Fusarium* wilt by non-pathogenic *Fusarium* and *Trichoderma* species [209, 240–242]. Several *Trichoderma* species, among which *Trichoderma viride*, *Trichoderma harzianum*, and *Trichoderma lignorum,* are better siderophore producers than *Fusarium solani* and *Fusarium oxysporum* and access more efficiently to low quantities of available iron [243]. *Trichoderma* species secrete a wide range of siderophores [244]. In an optimal medium for siderophore production in in vitro conditions, 18 different iron chelators were detected from one *Trichoderma* species. In addition, *Trichoderma harzianum*, known to have strong antagonistic activity, was the largest siderophore producer with 15 molecules. One of them, harzianic acid, promotes plant growth and has antifungal activity towards phytopathogens like *Pythium irregulare*, *Rhizoctonia solani,* and *Sclerotinia sclerotiorum* [17, 221]. Mycorrhizal and endophytic fungi known for their plant-growth-promoting effect also synthesize siderophores such as ferricrocin, and linear and cyclic fusigen [245–247]. Competition for iron could explain the suppressive effect of *Laccaria laccata* on pathogenic *Fusarium oxysporum* [248].

3.2 Competition for Root Colonization

Colonization of the root tissues is usually limited to penetration of the first or second layers of cells, and restricted to the intercellular spaces [223]. Attachment of the fungus to the root by appressorium-like structures is facilitated by several proteins localized on the outer surfaces of the hyphal and conidial cell walls [229, 235]. Then, root penetration takes place via the secretion of cellulolytic, hemicellulolytic, and proteolytic enzymes [232].

The proteins presently known to facilitate fungal root attachment are mostly hydrophobins (Table 3). Hydrophobins are small secreted proteins that have a characteristic domain of eight cysteine residues at conserved positions. They were initially divided into class I and class II hydrophobins based on their hydropathy patterns and solubility [249, 250]. However, recent bioinformatics analyses revealed an intermediate class in *Trichoderma* and *Aspergillus* species [251, 252]. In phyto-pathogenic fungi, hydrophobins are necessary to anchor fungal cells to the surface of the host plant [253, 254]. They could play a similar role in biocontrol agents such as *Trichoderma asperellum* and *Chlonostachys rosea*. Viterbo and Chet [229] isolated and characterized the *TasHyd1* hydrophobin gene from *Trichoderma asperellum*. They showed that attachment of germinating spores to root surfaces and intercellular root colonization were reduced in the *TasHyd1*-deletion mutants. In addition, normal spore attachment and root colonization were recovered by complementation, supporting a role for *TasHyd1* in the plant root colonization process, but none for direct mycoparasitic activity towards *Rhizoctonia solani*. However, the role of all hydrophobin genes is not fully understood yet. In the case of *Clonostachys rosea*, among the three *Hyd1*, *Hyd2*, and *Hyd3* hydrophobin genes recently identified in the

Table 3 Fungal proteins or molecules associated with antagonism and involved in competition

Protein/molecule	Encoding gene	Source fungus	Target pathogen	References
Competition for nutrients				
Di/tri-peptide transporter PTR2	*Ptr2*	*Chaetomium cupreum, Trichoderma harzianum*	*Botrytis cinerea, Rhizoctonia solani*	[106, 115, 220]
High-affinity glucose transporter Gtt1	*Gtt1*	*Trichoderma harzianum*	*Rhizoctonia solani*	[218, 219]
Siderophores				
Harzianic acid	N.A.	*Trichoderma harzianum*	*Pythium irregulare, Rhizoctonia solani, Sclerotinia sclerotiorum*	[17, 221]
Pulcherrimin	N.A.	*Metschnikowia pulcherrima*	*Alternaria spp., Aspergillus niger, Botryotinia fuckeliana, Botrytis cinerea, Gilbertella persicaria, Monilinia laxa, Mucor circinelloides, Mucor piriformis, Penicillium expansum, Rhizopus stolonifer* var. *stolonifer*	[222–224]
Rhodotorulic acid	N.A.	*Rhodotorula glutinis*	*Botrytis cinerea, Penicillium expansum*	[225, 226]
Competition for root colonization				
1-aminocyclopropane-1-carboxylate deaminase	*acdS*	*Trichoderma asperellum*	N.A.	[18]
Endopolygalacturonase Thpg1	*Thpg1*	*Trichoderma harzianum*	*Botrytis cinerea, Pythium ultimum, Rhizoctonia solani*	[227]
Hydrophobins	*Hyd3*	*Clonostachys rosea*	*Botrytis cinerea, Fusarium graminearum, Rhizoctonia solani*	[228]
	TasHyd1	*Trichoderma asperellum*	N.A.	[229]
	HFB2-6	*Trichoderma asperellum*	N.A.	[230]
	N.A.	*Phlebiopsis gigantea*	*Heterobasidion parviporum*	[231]

(*continued*)

Table 3 (continued)

Protein/molecule	Encoding gene	Source fungus	Target pathogen	References
Proteases	*PapA, PapB*	*Trichoderma asperellum*	N.A.	[232]
Swollenin	*TasSwo*	*Trichoderma asperellum*	N.A.	[233]
	swo1	*Trichoderma reesei*	N.A.	[234]
QID3 protein	*qid3*	*Trichoderma harzianum*	N.A.	[235]
QID74 protein	*qid74*	*Trichoderma harzianum*	*Fusarium solani*	[218, 236, 237]
SM2	*sm2*	*Trichoderma. virens*	N.A.	[238]
β-1,3-glucanase	N.A.	*Ulocladium atrum*	*Botrytis cinerea*	[239]

N.A. not available

fungus, only *Hyd3* is involved in plant root colonization by *Clonostachys rosea* [228]. In addition to hydrophobins, proteins such as *Trichoderma harzianum* QID74, an atypical cysteine-rich cell wall protein, also play a role in hyphal resistance to lytic enzymes and adherence to hydrophobic surfaces [236] or in root architecture and plant biofertilization [237]. QID74 promotes lateral root elongation and hair formation and elongation, and this increases the root absorptive surface and plant shoot biomass [237]. More recently, Crutcher and collaborators [238] highlighted the involvement of SM2, a paralog of the elicitor protein SM1, in the colonization of maize roots by *Trichoderma virens*. However, the role of SM2 in the colonization process is still unclear.

After attachment of the fungus to the plant root, lytic enzymes are secreted to facilitate root penetration. One of these enzymes is swollenin, a protein first isolated and characterized from *Trichoderma reesei* [234]. Swollenin contains an N-terminal fungal-type carbohydrate-binding module family 1 domain (CBD) with a cellulose-binding function, connected by a linker region to an expansin-like domain with homology to group 1 grass pollen allergens. Expansin proteins disrupt hydrogen bonding between cellulose and hemicellulose and thus loosen plant cell walls [234, 255]. Overexpression and silencing experiments showed that the *Trichoderma asperellum* swollenin gene *TasSwo* was essential for cucumber root colonization [233]. The swollenin CBD domain is indispensable for full activity of the enzyme in vivo and stimulates local defense responses of the plant that in turn protect it. Xylanases Abf1 and Abf2 as well as proteases PapA and PapB are also secreted by *Trichoderma asperellum* in response to cucumber root attachment [232]. The role of xylanases in colonization is not directly demonstrated, but they are upregulated during *Trichoderma*-plant interactions. Moreover, xylan is a major component of

hemicelluloses, and the second most important polysaccharide in plant cell walls. The role of proteases in colonization is not understood well enough yet. For some authors, they may be involved in the production of elicitors and the induction of plant defense reactions [232]. In *Trichoderma harzianum*, endogalacturonase Thpg1 is required for a beneficial *Trichoderma*-plant interaction (Table 3). A *Thpg1*-silenced line displayed lower galacturonase activity and a reduced ability to colonize tomato roots [227]. In other biocontrol fungi, root attachment and colonization are still poorly studied.

4 Mycoparasitism

Inhibition of fungal plant pathogen development in suppressive soils includes hyperparasitism by antagonistic fungi. Mycoparasitism consists in the secretion of a wide range of fungal cell-wall-degrading enzymes (CWDE) and proteases that enable the parasite to penetrate the pathogen's hyphae [256]. As chitin and glucan are the main fungal cell wall polysaccharides, CWDE are mostly chitinases and glucanases. Such processes are mainly described for *Trichoderma* spp. and for *Gliocladium* spp. that infect pathogens like *Botrytis cinerea, Rhizoctonia solani* and *Sclerotinia sclerotiorum,* or *Phytophthora* sp. and *Pythium* sp. [257–262] (Table 4). However, more data about the modes of action of other mycoparasitic fungi such as *Coniothyrium minitans* [25, 340] or *Sporidesmium sclerotivorum* [341] are becoming available.

4.1 Endochitinases GH 18

Chitinases belong to the glycosyl hydrolase (GH) group. Based on their amino acid sequence similarities, they are classified into three families: GH 18, GH 19, and GH 20 [262, 278, 294]. They include endo- and exochitinases. Endochitinases cleave chitin at internal sites into chitotetraose, chitotriose, and diacetylchitobiose. Exochitinases are further subdivided into chitobiosidases and *N*-acetyl-β-D-glucosaminidases. Chitobiosidases catalyze the progressive release of diacetylchitobiose in a stepwise fashion. *N*-acetyl-β-D-glucosaminidases split diacetylchitobiose into *N*-acetyl-glucosamine monomers [291].

Many endochitinase-encoding genes have been cloned and characterized, and their antagonistic activity has been tested against different plant pathogens (Table 4). The most studied chitinolytic system is the *Trichoderma* spp. system, especially in *Trichoderma harzianum* and *Trichoderma atroviride*. In particular, antifungal activity of Chit42 against *Botrytis cinerea*, *Rhizoctonia solani,* or *Fusarium solani* is well documented [218, 256, 266, 288]. Apart from *Trichoderma* spp., other fungi such as *Verticillium biguttatum*, *Talaromyces flavus*, *Clonostachys rosea*, *Chaetomium globosum, and Fusarium chlamydosporum* secrete chitin-degrading enzymes during mycoparasitism, as shown in Table 4. In the peculiar case of *Verticillium biguttatum*, chitinase is produced bound to the cell wall, suggesting a putative role in dissolving

and penetrating *Rhizoctonia solani* cell walls [23]. *Chaetomium globosum chi46* expression is triggered by *Rhizoctonia solani, Sclerotinia sclerotiorum,* and *Phytophthora sojae* cell wall fragments [297]. In addition, a 40-kDa chitinase from *Fusarium chlamydosporum* displayed biocontrol activity against *Puccinia arachidis* and inhibited its uredospore germination in a concentration-dependent manner [270].

4.2 Glucosaminidases GH 20

N-acetyl-β-D-glucosaminidases (NAGases) have mainly been isolated from *Trichoderma* spp., but other fungi such as *Chlonostachys rosea*, *Chaetomium cupreum,* or *Stachybotrys elegans* can also secrete these enzymes (Table 4). Dubey et al. [303] showed that disruption of the *eng18B* NAGase from *Trichoderma atroviride* affected the biocontrol activity of the fungus on the pathogens *Botrytis cinerea* and *Rhizoctonia solani*. The NAGase from *Stachybotrys elegans* (NAG-68) and *Chlonostachys rosea* (Cr-NAG1) also displayed antagonistic activity against *Rhizoctonia solani* and *Fusarium culmorum* [22, 308]. Synthesis of NAG-68 by *Stachybotrys elegans* was induced in the presence of purified *Rhizoctonia solani* cell walls, as well as during antagonistic interaction with this pathogenic fungus. In addition, the *Cr-nag1* gene was highly upregulated when *Chlonostachys rosea* was challenged by *Fusarium culmorum*.

4.3 Glucanases

Glucans are the glucose polysaccharides that cross-link chitin or chitosan polymers. There are two types of glucans according to the chemical bonding between glucose subunits. β-glucans are defined by β-(1, 3)- or β-(1, 6)- bonds and provide rigidity to the cell wall. α-glucans are characterized by α-(1, 3)- and/or α-(1, 4)- bonds and function as a part of the matrix. Various glucanases with putative mycoparasitic activity have been characterized. They are mainly β-1,3-glucanases (Table 4). For example, expression of *cmg1*, an exo-β-1,3-glucanase gene from *Coniothyrium minitans*, was induced during parasitic interaction with *Sclerotinia sclerotiorum,* and purified recombinant CMG1 strongly inhibited mycelial growth of the pathogenic fungus [312]. Gluc78 from *Trichoderma atroviride* P1 exhibited strong antifungal activity against *Botrytis cinerea* and acted synergistically with other fungal CWDEs [257]. In the post-harvest biocontrol agent *Pichia anomala*, disruption of the *PAEXG1* and *PAEXG2* exo-β-1,3-glucanase genes significantly reduced the efficiency of the biocontrol of *Botrytis cinerea* on apple [323].

The role of α-1,3-glucanases, α-1,4-glucanases, and β-1,6-glucanases during mycoparasitism is not so well documented. The best examples come from *Trichoderma* sp. studies. For instance, *Trichoderma harzianum* α-1,3-glucanase A13GLUC and β-1,6-glucanase B16GLUC were highly upregulated under *Sclerotinia sclerotiorum* challenge [277]. However, transcriptomic studies performed during antagonistic

Table 4 Fungal and oomycete enzymes associated with mycoparasitism and involved in degradation of fungal cell wall

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
Endochitinases (GH 18)				
32-KDa chitinase	*chit32*	*Talaromyces flavus*	*Alternaria alternata, Fusarium moniliforme, Magnaporthe grisea, Rhizoctonia solani, Sclerotinia sclerotiorum, Verticillium dahliae*	[263]
39-KDa chitinase	*chi1*	*Aphanocladium album*	Rust fungi	[264]
41-KDa chitinase	*chit41*	*Talaromyces flavus*	*Alternaria alternata, Fusarium moniliforme, Magnaporthe grisea, Rhizoctonia solani, Sclerotinia sclerotiorum, Verticillium dahliae*	[263]
Chitinase 1	N.A.	*Talaromyces flavus*	*Rhizoctonia solani*	[265]
Chitinase	*chit42*	*Trichoderma atroviride*	*Alternaria brassicola, Botrytis cinerea, Fusarium graminearum, F. oxysporum, Rhizoctonia solani, Sclerotinia sclerotiorum, Verticillium dahliae*	[266]
	chiA5, chiA6	*Clonostachys rosea*	*Botrytis cinerea, Rhizoctonia solani*	[267]
40-kDa chitinase	N.A.	*Coniothyrium minitans, Fusarium chlamydosporum*	*Puccinia arachidis, Sclerotinia sclerotiorum*	[268–270]
	N.A.	*Candida melibiosica*	*Botrytis cinerea*	[271]
	N.A.	*Metschnikowia pulcherrima*	*Botrytis cinerea*	[272]
30-KDa endochitinase	*ech30*	*Trichoderma atroviride*	*Botrytis cinerea*	[273]
33-KDa endochitinases	*chit33 (ech33)*	*Trichoderma atroviride, Trichoderma harzianum*	*Rhizoctonia solani, Fusarium solani, Sclerotinia Sclerotiorum*	[274–277]

(*continued*)

Table 4 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
	Tv-cht1, Tv-cht2	*Trichoderma virens*	*Rhizoctonia solani*	[278]
36-KDa endochitinases	*chit36*	*Trichoderma harzianum*	*Botrytis cinerea, Fusarium oxysporum* f. sp. *melonis, Sclerotium rolfsii*	[279]
	chit36Y	*Trichoderma asperellum*	*Alternaria alternata, Botrytis cinerea, Fusarium oxysporum* f. sp. *melonis, Rhizoctonia solani*	[280]
chit36P1	*Trichoderma atroviride*	N.A.	[280]	
37-KDa endochitinases	*cr-ech37, cr-ech42*	*Clonostachys rosea*	*Alternaria radicina, Botrytis cinerea, Fusarium culmorum*	[281, 282]
	chit37	*Trichoderma harzianum*	N.A.	[275]
42-KDa endochitinases	*chit42*	*Trichoderma atroviride, Trichoderma hamatum,*	*Botrytis cinerea, Penicillium digitatum, Rhizoctonia solani, Sclerotinia sclerotiorum*	[283–287]
	chit42 (ech42)	*Trichoderma harzianum*	*Botrytis cinerea, Gibberella fujikuroi, Fusarium solani, Rhizoctonia solani*	[218, 256, 275, 288–290]
	echi42	*Trichoderma asperellum*	*Rhizoctonia solani, Sclerotinia sclerotiorum*	[265]
	Tv-ech1, Tv-ech2	*Trichoderma virens*	*Rhizoctonia solani*	[278, 291]
43-KDa endochitinase	N.A	*Trichoderma harzianum*	*Sclerotium rolfsii*	[292]
44-KDa endochitinase	*sechi44*	*Stachybotrys elegans*	*Rhizoctonia solani*	[24, 293]
46-KDa endochitinase	*chi46*	*Chaetomium cupreum, C. globosum, Trichoderma asperellum, T. reesei*	*Fusarium oxysporum, Phytophthora sojae, Sclerotinia rolfsii, S. sclerotiorum, S. tritici, Rhizoctonia solani, Valsa sordida*	[106, 115, 294–297]
58-KDa endochitinase	*cr-ech58*	*Clonostachys rosea*	*Alternaria radicina, Fusarium culmorum*	[281]

(*continued*)

Table 4 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
Endochitinases	*crchi1*	*Clonostachys rosea, Trichoderma harzianum*	*Botrytis cinerea, Rhizoctonia solani*	[298, 299]
	trchi1	*Trichothecium roseum*	*Alternaria alternata, Cercospora nicotianae*	[300]
	N.A.	*Trichooderma asperellum*	*Phymatotrichopsis omnivora*	[301]
	N.A.	*Verticillium biguttatum*	*Rhizoctonia solani*	[23]
Glucosaminidases (GH 20)				
N-acetyl-β-D-glucosaminidases	*cr-nag1*	*Clonostachys rosea (G. roseum)*	*Botrytis cinerea, Fusarium culmorum*	[22, 302]
	eng18B	*Trichoderma atroviride*	*Botrytis cinerea, Rhizoctonia solani*	[303]
	exc1, exc2	*Trichoderma harzianum*	N.A.	[304]
	exc1Y	*Trichoderma asperellum*	*Alternaria alternata, Botrytis cinerea, Fusarium oxysporum* f. sp. *melonis, Rhizoctonia solani*	[280]
	exc2Y	*Trichoderma asperellum*	N.A.	[305]
	nag1	*Trichoderma atroviride, Trichoderma harzianum*	*Botrytis cinerea, Rhizoctonia solani, Sclerotinia sclerotiorum*	[256, 285, 306, 307]
	nag68	*Stachybotrys elegans*	*Rhizoctonia solani*	[308]
	Tvnag1, Tvnag2	*Trichoderma virens*	*Rhizoctonia solani*	[278]
	N.A.	*Trichoderma asperellum*	*Phymatotrichopsis omnivora*	[301]
	N.A.	*Trichoderma harzianum*	*Crinipellis perniciosa*	[309]
	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani*	[106]
Glucanases				
α-1,3-glucanase	*agn13.1*	*Trichoderma harzianum*	*Aspergillus niger, Botrytis cinerea, Colletotrichum acutatum, Fusarium oxysporum, Penicillium aurantiogriseum*	[310]

(*continued*)

Table 4 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
	agn13.2	*Trichoderma asperellum*	*Botrytis cinerea*	[311]
	a13gluc	*Trichoderma harzianum*	*Sclerotinia sclerotiorum*	[277]
	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
β-1,3-glucanases	*cmg1*	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[312]
	exgA	*Ampelomyces quisqualis*	*Sphaerotheca fusca*	[313]
	glu1	*Clonostachys rosea* f. *catenulate*	*Fusarium oxysporum* f. sp. *radicis-cucumerinum*	[261]
	gluc78	*Trichoderma atrovide*	*Phytophthora* sp., *Pythium* sp.	[257]
	lam1.3	*Trichoderma harzianum*	*Rhizoctonia solani, Sclerotium rolfsii*	[314]
	tag83	*Trichoderma asperellum*	*Rhizoctonia solani*	[315, 316]
	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[268, 269]
	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani*	[106, 115]
	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
	N.A.	*Stachybotrys elegans*	*Rhizoctonia solani*	[317]
29-KDa β-1,3-glucanase	N.A.	*Trichoderma harzianum*	*Pythium* sp., *Rhizoctonia solani, Sclerotium rolfsii*	[318, 319]
36-KDa β-1,3-glucanase	N.A.	*Trichoderma harzianum*	*Pythium* sp., *Rhizoctonia solani, Sclerotium rolfsii*	[318, 319]
74-KDa β-1,3-glucanase	N.A.	*Trichoderma harzianum*	*Sclerotium rolfsii*	[292]
78-KDa β-1,3-glucanase	*bgn13.1*	*Trichoderma harzianum*	*Botrytis cinerea, Gibberella fujikuroi, Phytophthora citrophthora, Rhizoctonia solani*	[401]
β-1,3-glucanase	*Tvbgn1, Tvbgn2*	*Trichoderma virens*	*Pythium oligandrum, Rhizoctonia oryzae, R. solani*	[278, 320]
	N.A.	*Trichoderma koningii*	*Rhizoctonia solani*	[321]

(*continued*)

Table 4 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
	N.A.	*Verticillium biguttatum*	*Rhizoctonia solani*	[23]
	N.A.	*Trichoderma harzianum*	*Fusarium solani*	[218]
	N.A.	*Aureobasidium pullulans, Wickerhamomyces anomalus*	*Botrytis cinerea*	[322]
β-glucanase	N.A.	*Candida melibiosica*	*Botrytis cinerea*	[271]
Endoglucanases	*cel12B, cel12D*	*Clonostachys rosea*	*Botrytis cinerea*	[282]
β-(1,4) endoglucanase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Endo-1,3 (4)-β-glucanase	N.A.	*Trichoderma asperellum*	*Rhizoctonia solani, Sclerotinia sclerotiorum*	[265]
Cell 5A endo-1,4-β-glucanase	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Exo-β-1,3-Glucanase	*PAEXG1, PAEXG2*	*Pichia anomala*	*Botrytis cinerea*	[323]
	N.A.	*Trichoderma asperellum*	*Phymatotrichopsis omnivora*	[301]
Mixed-linked glucanase, 1,3-1,4-β-glucanase/ 1,3-β-glucanase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
β-1,6-glucanase	*bgn16.1, bgn16.3*	*Trichoderma harzianum*	N.A.	[260, 324]
	bgn16.2	*Trichoderma harzianum*	*Botrytis cinerea, Rhizoctonia solani*	[325]
	b16gluc	*Trichoderma harzianum*	*Sclerotinia sclerotiorum*	[277]
	Tvbgn3	*Trichoderma virens*	*Pythium oligandrum, Rhizoctonia oryzae, R. solani*	[278, 320, 326]
Other glycoside hydrolases and polysaccharide lyases				
Exo-rhamnogalacturonase	N.A.	*Trichoderma harzianum*	*Fusarium solani*	[218]
Glycosyl hydrolase	N.A.	*Trichoderma harzianum*	*Fusarium solani*	[218]
1,4-α glucosidase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
α-glucosidase	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani*	[106, 115]

(*continued*)

Table 4 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
β -glucosidase	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani*	[106, 115]
	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Pectate lyase	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
TonB-like (glycoside hydrolase 1)	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Proteases				
Aspartic proteases	*P6281*	*Trichoderma harzianum*	*Botrytis cinerea, Pythium ultimum, Rhizoctonia solani*	[327]
	Sa76	*Trichoderma harzianum*	*Fusarium oxysporum, Phytophthora sojae, Rhizoctonia solani, Sclerotinia sclerotiorum, Valsa sordida*	[328]
	TaAsp	*Trichoderma asperellum*	*Alternaria alternata, Cytospora chrysosperma, Fusarium oxysporum, Rhizoctonia solani, Sclerotinia sclerotiorum*	[329]
	ASP55	*Trichoderma asperellum*	*Alternaria alternata*	[330]
	PAPA	*Trichoderma asperellum*	*Rhizoctonia solani*	[232]
	PAPA	*Trichoderma harzianum*	N.A.	[331]
	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani*	[106]
	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
	N.A.	*Trichoderma asperellum*	*Rhizoctonia solani*	[265]
Metalloendopeptidase	N.A.	*Trichoderma hamatum*	*Sclerotinia sclerotiorum*	[332]
Protease	N.A.	*Trichoderma harzianum*	*Botrytis fabae*	[333]
	N.A.	*Verticillium biguttatum*	*Rhizoctonia solani*	[23]

(*continued*)

Table 4 (continued)

Enzyme	Encoding gene	Source fungus or oomycete	Target pathogen	References
	N.A.	*Talaromyces flavus*	*Botrytis fabae*	[333]
	N.A.	*Aureobasidium pullulans*	*Botrytis cinerea*	[322]
Serine proteases	*prb1*	*Trichoderma harzianum*	*Rhizoctonia solani, Sclerotium rolfsii, Sclerotinia sclerotiorum*	[287, 334, 335]
	Spm1	*Trichoderma asperellum*	*Rhizoctonia solani, Sclerotinia sclerotiorum*	[265]
	SL41	*Trichoderma harzianum*	*Fusarium oxysporum, Phytophthora sojae, Rhizoctonia solani, Sclerotinia sclerotiorum, Valsa sordida*	[336]
	SS10	*Trichoderma harzianum*	*Alternbaria alternata, Cytospora chrysosperma, Fusarium oxysporum, Rhizoctonia solani, Sclerotinia sclerotiorum*	[337]
	ThSS45	*Trichoderma harzianum*	*Alternaria alternata*	[338]
	tvsp1	*Trichoderma virens*	*Rhizoctonia solani*	[339]
	N.A.	*Trichoderma harzianum*	*Fusarium solani*	[218]
	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani*	[106, 115]
Trypsin-like protease	*pra1*	*Trichoderma harzianum*	*Sclerotinia sclerotiorum*	[277]
Trypsin protease GIP-like	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Zinc metalloproteinase	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Nucleases				
Endonuclease	N.A.	*Trichoderma hamatum*	*Sclerotinia sclerotiorum*	[332]

N.A. not available

interactions between *Coniothyrium minitans* and *Sclerotinia sclerotiorum* suggest that such enzymes are also necessary for biocontrol by other antagonistic fungi [25]. Other glycoside hydrolases have been revealed by transcriptomic analyses, but their corresponding genes are not yet characterized (Table 4).

4.4 Proteases

Fungal proteases also play a significant role in cell wall lysis [333, 342]. They catalyze the cleavage of peptides that link amino acids to one another in proteins. Several studies evidence a role of extracellular proteases in enhanced fungal biocontrol by *Trichoderma virens*, *Trichoderma harzianum*, *Trichoderma asperellum*, *Trichoderma flavus,* or *Verticillium biguttatum* on pathogenic fungi and oomycetes such as *Rhizoctonia solani*, *Fusarium oxysporum*, *Botrytis cinerea*, *Sclerotinia sclerotiorum,* or *Pythium ultimum* (Table 4). Apart from breaking down the host cell wall, fungal proteases may act as proteolytic inactivators of pathogen enzymes involved in the plant infection process [343, 344]. The majority of mycoparasitic protease genes cloned so far are *Trichoderma* spp. genes. They encode several aspartic proteases and serine proteases. For example, *Trichoderma harzianum* P6281 and *Trichoderma asperellum* ASP55 are aspartic proteases [327, 330], whereas *Trichoderma harzianum* SL41 and *Trichoderma asperellum* Spm1 are serine proteases [265, 336].

5 Host Recognition and Genetic Reprogramming of Gene Expression

An antagonistic interaction starts with specific recognition of the target pathogen by the antagonistic fungus, followed by genetic reprogramming of its gene expression and subsequent control or destruction of the pathogenic fungus. These two steps are critical because they define the nature and the intensity of the antagonistic activities implemented by the biocontrol agent.

5.1 Host Recognition and Signaling Pathways

The ability of biocontrol fungi to sense and respond to different environmental conditions, including the presence of a potential host, is essential for them to successfully colonize soil, organic material, and developing plant roots. However, the host recognition and signaling pathways that lead to the effective antagonistic response are still poorly understood. Generally, heterotrimeric G-proteins composed of α, β, and γ subunits are involved in transducing signals from transmembrane G protein-coupled receptors to a variety of intracellular targets. Depending on the system, Gα or Gβγ transduces the signal by stimulating effectors such as adenylate cyclase or the Mitogen-Activated Protein Kinase (MAPK) cascade [345]. In

Trichoderma species, as in pathogenic fungi, some G-proteins, G-protein-coupled receptors, and adenylate cyclase are critical for the production of extracellular cell wall lytic enzymes, secretion of antifungal metabolites, and formation of infection structures. Following the silencing of the gene encoding the *Trichoderma atroviride* seven-transmembrane receptor Gpr1, adhesion of *Trichoderma* hyphae to the surface of *Rhizoctonia solani* was prevented, and two chitinase genes (*nag1* and *ech42*) and the protease gene *prb1* were not induced. These genes are known to be involved in mycoparasitism, therefore their downregulation in *Trichoderma atroviride* resulted in pathogen survival [346] (Table 5).

Tga1 and Tga3 are two G protein α subunits from *Trichoderma atroviride*. The *Δtga1* mutant thoroughly lost mycoparasitic activity against *Botrytis cinerea*, *Rhizoctonia solani,* and *Sclerotinia sclerotiorum* during direct challenge [347]. Yet the formation of infection structures was unaffected. Although secretion of 6-pentyl-α-pyrone and sesquiterpene-derived antifungal metabolites was reduced, low-molecular-weight antifungal metabolites were overproduced, suggesting opposite roles of Tga1 in the regulation of the biosynthesis of different antifungal substances in *Trichoderma atroviride* [348]. The *Δtga3* mutant was unable to form infection structures or mycoparasitize *Rhizoctonia solani* and *Botrytis cinerea* under direct challenge [349]. Moreover, chitinase activity was null despite a higher transcription rate of the chitinase-encoding genes *ech42* and *nag1* than in the wild-type strain. Addition of cAMP restored the formation of infection structures but not virulence [349]. In *Trichoderma virens*, the *ΔtgaA* mutant exhibited reduced ability to antagonize *Sclerotium rolfsii* but not *Rhizoctonia solani*, suggesting host-specific signaling pathways [351].

MAPK pathways transduce a large variety of signals, including those associated with pathogenesis. Several MAPKs involved in fungal mycoparasitism have been identified in *Trichoderma* species (Table 5). *Trichoderma virens Δtvk1* mutants displayed increased lytic enzyme secretion and were considerably more effective in disease control than the wild-type strain [356]. In addition, the *Trichoderma virens ΔtmkA* mutant fully antagonized *Rhizoctonia solani,* but it only partially antagonized *Sclerotium rolfsii* and failed to parasitize it [353]. Contrary to the wild type, *Trichoderma atroviride Δtmk1* mutants had reduced mycoparasitism activity against *Rhizoctonia solani* and *Botrytis cinerea* in the stage prior to direct mycoparasite–host interactions, but a specific regulation of *ech42* gene transcription is observed upon direct contact with *Rhizoctonia solani*. However, the authors report increased synthesis of antifungal metabolites and a higher ability to protect bean plants in *Rhizoctonia solani*-infected soils [354]. In *Trichoderma harzianum*, *hog1* overexpressing and silenced mutants were strongly affected in their antagonistic activity against *Phoma betae* and *Colletotrichum acutatum*, whereas no difference with the wild-type strain was noted against *Botrytis cinerea*, *Rhizoctonia solani,* and *Sclerotinia sclerotiorum* [355]. Therefore MAPKs and G proteins play a crucial role in fungus–fungus interactions. However, overlapping roles and host specificities are obvious, so more characterizations are necessary to fully understand the complexity of the signaling pathway related to biocontrol of specific fungal pathogens.

Cyclic adenosine monophosphate (cAMP) is an important regulator of growth, development, and pathogenicity in filamentous fungi [370–374]. cAMP is synthesized from ATP by a membrane-associated adenylate cyclase. The activity of adenylate cyclase is regulated by the α-subunits of heterotrimeric G-proteins in most fungi. cAMP generally stimulates a cAMP-dependent protein kinase made of two regulatory and two catalytic subunits [375] and regulates gene expression via phosphorylation of proteins such as transcription factors. In *Trichoderma atroviride* and *Trichoderma reesei*, G-protein α-subunits Tga3 and Gna3 positively stimulated the activity of adenylate cyclase and consequently mycoparasitism [349, 350, 376]. Moreover, in *Trichoderma virens*, deletion of *tac1*, an adenylate cyclase gene, abolished biocontrol activity against *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Pythium* sp., because of reduced secondary metabolite production [358]. This was the first demonstration that cAMP signaling positively regulates secondary metabolism and mycoparasitism in biocontrol fungi.

5.2 Transcription Factors Involved in Biocontrol

At the cellular level, the transcription factors (TFs) that regulate gene transcription during antagonism are still poorly investigated. TFs such as AreA/Nit2, Msn2/Msn4, or AceI, respectively involved in nitrogen repression, stress responses, and regulation of plant CWDE, may bind to specific motifs in the promoter of biocontrol genes from *Trichoderma* spp. However, there is no demonstration of their role in antifungal activity [287, 306]. The carbon catabolite repressor Cre1 is the first protein for which a role in mycoparasitic interactions was proved (Table 5). Interaction of *Trichoderma harzianum* with *Botrytis cinerea* revealed binding of Cre1 to the promoter sequences of the endochitinase-encoding gene *ech42* [359]. Later, Moreno-Mateos et al. [364] and Trushina et al. [363] showed that the pH-responsive transcription factors Pac1 (from *Trichoderma harzianum*) and PacC (from *Trichoderma virens*) were essential for their antifungal activity. Rubio et al. [366] pointed to the significant role of ThCtf1 in the production of secondary metabolites and in the antifungal activity of *Trichoderma harzianum*. The xylanase transcriptional regulator Xyr1 from *Trichoderma atroviride* is involved in mycoparasitism and is required to induce plant defense reactions [16]. Deletion of *xyr1* resulted in enhanced competition with the plant pathogens *Botrytis cinerea*, *Phytophthora capsici,* and *Rhizoctonia solani*. Moreover, induction of plant defense responses during the *Trichoderma atroviride*/*Arabidopsis thaliana* interaction was delayed.

Vel1 is a key regulator of morphogenesis and secondary metabolism in several filamentous fungi [377–383]. In *Trichoderma virens*, Vel1 is involved in conidium and chlamydospore formation, but also in antagonism [367]. Deletion mutants are defective in secondary metabolism, mycoparasitism, and biocontrol efficacy on *Rhizoctonia solani*. Moreover, *Aspergillus nidulans* VeA physically interacts with VelB and the regulator of secondary metabolism LaeA to form a complex that regulates secondary metabolism and sexual reproduction [380]. In *Trichoderma atroviride*, deletion and overexpression mutants were used to evidence that Lae1 is

Table 5 Fungal and oomycetes proteins associated with antagonism and involved in host recognition, signal transduction and genetic reprogramming of gene expression

Protein	Encoding gene	Source fungus	Target pathogen	References
Receptor proteins				
Integral membrane protein PTH11	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Seven-transmembrane receptor Gpr1	*gpr1*	*Trichoderma atroviride*	*Botrytis cinerea, Rhizoctonia solani, Sclerotinia sclerotiorum*	[346]
Seven-transmembrane receptor (secretin family)	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
WSC yeast cell wall integrity and stress response component proteins	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
G proteins				
G-protein beta WD-40 repeat	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
G-protein one	N.A.	*Trichoderma asperellum*	*Rhizoctonia solani, Sclerotinia sclerotiorum*	[265]
G-protein ypt3	N.A.	*Trichoderma asperellum*	*Rhizoctonia solani, Sclerotinia sclerotiorum*	[265]
G-protein rab2	N.A.	*Trichoderma asperellum*	*Rhizoctonia solani, Sclerotinia sclerotiorum*	[265]
α-subunit of G protein 1	*tga1*	*Trichoderma atroviride*	*Botrytis cinerea, Rhizoctonia solani, Sclerotinia sclerotiorum*	[347, 348]
α-subunit of G protein 3	*tga3*	*Trichoderma atroviride*	*Botrytis cinerea, Rhizoctonia solani*	[349]
	gna3	*Trichoderma reesei*	*Pythium ultimum*	[350]
α-subunit of G protein A	*tgaA*	*Trichoderma virens*	*Sclerotium rolfsii*	[351]
Mitogen-activated protein kinases				
Mitogen-activated protein kinases (MAPK) A	*smkA*	*Stachybotrys elegans*	*Rhizoctonia solani*	[352]
	tmkA	*Trichoderma virens*	*Rhizoctonia solani, Sclerotium rolfsii*	[353]
	N.A.	*Chaetomium cupreum*	*Rhizoctonia solani*	[106, 115]
MAPK 1	*tmk1*	*Trichoderma atroviride*	*Botrytis cinerea, Rhizoctonia solani*	[354]
MAPK	*hog1*	*Trichoderma hazianum*	*Colletotrichum acutatum, Phoma betae*	[355]
	tvk1	*Trichoderma virens*	*Pythium ultimum, Rhizoctonia solani*	[356]

(*continued*)

Table 5 (continued)

Protein	Encoding gene	Source fungus	Target pathogen	References
MAPK kinase kinase (MAPKKK)	*bck1*	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[357]
Cell wall integrity-related MAPK	*slt2*	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[357]
Similar to Wak1 protein (MAPKKK)	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Other proteins				
Adenylate cyclase Tac1	*tac1*	*Trichoderma virens*	*Sclerotium rolfsii, Rhizoctonia solani, Pythium* sp.	[358]
Microsomal signal peptidase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Myo-inositol-1-phosphate synthase	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Protein phosphotase 2a 65kd regulatory sububit	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Serine/Threonine protein kinases	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
TGF-beta receptor associated protein 1	N.A.	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[25]
Transcription factors				
Carbon catabolite repressor Cre1	*cre1*	*Trichoderma harzianum*	*Botrytis cinerea*	[359]
Heat shock factor 1	*hsf1*	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[360]
Methyltransferase Lae1	*lae1*	*Trichoderma atroviride*	*Alternaria alternata, Alternaria solani, Botrytis cinerea*	[361]
pH regulator PacC	*pacC*	*Trichoderma virens*	*Rhizoctonia solani, Sclerotium rolfsii, Sclerotinia sclerotiorum*	[130, 362, 363]
pH regulator Pac1	*pac1*	*Trichoderma harzianum*	*Phytophthora citrophthora, Rhizoctonia solani, R. meloni*	[364]
pH regulator CmPacC	*CmpacC*	*Coniothyrium minitans*	*Sclerotinia sclerotiorum*	[365]
Transcription factor ThCtf1	*ctf1*	*Trichoderma harzianum*	*Botrytis cinerea, Fusarium oxysporum* f. sp. *lycopersici, Rhizoctonia solani*	[366]
VELVET protein Vel1	*vel1*	*Trichoderma virens*	*Pythium ultimum, Rhizoctonia solani*	[367]
VibA	*vibA*	*Epichloë festucae*	*Drechslera erythrospila*	[368]

(*continued*)

Table 5 (continued)

Protein	Encoding gene	Source fungus	Target pathogen	References
Ste12	*ste12*	*Trichoderma atroviride*	*Botrytis cinerea, Rhizoctonia solani*	[369]
Xylanase transcriptional regulator Xyr1	*xyr1*	*Trichoderma atroviride*	*Botrytis cinerea, Phytophthora capsici, Rhizoctonia solani*	[16]

N. A. not available

essential for asexual development but also for antagonism toward *Alternaria alternata*, *Alternaria solani,* and *Botrytis cinerea* [361]. This, and the fact that Vel1 is very highly conserved across *Trichoderma* species [367], suggests that Vel1 and Lae1 may be master regulators of the antagonistic properties of *Trichoderma* and could be found in other biocontrol fungi.

Despite a few steps forward, our knowledge is still piecemeal and restricted to *Trichoderma* spp. To date, only *Coniothyrium minitans* PacC and heat shock factor 1 have been associated to antagonism [360, 365]. In addition, VibA transcription factor from the plant symbiotic fungus *Epichloë festucae* is required for antibiosis [368]. Mutants deleted for the *vibA* gene are affected in their antifungal activity against the grass pathogen *Drechslera erythrospila*. Conversely, overexpression transformants exhibit enhanced antifungal activity. Mutants are able to antagonize fungal pathogens that the wild type does not. Therefore more investigations are needed to fully understand the genetic reprogramming that underlies biocontrol.

6 Fungal Induction of Plant Defense Reactions

Two types of induced resistance are distinguished in plants. Systemic acquired resistance (SAR) is triggered by previous infections by avirulent pathogens, whereas induced systemic resistance (ISR) is triggered by previous colonization of the rhizosphere by beneficial microbes. Nonpathogenic plant-growth-promoting rhizobacteria (PGPR) and fungi (PGPF) influence soil and plant health; they induce a wide array of plant responses that result in enhanced defensive capacity of the whole plant against a broad spectrum of plant pathogens. The role of hyperparasitic fungi such as *Trichoderma* sp. in priming plant defenses has been extensively studied. Several secondary metabolites and proteins involved in mycoparasitism and antibiosis have been identified as ISR elicitors (Table 6). Expression of the *Trichoderma harzianum* chitinase gene *chit42* in tobacco and potato plants enhanced resistance to the foliar pathogens *Alternaria alternata, Alternaria solani, Botrytis cinerea,* and to the soil-borne pathogen *Rhizoctonia solani* [384]. Similarly, expression of the *Trichoderma atroviride* endochitinase gene *ech42* in barley resulted in increased resistance to *Fusarium* sp. infection [273]. *Trichoderma longibrachiatum*

Table 6 Fungal and oomycete proteins/molecules associated with antagonism and involved in the induction of plant resistance

Protein/molecule	Encoding gene	Source fungus or oomycete	Target pathogen	References
Proteins				
Chit42	*chit42*	*Trichoderma harzianum*	*Alternaria alternata, Alternaria solani, Botrytis cinerea, Rhizoctonia solani*	[384]
Epl1	*Epl1*	*Trichoderma asperellum*	*Rhizoctonia solani, Sclerotinia sclerotiorum*	[265]
Ech42	*ech42*	*Trichoderma atroviride*	*Fusarium* sp.	[273]
Elicitin-like protein 1 precursor	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Elicitin-like protein SOL13A	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Elicitin-like protein1, putative elicitin	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Elicitin-like protein RAL13D, Elicitin-like protein 1	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Endopolygalacturonase ThPG1	*Thpg1*	*Trichoderma harzianum*	*Botrytis cinerea, Pythium ultimum, Rhizoctonia solani*	[227]
Epl1	*Epl1*	*Trichoderma atroviride*	*Rhizoctonia solani*	[385]
Epl2	*Epl2*	*Trichoderma atroviride*	*Cochliobolus heterostrophus*	[386]
EplT4	*EplT4*	*Trichoderma asperellum*	*Cercosporidium sofinum*	[387]
FK506-binding protein 2 precursor transglutaminase elcitor family M81B	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Hydrophobin Hytlo1	*Hytlo1*	*Trichoderma longibrachiatum*	*Botrytis cinerea*	[115]
Methylisocitrate lyase	*mlc*	*Trichoderma atroviride*	*Botrytis cinerea*	[303]
Mitogen-activated protein kinase	*tmkA*	*Trichoderma virens*	*Sclerotium rolfsii*	[388]
NPP1-containing protein, Elicitin-like protein RAL13D	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Oligandrin	*oli-d1, oli-d2*	*Pythium oligandrum*	*Botrytis cinerea*	[389, 390]
	oli-s1	*Pythium oligandrum*	N.A.	[389]

(*continued*)

Table 6 (continued)

Protein/molecule	Encoding gene	Source fungus or oomycete	Target pathogen	References
PKS/NRPS hybrid enzyme	*tex13*	*Trichoderma virens*	N.A.	[147]
4-Phosphopantetheinyl Transferase	*ppt1*	*Trichoderma virens*	*Alternaria solani, Fusarium oxysporum, Fusarium* spp.*, Phytophthora capsici, Rhizoctonia solani, Sclerotium cepivorum, S. rolfsii*	[127]
Pod-1, Pod-2	*pod-1, pod-2*	*Pythium oligandrum*	*Botrytis cinerea, Fusarium oxysporum* f. sp. *radicis-lycopersici, Phytophthora parasitica*	[391–393]
Pos-1	*pos-1*	*Pythium oligandrum*	N.A.	[389]
Sm1 (Small Protein 1)	*sm1*	*Trichoderma virens*	*Colletotrichum* sp.	[394]
Sm2 (Small Protein 2)	*sm2*	*Trichoderma virens*	*Cochliobolus heterostrophus*	[386]
Swollenin TasSwo	*TasSwo*	*Trichoderma asperellum*	*Botrytis cinerea*	[233]
Thc6 protein	*Thc6*	*Trichoderma harzianum*	*Curvularia lunata*	[395]
Transglutaminase elicitor family M81BCBEL	N.A.	*Pythium oligandrum*	*Phytophthora infestans*	[21]
Xylanase Xyn2/Eix	*Xyn2/Eix*	*Trichoderma viride*	N.A.	[396]
Secondary metabolites				
Alamethicin (20mer peptaibol)	N.A.	*Trichoderma viride*	N.A.	[397]
Harzianolide	N.A.	*Trichoderma harzianum*	*S. sclerotiorum*	[398]
6-Pentyl-a-pyrone, harzianolide and harzianopyridone	N.A.	*Trichoderma* spp.	N.A.	[142]
Trichokonin (20mer peptaibol)	N.A.	*Trichoderma pseudokoningii*	N.A.	[265]

N.A. not available

cellulases, *Trichoderma viride* xylanase Xyn2/Eix, *Trichoderma harzianum* endopolygalacturonase ThPG1, or *Trichoderma asperellum* swollenin (expansin-like protein) TasSwo can also elicit systemic resistance [227, 233, 396, 399]. Nonenzymatic proteins such as *Trichoderma virens* and *Trichoderma atroviride* cerato-platanins Sm1/Epl1 also trigger plant systemic resistance. Secondary metabolites like 20-mer peptaibol (alamethicin and trichokinin), 18-mer peptaibol, 6-pentyl-a-pyrone, harzianolide, and harzianopyridone have antimicrobial effects at high doses but are ISR inducers at low concentrations (Table 6).

ISR triggering by other antagonistic fungi is well described. However, the nature of the elicitor is not always clearly established. The PGPF *Penicillium simplicissimum* enhanced resistance of barley to *Colletotrichum orbiculare* by inducing reactive oxygen species formation, lignification, salicylic acid accumulation, and activation of defense genes [402]. A number of *Pythium oligandrum* genes encoding elicitor-like proteins, functionally characterized or identified using transcriptomic data, are listed in Table 6. Mycorrhizal fungi are also powerful ISR elicitors [403]: colonization of *Oryza sativa* roots by *Glomus intraradices (*now named *Rhizophagus irregulare)* promoted systemic induction of defense-related genes and conferred resistance to *Magnaporthe oryzae* [404].

7 Conclusion

The diversity of metabolites produced by microorganisms, including fungi specifically mentioned in this chapter, is simply impressive. In the case of metabolites, it is not always possible to know if they are produced to affect other microorganisms, including pathogens, or whether they are metabolic by-products whose side effects are by chance useful for biocontrol. This is one of the criticisms that can be objected to all in vitro tests: they only show inhibition of pathogen growth in Petri dishes but do not allow investigators to know if the mechanism is reliable, durable, or only transient. A few studies use mutants to validate the genetic basis of the mechanism under focus and consider a role in the biological control of a pathogen. Knowledge of genes encoding these molecules and transcriptomic studies are actually much more informative and allow for a more efficient use of this potential. The microorganisms that harbor these genes become candidates that can be selected based on this criterion. Then it is also possible to determine the favorable conditions for the expression of these genes, and thus ensure that the environment where the selected agent should be introduced is favorable for the expected biocontrol activity. Some of the molecules identified so far are candidates for new fungicides. As fungi naturally produce them, the general public better perceives them. However, systematic phytotoxicity tests and environmental studies are needed if field use at high doses is envisaged. In this context, the direct use of biocontrol agents seems to be the most effective long-term solution, as these agents usually combine antibiosis, mycoparasitism, and induction of plant defense reactions to control pathogens without harming the environment or human health. Moreover, the diversity of the mechanisms of action prevents the development of new resistance. That is why it is important to keep studying and

deciphering the molecular mechanisms associated with antagonism to better understand how they are induced and possibly improve the biocontrol capacity of the currently available agents. However, the implementation of these mechanisms by microorganisms should be understood in natural situations. This is why these studies generally fall within the knowledge of the ecological requirements and ecological fitness of soil fungi, both plant pathogenic fungi and saprophytes. The control of the infectious activity of pathogenic agents can be achieved either by bioaugmentation (inoculation) of biocontrol agents selected on the basis of the metabolites they produce or by biostimulation of indigenous populations producing these metabolites of interest in situ. The two strategies can of course be combined, but in all cases, it is necessary to offer these biocontrol agents environmental conditions promoting their activity at the expense of deleterious pathogens.

Thus, knowing about the genes and metabolites involved in microbial interactions to control pathogens will be a major key to select the most interesting candidates and ensure their expression in the future environment of the biocontrol agent. This knowledge will also stimulate a preventive action to manage pathogen control more efficiently by providing indicators of the health status of soils and other growing substrates. Microarrays already exist to detect the presence of pathogens in soils. Complementary microarrays could be created, not based on the presence or absence of taxa but rather on the presence or absence of metabolites and proteins involved in pathogen control. This will help to assess the risk of cultivating a susceptible but high-value crop or to test the impact of an innovative agricultural practice on the resulting soil suppressiveness towards soil-borne diseases.

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Role of Quorum Sensing in Fungal Morphogenesis and Pathogenesis

3

Rohit Sharma and Kamlesh Jangid

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Abstract

Fungi are simple organisms yet complex in their morphology. They have evolved in several ways to cope with diverse environmental conditions which they encounter. Some produce dormant structures which help them to survive unfavorable conditions, while others, especially pathogens, have adopted dimorphic form to adapt to new conditions. In many pathogenic fungi, the hyphae are responsible for penetration either through natural openings or via invasion of tissue. Once inside the host, morphogenesis, which is many a times under quorum-sensing regulation, is triggered that enables the mycelium to switch to yeast phase that can now spread in the host with higher efficiency as well as evade host immune responses. Although very few fungi are known to regulate both morphogenesis and pathogenesis via quorum sensing (QS), it is believed that quorum-sensing regulation of at least morphogenesis is a universal phenomenon

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across all fungi. However, a systematic evidence for this is lacking. A wide array of inducer molecules, such as Butyrolactone I, phenylethanol, tyrosol, farnesol, oxylipin, and farnesoic acid among many others, have been identified in fungi till date. In addition to these chemical compounds, a calcium-binding protein (CBP) is also involved in quorum-sensing regulation of morphogenesis and pathogenesis in the genus *Histoplasma*. Quorum sensing has well-established applications in controlling the spread of diseases as an alternative strategy to the use of antibiotics and overcoming multidrug-resistant strains. While there are numerous potential inhibitors of quorum-sensing inducers, not even a single effective molecule, which is also economically viable, has been commercialized till date. The research in this field therefore demands a more systematic and coordinated effort to investigate quorum sensing and quenching molecules across the diverse taxa within fungi.

Keywords
Morphogenesis • Pathogenesis • Quorum sensing • Farnesol • Oxylipin • Butyrolactone I • Quorum-sensing inhibitor

List of Abbreviations

CDA	Czapek dextrose agar
MEA	Malt extract agar
MYA	Million years ago
PCA	Potato carrot agar
PDA	Potato dextrose agar
QS	Quorum sensing

1 Introduction

Fungi are among the oldest microbes known to have diverged from other life-forms at around 1.5 million years ago (MYA) [1]. They are eukaryotic and require an external supply of carbon for their nutrition. Of the more than 97,330 fungi known till date, majority are strictly saprophytic and remaining are parasitic [2]. Some fungi are also pathogenic to plants, animals, or humans and are able to evade the host defense mechanisms upon penetration, thereby causing symptomatic infection. In agriculture, fungi are important pathogens of crops causing heavier losses as compared to bacteria and viruses [3]. In humans, leaving aside superficial skin infection and dermatitis, most fungi cause infections in immunocompromised patients only. However, relatively recently there has been an increase in the human diseases caused by fungi, especially in the tropical habitats due to favorable conditions for the growth of fungal pathogen. It is the pathogenic fungi that have yielded most information about the mechanisms and modifications in structures that have evolved throughout their history. Interestingly, some fungi have evolved to move from saprophytic way of life to a pathogenic lifestyle even during the same life cycle or on a different host. Hence, it has become increasingly difficult to distinguish a pathogenic fungal strain

from a nonpathogenic one. Research has shown that at times a pathogenic fungus may lack virulence genes, whereas sometimes even though the gene is present, the fungus will not cause disease due to physiological adaptation of the host. Additionally, the fungal pathogen must complete its life cycle or part of it inside the host, such as plant tissue, skin, or lung alveoli, in order to produce symptomatic disease as well as to reproduce. Overall, during the course of their evolution, fungi have developed mechanisms to survive not only in harsh environmental conditions but also inside nutritionally rich host tissues.

Some fungi are known to have phenotypic plasticity which allows them to switch between morphologically different growth forms to achieve survival, dispersal, and reproduction. These pleomorphic fungi include *Aureobasidium*, *Ustilago*, *Puccinia*, etc., as well as some dimorphic yeasts, such as *Candida*, *Blastomyces*, *Saccharomyces*, *Cryptococcus*, etc. (Fig. 1). However, plant pathogenic fungi do not commonly have a dimorphic switch and are typically filamentous. Instead, additional structures are produced during infection of the host plant that help the fungi in penetration and infestation. These morphological changes are dependant upon various signals and thereafter secretion of various enzymes or local turgor pressure helps in the initial stages of infection. In contrast, dimorphism is common in animal or human pathogenic fungi, wherein yeast form is more pathogenic than the filamentous form. Most of these animal pathogens belong to *Ascomycota* but some are also reported from *Zygomycota* and *Basidiomycota.* For instance, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, and *Blastomyces dermatitidis* grow in yeast form inside the host, whereas some species of *Aspergillus*, *Trichophyton*, *Microsporum*, *Arthroderma*, *Pyronema*, and *Microdiplodia* grow as filaments. While most of these filamentous forms lose their ability to sporulate inside host tissue possibly because of high-nutrient conditions and easy spread through the body fluids, *Candida albicans* grows in both forms inside the host. Various environmental factors are known to trigger the shift from filamentous to yeast form. At 25 °C, the conidia of *Penicillium marneffei* germinate into filamentous form (which are nonpathogenic) and at 37 °C into the yeast form (which are pathogenic) [4].

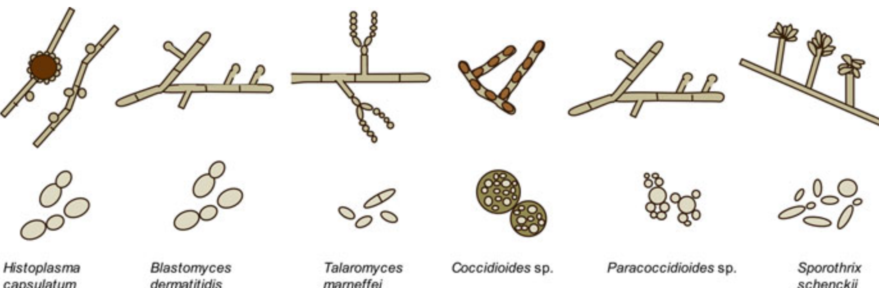

Fig. 1 Dimorphic fungi which form yeast and mycelial phases in their life cycle. Various fungi look morphologically different in different conditions (pH, temperature, inoculum density, etc.). Their nonpathogenic saprobic state is mycelial (*upper lane*), and pathogenic one is yeast (*lower lane*) (Adapted from Boyce and Andrianopoulos [95])

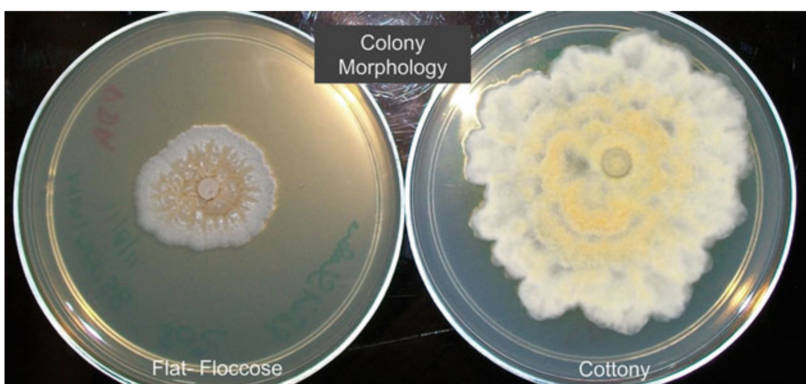

Fig. 2 Effect of nutrition on colony morphology of *Myceliophthora verrucosa*

Similarly, *Myceliophthora verrucosa* grows with cottony morphology forming conidia on malt extract agar (MEA) and Czapek dextrose agar (CDA), whereas it grows as flat and floccose on potato dextrose agar (PDA) (Fig. 2). Beside temperature and media, other factors which regulate the dimorphic behavior include pH, atmosphere, inoculum concentration, carbon, nitrogen, MAP kinase pathways, histidine kinases, and G-protein. Dimorphism in fungi has not only offered new opportunities to study their pathogenesis in various diseased conditions but also yielded significant information on the involvement of various molecular mechanisms.

Cell-to-cell communication is known to play a major role in morphogenesis in fungi. First discovered in prokaryotes and lower eukaryotes, it is typically achieved by the mechanism of quorum sensing in which extracellular concentration of a small signaling molecule, the autoinducer, mimics the cell density in the medium (Fig. 3). Upon achieving the "threshold" concentration, the molecule is transported back into the cell either actively or passively and activates transcription factors, which in turn trigger the signal transduction cascades to regulate the expression of genes involved in morphological differentiation, biofilm formation, virulence, etc. [5]. Different microbes employ different machineries for the transport and detection of molecules and reciprocal action through gene expression/transcription. In bacteria, quorum-sensing-regulated phenotypes include bioluminescence, exopolysaccharide production, virulence, antibiotic and exoenzyme production, biofilm formation, and growth inhibition [6]. However, in fungi it is only known to regulate morphogenesis of yeasts cells which in turn is associated with virulence capacity or pathogenesis (sporulation, pseudohyphae, biofilm formation). While some autoinducers (small molecules) are secreted and recognized by strains or population of same species (species-specific autoinducers), others are used to detect other species (interspecies). Moreover, there are reports about autoinducer detection between distantly related taxa, such as bacteria and fungi. In essence, quorum sensing helps the microbe to act in coordinated manner to adapt various environment conditions.

Compared to bacteria, cell-to-cell communication in fungi was only recently discovered. The role of quorum sensing in morphogenesis in dimorphic fungi was

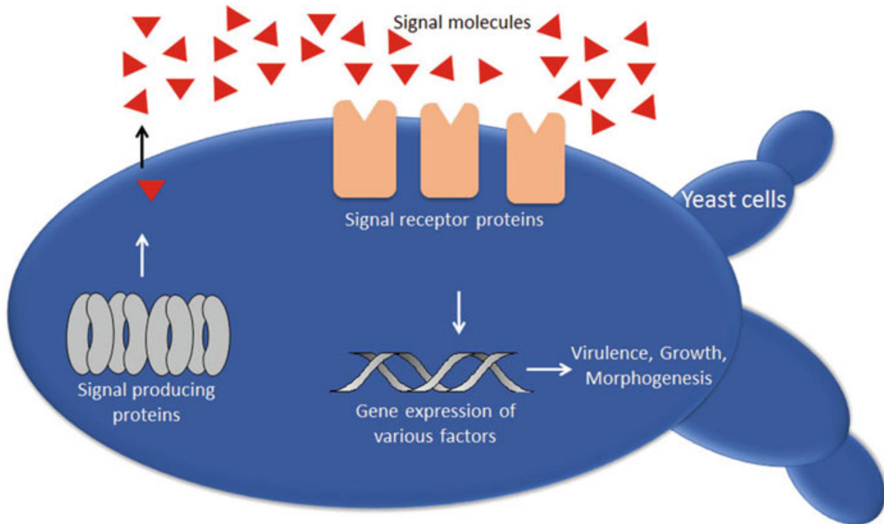

Fig. 3 General mechanism of quorum sensing in fungi. Similar to bacteria, signal-producing proteins are involved in the synthesis of signal molecules which are then detected by the signal receptor proteins which later on regulate the expression of various genes (Adapted from Sharma and Jangid [83])

established only in the last decade [7]. Most fungal studies on quorum sensing have focused on yeasts as a model system. Earlier, yeasts were thought to change the dimorphic behavior due to environmental changes especially physical, i.e., temperature, pH, etc. However, research on quorum sensing in fungi has shown its involvement in regulating many other phenotypes as well. In *Candida albicans*, the autoinducer farnesol blocks hyphal development and biofilm formation, while in stationary phase, tyrosol autoinducers (small numbers) stimulate formation of hyphae. In *Uromyces* (a mycelial fungus), methyl 3,4-dimethoxycinnamate inhibits the germination of uredospores [6]. Although very few fungi (both yeast and filamentous forms) have been experimentally studied for quorum-sensing system, new genomic data suggests that many fungi harbor quorum-sensing gene homologs. Hence, the possibility of discovering novel systems and phenotypes regulated by them is very high in fungi.

2 Morphological Changes During Pathogenesis

Fungi occupy various habitats and form diverse morphological structures to survive in environment. As discussed below, pathogenic fungi either make morphological changes (appressorium, peg formation) at the time of infection or show the phenomenon of dimorphism (yeast form, hyphal form, or pseudohyphae). Changes in environment generate some signals which are perceived by the fungi and trigger morphological changes (Table 1). These signals may be temperature, pH, nutrient, or

Table 1 Various dimorphic fungal pathogens (plant, mammal, insect) and their major stimuli for the morphological switch

Name of fungi	Phylum	Disease	Pathogenic to	Major stimuli for the morphological switch
Mucor circinelloides	*Zygomycota*	Mucormycosis	Mammalian pathogens	O_2 and CO_2 tension
Cokeromyces recurvatus	*Zygomycota*	Mucormycosis	Mammalian pathogens	Temperature, nutrients, O2 and CO2 tension
Ustilago maydis	*Basidiomycota*	Corn smut	Plant pathogens	Pheromones, plant lipids, plant hydrophobicity, pH, nitrogen
Malassezia furfur	*Basidiomycota*	Pityriasis versicolor	Mammalian pathogens	L-DOPA
Blastomyces dermatitidis	*Ascomycota*	Blastomycosis	Mammalian pathogens	Temperature
Coccidioides immitis	*Ascomycota*	Coccidioidomycosis	Mammalian pathogens	Temperature
Coccidioides posadasii	*Ascomycota*	Coccidioidomycosis	Mammalian pathogens	Temperature
Histoplasma capsulatum	*Ascomycota*	Histoplasmosis	Mammalian pathogens	Temperature, QS[#]
Emmonsia crescens	*Ascomycota*	Emmonsiosis	Mammalian pathogens	Temperature
Emmonsia parva	*Ascomycota*	Emmonsiosis	Mammalian pathogens	Temperature
Emmonsia pasteuriana	*Ascomycota*	Emmonsiosis	Mammalian pathogens	Temperature
Lacazia loboi	*Ascomycota*	Lacaziosis	Mammalian pathogens	Temperature
Paracoccidioides brasiliensis	*Ascomycota*	Paracoccidioidomycosis	Mammalian pathogens	Temperature

Paracoccidioides lutzii	*Ascomycota*	Paracoccidioidomycosis	Mammalian pathogens	Temperature
Sporothrix schenckii	*Ascomycota*	Sporotrichosis	Mammalian pathogens	Temperature
Hortaea werneckii	*Ascomycota*	Tinea nigra	Mammalian pathogens	Temperature, nutrition, inoculum size
Ceratocystis (Ophiostoma) ulmi	*Ascomycota*	Dutch elm disease	Plant pathogens	Nitrogen source, QS
Ceratocystis (Ophiostoma) novo-ulmi	*Ascomycota*	Dutch elm disease	Plant pathogens	Nitrogen source, QS
Holleya sinecauda	*Ascomycota*	Mustard seed rot	Plant pathogens	Unknown
Taphrina deformans	*Ascomycota*	Peach and almond leaf curl	Plant pathogens	Unknown
Beauveria bassiana	*Ascomycota*	White muscardine plant endophyte	Insect pathogens	Insect hemolymph
Ophiocordyceps unilateralis	*Ascomycota*	Zombie ant	Insect pathogens	Insect hemolymph
Metarhizium robertsii	*Ascomycota*	Locusts	Insect pathogens	Insect hemolymph
Saccharomyces cerevisiae	*Ascomycota*	*Saccharomyces cerevisiae* fungemia	Mammalian pathogens	Temperature, QS
Candida albicans	*Ascomycota*	Invasive candidiasis	Mammalian pathogens	Temperature, QS
Cryptococcus neoformans	*Ascomycota*	Cryptococcal meningitis, infects lung	Mammalian pathogens	Temperature, QS
Penicillium expansum	*Ascomycota*	Postharvest rots that infects apples	Plant pathogens	Temperature, QS
Penicillium digitatum	*Ascomycota*	Postharvest rots that infects citrus crops	Plant pathogens	Temperature, QS
Penicillium allii	*Ascomycota*	Pathogen of garlic (*Allium sativum*)	Plant pathogens	Temperature, QS
Penicillium fellutanum	*Ascomycota*	Penicilliosis	Mammalian pathogens	Temperature, QS

(*continued*)

Table 1 (continued)

Name of fungi	Phylum	Disease	Pathogenic to	Major stimuli for the morphological switch
Penicillium implicatum	*Ascomycota*	Infection to the eye; postharvest rots of pomegranate	Mammalian and plant pathogens	Temperature, QS
Penicillium marneffei	*Ascomycota*	Penicilliosis	Mammalian pathogens	Temperature, QS
Aspergillus terreus	*Ascomycota*	Aspergillosis in humans, infective endocarditis, pulmonary mycetoma, allergic bronchopulmonary aspergillosis; foliar blight of potatoes	Mammalian and plant pathogens	Temperature, QS
Aspergillus flavus	*Ascomycota*	Invasive aspergillosis; postharvest vegetable or fruit loss	Mammalian and plant pathogens	Temperature, QS
Aspergillus nidulans	*Ascomycota*	Chronic granulomatous disease	Mammalian pathogens	Temperature, QS
Aureobasidium pullulans	*Ascomycota*	Allergic infections like pneumonitis	Mammalian pathogens	QS
Aureobasidium melanogenum	*Ascomycota*	Allergic infections like pneumonitis	Mammalian pathogens	QS
Neurospora crassa	*Ascomycota*	Postharvest fruit crops	Plant pathogens	QS
Debaryomyces hansenii	*Ascomycota*	Not associated with any disease	Nonpathogenic	QS

#QS – Quorum sensing

some chemical molecule released by the fungus itself. In general, anaerobic environment supports yeast growth [8]. Many fungal pathogens especially those of animals and humans live in mycelial form outside (25 °C) and remain as yeast form inside host (37 °C) [9]. For clarity, we have discussed below two categories of fungal pathogens based on the host type, the mechanisms of invasion, morphogenesis, and pathogenesis.

2.1 Plant Pathogenic Fungi

The invasion of plant host by fungal pathogens is a complex multi-step process and is accompanied by several morphological changes during the process of pathogenesis. Our aim is not to discuss the steps in detail for which the reader may refer earlier work [10–13]. Briefly, the initial step involves the attachment of fungal spores to the host surface (Fig. 4). Recognition of the host surface is crucial for this attachment and is facilitated by certain signals on the host surface that may include the long-chain aliphatic fatty alcohols present in wax (hydrophobic) on the plant surface. After recognition of host, the spore germinates, and penetration begins via various modifications in the structure of fungal hyphae. Further, while some fungi like *Magnaporthe grisea* (rice blast fungus) directly penetrate the host surface (Fig. 4a), others, such as *Uromyces appendiculatus* (bean rust fungus), enter either through stomata or wound openings (Fig. 4b). After germination, the germ tube differentiates into an appressorium and forms a penetration peg that pierces into the cuticle and epidermal cell wall [14, 15]. In *Magnaporthe grisea*, penetration is the result of a coordinated effort of physical force as well as genetic regulation by the fungus [16]. Whereas the MPG1 gene is responsible for attachment, the cyclic adenosine monophosphate (cAMP) plays a role in signaling pathways enabling it to penetrate [17, 18]. Mutants for both fail to penetrate rice leaf [19, 20]. In addition, melanin and turgor pressure within the appressorium/penetration peg which are under genetic regulation also help in penetration [21]. While most plant pathogens enter through formation of appressoria, root pathogens like *Fusarium oxysporum* f. sp. *vasinfectum* produce netlike mycelia on root surface. Alternatively, fungi also secrete extracellular hydrolytic enzymes such as cutinase to enable mechanical penetration, such as in *Botrytis cinerea* and *M. grisea.* Although dimorphic switching occurs in basidiomycetous corn pathogen *Ustilago maydis*, it is not a general phenomenon in plant pathogenic fungi. It grows as yeast *in vitro* and its hyphal forms infect plants [22]. Hence, morphogenesis in plant pathogenic fungi plays an essential role in its pathogenesis.

2.2 Animal and Insect Pathogenic Fungi

Fungi cause a wide range of diseases in humans and animals: from superficial skin diseases caused by dermatophytes to invasive life-threatening infections. Most fungal pathogens are opportunistic, and their pathogenicity is dependent upon

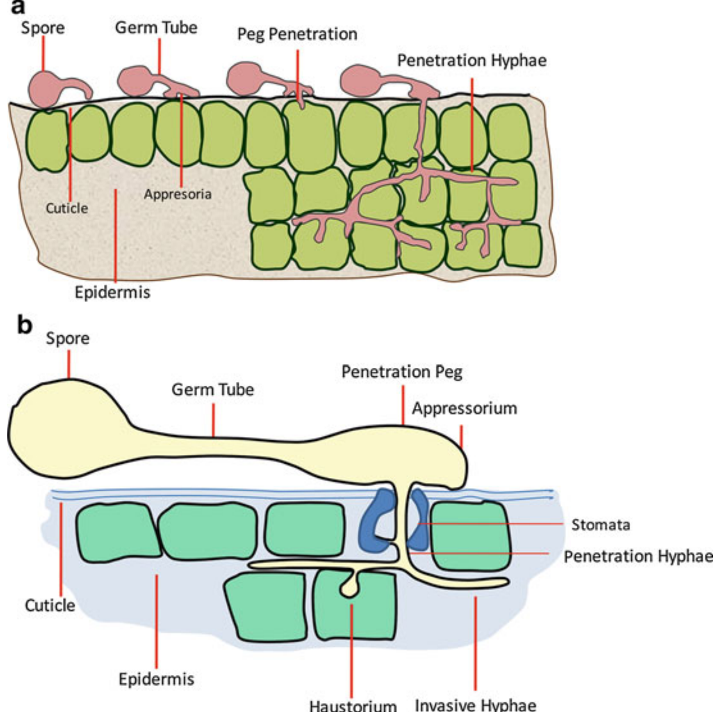

Fig. 4 Invasion of plant host by fungal pathogens. (**a**) Direct penetration and invasion. During this process, fungus first adheres to the surface of plant. It germinates by forming a germ tube and swollen structure (appressoria). With the help of turgor pressure and local extracellular enzyme, it penetrates the plant surface (After Agrios [101]). (**b**) Penetration and invasion through natural opening. Fungal mycelium enters the host by natural openings like stomata or wounds (After Meng et al. [102])

numerous factors, such as the health status of the host (healthy or immunocompromised), type of strain, and/or other environmental conditions (Fig. 5). Some common fungal pathogens of humans include *Candida albicans*, *Cryptococcus neoformans*, *Mucor circinelloides*, *Aspergillus flavus*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Malassezia furfur*, etc. Like plant pathogenic fungi, the morphogenetic switch from hyphal to yeast form helps the fungus to overcome the structural, thermal, and immunological barrier. The outside or epithelial barrier is overcome by the hypha; the conidia then pass the lung and enter the respiratory tract. Once inside the lung, conidia bind to inner wall and transform to yeasts. During this transition, the genes responsible for immune evasion, intracellular survival, and dissemination are upregulated, and the hyphae help the fungal pathogen to survive the environment, allow transmission to new host, and add genetic variability. For most fungi, upon dissemination, the sexual reproduction occurs outside the host when hyphae of opposite mating types come together and fuse implying that the pathogen must exit the host in order to complete its life cycle. In case of noninvasive

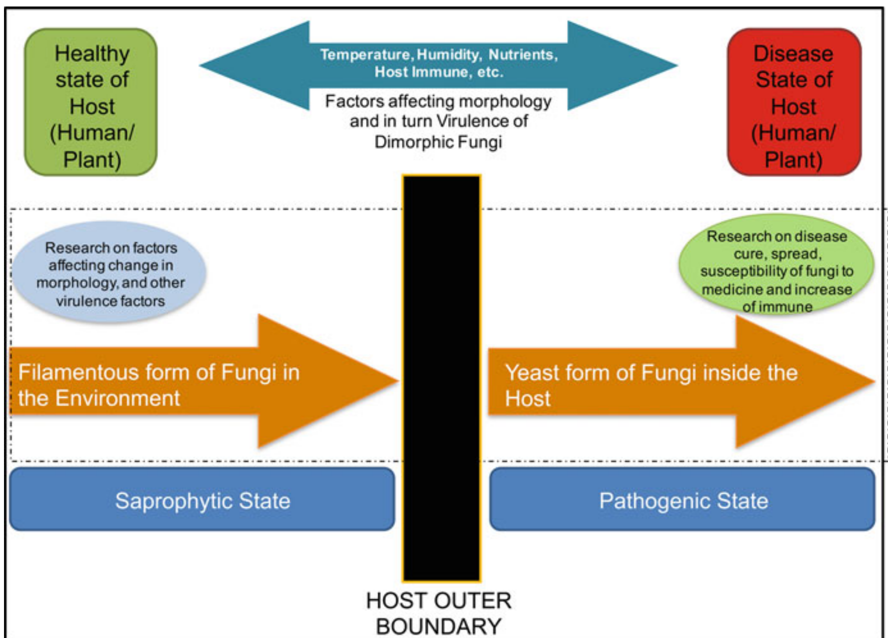

Fig. 5 An opportunistic fungus behaves differently in environment and host. A fungus can behave as saprophyte in the environment, and same fungus may behave as pathogen when it infects a host (mammal, plant, insect). Same fungal strain contains virulent factors which are only expressed inside the host

pathogens, such as dermatophytes, the pathogen grows on the skin superficially in mycelial form, and the conidia formed by them must be shed from the host upon degradation of the infected skin surface.

Likewise, morphogenesis plays an essential role during fungal pathogenesis in insect hosts. Host detection and adhesion, penetration of the cuticle, and non-self-recognition by host defense mechanism are crucial factors involved in the establishment of infections by entomopathogenic fungi, which mostly belong to the phylum *Ascomycota* and includes genera such as *Metarhizium*, *Beauveria*, *Nomuraea*, *Cordyceps*, etc. These fungi are not truly dimorphic but exhibit different morphological structures inside and outside the insect host. The penetration is by the hyphal form which then switches to the yeastlike form called blastospores inside the insect or at times remains mycelial (Fig. 6). The blastospores replicate by budding to promote dissemination, evading the immune system of insect [23]. Some fungi form ascomata outside the insect host.

In most cases, morphogenesis is associated with the virulence capacity of the fungal pathogen. For instance, *Metarhizium anisopliae* is a potent insect pathogen and a well-known biocontrol agent. It is cosmopolitan in nature and has metabolic and ecological variability and adaptability allowing it to establish itself as an entomopathogen or saprophyte or an endophyte as well. While inside insect

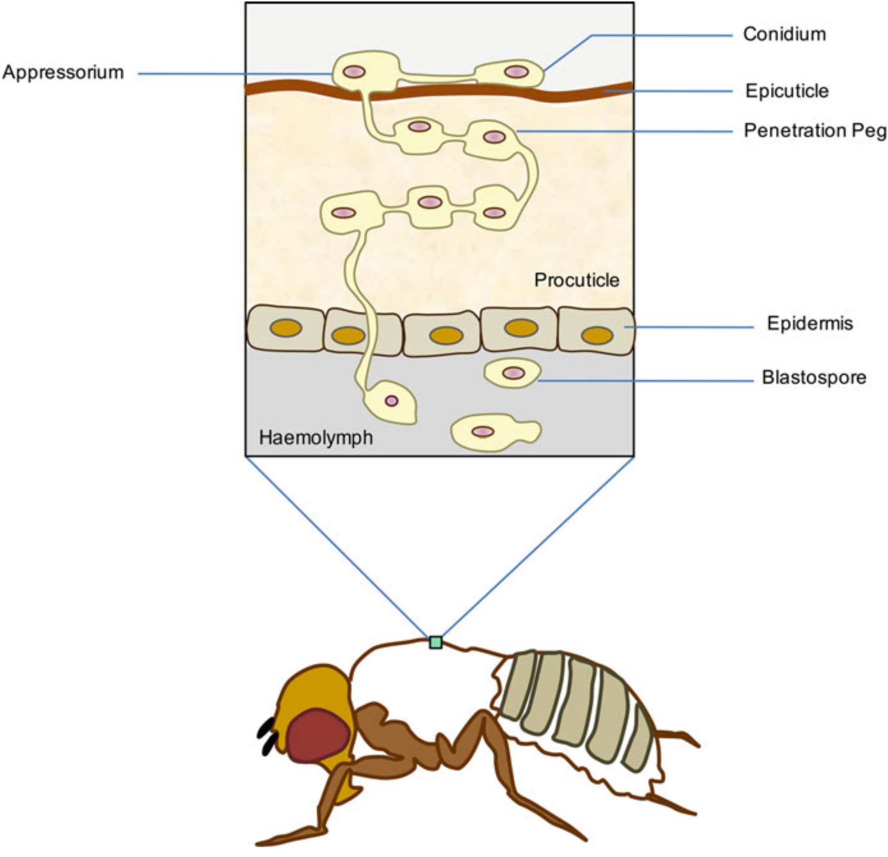

Fig. 6 Penetration and invasion of insect by fungi. Just like mammalian invasion, after landing on the surface of an insect, fungal conidia germinate and form penetration peg which in turn leads mycelia to the hemolymph

hemolymph and on plant root surfaces, the fungus produced two differentially induced proteins MAD1 and MAD2 which are involved in the recognition of surface structures by the fungal conidia [24]. MAD1 is involved in cytoskeletal organization and cell division. Increased production of MAD1 helps in insect surface recognition and blastospore formation thereby increasing the virulence. MAD1 deletion mutants have shown up to 90 % reduction in their capacity to adhere to a surface by affecting conidial germination. Other fungi like *Beauveria bassiana* and *Ophiocordyceps unilateralis* also alter their morphology for host infection [25, 26]. In *B. bassiana*, carbohydrate epitomes attached on the walls of various morphological structures help in avoiding host immune responses and enhancing subsequent infection [26]. Importance of surface carbohydrate in fungal-insect infection has also been demonstrated in *Paecilomyces farinosus* and *Nomuraea rileyi* [27, 28]. Thus, fungal pathogenesis in insect hosts is a complex process involving an array of genes and

signaling pathways that regulate phenotypic as well as structural changes within the pathogen.

3 Role of Quorum Sensing in Morphogenesis and Pathogenesis

Dimorphism, especially in fungal pathogens, is a unique adaptation for effective infestation and infection of the host. In most cases, this dimorphism is mediated by quorum sensing. However, interest in quorum-sensing system comes from the occurrence of dimorphism and other morphological changes among those fungi exhibiting pathogenicity toward plants and animals (Fig. 7). Several chemical and environmental factors have been reported to shift the yeast-mycelium dimorphism. Among these have been temperature, pH, glucose levels, nitrogen source, carbon dioxide levels, transition metals and chelating agents, and inoculum size or density [29]. Moreover, as compared to the total number of genera known, the number of genera studied for or showing quorum-sensing system is very few (Fig. 8). For instance, there are many plant pathogenic dimorphic fungi where quorum sensing is not known, such as in *Taphrina deformans*, *Mycosphaerella graminicola*, *Holleya sinecauda*, *Verticillium dahliae*, and *Verticillium albo-atrum*, all of which exhibit yeast-to-mycelial switching; no cell density-dependent regulation has been identified

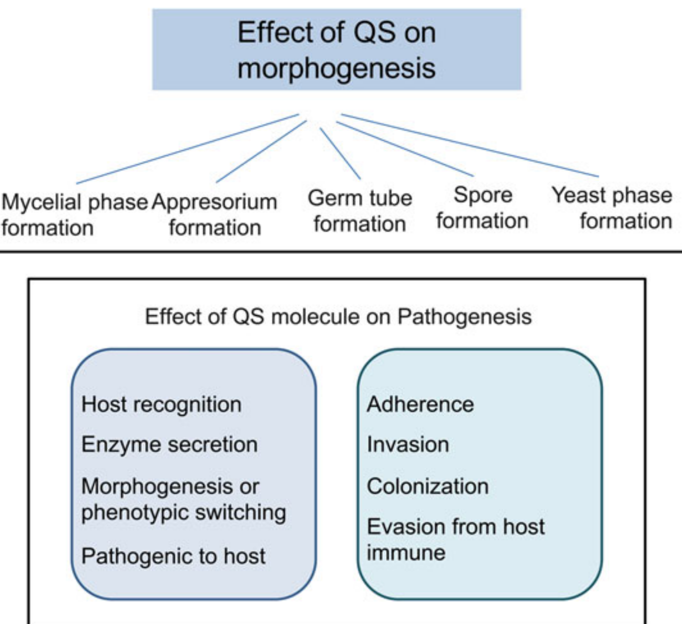

Fig. 7 Effect of quorum-sensing molecules on morphogenesis and pathogenesis

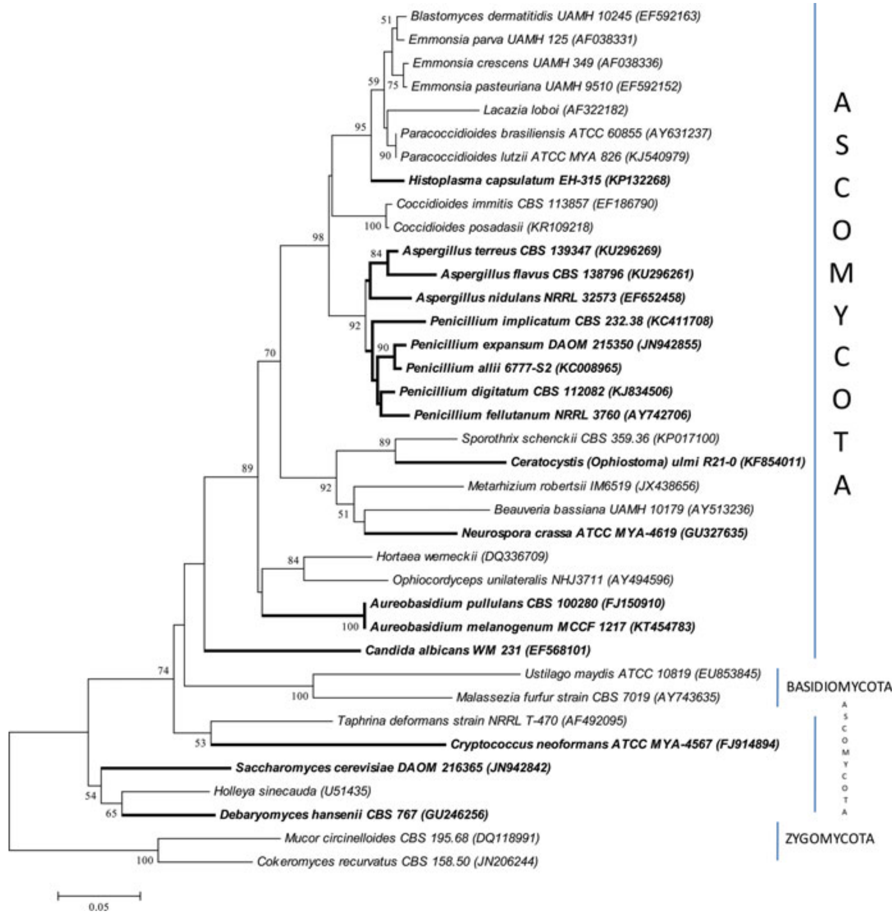

Fig. 8 Phylogenetic tree of pathogenic fungi (infecting plant, mammal, or insect) which show dimorphism. The fungi highlighted in bold are those for which quorum sensing is reported as one of the mechanisms for dimorphism

so far [30]. Fungi in which the morphological change is mediated by quorum sensing are discussed below based on the phylum it belongs.

3.1 *Basidiomycota*

3.1.1 *Cryptococcus*

Cryptococcus has more than 37 species, majority of which grow in soil and are not harmful to humans. Cryptococcal fungi grow as yeast form in culture and its sexual morph grows as filamentous form. However, *Cr. neoformans* is an animal and human pathogen of environmental origin causing life-threatening meningoencephalitis in

immunocompromised hosts [31]. Similarly, *Cr. laurentii* and *Cr. albidus* occasionally cause meningitis in immunocompromised human patients. Research has shown the involvement of short signaling peptides acting as quorum-sensing molecules in this fungus. The deletion of a global repressor TUP1 encoded by CQS1 gene resulted in less growth at both 25 °C and 30 °C but not at 37 °C. Further experimentation revealed that penetration by the TUP1 mutant was cell density dependant and was regulated by an 11-amino acid peptide (QSP1) which had effect on the morphology of the fungus [32]. Although small peptides are known inducers in many bacteria, such as *Streptococcus pneumoniae*, *Bacillus subtilis*, and *Staphylococcus aureus*, they have not been reported in any other fungi as a quorum-sensing molecule. It is possible that the oligopeptide may help the fungus to grow slow and survive the harsh environmental conditions by repressing the TUP1. However, once it enters animal or human body, the TUP1 is derepressed at the higher host body temperature and becomes fully functional, and hence full rate of cell multiplication may occur. Lee et al. [33] also found the patho-biological effect of TUP1 which helps in fast colonization of host, thus helping the fungus in pathogenesis. Although it has not been experimentally proven, it is very likely that this oligopeptide may regulate quorum-sensing system of other organisms.

The investigation of cell density-dependent behavior of *Cr. neoformans* in conditioned medium (medium in which *Cr. neoformans* has already grown) and medium containing pantothenic acid showed dose-dependent increase in the growth both as isolated cells and biofilm cells. The addition of conditioned medium also increased capsular synthesis (glucuronoxylomannan) and melanin synthesis which are considered as important pathogenesis factors [34]. All these mechanisms help the fungus in establishing itself in the host thereby causing severe infection. The same conditioned medium in which *Cr. neoformans* was grown stimulated the growth of *Candida albicans* and *Saccharomyces cerevisiae.* Conversely, conditioned medium from other fungal species, viz., *Candida albicans*, *Saccharomyces cerevisiae*, *Cryptococcus albidus*, and *Saccharomyces schenkii*, enhanced the growth of *Cr. neoformans* suggesting that the quorum-sensing system exists in the genus *Cryptococcus.* Further research on its social interactions, both inside and outside its host, will reveal more about the pathogenesis.

3.2 *Ascomycota*

3.2.1 *Aspergillus*

Aspergillus is one of the largest genera of phylum *Ascomycota* and *Eumycota.* They are one of the most cosmopolitan fungi colonizing almost all niches. Just like other filamentous fungi, species of *Aspergillus* (*A. terreus*, *A. flavus*, *A. nidulans*, etc.) also undergo morphological changes like sporulation (sexual, asexual morphs), as a result of nutrient and environment change. Species of genus *Aspergillus* are also known to harbor quorum-sensing system for regulating population-based functions like morphogenesis and production of secondary metabolite [35, 36].

A. terreus is one of the most important pathogens of both animal and plant causing invasive aspergillosis in humans, infective endocarditis, pulmonary mycetoma, and allergic bronchopulmonary aspergillosis. In plants, it causes infection and losses in rice, wheat, potato, maize and soybean. Under sufficient nutrition supply, it shows more of filamentous growth and sporulation by asexual means. However, once the nutrient is limiting, it forms sexual morph if both mating types are present. *A. terreus* produces Butyrolactone I (α-oxo-β-(*p*-hydroxyphenyl)-γ-(*p*-hydroxy-*m*-3, 3-dimethylallylbenzyl)-γ-methoxycarbonyl-γ-butyro-lactone) as the quorum-sensing signaling molecule (Fig. 9) that is known to increase its own production as well as lovastatin when externally added, thus confirming that quorum sensing is involved in increased production of secondary metabolite in *A. terreus* [37]. Butyrolactone I inhibits cyclin-dependent kinases which are protein kinases controlling cell cycle progress. In *A. terreus* morphogenesis also, it increases hyphal branching, extension, and sporulation [36]. While hyphal branching is important for fast and rapid colonization of a host or substrate, increased sporulation enhances rapid dispersal of the pathogenic fungi.

A. flavus is another common fungus which is responsible for huge losses in postharvest vegetables or fruits. In groundnut, it produces aflatoxins which are carcinogenic. In humans, it causes invasive aspergillosis when the conidial forms enter the lungs through inhalation. In *A. flavus* the fungus forms asexual spores (conidia) or resting structures (sclerotia), and their switching is population dependent wherein an increase in cell density (from 10^1 to 10^7 cells/plate) results in the decreasing numbers of sclerotia [38]. Extracts from low-cell density growth cultures induced a high-sclerotium-number phenotype, whereas high-cell density extract increased conidiation. While the involvement of quorum sensing is highly likely, the chemical nature of the compound is still not determined.

In addition to other compounds, lipids have also been identified as regulators of fungal growth and reproductive development. In *A. nidulans*, the cleistothecium-conidium switch is regulated by the lipid, oxylipin, which acts as the quorum-sensing molecule in this fungus [38–40].

3.2.2 *Aureobasidium*

Species of the genus *Aureobasidium* are black and yeast-like and occupy a wide diversity of environments, from soil to water, and exist as epiphytes as well as endophytes. Many species are also used as biological control agents. Two species, *Au. pullulans* and *Au. melanogenum* cause severe human infections like pneumonitis which are primarily allergic in nature. Although *Au. pullulans* shows phenotypic plasticity in response to temperature, pH, and nutrition status, the likelihood of this morphogenesis being under quorum-sensing regulation is high and needs further investigation.

3.2.3 *Candida*

Candida spp. are among the most common and medically important fungi, at times responsible for 70–80 % of fungal infections in humans. Species in this genus cause many invasive and noninvasive infections in healthy and immunocompromised patients.

QS Molecule	Fungal Genera	Structure
Butyrolactone I	*Aspergillus*	
Phenylethanol	*Debaryomyces, Saccharomyces*	
4-Hydroxyphenylacetic acid	*Ceratocystis/ Ophiostoma*	
Methylvaleric Acid	*Ceratocystis/ Ophiostoma*	
2-Methyl-1-Butanol	*Ceratocystis/ Ophiostoma*	
Tyrosol	*Candida, Debaryomyces*	
Farnesol	*Candida*	
Oxylipin	*Aspergillus*	
α-(1,3)- glucan	*Histoplasma*	
Calcium-binding protein (CBP)	*Histoplasma*	--
Tryptophol	*Saccharomyces*	
Farnesoic Acid	*Candida*	

Fig. 9 Structures of some known quorum-sensing molecules in fungi

While *Candida albicans* is the most commonly encountered species in nosocomial infections, others include *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* [41]. *C. albicans* is a polymorphic fungus which is a normal inhabitant of oral, gut, and vaginal microbiota of a healthy human being. However, in immunocompromised individuals, it is responsible for oral mycoses, skin and lung infections, etc. The increased pathogenicity is attributable to the changes in fungal morphology that allows the fungus to better adhere to medical devices and/or host cells, biofilm formation, and secretion of hydrolytic enzymes. Several genes are known to regulate these factors and are consequently involved in virulence [42].

Candida has been the most extensively studied fungi for quorum-sensing-dependent morphogenesis [6, 7, 43]. There are at least four quorum-sensing-related molecules identified so far, farnesol, farnesoic acid, tyrosol, and morphogenic autoregulatory substance (MARS) [44]. The switch from yeast to mycelial form in *C. albicans* occurs at cell densities lower than 10^6 cells/ml and is typically regulated by farnesol which is an isoprenoid molecule [45]. In the same year, farnesoic acid was identified from another strain of *C. albicans* that was farnesol negative [46, 47]. Farnesol blocks yeast-to-mycelium formation but does not stop elongation of preexisting hyphae [48, 49] and is also known to affect the formation of chlamydospore [50]. Expectedly, the strains also have the ability to form biofilms which is under the regulation of farnesol [6]. *Candida* biofilm plays an important role during infection; it inhibits lesion formation in oral candidiasis, inhibits murine macrophage activity, and causes apoptosis of human spermatozoa [51–53]. In contrast, *Candida* also produces tyrosol which enhances mycelial growth by initiating the germ tube formation [54, 55]. Hence, both farnesol (inhibits yeast-to-mycelium phase) and tyrosol (induces yeast-to-mycelium phase) act in opposite manner. Two other molecules, phenylethyl alcohol and tryptophol, have been reported in *Candida*, but their role in quorum-sensing regulation is yet to be confirmed [44].

Although the switch from yeast to hyphae is governed by environmental factors, it is largely dependent on transcriptional regulation of several signaling pathways, viz., the CEK1 mitogen-activated protein kinase pathway, the Ras/cyclic AMP-dependent pathway, the calcium signaling pathway, the Rim101-independent pathway, and the two-component signal transduction pathways which collectively transduce the environmental factors to dimorphism. Farnesol acts by blocking all or some of the signaling pathways. In response, the immune system of the host is also known to act against *Candida* invasion through the epithelial layers of the host, and this interaction is critical for the host as well as fungus [56–58]. Thus, *Candida* spp. are dependant on the dimorphic phase switch for its increased virulence with the hyphal phase, and its conidia required to enter the host either by penetration or through inhalation and later the yeast phase to colonize and spread within host.

3.2.4 *Ceratocystis (Ophiostoma)*

Ceratocystis (Ophiostoma) is an important plant pathogenic fungus belonging to the family *Ceratocystidaceae*. It is responsible for major diseases in trees, such as oak wilt and pineapple black rot. Unlike other fungi, nitrogen status in the surrounding environment is known to regulate dimorphism. For instance, in *Ce. ulmi* which

causes Dutch elm disease, the yeast form is induced in the presence of 10 mM proline with cell densities of $\geq 10^6$ cells/ml, whereas mycelial growth is induced at 10 mM ammonium or asparagines or arginine at cell densities between 10^3 and 10^8 cells/ml [59]. The quorum-sensing activity in *Ce. ulmi* is mediated by a lipophilic molecule which is species specific and does not cross-react with other fungi. Moreover, in the presence of this molecule, the fungus does not require the threshold cell density to switch between different dimorphic phases suggesting that dimorphism in *Ce. ulmi* is mediated by quorum sensing [29]. The regulation of germ tube formation in *Ce. ulmi* is also under quorum-sensing regulation and is mediated by 2-methyl-1-butanol, methylvaleric acid, and 4-hydroxyphenylacetic acid (also called as fusel alcohol and fusel oil) [60]. Fusel alcohols, viz., 3-methyl-1-butanol (isoamyl alcohol), 2-methyl-1-propanol (isobutyl alcohol), 2-methyl-1-butanol (active amyl alcohol), 2-phenylethanol, and 3-(2-hydroxyethyl)indole (tryptophol), are also known to control cell morphology in other fungi [61, 62].

There are several cyclic and noncyclic isoprenoid molecules, similar to farnesol, that are produced by various species of *Ceratocystis*, but their exact role is still unknown. At least in *Ceratocystis floccosum*, three cyclic sesquiterpenes are involved in quorum-sensing-mediated regulation of yeast-mycelium dimorphism [63]. Further, oxylipins could also be involved in yeast-to-mycelium transition as the strains grown on oxylipin showed decreased mycelium production [64]. It is likely that oxylipins act in a cell density-dependent manner to regulate morphogenesis and thereby in pathogenesis.

Members of *Ceratocystis* are less studied for quorum sensing with only partial characterization of the inducers. However, it is presumed that the actual molecular mechanism should be similar to other isoprenoid molecules as shown in *C.albicans* for farnesol. However, an extensive study involving the screening of all species of the genus *Ceratocystis* is required to understand their role in morphogenesis and pathogenesis.

3.2.5 *Debaryomyces*

Debaryomyces spp. are yeasts affiliated with the family *Saccharomycetaceae.* Its most common species, *Debaryomyces hansenii*, is characterized by its ability to grow in low pH and temperature but high NaCl concentrations [65]. These properties have enabled its use in cheese and food industry. Although earlier thought to lack dimorphism, the species was shown to exhibit this phenomenon during continuous fermentation of acid-hydrolyzed barley bran in 2000 by Cruz et al. [66]. Since then, research has shown ammonia-mediated quorum-sensing regulation of growth on agar plates in *D. hansenii* [67]. Later on, the authors showed N-dependent production of phenylethanol and tyrosol [68]. Also, high-cell density cultures produced more phenylethanol and tyrosol as compared to low-cell density cultures. These two alcohols are also responsible for biofilm formation suggesting a possible role of quorum sensing. Although *D. hansenii* is not yet reported as a pathogen, the quorum-sensing-dependent morphogenesis will help in fermentation of these cultures in food industry.

3.2.6 *Histoplasma*

Histoplasma capsulatum is one of the most studied pathogens of human. The fungus causes histoplasmosis, also called as cave disease, as the fungus is commonly found associated with bat guano and nowadays with poultry. *H. capsulatum* is a dimorphic fungus that lives as a saprophyte while in mycelial form and as an intracellular human pathogen in yeast form. It undergoes reversible morphogenesis from mycelia to yeast and vice-versa which is associated with increased virulence and successful pathogenesis. Although it is a common inhabitant of soil, its conidia and hyphal fragments are inhaled by humans. Inside the body, the fungus transforms into yeast form possibly triggered by the host body temperature. They survive inside the phagosome by changing to yeast form multiplying inside the phagosome. Studies on the genes expressed exclusively in yeast form have helped to understand its pathogenesis. *H. capsulatum* has two yeast phase factors, cell wall-bound α-(1,3)-glucan and a secreted calcium-binding protein, both of which are cell density dependent and involved in the morphogenesis and subsequent pathogenesis [69, 70]. While α-(1,3)-glucan is continuously produced by the fungus inside macrophages, the calcium-binding protein (CBP) is associated with the mycelia-yeast interchange. The protein is encoded by CBP1 at the level of transcription and secreted only at the time of infection of mammalian hosts. Moreover, Kugler et al. [69] showed that CBP1 is downregulated as the morphology shifts to mycelia form. Thus, morphogenesis in the fungus helps in the establishment of the infection and its subsequent spread.

3.2.7 *Neurospora*

Neurospora is one of the important genera of *Ascomycota*, and *Neurospora crassa* has been a model organism for fungal genetic studies. It is a saprophytic and a nonpathogenic fungus. At times, it has been reported to damage postharvest fruit crops. Hyphal fusions (anastomosis) are commonly observed between spore germ tubes and/ or between vegetative hyphae and are important for proliferation of the fungus. The anastomosis is reported to improve mycelial communication by transporting nutrients and water within the colony. Specialized hyphal cells produced by the conidia and conidial germ tube are called conidial anastomosis tubes (CATs). These are morphologically and physiologically distinct from germ tube. These CATs forming anastomosis structures show positive tropism. Although chemoattractant is unknown, the closeness of hyphae increases the frequency of formation of anastomosis [71, 72]. However, Roca et al. [72] have demonstrated that CATs in *N. crassa* are cell density dependent and induced by an extracellular molecule. Based on mutant analysis that lacked cyclic AMP (cAMP) synthesis, it was shown that it was not cAMP dependent; instead, it was regulated by putative transmembrane protein (HAM-2) and the MAK-2 and NRC-1 proteins of a mitogen-activated protein kinase signaling pathway. Mutation in the gene coding HAM-2, MAK-2, and NRC-1 did not form CATs. Since CAT formation was dependent on conidial concentration (at least 10^5 macroconidia/ml), it was hypothesized that it is under quorum-sensing regulation.

3.2.8 *Penicillium*

The genus *Penicillium* is the second most important genus of *Ascomycota.* It includes large number of species, some of which are pathogenic and some are industrially important, especially for the pharmaceutical industry. Species of *Penicillium*, viz., *P. expansum*, *P. digitatum*, and *P. allii*, are pathogenic to plants, and *P. fellutanum*, *P. implicatum*, and *P. marneffei* are pathogenic to humans and animals. *P. sclerotiorum* produces azaphilones, isochromophilone, polyketide, and γ-Butyrolactones containing multicolanic, multicolosic, and multicolic acid. In addition, it produces sclerotiorin, a yellow-colored secondary metabolite with phospholipase A2 inhibitor activity [73, 74]. Azaphilones and sclerotiorin help in binding to human epithelial layer. While Raina et al. [74] studied the possible mechanism of quorum sensing in controlling the production of sclerotiorin, its involvement in regulating morphogenesis and pathogenesis is not clear.

3.2.9 *Saccharomyces*

Saccharomyces is a dimorphic yeast commonly used in wine, brewing, and baking since ancient times. Although there is no direct relation of quorum-sensing activity in *Saccharomyces cerevisiae* morphogenesis related to pathogenesis, apoptosis is quorum-sensing dependent [75]. Apoptosis helps to remove unwanted old cells, keeps the colony healthy, and maintains the genetic stability of the colony, i.e., apoptosis is proportional to the degree of genetic damage. Pheromones and ammonia were reported as the signaling molecules along with some role of phenylethanol. During apoptosis, high concentration of α-mating factor was found along with ammonia [76, 77].

The nonpathogenic *S. cerevisiae* also undergoes morphological transition which is cell density dependent. Chen and Fink [78] demonstrated that cells of *S. cerevisiae* undergo morphogenesis by secreting aromatic alcohols by inducing the expression of FLO11 through a Tpk2p-dependent mechanism. The mutants defective in synthesis of these alcohols showed reduced filamentous growth. Like in many other fungi discussed above, this quorum-sensing activity was nitrogen dependent, and the aromatic alcohols would be released during N starvation. These alcohols were identified to be phenylethanol and tryptophol as the quorum-sensing molecules stimulating pseudohyphal growth in *S. cerevisiae*.

4 Application of Quorum Sensing in Disease Control/ Management

Ever since agriculture began, fungi have been a nuisance causing multiple pathogenic conditions in plants. In humans too, fungal infections have emerged as a major problem with the advent of immunocompromised diseases such as AIDS, H1N1, etc. Regardless of the host, morphogenesis in fungi is critical for pathogenesis. Because this shift is cell density dependent, blocking quorum-sensing-mediated regulation of morphogenesis in such pathogenic fungi is the most sought-after target nowadays. It becomes more important in the present day situation where more and more strains

are becoming resistant to treatment (whether plant or animal). Knowledge of these quorum-sensing molecules and the mechanism by which they stimulate growth may lead to improved measures for controlling fungal infection and provide important insights into potential antimicrobial treatments [79, 80]. Inhibiting morphogenesis, thereby decreasing the virulence capacity, is likely to become the most effective in disease management and control. Compounds capable of inhibiting the quorum-sensing signals could therefore establish a new generation of antimicrobial agents that would be useful in medicine, veterinary science, and agriculture [81]. Interference of quorum sensing can be achieved in several ways, by preventing signal production or release of quorum-sensing molecules, by signal degradation, or by preventing the accumulation of the quorum-sensing molecules [82]. Numerous fungi are known to produce such quorum-quenching compounds, and these have been discussed in detail very recently [83]. Abraham [84] discussed that certain compounds which control biofilm can be combined with established or novel antibiotics, and it may improve the treatment of biofilm infections.

5 Conclusion

Fungi exhibit several cell density-dependent behaviors. Many fungal spores inhibit their own germination in high cell density via regulation through phenolic molecules, viz., *cis*-ferulic acid methyl ester [85, 86]. Similarly, unsaturated fatty acids are known to affect fungal development in several genera. For example, in *Ustilago maydis* fatty acid signals trigger the initiation of filamentous growth to invade plant tissue; in *Neurospora crassa* linoleic acid induces perithecia; in *Cladosporium caryigenum* and *Aspergillus* spp., fatty acids inhibit sporulation and/or cleistothecial production [87–90]. These quorum-sensing molecules affect not only the growth of the fungi which are producing it but also that of other microbes [91–94]. More focused studies on the screening of all known fungal quorum-sensing molecules across fungal taxa will help to understand the scope of action of these quorum-sensing molecules.

Quorum sensing is a strategy used by pathogenic fungi to coordinate their activities (change in morphology and other virulence factors) for maximum damage to the host and protect themselves (Fig. 10). However, there are some dimorphic fungi in which the involvement of quorum sensing is not proven. So far, the involvement of quorum sensing in regulating both morphogenesis and pathogenesis has only been reported from the phylum *Ascomycota.* In *Zygomycota* and *Basidiomycota*, dimorphism and fungal morphogenesis are reported but not proved to be dependent on quorum sensing. For instance, in *Coccidioides* species (*C. immitis* and *C. posadasii*), *Paracoccidioides* species (*P. brasiliensis* and *P. lutzii*), and *Blastomyces dermatitidis* (teleomorph *Ajellomyces dermatitidis*), the dimorphic behavior is dependent on nutrition or physical factors [95]. Similarly, in *Mucor* (*M. circinelloides*), member of *Zygomycota*, and *Ustilago* (*U. maydis*), a member of *Basidiomycota*, dimorphism is reportedly due to anaerobic condition and pheromones, plant lipids, pH, and nitrogen [96–98]. Moreover, in *Uromyces*

Yeast	Pseudohyphae	Hyphae
• Colonization of mucosal surfaces • Dissemination to host tissues (in immunocompromised host)	• Enhanced nutrient scavenging • *De facto* motility	• Invasion of host tissues and mucosal epithelial cell layers •Breaching of endothelial cells •Thigmotropism •Antigenic variation
	Virulence gene expression	

Fig. 10 Virulence potential of various morphological structures formed by yeasts. Experiments have shown expression of various genes in different morphological structures of yeast

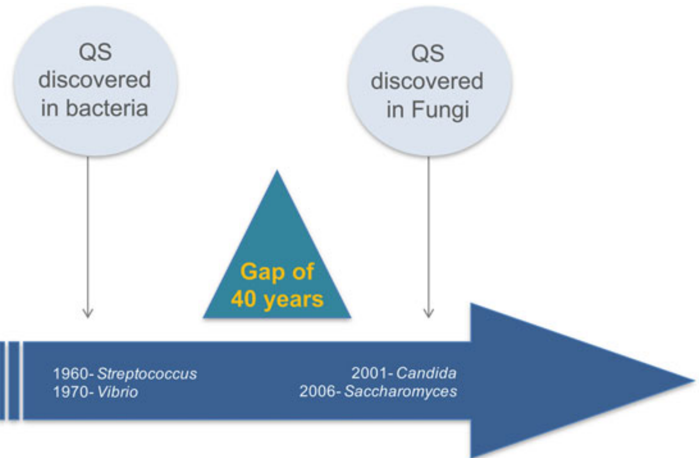

Fig. 11 Discovery of quorum sensing in fungi is relatively recent phenomenon

phaseoli and *Glomerella cingulata*, certain molecules autoregulate inhibition of spore germination and increase conidia formation, respectively [83, 99, 100]. However, they have not been studied further in the past four decades, and, hence, no further conclusion could be made about the presence of quorum sensing in these fungi.

Compared to bacteria, quorum sensing in fungi was discovered nearly 40 years later (Fig. 11). Since it is comparatively more recent, quorum-sensing system has been discovered in only few fungal genera. It thus requires a more systematic and coordinated effort. All further studies on discovering novel quorum-sensing system should follow a fixed criteria, similar to that established for bacteria. In order for any molecule to be regarded as regulator of quorum sensing, it must exhibit four properties: (1) accumulation during the fungal growth in a density-dependent manner; (2) upon achieving a threshold concentration, it should trigger a coordinated behavior in whole population; (3) the response should be restricted to a growth phase; and (4) exogenous addition of the molecule should reproduce the quorum-sensing behavior [34, 47]. If future studies use these criteria, the involvement of quorum sensing in fungi will be proved beyond doubt.

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Plant-Fungal Interactions: Special Secondary Metabolites of the Biotrophic, Necrotrophic, and Other Specific Interactions

4

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Abstract

Our environment is pervaded by a plethora of small exotic molecules, which are released without intermission by almost all organisms, like plants, microbes, or even animals. Plants and fungi are especially rich sources of these low-molecular-weight compounds, which are called secondary metabolites, and whose physiological functions are still mysterious in many cases. The number of the described compounds exceeds 100,000, and these molecules do not possess apparent importance in the producer's life but regulate, modulate, induce, hinder, or even kill organisms other than the producer. Of course, these often unexpected substantial biological effects make these molecules so interesting and valuable. In this chapter, secondary metabolites from a plant and fungal interactions are surveyed considering hormones, antifungal metabolites, as well as the metabolites of mutualistic interactions observed between plants. Special secondary metabolites from biotrophic, necrotrophic, and specific interactions are also presented here, and their physiological and ecological roles and significances are discussed.

Keywords

Host-pathogen interaction • Phytotoxin • Phytoalexin • Secondary metabolite • Mycotoxin

List of Abbreviations

ABA	Abscisic acid
AF	Aflatoxin
AF B1	Aflatoxin B1
AF B2	Aflatoxin B2
AF G1	Aflatoxin G1
AM	Arbuscular mycorrhiza
DMATS	Dimethylallyl tryptophan synthetase
DON	Deoxynivalenol
ET	Ethylene
FB1	Fumonisin B1
FB2	Fumonisin B2
JA	Jasmonic acid
IAA	Indole-3-acetic acid
ISR	Induced systemic resistance
HST	Host-selective toxin
MAPK	Mitogen-activated protein kinase
NHST	Non-host-selective toxin
NRPS	Nonribosomal protein synthase
PCD	Programmed cell death
PKS	Polyketide synthase

PR	Pathogenesis related
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SM	Secondary metabolite
TS	Tryptophan synthetase
VOC	Volatile organic compound
ZEA	Zearalenone

1 Introduction

Diverse and multilevel interactions do exist between plants and fungi, which are transmitted, in many cases, by the versatile products of the secondary metabolism of these organisms. Plant secondary metabolites (SMs) may function as defense molecules against microbes, viruses, or other competing plants or as signal molecules like hormones and even attractant compounds for pollinators or seed dispersal animals. Based on their biosynthetic origins, plant SMs can be divided into three broad groups, (i) flavonoids and allied phenolic and polyphenolic compounds, (ii) terpenoids, and (iii) nitrogen-containing alkaloids and sulfur-containing compounds, while other researchers classified plant SMs into more specific groups [1] (Table 1).

Nonpathogenic, plant growth-promoting microorganisms like rhizobacteria and mycorrhiza-forming fungi are with beneficial effects on plant performance in the rhizosphere. Microorganisms can stimulate plant growth in various ways including increasing tolerance to abiotic stress or by suppressing plant diseases [2]. In a close mutualistic association with plants, phyllosphere and rhizosphere microorganisms can even colonize plant tissues (endophytes). Symptomless endophytic fungi (e.g., black *Aspergillus* spp., *Penicillium* spp.) associated with plants have the capacity to develop as either pathogens or saprophytes. In any states, endophytic fungi can become producers of SMs like mycotoxins [3], and hence, they are rich sources of effector molecules. Phytopathogenic fungi classified as necrotrophic, hemibiotrophic, and biotrophic constitute one of the primary infectious agents in plants, causing alterations during developmental stages including the postharvest, gaining nutrients from the plants they invade, and resulting in enormous economic losses. In necrotrophic, hemibiotrophic, and obligate biotrophic fungi, the initial phases of pathogenesis do not differ fundamentally, but different strategies are used to acquire nutrients. Necrotrophic fungi have broader host ranges than biotrophs and often use cell-wall-degrading enzymes and small peptides or SM toxins [4]. In contrast to necrotrophic and hemibiotrophic fungal pathogens, obligate biotrophs are entirely dependent on living plant tissue and characterized by many sophisticated infection structures including appressoria, penetration hyphae, and infection hyphae. These are allowing the invader to suppress plant defense responses and to gain access to host nutrients (reviewed by [5,6]).

Table 1 Classification of plant secondary metabolites

Groups	Chemical structures	Examples
Phenolics with one aromatic ring	C6	Phenol, hydroquinone, pyrogallol acid
	C6-C1	Gallic acid, salicylic acid, methyl syringate, vanillic acid
	C6-C2	Acetophenones, apocynin
	C6-C3	Hydroxycinnamic acid, ferulic acid, sinapic acid, coumaric acid, eugenol, zosteric acid
Phenolics with two aromatic rings	C6-C1-C6 Xanthones	Mangosteen
	C6-C2-C6 Stilbenes	Resveratrol, chlorophorin
	C6-C3-C6 Flavonoids	Quercetin, glyceollin, sakuranetin
Quinones	Naphthoquinones Anthraquinones Benzoquinones	Alizarin, emodin
Flavonoid polymers and nonflavonoid polymers		Tannins
Terpenoids	C5 Hemiterpene	Isoprene, prenol, isovaleric acid
	C10 Monoterpene	Limonene, cineol, pinene, thymol, camphor, turpentine, carvacrol, citral, γ-terpinene, myrcene
	C15 Sesquiterpene	Abscisic acid, humulanes, culmorin, gossypol, zealexin
	C20 Diterpene	Gibberellin, taxol, oryzalexins, phytocassanes, momilactone, kauralexin
	C30 Triterpene	Brassinosteroids, squalene, lanosterol, avenacin
	C40 Tetraterpene	Carotenoids, lycopene
	$C > 40$ Polyterpenes	Rubber, glisoprenin
	Mixed origin (meroterpenes)	Cytokines, vitamin E
Nitrogen-containing	Alkaloids	Tomatin, solanin, nicotine
	Glucosinolates	Sinigrin, glucobrassicin
	Non protein amino acids	L-Canavanine
	Amines	Phenylethylamine, tyramine, morphine
	Cyanogenic glycosides	Amygdalin, sambunigrin, linamarin

2 Plant Secondary Metabolites

2.1 Hormone Production and Plant Resistance

In the regulation of plant development and resistance against necrotrophic or biotrophic pathogens and pests, hormone biosynthetic pathways are typically involved. Endogenous signaling molecules, e.g., salicylic acid (SA) [7], ethylene (ET) [8], jasmonic acid (JA) [2,9], abscisic acid (ABA) [10], or auxin indole-acetic acid (IAA) [11] (Fig. 1), have been associated with plant defense signaling against biotic stress.

salicylic acid (SA)

indole-3-acetic acid (IAA)

jasmonic acid (JA)

abscisic acid (ABA)

gibberellic acid

Fig. 1 Chemical structures of the main plant hormones (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

pipecolic acid

dehydroabietinal

azelaic acid

methyl salicylate

Fig. 2 Small metabolites as effectors in SAR signaling in addition to SA (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

Generally, SA synthesis is a crucial way in which a plant responds to a biological attack and is involved in both induced systemic resistance (ISR) and systemic acquired resistance (SAR) [7], whereas JA induces defense against insect herbivores and necrotrophic pathogens [12]. SAR induced after a local infection and conferred immunity throughout the plant to a broad spectrum of pathogens [13]. Increased SA production in the fungal pathogen-infected plant, e.g., cocoa tree invaded by *Moniliophthora perniciosa* [14], results in downregulated JA signaling pathway and vice versa [15]. During the onset of SAR [16], besides the increased levels of SA, additional small metabolites like methyl salicylate, abietane diterpenoid dehydroabietinal, pipecolic acid from lysine catabolism, dicarboxylic acid, azelaic acid (Fig. 2), and a glycerol-3-phosphate-dependent factor also appear as effectors. In response to subsequent exposure to the pathogen, some of these metabolites have been implicated in priming, a rapid activation of defenses in SAR [13].

The ISR pathway was not only stimulated by the necrotrophic bacterial attack but also was shown to protect plants against the necrotrophic fungal pathogens. For example, ISR was stimulated against *Alternaria brassicicola* [8], *Botrytis cinerea* [17], and *Plectosphaerella cucumerina* [18], where SAR was ineffective [2]. Further investigations revealed that JA and ET also had a role in the regulation of ISR [8,19,20]. ISR, similarly to SAR, was characterized by the coordinated activation of pathogenesis-related (PR) genes, many of which encode PR proteins [21].

ISR-related effect of methyl JA and SA also activated the reinforcement and lignification of the cell wall. Moreover, the increased production of some defense enzymes [22], which play a role in saving the plant cell wall and also raising the antioxidant capacity of plant cells [23], was detected. In ISR, the production of reactive oxygen species (ROS) and reactive nitrogen species increased the capability for the defense of plants. Soluble chitin fragments that originated from the cell wall of either pathogenic or symbiotic fungi and released by the action of plant PR or constitutively produced chitinases were identified as biotic elicitors of defense-related responses like phytoalexin synthesis in plants [24,25].

The main auxin in higher plants is IAA, which has main effects on plant growth and development [26]. It is considered that only the free form of IAA and related compounds are active; however, the majority of produced IAA is inactive because they are conjugated mainly to amino acids and sugars. IAA induces, e.g., the production of expansins, the proteins whose function is to loosen the cell wall. However, the loose cell wall is more vulnerable to the invasion of different types of pathogens. Hemibiotrophic or necrotrophic fungi produced IAA, similarly, to bacterial pathogens and also manipulated plant growth and subverted plant defense responses such as programmed cell death (PCD) to provide nutrients for fungal growth and colonization [27]. *Magnaporthe oryzae* secreted IAA actively in its biotrophic phase mainly in the area of the infection hyphae [28] and, in turn, provoked rice to synthesize its own IAA at the infection sites [29]. However, it is unclear whether IAA production is the manipulation of the host plant or also for the fungus's benefit. It is known that the host plant responds transcriptionally to the secreted auxin as the activation of an auxin-inducible promoter by fungal IAA indicated. The in vitro and in vivo effects of IAA were different because the IAA treatment of *Fusarium culmorum*-infected barley resulted in a reduction of symptoms and yield losses while IAA did not inhibit the growth of the fungus in vitro. The results also indicated that IAA increased gene regulation of defense-associated bacterium-primed genes [30].

Other important phytohormones are gibberellins (Fig. 1) that promote plant growth. Together with other several important terpenes, fungi can also synthesize gibberellins [31,32]. Fungal gibberellins involved in plant infection, e.g., as growth modulators similar to IAA, cytokinins, and ABA as it was demonstrated in *Aspergillus fumigatus* under salinity stress condition [32]. Gibberellic acids produced in the rice-infecting *Fusarium fujikuroi*, which is the causal agent of bakanae disease of rice (*Oryza* spp.), were good examples of phytohormone mimics [33]. Surveys of other members of the *F. fujikuroi* species complex identified the complete gibberellin gene cluster in nearly every species, but gibberellins were detected only in *F. fujikuroi*, *F. sacchari*, and *F. konzum*. Interestingly, other *Fusarium* species could have lost the ability to synthesize gibberellic, which is an advantage over other pathogens [34,35]. Interestingly, amino acid sequence homology analysis of the proteins in the gibberellic acid biosynthetic pathways [36] revealed that the higher plants and fungi have evolved their complex biosynthetic pathways convergently.

2.2 Antifungal Compounds from Plants

The antifungal-plant metabolites can be produced constitutively in healthy plants, and then they are called phytoanticipins, or they may be synthesized de novo in response to pathogen attack or various nonbiological stress factors (e.g., short-wavelength UV light, exposures to heavy metal ions), and in that case they are called phytoalexins. The classification is not so strict as the same compound may be a preformed antifungal substance in one species and can be phytoalexin in another. For example, the flavanone sakuranetin was a phytoanticipin in *Ribes nigrum* [37] and *Hebe cupressoides* [38] but was induced in the leaves of rice *Oryza sativa* [39]. These SMs can be constitutively present in one organ and can be induced in another. Since some plant SMs are even toxic to the producer, therefore, the accumulation of these compounds is regulated in a highly sophisticated manner in appropriate compartments. These compounds usually accumulate in smaller quantities than the primary metabolites (e.g., [40]); however, in particular tissues, they can build up to a higher concentration (e.g., [41]). Both primary and secondary transporters and many transporter genes are involved in the compartmentation and translocation processes. Especially genes belonging to transporter family of the multidrug and toxin extrusion types have been identified as responsible for the membrane transport of SMs [42,43].

Effective detoxification of plant SMs was detected in fungi in some studies in vitro (e.g., [44]). Some ABC transporters from phytopathogenic fungi, e.g., BcatrB from *B. cinerea*, were regarded as virulence factors, which increase the tolerance of the pathogen by controlling of the active transport of phytoalexins such as camalexin [45]. The three ABC transporters of *M. oryzae*, which were required for virulence, were also considered as factors to increase the susceptibility of rice plants to the fungus [46,47]. The rapid phytoalexin production is possibly one of the many tools, which confer resistance in concert with other defense mechanisms in resistant plants. Furthermore, all the rice phytoalexins may have a combined effect on fungitoxicity, which may even be synergistic.

The first identified phytoalexins (reviewed by [48]) were the momilactones A and B [49] in rice (Fig. 3), and momilactones exhibit antifungal activity against *M. grisea* [50]. These compounds were originally isolated and identified as plant growth inhibitors from rice seed [51]. Another group of diterpenoid phytoalexins is called oryzalexins (A–F) (Fig. 3), which were also isolated from rice [52–54]. Oryzalexins B, C, and D are *ent*-pimarane diterpenoids of *M. grisea* and are found in fungal-infected but also in healthy rice leaves [52]. Oryzalexin S [55] and phytocassanes A to E [56,57] are also labdane-related diterpenoid phytoalexins in rice. Overexpression of another rice flavonon phytoalexin sakuranetin resulted in an increased resistance to *M. grisea* [39,58] (Fig. 3).

In the last decade, against the phytopathogenic fungus *Rhizoctonia solani*, new chemical structures have been reported to have significant antifungal activity, e.g., isalexin, brassicanate A, and rutalexin from *Brassica napus* ssp. *rapifera* [59] as well as arvelexin isolated from *Thlaspi arvense* (stinkweed) [60]. Cauliflower (*Brassica oleracea var. botrytis*) produced other phytoalexins, caulilexins A, B, and C (Fig. 4),

Fig. 3 Phytoalexins isolated from rice (*Oryza sativa*) (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

which were also active against the economically important plant-pathogen fungi like *Leptosphaeria maculans* and *Sclerotinia sclerotiorum* [61]. Indole-3-acetaldoxime is an intermediate in the biosynthesis of diverse plant SMs such as indole-3-acetonitrile and brassilexin, brassinin, as well as the indole glucosinolate (glucobrassicin) and the plant hormone IAA in Cruciferae. In the detoxification processes of phytoalexins by phytopathogenic fungi, the metabolism of indole-3-acetaldoxime to IAA via indole-3-acetonitrile by fungi supported the development of plant diseases in crucifers [60,62].

In grapevine and berries, leaves produced phytoalexins such as resveratrol (*trans*-3,5,4'-trihydroxystilbene) [63] (Fig. 4) and related compounds after a fungal attack. These compounds have antifungal activity toward *B. cinerea* and also some other fungal pathogens including *Rhizopus stolonifer* and *Plasmopara viticola* [64]. The antifungal activity of carvacrol and thymol (Fig. 4) was also confirmed against *R. solani*, *B. cinerea*, *Fusarium moniliforme*, and *S. sclerotiorum* [65–67].

Terpenes are linear or cyclic and even saturated or unsaturated chains of isoprene units. These volatile organic compound (VOC) antimicrobials contribute to ISR, e. g., under invasion by *Cochliobolus sativus* and *F. culmorum* [68]. The resistance against *F. graminearum* (teleomorph: *Gibberella zeae*) in wheat was elicited by green plant volatile Z-3-hexenyl acetate (Z-3-HAC) priming; however, the mycotoxin deoxynivalenol (DON) production of the fungus also increased concomitantly [69]. The best-known terpenes are the odoriferous plant metabolites like turpentine and camphor. The industrially and medically important plant terpenes, e.g., the anticancer drug paclitaxel of *Taxus brevifolia* (taxol), are reviewed by [70].

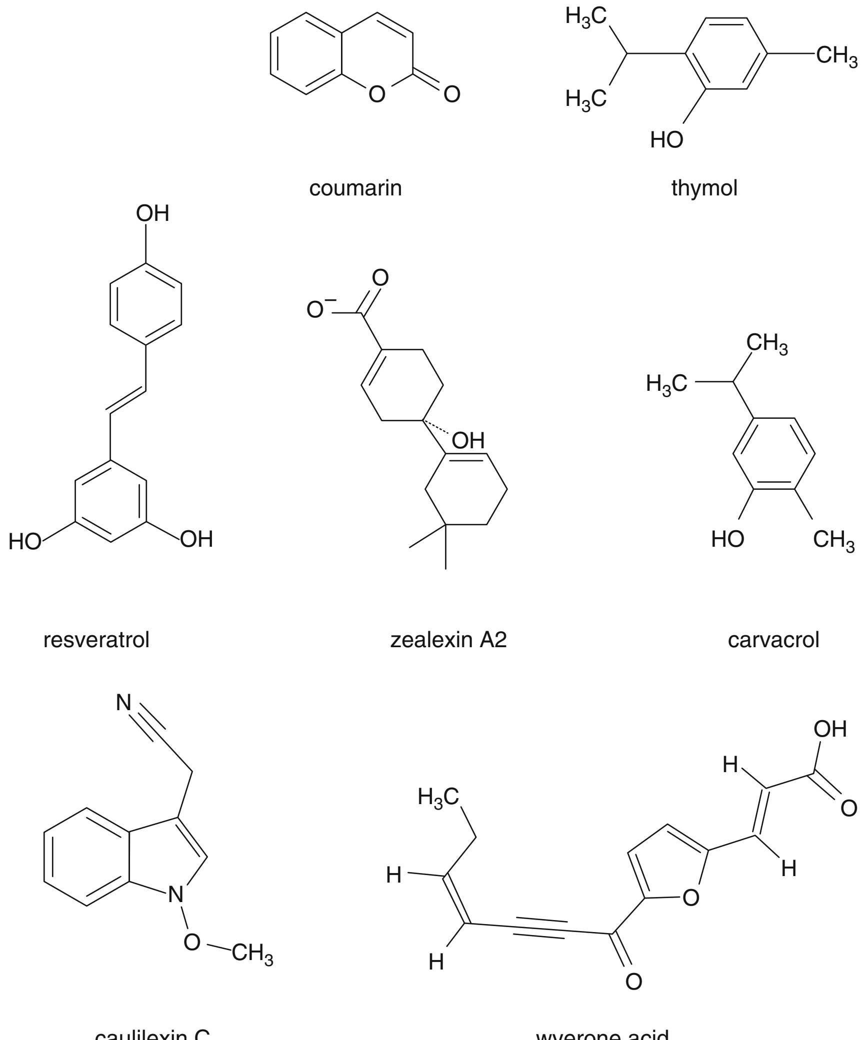

Fig. 4 Antimicrobial secondary metabolites of plants (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

The monoterpene γ-terpinene [71] and monoterpene citral showed high inhibitory activity against *B. cinerea* [66] and *Penicillium italicum* [72]. Essential plant oil components (e.g., camphor, D-limonene, cineole, β-myrcene, α-pinene, and β-pinene), which are well-known antimicrobials, showed significantly high antifungal activity against *B. cinerea* [73]. *Vicia faba* tissues also produced low-molecular-mass phytoalexins such as wyerone acid (Fig. 4) and wyerone furanoacetylenic acid as part of the postinfection defense response against the fungal pathogen. Wyerone acid accumulated in *B. cinerea* caused lesions, whereas in lesions caused by *Botrytis*

fabae, it started to accumulate but later tended to decrease. The enhanced ability of *B. fabae* to colonize broad bean tissues was therefore related to its capacity to detoxify broad bean phytoalexins [74].

Saponins are glycosylated triterpenoid (triterpenoids with sugar groups) antimicrobial compounds that are present in the cell membranes of many plant species and restrict the growth of pathogens in the apoplast. These compounds have detergent properties and disrupt the cell membranes, and, therefore, they have potent antifungal activities. The tomato saponin α-tomatine activated phosphotyrosine kinase and monomeric G-protein signaling pathways leading to Ca^{2+} concentration elevation and also ROS burst by binding to cell membranes followed by leakage of cell components in *Fusarium oxysporum* cells [75]. Different plant species produce a range of saponins, which are effective against a broad spectrum of pathogenic fungi [76]. For example, oats that contain avenacin were prevented from the infections by the wheat pathogen *Gaeumannomyces graminis*. However, some fungal pathogens, e.g., *B. cinerea, F. oxysporum*, and *Septoria lycopersici*, degrade saponins and as a consequence can cause disease in susceptible saponin-producing plants.

The flavonoids and allied phenolics, e.g., lignans, coumarins (Fig. 4), and polyphenolic compounds, including tannins and derived polyphenols, compose one major group of phytochemicals (reviewed by [77]). Flavonoids, allied phenolics, or their precursors are present in high concentrations in leaves and the skin of fruits. These molecules are involved in critical defense processes such as UV resistance, pigmentation, disease resistance, and stimulation of nitrogen-fixing nodules [78]. For the fungal phytopathogens, both positive and adverse effects have been described [79]. Phenolic compounds (reviewed by [80]) are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants. These compounds alter microbial cell permeability through interacting with membrane proteins, which cause deformation in the structure and functionality of these proteins. These adverse changes may lead to dysfunction and subsequent disruption of the membranes, including the following events: (i) dissipation of the pH gradient and electrical potential components of the proton motive force, (ii) interference with the energy (ATP) generating and conservation system of the cell, (iii) inhibition of membrane-bound enzymes, and (iv) prevention of substrate utilization for energy production [81,82]. Consequently, spore germination and hyphal growth were inhibited in a range of root pathogens [83]. Meanwhile, some flavonoids, e.g., from the exudates of pea and bean had a stimulatory activity on the associated pathogen, *Fusarium solani formae specialis = f.sp*. In this flavonoid-stimulated spore germination, cAMP-dependent protein kinase (PKA) signaling was involved [84], and the excreted flavonoids even had the potential to initiate interactions with pathogens which had developed an ability to cope with their inhibitory actions. For example, the isoflavonoid pisatin of pea induced *pda1* (pisatin demethylase protein) expression of *F. solani* f. sp. *pisi*, which, consequently, detoxified pisatin, and, therefore, this enzyme is a virulence factor of this fungus [85]. Similarly, the germination of the *F. oxysporum* spores was stimulated by tomato root exudates, and the fungus showed chemotropic growth toward the roots [86]. It is also well documented that in chemotropic sensing in *F. oxysporum*, tomato roots secreted class

III peroxidases (POX) functioned via the interaction with a pheromone receptor homologue and provoked mitogen-activated protein kinase (MAPK) signaling [87].

In maize, sesquiterpenoid phytoalexins called zealexins (Fig. 4) were discovered through characterization of physiological responses to the mycotoxin producer fungus *F. graminearum*. Importantly, zealexins exhibited antifungal activity against different phytopathogenic fungi (*A. flavus*, *F. graminearum*, *Rhizopus microsporus*) at physiologically relevant concentrations [88].

The high number of SMs is well characterized in cereals (reviewed by [89]) and in the families Fabaceae, Labiaceae, and Solanaceae [1]. Phytoalexins identified in Fabaceae and Rosaceae families and rice were evaluated by Grayer and Kokubun [90]. SMs in a range of crop plants from families Fabaceae, Cruciferae, Solanaceae [62], Brassicaceae, Vitaceae, and Poaceae (reviewed by [91]) have also been described recently.

In the infection site of *A. alternata*, high concentrations of alkaloid phytoalexin camalexin [92] have been detected [93] and also in the proximity of the lesions caused *Botrytis* species [94]. Camalexin biosynthesis was elicited by both biotrophic and necrotrophic plant pathogens in *Arabidopsis thaliana* leaves [95]. It is remarkable that *A. brassicicola* was able to detoxify camalexin but at a much slower rate than the phytoalexin brassinin from Brassicaceae [96].

Inhibition of toxinogenesis in *Fusaria* has also been studied. The efficacy of cinnamon, clove, lemongrass, oregano, and palmarosa essential oils (VOCs) was tested to prevent the accumulation of the myco-oestrogenic zearalenone (ZEA) and DON (Fig. 5) in *F. graminearum* infections [97]; however, it is notable that the assay was based on naturally contaminated maize grain. Dambolena et al. [98] studied the capacity of ten natural phenolic compounds to inhibit fumonisin B1 (FB1) (Fig. 5) synthesis by *F. verticillioides* and found that carvacrol, thymol (Fig. 4), isoeugenol, and eugenol were the most active. Plant phenols with antifungal activity are specifically induced upon attack by the soilborne pathogen *F. graminearum* in barley [99]. The phenol chlorophorin was the most efficient in reducing FB1 toxin production (94 % reduction), followed by caffeic acid (hydroxycinnamic acid), vanillic acid, ferulic acid, and iroko [100]. Aqueous extracts of host plants inhibited the fungal growth of *F. proliferatum* in a dose-dependent manner; however, the same extracts caused growth induction at low extract doses. Pea extract inhibited the FB production in most of the tested *Fusarium* strains [101].

The growth of another important mycotoxigenic fungus, *Aspergillus parasiticus*, and its aflatoxin (AF) B1 (Fig. 6) production were inhibited by methanolic extracts of *Ephedra major* roots, whereas the essential oil gained from the aerial parts of the plant did not cause any effect on AF B1 biosynthesis. The inhibition of the growth and AF B1 production of *A. parasiticus* were attributed to the presence of flavonoid compounds such as quercetin and *p*-coumaric acid in the extracts [102]. Methanolic extracts of banana pulp and peel, orange, eggplant, and potato pulp inhibited the AF B1 production in *A. flavus*; however, in case of the concomitant presence of banana and potato pulp extracts, the fungus produced AF B2, which was not detected in the control [103]. The crude essential oil of *Betula alba* inhibited both AF production and fungal growth simultaneously in *A. flavus* and *A. parasiticus*.

Fig. 5 Main mycotoxic secondary metabolites of *Fusarium* spp. (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

Jermnak et al. [104] obtained an active fraction after roughly purifying the oil by silica gel column chromatography, which was identified as methyl syringate. This compound strongly inhibited norsolorinic acid production, an early step in the AF biosynthetic pathway, and, consequently, it inhibited the AF B1 and also AF G1 (Fig. 6) production in a dose-dependent manner in submerged cultures of *A. parasiticus*, and it also inhibited AF B1 production by *A. flavus* on raw peanuts.

2.3 Metabolites in Mutualistic Interactions

Plants actively shape microbial communities either inhabiting their outer surface or colonizing the interior [105]. Growing plants secrete a broad range of chemicals, e.g., in root exudates, and they communicate with the rhizosphere microbes such as arbuscular mycorrhiza (AM) via the exudates [106–108]. In the root-microbe

aflatoxin B1 aflatoxin G1

Fig. 6 Aspergillus aflatoxins (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

communications, organic acids, amino acids, and phenolic compounds present in root exudates play an active role [29,109–111]. Moreover, stimuli such as microbial elicitors trigger compositional changes in the composition of root exudates [108]. For example, root exudates of *Arabidopsis thaliana* elicited by SA, JA, and chitosan as well as by two fungal cell-wall elicitors were studied. Among the several identified compounds, butanoic acid, *trans*-cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, ferulic acid, *p*-hydroxybenzamide, 3-indolepropanoic acid, methyl *p*-hydroxybenzoate, gallic acid, and vanillic acid were detected. These SMs inhibited the growth of *F. oxysporum*, *Phytophthora drechsleri*, and *Rhizoctonia solani* phytopathogenic fungi successfully [25].

The plant-derived sesquiterpene lactone hormone strigolactones, which are in the exudates of plants from diverse taxa, stimulate AM fungi by activating mitochondria and are regarded as natural essential signaling compounds for the establishment of symbiosis with AM [79,112,113]. Strigolactones acted as hyphal branching factors for AM fungi and stimulated root colonization [114] in the presymbiotic stage and not in the intracellular stage of fungal development [115]. Analyses of the effects of a strigolactone analog compound on fungi other than AM-forming species, including endomycorrhizal and biocontrol species in the genus *Trichoderma*, as well as the plant pathogens *B. cinerea* and *Cladosporium* sp., revealed consistent hyphal branching patterns and suggested strigolactones as distinct signals in the onset of AM symbioses [79].

AM-forming fungi change the mycorrhizosphere by altering exudation patterns of plants, changing the root size and architecture as well as altering physiology, and contributing to quantitative and qualitative microbial community changes by putative direct effects [116]. AM fungi are reducing damages caused by soilborne plant pathogens [117], meanwhile increasing the yields of the cultivar and decreasing needs for N and P fertilizers [118]. The activity of mycorrhizal fungi can even be increased by combining them with other beneficial microbes such as growth-promoting rhizobacteria or fungal biocontrol agents such as *Trichoderma* sp. [119]. Flavonoids have also been reported to stimulate the hyphal growth of

AM fungi in the presymbiotic stage [79]. However, their role as natural signaling compounds is questionable because different flavonoids can be found in various plant taxa.

3 Phytopathogenic Fungi

Plant-fungal pathogen interactions are complex processes that trigger molecular responses. Resistant plants can elicit responses in incompatible interactions. Meanwhile, susceptible plants can also launch a series of defense responses in compatible interactions. These interactions were characterized with similar gene expression profiles, and the only differences were found in the timing of transcriptome changes in the compatible interactions, where the defense gene induction occurred later than that in incompatible interactions [120].

3.1 Biotrophic Fungi

Biotrophic fungi and their metabolisms have been studied mainly on nonobligate biotrophs, for example, *Cladosporium fulvum* [121], *Mycosphaerella graminicola* [122,123], and *Magnaporthe grisea* [124]. Biotrophs establish haustoria for nutrient uptake [125], suppress the induction of host defense, and reprogram the host's metabolism [126]. Much less information is available about the obligate biotrophs, e.g., powdery mildews or rust fungi [127].

The biotrophic *Blumeria graminis* f. sp. *hordei* synthesizes only one iron-siderophore and one polyketide pigment of the cleistothecia [128]. Similar trends were observed in other biotrophs, e.g., in the basidiomycete corn smut fungus *Ustilago maydis* and the plant symbiotic fungus *Tuber melanosporum*. The loss of SM biosynthetic pathways is usually considered to be associated with biotrophy [128]. However, in *C. fulvum* (*Passalora fulva*), which infects tomato by growing extracellularly in close contact with host mesophyll cells, a twice higher number of key SM genes were identified in comparison to the closely related hemibiotroph *Dothistroma septosporum* (teleomorph *Mycosphaerella pini*). Moreover, these genes were organized into gene clusters along with other SM-related genes (Fig. 7) [129,130]. The number of the SM enzyme-encoding genes was comparable to those of *M. graminicola* but was lower than those in most other sequenced *Dothideomycete* [130]. In *C. fulvum*, the only known SM was cladofulvin [129,130]. Cladofulvin is an anthraquinone pigment, which did not cause necrosis on Solanaceae plants or showed any antimicrobial activity [130]. *C. fulvum* also has the potential to produce elsinochrome and cercosporin toxins; however, the corresponding core genes were not expressed in tomato infection, and they do not have any role in the pathogenic processes [130].

The *M. grisea* species complex includes many species [131], which cause diseases to at least 50 grass and sedge species, including important plants such as maize, rice, rye, wheat, barley, oats, finger millet, perennial ryegrass, weed, and

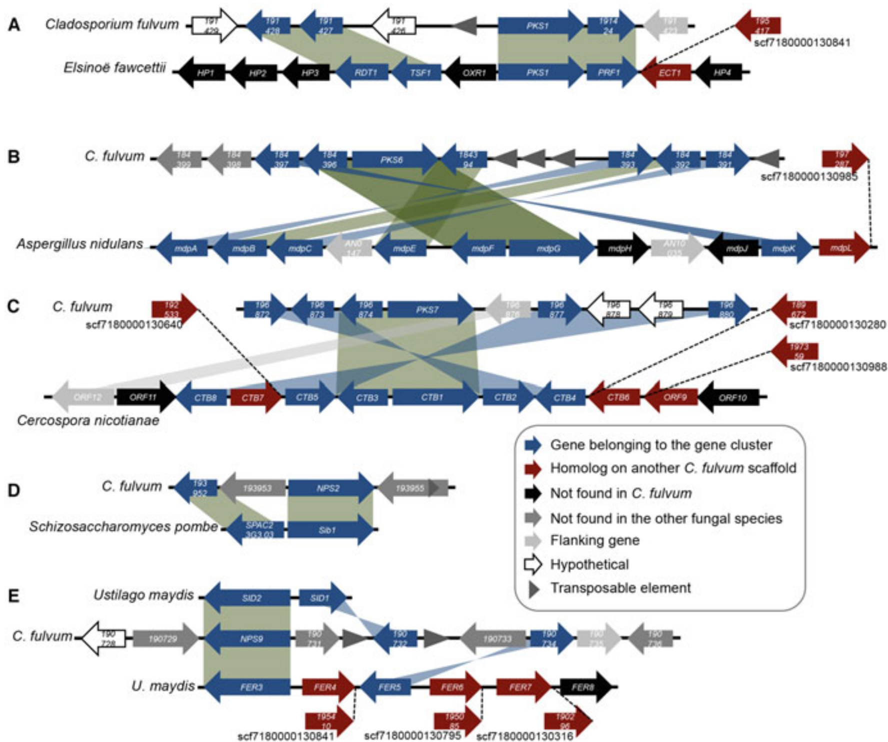

Fig. 7 Synteny and rearrangements of conserved secondary metabolism gene clusters in *Cladosporium fulvum*. The organization of gene clusters conserved in *C. fulvum* was compared to the previously described clusters involved in the biosynthesis of (**a**) elsinochrome, (**b**) monodictyphenone, (**c**) cercosporin, (**d**) ferricrocin, and (**e**) ferrichrome in other fungi. Genes are represented as *arrows*, indicating their orientation. Representation of genes is not to scale [130]

ornamental grasses. Within the species complex, *M. oryzae* (previously known as *M. grisea*) isolates form the pathotype *Oryza*, which causes rice blast disease. Approximately 10–30 % of the annual rice harvest is usually lost due to the *M. oryzae* infections. The fungus infects all aerial parts in rice, leading to leaf blast, collar rot, neck and panicle rot, and node blast (reviewed by [132]).

When the transcripts of rice (*Oryza sativa*) – *M. oryzae* interaction – were studied (Fig. 8), major rice phytoalexins were found to be produced intensively in response to the pathogen attack. The construction of the differential responsive expression patterns of compatible and incompatible fungal strains revealed more drastic reactions in the incompatible interaction, which were common at the initial infection stage, where many genes that are involved in the diterpene phytoalexin biosynthesis were upregulated at the initial infection phase. The expression levels of the genes *OsCPS2* and *OsKSL7*, taking part in phytocassane A–E biosynthesis, and *OsCPS4* and *OsKSL4*, in the biosynthetic pathway of momilactone A and B, showed incompatible-specific upregulation. Moreover, *OsKSL10* and *OsKSL8*, in the

biosynthetic pathway of oryzalexin A–F and S, showed induction in both the compatible and incompatible interactions. However, in the incompatible interaction, the fold changes of *OsKSL10* and *OsKSL8* gene expressions surpassed those calculated in the compatible interaction [133]. Furthermore, more abundant and more rapid phytoalexin accumulations have been reported in the resistant plants compared with the susceptible plants at 2 days after inoculation [44]. Nevertheless, intensive phytoalexin gene induction was observed in the incompatible interaction when compared it with the gene expressions in the compatible interaction in the same rice cultivar at the initial infection stage [133].

Claviceps purpurea is also a biotrophic fungus, which specialized in attacking the ovaries of young grasses exclusively, and produces the pharmaceutically important ergot alkaloids. During pollination of the host, the fungus forms huge black sclerotia [134], and, typically, ergot alkaloids are produced only in the sclerotia. Ergot alkaloids are nitrogen-containing SMs originating from L-tryptophane: the three broad structural groups are clavines, lysergic acid amides, and peptides (designated as ergopeptides or ergopeptines). The ergot alkaloid synthesis cluster consists of 14 genes spanning over approximately 68.5 kb of the genome [135].

Biotrophy is considered to be associated with a convergent loss of SM enzymes and also with a reduction in some genes encoding specific transporters of toxins and extrusion of host defense compounds, which is usual in necrotrophic fungi. Moreover, the downregulation of a high number of SM biosynthetic pathways may represent another mechanism associated with a biotrophic lifestyle [130].

3.2 Special Metabolites of Biotrophic Phytopathogens

Chemical signals are essential for appressorium formation in *M. grisea*. The appressorial glue of *M. grisea* contains glycoproteins, neutral lipids, and glycolipids [136]. The nontoxic plant metabolite zosteric acid (Fig. 9) [137] binds water and enhances the hydrophilicity of the surface, and, therefore, the binding capacity of the appressorial glue weakens. Zosteric acid inhibited spore adhesion and infection capability of *M. grisea* and *Colletotrichum lindemuthianum* on artificial hydrophobic surfaces and plant leaves [138]. In *M. grisea*, the two most efficient inducers of the germination and appressorium formation were 1,16-hexadecanedial and 1,16-hexadecanediol from cutin monomers [139].

Besides cutin monomers, surface waxes also activated developmental processes of fungi [140]. ET in ripened fruits [141] and fatty alcohols from cuticular waxes [142,143] induced conidia germination. Appressorium formation was induced by leaf wax or synthetic *n*-C22 fatty acid, fatty alcohol, or alkane [144].

In the appressorium, several important biochemical and morphogenetic events took place under the generation of the highest turgor pressure recorded in *M. grisea* (up to 8 MPa) to penetrate the tough rice cuticle [145]. This exceptionally high pressure and the mechanical penetration questioned the role of the secreted fungal cell-wall-degrading enzymes in the first steps of invasion of the natural host [146]. A thick melanin layer was deposited outside the cell wall of *M. grisea* to generate the

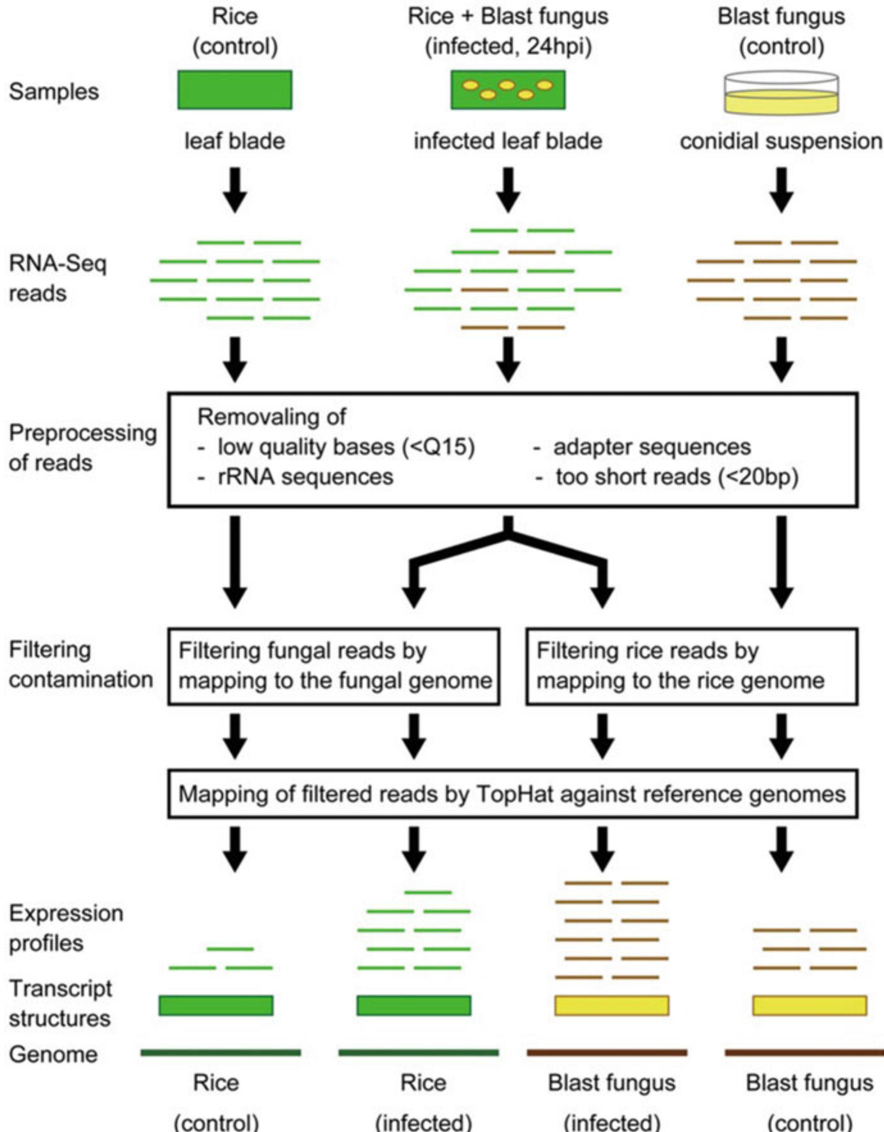

Fig. 8 Schematic representation of RNA-seq analysis of mixed transcriptome obtained from blast fungus-infected rice leaves. First, mRNA was extracted from the *Oryza sativa* ssp. *japonica* cv. Nipponbare (Pia) rice leaf blades 24 h after water treatment (rice, control) and inoculation (rice + blast fungus, infected, 24 h after inoculation) and also from conidial suspensions of the compatible and incompatible blast strains (blast fungus, control). RNA-Seq was conducted for each sample using the illumine GAIIx sequencer. In the preprocessing of reads, low-quality bases, adapter sequences, rRNA sequences, and too short reads (<20 bp) were removed. For the rice analysis, all of the preprocessed reads were mapped to the fungal genome to filter out contaminated fungal reads. For the fungal analysis, contaminated rice reads were removed by mapping all of the

Fig. 9 Schematic presentation of the nontoxic plant metabolite zosteric acid, which inhibits spore germination (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

high turgor pressure. Several natural compounds inhibited melanin biosynthesis in a secure manner, presumably hitting the same targets [147], for example, coumarin (Fig. 4), a standard plant SM [148], scytalol D of *Scytalidium* sp. [149], as well as the lipid biosynthesis inhibitor cerulenin [150]. The latter compound was first obtained from an isolate called *Cephalosporium caerulens*, conspecific to the phytopathogenic fungus of rice *Sarocladium oryzae* [151].

Colletotrichum shares similar lifestyles and infection strategies with *M. grisea*, particularly during the early stages of pathogenesis. However, unlike in the case of *M. grisea*, in the hemibiotrophic *C. gloeosporioides*, and in other *Colletotrichum* species, spore germination and appressoria development were not prevented by blocking the cell cycle [152]. The differentiation of fungal infection structures including appressoria preceded the mitosis and could proceed without nuclear division. Moreover, spore cell death did not occur during plant infection, and throughout the infection cycle, the primary infection structures of the fungus remained viable [152].

Potent autoinhibitory molecules of spore germination of many phytopathogenic fungi were isolated, and these compounds prevented the germination until they have been diluted out of the spore. *Colletotrichum* spp. is a rich source of these type of molecules. The first self-inhibitor isolated from conidia of *C. gloeosporioides* was gloeosporone (Fig. 10) [153] followed by (Z)-(E)-ethylidene-1,3-dihydroindole-2-one, which were active at much lower concentrations than gloeosporone [154]. At higher concentrations, both compounds inhibited the germination of conidia of other *Colletotrichum* sp. and *F. oxysporum* as well [154]. Mycosporine alanine is also a potent autoinhibitory molecule of conidial germination in *C. graminicola*, which was synthesized during the development of conidia in the fruiting body pycnidium, and it was quite effective in hindering germination of the spores until they have become dispersed [155]. Interestingly, the biosynthesis of mycosporines and mycosporine-like amino acids occurs in a broad range of bacteria, cyanobacteria,

Fig. 8 (continued) reads against the rice genome. Finally, all of the filtered reads were mapped to the reference genomes by TopHat software, and transcript structures are predicted by Cufflinks. For each rice and fungal transcript, expression levels were estimated using the numbers of uniquely mapped reads to the transcript structures [133]

gloeosporon

pyriculol

Fig. 10 Fungal self-inhibitor molecules of the germination (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

phytoplankton, and macroalgae and fungi, but not in animals because it needs the shikimate pathway [156]. *M. grisea* also produced self-inhibitors of the germination, which were related to the phytotoxin pyriculol (Fig. 10) [157]. Moreover, other fungi could also produce specific, nontoxic inhibitors of conidial germination and appressorium formation of *M. grisea* like flaviolin, tenuazonic acid, and glisoprenins [158]. *M. grisea* also produces the mycotoxin tenuazonic acid besides *A. tenuis* and *Phoma sorghina* (a pathogen of sorghum) [159].

3.3 Necrotrophic and Hemibiotrophic Fungal Interactions

The infection strategy of necrotrophic fungi is much simpler than that of obligate biotrophs. Typical necrotrophs, e.g., *Botrytis*, *Helminthosporium*, *Cercospora*, *Ramularia*, *Rhynchosporium*, *Alternaria*, *Fusarium*, *Sclerotinia*, or *Verticillium* species, form appressoria, which are inconspicuous, and infection hyphae formed within the host are quite uniform (reviewed by [160]). In several fungal pathogens, virulence correlates with the capability to synthesize a phytotoxin.

Fungal phytotoxins are classified as host-selective toxins (HSTs) and non-host-selective (NHSTs) toxins. Necrotrophs and hemibiotrophs are considered to employ fundamentally different mechanisms of promoting disease; however, the tools they utilize, e.g., host-selective toxins (HSTs), and protein effectors are quite similar or even identical [161].

The hemibiotrophic *Fusarium graminearum*, the causative pathogen of *Fusarium* head blight in wheat, causes a considerable economic loss. The defense against *Fusarium* head blight is sequentially regulated by SA and JA during the early and later stages of the infection, respectively [69,162]. In the first phase, a JA-mediated and ET-mediated defense mechanism is directed against fungal growth and sporulation and thus induces the transcription of a standard set of genes encoding antimicrobial peptides; PR proteins, e.g., lipid transfer proteins; defensins; and thionins in the resistant lines. Meanwhile, proteases and mycotoxins were induced in an alternative mechanism [163]. On the contrary, in maize cultivars, a total RNA-Seq-based transcriptome study elucidated that the induction of the SA-related genes in resistant and susceptible maize genotypes was insignificant at 3 days after inoculation with *F. verticillioides*. Interestingly, the activation of common JA- and ET-responsive PR genes and transcription factors, such as LOXs, PR10, and ACC oxidase (which regulate ET levels −1-aminocyclopropane-1-carboxylic acid oxidase) and chitinases, was observed with an enhanced induction in a resistant line (Fig. 11) [164].

F. verticillioides (teleomorph, *Gibberella moniliformis*) attacks stalks, kernels, and seedlings of maize. Maize maturation is a dynamic process, where distinct stages of the development can be observed, and the pathogen sensing a different environment (moisture, nutrients, fatty acids, flavonoid content of the seeds) shows a different gene regulation and transcription pattern.

Considering the maize developmental stages, silking (R1), blister (R2), milk (R3), dough (R4), dent (R5), and physiological maturity (R6) [165] can be differentiated. Infection at maturity stages R2–R5 with *F. verticillioides* revealed that the pathogen colonized the kernels equally well [166]. Nevertheless, significant sphingoid-derived FB1 production [166–168] occurred only in the R5-stage kernels where the normal acidic state also induced more FB1 toxin production [168]. Expression of the fumonisin biosynthetic genes FUM8 and FUM12, as well as moderate amounts of FB1, was detected in the R3 and R4 stages. In contrast, no FB1 or FUM gene expression was detectable in the R2 stage. Different maize genotypes were able to react differently to fungal attack when the infection rate was low, whereas the hybrid-related response ability decreased when more conducive conditions for fumonisin production occurred. Studies highlighted the importance and correlation of fatty acid composition with the contamination data, more linoleic acid (C18:2) correlated with higher fumonisin content [169].

In cotton-*Verticillium dahliae* interaction, an increased level of expression of lignin synthesis-related genes and increased phenylalanine ammonia-lyase and peroxidase enzyme activities as well as lignin accumulation were detected in the resistant plant line [170]. Similarly, microarray analysis demonstrated that in both sensitive and tolerant interactions between tomato and *V. dahliae*, increased gene

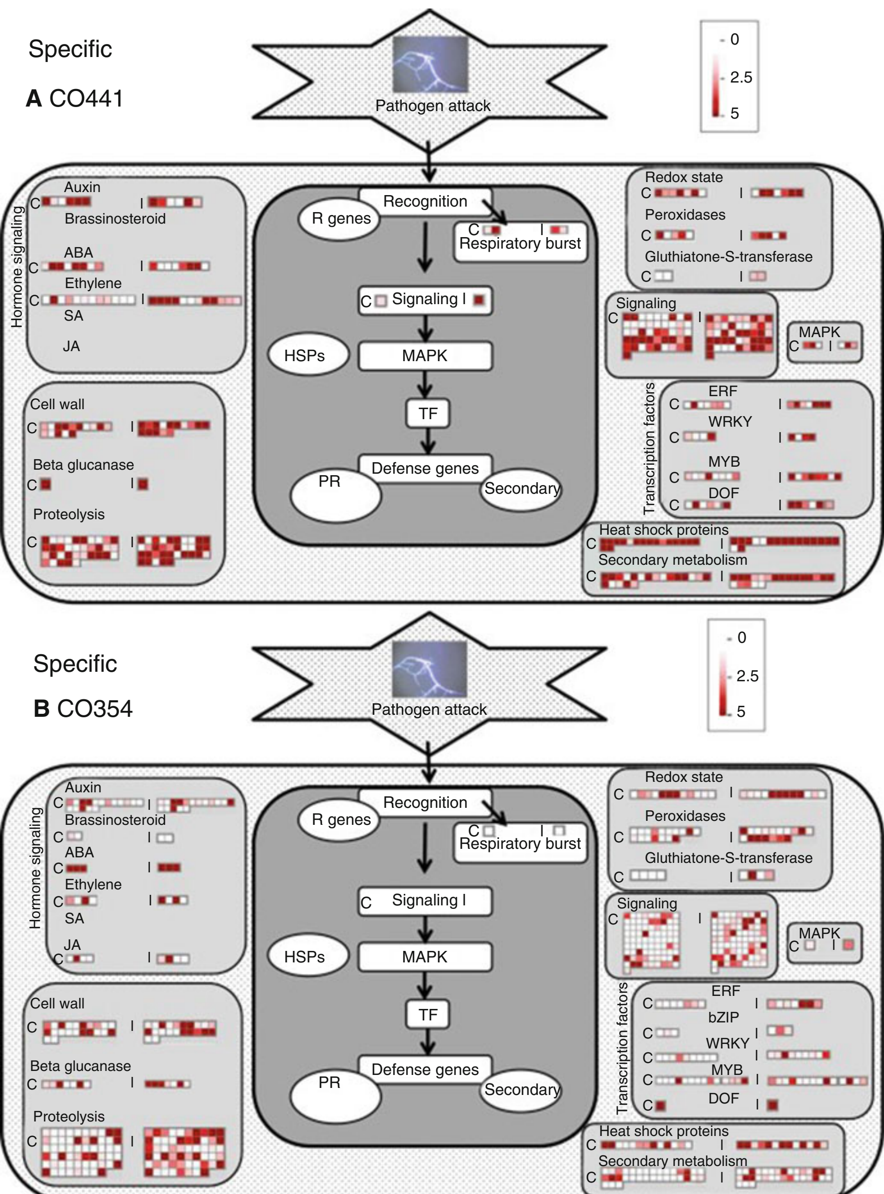

Fig. 11 Distribution of differentially expressed genes specific to resistant and susceptible genotypes related to biotic stress processes, visualized by MapMan. The abundance of each transcript was expressed as fragments per kilobase of exon model per million mapped reads (FPKM). Each *square* represented the FPKM expression value for one gene in control (heatmap *on the left* within each category) and inoculated (heatmap *on the right* within each category) resistant CO441 (**a**) and susceptible CO354 (**b**) genotypes of maize [164]

expressions of PR proteins were observable, but the genes that are associated with foliar necrosis and PCD in the susceptible interaction were suppressed in the tolerant interaction [171]. Plants infected by the pathogen not only expressed a series of essential defense-related genes but also activated phytohormone signal transduction and SM such as phenylpropanoid production [172].

In lettuce (*Lactuca sativa*)-*B. cinerea* interaction, global expression profiling by total RNA-Seq technology pronounced inductions of the host's phenylpropanoid pathway again, and terpenoid biosynthesis was detected, whereas the photosynthesis was globally downregulated at 48 h postinoculation. Both general and species-specific responses to the infection were identified; however, significant systemic transcriptional alterations could not be detected in the lettuce leaves at a distance from the inoculation site. Interestingly, the investigation of lettuce-*Bremia lactucae* (biotrophic pathogen fungus) interaction revealed the induction of the similar pathways [173].

In the chocolate tree (*Theobroma cacao*), the basidiomycete hemibiotrophic fungus *Moniliophthora perniciosa* causes witches' broom disease. *M. perniciosa* possesses a long-lasting and symptomatic biotrophic phase, which can endure for more than 60 days in the living cacao tissues. The biotrophic mycelia develop as long-term parasites that change plant metabolism to increase the availability of soluble nutrients before the death of the host plant. Several genes related to the biosynthesis of lignin, flavonols, anthocyanins, terpenoids, and alkaloids are strongly upregulated in green brooms. Meanwhile, *M. perniciosa* expressed an arsenal of genes encoding enzymes involved in detoxification and stress tolerance, e.g., ROS degrading enzymes. After this interplay between biotrophic hyphae and cacao plant cells, the infected tissues collapsed, and a senescence-like process was installed. Plant cells' death seemed to favor *M. perniciosa* and precede the fungal necrotrophic phase. The onset of cell death was considered as a physiological process of cacao caused by the metabolic disarrangement rather than a direct action of the pathogen. However, as a consequence, soluble nutrients from dead host cells became available to the pathogen, which later produced basidiomata and completed the disease cycle [174].

The genus *Colletotrichum* (*Glomerella*) comprises ~600 species attacking over 3,200 species of monocot and dicot plants. Biotrophy was confined in *C. higginsianum* to the first invaded host cell and was followed by a complete switch to necrotrophy. In contrast, in *C. graminicola*, which primarily infects maize, biotrophy extended into many neighboring host cells and persisted at the advancing colony margin while the center of the fungal colony became necrotrophic [175]. Five gene categories, which are relevant to pathogenicity (transcription factors, SM enzymes, secreted extracellular proteins, carbohydrate-active enzymes, and transporters) had significantly different expression patterns during infection. However, during early infection, 12 different SM gene clusters were induced in *C. higginsianum* before penetration and during biotrophy. The high number of activated gene clusters indicated a significant role for appressoria and biotrophic hyphae in synthesizing an array of small molecules and delivering them to the first infected plant cells. Because these cells initially remained alive, such molecules

cannot be phytotoxins and might function in host manipulation, similar to protein effectors [175]. Remarkably, the SM gene cluster with the strongest activation at this phase in *C. higginsianum* was induced in *C. graminicola* at any of the infection stages. Therefore, it can be concluded that a strict transcriptional regulation could also generate the diverse metabolite spectrum. Moreover, no evidence for specific transcriptional reprogramming of nutrient transporters was detected during biotrophy in *C. higginsianum*, suggesting that the biotrophic hyphae here function primarily to deliver protein effectors and SMs to the plant cell [175] (Table 2).

3.4 Saprophytic Aspergilli

Aspergillus species can be present as saprophytic or symptomless endophytes or weak and opportunistic phytopathogens. Black aspergilli are common soil organisms decomposing dead plant residues, but some of them are of a biotrophic endophytic existence in maize and onion. *A. niger var. niger* and *A. carbonarius* black aspergilli are the two primary producers of ochratoxin A (Fig. 12), which is carcinogenic, nephrotoxic, teratogenic, and immunosuppressive in animals, and they also synthesize FB1 mycotoxins (Fig. 5) [3].

A. flavus from the yellow aspergilli lacks any host specificity [176] as this fungus can attack successfully the seeds of both monocots and dicots (e.g., cotton, maize, groundnuts, and also other nuts like tree nuts such as Brazil nuts, pecans, pistachio nuts, and walnuts). It can cause ear rot on corn, and *A. flavus* together with *A. parasiticus* and *A. nidulans* is proposed to derive acetyl-CoA from fatty acids of the kernel for the biosynthesis of SM toxins (i.e., sterigmatocystin and AF) [4]. Preharvest contamination of these crops with SM aflatoxins (AFs) is common, but these fungi also caused spoilage postharvest resulting in significant economic losses to farmers. The fungus can attack maize kernels during all their six stages of their development in experimental circumstances. However, in the field, the infection of the non-injured kernels takes place during the R5 developmental stage just before physiological maturity (R6) [177]. Concomitantly with the increased expression of mycotoxin (sterigmatocystin and AF) biosynthetic genes in hyphae, *A. flavus* colonized the embryo and aleurone layer of the kernels as early as 4 days after inoculation [178]. The embryo and aleurone layer are the sites where most seed lipids are stored and induce SM production. In vitro supplemented oleic acid also induced the biogenesis of fungal peroxisomes, as well as catalase activity and β-oxidation. Meanwhile, AF precursor norsolorinic acid is accumulated in the peroxisomes [179].

A special task is to eliminate fungal toxinogenesis in crops by biological control tools, e.g., spreading endemic atoxinogenic isolates of a species or isolates of other fungal species that commonly contaminate the crops. Moreover, correlations of the mycotoxin production in pathogens, which infect concomitantly the same host plant, were also investigated. In a study, although a negative correlation between infections by *Aspergillus* spp. and *Fusarium* spp. was suggested in corn infections, a positive relationship between aflatoxin and fumonisin levels was observed although it was

Table 2 Distribution of secondary metabolite gene families in selected pathogenic fungi

Species	PKS[a]	PKS-like	NRPS[b]	NRPS-like	Hybrid[c]	DMAT[d]	Total	Ref.
A. alternata	10	n.d.[e]	n.d.	n.d.	n.d.	n.d.	~10	[198]
A. arborescens	29	n.d.	5	n.d.	2	n.d.	~36	[311]
C. fulvum	10	n.d.	10	n.d.	2	1	~23	[129]
C. lunatus CX-3	16	1	6	10	2	1	36	[202]
C. lunatus m118	14	1	5	9	2	2	33	[202]
C. heterostrophus C5	22	3	9	7	0	3	44	[202]
C. zea-maydis	11	2	7	8	1	1	30	[202]
P. nodorum	12	9	9	5	1	2	38	[202]
P. tritici-repentis	14	6	12	6	1	0	39	[202]
P. teres f. teres	18	1	27	n.d.	2	1	~49	[197]
B. cinerea	16	6	6	8	0	1	37	[193]
S. sclerotiorum	16	2	5	5	0	1	29	[193]
M. grisea	12	3	5	6	3	3	32	[193]
M. oryzae	23	2	8	6	5	3	47	[202]
A. flavus	25	3	18	14	2	8	70	[202]
A. niger	15	1	12	2	5	0	35	[197]
A. terreus	28	2	22	15	1	10	68	[206]
F. graminearum	12	2	10	10	0	0	34	[202]

(*continued*)

Table 2 (continued)

Species	PKS[a]	PKS-like	NRPS[b]	NRPS-like	Hybrid[c]	DMAT[d]	Total	Ref.
S. turcica	23	3	9	7	2	2	46	[202]
M. phaseolina	19	16	15	13	12	0	75	[193]
U. maydis	3	2	3	6	0	2	16	[206]
C. higginsianum	58	n.d.	12	n.d.	4	10	84	[175]
C. graminicola	39	n.d.	7	n.d.	7	7	60	[175]
R. solani AG1IA	1	n.d.	4	n.d.	n.d.	5	10	[299]

Listed organisms: *Alternaria alternata*; *Alternaria arborescens*; *Cladosporium fulvum*; *Cochliobolus lunatus* CX-3; *Cochliobolus lunatus* m118; *Cochliobolus heterostrophus* C5; *Cercospora zea-maydis*; *Phaeosphaeria nodorum*; *Pyrenophora tritici-repentis*; *Pyrenophora teres* f. *teres*; *Botrytis cinerea*; *Sclerotinia sclerotiorum*; *Magnaporthe grisea*; *Magnaporthe oryzae*; *Aspergillus flavus*; *Aspergillus niger*; *Aspergillus terreus*; *Fusarium graminearum*; *Setosphaeria turcica*; *Macrophomina phaseolina*; *Ustilago maydis*; *Colletotrichum higginsianum*; *Colletotrichum graminicola*; *Rhizoctonia solani AG1IA*

[a]Polyketide synthase

[b]Nonribosomal peptide synthase

[c]PKS-NRPS hybrid

[d]Dimethylallyl tryptophan synthetase

[e]Not determined

Fig. 12 Ochratoxin A produced by *Aspergillus* spp. (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

not statistically significant. Thus, natural infections with *Fusarium* spp. did not appear to protect against *Aspergillus* contamination and the production of aflatoxin [180].

In maize, transcriptional analysis revealed downregulated starch biosynthesis and upregulated starch hydrolytic enzymes like β-amylase of the plant together with downstream invertases and fructokinase after colonization of kernels by *A. flavus*. The hexoses produced by the hydrolytic activity flow through the upregulated shikimate pathway and the methylerythritol pathway and toward upregulated JA and oxylipin biosyntheses and feed the protein synthesis, e.g., production of PR proteins, including chitinases, peroxidases, and glutathione S-transferase (GST), which were also upregulated during the infection. Oxylipins, a diverse group of oxygenated polyunsaturated fatty acids, upregulate AF biosynthesis and sexual reproduction in *A. flavus* and downregulate fungal growth. Induction of the SM pathway leads to the production of the antifungal compounds flavonoids, phenylpropanoids, and phytoalexins and upregulated lignin production in maize. The upregulation of the plant hormones JA and ABA is crucial in these defense mechanisms [178]. A microscopy study of maize kernel tissue showed that cellular components such as cell walls were broken down in advance of *A. flavus* mycelia [181], which clearly indicated necrotrophic pathogenicity [182]. However, the colonization of kernel tissue from resistant maize lines exhibited increased levels of SA and unchanged levels of JA productions [183], and such patterns are typical of various plant species in resistance to biotrophic pathogens [12]. It was demonstrated that this facultative parasite might possess a unique pathogenicity mechanism, which did not fit clearly into the traditional biotrophic-necrotrophic classification scheme [184].

3.5 Genetic Background of Fungal SM Production

Based on their chemical structure, fungal SMs can be divided into four main classes: polyketides (e.g., AF and FBs), terpenoids, the shikimic acid-derived compounds, and nonribosomal peptides (e.g., sirodesmin, peramine, and metal-chelating

siderophores such as ferricrocin). Moreover, hybrid metabolites composed of moieties from different classes are also common, e.g., the meroterpenoids, which are formed by fusions between polyketides and terpenes [185]. Their metabolic diversity significantly contributes to the ability of fungi to colonize and penetrate plants. The metabolites required for the interaction are essential to access the cellular contents of the plants and, therefore, are considered to be essential for both growth and development of the fungus [31].

Comparative genome analyses shed light on *Ascomycetes* (Table 2) which have more genes putatively involved in secondary metabolism than *Archeo-Ascomycetes*, *Chytridiomycetes*, and *Basidiomycetes*, whereas *Hemiascomycetes* and *Zygomycetes* have none [185]. The majority of fungal SMs are products of nonribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), or terpene synthases (TSs). Ascomycete genomes code for an average 16 PKSs, 10 NRPSs, 2 TSs, and 2 dimethylallyl tryptophan synthetases (DMATS) with vital importance in SM synthesis (Table 2). These key genes of the SM syntheses code for signature enzymes usually enriched in secondary metabolism gene clusters and responsible for main synthesis steps of metabolites. Hybrid PKS-NRPS genes have been identified only in *Ascomycetes*, with an average of three genes per species. Many fungal species have more than 40 genes encoding PKS, NRPS, hybrids, TS, and DMATS biosynthetic enzymes in their genome, including *Magnaporthe grisea* (45 genes) [185]. *Neurospora crassa* and the human pathogens *Coccidioides* spp. and *Histoplasma capsulatum* have considerably less PKSs (1–9 genes), NRPSs (3–6 genes), and PKS-NRPSs (0–2 genes) than other *Ascomycetes*.

The synthesis of chelate-forming siderophores, a class of SMs specifically involved in iron uptake, is also based on NRPSs. The iron chelators are also crucial for the virulence of several fungi (e.g., *Cochliobolus heterostrophus*, *C. miyabeanus*, *F. graminearum*, and *A. brassicicola*) [186].

Comparative genome studies indicated that *Fusarium* and other filamentous fungi have the genetic potential to produce much more SMs than previously thought. Hansen et al. performed comparative analyses of PKSs and NRPSs from ten different *Fusarium* species including *F. acuminatum*, *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. verticillioides*, *F. solani*, *F. pseudograminearum*, *F. fujikuroi*, and *F. oxysporum* [187]. This study led to the identification of 52 NRPS and 52 PKSs orthology groups, respectively. A core collection of eight NRPSs (NRPS2–4, 6, 10–13) and two PKSs (PKS3 and PKS7), which were conserved in all investigated strains, was detected and analyzed. Meanwhile, whole-genomic analysis has identified 12–15 PKS genes in *F. graminearum* [188–191], where six of them were only found in this species. Most of the PKSs have no assigned products yet even though they are expressed under certain experimental conditions. In *F. graminearum*, the genes with known functions (13 SM genes) cover a minor fraction of the 51 predicted SM genes, among which 15 PKSs, 19 NPSs, and 17 TSs have been identified [191]. Besides the typical SM genes (TS, NPS, and PKS), 114 predicted genes encoding cytochrome P450 enzymes were also suitable

candidates for searching SM gene clusters. Cytochrome P450s play an essential role in many known biosynthetic pathways of fungal SMs, for instance, in the biosynthesis of gibberellins [36] and trichothecene mycotoxins [192].

From the ascomycetous fungal family *Botryosphaeriaceae*, *Macrophomina phaseolina* anamorphic fungus [193] possessed an exceptionally high number, altogether 75, of putative SM genes in comparison to 37 genes found in *B. cinerea*, 32 in *M. grisea*, 37 in *F. graminearum*, and 29 genes in *S. sclerotiorum* (Table 2). Numerous NRPSs, which catalyze the production of cyclic peptides including various toxins, were also found. In *M. phaseolina*, an NRPS, which is a homologue to *Cochliobolus carbonum* HST1, is the key enzyme responsible for the biosynthesis of the maize HST HC-toxin [194,195]. In comparison, analysis of the genome of the saprophytic model organism *A. nidulans* revealed 56 putative SM core genes, namely, 27 PKSs, two PKS-like genes, 11 NRPSs, 15 NRPS-like genes, and one hybrid NRPS-PKS gene [196].

The genomes of *B. cinerea* and *S. sclerotiorum* were sequenced and analyzed first by Amselem et al. [197]. The *B. cinerea* genome showed high sequence identity and a similar arrangement of genes to those of *S. sclerotiorum*. Moreover, both genomes contained a significant number of genes encoding key SM enzymes (Table 2). Nevertheless, taking into account that SM pathways usually have more than one key enzyme, these fungi have the potential to produce approximately 37 and 29 main SMs, respectively [193]. Therefore, despite the similarities of the genomes, the two fungi differed significantly in the number and diversity of SM gene clusters, which make them able to adapt to different ecological niches.

Alternaria species have different lifestyles ranging from saprophytes to endophytes and pathogens. Phylogenetic relations of the *Alternaria* complex were delineated, and these fungi were able to synthesize more than 60 SMs from which at least 10 PKS products were identified (Table 2) [198]. In melanin biosynthesis, a PKS was also characterized and named ALM (albino) [199]. For the production of SM siderophores and significance in virulence, a polypeptide analogous to fungal NRPS was demonstrated in *A. alternata* (AaNPS6) [200]. In the biosynthesis of the mycotoxin tenuazonic acid, which is produced by *A. tenuis* as well as *M. grisea* and *Phoma sorghina*, a gene for NRPS/PKS hybrid enzyme was identified [185].

The genus *Cochliobolus* (anamorph *Bipolaris/Curvularia*) [201] includes more than 40 closely related pathogenic species highly specific to their host plants [161]. Phylogenomic studies revealed that the highly virulent *C. lunatus*, which was evolved from *C. heterostrophus*, was capable of producing several SMs such as NHSTs and melanin, which can aid the fungus in niche exploitation and pathogenicity [202].

The ability of *C. heterostrophus* to produce HST T-toxin is based on three genes encoded at two unlinked loci [203]. However, further, six genes including two PKSs, one decarboxylase, five dehydrogenases, and one unknown protein, which were involved in the T-toxin production and high virulence to maize, were also reported [204]. In the biosynthesis of the cyclic tetrapeptide HC-toxin by *C. carbonum* [195],

one NPRS encoded by *HST1* played a crucial role. HC-toxin was also released by *Alternaria jesenskae* [205] and was also encoded in maize pathogen *Setosphaeria turcica* [161]. Six other known PKSs were also involved in the biosyntheses of various toxins, e.g., *A. alternata* ACT-toxin, *A. ochraceus* OTA, *F. graminearum* ZEA, *F. verticillioides* fumonisin, and the *C. heterostrophus* T-toxin. Genome level phylogenetic and modular analyses resulted in differences in the protein structures of *C. lunatus* CX-3 NRPSs, when compared to other known NRPSs, which are involved in the biosyntheses of mycotoxins such as HC-toxin of *C. carbonum*, similarly to the AM-toxin of *A. alternata*, the gliotoxin of *A. fumigatus*, and the enniatin of *F. equiseti* (Fig. 13) [202].

For some SM genetic elements, the horizontal gene transfer events [206] supported their spreading, e.g., homologues of the fumonisin and gibberellin biosynthetic gene clusters, which once were thought to be unique to *Fusarium* spp. [33,35] were found on a small scale of distantly related to *Ascomycetes* fungi, e.g., in *Aspergillus* spp. (*Eurotiomycetes*) [206] as well as in *Sphaceloma manihoticola* [207], and in *Phaeosphaeria* spp. (*Dothideomycetes*) [208].

Fig. 13 Gliotoxin of *A. fumigatus*, enniatin of *F. equiseti*, HC-toxin of *C. carbonum*, and AM-toxin of *A. alternata* (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

3.6 Stimuli in Fungal SM Production

For both the plant host and its fungal parasite, abiotic environmental stress such as drought or heat stress affects their interactions (e.g., [184]). Fungal genes that are having a role in stress-related responses, especially in oxidative stress, are overrepresented in phytopathogenic fungi [209]. Fungal SM toxins often play a role in triggering stress responses. Moreover, some nontoxic fungal SMs, such as mycosporines, polyols, and pigments, can take part in pathogenicity and/or fungal tolerance to several environmental effects, including temperature and UV light [156]. Hence, environmental factors (e.g., light, temperature, pH, calcium, and nutrients) regulate SM production concomitantly.

The plants need light for ROS production during the oxidative burst [4] elicited against the pathogens. However, light is also a requirement for the manifestation of the effect of the DON toxin similarly to the induction of PCD during *Botrytis* infections [210]. Meanwhile, light regulates fungi through a major regulatory protein complex, the velvet complex [211], which, in *Fusarium*, comprises, at least, FgVe1 (VeA homologue in *A. nidulans*) and FgVeB, while corresponding homologous components also have been identified in other fungi [212,213]. FgVe1 regulates trichothecene production at the level of the Tri4 and Tri5 of biosynthetic genes and the transcriptional regulator genes Tri6 and Tri10 [214,215]. The disruption of the *FgVeB* gene affected the regulation of Tri5 and Tri6 and led to different phenotypic defects, including defected aerial hyphae formation, reduced hyphal hydrophobicity, highly increased conidiation, and reduced DON biosynthesis [216]. Deletion of Lae1, a nuclear regulator from the velvet complex in *F. verticillioides*, decreased the expression of gene clusters responsible for the synthesis of the SMs bikaverin, fumonisins, fusaric acid, and fusarins. Analysis of SMs in the *F. verticillioides* and *F. fujikuroi* Lae1 mutants revealed differences in the regulation of SM production [217]. For example, bikaverin production was found to be reduced, but the amount of FB1 remained unchanged in *F. verticillioides* [218].

In the activation of the virulence functions in phytopathogenic fungi, nitrogen limitation is an essential stimulus. Their genetically encoded capability to metabolize different nitrogen sources makes fungi able to colonize various environmental niches and survive nutrient limitations [219]. Amino acids are required for SM biosynthesis, in particular for the NRPSs. Amino acid limitation in fungi resulted in the induction of a genetic network, which induced genes encoding the enzymes of multiple amino acid biosynthetic pathways as well as for aminoacyl-tRNA synthases. Meanwhile, inorganic N-sources also affected SM production. For example, ammonium ions activated the expression of AF genes [220], while nitrate inhibited AF biosynthesis in *A. parasiticus* [102]. In all fungal species studied, the major GATA transcription factor AreA and its co-repressor NmrA were central key players in the nitrogen regulatory network [219]. The importance of global nitrogen regulators in the development of pathogenicity was demonstrated in *M. grisea* [221] and also in many other fungal-plant pathogens, e.g., *C. lindemuthianum*, *C. acutatum*, and *F. oxysporum* [222]. In *F. graminearum*, which causes crop disease, nitrogen starvation activated the trichothecene pathway and induced the

biosynthesis of the DON toxin. DON was identified as a virulence factor [223,224], similar to the host-selective T-toxin from *C. heterostrophus* [225] and the cyclic peptide AM-toxin from *A. alternata* [226]. Low-nitrogen conditions also induce high levels of expression of fumonisin biosynthetic genes through AreA in both *F. fujikuroi* and *F. verticillioides* [227].

As it was mentioned above, SM production in fungi is also regulated by signals or even substrates from the plant. Plant lipoxygenase (LOX)-derived oxylipins regulate defense against pathogens. In plants, oxylipins such as the plant hormone JA and its immediate precursor 12-oxo-phytodienoic acid are formed enzymatically and accumulate in response to various stresses like wounding and pathogen infection [9]. These compounds are also formed nonenzymatically via the action of ROS [228], which accumulate in response to pathogen infection, heavy metal uptake, or other stresses.

Fungal oxylipins can mimic plant oxylipins, and, therefore, a reciprocal cross talk was proposed and has been shown between the plant and the pathogenic fungus [229]. The tomato-infecting *F. oxysporum* produced JAs using a lipoxygenase enzyme via a pathway similar to that present in plants suggesting that JA biosynthesis in pathogenic fungi also occurred [230]. The resistance of maize against *F. verticillioides* also depended on the overexpression of LOX pathway genes and the central regulatory role of JA [231].

In *A. flavus*, oxylipins are the molecules of quorum sensing, which means biomass density sensing of the fungus. At low extracellular oxylipin concentrations, increased sclerotia production, reduced conidiation, and concomitant increases in AF biosynthesis were detected [213,232,233]. Moreover, the pathogenicity of *A. flavus* decreased with deletion of the oxylipin-encoding dioxygenase genes (*ppo* genes) of the fungus. Exogenous plant oxylipins 9(S)-hydroperoxy-octadecadienoic (9(S)-HpODE) acid and 13(S)-hydroperoxy-octadecadienoic (13(S)-HpODE) acid affected the sporulation positively and also modulated the precursor sterigmatocystin and AF synthesis in *A. flavus*, *A. nidulans*, and *A. parasiticus* [234]. In a lipidomic approach, an important role of the maize oxylipins in the regulation of driving SM production in *A. flavus* has been demonstrated [235]; however, the mechanism of the plant oxylipin action has remained yet unsolved.

In addition to oxylipins, ROS, phytohormones, and other host-derived compounds also influence the onset of oxidative stress within fungal cells. Host-derived ET resulted in the reduction of ROS accumulation in *A. flavus* mycelia and reduced AF biosynthesis. The metabolic precursor of ET, 2-chloroethyl phosphoric acid, was capable of reducing the expression of *aflR* and *aflD*, which are key genes of the aflatoxin biosynthetic pathway. The repression decreased the accumulation of oxidative compounds and regulated the glutathione redox status in *A. flavus* mycelia [236]. Comparing a resistant and a susceptible maize cultivar after *A. flavus* inoculation, the expression of the ethylene-responsive factor 1 (ZmERF1) was found to be higher in the immature kernel tissues of the resistant maize inbred [184]. ZmERF1 is a key transcription factor involved in ET and JA signaling in maize.

3.7 Killing of the Host Plant Cells

Killing of the host plant cells by the secretion of low-molecular-mass compounds like peptide toxins or ROS [160,237] represents different fungal strategies. Toxin effectors of the necrotrophic fungi target one of the host's central signaling/regulatory pathways and trigger gene-mediated resistance (R) or downregulate defense enzymes, which increases thereby the host susceptibility to fungal attack [238]. From the host's side, free radical production in chloroplasts has a critical role in plant defense because these organelles are not only sites for the biosyntheses of the signaling compounds (SA, JA, and nitric oxide) but also ROS production. Therefore, chloroplasts are regarded as important players in the induction and regulation of PCD in response to both abiotic stresses and pathogen attack (e.g., [239]).

In the H_2O_2 detoxification machinery, plant ascorbate has an enormous role [240]. Of course, in fungi, different evolutionary ways were established to cope with and reduce ascorbate levels and, as a consequence, the deleterious effects of ROS generated by plant cells. For example, the SMs fusarenon, nivalenol, DON, T-2, HT-2, deacetoxyscirpenol, beauvericine, and neosolaniol from *Fusarium* spp. caused an alteration in the ascorbate metabolism in addition to the complete inhibition of seed germination and the induction of PCD, e.g., in tomato protoplasts [241]. The T-2 trichothecene toxin, which is produced by, e.g., *F. sporotrichioides* also induced H_2O_2 generation, PCD, callose deposition, and the accumulation of SA in the nonhost plant *A. thaliana* [242].

Light-dependent plant-damaging toxins also facilitate the progression of fungal infections and the development of disease symptoms. The production of NHST perylenequinone photosensitizers called cercosporins [243–245] by *Cercospora zeae-maydis* causes one of the most destructive foliar diseases of maize. Cercosporin belongs to a group of compounds, which are activated by light and generate ROS, i.e., singlet oxygen, H_2O_2, and the devastating hydroxyl radicals. ROS exert destructive effects on plant cells, primarily through oxidative lipid decomposition, DNA damage, nutrient leakage, and induction of PCD [243,245].

Another light-dependent plant-damaging toxin has been identified in *Ramularia collo-cygni*, the causal agent of *Ramularia* leaf spot disease on barley. Rubellin D, an anthraquinone derivative, induced peroxidation of α-linoleic acid in a light-dependent manner through singlet oxygen formation and, finally, chlorophyll bleach [246,247].

Besides SM toxins, ROS also play an important role in the cytotoxic effects of *B. cinerea*, as the fungus actively contributes to the elevated levels of ROS detected at the infection sites, which caused an oxidative burst during cuticle penetration and lesion formation [248,249]. SM toxins may also have an importance in ROS accumulation. For example, *Aspergillus* mycotoxin ochratoxin A induced necrotic lesions through oxidative burst induction by increased ROS levels and the concomitant downregulation of the expression of plant antioxidant defense enzymes

[250]. Rice leaf sheaths infiltrated with *R. solani*-toxin significantly expressed five new superoxide dismutase activities, which eliminated the antifungal oxidative burst in the plant tissues [251].

The ascomycete *Cochliobolus victoriae* is a necrotrophic fungal pathogen of *Arabidopsis* and oats and produces the HST victorine. Accumulation of victorine induced defense-related responses such as extracellular alkalization, phytoalexin synthesis, and PCD causing Victoria blight [252]. It was proposed that both JA and ET promote the *A. alternata* AAL-toxin-induced PCD [253] in detached leaves of *Solanum lycopersicum* via the disruption of sphingolipid metabolism [254]. Free sphingoid bases were shown to be involved in the control of PCD in *Arabidopsis* as well, presumably through the modulation of the ROS levels upon receiving different developmental or environmental cues [255]. In the response to changes in the oxidative environment, a crucial role of fungal SOD in H_2O_2 production was shown again in *Botrytis*-*Arabidopsis* as well as *Botrytis*-tomato plant interactions, where increased callose and oxylipin 12-oxo-phytodienoic acid productions [256] were detected in the response to changing the oxidative environment.

3.8 Toxic Effects of Phytotoxin SMs

As it was presented above, the release of phytotoxins and/or ROS leads to immediate membrane destruction in the plant cells, supplies the phytopathogens with nutrients, and also significantly hinders the activation of plant defense responses.

HSTs are typically active only against host plants and have unique modes of action and toxicity on the host [257]. Therefore, the production of the HSTs is vital for the virulence of these fungi [160,258,259]. Most of the known HSTs are produced by necrotrophic pathogens of the order of *Pleosporales* within the class of *Dothideomycetes* and especially in the genera *Alternaria* and *Cochliobolus* species [260,261]. These HSTs have diverse chemical structures ranging from low-molecular-weight compounds to cyclic peptides. For the biosynthesis of the HSTs, genes encoding polypeptides reside on a conditionally dispensable chromosome that controls host-specific pathogenicity [262]. The mechanism of host-selective pathogenesis caused by the HSTs is well understood, and about 20 HSTs have been documented [257,258]. Among them, at least, seven are from *A. alternata* pathotypes that were also characterized [257]. In some cases, gene-for-gene interactions mediated host sensitivity, and the sensitivity toward toxins was mandatory for disease development [263]. Contrarily, NHSTs are not crucial determinants of the host range and are not essential for pathogenicity either, although they sometimes contribute to virulence. These toxins have a wider range of activity, causing symptoms not only on the hosts of the pathogenic fungus but also on other plant species [258].

Several phytotoxic SM compounds either inhibited an aminotransferase or appeared to have such a mode of action, like cornexistin from *Paecilomyces variotii* [264], which was patented as an herbicide, or tentoxin, a cyclic tetrapeptide from

A. alternata, which indirectly inhibited the chloroplast development in cucumber and cabbage [265].

Alternaria species have been reported to cause diseases in nearly 400 plant species such as a broad range of economically important crops and caused severe economic problems. *A. alternata* alone can infect more than 100 plant species [266]. The production of several phytotoxins and HSTs is regarded as a key reason for the success of these pathogens [267]. The PKS gene ACRTS2 was found to be essential for ACR-toxin production and pathogenicity of the rough lemon pathotype of *A. alternata* [268]. The phytotoxin zinniol (Fig. 14), which is produced by *Alternaria* species and one *Phoma* species, bound plant protoplasts and stimulated Ca^{2+} entry into cells [269]. Some of these HSTs are outstandingly toxic even at low doses, e.g., 10^{-12} g of the HST AK-toxin from the pear pathogen *A. kikuchiana* was sufficient to disintegrating approximately 100 host cells [267]. *Alternaria* sp. also produced several NHSTs such as brefeldin A, altertoxin, and tentoxin and also other mycotoxins. Alternariol (Fig. 14) and alternariol-9-methyl ether (from *Nimbya* and *Alternaria* spp.) are also major NHSTs, which are common contaminants in food like fruits and fruit juices and cereals [270] and inhibit the electron transport chain of the host plants [271].

Tentoxin, which was mentioned above, is another NHST produced by *Alternaria* spp. and blocks ATP hydrolysis by certain chloroplast F1-ATPases through binding

alternariol

zinniol

AAL-toxin

tentoxin

Fig. 14 Schematic presentation of selected *Alternaria* toxins (Source: National Center for Biotechnology Information. PubChem Compound Database (accessed June 6, 2015))

to the protein surface between the α and β subunits of ATPase. The blockage leads to complete energy breakdown in the plant cells and, consequently, causes chlorosis in sensitive plants [272]. Further, the detailed experimental analysis revealed that a single molecule of the toxin can affect ADP release in a noncompetitive manner [273]. Fusicoccin from *Fusicoccum amygdali* (=*Phomopsis amygdali*) also activated irreversibly the plant plasma membrane H^+-ATPase [274]. Moreover, several analogs of the AAL-toxin of *A. alternata* and a series of structurally related fungal metabolites, e.g., FB1 of *Fusarium* spp., specifically inhibited ceramide synthase (sphinganine-*N*-acyltransferase) in plants [167]. Importantly, FB1 toxin also triggered the depletion of extracellular ATP reserves. Extracellular ATP functions as an endogenous external metabolite and affects plant cell viability. The deprivation of extracellular ATP altered the abundance of particular cytosolic, mitochondrial, chloroplast, and endoplasmic reticulum proteins and elicited a stress response, which ended in cell death. However, the process could be reverted by exogenous ATP in *Arabidopsis* [275].

Despite the metabolic diversity found within the *Fusarium* genus, relatively few metabolites have been reported in the individual species and isolates. *F. graminearum* is a worldwide pathogen of maize and grains such as wheat, barley, and oats. However, several reports indicated that this filamentous fungus produced only 7 out of 15 PKS (including fusarubins, zearalenone, aurofusarin, fusarielins, fusarins, aurofusarin, orcinol) and the NPRS-PKS hybrid fusaristatins only 3 out of 19 NRPS SM families (malonichrome, ferricrocin, fusarinin) [187].

Currently, more than 150 trichothecenes and trichothecene derivatives are known [276], which are all nonvolatile, low-molecular-weight sesquiterpene epoxides. Trichothecenes are divided into four groups (types A–D) according to their chemical properties and their origin [277]. In plants, trichothecenes cause necrosis, chlorosis, and mortality in plants, enabling the fungal pathogen to mediate a wide variety of plant diseases, including wilts, stalk rot, root rot, and leaf rot in many crops and ornamental plants [278] reducing crop yield and quality. However, only a few of the known trichothecenes (all of them are from types A or B) have importance in crops. Diacetoxyscirpenol (DAS) and T-2 toxin from type A and DON and nivalenol (NIV) from type B are the major products of the trichothecene biosynthetic pathway [277].

Fusarium species differ considerably in their trichothecene productions. Several phytopathogenic *Fusarium* spp. including *F. graminearum* and *F. culmorum* produce DON, which is one of the primary trichothecene metabolites found in wheat. Producers of type B trichothecenes can be separated further into two chemotypes by whether they produce DON (or its acetylated derivatives) or nivalenol [279,280]. Besides *Fusarium* spp., *Myrothecium verrucaria* and some other species also synthesize type C and D trichothecenes. The type C trichothecenes such as crotocin, e.g., in *Trichothecium roseum* and *Cephalosporium crotocinigerum*, are characterized by a second epoxide function at the C-7,8 or C-9,10 positions of the pentane ring, which is common to all trichothecenes [277]. Type D trichothecenes contain a macrocyclic ring between the C-4 and C-5 position of the pentane ring with two ester linkages. Type D trichothecenes include verrucarin (*Myrothecium*

verrucaria), satratoxin (*Stachybotrys atra*), and roridin (e.g., *Myrothecium roridum*, *Cylindrocarpon*) [277]. Verrucarin A is a known phytotoxin, and there is evidence that some unidentified metabolites of *M. verrucaria* also are involved in the observed bioherbicidal activity [281].

Isolates of the *F. graminearum* species complex exhibited wide strain-specific variability in both aggressiveness and trichothecene production on cultivars [282], and trichothecenes, especially DON, were inherently linked to the pathogenicity of *F. graminearum* [189,283]. The toxin inhibited translation without induction of the elicitor-like signaling pathway in the nonhost plant *A. thaliana* [242].

Although AAL-toxin (*A. alternata*) and fumonisins (*Fusarium* spp.) share common structural features and thus exhibit similar disrupting effects on the sphingolipid metabolism in plants and also in animals, fumonisins produced by *F. verticillioides* was shown to be dispensable for maize infection [284]. Meanwhile, as we mentioned before, at least in *F. graminearum*, DON is considered to have a critical role as a virulence factor. The spread of the fungus in the spikes and the production of DON correlated well with the presence of several polyamine compounds (e.g., putrescine) that are accumulated when the infection progressed through the spike and preceded the fungal production of DON [285]. The phytotoxic effect of six trichothecenes such as DON, 3-acetyldeoxynivalenol (3-ADON), NIV, DAS, T-2, and HT-2 were assessed and compared studying four wheat cultivars. DON and its chemotype 3-ADON were more cytotoxic than a T-2, HT-2, and DAS, and those toxins inhibited wheat coleoptile elongation [286].

F. culmorum, *F. graminearum*, and *F. venenatum* can produce SM culmorins, which are tricyclic sesquiterpene diols [287]. Culmorin showed weak phytotoxicity to wheat coleoptile tissue [288], but there are no reports on its role in the wheat head scab. There are no threshold limits for these SMs; therefore, *Fusarium*-contaminated grain stocks are usually not screened for culmorins. However, in naturally contaminated Norwegian wheat, barley, and oat samples, culmorin and hydroxyculmorins were detected at relatively high levels besides the high DON concentrations [289].

Several *Fusarium* species produce enniatins, which are cyclic hexadepsipeptides with phytotoxic properties and promote fungal virulence. Disruption of the enniatin synthase gene in *F. avenaceum* isolates caused defects in enniatin biosynthesis, and these isolates were less efficient in the colonization of potato tubers than the wild type [290]. Enniatins and the structurally related beauvericin function as cation chelators and transporters of divalent cations exerted across cell membranes [291].

Further phytotoxins can also cause significant economic loss. For example, diplodiatoxin produced as major SM by *Stenocarpella maydis* (*Diplodia maydis*) caused increased lesion lengths [292] and fungal dry rot of maize ears and was associated with diplodiosis, a neuro-mycotoxicosis in cattle grazing harvested maize fields mainly in Africa and South America [293]. The host-selective T-toxin, a family of C35 to C49 polyketides, from *C. heterostrophus* [204,294] inhibited mitochondrial respiration by binding to an inner mitochondrial membrane protein in sensitive plants. The binding caused pore formation, leakage of NAD^+ and and other ions, as well as subsequent mitochondrial swelling (reviewed by [295]).

The soilborne fungus *R. solani* (teleomorph *Thanatephorus cucumeris*) belongs to the phylum *Basidiomycota* and has economic importance. *R. solani* is a nonobligate necrotrophic pathogen, which causes diseases in many crops including species in the families Asteraceae, Brassicaceae, Fabaceae, and Poaceae, Solanaceae and ornamental plants and forest trees throughout the world [296]. The anamorph *R. solani* is a species complex including at least 14 different, genetically isolated populations (=anastomosis groups (AG)) that differ in their ecology and host range [297,298].

Interestingly, in genome analysis of the *R. solani* AG1-IA, only ten genes coding for SM biosynthetic enzymes were identified (a PKS, 4 NRPSs, and 5 DMATSs) (Table 2). The penta-functional AROM protein of the shikimate pathway, which takes part in the synthesis of phytotoxic phenylacetic acid, was also detected in the genome. Some genes in the biosynthesis of a putative phytotoxin enniatin and also DNA sequences featuring homology to putative trichothecene toxin citrinin, AF, and terpene biosynthesis genes (e.g., genes encoding sesquiterpene synthases) were also found. However, no other genes that are homologous to the mycotoxin biosynthesis genes in other *R. solani* AGs were identified [299]. Furthermore, three volvatoxin genes that are homologous to volvatoxin from *Volvariella volvacea* (*Basidiomycota*) were detected in *R. solani* AG1-IB [300]. In proteome analysis, a trichothecene 3-*O*-acetyltransferase, which is required for trichothecene biosynthesis and involved in the reduction the toxicity of DON in *Fusarium* spp. [224], was differentially expressed during the developmental stages in *R. solani* AG1 [301]. *R. solani* synthesizes both HSTs and NHSTs [302]. HSTs from *R. solani* strains increased the virulence of the pathogen (e.g., the HC-toxin on maize) [303]. Other *R. solani* AGs also produce various phytotoxic compounds like the phenylacetic acid, mentioned above, and its derivatives, a phenolic compound, and a carbohydrate [304], which support the broad host range and diversity observed within the *R. solani* species. From the fermentation broth of *R. solani* AG1 IA, Xu et al. identified eight compounds, from which *m*-hydroxymethyl phenyl pentanoate, (Z)-3-methylpent-2-en-1,5-dioic acid and 3-methoxyfuran-2-carboxylic acid also showed phytotoxicity in vitro [305].

The gray mold fungus *B. cinerea* is a typical necrotrophic phytopathogen with a very broad host range. It causes vast pre- and postharvest economic damages [197]. Two groups of its phytotoxic metabolites such as the sesquiterpene botrydial and related compounds [306] and botcinic acid and its derivatives [307] have been characterized. The sesquiterpene-derived phytotoxin botrydial has been implicated in virulence *in planta* because its addition facilitated fungal penetration and colonization of plants [308].

Some SMs and mycotoxin, including territrem A, citreoviridin, citrinin, gliotoxin, patulin, terrein, terreic acid, and terretonin, are produced by *A. terreus* [309], and in infected crops such as wheat, ryegrass, and potatoes, they were linked to the disease. The phytotoxic SM terrein found in *A. terreus* possessed ecological, antimicrobial, antiproliferative, and antioxidative activities and was highly induced in plant-derived media and elicited lesions on fruit surfaces [310].

4 Conclusions and Future Aspects

SMs play important roles in the development and lifestyles of the fungal-plant pathogens, but only approximately 25 % of the fungal SM gene clusters have been characterized thus far, and even fewer SMs have been identified on the host side. Fungal SM genes reflect remarkable diversity among species, and the differences in the SM spectrums may explain the differences observable in the lifestyles of the pathogens. Furthermore, horizontal gene and chromosome transfers may also broaden the host ranges of the pathogenic fungi.

Because a plethora of fungal genome sequences are publicly available by now, comparative genomics and transcriptomics tools can provide us with an array of genetic features characteristic of the fungal pathogens and, hence, can increase our knowledge on the regulation and adaptation mechanisms typical of the various pathogenic lifestyles (Fig. 15) [312].

The spread and availability of next-generation DNA sequencing tools also allows us to survey the SM gene clusters in a given fungus. Furthermore, next-generation RNA-Seq technologies are used routinely to screen the expression patterns of SM gene clusters at various stages of infection. RNA-Seq technologies are also suited perfectly for studying plant-pathogen interactions at the level of global transcriptional changes. Moreover, genetic manipulations of species- and strain-specific SM genes and gene clusters, which are associated with host-specific virulence, may provide us with the possibility to investigate further the roles of SMs in fungal-plant interactions. A deeper knowledge of fungus-plant interactions may also help us in resistance breeding in order to obtain new plant cultivars/hybrids with increased

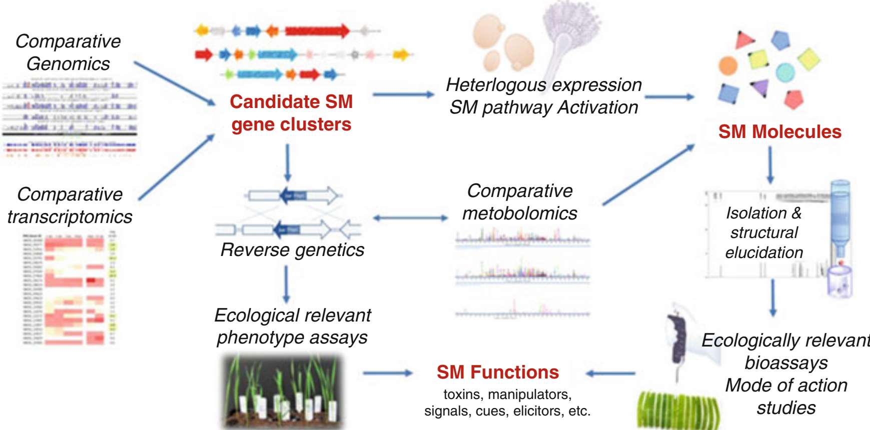

Fig. 15 An integrated chemical ecogenomic approach for understanding the functions of secondary metabolites (SMs) and bioactive molecule discovery. The strategy incorporates the common tools in ecological genomics, genome mining, and chemical ecology [312]

tolerance against various types of stress such as abiotic stress or invasion by fungal pathogens. Furthermore, well-characterized plant SMs could be used against various plant-pathogen fungi, e.g., in natural plant extracts in sprayable forms. Such technologies would have a great importance especially in organic agriculture, where no other chemicals are preferred.

Deciphering the biosyntheses of both plant and fungal SMs and especially those of mycotoxins and real phytotoxins together with current and future improvements in gene disruption techniques hopefully will allow us to develop new and effective molecular genetic tools to elucidate the roles of SMs in the progression of important plant diseases. The deeper our knowledge is, the clearer we can see the common features of plant-fungal interactions although these interactions can be very special or even unique. When all common and special features of plant-fungus interactions have been reveled successfully and the effects of various ecological parameters, e.g., changing environmental conditions or alterations in the rhizosphere biome, have also been assessed and analyzed in depth, we will be able to work out novel, effective, and reliable pretreatment and treatment strategies to prevent and cure plant diseases caused by fungi.

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Host and Guest: Vanilla Inhabited by Endophytes

5

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Abstract

Fungal endophytes are known to produce secondary metabolites. The synthesis of vanillin and its precursors have never been clearly elucidated. Given fungi can produce such metabolites, it is speculated that fungal endophytes in vanilla could be contributing to vanillin and its precursors. An investigation was thus carried to find whether fungal endophytes are present in *Vanilla planifolia*. Additionally, vanilla flavor varies across cultivation regions; hence, the distribution of endophytes across regions was also assessed and found to differ. The metabolic changes brought by the fungi on vanillin and its precursors in vanilla pods were also evaluated. Out of 434 isolated fungal endophytes, two candidates emerged: *Pestalotiopsis microspora* and *Diaporthe phaseolorum*. However, *P. microspora* increased the most the absolute amounts (quantified by ^{1}H NMR in μmol/g DW green pods) of vanillin (37.0×10^{-3}), vanillyl alcohol (100.0×10^{-3}), vanillic acid (9.2×10^{-3}), and *p*-hydroxybenzoic acid (87.9×10^{-3}) when cultured on green pod-based media. Given the physical proximity of fungi inside pods, endophytic biotransformation may contribute to the complexity of vanilla flavors.

Keywords

Endophytes • Distribution • Flavor • Biotransformation • Vanilla • Interaction

List of Abbreviations

DNA	Deoxyribonucleic acid
GC-O	Gas chromatography-olfactometry
H NMR	Proton nuclear magnetic resonance
HPLC	High-performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography-diode array detector
ITS	Internal transcribed spacer
MOTU	Molecular operational taxonomic unit
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PDA	Potato dextrose agar
p-HB acid	*p*-Hydroxybenzoic acid
p-HBAld	*p*-Hydroxybenzaldehyde
rDNA	Ribosomal deoxyribonucleic acid

1 Introduction

The genus *Vanilla* is a member of the Orchidaceae family and comprises of approximately 100 species [1]. Only two species, *Vanilla planifolia* Andrews and *Vanilla tahitensis* Moore, are allowed to be used in foods [2]. However, *Vanilla planifolia* is the most important source of natural vanilla flavor [1], a species which originates from Mesoamerica [2]. The plant requires 3–4 years to set the flower and afterward flowers once a year. The podlike fruit (vanilla bean) is allowed to develop for 8–10 months before harvesting (Fig. 1).

Fig. 1 Mature vanilla pod (Photo by H. Kodja)

Vanilla beans are harvested green and are initially flavorless. The green beans are subjected to a curing process for 3–6 months or longer, depending on various curing protocols in different production regions [2]. The product of the curing process is the dark pod which is then sold in the commerce. The objective of the curing process is to develop the prized vanilla flavor and to dry the cured beans to prevent microbial growth during transport and storage [2]. As mentioned before, the exact method for postharvest processing and curing of vanilla pods varies across regions of the world [3]. In Reunion Island, the traditional Bourbon curing method is used, and the process starts with the immersion of the pods in hot water at 65 °C for 3 min, a process known as scalding. The pods are then transferred to a wooden box lined with blanket, for sweating, and kept for 24 h at a temperature of 50 °C. Afterward, the pods are sun dried, under a temperature of about 50 °C for 1 week. The pods are then dried slowly for 2–3 months in racks placed in well-ventilated rooms maintained at 35 °C. The last step is known as conditioning and consists of storing graded and bundled pods wrapped in wax paper in closed boxes for a period of 8 months.

Natural vanilla flavor is the number one flavor tonality in the world as it is subtle but complex [4]. Vanilla is actually a versatile and dynamic flavoring substance, the potential of which has still not been fully realized. When vanilla is used in different foods, it renders different flavor profiles [2]. Natural vanilla flavor faces fierce competition from artificial vanillin, a replacement, on the international market given the lower price of the latter. However, companies such as Symrise, Firmenich, Takasago, and Givaudan still have active sustainability programs with local vanilla

bean farmers to improve both agricultural practices and wages in key producing areas because vanillin does not totally replicate the characteristic flavor profiles of high-quality vanilla extracts [5]. One of the factors that contribute to the higher price of natural vanilla compared to artificial vanillin is the labor cost and infrastructure required in the long curing process. One process that eliminates the curing process consists of a patent application from Givaudan. The process is a fermentation in which green ripe uncured vanilla beans are incubated with *Bacillus subtilis* in isolated form to convert glucovanillin to vanillin while consistently forming a fully developed well-balanced vanilla aroma without off-notes [6]. This is but one example that shows a connection between vanilla aroma and microorganisms.

Over 200 compounds which are associated to vanilla flavor have already been isolated and identified from vanilla beans. The contents of these compounds vary in the dark pods of the same vanilla plant species depending on the region of the world where the beans are harvested [7]. Four major flavor-related components (*p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillic acid, and vanillin) are used as marker compounds to determine quality and authenticity of vanilla products. For authentic unadulterated vanilla extracts, the ratios between the four components are fixed within a certain range [8]. In Réunion Island, vanilla plants are either cultivated in the undergrowth or in shade houses. Vanilla pods grown in the undergrowth appeared to display substantial qualitative differences of vanillin and vanillic acid contents in comparison to those grown under shade-house conditions. Additionally, cured vanilla pods from different regions of Réunion Island have different flavor qualities (Bertrand Come 2012, personal communication, 6 June). Parameters responsible for such a differences have not been identified yet [5] but could be due to climatic, edaphic factors or due to the endophytic microbial composition in the plant. One such candidate is endophytic fungi hence the investigation in this work.

Endophytic fungi are defined functionally by their occurrence within asymptomatic tissues of plants [7]. In spite of the ubiquitous features, the scale of their diversity, their host range, and geographic distributions much about endophytes is still unknown for many plants including vanilla. Hence, this work aimed at isolating fungal endophytes from vanilla. Endophytic fungi can either be transmitted vertically or horizontally. Vertical transmission occurs when fungi are transferred from the host to the offspring via host tissues. Horizontal transmission occurs when fungi are transferred to the host via spores, e.g., through aerial means. Endophytes can be involved in biomass production and nutrient cycling in the plant [7]. Previously, Porras-Alfaro and Bayman [9] isolated nonpathogenic fungi from inside asymptomatic roots of vanilla plants. Mycorrhizal fungi interact symbiotically with roots through an association of the mycelium (typically basidiomycete), while the hyphae form a mass around the rootlets or penetrate root cells. They are absent from the outer root cortex and hence differ from endophytes that are present deeper inside plant tissues. The mycorrhizal fungi *Ceratobasidium* spp., *Thanatephorus* spp., and *Tulasnella* spp. were found to be associated to different species of vanilla by Porras-Alfaro and Bayman [9]. Morphological identification followed by elongation factor gene sequence analysis showed that several *Fusarium* spp. are present in vanilla plants in Indonesia [10]. Roling et al. [11] have assessed the microbial diversity in

vanilla pods at different stages of postharvest processing in Indonesia. Microbial communities changed the most after the scalding of pods in hot water (65–70 °C for 2 min). Hence, an investigation was performed in this work to find microorganisms that survive post-scalding for pods from Reunion Island. Roling et al. [11] found a decrease in microbial diversity and of fungal growth.

In this chapter, we describe work in connection to vanilla and fungal endophytes: (1) finding if fungal endophytes are present in vanilla and their abundance; (2) finding distribution of endophytes, within plant, post-scalding, and across region; and (3) finding which species of endophyte affect vanilla aroma in green pod material.

2 Finding if Fungal Endophytes Are Present in Vanilla and Their Abundance

Fungal endophytes were isolated from green 8-month-old pods post-pollination and leaves ranks 1 (the youngest in the sample set), 3, 5, 10, and 15 (the oldest in the sample set) across seven regions in Reunion Island (St. André, St. Anne, St. Rose, Bois Blanc, Takamaka, Mare Longue, and Basse Vallée – Fig. 2) and under two culture conditions (shade house, in the field).

Fungal identification was carried out through morphological and DNA/RNA-sequencing methods. In this way, 23 MOTUs were identified (Table 1).

The total number of isolates recovered from all tissues was 434 isolates. The 23 different isolated MOTUs represent six classes (*Sordariomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Pezizomycetes*, *Agaricomycetes*, *Zygomycetes*). *Fusarium proliferatum* (MOTU1) was, by far, the most abundant fungus accounting for 37.6 % of the isolates (Table 2). *Botryosphaeria ribis* (MOTU16) and *Aspergillus fumigatus* (MOTU20) were the second most abundant taxa, each accounting for 5.8 % of the isolates. Sixteen fungal genera were isolated from *Holcoglossum* plants which, like vanilla, are also members of the family Orchidaceae, and the fungi belonged to three classes, *Sordariomycetes*, *Dothideomycetes*, and *Agaricomycetes* [13]. In comparison, a high number of 21 fungal genera were isolated from vanilla plants in this study representing six classes (*Sordariomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Pezizomycetes*, *Agaricomycetes*, *Zygomycetes*; Table 2) with *Sordariomycetes* being the dominant class (60 %, 14/23 MOTUs). This is in line with the fact that endophytic *Sordariomycetes* have a high frequency of occurrence within tropical plants [14].

The majority of the isolated endophytes belonged to the class *Sordariomycetes* (60 %, 14 out of 23 isolated MOTUs) which consist of members of the orders *Hypocreales* (consisting of 199 isolates making 5 MOTUs), *Xylariales* (consisting of 33 isolates making 3 MOTUs), *Diaporthales* (consisting of 25 isolates making 2 MOTUs), and *Glomerellaceae* (consisting of 23 isolates making 2 MOTUs). *Dothideomycetes* and *Eurotiomycetes* were the next most common classes both representing 11.8 and 9 % of all isolated MOTUs, respectively.

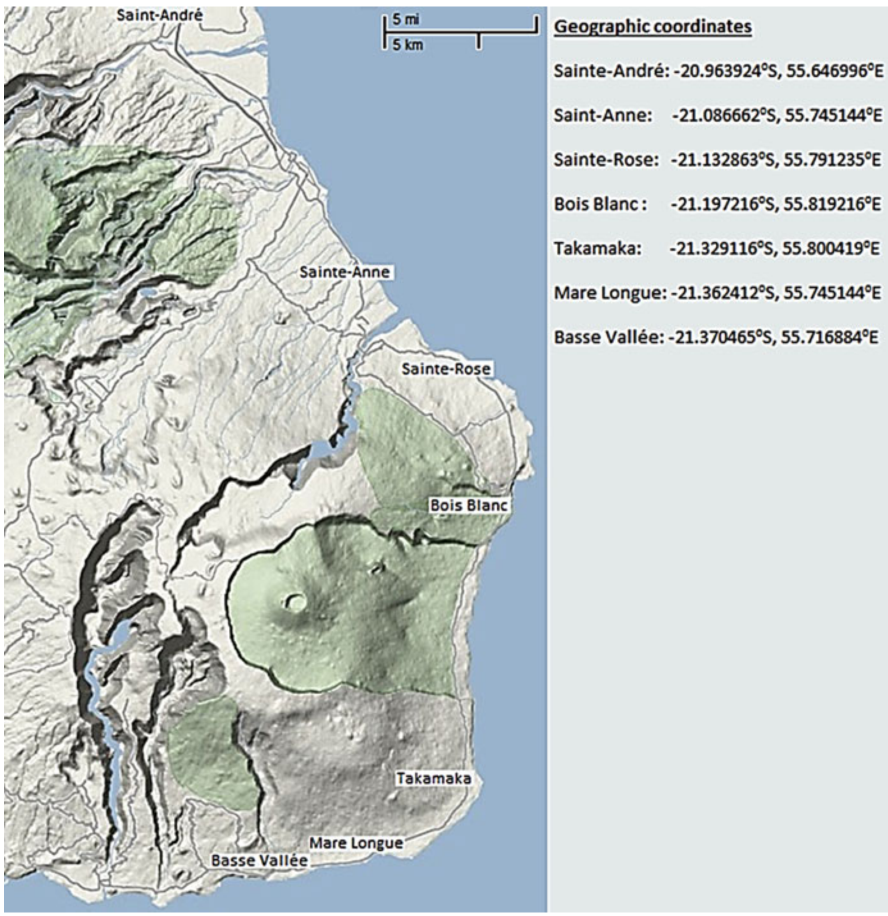

Fig. 2 The seven regions in Reunion Island from which vanilla organs were collected for fungal endophyte isolation

Classes *Pezizomycetes*, *Zygomycetes*, and *Agaricomycetes* were rare, with only one MOTU representative of each.

3 Finding Distribution of Endophytes: Within Plant, Post-scalding, Across Region

The ecology and distribution of fungal endophytes are known to vary based on the organ considered and the location of the host plant. Different fungal distribution in plant organs would cause specific metabolic changes in those organs. One important organ for vanilla commerce is the pod. In order to appreciate the distribution of fungal endophyte in pods, it is essential to understand fungal distribution in other

Table 1 Identification of endophyte MOTUs based on NCBI BLAST of 28S, ITS rDNA, EF-1α, or β-tubulin sequences (Source, Khoyratty et al. [12])

<table>
<tr><th>MOTU number</th><th>Isolate number</th><th>DNA region</th><th>BLast best match: GenBank accession number and ID</th><th>Class order</th></tr>
<tr><td>MOTU 1</td><td>9B (LCP5974)</td><td>EF-1α</td><td>>gi|149798252|gb|EF453149.1| Gibberella intermedia strain NRRL 43666 (Fusarium proliferatum)</td><td>Sordariomycetes Hyprocreales</td></tr>
<tr><td>MOTU 2</td><td>82D1</td><td>ITS</td><td>>gi|262476602|gb|GQ505743.1| Fusarium scirpi strain NRRL 36478</td><td>Sordariomycetes Hypocreales</td></tr>
<tr><td>MOTU 3</td><td>28</td><td>EF-1α</td><td>>gi|306412978|gb|HM347120.1| Fusarium oxysporum strain NRRL 26360</td><td>Sordariomycetes Hypocreales</td></tr>
<tr><td>MOTU 4</td><td>29A (LCP5979)</td><td>ITS</td><td>>gi|316980277|emb|FN706553.1| Acremonium implicatum MUCL 1412</td><td>Sordariomycetes Hypocreales</td></tr>
<tr><td>MOTU 5</td><td>3C1B (LCP5984)</td><td>28S</td><td>>gi|523713894|gb|KC157757.1| Purpureocillium lilacinum strain M4076</td><td>Sordariomycetes Hypocreales</td></tr>
<tr><td>MOTU 6</td><td>55E (LCP5980)</td><td>ITS</td><td>>gi|215490348|gb|FJ441623.1| Phomopsis phyllanthicola strain msy55</td><td>Sordariomycetes Diaporthales</td></tr>
<tr><td>MOTU 7</td><td>5 (LCP5978)</td><td>ITS</td><td>>gi|283856804|gb|GU066686.1| Diaporthe phaseolorum isolate 123 AC/T (Phomopsis sp.)</td><td>Sordariomycetes Diaporthales</td></tr>
<tr><td>MOTU 8</td><td>61B (LCP5982)</td><td>ITS</td><td>>gi|44893890|gb|AY541610.1| Nemania bipapillata strain CL8</td><td>Sordariomycetes Xylariales</td></tr>
<tr><td>MOTU 9</td><td>9B</td><td>ITS</td><td>>gi|387773616|gb|JQ846066.1| Xylaria sp. 5485</td><td>Sordariomycetes Xylariales</td></tr>
<tr><td>MOTU 10</td><td>61 F (LCP5983) 55A (LCP6051)</td><td>ITS</td><td>>gi|21310048|gb|AF377292.1| Pestalotiopsis microspora strain CBS364.54</td><td>Sordariomycetes Xylariales</td></tr>
<tr><td>MOTU 11</td><td>69D (LCP5988)</td><td>ITS</td><td>>gi|169135011|gb|EU482214.1| Colletotrichum gloeosporioides ICMP 17323</td><td>Sordariomycetes Glomerellaceae</td></tr>
<tr><td>MOTU 12</td><td>39</td><td>ITS</td><td>>gi|82799468|gb|DQ286216.1| Colletotrichum sp.</td><td>Sordariomycetes Glomerellaceae</td></tr>
<tr><td>MOTU 13</td><td>S101Z1 (LCP5987) 42a,b,c</td><td>ITS</td><td>>gi|317383391|gb|HQ631070.1| Nigrospora sp. TMS-2011 voucher SC9d1p7-1</td><td>Sordariomycetes Trichosphaeriales</td></tr>
<tr><td>MOTU 14</td><td>69H (LCP5985) S104Z1</td><td>ITS</td><td>>gi|383842765|gb|JQ316443.1| Fungal endophyte isolate EL-10 Australia</td><td>Sordariomycetes Trichosphaeriales</td></tr>
<tr><td>MOTU 15</td><td>S104Z1 Bis</td><td>28S</td><td>>gi|290889519|gb|GU390656.1| Delitschia chaetomioides strain SMH 3253.2</td><td>Dothideomycetes Pleosporales</td></tr>
</table>

(*continued*)

Table 1 (continued)

<table>
<tr><th>MOTU number</th><th>Isolate number</th><th>DNA region</th><th>BLast best match: GenBank accession number and ID</th><th>Class order</th></tr>
<tr><td>MOTU 16</td><td>25 (LCP6048) 61d,e,g, S104Z1ter</td><td>ITS</td><td>>gi|34328662|gb|AY236935.1| *Botryosphaeria ribis* isolate CMW7772</td><td>*Dothideomycetes Botryosphaeriales*</td></tr>
<tr><td>MOTU 17</td><td>51B (LCP5998)</td><td>ITS</td><td>>gi|330369659|gb|JF261465.1| *Guignardia mangiferae* strain CPC18848 (CBS128856 T)</td><td>*Dothideomycetes Botryosphaeriales*</td></tr>
<tr><td>MOTU 18</td><td>33B (LCP5989)</td><td>ITS</td><td>>gi|262386897|gb|GQ852747.1| *Mycosphaerella marksii* strain CPC:13273</td><td>*Dothideomycetes Capnodiales*</td></tr>
<tr><td>MOTU 19</td><td>8B2 (LCP6049)</td><td>ITS</td><td>>gi|310769695|gb|GU944569.1| *Penicillium citrinum* strain CBS 13945</td><td>*Eurotiomycetes Eurotiales*</td></tr>
<tr><td>MOTU 20</td><td>S102Z1 (LCP6050)</td><td>β-tub</td><td>>gi|110743536|dbj|AB248059.1| *Aspergillus fumigatus* strain: IAM 13869</td><td>*Eurotiomycetes Eurotiales*</td></tr>
<tr><td>MOTU 21</td><td>74A (LCP5981)</td><td>ITS</td><td>>gi|22023786|gb|AF485074.1| *Sarcosomataceous* endophyte E99297 strain E99297</td><td>*Pezizomycetes Pezizales*</td></tr>
<tr><td>MOTU 22</td><td>51A (LCP5976)</td><td>28S</td><td>>gi|353703687|gb|HQ848487.1| *Perenniporia* nanlingensis voucher Cui 7589</td><td>*Agaricomycetes Polyporales*</td></tr>
<tr><td>MOTU 23</td><td>3C1A (LCP5977)</td><td>ITS</td><td>>gi|15147895|gb|AF254932.1| *Cunninghamella blakesleana* strain CBS 133.27</td><td>*Zygomycetes Mucorales*</td></tr>
</table>

organs in comparison to pods. With regard to endophyte distribution across the host plant location, the distribution is significantly affected for horizontally transmitted endophytes. As a consequence, it was first necessary to find whether endophytes which were recovered in this work were horizontally transmitted to vanilla plants through aerial means.

3.1 Determining the Mode of Fungal Transmission

Symbiont transmission perpetuates symbioses through host generations. Horizontally transmitted symbionts are acquired through the environment, while vertically transmitted symbionts are often transferred through the female germ line, but mixed modes of transmission also exist. In order to establish the method of endophyte transmission in the plant, ovaries which have petals that are closed as well as those with petals that are open were collected under shade-house conditions at St. André. Five MOTUs were isolated (MOTU2 *F. scirpi*, MOTU13 *Nigrospora* sp1, MOTU15 *D. chaetomioides*, MOTU16 *B. ribis*, and MOTU20 *A. fumigatus*) from ovaries with opened petals only. Hence, fungi were recovered from ovaries with opened petals

Table 2 List and abundance of molecular operational taxonomic units (MOTUs) from endophytes identified in this study (Source, Khoyratty et al. [12])

MOTU number	Fungal species	Class/Order	Abundance (number of isolates)
MOTU1	*Fusarium proliferatum*	*Sordariomycetes/ Hypocreales*	163
MOTU2	*Fusarium scirpi*	*Sordariomycetes/ Hypocreales*	6
MOTU3	*Fusarium oxysporum*	*Sordariomycetes/ Hypocreales*	9
MOTU4	*Acremonium implicatum*	*Sordariomycetes/ Hypocreales*	12
MOTU5	*Purpureocillium lilacinum*	*Sordariomycetes/ Hypocreales*	9
MOTU6	*Phomopsis phyllanthicola*	*Sordariomycetes/ Diaporthales*	11
MOTU7	*Diaporthe phaseolorum*	*Sordariomycetes/ Diaporthales*	14
MOTU8	*Nemania bipapillata*	*Sordariomycetes/ Xylariales*	13
MOTU9	*Xylaria* sp.	*Sordariomycetes/ Xylariales*	7
MOTU10	*Pestalotiopsis microspora*	*Sordariomycetes/ Xylariales*	13
MOTU11	*Colletotrichum gloeosporioides*	*Sordariomycetes/ Glomerellaceae*	12
MOTU12	*Colletotrichum* sp.2	*Sordariomycetes/ Glomerellaceae*	11
MOTU13	*Nigrospora* sp.1	*Sordariomycetes/ Trichosphaeriales*	20
MOTU14	*Nigrospora* sp. 2	*Sordariomycetes/ Trichosphaeriales*	13
MOTU15	*Delitschia chaetomioides*	*Dothideomycetes/ Pleosporales*	1
MOTU16	*Botryosphaeria ribis*	*Dothideomycetes/ Botryosphaeriales*	25
MOTU17	*Guignardia mangiferae*	*Dothideomycetes/ Botryosphaeriales*	14
MOTU18	*Mycosphaerella marksii*	*Dothideomycetes/ Capnodiales*	11
MOTU19	*Penicillium citrinum*	*Eurotiomycetes/Eurotiales*	14
MOTU20	*Aspergillus fumigatus*	*Eurotiomycetes/Eurotiales*	25
MOTU21	*Sarcosomataceae* spp.	*Pezizomycetes/Pezizales*	13
MOTU22	*Perenniporia nanlingensis*	*Agaricomycetes/* Polyporales	8
MOTU23	*Cunninghamella blakesleana*	*Zygomycetes/Mucorales*	10
Total			434

only and not from ovaries with closed petals. Ovaries with opened petals are exposed to air, whereas those with closed petals are not.

Hence, fungal endophytes only entered the ovaries through aerial means when the petals are opened. Ovaries of *V. planifolia* seemed endophyte free at emergence. Thus, the five isolated fungal MOTUs from ovaries with opened flowers were most likely transmitted horizontally; however, endophytes may colonize fruits later in development. The discovery that MOTU1 *F. proliferatum* occurs in pods and leaves opens the way to the idea that some endophytes may issue from other vegetative tissues. Thus, further research is required to confirm which event occurred. The possibility for a horizontal transmission of endophytes in vanilla pods would be similar to the case of cacao where fruits are endophyte free at emergence but then accumulate diverse endophytes from spore rain in the environment [15]. With ovary maturation, endophyte populations in cranberries vary [16]. Similarly, the fungal MOTUs isolated from *V. planifolia* ovaries with opened petals in the shade house at St. André differed from those identified from 8-month post-pollination pods (MOTU1 *F. proliferatum* and MOTU7 *D. phaseolorum*) at the same location.

3.2 Endophyte Distribution Across Organs and Region

Fungal endophytes were not all equally distributed in all the sampled vanilla tissues. Over all 450 sampled tissues, 220 yielded endophytes. Hence, at least 48.9 % of sampled tissues were infected, given that some fungal endophytes may not be cultivable. This low percentage is due to young leaves (1 and 3 weeks old) that were free of endophyte, or a low infection level might have hampered isolation of fungi. The organ which was the most infected was the pods with 76 % of endophyte recovery. Pods from St. Rose had the highest endophyte recovery (100 %). Both *Fusarium proliferatum* and *Aspergillus fumigatus* (the two most recovered fungal species) occurred at all the nine sites sampled. Both were distributed over two sites, Saint André and Sainte Rose for MOTU16 and Saint André and Sainte Anne for MOTU20 (Table 3).

Other endophytes were rarely isolated, each occurring only in one site and one organ with MOTU15 *D. chaetomioides*, being the less abundant at 0.2 % of all isolates. The three mycorrhizal fungi isolated from roots of different species of vanilla by Porras-Alfaro and Bayman [9] are members of the class *Agaricomycetes*. Only one isolated fungus in this work belongs to the class *Agaricomycetes* although that fungus is an endophyte and not a mycorrhiza (MOTU22 *P. nanlingensis*). Furthermore, the fungus originated from inside the organ, and hence not through superficial contamination from the root of the plant, for instance, given that after surface sterilization, the organ surface was touched onto potato dextrose agar (PDA) media with no fungal growth obtained. Fungal growth was obtained only when the organ was split open and when the interior of the organ exposed to PDA.

Table 3 Types and abundance of MOTUs (shown in brackets) in relation to geographic origin, management types, and organs (Source, Khoyratty et al. [12])

Sites	Isolated MOTUS codes (number of times isolated)										Total
		St. Andre		St Anne		St Rose	Bois Blanc	Takamaka	Mare Longue	Basse Vallee	
	Organs	UC	SH	UG	SH	UG	UG	UG	UG	UG	
MOTU number/ (number of isolates)	Ovaries	Not detected	MOTU2(6) MOTU13(7) MOTU15(1) MOTU16(10) MOTU2O(11)	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	35
	Pods	MOTU1(9)	MOTU1(5) MOTIT(14)	MOTU3(9)	MOTU1(6) MOTU23(1O) MOTUS(9) MOTU11(12) MOTU14(13) MOTU2O(14)	MOTU1(13) MOTU4(12) MOTU8(13) MOTU1O(13) MOTU16(15)	MOTU1(9)	MOTU1(11)	MOTU1(9) MOTU18(11) MOTU6(11)	MOTU1(10)	228
	Leaf 1	Not detected	MOTU1(6)	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected	6
	Leaf 3	Not detected	MOTU1(9)	Not detected	Not detected	Not detected	Not detected	MOTU1(3)	Not detected	Not detected	12
	Leaf 5	Not detected	MOTU1(12) MOTU13(13)	MOTU1(5)	Not detected	MOTU1(7)	Not detected	MOTU1(4)	Not detected	Not detected	41
	Leaf 15	Not detected	MOTU1(12) MOTU9(7) MOTU12(11) MOTU19(14)	MOTU1(11)	Not detected	MOTU1(8)	Not detected	MOTU1(7) MOTU21(13)	MOTU1(7) MOTU22(8) MOTU17(14)	Not detected	112
Grand total											434

Management type: undergrowth (*UG*), shade house (*SH*)
Footnote: The first youngest leaf collected is leaf rank 1 followed by 3, 5, and 15 leaves on the same branch with the 15th being the oldest.

3.3 Endophyte Diversity After Scalding Treatment

Microbial diversity and fungal growth changed drastically after pod scalding [11]. As a consequence, in this work, fungal endophyte isolation was performed on pod post-scalding. Only the fungal endophyte *Hypoxylon investiens* was recovered post-scalding from pods originating from St. Rose at 47 % (isolated from 7 pods on a total of 15 collected pods).

4 Finding Which Species of Endophyte Affect Vanilla Aroma

In order to find metabolic changes in the pod due to fungal endophytes, metabolomics analysis techniques were applied. Metabolomics is defined as both the qualitative and quantitative analysis of all primary and secondary metabolites of an organism [17]. Two chemical analysis techniques used in metabolic profiling include proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and high-performance liquid chromatography (HPLC). Both methods are well adapted for works on plant-fungal endophyte interactions. For instance, high-performance liquid chromatography-diode array detector (HPLC-DAD) analysis showed that the inoculation of in vitro plantlets of *Hyptis marrubioides* Epling with bacterial and fungal endophytic isolates induced the production of metabolites 3,4-O-(Z)-dicaffeoylquinic acid and quercetin-7-O-glucoside in the plant [18]. Hence, similar metabolomics methods can be effective to decipher the potential involvement of endophytic fungi in the production of secondary metabolites in this work. Despite being a simple molecule, natural vanillin biosynthesis from *V. planifolia* plants remains controversial. In fact, there is still some disagreement over the exact cell types that produce vanillin. A possible reason for such controversy stems from the fact that vanillin is a simple structure that lends itself to multiple possible theoretical biosynthetic pathways and due to the general promiscuity of many enzymes of plant phenolic metabolism; it is possible to find evidence to support any of these pathways from in vitro biochemical approaches [19].

Vanillin can also be produced from natural sources other than from vanilla pods. For instance, vanillin can either be produced through the biotransformation of an existing precursor compound or by de novo synthesis of a precursor where the organism produces an intermediate in vanillin biosynthesis. Biotransformation of vanillin precursor can also be achieved with microorganisms (Fig. 3), e.g., fungi.

Indeed, the involvement of fungi in the production of metabolites related to vanilla aroma is observed for several compounds such as *p*-hydroxybenzaldehyde, vanillic acid, and vanillyl alcohol. In this way, the fungus *Paecilomyces variotii* cultured on minimal medium converts *p*-coumaric acid to *p*-hydroxybenzaldehyde [20]. Vanillic acid is formed from vanillin by *Hormodendrum* sp. grown on basal medium [21]. Vanillyl alcohol is made by *Pestalotia palmarum* from ferulic acid grown on synthetic medium supplied with glucose [22]. The aforementioned compounds are all involved with vanilla aroma.

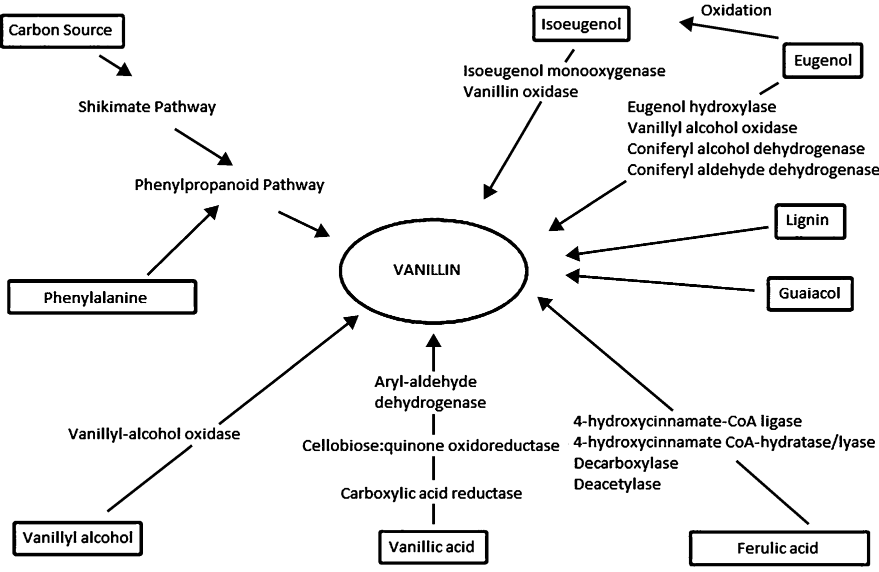

Fig. 3 Different microbial routes to vanillin (Source, Khoyratty et al. [12])

Additionally, the simplicity of the vanillin structure has led to the use of various precursors in the microbial/fungal or enzymatic process of vanillin production: lignin, curcumin, siam benzoin resin, phenolic stilbenes, isoeugenol, eugenol, ferulic acid, aromatic amino acids, and glucose via de novo biosynthesis. Hence, comparable biotransformation reactions with regard to compounds related to vanilla aroma occur in fungi when compared to vanilla plants. Given such similarities in the biosynthetic pathways of polyphenols in vanilla plants and fungi, it is not surprising that a possible role of microorganisms in vanilla has been investigated before. Roling et al. [11] and Dunphy and Bala [4, 23], for example, studied a possible involvement of microorganisms during the curing of the pods all pointing principally to the occurrence of bacteria and actinomycetes. The current study concerns another aspect though: the possible role of fungal endophytes in the vanilla plant and the green pods in the formation of vanilla flavor-related compounds.

4.1 Identifying Flavor-Related Metabolites

After isolating and characterizing the fungal endophytes from vanilla, a series of experiments were conducted to elucidate their biotransformation abilities. The cultural conditions in which fungi are placed affect their biotransformation reactions, e.g., the amount of the bioactive secondary metabolite arundifungin produced by the endophytic fungus *Arthrinium* isolated from plant roots of *Apiospora montagnei* Sacc. changes depending on the time of incubation, temperature, and pH of the culture medium [24]. As a consequence, the media on which the fungi were cultured

in the laboratory was made to be the closest to that of the conditions in the green pod where they were isolated. Thus, to investigate the potential changes that fungal endophytes produce on flavor-related metabolites in green vanilla pods, experiments were conducted where fungi isolated from mature green pods (8 months after pollination) that were cultured on a medium composed of lyophilized and autoclaved green pod material. The lyophilized green pod material was the only source of available nutrients for fungi to grow in the experiments. Consequently, any fungal growth is due to the ingredients of the green pods, which includes various primary metabolites, including sugars and amino acids, as well as the various phenolics including vanillin glycoside.

Endophytic fungi *D. phaseolorum* (MOTU7), *P. microspora* (MOTU10), *F. oxysporum* (MOTU24), *Nigrospora* sp. (MOTU13), *N. bipapillata* (MOTU8), *M. marksii* (MOTU18), *A. implicatum* (MOTU4), *B. ribis* (MOTU16 – 61G1 isolated at St. Rose, Reunion Island), *C. gloeosporioides* (MOTU11), *F. proliferatum* (MOTU1), *B. ribis* (MOTU16 – 25 isolated at St. Anne, Reunion Island), *P. phyllanthicola* (MOTU6), and a vanilla pathogen *F. oxysporum* (MOTU24) were used for such experiments. Preliminary experiments were carried out to find the growth rate on 8-month-old pod-based media of the fungi selected for this work. It was found that on average, the fungi covered a 90 mm petri dish in 30 days. Hence, 30 days of culture was the time retained for the experiments. After 30 days of culture, the medium was analyzed through ^{1}H NMR. In all the experiments conducted, the same biotransformation medium containing grind green pod material was used. However, different fungi were cultured on this common media. Before any further investigations can be pursued into the biotransformation abilities of the fungi with respect to flavor compounds, it is essential to confirm the identity of the metabolites that had been biotransformed and the new products that are formed. In order to identify the products in the medium after 30 days of fungal growth, two approaches were adopted. The first approach consisted in identifying flavor-related metabolites and sugars present in green pods by comparison of the NMR spectra of medium extracts against the NMR spectra of reference compounds. In this way in the control medium made of green pods as well as in the spent medium, eight molecules of interest were identified (vanillyl alcohol, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillic acid, vanillin, glucovanillin, glucose, and sucrose) (Fig. 4 a, b, c, and d show an identification example of the ^{1}H NMR spectra of the medium on which *P. microspora* was cultured compared to the control).

The second approach was to perform an HPLC analysis on the same samples so as to confirm the identity of the compounds found in the ^{1}H NMR analysis (Fig. 5 a, b). Additionally, *p*-coumaric acid was identified by HPLC but not by ^{1}H NMR due to the higher sensitivity of HPLC to detect compounds present at a lower concentration of detection than NMR can detect (*p*-coumaric acid concentration, 0.214 mmol/L of medium in the control and 0.156 mmol/L of medium in the spent medium on which *P. microspora* was cultured). Based on the signal intensity in the ^{1}H NMR spectra, sucrose disappeared completely from the medium, while the level of glucose increased (Fig. 4 (c, d)).

4.2 Comparing the Biotransformation Reactions Across Fungi

The scatter score plot of the principal component analysis (PCA) of the ^{1}H NMR spectral data of the pod-based media shows that metabolites present from the control medium (green pod media only without any fungal culture initiated) place it alone in quadrant 1 relative to the experimental samples. This implies that there were significant differences between metabolites present in the control compared to the experiments where 12 fungi were cultured individually on the same media made of green pod material (Fig. 6).

^{1}H NMR spectral data from the media on which *D. phaseolorum* (MOTU7), *P. microspora* (MOTU10), and *F. oxysporum* (MOTU24) were cultured shows that they cluster together in quadrant 2, whereas the spectral data for the media on which *Nigrospora* sp. (MOTU13), *N. bipapillata* (MOTU8), and *M. marksii* (MOTU18) were cultured shows that they cluster together in quadrant 3. Finally, the spectral data for the media on which *A. implicatum* (MOTU4), *B. ribis* (MOTU16 – 61G1), *C. gloeosporioides* (MOTU11), *F. proliferatum* (MOTU1), *B. ribis* (MOTU16 – 25), and *P. phyllanthicola* (MOTU6) were cultured shows that they cluster together in quadrant 4. The different fungi did not cluster based on their order or genus, for instance, despite belonging to the same genus and based on metabolite composition, medium on which *F. oxysporum* (MOTU24) was cultured occurs in quadrant 2, whereas medium on which *F. proliferatum* (MOTU1) was cultured occurs in quadrant 4. This is so despite quadrants 2 and 4 being antagonistic in terms of metabolites, i.e., metabolites which are present in a higher concentration in quadrant 2 would be in a lower concentration in quadrant 4 and vice versa. The results thus show that it is not possible to predict the biotransformation abilities of the fungus based on the order and genus that they belonged to and that such empirical data from experiments are important to decipher the connection between specific endophytes and flavor development in vanilla pods. This thus renders building a hypothesis from literature for potential fungal endophytes with specific effects on flavor compounds difficult. Furthermore, *F. oxysporum* (MOTU24) is a pathogen [25] which was introduced in this work so as to compare the differences in biotransformation abilities of a pathogen from vanilla compared to endophytes from the same plant and on the same green pod-based medium.

It is essential to know which metabolites contribute significantly to separate the fungi in the PCA score plots as well as finding the relationship between such metabolites. This would then form an indirect method of assessing the differences in biotransformation abilities of the fungi, in terms of metabolites converted and products formed. Particularly, it is necessary to find whether such metabolites that demarcate the fungi on the PCA score plot in Fig. 7 are flavor-related molecules in vanilla. In order to elucidate the identity of such molecules, a scatter loading plot was constructed based on the PCA score plot results from the ^{1}H NMR analysis of medium on which endophytic fungi as well as a pathogen (MOTU24 *F. oxysporum*) was cultured (Fig. 7).

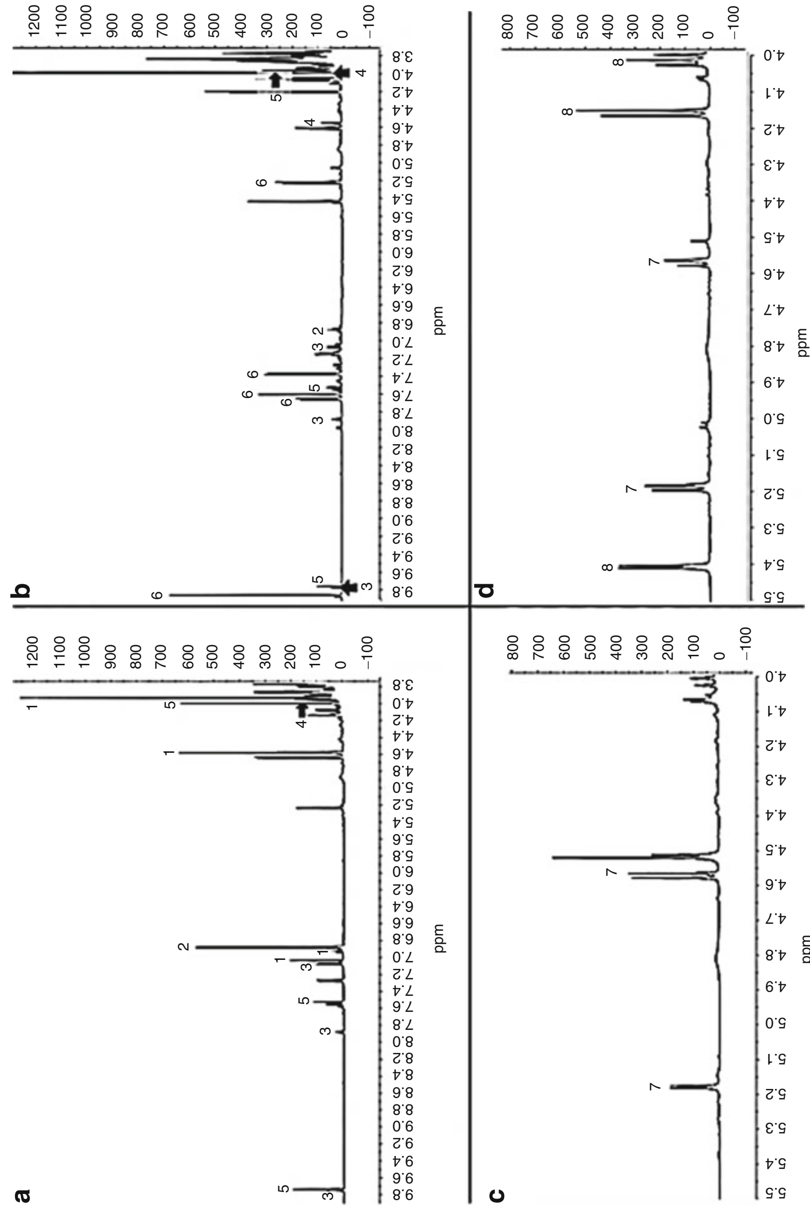

Fig. 4 (continued)

In terms of events, the fungal endophytes isolated from mature green pods are present before the time of pod curing. The very presence of such endophytes in green pods imply that the fungi are all able to feed onto pod material while being unaffected by the antimicrobial properties of *V. planifolia* [26]. The metabolites vanillin, *p*-hydroxybenzaldehyde, and vanillyl alcohol contribute to the final dark pod vanilla flavor. The scatter loading plot in Fig. 7 shows that all three metabolites occur in quadrant 2 only. Hence, media on which fungi *D. phaseolorum* (MOTU7), *P. microspora* (MOTU10), and *F. oxysporum* (MOTU24) were cultured also clustered in quadrant 2 on the PCA (Fig. 6). The three aforementioned fungi are thus associated with a higher concentration of vanillin, *p*-hydroxybenzaldehyde, and vanillyl alcohol essential to vanilla flavor compared to the control and to all other fungi tested in this part of the work. Although within the green pod, the three aforementioned molecules do not occur freely and rather occur in glycosylated form; it is possible to imagine a situation where the fungi would affect the relative ratios at which the three flavor-related molecules would occur in the pod prior to curing. However, flavor-related phenolics occur in glycosylated form in the mature green pods in order to render them less toxic. Despite the results here do not show this, it is possible that the biotransformed molecules would be again glycosylated by the green pod living plant material, but this time, the ratio at which such molecules occur in the green pod would differ due to the biotransformation intervention of the fungi.

However, this later hypothesis can be checked with further research. If quadrant 2 harbors those fungi that could potentially have an effect on vanilla flavor (Fig. 6), the antagonistic fungi to those in quadrant 2 (based on the final metabolites present in the medium after the experiment) are those in quadrant 4, i.e., *A. implicatum* (MOTU4), *B. ribis* (MOTU16 – 61G1), *C. gloeosporioides* (MOTU11), *F. proliferatum* (MOTU1), *B. ribis* (MOTU16 – 25), and *P. phyllanthicola* (MOTU6). Given that the aforementioned fungi are antagonistic to those (*D. phaseolorum* (MOTU7), *P. microspora* (MOTU10), and *F. oxysporum* (MOTU24)) that influence the quality of the green pods with regard to vanilla flavor, it is possible that controlling fungi present in quadrant 4 (Fig. 6) through the application of targeted fungicides might improve the quality of the green pods. Further research in this direction may be conducted. In extreme cases of infection, the pathogenic fungus *F. oxysporum* (MOTU24) kills vanilla plants. However, the results in the PCA score plot (Fig. 6) show that the medium on which this fungus was grown occurs in quadrant 2 in Fig. 6, which is the quadrant associated with a major

Fig. 4 ^{1}H NMR spectra (methanol-*d*4-KH_2PO_4 in D_2O, pH 6.0 extract) of medium on which *P. microspora* was cultured (Medium 1) and of the control. (**a**) Spectrum of Medium 1 in the range δ 3.7–9.9. (**b**) Spectrum of the control media in the range δ 3.7–9.9. (**c**) Spectrum of Medium 1 in the range δ 4.1–5.5 (carbohydrates range). (**d**) Spectrum of the control medium in the range δ 4.1–5.5 (carbohydrates). The assigned peaks are as follows: *1*, vanillyl alcohol; *2*, *p*-hydroxybenzoic acid; *3*, *p*-hydroxybenzaldehyde; *4*, vanillic acid; *5*, vanillin; *6*, glucovanillin; *7*, glucose; and *8*, sucrose (Source, Khoyratty et al. [12])

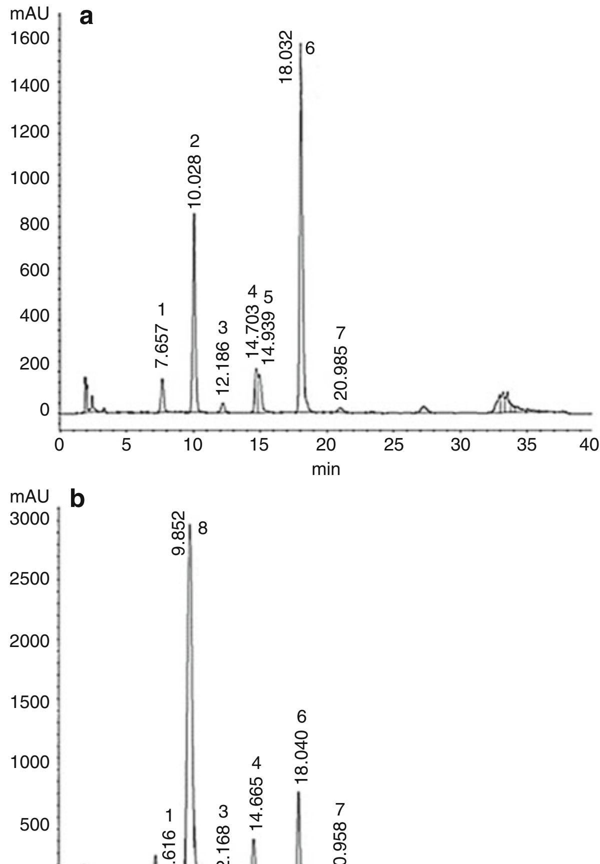

Fig. 5 HPLC profile with a retention time range of 0–40 min. (**a**) Spectra of medium on which *P. microspora* was cultured (Medium MOTU10); (**b**) Spectra of the control medium. The assigned peaks are as follows: *1*, *p*-hydroxybenzyl alcohol; *2*, vanillyl alcohol; *3*, *p*-hydroxybenzoic acid; *4*, *p*-hydroxybenzaldehyde; *5*, vanillic acid; *6*, vanillin; *7*, *p*-coumaric acid (detected in HPLC only); and *8*, glucovanillin. The retention times are shown next to each peak (Source, Khoyratty et al. [12])

amount of three vanilla flavor-related compounds in the experimental medium (Fig. 7). This implies that *F. oxysporum* (MOTU24) has the ability to influence the amount of flavor present in green pods. This would then affect the amount of flavor metabolites in the mature green pods that would be available for postharvest curing. It is to be noted that the fungal endophytes used in this work were isolated from such mature green pods.

Fungi need a carbon source to grow and develop and can use various sources. However, they vary in their ability to utilize different carbon sources and thus show some form of adaptation to their environment. For instance, different fungal taxa target different carbon sources [27]. However, not all fungi tested consume the same type and amount of carbon sources. For instance, quadrant 3 on the scatter loading plot (Fig. 7) consists of those fungi that consume sucrose and glucose as carbon sources. Such fungi include *Nigrospora* sp. (MOTU13 *N. bipapillata* (MOTU8), and

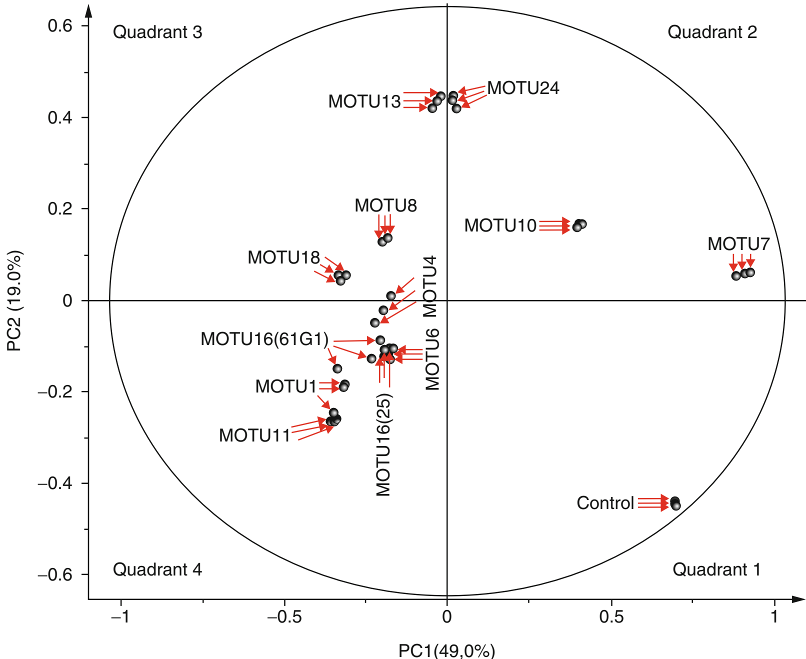

Fig. 6 Scatter score plot of principal components (*PC*) 1 and 2 of the principal component analysis (*PCA*) results obtained from ^{1}H NMR spectral data of the pod-based media on which fungi were cultured and scaled to Pareto distribution. Twelve fungi were used for culture; additionally, a control was included: MOTU1, *Fusarium proliferatum*; MOTU4, *Acremonium implicatum*; MOTU6, *Phomopsis phyllanthicola*; MOTU7, *Diaporthe phaseolorum*; MOTU8, *Nemania bipapillata*; MOTU10, *Pestalotiopsis microspora*; MOTU11, *Colletotrichum gloeosporioides*; MOTU13, *Nigrospora* sp.; MOTU16–25, *Botryosphaeria ribis*; MOTU16-61G1, *Botryosphaeria ribis*; MOTU18, *Mycosphaerella marksii*; and MOTU24, *Fusarium oxysporum*.) (Source, Khoyratty et al. [12])

M. marksii (MOTU18) according to the scatter score plot in Fig. 6. Additionally, quadrant 4 on the scatter loading plot (Fig. 7) is characterized by those fungi that consumed vanillin as a carbon source and, according to the scatter score plot (Fig. 6), include fungi *A. implicatum* (MOTU4), *B. ribis* (MOTU16 – 61G1), *C. gloeosporioides* (MOTU11), *F. proliferatum* (MOTU1), *B. ribis* (MOTU16 – 25), and *P. phyllanthicola* (MOTU6).

Le Comité d'experts FAO/OMS sur les additifs alimentaires [28] states that the organoleptic property of vanillyl alcohol is defined as balsamic, vanilla-like flavor. Although vanillyl alcohol is not the only molecule in vanilla to have an organoleptic balsamic flavor, an increase in its amount would certainly contribute to this flavor. Furthermore, gas chromatography-olfactometry (GC-O) analysis shows that although vanillyl alcohol is present in pods at a much lower concentration than vanillin, its contribution to aroma is as intense [29]. According to Ranadive [8], the Bourbon-type vanilla, which includes pods from Réunion Island, is characterized by

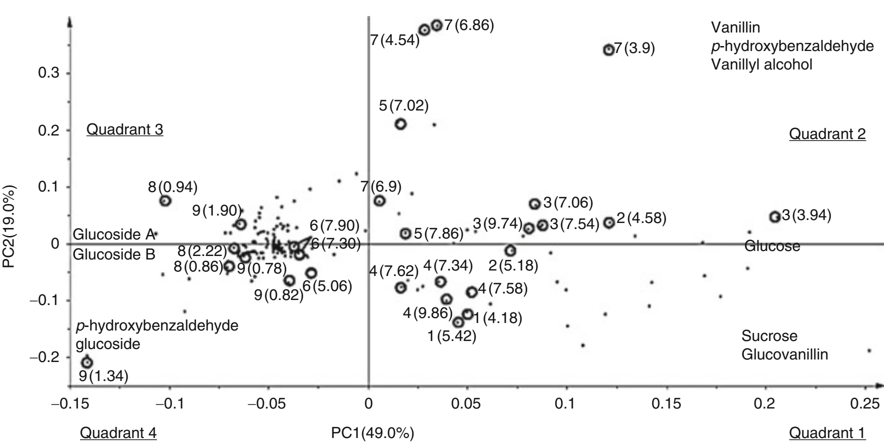

Fig. 7 Scatter loading plot of principal component (*PC*), one and two of the principal component analysis (*PCA*) results obtained from ^{1}H NMR spectral data of the pod-based media on which fungi were cultured and scaled to Pareto distribution. Chemical shifts identified for each metabolite are shown in bracket in the figure; the chemical shifts from literature (all from [17]) are shown in brackets in this figure legend, as follows, next to the numbers (one to nine) that identify the metabolites in the above figure: 1, sucrose (chemical shifts, δ 5.4, δ 4.17); 2, glucose (chemical shifts, δ 5.18, δ 4.57); 3, vanillin (chemical shifts, δ 9.73, δ 7.52, δ 7.49, δ 7.04, δ 3.93); 4, glucovanillin (chemical shifts, δ 9.82, δ 7.62, δ 7.57, δ 7.34, δ 5.19, δ 3.95); 5, *p*-hydroxybenzaldehyde (chemical shifts, δ 9.75, δ 7.85, δ 7.04); 6, *p*-hydroxybenzaldehyde glucoside (chemical shifts, δ 9.84, δ 7.94, δ 7.29, δ 5.01); 7, vanillyl alcohol (chemical shifts, δ 3.75, δ 4.38, δ 5.02, δ 6.71, δ 6.72. δ 6.89, δ 8.79); 8, bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-isopropyltartrate (glucoside A) (chemical shifts, δ 2.20, δ 0.92, δ 0.86); and 9, bis[4-(β-D-glucopyranosyloxy)-benzyl]-2-(2-butyl)tartrate (glucoside B) (chemical shifts, δ 1.90, δ 1.35, δ 1.10, δ 0.84, δ 0.77). Quadrants were labeled with metabolite(s) that predominate in amount in each (Source, Khoyratty et al. [12])

an intense balsamic flavor. The presence of high amounts of vanillyl alcohol in the media (Figs. 6 and 7) on which *D. phaseolorum* (MOTU7), *P. microspora* (MOTU10), and *F. oxysporum* (MOTU24) were cultured is hence of interest with regard to vanilla flavor. This may then contribute to the intense balsamic flavor which is characteristic of Bourbon-type vanilla.

4.3 Amounts of Biotransformed Flavor Metabolites by Fungi Grown on the Same Media

Catabolism of the compounds present in the same green pod-based media by each fungus alters the amounts of flavor-related metabolites present in the medium, in which the mature green pod material was the only nutrient for the fungus. In order to quantify the bioconversion by a selection of the tested fungi, calculations on the ^{1}H NMR analysis of metabolites from the media (Table 4) were performed.

The quadrant in which each medium occur in the principal component analysis (PCA) (Figs. 6 and 7) is also indicated below. The quantification was performed

through ^{1}H NMR (micromoles per gram DW green pods, n = 3 biological replicates).

Six media, including the control, were chosen on which the fungi MOTU10 *P. microspora*, MOTU7 *D. phaseolorum*, MOTU24 *F. oxysporum*, MOTU8 *N. bipapillata*, and MOTU1 *F. proliferatum* were cultured. The choice was made based on the PCA score plot results (Fig. 6) where at least one representative media from each quadrant was chosen, in order to have a quantitative overview of metabolites that characterize each quadrant. However, all media in quadrant 2 (Fig. 6) were selected given that media in this quadrant were clustered together based on the presence of high amounts of vanilla flavor-related metabolites according to the scatter loading plot (Fig. 7). The quadrant from which the chosen media belongs to on the PCA score plot is indicated in Table 4 (quadrant in PCA in which media occurs, 1, 2, 3, and 4).

The amounts of nine metabolites in the samples was calculated from ^{1}H NMR data: six compounds related to vanilla flavor that were identified by ^{1}H NMR (vanillin, glucovanillin, *p*-hydroxybenzaldehyde, vanillyl alcohol, vanillic acid, *p*-hydroxybenzoic acid) as well as three carbohydrates (sucrose, glucose, and fructose), all of which separate the samples onto the different quadrants on the PCA score plot (Fig. 6).

The amounts of the selected nine metabolites present in the medium differed depending on the fungus that was grown on that medium (Table 4). There was no fructose in the spent media on which *F. oxysporum* (Medium MOTU24) and *F. proliferatum* (Medium MOTU1) were cultured. It is possible that the fructose was consumed by the fungi given that *Fusarium* spp., such as *F. oxysporum* f.sp. *cubense*, consume fructose [30]. However, the amount of fructose in the spent Medium MOTU7 on which *D. phaseolorum* was cultured is almost that of the amount of sucrose in the control medium. Given that sucrose was completely consumed, the fructose may thus have come from the hydrolysis of sucrose. Depending on the length of time of culture, the fungi would deplete the media completely of sugars as was the case in this work. The amounts of the flavor-related metabolites vanillin, vanillyl alcohol, vanillic acid, and *p*-hydroxybenzoic acid increased in the medium on which *P. microspora* (Medium MOTU10) was cultured compared to the control. Whereas in Medium MOTU7 (cultured with *D. phaseolorum*), the amounts of vanillin, vanillic acid, and *p*-hydroxybenzoic acid, but not vanillyl alcohol, were higher than in the control medium. The amounts of vanillin in Medium MOTU24 (on which the vanilla pathogen *F. oxysporum* was cultured) as well as that in Medium MOTU8 (on which *N. bipapillata* was cultured) were lower than that of the control. Both Media MOTU8 and MOTU1 (on which *F. proliferatum* was cultured) had lower amounts of flavor-related metabolites compared to the control with the exception of vanillic acid and *p*-hydroxybenzoic acid which were higher. Overall, the quantity of flavor-related metabolites in the medium tends to decrease from medium that are placed in quadrant 2 (highest quantity, Table 4), moving to lower amounts in quadrant 3 and quadrant 4 (lowest quantity, Table 4). Medium MOTU7 shows the highest vanillin amount, whereas Medium MOTU10 shows the highest vanillyl alcohol amount.

Table 4 Average amounts with standard deviation of different forms of vanillin and related compounds in media as well as carbohydrates on which *Pestalotiopsis microspora* (Medium MOTU10), *Diaporthe phaseolorum* (Medium MOTU7), *Fusarium oxysporum* (Medium MOTU24), *Nemania bipapillata* (Medium MOTU8), and *Fusarium proliferatum* (Medium MOTU1) were cultured for 30 days (Source, Khoyratty et al. [12])

Quadrant in PCA in which media occur	1	2	2	2	3	4
Metabolite/medium	Control	MOTU10	MOTU7	MOTU24	MOTU8	MOTU1
Vanillin	$(17.0 \pm 0.6) \times 10^{-3}$	$(37.0 \pm 1.61) \times 10^{-3}$	$(56.0 \pm 11.2) \times 10^{-3}$	$(1.9 \pm 0.5) \times 10^{-3}$	$(1.4 \pm 2.4) \times 10^{-3}$	0
Glucovanillin	$(127.0 \pm 2.1) \times 10^{-3}$	0	0	$(0.9 \pm 0.2) \times 10^{-3}$	0	0
p-Hydroxybenzaldehyde	$(6.2 \pm 0.7) \times 10^{-3}$	$(3.9 \pm 0.5) \times 10^{-3}$	$(9.0 \pm 1.7) \times 10^{-3}$	$(1.2 \pm 0.3) \times 10^{-3}$	0	0
Vanillyl alcohol	$(15.5 \pm 7.0) \times 10^{-3}$	$(100.0 \pm 6.3) \times 10^{-3}$	$(38.6 \pm 6.6) \times 10^{-3}$	$(11.7 \pm 2.6) \times 10^{-3}$	$(7.7 \pm 5.5) \times 10^{-3}$	$(3.1 \pm 1.2) \times 10^{-3}$
Vanillic acid	0	$(9.2 \pm 6.1) \times 10^{-3}$	(7.1 ± 2.7) x 10^{-3}	$(1.0 \pm 0.2) \times 10^{-3}$	$(1.9 \pm 1.5) \times 10^{-3}$	$(2.9 \pm 2.6) \times 10^{-3}$
p-Hydroxybenzoic acid	$(7.4 \pm 0.9) \times 10^{-3}$	$(87.9 \pm 12.7) \times 10^{-3}$	$(16.5 \pm 4.3) \times 10^{-3}$	$(13.3 \pm 2.5) \times 10^{-3}$	$(17.1 \pm 12.1) \times 10^{-3}$	$(1.0 \pm 1.7) \times 10^{-3}$
Sucrose	350.5 ± 82.7	0	0	0	0	0
Glucose	54.3 ± 7.3	143.5 ± 8.7	0	6.6 ± 2.1	23.1 ± 19.5	43.1 ± 17.6
Fructose	0	118.9 ± 2.5	279.9 ± 75.3	0	0	0

Hence, in Medium MOTU10, most of the vanillin glucoside was converted into vanillyl alcohol, by *P. microspora*, which increased more than sixfold relative to the control. Vanillin increased more than twofold. But the overall picture is that in the mass balance, only a small amount of about 10 % vanillin is lost through the bioconversion by *P. microspora*, whereas with *F. proliferatum* (Medium MOTU1), a 100 % loss of vanillin was observed. Sucrose was completely consumed by all fungi tested, whereas glucose amount increased in Medium MOTU10. Overall, from the ^{1}H NMR analysis, it was observed that all fungi tested catabolized either all or almost all (as in the medium MOTU24) glucovanillin present in the media without an increase in vanillin content, i.e., the amount of vanillin in the experiments were lower than that in the control except in the case of MOTU10 *P. microspora* and of MOTU7 *D. phaseolorum*, where the medium showed an increase in vanillin amount in the experimental medium compared to the amount present in the control.

As a consequence, not all fungi contribute equally to a change in vanilla flavor in tests conducted under laboratory conditions. It is thus conceivable that some fungi would improve the aromatic quality of the pods, while others would decrease. Moreover, the different fungi tested would interact together in a concerted manner in the pod. However, the latter situation is beyond the scope of the experiments carried out in this work but could prove as a potential future work conducted on aseptic living green pods and thus possibly bypass the time-consuming curing process. The experiments conducted here concern dead aseptic green pod material.

4.4 Ratios of Quality Marker Metabolites After Biotransformation

In view of evaluating the quality of the material obtained after *P. microspora* (Medium MOTU10), *D. phaseolorum* (Medium MOTU7), *F. oxysporum* (Medium MOTU24), *N. bipapillata* (Medium MOTU8), and MOTU1 *F. proliferatum* (Medium MOTU1) cultures, a comparison is necessary to reference parameters that define quality in cured vanilla pods, the final product of vanilla production.

A method of evaluating quality involves a calculation based on the ratios in which the four quality markers (*p*-hydroxybenzoic acid (p-HB acid), *p*-hydroxybenzaldehyde (p-HBAld), vanillic acid, and vanillin) occur in cured pods [8]. Relative to the ratios in the control medium, not all fungal biotransformations produced the same ratios R1 (vanillin/p-HBAld), R2 (vanillic acid/p-HBAld), R3 (p-HB acid/p-HBAld), R4 (vanillin/vanillic acid), and R5 (vanillin/ p-HB acid) (Table 5) where p-HBAld is *p*-hydroxybenzaldehyde and p-HB acid is *p*-hydroxybenzoic acid.

The ratios associated to quality in dark pods that are indicated were *p*-hydroxybenzaldehyde (*p*-HBAld) and *p*-hydroxybenzoic acid (*p*-HB acid). The values from Medium MOTU10, MOTU7, MOTU24, MOTU8, and MOTU1 which are either within the range of closest to the range of the references are underlined and in bold.

Table 5 Ratios of various vanilla compounds related to quality in the control and in the spent medium on which MOTU10 *Pestalotiopsis microspora*, MOTU7 *Diaporthe phaseolorum*, MOTU24 *Fusarium oxysporum*, MOTU8 *Nemania bipapillata*, and MOTU1 *Fusarium proliferatum* were cultured for 30 days (Source, Khoyratty et al. [12])

Sample	R1 = vanillin/p-HBAld	R2 = vanillic acid/p-HBAld	R3 = p-HB acid/p-HBAld	R4 = vanillin/vanillic acid	R5 = vanillin/p-HB acid
Control	2.73	–	1.19	–	2.30
Medium MOTU10	9.54	2.37	22.82	4.02	0.42
Medium MOTU7	6.24	0.78	1.83	7.98	3.42
Medium MOTU24	1.57	0.83	11.07	1.89	0.14
Medium MOTU8	–	–	–	0.74	0.08
Medium MOTU1	–	–	–	–	–
Reference ratios (cured pods, [8])	10–20	0.53–1.5	0.15–0.35	12–29	40–110

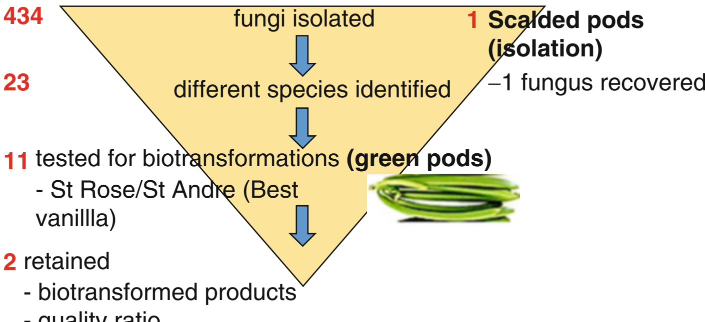

Fig. 8 Number of fungi isolated in relation to the number of fungi found to affect content of vanilla aroma-related compounds

Medium MOTU10 had the closest ratio R1 to the reference, Medium MOTU7 and 3 to that of R2, and Medium MOTU7 to that of R4. However, the ratios R3 and R5 for Medium MOTU10, MOTU7, MOTU24, MOTU8, and MOTU1 are well outside the acceptable reference range for good quality cured beans.

5 Conclusions

Out of 434 isolated fungi, 23 species were identified. Eleven fungi were tested for their abilities to biotransform green pod material into compounds related to vanilla aroma. Two were retained for having such abilities (Fig. 8).

In this case, only 0.4 % of isolated fungi were related to the biotransformation reaction function of interest. This is so given that endophytic fungi present inside a plant do not all interact with the same metabolic pathways of the plant. As a consequence, it is always necessary in such type of work, to search from a large set of fungi for the few fungi of interest. The two fungi that were retained (*P. microspora*, *D. phaseolorum*) are promising toward influencing vanilla aroma. It is thus possible to infect plants with both fungi to improve vanilla aroma in pods. The latter possibility is subject to further research.

Furthermore, as with the patent application from Givaudan [6], the two fungi could aid to skip the laborious and costly curing process, i.e., the harvested green pods may be transformed by fungal culture into a material comparable to dark pods within a short lapse of time. This process can decrease costs and the time for the final product to be ready by bypassing the curing process.

Additionally, the fungus recovered post-scalding was different from those recovered pre-scalding. This is important given the different events that occur during the curing process of vanilla pods. Hence, it might be possible that a difference in endophytic composition at different stages in curing is correlated to different aroma development. The latter can form part of further research work.

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Aflatoxin in Rice Crop: Prevalence and Assessment of Daily Exposure

6

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Abstract

Aflatoxin B1 is one of the most hepatocarcinogenic naturally occurring compounds known, produced by toxic species of fungi in different types of food including rice. The contamination of food with this toxin could lead to a series of health problems and huge economic losses. Rice is the second largest quantity staple food and internationally traded cereal. Aflatoxin is produced in areas where climatic conditions are favorable to fungal growth and the production of aflatoxin affects plant growth and rice yield. The aim of this review article is to show and explain the levels of aflatoxin contamination of rice worldwide during the period 2004–2014. In general, aflatoxin levels in rice are low and vary from country to country. However, the high daily intake of rice makes even these lower levels of concern, as aflatoxin B_1 (AFB1) is carcinogenic and has been correlated with hepatocellular carcinoma (HCC) incidence in some countries. In addition to the increased distribution of aflatoxins in rice being addressed, the analytical procedures and the local and global permissible limits for aflatoxins are presented and discussed.

Keywords

Aflatoxin • Rice • Worldwide contamination • Health • Economy

1 Introduction

1.1 The Toxicity Aflatoxin

The aflatoxins (AFs) are natural carcinogenic secondary metabolites produced by several *Aspergillus* species including *A. flavus* and *A. parasiticus* (Rasooli and Abyaneh 2004) and rarely by *A. nomius* ([56, 57, Ok et al. 2014; [1]). AF has been discovered in 1961 after epizootic of "turkey X" disease in England that resulted from feeding peanut meal contaminated by this metabolite's molds. Investigations on these outbreaks showed the presence of the four main aflatoxins, named aflatoxin B_1, B_2, G_1, and G_2, the letters standing for the fluorescence color of blue and green and the numbers of the sequences of Rf values on thin layer chromatography plates [52]. Since that time, the occurrence and toxicity of AFs have been investigated in detail. AFB1 was classified by the International Agency for Research on Cancer (IARC) as a class 1A human carcinogen, because it has been found to be the most potent naturally occurring carcinogen to animals with a very strong association with hepatitis cellular carcinoma (HCC) [43, 45, 48, 59].

The order of potency for both acute and chronic toxicity of AFs is aflatoxin B_1 (AFB1) > G_1 (AFG1) > B_2 (AFB2) > G_2 (AFG2) [58]. It is known now that AFB1 is the most hepatocarcinogenic substance known to man [62, 55]. What is far more worrying is that death may result from the consumption of food that is heavily contaminated with this mycotoxin. Outbreaks of acute aflatoxicosis with high mortalities have been documented among humans and animals. Several reports indicate that the largest reported outbreak of aflatoxicosis in humans occurred in eastern Kenya in 2004 with 317 cases identified with a very high mortality rate of 68 % (215 deaths) [60, 61].

1.2 The Importance of Aflatoxin in Rice

Aflatoxin in many crops and food commodities, e.g., peanut, corn, nuts, and milk, has been widely investigated, but there are a limited number of reports on the incidence and levels of aflatoxins in rice worldwide. According to the Global Environmental Monitoring System of the World Health Organization [2], rice (*Oryza sativa* or *Oryza glaberrima*) is the second level of cereal staple consumed food worldwide after wheat and consists of the major part of the diets for half of the world. It is also reported that the rice forms 27 % of the global diet and 20 % of dietary protein intake in the developing countries (Ok et al. 2014). Further, the Food and Agriculture Organization (FAO) of the United Nations (UN) has reported in 2004 that rice is the major staple food for 17 countries in Asia, 9 countries in North and South America, and 8 countries in Africa [3].

The FAO in 2012 reported that there are 156 million cultivated hectares of rice, producing 721 million tons globally in 2011 [4]. China was the highest producer with 202.3 million tons followed by India with 154.5, whereas Latin America, the Caribbean, and Brazil produced the larger amount of rice of ~12 million tons in 2009/2010 [3]. In Africa, rice is mainly produced in Egypt and Nigeria [5], whereas in Europe it is mainly produced in France and Spain [4]. In some countries like the Philippines, rice formed 35 % of total food intake with the provisional tolerable daily intake (PTDI) of up to 280 g per adult person [2] and in Vietnam of up to 500 g per adult [6]. Rice is usually produced in climatic conditions favorable for fungal infection and growth, and hence potential mycotoxin contamination can occur before and after harvest [2]. However, the contamination reported for rice with fungi is lower than that for many other cereals [1]. Even so, that does not mean that fungal infection and mycotoxin contamination do not pose a real problem, as chronic intoxication can occur due to the exposure of low levels of AFB1 over a long period of time. An example of this was reported by Almeida et al. [3] who recorded approximate levels of 13.13 μg/kg of ABB1 with co-occurrence with other mycotoxins during outbreak of beriberi disease in 2006 in Brazil. This was attributed to the consumption of contaminated rice stored in inappropriate conditions.

2 Analytical Procedures for Determining of Aflatoxin in Rice Samples

2.1 Sampling

The sampling of materials, especially large bulk cereals, for contaminants is not easy and, if done correctly in a statistical manner, can be expensive. To take a sample(s) representing a ship's cargo or a bulk load being off-loaded from a train or trucks or from large storage silos, particular tools and equipment are required such as grain sampling spears, and these can be used for sampling different parts of the lot [7]. Moreover, the collected samples must be mixed well, which can include grinding before subsampling to give an appropriate sample size. The final samples are ground to small particle size and kept in appropriate condition for subsequent extraction and analysis. The bulk of rice sometimes needs to be stored in the field or retail stores before exporting and marketing. Researchers examining rice crops in countries of production are more likely to do sampling of the crop in the field after harvesting or in the retail stores rather than to collect them from the markets or homes. Even so, mycotoxin levels can increase during all these storage stages and/or transportation after initial sampling. Another important factor to consider is seasonal sampling, particularly in the regions affected by humid and hot seasons. Furthermore, the results can be improved by the care of following factors:

1. Specific geographical area in large countries such as China and India, as sometimes the environmental conditions may be completely different even if the crop produced in the same region
2. Number and distribution of collected samples, as it is well known that mycotoxins do not occur evenly distributed in seeds, individual plants, or plant populations
3. The size of the collected samples should, before subsampling, be at least 0.5 kg, also to decrease the error of mycotoxin distribution in the subsample.

An examination of the literature shows that Ok et al. [51] collected their 80 samples of rice from the farms in different areas, while Park et al. [21] in Korea took 88 samples from supermarkets and kept them at –20 °C before subsampling. Nguyen et al. [6] collected 100 field samples in two seasons (rainy and dry seasons) from five provinces of Vietnam. These were also kept at−20 °C before subsampling. In Vienna, 81 samples were purchased from different markets; 71 out of them were long grain rice, 5 were short grain rice, and 5 were puffed rice (Reiter et al. [16]). In China, 29 samples from households were collected [8], whereas Wang and Liu [9], collected 74 samples from eight regions, packed in dry and sealed containers. Reddy et al. [10] purchased 13 rice samples from different supermarkets in Penang (Malaysia), while Ghali collected 11samples from local markets of Tunisia and stored them in plastic bags at 4 °C until grinding and analysis [11]. In the Ivory Coast, Sangare-Tigori et al. [12] followed a statistically planned protocol for subsampling; ten rice samples from markets were collected, and from a 50 kg batch, a 5 kg subsample was

taken, ground, and mixed from which a final subsample was used for analysis, after storage at −20 °C.

In Canada, 200 samples were collected from markets (imported from the United States and Asian countries) over 2 years. The samples (1 kg) were ground and (400 g) were frozen at −20 °C before analysis [13]. In Brazil, 230 samples of rice (bran, rice husk, and broken) were collected from different regions in 2007–2009 [3]. In Spain and Mexico, a total of 67 rice samples (1 kg) were collected from local stores and supermarkets in 2008–2009. They were kept after drying at 60 °C in plastic bags before analysis [4]. Seventy-eight polished and nine brown rice samples (1 kg/t) were obtained in June 2003 in Southern and Central Mindanao, the highest rice-producing regions of the Philippines, using a good protocol of quarterly subsampling. In fact, they mixed the sample in an electric mixer, quartered it, took the two opposite quarters, and repeated this step until, approximately, a 250 g sample was obtained [2]. Makun et al. [5] collected 21 rice samples (about 0.5 kg each) from the fields during December 2008 from 21 villages in the traditional rice growing area of Niger and stored them in the deep freezer at −20 °C before analysis. Rice samples (99) from retail outlets in the south and central parts of Sweden were collected and then pooled, mixed, ground to a fine powder, and stored at room temperature before analysis [14].

2.2 Extraction

Aflatoxins are moderately polar compounds and, therefore, are likely to be soluble in and extracted in solvents of medium polarity. In term of polarity, AFG2 is the more polar followed by AFG1, AFB2, and then AFB1. Chloroform is the ideal extraction solvent; however, its use is banned due to its toxicity and it also tends to extract other oils such as vegetable oils [38, 39, 41]. Consequently, aqueous methanol or acetonitrile is the choice for extracting aflatoxin from rice as indicated by the literature. Methanol is the more commonly used solvent as it is also cheaper than the others; for example, a solvent mixture of methanol/water with the ratio rate of 80:20 v/v is widely used. Several studies have used different volumes of this mixture with respect to the sample weight to extract the AFs. Ok et al. used 100 ml to extract the toxins from 25 g of milled rice (Ok et al. 2014), whereas 20 ml for 10 g was used by Villa and Markaki [15], 200 ml for 50 g [32], 100 ml for 50 g [16], 40 for 10 g [11], 250 ml for 50 g [13], 120 ml for 25 g [4], 300 ml for 50 g [3], and 60 ml for 20 g [10]. Only in one study, a different ratio was used and this was 125 ml methanol/water with a ratio of (50:50, v/v) to extract 25 g of milled rice [8].

The other useful extraction solvent for AFs is aqueous acetonitrile where 100 ml of acetonitrile – 4 % aqueous solution of potassium chloride (9:1) was used to extract both AFB1 and ochratoxin A from 20 g of sample [6]. A 80 mL acetonitrile/water solution (84/16) for 20 g sample was used [9], 200 ml of 84 % acetonitrile for 50 g [14], and Chloroform (150 ml) was used in one study to extract 50 g of sample in the presence of water (25 ml) and diatomaceous earth [17].

2.3 Cleanup and Derivatization

In general, for precise and accurate monitoring of AFs in food commodities, the extraction step must be followed by a cleanup process. This step is very important in order to remove interfering substances and enhance the performance of detection, and to this effect, several techniques has been used. Immuno-affinity column chromatography [3, 4, 11–13, 16, 18, 49] and solid-phase extraction (SPE) chromatography ([14, 15, 17, 19, 20, 40, 51]) were employed by various workers. Although the immune-affinity column chromatography is relatively costly, it is the more predominant technique. Followed by SPE and more rarely used is classical liquid-liquid chromatography [42].

In case of use of HPLC, the derivatization of AFB1 and AFG1 to the more fluorescent AFB2a and AFG2a is required (Fig. 1). As AFB1 and AFG1 have less fluorescence properties than that of AFB2 and AFG2, they can reduce the efficiency of the detection. The modern technique of the Kobra cell has been used widely in several investigations [3, 5, 6, 11, 14, 16]. This technique of derivatization is based on brominating the aflatoxin type of interest using an electrochemical cell. The derivatization occurred during the run of samples, so it is a time-saving technique. However the cheaper and classical derivatization using trifluoroacetic acid (TFA) is still the predominant derivatization method used ([2, 4, 15, 18, 19]; [9, 51]). TFA works by hydroxylating the 8,9 alkene group in aflatoxin.

2.4 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is the most predominant technique used for separation, detection, and quantitation of aflatoxins in rice crop ([2–6, 11, 13–16, 18–20]; [9, 51]). Aflatoxins are fluorescent compounds and occur naturally in very small amounts of μg/kg. Therefore, the analysts adopt the fluorescence detector which is more sensitive than UV absorption, as the detector with the HPLC system. In general, the fluorescence detector is approximately 1,000 times more sensitive than the UV-Vis detector. Recently, the HPLC coupling with mass spectrometry is also becoming common but not in developing countries, as the technique is extremely expensive. A reverse phase column is normally used (C18), the main advantage being the use of water as a mobile phase constituent. This decreases the cost of separation and enhances the aflatoxin detection, as water is an important solvent for water-soluble fluorescent compounds. However, AFB1 and AFG1 have less fluorescence properties.

The retention time (RT) of aflatoxins in HPLC chromatography is dependent on the eluent and column used, temperature, pumping pressure, and rate of eluent passage. In reverse phase RP-HPLC, the derivatized AFG1 is eluted first, followed by the AFB1 derivative, then AFG2, and AGB2 last. The presence of one more oxygen in AFG2 and AFG1 makes them into more polar compounds, and the

Fig. 1 Aflatoxin B1 and G1 derivatization to the more fluorescent compounds using the trifluoroacetic acid (TFA) and Kobra cell

presence of an alkene group in AFG1 makes it less polar than AFG2, whereas in the derivatized toxin, a polar group is replaced by the alkene group. So the normal elution sequence of the four aflatoxins is AFG2, AFG1, AFB2, and AFB1 in reverse phase column chromatography.

2.5 Enzyme-Linked Immunosorbent Assays

Other methods used for aflatoxin monitoring and survey include the technique of enzyme-linked immunosorbent assays (ELISAs) [8, 10, 12, 21, 22]. ELISA is a simple and easy technique and is being rapidly developed for aflatoxin detection. It used for general screening as well as quantification. The disadvantage of ELISA is that it is temperature sensitive and therefore considered as a semiquantitative technique [23].

3 Occurrence of Aflatoxins in Rice

3.1 Aflatoxin B1

AFB1 is the most harmful mycotoxin found in rice samples in several investigations. Park et al. [21] reported that 5 out of 88 rice samples had an average contamination level of 4.8 μg/kg AFB1; a higher frequency of contamination of 51 out of 100 samples with an average of 3.31 μg/kg was reported by Nguyen et al. [6]; 29 samples analyzed by Sun et al. had levels with a mean concentration of approximately 0.57 μg/kg [8]. In another study, 16 out of 84 rice samples were contaminated with range of 0.15–3.22 μg/kg [9], and 9 of 13 rice samples analyzed by Reddy et al. had a mean value of 1.75 μg/kg [10], whereas Reddy et al. [22] found that 814 out of 1,200 rice samples had detectable levels and 50 % of 199 rice samples were found to be contaminated with average range of 0.34–0.39 μg/kg [13]. AFB1 were recorded in 23 out of 81 Austrian samples with an average of 1.97 μg/kg [16]. AFB1 was also detected in ten out of ten rice samples with an average concentration of 4.5 μg/kg [12]. AFB1 was detected in ten field samples with an average concentration of 60.3 ± 28.2 μg/kg and range of 4.0–304.4 μg/kg, in six stored samples with an average 12.2 ± 1.9 μg/kg and range of 5.6–17.6 μg/kg, and in five samples from markets with an average of 21.0 ± 4.7 μg/kg and mean level of 9.9–34.1 μg/kg [5].

These results indicate occurrence of AFB1 in rice samples with varied concentration but generally less than 5 μg/kg. However, it was observed in some samples that the AFB1 levels were extremely high as reported by Makun and coworkers above the EC regulation of 2 μg/kg, which is considered the highest permitted level for aflatoxin in any food worldwide. The intake of rice in Europe is very small compared to that of Asian, some Latin American, and African countries, where rice is the main dietary food with the consumption of at least one meal per day. Hence, the toxic and carcinogenic effects of AFB1 on humans in such countries are expected to be high due to long and repeated chronic exposure. The highest reported value for AFB1 in rice was 308 μg/kg [22], 153 times higher than the EC's permitted level, followed by 30 μg/kg [6], 10 μg/kg [12], and 8 μg/kg [21] in other samples. These levels of AFB1 are high with the highest likely to cause acute symptoms in humans. The presence of these levels in the rice is alarming and hence is of importance to specialists, governmental bodies, producers, and consumers. They would constitute

a leading role of AFB1 in rice in the spread of hepatocellular carcinoma (HCC) in countries with a high intake of rice. Thus the outcome of these investigations imposes a responsibility on member states of such populations, toward food safety for this staple food commodity.

3.2 Total Aflatoxin

The total aflatoxin content (AFB1+ AFG1 + AFB2 + AFG2) is no less important than the reporting on AFB1. The majority of regulations throughout the world cover the presence of total aflatoxin, but there are a few countries that also consider AFB1 individually such as EC laws. The carcinogenicity of AFG1, although not as commonly found as AFB1, should also be considered as it has been showed to form DNA adduct similar to that of AFB1 [24]. In addition, recent experimental work shows that AFG1 induces oxidative DNA damage [25]. The occurrence of several aflatoxins together was detected in rice samples, as indicated in the literature. However, the estimated total contamination levels are less with regard to other commodities, such as peanut and peanut products. The highest value of contamination with several aflatoxins in recent data (during 2004–2014) was reported by Makun et al. [5]. Aflatoxins were found in all 21 samples analyzed with contamination range of 27.7–371.9 μg/kg and average concentration of 82.5 ± 16.9 μg/kg. Similarly, 135 rice samples out of 230 were contaminated with an average concentration of 13.13 μg/kg [3]. Iqbal et al. [19] reported 185 out of 413 rice samples contaminated with mean concentration of 11.2 ± 3.91 μg/kg. Further, in limited survey, one sample out of 3 rice samples was contaminated with 14.7 μg/kg [20]; aflatoxins were detected in 24 out of 81 rice samples with an average contamination of 1.97 μg/kg [16]. Also 74 out of 78 and 23 out of 84 were found contaminated with mean concentration of 1.53 μg/kg [2] and 0.79 μg/kg [9], respectively. It is clear that the majority of rice samples were heavily contaminated with these mycotoxins. The reported higher value of 371.9 μg/kg was 92 times greater than 4 μg/kg legal limit of aflatoxin in rice prescribed by EC legislation. Other workers also reported very high incidence of aflatoxin in rice such as 68 μg/kg [19], 9.9 μg/kg [16], and 8.7 μg/kg [2]. Although 9.9 and 8.7 μg/kg is less than the US FDA limit of 20 μg/kg, these concentrations are expected to pose serious problems particularly in the region of high intake of rice, such as Asian countries.

3.3 Other Aflatoxins (AFG1, AFG2, and AFB2)

Special focus to these aflatoxins (AFG1, AFG2, and AFB2) is very rare as there are no regulations for these aflatoxins individually. The toxicity of AFG2 and AFB2 gets less attention; hence little is known about these aflatoxins. Moreover, the toxicity of AFG1 becomes known recently [25], which is considered within the total aflatoxins. The international regulatory bodies consider the total aflatoxins as harmful substances, including all the four aflatoxins: AFB1, AFG1, AFG2, and AFB2.

Some studies were focused on these individual aflatoxins (AFG1, AFB2, and AFG2) and their levels were demonstrated. Of the 78 analyzed rice samples, 74 were found to be contaminated with AFG1 which ranged from "not detected" to "0.93 μg/kg" with an average value of 0.08 μg/kg. In these samples, AFB2 ranged from "not detected" to 0.33 μg/kg with the average value of 0.08 μg/kg [2]. Wang and Liu [9] have reported AFG1 in 7 out of 84 samples with contamination range of 0.36–1.59 μg/kg. AFB2 was found in three samples with contamination range of 0.06–0.24 μg/kg, while AFG2 was not detected in all the samples. Another investigation carried out using 32 rice samples detected AFB2 in 1 sample (0.7 μg/kg), AFG1 in 20 samples (1.6–16.3 μg/kg), and AFG2 in 31 samples (1.6–25.9 μg/kg) [4]. Further, of the 24 analyzed samples, AFG1 was not detected in all the samples, while AFG2 was recorded in only one sample. The concentration was less than the limit of quantification. Though AFB2 was found in 14 samples, the concentration was less than the limit of quantification, and only one sample had detectable concentration of 1.53 μg/kg. However, these results could be improved by increasing the concentration of extract residue as well as increasing the volume of injected sample [16]. Another study had considered only AFB2 in the investigated samples. AFB2 was detected in 23 out of 100 rice samples with contamination value ranging from 0.002–0.63 μg/kg and average value of 0.08 μg/kg [13].

These studies have clearly demonstrated the occurrence of all types of natural aflatoxins in rice crops. However, as expected, the level is less than the reported levels of aflatoxin B1. This is in consistence with the literature showing the predominance of AFB1 over other aflatoxins. Hence, the rice crop is contaminated with all the four types of aflatoxins.

4 Levels and Exposure to Aflatoxins Globally

4.1 Asia

Rice crop is a favorable food diet in the majority of Asian countries. According to report published by FAO, the average daily intake of rice in selected Asian countries is 269 g per day per person. The average contamination ranged from 0.5 μg/kg in China [8] to 45 μg/kg in India [17].

AFB1 was investigated in rice samples in several countries of the Western Pacific Region. The higher value of contamination was reported in samples from South Korea, followed by Malaysia, and then China. In Korea, two investigations were conducted. [21] reported the presence of AFB1 in 5 out of 88 samples with the average concentration of 4.8 μg/kg. Recently, aflatoxin was detected in six rice samples in range between 0.7 and 2.7 μg/kg which was lower than the previously published values (Ok et al. 2014). In Malaysia, AFB1 was detected in 9 out of 13 rice samples with an average contamination of 1.75 μg/kg [10]. These levels were higher than that reported from China. AFB1 was detected in all the 29 rice samples analyzed from different locations of China (Huantai, Huaian, and Fusui). The average contamination range recorded was from 0.5 to 0.6 μg/kg [8]. This reported value of

contamination was slightly lower than the results obtained before which showed an average concentration of 0.79 μg/kg [9].

The daily intake reported for these three countries is 285 g/day/person in China, 268 g in Korea, and 230 g in Malaysia. According to this intake value, the provisional tolerable daily intakes (PTDIs) for these countries are 2.6, 7.6, and 6.7 ng/kg bodyweight, respectively. These are higher than the reported PTDI for aflatoxins in vegetable oils and comparable or slightly less than reported for aflatoxins in peanut [26]. The calculation of PTDI is based on speculation of 60 kg weight per person.

In South Asia, aflatoxin levels in rice have been reported from India, Pakistan, Vietnam, and the Philippines [2, 6, 19, 22]. In Japan, 83 analyzed rice samples were reported to be aflatoxin-free samples [27] similar to analyzed samples reported previously [28].

India represents one of the most important producers and exporters of rice, particularly to the Middle East. Aflatoxin levels in rice in India were considered in three separate research investigations, out of which two studies showed high value of contamination. In more than 1,500 rice samples collected and analyzed in India, aflatoxins were found in higher value of contamination. The samples representing the rice of 12 states had shown that the aflatoxins were greater than 5 μg/kg in 930 samples with a median concentration of 45 μg/kg. This value exceeds the Indian permissible limit of 30 μg/kg for total aflatoxin in 256 analyzed samples [17]. Recently, a survey covering 20 states has been conducted. In this research work, 1,200 rice samples were analyzed, and AFB1 was found in contamination range of 0.1–308 μg/kg with the frequency of contamination being up to 67.8 % [22]. In a more recent investigation, less aflatoxin contamination was reported in rice from Punjab in India [18]. These values of contamination may pose a health risk problem, considering the high daily intake of 186 g rice and aflatoxin value ng/kg bodyweight. This justifies more investigations for aflatoxin in rice in India. The professional and/or regional bodies in India recommended to reconsider these results once again.

The level of contamination in rice in Pakistan is lower than that recorded in Indian rice. Of the 314 samples analyzed, aflatoxins were recorded in 185 samples with an average concentration of 11.2 ± 3.91 μg/kg. This value of contamination was observed in several types of rice including the paddy, parboiled, brown, white, and broken rice. The value has ranged from 7.10 to 16.35 μg/kg [19]. The daily intake of rice in Pakistan is not clearly known. Considering the lowest value of 107 g/day/person in selected rice-eating countries by FAO, the calculated PTDI of aflatoxin is 23.2. This value of exposure is very alarming and very high.

The distribution of aflatoxins in the Philippines and Vietnam are comparable with that reported in the Western Pacific Region. In the Philippines, the total aflatoxins were investigated and were recorded in 74 out of 78 samples with a mean level of 1.53 μg/kg [2]. This was less than that reported in rice from Vietnam, where aflatoxins were detected in 51 out of 100 samples, with a mean level of 3.31 μg/kg [6]. The daily intake of rice in these countries is 252 and 403 g/day/person in the Philippines and Vietnam, respectively. The recent estimation of daily intake of rice in Vietnam is 500 g/day/person according to [6]. The calculated PTDI of aflatoxin in rice is 6.4 and 22.2, respectively. The estimated PTDI of aflatoxin in rice in the Philippines is high

and comparable with that estimated for Korea and Malaysia, whereas in Vietnam, it is very high and alarming as that estimated for India and Pakistan.

The last region in Asia that we will discuss here is West Asia. Herein the aflatoxin in rice samples was investigated in Turkey and Iran. The contamination level in Turkey ranged between 0.05 and 21.4 μg/kg, which is 32 % above the legal limit of the EC regulation of 4 μg/kg. AFB1 was also considered in this investigation and it was found in 58 out of 100 samples analyzed with 14 % samples containing values higher than the EC regulation of 2 μg/kg for AFB1 intended for direct human consumption [29]. In Iran, the aflatoxin B1 in rice was found in 59 out of 71 samples with an average contamination level of 1.89 μg/kg [32]. In another investigation in Kashkineh in Iran, aflatoxin was detected in one out of 41 rice samples with a contamination level of 0.64 μg/kg [53].

The estimated risk for these levels in West Asia depends on the food habit in these countries. The data of the daily intake of rice for Turkey and Iran are not available. However, the general overview of these levels of contamination suggests posing mycotoxicosis as a health problem to the rice consumers in these countries.

4.2 Africa

The FAO report has shown selection of nine rice-eating countries in the African continent. The highest daily intake of rice is in Guinea-Bissau (318 g/day/person), followed by Madagascar (304), Liberia (301), Gambia (268), Sierra Leone (244), Comoros (214), Mauritius (195), Ivory Coast (173), and Guinea (162). This report did not cover all the rice-eating countries.

Aflatoxin data in rice samples from Africa and especially in these countries is very limited. In the Ivory Coast (West Africa), aflatoxin was detected in all the ten samples analyzed with an average levels of 4.5 μg/kg of AFB1 [12]. Similar results were reported from Nigeria with aflatoxin detected in 21 samples of rice with mean level of 82.5 μg/kg [5], while in Tunisia, there was no aflatoxin detected in any of the 10 rice samples [11].

The update data of daily intake of rice in the Ivory Coast has increased up to 429 g/day/person [12]. Therefore, the PTDI is 32.2 ng/kg/bodyweight/person. The level of PTDI reported here is very alarming and may pose a series of health risks to the consumers. Nigeria is a producer as well as a consumer country. The reported aflatoxin level is higher or comparable with that reported in India. So we expect comparable risk associated to the exposure to aflatoxin in rice. We recommend more comprehensive investigation for aflatoxin in rice in Africa, particularly in these rice-consuming countries.

4.3 Latin America

The order of rice-consuming countries in Latin America as shown in the FAO report is as follows: Guyana has the highest value with daily consumption rate of

236 g/day/person, followed by Dominican Republic (121), Brazil (112), Colombia (99), and Belize (68) at Central America. The update of these values was reported for the daily intake in Brazil with slight decrease of 100 g/day/person [3].

The aflatoxin in rice in Latin America is limited to one reported investigation carried out in Brazil. The total aflatoxin detected was in 135 out of 230 samples (59 %) with a mean contamination of 13.13 μg/kg [3]. The PTDI of aflatoxin in rice belonged to this value of contamination and is estimated to be 22 ng/kg/bodyweight/person based on daily intake of 100 g/day/person. This value is similar to the reported values in South Asia in Pakistan and Vietnam.

4.4 Europe and North America

Aflatoxins (AFB1 and/or AFB1 + AfB2) in rice in Canada (imported from the United States and Asian countries) were found in 99 out of 200 samples with an average level of 0.34–0.39 μg/kg for AFB1 and 0.08 μg/kg for AfB2 [13].

Only four papers represented the aflatoxin in rice (locally or imported) in Europe in four countries including Scotland, Austria, Spain, and Sweden. Aflatoxin was found to be reported in all samples from these countries. In Scotland, the brown rice (Asian origin sourced) had a contamination with an average value of 14.7 μg/kg of total aflatoxin [20]. The results in Austria in central Europe showed that 15 out of 81 samples (imported mainly from Asian countries, only one sample from Spain and one from Egypt) were contaminated with an average level of 1.97 μg/kg of total aflatoxin [16]. Similar results were found in Sweden (imported rice) with the contamination level ranging between 0.1 and 50.7 μg/kg of total aflatoxins [14]. However, the level of contamination was higher in both imported and locally sampled rice in Spain and Mexico; the aflatoxin was detected in 66 out of 67 samples with a mean concentration of 37.3 μg/kg [4].

Generally, the levels of aflatoxins in rice are varied throughout the world even within some countries in the same region. For example, Japan did not suffer from any high values of contamination, but in nearby India and Pakistan, the cases are completely different. This is due to good strategies followed by the Japanese to avoid fungal growth and aflatoxin production [1]. On the other hand, the rice in such countries like the Philippines, India, Nigeria, and Spain contains high levels of contamination, which may lead to aflatoxicosis and/or contribute to HCC in these countries. The effect of these levels may be extended to other countries importing this rice without serious import controls.

Specifically, the highest reported contamination was found in India [17], followed by Spain (locally and imported rice) in Europe [4], and Nigeria in West Africa [5]. The reported levels have exceeded the US FDA legal permissible limit of total AFs in food of 20 μg/kg. Significant levels higher than the EC regulation of 10 μg/kg for total aflatoxin in food were found in Scotland (rice imported from Asia) [20], Brazil [3], and Pakistan [19]. The remaining countries have reported the lowest value of contamination.

5 The Provisional Tolerable Daily Intake (PTDI)

The term provisional tolerable daily intake (PTDI) was developed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The term expresses the possible accumulation of contaminants in the body. The PTDI gave a precise description and accurate measurement of the suspected risk, based on the daily intake of selected food. The PTDI is the result of multiplying the aflatoxin level and the average of the consumption per day per bodyweight [30].

The data about rice consumption by humans is mentioned in eight works listed in Table 1. Rice is the principal staple cereal in Asia, parts of Africa, and South America. The provisional tolerable daily intake is calculated by assuming that 60 kg is the average of bodyweight of persons in these areas. To determine the hepatocarcinogenic effect of aflatoxin in rice, we have to consider the PTDI and the HCC cases in selected countries. The data concerning HCC in these countries was for global occurrence and taken from the Globocan database [31]. The coefficient of correlation (linear regression) between the occurrence of HCC and the PTDI is weak ($R^2 = 0.0002$) but becomes more significant when data from Asian countries was plotted alone ($R^2 = 0.6189$). When data from Spain, Brazil, and Ivory Coast are included, as they are thought to have high PTDI values, the curve is diffused again, suggesting that there are special local nutritional habits skewing the results and/or other factors are involved, such as hepatitis B virus and aflatoxin in other consumed commodities such as peanut.

Even though this correlation is low, the reported and calculated value of the PTDI of aflatoxins in rice highlights its importance. Sometimes the PTDI even at a low level showed the risk factor of cancer, due to its high consumption. The reporting of

Table 1 The provisional tolerable daily intake (PTDI) of aflatoxin in rice/ng/60 kg bodyweight/person in various countries [50]

Region	Country	AF in rice (μg/kg)	Rice daily intake/g	PTDI	HCC cases[a]	Reference
West Africa	Ivory Coast	4.5	429	32.18	2,237	[12]
South America	Brazil	13.13	100	21.88	9,678	[3]
Southeast Asia	Philippines	0.37	280	1.73	7,734	[2]
	Vietnam	3.31	500	27.58	21,997	[6]
Western Pacific (Asia)	Korea	3.25	181	9.8	16,900	[51]
	China	0.6	210	2.1	394,770	[8]
Central Europe	Austria	1.97	10	0.33	955	[16]
Southwestern Europe	Spain	37.3	16	9.95	5,522	[4]
Northern Europe	Sweden	2.2	9	0.33	490	[14]

AF total aflatoxin, *PTDI* provisional tolerable daily intake of AF in rice, *HCC* hepatocellular carcinoma

[a]HCC cases reported in 2012 by the international agency for research on cancer (IARC)

aflatoxin in rice without considering the daily intake will not provide a clear understanding to the real aflatoxin exposure.

6 Legal Limit

The conclusion of the selected works indicates that there is no common regulation related to aflatoxin in rice. The data are prepared and compared with local permissible levels designed by regulatory authorities of the area of investigation and/or with the European Commission, which is 2 μg/kg for AFB1 or 4 μg/kg for total aflatoxins in food [2, 4, 5, 11, 14, 20].

The WHO has set 30 μg/kg as the permissible limit for AFs in food, and this high limit has been used in several countries for aflatoxin in rice without any regard to the daily intake of rice [6]. A comparable regulation has been reported in Malaysia (35 μg/kg) [10] and in India (30 μg/kg) [22]. These limits have been set for food in general but it may not be suitable for a global staple food like rice.

The lowest legal limit for AFB1 has been set in Tunisia (2 μg/kg) in line with that of the EU [11], then in Iran (5 μg/kg), and followed by South Korea (10 μg/kg) [21, 32].

7 Conclusion

Aflatoxin has a high impact on human and animal health. Therefore, a large economic loss in the international trade of cereal and crops could be attributed to legislation controlling the permissible levels in these commodities related to the safety of their consumption by humans and animals [37, 42, 44, 46, 47]. Herein, all the presented studies have shown the widespread occurrence of aflatoxin AFB1 and total AFs in rice everywhere in the world, except in Tunisia and Japan. In Tunisia, it may be due to a small number of samples (11) analyzed, whereas in Japan, it may be due to special care to control and counter the fungal growth and aflatoxin production.

The determined levels of aflatoxin in rice are not high as in peanut or other cereal commodities; for example, the average of total aflatoxin in peanut butter in Sudan is up to 200 μg/kg [33], and the average of AFB1 in groundnut in DR Congo is 229.07 [34]. However, that does not mean the aflatoxin in rice has no contribution in general health, because of high daily intake of rice in certain regions of the world (Table 1). The values of PTDI have shown significant correlation with HCC incidence in Asian countries, which includes the Philippines, Vietnam, Korea, and China. The most important point here is that the average contamination in these countries does not exceed the EC limit of 10 μg/kg for total aflatoxin in food according to the updated regulation of 2006 in 2010. That is indicating that the PTDI of AFs in rice as shown in Table 1 could be a major factor for the chronic disease causative role of aflatoxin. This observation suggested the importance for the particular regulation of aflatoxin in rice with respect to the PTDI and/or with health risk assessment. Of these factors in reulation means: the care of aflatoxin levels, the data of daily intake, the average

data of bodyweight of consumers in the area of interest, and the health status of the people if they are HBV carries or not. This fact has been based on the carcinogenic potency of aflatoxin B1 as estimated to be 0.01 and 0.3 HCC cases per year per 10^5 people per ng per kilogram of bodyweight per day for hepatitis B surface antigen-negative (HBsAg-) and surface antigen-positive (HBsAg+) individuals, respectively [35, 36].

In terms of consumption, rice is consumed widely in Vietnam as shown in Table 1 [6], then Ivory Coast [12], the Philippines [2], China [8], Korea (Ok et al. 2014), and Brazil [3]. Rice consumption in Europe is rare, and this may explain the lesser attention in European countries to manage and control AFs in rice, as the high value of contamination has been reported.

The aflatoxin contamination in rice worldwide varies from countries to countries. In some regions of the world, it has shown lowest values of contamination, but in the others, it has become significantly alarming. An example of the latter countries is Nigeria where levels of AFs are higher and consequently may pose a series of health problems. Further, the effect attributed to AFs in rice in other regions of the world is still unclear, as the lower levels do not absolutely mean safer rice without considering all factors involved in their toxicity.

Lastly, in spite of lower levels of aflatoxins permitted by regulations in particular countries, further investigations are required to find out the effects of long-time exposure and estimates of daily intake of contamination, especially with respect to rice as the data about aflatoxin in rice is lacking in several rice-consuming countries worldwide.

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Impact of Mycotoxins on Human Health

7

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Abstract

Mycotoxins are secondary metabolites produced by filamentous fungi which contaminate a large fraction of the world's food, mainly staple foods such as corn, cereals, groundnuts, and tree nuts, besides meat, milk, and eggs. This worldwide contamination of foods is an enormous problem to human populations, principally in less industrialized countries and in the rural areas of

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some developed countries. The adverse effects of mycotoxins on human health can be both acute and chronic, provoking problems such as liver cancer, reduction of immunity, alterations in the protein metabolism, gangrene, convulsions, and respiratory problems, among others. The economic impact of mycotoxins in foods includes increased health care costs and premature deaths. Some factors which influence the presence of mycotoxins in foods are related to environmental conditions, such as storage, that can be controlled without too much expense. The cleaning of contaminated foods, on the hand, is economically costly and rarely implemented, so it tends to be carried out mainly in developing countries. Aflatoxins, ergot alkaloids, ochratoxins, 3-nitropropionic acid, fumonisins, trichothecenes, and zearelenone, are the most important economically, although dozens of other mycotoxins can also be associated with human health risks. Despite international attempts to improve and implement legislation to control the presence of mycotoxins in foods, its implementation has been ineffective.

Keywords
Fungi • Secondary metabolites • Human health risks • Mycotoxicoses • Developing countries

List of Abbreviations

AF	Aflatoxins
BEN	Balkan endemic nephropathy
CTN	Citrinin
DAS	Diacetoxyscirpenol
DON	Deoxynivalenol
F	Fumonisins
FUS-X	Fusarenon X
HBV	Hepatitis B virus
HT-2	Toxin HT-2
IPH	Idiopathic pulmonary hemosiderosis
LEM	Equine leucoencephalomalacia
LSD	Lysergic acid diethyl amide
NIV	Nivalenol
OTA	Ochratoxin A
T-2	Toxin T-2
TCT	Trichothecene
ZEN	Zearalenone

1 Introduction

Despite the difficulty defining and classifying them, all mycotoxins represent natural secondary metabolites of low molecular weight products produced by filamentous fungi. Although the term mycotoxin was only coined in 1962, after the sudden death of 100,000 young turkeys in England, who had ingested peanut meal contaminated

with aflatoxins from *Aspergillus flavus*, their occurrence can be traced back at least two millennia, when the development of settled agricultural communities relied on the storage of grains [1]. The writings of the Dead Sea Scrolls contain references to them, as to and in the Middle Ages, when ergot alkaloids were prepared and used by Asian and European people over 500 years ago; there were descriptions of "St. Anthony's Fire," caused most probably by the ingestion of ergot alkaloids from *Claviceps purpurea*, a fungus which contaminates staple food grains of wheat and rye [2, 3]. More recently, the deaths during the Second World War, the deaths of thousands in the former Soviet Union from the haemorrhagic syndrome known as alimentary toxic aleukia, caused primarily by T-2 toxin produced by *Fusarium sporotrichioides* and *F. poae* which had contaminated cereal overwintered in fields, have also drawn attention to this important human health problem [3].

As secondary metabolites, mycotoxins have no apparent significance in fungal development and growth, but represent a wide range of compounds, and are mainly produced, although not exclusively, when the fungus reaches maturity. Many have bizarre molecules with structures ranging from single heterocyclic rings with molecular weights of scarcely 50 Da to groups of 6–8 irregularly arranged heterocyclic rings with a total molecular weights greater than 500 Da, as a simple C4 moniliformin (produced by *Fusarium* spp.) and structurally complex substances, such as phomopsins (produce by *Phomopsis* spp.) [4]. Probably because they are so small, such small molecules induce no response in the human immune system, and until recently mycotoxins were considered metabolites produced only by filamentous fungi. However, agaric acid (hydroxylated tribasic acid), produced by the macrofungus *Fomes officinalis*, has been included among the mycotoxins under regulation in some countries of Asia and Oceania [5]. Nowadays, approximately 400 mycotoxins have been recognized, although attention has been given mainly to those proven to be carcinogenic and/or toxic to people [6]. Reliable calculations indicate that approximately 25–50 % of world crops, in particular staple crops, are contaminated with mycotoxins [7]. Human food can be contaminated with mycotoxins at various stages in the food chain, with mycotoxins occurring as a result of consumption of contaminated plant-derived foods as well as from the consumption of meat, eggs, milk, and other animal products and air and dust containing toxigenic fungi or their toxins (sick buildings) [8–11]. Adverse effects on human health from the consumption of agricultural products contaminated with mycotoxins occur everywhere, although they are far more common in developing countries. Indeed, in developed countries, the heavy economic costs ensure low concentrations of mycotoxins, and legislation on food processing and the marketing system help to significantly reduce the exposure of the population to mycotoxins [12]. In nonindustrialized countries, however, substandard nuts, maize, and other products may be consumed without any form of sorting or inspection, and this means that mycotoxins ingestion remains far too high, especially in rural areas. In addition, such contaminations have more serious consequences, affecting agricultural economics and reducing annual production. While good quality products are exported, the substandard produce, unacceptable to foreign buyers (because they exceed regulatory limits for mycotoxin contents) are sold on the domestic market [13, 14].

However, the occurrence of mycotoxins in food and derivatives is not only a problem in developing countries. Data from the World Health Organization show that the presence of mycotoxins produced by molds in food for human consumption is not on the decrease. Due to the clear increase in the number of diseases caused by mycotoxins, which have been implicated as potential etiological factors, a great effort is made to identify mycotoxins present in food and thereon to eliminate them. The presence of mycotoxins in food is a problem which has been faced by mankind, since ancient times. Regardless of the fact that there is a big difference between developed and underdeveloped countries, this problem affects all people, as everybody is constantly exposed to acute or chronic mycotoxicosis. However, due to the ongoing difficulties in obtaining enough food to eat in some of these underdeveloped regions, it would be unrealistic to expect a solution to the problem of mycotoxicosis in a short time. People in developed or highly developed areas of the world are less exposed to mycotoxins, primarily due to geographic and climatic conditions. In addition, there are considerable food resources, modern processing, the storage of food is constantly monitored, strict legislation is introduced, and there is therefore a very strict control of the presence of mycotoxins in food [15]. In developing countries, despite the existence of good legislation with regard to food safety which aims to control the presence of mycotoxins in food and feed, there is a great problem which is less evident in developed countries. There is less emphasis on legislating maximum tolerated levels and even when such legislation exists, the capacity to enforce it is frequently lacking due to corruption, namely, the bribery of governmental personnel who allow foods recognizably contaminated by high levels of mycotoxins to be sold and traded as good ones. Then, in less industrialized countries (Africa, Asia, Central, and South America), the problem of mycotoxicosis is basically educational, as in such low-income countries mycotoxins have not been widely prioritized from a public health perspective. Where attention has been paid, it has been largely driven by the need to meet the stringent import regulations on mycotoxin contamination in place in the richer nations of the world, rather than by the desire to protect the local population from producing and consuming the contaminated crops [16].

In this chapter, we discuss the problems of mycotoxins in food, the main producer fungi, the possible effects of mycotoxins on human health as well as the recent advances in our understanding of the approaches to improve the food safety, in order to minimize consequences of human exposure and to manage the problem.

2 Mycotoxigenic Fungi and Toxins They Produce

According to Frisvad et al. [17], the modern literature is full of examples of fungi species supposedly responsible for producing particular mycotoxins, although many of these associations are clearly incorrect. The same authors exclude Basidiomycete toxins, because these are ingested by eating fruiting bodies, a problem different from the ingestion of toxins produced by microfungi. However, it is worth mentioning that agaric acid (hydroxylated tribasic acid), produced by the macrofungus *Fomes*

Table 1 Species of fungi that are known as mycotoxin producers

Fungi	Mycotoxins	References
Alternaria alternata	Alternariol	[20]
Aspergillus carbonaris	Ochratoxin	[21]
Aspergillus clavatus	Patulin	[22]
Aspergillus flavus	Aflatoxins	[13]
Aspergillus fumigatus	Gliotoxin	[23]
Aspergillus niger	Fumonisins	[24]
Aspergillus parasiticus	Aflatoxins	[14]
Aspergillus tamarii	Aflatoxins B_1 and B_2 and cyclopiazonic acid	[25]
Aspergillus oryzae	Aflatoxin and cyclopiazonic acid	[26]
Aspergillus versicolor	Sterigmatocystins	[27]
Claviceps purpurea	Ergot alkaloids	[28]
Fusarium moniliforme	Fumonisins	[29]
Fusarium oxysporum	Fusaric acid	[30]
Fusarium graminearum	Trichothecenes	[31]
Penicillium citrinum	Citrinin	[32]
Penicillium brevicompactum	Mycophenolic acid	[33]
Penicillium expansun	Patulin	[34]
Penicillium nordicum	Ochratoxin A	[35]
Penicillium verrucosum	Ochratoxin and citrinin	[36]
Penicillium viridicatum	Citrinin	[37]
Stachybotrys chartarum	Roridin E, satratoxin H, sporidesmin G, trichoverrins, verrucarol	[38]

officinalis (Basidiomycete), has been included among the mycotoxins under regulation in some countries of Asia and Oceania [5]. Undoubtedly, the main genera producing mycotoxins in food in the world are: *Aspergillus, Fusarium*, and *Penicillium*. Other genera also considered important mycotoxins producers are *Alternaria, Claviceps*, and *Stachybotrys* (Table 1). Attention should be drawn to the fact that some mycotoxins are common to both the *Aspergillus* and *Penicillium* species [18]. Lower fungi belong to the genera *Rhizopus* and *Mucor* (*Mucorales*) are not excluded, but compounds of sufficient toxicity to be termed mycotoxins have not been found in these genera, except perhaps for rhizonin A and B from *Rhizopus microspores* [19].

2.1 Main Mycotoxins and Their Possible Effects on Human Health

The impact of ingestion of mycotoxins on human health depends on several factors such as: the concentration of mycotoxins in food and the period during which the person was exposed to the toxin; it depends on whether the compound is causing an acute or chronic infection in the human body; it also depends on the weight of the

person, the presence of other mycotoxins (synergistic effect), and environmental factors (mainly the storage conditions of commodities). Although about 300 mycotoxins are presently known, the attention of scientists has been directed to those which can have been proved to be toxic or which cause cancer in humans. People are exposed to mycotoxins after consuming plant foods contaminated with mycotoxins or through the ingestion of food of animal origin (eggs, meat, dairy products), as well as by the inhalation of dust containing mycotoxigenic fungal spores [9, 11] which can cause a reduction of immunity, bronchitis, asthma, and fatigue [39]. This is the case in sick buildings syndrome, in which *Stachybotrys chartarum* fungus has been blamed for the development of idiopathic pulmonary hemosiderosis (IPH) in children, although the manner in which the infection occurs has not yet been elucidated [40]. The main mycotoxins are aflatoxins, ergot akaloids, citrinin, fumonisins, ochratoxins, 3-nitropropionic acid, trichothecenes and zearalenone. A wide variety of commodities may be contaminated with mycotoxins, such as cereals, oilseeds, almonds, dried fruits, wine, spices, beans, roasted coffee and cocoa, malt and beer, bread and bakery products, wines and grape juices, as well as animal products (poultry meat and kidneys, pig kidneys, and pork sausages). A complicating aspect in the case of food contaminated with mycotoxins is the fact that several mycotoxins can occur simultaneously on the same food. In addition, the vast majority of mycotoxins are thermostable, i.e., they are not destroyed during the industrial processing and only a small, partial destruction occurs when food is boiled, fried, or even pasteurized.

2.2 Aflatoxins

Aflatoxins are the most significant worldwide mycotoxins because of the widespread human exposure to high levels and their carcinogenic properties. They are produced by *Aspergillus flavus*, *A. parasiticus*, and, apparently, *A. nomius* [41] and contaminate a variety of staple foods, particularly maize and groundnuts, in low-income countries. It has been estimated that approximately 4.5 billion people of the world's population are exposed to aflatoxins [42]. These dangerous mycotoxins occur mainly in tropical countries, where there are high temperatures and humidity, and where poor postharvest conditions of stored commodities increase fungal growth. Aflatoxins were first identified and characterized chemically in 1960 and since then have been linked to liver cancer in humans. The aflatoxins B_1, B_2, G_1, G_2, and naturally occurring AFB_1 are considered the most abundant, the most toxic, and highly carcinogenic [43]. The aflatoxins M_1 and M_2 correspond to the hydroxylation product of B_1 and B_2, respectively, and are found in milk and its derivatives. AFB_1 is the most toxic in both acute and chronic aflatoxicoses whereas AFM_1 is as acutely hepatotoxic as AFB_1 but not as carcinogenic [44].

Various types of symptoms are observed when people eat food contaminated with aflatoxins. The intensity of the symptoms observed depends on the amount of aflatoxin in food, the time period during which the person has ingested the contaminated food, the general state of health of the person, and the person's age (children

and the elderly are more susceptible). One of the main problems caused by AFB_1 is liver cancer, as AFB_1 is recognized as one of the most efficient inducers of this cancer. In areas where contamination of food with AFB_1 is high, the occurrence of liver cancer is coincidentally also higher [45]. For these reasons, the International Agency for Research on Cancer [46] included AFB_1 in group 1 of carcinogens, considering the high degree of risk of this toxin to human health. Another serious risk to human health, from the consumption of food contaminated with AFB_1, is acute toxic hepatitis, mycotoxicose. Consumption of food contaminated with aflatoxins can also induce a protein deficiency in the human body, known as kwashiorkor, whose main feature is a hypoalbuminemia, enlarged fatty liver, dermatosis, and generalized edema, which can lead people to death, as happened with several children [47, 48]. Ingesting aflatoxin is suspected of being associated with impairment of T4 lymphocytes [49]. Aflatoxins have been found in breast milk in Brazil and other countries [50].

According to calculations conducted by some researchers, the carcinogenic effects of aflatoxin B_1 is much higher in populations of less industrialized countries compared to the population of developed countries. Indeed, the poorest people have a less varied diet, are weaker, and are more subject to the synergistic action of AFB_1 with other diseases, such as hepatitis B virus (HBV), which has widespread occurrence in developing countries [51]. Aflatoxicosis can induce the development of hepatitis and may, in severe cases, lead to death. Recent cases of hepatitis have occurred in Kenya, where about 125 deaths were confirmed among people who consumed corn contaminated with AFB_1 [52, 53].

2.3 Citrinin

Citrinin (CTN) is considered a nephrotoxic mycotoxin and was isolated initially from *Penicillium citrinum*, but it can also be produced by other species of *Penicillium* (*P. camemberti* as used in cheese production) *Aspergillus* (*A. terreus, A. niveus*, and *A. oryzae*, used in the production of Asian ethnic foods (miso, sake, and soy sauce)) and *Monoascus* (*M. ruber* and *M. purpureus*, industrial species used to produce red pigments). CTN presents as yellow crystals, being a compound derived from phenol. This can contaminate various commodities, especially cereals, such as barley, corn, rice, oats, and wheat. Citrinin has been detected in certain vegetarian foods colored with *Monascus* pigments and in naturally fermented sausages from Italy [54]. It is usually found in association with another nephrotoxic mycotoxin, ochratoxin A (OTA). Besides its nephrotoxicity, CTN is also embryocidal and fetotoxic and, together with OTA, is believed to be responsible for the etiology of nephropathy. It is highly likely that when citrinin and ochtratoxin A occur in combination in grains their effects may be exacerbated due to the similarity of the effects of both toxins. CTN was associated with "yellow rice" syndrome in Japan, in 1971, due to the frequent presence of *P. citrinum* in this food [55]. Fortunately, field contamination with CTN is a rare event. However, it is reasonable to believe that humans are much more frequently exposed to CTN than generally accepted, because

this mycotoxin is produced by the same fungi as OTA, which is a common contaminant of human food all over the world. As for with almost all other mycotoxins, CTN contamination in grains occurs during storage. By maintaining moisture content below 14 % and avoiding insect damage, grains can be kept free from mycotoxin formation during storage [56].

2.4 Ergot Alkaloids

The genus *Claviceps* is a group of phytopathogenic ascomycetes which is composed of approximately 36 different species of filamentous fungi, capable of parasitizing over 600 monocotyledonous plants of the families Poaceae, Juncaceae, and Cyperaceae, including forage grasses, corn, wheat, barley, oats, millet, sorghum, rice, and rye. The term ergot derives from the French word argot (a spur) and represents the dark brown, horn-shaped pegs (sclerotia) that replace the seed or kernel of a plant after infestation [57]. Other fungi, although to a lesser extent, can also produce ergot alkaloids, such as some species of *Penicillium*, *Aspergillus*, and *Rhizopus* spp. [58]. The ergot alkaloids isolated from *Claviceps* sclerotia are structurally related to the hallucinogenic drug known as lysergic acid diethyl amide (LSD) are usually divided into three groups: derivatives of lysergic acid (e.g., ergotamine and ergocristine); derivatives of isolysergic acid (e.g., ergotaminine), and derivatives of dimethylergoline (clavines, e.g., agroclavine) [59]. The species which produce these alkaloids, in addition to *C. purpurea* (rye and other cereals) include *C. paspali* (forage grass), *C. fusiformis* (*Pennisetum typhoides*), *C. gigantea*, and *Sphacelia sorghi* (anamorphic form of *Claviceps*), are responsible for the form of ergotism known as gangrenous, which is caused by the ergotamine-ergo-cristine alkaloids. These mycotoxins have a strong vasoconstrictive activity. The symptoms range from edema of the legs, with severe pains, to paraesthesias, which can cause gangrene, often leading to amputation of legs and sometimes to death [60]. The other type of ergotism, a convulsive form, which is caused by clavine alkaloids from *C. fusiformis*, has gastrointestinal symptoms (nausea, vomiting, and giddiness), followed by effects on the central nervous system (drowsiness, prolonged sleepiness, twitching, convulsions, blindness, and paralysis) [61, 62]. Because of the techniques used in food industries, such as grain cleaning and milling processes, most of the ergot is removed so that ergotism is extremely rare today, especially in developed countries. Besides, the ergot alkaloids are relatively labile and are usually destroyed during baking and cooking [63].

2.5 Fumonisins

Fumonisins had their structure elucidated in the late 1980 after many years of study of the disease known as equine leucoencephalomalacia (LEM) [64–66]. Fumonisins consist of a 20-carbon aliphatic chain with two ester-linked hydrophilic side chains.

This structure resembles sphingosine, an essential phospholipid in cell membranes. The most dangerous is FB_1, which is a diester of propane 1,2,3-tricarballylic acid and 2-amino-12, 16 dimethyl-3,5,10,14,15-pentahydroxycosane [64]. Because of this similarity, investigators suggest that the toxic action of fumonisins appears to result from competition with sphingosine in sphingolipid metabolism [67, 68]. The inhibition of sphingolipid biosynthesis may cause serious problems related to cell activity, as these substances are essential for membrane composition, for cell-to-cell communication, for intracellular and cell-matrix interactions, and for growth factors [69]. These mycotoxins are mainly produced by several species of *Fusarium*, such as *F. verticillioides* (*F. moniliforme*), *F. proliferatum*, and *F. nygamai*, besides *Alternaria alternata* f.sp. *lycopersici* [10]. *F. anthophillum, F. dlamini, F. napiforme, F. subglutinans, F. oxysporum,* and *F. polyphialidicum* have also been implicated as producers of fumonisins [70]. Fumonisins constitute a group of distinct substances, as follows: B_1 (FB_1, FB_2, FB_3, and FB_4), A1, A2, A3, AK1, C1, C2, C3, C4, P1, P2, P3, PH1a, and PH1b [71, 72]. The occurrence of corn grains with elevated levels of fumonisins has been correlated with cases of esophageal cancer in inhabitants of Transkei (southern region of South Africa), in China, and in Northeastern Italy [63]. Besides having been associated with leucoencephalomalacia in equines and rabbits [65, 73, 74], fumonisins have also been associated with pulmonary edema and hydrothorax in pigs [75] and hepatotoxic, carcinogenic, and apoptosis effects in the liver of rats [76–78]. From corn sold in a supermarket in Charleston (South Carolina), high levels of these mycotoxins were detected; coincidentally, Charleston is the American city with highest incidence of esophageal cancer among Afro-Americans [79]. While the other mycotoxins are soluble in organic solvents, fumonisins are hydrosoluble. Such characteristic makes them more dangerous to human health, as they can remain undetectable most of the time.

2.6 Moniliformin

Chemically, the mycotoxin moniliformin is a salt of 1-hydroxycyclobut-1-ene-3,4-dione, and was first described by Cole [80] and colleagues in 1973, while screening for toxic products of a North American isolate of *Fusarium moniliforme* (now *F. verticillioides*) cultured on corn. The same investigators characterized its structure in the following year. The occurrence of moniliformin in cereals and cereal products has been described for different regions worldwide, such as Argentina, Austria, Canada, Germany, Italy, Poland, New Zealand, Peru, South Africa, USA, and parts of Africa and Asia. Analyses of 22 samples of corn from the Brazilian state of Sao Paulo did not confirm the presence of this mycotoxin in Brazil. Moliformin has since been shown to be produced also by *F. moniliforme* var. *subglutinans*, *F. sacchari* var. *subglutinans*, *F. avenaceum*, *F. acuminatum, F. anthophyllum, F. concolor, F. denticulatum, F. equiseti, F. fujikuroi, F. fusarioides, F. oxysporum, F. proliferatum, F. ramigenum, F. sambucinum, F.semitectum. F. succisae, F tricinctum*, and *F. thapsinum* [81].

2.7 Ochratoxin A

Ochratoxin (OTA) has a chemical structure similar to that of aflatoxins, with an isocoumarin substitute bound to an L-phenylalanine group. OTA was discovered in 1965, during a study to detect new mycotoxins, as a secondary metabolite of *Aspergillus ochraceous*, a fungus usually present in drying or decaying vegetation, seeds, nuts, and fruits [82]. Other fungal species capable of producing this toxin are *A. alliaceus, A auricomus, A.carbonarius, A. glaucus, A. meleus,* and *A. niger*, besides *Penicillium nordicum* and *P. verrucosum*. The *A. niger* is an efficient producer of enzymes and citric acid for human consumption, so the isolates used in industry must be checked as far as their capability of producing OTA is concerned [83, 84].

Besides being recognized as an acute nephrotoxin, OTA also shows hepatotoxic, immunosuppressive, teratogenic, and carcinogenic behavior [85–88]. OTA has a significant relationship with Balkan endemic nephropathy (BEN) which is a chronic kidney disorder, with lethal results. BEN has an endemic character, and it has been recorded in rural regions of the Balkan countries [89, 90]. OTA has been found in barley, coffee grains, oats, and wheat. There is some indication that OTA may occur in wines in which the fruits have been infected by *A. carbonarius* [41, 91, 92]. In humans, OTA has been found in serum and in milk [93]. The International Agency for Research on Cancer [46] classified OTA as a potential carcinogenic for the human population (group B). In Brazil, approximately 50 % of samples of bean, corn, rice, and wheat contained significant levels of OTA [94]. This mycotoxin was also confirmed in roasted, soluble, and ground coffee, in Belo Horizonte city (Brazil) [95].

2.8 Patulin

Chemically, patulin is known as 4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one, and was first isolated as an antimicrobial active principle during the 1940s from *Penicillium patulum* (later called *P. urticae*, now *P. griseofulvum*). This compound is a mycotoxin that forms the smallest group of toxic metabolites referred to as polyketides and is reported to be produced by other fungi such as *Aspergillus clavatus*, *A. giganteus*, *A. terreus*, *Paecilomyces* sp., *Byssochlamys nivea,* and *B. fulva*. Patulin was also isolated from other species and given the names clavacin, claviformin, expansin, mycoine c, and penicidin [96]. This metabolite was used as a spray for treatment of nose and throat common cold, as well as an ointment for the treatment of skin infections [96, 97]. However, during the 1950s and the 1960s, it became clear that, despite the action of patulin as antimicrobial, antiviral, and antiprotozoan, it was also toxic to both plant and animal cells, and it was then reclassified as a true mycotoxin [10]. Nowadays, patulin plays an important role as a method for monitoring the quality of apple juices and concentrates in apple processors. Its presence in high amounts indicates that moldy apples were used in the production of the juices. Patulin is considered to be the most dangerous mycotoxin in fruits, particularly

apples, pears, and their products [38]. The most efficient producer of patulin, the fungus *P. expansum*, is the causative agent of the disease known as "blue mold," which is extremely common on apple, cherry, pears, and other fruits. This mycotoxin is frequently detected in nonfermented apple juices, although fortunately it does not survive fermentation in cider derived products, since it is efficiently metabolized by yeasts [98]. Despite the fact that its effects on human health have not been conclusively proven, the Joint Food and Agriculture Organization–World Health Organization Expert Committee on Food Additives has established a provisional maximum tolerable daily intake for patulin of 0.4 mg/kg of body weight [99].

2.9 Trichothecenes

Trichothecene mycotoxins (TCT) comprise a vast group of over 150 metabolites produced by more than 350 species of fungi, although the main producer genera are *Fusarium, Myrothecium, Phomopsis, Stachybotrys, Trichoderma, Trichotecium,* and *Verticimonosporium* [100–102]. Trichothecenes have low molecular weight (250–500 Da), are nonvolatile, and share a common tetracyclic$_{12,13}$ -epoxy skeleton, which is responsible for their toxicological activity. The term trichothecene stems from trichothecin, the first member of the family identified. According to their characteristic functional groups, the TCT are divided into four groups: Type A and B trichothecenes are the most common. Type A is represented by HT-2 toxin and T-2 toxin, and type B is most frequently represented by DON, 3-acetyl-DON (3-Ac-DON), 15-acetyl- DON (15-Ac-DON), nivalenol (NIV), and fusarenon X (FUS-X). Type C and D trichothecenes are characterized by a second epoxide (C-7,8 or C-9,10) or an ester-linked macrocycle (C-4,16), respectively [103]. The trichothecenes as a group are immunosuppressive. Despite the high number of trichothecenes chemically characterized, only some of them occur in nature. The main trichothecenes are deoxynivalenol (DON), nivalenol (NIV), toxin T-2, toxin HT-2, and diacetoxyscirpenol (DAS). These mycotoxins show a strong capability to inhibit protein synthesis in eukaryotic cells, hampering the initiation, the elongation, and the termination steps of protein synthesis. TCT were the first compounds proven to be involved in the inhibition of peptidyl transferase activity [104, 105]. TCT mycotoxins occur worldwide in grains (barley, corn, oats, rice, rye, wheat), vegetables, and other crops [41].

DON (12, 13-epoxy-3, 4, 15-trihydroxytrichothec-9-en-8-one) or RD-toxin, is also known as vomitoxin, although less toxic than many other major trichothecenes, DON is the most prevalent and is commonly found in barley, corn, rye, safflower seeds, and wheat [106]. This mycotoxin is considered to be extremely stable, surviving most of the processing methods such as powdering and milling. However, the effects of DON on human health are not yet well understood [107]. Studies conducted by Minervini [108] suggest that human blood cells are sensitive to mycotoxin exposure, that NIV is more toxic than DON which is more toxic than FB_1, and that DNA damage and apoptosis rather than plasma membrane damage and necrosis may be responsible for the observed cytotoxicity.

T-2 toxin was first isolated from the mold *Fusarium sporotrichioides* [109]. It belongs to nonmacrocyclic type A trichothecenes. *F. sporotrichioides*, the major producer of T-2 toxin, occurs mainly in temperate to cold areas and is associated with cereals. A large-scale human toxicosis which occurred in India in 1988, and in China, Japan, and Korea, was imputed to T-2 toxin [110]. T-2 toxin poisoning occurred in Kashmir, India, in 1987 and was attributed to the consumption of bread made from moldy flour. Symptoms commonly observed were anorexia, nausea, vomiting, headache, abdominal pain, diarrhea, chills, giddiness, and convulsions [107]. This mycotoxin is so far the only one known to have been used as a biological weapon [111]. T-2 toxin is well absorbed by topical, oral, and inhalational routes. Its toxic action is thought to disrupt DNA polymerase, terminal deoxynucleotidyl transferase, monoamine oxidase, and several proteins involved in the coagulation pathway [112]. HT-2 toxin is also an A type trichothecene, which occurs mainly on small cereal grains and corn, and is produced by *Fusarium sporotrichoides, F. poae*, and *F. langsethiae*. Analyses conducted in corn-based products, commercialized in the city of Sao Paulo (Brazil), indicate a low occurrence of trichothecene mycotoxins. However, despite the high levels of T-2 and HT-2 found in one sample the investigators suggested that there is no immediate cause of concern as far as the public health is concerned [113].

The trichothecenes produced by *Stachybotrys chartarum* are the ones which have recently received the most attention of public health authorities. Macrocyclic trichothecenes and related trichoverroids: roridin E and L-2; satratoxins F, G, and H; isosatratoxins F, G, and H; verrucarins B and J; and the trichoverroids, trichoverrols A and B and trichoverrins A and B are highly toxic compounds with a potent ability to inhibit protein synthesis [114]. Numerous studies have demonstrated the toxicity of toxins from *S. chartarum* on animals and animal and human cells [115]. Yang [116] reported that satratoxin G was the most cytotoxic of eight trichothecenes tested on mammalian cells. These authors concluded that this toxin is far more toxic than the T-2 toxin associated with alimentary toxic aleukia. The presence of *S. chartarum* has been associated with pulmonary bleeding in children [38]. Stachybotryotoxicosis was first described as an equine disease of high mortality associated with moldy straw and hay. Until recently, human Stachybotryotoxicosis was considered a rare occupational disease, limited to farm workers who handle moldy hay. On the contrary, it has become evident that *S. chartarum* grows well on almost all wet building material, such as water-damaged gypsum boards, wood fiber boards, ceiling, wall paints, and even dust-lined air conditioning ducts [117].

2.10 Zearalenone

The classification of this secondary metabolite as a mycotoxin is actually considered inadequate since, despite being biologically potent, it is barely toxic. In fact, its structure resembles 7β–estradiol, the principal hormone produced in the human female ovaries. This secondary metabolite is an estrogenic (US spelling) toxin, produced mainly by *Fusarium graminearum,* although other species of the same

genus can also produce this substance (*F. culmorum, F. equisetii, F. crookwellense, and F. sporothichioides*). Zearelenone is better classified as a nonsteroidal estrogen or mycoestrogen. Sometimes it is called a phytoestrogen compound, known as 6-(−10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid μ-lactone [118].

Among the human population, children are the most affected due to the consumption of ZEN-contaminated cereal-based food products (corn, barley, and wheat grains). Due to its estrogenic structure, it is considered that zearalenone and/or its derivatives, especially zearalanol, have been implicated in several incidents of precocious puberty in children at the age of 7–8 [119]. There is, however, inadequate evidence in humans for the carcinogenicity of zearalenone.

3 Mycotoxins Legislation in Different Countries

Laws have been adopted in many countries in order to protect consumers from the harmful effects of mycotoxins in fresh and processed food. The best known are those laws that regulate the levels of aflatoxins, despite the fact that legislation for other mycotoxins is also being implemented. There are several factors leading to the preparation of legislation. For example, there are scientific aspects such as the availability of toxicological information, knowledge about the distribution of mycotoxins in foods, in addition to analytical methodology. The political and economic aspects should also be taken into consideration, particularly with respect to commercial interests and the impacts on the availability of food supply [120–122].

Information gathered by the year 2003 showed that about 100 countries now have legislation to regulate the limits of mycotoxins in food, feed, and commodities, representing a 30 % increase compared to 1995 [123]. The countries covered by these laws include approximately 90 % of the world population [124]. This survey confirms that the increase in the population now protected by mycotoxin legislation occurred thanks to a slight increase observed in Latin America and Europe and a significant increase in population coverage in Africa and Asia/Oceania. Moreover, all countries which had legislation for mycotoxins before 2003 have at least regulatory limits for the presence of aflatoxin B_1 or the sum $B_1 + B_2 + G_1 + G_2$. However, several other mycotoxins have also fallen under legislation. Among them stand out aflatoxin M_1; deoxynivalenol trichothecenes; diacetoxyscirpenol; toxins T-2 and HT-2; fumonisin B_1, B_2, and B_3; ochratoxin A; patulin; sterigmatocystin; zearalenone; ergot alkaloids; and even the agaric acid and phomopsins. In 2003, it was observed that a greater number of mycotoxins were controlled by law, and there were also a high number of analyzed products and commodities. The tolerance limits have remained at the same levels or have shown a tendency to be reduced, while sampling and analysis methods have become more diversified and more detailed. An extremely interesting trend is the harmonization of the laws in the countries belonging to different economic blocks such as Australia/New Zealand, the European Union, and Mercosul [122].

In most African countries, where there is no legislation in place, the population is exposed to mycotoxin contamination, particularly with respect to subsistence

farming, where crops are consumed in their own areas of production or in their vicinity. African countries have some legislation but it only covers aflatoxins. Among the countries of that continent, Morocco has the most advanced legislation. Regarding Asia/Oceania, about 26 countries have legislation for mycotoxins, representing 88 % of the population of that region. New Zealand, however, has its own legislation, with some differences in relation to Asia and northern Australia. Currently, Australia and New Zealand are harmonizing their laws which include limits on exotic mycotoxins, such as agaric acid and phomopsins. In this extensive continent, the laws of China and the Islamic Republic of Iran are the most complete and detailed.

On the European continent, 39 countries, representing 99 % of the European population, have legislation for the regulation of mycotoxins in food and feed. Compared to other world regions, Europe has the most complete and detailed legislation on mycotoxins in food. In the European Union, legislation has been harmonized for aflatoxins in various foods, for aflatoxin M_1 in milk, ochratoxin A in cereals and dried fruit, for patulin in apple juice and products derived from apples, and aflatoxin B_1 in various rations. Preliminary actions have been initiated regarding deoxynivalenol in cereals and products derived from cereals [122, 124].

In North America, the United States and Canada have had legislation for mycotoxins for many years and continue perfecting the methods of sampling and analysis. In both countries the limits for aflatoxins are established for the sum $B_1 + B_2 + G_1$ and G_2. In Canada, beyond the limits imposed for fusarium toxins, there are also tolerance percentages for damaged grains of wheat spikelets, both the soft type and the hard type, in addition to other grain boundaries. In this country, there are also limits to the presence of sclerotia of *Claviceps purpurea* in various cultures (it is in the sclerotia where the ergot alkaloids accumulate). In the United States, there are detailed tolerance limits for the sum of fumonisins B_1, B_2, and B_3 in a wide variety of corn products. This is the only country in the world where there are limits to the sum of these three fumonisins.

In Latin America, 19 countries have legislation for mycotoxins, representing almost 91 % of the continental population. Legislation to aflatoxins is harmonized in Mercosur, which includes Argentina, Brazil, Paraguay, and Uruguay. Uruguay has the most comprehensive legislation in Latin America with ergot alkaloids limits in feed, which is unprecedented in any legislation in the world. In South America the legislation is available for the following mycotoxins: aflatoxin B_1, B_1/G_1 aflatoxin total aflatoxins ($B_1 + B_2 + G_1 + G_2$), fumonisin B_1, deoxynivalenol, ochratoxin A, patulin, and zearalenone (Table 2).

4 Conclusions and Perspectives

Mycotoxicoses due to the ingestion of mycotoxin contaminated foods will remain a huge public health problem worldwide, mainly in less industrialized countries. Acute and chronic contaminations and their effects have been well documented. For instance, AFB_1 is synergistic with hepatitis B virus (HBV) infection, which has a

Table 2 Legislation for mycotoxins in food and feed in different continents (Adapted from Refs. [5, 125])

Region	Mycotoxin	Substrate/limit
Africa	Afl. B_1[c] Afl. G_1[a] Afl. $B_{1+}G_1$[b] Afl. M_1[a] Afl. $B_1 + B_2 + G_1 + G_2$[c] Ochratoxin A[c] Patulin[a] Zearalenone[c]	For all foods: B_1: 5 ppb; $B_1 + B_2 + G_1 + G_2$: 10 ppb Peanuts for export: B_1: 5 ppb Peanut and its products, vegetable oils: $B_1 + B_2 + G_1 + G_2$: 20 ppb Baby food: B_1: 0 ppb Fluid milk: M_1: 1 ppb Feed: B_1: 50 ppb Peanut products as feed: B_1: 50 ppb Peanut products as ingredients for animal feed: B_1: 300 ppb Peanuts, corn, and sorghum: B_1: 5 ppb; G_1: 4 ppb Poultry feed: B_1: B + G1: 10 ppb Rice flour: B_1: 5 ppb ; G_1: 4 ppb
Asia/ Oceania	Agaric acid[a] Afl. B_1[c] Afl. M_1[a] Afl. $B_1 + B_2 + G_1 + G_2$[c] Diacetoxyscirpenol[a] Deoxynivalenol[b] Phomopsins[a] Fumonisin B_1[a] Fumonisin $B_1 + B_2$[a] Ochratoxin A[c] Patulin[a] T-2[c] Zearalenone[c]	All foods: $B_1 + B_2 + G_1 + G_2$: 5 ppb ; Phomopsins: 5 ppb Peanut butter, nuts in general: $B_1 + B_2 + G_1 + G_2$: 15 ppb Nuts and their products: $B_1 + B_2 + G_1 + G_2$: 20 ppb Brazil Nut: $B_1 + B_2 + G_1 + G_2$: 15 ppb Rice, edible oils: B_1: 10 ppb Oats, barley, beans, sorghum, wheat, other grains, and fermented foods: B_1: 20 ppb Fluid milk and milk products: B_1: 0.5 ppb Peanuts and products: $B_1 + B_2 + G_1 + G_2 + M_1 + M_2$: 20 ppb All foods: 30 ppb Peanut meal for export: B_1: 120 ppb, Feeds: B_1: 10 ppb Peanut butter, beans peanuts, tree nuts: $B_1 + B_2 + G_1 + G_2$: 15 ppb Food for children up to 3 years old $B_1 + B_2 + G_1 + G_2$: 1 ppb Feeds: B_1: 1000 ppb Dried coconut kernel in feed for cows, pigs, ducks, sheep: $B_1 + B_2 + G_1 + G_2$: 1000 ppb Peanut bran, sesame, rapeseed, cassava in feed for chickens: $B_1 + B_2 + G_1 + G_2$: 200 ppb
Latin America	Ergot alkaloids[b] Afl. B_1[c] Afl. $B_1 + G_1$[a] Afl. M_1[a] Afl. $B_1 + B_2 + G_1 + G_2$[c] Deoxynivalenol[c] Fumonisin B_1[a] Ochratoxin A[a] Patulin[a] Zearalenone[c]	Foods: $B_1 + B_2 + G_1 + G_2$: 20 ppb Peanuts shelled or unshelled, raw or toasted, and peanut butter paste or butter: $B_1 + B_2 + G_1 + G_2$: 2 ppb Corn grain, corn meal, flour, and semolina: $B_1 + B_2 + G_1 + G_2$: 20 ppb Fluid milk: M_1: 0.5 ppb Powdered milk: M_1: 5 ppb Baby food: B_1:0 ppb Fluid milk and powder: M_1: 0.05 ppb Milk products: M_1: 0.5 ppb Foods and spices: $B_1 + B_2 + G_1 + G_2$: 20 ppb Soy products, peanuts, dried fruit: $B_1 + B_2 + G_1$ + G2: 30 ppb

(*continued*)

Table 2 (continued)

Region	Mycotoxin	Substrate/limit
		Cocoa beans: $B_1 + B_2 + G_1 + G_2$: 10 ppb Infant foods: $B_1 + B_2 + G_1 + G_2$: 3 ppb Corn and barley: Zearalenone: 200 ppb Fruit juices: Patulin: 50 ppb Rice, coffee, barley, and corn: Ochratoxin A: 50 ppb Feeds: B_1: 20 ppb; $B_1 + B_2 + G_1 + G_2$: 50 ppb Rice flour: $B_1 + B_2 + G_1 + G_2$: 5 ppb
North America	Ergot alkaloids[b] Afl. M_1[a] Afl. $B_1 + B_2 + G_1 + G_2$[c] Diacetoxiscirpenol[b] Deoxynivalenol[c] Fumonisin $B_1 + B_2 + B_3$[c] HT-2[b] Ochratoxin A[b] Patulin[a] T-2[b] Zearalenone[b]	Foods: $B_1 + B_2 + G_1 + G_2$: 20 ppb Nuts and products: $B_1 + B_2 + G_1 + G_2$: 15 ppb Prepared food wheat: Deoxynivalenol: 1000 ppb Wheat: Deoxynivalenol: 2000 ppb Dairy: M_1: 0,5 ppb Feeds: $B_1 + B_2 + G_1 + G_2$: 20 ppb Grains, livestock, and poultry: Deoxynivalenol: 5000 ppb; Toxin HT-2: 100 ppb Feed for pigs, sheep, and lactating animals: Deoxynivalenol: 1000 ppb; Toxin HT-2: 25 ppb
Europe	Afl. B_1[c] Afl. $B_1 + G_1$[c] Afl. M_1[a] Afl. $B_1 + B_2 + G_1 + G_2$[c] Diacetoxyscirpenol[b] Deoxynivalenol[c] Fumonisin B_1[a] Fumonisin$B_1 + B_2$[a] Ochratoxin A[c] Patulin[a] Sterigmatocytin[a] T-$_2$[c] Zearalenone[c]	All foods: B_1: 10 ppb All foods: $B_1 + B_2 + G_1 + G_2$: 5 ppb; Patulin: 50 ppb Food for children and adolescents: $B_1 + B_2 + G_1 + G_2$: 0.05 ppb; M_1: 0.05 ppb Milk: M_1: 0,05 ppb Peanuts, nuts, and dried fruit for direct consumption or as food ingredients: B_1: 2 ppb; $B_1 + B_2 + G_1 + G_2$: 4 ppb Nuts and dried fruit subjected to selection or physical treatment: B_1: 5 ppb; $B_1 + B_2 + G_1 + G_2$: 10 ppb Cereals and processed products for direct consumption or as food ingredient: B_1: 2 ppb; $B_1 + B_2 + G_1 + G_2$: 4 ppb Cereal products for direct consumption: Ochratoxin A: 3 ppb; Zearalenone: 100 ppb Raw cereals: Ochratoxin A: 5 ppb; Dry fruits: Ochratoxin A: 10 ppb Brazil Nuts: $B_1 + B_2 + G_1 + G_2$: 4 ppb Spices and seasonings: B_1: 5 ppb; $B_1 + B_2 + G_1 + G_2$: 10 ppb Beer: Ochratoxin A: 0.2 ppb Herbs for teas: B_1: 5 ppb; $B_1 + B_2 + G_1 + G_2$: 10 ppb Fresh milk or for the production of dairy products and heat-treated milk: M_1: 0.05 ppb Apple juice and other fruit: Patulin: 50 ppb Kidneys of pigs: Ochratoxin A: 25 ppb Raw material for feed: B_1: 50 ppb Ready feed: B_1: 10 ppb Complete feeds for poultry and pigs, except for young animals: B_1: 20 ppb

(*continued*)

Table 2 (continued)

Region	Mycotoxin	Substrate/limit
		Complete feeds for calves and lambs: B_1: 10 ppb Feed supplements for poultry and swine: B_1: 30 ppb Supplements for animal feed in general: B_1: 5 ppb Peanut products, cotton, babassu, shopping, dried coconut kernels, and corn: B_1: 20 ppb Feed supplements for cattle, goats, and sheep, except for lactating animals, lambs, kids, and calves: B_1: 50 ppb

[a]Mycotoxin with legislation only for food
[b]Mycotoxin with legislation just to feed
[c]Mycotoxin with legislation both for food and for feed

greater prevalence in the developing world, besides being linked to liver cancer and immune suppression. At the same time, richer countries have a wealth of information that could significantly reduce the exposure of poor people to mycotoxins. We agree with many researchers when they say that mycotoxin contamination in foods is not decreasing. Actually, the problem tends to become exacerbated as the global population increases. The Food and Agriculture Organization (FAO) has estimated that by 2050 the world will have to feed nine billion people. Increased production of foods will be needed in the future to satisfy growing food demand in developing countries and feed demand in the newly industrializing countries. This situation is further aggravated if we remember the climate changes, droughts, the water scarcity, the wars, the great fluxes of migration, and the growing poverty in Africa and in other parts of the tropical world. In such situations, nobody will pay attention if the food is moldy or not or if the level of mycotoxins (when they are aware of this problem) is exceeding the limit of legislation. The main purpose of these people will just be to have something to eat and survive until the next day. Therefore, the strict control of food quality in both industrialized and developing countries would be desirable to avoid mycotoxicoses outbreaks. Under such circumstances, occurrence of mycotoxins in agricultural commodities in the poorest areas of the world will continue to remain, unfortunately, a largely ignored health and economic issue.

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Melanin Pigments of Fungi

8

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Abstract

Fungi possess all kinds of melanins found in nature. These pigments are products of polymerization of phenolic compounds. Phenolic precursors determine the polymerization products. Though formed via different precursors, polymerized melanins possess common properties. Melanin multifunctionality is well documented in fungi. Such functions of melanins as creating a tolerance to harsh environments are fulfilled in both saprophytes and parasites.

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But pathogenic fungi face double impact – hostile environment plus defenses of their hosts. Fungal melanin affords remarkable protection from many such factors and thus favors pathogenicity. Besides, pathogen melanins may act as an arm of aggression through involvement in parasite's penetration and suppression of host's responses. In outline, natural polymers of this group have irregular structure and perform effectively many biologically important functions related to high adaptive flexibility of their carriers.

Keywords
Melanins • Biosynthesis • Localization • Properties • Extremophylic fungi • Pathogenesis

List of Abbreviations

Bq/kg	Becquerel/kg
DHI	5,6-Dihydroxyindole
DHICA	5,6-Dihydroxyindole-2-carboxylic acid
DHN	Dihydroxynaphthalene
DOPA	L-Dihydroxyphenylalanine
ESR	Electron spin resonance
eV	Electron volt
GHB	Glutaminyl-4-hydroxybenzene
GDHB	Glutaminyl-3,4-dihydroxybenzene
$^1O_2$	Singlet oxygen
OH	Hydroxyl free radical
PKS	Polyketide synthase
ROS	Reactive oxygen species
1,3,6,8-THN	1,3,6,8-tetrahydroxynaphthalene
UV	Ultraviolet
W/m^2	$Watt/m^2$

1 Introduction

In order to survive extremes of pH, temperature, salinity, radioactivity, and host defenses, microorganisms have been found to develop unique protective mechanisms. Melanization occurs in the environment and is important for adaptation to unfavorable life conditions. The term "melanin" originates from melanos – a Greek word for black. Melanin is a class of compounds found in plants, animals, fungi, and protists. The presence of various kinds of melanins in representatives of almost every large taxon suggests an evolutionary importance of melaninogenesis [1]. In general, melanins are hydrophobic pigment biopolymers formed by oxidative polymerization of phenolic or indolic compounds. Exact structures of melanins are unidentified.

Fungi show successful examples of adaptation to extreme conditions, especially toward two or more extreme factors. They demonstrate all the variety of melanins found in nature: eumelanins (black or dark brown), pheomelanins (yellow or red),

and the most heterogeneous group of allomelanins, including soluble piomelanins and melanins formed from dihydroxynaphthalene compounds (DHN) [1, 2]. Though formed via different precursors, polymerized melanins possess common properties [3]. The melanin is produced after cessation of active growth and is therefore a likely secondary metabolite.

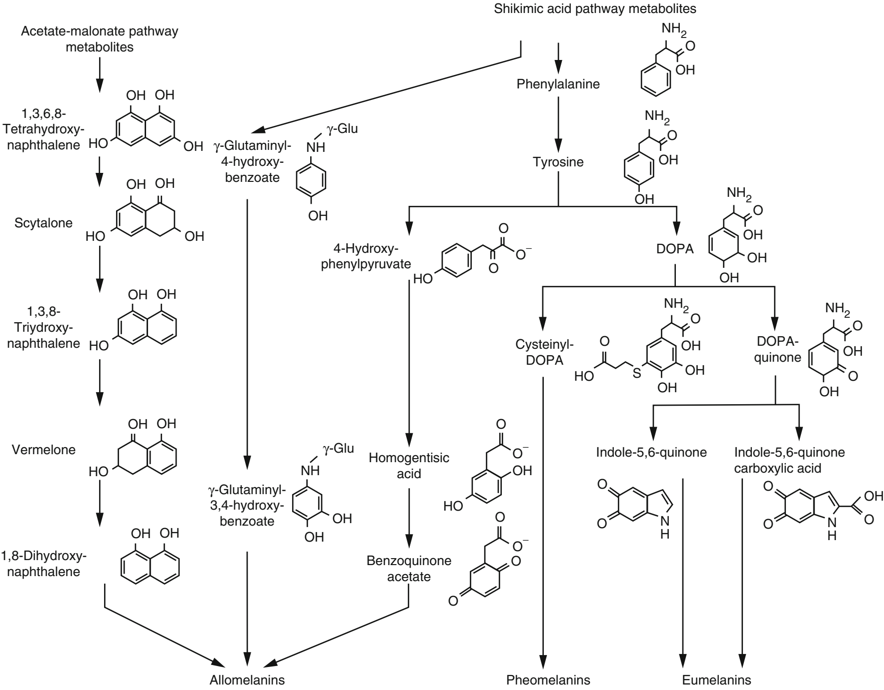
Melanin in fungi has been postulated to be involved in a range of virulence-associated properties, including interactions with hosts, oxidative stresses, UV light, and hydrolytic enzymes; resistance to antifungal agents; iron-binding activities; and even the harnessing of ionizing radiation in contaminated soils [4–7]. The photochemical properties of melanin make it an excellent photoprotectant. It absorbs harmful UV radiation and transforms the energy into harmless amounts of heat. These properties eventuate adaptations of fungi to stressful environments: high insolation, low temperature, low water content, starvation, elevated ROS, and increased radioactivity. Melanin protects fungi from presence of toxins [8–10]. Melanin as an element of pathogenic mechanisms includes sequestration of host defensive proteins, redox buffering, trapping of single electrons, dismutation of superoxide anion radical, an osmotic role in penetration of the (plant) cell wall by the appressorium, and protection against hydrolytic enzymes [4–6, 11]. Additionally, melanin provides defense from environmental predation by microorganisms such as the nematode, *Caenorhabditis elegans*, and the amoeba, *Acanthamoeba castellani* [12, 13]. Thus, melanin has a protective role in fungi both in the host and in the environment. Melanized fungi have been discovered in extreme cold, dry, salty, acidic climate; deep-sea habitats; zones depleted of nutrients; etc. Moreover, quite a wide taxonomic range of yeast-like and mycelial fungi have evolved potent radiation resistance [14, 15].

This chapter highlights the properties of melanins allowing extremophilic fungi to expand widely, in detrimental to the majority of other species habitats, even under significantly different extreme conditions imposed as a consequence of human insult of the environment. We have also touched on melanin types in fungi, their biosynthesis and localization, melanin precursors in fungal cells, and role of melanin in parasitic fungi, mainly the plant pathogens.

2 Melanin Biosynthesis in Fungi

Fungal melanin diversity is achieved through two biosynthetic pathways: acetate-malonate pathway and shikimic acid one (scheme). High molecular weight melanins are formed by oxidative polymerization of phenolic compounds (scheme). These reactions are catalyzed by copper-based enzymes. Copper is important for the production of melanin by both the DHN and L-dihydroxyphenylalanine (DOPA) pathways [16, 17] (Scheme 1).

Depending on the precursors, the resulting products of polymerization are the brown-black eumelanin, the yellow-red pheomelanin, and a heterogeneous group of allomelanins, including piomelanins and, a very common in fungi, DHN-melanin formed via the polyketide pathway. Eumelanins are dark brown to black pigments



Scheme 1 Melanin biosynthetic pathway in fungi

with 6–9 % nitrogen and 0–1 % sulfur. They are the oxidation products of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [5, 18]. In contrast, pheomelanins are reddish-brown pigments with 8–11 % nitrogen and 9–12 % sulfur, composed of benzothiazine monomer units [18, 19]. Allomelanins show a heterogeneous group of pigments derived from metabolites of homogentisic or *p*-hydroxyphenylpyruvic acid (piomelanins), γ-glutamyl-4-hydroxybenzene, and catechols [4, 20–24]. Melanins formed from DHN also belong to allomelanins. They are very common in fungi and typically do not contain nitrogen.

2.1 The DHN-Melanin Biosynthesis

Most fungal melanins are derived from the precursor molecule 1,8- DHN and are known as DHN-melanins. The way which furnishes DHN has been termed the polyketide pathway and resides primarily in ascomycetes and related deuteromycetes [23]. Recognized human pathogens which form melanin precursors by the polyketide pathway include *Aspergillus nidulans*, *A. niger*, *Alternaria alternata*, *Cladosporium carionii*, *Exophiala jeanselmei*, *Fonsecaea compacta*, *F. pedrosoi*, *Hendersonula toruloidii*, *Phaeoannellomyces wernickii*, *Phialophora*

richardsiae, *P. verrucosa*, *Wangiella dermatitidis*, *and Xylohypha bantiana* [25–27].

Malonyl-CoA serves as the starter and extender units for the polyketide synthase (PKS1) catalyzing the first step in the biosynthesis pathway. PKS converts malonyl-CoA to the first detectable intermediate of the pathway, 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). Following this, 1,3,6,8-THN is reduced by a specific reductase enzyme to scytalone. It was discovered that a specific reductase inhibitor, tricyclazole, produced the same defect as a mutation in the reductase gene, namely the accumulation of flaviolin, a shunt product of 1,3,6,8-THN [5]. Scytalone is dehydrated enzymatically to 1,3,8-trihydoxynaphthalene [27], which is in turn reduced, possibly by a second reductase, to vermelone [27, 28]. This reductase can also be inhibited by tricyclazole. A further dehydration step [24], possibly also catalyzed by scytalone dehydratase, leads to the intermediate 1,8-DHN, for which this pathway was named. Subsequent steps are thought to involve a dimerization of the 1,8-DHN molecules, followed by polymerization, possibly catalyzed by laccases, phenol oxidases, peroxidases, and catalases [29–32]. This is a general model for DHN-melanin biosynthesis, but the pathway (and the resulting color) may vary in different fungi. Interestingly, several by-products of the fungal DHN-melanin pathway have been shown to have antibacterial or immunosuppressive properties [33].

In fungi, the melanin biosynthesis could begin through metabolites of shikimic acid pathway.

2.2 L-3,4-Dihydroxyphenylalanine-Melanin Biosynthesis

DOPA melanin-synthesizing fungi include many model organisms such as *Neurospora crassa*, *Podospora anserina*, *A. nidulans*, *A. oryzae* and also pathogenic fungi such as *Cryptococcus neoformans*. A biosynthesis pathway for fungal DOPA-melanin strongly resembles the pathway found in mammalian cells, though details may differ [5].

Eumelanins are formed from tyrosine or phenylalanine oxidized by tyrosinase (EC 1.14.18.1) or laccase (EC 1.10.3.2) into DOPA. Tyrosinase (monophenol: diphenol oxygen oxidoreductase) catalyzes the formation of DOPA from tyrosin; it was found in *Agaricus bisporus*, *N. crassa*, *Tuber melanosporum*, *T. manatum*, and many other fungi [1, 29, 34]. The expression of this enzyme is closely related to the developmental stages and pathogenesis of fungi [4, 35]. DOPA can also be converted into melanin with the participation of laccases, for example, in *Lentinula edodes* and *C. neoformans* [36].

The second DOPA oxidation step is manifested in DOPA quinone formation, followed by the cyclization and building up DHI or DHICA with their following oxidation to indole-5,6-quinone or indole-5,6-quinone carboxylic acid (scheme) [5, 37]. The latter compounds polymerize to form brown and black pigment eumelanins. This pathway of melanin synthesis was found in *Candida albicans*, *Paracoccidioides brasiliensis*, *C. neoformans*, and *Sporothrichum* (syn. *Sporotrix*) *schenckii*.

The pheomelanin synthesis pathway involves sulfur compounds, the amino acid cystein or glutathion, that liberate cysteins through the action of a glutamyl-transpeptidase. In presence of cysteins, DOPA-quinones connect with cysteins to form 5-S-cysteinyl-DOPA and 2-S-cysteinyl-DOPA which give benzothiazin intermediates that polymerize to produce pheomelanins – brown, red, or yellow pigments (scheme) [19, 38, 39]. The pathway shows biosynthesis of sulfur-containing melanins in the truffle *T. melanosporum* [34].

One more DOPA pathway of melanin biosynthesis in fungi includes tyrosine transaminase (EC 2.6.1.5.), providing formation of 4-hydroxyphenylpyruvate, which is subsequently converted into homogentisic acid by dioxygenase (EC 1.13.11.2) and is then spontaneously oxidized to benzoquinone acetate and polymerized resulting in the formation of soluble brown piomelanins (scheme) [1]. The formation of soluble piomelanins from tyrosine via hydroxyphenylpyruvate and homogentisic acid was found in *A. fumigatus*, *A. kawachii*, *Madurella mycetomatis*, and *Yarrowia lipolytica* [2, 39]. In fungal parasite *Ustilago maydis*, polymerization of catechol dimers with the formation of melanin fibrils was found [40].

2.3 Glutaminyl-4-Hydroxybenzene Melanin

There is good evidence that the basidiospore wall melanin of *A. bisporus* is generated from the precursor glutaminyl-4-hydroxybenzene (GHB), synthesized via the shikimate pathway [41]. GHB is apparently converted to glutaminyl-3,4-dihydroxybenzene (GDHB). Peroxidase and/or phenolase oxidize this compound to form γ-glutaminyl-3,4-benzoquinone, which is polymerized later [20, 21]. It is the immediate precursor to the spore wall melanin. GDHB is found only in reproductive hyphae that form the melanized spores. Thus products of polymerization of γ-glutaminyl-3,4-benzoquinone, benzoquinone acetate, and 1,8-DHN form a heterogeneous group of allomelanins (scheme).

Other basidiomycetous mushrooms contain GHB and GDHB [42], which leads to the proposal that DHN melanins may be exclusively produced by ascomycetous fungi, while GDHB melanins may be restricted to the basidiomycetous fungi [43]. However, the black yeast *Phaeococcomyces* spp. shows all of the physiological and ultrastructural factors for designation as being of basidiomycetous affinity, but it produces DHN melanin [44] which enhances pathogenesis of fungi [4, 36].

2.4 Two Pathways of Melanin Biosynthesis in One Fungus

Some fungi have more than one biosynthetic pathway of melanins. For example, in *A. fumigatus*, piomelanins, synthesized from homogentisic acid (L-DOPA pathway), protect the hyphae cell wall from ROS, and gray-green DHN-melanins establish the structural integrity of the cell wall of conidia and their adhesive properties [45, 46]. In *A. bisporus*, melanins are formed from DOPA by tyrosinase and from

γ-glutaminyl-4-hydroxybenzene by peroxidase and phenolase [47]. In *Hormoconis resinae* and *Aureobasidium pullulans*, melanins were found in the cell wall, and exogenous melanins were found in culture fluids [48]. The genus *Aspergillus* comprises many species, which possess pigmented conidia of various colors. Effects of inhibitors on DHN-melanin synthesis (tricyclazole and phthalide) and DOPA-melanin synthesis (kojic acid and tropolone) in a range of *Aspergillus* species demonstrate differences in the amounts and types of melanins synthesized by related species [49, 50].

Talaromyces marneffei (Basionym: *Penicillium marneffei*) is capable of synthesizing DHN-melanins in conidia and DOPA-melanin in yeast cells and mycelia depending on growth conditions and supply of precursors [51]. There are also numerous enzymes, such as laccase, polyphenoloxidases, and perhaps peroxidases and catalases that are found in the cell walls and environs of normally nonmelanized fungi that will form black polymers from applied solutions of DOPA. This may lead to erroneous assumptions about the nature of the monomer of fungal melanins. The native melanin of the black yeast *Phaeococcomyces*, for instance, is known to be of DHN origin [44, 52], but albino mutant colonies on agar will blacken when overlaid with drops of L-DOPA solution. Numerous fungi now known to produce DHN melanin were previously reported to produce DOPA melanins [24].

In *C. neoformans,* melanins are synthesized from various exogenous substrates, e.g., D- and L-dopamine [53], homogentisic acid [54], catecholamines, and other phenolic compounds [55]. Polymerization of exogenous substrates in *C. neoformans* occurs as a result of laccase action. High concentrations of exogenous substrates (above 1 mM) inhibited the growth of the fungus and the formation of melanins, probably due to the toxicity of the substrates themselves [56].

Genes of melanin synthesis often assembled into clusters, thereby coordinating their expression at different stages of development of fungi [32, 45, 50, 57].

3 Localization of Melanins in Fungi

Early works on melanin localization have shown that these pigments in fungi may be detected in the cell wall or secreted into the environment [29]. Fungal cell wall incorporates a mix of cross-linked fibers (the polysaccharides glucan and chitin) and matrix components (primarily proteins and mannans). The outer layer contains high levels of different types of mannoproteins. The inner layer is made mainly of polysaccharides (beta-glucans and chitin) and small amounts of proteins [58]. Melanin can be found in the inner or outer layers of the cell wall depending on the fungal species [6]. In pathogenic fungi, melanins are often reported to be associated with or “in” the cell wall [30, 35, 59–61]. However, there is variation between species: the melanin may be located external to the wall, e.g., in *P. brasiliensis* [5, 62]; within the wall itself; or as a layer internal to the wall and external to the cell membrane, e.g., in *C. neoformans* [21, 56, 63].

In the halophilic fungus *Hortaea werneckii* at an optimal concentration of NaCl (0.86 M), the melanized dark layer is on the outer part of the cell wall, but the whole cell

wall is melanized upon the increased salt concentration [64]. Dark melanin granules were found in the fibrillar matrix on the surface of the cell wall in *A. pullulans*, *Verticillium dahliae*, and *Phomopsis* spp. [65]. In the presence of a melanin precursor DOPA, melanin granules are formed on the surface of conidia and yeast cells of the dimorphic pathogenic fungi *Histoplasma capsulatum* and *Blastomyces dermatitidis* and the cell surface becomes rilled [62, 66]. According to electron microscopy of the soil fungus *Gaeumannomyces graminis*, the melanin layer composes almost half of the thickness of the cell wall and is located between the cell wall and the inner chitinous layer [65]. In fungi, melanin is deposited in the cell wall and cytoplasm, and melanin particles ("ghosts") can be isolated from these fungi that have the same size and shape as particles in the original cells [63].

A combination of SEM and TEM microscopy shows that melanin has an overall granular structure. In various fungi, the granules are localized to the cell wall where they are likely cross-linked to polysaccharides [6, 63, 67]. In *C. albicans* the melanin clumps appeared as variably sized extracellular granules loosely adherent to the pseudohyphae and yeasts, and chitin synthase enzymes are directly involved in the synthesis of melanin. Thus melanin externalization in *C. albicans* depends on cell wall chitin structures [68].

There are exogenous soluble melanins, for example, piomelanins of *Ophiocordyceps sinensis* which parasitizes on insects [69]. The soluble melanins are characteristic of *A. bisporus* and a number of basidiomycetes [70]. Exogenous melanins are also found in culture fluids of *Cladosporium resinae* and *A. pullulans* [48]. Soluble melanins are a significant part of the extracellular matrix of *Botrytis cinerea* [71]. In *Sclerotinia sclerotiorum*, melanins are localized in the outer layer of sclerotia, forming a solid protective cover [72]. In the multicellular conidia *A. alternata* [73], melanin was localized in the outer layer of the cell wall and in septa [74].

Evidence exists that fungal melanin precursors occur inside the cells in lipid vesicles, analogues of melanosomes of animal tissues [6]. They are subsequently transported to the cell wall. The existence of fungal melanosomes has been suggested for *Fonsecaea pedrosoi*, where electron microscopy has shown electron-dense cytoplasmic structures in melanized cells [74]. Studies in *C. neoformans* suggest that melanin may also be synthesized in vesicles in this fungus. Laccase activity is associated with extracellular vesicles secreted from *C. neoformans* [75].

It was shown that melanized cell walls are considerably less porous than nonmelanized ones. Hence, melanin incorporation into the cell wall determines the size of the cell wall pores [76]. In some plant pathogens, melanin accumulated in appressoria again works as a diffusion barrier. It is located between fungal plasma membrane and cell wall except for the pore where the penetration peg emerges. Appressoria accumulate glycerol; its diffusion is restrained by the pigment to create conditions necessary for host penetration (see the Sect. 6.2).

Therefore, melanin localization on the cell surface and its participation in cell wall porosity makes these secondary metabolites an excellent defense system against detrimental environments and antifungal drugs, as well as a factor of host-parasite interactions.

4 Properties of Melanins

Melanins represent a group of related pigments similar in physical and chemical traits that allow them fulfilling their protective functions. Melanins are among the most stable and resistant of biochemical materials [77]. Melanins have been extensively studied and characterized as negatively charged amorphous compounds with quinone groups, hydrophobic and insoluble in organic solvents [4, 5].

The polymer net structure of melanins formed by the enzymatic and autoxidative polycondensation of various hydroaromatic precursors may additionally include other organic molecules. Usually, melanins are associated with proteins (melanoproteins) or with glycoproteins (melanoglycoproteins) [29]. The presence of carbohydrates and fatty acids, covalently and noncovalently bound to melanin, was confirmed by NMR methods [6, 67, 78–80]. It was demonstrated that in cells of *C. neoformans,* the polysaccharides and/or chitin that are associated proximally with lipid membrane constituents form a chemically resistant framework that could serve as the scaffold for melanin synthesis. The pyrrole aromatic carbons of the pigments bind covalently to the aliphatic framework via glycoside or glyceride functional groups [67]. The results of X-ray analysis of four different fungi melanins suggested that they are composed of planar structures that can differ in stacking distances. The distance between monomers might be an important feature of melanin pigments [6]. As melanins contain unpaired electrons, they can be detected by electron spin resonance (ESR) [81]. A comparison of the ESR spectra of melanin in various fungi showed that the g-factor of the signal was in the range 2.0036–2.0042 with a halfwidth of 4–7 eV. Melanin pigments retain the ability to deactivate free radicals and peroxides and absorb heavy metals and toxic electrophilic metabolites. Sequestration of iron ions has been identified as a major mechanism for the inhibitory effects of melanin on lipid peroxidation [82]. Thus these pigments exhibit profound antioxidant activity [83–85]. The gene expression of melanin synthesis enzymes increases the resistance of fungi to oxidants [50, 86].

Melanins exhibit unusual electronic properties due to the presence of mobile π-electrons [87]. It was proposed that due to melanin's numerous aromatic oligomers containing multiple π-electron systems, a generated Compton recoil electron gradually loses energy while passing through the pigment, until its energy is sufficiently low that it can be trapped by stable free radicals present in the pigment. Controlled dissipation of high-energy recoil electrons by melanin prevents secondary ionizations and the generation of damaging free radical species [88].

According to Blois experiments in the 1960s, it was postulated that melanins were electrical conductors showing photoconductivity in the solid state [89, 90]. Later, the possibility of tunneling electrons between photoinduced paramagnetic centers was shown [91]. Melanin chemistry is largely defined by its many ionizable moieties – carboxylic acids, amines, and catechols in various states of oxidation within the macromolecular structure [4]. One may therefore anticipate that melanin (like many other functional biomacromolecules) will exhibit generic polyelectrolyte behavior in which its weakly acidic nature plays an important role. Recent studies have demonstrated that melanin's ionic conductivity is facilitated by binding of water to the

pigment [92]. Melanins are supposed to be electronic-ionic hybrid conductor. The presence of these unique properties makes melanin a promising material in bioelectronics, especially when one takes into account its strength, resistance to high temperatures, and biocompatibility [92].

Melanin pigments absorb light in a wide spectrum range enclosing UV. Absorption intensity decreases slowly with increasing wavelengths [93, 94]. Melanins absorb light with the conversion of photon energy into heat [93, 95]. The mechanism of photoprotective action of melanins is of great interest. The possible transfer of protons inside a monomer as a result of energy dissipation during the photoexcitation of a pigment was confirmed in a set of experiments [96]. The polymerization of monomers resulted in an increase in the lifetime of the excited state of the oligomer from 100 ps to 3 ns [97]. Upon interaction with hard UV radiation (240–300 nm), photoionization and subsequent partial destruction of melanins can be observed [93]. Experiments on synthetic melanins showed that their electron emission was far less than 1 % [94, 98]. This suggests that fast thermal relaxation of absorbed radiation energy occurs in melanins, and the risk of dangerous photochemical reactions decreases [93]. These properties allow melanins to be effective protectors against UV- and solar radiation. However, the formation of cytotoxic products during hard UV radiation cannot be excluded [98, 99].

Although the structure of melanins was unaffected by X-ray, γ, or UV radiation, some signal changes were detected by the ESR, indicating an increase in semiquinone radical number [88, 93].

Thus, the presence of unpaired electrons in the highly polarized structure of melanin pigments and their ionic conductivity determines a wide range of properties: the ability to convert all types of heat radiation, adsorb electrophilic compounds, exhibit antioxidant properties, and, probably, to use the energy of radiation in the redox reactions of living cells. These properties provide for survival of fungi under extreme conditions, such as high insolation, low temperature, a low content of water and organic substrates, high concentrations of reactive oxygen species, and enhanced radiation doses.

5 Melanins in Extremophilic Fungi

Melanized fungi inhabit some remarkably extreme environments such as excessive heat or cold, extreme pH or osmotic conditions, polychromatic radiation, simulated outer space, desiccation, and it also seems to mediate tolerance toward metals, hypersaline environments, radionuclides [100, 101]. Dominance of melanin-containing fungal species has been observed around Chernobyl lately [101]. Melanins ensure fungal survival in desert soils, uplands, and on plant surface [14, 101]. In aerial environment, melanized spores prevail over soil ones [14, 101]. Melanins provide high spore survival under hard UV radiation while nonpigmented forms entirely die within a few minutes. Microcolonial yeast-like fungi are highly melanized. They keep ability to grow on the surface of stones under marked temperature differences, hypersaline environments, drought, low concentrations of organic

compounds [14, 101–103]. Viable cells of these fungi were found in the extreme climate of the Antarctic [104, 105]. Inhabiting rock cavities and fossils under harsh environment of Antarctic, the microscopic fungi *Cryomyces antarcticus* and *Cryomyces minteri* showed high resistance to UV radiation (280–360 nm, 3 W/m^2), which they were able to sustain for few hours, whereas nonpigmented *Saccharomyces pastorianus* cells died after 30 min of exposure [102].

In the hypersaline waters, a surprisingly rich diversity of fungi was discovered. Such waters in salterns offered natural ecological niches for halophilic black yeasts. *H. werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum*, *A. pullulans,* and *Cladosporium* spp. were detected with the highest frequency in hypersaline water (3–30 % NaCl) [64, 106, 107]. Among various adaptation mechanisms to hypersaline environments is melanization of the cell wall as has been shown for *H. werneckii*. The outer part of its melanized cell wall has a continuous layer of melanin granules that minimizes glycerol loss from the cells, as this layer creates a mechanical permeability barrier for glycerol by reducing the size of the pores in the cell wall [76]. In terrestrial environments, extremophilic fungi form facultative lichen-like associations with algae or cyanobacteria [100, 103]. 58 % of fungal endophytes associated with leaves of *Colobanthus quitensis*, a dicotyledonous plant that lives in Antarctica, were able to produce melanin in their hyphae [108].

Melanized microscopic fungi have an advantage to survive in regions of technogenic pollution. In industrial and roadside areas, melanin-containing fungi were the most abundant. In these habitats, they showed highest resistance to contamination by heavy metals and unsaturated hydrocarbons [109, 110]. *Cladosporium* and *Alternaria* spp. predominate in air and snow samples of the city settlement areas [111].

Fungi appear to be highly resistant to radionuclides in the environment. Many (25 %) fungal species from the Nevada Test Site contained melanin or other pigments [101], and up to 40 % of all fungi isolated from the Chernobyl 4th block reactor contained melanin or other pigments [112]. These noticeably exceeded the ratio of melanin-containing fungi, found in environments with background radioactivity. The most frequently occurring pigmented species were *C. sphaerospermum*, *C. herbarum*, *H. resinae*, *A. alternata,* and *A. pullulans*. Despite differences in the habitat between sampling sites, there was a trend of change in dominance of fungal species with radiation level [112–116]. Both *Chaetomium aureum* and *Purpureocillium lilacinum* (*Paecilomyces lilacinus*) were indicators of high levels of radionuclide contamination (3.7 × 106–3.7 × 108 Bq/kg) of soil in woodland ecosystems. Though they were among the light-colored fungi, *P. lilacinum* strains from radionuclide-contaminated soils had melanin content about 2–2.5 times higher than its content in related strains isolated from the areas with background radioactivity [117, 118].

Melanin has been shown to account for between 45 % and 60 % of ^{60}Co and ^{137}Cs incorporation into fungal hyphae [119]. Thus melanized fungi are proposed to be good candidates in bioremediation, since the organisms can potentially bind radionuclides and many other toxic substances. The occurrence of melanized fungi in areas with high levels of radiation undoubtedly reflects their advantage relative to nonmelanized species. Fungal melanin subjected to ionizing radiation showed

changes in its ESR signal, which consists with changes in electronic structure. Irradiated melanin showed an increase by four in capacity to reduce NADH relatively to nonirradiated one [120]. Gamma radiation-induced oxidation of melanin resulted in electric current production, especially in the presence of a reducing agent [121]. These properties apparently explain the increased metabolic activity and enhanced growth of fungal hyphae under different types of radiation, found in melanin-containing fungi [122, 123]. Observations of enhanced growth of melanized fungi under low-dose ionizing radiation in the laboratory and in the damaged Chernobyl nuclear reactor suggest they have adapted the ability to survive or even benefit from exposure to ionizing radiation [123]. Thus the possibility of participation of melanin in active electron transport in living cells leads to a hypothetical mechanism of radiation energy utilization for the increase in metabolic activity. Further research in this area can provide a better understanding of the nature of the radio- and UV-protective effect of melanin [14, 15].

6 Fungal Melanin as a Factor of Pathogenesis

Indeed, melanin is not sufficient condition of fungal pathogenicity since saprophytes contain it as well. However, for melanized parasites the pigment plays roles, sometimes indispensible, in colonization of either plant or animal hosts. Pathogenesis-related properties of melanins are well reviewed [4, 5, 24, 80, 124].

6.1 Evidence That Fungal Pathogenicity Depends on Melanin

Importance of melanin for pathogenicity is evidenced by complete or partial loss of this ability when its biosynthesis is disturbed on several occasions.

6.1.1 Genetically Impaired Melanin Biosynthesis Attenuates Pathogenicity

The role of melanin for blast fungus *Magnaporthe oryzae* (synonyms are *Magnaporthe grisea* and *Pyricularia oryzae*) that affects rice leaves and panicles is being studied rather thoroughly. The fungus accumulates DHN melanin in spores (conidia), mycelia, and appressoria. Unlike the parental strain, its melanin-deficient mutants are not virulent under natural conditions. Three such mutants were recovered by Chumley and Valent [125], namely, albino (Alb$^-$), rosy (Rsy$^-$), and buff (Buf$^-$). Similar nonpathogenic mutants albino (alb-1) and rose (ros-1) were derived from the wild-type strain H5-3 by Dzhavakhiya et al. [126].

The hemibiotrophic fungus *Mycosphaerella fijiensis* causes a very harmful black Sigatoka disease of banana leaves. Its mycelium contains deep green DHN melanin, whose amount in infected leaves correlates positively with the disease stage. The melanin content is very low in pink-pigmented isogenic mutants. They penetrate leaves, but the infection is blocked soon probably due to hypersensitive response of

the host [127]. The fungus *G. graminis* producing DHN melanin causes take-all disease of wheat and barley; its albino mutant is nonpathogenic [29].

Among human pathogens, yeast-like *C. neoformans* in the presence of DOPA synthesizes the corresponding melanin detectable by ESR. Its mutant Mel^- does not show ESR signal and is less virulent for mice than the wild type. *C. neoformans* strains deficient in melanin survive poorly in infected animals [124]. In this fungus, the genes of melanization contribute to dissemination of the pathogen over the host and death of the latter [4, 128]. The similar genes of *C. gattii* are greater expressed (in concert with the higher melanin production) in more virulent than in less virulent strains. Melanized cells of *P. brasiliensis* affect animals more severely than nonmelanized cells, and the infection is accompanied with the increased expression of melanin synthesis genes [80]. The reduced virulence toward mice was reported for Mel^- mutants of *W. dermatitidis* and *A. fumigatus*. Melanizing, but not nonmelanizing, strains of *Basidiobolus* sp. are associated with human disease [4].

Opposite examples are known, namely, rise in (or appearance of) pathogenicity in nonpigmented parasite after transfer of melanin biosynthesis genes to its genome. Entomopathogenic fungus *Metarhizium anisopliae* is amelanotic. Genes of polyketide synthase, scytalone dehydratase, and 1,3,8-trihydroxynaphthalene reductase were transformed to it from *A. alternata*. The transformant was more virulent than the wild type in killing diamondback moth (*Plutella xylostella*) larvae [129]. Transfer of *A. alternata* genes *ALM*, *BRM1*, and *BRM2* to melanin-deficient nonpathogenic albino (Alb^-) and buff (Buf^-) mutants of *M. oryzae* restores their mycelial and appressorial pigmentation along with pathogenicity [130].

Melanoprotein complexes isolated from *Venturia inequalis* (causing apple scab) and applied to apple leaves inoculated with this fungus increased the numbers and size of lesions [131]. In general, there are few examples of increased virulence achieved by exogenous melanin addition to inocula. Obviously, it follows (taking melanin multifunctionality into account) that pigment involvement in pathogenesis requires not merely its presence in the host-parasite system but depends on its localization, stage of disease, etc.

6.1.2 Chemical or Physical Agents Altering Melanization Affect Pathogenicity

The necessity of melanin for rice blast pathogenicity is witnessed convincingly by usage of inhibitors of its biosynthesis tricyclazole, ftalide, pyroquilon, carpropamid, etc. as effective commercial antiblast fungicides [132]. Strictly speaking, they are not fungicides because they do not kill the fungus. It, despite the abnormal pigmentation, develops normally in artificial culture like pigment mutants. But it is no more infective [24]. Interestingly that sodium diethyldithiocarbamate, the known inhibitor of Cu-Zn superoxide dismutase, when added to the nutrient medium of *M. oryzae* not only suppresses the enzyme but also alters the pigmentation. The color of colonies and conidia shifts from deep gray to thin gray. The compound does not affect growth of mycelium and germination of conidia harvested from it but deprives them of pathogenicity. Such lightened conidia can infect wounded leaves but give rise to

lesions about 2–3 times smaller in size than in the untreated control. Spray of rice plants with diethyldithiocarbamate expectedly controls the disease [133].

In the zoopathogen *W. dermatitidis*, tricyclazole decreases pigmentation and virulence. The capacities of albino mutants of this fungus are restored by exogenous scytalone, the intermediate in DHN melanin synthesis. Glyphosate inhibits melanization in *C. neoformans* and improves survival of inoculated mice [4].

The consequences of pigment mutations and chemical inhibition are not always the same as to pathogenicity. For example, an albino mutant of *G. graminis* is nonpathogenic for plants. However, its wild type does not lose the virulence upon inoculation in the presence of melanogenesis inhibitors [29]. Presumably, the inhibitor does not have enough time for the full effect.

In wild type fungi, the environment may shift, although less dramatically, melanin content with significant consequences for the pathogenic competence. As known, long maintenance of facultative phytopathogens on artificial media weakens the spore aggressiveness. The latter, however, is regained after inoculation of the host plant and re-isolation of the parasite. This was confirmed for *M. oryzae*, and its aggressiveness changed in parallel with mycelium and spore melanization [134]. The fungus grown on a carrot broth produced more aggressive and more melanized spores than that from a potato broth. On the latter medium, both properties of spores were stronger in illuminated than in blackout culture [133]. So, pigmentation changes might be among reasons of aggressiveness changes under these conditions.

6.2 Mechanisms of Melanin Involvement in Fungal Pathogenicity

Modes of melanin action in fungal pathogenesis are diverse as well as general biological roles of the pigment. Such functions of melanins as creating a tolerance to harsh environment are fulfilled both in saprophytes and parasites similarly. But some activities are specific to the second group, for instance, involvement in pathogen's entry into tissues of the host or impeding defense responses of the latter.

6.2.1 Intrusion into Host

Appressorium is an anchoring organ, and melanin present here may be implicated in the parasite's adherence to the plant surface. This capacity necessary for penetration was evaluated for *M. oryzae* by count appressoria remained on inoculated barley leaves after shaking with water. Appressoria began to adhere at the onset of their melanization, and both features were reduced by leaf treatment with tricyclazole [135].

At early stages of plant diseases caused by pathogens *M. oryzae*, *Colletotrichum lagenrium*, *C. lindemuthianum* [24], and *C. kahawae* [80] melanin present in appressoria plays a special role. Namely, it enables local accumulation of glycerol resulting in high osmotic pressure inside appressorium. This allows the penetration peg to prick mechanically epidermal cuticle and cell wall. The significance of this mechanism is evidenced by the facts that pigmentless appressoria of *M. oryzae*

melanin-deficient mutants form penetration pegs, which grow poorly through either plant epidermis or artificial membranes. Accordingly, the mutants are able to infect mechanically damaged leaves. Wild type of *M. oryzae* treated with tricyclazole or other inhibitors resembles the mutants: it loses both appressorium pigmentation and capacity to penetrate plant cells or artificial membranes. Expectedly, such conidia are pathogenic for wounded leaves [24].

Although zoopathogenic fungi do not seem to form appressoria, their melanin might play the penetrative role. In fact, hyphae of *W. dermatitidis* wild type grow through dense agar faster than those of Mel^{-} mutants. Moreover, tricyclazole slows the wild type whereas scytalone accelerates the growth of the mutant [4].

Some phytopathogens are melanized and penetrate their hosts via appressoria but without help of melanin. As mentioned above, pigment biosynthesis genes transferred from *A. alternata* to melanin-deficient mutants of *M. oryzae* restored their appressorial pigmentation and pathogenicity. But appressoria of *Alternaria* itself are colorless! Furthermore, its melanin-deficient mutants are as virulent as the wild type. Melanin of this fungus is present only in mycelium and conidia. It is assumed to contribute to pathogenicity as a factor of the parasite survival in nature [130].

6.2.2 Resistance to Host Defense

Melanin multifunctionality in fungal pathogenesis is illustrated, in particular, by *M. oryzae* melanin-deficient mutants alb-1 and ros-1. In fact, they are still nonpathogenic upon inoculation by injection, which should bypass mechanical barriers [126]. Correspondingly, the wild type depigmented by tricyclazole does not necessarily infect damaged leaves. Actually, tricyclazole or ftalide still protect wounded leaves of cv. Sha-tiao-tsao from the virulent strain H5-3 [136]. Therefore, the fungus recruits melanin not only as an arm for penetration. In many cases, the pigment protects the pathogen from adverse abiotic environment and antifungal responses of the host.

The development of blast fungus is suppressed before its penetration on rice leaves of certain cultivars. Here spore germination and appressorium formation of melanin-deficient mutants alb-1 and ros-1 are affected stronger than those of the parent H5-3 [126]. The suppression is driven by fungitoxic exo-metabolites of leaves since leaf diffusates show the same effect on spores in vitro. Diffusates of infected leaves are more toxic than those of healthy ones; the toxicity is higher in incompatible than in compatible combinations [137]. The agents causing acquired disease resistance increase the toxicity [138]. Therefore, this antifungal effect acts as one of defense responses. Spores of the wild type H5-3 are the most tolerant to a diffusate of rice healthy leaves of the susceptible cultivar while the albino spores alb-1 are the most sensitive; the defectively pigmented ros-1 is intermediate. Inoculation with any strain increases the leaf diffusate toxicity, to which the mutants are again more sensitive than the wild type [139]. Therefore, the plant hinders ectophytic development of the pathogen; melanin-deficient mutants are more liable to this impact that may contribute to their nonpathogenicity.

Like the mutations, spoiling pigmentation of the wild type fungus by tricyclazole, fthalide [136], or diethyldithiocarbamate also sensitize spores to leaf diffusates. On

the contrary, spores with melanization enhanced by maintenance on carrot medium (as against potato medium), under light (as opposed to darkness) [133], or by reisolation (in comparison with an old culture) [134] are more tolerant.

The antidotal action of endogenous melanin of blast fungus is confirmed by the same activity of the exogenous pigment. Melanin extracted from the wild type H5-3 mycelium and added to albino spores alb-1 restores their germination in leaf diffusates. The specimen isolated the same way from ros-1 protects weaker while the stuff from alb-1 does not protect at all [139]. The aforementioned rice leaf diffusate toxicity to blast spores is abolished or strongly diminished by antioxidants destroying hydrogen peroxide, superoxide, or hydroxyl radicals; so it is mediated by the reactive oxygen species [137]. Consistently, leaf diffusates generate chemically assayed superoxide, and its level increases after inoculation, especially on the resistant cultivars [140]. Since the defense is prooxidative in nature, the melanin-mediated tolerance to it is apparently based on the well-documented (see the Sect. 4) antioxidant action of the pigment.

The responsibility of this action for *M. oryzae* spore resistance to leaf diffusate agrees with the higher (than that of H5-3) sensitivity of ros-1 and, notably, alb-1 to hydrogen peroxide and model chemical sources of superoxide or hydroxyl radicals [141] or singlet oxygen [142]. Any artificial ROS are detoxified by exogenous melanin from H5-3, the preparation from ros-1 protects from $^{\cdot}OH$ and $^{1}O_2$ weaker while that from alb-1 does not protect. In the experiments, singlet oxygen was generated by illuminated photodynamic dies. The revealed difference in sensitivity of the strains was not due to different amounts of dyes absorbed by cells. Melanin added to spores after switching off the light did not protect them; hence, the pigment prevented rather than repaired an oxidative damage. The protective ability of exogenous pigment was not a result of light shielding by it [142]. These observations are in line with singlet oxygen quenching by melanins [143, 144].

Antioxidant properties of *M. oryzae* melanin are shown not only by protection of cells from oxidative damage but also chemically. The isolated melanin exhibits superoxide dismutase and catalase activities, although their specific values are lower than those of the corresponding enzymes. It also decreases yield of hydroxyl radical in Fenton system (Fe^{2+} + H_2O_2). The latter effect, as well as cell protection in this system, might be caused by scavenging of both hydroxyl radical and iron ions [141].

The intermediate position of the strain ros-1 poses a question of the nature of its pigment. As stable free radicals, melanins possess paramagnetic properties assayed by ESR (see the Sect. 4). Expectedly, the corresponding signal is found in spores, mycelium, and isolated pigment from the wild type but not in the albinos. However, the samples from rose mutant ros-1 also give a signal although weaker than that of H5-3 [145]. In ros-1, the polykedide pathway of melanin synthesis is blocked at the stage of 1,3,8-trihydroxyphthalen. This may lead to accumulation of scytalone or 3,4-dihydro-3,4,8-trihydoroxy-1(2H)-napthalenone. But both products are not paramagnetically active. Presumably, ros-1 cells contain a polymer of earlier or side intermediates of melanin biosynthesis or certain melanin with changed color like animal feomelanin. Such product appears to have some paramagnetic but, all the same, too weak antioxidant properties that leads to the infection failure.

Apparently, the lack of pathogenicity joined with a normal, at first sight, pigmentation may also be related to the inadequate antioxidant activity of melanin. In particular, the nonpathogenic strain H-9 is normally pigmented as H5-3 [139] and, furthermore, shows the same ESR signal [145]. However, its spore germination and, notably, appressorium formation on rice leaves are suppressed approximately at the same rate as those of the strain ros-1. The pigment isolated from H-9 mycelium and in excess added to leaf diffusates or Fenton system protects spores just weakly, at the level of ros-1 [139].

Like the mutants, wild type blast spores demelanized by tricyclazole, fthalide, or diethyldithiocarbamate are more sensitive than untreated ones to artificially generated ROS. Similar differences are also found in spores from cultures grown under different conditions [133, 134].

Other fungi, for example, *C. neoformans*, *W. dermatitidis*, and *A. alternata* also demonstrate increased sensitivities of their albino mutants to ROS and other strong oxidants, and protection from these agents by the wild-type exogenous melanins [29]. White mutants of *A. fumigatus* are about one order more sensitive to exogenous oxidants than the wild type is [4]. *C. neoformans* resists the toxicity of artificial oxygen- and nitrogen-derived radicals better, if it is supplied with DOPA enabling melanin synthesis [128]. Chemical inhibition of pigmentation sensitizes the fungus to oxidative damage. Another human pathogen, *F. pedrosoi* constitutively produces DHN-melanin. The suppression of colony growth by H_2O_2 or S-nitroso-N-acetylpenicillamine (a donor of NO) is stronger in a tricyclazole-treated culture [85].

Endogenous melanin renders zoopathogenic fungi tolerant not only to phagocytosis-mimicking model ROS but also to phagocytosis itself. It is reported for *C. neoformans*, *P. brasiliensis*, *S. schenkii*, *and F. pedrosoi* [85, 124, 128]. Melanized strains of *W. dermatitidis* better than nonmelanized ones resist killing by human neutraphils. Acidic growth medium, which inhibits melanization of the wild type, diminishes its survival down to the level of albino strain. But the latter acquires resistance if supplied with scytalone [4]. Nonpigmented mutant conidia of *A. fumigatus* are more susceptible to killing by human monocytes and exhibit more severe structural injury compared to wild-type conidia [124].

Conidial melanin of *A. fumigatus* inhibits apoptosis in phagocyting macrophages [80]. Besides, the pigment damps the phagocytic oxidative burst. In the course of the latter, macrophages produce chemically assayed superoxide radical in contact with, for example, *Saccharomyces cerevisiae*. This is also valid for *F. pedrosoi* conidia but only for the fungus demelanized by tricycazole [85]. Upon incubation of *A. fumigatus* conidia with phagocytes, pigmentless mutants bring about eightfold more ROS than the wild type. Pigment revertants regain resistance to oxidants and suppression of oxidant production of phagocytes as well as virulence [4]. Thus, the pigment can reduce both the yield of toxic products from the host's immune system and the parasite's sensitivity to the intoxication.

The counteraction of microbial melanins to induction of phagocytic oxidative burst may rest on fungal pigment-elicitor relations. Macrophages contain receptors sensing glucans of fungal cell wall to promote the synthesis and release of NO and ROS. Melanin is suggested to mask antigens/glucans, but this function is disturbed

in tricyclazole-treated *F. pedrosoi*, which elicits the macrophages' oxidative response [85]. In zoopathogenic *A. fumigatus,* deletion mutant conidia, unlike those of wild type, activate human dendritic cells and the subsequent cytokine production [146]. It is not excluded (but perhaps not yet tested) that melanin layers could hide elicitors in fungal cell wall to prevent recognition by defense systems of plants [29]. However, in animals, melanin itself is immunologically active: cryptococcal pigment elicits generation of specific antibodies in mice [124].

It is also possible that melanins detoxify not only ROS but also plant organic defensive compounds, for example, phytoalexins [29]. The negatively charged pigment of zoopathogenic *C. neoformans* neutralizes neutrophil defensin and other cationic antimicrobial peptides [124]. The similar activity of melanin against antifungal therapy is reported. The knockout of *PKS1* gene of *W. dermatidis* inhibits melanin synthesis and sensitizes the fungus to voriconazole and amphotericin B. Melanized cells of *Histoplasma capsulatum* and *C. neoformans* are more resistant to amphotericin B and caspofungin. This resistance can be accounted for by drug binding and deactivation by melanins [80]. However, Mel^+ and Mel^- strains of *W. dermatidis* do not differ in their sensitivity to antifungals [4].

Melanin as a structural element of fungal cell wall plays different roles essential for pathogencity. Pigmentless mutants of *A. fumigatus* have abnormally smooth conidial surface [4]. It is suggested that the pigment is required for correct assembly of the cell wall layers and the expression at the conidial surface of adhesins and other virulence factors [46]. In this fungus, the melanin renders conidia immunologically inert because of proper surface charge and hydrophobicity [146].

6.2.3 Resistance to Hostile Environment

In addition to host's defenses, abiotic extreme environment impacts parasites so that the fate of infection depends on survival of the causal agent. For example, diurnal light is natural for plant shoot microflora. But the excessive insolation represses both fungal pathogens and, in many cases, disease development [147]. The vulnerability of pathogenic fungi to intense UV and visible light in consequence of altered melanization is well known [4]. In many cases, photodamage is oxidative. For example, photoinhibition of *M. oryzae* spore germination and appressoria formation is rescued by exogenous superoxide dismutase or catalase pointing to involvement of O_2^- and H_2O_2. So, melanin may photo-protect cells not only as a light shield but also as an antioxidant. Photosensitivity of the wild type strain H5-3 and its melanin-deficient mutants ros-1 and alb-1 are ranged in the same sequence [148] as sensitivity to leaf diffusates or model ROS (see above). Similar differences in light sensitivity are revealed for wild type fungus treated with tricyclazole or diethyldithiocarbamate or grown under different conditions [133, 134]. Addition of melanin biosynthesis genes from *A. alternata* to melanin-less entomopathogenic fungus *M. anisopliae* increases its tolerance to UV-B together with its virulence [129].

Melanin protects pathogenic fungi also from other abiotic stressors, sometimes complex. One example is water deficit. Microsclerotia of albino strain of *Verticillium* spp. badly suffer from desiccation in comparison with wild type or albinos whose pigmentation was restored by exogenous scytalone. The resistance of the wild type

may be essential for its overwinter survival. Tolerance of *Phyllosticta* sp. to desiccation is related with creation of turgor necessary for fungus penetration into host tissues. Conidia of albino *Monilinia fructicola* perish faster than wild type under desiccation as well as at high (40 °C) temperature or under UV [29]. Exogenous DOPA added to a wild type *C. neoformans* increases its heat tolerance [4].

Melanin of pathogenic fungi helps their withstanding unfavorable biotic challenges from not only hosts but also other microbes. Many examples are given in the review of Butler and Day [29]. Scletotia of *Botrytis cinerea* depigmented by tricyclazole acquire sensitivity to attack of mold and mycophilic fungi. After the same treatment, scletotia of *Sclerotinia* germinate while untreated ones remain dormant. The antilysis tolerance is another way of protection by melanins from biotic damages occurring, in particular, in soil. It is reported for such phytopathogenic fungi as *Rhizoctonia* sp., *Sclerotinia* sp., *Verticillium* sp., and *Cochliobolus sativus*. Presumably there are no microbes capable of damage the heavily melanized sclerotia of *Sclerotinia rolfsii*. High resistance of *Rhizoctonia solani* to bacterial lysis is probably due to melanin resistance to lytic enzymes. In agreement with this, synthetic melanin protects casein from proteases. Rise in melanin content in *Alternaria kikuchiana* treated with polyoxin accompanies the rise in the fungus resistance to lytic enzymes. Albino chlamidospores of *Thielaviopsis basicola* are more sensitive to enzymatic lysis than normally pigmented ones. In contact with litic enzymes or soil, death of nonmelanized isolates of *Cochliobolus sativa* occurs earlier than that of normally melanized. In preparing *M. oryzae* protoplasts, the tolerance to hydrolytic enzymes correlates positively with melanin content [126]. Albino conidia of *Monilinia fructicola* are several times more sensitive to lysis than the wild type. Enzyme binding and inactivation by melanins may be responsible for the cell protection from lysis [4].

6.2.4 Other Functions

Melanin seems to participate in pathogenesis by somewhat unusual way, namely, as a source of oxidative damage rather than protector from it. There was a recommendation in the 1940s to grow bananas under partial shade. That was because light favors the disease of this and other crops caused by *Cercospora* species. The light effect belongs to the fungal toxin cercosporin, which is a photosensitizer yielding singlet oxygen and other ROS [149]. Black Sigatoka disease of banana is also promoted by light but the causal agent *M. fijiensis* does not produce cercosporin. Beltrán-García and coworkers [127] hypothesized that melanin of *M. fijiensis* behaves as a light-activated toxin. They assayed singlet oxygen by IR emission (at 1270 nm) in laser-excited (at 532 nm) samples. This ability was actually found in whole mycelium as well as in melanin isolated from it; mycelia of albino mutants were less productive. Photogeneration of $^1O_2$ also occurred in the secreted fraction of melanin and in the mixture of intermediates of melanin biosynthesis releasing into medium amended with tricyclazole, tropolone, or pyroquilon. The authors suggest the mycelial melanin protects the fungus by deactivating singlet oxygen and oxygen radicals. At the same time, the pigment and its soluble intermediates may play the

opposite, i.e., pro-oxidative role producing ROS that injure host tissues and resulting in the destructive disease symptoms.

The idea of double faced melanins is not contradictory. ROS generation by melanins in their autoxidation [82] or upon UV irradiation along with accompanying cytotoxic effects [99, 150] are known. However, prooxidative role of melanin in pathogenesis deserves detailed consideration. Because the IR spectrometry is rather sensitive, one would wonder if naturally illuminated melanin produces $^1O_2$ in amounts sufficient for biological effects. It would be interesting to find out whether melanin intermediates posses light-depended phytotoxicity; inhibitor-based studies with appropriate antioxidants would also be desirable.

Another disruptive role of fungal melanins against host plant is related with a lytic activity of the fungus. Cell wall melanin of the apple scab pathogen *V. inequalis* was found to bind fungal extracellular hydrolytic enzymes RNAase, DNAase, acid phosphatase, and phenoloxidase. The enzymes are released from melanin afterwards and retain high activity that allows concentrating their action at particular sites [29]. By the way, shunt products from DHN melanins may also be phytotoxic and involved in pathogenicity. This is, for example, alteichin, a phytotoxin of *Alternaria eichhorniae*: its synthesis is blocked by tricyclazole [24].

Properties of melanins are really unique, but they should not be overrated because the pigments share certain functions (antioxidative, for instance) with other metabolites. It follows that once pigmentation is altered, this is not necessarily the sole cause of accompanying effects. The inhibitors of melanin biosynthesis may have auxiliary modes of action. For example, the antiblast fungicide carpropomide stimulates host defense responses including lignification [151]; tricyclazole and fthalide promote oxidative burst in rice leaves [136]. In general, fungal melanins act as universal protectors from numerous antimicrobial factors, but the harm resulted from melanized pathogens is not controlled universally by inhibitors of melanin biosynthesis.

7 Conclusions

New fungal extreme ecosystems continue to be discovered and investigated including the deep biosphere, new regions of technogenic pollution, and latest human and plant parasites. Thus physiological characteristics of extremophilic organisms stated in the twentieth century must be updated. Melanin pigments represent an important point in this field. Achievements in melanin research, based on organic chemistry, advanced spectroscopic and imaging techniques, theoretical calculations, and methods of solid-state physics, unraveled unique structural and optoelectronic properties of melanins, their localization in the cells, intracellular transport of melanin precursors and reactions of their polymerization. The ever-growing knowledge not only gains insight into multifunctionality of melanins in extremophilic fungi but may also be used in effective strategies for exploiting their properties to create a new class of biologically provided high technological materials and new antifungal drugs.

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Secondary Metabolites in Cheese Fungi

9

Juan F. Martín and Paloma Liras

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Abstract

Several filamentous fungi grow on the surface or inside different types of cheese, produce secondary metabolites, and contribute to the organoleptic characteristics of mature cheese. Particularly relevant is the contribution of *Penicillium roqueforti* to the maturation of blue-veined cheeses (Roquefort, Danablu, Cabrales, etc.). *P. roqueforti* is inoculated into these cheeses as a secondary starter. This fungus is closely related taxonomically to *Penicillium carneum* and *Penicillium paneum*, but these two species are not used as starters because they

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produce the potent toxin patulin. *P. roqueforti* Thom has the capability to produce about 20 secondary metabolites of at least seven different families, but it seems that only some of them are produced in microaerobic conditions and accumulate inside the cheese (e.g., andrastins). This article focuses on the biosynthetic pathways, gene clusters, and relevance of the known metabolites of *P. roqueforti* including roquefortines, PR-toxin and eremofortins, andrastins, mycophenolic acid, clavines (agroclavine and festuclavine), citreoisocoumarin, and orsellinic acid. In addition the biosynthesis of patulin (a *P. paneum* and *P. carneum* product) is discussed. *Penicillium camemberti* grows on the surface of Camembert, Brie, and related white rind cheeses, and the penetration of secondary metabolites inside the cheese is relevant. One of the *P. camemberti* metabolites, cyclopiazonic acid, is important because of its neurotoxicity and its biosynthesis is reviewed. The removal of toxic metabolites gene clusters by precise gene excision while preserving all other characteristics of the improved starter strains, including enzymes involved in cheese ripening and aroma formation, is now open. A possible strain improvement application to the cheese industry is of great interest.

Keywords

Cheese fungi • Blue-veined cheeses • *Penicillium roqueforti* • *Penicillium camemberti* • Secondary metabolites biosynthesis • Roquefortines • PR-toxin • Eremofortins • Andrastins • Mycophenolic acid • Clavine alkaloids • Cyclopiazonic acid

List of Abbreviations

ACP	Acyl-carrier protein
AT	Acyltransferase
ATCC	American Type Culture Collection
DMA-PP	Dimethylallyl diphosphate
DMAT	Dimethylallyltryptophan
DMOA	3,5-Dimethylorsellinic acid
FPP	Farnesyl diphosphate
KS	Ketosynthase
MFS	Major facilitator superfamily
MPA	Mycophenolic acid
6-MSAS	6-Methyl salicylic acid synthase
MT	Methyltransferase
nr-PKS	Non-reductive polyketide synthase
NOX	N1 hydroxylase
RAPD	Random amplified polymorphic DNA
RDH	Roquefortine D dehydrogenase
RPT	Roquefortine prenyltransferase
SAR	Starter unit acyltransferase

1 Introduction

Many *Penicillium roqueforti* strains are used in different countries in the world as secondary starters for the production of blue-veined cheese. More than one hundred of these strains have been characterized morphologically [1], and all of them are closely related to the original type strain described by Charles Thom [2] as *P. roqueforti* Thom ATCC10110. Taxonomically, this strain is referred as *P. roqueforti* subspecies *roqueforti*. Some strains of the *P. roqueforti* cluster (initially identified as *P. roqueforti*) differ in the metabolites that are separated by thin layer chromatography [3] and in the pigmentation of the reverse of the colonies [4]. These authors found a group *of P. roqueforti* strains that are less pigmented and produce patulin instead of PR-toxin and named this group *P. roqueforti* subspecies *carneum* because they are found associated with spoiled meat products.

Later Boysen et al. [5] using rRNA sequences and RAPD (random amplified polymorphic DNA) techniques divided the "*P. roqueforti*" strains into three species, namely, *P. roqueforti* sensu stricto, *Penicillium carneum*, and *Penicillium paneum*. The last one was associated with molded bread, flour, and cereal grains.

Recently Houbraken et al. [6] discovered a new member of the *P. roqueforti* series in cold-preserved apples. This strain, which grows and forms sexual cleistothecia at low temperature, has been classified as *Penicillium psychrosexualis*. It produces patulin as *P. carneum* and *P. paneum* at difference of *P. roqueforti* (Table 1). So far, *P. psychrosexualis* has not been found in cheeses and appears to be mainly associated with fruits such as apples and pears, in which it may produce pigmented spots. Therefore, the relevance of the secondary metabolites of this species in cheese is lower than that of *P. roqueforti*, *P. carneum*, and *P. paneum*.

1.1 Fungal Secondary Metabolites

Frequently filamentous fungi produce a few dozens of molecules belonging to different classes of secondary metabolites (polyketides, terpenes, nonribosomal peptides, aromatic compounds, heterocyclic metabolites, etc.) [7]. Usually they are produced as mixtures of chemically related molecules (e.g., roquefortines C, D, L, M or andrastins A to D). Each family of these compounds derives from a set of enzymes encoded by a gene cluster. Genetic information, in the form of gene clusters, for about 15 to 30 secondary metabolites have been found in the sequenced genome of ascomycetes [8, 9]. In some fungi, several gene clusters have been characterized by genetic and biochemical analysis (e.g., *A. nidulans*, *A. fumigatus*), whereas in others only a small number of secondary metabolite gene clusters has been identified so far (e.g., *P. chrysogenum* or *P. roqueforti*) and many other gene clusters remain cryptic, i.e., encoding unknown products [7, 10]. In addition, a number of gene clusters remain fully silent or nearly silent, although in some cases their expression may be activated by specific methods [10, 11]. In this article, we focus on the study of the

Table 1 Secondary metabolites produced by *P. roqueforti* and the closely related *P. carneum*, *P. paneum*, and *P. psychrosexualis*

Metabolites in *P. roqueforti*	Metabolites of *P. carneum*	Metabolites of *P. paneum*	Metabolites of *P. psychrosexualis*
Agroclavine	Agroclavine	Agroclavine	
Andrastins A, B	Andrastins A, B	Andrastins A, B	Andrastin A
Citreoisocoumarin	Citreoisocoumarin	Citreoisocoumarin	
Eremofortins A, B			
Festuclavine	Festuclavine	Festuclavine	
16-Hydroxyroquefortine	16-Hydroxyroquefortine	16-Hydroxyroquefortine	
		Marcfortins A, B, C	
Mycophenolic acid	Mycophenolic acid	Mycophenolic acid	Mycophenolic acid
Orsellinic acid	Orsellinic acid	Orsellinic acid	
PR-toxin			
Roquefortines C, D, L	Roquefortines C, D, L	Roquefortines C, D, L	Roquefortine C
	Patulin	Patulin	Patulin
	Penitrem A	Penitrem A	
		VM55599	
			"Fumu", uncharacterized

(1) *P. carneum* and *P. paneum* may produce variable amounts of all other *P. roqueforti* secondary metabolites with the exception of eremofortins and PR-toxin (see text). An early description of penicillic acid production in a *P. roqueforti* strain is now explained due to a misclassification of the producer strain

secondary metabolites produced by the cheese fungus *P. roqueforti* (Table 1). The metabolites produced by the related fungi *P. paneum* and *P. carneum*, which are only rarely found in blue cheeses, and *P. psychrosexualis* are listed in Table 1, but they are reviewed succinctly at the end of the chapter (e.g., patulin produced by *P. paneum* and *P. carneum*) [12]. Also the biosynthesis of the neurotoxin cyclopiazonic acid by *Penicillium camemberti* is included in this article.

2 Secondary Metabolites Produced by *P. roqueforti*

In the last decades, increasing evidence has been reported on the ability of *P. roqueforti* to produce secondary metabolites in different culture media and inside the blue cheeses [13, 14]. The biosynthetic pathway of some of these metabolites and the gene clusters encoding their pathways have been located in the genome of the producer fungi [15–18], although the pathways for some of the rare secondary metabolites remain unknown. The full genome sequence of *P. roqueforti* FM164 has been made available [19], and this information will contribute to a better understanding of the ability of this fungus to express the genes encoding secondary metabolites under different growth conditions. So far the information available about

the expression of the genes encoding enzymes for secondary metabolites biosynthesis inside the blue-veined cheeses is very scarce.

2.1 Roquefortines

Roquefortines were discovered many decades ago [20, 21] and are among the best known *P. roqueforti* secondary metabolites [22, 23]. The roquefortine family includes roquefortine C and the related roquefortines D (3, 12-dihydroroquefortine C), 16-hydroxyroquefortine C, roquefortine L, and some other minoritary roquefortines [24]. These compounds are members of the prenylated indole alkaloid class of compounds (Fig. 1) (reviewed in reference [25]). Roquefortine C is produced by *P. roqueforti* growing in a variety of solid substrates, but its formation in blue cheeses does not occur in significant amounts, and there is a consensus that roquefortines in cheese do not pose a health problem for humans [12, 14, 22, 26].

The compounds of the roquefortine family derive from L-tryptophan, L-histidine, and mevalonate [25]. Roquefortines are produced by several *Penicillium* species including *P. roqueforti*, *P. chrysogenum* [15, 16], and other plant-associated or saprophytic fungi [27, 28]. However, it was unknown if the biosynthetic pathway of roquefortine alkaloids is identical in all these fungi. Recent evidence [18] demonstrated that the roquefortine pathway in *P. roqueforti* is shorter than that for roquefortine/meleagrin in *P. chrysogenum* (see below).

2.1.1 Biosynthesis of Roquefortine and Meleagrin

One of the common early intermediates of prenylated indole alkaloids is a molecule of dimethylallyl-tryptophan (DMAT) that is formed by a prenyltransferase that uses L-tryptophan (or a L-tryptophan-containing cyclopiperazine dipeptide) and dimethylallyl diphosphate (DMA-PP) as substrates [15, 16, 25]. In roquefortine alkaloids, precursor condensation of these substrates occurs at C-3 of L-tryptophan with the 3′carbon atom of DMA-PP (named “reverse condensation”) [29].

Fig. 1 Structure of the major secondary metabolites produced by *P. roqueforti*. The metabolites produced by *P. carneum*, *P. paneum*, and *P. camemberti* are not included in the figure

Following initial precursor studies in *P. roqueforti* [30, 31], it was established that the tryptophan-histidine cyclopiperazine nucleus of roquefortine C and the related compounds glandicolines A and B, meleagrin, and neoxaline derive from the precursor compounds L-tryptophan and L-histidine. These two amino acids are condensed by a dimodular nonribosomal peptide synthetase, named RDS (roquefortine dipeptide synthetase) consisting of two similar modules with the domain sequence ATCATC, where A indicates adenylation domain (amino acid activation), T thiolation (peptidyl carrier) domain, and C condensation domain. The amino acid specificity of each domain has been elucidated [15, 25]. The cyclodipeptide (*cyclo*-trp-his) is then prenylated by the roquefortine prenyltransferase (RPT) that introduces an isopentenyl group at C-3 of tryptophan (Fig. 2b). The resulting prenylated compound is roquefortine D (3, 12-dihydroroquefortine C). In the last step of the roquefortine pathway, roquefortine D is oxidized by the roquefortine D dehydrogenase (RDH), losing two H atoms with the formation of a double bond between carbons 3 and 12 resulting in roquefortine C (Fig. 2b). The order of the second and third biosynthetic reactions

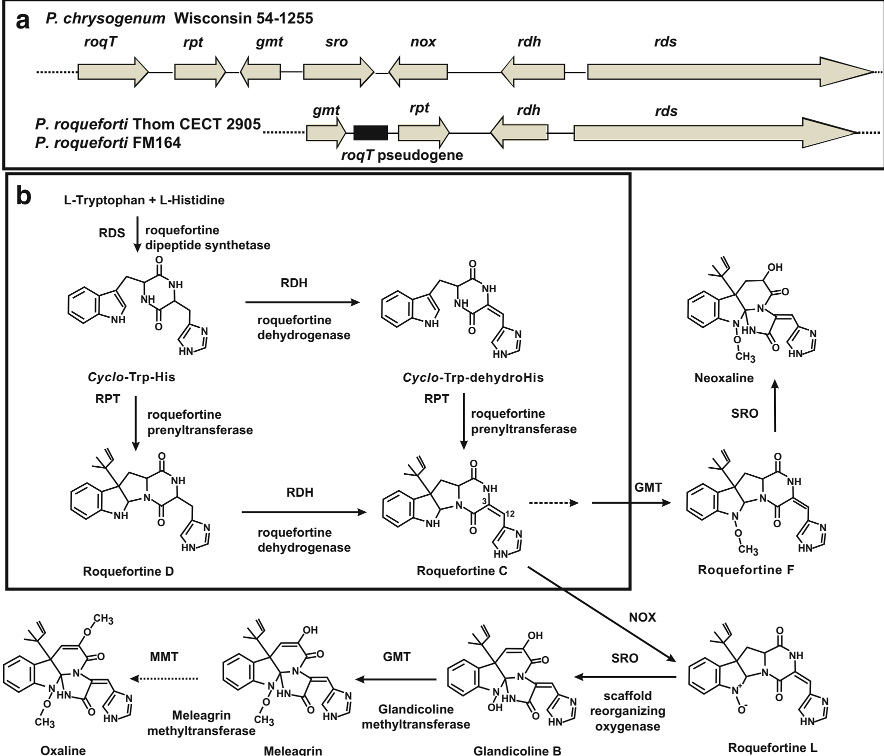

Fig. 2 (**a**) Gene cluster for roquefortine/meleagrin in *P. chrysogenum* compared to the roquefortine cluster in *P. roqueforti*. (**b**) Biosynthetic pathway of roquefortine C (*boxed area*), meleagrin, neoxaline, and oxaline in *P. chrysogenum* (Modified from Ref. [28])

(prenylation and roquefortine dehydrogenation) is indifferent [16], and therefore, a metabolic grid occurs in these early steps of the pathway [25].

In some fungi (e.g., *P. chrysogenum* and *P. oxalicum*), roquefortine C is later converted to roquefortine L (or in other fungi to glandicoline A) and then to glandicoline B and meleagrin (see structure in Fig. 1) [31]. The conversion of roquefortine C (or roquefortine L in *P. chrysogenum*) to the late pathway products involves (i) a carbon scaffold reorganizing oxygenase (SRO), similar to the FtmG oxygenase of *Aspergillus fumigatus* involved in fumitremorgin biosynthesis, and (ii) a N1 hydroxylase (NOX) and a N-OH methyltransferase (Fig. 2b). The role of each of these enzymes in the roquefortine and meleagrin pathways has been reviewed elsewhere [25, 28].

2.1.2 The Roquefortine/Meleagrin Gene Cluster

Molecular genetic studies on the roquefortine/meleagrin gene cluster (*roq/mel*) were performed first in *P. chrysogenum* Wis54-1255 leading to the characterization of the gene cluster and the proposal of a roquefortine/meleagrin biosynthetic pathway [15]. The roquefortine/meleagrin gene cluster was later confirmed by Ali et al. [16] and Ries et al. [24], who reported the formation in *P. chrysogenum* of roquefortine L (instead of glandicoline A) as an intermediate in the pathway, in addition to other minoritary roquefortines derived from late branches of the pathway. The entire pathway in *P. chrysogenum* is encoded by a seven-gene cluster (Pc21g15420 to Pc21g15480) (Fig. 2a).

An important question is if the many natural isolates (strains) of *P. roqueforti* obtained from different geographical areas [1] have genetic differences in their capability to synthesize roquefortine and the related indole alkaloids [14, 26] and whether these differences are due to changes in the roquefortine gene cluster.

The roquefortine gene cluster of *P. roqueforti* has recently been investigated [18]. The initial steps of roquefortine biosynthesis in *P. roqueforti* are identical to those of *P. chrysogenum*, but we found that *P. roqueforti* lacks the genes that encode the enzymes for the “late” conversion of roquefortine C to roquefortine L, glandicoline B, and meleagrin [18]. A natural short pathway was found in *P. roqueforti* that is dedicated to the production of roquefortine C but is unable to form derivatives containing the meleagrin scaffold [18, 28].

A comparative analysis of the *roq* cluster of *P. roqueforti* and the *roq/mel* cluster of *P. chrysogenum* revealed that two key genes located in the central region of the *roq/mel* cluster in *P. chrysogenum* (*sro* and *nox*) have been lost in *P. roqueforti* during evolution and the order of two of the conserved genes has changed during gene reorganization. Furthermore, the *roqT* gene, encoding a transmembrane transport protein in *P. chrysogenum*, has been rearranged into a pseudogene (Fig. 2a) that encodes only residual peptides [18, 28]. As a result of the *roq/mel* cluster reorganization, *P. roqueforti* is unable to convert the roquefortine-type carbon skeleton into a meleagrin-type scaffold and is incapable to produce glandicolines. The cluster reorganization is not a recent event derived from “industrial” strain selection. Rather, it seems to be an ancient phenomenon that occurred probably millions of years ago during adaptation of a progenitor *Penicillium* to cheese environments [28].

PR-Toxin and Eremofortins PR-toxin and eremofortins are isoprenoid secondary metabolites. PR-toxin is probably the most potent mycotoxin produced by *P. roqueforti* [12, 23]. This isoprenoid mycotoxin is clearly toxic for mice, rats, hamsters, and some domestic animals in vivo. Furthermore, PR-toxin has mutagenic action in vitro, as shown in studies using the Ames test. Actually, PR-toxin is considered to be the causative agent of cow toxicosis produced by poorly conserved moldy silages [12]. Fortunately, PR-toxin is modified to less toxic derivatives by *P. roqueforti* cells, and its toxic form does not seem to be accumulated in large amounts in blue cheese [22].

Recently, we have studied the biosynthesis of PR-toxin and its intermediates the eremofortins [17]. PR-toxin derives from the sesquiterpene (15 carbon atoms) aristolochene; this first intermediate is formed by aristolochene synthase (encoded by the gene *ari*1). Hidalgo et al. [17] cloned and sequenced a partial PR-toxin cluster containing four genes that include the *ari*1 (*prx2*) gene reported previously in *P. roqueforti* (Fig. 3a). Gene silencing of each of the four genes, named *prx*1 to *prx*4 (*prx,* abbreviation for PR-toxin), caused a reduction of 65–75 % in the production of PR-toxin indicating that these four genes encode enzymes involved in PR-toxin biosynthesis. An eleven gene cluster (Pc12g06260 to Pc12g06370) that includes the above-mentioned four *prx* genes and a 14-TMS (transmembrane spanner domain) drug/H^+ antiporter of the MFS family was found in the genome of *P. chrysogenum* (Fig. 3a). A detailed analysis of the published genome sequence of *P. roqueforti* FM164 [19] revealed that this strain contains in two subclusters 10 of

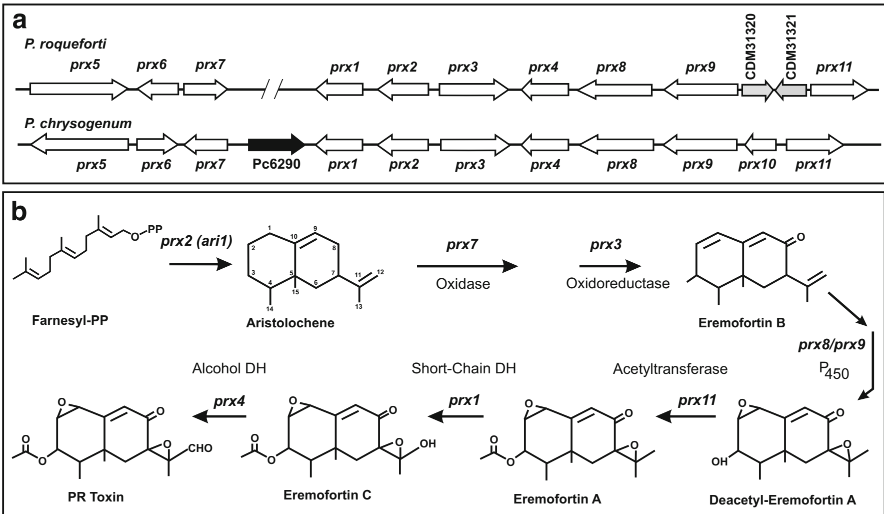

Fig. 3 (**a**) Gene clusters of PR-toxin in *P. chrysogenum* Wis 54–1255 and *P. roqueforti* FM164. The location of a pseudogene in the *P. chrysogenum* cluster (*shadowed in black*) is shown. In *P. roqueforti*, three of the *prx* genes (*prx*5 to 7, proteins CDM35712 to 35710) are located elsewhere (Contig Proq06g) in the genome in relation to the *prx*1 to *prx*11 gene cluster (contig Proq02g, proteins CDM31314 to 31322). (**b**) Proposed PR-toxin biosynthetic pathway (see text for details)

the 11 *prx* genes described in *P. chrysogenum*. The exception is *prx*10 that was reported as encoding a protein of unknown function [17]. As shown in Fig. 3a, seven of the 10 *prx* genes (*prx* 1 to 4, *prx* 8, 9, and 11) are clustered together in contig Proq02g of *P. roqueforti*, whereas the three remaining genes (namely, *prx*5, 6, and 7) are located elsewhere (contig Proq06g) in the genome.

PR-toxin biosynthesis pathway from farnesyl diphosphate was proposed based on all available evidence [17]. It proceeds to PR-toxin through aristolochene and the eremofortins (Fig. 3b). The PR-toxin pathway is divided in two parts. The first part corresponds to the conversion by oxidative enzymes of the 15-carbon atom aristolochene to 3-hydroxy, 8-oxo, 12-dehydroaristolochene, eremofortin B, and deacetyl-eremofortin A (DAC-EreA), all containing 15 carbon atoms. In the second half, DAC-eremofortin A is acetylated to the 17-carbon eremofortin A by an acetyltransferase encoded by *prx11*, and then eremofortin A is converted to eremofortin C and finally to PR-toxin.

Both eremofortins and PR-toxin are probably secreted by the MFS transporter encoded by the *prx5* gene in the *prx* cluster, as proposed for several antibiotics and other secondary metabolites [32].

The PR-toxin is converted in vitro and probably also in vivo to PR-amide and PR-imine by reaction of the PR-toxin carboxylic group with ammonium ions or primary amines in the culture medium or in the cells [33, 34], and these derivatives appear to be less toxic than the PR-toxin itself.

2.2 Andrastins

Another important family of *P. roqueforti* secondary metabolites is the andrastins that belong to the polyketide-isoprenoid class. They are inhibitors of the farnesyl-transferase of the *ras*-encoded oncogenic protein [35, 36]. Prenylation (farnesylation) of the human Ras protein is essential for its biological activity that may cause tumor formation. Therefore, inhibitors of the Ras prenyltransferase activity are interesting for their use as potential antitumor agents [37].

The andrastins belong to the meroterpenoid class of secondary metabolites that include compounds with interesting pharmacological activities [38]. Andrastins A, B, C, and D were discovered by S. Omura (Nobel Prize 2015) and coworkers at the Kitasato Institute in Japan in a screening of antitumoral agents. These compounds were first identified in the culture broth of *Penicillium* sp. FO4259 [36, 39, 40], and they are produced by several other *Penicillium* species [41].

Nielsen et al. [13] and Fernández-Bodega et al. [14] found that *P. roqueforti* produces andrastins and that andrastin A (the final product of the biosynthetic pathway) is accumulated inside blue cheeses inoculated with *P. roqueforti* as a secondary starter. Andrastin A concentrations in different blue cheeses such as Roquefort, Danablu, Cabrales, Bejes-Tresviso, and Valdeón vary depending on the particular *P. roqueforti* strain used as starter and ripening conditions [14]. Andrastins are considered to be beneficial for human health because of their *ras*

prenyltransferase inhibitory activity, but there are no studies that support its lack of toxicity when accumulated in high concentrations in cheese.

2.2.1 Biosynthesis of Andrastins

Initial precursor incorporation studies showed that the andrastins derive from 3, 5-dimethylorsellinic acid (DMOA) and the terpene precursor farnesyl diphosphate (FPP) [39]. In fungi, orsellinic acid is formed by the condensation of one unit of acetyl-CoA (starter unit) and three units of malonyl-CoA (elongation unit) followed by cyclization of the tetraketide to form the aromatic ring of orsellinic acid. These reactions are catalyzed by a specific nonreducing polyketide synthase (nr-PKS). The precursor incorporation studies suggested that the two methyl groups of DMOA derive from methionine [39] although it is not entirely clear if the incorporation of the methyl groups occurs during polyketide elongation or after orsellinic acid is formed. Based on the information available on the molecular genetics of the biosynthesis of other farnesylated-DMOA-derived fungal metabolites (e.g., austinol or terretonin), Matsuda et al. [42] identified a gene cluster encoding enzymes for andrastin biosynthesis in *P. chrysogenum*. The biosynthetic pathway of farnesyl-DMOA containing meroterpenoids [38] indicates that DMOA is converted to farnesyl-DMOA by a specific farnesyltransferase and then the farnesyl-DMOA is converted into farnesyl-DMOA methyl ester by the action of methyltransferase. A FAD-dependent monooxygenase converts the terminal double bond of farnesyl-DMOA methyl ester into its epoxy derivative (Fig. 4b).

The epoxy farnesyl-DMOA methylester is later cyclized to a polycyclic meroterpenoid by a characteristic terpene cyclase. The cyclases of each meroterpenoid gene cluster may yield a (slightly) different cyclic structure [43]

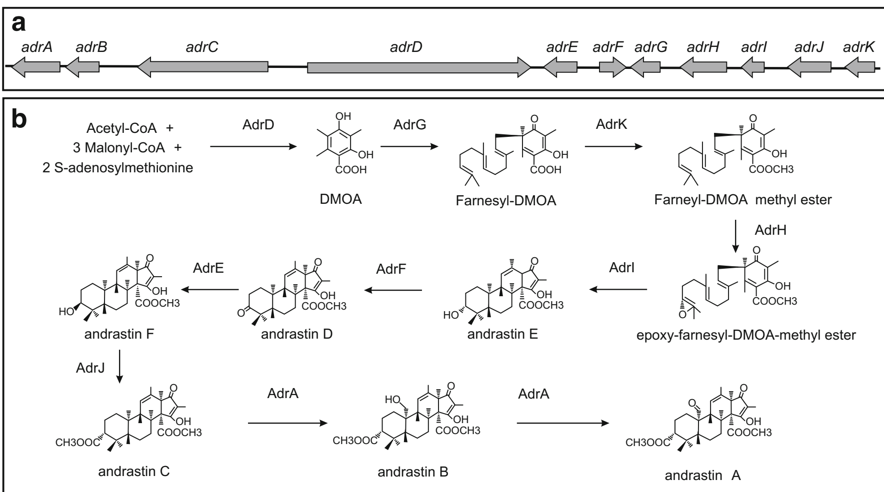

Fig. 4 (**a**) Gene cluster for andrastin in *P. chrysogenum* (see text for details). (**b**) Biosynthetic pathway of andrastins (Modified from Ref. [42])

that are converted to different final meroterpenoid molecules by “late” modification enzymes (so-called tailoring enzymes) (Fig. 4b).

2.2.2 Andrastins Gene Cluster

The andrastin A gene cluster of *P. chrysogenum* (Fig. 4b) comprises eleven genes (Pc22g22820 to Pc22g22920) of which nine correspond to enzymes that are directly involved in andrastin A (the most modified final product) biosynthesis. These nine enzymes include (i) an iterative type I, nonreductive polyketide synthase that forms DMOA (named AdrD); (ii) a prenyltransferase that attaches the farnesyl group of FPP to the DMOA moiety (AdrG); (iii) a methyltransferase that methylates the carboxyl group of farnesyl-DMOA forming farnesyl-DMOA methyl ester (AdrK); (iv) a FAD-dependent monooxygenase that converts farnesyl-DMOA methyl ester to epoxy-farnesyl-DMOA methyl ester (AdrH); (v) a terpene cyclase (AdrI) that cyclizes the epoxyfarnesyl-DMOA methyl ester intermediate to form andrastin E, the first member of the andrastin family; and (vi) four additional tailoring enzymes that convert andrastin E to andrastins D, F, C, B, and A (final product) (Fig. 4b). These tailoring enzymes include a short-chain dehydrogenase (AdrF), a ketoreductase (AdrE), an acetyltransferase (AdrJ) forming andrastin C, and finally a P450 monooxygenase (AdrA) involved in the consecutive oxidations of the C-23 methyl group of andrastin C to form andrastin B and then andrastin A that contain an alcohol and an aldehyde group at the C-23 position, respectively (Fig. 4b). The involvement of these 9 genes in andrastin A biosynthesis was confirmed by heterologous expression of a reconstructed gene cluster in *Aspergillus oryzae* that resulted in the production of andrastin A [42]. The andrastin gene cluster of *P. roqueforti* has not been characterized so far, although is likely to be similar to that of *P. chrysogenum.*

2.3 Mycophenolic Acid

Another important secondary metabolite of *P. roqueforti* is mycophenolic acid (MPA). This compound was already known at the beginning of the twentieth century, before the discovery of penicillin, as an antibiotic active against *Bacillus anthracis*, produced by a *Penicillium* sp. strain. Production of MPA in liquid cultures has been studied in *Penicillium brevicompactum* [44], the fungus which is used for MPA industrial production and in *P. roqueforti* [17]. Mycophenolic acid, discovered initially as antibacterial agent, was later found to have other important biological activities [45]. Particularly relevant is its activity as immunosuppressant used successfully to prevent organ rejection in transplants [46]. In addition, MPA has antitumor, antiviral, and antifungal activities and is used in the treatment of psoriasis [47–51].

2.3.1 Mycophenolic Acid Biosynthesis and Resistance Genes

Initial precursor incorporation studies [52] suggested that MPA is a compound synthesized through the hybrid polyketide-terpene pathway. Recently, the *mpa*

gene cluster was cloned from a *P. brevicompactum* strain [53] and later confirmed in the sequenced genome of a different *P. brevicompactum* strain [54]. In both strains, the *mpa* cluster comprises seven genes (Fig. 5), namely, *mpaA* (encoding a prenyltransferase), *mpaB* (encoding a protein of unknown function), *mpaC* (encoding a polyketide synthase), *mpaDE* (encoding a bifunctional fused protein with two domains corresponding to a P450 monooxygenase and a hydrolase), *mpaF* (encoding an inosine-5′-phosphate dehydrogenase), *mpaG* (encoding an O-methyltransferase), and *mpaH* (encoding an oxidative cleavage enzyme).

A key enzyme in MPA biosynthesis is the non-reductive iterative PKS encoded by *mpaC*. This protein contains the following domains: a starter unit acyltransferase (SAT), a ketosynthase (KS), an acyl-carrier protein (ACP), a methyltransferase (MT), and a standard acyltransferase (AT). These activities are required for the synthesis of the MPA intermediate 5-methylorsellinic acid from one starter acetyl-CoA, three malonyl-CoA extender units, and a methyl group.

Involvement of the *mpaC* gene encoding the non-reductive PKS and *mpaDE* encoding the bifunctional P450 monooxygenase-hydrolase in MPA biosynthesis has been confirmed by disruption of these genes in *P. brevicompactum* and by their expression in the heterologous host *Aspergillus nidulans*, a nonproducer of MPA that lacks the orthologous genes [53, 55]. More recently Zhang et al. [54] proved that *mpaG* encodes a S-adenosylmethionine (SAM)-dependent O-methyltransferase that converts in vitro demethylmycophenolic acid to MPA, the last step in the pathway (Fig. 5). This methyltransferase was purified after expression of the *mpaG* gene in *E. coli*. The enzyme showed similar substrate kinetics to O-methyltransferase

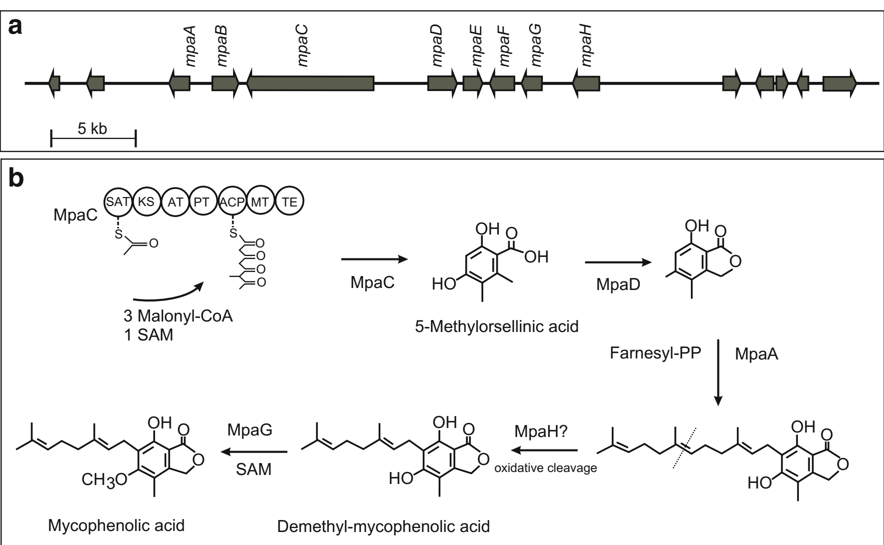

Fig. 5 (**a**) Gene cluster of mycophenolic acid in *P. brevicompactum*. (**b**) Proposed biosynthetic pathway for mycophenolic acid (Modified from Ref. [53])

obtained from *P. stoloniferum* (gene not yet cloned), another mycophenolic acid-producing fungus.

The MPA-producing fungi have to protect themselves against the antifungal activity of MPA. This resistance to MPA appears to be exerted by an IMP-dehydrogenase encoded by *mpaF* [56], although other mechanisms such as active MPA secretion and lack of uptake of the secreted extracellular MPA may also contribute to the resistance as occurs with other secreted metabolites [32].

Mycophenolic acid is active against fungi and human lymphocytes (involved in immune response) because it exerts a strong inhibition of the inosine-5′-phosphate (IMP) dehydrogenase, a key enzyme in *de novo* purine biosynthesis in those cells that lack the purine recycling pathway (as it is the case in lymphocytes). Overexpression of the *mpaF* gene in *A. nidulans* drastically increases the resistance to MPA in this fungus [55, 56]. Indeed, these authors reported that six different fungi, including those that produce MPA and also some putative nonproducers, contain two IMP dehydrogenase genes, one of them presumably located within the *mpa* gene cluster [56].

While this manuscript was in the proof stage, a recent report described the mycophenolic acid gene cluster in *P. roqueforti* (see note added in proof).

2.4 Agroclavine and Festuclavine

There are two subgroups of alkaloids produced by fungi: (i) the clavine alkaloids represented by fumigaclavine, synthesized by *A. fumigatus* [57], and agroclavine and festuclavine produced by *P. roqueforti*, and (ii) the lysergic acid-containing ergot peptide alkaloids produced by species of *Claviceps* [58]. Several *P. roqueforti* strains of different origins produce the clavine-type alkaloids agroclavine and festuclavine (Fig. 1). Festuclavine is also produced by *P. carneum* [12].

The clavine alkaloids have a tricyclic or tetracyclic structure with small structural differences between them. There are no detailed studies on the biosynthesis of agroclavine and festuclavine in *P. roqueforti*, but the biosynthesis of fumigaclavines in *A. fumigatus* [57] and ergot alkaloids in *Claviceps purpurea* [58] has been extensively studied.

2.4.1 Biosynthesis of Agroclavine and Festuclavine

All these compounds derive from the precursors L-tryptophan, dimethylallyl diphosphate, and the methyl group of methionine. The first step in the biosynthesis of these clavine alkaloids is prenylation of L-tryptophan at C-4 to form 4-dimethylallyltryptophan (DMAT) by the enzyme DMAT synthase, a prenyltranferase encoded by the gene named *fga*PT2 in *A. fumigatus* (or *dmaW* in *Claviceps*). In the second step, the primary amino group of DMAT is methylated using S-adenosylmethionine as methyl donor. The N-methyltransferase is encoded by the *fga*MT gene located in an 11-gene cluster that comprises all genes involved in the pathway [59, 60]. The N-methyl-DMAT is then cyclized and oxidized to chanoclavin-1 by a FAD-containing oxidoreductase named chanoclavine synthase,

encoded by the *casA* gene (also named *case* by other authors) [61]. This conversion also requires the product of a cluster-located gene, *casC*, encoding a catalase-like protein that is involved in oxidation of the 3′-methyl group of the dimethylallyl moiety (derived from DMA-PP) to a CH_2OH [62]. Disruption of this catalase-like gene results in the interruption of the clavine or ergot alkaloids biosynthetic pathways in the producer organisms with accumulation of the N-methyl-DMAT intermediate. The exocyclic alcohol group of chanoclavine-I is then oxidized to form chanoclavine-1-aldehyde by the enzyme chanoclavine-1 dehydrogenase encoded by the *fgaDH* gen (also named *casD*) [58, 63]. The chanoclavine-1-aldehyde is the branching point intermediate in the biosynthesis of the different clavines and lysergic acid-containing alkaloids in different fungi. *P. roqueforti* produces the tetracyclic compound festuclavine that in *A. fumigatus* (but apparently not in most *P. roqueforti* strains) is later converted to fumigaclavine. The conversion of the tricyclic intermediate chanoclavine-1-aldehyde to festuclavine requires two enzymes encoded by the genes *fgaFS* and *fgaOx3* [64]. The exact mechanism by which these two enzymes convert chanoclavine-1-aldehyde to festuclavine is still a matter of debate [64, 65]

The second tetracyclic clavine alkaloid produced by *P. roqueforti* is agroclavine. Agroclavine differs from festuclavine in that the former has a double bond between carbons 8 and 9 that is already present in the previous intermediate chanoclavine-1-aldehyde but is saturated (reduced) in festuclavine (Fig. 1), suggesting that there is an enzyme activity involved in the reduction of the double bond. At difference of *P. roqueforti*, *A. fumigatus* does not seem to accumulate agroclavine, probably because the pathway continues to fumigaclavine.

Formation of agroclavine from chanoclavine-1-aldehyde (the branching point intermediate) has been reported in *Claviceps* [66]. The agroclavine synthase EasG of *Claviceps* is a homologous enzyme to festuclavine synthase of *A. fumigatus.* Indeed, Cheng et al. [65] reported that the festuclavine synthase of *A. fumigatus* (about 65 % similarity to the agroclavine synthase of *Claviceps*) is able to produce agroclavine when incubated with the substrate chanoclavine-1-aldehyde in the presence of a FgaOx3 enzyme from *Neotyphodium lolii.* The difference between both homologous enzymes may explain the lack of the hydrogenase (reductase) activity characteristic of *A. fumigatus* (a festuclavine producer) in the agroclavine producers, such as *Claviceps purpurea*. Interestingly, *P. roqueforti* produces both agroclavine and festuclavine [12]. In summary, it seems likely that *P. roqueforti* synthesizes agroclavine by the action of a FgaFS-homologous enzyme as a less reduced product of the pathway.

Other *P. roqueforti* Metabolites Several strains of *P. roqueforti* are also known to produce citreoisocoumarin and small amounts of orsellinic acid. Very little is known about the biosynthesis of these compounds.

Orsellinic Acid. The biosynthesis of orsellinic acid is related to that of methylorsellinic acid and dimethylorsellinic acid described above. In the absence of experimental information in *P. roqueforti*, it is unclear if there is a separate polyketide synthase without the methylation domain, specific for orsellinic acid biosynthesis or

whether this compound is formed by the 5-methylorsellinic acid PKS of the mycophenolic acid pathway (encoded by *mpaC*) when the methyltransferase domain is bypassed by the "domain skipping" mechanism occurring in some of these synthases. In support of this last possibility is the fact that orsellinic acid is produced in very small amounts in the tested strains of *P. roqueforti* [12]. A similar nr-PKS is the DMOA synthase involved in andrastins biosynthesis (see above).

Citreoisocoumarin. Another metabolite produced by *R. roqueforti* is citreoisocoumarin. There is no information on the biosynthesis of this compound in *P. roqueforti*, but in *Fusarium* species, citreoisocoumarin is known to be a byproduct of the biosynthesis of aurofusarin. Both compounds derive from a precursor polyketide that may be cyclized by (i) a carbon-to-carbon (C-C) Claysen-type condensation giving aurofusarin or (ii) by formation of an internal lactone resulting in citreoisocoumarin [67]. This last type of cyclization (lactone formation) appears to predominate in *P. roqueforti*, but the enzyme(s) and molecular basis underlying citreoisocoumarin biosynthesis are still unknown.

3 Metabolites of *Penicillium carneum* and *Penicillium paneum*

P. carneum and *P. paneum* are closely related to *P. roqueforti* Thom, although as indicated in the Introduction section, they are classified as separate species [5, 12]. Indeed, *P. carneum* and *P. paneum* differ from *P. roqueforti* and among themselves in their ability to produce some secondary metabolites (Table 1). *P. carneum* predominates in some spoiled meat products, whereas *P. paneum* is associated with molded bread or grains and grass silages. Both fungi may occur in the surface of some cheeses, but they are not used as secondary starters in blue-veined cheese [1, 23, 26], although they may be present in homemade artisanal blue cheeses, particularly in those produced in some developing countries. *P. carneum* and *P. paneum* may be included in the group of cheese contaminant fungi [22], and their absence in most blue cheeses makes their secondary metabolites less relevant for human health.

The main mycotoxins produced by these fungi are patulin, marcfortins, penitrem, and botryodiplodin (Table 1). *P. paneum* is more different from *P. roqueforti*; it lacks the ability to produce PR-toxin but synthesizes patulin that is not found in *P. roqueforti*. *P. carneum* produces most of the described metabolites of *P. roqueforti* and also patulin. Penitrem A is produced by *P. carneum* but not by *P. roqueforti* or *P. paneum*. The marcfortins A, B, C are produced only by *P. paneum*. On the other hand, PR-toxin and the intermediates eremofortins A, B, and C are produced exclusively by *P. roqueforti* [12]. In summary, *P. paneum* is different from the other two related species in its set of secondary metabolites, whereas *P. carneum* is more similar to *P. roqueforti*. The biosynthesis of these metabolites is poorly known with exception of that of patulin.

3.1 Patulin

Patulin is a potent mycotoxin that causes neurological and immunological disorders and gastrointestinal alterations in humans [68]. Patulin is a common mycotoxin produced by many species of *Penicillium* (including *P. paneum* and *P. carneum*), *Aspergillus*, *Paecilomyces*, and *Byssochlamys nivea* [69, 70]. Patulin is frequent in fungi-spoiled apples, and the levels of patulin allowed in apple-derived products, such as cider, apple jellies, or apple-derived infant foods, are strictly limited by the food safety agencies of many western countries. Among the producer fungi, *Penicillium expansum* is known to cause the soft rot of apples and pears and appears to be the major producer of patulin in fruits [71].

Fragmented evidence reported over the last three decades has shown that the first intermediate, 6-methylsalicylic acid (6-MSA), is converted to patulin through the intermediates m-cresol, m-hydroxybenzyl alcohol, and isoepoxydon. Biochemical and genetic studies in different producer fungi, including *P. paneum*, identified two enzymes, namely, 6-methyl salicylic acid synthase (6-MSAS) and isoepoxydon dehydrogenase (IDH) involved in patulin biosynthesis [72–74]. More recently two P450 monooxygenases have been found to be involved in the conversion of 6-MSA to m-cresol and m-hydroxybenzyl alcohol [75]. When the genome of the patulin producer *Aspergillus clavatus* was sequenced (TIGR http://www.aspergillus.org.uk/indexhome.htm?secure/sequence_info/index.php ~ main), a 15-gene cluster putatively encoding the entire patulin pathway was identified. This cluster includes the *msas* gene (encoding 6-MSA synthase), the *idh* (encoding the IDH), and the two P450 monooxygenase encoding genes. The patulin gene cluster of *P. paneum* or *P. carneum* has not been reported yet but is likely to be similar to that *of A. clavatus.*

4 *Penicillium camemberti:* Cyclopiazonic Acid

Several filamentous fungi may grow on the surface of cheeses during the ripening process. Most of them are strictly external, but some of them, e.g., *P. camemberti* and *P. nalgiovense*, may contribute to the organoleptic characteristics of mature cheeses.

P. camemberti is associated with ripening of white rind soft cheeses such as Camembert and Brie cheeses [76]. Selected strains are routinely used in production of soft cheeses. Although many of the *P. camemberti* secondary metabolites remain unexplored, one of them, cyclopiazonic acid, acquired relevance because of its well-known neurotoxicity. Cyclopiazonic acid (CPA) is a highly active inhibitor of Ca^{2+}-dependent ATPases of animal cells and is a potent neurotoxin for humans and other mammals [77].

Cyclopiazonic acid is a prenylated indole alkaloid containing a tetramic acid ring produced by several *Aspergillus* and *Penicillium* species, including *P. camemberti.* The biosynthesis of CPA has been studied in *Aspergillus flavus* and *A. oryzae* but not

in detail in *P. camemberti*, although the biosynthetic pathway is likely to be conserved in all fungi.

CPA derives from L-tryptophan, a four-carbon unit, and a dimethylallyl diphosphate units [77]. The four-carbon unit is formed by condensation of an acetyl-CoA and a malonyl-CoA. The first step in the CPA biosynthetic pathway is catalyzed by a hybrid polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) that activates L-tryptophan and condenses this amino acid with acetyl-CoA and malonyl-CoA units forming the acetoacetyl-L-tryptophan (AA-L-trp) intermediate. This key enzyme is encoded by the *cpaA* (also named *cpaS*) gene. The NRPS component of the hybrid PKS-NRPS has four domains C-A-T-R* with activities for condensation, L-tryptophan activation and thiolation (peptidyl/acyl carrier), and peptide product release. The R* domain is a reductase that lacks the catalytic triad of NADH-dependent reductases and is proposed to release the N-acetoacetyl-tryptophan intermediate by cyclization via a Dieckmann condensation to form the cyclo-acetoacetyl-L-tryptophan intermediate that is released from the phosphopantetheinyl arm of the T domain of the enzyme [78].

In the second step of the CPA biosynthetic pathway, the cyclo-acetoacetyl-L-tryptophan intermediate is prenylated at C-4 of the indole nucleus by the enzyme cyclo-AA-trp prenyltransferase that introduces an isopentenyl group from the DMA-PP donor. The enzyme encoded by the *cpaD* gene is a member of the well-known family of prenyltransferases [79]. In *A. clavatus* and *A. oryzae*, there is a third step of the pathway catalyzed by a FADH-dependent oxidoreductase *cpaO* that converts β-cyclo-acetoacetyl-tryptophan into the final product, CPA [77, 80, 81]. The *cpa* gene cluster of *A. flavus* includes in addition to *cpaA-epaD-cpaO* structural genes two additional genes, *epaM* that encodes a transmembrane protein of the MFS family and *cpaR* that encodes a regulatory protein of the C6 Zn^{2+} finger type [82]. The CpaM transporter presumably is involved in CPA secretion [32].

CPA is the final product of the biosynthetic pathway in *A. flavus* and probably also in *P. camemberti* since CPA is secreted and accumulated in these two fungi. However, *A. oryzae* that is considered to be a "domesticated" variant of *A. flavus* widely used in Japanese food industries contains an additional gene in the cluster, *cpaH*, that encodes a P450 monooxygenase which converts CPA to 2-oxo-CPA. This compound is much less toxic than CPA [83], and the authors proposed that this P450 monooxygenase is a toxicity-reducing "safeguard" enzyme evolved in *A. oryzae* during its adaptation to grow in fermented foods [83]. The mechanism of adaptive safeguard appears to be more complex since some *A. oryzae* strains have lost completely the ability to produce CPA and 2-oxo-CPA due to a mutation in the N-terminal region of the CpaA hybrid PKS-NRPS. It will be interesting to confirm that *P. camemberti* lacks the "safeguard" *cpaH* gene and therefore maintains the high toxicity characteristic of CPA.

Other secondary metabolites have been reported to be produced by *P. camemberti*, e.g., asperenone, asperrubrol-like compounds, methyl-isoborneol, and hadacidin [27], but it is unknown if these compounds pose a health problem for humans.

5 Conclusions and Future Perspectives

Blue-veined cheese is a gourmet food consumed all over the world. These cheeses are maturated in different countries with *P. roqueforti* as a secondary starter that grows inside the cheese in microaerobic conditions. As described in this article, *P. roqueforti* is able to produce about 20 different secondary metabolites belonging to at least seven different families. Some of these compounds are highly toxic, e.g., PR-toxin, whereas others, e.g., andrastins, are considered to be beneficial for human health. Selection of fungal strains over centuries has favored the use of *P. roqueforti* Thom that lacks production of patulin, over the closely related *P. carneum* and *P. paneum* that produce this mycotoxin. Most *P. roqueforti* secondary metabolites are produced in rich solid (agar plates) and liquid cultures, but there is very limited information on the production and accumulation of secondary metabolites inside the cheese, under the microaerobic conditions. Andrastin A is known to be formed and accumulated in the blue-veined cheeses [13, 14]. Humans and other animals consume a variety of plants and fungal secondary metabolites; many of them are probably nontoxic, but in other cases, animals reject certain plants or mushrooms because they contain toxic secondary metabolites known as feeding deterrents. It is possible now to construct strains lacking certain secondary metabolite gene clusters. The possible removal of toxic metabolites gene clusters by precise gene excision, while preserving all other characteristics, including enzymes involved in cheese ripening and aroma formation, is now a possible application of the molecular genetics of *P. roqueforti* to the cheese industry. This "domestication" process would be equivalent to the "natural domestication" process that has evolved in *A. oryzae* for removal of CPA.

6 Authors' Note

When this article was in press Del Cid and coworkers reported the mycophenolic acid gene cluster of *P. roqueforti* (Del-Cid A, Gil-Durán C, Vaca I, Rojas-Aedo JF, García-Rico RO, Levicán G, Chávez R. (2016) Identification and Functional Analysis of the Mycophenolic Acid Gene Cluster of *Penicillium roqueforti*. PLoS One. 1(1):e0147047.

The *P. roqueforti mpa* cluster is almost identical to that known for *P. brevicompactum* (Fig. 5) and the conclusion obtained by these authors are similar for both fungi.

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Part II

Methods and Biotechnology

Solid-State Fermentation: Special Physiology of Fungi

10

Javier Barrios-González and M. Rosario Tarragó-Castellanos

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Abstract

The evolution of higher fungi and actinomycetes took place on solid growth substrates, so these microorganisms are perfectly adapted to grow in a solid environment. This implies that their cultivation in liquid culture may impair their metabolic efficiencies. However, conventional technology for the production of valuable fungal products is liquid submerged fermentation. In recent times, solid-state fermentation has become an alternative industrial production system to produce enzymes, primary and secondary metabolites. There are several advantages in employing many solid-state fermentation processes over the conventional submerged fermentation ones, like higher yields of secondary metabolites or enzymes. Moreover, certain enzymes and secondary metabolites can only be produced in solid-state fermentation. The main advantages of this culture system are related to the special physiology displayed by fungi when growing in solid culture. This chapter describes and analyzes recent advances in our understanding of this special physiology (the physiology of solid medium) and the underlying molecular mechanisms. It is also discussed how this knowledge can be applied to create novel technological advances.

Keywords

Physiology of solid medium • Advantages • Gene expression differences • Regulation • Applications

List of Abbreviations

Aw	Water availability
cDNA	Complementary DNA
CM	Cellulase carboxymethyl cellulose
dsDNA	Double-stranded DNA
GFP	Green fluorescent protein
glaA	Glucoamylase A
glaB	Glucoamylase B
GSH/GSSG	Redox balance
HSE	Heat shock element
MSLC	Membrane-surface liquid culture
PSM	Physiology of solid medium
ROS	Reactive oxygen species
SmF	Submerged fermentation
SMs	Secondary metabolites
SSF	Solid-state fermentation

1 Introduction

Fungi are relatively simple eukaryotic organisms that perform biological processes that are essentially based on the same principles as biological processes in higher eukaryotes. These are interesting and useful microorganisms that show high levels of protein secretion, metabolite production, and metabolic versatility.

For these reasons, they are being increasingly used for the large-scale production of enzymes and a wide range of (semi)bulk chemicals and pharmaceuticals, as well as other products like high-value nutraceuticals (health and medical benefits). Conventional technology for the production of these fungal products is liquid submerged fermentation. However, the evolution of higher fungi and actinomycetes took place on solid growth substrates, i.e., spent their evolutionary history as terrestrial. In other words, they were designed by evolution to grow on moist solids, so they are perfectly adapted to grow in a solid environment. This implies that their cultivation in liquid culture may impair metabolic efficiencies and bring about certain shortcomings.

Solid-state fermentation (SSF) is an ancient culture method that has been modernized to become an alternative industrial production system. SSF has been used to produce enzymes, primary and secondary metabolites.

There are several advantages in employing many SSF processes over the conventional submerged fermentation (SmF) ones, like higher yields of secondary metabolites or enzymes. Moreover, certain enzymes and secondary metabolites can only be produced in SSF. The main advantages of SSF are related to the special or different physiology displayed by fungi and other microorganisms when growing in solid culture. This physiology (i.e., a behavior that deviates from the one displayed by the fungus in liquid medium) is sometimes referred to as "physiology of solid medium."

This physiology is just beginning to be understood, but recent advances show a very wide and interesting scientific panorama with great technological potential.

In this chapter, general aspects of SSF are described, and how this different physiology became apparent, by reviewing examples of proteins, enzymes, and metabolites production. The last sections review recent advances in our understanding of the molecular mechanisms underlying the physiology of solid medium, and how this knowledge can be applied to create novel technological advances.

2 Solid-State Fermentation: Definition and Background

SSF is an alternative microbial culture system that has been used since antiquity, but that has been modernized by research carried out in the last 25 years. By applying modern concepts of microbiology, biochemistry, and biochemical engineering these are more controlled processes that can be applied to more sophisticated ends.

Solid-state culture or solid-state fermentation (SSF) is defined as a microbial culture that develops on the surface and at the interior of a solid matrix and in the absence of free water [1].

At this point, it is important to clarify the use of the term *fermentation*, which in the field of biochemistry is described as an anaerobic process: an energy production process where the final acceptor of the electrons is not the oxygen but an organic compound, pyruvate or a derivative. These compounds when reduced by the electrons form useful products like ethanol or lactic acid. However, the term *fermentation*, in industrial environments, is used as any type of microbial culture (aerobic or not).

The origins of SSF are related to the koji process in the Far East. The use of koji in China was reported in the year 1000 BP but was probably used since 3000 BP [2], and Buddhist priests took this tradition to Japan in the seventh century. Koji process can be considered as a prototype of SSF. It consists of the cultivation of *Aspergillus oryzae* on soybeans and other grains to produce proteases and amylases, which degrade proteins and transform starch in sugars. In this way, the fermented material is used for the production of soy sauce or, in a second stage, rice wine or saké.

In the West, the types of SSF had been used for the production of vinegar, gallic acid for the tanning process. Even in the beginning of the twentieth century there was production of primary metabolites such as enzymes and organic acids by microorganisms in SSF.

Takamine [3] adapted the koji process, using wheat bran for the production of taka-koji or "moyashi" obtained by drying the spent moldy bran. He later patented the making of an alcoholic precipitate of a water extract of taka-koji [4] called "distasic enzyme." This precipitate was sold as a digestive aid called "taka-distase" by Sakyo Co and was the pioneer of crude amylolytic extracts produced by SSF [5].

Underkofler [6] patented the use of rotating drums in order to produce amylase by "moldy brans" or wheat bran fermented by *A. oryzae*. Underkofler et al. [7] commented the advantage of koji moldy bran over malting or acid hydrolysis, to improve the conversion of starch into ethanol. Later, Underkofler et al. [7] reported the successful industrial scale-up of the moldy bran process, but using trays instead of rotating drums because of the slow damage problems of fungal biomass by mechanical stirring. The process was used to produce a crude amylolytic preparation blended with cooked cereal grains that was the input for ethanol production. The final ethanol yields were around 80 % of the theoretical yield. Hence, Takamine and Underkofler planted the seeds of present bioethanol industries.

Although the earliest commercial production of enzymes depended on SSF technology, the advent of sterile submerged culture techniques or liquid submerged fermentation (SmF) in the 1940s displaced the solid-state methods in the western countries. The discovery of penicillin in the 1930s, followed by streptomycin, chloranphenicol, and tetracycline in the early 1950s, overshadowed the emerging SSF process and emphasized on SmF.

In the 1970s, work performed by Hesseltine et al. [8–10] studied and described the traditional SSF processes of the East, and informed the West about the great technological importance of these culture systems. These reports are probably responsible, in great part, for the renewed interest in SSF observed during the last 30 years, with important research woks that have transformed and modernized SSF.

3 Modern SSF Systems

As a consequence, solid-state fermentation (SSF) is being transformed for new purposes, using new approaches. This has resulted in new SSF systems that often present several advantages over submerged fermentation (SmF).

3.1 SSF Modern Applications

Recent years have witnessed a boom in the development of bioprocesses such as production of feed (biotransformation of crops and crop residues for nutritional enrichment), fuel, food, and industrial chemicals and biopesticides, as well as in bioremediation, biobleaching, and biopulping [11].

Importantly, also the development of SSF production processes for high value, low volume, high cost products like biopharmaceuticals or compounds of use in food industry or agriculture, such as biologically active secondary metabolites including antibiotics, cholesterol lowering drugs, alkaloids, plant growth factors, aroma compounds, etc. Also, developments of applications for the production of proteins: recombinant proteins as well as enzymes.

Lately, some problems of SmF, such as high-energy consumption and serious pollution problems, are becoming increasingly notorious, significantly limiting the sustainable development of fermentations.

Academic or industrial researchers are again taking notice of advantages of SSF such as water saving, energy saving, and low costs. SSF has begun to play a role in the chemical, pharmaceutical, and environmental fields, which points out a clear direction for the sustainable development of the entire biological and chemistry industry [12].

As mentioned before, typical advantages of SSF process include lower capital investment, low energy requirements, less water output, and very superior productivity.

Nevertheless, large-scale applications are less abundant than expected due to relative difficulty to control process parameters (pH, heat, nutrient conditions, etc.), as well as less knowledge of the SSF process by western scientists.

Commercial operations using SSF processes have been developed in countries such as Japan, India, U.S.A., and France. The successful operation of the companies utilizing the SSF process ensured that the process gets its due attention.

Research on SSF is in great part devoted to search for substrates and optimization of the production of different products, as well as potential new applications. The deeper and more basic research is generally engineering oriented and studies fungal physiology mainly from the standpoint of the effect of the main fermentation parameters (T, moisture content, aeration, etc.) on growth. This has been the basis for mathematical models that have been very useful for scale-up as well as for reactor design and control.

However, much less attention has been paid to the special physiology shown by fungi in SSF, i.e., a behavior that deviates from the one displayed by the fungus in liquid medium and that includes the major advantages of this culture system, and sometimes it is referred to as “physiology of solid medium.”

3.2 Bioreactors for SSF

The delay in SSF being the major mode of fermentation can be partially attributed to the bioreactors initially available. During the initial phases mostly tray-type

fermenters were used with poor instrumentation support. Heat generated during the culture was poorly dissipated.

However, research in this area has made important advances in fermenter design and in developing sensors and measurements in SSF processes [13, 14].

Different bioreactor configurations include periodic pressure solid-state fermenter, immersion, expanded bed and tray-type reactor, intermittent agitation rotating drum type, and "Plafractor" [15], developed for production of enzymes, biocontrol agents, and pharmaceuticals at industrial level.

3.3 Types of SSF

Besides the koji-type systems, SSF cultures are now performed on other amylasic substrates like roots (cassava or potato flour), bananas, etc. Generally, agroresidues including lignocellulosic materials, but also wastes of industries such as potato chips, spent brewing grain, paper, and wood processing industries. This type of SSF can be seen as a technique to utilize organic wastes as raw materials to produce value-added products [16]. SSF on natural solid substrates has the advantage of reducing process costs by using agricultural wastes, additionally contributing to solve the environmental problems caused by their disposal.

Another type of SSF uses an inert support with absorbed liquid medium. The support can be of natural origin like sugarcane bagasse, or artificial like polyurethane, amberlite or vermiculite, etc.

Hence, today, two types of SSF systems can be distinguished, depending on the nature of the solid phase used: (a) SSF on natural solid substrates and (b) SSF on impregnated inert supports [17].

This last SSF system was initially used for basic studies of SSF, since the composition of the absorbed medium could be designed, and its constitution determined at any time of the culture. In addition, biomass concentration was more easily quantified. Remarkably, production yields at least as high as the ones obtained in the more common SSF on solid natural substrates are also reported in these systems [17–20]. Moreover, its advantages for basic studies, as well as for industrial production of high-value products such as metabolites, enzymes, and biocontrol agents, have been assessed, as well as its economic feasibility [21].

In this way, in the SSF on inert support product recovery is less complicated because the extracellular product can be easily extracted from the inert support and products are obtained with fewer impurities [11], but medium costs are higher than in the previous case.

3.4 Other Systems with Certain SSF-Type Behavior

Some culture systems have been developed as models to study basic aspects of SSF or other biological phenomena, but also for applied purposes. These are systems

where the fungi display a physiology different from the one shown in SmF, although not exactly the one in SSF; probably a midway physiology.

Interestingly, *A. oryzae* and other fungi, grown on a nylon membrane, placed over an agar plate medium, show a SSF-type physiology. This has been very useful for certain experiments and also because mycelia can be recovered and much more easily prepared than from native SSF.

However, it has been shown that the presence of the membrane filter reduces maximum respiration rate and biomass and α-amylase production [22]. This is further supported by our observations that, although lovastatin specific production increases in membrane cultures (in relation to normal petri dish cultures), this value is still very far from the one displayed in real SSF [23] (see Sect. 8.4).

On the other hand, in what could be seen as applications of the physiology of solid medium (PSM), some novel types of culture systems have been developed, for applied purposes. Two interesting examples are the membrane-surface liquid culture, and the so-called biofilm (liquid) reactors, both with potential industrial applications, and are described in a later section.

4 Production of Primary Metabolites

Primary metabolites are those which have identifiable functions and play specific roles in normal physiological processes, like amino acids and nucleic acids. These include intermediates and end products of anabolic metabolism. Other primary metabolites (e.g., citric acid, acetic acid, and ethanol) result from catabolic metabolism and their production is related to energy production [24].

Organic acids (like citric acid) are widely used in the food and beverage industries, but also find application as additive in detergents, pharmaceuticals, cosmetics, and toiletries.

Recently, the importance of citric acid and fermentation (in the biochemical sense) products, like ethanol, lactic acid, and others, is increasing due to the concepts of white biotechnology and biorefineries. The idea of replacing oil and gas with (renewable) biomass, mainly lignocellulosic agricultural residues, for biofuel production and replacement of chemical synthesis by fermentation or biocatalysis products, is very attractive. Functional groups that must be introduced by costly oxidative process steps into naphtha are already present in organic acids, so these compounds can be produced by fermentation and can then be used as building blocks to obtain polymers, bioplastics, etc. Consequently, the field that investigates microbial organic acid production is currently moving fast [25].

Examples are citric acid that has been on the market for some time, lactic acid, which came to market in large scale only recently, and succinic acid, which (despite the fact that a feasible industrial bioprocess has not yet been developed) has huge potential.

The use of SSF is being reconsidered in this field. In fact, citric acid has been produced under SSF conditions for many years, so this would facilitate the technology transition needed to produce other organic acids by SSF. An important

advantage of SSF over SmF for citric acid production is that the presence of trace elements does not affect the yield, as it does in SmF. Therefore, substrate pretreatment is not required, saving time and energy [26].

Lactic acid and its derivatives are widely used in food, medicine, feed, chemicals, and environmental protection. Recently, there has been increased interest because of its applications in the production of polylactic acid that can be used to synthetize biodegradable, biocompatible plastics and coatings. There have been reports of SSF processes, using lactic acid bacteria or fungal strains (*Rhizopus*) by SSF, that claim that the use of agroresidues in SSF could be significant in reducing production costs [27, 28].

Itaconic acid is another promising building block for the polymer industries that has also been produced by SSF on sugarcane bagasse with strains of *Aspergillus terreus* [29].

5 Production of Enzymes and Proteins

Besides other applications, modern SSF systems have a record of successful applications for the production of microbial enzymes and secondary metabolites or bioactive compounds [30].

There has been growing interest in SSF because the amounts of enzymes (or heterologous proteins) secreted by filamentous fungi in this system are large and very frequently exceed those secreted in submerged fermentation (SmF) [19, 20, 31, 32]. For example, it has been reported that in SSF on wheat bran *Aspergillus oryzae* produced 500-fold higher yield of heterologous protein (chymosin) than in SmF [33].

The enzymes produced by SSF include cellulases, hemicellulases, pectinases, amylases, α- and β- galactosidases, caffeinase, tannase, proteases, etc. The major support matrices used include brans (wheat, rice, barley), oil cakes (sesame, soy olive, coconut, mustard), bagasse (sugarcane, cassava, orange) [34].

Regarding enzymes production, several evident differences between SSF and SmF have been reported (Table 1). These include higher productivities and somehow less regulated (probably higher regulation thresholds). In many cases, enzymes produced in SSF have different characteristics in relation to the ones produced in SmF: like higher optimal temperature or pH stability, different kinetic parameters, or

Table 1 Enzymes production. Special physiology shown by fungi in SSF that contrasts with the one shown in SmF, in this table, characteristics related to enzymes production

1	Enzymes are generally produced in much higher concentrations in SSF
2	Some enzymes from SSF show different characteristics (molecular weight, kinetic parameters, optimal conditions) in relation to the ones obtained in SmF
3	Some enzymes that are intracellular in SmF are extracellular in SSF
4	Strains that are good producers in SmF are not so good in SSF and vice versa

Modified from Ref. [23]

even enzymes that were intracellular in SmF that are secreted to the medium in SSF [19, 35].

Moreover, in recent years SSF-specific enzymes like glucoamylase B or the protease PepA have been identified (see Sect. 8.4).

6 Microbial Secondary Metabolites

6.1 General Aspects

Secondary metabolites are compounds with varied and sophisticated chemical structures, produced mainly by actinomycetes and fungi, usually late in the growth cycle. These compounds do not play a physiological role during exponential phase of growth, so they have been described as SMs in opposition to primary metabolites that are essential for growth. Although antibiotics are the best-known secondary metabolites (SMs), there are other such metabolites with an enormous range of biological activities, hence acquiring actual or potential industrial importance.

SMs are usually not produced during the phase of rapid growth (trophophase), but are synthesized during a subsequent (production) stage: idiophase. From studies in liquid medium, it is now known that production of SMs starts when growth is limited by the exhaustion of one key nutrient: carbon, nitrogen, or phosphate source. For example, penicillin biosynthesis by *Penicillium chrysogenum* starts when glucose is exhausted from the culture medium and the fungus starts consuming lactose, a less readily utilized sugar.

6.2 New Biological Activities

The last 25 years have been a phase of rapid discovery of new activities and development of major compounds of use in different industrial fields. This new stage has been driven by modern strategies to find microbial SM. Earlier whole cell assay methods, like bioassays, are being replaced by new and sophisticated, target-directed, mode-of-action screens. In this way, culture broths of new isolates are tested in key enzymatic reactions or as antagonistic or agonistic of particular receptors. This new approach relies on the knowledge of the biochemical and molecular details of different diseases or physiological processes [36, 37].

This has allowed the development of major compounds of use in different industrial fields, mainly pharmaceutical and cosmetics, food, agriculture, and farming. Some examples are anti-inflammatory, hypotensive, antitumor, anticholestrolemic, but also insecticides, plant growth regulators, and environmental-friendly herbicides and pesticides. This growing wealth of bioactive compounds is usually produced by SmF but many of these metabolites could be advantageously produced by SSF.

7 Secondary Metabolites Production by SSF

In the production of SMs, SSF presents advantages like higher product yield, often in shorter times and higher product stability, while some disadvantages are related to more complicated scale-ups [38–40]. Perhaps an additional disadvantage or weakness is the limited knowledge of PSM, so its full potential is still to be developed.

In any case, industrial SMs production is now a reality. Several years ago an Indian company started industrial production of several SMs, and the Food and Drug Administration approved this technology (SSF) for the production of metabolites for human application [15].

In relation to production of SMs by SSF, the last years have witnessed not only an increase in the number of publications but also by the increase in the proportion of SMs with biological activities different from antibiotics. Another interesting feature of this stage is the surprisingly high productivity of SMs obtained in the processes designed in these studies, i.e., relatively high yields are quite common in recent works. Interestingly, also the production of SMs from actinomycets, and even from *Bacillus* species, have also been produced by SSF [17].

Other fungal SMs that have been produced in this culture system include the cyclodepsipeptides dextruxins A and B, compounds that display insecticidal and antiviral activity [41]. Also, the novel tetramic acid antibiotic conoicetin, which shows a pronounced antibacterial and antifungal action, inhibiting even multidrug-resistant strains of *Staphylococcus aureus*, has been produced by SSF. Interestingly, the producing fungus, *C. ellipsoida*, synthesizes this antibiotic only in SSF, although it grows well in SmF [42]. Another interesting case where new antibiotics were only produced under SSF-type conditions was reported by Bigelis et al. [43]. Pyrrocidienes A, B and acremonidins A–E were only detected when the corresponding fungal strains were grown on agar bearing moist polyester-cellulose paper. Differences were also apparent in bioassays of the extracts against antibiotic-resistant Gram-positive bacteria. These antibiotics that are produced only in SSF remind the SSF-specific enzymes described in Sect. 8.4.

7.1 Secondary Metabolism in SSF: Physiological Studies

The physiological responses of fungi to growth in a solid environment could be divided into responses that are similar to the ones displayed by the microbe in liquid medium, and responses that are particular of growth in SSF. Although the use of the concept PSM generally refers to the latter, the former are also integral part of this physiology and have implications in the control of secondary metabolism in SSF.

7.1.1 Similarities with SmF

It has been observed that physiology of idiophase or secondary metabolism in solid culture shares several basic characteristics with the well-known physiology studied in liquid environment (SmF).

Using SSF on inert support, i.e., sugarcane bagasse impregnated with liquid medium, our group performed basic studies on secondary metabolism in SSF. This research showed that the same culture medium used for penicillin production by *Penicillium chrysogenum* in SmF can be used to impregnate this kind of inert support SSF, although using a higher medium concentration. The use of 2X concentrated medium caused a fivefold penicillin production increase in SSF, while it was detrimental in SmF [44].

Moreover, respirometric studies together with glucose concentration kinetics in this system showed that idiophase (in the case of penicillin and several other SMs) in SSF also started exactly when growth was limited by exhaustion of one key nutrient, as is the case in SmF. Results also suggested that the same mechanisms that regulate penicillin biosynthesis in SmF (e.g., catabolite repression) regulated its production in solid culture. In this way, C, N, or P regulation mechanisms are also operating in SSF.

Moreover, experiments on cephalosporin C production have shown that pH regulation of cephalosporin C is also active in SSF, and works in a similar way as in SmF.

Studies in SmF have shown that cephalosporin C biosynthesis is regulated by pH, and that it occurs in a relatively narrow pH range. Cuadra et al. [45] reported that in SSF using sugarcane bagasse as inert support, biosynthesis of cephalosporin C only took place in a defined range of pH. In a subsequent work, comparative experiments SmF versus SSF showed that the antibiotic synthesis occurred at fairly the same pH range (6.4–7.8) in both culture systems [46].

This basic knowledge has practical implications for SM production in SSF: the same strategies of medium design, considering limiting nutrient, avoiding repressing components, while including inducers and precursors, should be used in SSF [47, 48].

7.1.2 Particularities of Secondary Metabolism in SSF

Besides the nutritional and environmental factors that bring about metabolic reactions similar to the ones observed in SmF, there are other factors in SSF conditions that induce higher SMs production, or even biosynthesis of metabolites that are not produced in SmF. These characteristics are part of the special physiology shown by fungi in SSF, i.e., a behavior that deviates from the one displayed by the fungus in liquid medium. Table 2 summarizes the main characteristics of this physiology in relation to secondary metabolism. As can be seen, another apparent difference is the effect of using concentrated media, which strongly stimulates the metabolite production in SSF on impregnated support, while causing the opposite effect when used in SmF.

A further confirmation that physiology required in solid medium is different from the one in SmF came from the finding that enzymes or secondary metabolites overproducing strains, generated for SmF, generally do not perform well in SSF [49], and vice versa. In fact, this has dictated the need for methods to generate overproducing strains particularly suited for SSF [17, 18, 39] particularly since continuous improvement of the production strain(s) is a condition to make and keep a fermentation industry competitive.

Table 2 Physiology of solid medium. Special physiology shown by fungi in SSF that contrasts with the one shown in SmF, in this table, characteristics related to secondary metabolites production

1	Higher production, often in shorter times
2	Some secondary metabolites are only produced in SSF, like coniosetin, pyrrocidienes A, B and acremonidins A-E
3	Concentrated medium increase production in SSF
4	Strains developed for SmF don't perform well in SSF, indicating different functions are needed to produce in SSF
5	Molecular differences in biosynthetic genes expression

Modified from Ref. [23]

8 Physiology of Solid Medium

The abovementioned cases, of different physiological responses to solid culture conditions, raises questions the study of PSM strives to answer:

- Why are enzymes and secondary metabolites generally produced in much higher concentrations in SSF?
- Why do some enzymes from SSF show different characteristics (molecular weight, kinetic parameters, optimal conditions) in relation to the ones from SmF; also, why many are enzymes that are intracellular in SmF extracellular in SSF?
- Why are strains that are good producers of enzymes or metabolites in SmF not so good in SSF and vice versa?

8.1 Growth Model

A current model of the microscopic events taking place during fungal growth in SSF helps visualize some characteristics of growth in SSF. After germination, filamentous fungi form hyphae that elongate at the tips and periodically form new branches. Their morphology allows filamentous fungi to colonize the surface of the substrate and penetrate into the substrate matrix in search of nutrients. The microbial biomass inside and on the surface of the substrate secretes metabolites and enzymes and consumes the liberated nutrients. Concentration gradients are needed to supply the substrates and remove the products. Likewise, gradients in the concentration of inducers and repressors may affect enzyme production [23, 50]. In this way, these gradients are one of the noticeable differences between SSF and SmF and therefore might be contributing to the observed differences in physiology [50].

It is considered that in SSF fungal hyphae form a layer of biomass on the substrate particle, i.e., a three-dimensional net of hyphae with pores in between: (1) an upper layer with aerial hyphae and air-filled pores and (2) a lower layer with densely packed hyphae and liquid-filled pores; (3) underneath, hyphal tips penetrate the substrate forming the third layer: penetrative mycelia [51] (Fig. 1). It has been

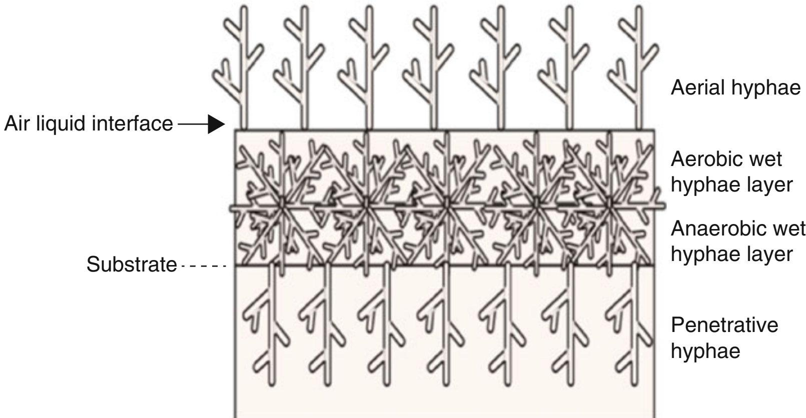

Fig. 1 Model or schematic drawing of fungal growth in SSF, showing the different mycelial layers (From Barrios-González [23])

proposed that *A. oryzae* might overcome oxygen limitation, in the deeper parts of the wet mycelia layer, by forming abundant aerial mycelia, so rapid diffusion of oxygen is expected there, but diffusion of nutrients and enzymes in the cytoplasm of the hyphae is likely to be comparatively slow [50]. Recently, Viniegra-González et al. [19] compared the productivity of three fungal enzymes using SSF and SmF techniques. Thus, they proposed that the higher titers found in SSF than in SmF were due to SSF cultivation works as a fed batch culture with fast oxygenation but slow sugar supply.

8.2 General Physiology

A few works have studied deeper physiological differences that arise during the growth of microbial cells in the two types of culture systems. In *A. oryzae*, low water activity and osmotic stress are important environmental conditions particular of SSF that bring about physiological differences. This is apparent from the accumulation of polyols (glycerol, erythritol, and arabitol) in the cells under SSF conditions, while only mannitol was accumulated under SmF conditions [52]. It is important to note that glycerol and these other polyols are synthesized as a defense against osmotic stress, suggesting that growth in SSF proceeds with this kind of stress. Moreover, we have observed a strong expression of the osmotic stress defense gene *gldB* during lovastatin SSF, indicating that *Aspergillus terreus* senses osmotic stress during the course of SSF, but not in SmF [53].

Recently, we found that mycelium, growing in SSF, had a more reducing intracellular environment than mycelium from SmF. Redox balance (GSH/GSSG) kinetics were performed during lovastatin SmF and SSF. A high redox balance was

observed, in both culture systems, during the growth phase that dropped to a low value during idiophase (around sixfold) [54]. It was later seen that this acute reduction was related to regulation of lovastatin biosynthesis (see Sect. 7.1.2).

However, there was a difference between the culture systems, a fourfold higher redox balance (in both phases) was observed in the mycelium cultured in SSF. The meaning and consequences of this in the PSM remains to be unveiled.

8.3 Secondary Metabolism in SSF: Molecular Studies

Molecular studies related to secondary metabolism in SSF are very scarce. Our group performed a series of studies at this level with the intention of obtaining a deeper understanding of PSM, using lovastatin production as a model.

Lovastatin is a valuable SM produced industrially by *Aspergillus terreus* that holds medical and industrial importance since it decreases cholesterol levels in blood. In more recent research we developed a novel SSF production process on polyurethane foam (artificial inert support). This system not only facilitates basic studies but also induces very high lovastatin productivity. A 30-fold higher production was obtained in SSF as compared with SmF conditions under analogous conditions (Fig. 2). Specific production calculations revealed that each milligram of (dry) biomass produced 815 μg of lovastatin in SSF vs 54.7 mg^{-1} in SmF. In this way, physiology of solid medium is clearly manifested in this system [55, 56].

Since higher SMs production is a clear characteristic of PSM, in these studies we used this parameter to identify PSM, particularly specific production, to avoid divergences only due to differences in growth between the culture systems.

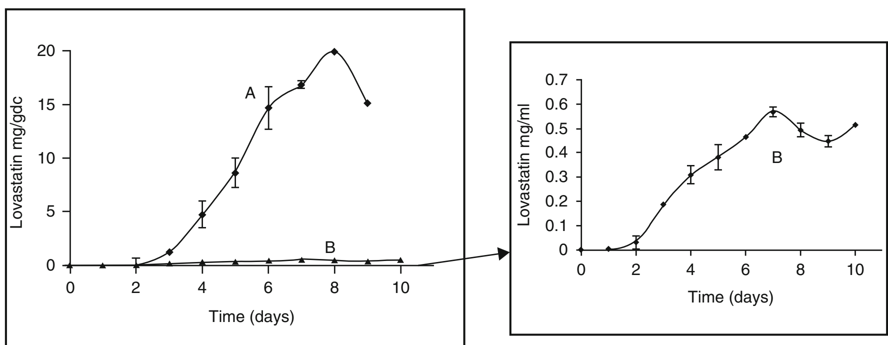

Fig. 2 Comparison of lovastatin production kinetics by *Aspergillus terreus* in SSF (**a**), and in SmF (**b**), using the same culture medium. Lovastatin production in SmF (**b**) is shown again in the *left*, using a much smaller scale of lovastatin concentration. Both cultures showed similar pH kinetics (not show) (From Barrios-González [23])

8.3.1 Higher SMs Production and Higher Expression Levels of the Biosynthetic Genes

A subsequent study was performed to establish the causes of the superior lovastatin productivity in SSF. Results showed that the higher production in SSF was related to a higher expression of the lovastatin biosynthetic genes and, most importantly, that these higher transcription levels were the result of higher (4.6-fold) expression of the regulatory gene *lovE*. This gene encodes the specific transcription factor for the lovastatin biosynthetic genes cluster (Fig. 3). Moreover, it is very probable that this higher expression of biosynthetic genes is an important underlying cause of the higher production reported for other secondary metabolites [55].

These experiments showed that higher expression of the biosynthetic genes is an important cause underlying higher SMs production.

In a later work we compared the cephalosporin C biosynthesis-related genes expression in the SmF versus SSF. Results showed the existence of higher expression levels of the genes encoding for epimerase, expandase/hydroxylase, and metabolites exporting activities in SSF, probably explaining the increased antibiotic production in this system [46].

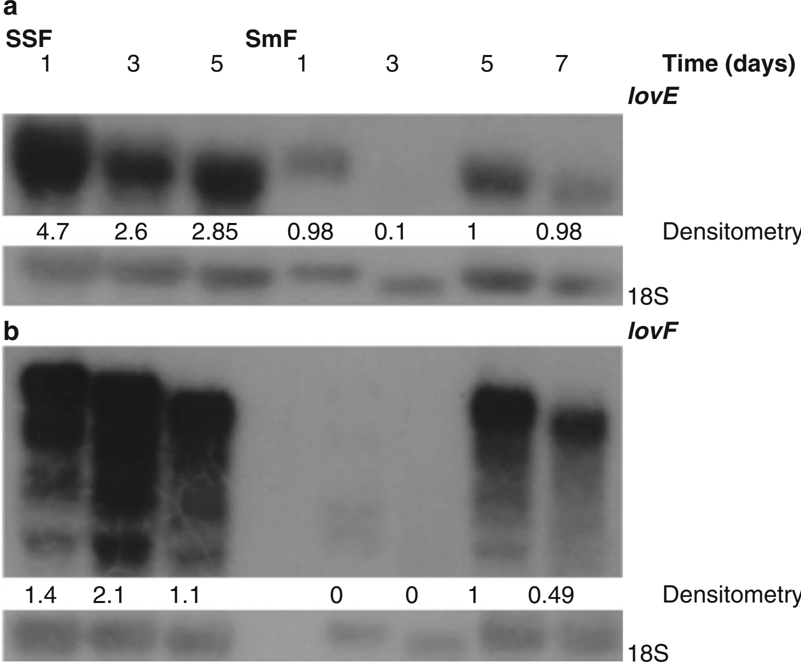

Fig. 3 Northern blot analysis comparing the expression of genes lovE and lovF during the course of lovastatin solid-state and submerged fermentations. Intensity of the bands was quantified by densitometry and normalized values were expressed as a fraction of the highest expression value in SmF, to which a value of one was assigned (From Barrios-Gonzalez [23])

These and other works indicate that there are certain environmental cues that notify the fungus it is a solid medium. These stimuli must be sensed and then transduced, triggering a number of events at a molecular level. These reactions upregulate certain transcription factors, which in turn upregulate different groups of genes. That is, groups of genes must be upregulated and others downregulated, giving rise to physiology of solid medium [23].

8.3.2 Environmental Stimuli that Trigger PSM

In search for the solid culture environmental stimuli inducing PSM, Avila & Barrios-González [57] showed that (1) direct contact with air, (2) the stimuli of the support, and (3) water availability (Aw) are important environmental signals. This work studied the effect of these stimuli on lovastatin specific production, which was taken as the parameter indicative of either PSM or normal liquid environment physiology. Results showed that direct contact with air is a major environmental cue that induces the PSM, at least in relation to secondary metabolism.

It was also observed that the contact with a support (or with the "stimuli of the support") also induced a higher lovastatin specific production, although increases were smaller than the ones observed with direct contact with air. It was concluded that the added effects of stimuli of direct contact with air, the support, and in smaller proportion low water availability, contribute to generate the PSM, i.e., the very high lovastatin specific production obtained in SSF.

In relation to direct contact with air (identified as a major environmental cue), it was considered that its effect on fungal physiology could be mediated by reactive oxygen species (ROS).

This was an interesting idea since, although high ROS concentrations in the cell are harmful, lower levels can function as signaling molecules in the cell. In recent years, many authors have found evidence of a close association between ROS and development and differentiation in fungi, for example, increased ROS levels have also been detected during cell differentiation in *A. nidulans* [58, 59].

Regarding lovastatin biosynthesis, we recently showed a link between ROS and lovastatin biosynthesis. It was shown that *sod1* gene (oxidative stress-defense enzyme) was intensely expressed during rapid growth phase (or trophophase), but it was downregulated in production phase (idiophase). In that moment ROS levels increased to high levels that remained during all production phase [54]. In a subsequent work it was shown that ROS regulate lovastatin biosynthesis at a transcriptional level, in both culture systems [60]. It is considered that transcription factors, associated to signal transduction pathways related to oxidative stress, could be the link between ROS and lovastatin biosynthetic genes.

Although ROS accumulation in idiophase happens in both culture systems, there are differences that could explain higher lovastatin production in SSF: Surprisingly, ROS concentration in idiophase was ten times higher in SmF in relation to SSF. Probably equally important, ROS kept a much more steady level in SSF.

This agrees with the relatively lower *sod1* expression level in SSF, as well as the higher redox balance found in this culture system (see above).

Evidently, signal transduction pathways play a central role in sensing these stimuli and triggering a chain of events resulting in the massive change in gene expression observed in fungi growing in SSF. Our group has carried out studies in this area (SAPK/MAPKinase and cAMP-PKA) and identified a different behavior of some components of cAMP-PKA pathway (manuscript in preparation). LaeA is a global regulator of secondary metabolism in fungi that is associated to this signaling pathway. In a very recent work we overexpressed this gene in *A. terreus* and increased lovastatin production was obtained. Lovastatin overproducing mutants for SSF were much more abundant in the transformant population. In SSF, the constitutive promoter-containing transformant T2laeAcons reached 30.6 mg of lovastatin g dry culture^{-1} [61].

We have performed research exploring the relation between ROS profiles and lovastatin biosynthesis. Manipulation of ROS accumulation profile by genetic or environmental means greatly impacted the metabolites production level. Silencing genes encoding transcription factors involved in these signaling pathways generated transformants displaying lovastatin production increases of 60 % in SmF and 70 % in SSF (Pérez-Sanchez et al. Manuscript in preparation). The role of ROS in the induction of the physiology of solid medium caused by direct contact with air is a subject still under investigation.

8.4 Enzyme and Protein Production in SSF: Molecular Studies

Filamentous fungi are eukaryotic microorganisms commonly used for the production of enzymes and metabolites. They are also considered as suitable hosts for extracellular recombinant protein production, due to their high secretion potential and their ability to perform posttranslational modifications [62].

As described in a previous section, *Aspergillus oryzae* is widely used in Japan and other oriental countries to produce traditional fermented foods. Since it is known that under SSF conditions it produces greater amounts of hydrolases (like amylases and proteases) than in SmF, it is also used in commercial enzyme production in Koji-type cultures.

Because of its industrial importance, it has been subjected to many molecular biology studies that have significantly contributed to understand PSM. These studies have contributed or helped to identify SSF-specific genes and given a deeper insight in the genetic expression and regulation mechanisms involved, determining important differences with SmF [23].

A series of studies on the glucoamylase *glaB* gene of *A. oryzae* became an emblematic case, not only because it was the first SSF-specific enzyme identified but because subsequent studies gave a deep and exciting view of its genetic regulation, identifying environmental factors that induced its expression.

In SSF, this fungus produces great quantities of amylases and proteases [63], but in SmF glucoamylase productivity is much lower. Although it was originally thought that it was the same enzyme, Hata et al. [64] found that the enzyme produced in SSF had a different amino acid sequence, and that this organism has two glucoamylase-

encoding genes: *glaA* and *glaB*. However, the expression of *glaB* is very high in SSF but repressed in SmF, while *glaA* is mainly expressed in SmF. This meant that *glaB* is a SSF-specific gene.

Ishida et al. [65] measured individual transcriptional efficiency of *glaA* and *glaB* promoters under various culture conditions, by means of reporter genes (the *glaB* or *glaA* promoter fused to the coding sequence of an easily detected gene product). As expected both genes were induced by starch, but other physical factors were required for maximal expression of *glaB*. They showed that this SSF-specific gene was also induced by three environmental factors: (1) low water availability (Aw), (2) high temperature, and (3) physical barriers to hyphal extension, that is, SSF-specific conditions. In addition, it was shown that glucoamylase production in SSF was regulated at a transcriptional level. It is important to note that during the koji traditional process more amylase is generated at high temperature (40 °C) than at low temperature (30 °C).

In relation to the third environmental factor, the authors observed that *A. oryzae* grown on a nylon membrane, placed over an agar plate medium (physical barriers to hyphal extension), showed significant induction of the SSF-specific glucoamylase gene (*glaB*). Small pore size (0.2 mm) of the membrane (not letting the hyphaes pass through) and high concentration of maltose (high Aw) in the medium were important for strong induction.

This method has been useful because of the SSF-like conditions it simulates and because mycelia can be recovered and more easily prepared than in a real SSF. However, it is important to note that it has been shown that these model systems present certain differences in metabolism and kinetics, in relation with SSF [23].

Interestingly, what these authors called “physical barriers to hyphal extension” corresponds to the environmental cue that was called “support stimuli” in the work on secondary metabolism (lovastatin production) described before. Moreover, in that work, Aw was also identified as one of the environmental stimuli inducing PSM, although its effect was smaller in the case of lovastatin.

It is worth remembering that a cis-acting factor is a short DNA sequence in the promoter region of a gene, shown to be a binding site for a transcription factor (regulatory protein), to control this gene’s expression.

In a later work of this group [66] cis-acting factors in the *glaB* promoter necessary for high-level expression in SSF were identified. A deletion analysis indicated that removal of a short region of the promoter (−350 to −324) (Region A) produced fatal loss of the promoter activity in SSF, but only loss of regulation in SmF. This region contains two heat shock element motifs (HSE) (5′ -AGAAN-3′) and a GC box (−335 to −324). Substitution of the first HSE brought about lower promoter activity although regulation was conserved, but substitution of the GC box caused lower expression in SSF and induction by starch, Aw, and T was lost.

This region, together with the neighboring B Region, was used to construct improved promoters for SSF. When eight copies of this 97-bp fragment (−350 to −254) were tandemly fused to the *glaB* promoter, a 4.6-fold increase in promoter activity was observed. This improved promoter showed a 4.1-fold increase in recombinant glucoamylase production (*glaA*) in SSF, reaching 1,524 mg/kg-koji.

Table 3 Chronology of important events related to molecular basis of physiology of solid medium.

Year	Secondary metabolites production
1990–2008	Classical regulatory mechanisms of secondary metabolites, like carbon or nitrogen regulation, are also active in SSF. So, like in SmF, these mechanisms should be by-passed (or taken advantage of) by manipulating the culture medium. However, a higher medium concentration (at least 2X) is needed for high production of secondary metabolites in SSF
2008	Lovastatin high production system: SSF on inert artificial support: 14-fold higher specific production in SSF
2008	Higher lovastatin production in SSF is related to a fourfold higher expression of pathway-specific transcription factor (*lovE*), and lovastatin biosynthetic genes
2011	Environmental stimuli for high lovastatin production in SSF include contact with air, water activity (Aw) and physical barriers against hyphal extension
	Enzymes and protein production
1997	Discovery of first SSF-specific enzyme: glucoamylase, and corresponding gene: *glaB*
1998	Regulation of *glaB* gene: environmental stimuli that induce its production: low water activity (Aw), physical barriers against hyphal extension and/or high temperature (42 °C)
2000	Identification of *cis*-acting factors (functional elements) in *glaB* promoter, necessary for high-level expression in SSF: GC box and two heat shock elements (f-HSE)
2002	Discovery of other SSF-specific enzymes like *pepA* gene. Its promoter does not have a GC box, but it contains a HSE
2002–2010	Subtractive cloning, cDNA microarrays and proteomic analysis: This special physiology is supported by a major change at a molecular level 4,628 genes are differentially expressed between SSF and SmF. About half of the genes, expressed only in SSF, are annotated as functionally unknown
2006	Protein production in SSF is regulated in a complex form, with different regulatory circuits for different gene groups; one of which is similar to SmF. Other genes groups respond exclusively to SSF- or SmF-environmental conditions, at transcriptional and/or posttranscriptional levels

Modified from Ref. [23]

After that, other SSF-specific genes have been found. The *pepA* gene, which encodes an acid protease, is also specifically expressed in SSF [67]; but unlike *glaB* gene, *pepA* was not found to contain a GC box; but its promoter contains a HSE. Hence, the molecular mechanisms for regulation of gene expression in SSF were not as simple as originally thought (Table 3).

8.4.1 SSF-Specific Genes and Genes Expressed Differentially

Several years later (between 2002 and 2007), in the search for SSF-specific genes, Japanese groups performed subtractive cloning on *A. oryzae* grown in SSF and SmF. This method compares mRNAs from two conditions. cDNAs are generated and it allows for PCR-based amplification of only cDNA fragments that differ between both populations. The technique relies on the removal of dsDNA formed, by

hybridization between cDNAs common to both conditions, leaving only the cDNAs that are differentially expressed.

Several SSF-specific genes were detected, but about half of them were annotated as encoding functionally unknown proteins. This has been also found in other studies and is indicative of the lack of knowledge in this field and also of the great difference between these two physiologies.

On the other hand, about one half of genes expressed specifically in SSF encoded for secreted or internal enzymes and for transport proteins. These transporters might be necessary for the growth in vast amounts of the limited variety of raw materials, with slower diffusion processes.

In 2005, a Dutch group [68] performed similar experiments with *A. oryzae*, focusing on genes related to the different mycelial growth phenotype required in SSF (polarized growth for colonization of the solid substrate). They identified 29 genes, strongly upregulated in SSF: Six encoded proteins were functionally related to polarized growth, four encoded products involved in morphogenesis, and three coded for cell wall composition proteins. The rest were unknown proteins. The authors interpreted these findings as suggestive of important differences in the organization of the cytoskeleton during growth in SSF, with potential impact on intracellular distribution of several organelles and in polarized secretion of proteins. Genes encoding proteins related to formation of aerial hyphae and attachment to surfaces were also differentially expressed in solid culture.

These findings substantiate the idea that mycelium from solid culture is physiologically different from the one from SmF, and relate to the concept that support-related stimuli are important environmental cues inducing PSM. In addition, these advances have been the basis for genetic improvement methods described in Sect. 8.5.

After this, and well into the omics era, many genes that are differentially expressed in SSF have been identified by cDNA microarrays [69, 70] and proteomic analysis [71]. More recently Wang et al. [72] applied a high-throughput RNA-sequencing methodology (RNA-Seq) to a full-scale transcriptome analysis. In this way, it was revealed that this special physiology is supported by a major change at a molecular level: 4,628 genes are differentially expressed between SSF and SmF.

These include 2,355 upregulated and 2,273 genes downregulated on SSF. Upregulated genes were specifically located in the pathways of ribosome, DNA replication, oxidative phosphorylation, and the TCA cycle. The authors interpreted this as the capacities for protein translation/modification and energy production were much more powerful in the fungus grown on SSF compared to SmF. They suggested this could be due to hyphal differentiation and the faster growth observed in SSF [23].

Again, about half of the genes that were expressed only in SSF are annotated as functionally unknown.

Upregulated genes in SSF also suggested that protein folding is more efficient under SSF conditions. In addition, results also indicated that the capacity for protein glycosylation was greater under SSF conditions. These are all valuable

characteristics that could make SSF a competitive system for homologous and heterologous protein production.

These findings can explain the results of Maruyama et al. [73] in an application of SSF for the production of antigens for use in humans. They found that a better glycosylation pattern in the pre-S2 antigen (hepatitis B virus) produced in SSF. In SmF, *A. oryzae* secreted a heterogeneously glycosylated form of the fusion protein that was partially degraded. Contrasting, wheat bran SSF resulted in the secretion of a homogeneously glycosylated form of the whole fusion protein.

8.4.2 SSF: Proteomic Studies

Transcriptomic studies are of key importance to understand differences in gene expression due to different cultural conditions. However, RNAs do not always correlates with protein content. It is now known that mRNA is not always translated into protein, and the amount of protein produced for a given amount of mRNA also depends on other factors Proteomics confirms the presence of the protein and provides a direct measure of the quantity present. In addition, many proteins can undergo a wide range of post-translational modifications. Many of these post-translational modifications are critical to the protein's function. Hence, proteomics provides a good complement to transcriptional analysis, since it reveals additional information on posttranscriptional and secretion regulation.

Oda et al. [71] carried out a proteomic analysis comparing extracellular proteins in SSF and in SmF. Although this work only focused on the secreted proteins (secretome), it improved the global view of the complex way in which *A. oryzae* controls protein production in response to solid-culture conditions. The authors were able to identify 29 proteins. Taking into account the conditions under which they were synthesized or produced, the authors divided these proteins into four groups: Group 1 consisted of enzymes specifically produced in SSF, such as glucoamylase B (*glaB*). Group 2 is formed by extracellular proteins specifically produced in SmF, such as glucoamylase A (*glaA*). Group 3 consisted of proteins produced in both culture conditions, such as xylanase G1. Group 4 consisted of proteins that were secreted to the medium in the SSF, but trapped in the cell wall in the liquid culture, such as amylase (TAA).

It was also observed that secretion of GlaB was regulated at transcriptional level, while GlaA was regulated at the posttranscriptional level in SSF. This work revealed that not only transcriptional regulation but also posttranscriptional regulation plays important roles in protein production in SSF.

On the other hand, the regulation of Group 3 proteins substantiates the notion, described before (Sect. 7.1.1), that one part of physiology of fungi in solid culture shares a number of basic characteristics with the physiology displayed in liquid environment (SmF).

Since then, different groups have reported several proteomic studies on this and other fungal species. However, these studies have mainly been driven by the "biorefinery" idea, and have focused on lignocellulosic degrading enzymes in the secretome. Others have performed intra- and exaproteomics. Unfortunately, these studies have been carried out either in SSF or in SmF, but very few have performed

comparative studies. In this way the panorama of this area has not changed substantially. Comparisons of the whole proteome (intra- and exa-) in SSF vs SmF are badly needed to complement the transcriptomic data available.

In a recent study Li et al. [74] compared cellulase activities and the secretomes of *Neurospora sitophila* cultured in SSF and SmF using steam exploded wheat straw as carbon source and enzyme inducer. The very high capacity of proteins secretion in SSF was again seen. The total amounts of protein and biomass in SSF were respectively 30 and 2.8 times of those in SmF. A great difference in these enzymes' production was also confirmed. The CMCellulase, FPA, and β-glucoside activities in SSF were 53–181 times of those in SmF. Many of the critical enzymes were produced in both culture systems, although a β-xylosidase was exclusively identified in SSF.

Interestingly, the nonenzyme proteins in SSF were involved in fungal mycelia growth and conidiation while those in SmF were more related to glycosemetabolism and stress tolerance. The authors discuss that SSF more likely serves as a natural habitat for filamentous fungi to facilitate the enzyme secretion.

The fact that an important part of nonenzyme proteins, more actively produced in SmF, were related to stress tolerance suggests that, against what has been thought in the past, fungal life in SmF is more stressful that in SSF. This agrees with very recent results of our group indicating that mycelium was subjected to cell wall stress in SmF, but not in SSF (Bibián et al. manuscript in preparation).

8.5 Applications

Advancement in the understanding of PSM could be applied to better control metabolism and direct product formation in SSF processes. In fact, some of these basic findings are starting to be applied to the construction of strains, particularly suited for SSF.

Based on the model of fungal growth in SSF, where there can be an oxygen limitation, in the deeper parts of the wet mycelia layer, described in Sect. 8.1; Te Biesebeke et al. [68] identified a hemoglobin domain of gene *fhbA*, encoding a flavohemoglobin, i.e., a protein that can attract and bind O_2. The authors cloned and overexpressed this protein domain in *A. oryzae*. The transformants displayed slightly higher growth on SSF, as well as higher amylase, protease, and particularly glucoamylase activities. This indicated that this strategy could be used as a molecular genetics strain improvement method for protein production under SSF.

Findings related to SSF-specific enzymes in *A. oryzae* have also been used in the construction of improved promoters for SSF that can be used in the production of enzymes or recombinant proteins.

Examples of applications, derived from the advances in the PSM in *A. terreus*, include the overexpression of a component of a signal transduction pathway (cAMP-PKA) apparently involved in transducing stimuli of solid medium. Transformants overproducing lovastatin in SSF were very abundant and displayed important lovastatin production increases (described in Sect. 7.1.2). In another work, the

silencing of a gene, encoding a transcription factor associated to the SAPK/ MAPKinase signaling pathway, changed the ROS accumulation profile during the course of the culture, inducing 70 % higher lovastatin production in the transformant (described in the same section).

A deeper understanding of the PSM can also be applied to the development of new types of SSFs, hybrid SSF–SmF systems, or even novel SmF systems that will include some solid culture stimuli. One example of the latter is the one developed by Shoji et al. [75]. The authors found that higher enzyme productions were obtained when whole barley grains were used in SmF, instead of milled whole barley glucoamylase and α-amylase very probably involving support stimuli.

The group of Nakanishi has reported that fungi cultivated by membrane-surface liquid culture (MSLC) show cultivation behaviors that are similar to those cultivated on agar-plate culture [76–78]. They showed that neutral protease, α-glucosidase, and kojic acid are produced at much higher levels by MSLC than by SmF, in a manner similar to SSF. The authors claim that MSLC could be an efficient production system [78].

Recently the term *biofilm* has been extended to the surface-associated growth of filamentous fungi. In opposition to immobilized-cell reactors, in a fungal biofilm reactor, biomass naturally adheres and colonizes the surface of an inert support in contact with a liquid medium [79, 80]. In other words the cells will sense what was called here the “support stimuli.”

In a very recent study, Zune et al. [81] tried to apply concepts of PSM to the production of recombinant proteins by *A. oryza*e. Considering that BfR conditions were at least partially similar to SSF the authors studied the production of a GlaA:: GFP (Green Fluorescent Protein) fusion protein, under the control of the *glaB* promoter, that is, an SSF-specific promoter. In addition, they compared this system with a typical SmF (stirred tank bioreactor). Although this work represents an advance in the right direction, results were unpredicted. Unexpectedly, the fusion protein was also produced in SmF, despite the use of the *glaB* promoter. Moreover, the best yield was obtained in the classical stirred tank reactor, but involved alteration of the recombinant product. On the other hand, production in the BfR enhanced stability of the recombinant product. Expression of the *glaB* promoter in SmF appears to be related to the shear rate, since when this reactor was operated at 200 rpm (instead of 800 rpm) no excretion of the fusion protein was observed.

In addition, it has become apparent that basic aspects of the PSM, found in studies with *A. oryzae*, can be applied to other fungal species, even phylogenetically distant fungi like *Rhizopus*. Xu et al. [82] found that *R. chinensis* produced a wide range of lipases that were able to synthesize useful flavor esters from free fatty acids and alcohols. Particularly, this fungus produced lipase Lip1 with high synthetic activity and that turned out to be a solid-state specific enzyme, so it was produced in large amounts in SSF [83]. As in the case of GlaB, low water activity played a significant role in the induction of Lip1, and they were able to increase the expression level of this lipase in SmF (20–46 mg g dm^{-1}) when decreasing water activity of the liquid medium. Again, physical barriers against hyphal extension were found to be another required factor. Expression of Lip1 was significantly enhanced (in agar plates) by

threefold, so enzyme production reached 388.4 mg g dm^{-1}. When this growth barrier effect was combined with low water activity (in petri dish), specific production increased to 921.5 mg g dm^{-1} [84].

This study on enzymes production, together with others, and our own work on SMs, indicate that the knowledge of physiology of solid medium, generated in studies with *A. oryzae* or *A. terreus*, is of general nature and can be applied to processes with other fungi.

9 Conclusions

Many primary metabolites show advantages when produced by SSF. However, the most impressive results are seen in the area of SMs and enzymes production by SSF. These compounds are often produced at much higher yields in SSF, and certain SMs and certain enzymes are only produced under SSF conditions. This is considered to be part of the special physiology displayed by fungi (and other microorganisms) in SSF and that has been called physiology of solid medium [23].

These capacities have called the attention of researchers and made that recently PSM has acquired importance in hot research topics like lignocellulosic residues biotransformation (to biofuels or bulk chemicals), or the search for conditions to awake SM biosynthetic gene clusters, which have been found by genomic mining.

Studies on the biosynthesis of secondary metabolites in solid culture, mainly using the model of lovastatin production, have shown that the solid culture environment induces higher transcription of the specific transcription factor (LovE) as well as the biosynthetic genes, and hence higher production. These studies showed that important environmental factors, inducing this different physiology, are (1) direct contact with air, (2) support stimuli (physical barriers to growth), and with a lower impact, (3) low Aw.

The last two stimuli have also been identified in the field of enzymes (and proteins) production. Research using SSF-specific enzyme glucoamylase GlaB as a model identified physical barriers to growth and low Aw as inducing environmental cues. Moreover, the study of cis-regulatory elements, identified in the promoter of this and other SSF-specific genes, together with research described below, have given a deeper insight in the genetic expression and regulation in SSF.

Transcriptomic and proteomic studies have revealed that this special physiology is supported by a major change in gene expression. An impressive figure of 4,628 genes was found to be expressed differentially between SSF and SmF. Also, that protein production is controlled in response to solid-culture (or liquid) conditions. Genetic expression is regulated in a complex form, with at least four different regulatory circuits, one of which is similar to SmF, i.e., does not respond to culture system, but only to medium components. Other gene groups respond exclusively to SSF- or SmF-environmental conditions, at transcriptional and/or posttranscriptional levels.

A great proportion of molecular studies were performed on *A. oryzae*, but this knowledge has been found to explain the behavior of other fungi in SSF. These basic

findings have already found applications in technologies like genetic improvement methods to generate overproducing strains for SSF. Moreover, it is also starting to be applied to design novel culture systems, and other technological advances. Eventually, this understanding will surely start having an impact in other applications of SSF like production of feed, fuel, food, and industrial chemicals.

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Bioproduction of Fungal Cellulases and Hemicellulases Through Solid State Fermentation

11

Amita Shah, Harshvadan Patel, and Madhuri Narra

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Abstract

Fungi produce extensive set of enzymes to degrade lignocellulosic plant biomass. Fungal (hemi)cellulases are among the most widely exploited microbial enzymes for many industrial and environmental applications. However, in biofuel

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industries and few other sectors, the cost of the enzymes is a big hurdle in the development of successful technology. So far industrial production of (hemi) cellulases is mainly achieved by submerged fermentation technique. But solid state fermentation (SSF) is an alternative low-cost and less energy-intensive technology which can lead to reduction in the cost of these enzymes. The chapter initially describes structure and occurrence of plant cellulose and hemicellulose and their degradation by fungal enzymes. Extracellular multienzyme systems of wood-rotting fungi, plant-pathogenic fungi, and thermophilic fungi are also reviewed. Production of (hemi)cellulases by SSF is explained with discussion on critical factors affecting the process and their optimization. Additionally, attempts to develop large-scale SSF processes using bioreactors are also described. Improvements of fungal (hemi)cellulases by genetic approaches and the current applications of (hemi)cellulases along with bioconversions of ligno-cellulosic waste into valuable products for use as energy source or food additives are briefly narrated.

Keywords
Cellulase • Hemicellulase • Ascomycete • Basidiomycete • *Trichoderma reesei* • *Aspergillus niger* • Bioconversion • Lignocellulosic biomass

List of Abbreviations

a_w	Water activity
BBD	Box-Behnken design
CBH	Cellobiohydrolase
CBM	Carbohydrate-binding module
CCD	Central composite design
CCFD	Central composite face-centered design
CFC	Cellophane film culture
CMCase	Carboxymethyl cellulase
g	Gram
GH	Glycosyl hydrolase
LCM	Lignocellulosic Material
LPMO	Lytic polysaccharide monooxygenases
PBD	Plackett-Burman design
SmF	Submerged fermentation
SSF	Solid state fermentation
U/g	Unit per gram of substrate

1 Introduction

With the ever-increasing world population, human beings are facing extreme pressure on conservation of environment, energy, and resources for present and future generations. One of the safer ways to handle such issues is to minimize the use of chemicals and more energy-intensive processes by replacing them with enzyme-driven processes.

Enzymes can play crucial role in enhancing the quality of life as well as maintaining sustainable environment by having a wide array of catalytic activity. Majority of the enzymes used for various technical and nontechnical applications are hydrolytic enzymes, viz., proteases, carbohydrases, and lipases. Microorganisms are the most powerful and convenient sources of industrial enzymes wherein fungi being the source of a wide range of extracellular enzymes including cellulases and hemicellulases. In spite of having very good efficiency and functionality, economic feasibility of enzyme-based processes is often prohibitive for their applications. Hence, research and developments in this field are focused on production of efficient enzyme formulations by cost-effective processes.

Cellulose and hemicellulose of plant biomass are the most abundant renewable bioresources on the earth. Huge quantities of plant biomass are being wasted or underutilized in various countries. To enable efficient utilization of plant biomass, fungi produce an extensive set of carbohydrate-active enzymes to degrade cellulose and hemicellulose. Fungi are the most important and widespread group of organisms responsible for the recycling of plant material back into the ecosystem and are therefore essential components of the global carbon cycle. One of the reasons for this seems to be the "side-by-side" evolution of plants and fungi. The variety of the enzymes produced by each fungus differs and often corresponds to the requirements of its habitat.

Cellulolytic and hemicellulolytic enzymes from fungi are currently being produced on large scale for various applications like textile processing, food processing, detergents and laundry, animal feeds improvement, and biofuel production. The development of second-generation biofuels derived from lignocellulosic raw materials is now expected to drive the demand of industrial (hemi)cellulases. Enzyme-based bioconversion of lignocellulosic biomass into variety of valuable products can be the most efficient and environmentally friendly approach. However, high cost of cellulases is one of the largest obstacles for commercialization of biomass biorefineries [1, 2]. Cellulase production can be made more economical by increasing volumetric productivity, producing enzyme preparations with greater stability for specific processes, producing cellulases with higher specific activity, and using cheaper solid substrates. Considering these aspects, solid state fermentation (SSF), although an old concept, is now being reconsidered by scientists for production of such enzymes at least for biofuels and biorefinery. Because of some inherent technological problems of solid state fermentation, submerged or liquid state fermentation is still the dominating technology for (hemi)cellulase production at industrial level. So commercialization of SSF processes is a challenging task, and transition from an "ancient art" to a modern technology is necessary.

2 Occurrence and Structure of Cellulose and Hemicellulose

Major polysaccharide in plant lignocelluloses is cellulose – typically in the range of 35–50 %. Cellulose makes up 15–30 % of the dry biomass of primary and up to 40 % of the secondary wall. Cellulose fibers are embedded in a matrix of other structural

Table 1 Composition of lignocellulosic biomass

Substrate	% Cellulose	% Hemicellulose	% Lignin	References
Corn stover	39	19.1	15.1	[4]
Corn cobs	45	35	15	[5]
Wheat straw	41.3	30.8	7.7	[6]
Rice straw	39	15	10	[7]
Rice hulls	24–29	12–14	11–13	[8]
Sugarcane bagasse	43	25	24	[7]
Sawdust	45	15.1	25.3	[9]
Coconut fiber	17.7	2.2	34	[9]
Switch grasses	31	22	18	[7]
Grasses	25–40	25–50	10–30	[5]

biopolymers, primarily hemicellulose and lignin. Hemicellulose comprises 20–35 % and lignin 5–30 % of plant dry weight. Hemicellulose is found in the spaces between cellulose microfibrils in primary and secondary cell walls, as well as in the middle lamellae [3]. Lignocellulosic material (LCM) consisting of these carbohydrate polymers (cellulose and hemicellulose) and lignin is a highly recalcitrant structure and difficult to deconstruct. Table 1 shows typical composition of various plant biomass.

Plant cellulose is a linear homopolysaccharide synthesized from D-glucose residues linked by β-(1,4) glycosidic linkage. The degree of polymerization (DP) in a single chain can be as high as 14,000 in secondary cell wall and 6000 in primary cell wall. The linear chains undergo self-assembly by inter- and intrachain hydrogen bonds leading to the formation of a microfibril. Microfibrils (diameter 2–4 nm) are reported to be made up of around ~36 cellulose chains coated with other noncellulosic polysaccharides. Such microfibrils are cross-linked by hemicelluloses/pectin matrix covered by lignin to form macrofibrils of varying diameters that mediate structural stability in the plant cell wall. This special crystalline structure makes cellulose resistant to both biological and chemical treatments [10, 11] as well as impermeable to water. In this polymeric structure, there are both crystalline and amorphous regions in addition to several types of surface irregularities. The relative amount of crystalline and amorphous regions is varied in different sources of cellulose. In native cellulose two crystalline forms 1α and 1β exist. The 1α form is more susceptible to hydrolysis, but plant cellulose mainly contains 1β form [12].

Hemicelluloses are heteropolysaccharides with more complex, branched structure formed from various monomeric pentose and hexose sugars attached through different linkages. Carbohydrate substituents and noncarbohydrate components occur in hemicelluloses either on the main chain or on the carbohydrate branches. Such a complex structure of the hemicellulose is believed to confer a wide range of biophysical and biomechanical properties to the plant tissues in which they occur. Classification of hemicelluloses is based on the main residues of sugars present in the backbone of the structural polymer. Various types of hemicelluloses and their main

Table 2 Occurrence of various hemicelluloses in plant biomass and their chemical composition [13]

Polysaccharides/form of xylan	Plants	Backbone	Side chains
Arabinoxylan	Cereals	β-D-Xylp	α-L-ArafFeruloy
Arabinogalactan	Softwood	β-D-Galap	β-D-Galp α-L-Araf β-L-Arap
Arabinoglucuronoxylan	Grasses, cereals, softwood	β-D-Xylp	4-*O*-Me-α-D-GlcpAβ-L-Araf
Glucuronoxylan	Hardwood	β-D-Xylp	4-*O*-Me-α-D-GlcpA Acetyl
Glucuronoarabinoxylan	Grasses and cereals	β-D-Xylp	α-L-Araf 4-*O*-Me-α-D-GlcpA Acetyl
Glucomannan	Softwood and hardwood	β-D-Manp β-D-Glcp	
Galactoglucomannan	Softwood	β-D-Manp β-D-Glcp	β-D-Galp Acetyl
Xyloglucan	Hardwood, grasses	β-D-Glcp β-D-Xylp	β-D-Xylp β-D-Galp α-L-Araf α-L-Fucp Acetyl
Homoxylan	Algae	β-D-Xylp	

Table 3 Monomeric sugar composition (g/100 g of dry materials) of xylan from some agroresidues

Lignocellulosic substrate	Xylose	Arabinose	Mannose	Galactose	Acetyl group	Reference
Wheat straw	19.2–21.0	2.4–3.8	0–0.8	1.7–2.4	–	[4, 14]
Wheat bran	16	9	0	1	0.4	[15]
Rice straw	14.8–23	2.7–4.5	1.8	0.4	–	[4, 16]
Rice husks	17.7	1.9	–	–	1.62	[17]
Corn stover	14.8–25.2	2–3.6	0.3–0.4	0.8–2.2	1.7–1.9	[4, 18, 19]
Corn stalks	25.7	4.1	<3.0	<2.5	–	[20]
Corn cobs	28–35.3	3.2–5.0	–	1–1.2	1.9–3.8	[15, 18, 21, 22]
Sugarcane bagasse	20.5–25.6	2.3–6.3	0.5–0.6	1.6	–	[23, 24]

chain and backbone residues are shown in Table 2, and monomeric sugar composition of various agroresidues is given in Table 3.

Xylan is the most abundant hemicellulose polymer in cereals and hard woods. In land-based plants, it constitutes up to 30 % to 35 % of total dry weight. These complex heteropolysaccharides consist of β-1,4-linked D-xylopyranosyl backbone

and can be substituted with different side groups, such as L-arabinosyl, D-galactosyl, acetyl, feruloyl, *p*-coumaryl, and glucuronosyl residues. The frequency and composition of the branches are dependent on the xylan source [25].

Another two major hemicelluloses in plant cell wall are galacto(gluco)mannans and xyloglucans. Galacto(gluco)mannans consist of a backbone of ß-1,4-linked D-mannose (mannans) and D-glucose (glucomannans) residues with D-galactose side chains. Homo- and heteromannans are based on variations of β-mannan backbone, which might be interrupted with D-glucose (glucomannan) and/or branched with α-1,6-linked D-galactose (galactomannan/galactoglucomannan). The mannose and glucose residues in the backbone are sometimes acetylated at C-2 or C-3. Linear mannan and glucomannan chains containing more than 5 % (w/w) D-galactose are called galactomannans and galactoglucomannans. Xyloglucans that consist of a ß-1,4-linked D-glucose backbone substituted by D-xylose [26]. In xyloglucan, L-arabinose and D-galactose residues can be attached to the xylose residues, and L-fucose can be attached to galactose residues. The diversity of side groups that can be attached to the main backbone of xyloglucans confers to high structural complexity and variability [27].

3 (Hemi)cellulolytic Enzymes of Fungi

3.1 Cellulases

Cellulases are known to be produced not only by fungi but also by bacteria, protozoa, plants, and some members of animal kingdom. The insoluble and recalcitrant nature of plant cellulose poses great challenge for biochemical degradation of cellulose into monomeric sugars. For microorganisms to hydrolyze and metabolize insoluble cellulose, extracellular cellulases must be produced that are either free or cell associated. A cellulase system from aerobic and anaerobic bacteria and fungi has been extensively studied during the past two decades. Cellulolytic enzyme system can be complexed or noncomplexed. Noncomplexed cellulases are found in aerobic fungi, bacteria, and actinomycetes. These organisms secrete cellulases as free enzymes in the exterior, while in anaerobic bacteria and fungi, these enzymes are organized as high-molecular weight complexes called cellulosomes. Structure and organization of such cell wall associated cellulosome is well elucidated in *Clostridium thermocellum*. However, anaerobic fungi, viz., *Neocallimastix*, *Orpinomyces*, and *Piromyces* cellulosomes, are known, but molecular arrangements are not clearly established [28].

Components of cellulase systems have been classified based on their mode of catalytic action and structural properties [29]. Three major types of enzymatic activities are found:(i) Endoglucanase or β-1,4-D-glucan-4-glucanohydrolases (EC 3.2.1.4) cut the internal glycosidic linkages in amorphous cellulose randomly and generate oligosaccharides of various chain lengths and consequently open new chain ends.(ii) Exoglucanases including β-1,4-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and β-1,4-D-glucan cellobiohydrolases

(EC 3.2.1.91) act in a processive manner (successive cleavage of products) on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. (iii) β-D-Glucosidases or β-D-glucoside glucohydrolases (EC 3.2.1.21) act on cellobiose and cellodextrins and release D-glucose units. *T. reesei* produces at least two exoglucanases (CBHI and CBHII). Both CBHs, CEL6A and CEL7A, have been shown to act processively, whereby CEL6A cleaves the cellobiose dimers from the nonreducing end of the cellulose chain and CEL7A from the reducing end [30], five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV), and two β-D-glucosidases (BGLI and BGLII) [31, 32]. *A. niger* has 5 EGLs within GH families 5 and 12, 4 CBHs in families 6 and 7, and 13 BGLs in families 1 and 3 [33].

Analyses of the genome sequences of more than 40 ascomycete and basidiomycete species show that these enzymes are confined to a relatively low number of GH families [34]. Strictly processive exocellulases (cellobiohydrolases) are found in GH families 6 and 7, while endoglucanases are distributed in large number of GH families (GH families 5, 7, 12, and 45). β-Glucosidases are predominantly found in the GH1 and GH3 families.

In addition to glycoside hydrolases, oxidative enzymes called lytic polysaccharide monooxygenases (LPMOs) are also shown to play important role in cellulose degradation. Recently some of the members of GH61 family now included in auxiliary activity, families 9 and 11 (AA9 and AA11) which encode a novel class of copper-dependent enzymes, are now referred to as lytic polysaccharide monooxygenases (LPMOs) [35, 36]. LPMOs catalyze an oxidative cleavage of cellulose in the presence of an external electron donor, thus exhibiting synergy with hydrolytic biomass depolymerization.

Cellulose hydrolysis is also facilitated by “non-hydrolytic” accessory proteins. The expansin (plant protein produced during growth)-like protein swollenin, identified in *Trichoderma reesei*, synergistically raises the activity of the cellulases but does not exhibit any enzymatic activity on cellulose themselves [37]. They presumably act through their ability to disrupt hydrogen bonds and thereby reduce cellulose crystallinity and increase cellulase accessibility. Additionally, swollenin has also been reported to synergize with xylanases in the release of xylose from steam-pretreated corn stover [38]. However, high amount of protein is required along with hydrolytic enzymes. Fungal swollenin has successfully achieved recombinant expression in several eukaryotic and prokaryotic hosts [39].

Most of the aerobic fungi have cellulases having two domains: catalytic domain and carbohydrate-binding domain (CBM). The CBM affects binding to the cellulose surface, to facilitate cellulose hydrolysis by bringing the catalytic domain in close proximity to the insoluble cellulose. The presence of CBMs is particularly important for the initiation and processivity of exoglucanases [40]. In aerobic fungi, the CBM is invariably from family 1, which is very small (~30–35 amino acid residues). All types of CBMs exhibit a planar array of highly conserved aromatic side chains located on its relatively flat surface which are believed to align with the hydrophobic faces on the cellulose surface and facilitate substrate binding of the CBM. According to Bayer et al. [41], the crystalline form of cellulose requires CBM for its hydrolysis,

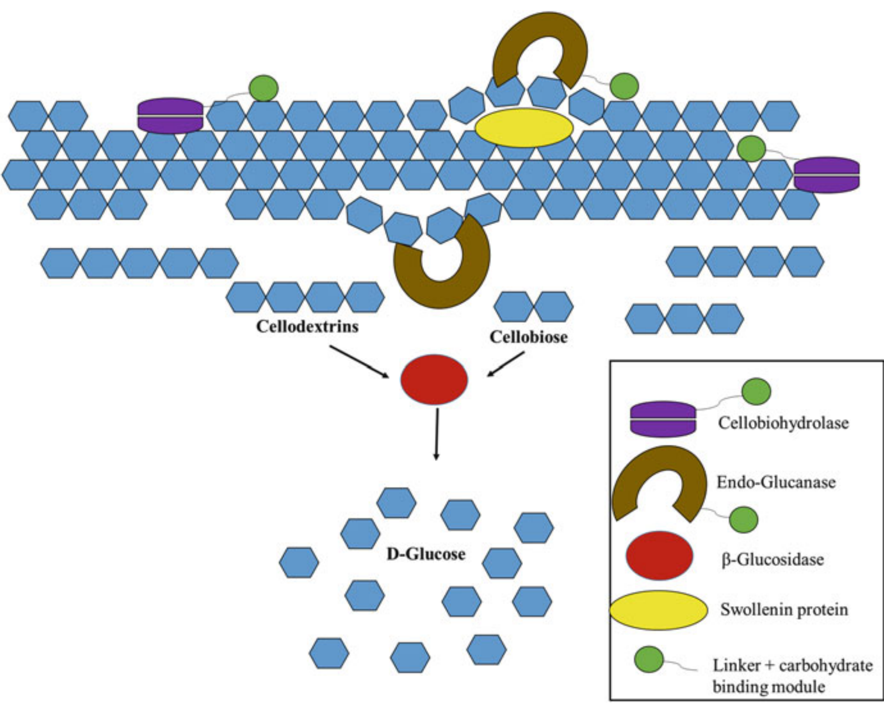

Fig. 1 Schematic presentation of action of cellulases and role of swollenin and CBM in hydrolysis of cellulose to glucose

but on the other hand, it has been observed that CBM may lead to unproductive binding to cellulose and lignin and thereby reduce the rate of cellulose hydrolysis. The CBMs are divided into 59 families on the CAZy database. Figure 1 shows a schematic presentation of action of cellulases and role of swollenin and CBM in hydrolysis of cellulose to glucose.

3.1.1 Induction of Cellulase Production

Cellulase production by fungi is mainly regulated at the transcriptional level. Substrates such as cellulose, lactose, and sophorose act as inducers, while glucose normally acts as gene repressor. The presence of a low-level constitutive enzyme is required to initiate cellulose degradation, which generates soluble inducers that can enter the cell and activate cellulase gene transcription [42, 43]. The cellulolytic system of *T. reesei* is coregulated which means that they are expressed under all conditions at the same relative amounts [44, 45]. The mechanism by which filamentous fungi sense the substrate and initiate the enzyme production is still not resolved. Some scientists also studied the role of carbon starvation, in activation of cellulase/ hemicellulase expression [46]. The most powerful inducer of cellulases in *T. reesei* is sophorose, a disaccharide composed of β-1,2-linked glucose units. Sophorose appears to be formed from cellobiose through transglycosylation activity of

β-glucosidase [47, 48]. In addition to *T. reesei*, sophorose is known to induce cellulase expression in *A. terreus* and *P. purpurogenum* [49, 50]. Cellobiose appears to induce cellulase expression in many species of fungi. Another inducer cellobiose is formed as the end product of cellobiohydrolases activity, and it has been shown to induce cellulase expression in *T. reesei*, *Volvariella volvacea*, *P. janthinellum*, and *A. nidulans* [45, 51]. However, according to Aro et al. [27], the inducing effect of cellobiose on cellulase expression is controversial, because cellobiose can be transglycosylated by β-glucosidases, producing sophorose. Besides, β-glucosidases can cleave the cellobiose into glucose, which may cause catabolite repression.

Lactose (1,4-*O*-β-D-galactopyranosyl-D-glucose) is another most widely used inducer of cellulose expression in *T. reesei*. In filamentous fungi, lactose is cleaved by extracellular β-galactosidase into glucose and galactose. However, the mechanism of induction is not clear. In addition, induction of cellulase genes could also be achieved by laminaribiose, gentiobiose, xylobiose, L-sorbose, and δ-cellobiono-1,5-lactone. L-Arabitol and different xylans have been shown to induce expression of cellobiohydrolase 1 (*cbh1*) in *T. reesei* [27].

3.2 Hemicellulases

Due to the heterogeneity and complex chemical nature of hemicelluloses as described earlier, hydrolysis of hemicelluloses into simple constituents like monomeric and dimeric sugars or oligosaccharides requires the action of a wide spectrum of enzymes with diverse catalytic specificity and modes of action (Table 4). The enzymes can be categorized as main-chain-degrading enzymes (xylanase, β-xylosidase, mannanase, β-mannosidase, arabinase) and side-chain-cleaving enzymes (α-L-arabinofuranosidase, esterases). Cooperative action of both types of enzymes is required for complete and biodegradation of hemicelluloses.

Endo-xylanases (EC 3.2.1.8) are extracellular enzymes produced by various fungi. They cleave internal β-(1–4) linkages in xylan backbone and release short-chain xylooligosaccharides of varying lengths including xylose [25]. Xylanases have been classified in GH families 5, 7, 8, 10, 11, and 43 on the basis of their amino acid sequences, structural folds, and mechanisms for catalysis [52, 53]. GH10 and GH11 family xylanases represent the best studied xylanase families. GH10 family xylanases have four or five subsites, and GH11 family xylanases have at least seven subsites [54]. Moreover, generally family 10 xylanases are characterized by high molecular weight (usually >30 kDa) and acidic pI, while the members of family 11 have low molecular weight and basic pI [55, 56]. Xylanases from families 10 and 11 can be differentiated on the basis of lower or higher substrate specificities, respectively. Family 10 xylanases are able to catalyze the hydrolysis of pNP-cellobioside at a gluconic linkage, while the members of family 11 xylanase failed to recognize this as substrate [52, 57]. Another feature that distinguishes GH10 and GH11 xylanases is the nature of the reaction products released from decorated xylans. GH11 xylanases produce substituted xylooligosaccharides both at the aglycone and glycone subsites [58]. *Aspergillus niger* reported to have five

Table 4 Various hemicellulases and their mode of action

Enzymes	EC No	Mode of action
Endo-xylanase	EC 3.2.1.8	Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone
Exo-xylanase		Hydrolyzes β-1,4-xylose linkages releasing xylobiose
β-Xylosidase	EC 3.2.1.37	Releases xylose from xylobiose and short chain xylooligosaccharides
α-Arabinofuranosidase	EC 3.2.1.55	Hydrolyzes terminal nonreducing α-arbinofuranose from arabinoxylans
α-Glucuronidase	EC 3.2.1.139	Releases glucoronic acid from glucuronoxylans
Acetylxylan esterase	EC 3.1.1.72	Hydrolyzes acetyl ester bonds in acetyl xylans
Ferulic acid esterase	EC 3.1.1.73	Hydrolyzes feruloyl ester bonds in xylans
p-Couramic acid esterase	EC 3.1.1.74	Hydrolyzes *p*-coumaroyl ester bonds in xylans
β-Mannanase	EC 3.2.178	Attacking the internal glycosidic bonds of the mannan backbone chain, releasing short β-1,4-manno-oligosaccharides
β-Mannosidase	EC 3.2.1.25	Hydrolysis of mannose units from the nonreducing end of manno-oligosaccharides
α-Galactosidase	EC 3.2.1.22	Hydrolyze galactosyl side groups from oligomeric and polymeric mannan
β-Glucosidase	EC 3.2.1.21	Hydrolyze nonreducing end glucose from oligosaccharides released by β-mannanase
Acetyl mannan esterase	EC 3.1.1.6	Hydrolyze galactosyl side groups from oligomeric and polymeric mannan

xylanase genes. One of them belongs to GH10 family and other four genes belong to GH11family [59]. In *T. reesei* two forms of GH11 xylanases, Xyl-I and Xyl-II, have been reported [60].

β-Xylosidases (1,4-*β*-D-xylanohydrolase, EC 3.2.1.37) are exo-type glycosidases which hydrolyze xylobiose and short-chain xylooligosaccharides from the nonreducing end to xylose. β-Xylosidases play a crucial role in alleviating end product inhibition of endo-xylanases caused by xylooligomers and increase the efficiency of xylan hydrolysis [61]. In filamentous fungi this enzyme remain associated with the mycelia during early stages of growth and can be released later into the medium either by secretion or as a result of cell lysis, while in some fungi, they remain associated with cell surface through their growth cycle [62]. Although xylose is the end product inhibitor of β-xylosidases, it can act as inducer of xylanolytic gene expression. High yields of β-xylosidase on xylose were observed with *T. reesei* [63] and *A. versicolor* [64]. β-Xylosidases from fungi are often monomeric glycoproteins, but some have been reported to possess two or three subunits [65, 66]. They are grouped into five different families (GH3, GH39, GH43, GH52, and GH54); however, the best characterized β-xylosidases are from GH3 and GH43 [54].

α-L-Arabinofuranosidases (EC 3.2.1.55) are accessory exo-type enzymes which release arabinose through the cleavage of the α(1–2), α(1–3), and α(1–5) bonds that link L-arabinosyl side-chain decorations to the main chain of polysaccharides such as arabinoxylan, arabinogalactan, and arabinan. These enzymes can hydrolyze arabinosyl linkage of arabinan. The α-L-arabinofuranosidases are essential part of microbial xylanolytic systems necessary for complete breakdown of arabinoxylans. α-L-Arabinofuranosidases act synergistically with other hemicellulases and pectic enzymes for the complete hydrolysis of hemicelluloses and pectins [67]. The arabinan-degrading enzymes that act in an endo fashion are called endo-1,5-α-L-arabinanases (EC 3.2.1.99). Bifunctional α-L-arabinofuranosidases possessing β-xylosidase activity or xylanase activity have also been described [68]. These enzymes expedite the hydrolysis of the glycosidic bonds by more than 10^{17}-fold, making them one of the most efficient catalysts. α-L-Arabinofuranosidases exist in monomeric, dimeric, and multimeric forms [69]. They are classified into five GH families, viz., GH3, GH43, GH51, GH54, and GH62 [70]. α-L-Arabinofuranosidases belonging to GH51 and GH62 families release O-2- and O-3-linked arabinofuranosyl units from monosubstituted xylose while α-L-arabinofuranosidases of GH43 family arabinose from disubstituted xylose also [71].

Acetylxylan esterases (EC 3.1.1.6) remove *O*-acetyl group from the C-2 and C-3 positions of xylose and xylooligosaccharides. Biely et al. [57] first reported the presence of acetylxylan esterases in various fungal cellulolytic and hemicellulolytic systems, such as *Trichoderma reesei*, *Aspergillus niger*, *Schizophyllum commune*, and *Aureobasidium pullulans*. Acetylxylan esterases are enzymes that are able to hydrolyze the ester linkage between acetyl and xylose residues in xylans. As the acetyl side groups can interfere with the approach of enzymes that cleave the backbone by steric hindrance, their elimination facilitates the action of endo-xylanases [72]. The degradation of acetylxylan with endo-xylanases proceeds faster and to a higher degree in the presence of acetylxylan esterases. They also deacetylate the partially acetylated xylooligosaccharides which makes the oligosaccharides fully susceptible to the action of β-xylosidases [71]. These enzymes may contribute to lignin solubilization by cleaving the ester linkages between lignin and hemicelluloses [73].

Ferulic acid and *p*-coumaric acid are common constituents of animal feed and may represent up to 2.5 % by weight of the cell walls in temperate grasses. Many of the arabinose residues in various arabinoxylans are esterified with ferulic acid and *p*-coumaric acid residue. Barley straw arabinoxylan contains approximately one *p*-cupric acid per 31 arabinose residues and one ferulic acid per 15 arabinose residue. Ferulic acid esterases cleave ester linkages between ferulic acid and arabinose in xylan. Most of the feruloyl esterases are extracellular and are active against xylan and xylan-derived oligosaccharides, from which they are able to release ferulic acid. Ferulic/coumaric acid esterases belong to the carbohydrate esterase (CE) family 1 [74].

α-Glucuronidase (α-D-glucuronidase, EC 3.2.1.131) is an important accessory enzyme which cleaves the α-1,2-glycosidic linkage between xylose and glucuronic acid or its 4-*O*-methyl ether. Hardwood xylans possess an average of one

α-1,2-linked uronic acid side group per ten xylose units, and softwood xylans contain one per five xylose units. α-Glucuronidases (EC 3.2.1.131) have been grouped in family GH67, and it removes only the glucuronosyl group that is attached to the terminal residue at the nonreducing end of xylo-oligosaccharides [75].

β-Mannanase (EC 3.2.178) is an enzyme responsible for the conversion of heteromannans to mannooligosaccharides and small amount of mannose, glucose, and galactose. The β-mannanases are endo-acting hydrolases, attacking the internal glycosidic bonds of the mannan backbone chain, releasing short β-1,4-mannooligosaccharides. β-Mannanase is the key enzyme that catalyzes the random hydrolysis of β-mannosidic linkages in mannan and heteromannans. Multiple extracellular mannanases have been reported among many fungi like *T. reesei*, *T. harzianum*, and *Aspergillus* sp. [76].

β-Mannosidases (β-1,4-D-mannoside mannohydrolase, EC3.2.1.25) catalyze the hydrolysis of mannose units from the nonreducing end of mannosides. However, some β-mannosidases are also active on glucosides [77]. β-Mannosidases are described in GH families 1, 2, and 5 [78]. β-Mannosidases from *T. reesei* belong to family 5 [60]. α-Galactosidase (EC 3.2.1.22) cleaves α-(1 → 6)-linked nonreducing galactose residues. α-Galactosidase releases galactosyl side groups from oligomeric and polymeric mannan substrates. Some of the α-galactosidases in family 27 can release galactose from polymeric substrates [79].

3.2.1 Induction of Hemicellulase Production

Since long it has been observed that the presence of the hemicelluloses like xylan, xyloglucan, arabinan, and mannan usually induces a high production of hemicellulases, but the mechanism of sensing is not clear. Usually, small hemicellulose-derived molecules are able to induce the expression of a wide range of hemicellulases. The monosaccharide D-xylose is a well-known inducer of xylanolytic enzymes in *Aspergillus* species. In *A. niger*, D-xylose appears to induce the accessory enzymes also like α-glucuronidase (*aguA*), acetylxylan esterase (*axeA*), and feruloyl esterase (*faeA*) [80, 81]. On the contrary it was also demonstrated that xylose can act as a repressor of hemicellulase production at high concentrations [82]. In addition to xylose, xylobiose and D-glucose-β-1,2-D-xylose have been demonstrated to induce expression of xylanolytic genes in *A. terreus* [50]. In *A. niger*, arabinoxylan-degrading enzymes were found to be induced by arabinose and L-arabitol [27]. The arabinolytic system of *A. niger* was found to be independent from xylanolytic system [83].

3.3 Multiplicity of (Hemi)cellulases

Culture supernatants of many cellulolytic fungi generally show multiple forms of cellulases and hemicellulases. Perhaps the structural complexity and variability of lignocelluloses have resulted in the need for these multiple forms. Multiplicity is considered to be due to mRNA heterogeneity which may be the result of differential splicing of primary mRNA and due to multiple initiations of transcription.

Another reason for multiplicity is differences in posttranslational modifications of the same protein either by protease cleavage or at the secretory level due to differential glycosylation [84]. *Schizophyllum commune* is reported to produce all the three principal cellulases in multiple forms. There are two distinct types of cellulases expressed from each mRNA with different molecular weights (exoglucanases 59.3 and 58.2, endoglucanases 40.6 and 39.4, and β-glucosidases 95.7 and 93.8 KDa). In the case of xylan, since the pattern of cleavage of xylosidic bonds is different in xylanases, to carry out efficient hydrolysis of xylan fungi needs a multienzyme system of xylanases, in which each enzyme has a special function. Production of multiple xylanases from thermophilic fungus *Myceliophthora* sp. was observed in response to the type of carbon source as well as culture condition. Rice straw induced expression of three xylanase isoforms under shake flask cultivation, while five xylanase isoforms produced solid state fermentation [85].

Multiplicity has also been observed in β-xylosidases, α-L-arabinofuranosidases, and acetylxylan esterases and feruloyl esterases [26]. Two β-xylosidases liberated from the cell surface of *P. herquei* were purified and identified as GH43 enzymes [86]. Multiplicity of β-xylosidases originates from the differences in efficiency to hydrolyze different heterogenous xylooligomers released by the action of different endo-xylanases [56, 62]. Three different forms of α-L-arabinofuranosidases from *P. purpurogenum* were separated by isoelectrofocusing and detected using the zymogram technique. Some of the thermophilic fungi like *H. insolens*, *Chaetomium thermophilum*, and *Melanocarpus* sp. were found to produce high titers of multiple esterases that were putatively classified as xylan acetyl esterase and feruloyl esterase [87].

3.4 Synergism Between (Hemi)cellulolytic Enzymes

Efficient degradation of plant cell wall polysaccharides requires cooperative or synergistic actions between the enzymes responsible for cleaving different linkages. A highly balanced cocktail of cellulolytic and hemicellulolytic enzymes is required for rapid and efficient degradation. Cellulase systems often exhibit higher collective activity than the sum of the activities of individual enzymes, a phenomenon known as synergism [88]. Four forms of synergism have been reported: (i) endo-exo synergy between endoglucanases and exoglucanases, (ii) exo-exo synergy between exoglucanases processing from the reducing and nonreducing ends of cellulose chains, (iii) synergy between exoglucanases and β-glucosidases that remove cellobiose (and cellodextrins) as end products of the first two enzymes, and (iv) intramolecular synergy between catalytic domains and CBMs. Very high degrees of synergy were observed between endoglucanase and cellobiohydrolases of *Trichoderma* sp. on highly crystalline cellulose such as bacterial cellulose (5–10) and cotton (3.9–7.6), while more amorphous celluloses generally display lower degrees of synergy (0.7–1.8) [89, 90]. In contrast, Andersen et al. [91] reported opposite observations where synergy was displayed by cellulases on phosphoric acid-swollen cellulose (3.1), and no synergy was displayed on Avicel. The degrees of

synergy therefore appear to vary depending on the nature of the substrate, the specific nature of the enzymes, the ratios of enzymes involved, and the assay conditions [92, 93].

With respect to hemicellulases, three types of synergies have been identified, namely, homosynergy, heterosynergy, and anti-synergy [94, 95]. Homosynergy occurs between main-chain cleaving enzymes (e.g., β-mannanase and β-mannosidase), while heterosynergy occurs between main-chain cleaving and debranching enzymes (e.g., β-mannanase and α-galactosidase). Anti-synergy means inhibition of one enzyme by another enzyme. This may be possible when a main-chain cleaving enzyme requires a substituent for its activity and a debranching enzyme removes that substituent [95]. During xylan hydrolysis, synergism has been observed between enzyme action on the 1,4-β-D-xylan backbone (β-1,4-endo-xylanase) and side-chain cleaving enzymes (α-L-arabinofuranosidase, acetyl xylan esterase, and β-glucuronidase). The synergistic action between acetyl xylan esterase and endo-xylanases results in the efficient degradation of acetylated xylan [96]. The release of acetic acid by acetyl xylan esterase increases the accessibility of the xylan backbone for endo-xylanase attack, and on the other hand, the endo-xylanase creates shorter acetylated polymers, which are preferred substrates for esterase activity [97]. It was argued that a main-chain cleaving enzyme will have enhanced activity if substituents are first removed through debranching enzymes [98] because the substituent poses a steric hindrance to the main-chain cleaving enzyme. However, there are variations among xylanases from different families with respect to their active sites. Family 11 xylanases have a large active site and prefer cleaving main chains in unsubstituted regions, while family 10 xylanases have a smaller active site and are able to cleave main chains closer to the substituent [98]. Many such synergistic interactions have been identified between feruloyl esterases and endo-xylanase [98, 99], endo-xylanase, β-xylosidase, and α-L-arabinofuranosidase synergy on arabinoxylan [100, 101]. Synergistic activities are also found in mannan-degrading enzymes [94].

4 (Hemi)cellulolytic Fungi

Filamentous fungi of class *Ascomycete*s are well-known and well-established sources of cellulases and hemicellulases. *Trichoderma reesei* was one of the first cellulolytic organisms isolated in the 1950s, and by 1976, more than 14,000 fungi active against cellulose and other insoluble fibers were collected throughout the world [102]. However, industrial cellulases are almost all produced from aerobic cellulolytic fungi, such as *Hypocrea jecorina* (*T. reesei*) or *Humicola insolens* and *Aspergillus niger* [103]. This is because engineered strains of these organisms produce extremely large amounts of crude cellulase (over 100 g per liter) with a relatively high-specific activity on crystalline cellulose. Several other mesophilic strains producing cellulases like *Fusarium oxysporum*, *Piptoporus betulinus*, *Penicillium echinulatum*, *P. purpurogenum*, *A. fumigatus*, etc. have also shown high potential [104–106]. The cellulases from *Aspergillus* usually have high

β-glucosidase activity but lower endoglucanase levels, whereas *Trichoderma* has high endo- and exoglucanase components with lower β-glucosidase levels, which restricts their efficiency in cellulose hydrolysis. Hemicellulase expression has been studied mostly in *Aspergilli* and *T. reesei*. However, a comparison of the genome sequences of *T. reesei* [107] and *Aspergillus niger* [59] demonstrated that *A. niger* is more versatile in the range of hemicellulases, and therefore this species has been used extensively for basic research in recent years. *Penicillium* species are also found interesting as they have the ability to produce both cellulase and hemicellulase in higher amounts, and specifically they have higher beta-glucosidase activity than *Trichoderma* sp. [108]. The enzyme from *Penicillium* sp. ECU0913 was found very efficient in hydrolysis of pretreated corn stover without any accessory enzymes [109].

In the past few decades, increased interests have been found in phytopathogenic fungi. These fungi are naturally capable of expressing highly effective cellulases/hemicellulases for invasion through the plant cell wall and helping them in pathogenesis. Such enzymes are also required in the later stages of invasion as they provide monosaccharides and oligosaccharides for growth and reproduction. Recently, hydrolytic profile of plant pathogens was found to be more active than *Trichoderma* sp. [110]. As these plant cell wall-degrading enzymes (CWDEs) of pathogen have to face and overcome the inhibitors produced by plants as a defense mechanism, their enzymes are more potent, robust, and unique [111]. A number of plant pathogenic fungi (*Fusarium oxysporum*, *Phoma betae*, *Colletotrichum gloeosporioides*, etc.) have been reported to elaborate high levels of cellulases [112]. Production of cellulases and hemicellulases by *Chrysoporthe cubensis*, a well-documented pathogen of various tree species, was examined in solid state fermentation using different carbon sources. *C. cubensis* was able to produce high titers of endoglucanase, β-glucosidase, FPase, and xylanase activities under SSF using wheat bran as substrate. The (hemi)cellulolytic extract from *C. cubensis* showed great potential to be applied in biomass saccharification processes [113].

Wood-rotting fungi, viz., white rot fungi (WRF) and brown rot fungi (BRF), are another important groups of fungi as a source of (hemi)cellulolytic enzymes. Some of the wood-rotting fungi, viz., *Phaenerochaete chrysosporium* and *Gloeophyllum trabeum*, are very competent cellulase producers. Most of the WRF are categorized as *Basidiomycetes*, while only a few as *Ascomycetes* [114]. On the other hand, all the BRF belong to class *Basidiomycetes* [114]. Both of them degrade cellulose and hemicelluloses [115]. But the decay pattern of BRF and WRF is different. The WRF in addition to cellulose and hemicelluloses also degrade lignin [116], whereas BRF can only modify the lignin [114, 115]. The BRF have a more superior performance in cellulase production because of different enzyme system. According to Tewalt and Schilling [117], BRF degrade lignocellulosic substrate in two steps. In the initial stage, the cell wall of lignocellulosic substrate is modified in the absence of enzymes by hydrogen peroxide (H_2O_2). Thereafter, cellulase enzyme is secreted by BRF to break down the cellulose into glucose [118]. The production of hydrogen peroxide by BRF was induced by cellulose, preferably the crystalline cellulose [119]. These low molecular weight agents penetrate through the cell wall of the lignocellulosic

substrate and react with endogenous iron or other transition metals to produce hydroxyl radical via Fenton reaction [119, 120]. The hydroxyl radical produced degrades the lignocelluloses in the substrate by oxidative degradation. Generally cellulase system of BRF has sufficient level of endoglucanase and β-glucosidase, but it is deficient in exoglucanase. Hence, the oxidative mechanism plays a crucial role in improving bioconversion of cellulose.

For commercial applications, industrial enzymes must be more stable, robust, and efficient. In search of such enzymes, researchers have also focused on thermophilic fungi and their cellulolytic enzymes. These fungi grow at a much faster rate and show high productivity of hydrolytic enzymes. The enzymes produced by such fungi show significantly high thermostability which is highly advantageous in biocatalytic processes in biorefineries and in industrial processes like textile industry. Several thermophilic fungi and their biomass-degrading cellulases and hemicellulases have been reported by scientists. Some of the potential thermophilic fungi include *Humicola grisea* var. *thermoidea*, *Humicola insolens*, *Aureobasidium pullulans*, *Candida peltata*, *Chaetomium thermophilum*, *Coprinopsis cinerea*, *Ganoderma colossum*, *Melanocarpus albomyces*, *Myceliophthora thermophila*, *Myriococcum thermophilum*, *Penicillium duponti*, *Sporotrichum thermophile*, *Stilbella thermophila*, *Talaromyces emersonii*, *Thermoascus aurantiacus*, *Thermomyces lanuginosus*, and *Thielavia terrestris*. Hydrolytic enzyme profile of *H. grisea* was shown to catalyze complete saccharification of lignocellulose from different kinds of substrates, including sugarcane bagasse, ball-milled straw [121, 122], brewers' spent grain, and wheat bran [123]. *H. grisea* thermostable enzymes have already been employed as bleaching agents in the paper and pulp industry [124, 125]. *Melanocarpus albomyces* an ascomycete fungus is also a promising candidate to provide industrial cellulases. These fungus culture supernatants presenting two endoglucanases (20 and 50 KDa) and one cellobiohydrolase (50 kDa) activities were employed for denim fabrics indigo dye release (biostoning) [126]. This fungus also produces seven thermostable xylanases active at temperature from 55 °C to 70 °C [127] and was reported to produce high titer of xylanases under solid state fermentation [128]. *Penicillium duponti (Talaromyces thermophilus)* was first isolated from compost in Japan [129]. *P. duponti* produces β-hydrolases with interesting features for industrial use. The organism also produces thermostable xylanase, α-L-arabinofuranosidase, and β-xylosidase.

5 Production of (Hemi)cellulases by Solid State Fermentation

Solid state fermentation refers to a fermentation process using moist solid substrates in the absence or near absence of free water. The growth of filamentous fungi during solid state fermentation very much resembles its natural way of life in terrestrial environments. Solid state fermentation is an important technology for solving energy crisis and environmental pollution [130]. A wide variety of low-cost or no-cost solid residues of agro-industrial origin can be used to cultivate fungi under appropriate

conditions for production of plant cell wall-degrading enzymes like cellulases and hemicellulases. So far submerged fermentation (SmF) has been a dominating technology for industrial production of many hydrolytic enzymes along with (hemi) cellulases. However, solid state fermentation has many superior and attractive features over submerged or liquid fermentation. SSF is strongly recommended for (hemi)cellulase production as higher titer and higher volumetric productivity are achieved as compared to submerged fermentation [131–133]. Recent studies have shown that better product formation in SSF as compared to SmF is considered to be part of the different physiology displayed by fungi [134]. Enzyme production by SSF is less prone to substrate inhibition and catabolite repression. Also the fermentation time may be shorter, and degradation of enzymes by proteases is minimized during SSF [131]. There are reports on better temperature and pH stability of enzymes also as compared to the enzymes produced by SmF. A distinctive advantage of SSF in cellulase production is the possibility of using mixed cultures, thereby exploiting metabolic synergisms among various fungi which ultimately leads to better composition of enzymes. It has been observed that the microbial behavior (growth and metabolic profile) in SSF and SmF changes significantly. Li et al. [135] compared the cellulase production by *Neurospora sitophila* in SSF and SmF using steam-exploded wheat straw as carbon source. Compared to SmF, not only the CMCase, FPA and β-glucosidase activities in SSF were higher (53–181 times), but the culture also produced β-xylosidase exclusively in SSF. The authors concluded that SSF provides near natural growth conditions for the fungus and facilitates more enzyme secretion.

Studies have also proven that cost of enzymes can be greatly reduced when SSF was used [136]. Cost reduction is achieved as a result of cheaper substrates, lesser requirements for sterility, lesser cost of downstream processing (because crude enzyme solution is concentrated), and lesser energy expenditure as compared to submerged fermentation. The process also generates lower volumes of effluents as compared to submerged fermentation. SSF proves to be an efficient technology for (hemi)cellulases to be used for bioconversion/biorefinery of lignocellulosic biomass or other technical applications where concentrated crude product can be directly used without purification.

In spite of many attractive features of SSF, there are some critical problems associated with the use of SSF at commercial scale. The major constraints faced are poor heat and mass transfer, heterogeneity of substrate, difficulties in control of the process parameters, and problems in the scale-up of SSF processes. As a result of these constraints, reproducibility of the process is very less which leads to significant risk of batch variations [132].

At laboratory scale, many studies have been conducted to produce cellulases and hemicellulases using filamentous fungi. Tables 5 and 6 show, respectively, the cellulase and hemicellulase yields under SSF on suitable substrates by various fungi.

Enzyme production by solid state fermentation is strongly governed by various physicochemical parameters which include substrate, inducers, nitrogen sources, moisture content, aeration, mixing, temperature, and pH. It is very much necessary to understand the impact of each factor on growth and enzyme production in order to develop an optimized process for maximum production of desired enzymes.

Table 5 Production of cellulolytic enzymes under solid state fermentation by fungi

Organism	Substrate	Enzyme activities (U g^{-1})			References
		FPase	Endoglucanase	β-Glucosidase	
Trichoderma reesei	Sugarcane bagasse	5.3	9.1	22.6	[137]
Trichoderma reesei ZU 02	Corncob residue	158	NA	NA	[138]
Trichoderma reesei	Sawdust	5.66	0.775	NA	[139]
Trichoderma reesei + *Aspergillus niger*	Wheat bran	22.8	NA	NA	[140]
Aspergillus niger	Soybean bran	5.6	6.5	152.0	[141]
Aspergillus terreus	Corncob	243	581	128	[142]
Aspergillus terreus	Rice straw	10.96	134	55	[143]
Thermoascus aurantiacus	Wheat straw	4.4	48.8	987	[144]
Penicillium decumbens	Wheat straw/bran	17.7	52.8	NA	[145]
Penicillium echinulatum 9A0251	Wheat bran and pretreated sugarcane bagasse	32.89	58.95	282.36	[146]
Fusarium oxysporum	Corn stover	304	ND	0.140	[147]
Myceliophthora sp.	Rice straw	2.44	7.48	32.9	[148]
Pleurotus dryinus	Wheat straw	41.0	NA	401	[149]
Fomitopsis	Wheat bran	3.492	53.679	71.699	[150]

Table 6 Production of hemicellulolytic enzymes under solid state fermentation by fungi

Enzymes	Cultures	Substrate	Enzyme yield	References
Xylanase	*Aspergillus foetidus* MTCC 4898	Wheat bran	8000 U g^{-1}	[151]
Xylanase	*Trichoderma reesei* QM 9414	Wheat bran	1000 U g^{-1}	[152]
β-Xylosidase	*Aspergillus tamarii*	Wheat bran	600 U g^{-1}	[153]
α-L-Arabinofuranosidase	*Aspergillus niger* ADH-11	Wheat bran	22 U g^{-1}	[154]
α-L-Arabinofuranosidase	*Thermoascus aurantiacus*	Sugar beet pulp	1083.3 $nkatg^{-1}$	[155]
Feruloyl/*p*-coumaroyl esterase	*Aspergillus niger* I-1472	Sugar beet pulp	19.5 $nkatg^{-1}$	[156]
Feruloyl/*p*-coumaroyl esterase	*Sclerotium rolfsii*	Locust bean Guar gum Copra	2591 nkat g^{-1} 2236 nkat g^{-1} 1921 nkat g^{-1}	[157]
β-Mannosidase	*Aspergillus oryzae*	Copra	19.4 U mg^{-1}	[158]
β-Mannosidase	*Aspergillus niger* FTCC 5003	Palm kernel cake	2117.89 U g^{-1}	[159]

5.1 Physicochemical Parameters Influencing Solid State Fermentation

5.1.1 Choice of Substrate

Choice of suitable substrate is a very crucial aspect in SSF. The solid substrate is not only the source of nutrients and inducers, but it also serves as an anchorage for the microbial growth. The fungi grow in mycelial form where both aerial and substrate penetrating hyphae are produced. Cellulase production by SSF has been attempted over a wide range of solid substrates which include various agroresidues, animal wastes, food processing wastes, and even nutrient-impregnated inert support materials such as vermiculite. The composition of enzyme complex produced by the fungus depends on the chemical composition and complexity of substrates. Generally for (hemi)cellulase production, substrate containing accessible inducer along with low level of free monomeric sugars is preferred [160]. Generally herbaceous and woody substrates are the two major categories of substrates used for large-scale fungal solid state fermentation. Herbaceous substrates like wheat straw, rice straw, corn stalks, etc. are many times more preferred as they are abundantly available as well as cheaper. Woody substrates like eucalyptus wood chips may change with age and hence reproducibility may be a problem. Moreover, woody biomass generally has higher content of lignin, and hence accessibility of cellulose and hemicellulose is lesser which may have negative impact on growth and enzyme production by fungi [161].

Wheat bran is considered as the universal substrate because it acts as a complete nutritious feed for microorganisms having all the ingredients and remains loose even

under moist conditions providing a large surface area [162]. Biochemical characterization of wheat bran indicates that it contains predominantly non-starch carbohydrate polymers (~58 %), starch (~19 %), and crude protein (~18 %), the non-starch carbohydrate polymers being arabinoxylans (~70 %), cellulose (~24 %), and β-(1,3)(1,4)-glucan (~6 %) [163].

Apart from chemical composition, physical properties of substrate like crystallinity, particle size, surface area, porosity, water absorption, and tendency to agglomerate when moistened are also important aspects to be considered in selection of substrates for solid state fermentation. Xylanase production was significantly affected by variations in size (0.125–0.8 mm) of oil palm trunk by *Aspergillus fumigatus* [164].

Owing to the recalcitrant and crystalline structure of lignocellulosic substrate which might impede the accessibility of microorganisms to the cellulose and hemicellulose portion of the substrate, pretreatment is often viewed as a means to alter the originally complex and recalcitrant chemical structure of lignocellulosic substrate. However, it is not found to be a prerequisite for cellulase production as well as xylanase production, and many times rather better yield of enzymes have been reported on untreated substrates [165]. High accessibility of the substrate may lead to quick degradation of polysaccharides, and concurrent release of monomeric sugars would lead to a repression of enzyme synthesis in some microorganisms.

5.1.2 Inoculum

(Hemi)cellulase production by filamentous fungi is strongly influenced by inoculum size. At low dose of inoculum, colonization of fungi on solid lignocellulosic substrate may require a longer time and thereby increase the risk of contamination. Hence, normally high ratio of inoculum is desirable especially to prevent contaminants which may allow SSF process at low standards of sterility. On the other hand, at higher inoculum size, rate of nutrient depletion is very high which may adversely affect growth and enzyme production. Therefore, optimization of inoculum size is a crucial step in developing SSF process. Various methods of inoculation have been adopted for filamentous fungi. Generally for sporulating fungi, the use of spore suspension as inoculum was commonly employed. However, mycelial suspension, mycelia disk, and pre-inoculated substrates as inoculum are found beneficial for different fungi. For preparation of spore suspension, fungal biomass/growth on the solid media is macerated using suitable sterile liquid. A high inoculum density ($\geq 10^6$ spore/ml) can be adjusted to achieve the desired spore count. One of the drawbacks of spore inoculums is that fungal growth may be delayed due to longer lag phase. For basidiomycete and ascomycete fungi, mycelia disk is prepared by cutting agar plug from the periphery of actively growing fungi. This is a more convenient method but might not be advisable for comparing enzyme production by different fungi. Mycelia suspension is often preferred for sporeless white rot and brown rot fungi in SSF. Lag phase can be reduced, but method is tedious and time-consuming.

Pre-inoculated substrate as the inoculum has also been preferred by some researchers [166]. In this method, inoculum size is difficult to determine, and comparison of the performance of the different fungi for cellulase production is

not reliable. On the other hand, it is advantageous that substrate after SSF can be blended with fresh inoculum and can be used to inoculate fresh batch of the substrate. In recent years a novel method was developed specially for SSF called cellophane film culture (CFC) [164]. In this technique agar medium is overlaid with cellophane film for easy separation of fungal biomass. It has been claimed that colonization of fungi occurs quickly when CFC is inoculated into solid substrate. Moreover, this method has lower risk of contamination during inoculum preparation as compared to the spore and mycelial suspension.

5.1.3 Moisture Content

Moisture content of the solid medium influences the vegetative growth, sporulation, spore germination, as well as enzyme production and enzyme activity. Filamentous fungi can generally grow at lower water activity levels than bacteria or yeasts. They optimally grow in the range of 0.87–0.80 a_w. The lowest water activity at which molds are capable of growing is about 0.6. In terms of moisture content, it is 10–20 %. Hence, it was concluded that SSF can be possible in the range of 10–20 to 80 % moisture content [167]. It has been reported that water activity levels required for growth are lower than those required for metabolite formation. The moisture level at which free moisture occurs varies considerably among substrates. It depends on water-binding characteristics of substrates. The porosity and specific area of the solid particle govern the water holding capacity of the substrate. So the amount of liquid required is directly related to the moisture (water activity, a_w) requirement of the organism and structure of the lignocellulosic substrate. If moisture content is too low, the solubility of nutrient is limited which hinders the nutrient uptake by the fungi, and if moisture content is too high, the particles may get agglomerated which limits the air diffusion. Several studies have been conducted to find out optimum initial moisture content of SSF processes for cellulase and xylanase production. Generally optimum moisture levels are found in the range of 35 % to 90 % for (hemi)cellulase production [168]. However, according to the type of solid substrate and the organism, it may vary. Singhania et al. [169] reported maximum cellulase (FPase) production by *T. reesei* Rut-C30 at initial moisture content of between 37 % and 38 % on wheat bran as a substrate. However, Latifian et al. [170] reported 55–70 % moisture content optimum for cellulase (FPase) production by *T reesei* QM9414 and MCG77.

Looking at various studies, it can be understood that the optimal moisture content used for SSF depends not only on the solid substrate and the microorganism but also on the other process conditions like temperature and provision for aeration, air flow rate, relative humidity of the atmosphere, and type of bioreactor system. Therefore, optimization of the optimum operational conditions for (hemi)cellulolytic enzyme production using SSF requires an integrated study of the abovementioned variables.

5.1.4 Moistening Agents

The amounts of nitrogen and/or essential nutrients are often too low in lignocellulosic substrates to support good growth and enzyme production. Hence, liquid media formulation for fermentation is of significant concern for optimum growth and

cellulase production. Also the media used are mostly specific for the organism concerned. In *T. reesei*, a basal medium like Mandels and Reese [171] or Mandel and Weber [172] or Mandels and Sternberg (MS) [102] has been most frequently used with or without modifications. Fermentation media used by most of the researchers for cellulase production by SSF consist of suitable nitrogen source, phosphorus source, and other minerals. Cellulose in lignocellulosic substrate acts as essential carbon source with a role of inducer for cellulase production. In addition to that, suitable nitrogen source can stimulate the production of all the components in a complete cellulase system. Peptone was most commonly used to enhance cellulase production in different lignocellulosic substrates. Type of suitable nitrogen source may differ with different lignocellulosic substrates because of the interactive effects with the lignocellulosic substrate. Therefore, it is essential to have proper combination of nitrogen source, lignocellulosic substrate, and fungal strain for maximum production of cellulase via SSF. In case of nutritionally rich substrates like wheat bran, only distilled water may also be used as moistening agent [173]. Furthermore, enzyme production can be enhanced by adding surfactant such as Tween 80 of Triton X-100 in the fermentation media. These nonionic surfactants allow faster secretion of (hemi)cellulases by increasing permeability of fungal cell membrane [174].

The cost of cellulase production can be reduced by replacing fermentation media with liquid waste or by-products of industry. Some attempts have been made to use diluted anaerobically treated distillery spent wash, and very high xylanase yield was achieved using *Aspergillus foetidus* MTCC 4898 [151]. Similarly, cellulase production was also reported using *Aspergillus ellipticus* strain [175]. Haapala et al. [176] reported the use of industrial by-product-based medium containing spent grains and whey for xylanase and endoglucanase production. Shah et al. [133] also attempted xylanase production using cheese whey as moistening agent by *A. foetidus* MTCC 4898.

5.1.5 Temperature and pH

Like any biological process, temperature is one of the strong factors affecting enzyme production by SSF. Because of the aerobic and exothermic fungal growth on moist substrates, control of temperature is a critical aspect in this technology. As SSF is normally carried out in the absence of free water and in static conditions, it is difficult to remove heat generated by metabolism due to poor thermal conductivity of the solid substrate and the low thermal capacity of air [177]. This problem can be even more serious during scale-up of SSF processes. Temperature also has great influence on time of incubation during solid state fermentation. Many studies have shown influence of temperature on cellulase production. Jecu [178] studied the effect of temperature on endoglucanase production by an *A. niger* strain, using wheat bran and wheat straw as substrates. It was noticed that variations in temperature between 25 °C and 37 °C did not strongly affect enzyme production. Optimum endoglucanase production was achieved between 28 °C and 34 °C. (Hemi)cellulase production has been largely carried out using mesophilic fungi of the class ascomycetes and basidiomycetes in the temperature range of 25–40 °C [168]. However, many laboratory-scale SSF processes using thermotolerant and thermophilic fungi

are reported at higher temperature. A thermotolerant strain of *A. terreus* was reported to produce cellulases and hemicellulases at 45 °C using rice straw as a substrate under solid state fermentation [143]. Another thermotolerant strain *A. fumigatus* gave maximum exoglucanase production at 55 °C [179].

Initial pH during fermentation is another important parameter which regulates the growth and metabolism of fungi. Generally, initial pH of fermentation medium is adjusted, but variations are likely to occur during the course of SSF. A decrease in pH has been observed in some processes because of the excessive production of organic acids and consumption of ammonium salt in the fermentation medium. Similarly increase in pH may also be observed due to utilization of organic acids. Normally, changes in pH are resisted by the buffering properties of the lignocellulosic biomass, and pH is normally not a controlled parameter during SSF. In addition to fungal growth and metabolism, enzyme activity is also very sensitive to pH. For ascomycetes and basidiomycetes fungi, initial pH of 5.0 is preferable [168]. Several studies have shown the importance of pH on cellulase and xylanase production. It was observed that in the range of 3.66–5.34, only a small effect on cellulase activity occurred during SSF using coculture of *T. reesei* and *A. oryzae* [180]. But Zhang et al. [181] reported that pH had a significant effect on cellulose production by *A. niger* with an optimum initial culture pH of around 4.6. Such studies show the need for instrumentation to monitor and control the pH.

5.1.6 Aeration and Mixing

Since cellulase- and hemicellulase-producing fungi are mostly aerobic in nature, aeration is a very critical factor. In addition to provide sufficient oxygen, aeration rate also influences other physicochemical parameters of the process. Aeration has many functions: maintaining aerobic conditions, removing CO_2, dissipating heat (regulating the temperature of the medium), distributing water vapor (regulating humidity), and distributing volatile compound produced during metabolism. The aeration rate depends on the porosity of the medium, and pO_2 and pCO_2 should be optimized for each type of medium, microorganism, and process [182]. Since thermal conductivity of solid medium is poor, there is a severe problem of heat removal and buildup of thermal gradient in the substrate which may adversely affect fungal growth and activity. One of the limitations of SSF is the ability to remove excess heat generated by metabolism by microorganism due to low thermal conductivity of the solid medium. In practice, SSF requires more aeration for heat dissipation than as a source of oxygen [183]. Therefore, the rate of aeration has been integrated with the control of temperature and moisture content by evaporative cooling water [184]. Mo et al. [145] found that forced aeration had a positive effect on cellulase production by *P. decumbens* cultivated under SSF. Cellulase production increased with increasing air flow rate up to a certain point and then decreased with further increase in air flow rate. At higher air flow rates, loss of moisture from the substrate adversely affected the growth of the microorganisms. Farinas et al. [185] found that endoglucanase production could be increased by using forced aeration, instead of static conditions, when *A. niger* was cultivated under SSF using a column-type instrumented lab-scale bioreactor. Pirota et al. [186] studied xylanase production by

a strain of *A. oryzae* P6B2 cultivated under SSF using an instrumented lab-scale bioreactor and found a substantial positive effect of controlled forced aeration, compared to static conditions.

Mixing is an additional control parameter used in connection with aeration. Mixing of the fermenting mass has beneficial effects like provision of new surface to aeration, distribution of inoculum, promotion of homogeneity and growth on individual particles of the substrate, and prevention of aggregate formation and of localized change [187]. However, mixing may disrupt shear-sensitive mycelial morphology of filamentous fungi and also the contact of mycelia to solid substrates which may eventually lead to decrease in product yields.

5.2 Optimization of (Hemi)cellulase Production Under SSF

Optimization can be carried out by changing one variable in a particular range and keeping other parameters constant. But in view of the large number of factors affecting SSF, it is quite laborious. Moreover, strong interactive effects may also be possible which may not be revealed. Hence, statistical optimization method like response surface methodology (RSM) is widely used for optimizing physicochemical parameters affecting SSF for (hemi)cellulase production. By employing such optimization methods, significant improvement in the yield of enzymes can be obtained. Table 7 shows some of the attempts for optimization of (hemi)cellulase production using statistical methods and the fold increase in the enzyme yield.

5.3 Coculturing of Fungal Strains

Improvement in cellulase production can be achieved via coculture of suitable and compatible fungi. In nature also multiple fungi coexist in various habitats and symbiotically degrade and utilize such polymers of solid substrates [131]. During cocultivation of different fungi, some of the individual enzyme activities may show synergistic increase. It also offers many advantages such as higher specific activity, increased adaptability to changing conditions, better substrate utilization, greater overall growth, and increased resistance to contamination by unwanted microbes as compared to pure monoculture [192]. It has been proven by many studies that the enzyme system produced by coculturing different fungi could complement each other and form a complete cellulase system that is favorable for hydrolysis of cellulosic substrate. However, when the fungi are cocultured for cellulase production, care should be taken in selection of fungi. The strains used should not compete with each other and should not cause any significant negative effect on growth of each other. Additionally timings of inoculation should also be determined for successful cocultivation [193].

Coculturing has been successfully employed for production of cellulases. Improved titers of β-glucosidase were reported by the cultivation of *Aspergillus ellipticus* and *Aspergillus fumigatus* [194]. The coculture of *Trichoderma viride* and

Table 7 Optimization of solid state fermentation processes for cellulases and hemicellulases

Enzyme	Organisms	Substrate	RSM design	Optimized parameters	Yield	Fold increase in production	References
Cellulases	*Scytalidium thermophilum*	Rice straw/ wheat bran (1:3)	BBD	$(NH4)_2SO_4$ 0.39 %, pH 5.75, and inoculum 2 ml	Endo-glucanase 23 U g^{-1}, FPase 3.0 U g^{-1}, β-glucosidase 151 U g^{-1}	2.12-fold β-glucosidase and 2.14-fold FPase	[188]
Cellulases	*Aspergillus terreus*	Wheat straw	BBD	Substrate 5 g, moisture ratio 1:679 (w/v), inoculum size 1×10^5 spores ml^{-1}, and pH 5.	10.96 U g^{-1}FPase	1.5-fold	[143]
Cellulases	*Trichoderma reesei* RUT C30	Sugarcane bagasse	BBD	Temperature 33 °C, time 67 h, inducer (cellulose hydrolysate) 0.331 ml g^{-1}	25.6 U g^{-1}FPase	4.7-fold	[189]
Xylanase	*Thermoascus aurantiacus*	Sugarcane bagasse	CCD	81 % initial moisture, 17 g bagasse, time 10 days, and inoculums 10^4 spore g^{-1} substrate	2700 U g^{-1}	–	[190]
Xylanase	*Aspergillus niger* XY-1	Wheat bran	PBD and CCD	Urea 41.63 g L^{-1}, Na_2CO_3 2.64 g L^{-1}, $MgSO_4$ 10.68 g L^{-1}, time 48 h	14637 U g^{-1}	–	[191]
β-Xylosidase	*Aspergillus niger* FTCC 5003	Palm kernel cake	CCD	Temperature 32.3 °C, initial moisture 59.7 %, and aeration rate 0.5 L min^{-1} after 7 days aerated pack bed bioreactor	6.13 U g^{-1}	–	[159]

(*continued*)

Table 7 (continued)

Enzyme	Organisms	Substrate	RSM design	Optimized parameters	Yield	Fold increase in production	References
α-L-Arabinofuranosidase	*Aspergillus niger* ADH-11	Wheat bran	PBD and BBD	$MgSO_4$ 1.59, urea 2.55, $CaCl_2$ 1.55, and ammonium sulfate 5 g L^{-1} at pH 5.5 and fermentation time of 180 h	22.14 U g^{-1}	2.34-fold	[154]
β-Mannanase	*Aspergillus niger* FTCC 5003	Palm kernel cake	CCFD	Incubation temperature 32 °C, initial moisture content 59 %, and aeration rate 0.5 L min^{-1} after 7 days aerated pack bed bioreactor	2231.26 U g^{-1}	–	[159]
Mannanase	*Scytalidium thermophilum*	Rice straw/ wheat bran (1:3)	BBD	$(NH4)_2SO_4$ 0.39 %, pH 5.75, and an inoculum 2 ml	4.9 U g^{-1}	1.95-fold	[188]

BBD Box-Behnken design, *PBD* Plackett-Burman design, *CCD* central composite design, *CCFD* central composite face-centered design

Ganoderma lucidum has shown positive results in exoglucanase and β-glucosidase production [195]. Hu et al. [194] reported that β-glucosidase and cellobiohydrolase activities were enhanced when *Phanerochaete chrysosporium* was cocultured with *Aspergillus* sp. on wheat bran. In another study by Kalyani et al. [196], it was shown that the deficiency of β-glucosidase in the cellulase system of *Sistotema brinkmannii* caused the accumulation of cellobiose, and this accumulated cellobiose served as a strong inducer for β-glucosidase production by the cocultured strain, *Agaricus arvensis*. As a result of cocultivation, 2.3–3.0-fold rise in filter paper activity was observed.

5.4 Bioreactors for Production of (Hemi)cellulases

Cellulase production by SSF has mainly been confined to laboratory scale. However, limited attempts have been done using bioreactors for large-scale production. The most commonly used bioreactors at lab scale are tray-type bioreactor, packed bed bioreactor or column-type bioreactor, horizontal rotary drum bioreactor, and fluidized bed reactor [197, 198]. Selection of an appropriate type of bioreactor is a very crucial aspect of any fermentation.

5.4.1 Tray-Type Bioreactor

Tray fermentation is one of the simplest approaches for SSF. Multiple trays containing substrate are incubated in a temperature-controlled humidity chambers with a circulation of moist air. The height of the substrate is a very important factor in tray fermentation to overcome heat and mass transfer problems. Since mixing is not required, the energy requirement becomes low (Fig. 2a). The tray-type bioreactors have been widely employed at both laboratory and commercial scale for the production of β-glucosidase, FPase, and xylanase by SSF with mono- and cocultivation of fungal cultures [199–201]. M/s. Alltech uses tray fermentation for the production of cellulases and xylanases for application in improvement of chicken broiler feed. The tray fermentation has some disadvantages also. The process needs large number of trays and large room for installation of multiple trays. Moreover, it is labor intensive. Double dynamic system air pressure pulsation and internal circulation of air can effectively control temperature and remove excess heat [168].

5.4.2 Packed Bed-Type Reactor

Another most commonly employed SSF bioreactor configuration is the packed bed bioreactor (Fig. 2b). It is also known as column-type bioreactor. Typically, the packed bed-type reactor consists of long and thin columns where the solid substrate is placed over a perforated plate and air is blown from the bottom and is discharged from the top. This kind of reactor is commonly used where mixing is not desirable. Temperature during fermentation can be controlled by providing water jackets or heat transfer plates. Many researchers have tried packed bed reactor for the production of endoglucanase and xylanase by SSF [185, 202, 203]. Scale-up of packed bed

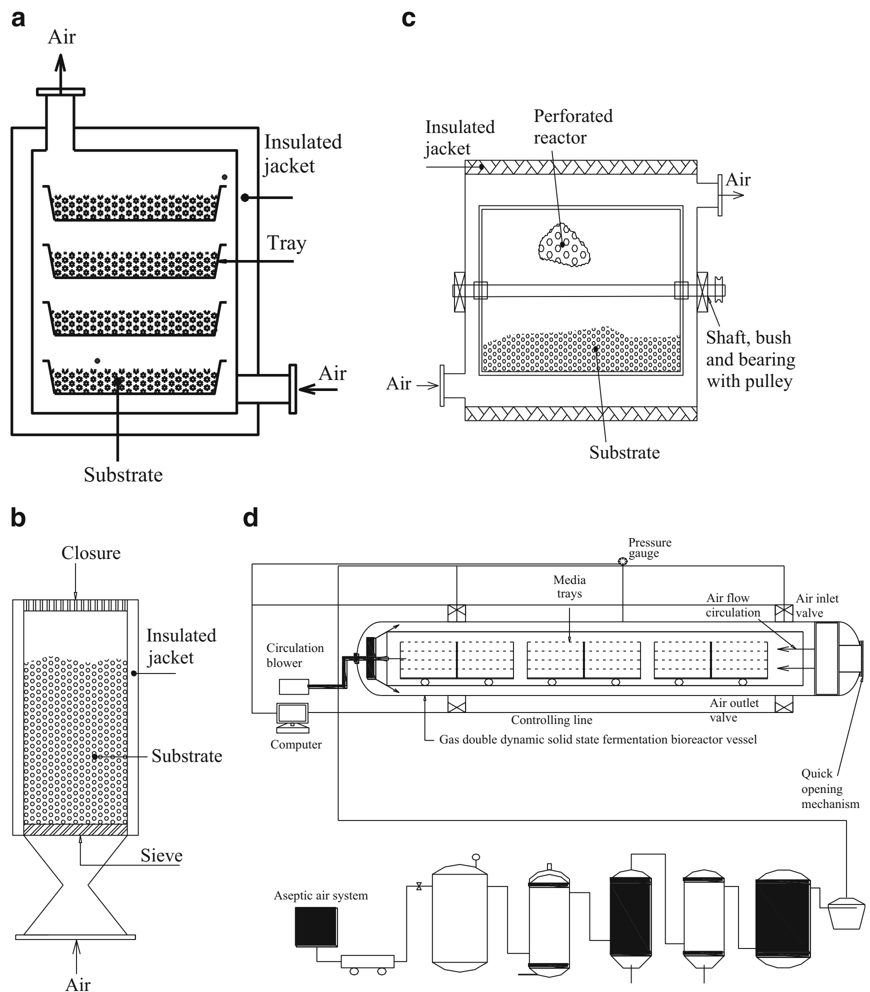

Fig. 2 Schematic of different types of bioreactors for SSF. (**a**) Tray-type of bioreactor. (**b**) Packed bed-type reactor. (**c**) Rotating drum-type bioreactor. (**d**) Gas double dynamic solid state fermentation

reactor is still not feasible because of the problem of heat removal, nonuniform growth of microorganism, pressure drop, etc.

5.4.3 Rotating Drum-Type Bioreactor

The reactor consists of a horizontal or inclined drum with or without baffles, which rotates along its axis slowly to mix the contents inside the reactor (Fig. 2c). During the rotation process, small particles form groups of knots, which affect the heat and mass transfer in the entire fermentation process. This kind of problem can be overcome by employing rotating drum bioreactor with baffles or paddles.

Diaz et al. [204] tried 10 L capacity horizontal bioreactor for cellulase and hemicellulase production by the thermophilic fungus *Thermoascus aurantiacus* under SSF. Alam et al. [205] evaluated the production of cellulases by *Trichoderma harzianum* T2008 from palm oil biomass as the main substrate and reported higher cellulase activities by employing horizontal rotary bioreactor (50 L capacity). Panagiotou et al. [147] also reported higher (hemi)cellulolytic enzyme activities from corn stover by *Fusarium oxysporum*. Commercial enzyme-producing companies, namely, Biocon and Lyven, use agitated solid state systems based on the rotating drum-type reactor with temperature and humidity controllers [206].

5.4.4 Gas Double Dynamic Solid State Fermenter

New gas double dynamic solid state fermenter was developed and reported by Zeng and Chen [207] (Fig. 2d). It is characterized by internal air circulation and periodic pulsation of air pressure. Its main purpose is to strengthen internal heat transfer. At different fermentation stages, the rate of air circulation in the bioreactor changes according to the speed of metabolic heat production. Thus, this reactor not only takes care of oxygen transfer but also improves heat transfer. The shortcomings of traditional solid state fermentation such as heat and mass transfer problems can be easily overcome by employing gas double dynamic solid state fermentation. Gas double dynamic solid state fermentation process was used for the production of cellulases by *Penicillium decumbens* JUA 10 in a 50 L fermenter and observed that the yield of cellulases was almost doubled than the static SSF [208]. Similar observation was made by Zeng and Chen [207], where feruloyl esterase production was higher in 25 L capacity fermenter. In both studies time was also reduced.

In addition to the above mentioned reactors, fluidized bed bioreactor and gas-solid fluidized bed bioreactors have also been developed for enzyme production. However, these reactors have not been used for extensive research in the aspect of cellulase production. A bioreactor named “Fermostat” has been developed for cellulase and xylanase production by *Aspergillus niger* USMAI 1 under SSF by controlling the temperature and agitation as well as by controlling the inlet and outlet of inoculum and substrate [206]. It can be seen from the published reports that, the production of cellulases and hemicellulases using different types of SSF bioreactors is quite encouraging, but at the same time, all types of reactors suffer from some kind of engineering limitation. However, studies concerning the novel bioreactor designs for large-scale production of cellulase and hemicellulases with advanced control mechanism remain a great challenge in order to achieve significant advancement in the application of this technology.

6 Improvement of (Hemi)cellulases by Genetic Approaches

Cellulases have been successfully used since long in cotton processing, in paper recycling, in detergent formulations, and in food as well as in animal feed processing. However, their use in bioconversion of cellulosic biomass to bioethanol and other value-added chemicals is limited by problems of their lesser efficiency on

many feed stocks and higher costs. Although extensive work has been done on the improvement of cost and enzyme yield by modifications of fermentation technology, some of the improvements related to synthesis of robust (hemi)cellulases and novel mixtures of cellulases and hemicellulases are possible only through genetic modifications of fungal strains and enzyme engineering by rational designs and directed evolution.

Various genetic tools have been used to improve cellulases and their production by fungi. The most common approach is random mutagenesis by chemical agents or ultraviolet light. Random mutagenesis is often limited by genetic makeup of microbes. With respect to *Trichoderma reesei*, it is now believed that this technique has reached its limit, and now for further improvements, precise genetic engineering approaches are needed [103]. Site-directed mutagenesis has been successful to add desirable characteristics to enzymes. Recently Novozymes has developed a versatile enzyme cocktail with increased catalytic activity and thermostability by introducing improved CBH II and β-glucosidase to *Trichoderma* enzyme mixture [209].

Attempts have been made to clone potent cellulase genes from different fungi and expressed in *Trichoderma* and *Aspergillus* to get efficient synergistic mixtures. CBH 1 promoter of *Trichoderma reesei* is a highly efficient promoter with unusually high rate of expression under cellulase induction condition. This promoter has been used to express BGL and EG [210, 211]. Cellulase system of many fungi including *Trichoderma reesei* is deficient in BGL and sensitive to feedback inhibition by glucose. Considering this aspect, attempts were made to increase the copy number of BGL gene and introduction of glucose tolerant BGL gene in *Trichoderma reesei* [210, 212]. Glucose repression was also addressed by using truncated CBH I promoter lacking binding sites for the carbon catabolite repressor CRE 1 [213]. Another strategy employed for improvement in cellulase production is by using promoters that are insensitive to glucose repression [214].

One of the major bottlenecks in enzymatic hydrolysis of biomass is inactivation of cellulases by lignin. To overcome this problem, six amino acids were replaced in *Trichoderma reesei* Cel6A, and its resistance was improved by 15 % (Lys-129 to Glu-129, Ser-186 to Thr-186, Ala-322 to Asp-3, Gln-363 to Glu-363, Ser-413 to Pro-413, and Arg-410 to Gln-410) [215]. The same enzyme was altered by a single change in amino acid (Ser-413-Pro) which increased optimal temperature by +5.6^{o}C, alkalophilicity by +1.25 pH units, and thermostability by 11.25 times as compared to original cellulase [216]. Similarly endoglucanase 5 from *Humicola insolens* was engineered to resist surfactants at alkaline pH for application in detergent [217]. Two single (Ala-162-Pro; Lys-166-Glu) and one double mutant (Ala-162-ProLys-166-Glu) were generated. These protein variants on an average showed 3.7 times higher activities when compared to the original cellulase [217].

Like cellulases extensive efforts have been carried out to improve xylanases also in order to meet the industrial requirements of titer, substrate specificity, thermostability, alkalophilicity, enantioselectivity, stereospecificity, and tolerance to toxic reagents. Improved expression of *Trichoderma reesei* M2C38 endo-xylanase was done by creating a new *N*-glycosylation site in the coding sequence by amino acid replacements. Introducing Asn at position 131 in association with Thr/Ser at position

133, a conserved feature for family 11 xylanases created an *N*-glycosylation site Asn-Xaa-Thr/Ser. The Asn 131 variant showed 40 % enhanced protein expression in comparison with wild type [218]. In another attempt, *Trichoderma reesei* endo-xylanase II was engineered to improve its alkaliphilicity and thermostability for applications in paper and pulp industries by three strategies, viz., (1) the native amino acids at positions 10, 27, and 89 were replaced with histidine, methionine, and leucine, respectively; (2) the N-terminal amino acid sequence was replaced by the N-terminal sequence of *Thermomonospora fusca*; and (3) the N-terminal amino acid sequence was added a tripeptide of glycine–arginine–arginine or ten extra amino acids from N-terminus of *Clostridium acetobutylicum* xynB. All the three strategies increased the thermophilicity of the enzyme from 55 °C to 75 °C and the alkalophilicity from pH 7.5 to 9.0 [219].

Above-cited studies clearly show the potential of genetic approaches for improvements in cellulase and xylanases which can improve their suitability and performance in various applications,

7 Current Industrial Applications of Cellulases and Hemicellulases

Applications of enzymes in industrial processes and synthesis of high value compounds started since long. Technical enzymes are in great demand especially for detergents, starch, textile, leather, pulp and paper, and personal care industries. Next important group of enzymes are food enzymes which are used in dairy, brewing, juice, fats and oils, and baking industries. In animal feed industry, microbial enzymes are also in significant demand. In 2012, the latest estimation of the global market for industrial enzymes grew by 7 % to reach 3.75 billions in US dollars [220].

Commercial cellulases and xylanases are industrially produced by mainly *Trichoderma* sp., *Aspergillus niger*, and *Humicola insolens* [221]. Cellulases are the third largest group of industrial enzymes. Cellulases and hemicellulases have been used for number of technical applications as well as for food and animal feed processing industries. Textile industry is one of the first sectors to benefit from cellulases. Cellulases can catalyze selective removal of impurities and modifications of physicochemical properties of textile fibers. Commercial enzymes, namely, Cellusoft AP, and Cellusoft CR from Novozyme are available in the market for bioblasting in textile mills, whereas Denimax 6011 for bio-stoning. Many enzyme manufacturers like Ab enzymes, Finland (Rohament CL/CEP); Yakult Co. Ltd., Japan (Onozuka series); Amano enzymes, Japan (Cellulase T/AP); and Quest Int. USA (Biocellulase TRI) have developed commercial cellulase products for food industries like baking and brewing. Novozyme, a leading manufacturer in cellulase market, is supplying Carezyme and Celluclean for laundry detergent. In the pulp and paper industry, microbial enzymes have to be efficient under various operating conditions since it is emerging as one of the potential large markets for enzyme application. Cellulases and hemicellulases have been employed for biochemical pulping, de-inking of recycled fibers, and improving drainage and run ability of

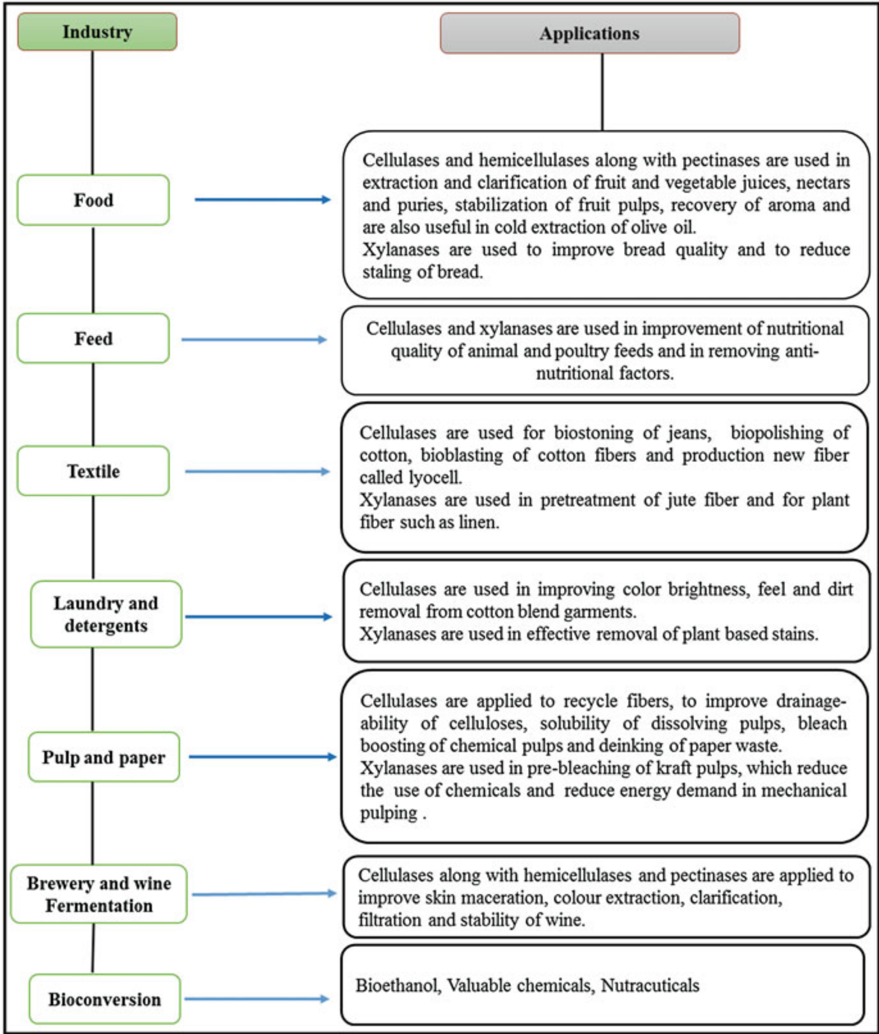

Fig. 3 An overview of various current applications of cellulases and hemicellulases

paper mills. Cellulase-free xylanases are also required for pre-bleaching of kraft pulps [222]. Apart from all these applications, the use of cellulases and hemicellulases in bioconversion of plant biomass into valuable products especially as bioenergy sources, food additives, some pharmaceuticals, and nutraceutical products is of immense interest. The overview of various current applications of cellulases and hemicellulases is shown in Fig. 3. Detailed chart of bioconversion of lignocellulosic biomass into valuable products is given in Fig. 4.

Bioconversion of lignocellulosic biomass into bioethanol is being extensively studied, and so far the technology has not gained commercial status. The major

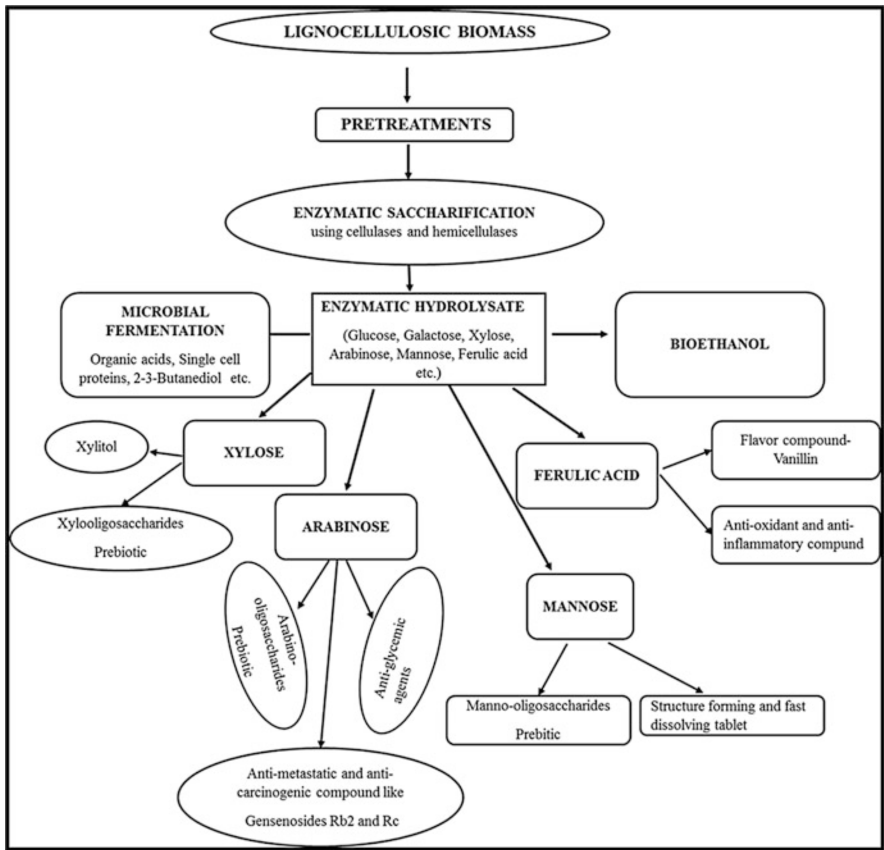

Fig. 4 Detailed chart of bioconversion for lignocellulosic biomass into valuable products

bottleneck in the technology is the cost and effectiveness of available commercial enzymes. Moreover, each biomass needs tailor-made enzyme cocktail because of differences in physicochemical structure [88]. Enzymes for biomass conversion could be a blend or enzyme cocktail containing endo- and exo-cellulase, xylanase, β-glucosidase, pectinase, etc. which could vary for different biomass on the basis of their composition. Enzyme cocktails can also be developed by mixing commercial cellulases with indigenously produced auxiliary enzymes including xylanases, β-xylosidase, α-L-arabinofuranosidases, pectinases, β-glucosidases, etc. for efficient and cost-effective hydrolysis of various feedstocks [223]. The use of hemicellulases and other auxiliary enzymes, in conjunction with cellulolytic enzymes, can improve cellulose conversion by removing hemicellulose and increasing the access of cellulases to the substrate. Bioethanol technology from plant biomass can be economically feasible if monomeric sugars from cellulose as well as hemicelluloses are utilized. Hence, highly balanced cocktails of cellulases and hemicellulases are in great demand [142].

Currently, there are number of programs in many countries which focus on production of biofuels such as biogas, bioethanol, biodiesel, and fuel cells from renewable sources [224]. It has been estimated that the global fuel ethanol demand could grow to exceed 125 billion liters by 2020 [225]. At present, companies such as Danisco-Genencor, Novozymes, and Dyadic are producing and marketing cellulase and hemicellulase preparations for biomass conversion.

8 Conclusion

Cellulases and hemicellulases are widely distributed among saprophytic, wood-rotting, and plant-pathogenic terrestrial fungi. Compared to bacteria, fungi are rich sources of multiple plant cell wall-degrading enzymes including several accessory debranching enzymes, and as a result, they are more efficient in complete degradation of celluloses and hemicelluloses. Fungal (hemi)cellulases can replace many pollution-prone industrial processes and thereby help in generating sustainable environment. The demand of (hemi)cellulases is increasing especially in the emerging biofuel industry, which has accelerated research and development for effective and economical methods to produce cellulases on large scale. Looking at the large number of laboratory-scale (hemi)cellulase production attempts and comparison with submerged processes, it seems that solid state fermentation holds the key to production of these enzymes in high titer, in high volumetric productivity, and still at lower production cost. However, production of (hemi)cellulases by SSF at commercial scale is highly challenging because of inherent problems in scale-up and poor reproducibility of the system. Still much more attention is required to be paid in searching for more potent (hemi)cellulolytic fungal strains producing balanced ratio of cellulases and hemicellulases, optimum process conditions, and suitable bioreactors with advanced control mechanisms.

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Biosynthesis of Nanoparticles by Fungi: Large-Scale Production

12

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Abstract

Nanoparticles are structures in nanoscale with a wide range of applications across various fields of technology, industry, environment, medicine, and science. Increasing demands for NPs caused to develop their production based on chemical and physical approaches, recently. These approaches

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carry health and environmental disadvantages with themselves. Need for safer alternatives in large-scale production of NPs ended up with development of eco-friendly methods. Industrial nanobiotechnology takes advantage of biological-based approaches to produce nanomaterial using biological renewable resources. Decreasing energy intake, greenhouse gas (GHG), and hazardous waste production are the main advantages of nanomaterial biosynthesis. In contrast, the other synthesis methods bring environmental drawbacks. Among the nanomaterials, nanoparticles have attracted the attention because of their wide spectrum of application. Microorganisms and in particular bacteria and fungi are used as the biological agents and showed a promising potential for biosynthesis of nanoparticles. Here we highlight different aspects of industrial production of NPs by fungi including advantages and disadvantages. Also, we discuss the application of different technologies in development of high-scale production of NPs by fungi-like protein engineering, metabolic engineering, synthetic biology, systems biology, and downstream processing.

Keywords

Nanoparticles • Fungi • Nanotechnology • Nanobiotechnology • Biotechnology • High-scale production

List of Abbreviations

CNS diseases	Central nervous system disease
GHG	Greenhouse gas
NP	Nanoparticle

1 Introduction

Nowadays, high-scale production of nanomaterial is an unavoidable requirement of our society. The chemical methods of nanoparticle production have their own intrinsic drawbacks for human health and environment. This is a consequence of using industrial process by means of toxic substances and high amount of energy. The requirement of high energy has been fulfilled by fossil fuels burning. Its direct consequents are GHGs that are dramatically increasing from the beginning of industrial revolution. Therefore, new approaches are needed to produce the nanomaterials in high scale while reducing the health risk issues and environmental downsides. The sustainable growth of industrial production requires methods efficient in market and economy. There are promising alternatives to avoid these challenges in large-scale production of nanomaterials. Industrial nanobiotechnology uses biotechnology and biological-based process for high-scale production of nanomaterials.

2 Nanotechnology

The term nanotechnology refers to production and study of material in the 1–100 nm scale [1, 2]. Because of variation in physical characteristics, material with the nanometric scale exhibits different properties from their original properties [3, 4]. The possibility to have various size- and shape-dependent properties for different materials provides a unique opportunity for scientists to develop new form of material with activity in wide-spectrum fields of science and technology. Nanoparticles are a wide sector of nanomaterials owning new structure and properties (Fig. 1) with extensive application in different aspects of our life such as science, technology, medicine, industry, and environment [5, 6]. Diversity of different

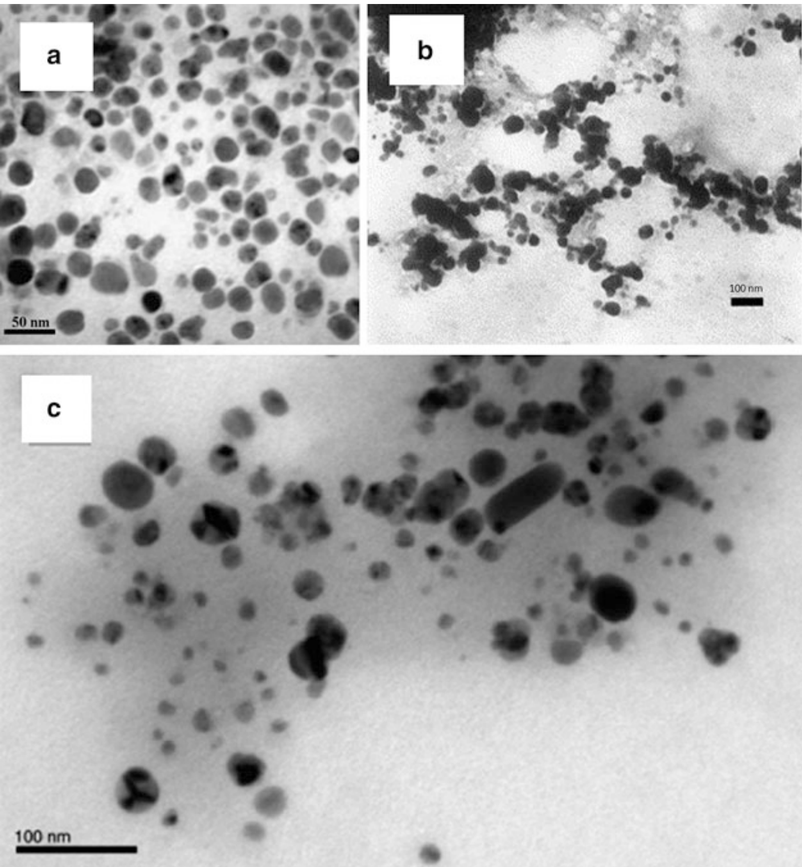

Fig. 1 Nanoparticle biosynthesis by *Trichoderma reesei* [23], *Fusarium oxysporum* [24], and *Trichoderma viride* [25]

physical and chemical properties has attracted the attention toward nanoparticle production more than other sectors of nanomaterial [7]. At the moment, some nanomaterial productions have reached to the industrial scale and their number is growing with their developing applications. Nanoparticles are a promising sector of nanomaterial that have been forwarded to industrial production because of their significance and efficiency in various aspects of our life [8]. Growing interest in different nanoparticles highlights the requirement of safe and efficient procedures for their high-scale production.

3 Nanoparticles

NPs' physical and chemical properties are different from their bulk material due to their nanoscale structure. Various types of NPs can be differentiated by their material content.

Organic NPs (liposomes, polymeric, micelles, and solid lipids) mostly are biodegradable and compatible with biologic systems with low toxicity rate [9, 10]. Liposome NPs (20–100 nm) are made of two phospholipid layers [11]. A polymeric structure is the main body of polymeric NPs [12]. Organic NPs are suitable for delivery of hydrophilic and hydrophobic molecules such as drugs. Liposome NPs have been mainly used as antimicrobials such as Ambisome® [10]. Polymeric NPs such as polylactic-co-glycolic acid have been approved by FDA as drug delivery systems [13].

Inorganic NPs (1–100 nm) are based on different inorganic oxides and exhibit variation in morphology and chemical properties like solubility [14]. Synthesis of inorganic NPs such as metallic NPs performs via reduction of the salt mediated by reducing molecules such as biopolymers [15]. Control of NPs' chemical and physical properties is possible via modification of their synthesis condition like temperature, pH, reaction duration, and reducing molecules [16]. Higher loading capacity and smaller size of metal NPs make them better option for distributing drugs in human body [14]. But they have weaknesses like aggregation and accumulation over the time and heterogeneity of the size and shape [17, 18]. Also, their excretion from human body is a time consuming process [18].

NPs based on biopolymers such alginate, albumin, or chitosan are more compatible with human body as drug delivery system because of their low immunogenicity in comparison with synthetic polymers [19]. Biological-based synthesis of NPs is not only important from economy and environmental point of view; also it affects the NPs' biocompatibility in pharmacy and medical application. It is critical for drug delivery purposes to use a biocompatible reducing molecule because it plays a binding role between drug and NPs [20]. Also, some biomolecules have synergistic effects on other important properties of NPs like cytotoxicity and antimicrobial and controlled release of drugs [21].

Nevertheless, some NPs like heavy metal NPs exhibit toxic properties for human body. The level of metal NP toxicity is different based on the metal ion composition;

in some cases, they can be absorbed and excreted through normal physiological process [22].

3.1 NP Applications

Nowadays, by advancement in nanotechnology it is possible to detect and cure infections and diseases more precisely, rapidly, and with lower side effects. This is feasible via development of complicated techniques that assemble drug and diagnostic packages on nanostructures and target specific tissue and cells. Application of polymeric NPs and nanocapsules results in higher availability of different therapeutic molecules [26]. Infections resistance against traditional antibiotics is conquered by antmicrobial nanoparticles that newly developed [10]. Nanotech-based drugs can fulfill the human requirements not only for more powerful and unbreakable antimicrobial agents but also for cell- and tissue-directed drug packages in very low doses. This provides new opportunity for the healthcare system to control the growth of multidrug-resistant pathogens and parasites. It also gives us the unique opportunity to cure cancer cells with very toxic medicines without severe side effects on patients.

Silver NPs (AgNPs) are famous for their antimicrobial properties and have been extensively used in this area [27]. These NPS are applied in medical devices, dental implements, bone cement, cancer treatment, and imaging as well. In addition to silver NPs, most drug molecules, DNA, and iRNA can bind to the gold NPs (AuNPs) because of its highly specific surface [28].

There are other inorganic NPs like Pt, Al, Zn, Ti, Pa, Fe, Cd, Si, and Cu, which have been used as delivery agents to target specific tissue. Inorganic NP ceramics (porous Si, Al, and Ti NPs) have been widely used in drug delivery because of their high capacity as drugs carrier. Also, silica nanoceramics are very good candidates for medical and therapeutic applications because of their biocompatible nature [29]. Cu NPs are a good option to reduce agglomeration and oxidation in the presence of stabilizer. Also, Cu NPs are cheap in price, easy to prepare, and exhibit antimicrobial activity. The homeostasis challenges with this NP are less because of human Cu transporters [30]. Fe NPs with magnetic activity showed good ability for rapid and precise diagnosis of microbial infection [20]. Zn and Cd are normally used in the production of quantum dots to be used in optic-related products [31].

Besides the medical, food, and pharmaceutical applications, noble metal NPs like Au and Ag are applied in different areas of science in recent years such as electron microscopy, analysis, biosensors, electronics, dyes, conductive coatings, optic, electronic catalysis, and basic research [32].

Seventy-six percent of nanotechnology researches have been invested in NP application in life science and pharmacy that reach to billions of dollars [29]. Unlike traditional drugs, NP-based drugs have benefits like higher treatment efficacy and less health drawbacks. It has been documented that NPs are effective in therapy of different diseases such as oncology, infections, CNS diseases, cardiovascular, Alzheimer, and ocular pathologies [27].

3.2 Metal NP Synthesis

Nanoparticles can be produced by two general top-down (etching, milling, sputtering, laser ablation, lithography, thermal decomposition) and bottom-up (green synthesis, spinning, pyrolysis, sol–gel processes, supercritical fluid synthesis, chemical vapor deposition) approaches (Fig. 5). Bottom-up approaches have proven the higher efficiency and precision [23]. Among different bottom-up procedures, chemical- and physical-based methods like lithography, laser ablation, aerosol, radiolysis, and photochemical reduction have been known as expensive, high energy consuming, and hazardous for health and ecosystem [33, 34]. Chemical methods have high efficiency relative to physical ones but involve some toxic substances like dispersants, surfactants, or chelating agents for stabilizing nanoparticles. These substances are mainly toxic and pollutants; therefore, the chemical methods are not good for high-scale production [35]. Our critical situation forces us to design environmental-friendly production methods that are efficient in expenses, energy, GHG emission, and pollutant waste production [36]. This has been a challenging step for sustainable production of nanomaterials in large scales without high costs and toxic waste production [7, 33]. Recently, biology-based synthesis of NPs has attracted attentions because of their efficiency, low health, and environmental drawbacks plus high output. In this approach, an organism or a biomolecule plays a main role in the synthesis of NPs. Biosynthesis of NPs is a promising alternative for chemical methods, which carry ecological consequences [37], because it uses biocompatible and nontoxic solvents, agents, and stabilizers [38, 39]. Although biosynthesis of NPs is environmentally safer than chemical methods, it has not industrially ideally developed [40, 41].

4 Industrial Nanobiotechnology

In recent decades, industrial biotechnology could show a significant potential in reduction of CO_2 and other GHG emissions using renewable resources. Its outcomes are compatible with environment and do not result in accumulation of pollutions in ecosystem. Furthermore, it has the potential of remediation of other pollution in long term. In industrial biotechnology, biomass input is used under the process of biological agents like microbes and biomolecules to create a wide spectrum of products. [42]. There is a global interest to push the production of different nanomaterials on biotechnological lines because of its powerful tools for modification, improvement, and development of feedstock, biological agents, and products. Industrial nanobiotechnology is the application of different biological-based procedures in large-scale production of nanomaterials. It takes advantage of different biotechnology and bioinformatics tools to facilitate and improve production of interested materials.

4.1 Biotechnology Manipulation Platform

Protein engineering plays an important role in modification and adaptation of natural form of proteins using rational design and directed evolution. This optimization is possible via engineering of protein performance, selectivity, thermal and solvent stability, enantioselectivity, and substrate/product inhibition [43]. Metabolic engineering is another powerful tool to control and modify the cellular transportation of enzymes and their expression level, readdressing metabolic flux and gene regulation [44]. It is normally effective for overproduction of valuable metabolites that are hard to extract like anticancer metabolite, taxol, with US$1 billion market. A simple manipulation in the taxol competitive regulation pathways leads to enhancement of the metabolite yield up to 40-fold in *Taxus brevifolia* [45]. Likewise, synthetic biology can end up in production of new metabolites by redesigning of regulatory networks [46]. Synthetic biology creates artificial organisms by assembly of de novo created genome into a biological frame like genome-free cell [47]. It is also able to take advantage of unnatural amino acids in combination with expanded genetic code to form new molecular properties such as catalytic and binding activities with unique properties. This provides us a powerful method to create and design new regulatory networks in different levels of genome, transcriptome, proteome, and transductome [48]. Its combination with metabolic and protein engineering opens new pathways with novel products in higher level of outcomes [49, 50] as it has been done by introducing synthetic artemisinic acid (a precursor of antimalarial drug) pathway into the *Escherichia coli* [51]. Systems biology integrates the genomics, transcriptomics, proteomics, and metabolomics data to draw comprehensive picture of complex cellular process and regulatory networks. This helps to understand, monitor, and simulate cellular and molecular regulatory networks during biotechnology projects [52]. For instance, a systems biology approach enabled scientists to increase lysine production up to 40 % in *Corynebacterium glutamicum* [53].

Combination of different biotechnology tools such as genome sequencing; genetic, protein, and metabolite engineering; synthetic biology; and systems biology has suitably served to increase total performance of different microbes in industrial biotechnology [54, 55]. More investment on this area helps to overcome on the critical challenges like global warming. Since CO_2 emission has been the main threat among GHG increase, it is possible to imagine that industrial biotechnology will be able to reduce atmospheric CO_2 in long term because it has low energy consumption unlike other industrial methods [56]. Microbes are main agents of industrial biotechnology that facilitate the conversion of different feedstock to desired products as part of their growth process. Microbial-based high-scale production of compounds gives us almost endless choices with ecological and economic advantages. Various microorganisms exhibit different abilities in industrial biotechnology. Fungi have shown great potential in this area. Bacteria normally need complicated and expensive media for optimal high-scale production [57]. Unlike bacteria, fungi are easy to use and require simple and cheap media for high-scale cultivation (Chap. 19, "▶ Aspergillus Lipases: Biotechnological and Industrial Application"). Also fungi

surface and submerse cultures in large scale are possible with simple and large bioreactors [58]. Normally, agricultural wastes and biomasses from different sources can be used directly as fungi feedstock to get valuable proteins and metabolites as their products.

4.2 Fungal Biotechnology

Fungi are easy, flexible, tolerant, and economic biologic system for industrial biotechnology and have been used extensively in high-scale production of different metabolites (primary and secondary (see more)). Their tremendous ability in secretion of proteins up to100g/L, metabolic diversity, and high production capacity have made them unique option for industrial biotechnology for decades [59, 60]. Moreover, the ability of running posttranslational modification implemented in fungi by genetic and protein engineering turns them in cell factories of overproduction of engineered proteins. Some species like *Trichoderma reesei* and *Aspergillus niger* have extensively been used in different industrial and medical and food sectors [60–62]. Fungi also have proved that they are the trustable candidates to produce succinic acid (SA), alternative molecule to replace petroleum, from cheap and renewable row materials. SA plays a main role as the building block in biodegradable polymers. It has been shown that some fungi like *Fusarium, Aspergillus,* and *Penicillium* species can produce SA in high scale [57].

Aspergillus spp. is very useful in industrial biotechnology for its valuable proteins and organic acids such as citric and itaconic acid. Extraction of citric acid from other sources like citrus fruits and bacteria is more expensive in comparison with *A. niger*-based production. Also diversity of metabolites can be controlled over variation of *Aspergillus* strains [63, 64].

1600 different antibiotics along with various medical drugs are synthesized by fungi at present (see more). Anticholesterol statins are another famous example of drug production by fungi such as pravastatin (*Nocardia autotrophica*), lovastatin (*Aspergillus terreus*), and mevastatin (*Hypomyces*, *Paecilomyces*, *Trichoderma*, *Penicillium citrinum*) (see more). Other fungal species are involved in biosynthesis of steroids (*Rhizopus nigricans)* and immunosuppressant cyclosporins used in organ transplanting (*Trichoderma, Tolypocladium* and *Cylindrocarpon*) (see more). *Ashbya gossypii* fungus naturally carries the ability of high-scale production of the vitamin riboflavin, which along with its small haploid genome turns this plant pathogenic fungi to an important industrial biotechnology option [65] (see more).

Fungi abilities for the enzymatic cellulose alteration in plant cell walls make their industrial cultivation economically efficient (see more). Also, same enzyme activity has other applications like fiber treatment and modification like cotton alteration by fungi catalases. In addition, enzymes like cellulases and xylanases from *Trichoderma* are used in fabric and leather industries. Biological bleaching of xylemn in pulp and paper industry by peroxidase and xylanase enzymes from Trametes and Phanerochaete fungi is safe and economic alternative for chemical

bleaching. Fungal-based synthesis of vitamin B2 has taken over its chemical synthesis process over 25 years ago [66].

The extensive application of fungi in industrial biotechnology has made them best options for large-scale bioprocessing and production of organic products such as protein, polysaccharides, lipids, metabolites, pigments, and organic acids. Since the biosynthesis of nanomaterial is the safest economic approach for high-scale nanotechnology, it is convenient to use fungi as the most efficient industrial biotechnology agents, to meet competent industrial nanobiotechnology. In this case, the natural potential of fungi and the enormous diversity of their outcomes will be ideally effective by having the possibility of fungi manipulation. This provides us a binary tool to shift the fungi toward the desired biological platform on the one hand and on the other hand to adapt and simplify the industrial complicated process upon the fungi growth condition. This brings highest efficiency of industrial production of nanoparticles in parallel to lowest health and environmental drawbacks.

5 Biosynthesis of Metal NPs

During biosynthesis of NPs, reduction of precursor (mainly metal salts ion) by reducing agents (a biomolecule or a biological process) normally results in accumulation of reduced ions and formation of NPs. Therefore, the condition of ion reduction strongly affects the size, shape, and stability of NPs. This is the main key factor to control different properties of NPs. Because of the biotechnology abilities, modification in precursor and reducing agent or their interaction condition provides almost unlimited toolbox for control of NP characteristics, production rats, and also waste minimization [67–69]. Ions reduction occurring by transfer of electrons from biomass which contain polysaccharides, proteins (such as reductive enzymes), natural polymers that carry enormous hydroxyl and other functional groups [5, 70]. This material and functional groups are frequently available in raw material of fungi, bacteria, or plant cultivations.

Fungi are commonly used in the biosynthesis of inorganic NPs in comparison to bacteria because of higher output and their easy handling [40, 41]. Besides nontoxic feedstock like agricultural row material, the wastes of NP biosynthesis are media and biomass of the fungi that are biodegradable and simply can be used as organic fertilizers. Recovery of NPs from fungi media is very simple by pure water washing [23, 71]. In contrast, the chemical methods use toxic solvents like 1,2 hexadecanediol, oleylamine, phenyl ether [72], 1-hexadecene, octyl ether, 1-octadecene, 1-eicosene, and trioctylamine [73] to recover NPs. This produces NPs with hydrophobic surfaces that should be converted to hydrophilic by applying extra steps [37].

5.1 Biosynthesis of Nanoparticles by Fungi

70000 of fungi species have been identified among a potential number of fungi species that have been estimated up to 1.5 million. This population contains

enormous diversity of biological, physiological, and molecular properties [74]. They mainly feed on small molecules resulted from biodegradation of complicated organic resources by enzymatic activities [74]. Fungi have been well known for biosynthesis of NPs and more specifically for metal NPs [75]. They are able to grow in a thin layer of material and produce a huge amount of extracellular enzymes. This makes them best candidates as industrial agents for enzyme and metabolite production [76]. High atom economy, simplicity of biomass applications, considerable wall-binding, intracellular metal absorption, and easy propagation along with their fast growth rate are other advantages of fungi as green choice for NP large-scale production [77]. High atom economy refers to maximum yield relative to initial raw material used in the reaction.

In the fungal-based NP biosynthesis, a biomineralization process is done via reducing different metal ions by intracellular and extracellular enzymes and biomolecules [78].

Among different metals, silver has been more used for production and study of NPs. Also, Au, Ti, and Zn have been reported as the next more considerable metal ions used in biosynthesis of NPs by fungi (Fig. 2).

Fusarium, *Aspergillus*, *Trichoderma*, *Verticillium*, *Rhizopus,* and *Penicillium* species are the fungi with more studies in NP biosynthesis (Fig. 3). Most of the studied fungal species for NP production have been reported as pathogens of plants or human. This has been the main obstacle in large-scale biosynthesis of NPs by fungi [79]. In contrast to pathogenic fungi, *Trichoderma* species such as *T. reesei* have been more attractive for high-scale production of AgNPs. *T. reesei* is industrially adapted species with no report as a harmful fungus [23, 71, 79].

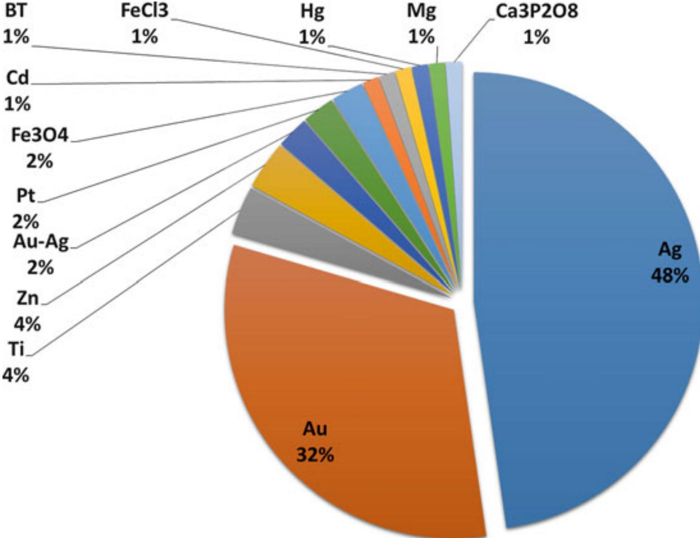

Fig. 2 Application of different metals in biosynthesis of NPs by fungi

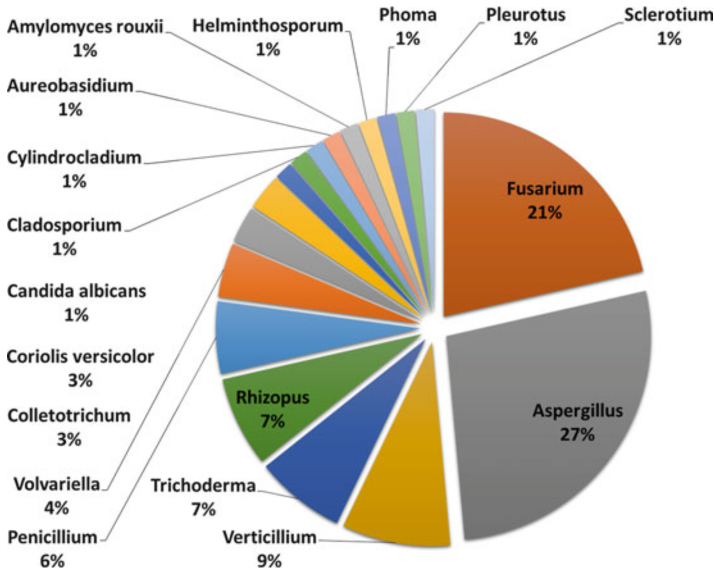

Fig. 3 Frequency of different fungi used for biosynthesis of NPs

Fungi produce NPs in a wide variation of shape and size ranging from >1 nm [79, 80]. Morphology of NPs has great effect on their properties and accordingly it can be reflected in the applications like antimicrobial, therapeutic, and drug delivery uses. This wide range of size and shape plays the role of useful toolbox to select the right species for high-scale production. Morphology of NPs produced by fungi can be very limited in the size and shape [81, 82] or it can be very diverse like AuNPs produced by *Verticillium luteoalbum* [83] and AgNPs produced by *Penicillium strain* [84].

Nanoparticles produced by fungi have been used for different purposes (Fig. 4) such as medicine, anticancer drug, antibiotic, antifungal, antimicrobial, antiviral against HIV, diagnostic, engineering, bioimaging, biosensor, agricultural, and industrial applications [79, 80]. The main applications of NPs have been referred to agricultural and medical applications, subsequently (Fig. 4).

5.2 Mechanism of NPs Biosynthesis

Fungi produce NPs as part of their defense response against environmental pollutions. They reduce different ion toxicities by precipitating, immobilization, ion form modification, co-precipitation, and coupling them to biological molecules [85, 86]. Reduction of ions results in precipitation of metals as nanomaterial in the intra- or extracellular spaces [87]. Human takes advantage of microbial response system against toxic environment to produce nanomaterial and to clean wastes and ecosystem via bioremediation process [88]. The key step in the biosynthesis of NPs by

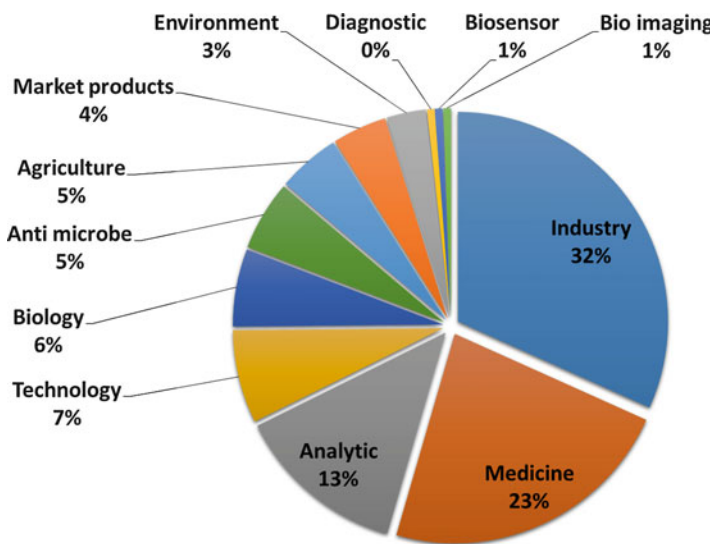

Fig. 4 Application of fungal-based NPs in different aspects of our life

fungi is exchanging electron from a donor molecule to the ion that results in ion precipitation as nanoparticle. The electron exchange can be done via biological process by fungi enzymatic system or by fungal-originated molecules. Different biological molecules have the potential of electron exchanging, for instance, polysaccharides, peptides, amino acids, vitamins, enzymes, alkaloids, flavonoids, saponins, steroids, tannins, carboxylic acids, quinones, and other secondary metabolites [80, 89]. Also, some enzymes like α-NADPH-dependent nitrate reductase, phytochelatin, and glutathione reductase FAD-dependent are able to reduce ions of toxic metal and produce nanoparticles from this reduced ions [90]. The biosynthesis is possible by direct contact of ions with fungi biomass [23, 75] or interaction of metal ion with biomass-free extracts [91] such as enzymes and other biomolecules secreted from fungi [92]. Important factors in controlling size and shape of NPs have been mentioned as fungi species, reducing biomolecules, reaction conditions, concentration of precursors, incubation time, and PH [83, 93]. As presented in Fig. 5, the application of fungi in biosynthesis of NPs is not only limited to their direct role in synthesis of NPs but also it includes all biological-based synthesis methods that use different fungi biomolecules such as peptides, organic acids, enzymes, and polysaccharides with high atom economy [94].

5.3 Advantages and Disadvantages

Important advantages for biosynthesis of nanoparticles are high output, biodegradable feedstock, low costs, simple procedure without hazardous wastes,

morphological reproducibility, application of safe material, low energy consumption, simple control in large-scale process, and easy recovery of NPs [37]. Unlike chemical process that needs high temperatures more than 250 °C [95, 96], biosynthesis of NPs is performed in biological reaction temperature <60 °C [37]. Biosynthesis of NPs is an unavoidable prerequisite specifically for their application in medical and pharmacy [97]. The general advantages in all biological-based sustainable processes are low waste generation, high atom economy [94], using safe process and substances [98], low energy consumption [99], using renewable feedstock [100, 101], using enzymes as powerful catalysts [102], no derivatives, and no need for postproduction treatment [103]. Furthermore, in biosynthesis of NPs, scaling up the process considerably reduces the price of product because feedstock and raw material are cheap, biodegradable, and safe. These advantages are somehow fitted to the major green chemistry principles [67].

Besides the general advantages of green synthesis of NPs (Fig. 5), specific advantages of fungal-based synthesis in high-scale production of NPs are dependent

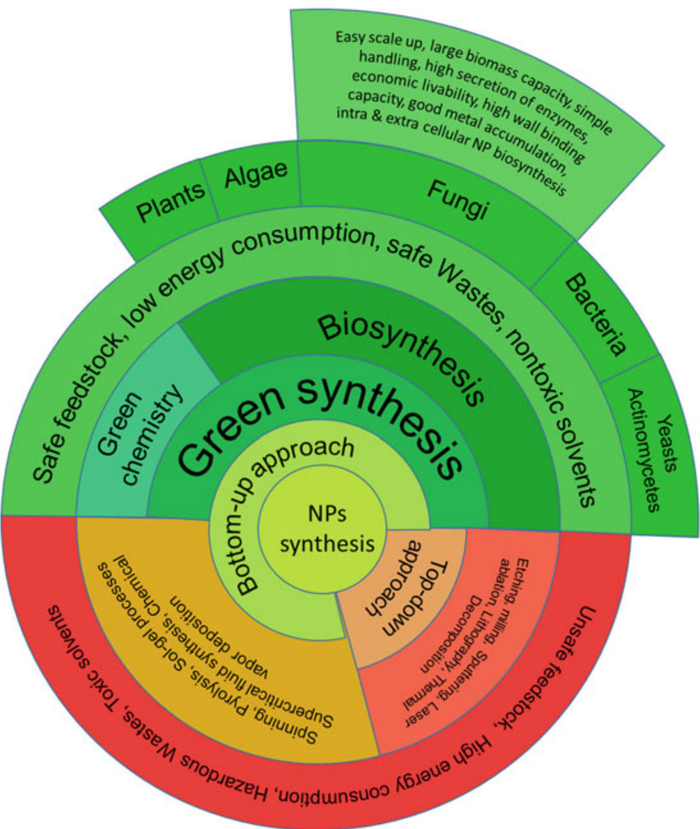

Fig. 5 An overview of different approaches for synthesis of NPs

on the NP composition, fungi strains, feedstock, and reaction condition. In biosynthesis of NPs by fungi or fungal-based material, there is no need for toxic agents during NP recovery and purification process [103–105]. Finally, industrial nanobiotechnology takes advantage of all-powerful tools in biotechnology to manipulate the protein structure, gene regulation, and metabolic pathway for enhancing and improving NP production by fungi.

6 Conclusions

Application of NPs in different aspects creates a growing need for NP high-scale production. The sustainable production of NPs requires procedure with minimum pollution production and high efficiency in large-scale synthesis. Conventional methods of NP production inherit disadvantages like using toxic solvent and hazardous inputs, high energy consumption, producing toxic wastes, and pollution. Biological-based methods for synthesis of NPs offered new perspective in high-scale production with safer feedstock, less energy usage, and without harmful solvents and wastes. In industrial nanobiotechnology, different biological agents are involved in the production of nanoparticles like bacteria, fungi, and algae or their originated material in order to facilitate production of nanomaterial in high scale. It owns advantages like less energy consumption, using safe material and feedstock, plus biodegradable and safe outputs. Among different biological agents, fungi have emerged as favorable and flexible organisms to be used in high-scale production. Easy procedure, no expensive media and material, enormous production of biomass, high amount of output, and secretion of protein and metabolites are some advantages of fungal-based industrial nanobiotechnology over other methods. Fungi have been extensively used in the production of different NPs. The diversity of metabolites, ease of harvest, and flexibility of fungi growth condition in combination with different biotechnology tolls to improve and manage enzymes and metabolites provided almost a limitless platform for researchers. Fungal-originated NPs demonstrate the unique diversity of physical and chemical properties that in combination to their compatibility with human body makes them one of the best options for medical and therapeutic applications. Also, they have shown promising results for their antimicrobial and delivery activity. We have described the growing needs for high-scale production of NP and we discussed the abilities of fungi as one of the best agents for high-scale production of NPs. Finally, the success of fungi in large-scale production of NPs is correlated with other determining factors such as demand rate, feedstock prices, final price, global warming, international policies, company's competition, consumer choices, governmental investments, technology advancement, application developments, and our knowledge about NP consequences. As final remarks, it is important to mention that our knowledge about NP toxicity, metabolism, bioremediation, and their drawbacks in human body and environment is limited to a short period since NPs have been used. It has been documented that some NPs under certain circumstances exhibit problems like cytotoxicity, pollution, high surface charge, and reactive oxygen radical formation [106]. Increasing and

exchanging the paraclinical studies at the international scale will result in tracking NP consequences and control subsequent problems. This along with increasing financial investments and adjusting governmental policies for biological-based NP synthesis will help in sustainable development in NP large-scale biosynthesis.

7 Cross-References

► Aspergillus Lipases: Biotechnological and Industrial Application
► Cordycepin: A *Cordyceps* Metabolite With Promising Therapeutic Potential
► Lanostanoids From Fungi as Potential Medicinal Agents
► Solid-State Fermentation: Special Physiology of Fungi

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Analytical Techniques for Discovery of Bioactive Compounds from Marine Fungi 13

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Abstract

Marine fungi have been a rich source of bioactive natural products with interesting pharmaceutical activities and potential therapeutic applications. This chapter reviews the recent analytical techniques for discovery and the characterization of bioactive compounds derived from marine fungi, which are highly diversified and are less explored. An overview about bioprospecting, collection, preparation, and preservation of fungi samples are also presented, as well as different methods and strategies used for extraction, fractionation, and structural characterization of the bioactive compounds are discussed, including their advantages and the disadvantages. Possible roles of these natural compounds in several interesting biological activities are also covered in this chapter.

Keywords

Analytical methodologies • Fungus • Bioprospection • Preservation • Collection • Extraction • Fractionation • Chromatography • Bioactivity • Bioassay • Structural characterization • Online combination

1 Introduction

The oceans dominate the surface of the Earth and contain the greatest known biodiversity of life [1]. With the increase of oceans exploration, a growing number of bioactive natural products are being isolated from several marine organisms [2, 3]. The marine environment represents a rich source of both biological and chemical diversity [1]. Recent reports estimate hundreds of millions of marine species depicting over 90 % of total marine biomass containing unique molecules. For marine fungi, only about 465 species are referenced; however, it is estimated that there are 1.5 million species [4, 5]. Marine fungi provide a diverse and remarkable supply of promising bioactive molecules, often with interesting applications in medicine, such as penicillin, caspofungin, mevinolin, and fingolimod [6–8]. These compounds showed several biological properties, including antibacterial, antifungal, and immunomodulatory activities, as well as cholesterol synthesis inhibition [9–11]. Furthermore, in the health care area, studies revealed promising bioactive compounds (BC) isolated from marine fungi sources, with proven anticancer activity: penicisteroid A **(1)** is a new polyoxygenated steroid isolated from the *Penicillium chrysogenum* QEN-24S, obtained from a marine red algae. It showed a distinctive chemical structure with tetrahydroxy and C-16-acetoxy groups and exhibited potent cytotoxic activity against the tumor cell lines HeLa, SW1990, and NCI-H460 [12]. From fungus strain KT29 isolated from the red seaweed *Kappaphycus alvarezii*, one compound, named 2-carboxy-8-methoxy-naphthalene-1-ol **(2)**, was obtained and showed in vitro cytotoxicity against the human bladder carcinoma cell line 5637 [13]. Two other new alkaloids, 2-(3,3-dimethylprop-1-ene)-costaclavine **(3)** and 2-(3,3-dimethylprop-1-ene)-epicostaclavine **(4)**, were isolated from the marine-derived fungus *Aspergillus fumigatus*. Both compounds showed cytotoxicity against a mouse leukemia cell line (P388) [14].

The BCs are considered chemical compounds derived and isolated from biological sources. Lately, the characterization of the compositional, structural, and sequential features of BC has been the main focus. Structural information can be used to organize these compounds according to Schmitz's chemical classification into six major chemical classes, namely, alkaloids, peptides, polyketides, shikimates, sugars, and terpenes [1]. However, marine fungi have not been given the attention they deserve, and a very limited insight into the capabilities and bioactive potential of marine microorganisms is yet available in the scientific literature. There is still scope for more research to explore the potential of marine microorganisms as producers of novel drugs, which are naturally accepted by consumers unlike chemically synthesized drugs [15].

This chapter summarizes different methodologies used to isolate pure bioactive compounds. Different approaches for the collection and preservation of marine fungi samples are presented, as well as some possible techniques for extraction, fractionation, and structural characterization of bioactive compounds.

2 Prospection, Collection, and Preservation of Marine Fungi

2.1 Bioprospecting in Marine Fungi

Bioprospecting is the process of discovery and commercialization of new products based on biological resources [16]. It is the systematic search for and development of new sources of chemical compounds, genes, microorganisms, macroorganisms, and other valuable products from nature. Bioprospecting involves the incessant research for biochemical and genetic sources with high commercial value from nature resource and that have never been used in traditional medicine before [17, 18]. Thus, bioprospecting means looking for ways to commercialize biodiversity. Currently, the development on indigenous knowledge associated to the exploitation and administration of biological resources has also been incorporated into the concept of bioprospecting. Consequently, bioprospecting comprises the conservation and sustainable use of biological resources and the rights of indigenous and local populations [19, 20]. Bioprospecting, when well-managed, can be beneficial, leading to the development of new BC. In contrast, bioprospecting also can lead to environmental problems relating to unauthorized overexploration, as well as social and economic complications [20, 21]. Bioprospecting generates environmental disruption problems during the extraction procedure. Special attention must be given to ethical questions and conservation policies, i.e., research must honor host organizations regarding new discoveries, including new habitats of rare and endangered species [21, 22].

Marine bioprospecting has mainly focused on macroorganisms because of their easy availability, ease of capture, rich biodiversity, and a variety of unique molecules that they developed in response to hostile habitats and environmental conditions. However, a growing effort has increased the research and exploitation of the deep

ocean, mostly around hydrothermal vents, due to unexplored biodiversity developed in extreme conditions [23, 24].

Extreme environments such as Antarctica or the high seas, particularly the deep seabed, provide locales for "extremophiles," which are organisms with unique metabolic properties, which makes them promising and interesting sources of bioactive compounds [25–27]. The biological circumstances which enable these extremophiles to survive in extreme pressures, temperatures, pH, light, salinity, and other exceptional conditions are sources of new prospective for scientific exploration and commercial utilization [28–30]. However, tropical environments and shallow temperate have been the most explored and studied so far [24, 31]. Another challenge to bioprospecting in these habitats, as in all marine ecosystems, is to have access to a sufficient quantity of biological substances in order to get pure bioactive compounds [32, 33]. Bioprospecting of marine compounds commonly depends on collecting wild specimens [34]. Novel and less troublesome solutions can be found through bioprospecting the oceans, especially in mostly unexplored microscopic marine organisms. The combination of modern advances in DNA technologies and increased consciousness of environmental problems, such as global warming, have stimulated the science of marine microbiology [23, 35].

2.2 Collection of Marine Fungi

In order to have a full and undisputable natural compound assessment and to assure the properties discovered, different sampling strategies, depending on the type of habitat and the species ecology, have to be taken into account to ensure the appropriate collection of the mixed cultures from natural environments. Since the collection of microorganisms from the marine environment is not always easy, it is often necessary to also harvest the supporting materials in order to keep the fungi viable until arriving to the laboratory [36].

The biodiversity preservation should be taken into account, reducing the impacts as much as possible, in order to protect marine species from environment disorders during collection. Harvesting of organisms must be restricted to minimum quantities and attention with rare or endangered species must also be taken into account [37].

Distinct microorganisms can be found in different areas of the marine environment, such as (1) in free suspension in the water column; (2) in flocculated particulates in the water column; (3) in the sediment; (4) on surfaces of both living and nonliving bodies; and (5) in endophytic/symbiotic associations [36, 38]. The biggest risks concerning the collection of these organisms are contamination and cross-contamination. Thus, the most appropriate techniques rely on sampling the material together with supporting materials, which include water, sediment, portions of plants and other macroorganisms, or other subtracts [36]. In the case of water samples, in order to avoid contamination and proliferation of cultures, they must be collected with sterilized glass containers. For sediments from intertidal areas, sampling can be performed by removing core samples, while in subtidal regions, sediment grabs, such as Ponar and Van Veen grabs, can be used. Nevertheless, in more hostile

environments such as the deep sea, more refined procedures are essential, such as the system developed by Parkes et al. [39]. DeepIsoBUG system was developed for high-pressure systems, allowing the collection of cores and slices of the sediment, with each slice being moved to a low pressure container, thus minimizing the chance of contamination and keeping temperature and pressure [37, 39]. After the collection, preservation must be performed as quickly as possible to avoid genetic and phenotypic modifications of the cultures [36, 37]. It is also important to consider that wild harvest only partially satisfies the demand and is an unsuitable production way [34]. Using big quantities of biomass may have an impact on the number of specimens, and for rare species, it can be impossible to collect enough organisms for the research. A solution to these problems can be the aquaculture of target species, allowing the continuous production of biomass using standardized conditions [40]. When it comes to microorganisms and if the compounds are needed on a commercial scale, the fermentation also is a suitable process for bioactive compounds production [41]. Thus, bioactive compounds can only be obtained by collecting from natural sources, aquaculture, or synthesis [42].

2.3 Preservation of Marine Fungi

Conservation of marine specimens is a prerequisite for field studies in faraway zones or when there are restrictions to return the samples to the lab in due time. The harvesting of marine organisms for the study of BCs is frequently performed in open sea, where assays are often difficult or even impossible [43]. In the case of microorganisms, such as fungi, there often is a macro-host which must also be collected during sampling, avoiding sample degradation until arrival at the laboratory [44]. As most marine organisms are quite vulnerable to fast degradation, the samples should be quickly frozen with dry ice and stored at −20 °C as soon as possible until the next step in processing [45]. Once in the lab, the collected fungi need to be preserved pure and viable for additional studies. Usually, they are brought into pure culture and stored in liquid nitrogen in order to maintain the viability of these cultures, preventing genetic and phenotypic changes induced by repeated passages of the cultures [36, 38].

3 Preparation, Extraction, and Fractionation of Marine Fungi

3.1 Preparation of Bioactive Compounds from Fungi Samples

The bioactive compounds isolated from marine fungi samples must follow a multistep process. One of the first steps to be considered is the culture conditions, since marine fungi samples collected will be cultured in nutritional media at the laboratory. Therefore, special attention must be taken into account for the temperature, incubation time, aeration, media composition, and pH, since less favorable conditions can affect the output and yield of the wanted bioactive compound [46].

The next step includes the biological activity screening. The bioactivity assay is an essential parameter in the development of new drugs, usually conducted to measure the effects of a biopharmaceutical drug on a living organism. Extraction of active samples is the next step, followed by the fractionation, separation, and purification of pure compounds. Finally, it follows the structural characterization of the bioactive compounds [47].

Due to the existence of symbiotic relationships between organisms, some marine fungi may be isolated from other marine organisms, such as algae. In order to get BC, including 1-O-(α -D-mannopyranosyl)chlorogentisyl alcohol **(5)**, Yun et al. [48] isolated the fungi *Chrysosporium synchronum* from a brown algae *Sargassum ringgoldium*, which showed a radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) [48].

Before delineating the steps of the isolation methodology, the characteristics of the target compound, i.e., molecular size, charge, stability, solubility, and acid–base properties, should be regarded, since the selection of the best procedure for additional separation allows a faster isolation protocol [47, 49].

3.2 Extraction of Bioactive Compounds from Fungi Samples

The bioactive metabolites extracted from marine fungi can be divided into alkaloids, amino acids, polyketides, sugars, sterols, saponins, peptides, terpenoids, hydrocarbons, and fatty acids [50]. Since the chemical nature of bioactive substances in a mixture is unknown, it is not possible to delineate any specific technique for the separation of these components from the complex mixture. However, a wide separation of the mixture can be obtained by extraction with organic solvents. Other methodologies have also been developed to improve the isolation of BCs which include several extraction techniques. The most common extraction methodologies and the main BCs isolated reported in recent literature are discussed in the ensuing sections.

3.2.1 Extraction by Solvents

Bioactive compounds are generally extracted from mycelium and/or culture medium using a variety of aqueous or organic solvents. After sampling from marine habitat, fungi cultures are submitted to extraction using solvents with different polarities [36]. Examples of bioactive molecules and related solvents usually used for extraction are shown in Table 1.

The extracts of marine fungi showing biological activities could be a mixture of different molecules. Most marine fungi yield hydrophobic compounds, when extracted with organic solvent, such as ethanol (EtOH), methanol (MeOH), chloroform ($CHCl_3$), acetone, and ethyl acetate (EtOAc) [2, 51]. However, bioactive hydrophilic compounds can also be extracted from marine fungi using solvents, such as hexane and carbon tetrachloride [52]. New marine-derived compounds, named hypochromins A and B, were obtained from the *Hypocrea vinosa*, showing great tyrosine kinase inhibitory activity, when isolated from the ethanol extract [53].

Table 1 Bioactive compounds and some solvents usually used for their extraction

Class of bioactive compounds	Bioactive compounds	Solvents
Polar organic compounds	Alkaloids Amino acids Shikimates Polyhydroxysteroids Polyketides Saponins Sugars	Acetone Chloroform Ethanol Ethyl acetate Methanol *N*-butanol Water
Medium-polarity compounds	Peptides	Carbon tetrachloride Dichloromethane Methanol
Low-polarity compounds	Fatty acids Hydrocarbons Terpenes	Carbon tetrachloride Hexane

Fractions of different polarities are then submitted to biological assays. Sometimes, biological activity is spread across multiple fractions. However, if the isolation is good, the biological activity may be condensed on a single fraction, thus maximizing time and resources. In contrast, when ideal conditions are not respected and biochemical characteristics not investigated, low recoveries are obtained and additional extraction must be made in order to obtain the best association of extraction solvents to obtain better extraction purity [54]. Low processing cost and ease of operation are some of the advantages of using solvent extraction. In turn, the disadvantages are low extraction efficiency, low selectivity, and production of solvent residues [47, 54].

3.2.2 Extraction by Other Modern Methodologies

Due to the limitations presented by extraction with organic solvents, other methods are also applied in the separation of BC. Among them stand out microwave-assisted extraction (MAE); ultrasound-assisted extraction (UAE); supercritical fluid extraction (SFE); subcritical water extraction (SWE); and pressurized liquid extraction (PLE) which are fast and efficient unconventional extraction methods developed for extracting bioactive compounds from microorganisms [55].

MAE is based on the direct effect of microwaves on molecules of the extracted system caused by two mechanisms: ionic conduction and dipole rotation [56]. MAE heats the extracted system directly by friction between polar molecules, leading to very short extraction times. Intracellular heating of the matrix induces pressurized effects that damage cell walls and membranes, as well as cause electroporation effects. Consequently, a quicker transfer of the molecules from the cells into the extracting solvent is observed [56–58]. Polar solvents are better MAE extracts than nonpolar in the following order: water > methanol > ethanol > acetone > ethyl acetate > hexane [57].

UAE notably decreases isolation time and increases extraction efficiency of several natural compounds, due to the formation of cavitation bubbles in the solvent [55, 59]. This ability is influenced by the properties of ultrasound wave, the solvent

characteristics, and the ambient conditions. After a cavitation bubble is formed, it collapses throughout the compression cycle, which pushes the liquid molecules together, and a high-speed micro-jet is created towards the matrix particle, promoting the mixture of the solvent with the matrix. Temperature and high pressure involved in this procedure breaks membranes and cell walls. After cell damage, the solvent can easily penetrate into cells, releasing the intracellular contents [55].

SFE is another extraction method that yields extracts with none or less polar impurities than the traditional organic liquid extracts [60]. It is based on the use of a gas compressed at a pressure and temperature above a critical point, comprises a dense gas state in which the fluid combines hybrid properties of liquid and gas. The supercritical CO_2 has properties, such as high diffusivity to extract organic compounds, low viscosity, nonflammable, low cost, easily accessible, critical point conditions, decompression directly to the atmosphere, and harmless to the environment. In order to overcome limitations in the extraction of polar compounds, the addition of an organic modifier, such as ethanol, is recommended [61]. The greatest limitation of supercritical CO_2 is to not be adequate to extract polar compounds. Nevertheless, the addition of an organic solvent, such as EtOH or MeOH, can largely improve extraction yield [55].

SWE, also known as pressurized hot water extraction, besides using an environmentally friendly solvent also allows the adjustment of the dielectric constant of the water, and thus the solubility of organic substances, which allows the extraction of polar and medium-polar compounds [62]. Polar molecules with high solubility in water are extracted with more efficiency at lower temperatures, while medium-polar and nonpolar compounds need a less polar medium induced by higher temperature [63]. Based on the scientific results published in the last years, it has been demonstrated that the SWE is quicker, cheaper, and cleaner than the traditional extraction techniques [64].

PLE, also named accelerated solvent extraction, is a developing technology that uses very low volumes of liquid solvents such as acetone, ethanol, and hexane to retrieve target analytes in a short extraction time. This emerging technology combines both high pressures and temperatures to improve the solubility in the pressurized liquids and increase the desorption kinetics of compounds from the matrices [65].

3.3 Fraction of Bioactive Compounds from Fungi Samples

In order to obtain fractions of increasingly pure bioactive substances from a mixture of extract, the fractionation technique is used. The two main approaches to screening BCs from the extracts are the bioassay-guided fractionation and pure compound screening [66]. In the bioassay-guided fractionation procedure, it is possible to exclude the extracts and fractions that do not show bioactivity. In turn, pure compound screening is used less frequently than bioassay-guided fractionation, and it is necessary to select extracts containing compounds, which are not present in the available libraries of pure compounds, since bioactivity is only checked after isolation and structural elucidation [36, 66]. Table 2 shows the extraction and

Table 2 Extraction, Fractionation, and Bioactivities of Novel Bioactive Compounds from Marine Fungi

Marine fungi	Bioactive compounds	Extraction	Fractionation and purification	Bioactivity	References
Penicillium chrysogenum QEN-24S	Penicisteroid A ($C_{30}H_{50}O_6$) **(1)**	Ethyl acetate	Silica gel, Sephadex LH-20 and Lobar LiChroprep RP-18 column chromatography (MeOH).	Antifungal activity (against *Aspergillus niger*, *Alternaria brassicae*). Cytotoxicity activity (against tumor cell lines HeLa, SW1990, and NCI-H460).	[12]
Fungal strain (KT29) (isolated from macroalgae *Kappaphycus alvarezii*)	2-carboxy-8-methoxy-naphthalene-1-ol ($C_{12}H_{10}O_4$) **(2)**	Ethyl acetate	Sephadex LH-20 column chromatography (MeOH). TLC (silica gel).	Antibacterial activity (against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*). Antifungal activity (against *Candida maltose*). Cytotoxic activity (against the human bladder carcinoma cell line 5637).	[13]
Aspergillus fumigatus	2-(3,3-dimethylprop-1-ene)-costaclavine ($C_{21}H_{28}N_2$) **(3)**	Acetone–MeOH	Silica gel column chromatography (*n*-hexane–EtOAc). PTLC (dichloromethane- MeOH). HPLC (MeOH in H_2O).	Cytotoxicity activity (against mouse leukemia cell line (P388)).	[14]
	2-(3,3-dimethylprop-1-ene)-epicostaclavine ($C_{21}H_{28}N_2$) **(4)**				

(*continued*)

Table 2 (continued)

Marine fungi	Bioactive compounds	Extraction	Fractionation and purification	Bioactivity	References
Chrysosporium synchronum	1-*O*-(α -D-mannopyranosyl) chlorogentisyl alcohol ($C_{13}H_{17}ClO_8$) **(5)**	Ethyl acetate	Silica gel column chromatography (*n*-hexane–EtOAc). MPLC (H_2O–MeOH). HPLC (MeOH in H_2O).	Radical scavenging activity (against DPPH).	[48]
Myceliophthora lutea	Isoacremine D ($C_{12}H_{14}O_3$) **(6)**	Ethyl acetate and dichloromethane	Silica gel (hexane–EtOAc) and Sephadex LH-20 ($CHCl_3$) column chromatography. TLC (silica gel).	Antimicrobial activity (against *Staphylococcus aureus*). Cytotoxic activity (against *Strongylocentrotus nudus* sea urchin embryos and sea urchin sperm cells).	[72]
	Acremine A ($C_{12}H_{18}O_4$) **(7)**			Cytotoxic activity (against *Strongylocentrotus nudus* sea urchin sperm cells).	
Ascomycetous	Balticolid ($C_{12}H_{16}O_4$) **(8)**	Ethyl acetate	TLC (silica gel). Silica gel column chromatography (EtOAc/hexane/MeOH, EtOAc/MeOH, EtOAc/MeOH, and MeOH and DCM/EtOAc, and EtOAc). RP-HPLC (MeOH).	Antiviral activity (anti-HSV-1 activity).	[73]

fractionation methods used for the isolation of bioactive compounds from fungi organisms.

3.3.1 Fractionation by Solvent Partition

The fractionation by solvent partition of active extracts is currently performed using the bioassay-guided fractionation procedure [12, 67–69]. This type of approach is the most used standard procedure and is characterized by several steps: (1) assessment of the potential bioactivity of the sample using a bioassay; (2) extraction using different solvents followed by assessment of bioactivity; (3) repeated fractionation of bioactive extracts and fractions in order to obtain the successful isolation of the bioactive compounds; and (4) structural characterization of the bioactive compounds by spectroscopic techniques, followed by pharmacological and toxicological assays [36, 47, 66].

Selected marine extracts contain compounds of different polarities, thus the fractionation by solvent partition separates the active compound from the inactive according to the different partition coefficients of analytes, resulting in full recovery of target compounds [70]. Compounds, such as alkaloid, shikimates, polyketides, sugars, amino acids, polyhydroxysteroids, and saponins are generally obtained in water soluble fractions; peptides are extracted in medium-polarity fractions, and substances like terpenes, hydrocarbons, and fatty acids are found in low-polarity fractions [36]. Each of the obtained fractions is then subjected to purification.

3.3.2 Separation and Purification by Chromatography

After fractionation by solvent partition, the active fractions are separated and purified by chromatography, in order to find pure bioactive molecules. The active fractions can be subjected to fractionation by column chromatography of several types, such as adsorption on silica gel and gel permeation, applying a range of solvents suitable to the polarity of the active fraction [54]. Silica gel column is the most common stationary phase used in the chromatography technique, and gel permeation chromatography (GPC) is a type of size exclusion chromatography (SEC), which separates compounds on the basis of size. In both techniques, for the successful separation, large amounts of organic solvents are needed [71]. In the final step of separation of pure compounds, other methods, such as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), should be used. Thin-layer chromatography (TLC) is a simple, fast, and cheap procedure, capable of processing large amounts of samples in one chromatography run. TLC has the advantage that it can be used after the column chromatography and before the HPLC technique usually for obtaining phenolic compounds and steroids [13, 72, 73]. Smetanina et al. [72] used the thin-layer chromatography to fractionate two new secondary metabolites (isoacremine D **(6)** and acremine A **(7)**) from fungus *Myceliophthora lutea* [72]. Reversed-phase high-performance liquid chromatography (RT-HPLC) is a technique, also commonly used to separate molecules according to their hydrophobicity. The analytes in a mixture are eluted with a pressurized liquid solvent through a column filled with an adsorbent stationary phase containing hydrophobic groups. As reported in many studies, the HPLC

technique has been used as a final purification step to obtain pure bioactive compounds (Table 2) [69, 73, 74].

4 Bioassays for Bioactivity Screening

Bioactivity is the ultimate goal desired throughout the extraction, separation, and purification process of marine fungi organisms. Thus, the designed bioassay is crucial for the detection of potential therapeutic applications. Throughout the isolation process, extracts, fractions, and end products are subjected to bioactivity assays in vitro and/or in vivo. Therefore, screening systems must include a broad range of biological assays in order to unravel potential substance-related activities [75].

Commonly, the bioactivity assays can be categorized into primary and secondary bioassay screens. The primary bioassay screens can be applied to a large number of samples in order to evaluate their bioactivities. The general requirements of these bioassays comprise high capacity, providing quick results, being cheap and not quantitative. During the selection of bioactivity assay, other basic qualities, such as validity, reproducibility, sensitivity, accuracy, cost effectiveness, simplicity, lack of ambiguity, and selectivity (in order to narrow the number of substances for secondary bioassay and reject false positives), should be taken into consideration. If a positive result is detected in the primary screening, a secondary screen, which is more accurate and precise, is executed. However, the secondary screening, having low capacity, is time consuming and expensive. In this bioassay, the pure compounds are assessed in various models and test circumstances in order to choose potential candidates for clinical trials [75, 76]. Whenever possible, available information on the target marine organism should be consulted in order to help in selection of the bioactivity screening assay.

The bioactivity screening of extracts of marine sources is an important and indispensable part of any pharmaceutical agent discovery platform.

5 Tools for Structural Characterization and Determination of Bioactive Compounds

Structural elucidation of active molecules from marine fungi sample is not an easy task, especially when considered the diversity of chemical structures comprised in each mixture. Thus, the chemical characterization of a molecule can be easier using literature reports.

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are among the most promising methods for thoroughly characterizing the structure and composition of marine bioactive compounds, such as amino acids, fatty acids, phenols, sterols, or sugars [77, 78]. However, the inherent complexity of these mixtures hinders the structural determination by means of those high-resolution techniques. Therefore, it is very useful to perform any separation steps before structure analysis in order to decrease the complexity of the marine extracts and in

turn provide additional information about the existing active components in the extract [79].

MS has been demonstrated its power and utility to elucidate unknown molecules in several marine organisms, as well as in marine fungi samples. Shushni et al. [73] identified a new 12-membered macrolide, named balticolid **(8)**, using MS spectra. Diverse methods based on high-resolution 1D and 2D NMR spectroscopy are used for the structural characterization of the bioactive compounds [68, 80].

In search of better characterization of BCs, multidimensional separation systems have become visibly interesting techniques for the analysis of complex mixtures [81, 82]. Multidimensional chromatography combines two or more separation techniques that fractionate complex mixtures based on different and independent properties. The major advantage of combining two separation techniques with different selectivity is the reduction of analytes overlap. Two-dimensional (2D) chromatography is the simplest example of a multidimensional separation scheme. Two-dimensional nuclear magnetic resonance spectroscopy (2D NMR) provides more information about a molecule than one-dimensional NMR spectra and is particularly useful in determining their chemical structure, particularly for analytes that are too complex using one-dimensional NMR. Types of 2D proton NMR include correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), exchange spectroscopy (EXSY), and nuclear overhauser effect spectroscopy (NOESY), which allows the determination of the conformation of the molecule or the relative location of the protons [83].

The complexity of the samples often exceeds the separation capacity of chromatographic systems. This challenge drives researchers all over the world to develop more sophisticated chromatographic methods that enable a greater resolution and peak capacity [79]. The progresses in the improvement of analytical methods linked to fast access and reliable data bases can be used as tools for rapid discovery of known bioactive molecules, thus needing less amounts of sample and simplifying sample preparation [84–86].

6 Online Combination of Bioassays for Detection of Bioactive Compounds

The analysis and isolation of bioactive molecules from complex mixtures without requiring cumbersome purification steps is hard and demanding [87]. The traditional analytical procedure consists in research with laborious bioassay-guided fractionation to isolate an individual bioactive analyte. On the other hand, the online combination or concurrent surveying of bioassays with chemical and structural characterization enables quick analysis and identification of single bioactive agents with various biological activities without needing previous purification procedures [88].

A number of approaches have been tried in analyzing bioactive molecules in marine extracts, containing total or partial online screening. These procedures combine separation techniques, chemical detection techniques, such as mass

spectrometry, nuclear magnetic resonance, and biochemical assays [4]. Two predominant approaches, high-throughput screening (HTS) and high-resolution screening (HRS), have been employed by researchers, both with advantages and disadvantages. The strategies used for HTS can normally be classified into precolumn and postcolumn methods. Precolumn techniques have been undertaken based on the fact that a bioactive analyte in a complex mixture is required to interact first with a target protein prior to separation, followed by chemical detection and identification [89, 90]. HTS postcolumn approaches consist of fractionating complex mixtures, recovering the fractions and their evaporation, and detecting bioactive fractions with parallel chemical detection and identification by microplate-based bioassays. HRS usually includes the online coupling of a bioassay of the chromatographic separation [91]. The high resolution achieved with the chromatographic separation stages in HTS postcolumn screening is frequently lost in lower resolution fraction obtained for the bioactivity screen [88]. Obtaining high resolution and sensitivity in HRS requires the integration of fast and simple online biochemical detection assays (BCD), such as enzymes, antioxidant screening assays, and receptor-based assays. The basis of BCD assays is the detection of bioactive molecules in simulated and nonsimulated biochemical reactions [91, 92]. This analysis approach is a mean to overcome preisolation limitations since it directly evaluates the effects of bioactive molecules after separation (postcolumn) and reduces in vitro assays, since only fractions with specific activities need to be isolated and tested. Some development time and effort in order to improve and implement more sensitive and faster novel methodologies is required to reduce the amounts of solvents used.

7 Conclusion

Obtaining a pure compound is a difficult process, requiring long periods of time, significant amounts of work, and large numbers of solvent-consuming steps. The isolation of marine fungi compounds depends on the quality and quantity of the sample, collection, preservation of samples, preparation of fungal cultures, extraction, fractionation, separation, purification, and bioactivity assays screening. There is no specific methodology that can be followed for the separation of BCs in a mixture of marine fungi. However, the marine bioactive compounds are mainly obtained by solvent extraction with different polarities. Fraction of bioactive compounds from fungi samples can be achieved by solvent partition or combining chromatographic techniques. If the purification was effective, the biological activity may be concentrated in a particular fraction; however, sometimes, the compounds may be already known or not show activity. Therefore, the bioactivity assays screening is an important step along the entire separation process. The structural characterization of BCs is also an important step in which MS and NMR play an important role in their determination. An effort to apply quicker and more sensitive techniques in structural analysis will accelerate the discovery of new bioactive compounds. The use of online screening approaches can rapidly provide a great deal of information

about the nature of compounds, which is very useful when large numbers of samples need to be processed avoiding unnecessary isolation of certain compounds. The successful investigation reports using marine organisms as potential sources of bioactive compounds encourage the incessant research of new molecules with interesting pharmaceutical applications. However, there is still much to research and explore the potential of marine fungi as source of novel agents, as well as develop strategies based on green analytical chemistry in order to reduce the quantity of solvents used.

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Bioengineering of Value-Added Wood Using the White Rot Fungus *Physisporinus vitreus*

14

Francis Willis Mathew Robert Schwarze and Mark Schubert

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Abstract

Over the past 10 years, we have intensively investigated the potential of the white-rot fungus *Physisporinus vitreus* for engineering value-added wood products. Because of its exceptional wood degradation pattern, i.e., selective lignification without significant wood strength losses and a preferential degradation of bordered pit membranes, it is possible to use this fungus under controlled conditions to improve the acoustic properties of resonance wood (i.e., "mycowood") as well as to enhance the uptake of preservatives and wood modification substances in refractory wood species (e.g., Norway spruce), a process known as "bioincising." This chapter summarizes the research that we have performed with *P. vitreus* and critically discusses the challenges encountered

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during the development of two distinct processes for engineering value-added wood products. Finally, we peep into the future potential of the bioincising and mycowood processes for additional applications in the forest and wood industry.

Keywords
Bioincising • Mycowood • Wood permeability • Acoustic properties • Resonance wood • Value-added wood products

1 Introduction

Using wood decay fungi for biotechnological applications in the forest products industry has been studied for several decades because the specificity of their enzymes and the mild conditions under which degradation proceeds make them potentially suitable agents for wood modification [1–3]. For example, fungi are successfully used in the biopulping or biobleaching of kraft pulp [4] or in bioremediation and detoxification of preservative-treated waste wood because of their tolerance and ability to degrade creosote, toxic polyaromatic hydrocarbon compounds, and pentachlorophenol [5–8]. The alterations in the woody cell wall structure reflect the plasticity of the degradation modes of wood decay fungi and can be used for the purpose of wood engineering [3, 9, 10]. During the early 1960s, industrially cultivated white-rot fungus (*Trametes versicolor* L.) was used in the German Democratic Republic, mainly on beech wood for pencil or ruler production (i.e., "mykoholz") [4, 11]. More recently, we have investigated the potential of a range of wood decay fungi for biotechnological applications in the forest product industry. In Switzerland, 65 % of the forest stand consists of Norway spruce [*Picea abies* (L.) Karst.] and European silver fir (*Abies alba* Mill.). The wood of either of these species to be used outdoors requires preservative treatment, which involves impregnating the wood cells with chemical preservatives or wood modification substances to suppress colonization by wood decay fungi. In most cases, the substance is infused into the wood cells using vacuum pressure impregnation, but the wood of difficult-to-treat (refractory) species such as *P. abies* and *A. alba* must be incised to enhance the uptake and distribution of the chemicals in the wood. Incising is a pretreatment process in which small incisions, or slits, are made in the wood surface to increase the exposed end and side grain surface area [12]. "Bioincising" is a biotechnological process that has been developed to improve the permeability of refractory wood species by incubation under controlled conditions for short periods with a white-rot fungus, *Physisporinus vitreus*. Our studies show that isolates of *P. vitreus* have an extraordinary capacity to induce substantial permeability changes in the heartwood of *P. abies* without causing significant loss of impact bending strength [10, 13–15]. In fact, wood durability of *P. abies* and *A. alba* is enhanced by the bioincising process, which is a promising technology for efficiently distributing wood modification substances, promoting desired improvements in wood properties, as well as leaving the wood surface aesthetically pleasing and the mechanical wood properties unaltered [16]. Another application of the controlled use of the

degradation pattern of *P. vitreus* is the production of mycowood with improved acoustic properties to overcome the shortages of natural wood with the superior tonal qualities desired by traditional musical instrument makers. The objective of this chapter is to summarize the work that has been conducted to implement the ambitious goal of transferring a standardized biotechnology process using *P. vitreus* from "science to market."

2 Wood Anatomy

2.1 Anatomy of Gymnospermous Wood

Gymnospermous wood is relatively homogeneous in structure and consists primarily of tracheids, uniseriate xylem rays, and, in some genera, axial parenchyma and epithelial cells surrounding resin canals (Fig. 1a–e). Tracheids are dual-purpose cells, combining properties of both structural support and water conduction. By comparison, angiospermous wood is more heterogeneous and its water-conducting functions are served by vessels, whereas fibers or fiber tracheids mainly supply strength and support [3]. Parenchyma is a more prominent feature in angiospermous than in gymnospermous wood, with most genera having multiseriate xylem rays and varying amounts of axial parenchyma. At the microscopic level, the woody cell wall is organized in layers with different thicknesses and different ratios of cellulose, the matrix material lignin, and hemicellulose [17–19]. The structure of woody cell walls is shown in Fig. 1. The cell wall proper consists of a thin primary wall, to which a much thicker secondary wall, consisting of three layers (S_1, S_2, and S_3), is added after initial formation of the cell. As in plant cells generally, a layer, termed the middle lamella (Fig. 1a), bonds the walls of adjacent cells together. It consists of calcium and pectic substances, which are polymers of galacturonic acid and its derivatives, acting like glue between adjacent walls. These compounds are amorphous and are therefore non-birefringent.

The main structural component of the walls of young wood cells is cellulose, a polysaccharide made up of long thread-like glucose molecules joined end to end by hydroxyl linkages without any side branching. This forms a largely crystalline structure, which has the optical property of birefringence and so appears bright when viewed between crossed Nicols (Fig. 1b). Within the different cell wall layers, cellulose exists as a system of fibrils with diameters of 3–4 nm aggregated in larger structural units [20–22].

The cellulose microfibrils are helically wound at different angles in the various layers of the cell wall. In a supporting cell (i.e., a fiber or tracheid), the first layer of the secondary wall (S_1) forms a thin outer shell of cross-laminated cellulose microfibrils aligned at a low angle to the cell axis. The second, or middle, layer (S_2) is thick and its microfibrils are aligned at a steep angle, while the third layer (S_3), bordering the lumen, has its microfibrils at a relatively low angle (Fig. 1c). These different helical windings are thought to contribute to the mechanical resilience of the wood. The degree of polymerization is also structurally important because it is highly

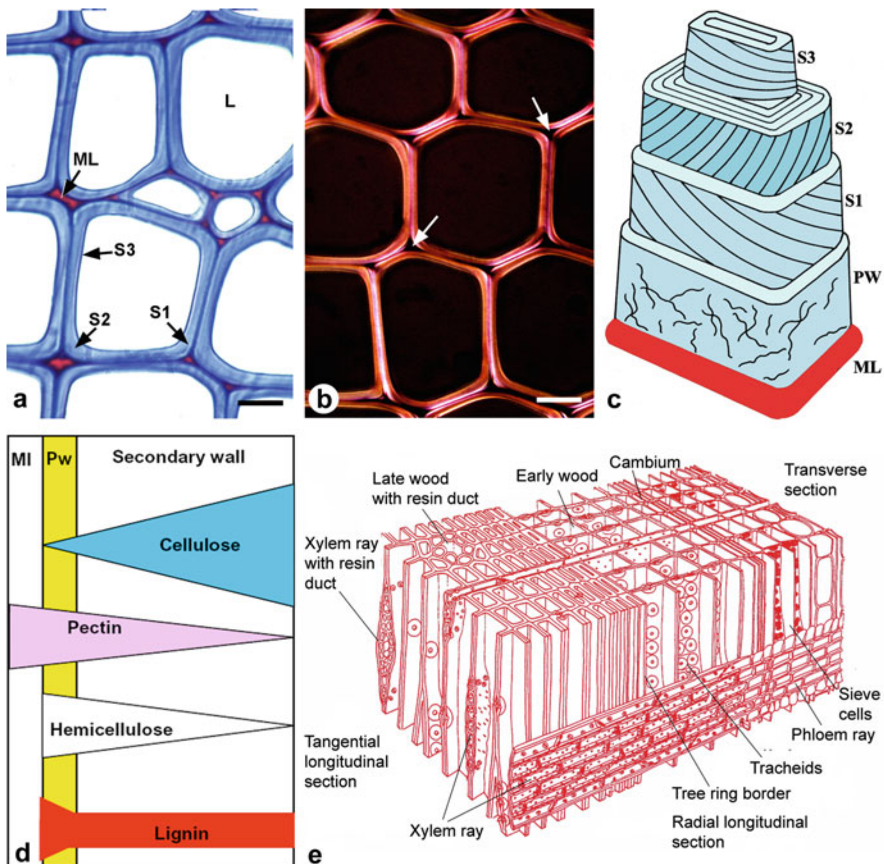

Fig. 1 (**a**–**e**) (**a**) Transverse section (T.S.) of early wood tracheids. *ML* middle lamella, *S1, S2, S3* outer, middle, and inner layers of secondary wall, *L* lumen. Bar, 10 μm. (**b**) T.S. of early wood viewed between crossed Nicols. Note secondary walls appear bright, whereas the amorphous compounds in the middle lamella are non-birefringent (*arrows*). (**c**) Conventional cell-wall model, which distinguishes five cell-wall layers: middle lamella (ML), the primary wall (PW), and the three-layer secondary wall (S) of outer (S1), middle (S2), and inner layer (S3). Bar, 10 μm. (**d**) Diagram of the relative distribution of the main cell wall constituents within the different layers of the cell wall. (**e**) Schematic of conifer wood showing cell types within the xylem and phloem (Reproduced from Schwarze [3])

correlated with tensile strength. The cellulose molecules are surrounded by the polymers lignin and hemicellulose. The major hemicelluloses in coniferous wood are galactoglucomannan, glucomannan, and arabinoglucuronxylan (Fig. 1d). Other softwood hemicelluloses are arabinogalactan, xyloglucan, and other glucans. Other polysaccharides are pectins, which are composed mainly of linearly connected β-1,4-d-galacturonase acid units and their methyl esters, interrupted in places by 1,2-linked l-rhamnose units [23]. In woody cells, a major part of the pectic substances occurs as polygalacturonic acid in the middle lamella, usually together with

Ca^{2+} ions in the form of calcium pectate. The term pectin or pectic compounds is used either strictly for the component rhamnogalacturonan or more generally for the group of components comprising the rhamnogalacturonans, galactans, and arbinans [24]. The wood of conifers is more primitive than that of angiosperm trees. In coniferous or softwood species, the wood cells are mostly of one type, tracheids, and as a result the material is much more uniform in structure than that of most angiosperms [3].

2.1.1 Tracheids

There are no vessels in coniferous wood, such as are seen so prominently in oak and common ash, for example [3]. Tracheids are dual-purpose cells combining the properties of both strength and support and water conduction (Fig. 1e). They constitute the greater part of the structure of softwoods (90–95 %), whereas the xylem rays, axial parenchyma, and resin ducts make up only 5–10 %. The presence of resin ducts that are surrounded by epithelial cells, which synthesize and secrete resin into the ducts, is a unique feature of most conifers. In some conifers, resin ducts are absent (e.g., white fir: *Abies alba*) and yew (*Taxus baccata*) are only formed in the xylem after damage (i.e., traumatic resin ducts). There is generally less axial and ray parenchyma in conifer wood than in the wood of angiosperms and the latter cells are mostly uniseriate. Tracheids are considered to be the most ancient woody cell type from which all other wood cells have derived [3]. They are dead, elongated, lignified cells with pointed ends and are a few millimeters in length. In transverse section, tracheids appear in homogeneous radial cell rows. The diameter of the cell lumen decreases from the early to the late wood, as the cell wall becomes thicker. The latter differences are responsible for the conspicuous appearance of the annual rings in the wood of conifers, as the dense late wood is darker than the less dense early wood. In most native conifers, the transition between early and late wood is gradual, but in other species, such as *Larix* and *Pseudotsuga*, it is abrupt. The most striking feature of conifer tracheids is the bordered pits, which predominantly occur in the radial cell walls.

2.1.2 Pit Types

Bordered Pits

The pits between tracheids typically have overarching walls that form a bowl-shaped chamber, giving them the name “bordered pits” (Fig. 2). The construction is more variable and complicated than that of simple pits. The pit cavity consists of a canal and a chamber [3]. The canal expands from the inside (porus) to the outside where the membrane is located (torus). When viewed in longitudinal sections the pits are often surrounded by a halo, hence the name bordered pits. Their structure can be discerned best in a transverse section through adjacent cells. In tree species of the genera *Abies, Larix, Picea,* and *Pinus,* the center of the membrane is made of a thickened disc of primary cell wall material called torus. No secondary walls exist in the pit’s structure. The area between the torus and wall (the former middle lamella) is called margo and is very porous, allowing the movement of water and ions from

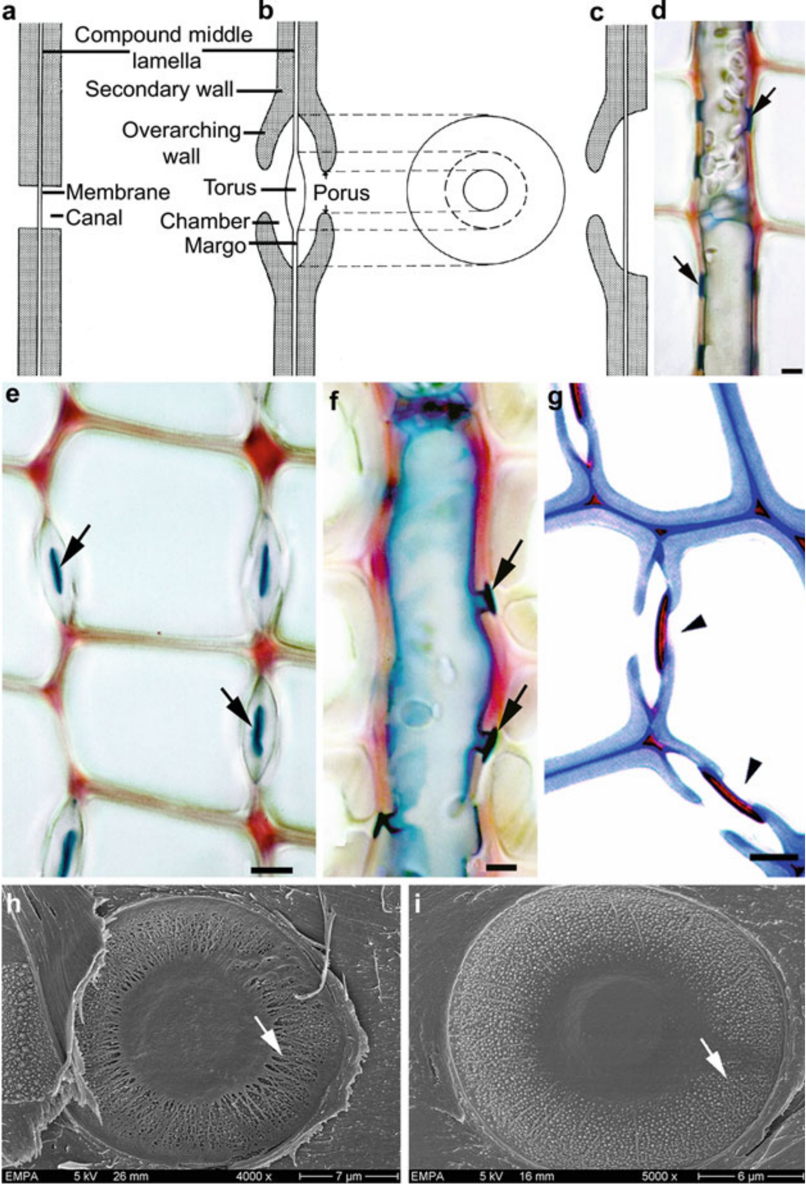

Fig. 2 (**a**–**i**) **Pit types:** Schematic of a simple pit between adjacent parenchyma cells (**a**), bordered pit between two tracheids (**b**), and a half-bordered pit between a tracheid and a parenchyma cell (**c**) (Reproduced from Grosser [25]). Transverse section (T.S.) of *Picea abies* stained with safranine and astra *blue* showing non-lignified simple (**d**, *arrows*), bordered (**e**, *arrows*) and half-bordered pits (**f**, *arrows*). Note that within the sapwood non-lignified pit membranes are stained blue. T.S. of *Picea abies* showing heartwood with aspirated and lignified tori that are stained *red* with safranine (**g**, pointers) within the bordered pits. FE-REM showing longitudinal radial sections of bordered pits

tracheid to tracheid. The torus acts as a valve, closing tightly against one side or the other of the bordered pit in response to small pressure changes in the xylem [3].

Early-wood tracheids have larger bordered pits and a round pit aperture (porus). In late-wood tracheids, they are smaller, with slit-like apertures. Pits are mostly arranged singly in the cell wall of tracheids. Based on the shape of the chamber, two different types of pits can be distinguished: simple pits and bordered pits [3]. Two supplementary pits are termed the complementary pits and make a pit-pair. The most important types of pit pairs (simple pit pair, bordered pit pair, and a half-bordered pit pair) are illustrated in Fig. 2a–f.

Half-Bordered Pits

Between dead tracheids and living parenchyma cells, the half-bordered pits exist with only one pit chamber towards the side of the tracheid (Fig. 2f). Species of *Pinus* have very large and conspicuous pit membranes, also termed window pits. These pits can be observed in radial longitudinal sections within the cross fields of xylem ray parenchyma [3].

2.2 Wood Decay

Despite the inherent resistance of wood, fungi will degrade woody tissues and decay types fall into three categories according to their mode of degradation of the woody cell walls. Traditionally, wood decomposition by fungi was separated into two categories based on the micro-morphological and chemical characteristics of decay, which result in different patterns of degradation of the compound middle lamella, S_1, S_2, and S_3 layers: brown rot and white rot, the latter subdivided into simultaneous rot and selective delignification. The ability to oxidize phenolic compounds extracellularly was used to differentiate white-rot fungi from brown-rot species. Several enzymes, including tyrosinase (oxidation of monophenols) and laccase, show polyphenol oxidase activity, which oxidizes mono- and diphenols. For this purpose, a rapid screening test for white-rot fungi based on polyphenol activity, the Bavendamm test, which monitors the development of a brown coloration on agar plates containing guaiacol or gallic acid, has been used to distinguish white- from brown-rot fungi [26]. Today there are many reagents used to identify the phenoloxidases of wood-decay fungi in pure culture [27–31].

The work of Savory [32] marked an important further step in the understanding of decomposition processes by lignolytic fungi. His description of decay by ascomycete and deuteromycete fungi in wood from industrial cooling towers revealed a particular pattern that had been formerly observed by Bailey and Vestal [33],

Fig. 2 (continued) in sap (**h**) and heartwood (**i**) of *Abies alba*. Note that in the sapwood the structure of the margo (*arrow*) in the bordered pits is discernible, whereas in the heartwood it is strongly encrusted (*arrow*) and hardly visible (Reproduced from Schwarze [3])

Tambyln [34] and Barghoorn and Linder [35] in wood from other sources. This type of decay was termed “soft rot” because of the spongy texture of the wood surface. Soft rots differ from both brown and white rots in its pattern of development, which involves a process of hyphal tunnelling inside the lignified cell walls. Soft rot was therefore described as distinct from brown- and white-rot forms of wood decay normally attributed to ligninolytic basidiomycetes and some of the larger ascomycetes. On the basis of polyphenol activity, certain fungi, including members of the genera *Daldinia*, *Hypoxylon*, *Kretzschmaria,* and *Xylaria*, were previously considered to be white-rot fungi, but light microscopy studies show that their mode of degradation is more correctly classified a Type 1 or Type 2 form of soft rot [36–39]. Increasing evidence also indicates that a range of brown- or white-rot fungi cause a soft rot in addition or alternatively to their more typical mode of degradation Thus the boundaries between these three types of fungal decay are less clear cut and recent studies suggest that there is a much greater diversity in the way different decay fungi challenge their hosts and substrates. There is evidence that the terms brown rot, white rot, and soft rot may not be obsolete, but rigid definitions of fungi that are placed into these categories may be less appropriate than thought previously [40].

2.2.1 White Rot

White rots are caused by basidiomycetes and certain ascomycetes. The common feature of all these fungi is that they can degrade lignin as well as cellulose and hemicellulose. However, the relative rates of degradation of lignin and cellulose vary greatly according to the species of fungi and the conditions within the wood. As with brown rots, there is additional variation related to the preferential decay of different zones within the annual ring. The adaptation of white-rot fungi to the more heterogeneous structure of the wood of angiosperms, plus their ability to degrade all the cell wall constituents extensively, leads to a multiplicity of patterns of wood decay. Within this range of variation, two broad divisions are widely accepted: selective delignification and simultaneous rot. White-rot fungi degrade lignin by oxidative processes, which involve phenoloxidases such as laccase, tyrosinase, and peroxidase. They degrade cellulose in a less drastic way than brown-rot fungi because their cellulolytic enzymes attack the molecules only from the ends, splitting off glucose or cellobiose units.

Selective Delignification

In selective delignification, lignin is degraded earlier in the decay process than cellulose or hemicellulose. The hyphae grow in the cell lumina in some cases, so that the lignin is dissolved out of the adjacent cell wall. In other cases, hyphae penetrate the cell wall and initially delignify the middle lamella so that the cells tend to separate. Cellulose is left relatively unaltered during selective delignification, at least in the early stages of decay [39]. Some white-rot fungi (e.g., *Meriplus giganteus*) have an extraordinary capacity to hydrolyze the pectin in the middle lamella of xylem during the incipient stages of selective delignification [41]. Sections stained with ruthenium red and hydroxylamine-ferric chloride reveal that

M. giganteus preferentially degrades the pectin-rich regions of the middle lamellae in xylem ray cells of beech [42]. In the wood of large-leaved lime, such regions are uniformly located in the middle lamellae of axial and ray parenchyma. In beech wood, degradation of pectin-rich middle lamellae commences after the delignification of secondary walls and results in a conspicuous hollowing of multiseriate xylem rays [42]. The effects of treating conifer wood with commercial pectinases or bacteria to improve penetration of preservatives have been studied in detail [4, 43–46].

Commercial pectinase treatment improves preservative penetration of the sapwood of Douglas fir by opening pit apertures, as long as the treatment is combined with either a low pH or a calcium chelator, such as ammonium oxalate or sodium hexametaphosphate [47]. The most effective tested on finely ground wood of Norway spruce are hydrolases with a broad spectrum of cellulolytic and hemicellulolytic activity [48, 49]. The application of these enzymes, however, has failed to enhance the permeability of solid wood to any useful extent because of the slowness of their diffusion into wood and to the effect of extractives, adhering to aspirated pits, making them resistant to decomposition [48, 49].

2.2.2 Enzymatic Activity of *Physisporinus vitreus*

Elucidation of the enzymatic processes behind this heterogeneous degradation pattern of *P. vitreus* is still in progress. Under lignolytic conditions, laccase was the predominant phenoloxidase secreted by *P. vitreus* and neither mangan peroxidase nor lignin peroxidase were produced [50]. These findings indicate that the enzyme laccase plays an essential role in the selective delignification process. Laccase belongs to the copper oxidase family and as with other extracellular enzymes, the laccase is glycosylated. Laccases catalyze the one-electron oxidation of substrates at the T1 Cu-site, coupled to the reduction of oxygen to water at the trinuclear Cu-site [51].

Interestingly, *P. vitreus* produces a particular laccase system with only one isoform. After purification, SDS-PAGE revealed that the laccase has a mass of about 53 kDa, which is similar to a bacterial laccase (40–60 kDa, [52]) than to a fungal laccase (60–80 kDa, [53]). The ability to oxidize lignin model compounds according to the method of Kudanga et al. [54] showed reduced reactivity of the laccase of *P. vitreus* in comparison with the commercially available laccase of *T. versicolor* (Sigma Aldrich) or that of *Myceliophthora thermophila* (Novozymes). Because of the low redox potential, the action of the enzyme laccase is restricted to the oxidation of the phenolic lignin moiety, whereas non-phenolic substrates with a redox potential above 1.3 V cannot be oxidized by laccases directly [55]. Our results are in good agreement with previous studies [55] and revealed that *P. vitreus* degrades phenolic lignin much faster than non-phenolic lignin. However degradation of non-phenolic lignin substructures is definitely involved in the selective delignification process of *P. vitreus*. Whether the degradation of non-phenolic lignin substructures is caused by other enzymatic or non-enzymatic reactions or laccase-mediator systems warrants further investigation [56]. Mediators are substrates for laccases, and after oxidation, they can oxidize a secondary substrate in a distance from the active site of the enzyme. The presence of such small molecular weight compounds expands the substrate range of laccase towards more recalcitrant

compounds such as non-phenolic lignin units [57]. Camarero et al. [58] demonstrated that lignin-derived compounds can function as natural mediators. We hypothesize that *P. vitreus* uses liberated phenolic lignin fragments as mediators. However, further studies are needed to reveal the detailed mechanisms of laccases (and potential mediators) in the selective delignification process of *P. vitreus*.

During incipient stages of degradation, *P. vitreus* apparently excretes small amounts of polygalacturonase beside the phenoloxidase laccase. Polygalacturonase might be involved in the degradation of the tori of bordered pit membranes, which consist predominantly of α-1,4-linked galacturonic acid units [24]. Only at a later stage of degradation, cellulases were detectable.

Supposedly because of its specific enzyme composition, *P. vitreus* is a relatively weak lignolytic fungus that causes only small losses of mass during the incipient period of substrate colonization and it selectively delignifies the secondary walls without affecting the middle lamellae, even at advanced stages of degradation (Fig. 3b).

Genetic engineering could be a powerful approach to increasing productivity, minimizing unwanted effects, and optimizing the biotechnological use of filamentous fungi, but the challenge is establishing a suitable transformation method [59]. Genetic manipulation of a fungus of interest requires the development of a plasmid-mediated transformation system that includes: (1) infusion of exogenous DNA into recipient cells, (2) expression of genes present on the incoming DNA, and (3) stable maintenance and replication of the inserted DNA, leading to expression of the desired phenotypic trait [60]. Thus, to design an optimal engineering strategy, it is necessary to first identify the target property or negative side effect that should be increased or decreased, respectively. In the case of *P. vitreus*, identifying such a target property or side effect is very complicated because of the complex wood–fungus system. Therefore, in a project 205321-121701 funded by the Swiss National Science Foundation, we developed a transformation protocol for *P. vitreus* [60], several visualization techniques [61, 62], and a fungal growth model for *P. vitreus* [63, 64]. By comparing the macro- and microscopic system properties obtained from computer simulations with results of laboratory experiments, we could improve our understanding on how a complex and difficult to observe system, such as fungus-wood, interacts under defined conditions [109].

3 Bioincising: Improving the Permeability of Refractory Wood Species

P. vitreus is a basidomycete (Polyporales, Meripilaceae) that belongs to the large and puzzling *Polyporus lignosus* complex. It can be very easily confused with *Poria nigrescens* and *Physisporinus sanguinolenta* but is most easily distinguished by its characteristic decay, a conspicuous white pocket rot. When fresh, the basidiocarp

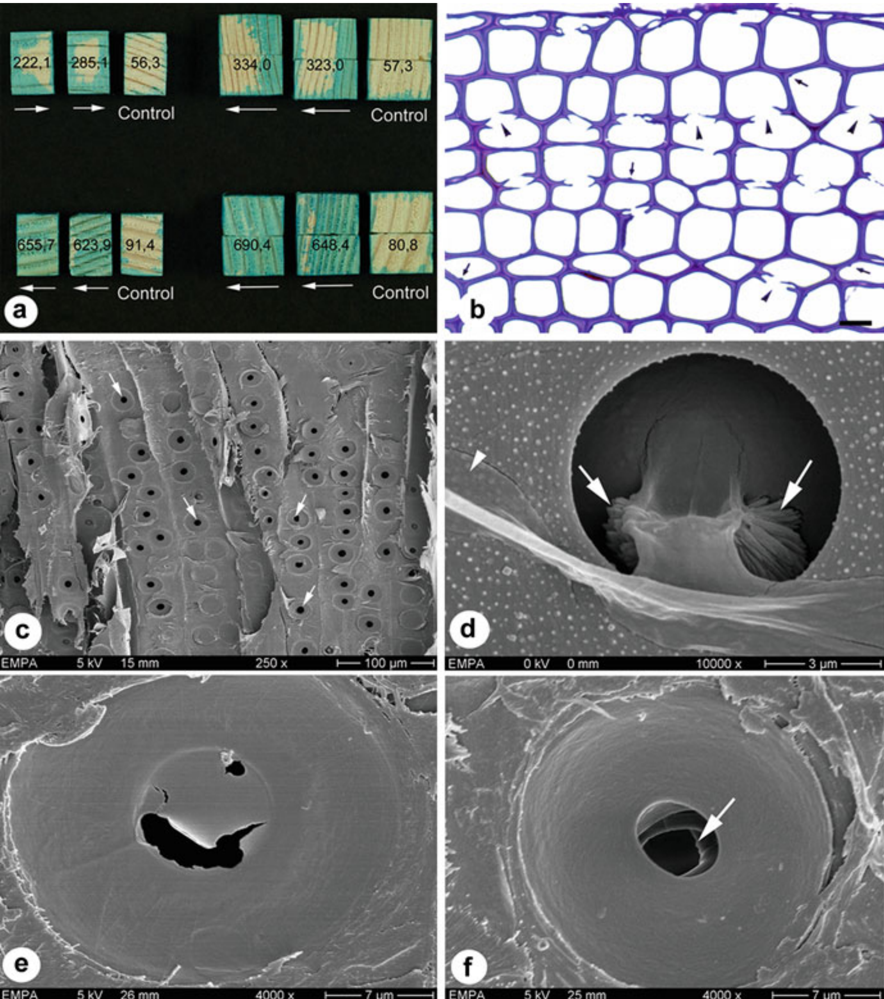

Fig. 3 (**a**–**f**) (**a**) Heartwood specimens of Norway spruce (*top*) and white fir (*bottom*) impregnated with the bluish dye Neolan Glaucin E-A after 6 weeks' incubation with *Physisporinus vitreus*. Numbers refer to radial and tangential uptake of water in kg/m^3. (*Arrows*) Direction of hyphal colonization. (**b**) Transverse section (T.S.) of Norway spruce showing selective degradation of bordered pits (*arrowheads*). Bar, 20 μm. (**c**) Scanning electron micrographs (5 kV) showing bordered pits in Norway spruce and white fir after 6 weeks' incubation with *Physisporinus vitreus*. (**d**) Note degraded tori (*arrows*) within bordered pits. (**e**) Hyphae enter the bordered pits via the aperture and degrade the torus; lysis of the warty layer and calcium oxalate crystals in close proximity to hyphae within bordered pit chambers (*arrowheads*). (**f**) After 12 weeks, most bordered pits showed partial to complete dissolution of pit membranes, such that both apertures of pit of pit pairs are occasionally exposed (*arrow*) (Reproduced from Schwarze et al. [15])

appears much like *P. sanguinolenta*, which differs in usually turning reddish where bruised and on drying and in having somewhat larger pores. *P. sanguinolenta* var. *expallescens*, however, is so similar that in the absence of decayed wood, a clear distinction is difficult. *P. vitreus* occurs on angiosperms and more rarely on gymnosperms, in the USA apparently more abundant southward but known from Ontario southward in eastern North America to Missouri, in Alaska, Idaho, British Columbia, Washington, Puerto Rico, Europe, and New Zealand. Interestingly, *P. vitreus* decomposes water-saturated timber in cooling towers by a fibrous, white pocket rot [65–68]. In the laboratory, the fungus reveals a remarkable pattern of colonization. In crosswise piled water-saturated pine wood, the fungus decomposes only those parts of the substrate not surrounded by air [66, 67].

Some isolates of this fungus have an extraordinary capacity to induce significant permeability changes in the heartwood of Norway spruce and silver fir after hydrolysis of bordered pit membranes without causing significant loss of wood strength [15].

The *P. vitreus* strain (Empa No. 642) used in all of our studies was assigned by PCR amplification and sequencing of the ITS1-5.8S-ITS2 region of the rDNA followed by alignment with published sequences using nucleotide BLAST [69]. The ITS1-5.8S-ITS2 sequence of the *P. vitreus* (Empa strain No. 642) was submitted to the EMBL databank under the following accession number: FM202494.

Even after 6 weeks incubation, when the mass loss induced by *P. vitreus* was slight (>1 %), wood permeability increased to approximately 300–400 kg/m^{-3} in Norway spruce and 400–680 kg/m^{-3} in silver fir (Fig. 3a; [15]). Conspicuous, qualitative changes in permeability were also apparent from the uptake of the bluish dye Neolan Glaucin E-A. Uptake of the dye within test blocks of silver fir incubated with *P. vitreus* was visually homogeneous but less so in Norway spruce (Fig. 3a; [15]). FE-REM studies revealed that uptake of Neolan Glaucin E-A was attributable to degradation of pit membranes (Fig. 3b–f). The hyphae entered the pit chamber via the apertures, and the membranes were subsequently degraded (Fig. 3d–f). Degradation commenced from the thickened, central part of the membrane (the torus). Calcium oxalate crystals were regularly observed on the hyphae (Fig. 3d), and in the wood of *A. alba*, they often accumulated within bordered pits in close proximity to the hyphae.

4 Mycowood: Improving the Acoustic Properties of Wood for Violins

An interesting field in which wood modification by *P. vitreus* can be used is the improvement of the acoustic properties of wood for contemporary violin making. Musical instruments produced by Antonio Stradivari during the late seventeenth and early eighteenth centuries are reputed to have superior tonal qualities than more recent instruments. Dendrochronological studies show that during his later decades, Stradivari used Norway spruce wood that had grown mostly during the Maunder

Minimum [70, 71], a period of reduced solar activity when relatively low temperatures caused trees to lay down wood with narrow annual rings, creating a high modulus of elasticity and low density [72]. Traditionally, wood used in the manufacture of musical instruments is treated with primers, varnishes, or minerals to stiffen it. Such treatments can strengthen the adhesion between cell layers but increase the density and vibrating mass because the cells' lumina become occluded by the substance [73–76], which ultimately reduces the speed of sound. The increase in density has an adverse effect on the radiation ratio [R = speed of sound (c)/ density (ρ)], reducing the speed of sound and its resonance frequencies [73, 77, 78]. Tests of other chemical treatments have shown that they increase the dynamic modulus of elasticity (EL and ER) and decrease the damping factor (δL and δR, [79–81], but although these treatments do not alter wood density, they increase the crystallinity of the cell wall, which is considered disadvantageous for wood processing [80]. Other authors have suggested that the wood used by Guarneri and Stradivari to make the violins was chemically treated to kill woodworm and fungi [82].

An alternative approach to improving the acoustic properties of wood is to reduce its density by fungal or bacterial degradation. During the seventeenth and eighteenth centuries, some degradation probably resulted from the practice of floating tree trunks in water [83], but there is no evidence that this caused any noticeable reduction in wood density. According to Nagyvary [84], the microbial degradation of pit membranes that occurred during this treatment would have resulted in an increase in wood permeability such that subsequent penetration of varnish would be enhanced. Recently, Wagenführ et al. [85, 86] used a new thermal treatment to improve the acoustic properties of resonance wood. Treatment at high temperatures results in a reduction in density because of decomposition of hemicellulose and cellulose, but E is reduced. A negative side effect of heat treatment is that the material becomes brittle, causing problems during the manufacture of the instruments. Most of the described treatments alter the woody cell wall and adversely affect the properties of the compound middle lamellae, both of which have a pivotal role in determining the overall stiffness of wood. In a homogeneous bulk material, ignoring surface effects, the speed of sound, c, is governed by two mechanical properties: E and the density. In wood, which is strongly anisotropic, c varies directionally and is decreased by any discontinuities in the compound middle lamella, such as those resulting from microbial degradation. Using the formulae shown in Table 1, it can be deduced that such degradation, even if very slight, results in an abrupt reduction in both E and c and has a negative impact on the acoustic properties of the wood [87].

The compound middle lamella is penetrated or otherwise altered by most species of wood decay fungi, except for members of the Xylariaceae (e.g., *Kretzschmaria deusta* and *Xylaria longipes*), which have little ability to degrade guaiacyl, found in very high concentrations in the compound middle lamella. As a result, this layer remains as an intact skeleton, even at a quite advanced stage of decay [10, 36, 87], which explains why the speed of sound through the wood is little affected until that stage [10, 87] and is the reason why decay caused by *K. deusta* is hard to detect in trees by means of acoustic devices [10, 39, 87].

Table 1 Principal acoustic properties used for the assessment of resonance wood quality of axial (L) and radial (R) samples

Property	Assessment
Density ρ (kg/m^3)	ρ for the specimens in L and R directions
Young's modulus of elasticity E (MPa)	E for L and R directions
Speed of sound c (m/s)	$c = \sqrt{\frac{E}{\rho}}$ for L and R directions
Radiation ratio R (m^4/kg s)	$R = \frac{c}{\rho} = \sqrt{\frac{E}{\rho^3}}$ for L and R directions
Damping factor δ_L for L direction and δ_R for R direction	$\delta = K\frac{\Delta f}{f_r}$ where f_r is the resonance frequency, Δf the associated damping, and K is a coefficient which varies between $\frac{1}{x}$ and $\frac{\pi}{\sqrt{3}}$

Incubation of Norway spruce wood with *P. vitreus* causes marked density loss and cell wall thinning (Fig. 4); that is, the partly degraded wood resembled superior resonance wood grown under cold climate conditions. By assessing the incubated specimens microscopically and measuring five physical properties (Table 1), we found alterations in the wood structure that resulted in a reduction in density, accompanied by relatively little change in the speed of sound. After 20 weeks incubation, the wood had reduced in density by more than 10 % and showed a concurrent increase in sound radiation, without any weakening of the structure [88]. This finding is in good agreement with other research that shows that the gradual decomposition and loss of hemicellulose with time decreases wood density without affecting its Young's modulus, subsequently increasing the radiation ratio [89]. In Fig. 5, the density differences in the top plate of the Opus 58 before and after 9 month fungal treatment are apparent.

The increase in the radiation ratio of mycowood from “poor” to “good” puts it on a par with “superior” resonance wood grown in a cold climate [88]. Moreover, differences in wood density between early and late growth were reduced, improving the vibrational efficacy and the production of sound similar to that of ancient violins. Norway spruce wood treated with wood decay fungi also damps sound more efficiently, giving it a more mellow timbre. The significant increase in the damping factor (340 % in the radial direction) that was recorded after 20 weeks incubation can be attributed partly to selective degradation of pit membranes.

5 Process Optimization by Characterization of Fungal Activity and Assessment of Fungally Modified Wood

Despite initially promising results on small wood samples, the biotechnological use of wood decay fungi for modification of larger wood profiles and the transfer to industrial application displays particular challenges because of the variable

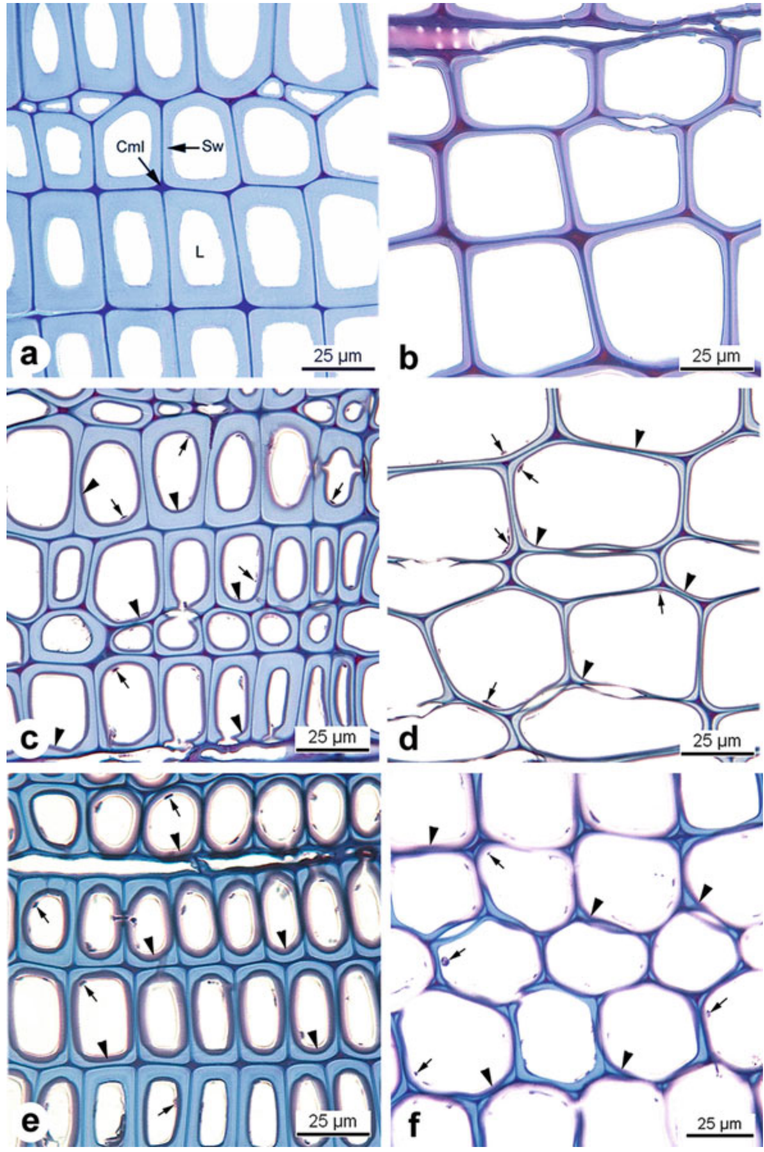

Fig. 4 (**a**–**f**) Transverse sections of untreated controls and Norway spruce wood incubated with *Physisporinus vitreus*. (**a**) Late- and (**b**) early-wood tracheids of control specimens. *L* cell lumen, *S* secondary wall, *Cml* compound middle lamella. (**c**) After 12 weeks' incubation, delignification of secondary walls (*arrowheads*) commenced from hyphae (*arrows*) growing within the cell lumen of late- and early-wood (**d**) tracheids. (**e**) After 20 weeks' incubation, secondary walls are strongly delignified (*arrowheads*) and cell wall thinning is apparent in late- and early-wood tracheids (**f**) (Reproduced from Schwarze et al. [88])

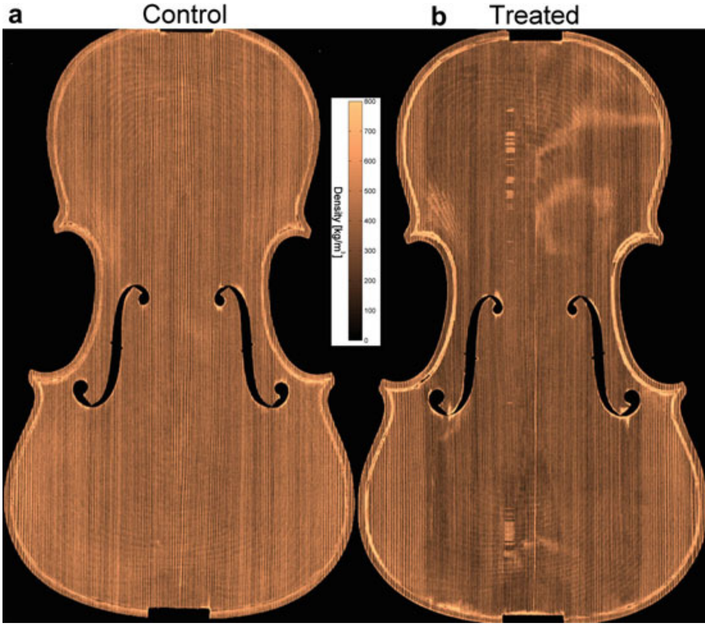

Fig. 5 Density distribution in top plates made of Norway spruce wood for the Opus 58, before and after 12 months incubation with *P. vitreus*, (**a**) density map before and (**b**) density map after fungal treatment in kg/m^3. Note: lower densities (*darker* areas) in the center of the fungal treatment were apparent after X-ray radiography analysis. The images were reconstructed with a Toshiba medical scanner at Leiden University Medical Center/Netherlands (Image analysis was performed based on an algorithm in Matlab as explained in Stoel et al. [90])

performance of the fungus and the heterogeneity of the substrate. Highly efficient modification of wood by *P. vitreus* is based on optimal development of the mycelium, including the lag phase, hyphal elongation, and branching [91]. Mycelial growth is greatly influenced by environmental conditions [31], and the effect of the fungus is inextricably linked to the underlying substrate, wood, which is a complex anisotropic material with several hierarchical levels of organization from the macroscopic (e.g., growth rings), the mesoscopic (e.g., the set of wood cells) down to the microscopic, and nanoscopic scales (e.g., wood cells and fibrils). The interplay among the chemical composition of the substrate, its geometric structure, and the enzymatic activity influence the performance of *P. vitreus* and thus the outcome of the wood modification process. Successful upscaling of biotechnological processes in which *P. vitreus* is used to improve the substrate's properties requires a set of investigations to identify and quantify the important physical, chemical, and biological parameters and their sensitive control during fungal incubation. In addition to characterizing the fungal activity, the fungally modified wood has to be assessed, so an industry cooperation project was launched with the objective of scaling up the bioincising process using *P. vitreus* (CTI No. 8593.1). Improving the effectiveness of wood modification by ensuring variable performance is minimized

and development of the fungus is optimized requires identification of the principal parameters and understanding how these factors influence wood colonization by *P. vitreus*. Therefore, we determined the lag phase and the specific growth rate of *P. vitreus* under combined effects of a range of parameters (i.e., temperature, water activity (aw), and pH) [91, 92].

Our results show that temperature and aw, in particular, are key determinants of the development of *P. vitreus*, and pH plays a secondary role [92]. The results are in good agreement with those of Griffin [93] and Anagnost [94], who maintain that most wood decay fungi are hydrophilic organisms (aw > 0.90) and require aw to be at least 0.97. Furthermore, Schmidt et al. [66, 67] observed an extraordinarily strong dependence of *P. vitreus* on high moisture levels. They reported preferential colonization and degradation of water-saturated wood [wood moisture content (WMC) >100 %] of *Pinus sylvestris*, *P. abies*, and *Fagus sylvatica*, with low air content. At WMC > 90 %, fungal growth within wood is normally impeded by a lack of oxygen, which highlights the remarkable biological capacity of *P. vitreus* to degrade timber in the special habitat of cooling towers. Taken together, these findings indicate that the amount of water available in the substrate (wood) is a very important factor for ensuring colonization and fungal growth by *P. vitreus*. Until the fiber saturation point (FSP) is reached, moisture in wood exists as bound or hygroscopic water within the cell wall because of hydrogen bonding of the hydroxyl groups mainly in the cellulose and hemicellulose [68]. At about the FSP, which ranges between 25 % and 30 % depending on the wood species, the content of bound water is maximal and free capillary water in liquid form is available in the cell lumen, as well in other voids in the woody tissue [68, 94]. To enable a short lag phase and high growth rate, superficial drying of wood must be avoided by setting low air flow around the wood and maintaining a high relative humidity. In addition to abiotic parameters, biotic stress caused by other microorganisms influences the performance of *P. vitreus*. Therefore, the competitiveness and growth mode of *P. vitreus* against a range of blue stain fungi and *Trichoderma* species (challenge species) was investigated in dual culture tests and a spatially heterogeneous system of tessellation agar on different media. In addition, the robustness of wood colonization by *P. vitreus* against biotic influences was determined by controlled interaction tests with different inocula of a range of ascomycetes on Norway spruce heartwood [95]. The selection of the challenge fungi was based on the fact that in pre-tests of wood incubation with *P. vitreus*, the selected fungi occurred as contaminants, or they are known to be wood-inhabiting pioneer colonizers [31] or, in the case of *Trichoderma* spp., to have high antagonistic potential against basidiomycetes [96–98]. A comparison of the lag phase and growth rate of *Trichoderma* sp. and *P. vitreus* revealed that even under optimal growth conditions, *Trichoderma* sp. showed a significantly shorter lag phase and higher growth rate [91, 92, 99, 100]. Apart from their ability to overgrow and parasitize the mycelium, their high spatial and nutrient competitive abilities make *Trichoderma* spp. a decisive competitor of *P. vitreus*. Schubert and Schwarze [95] also showed that *Trichoderma* spp. have by far the most significant negative effect on the performance of *P. vitreus*, even with a low inoculum potential. Synthesis of the results of the influence of abiotic and biotic parameters provides the basis for

successful scaling up of the technologies and indicates that efficient heartwood modification by *P. vitreus* depends on optimal growth conditions as well as inhibiting contaminants, particularly competition by *Trichoderma* spp. during the lag phase of *P. vitreus*.

Intensive investigations to characterize fungally modified wood as well as to evaluate bioincised wood as an improved substrate for subsequent treatment with property-improving substances have been conducted [13, 16, 101–103]. Despite the higher uptake of wood modification agents after brushing or dipping and particularly after impregnation, a negative effect of bioincising on the target properties of the agents, regardless of the application method, has been found [13, 102]. Only the performance of biocides is enhanced in bioincised wood [103]. Lehringer et al. [101] showed that under incubation conditions using a malt agar nutrient medium (narrow C/N ratio of approximately 50/1), the colonization of the wood was very inhomogeneous and degradation of bordered pits was accompanied by cell wall degradation (selective delignification and hotspots of soft rot types I and II). These alterations in cell wall structure, particularly both types of soft rot, may be responsible for the enhanced flammability of bioincised wood. As Lehringer et al. [101] discussed in detail, a high nitrogen concentration (e.g., malt agar) seems to reduce the selective lignin degradation rate [104] and additionally stimulates polysaccharide breakdown [105–107]. The occurrence of selective delignification and simultaneous degradation of lignin, cellulose, and hemicellulose were furthermore demonstrated by ultramicrospectrophotometry, Fourier transform infrared spectroscopy [16], and wet chemical analysis [102]. Vermiculite (wide C/N ratio of approximately 400/1) has been used in other studies [14, 88], and degradation of pit membranes only coincided with selective delignification of the tracheids without further cell wall damage.

Taking all important parameters (e.g., water activity, biotic stress, C/N ratio) into consideration, we developed a controlled wood incubation system for *P. vitreus* in which all important parameters can be adjusted accordingly. The required wood incubation time could be significantly reduced with this incubation system. However, the larger the wood specimens, the higher the costs and the more difficult it is to obtain a homogeneous wood colonization and to improve the wood properties. Thus, according to the current state of knowledge, the developed wood modification process appears to be most promising for specialized high end products (e.g., resonance wood or “mycowood”).

6 Conclusions

6.1 Benefits of Technology Transfer of the “Bioincising” and “Mycowood” Technology for the Forest and Wood Industry

Technology transfer does not mean simply movement or delivery; transfer can only happen if a technology is used. In other words, it is the application of knowledge and considered a process by which a technology developed for one purpose can be used

in either a different application or by a new user. One vision of high-technology wood preservation is the chemical modification of wood to render it impervious to natural degradation processes. Successful chemical modification of refractory wood species can only be accomplished if wood permeability is enhanced, which is the reason bioincising was developed. The estimated costs for the production of bioincised wood are currently approximately US $5,750 per m^3. The technology breakthrough in this area might not be the meeting of the economic criteria that would make it applicable to traditional markets for treated wood but rather the opening up of new markets for value added decorative products. Low maintenance, durable wood products have been clearly identified as having considerable market potential. The best added value is derived from products that customers buy because they want them, not because they have to. More is paid for beauty than for practicality. In this context, musical instruments such as the violin are high-end products and a unique investment. Facing volatile equity markets, investors often look to gold and silver, but an updated study of classical instrument valuations by Graddy and Margolis [108] shows that violins may be among the most stable of investments. Their data indicate that between 1850 and April 2009, the value of professional-quality instruments rose in real terms (i.e., after inflation) approximately 3 % annually. High-end violins have appreciated at much higher rates, particularly the rare instruments made by Italian masters such as Stradivari, Amati, and Guarneri del Gesù. Since the beginning of the nineteenth century, Stradivarius violins have been compared with contemporary instruments made by other violin makers in so-called blind tests, the most serious of all probably being that organized by the BBC in 1974. In that test, the world famous violinists Isaac Stern and Pinchas Zukerman, together with the English violin dealer Charles Beare, were challenged to identify blind the “Chaconne” Stradivarius made in 1725, a “Guarneri del Gesu” of 1739, a “Vuillaume” of 1846, and a modern instrument made by the English master violin maker Roland Praill. The result was rather sobering; none of the experts were able to correctly identify more than two of the four instruments, and in fact, two of the jurors thought that the modern instrument was actually the “Chaconne” Stradivarius.

In a blind test at the Osnabrücker Baumpflegetage in 2009, the British star violinist Matthew Trusler played five different instruments behind a curtain so that the audience did not know which was being played. One of the violins Trusler played was his own “Strad,” worth US $2 million. The other four were all made by Michael Rhonheimer: two from mycowood and the other two from untreated wood. A jury of experts, together with the conference participants, judged the tonal quality of the violins. Of the more than 180 attendees, an overwhelming number (90 persons) considered that the tone of the fungally treated violin “Opus 58” to be the best. Trusler’s Stradivarius reached second place with 39 votes, but, amazingly, 113 members of the audience thought that “Opus 58” was actually the Strad! “Opus 58” was made from wood that had been treated with fungus for the longest time, 9 months. In comparison with the untreated wood instruments, the violins made of mycowood had a warmer, rounded mellow sound. Many participants at the Osnabrücker Baumpflegetage stressed that the high notes of the untreated violins are irritating

on the ear, unpleasant, and shrill, whereas these high notes of the treated wood violins were dampened and the violins sounded more mellow and warmer. The quality of the resonance wood is very important for the acoustic quality of the violin. The method described here for modifying wood is intended primarily to allow better solo instruments to be made. A solo violinist prefers an instrument that is suitable for playing “against” the orchestra. Its tonal properties include high projection ability, high volume, and dynamic range, together with a sensitive modulation of tonal colors. These properties directly depend on the material quality of the resonance plates of the violin. This quality correlates positively with the velocity of the longitudinal sound waves (both across and along the grain) and negatively with wood density. A material with a high ratio of sound velocity to density increases the sound emission of the instrument, which means that the plate amplitudes are high in relation to the force that excites the strings. This enhancement of resonance makes the difference between a violin of average quality and one that is suitable for a top soloist. Future acoustic instruments, made from wood modified by the described procedure, are desirable because of the enormous size of today’s concert halls and the needs of soloists. Instead of seeing the forest as a mass source of low-grade pulp wood that yields only a few US dollars per cubic meter, the trees can be nurtured to their highest potential, where a single violin top (a piece of wood 2.5 cm thick, 6 cm wide, and 35 cm long) can command a price of hundreds of US dollars. That same piece of wood, after leaving the hands of a skilled violin maker, may then be priced in the many thousands of US dollars. Admittedly, not all trees will measure up to these standards, and a large forest industry cannot rely on the demand from violin makers alone. But the point is that it is the highest possible potential of the forest that should be kept in mind as the guiding principle of forest stewardship, not necessarily the most immediate short-term liquidation of the resource, which seems to have been the objective of much of the forest policy of the past. The mycowood method described here allows improvement in the acoustic properties of resonance wood at a time when it is becoming increasingly difficult to find naturally grown, superior-quality resonance wood because of the impact of global warming. For the professional musician, a violin or cello made in northern Italy’s Cremona is the ultimate status symbol. Prices can run as high as US $27,000 for a violin, double for a cello. As with other crafts in Europe, lutherie is coming under threat from inexpensively made Chinese products. Xiqiao in southeast China has more than 40 companies turning out a violin, bow and case for US $25. The Taixing Fengling Musical Instrument Co., one of the largest, made 300,000 violins, violas, cellos, and basses in 2009. To compete, Cremona and other European violin makers must compete on quality. The estimated costs for the production of mycowood (i.e., for a top and bottom plate) are currently approximately US $2,500. Thus the mycowood technology meets the economic criteria and enables acoustically superior instruments with same tonal quality to be available to talented young musicians who would never be able to afford their own Stradivarius. These studies have sparked the worldwide interest of the media, music lovers, and violin makers. A number of companies that produce acoustic panels and resonance wood suppliers are interested in using wood decay fungi to improve the acoustic properties of wood, which will revolutionize the

use of wood in traditional musical instrument making of guitars, violas, hammered dulcimers, acoustic panels, and/or wood cones for loud speakers. The appreciation of wood in the world of acoustics and sounds will continue to increase. Thus, wood can improve its competitive edge over synthetic materials such as carbon fibers that are increasingly being used in musical instrument making. Further studies are currently in progress with the objective of developing a quality assurance program to maximize the probability that minimum standards of quality are being attained by the mycowood process.

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Lactone Formation in Yeast and Fungi

15

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Abstract

Lactones are important secondary metabolites for fungi. In this chapter are presented some lactones that are important in biotechnology such as flavoring lactones or fragrance macrocyclic musk compounds, whereas others are important for quorum sensing and health (mycotoxins). Different pathways or enzymes can give rise to lactones, and the pathways going through β-oxidation and ω-oxidation and the fungal polyketide pathway (relatively similar to the fatty acid synthesis pathway) are presented as well as the activity of Baeyer–Villiger monooxygenases and lactonases and their potential use in biotechnology.

Keywords

Lactones • Macrocyclic musk fragrances • Butyrolactone • Quorum sensing • Mycotoxins • Patulin • Aflatoxins • β-Oxidation • ω-Oxidation • Polyketide synthase • Baeyer–Villiger monooxygenase • Lactonase

List of Abbreviations

6MSA	6-Methylsalicylic acid
AHL	Acyl-homoserine lactone
AF	Aflatoxin
AFL	Aflatoxicol
ATP	Adenosine triphosphate
BVMO	Baeyer–Villiger monooxygenase
CDK	Cyclin-dependent kinase
DALs	Dihydroxyphenylacetic acid lactones
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
FAS	Synthesis of fatty acids
HSP	Heat shock protein 7
IC_{50}	Half maximal inhibitory concentration
MAP, MAPK, MAPKKK	Mitogen-activated protein kinase (MAP), kinase (K)
mRNA	Messenger ribonucleic acid
PKS	Polyketide synthase
QS	Quorum sensing
RALs	Resorcylic acid lactones
TAK	Transforming growth factor-activated kinase
ZAL	Zearalanol
ZAN	Zearalanone
ZEN	Zearalenone
ZEL	Zearalenol

1 Introduction

The lactone function is characterized by the presence of an ester in a cycle. A lactone is thus an oxygenated heterocycle resulting from the cyclization (or lactonization) of hydroxy acids. γ-Lactones and δ-lactones, also corresponding to 4- and 5-olides, represent the two structures most frequently identified in the composition of aroma compounds. They result from the cyclization of acids hydroxylated in 4 or 5. But there are several other groups of lactones exhibiting various important properties. Among them are volatile lactones exhibiting flavoring and perfuming properties as well as lactones involved in cell-to-cell communication, but lactones can exhibit other bioactivities such as antimicrobial, anti-inflammatory, and anticancer ones. Fungi are able to produce several types of lactones. Yeast is the reference organism for the biotechnological production of musk macrocyclic lactones and of the flavoring γ-decalactone. Besides, communication homoserine lactones, although having been more studied in bacteria, exist also in fungi. Less-known lactones exhibiting antimicrobial effects have also been identified in fungi. Eventually, fungal polyketide synthases are versatile tools to produce lactones.

This chapter aims to introduce pathways for lactone formation in yeast and other fungi, and, after giving a short introduction on the principles of lactone formation, it will present the pathways for the production of the various lactones cited above.

2 General Ways of Lactone Formation

Lactone can result from different enzymatic pathways, and three main biosynthetic pathways are presented in this part. Some of these ways (β- or ω-oxidation) can be related to the main metabolic pathways of oxidation of lipids in fungi (Fig. 1). Main pathways for catabolism of hydrophobic compounds give potentially rise to lactones, while another is specific for the synthesis of polyketides. Besides, some enzymes involved in various pathways (Baeyer–Villiger monooxygenases, lactonases) can also catalyze the synthesis or hydrolysis of lactones.

2.1 Lactone Resulting from Intra-esterification of Hydroxy Acids

This pathway is a very common way to get lactones [1]. When the hydroxyl group of a fatty alcohol can be in contact with the hydroxyl from the acid group, esterification can occur readily, especially in acidic conditions.

Different types of enzymes can be involved in this reaction. First, fatty acids have to be hydroxylated which is catalyzed by oxygenases, hydratases, or hydroxylases. Monooxygenases are available in the ω-oxidation pathway. They can oxidize alkanes at one end of the molecule or fatty acids in the ω-end of the molecule.

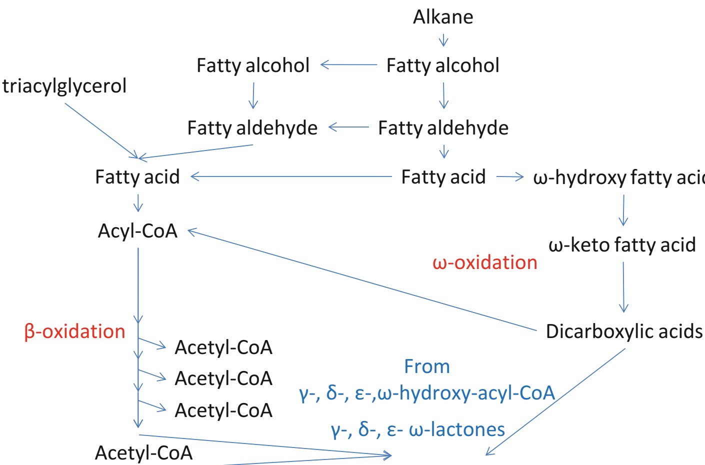


Fig. 1 Main pathways for catabolism of hydrophobic compounds giving potentially rise to lactones

Their role in metabolism and in biotechnology will be given in the part on macrocyclic lactones, and some fundamental or applied aspects have already been given in [2, 3]. Beside these α- and ω-regiospecific enzymes, there is a great deal of cytochrome P450 enzymes catalyzing oxidation of various substances [4]. However, in the case of fatty acid hydroxylation, most enzymes are not specific, giving rise to several different hydroxy acids with low yields. For instance, *Mortierella* sp. were used to transform caprylic acid into octalactone but gave rise to several different lactones [5].

If the hydroxyl group is not well located to react with the acid group, β-oxidation can occur first, resulting in a shortening of the distance between the two groups. This oxidation system involves a set of four reactions occurring in a cyclic way on energized fatty acids, acyl-CoA (Fig. 2). At each cycle, the length of the fatty acyl decreases of two carbons and an acetyl-CoA is created. Through this mechanism, a 10-hydroxylated fatty acid like ricinoleic acid **1** on Fig. 2 can be shortened of 6 or 8 carbons (3 or 4 β-oxidation cycles), giving rise to an ε- or γ-lactone, respectively. In the set of β-oxidation enzymes, there is an enoyl-CoA hydratase catalyzing the 3-hydroxylation of the acyl-CoA which is later dehydrogenated into its ketone. When lactonization occurs during this β-oxidation cycle, it can thus give rise to functionalized lactones. The catabolism of ricinoleic acid **1** on Fig. 2 giving rise to various lactones has been discussed in [6], and more information on biotechnological applications of β-oxidation will be given below, in the part on flavoring lactones.

In many cases, intra-esterification occurs readily, but some esterases have also been shown to exert a lactonase activity catalyzing cyclization or the opening of the lactone cycle. Actually, hydrolysis has been more investigated as it is involved in the catabolism of active compounds, whereas for esterification it is often difficult to state whether it has occurred readily or through an enzymatic catalysis. Readers interested in microbial lactonase activities can find information in a review on this subject [7].

Fig. 2 β-Oxidation and synthesis of lactones (**a**) and synthesis of lactones from intermediates of β-oxidation cycle (**b**)

2.2 The Polyketone Pathway

Beside the hydroxyl fatty acid pathway, a very common pathway in the synthesis of lactones is through the polyketide synthase pathway [8]. Polyketides are a family of complex secondary metabolites built from carboxylic acid building blocks. In microorganisms, they are produced by large, multifunctional proteins termed polyketide synthases (PKS). This pathway involves a set of basic reactions that are often

compared to the synthesis of fatty acids (FAS) as there is a start with a keto-synthase with an acyl group which condensates with acyltransferase-catalyzed loaded malonyl units onto an acyl carrier protein. Ketoreductase, dehydratase, and enoyl-reductase catalyze the processing of the compounds which is eventually terminated by a thioesterase. In fungi, this pathway has first been observed in the synthesis of patulin (see below) but is also at the origin of aflatoxins and many compounds. The better understanding of the PKS pathway in fungi enabled evolutionists to investigate the relationship between the various PKS and FAS systems [9]. This confirmed that the iterative fungal PKS-I system is directly related to the animal FAS-I system and far from the fungal FAS-I system.

2.3 Baeyer–Villiger Monooxygenases

Baeyer–Villiger (BV) oxidation consists in the transformation of a linear or cyclic ketone into its corresponding ester or lactone by insertion of an oxygen atom next to the carbonyl group (Scheme 1). It is catalyzed by Baeyer–Villiger monooxygenases (BVMOs, EC 1.14.13.x), which were first isolated in the 1960s, and their encoding genes, identified in the 1990s. There are different types of BVMO but the majority is sequence related (type I BMVOs), and they belong to the subclass B flavoprotein monooxygenases; the FAD cofactor is the prosthetic group, and they depend on NADPH as electron donor. An exhaustive review on the subject has been published some years ago [10].

The role of this enzyme is not fully elucidated, but it is remarkable that it has been only described in microorganisms where BMVO-specific protein sequence motifs have been found in each microbial genome investigated. However, some specific

Scheme 1 Degradation of limonene by the mountain pine beetle-associated pathogen *Grosmannia clavigera* (Adapted from Wang et al. [13])

filamentous fungi possess families of BMVO-encoding genes. These enzymes can fulfill a variety of functions, such as catabolic properties enabling microorganisms to grow on and degrade various ketones (shown for *Candida* sp. in [11]), cyclic alkanes, alcohol, or terpene into dicarboxylic acids (shown for bacteria in [12] and for *Grosmannia clavigera* in [13] (Scheme 1)), and, as shown below, they are involved in the synthesis of secondary metabolites.

3 Lactones as Flavors and Perfumes

3.1 Lactones as Flavors

3.1.1 Historic of Production

Short- and medium-sized length lactones resulting from the esterification of hydroxy fatty acids are an important family of aroma compounds. From butyrolactone (butyrolactone corresponds to two different molecules depending on the field, flavors, or quorum sensing; here it stands for molecule **3** in Fig. 2 with R = 0, while in the field of quorum sensing, butyrolactones correspond to lactones with a 4-carbon ring (or γ-lactones or 4-olides) like butyrolactone-I **10**) to C12 γ- or δ-lactones that can be functionalized or desaturated, there are several lactones possessing flavoring properties especially with fruity, fatty, and oily notes (Fig. 3). These lactones are present in many fruits where they are likely to result from the 1.1 pathway shown above. Yeasts are also able to synthesize these compounds that can be encountered in fermented food such as bread, beer, or whisky [14]. During fermentation, the pathway of synthesis involves first a hydroxylation step which can be fulfilled by lactobacilli before the β-oxidation step carried out by yeast [15]. With similar ways of synthesis from plants and

δ-decalactone: peach, oily, creamy

4 6-pentyl-α-pyrone: coconut

5 δ-jasminolactone: oily, fruity, floral, jasmin, peach

6 γ-octalactone: coconut, caramel

γ-nonalactone: coconut, fatty, fruity, aniseed

γ-decalactone: peach, fatty, fruity

7 γ-dodecalactone: peach, butter, fatty, musk

Fig. 3 Some lactones possessing flavoring properties and their characteristic notes

from yeasts, these latter catalysts have been employed to produce lactones with the natural label from the first observation of their capability to produce lactones which occurred from investigation of the metabolism of hydroxy fatty acids. When using *Candida* sp., γ-decalactone **2** accumulated during the catabolism of ricinoleic acid (hydroxylated in C10) [16]. In this study, ricinoleic acid **1** had been chosen because it was the only hydroxy acid available at low cost and in high amount. Indeed, due to a specific evolution in castor beans, castor oil possesses about 90 % ricinoleyl moieties. From ricinoleic acid, γ-decalactone will be produced. This lactone benefited from technology developments becoming the aroma compounds the most produced through biotechnology. Its cost followed the trend from a fine chemical price (around $12000/kg in 1986) to a low cost of natural aroma compounds ($500/kg in 1998) [17]. The development of research to produce lactones through biotechnology has also to be related to the consumers' trend toward natural compounds. There has been a first period of interest in the 1980s–1990s which followed the demand of European consumers (mainly in German-speaking countries in the beginning). During this period, most pathways of synthesis of aroma compounds had been investigated, and only some had been selected as potential sources of realistic price compounds [3]. Other lactones produced at this time were 6-pentyl-α-pyrone **4**, δ-jasminolactone **5**, γ-octalactone **6**, γ-dodecalactone **7**, δ-dodecalactone, etc. (Fig. 3). Finally, after a time of decrease in the interest, activities in lactone production began to rise again with the world interest for natural flavors in the 2000s. In the meantime, the price decreased again, making it more difficult for new companies aiming at beginning production. Moreover, despite some rare new biotechnological strategies or the identification of some efficient catalysts, most of the strategies published in the present period cannot address the real issues in the field which are described in the next part. Instead of this, most of the works published recently concern the optimization of production with a specific medium or the repetition of previous studies on the effect of aeration or on the performances of *POX*-mutants from *Yarrowia lipolytica*.

3.1.2 Limiting Steps of Lactone Production

Hydroxylation

As shown above, from fatty acids hydroxylated at various carbons, it would be possible to produce several lactones. However, production is limited to natural precursors that are already available at a low cost, such as ricinoleic acid **1**, and to precursors that can be specifically hydroxylated, such as unsaturated fatty acids that can be hydroxylated by some fungal activities. The ability to hydroxylate a fatty acid at a specific level and with good yields could have a high impact on lactone production (see [1] for review), but this step, which has been a real challenge in the first period of development of lactones, does not receive much attention now. Fungi able to hydroxylate before carrying out β-oxidation could be particularly interesting as the two steps of biotransformation could be carried out in one reactor. Some examples are shown in Fig. 4. Another strategy to overcome this problem is to

Fig. 4 Lactones produced from non-hydroxylated fatty acids (Adapted from Romero-Guido et al. [1])

caplyric acid → γ-octalactone R=4
linoleic acid → γ-nonalactone R=5
linoleic acid → 6-pentyl-α-pyrone R=5
linolenic acid → δ-jasminlactone
oleic acid → γ-dodecalactone R=8

modify plant substrates to make them produce hydroxylated fatty acids. Indeed, in the case of castor beans, it is remarkable that it is only a 4-amino acid evolution which changed the desaturase activity into a hydroxylase one [18]. From these facts, it seems promising to modify further enzymes of the fatty acid synthetic pathways to get a higher diversity of plant fatty acids.

β-Oxidation Control

The other crucial point is to control the flux of β-oxidation in yeast cells. Indeed, β-oxidation can go on after the stage of lactone formation, or the synthesized lactone can be further degraded by the producing cells. The fluxes of production have thus been investigated with several models for the production of γ-decalactone. Some interesting works have shown with *Sporobolomyces* sp. that several different models of channeling were present in the different strains tested [19]. In the meantime, works were carried out with mutant of the yeast *Yarrowia lipolytica*. This species is, for many producers, the γ-decalactone-producing species. It is also a reference species for the study of lipid metabolism as it possesses many multigene families, and it can be genetically manipulated [2]. The role of the various enzymes has thus been characterized [20–22], enabling to genetically engineer new strains [23] and to study fluxes in this species [24].

A physicochemical approach was also used to decrease both the lactone toxicity toward and the lactone degradation by the producing cells. Lactone was trapped using various materials exhibiting affinity, and a reactor was designed [25–27].

Finally, these studies show a diversity of yeast behavior with some strains exhibiting very efficient β-oxidation that requires non-favorable conditions to produce lactones [1, 3, 6, 28–30], whereas other strains exhibit a channeled production with an increased production in better conditions (Alchihab, personal communication).

Recently a strain of *Waltomyces lipofer* exhibiting no limitation of lactone production has been reported and patented [31, 32]. Depending on the hydroxylated

substrate, this strain was able to produce γ-dodecalactone, γ-decalactone, and γ-butyrolactone. This strain revolutionized the knowledge acquired on lactone production as (i) it was not sensitive to lactone toxicity and could produce more than 50 g/l of the highly toxic γ-dodecalactone; (ii) it could produce in a state of permeabilization with a 70 % ethanol treatment and other drastic treatment which did not seem to alter the β-oxidation pathway and all the cofactor regeneration required, if this pathway was also the one involved in this strain; and (iii) in some cases, accompanying the 100 % conversion of hydroxy acid into lactone, the cell produced de novo about 20 % of the same lactone from glucose, reaching yields about 1.16 to 1.22. This latter point suggests the ability to produce C10 hydroxylated fatty acids by this yeast.

3.2 Macrocyclic Musk Lactones

3.2.1 Macrocyclic Musk Fragrances

Musk-like fragrances have been used from the late antiquity and still have wide applications in the world of perfumes as bottom notes exhibiting warm, sweet, powdery, or animal notes that are long-lasting, tenacious, and substantive. They are particularly interesting in cosmetics where musk is among the most popular perfumes for shower gels and deodorants [33]. Three main categories of compounds exhibit these musk notes: aromatic nitro-musks, polycyclic musk compounds (e.g., galaxolide, which, with its low cost and strong and sweet floral musk smell, has been used in many perfumes), and macrocyclic musk compounds. The two first groups are used in the cosmetics and detergent industries, but their detection in human tissues and in the environment in addition to a suspicion of carcinogenic properties gave rise in the 1990s to a public debate on safety concerns resulting in their progressive replacement by compounds belonging to the 3rd group.

This latter group consists of macrocyclic ketones and lactones that are synthesized from fatty acids (Fig. 1). The compounds responsible for these sensorial notes were at the origin extracted from glands of animals such as the Asian musk deer (*Moschus moschiferus*, Moschidae) for macrocyclic ketones and from plant sources for macrocyclic lactones. From animal origin, the price was very high as about 30–50 animals had to be sacrificed to get one kg of musk grains (without the possibility of eating the meat due to the strong musk odor). Despite the high price, this source was common in many popular perfumes until the protection in 1979 of musk deer as an endangered species (CITES). In plants, macrocyclic lactones (or macrolides) were isolated from angelica root (e.g., 15-pentadecanolide), ambrette seed oil, galbanum resin, orchids, etc. Although most of these ketones and lactones were considerably more expensive than musk fragrances from the two other groups, interest to them increased with the process of replacement of synthetic compounds that begun in the 1990s. However, as the synthesis of precursors of macrocyclic ketones and lactones was possible through a fungal metabolic pathway, yeast cells were rapidly preferred to plant and animal extraction for production. A set of compounds produced through yeast biotechnology was soon available with ketones

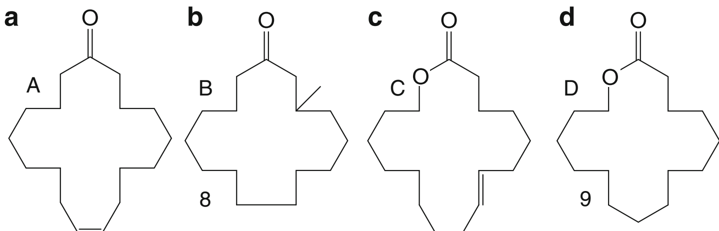


Fig. 5 Some macrocyclic musk produced commercially with their commercial names. (**a**) Civettone or civetone; (**b**) muscone; (**c**) ambrettolide; (**d**) exaltolide, muskalactone, pentalide, thibetolide (Adapted from Sommer [34])

such as muscone **8** and exaltone resulting from α,ω-dicarboxylic acids and macrocyclic lactones resulting from acid–alcohol. The pentadecanolide **9** isolated from angelica root was thus proposed by several fragrance producers under different commercial names (e.g., exaltolide, thibetolide, macrolide, pentalide, etc.) (Fig. 5). The fragrance of the compounds depends on their structure as well as on their chain length. With 14 carbons, a weak musk scent is exhibited, but the musk odor is strong and nice with 15- and 16-carbon compounds [34].

3.2.2 Pathway of Synthesis

The complete pathway of synthesis of macrocyclic molecules goes through the synthesis of a 15– 16-carbon-long fatty acid that is oxidized into ω-hydroxy fatty acids or α,ω-dicarboxylic acids and then cyclized into ketone or lactone macrocycles. The pathway of production is rather similar for the homologous cycles exhibiting animallike fragrances and for the heterologous cycles exhibiting plantlike fragrances, the first one going through the intra-esterification of ω-hydroxy fatty acids and the second one through the one of α,ω-diacids (Scheme 2). From Baeyer's strain theory and entropy studies, it has long been thought that such molecules with ring sizes over seven carbons were impossible to get [147]. However, they were not only present in nature but also possible to synthesize with the help of fungi catalysis. It must be stressed, however, that, contrary to the flavor lactones presented above, macrocyclic compounds result from a minor metabolic pathway which gives only rarely rise to macrocyclic ketones or lactones in the culture medium for wild-type yeasts. This pathway, through ω-oxidation (Fig. 1 and Scheme 2), enables the cell to oxidize alkanes and fatty alcohols into fatty acids that can enter the β-oxidation pathway. However, beside β-oxidation, alkanes and fatty acids can undergo the ω-oxidation pathway which is catalyzed by a cytochrome P450 oxygenase encoded by an *ALK* gene. The third step of the pathway, cyclization, is based on the esterification of the alcohol–acid or diacid molecule. This reaction occurs readily in many conditions, but the intra-esterification giving rise to macrocyclic compounds is competing with interesterification which results in polymers. The conditions and concentrations of precursors must be carefully chosen to favor the first reaction. It is also possible to favor lactonization with biocatalysis as several fungal lipases exhibit lactonase activity.

ω-oxidation ω-oxidation

intramolecular esterification
(non yeast-catalysed or
fungal-lipase catalysed)

Scheme 2 Pathway for the synthesis of macrocyclic lactone and other α,ω-cyclic fragrances from a carboxylic acid

3.2.3 Biotechnological Developments

Due to the growing interest in natural and safe macrocyclic musk fragrances and to the limitation in the possibility of extraction from animal or plant tissues, biotechnologists have begun to imagine new ways to obtain these compounds in the beginning of the 1990s. The starting materials were natural C15 and C16 fatty acids available at a relatively low cost. The biocatalytic part began at this stage with yeast ω-oxidation. As mentioned earlier, this pathway is rather a minor one, and C15 or C16 fatty acids are likely to be oxidized in the β-oxidation loop in most yeast possessing an efficient ω-oxidation biocatalytic potential. As a result, the pioneering work by Picataggio [35, 36] consisted in engineering genetically a strain of *Candida tropicalis* to block the β-oxidation pathway and, in the meantime, to amplify the ω-oxidation monooxygenase enzyme. To block the β-oxidation pathway, the *POX* gene encoding the enzyme catalyzing the first reaction, the acyl-CoA oxidase (Aox) was deleted. As this strain possessed two copies of a family of two *POX* genes (*POX4* & *POX5*), the authors had to delete 4 genes. This first step was required to avoid degradation of the macrocycle precursors in this pathway, but, to increase the ω-oxidation pathway, the monooxygenase encoding gene (*ALK1*) and the *CPR* genes coding for the NADPH–cytochrome P450 reductase had to be amplified. This strategy was a success which limited the access to yeast macrocyclic musk to competitors. A second group in Japan working with a related strain belonging to *Candida maltosa* investigated another strategy based on repeated mutagenesis and screening for higher dicarboxylic acid production. They selected strains overproducing dicarboxylic acids, and, through analysis of the resulting strains [37], it was shown that the overproducing strain of *C. maltosa* exhibited decreased level of β-oxidation proteins and an increased induction of synthesis of Alk proteins in the presence of alkanes.

4 Lactone for Quorum Sensing (QS) in Yeast and Fungi

4.1 Introduction to Lactones for Quorum Sensing

Quorum sensing (QS) is a phenomenon of the microbial communication whereby the accumulation of certain chemical compounds (signal molecules) enables a single cell to sense the population density. This phenomenon is widespread in microbial communities and mostly studied in bacteria. QS enables bacteria to coordinate gene expression according to the density of their local population and to coordinate certain of their behaviors such as biofilm formation, virulence, and antibiotic resistance. These responses include adaptation to availability of nutrients, defense against other microorganisms which may compete for the same nutrients, and the avoidance of toxic compounds potentially dangerous for bacteria. Quorum sensing is also prevalent in the unicellular (yeast) and filamentous fungi. It has been observed fifteen years ago with the discovery that farnesol controls filamentation in the pathogenic polymorphic fungus *Candida albicans* [38] and that phenylethanol and tryptophol stimulate morphogenesis and pseudohyphal growth formation in *Saccharomyces cerevisiae* [39]. Furthermore, quorum-sensing mechanisms are reported in various filamentous fungi including *Aspergillus nidulans*, *Aspergillus terreus*, *Penicillium chrysogenum*, and *Penicillium sclerotiorum* [40–42].

Lactone-containing compounds are widespread in nature and are involved in acting as signaling molecules in bacteria and fungi. A large group of QS signals including lactone-containing molecules such as acyl-homoserine lactones (AHLs), butyrolactone-I **10**, and γ-heptalactone **11** are found in several gram-positive and gram-negative bacteria and filamentous fungi (e.g., *A. nidulans*) [43]. AHLs, composed of a lactone ring and different-length and different-substituent acyl side chain, are the major class of QS molecules in bacteria and are produced by more than 50 different bacterial species. Each AHL is catalyzed by a specific AHL synthase enzyme belonging to the *LuxI* family and corresponds to a particular cytoplasmic DNA-binding regulator *LuxR*-type protein in bacteria such as *Pseudomonas aeruginosa* [44]. At high cell densities, the accumulated autoinducer AHLs bind to regulatory proteins *LuxR*, and then this complex recognizes and binds specifically to a QS-regulated promoter, thus activating the transcription of target genes (DNA sequences) and inducing a particular QS response [45].

Other lactone-containing compounds such as γ-butyrolactone are found as signaling molecules in filamentous bacteria *Streptomyces* sp. [46]. The high similarities between the filamentous bacteria and filamentous fungi triggered researchers to investigate the presence and role of γ-butyrolactone-containing molecules in filamentous fungi [43]. It was later reported that several γ-butyrolactone-containing molecules such as butyrolactone-I **10**, γ-heptalactone **11**, and multicolanic acid **12** act as putative QS molecules in filamentous fungi.

The study of quorum sensing belongs to microbial ecology and population biology. It is still very little known about the mechanisms of synthesis and

metabolism of signaling lactone-containing molecules involved in different bacterial and fungal species. The understanding of the above phenomena could have great potential for enhancing industrial production of commercially useful bacterial and fungal products. The genetic manipulations of the genes involved in the lactone QS and its metabolism processes could result in generation of engineered bacterial and fungal strains having implication in medicine, agriculture, and biotechnology with more specificity, i.e., antibiotic therapy, preventative therapy for plant disease, biosynthesis of antibiotics, and luminescent biosensors.

4.2 Lactone-Containing Molecules for Quorum Sensing in Fungi

The lactone-containing molecules for QS phenomenon in filamentous fungi have only recently been observed; however, the criteria for existence of this system in fungi are based on proposals which are particularly verified in bacteria and yeast [47]. One of the fundamental characteristics of quorum-sensing signaling molecules is the increase in concentration as the microbial population grows and the subsequent autoinduction when the population density threshold has been reached, which ensures the correct timing of the physiological response [48]. For a molecule to qualify as a quorum-sensing entity, it should satisfy some critical characteristics: the molecule should be produced throughout the growth of the organism; however, the quorum-sensing response is only initiated at a certain stage of the growth [49]. It is at this stage of growth that the increase of the QS molecule reaching a specific concentration alters a coordinated response in the entire population's behavior, i.e., secondary metabolite production. In many fungi such as *Aspergillus nidulans*, *Aspergillus terreus*, *Penicillium chrysogenum*, and *Penicillium sclerotiorum*, oxylipins and lactone-containing molecules have been considered as signaling molecules and are reported to induce physiological responses including morphological changes, sporulation, and secondary metabolite production including mycotoxins and antibiotics [40–42]. Table 1 provides further evidences of various lactone-containing signaling molecules utilized by fungi and their biological functions.

Table 1 Filamentous fungi and lactone-containing QS with their corresponding target functions

Filamentous fungi	QS lactone-containing molecules	Physiological response	References
Aspergillus terreus	Butyrolactone-I **10**	Hyphal branching, submerged sporulation, secondary metabolite production (lovastatin and sulochrin)	[40]
Aspergillus nidulans	γ-Heptalactone **11**	Increases penicillin production	[50]
Penicillium sclerotiorum	Multicolanic acid **12** and derivatives	Sclerotiorin production (antibiotic)	[51]

4.2.1 Butyrolactone-I

Butyrolactone-I **10** is produced as a secondary metabolite by *Aspergillus terreus*. Because small butyrolactone-containing molecules act as self-regulating factors in some bacteria, the effects of butyrolactone-I addition on the producing organism, specifically changes in morphology, sporulation, and secondary metabolism, were recently studied [40, 52]. Threefold or greater increases in hyphal branching, submerged sporulation, and secondary metabolism were observed when butyrolactone-I was added to cultures of *A. terreus*. Schimmel and co-workers observed that butyrolactone-I acts as a signaling molecule to enhance the production of the secondary metabolite lovastatin (threefold increase) and sulochrin (twofold increase) in similar growth conditions when compared to the control without butyrolactone-I addition [40]. It was also found to have an auto-stimulatory function as well as induction of lovastatin biosynthetic genes [51]. Lovastatin is known therapeutically important for the prevention of cardiovascular disease [53, 54], and sulochrin has weak antibacterial and antifungal properties [55]. Furthermore, these findings indicate that butyrolactone-I induces morphological and sporulation changes in *A. terreus* and enhances secondary metabolite production in a manner similar to the changes that were observed with small γ-butyrolactone-containing molecules in filamentous bacteria of the genus *Streptomyces*. This observation is in accordance with the idea that butyrolactone-I may function as a QS molecule in *A. terreus*. The practical application of these studies is the possibility that butyrolactone-I could be used to increase or promote the production of desired secondary metabolites in *A. terreus*, i.e., lovastatin and sulochrin production. Moreover, the mechanism by which butyrolactone-I is produced or diffused out of the fungal cells during the growth process is not known.

Butyrolactone-I **10** γ-Heptalactone **11** Multicolanic acid **12**

Besides, butyrolactone-I is known as an antitumor and anticancer molecule. Indeed, it is a potent and selective inhibitor of the cellular roles of cyclin-dependent kinase (CDK) enzymes, specifically inhibiting Cdk2 and Cdc2 kinase [56]. CDKs are protein kinases that control cell cycle progression in all eukaryotes and are regulated by phosphorylation and dephosphorylation of critical serine, threonine, or tyrosine residues. The inhibitory effect of butyrolactone-I due to competition with ATP binding at CDK is to block the phosphorylation of the transcription factor E2F-1. Therefore, it inhibited Cdc2 of unsynchronized cultured prostate cancer cells and interrupted the cell cycle progression toward cell division [57].

4.2.2 γ-Heptalactone

Another γ-butyrolactone-containing molecule, γ-heptalactone **11**, is an endogenously produced QS molecule regulating growth and secondary metabolite production by *Aspergillus nidulans* [50] that is a filamentous fungus well known for its ability to produce the secondary metabolite penicillin [58]. This fungus produces γ-heptalactone at a high cell density, and it can alter the organism's behavior at a low cell density, i.e., altering the organism's growth profile by shortening the lag phase. It also induces the production of the secondary metabolite penicillin. Indeed, the addition of this γ-butyrolactone-containing molecule to the wild-type *A. nidulans* strain led to a 31.9 % increase in penicillin production [50]. Because fungi coexist with bacteria in the environment, so they must rely on chemical defense mechanisms due to their lack of an active immune system. It can be suggested that *A. nidulans* has adapted a QS process and uses a range of regulatory circuits to adjust gene expression and coordinate cell-to-cell interactions.

The identification of γ-heptalactone as a QS molecule in *A. nidulans* can be further explored and hence exploited by the biotechnology industry to enhance yields of penicillin production. In flavor and fragrance industries as shown above, this lactone is widely used in peach, nut, maple, almond, caramel, and cream flavors, for a creamy finish in most vanilla, and in coconut and gardenia fragrances.

4.2.3 Multicolanic Acid 12 and Derivatives

Multicolanic, multicolosic, and multicolic acids were isolated by Gudgeon et al. [59] from *Penicillium sclerotiorum*. These compounds belong to a small group of chemicals called tetronic acid metabolites [60] which contain a γ-butyrolactone molecule. These γ-butyrolactone-containing compounds are synthesized by oxidative cleavage of an aromatic precursor 6-pentylresorcylate [61] and classified as hexaketides because of their polyketide origin [62].

In order to test whether γ-butyrolactone molecules produced by *P. sclerotiorum* exerted a physiological response in the cells, the effect of these potential quorum-sensing molecules on sclerotiorin production in these fungus was investigated [51]. This study suggests that addition of spent medium containing the putative quorum-sensing molecules has the ability to initiate production of sclerotiorin in a low-sclerotiorin-producing strain. The presence of γ-butyrolactone-containing molecules (multicolic acid, multicolosic acid, multicolanic acid, and related derivatives) in the spent medium increased sclerotiorin yield (6.4-fold). These data suggest that addition of γ-butyrolactone molecules had created an environment for the cells to respond similarly to the conditions where the threshold cell concentration was achieved, allowing for the expression of genes under quorum-sensing control [51]. However, the chemical structure of the molecule(s) responsible for the regulation of sclerotiorin was not precisely determined in this study.

The investigation in the effect of multicolanic acid and derivatives (i.e., dimethyl-*O*-methylmulticolosate, dimethyl dihydromulticolosate, and methyl-*O*-methylmulticolate acetate) as QS molecules in *P. sclerotiorum* open up a possible new way to enhance the ability of sclerotiorin production of this fungus. Thereon,

sclerotiorin is known as an aldose reductase inhibitor as well as a potent reversible lipoxygenase inhibitor [63, 64].

4.3 Perspectives

Few findings of lactone-containing compounds acting as quorum sensing on fungi have opened up a new front in further investigating this question with the potential for further basic and applied research. Once the importance of quorum sensing is established in pathogenic fungi and the mechanistic details are uncovered, the value of QS pathways as potential therapeutic targets can be assessed [65]. Hence, further elucidation of the mechanisms of QS in these pathogens and its effects on various metabolic pathways will lead to a better understanding of fungal pathogenesis facilitating the development novel antifungal approaches to combat human diseases. The mechanism of signal transduction in QS may be clarified by identification of the receptor proteins to which the γ-lactone binds to on the cell surface to enable signal perception.

As said above, the role QS lactones have in the organisms was more studied in bacteria than in fungi. For example, a mechanism involved in signal transduction from the detection of γ-lactone substrates/N-acyl-homoserine lactones (NAHSL) signals to the transcription of the *qsdA* operon in *Rhodococcus erythropolis*; an environmental gram-positive bacterium was illustrated in the review of Latour et al. [66]. A similar mechanism is presumed to control the qsdA operon with γ-lactone in the role of tetracycline [66] (Fig. 6). In the absence of a γ-lactone source, the QsdR (quorum-sensing signal degradation) regulator protein forms dimers that bind to the operator region, switching off the biosynthesis of catabolic enzymes. But the presence of γ-lactone binding to *qsdR*, a putative TetR family transcriptional regulator gene, changes the conformation and causes TetR detachment from the operator region and results in the expression of the gene encoding catabolic enzymes.

The industrial exploitation of QS lactone-containing molecules requires an optimization of their production from producing strains. The possible medium conditions were identified to maximize the production of butyrolactone-I from a butyrolactone-overproducing strain (Bty345) that had been derived by mutagenesis from *Aspergillus terreus* ATCC 20542 and selected for increased butyrolactone production which was available in the Merck (Elkton, VA) culture collection [67]. In this study, the yield of butyrolactone-I using optimized medium concerning the source and concentration of carbon and nitrogen represents a tenfold increase over the butyrolactone-I produced using the original, basic medium.

5 Lactone Mycotoxins and Other Bioactive Macrocyclic Lactones

The large group of lactones, apart from compounds that are flavoring components of food products (γ- and δ-lactones) [68, 69] or that reflect desirable aromas in the fragrance industry (coumarin, exaltolide) [70, 71] and that have been described

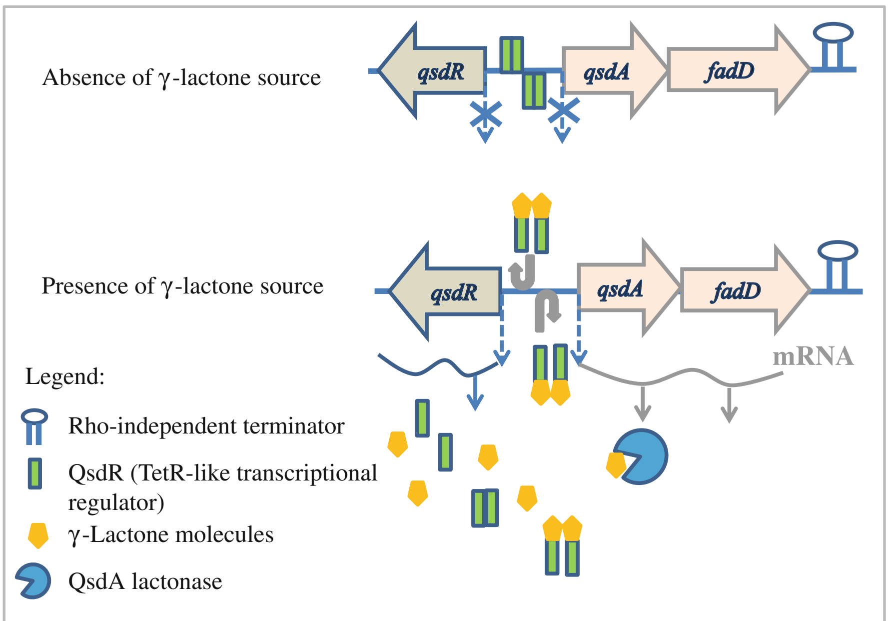


Fig. 6 The *qsdA* operon of *R. erythropolis* and its putative mechanism of regulation (Adapted from Latour et al. [66])

above, comprises lactones of diversified biological activity, including toxic (carcinogenic, teratogenic, mutagenic) and antitumor or anti-inflammatory effect.

Lactones having biological properties, isolated from natural sources, are currently a subject of study of many research centers. Research laboratories conduct ongoing works on the isolation and identification of active lactones, determine relationships between the structure of compounds and their biological properties, and in many cases synthesize analogs of these compounds characterized by a higher activity and stronger effect or try to conduct their inactivation.

5.1 Lactone Mycotoxins

Some of naturally synthesized lactones exhibit strong toxic activity. They are mainly compounds which are low-molecular-weight ($M < 1.5$ kDa) secondary metabolites of filamentous fungi, or so-called mycotoxins, of different levels of toxicity both to humans and to animals, plants, and microorganisms. Toxic lactones can be stored as endotoxins in mycelium and conidia or can be excreted to the medium. These compounds cause contamination of raw materials and products of the food industry, fodders, and food of animal origin. The synthesis of lactones by molds is determined both genetically (metabolism of amino acids or fatty acids) and phenotypically (environmental factors).

In the group of mycotoxins, most of the studies were devoted to aflatoxins, comprising approximately 20 heterocyclic difuranocoumarin derivatives (coumarin is a lactone of *O*-hydroxycinnamic acid) produced by toxigenic strains of *Aspergillus* fungi, especially *A. flavus*, *A. parasiticus*, and *A. nominus* [72]. The pathway of biosynthesis of aflatoxins comprises at least 23 reactions catalyzed by enzymes. So far it was possible to identify 15 intermediates of these reactions. Genetic studies on the mechanism of the synthesis of aflatoxins by *A. flavus* and *A. parasiticus* allowed for cloning 29 genes responsible for the formation of enzymes necessary for this metabolic pathway [72, 73]. Aflatoxins are classified into two broad groups according to their chemical structure, and they include the difurocoumarocyclopentenone series (AFB_1, AFB_2, AFB_{2A}, AFM_1, AFM_2, AFM_{2A}, and aflatoxicol) and the difurocoumarolactone series (AFG_1, AFG_2, AFG_{2A}, $AFGM_1$, $AFGM_2$, $AFGM_{2A}$, and AFB_3) (Table 2) [74, 75]. These compounds have closely related structures (Scheme 4). Aflatoxin B_1 is formed by, among others, a lactone ring, which is adjacent to a benzene ring and forms the same system as in coumarin, and two furan rings, including the extreme one with double bond. In aflatoxin G_1 **15** the extreme ring with the ketone moiety is enriched with one atom of oxygen to form a lactone ring. Aflatoxins B_2 **14** and G_2 **16** are hydroxyl derivatives of aflatoxins B_1 **13**

Table 2 The most important aflatoxin produced by the *Aspergillus* species [10, 12, 77]

Difuranocoumarins	Type of aflatoxin	*Aspergillus* species
Difurocoumarocyclopentenone series	Aflatoxin B_1 **13** (AFB_1)	*A. flavus, A. arachidicola, A. bombycis, A. minisclerotigenes, A. nomius, A. ochraceoroseus, A. parasiticus, A. pseudotamarii, A. rambellii*
	Aflatoxin B_2 **14** (AFB_2)	*A. arachidicola, A. flavus, A. minisclerotigenes, A. nomius, A. parasiticus*
	Aflatoxin B_{2a} (AFB_{2a})	*A. flavus*
	Aflatoxin M_1 **17** (AFM_1)	*A. flavus, A. parasiticus*
	Aflatoxin M_2 **18** (AFM_2)	Metabolite of aflatoxin B_2
	Aflatoxin M_{2A} (AFM_{2A})	Metabolite of AFM_2
	Aflatoxicol (AFL)	*A. flavus*, metabolite of AFB_1
Difurocoumarolactone series	Aflatoxin G_1 (AFG_1)	*A. arachidicola, A. flavus, A. minisclerotigenes, A. nomius, A. parasiticus*
	Aflatoxin G_2 (AFG_2)	*A. arachidicola, A. flavus, A. minisclerotigenes, A. nomius, A. parasiticus*
	Aflatoxin G_{2A} (AFG_{2A})	Metabolite of AFG_2
	Aflatoxin GM_1 ($AFGM_1$)	*A. flavus*
	Aflatoxin GM_2 ($AFGM_2$)	Metabolite of AFG_2
	$AFGM_{2A}$	Metabolite of $AFGM_2$
	Aflatoxin B_3 (AFB_3)	*Aspergillus* species not defined

and G_1 **15**, respectively, while aflatoxins M_1 **17** and M_2 **18** are 4-hydroxyderivatives of aflatoxins B_1 and B_2 [72]. Among the aforementioned mycotoxins, aflatoxin B_1 is the most toxic. It is classified by the WHO as a group 1 carcinogen. Based on the toxicity, carcinogenicity, and mutagenicity of mycotoxic lactones of the aflatoxins group, they are classified in the following order: $AFB_1 > AFM_1 > AFG_1 > AFB_2 > AFG_2$ [74, 76, 77].

Aflatoxin B_1 13 Aflatoxin B_2 14 Aflatoxin G_1 15

Aflatoxin G_2 16 Aflatoxin M_1 17 Aflatoxin M_2 18

The toxicity of these compounds is determined mainly by the lactone ring present in the coumarin moiety [78] and the double bond at position 8 and 9 of the furan ring. In the body, aflatoxins are transformed in the liver by cytochrome P450 enzymes into various metabolites and in case of AFB1 into particularly toxic AFB1-exo-8,9-epoxide (AFBO). These compounds interact with nucleic acids such as DNA or RNA and interfere with protein synthesis and glycolysis pathway. The formation of DNA adducts contributes to genetic mutations and cancer [77, 79].

The reduction of double bond in the extreme furan ring and the opening of the lactone ring and decarboxylation of the resulting –COOH group are substantially important to reduce the toxicity of aflatoxins. Inactivation of aflatoxins by ring opening can be conducted using, among others, acid or base hydrolysis. Additionally, the increase in temperature under these conditions to approx. 100 °C results in the removal of the methoxy group from the aromatic ring. Other chemical factors which cause a decomposition of aflatoxin structure are sodium hypochlorite, chlorine, and oxidizing agents such as hydrogen peroxide, ozone, and sodium metabisulfite [75].

The group of mycotoxic lactones comprises also patulin **19** produced by fungi of the *Penicillium* and *Byssochlamys* species. This compound was first isolated in 1940 from the culture of *Penicillium patulum*. In terms of chemical structure, patulin is a bicyclic lactone of the name 4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one, soluble in water [80, 81]. This compound is a polyketide metabolite, the first for which the polyketide pathway has been characterized, synthesized in a

Scheme 3 Pathway of synthesis of patulin in *Penicillium* and *Aspergillus* sp. (Adapted from Puel et al. [82]). The Pat enzyme-encoding genes are organized in clusters in many fungi

10-step pathway, starting from 6-methylsalicylic acid (6MSA compound **20** in Scheme 3) formed by the condensation of acetyl-CoA with three units of malonyl-CoA (Scheme 3). The reaction is catalyzed by a multifunctional enzyme, composed of four identical polypeptide chains of 176 kDa each, having the activity of acetyl- and malonyltransferase, ketoacyl synthase, ketoreductase, and dehydratase [82].

In the initial period of the study, patulin was tested for antibiotic properties, but because of its strong neurotoxic and teratogenic activity discovered in a later period, it was excluded from clinical use and in 1960 qualified as a mycotoxin [80]. In 1986, this compound was recognized by the IARC (International Agency for Research on Cancer) as a group 3 carcinogen. Patulin belongs to very reactive compounds that interact with nucleic acids and proteins. It exhibits strong affinity especially to thiol groups, which can result in severe damage to cells [82].

The process of detoxification of patulin employs chemical compounds based on oxidation and reduction of this lactone or the formation of less toxic thiol adducts. Detoxification of patulin using ammonia or potassium permanganate was performed with almost 100 % efficiency [83]. Sulfur dioxide was also an effective inhibitor of this toxin. At a concentration of 2000 ppm, a reaction of sulfur dioxide to the

hemiacetal ring of patulin, forming a carbonyl hydroxysulfonate and opening of the lactone ring structure at the double bond, was observed. Reduction of patulin toxicity was also possible thanks to the use of organic acids and vitamins, including ascorbic acid and vitamins of B group: thiamine hydrochloride, pyridoxine hydrochloride, and calcium-d-pantothenate [84].

A lactone with proven toxic properties, including carcinogenic properties, is penicillic acid **21** produced by fungi of the *Penicillium* and *Aspergillus* species. This compound was first isolated in 1913 from *Penicillium puberulum*. The carcinogenicity of this compound is determined by an α,β-unsaturated ring with a conjugated double bond at position 4 [81]. Penicillic acid, similarly to patulin, is a carcinogenic factor of group 3 (IARC 1998) [85]. It has been proven that this compound induces DNA strand breaks in HeLa cells [86].

19 21

Mycotoxins produced by fungi of the *Fusarium graminearum* species (teleomorph *Gibberella zeae*) include zearalenone **22** (ZEN) – a lactone of resorcylic acid, chemically described as 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone [80]. This compound is synthesized in the polyketide pathway involving polyketide synthases (PKSs), which catalyze sequential condensation reactions of acetate units to polyketide [87]. Zearalenone belongs to the compounds which disrupt a normal activity of the reproductive system. Because of its estrogenic properties, it is referred to as a nonsteroidal estrogen or mycoestrogen. The molecular structure of ZEN and its derivatives (α-zearalenol **24** [α-ZEL], β-zearalenol **25** [β-ZEL], α-zearalanol **26** [zeranol, α-ZAL], β-zearalanol **27** [teranol, β-ZAL], and zearalanone **23** [ZAN]) determines their ability to bind to estrogen receptors. ZEN is absorbed from the gastrointestinal tract and metabolized to ZEL or conjugated with glucuronic acid [88]. Estrogenic activity of ZEN depends on metabolic processes occurring in the body and on the immunologic status of the reproductive system of the contaminated organisms. It was demonstrated that ZEN affects the maturation and degree of degeneration of oocytes depending on the dose and time of exposure. In vivo and in vitro studies also show that ZEN reduces the activity of many enzymes, including those that are involved in the process of steroidogenesis in animals and belong to cytochrome P450scc and hydroxysteroid dehydrogenases of 3β- or 17β-type and their isomers, which are involved in conversion process of pregnenolone to progesterone or estrone to estradiol. In recent years, exposure to ZEN is associated with the occurrence of hormone-dependent cancers, including breast, cervical, and prostate cancer [89, 90].

Zearalenone 22 α-zearalenol 24 β-zearalenol 25

Zearalanone 23 α-zearalanol 26 β-zearalanol 27

5.2 Other Bioactive Macrocyclic Lactones

The wide range of lactones includes a series of macrocyclic esters having a diversified biological activity, including antitumor, antimicrobial, antimalarial, or immunosuppressive activity [91]. They are a group of natural macrolides, synthesized in a pathway of polyketide synthase (PKS). Macrolides form a group of homologous compounds, which includes resorcylic acid lactones (RALs) (such as zearalenone and its derivatives) and dihydroxyphenylacetic acid lactones (DALs). Structurally RALs **28** and DALs **29** are formed by resorcinol fused to a lactone ring, at α- and β- or β- and γ-position, respectively.

RALs 28 DALs 29

Resorcinol macrolides were discovered in 1953, when radicicol **30**, known initially as monorden, was first isolated [92]. Radicicol was initially identified as an antifungal antibiotic, and later studies assigned to it also several other biological activities, including a mild sedative effect [93]. In 1992, a group of scientists from Harvard University showed the inhibitory effect of radicicol in relation to the oncogenic Src kinase [94, 95]. Subsequently it was demonstrated that radicicol is a strong and selective inhibitor of heat shock protein HSP90, responsible for maturation and stability of many other oncogenic cellular proteins. It was shown that it contributes to the inhibition of tumor cell growth and their apoptosis by blocking HSP90 (radicicol blocks the ATP bond in N-terminal pocket of HSP90, thus preventing the conversion into a mature complex) [96].

The family of resorcinol macrolides, which are conjugated *cis*-enones, comprises also other lactones of biological activity, including radicicol A **31**, LL-Z1640-2 **32**, and LL-783277 **33**. It was proved that these compounds inhibit irreversibly mitogen-activated protein kinases – MAP kinases, which are responsible for the regulation of many intracellular processes, including gene transcription, protein biosynthesis, cell division, cell differentiation, and survival or apoptosis [91]. Radicicol A inhibits the activity of cytokines IL-1β and accelerates the degradation of specific mRNA sequences containing adenylate-uridylate-rich elements [97]. LL-Z1640-2 exhibits the inhibitory effect in relation to TAK1 kinase (transforming growth factor-activated kinase 1) (IC_{50} = 8.1 nM) of the MAPK KK family [98] and ERK kinase (extracellular signal-regulated kinase) (IC_{50} = 8 nM) of the MAPK family [99], while L-783277 isolated from fungi of the *Phoma sp*. genus is characterized by a specific, strong inhibitory activity in relation to MEK1 kinase (4 nM) [100].

Radicicol 30 Radicicol analogue A 31 LL-Z1640-2 32 L-783277 33

RALs which inhibit the activity of protein kinases include also hypothemycin **34**. This compound was first identified in 1980, after its isolation from the fungi *Hypomyces tricothecoides* [101]. Hypothemycin exhibits antifungal [102] and antimalarial activity, as well as cytotoxicity against various human cell lines [103]. According to a study of Fukazawa et al. [104], this compound contributes by binding cysteine, resulting to the inactivation of several protein kinases, including MEK1 (mitogen-activated protein kinase, whose activity is regulated by extracellular factors (IC50 15 nM)), ERK (extracellular signal-regulated kinase), and platelet-derived growth factor receptor. Solit et al. [101] demonstrated a strong activity of this lactone in the inhibition of protein BRAF mutation (BRAF V600E mutation is a point mutation, affecting the change in the protein activity, based on the replacement of valine 600 by glutamic acid). An analog of hypothemycin, 4-.-demethylhypothemycin **35**, isolated from *Hypomyces subiculosus* showed an equally strong cytotoxicity against a number of mutations of BRAF protein [105].

Hypothemycin 34

4-*O*-demethylhypothemycin 35

The group of less active lactones comprises also pochonins A–E **36–40**, isolated in 2003 from *Pochonia chlamydosporia* var. *catenulate* fungi. These compounds exhibit antiviral activity against, among others, herpes simplex virus 1 (the strongest activity is exhibited by pochonin C **38**) and are active against parasitic intestinal protozoa *Eimeria tenella* [106]. From 2009, the group of pochonins additionally comprises K–P analogs (K **41** L **42** N **43** O **44**) inhibiting expression of the WNT-5A protein and showing cytotoxicity against dermal papilla cells [107].

36 Pochonin A

37 Pochonin B

38 Pochonin C

39 Pochonin D

40 Pochonin E

41 Pochonin K

42 Pochonin L

43 Pochonin N

44 Pochonin O

A diversified biological activity is also characteristic for aigialomycins A–E (A **45**, C **46**, D **47**), macrolides isolated in 2002 from a marine species of fungi – *Aigialus parvus*. Aigialomycin D has antimalarial activity (IC_{50} 6.6 μM) and exhibits cytotoxic effects against the cells of the KB type and BC-1 protein (which inhibits apoptosis) [108].

Aigialomycin A 45 · Aigialomycin C 46 · Aigialomycin D 47

Another group of RALs comprises paecilomycins A **48**, B **49**, E **50**, F **50** [109], G-I **51–53** [110], and J–M [111] isolated from the solid medium of the fungus *Paecilomyces* sp. SC0924 in the years 2010–2013. These compounds exhibit inhibitory activity against a protozoan of the *Plasmodium* genus – *Plasmodium falciparum* – causing the most severe form of malaria in humans. Paecilomycin E is a strong inhibitor of the 3D7 strain of *Plasmodium falciparum* (IC_{50} 20 nM), while paecilomycin F inhibits the proliferation of the Dd2 strain.

Paecilomycin A 48 · Paecilomycin B 49 · 50 Paecilomycin E - R_1=H, R_2=OH, Paecilomycin F - R_1=OH, R_2=H · Paecilomycin G 51 · Paecilomycin H 52 · Paecilomycin I 53

Neocosmosins A–C **54–55** belong to another recently identified group of resorcylic acid lactones synthesized by fungi of the *Neocosmospora* genus. These compounds, especially neocosmosin C, exhibit activity of agonists of opioid and cannabinoid receptors [112].

Neocosmosin A 54 · 55 Neocosmosin B R=H, Neocosmosin C R=Me

In 2011, the group of Shao [113] managed to determine the structure of three natural lactones – cochliomycins A–C **56–58** isolated from a broth culture of the *Cochliobolus lunatus* fungus originated from a gorgonian *Dichotella gemmacea* inhabiting the South China Sea. The studies involving cochliomycins showed,

among others, antibacterial activity of these compounds. It was demonstrated that these lactones exhibit inhibitory activity against bacteria *Staphylococcus aureus* and a lichen organism *Balanus amphitrite*.

56 Cochliomycin A

57 Cochliomycin B

58 Cochliomycin C

The family of lactones also comprises compounds which influence, among others, the regulation of plant growth. This activity was assigned to 12-membered RALs – lasiodiplodin **59** and de-*O*-methyllasiodiplodin **60**, first isolated in 1971 from the culture broth of fungi *Lasiodiplodia theobromae* [114, 115]. Later, both these lactones were also identified in plants. Based on several studies, it was demonstrated that lasiodiplodin exhibits an antileukemic activity, while de-*O*-methyllasiodiplodin was recognized, among others, to be an inhibitor of prostaglandin synthesis [116], a potential inhibitor of pancreatic lipase (IC 4.5 μM), and an antagonist of mineralocorticoid receptors, which may be effective in the treatment of hypertension and other cardiovascular disorders [117]. In 2011 also, a cytotoxic activity of de-*O*-methyllasiodiplodin against the KB (nasopharyngeal carcinoma cell line), BC1, and NCI-H187 (retinoblastoma cell line) cell lines was demonstrated [118]. According to the team of Buayairaks et al. [118], the group of lasiodiplodin derivatives comprises also 6-oxo-de-*O*-methyllasiodiplodin **62**, (3*R*),(5*R*)-5-hydroxy-de-*O*-methyllasiodiplodin, and (3*R*),(5*S*)-5-hydroxy-de-*O*-methyllasiodiplodin **61** isolated from fungi *Syncephalastrum racemosum*. To the latter lactone, a toxic activity against several tumors cells is attributed, especially against cholangiocarcinoma KKU-M139, KKU-M156, and KKU-M213.

59 Lasiodiplodin

60 de-*O*-methyllasiodiplodin

61 (3*R*),(5*S*)-5-hydroxy-de-*O*-methyllasiodiplodin

62 6-oxo-de-*O*-methyllasiodiplodin

Further RALs are two isomers, *trans*- **63** and *cis*-resorcylide **64**, isolated from fungi of the *Penicillium spp*. [119], *Pyrenophora teres* [120], and *Acremonium zeae* [121] genera, which also exhibit a broad biological activity. These lactones are

regarded as inhibitors of plant growth (*trans*-resorcylide isomer exhibits about tenfold stronger inhibitory activity than *cis*- isomer). Furthermore, *trans*-resorcylide is cytotoxic against a wide range of cancer cell lines, is considered to be an inhibitor of 15-hydroxyprostaglandin dehydrogenase (a key enzyme in the catabolism of prostaglandins), and is characterized by antimicrobial activity against *Pyricularia oryzae* [122]. *Cis*-isomer exhibits inhibitory activity against the coagulation factor XIIIa, responsible for the stabilization of fibrin [123].

63 *trans*-resorcylide 64 *cis*-resorcylide 65 Dihydroresorcylide

Dihydroresorcylide **65** is a saturated analog of *cis*-resorcylide. It was identified by the team of Polling et al. [121] in an endophyte *Acremonium zeae*. Previous studies of this macrolide demonstrated its antifungal activity [124].

Lactones which are interesting in terms of their structure and biological activity are (*3R,5R*)-sonnerlactone **66** and its diastereoisomer (*3R,5S*)-sonnerlactone **67** colonizing a plant *Sonneratia apetala* [125]. Sonnerlactones exhibit antiproliferative activity against oral cavity cancer cell lines, resistant to numerous drugs.

66 (*3R,5R*)-sonnerlactone 67 (*3R,5S*)-sonnerlactone

The group of DAL macrolides which are derivatives of dihydroxyphenylacetic acid lactones comprises a number of compounds of various biological activities, including curvularin **68**, 10,11-dehydrocurvularin **69**, and two epimers 11-α-methoxycurvularin **70** and 11-β- methoxycurvularin **71**. These lactones were identified in several fungal species, including *Penicillium* sp. such as *Penicillium citreoviride* [126–128], *Curvularia* sp. [129], *Chrysosporium lobatum* [130], *Eupenicillium sp*. [131], and *Nectria galligena* [132]. Curvularin is characterized by an antibiotic activity against numerous fungal species. It is an inhibitor of nitric oxide synthase [96] and an effective anti-inflammatory compound, inhibiting Janus kinases, which allows for its use in the development of drugs against chronic rheumatoid conditions [127]. Both curvularin and 10,11-dehydrocurvularin exhibit similar levels of cytotoxicity against several cancer cell lines including breast (MDA-MB-231 and MCF-7), cervical (HeLa), and lung (A549) cancer cell lines. In addition, 10,11-dehydrocurvularin is active against colon cancer cell line COLO 205 [130]. The other two abovementioned compounds of the curvularin group are also characterized by cytotoxic activity against, among others, lung NCI-H460, breast MCF-7, and pancreatic MIA Pa Ca-2 cancer cell lines [96, 130, 131].

A lactone of the DAL group, obtained in the polyketide synthase (PKS) pathway, is also citreofuran **72**. This compound is a metabolite of a hybrid strain *Penicillium citreoviride* ME 0005, isolated by Nakada and Yamamura [133]. However, its biological activity has not been reported yet.

Sporostatin **73** is another example of a mycotoxic lactone, isolated from the fungus *Sporormiella* M5032. This compound exhibits strong inhibitory activity against tyrosine kinase of epidermal growth factor receptor. In addition, it is an inhibitor of a phosphodiesterase specific for cyclic adenosine-3′,5′-monophosphate [134].

68 Curvularin; 69 10,11-dehydrocurvularin; 70 11-alfa-methoxycurvularin

71 11-beta-methoxycurvularin; 72 Citreofuran; 73 Sporostatin

The DAL family also comprises xestodecalactones A–C **74–76**, isolated from the fungus *Penicillium cf. montanense* originated from marine sponges *Xestospongia exigua* [135], and D–F **77–79**, identified in fungi *Corynespora cassiicola* [136]. These compounds exhibit antifungal activity, and xestodecalactone B inhibits the growth of, among others, fungi *Candida albicans* [135].

74 Xestodecalactone A; 75 Xestodecalactone B; 76 Xestodecalactone C

77 Xestodecalactone D; 78 Xestodecalactone E; 79 Xestodecalactone F

This review of macrocyclic lactones indicates a diversified structure and broad spectrum of biological activity. Valuable biological properties of lactones isolated from natural sources are the inspiration for the research works related both to the isolation of consecutive natural lactones occurring in nature and to the synthesis of new compounds containing lactone moiety in their molecules. Given the increasing

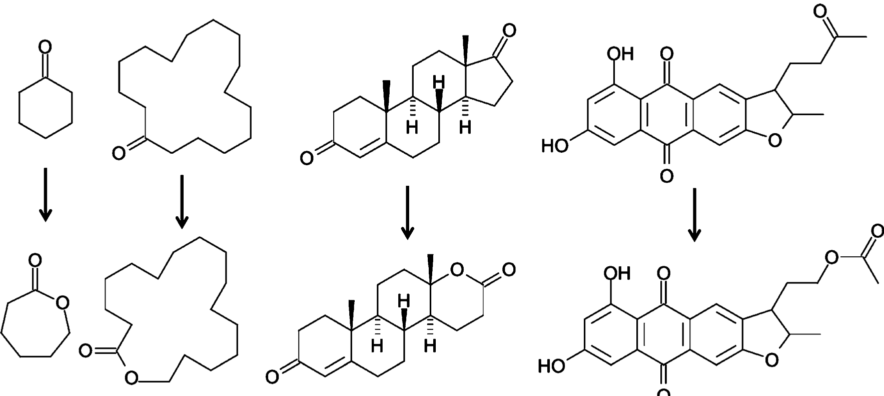
number of people suffering from cancer and continuous mutations of pathogenic microorganisms, it is understandable that many research centers began the search for natural and synthetic biologically active compounds, which in the future may become approved drugs.

6 Fungal Biocatalysts

Some of the fungal systems described above are used out of fungal metabolism for biotechnological applications. It is the case for Baeyer–Villiger monooxygenases, lactonases, and the polyketide synthase pathways, although more applications have been carried out from bacterial systems.

6.1 Baeyer–Villiger Monooxygenases

Baeyer–Villiger (BV) oxidation which consists in the transformation of a linear or cyclic ketone into its corresponding ester or lactone by insertion of an oxygen atom next to the carbonyl group is a precious reaction for oxidation of carbon chains or cycles (Scheme 4). The chemical reaction has been first described in 1899 by Baeyer and Villiger. In its traditional chemical catalysis, this reaction is not enantioselective, and catalysts are thus required that can result in enantiopure lactones. This property is exhibited by enzymes that are called Baeyer–Villiger monooxygenases (BVMOs – EC 1.14.13.x). First described in 1953 after studies on the degradation of steroids [137, 138], most of the known enzymes are bacterial, and the amount of studies resulted in a characterization of the enzyme



Scheme 4 Some lactone-related reactions catalyzed by Baeyer–Villiger monooxygenases. From left to right: the much studied (in *Acinetobacter* sp.) cyclohexanone oxygenation and the oxygenation of cyclopentadecanone, a macrocyclic ketone, in a way different from what described in (2), of androstenedione (steroid), and of hydroxyversicolorone (aflatoxin precursor) (Inspired from Torres Pazmiño et al. [144]

with the identification of a sequence motif [139] and a better knowledge of the role of BVMOs in metabolic pathways. All this aimed at developing biotechnological applications especially toward the enzymatic properties of regioselectivity and stereopecificity. Many important reactions can be catalyzed by BMVOs (Scheme 4). Recent work aimed at using directed evolution techniques to modify BVMOs and find new activities. It was thus found that a modification of only one amino acid could turn the BVMO of *Thermobifida fusca* into a NADPH oxidase [140], but some works have also been carried out to find new activities through chemical screening, genome mining, or evolution studies [141]. Several reviews on chemical and biotechnological applications of BVMO have been published [10, 12, 142–144], but most of them concerned bacterial catalysis. Although fungal BVMOs have been discovered in early research on the subject, the number of characterized fungal BVMOs is still low, although new attention has been devoted to these organisms with the possibility of genome mining investigation [145].

6.2 Lactonases

Due to the important place that lactones have in microbial metabolism, enzymes exhibiting the capability to catalyze their degradation through the opening of the cycle are important. The opening of the ring takes place usually through the hydrolysis of the ester bond, and enzymes able to open this belong to the esterase family and are called also lactonases. Different lactone rings can be hydrolyzed with lactonase catalysis, and an example is given Fig. 3 on the degradation of limonene which is first oxidized into a lactone with a BMVO catalysis and then hydrolyzed by a lactonase [13]. Like other esterases, lactonases may be highly enantiospecific which results in biotechnological applications in the resolution of racemics. As an example, lactonases are used for the resolution of racemics of pantoyl lactone (Scheme 5) (this example is related in the review on lactonases by [7]). Lactonases of *Fusarium oxysporum* or from *Agrobacterium tumefaciens* can be used, reaching the different enantiomers with enantiomeric excess (ee) at about 90–95 %. However, for the industrial reaction, it was easier to work with *Fusarium* lactonase and to immobilize it to keep activity.

6.3 The Polyketide Synthase Pathway

This system has been developed as a modular enzyme system enabling technologists to select the interesting activities to synthesize molecules. In terms of biocatalysis, this system is probably one of the most complex system developed. Several reviews have reported the advances in the field [9, 146, 148]. However, this part will not be developed in the present review as those megasynthases concern the bacterial system and not the fungal one.

Scheme 5 Resolution of D/L-pantoyl lactone racemic with *Fusarium oxysporum* and *Agrobacterium tumefaciens* lactonases

7 Conclusion

Lactones are important bioactive compounds for fungi. They play a significant role in fungal ecology as communication and antimicrobial molecules, but they have also a great impact on our lives through mycotoxins and can also be involved in positive aspects of human health as some compounds are active against cancer cells and other diseases. Beside health, many lactones are active on human senses such as flavor and fragrance lactones that can be produced by fungi in a natural way. In addition, pathways of production of lactones, β- and ω-oxidation, polyketide synthases, Baeyer–Villiger monooxygenases, and lactonases have been studied to understand and control the synthesis of fungal lactones, but they can also be used for industrial synthesis of building blocks or fine chemicals as they exhibit interesting properties for region-specific and region-selective oxidation.

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Pigments and Colorants from Filamentous Fungi

16

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Abstract

With the impact of globalization on research trends; the search for healthier lifestyles; the increasing public demand for natural, organic, and "clean labelled" products; as well as the growing global market for natural colorants in economically fast-growing countries all over the world, filamentous fungi started to be investigated as readily available sources of chemically diverse pigments and colorants. The formulation of recipes containing fungal pigmented secondary metabolites has steadily increased over recent years. For all of these reasons, this chapter highlights exciting findings, which may pave the way for alternative and/or additional biotechnological processes for industrial applications of fungal pigments and colorants. The fungal biodiversity from terrestrial and marine origins is first discussed as potential sources of well-known carotenoid pigments (e.g., β-carotene, lycopene) and other specific pigmented polyketide molecules, such as *Monascus* and *Monascus*-like azaphilones, which are yet not known to be biosynthesized by any other organisms like higher plants. These polyketide pigments also represent promising and yet unexplored hydroxy-anthraquinoid colorants from Ascomycetous species. The putative biosynthetic pathways of the carotenoids and polyketide-derivative colored molecules (i.e., azaphilones, hydroxyanthraquinones, and naphthoquinones) in pigment-producing fungal species are investigated herein. As an additional aspect, this chapter describes biotechnological approaches for improving fungal pigment production and identifying new clean opportunities for the future. Alternative *greener* extraction processes of the fungal colored compounds are also further explored. The current industrial applications along with their limits and further opportunities for the use of fungal pigments in beverage, food, pharmaceutical, cosmetic, textile, and painting areas are, then, presented.

Keywords
Pigments • Filamentous fungi • Ascomycetous • Polyketides • Azaphilones • Anthraquinones • Carotenoids • Biosynthetic pathway • Natural colorant • Food colorant • Biotechnology

List of Abbreviations

ADI	Acceptable daily intake
ASE	Accelerated solvent extraction
ATPS	Aqueous two-phase system
BIK	Bikaverin polyketide synthase
CoA	Coenzyme A
CWD	Cold-water-dispersible
DOE	Design of Experiment
EAE	Enzyme assisted extraction
EFSA	European Food Safety Authority
EU	European Union
FDA	Food and Drug Agency
GMO	Genetic Modified Organism
GMP	Good Manufacturing Practices
GRAS	Generally Recognized As Safe
HPLC	High performance liquid chromatography
IL	Ionic liquids
IPP	IsoPentenyl-pyrophosphate
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MAE	Microwave assisted extraction
PCR	Polymerase chain reaction
PKS	Polyketide synthase
PLE	Pressurised fluid extraction
PUFAs	Polyunsaturated fatty acids
SFE	Subcritical fluid extraction
SWE	Subcritical water extraction
TLC	Thin layer chromatography
UAE	Ultrasound assisted extraction
UV	Ultraviolet

1 Introduction

Molecules and ingredients derived from microbial fermentation are steadily gaining ground in industry. Thickening or gelling agents (e.g., polysaccharides such as xanthan, curdlan, gellan), flavor enhancers (yeast hydrolysate, monosodium glutamate), polyunsaturated fatty acids (PUFAs), flavor compounds (gamma-decalactone, diacetyl, methyl-ketones), vitamins, essential amino acids, and acidulants (lactic acid, citric acid) are some examples illustrating this trend. Efforts have been made

and continue to be made in order to reduce the production costs of pigments produced by fungal fermentation, since synthetic pigments or those extracted from natural plant sources can often be produced more economically [1]. The successful marketing of natural pigments such as β-carotene, lutein, and astaxanthin derived from algae (i.e., nonconventional sources) or extracted from flowering plants (conventional sources), both as food colorants and nutritional supplements, reflects the presence and importance of niche markets in which consumers are willing to pay a premium for "natural healthy ingredients."

Among other nonconventional sources, filamentous fungi are known to produce an extraordinary range of pigments that include several chemical classes such as carotenoids, melanins, azaphilones, flavins, phenazines, quinones, and more specifically, monascins, violacein, and indigo [2]. The success of any class of pigment produced by fermentation depends on its acceptance by the consumers, regulatory approval, and the capital investment required to bring the product onto the market. Twenty years ago, influential representatives from industry expressed doubts about the successful commercialization of algae-derived and fermented food grade pigments due to the high investment required for open ponds, photobioreactors and fermentation facilities, and the extensive and lengthy toxicity studies requested by the regulatory authorities. Nonexistent or poor public perception of fungal-derived products for food use had also to be taken into account. Nowadays, some food grade pigments obtained by fermentation exist on the market worldwide. Among them, fungal *Monascus* pigments, Arpink red™ (now Natural Red™) produced by *Penicillium oxalicum*, microalgal phycocyanin from *Arthrospira* (*Spirulina*) *platensis*, riboflavin from the mold fungus *Ashbya gossypii*, lycopene and β-carotene from the tropical mold *Blakeslea trispora*, β-carotene from the microalgae *Dunaliella salina*, and astaxanthin from the bacterium *Paracoccus carotinifaciens* and microalgae *Haematococcus pluvialis*, respectively. As an example, the production yield of β-carotene may be as high as 17 g/L of the *Blakeslea trispora* culture medium [3].

Thus, the present chapter emphasizes the crucial role that fungi are currently playing and are likely to continue to play in the future as microbial cell factories for the production of pigments for the industry. This is due to the versatility in their pigment color and chemical profile, amenability for easy large-scale cultivation, and a long history of production by well-investigated production strains.

2 Natural Polyketide Pigments Produced by Filamentous Fungi

Among nonconventional sources, filamentous fungi are known to produce an extraordinary range of fungal pigments that are often more stable and soluble than plant-derived pigments [3, 4]. Fungal secondary metabolites like fungal pigments can be grouped into four different classes depending on their structural properties: terpenes, polyketides, nonribosomal peptides, and amino acid-derived compounds. These fungal secondary metabolites, also known as exometabolites, are small molecules produced during morphological and chemical differentiation that are

outward directed, i.e., secreted or deposited in or on the cell wall, and accumulated in contrast to endometabolites (primary metabolites) that are fluctuating in concentration, and either transformed into other endometabolites or feeding into exometabolites, exoproteins, and exopolysaccharides. While endometabolites can be found in almost all species of fungi, exometabolites are taxonomically restricted, being produced in species-specific profiles [5].

Biosynthetically, many exometabolites produced by ascomycetous filamentous fungi are polyketides. Polyketides are typically synthesized by multifunctional polyketide synthases (PKS) from small carboxylic acid derivatives (acetyl-coenzyme A (CoA) and malonyl-CoA) in a manner similar to the synthesis of fatty acids. Nonreducing polyketide synthases synthesize polyketides in which carbonyl groups are not reduced, and reducing polyketide synthases synthesize polyketides in which the carbonyl groups are partially or fully reduced [6, 7]. Polyketides represent an array of often structurally complex natural products and include such classes as anthraquinones, hydroxyanthraquinone pigments, naphthalenes, naphthoquinone pigments, flavonoid pigments, macrolide antibiotics, polyenes antibiotics, tetracyclines, and tropolones. Azaphilone pigments, namely pigments with pyrone-quinone structures and a chiral quaternary center, can also be considered as polyketide derivatives. Polyketide-based pigments with different shades (red, yellow, orange, brown) have been found abundantly in ascomycetous filamentous fungi as exemplified in case of pigments produced by species belonging to *Monascaceae*, *Trichocomaceae*, *Pleosporaceae,* and *Nectriaceae* families [3, 8].

2.1 Species of the Monascaceae Family Producing Pigments

Monascus has been used to produce natural colorants and food supplements for more than one thousand years in Asia, and approximately more than one billion Asian people consume *Monascus*-fermented products with their daily diet. The first known source reporting the use of these red colorants was a recipe for the preparation of red pot-roast lamb, in which meat was simmered with *hong qu* (red rice koji, made with *Monascus purpureus*), as handed down in the Qing Yilu in AD 965. *Monascus* species are known to produce six major azaphilone pigments, namely the yellow monascin and ankaflavin, the orange monascorubrin and rubropunctatin, and the red monascorubramine and rubropunctamine. To date, more than 50 different chemical structures have been identified as azaphilones easily combine with nitrogen-containing compounds [9]. Using next-generation sequencing and optical mapping approaches, a 24.1-Mb complete genome of a *Monascus purpureus* YY-1 industrial strain has been described for the first time and this will allow huge improvements in the process in the coming years [9]. It consists of 8 chromosomes and 7491 genes. *M. purpureus* should belong to the *Aspergillaceae*, mainly comprising the genera *Monascus*, *Penicillium*, and *Aspergillus*. Phylogenetic analysis at the genome level provides the first comprehensive prediction of the biosynthetic pathway for *Monascus* pigments. Comparative genomic analyses demonstrated that the genome of *M. purpureus* is 13.6–40 % smaller than that of closely related filamentous fungi

and has undergone significant gene losses, most of which likely occurred during its specialized adaptation to starch-based foods. Some polyketide synthases (PKS) are expressed at high levels under high pigment-yielding conditions. The citrinin PKS C6.123 has also been found in the genome [9], paving the way for research aiming at non-mycotoxin-producing strains, if suppression of the citrinin gene does not change the ability of the strain to produce pigments, which seems to be feasible, as described by Fu et al. [10]. The latter group has shown that monascorubrin and citrinin are synthesized by two separate pathways, because, when the PKS gene responsible for synthesis of citrinin was disrupted, red pigment production from the fungus was not affected. Comparative transcriptome analysis revealed that carbon starvation stress, resulting from the use of relatively low-quality carbon sources, contributed to the high yield of pigments by suppressing central carbon metabolism and augmenting the acetyl-CoA pool. As for other pigments produced by biotechnology, the problem is to have enough carbon oriented in the correct pathway, i.e., the pigment pathway.

Woo et al. [11] investigated another filamentous fungus, *Penicillium marneffei*, for the production of azaphilones exhibiting black, yellow, and red hues. The polyketide gene cluster and biosynthetic pathway were reported for monascorubrin in this red pigment-producing, thermal dimorphic fungus, taking advantage of available genome sequence and faster growth rate compared to *Monascus* species [11]. The red pigment of *P. marneffei* has been shown to consist of a mixture of more than 16 chemical compounds, which are amino acid conjugates of monascorubrin and rubropunctatin, as amino acids can be conjugated under specific conditions without enzymatic catalysis, i.e., by Schiff base formation (Fig. 1) [11].

The aforementioned polyketide gene cluster and pathway have been shown to be also responsible for the biosynthesis of ankaflavin and citrinin, the latter being a mycotoxin exerting nephrotoxic activity in mammals [12]. Twenty-three putative PKS genes and two putative PKS-nonribosomal peptide synthase hybrid genes were identified in the *P. marneffei* genome [11]. Woo et al. [11] systematically knocked out all 25 PKS genes of *P. marneffei.* They also knocked out genes located up and downstream of the PKS gene responsible for red pigment production and characterized the pathway for biosynthesis of the red pigment. However, it is still questionable whether it will be possible to produce mevinolin/lovastatin-free (a cholesterol-lowering drug that is undesired in normal foods) and citrinin-free red pigments from *P. marneffei*, as the latter, a mycotoxin, appears to be an early by-product of the biosynthetic pathway.

2.2 Species of the Trichocomaceae Family Producing Pigments

2.2.1 Pigments from *Penicillium* and *Talaromyces* Species

Fungi belonging to the genera *Penicillium* and *Talaromyces* are cosmopolitan, classified under the family *Trichocomaceae* and contain 354 and 88 species, respectively. Species of the genera are commonly found in soil, decaying organic materials, animal feed, stored grains, and other materials [13]. Species of *Penicillium* and *Talaromyces* are extraordinarily productive concerning exometabolites. They are

Fig. 1 Hypothetical pathway of monascorubrin, ankaflavin, and citrinin biosynthesis in *P. marneffei* (Adapted from Woo et al. [11])

among the most chemically inventive of all fungi. A comparison with other genera shows that most exometabolites have been reported from *Aspergillus* (1984 exometabolites), next-most from *Penicillium* (1338), and fifth-most by *Talaromyces* (316), with only *Fusarium* (507) and *Trichoderma* (438) producing more exometabolites [14]. These fungal genera produce various compounds, which are beneficial to the society, but amongst all, they are potential producers of natural pigments, thus they could be used for various industrial purposes. Many workers have studied the pigment production of various species of *Penicillium* and *Talaromyces* isolated from different environments. In addition, some researchers have made experiments to optimize the pigment production from these fungal species using different media by modifying the intrinsic and extrinsic factors. The most common hues produced by both genera include yellow, red, orange, and reddish-brown. According to Teixeira et al. [15], it was found that the yellow pigment is predominant in most of the *Penicillium* species. Strains collected from Amazon forest such as *P. simplicissimum* DPUA 1379, *P. melinii* DPUA-1391, and *P. atrovenetum* yielded yellow pigment with antibiotic activity [15]. As a first commercial fungal red colorant, the Arpink red™ pigment (now Natural red™) has been claimed to be produced by fermentation and bioprocess engineering using the strain *Penicillium oxalicum* var. armeniaca CCM 8242 obtained from soil. On the second day of cultivation in liquid broth containing carbohydrates, zinc sulfate, and magnesium sulfate, a hydroxyanthraquinone red colorant is released in the liquid medium, and its concentration keeps increasing up to 1.5–2.0 g/L of broth after 3–4 days [2, 16, 17].

In *Penicillium* and *Talaromyces* species, polyketide-based pigments are very common, and particularly the azaphilone (hexaketide) ones, like the derivatives of monascorubrin and rubropunctatin from *P. marneffei* [11]. In similar lines, two azaphilone pigments, monascorubramine homologues PP-V [(10Z)-12-carboxyl-monascorubramine] and PP-R [(10Z)-7-(2-hydroxyethyl)-monascorubramine], are isolated from a strain of *Penicillium* sp. AZ [18]. PP-V and PP-R are slight modifications of monascorubramine. Over the past 5 years, very few reports have been published on the *Monascus*-like azaphilone pigments produced by non-mycotoxigenic strains of *Talaromyces* species (formerly *Penicillium sp.*) [19–24] (Fig. 2). *Penicillium purpurogenum* is an important species in biotechnology for its ability to produce enzymes and pigments, which are used as natural colorants [24–27]. Recent revision of the taxonomy of *P. purpurogenum* showed that this species is a complex consisting of four taxa: *T. purpurogenus*, *T. ruber* (syn. *P. rubrum*), *T. amestolkiae*, and *T. stollii* [28]. From a biotechnological point of view, it is recommended to use *T. ruber* for enzyme production, because *T. purpurogenus* produces four types of mycotoxins and *T. amestolkiae* and *T. stollii* are potentially pathogenic to immunocompromised individuals [23]. As reported by Méndez et al. [20], the strain *P. purpurogenum* GH2 can produce *Monascus*-like pigments with no coproduction of toxic citrinin, which paves the way for producing water-soluble red pigments at an industrial level to be used in food industry [19–21]. *N*-glutarylmonascorubramine and *N*-glutarylrubropunctamine were the water-soluble *Monascus*-like polyketide azaphilone pigments discovered in the extracellular pigment extract obtained from the liquid medium of *P. purpurogenum* [29] (Table 1). More recently, an European patent has been granted for a submerged cultivation method for some of the non-mycotoxigenic strains of *Talaromyces* sp. whereby the concentration of pigments was significantly enhanced, and the number of pigmented constituents was significantly reduced with the polyketide azaphilone purple pigment PP-V being the major compound [30].

Talaromyces is the telemorph genera, which comprise a monophyletic clade that is distinct from *Penicillium*. Some species of *Talaromyces* produce red pigments while a few other synthesize yellow pigments of azaphilone series [31]. Studies have shown that *Monascus*-like azaphilone red pigments and/or their amino acid derivatives are naturally produced by *Talaromyces aculeatus*, *T. pinophilus*, *T. purpurogenus,* and *T. funiculosus*. *Talaromyces amestolkiae*, *T. ruber,* and *T. stollii* also produce azaphilone pigments, as recently described by Yilmaz et al. [23], but in those three species the pigments are not diffusing into the growth medium. *T. atroroseus* can secrete large amounts of *Monascus*-like azaphilone red pigments, without the production of any known mycotoxins. On the other hand, *T. purpurogenus* produces mycotoxins such as rubratoxins A and B, rugulovasins, and luteoskyrin [23]. These factors limit the use of these species for biotechnological production of azaphilone pigments. However, some *Talaromyces* species can be used to produce pigments at industrial scale if no coproduction of toxins has been concluded. Red pigment producers, such as *T. atroroseus, T. albobiverticillius, and T. purpurogenus*, produce rubropunctatin and other *Monascus* pigments while other species such as *T. cnidii* and *T. coalescens* produce red soluble pigments in some isolates (Table 1).

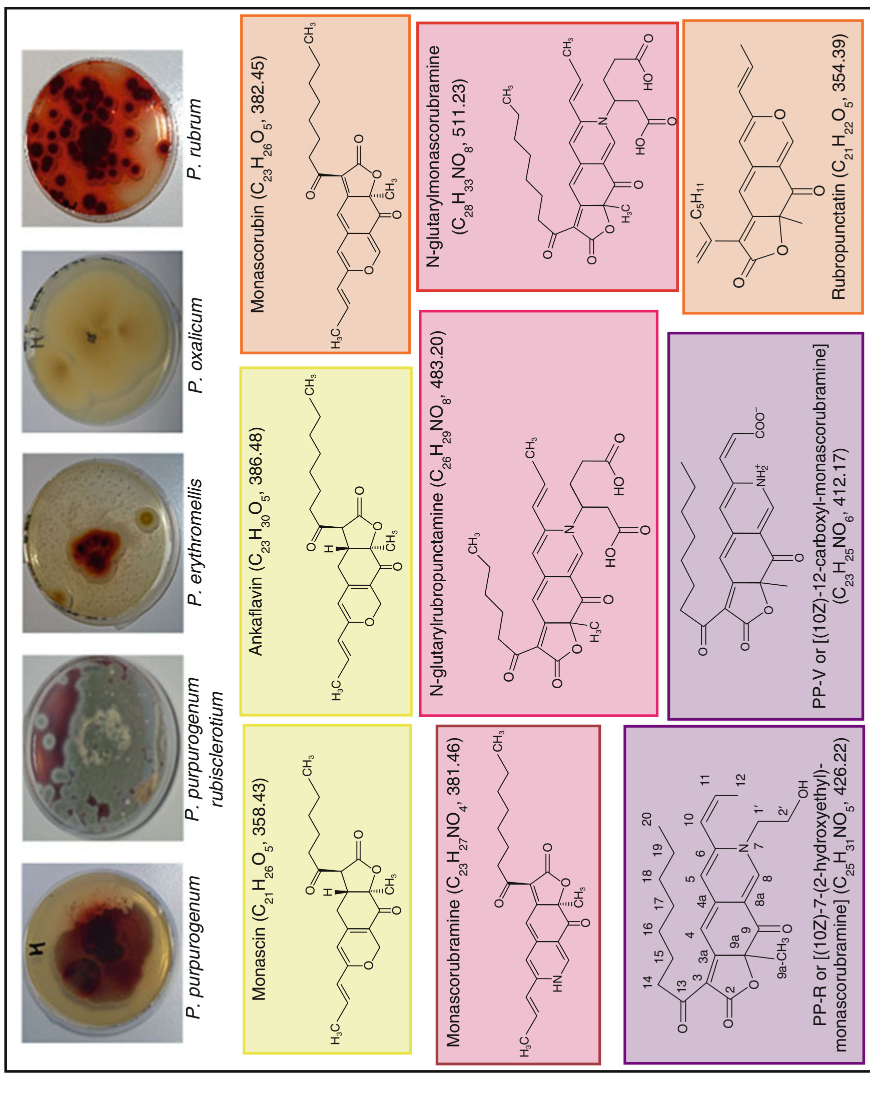

Fig. 2 Main *Monascus*-like azaphilone pigments produced by *Penicillium* and *Talaromyces* species

Table 1 Polyketide pigments produced by *Penicillium* and *Talaromyces* species

Fungal species	Pigment composition (colour) (Toxic colored compounds in bold)	Major known mycotoxic uncolored metabolites
P. atramentosum	Uncharacterized dark brown	Roquefortine C
		Rugulovasine A and B
P. atrosanguineum	Phoenicin (red)	Unknown
	Uncharacterized yellow and red	
P. atrovenetum	Atrovenetin (yellow)	beta-nitropropionic acid
	Norherqueinone (red)	
P. aurantiogriseum	Uncharacterized	Nephrotoxic glycopeptides
		Penicillic acid
		Verrucosidin
P. brevicompactum	Xanthoepocin (yellow)	Botryodiploidin
		Mycophenolic acid
P. chrysogenum	Sorbicillins (yellow)	Roquefortine C
	Xanthocillins (yellow)	
P. citrinum	Anthraquinones (yellow)	Unknown
	Citrinin (yellow)	
P. cyclopium	**Viomellein (reddish-brown)**	Penicillic acid
	Xanthomegnin (orange)	
P. discolor	Uncharacterized	Chaetoglobosin A, B, and C
P. echinulatum	Uncharacterized (yellow)	Territrems
P. flavigenum	Xanthocillins	Unknown
P. freii	**Viomellein (reddish-brown)**	Unknown
	Vioxanthin	
	Xanthomegnin (orange)	
P. herquei	Atrovenetin (yellow)	Unknown
	Herqueinones (red and yellow)	
P.oxalicum	Arpink red™- anthraquinone derivative (red)	Unknown
	Secalonic acid D (yellow)	
P. paneum	Uncharacterized (red)	Botryodiploidin
		Patulin
		Roquefortine C
P. persicinum	Uncharacterized cherry red	Roquefortine C
P. viridicatum	**Viomellein (reddish-brown)**	Penicillic acid
	Vioxanthin	Viridic acid
	Xanthomegnin (orange)	
T. macrosporus	Mitorubrin (Yellow)	Duclauxin
		Islanditoxin
P. aculeatum	Uncharacterized	Unknown
P. crateriforme	Uncharacterized	Rubratoxin
		Rugulovasine A and B
		Spiculisporic acid

(*continued*)

Table 1 (continued)

Fungal species	Pigment composition (colour) (Toxic colored compounds in bold)	Major known mycotoxic uncolored metabolites
P. funiculosum	Uncharacterized	Unknown
P. islandicum	**Emodin (yellow)**	Cyclochlorotine
	Erythroskyrin (orange-red)	Islanditoxin
	Luteoskyrin (yellow)	Rugovasin A and B
	Skyrin (orange)	
	Rugulosin (yellow)	
P. marneffei	Monascorubrin (red)	Unknown
	Rubropunctatin (orange)	
	Mitorubrinol	
	Monascorubramine (purplered	
	Secalonic acid D (yellow)	
P. pinophilum	Uncharacterized	Unknown
P. purpurogenum	Mitorubrin (yellow)	Unknown
	Mitorubrinol (orange-red)	
	Purpurogenone (yellow-orange)	
	PP-R (purple red)	
P. rugulosum	**Rugulosin (yellow)**	Unknown
P. variabile	**Rugulosin (yellow)**	Unknown

2.2.2 Pigments from *Aspergillus* and *Emericella* Species

Aspergillus and *Emericella* are two genera consisting of a few hundred mold species found worldwide in various climates. They are well known to produce different secondary metabolites such as polyketide-based pigments in solid and liquid cultures. The most important colored metabolites produced by *Aspergillus* and *Emericella* species are, respectively, hydroxyanthraquinone (octaketide) pigments and azaphilone (hexaketide) ones (Fig. 3). From the genus *Aspergillus*, 18 different hydroxyanthraquinone pigments, at least, were identified. Some strains of this genus, such species as *A. glaucus*, *A. cristatus,* and *A. repens,* share many common secondary metabolites and are found to produce hydroxyanthraquinone pigments such as emodin (yellow), physcion (yellow), questin (yellow to orange-brown), erythroglaucin (red), catenarin (red), and rubrocristin (red) (or dimer of emodin and physcion) [17, 32]. Other hydroxyanthraquinone pigments, such compounds as averufin, norsolorinic acid, versicolorin, variecolorquinone, ascoquinone A, averantin, chrysophanol (orange), cynodontin (bronze), and tritisporin (brownish-red), are rarer products revealed from this genus. Presently, *A. glaucus* (group) seems to be the best producer according to the diversity of hydroxyanthraquinone compounds produced (11 different) [17, 32]. In addition, other polyketide-based pigments, e.g., viomellein (reddish-brown), viopurpurin (purple), xanthomegnin (orange), and rubrosulfin (red), are produced by *A. ochraceus*, *A. melleus*,

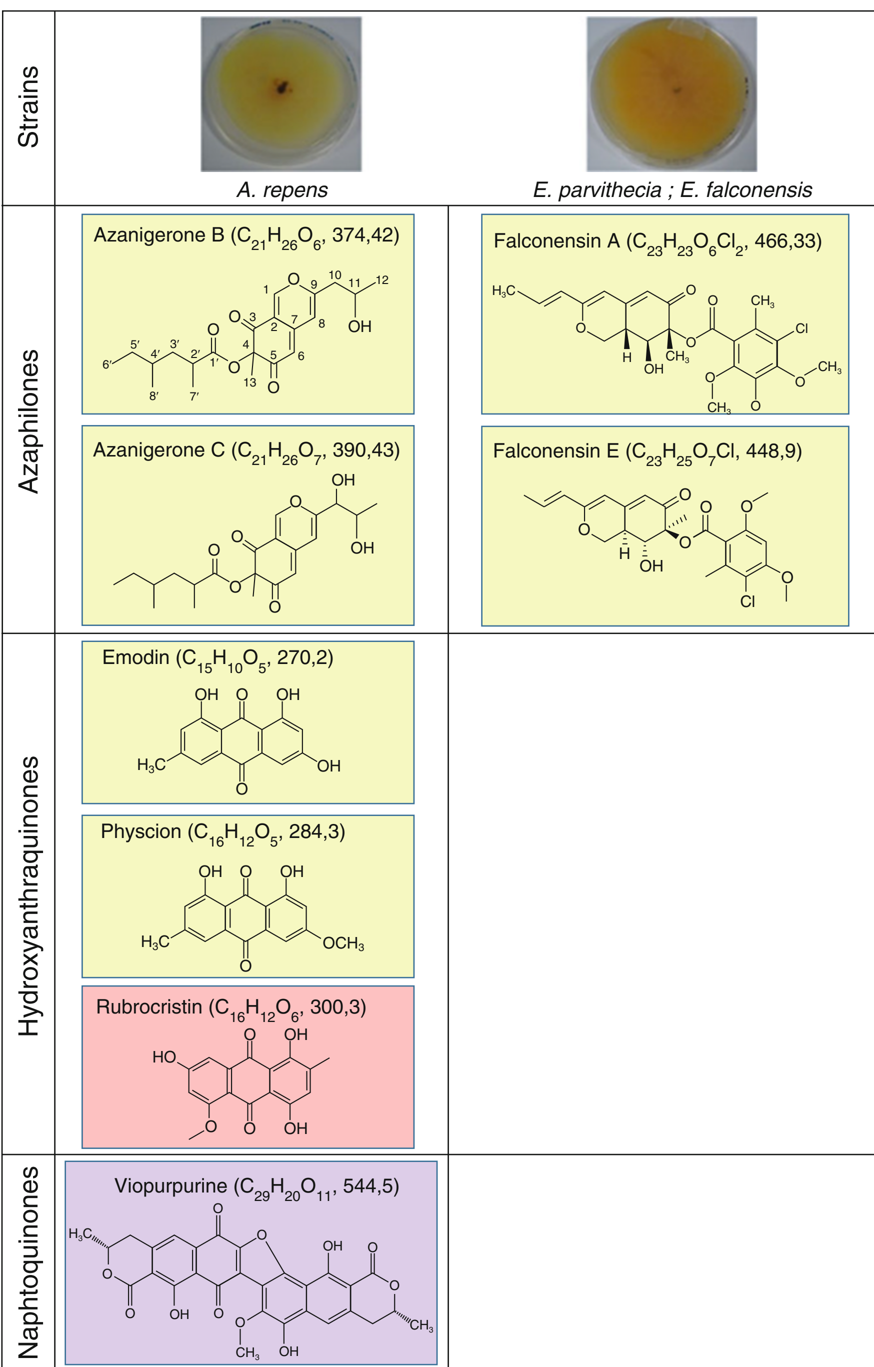

Fig. 3 Main azaphilone, hydroxyanthraquinone and naphthoquinone pigments produced by *Aspergillus* and *Emericella* species

A. sulphureus, and *A. westerdijkiae* [25]. The fungus *Aspergillus nidulans* is also known to produce dark-brown melanin pigments. Melanin pigments appear to be essential for the protection of the fungus against environmental stress [33]. The conidial pigment of *Aspergillus fumigatus* contains the 1,8-dihydroxynaphthalene-like melanin pigment (a complex aggregate of polyketides). This pigment plays a major role in the protection of the fungus against immune effector cells; for example, it is able to scavenge reactive oxygen species generated by alveolar macrophages and neutrophiles [34]. From studies performed on *A. niger*, secondary metabolite profiling of the color mutants revealed a close relationship between polyketide synthesis and conidial pigmentation in the fungus [35]. The production of the 1,8-dihydroxynaphthalene, precursor of melanin pigment, and the naphtho–γpyrone subclass of polyketides (commonly found in significant quantity in *A. niger* culture extracts) were dependent on polyketide synthases [35, 36]. More recently, six novel compounds belonging to the family of azaphilones, azanigerones A-F, were isolated and characterized from culture of *A. niger* [37], which indicates the presence of an azaphilone biosynthetic pathway in *Aspergillus* species.

The genus *Emericella* comprises 34 species and the name *Emericella* refers to the sexual phase (teleomorph) of these fungal species. Some of these fungi are well known to produce yellow azaphilone pigments. As example, eight azaphilone-based pigments, named falconensins A-H (yellow), have been isolated from the mycelium of both *Emericella falconensis* and *Em. fructiculosa,* along with other yellow pigments, falconensones A1 and B2 (i.e., cyclopent-2-enone derivatives), and hopane-type triterpene, zeorin (yellow) [25, 38]. Three dicyanide derivatives, epurpurins A to C (yellow) were also isolated from *Emericella purpurea* [39, 40]. The yellow pigment sterigmatocystin, a carcinogenic polyketide compound, has been reported in several *Emericella* species (e.g., *Em. rugulosa*, *Em. parvathecia*, and *Em. nidulans*) and also in the fungus *Aspergillus versicolor* [25, 41] (Fig. 3). The effect of different wavelength of light (daylight, darkness, blue 492–455 nm, green 577–492 nm, yellow 597–577 nm, and red 780–622 nm) on growth, intracellular and extracellular pigment production by the fungus *Emericella nidulans* has been reported by Velmurugan et al. [42], and total darkness was concluded to favor biomass, extracellular and intracellular pigment productions.

2.2.3 Pigments from *Eurotium* and *Paecilomyces* Species

The common genus *Eurotium* consists in teleomorphic often xerophilic species, usually related to *Aspergillus* anamorphs, especially from *A. glaucus* group. The genus *Eurotium* contains several species and is also an important polyketide producer. Some species of *Eurotium* including *E. amstelodami*, *E. chevalieri,* and *E. herbariorum* are found to produce hydroxyanthraquinone pigments. The pigments most frequently identified are physcion (yellow) and erythroglaucin (red); however, the strains produce in addition two benzaldehyde colored compounds, e.g., flavoglaucin (yellow) and auroglaucin (orange-red), and the mycotoxin echinulin (colorless) [17, 43]. Sixteen more species of *Eurotium* are able to synthesize hydroxyanthraquinone pigments. Physcion and erythroglaucin are the

most widespread pigments throughout the *Eurotium* studied strains [17, 32]. From Anke study [44], within this genus, *E. rubrum* and *E. cristatum* produce the highest diversity of compounds regarding hydroxyanthraquinones: erythroglaucyn, physcion, catenarin (red), rubrocristin (red), and emodin (orange) were identified in their cultures. *E. umbrosum*, *E. spiculosum*, *E. glabrum*, *E. echinulatum, and E. chevalieri* synthesize the first four compounds out of the five mentioned. *E. tonophilum*, *E. acutum*, *E. herbariorum*, *E. intermedium*, and *E. leucocarpum* produce only either physcion or erythroglaucin (Fig. 4). The study also demonstrated that within a same species there was a great variability

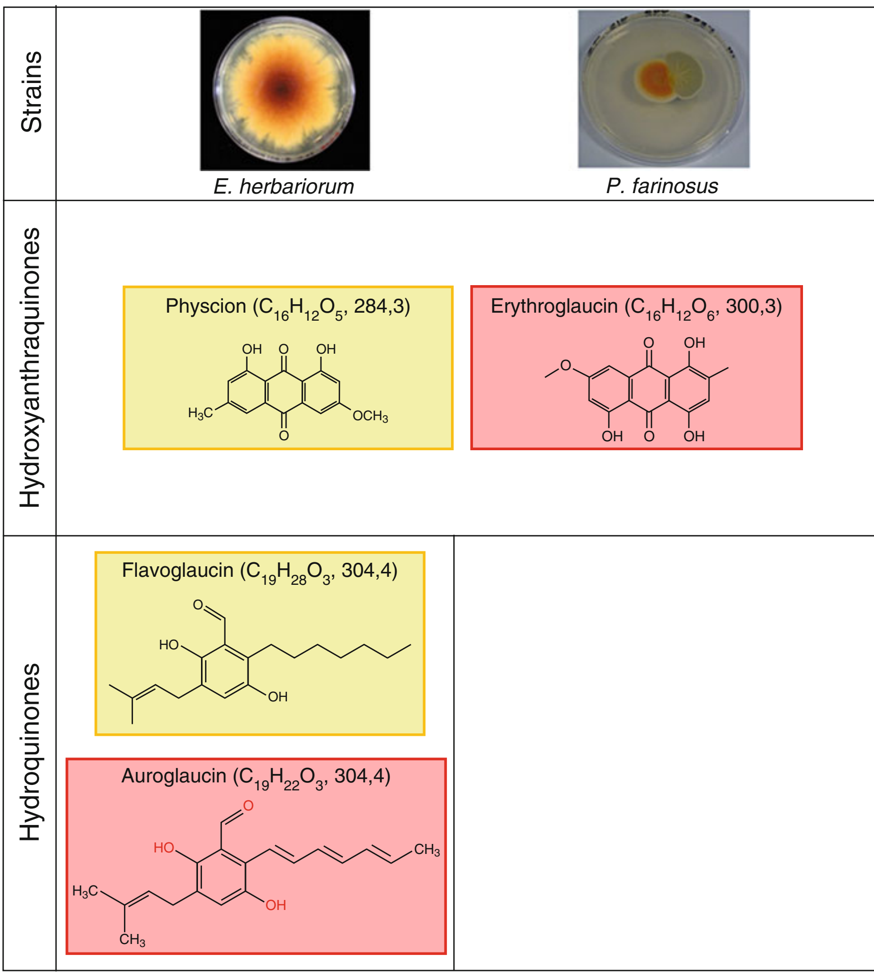

Fig. 4 Main hydroxyanthraquinone and hydroquinone pigments produced by *Eurotium* and *Paecilomyces* species

toward hydroxyanthraquinones production, as some strains of *E. rubrum*, *E. niveoglaucum*, *E. leucocarpum*, *E. intermedium*, *E. herbariorum*, *E. pseudoglaucum*, *E. appendiculatum*, *E. echinulatum*, and *E. acutum* behaved differently when grown under the same culture conditions [44]. Moreover, some of the strains of *E. amstellodami*, *E. heterocaryoticum*, and *E. montevidensis* included in this study did not produce any hydroxyanthraquinone pigments under the conditions of the experiment [44]. Another *E. rubrum* strain (QEN-0407-G2) isolated from the inner tissue of the stem of the marine mangrove plant *Hibiscus tiliaceus* synthesized the pigment questin (orange), a glycosyled derivative of questin [6, 3-*O*-(α-D-ribofuranosyl)- questin; orange], and three other fungal metabolites (eurorubrin, and two *seco*-anthraquinone derivatives [3, 2-*O*-methyl-9-dehydroxyeurotinone and 4, 2-*O*-methyl-4-*O*-(α-D-ribofuranosyl)-9-dehydroxyeurotinone]) [45].

The genus *Paecilomyces* may be distinguished from the closely related genus *Penicillium* by forming colonies that show various color shades. Colonies are fast growing, powdery or suede-like, gold, yellow-brown, lilac, or tan, but never green or blue-green as in *Penicillium*. A red uncharacterized pigment has been isolated from the fungus *Paecilomyces sinclairii* and is certainly an amino group linked to a hydroxyanthraquinone structure [46] (Fig. 4).

2.3 Species of the Nectriaceae Family Producing Pigments

Fusarium is a diverse group of fungi of the *Nectriaceae* family. The name *Fusarium* refers to the asexual phase (anamorph) of the fungus. In accordance with their genetic potential, *Fusarium* species have been found to produce a wide range of fungal pigments that are diverse in structure and biological activity. However, among the *Fusarium* secondary metabolites, numerous toxic compounds have been identified (e.g., fumonisins, zearalenone, fusaric acid, fusarins, and beauvericins) [25, 47, 48]. Numerous terpenes have been characterized from *Fusarium* species, and a representative example is *Fusarium fujikuroi*, which is able to produce orange carotenoids (neurosporaxanthin) [49, 50]. Among the *Fusarium* secondary metabolites, numerous polyketide pigments have also been identified, such as the naphthoquinone pigments which form the most abundant group [51–62] and the hydroxyanthraquinone ones [17, 63]. Previously, Cajori et al. [64] have isolated the red naphthoquinone pigment, bostrycoidin, produced by a *F. oxysporum* (formerly *F. bostrycoides*) strain. Tatum et al. [51] reported that six napthtoquinone pigments of the naphthazarin structure were produced by *F. oxysporum*. According to the authors, the major pigment isolated was the 9-*O*-methylfusarubin (formerly 8-*O*-methylfusarubin), with 5-*O*-methyljavanicin (formerly 8-*O*-methyljavanicin), 8-*O*-methylbostrycoidin, 1,4-naphthalenedione-3,8-dihydroxy-5,7-dimethoxy-2-(2-oxopropyl) (formerly 8-*O*-methyl-13-hydroxynorjavanicin), 5-*O*-methylsolaniol (formerly 8-*O*-methylsolaniol), and 9-*O*-methylanhydrofusarubin (formerly 8-*O*-methylanhydrofusarubin) in decreasing concentration. All of these pigments are red, except 8-*O*-methylanhydrofusarubin, which is purple (Fig. 5). Then Baker and Tatum [63] have isolated two yellow/orange hydroxyanthraquinone pigments among

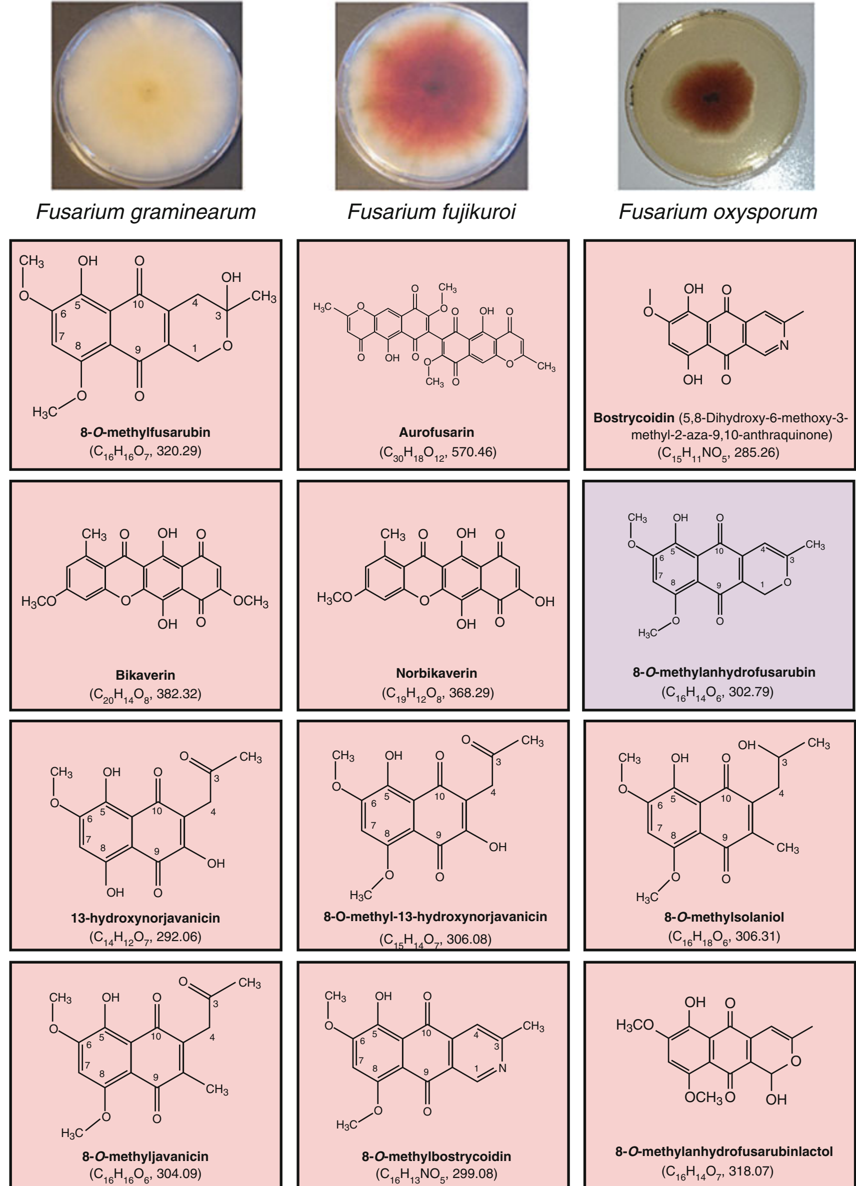

Fig. 5 Main naphthoquinone pigments produced by *Fusarium* species

the secondary metabolites produced by *F. oxysporum*. Nowadays, the most thoroughly studied *Fusarium* polyketide pigments are the red dimeric naphthoquinone pigment, aurofusarin, from *F. graminearum*, the red naphthoquinone pigment, bikaverin, and its minor coproduct, nor-bikaverin, from *F. fujikuroi* [52–58]. Over the past decade, few

reports have been published on the aurofusarin and bikaverin biosynthetic pathways. Their polyketide nature was confirmed through identification of polyketide synthase genes responsible for their biosynthesis. Aurofusarin was first described as a *Fusarium culmorum* pigment by Ashley et al. [65]. The color of aurofusarin is dependent on the pH value of the solvent, ranging from golden yellow in acidic solvents to red/purple in alkaline solvents [65]. The pigment is produced in high quantities, continuously during mycelium development, resulting in the increasing staining of both mycelium and medium (the mycelium shifts from white to yellow and finally to a deep red color). Known producers include *Fusarium acuminatum*, *F. avenaceum*, *F. crookwellens*, *F. culmorum*, *F. graminearum*, *F. poae*, *F. pseudograminearum*, *F. sambucinum*, *F. sporotrichioides,* and *F. tricinctum* [66]. Aurofusarin is produced under various suboptimal conditions, such as high or low pH, high temperatures, and phosphate starvation. The genes responsible for the production of aurofusarin could be regulated by a global pH regulatory factor. The ambient pH is the most critical parameter, as many naphthoquinones are cytostatic at neutral pH [67].

Concerning the red naphthoquinone pigment, bikaverin, it has been shown that its production is strongly dependent on culture conditions and its regulation has been investigated in detail in *F. fujikuroi*. The bikaverin production is repressed in culture media containing high nitrogen levels and under alkaline conditions; aeration also stimulated its production [54–56]. Bikaverin was first discovered as a pigment in cultures of *F. lycopersici* and *F. vasinfectum* [55, 68]. Limón et al. [55] have reported no negative incidence of bikaverin-contaminated products on human health, even if bikaverin is often considered a mycotoxin today [53]. Biological effects of bikaverin differ largely between different organisms, and bikaverin was not genotoxic according to a DNA synthesis assay [52]. Bikaverin is a fungal polyketide-based pigment with antibiotic activity against fungi [69] and antitumor action [70]. Some reports on isolation of aurofusarin and bikaverin have also been published for other *Fusarium* species such as *F. solani* [51] and *F. verticillioides* [42, 61]. More recently, Sørensen et al. [71] have discovered a medium with low nitrogen content that partially redirects the aurofusarin and bikaverin biosynthetic pathways to produce the lactones citreoisocoumarin and SMA93, respectively, in some aurofusarin and bikaverin producing *Fusarium* species; the redirection seems to be regulated by the same mechanism, which is triggered by some organic nitrogen source (glutamine, arginine) and acidic conditions (with an optimum at pH 5–6). According to Medentsev et al. [72], who have studied the biosynthesis of colored naphthoquinone metabolites by *Fusarium decemcellulare*, *F. graminearum*, and *F. bulbigenum* fungi, the biosynthesis of naphthoquinone pigments in *Fusarium* species was shown to be the main response of the fungi to stress, observed under conditions of growth inhibition or arrest. Depending on the conditions of cultivation, *F. bulbigenum* and *F. graminearum* synthesized bikaverin and aurofusarin, respectively, whereas *F. decemcellulare* synthesized soluble extracellular naphthoquinones of the naphthazarin structure (javanicin, anhydrojavanicin, fusarubin, anhydrofusarubin, bostrycoidin, and novarubin) or extracellular dimeric naphthoquinone aurofusarin [72] (Fig. 5).

Concerning the *Fusarium* perithecial pigments, a violet pigment that accumulates in the walls of sexual fruiting bodies (perithecia) has been isolated from both cultures of *F. verticillioides* and *F. graminearum* [60, 73]. In contrast, the pigment that accumulates in the perithecial walls of *F. solani* is red and results from the activity of a different polyketide synthase [59]. The fungus *F. fujikuroi* has been reported to produce particular naphthoquinone pigments, i.e., red fusarubins [62]. Five main red fusarubin-like naphthoquinone pigments have been isolated: 8-*O*-methylfusarubin, as the main product (interestingly, with the same structure of the major pigment isolated by Tatum et al. [51] in culture of *F. oxysporum*), 8-*O*-methylnectriafurone, 8-*O*-methyl-13-hydroxynorjavanicin, 8-*O*-methylanhydrofusarubinlactol, and 13-hydroxynorjavanicin that are produced under specific culture conditions, which are different from those for red bikaverin [62] (Fig. 5). Naphthoquinone pigment related to fusarubin was initially isolated from the fungus *F. solani* [74]. Although the functional characterization of most of the cluster in *F. fujikuroi* provides strong evidence that fusarubins (red) are the precursors of the perithecial pigment (violet), the structure of the violet perithecial pigment has yet to be determined [62, 75]. Thus, studies are in progress to determine the relationship between fusarubin pigments and the violet perithecial one.

2.4 Species of the Hypocreaceae Family Producing Pigments

Fungal strains of the genus *Trichoderma* from the *Hypocreaceae* family are well-known producers of secondary metabolites with antibiotic activity [76] and are important biocontrol agents successfully applied as biopesticides worldwide. Strains of *Trichoderma aureoviride*, *T. harzianum*, *T. polysporum*, and *T. viride* all produce the hydroxyanthaquinone pigments pachybasin (yellow) and chrysophanol (orange-red). The fungus *T. viride* also synthesizes emodin (yellow), 1,3,6,8-tetrahydroxyanthraquinone, and 2,4,5,7- tetrahydroxyanthraquinone. Only emodin is known from culture of *T. polysporum* [17] (Fig. 6). In 2012, Lin et al. [77] reported that the pachybasin and emodin compounds are secreted by *T. harzianum*. In the genus *Trichoderma*, the increase of the concentration of excreted emodin or pachybasin increases the concentration of cyclic AMP indicating that these hydroxyanthraquinone pigments are key substances in the regulation of this secondary messenger and also suggest that pachybasin and emodin play roles in the biocontrol mechanism of *Trichoderma sp.* [32, 77]. The major secondary metabolites produced by *Trichoderma harzianum* T22 and T39, two commercial strains successfully used as biopesticides and biofertilizers in greenhouse and open field production, are hydroxyanthraquinones (e.g., 1-hydroxy-3-methyl-anthraquinone and 1,8-dihydroxy-3-methyl-anthraquinone), azaphilone (T22azaphilone), and three other metabolites (T39-butenolide, harzianolide, and harzianopyridone) [78].

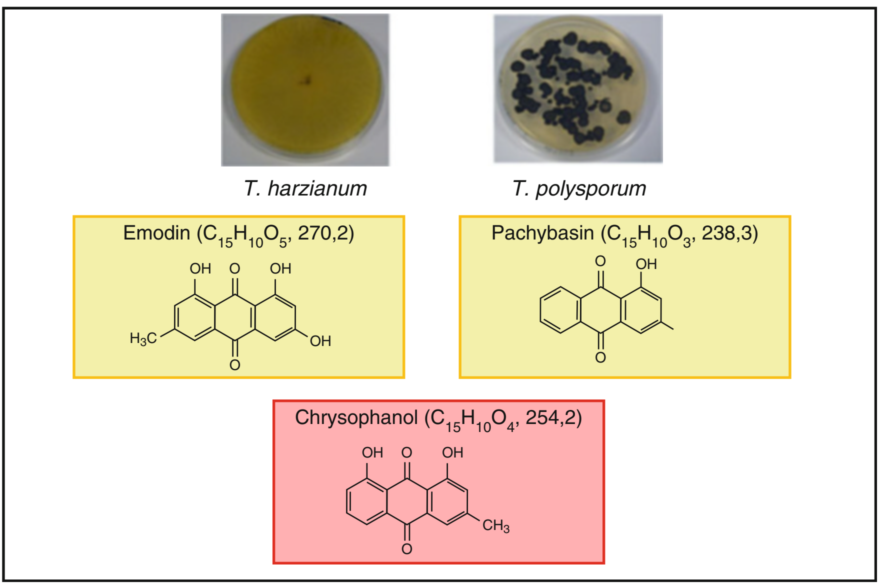

Fig. 6 Main hydroxyanthraquinone pigments produced by *Trichoderma* species

2.5 Species of the Pleosporaceae Family Producing Pigments

In the *Pleosporaceae* family (*Alternaria*, *Curvularia*, *Drechslera*...), both hyphae and conidia are heavily pigmented. Hydroxyanthraquinone pigments are produced by a broad range of *Alternaria*, *Curvularia*, *and Drechslera* species. As example, the main hydroxyanthraquinone pigments characterized from the fungus *Curvularia lunata* are chrysophanol (orange-red), cynodontin (bronze), helminthosporin (maroon), erythroglaucin (red), and catenarin (red) [17, 25]. Cynodontin extracted from the biomass of *C. lunata* has been converted successfully to two anthraquinone biodyes (Disperse blue 7 and Acid Green 28). The properties of these biodyes applied to knitten polyamides were compared with those of conventional dyes and found to be identical to all-important aspects. Several species of *Drechslera* (e.g., *D. teres*, *D. graminea*, *D. tritici-repentis*, *D. phlei*, *D. dictyoides,* and *D. avenae*) produced the following hydroxyanthraquinone pigments: catenarin (red), cynodontin (bronze), helminthosporin (maroon), tritisporin (reddish-brown), and erythroglaucin (red), without coproduction of known mycotoxins [17, 79]. In similar lines, three species of *Alternaria*, e.g., *Alt. solani*, *Alt. porri,* and *Alt. tomatophila,* can produce a yellow-orange polyketide pigment, altersolanol A, without mycotoxin's production [80] (Fig. 7). These fungal strains can be investigated as a possible potentially safe source of pigments [25].

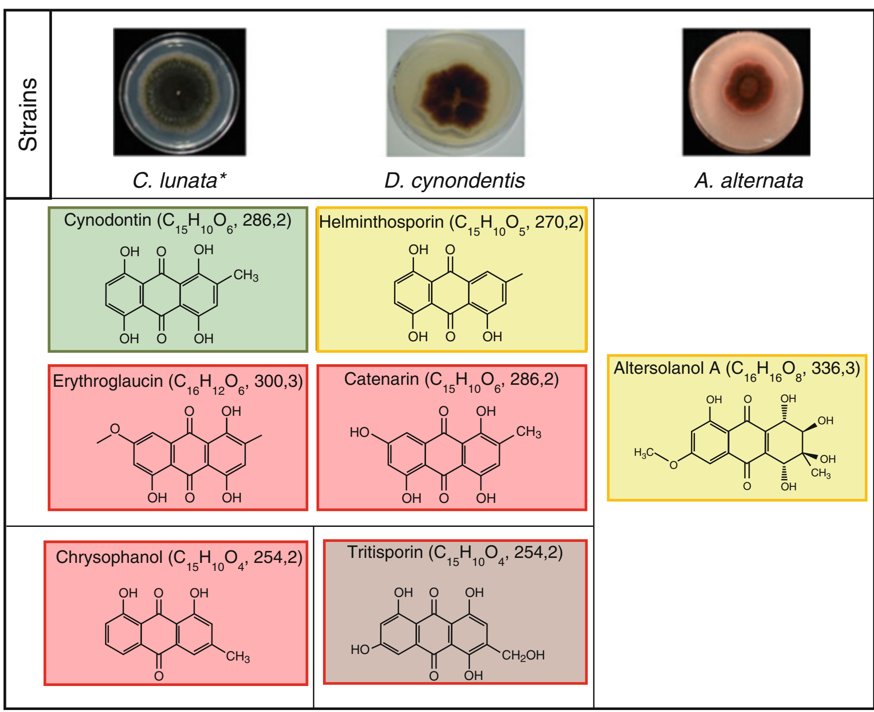

Fig. 7 Main hydroxyanthraquinone pigments commonly and specifically produced by *Curvularia*, *Dreschlera* and *Alternaria* species

2.6 Species of the Cordycipitaceae Family Producing Pigments

Fungal strains belonging to the *Cordydipitaceae* family are often characterized as insect pathogens, and some of them can be used as biological control against crop pests, such as aphids [81]. From the 18 genera classified in this family, shades of orange to deep-red pigments have been described, and mainly found in the 5 following clades: *Beauveria*, *Cordyceps* (the sexual states (teleomorphs) of *Beauveria* species), *Hyperdermium*, *Isaria* (formely *Paecilomyces*), *and Lecanicillium* (former *Verticillium*). The fungus *Cordyceps unilateralis* can produce six extracellular red naphthoquinone pigments, with erythrostominone as the major one, followed by 4-*O*-methyl erythrostominone, deoxyerythrostominol, deoxyerythrostominone, epierythrostominol, and in a smaller proportion 3,5,8-trihydroxy-6-methoxy-2-(5-oxohexa-1,3-dienyl)-1,4-naphthoquinone (shortened to 3,5,8-TMON) [25, 82] (Fig. 8). Interestingly, the 3,5,8-TMON presents the most intense red hue and the lowest cytotoxic properties among the six naphthoquinones mentioned, rendering this latter promising for food and cosmetic applications. Moreover, it has been shown that erythrostominone can be chemically converted to 3,5,8-TMON by heating up the fermentation broth (100 °C) under acidic conditions (pH 4).

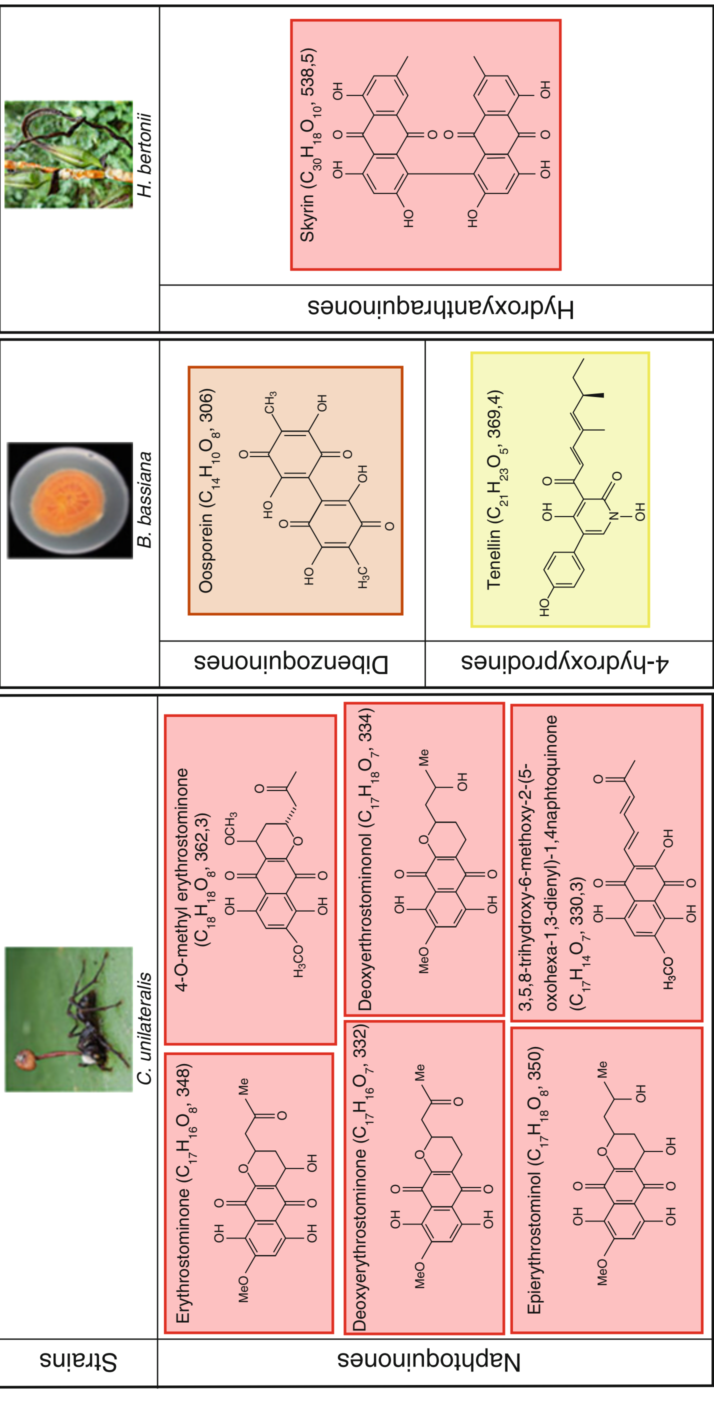

Fig. 8 Main naphtoquinone, dibenzoquinone and hydroxyanthraquinone pigments produced by *Cordyceps*, *Beauvaria* and *Hyperdermium* species

A blood-red dibenzoquinone mycotoxin, oosporein, was isolated in *Beauveria bassiana* [83] and *Lecaniccilium aphanocladii* [84]. This molecule has a wide range of bioactivities from antifungal, antimicrobial, and phytotoxic effects to growth inhibition in plants. Additionally, kidney damage and even death were noticed in poultry exposed to oosporein. Yellow pigments 2-pyridone tenellin [85] and bassianin [86] have been described in *Beauveria sp*. (Fig. 8). Little has been investigated on these components. Tenellin have also been used as biocontrol agent for agrochemical pests, such as thrips, bollworms, whiteflies, mealybugs, and mites. Similarly, the known orange-red anthraquinone dimer, skyrin, was found in *Hyperdermium bertonii*, and was demonstrated to have selective toxicity toward insect cells, which suggests potentialities for agrochemical applications of such pigments [87]. Other alkaloid-type yellow pigments, torrulbiellone A, as well as brownish isocoumarine glucoside compounds, were described in *Torrubiella sp*. [88, 89], and were concluded to have strong activity against human cancer cell lines, in particular regarding breast cancer and epidermoid carcinoma.

A red pigment produced by a strain of *Isaria farinosa* (formerly *Paecilomyces farinosus*) was elucidated as a chromophore of the hydroxyanthraquinone type [17, 42]. This pigment was excreted in the fermentation broth and was relatively easily extracted with a mixture of water and ethanol (1:1, v/v). The ready availability of this pigment along with its heat, pH, and temperature resistance offer new insights for food coloring applications.

Thus, the *Cordycipitaceae* family displays promising fungal strains producing relatively high concentrations of reddish bioactive pigments. Most of them are also mycotoxins, whose activities against insects have been widely demonstrated, suggesting that further toxicity tests would be required to ensure they would remain harmless to human health whatsoever their final industrial application and discard.

2.7 Species of the Xylariaceae Family Producing Pigments

The *Xylariaceae* is one of the largest families of filamentous fungi isolated from plant material. This is due to the fact that many members, as *Hypoxylon* or *Daldinia*, can develop an endophytic stage during their life cycle. Nowadays, around 1300 species are accepted in this family. They essentially grow under the form of mycelial structure, as their fruiting bodies (stromata) seem to form only when their host is stressed or unhealthy. Thereby, the elucidation of their life cycle and their phylogeny have been recently achieved, thanks to molecular studies. They are known to produce several novel and interesting secondary metabolites such as antiparasitic agents, enzyme inhibitors, immunomodulators, antimicrobial substances, or pigments, mainly extracted from their fruiting stages [90].

The subfamily *Hypoxyloideae* includes many genera whose stromata show bright colors, depending on their cycle stage. The stromata of many *Xylariaceae*, above all the genera *Hypoxylon*, *Daldinia*, and *Annulohypoxylon*, are rich in characteristic pigments that also serve as chemotaxonomic marker molecules [91, 92]. Many of them are azaphilones containing a highly oxidized pyrone–quinone bicyclic core

with a chiral quaternary center. *Hypoxylon lechatii*, collected in French Guiana, produced vermelhotin and three novel congeners hypoxyvermelhotins A–C. Like vermelhotin, these compounds constitute orange-red pigments and a preliminary biological characterization revealed rather strong cytotoxic and moderate to weak antimicrobial effects [93–95]. High-performance liquid chromatography (HPLC) profiling of *H. fragiforme* (a common fungus associated with *Fagus sp.* in the Northern hemisphere) by Stadler et al. [96] revealed changes in the pigment composition during stromatal development. The white cytotoxic mycotoxin, cytochalasin H, and two new cytochalasins (fragiformins A–B) were identified as major constituents of the young, maturing stromata, whereas mature, ascogenous material yielded large amounts of mitorubrin-type azaphilones. Indeed, the red color of mature *H. fragiforme* as well as in *H. howeanum* is due to four mitorubrin azaphilones (mitorubrin, (+)-mitorubrinol, mitorubrinol-acetate, and (+)-mitorubrinic acid) which are concentrated in orange-brown granules located beneath the stromatal surface [97]. Mellein derivatives and the green pigment hypoxyxylerone have also been isolated from cultures of this fungus [98, 99]. Two apparently specific mitorubrinol derivatives were, moreover, identified from the stromata of a strain of *H. fulvo-sulphureum*, belonging to the *H. rubiginosum* complex [100]. Bodo et al. [101] identified a novel naphthyl-naphthoquinone: 5-hydroxy-2- (1′,8′-dihydroxy-4′-naphthyl)-1,4-naphthoquinone, named hypoxylone (orange), in *H. sclerophaeum*. From *H. rickii*, rickenyl B and D (red and brown) were obtained. These compounds belong to the class of parasubstituted terphenyls, which are widespread in the kingdom Fungi without any preferences for specific taxonomic groups [94]. Seven new pigmented azaphilones: lenormandins A–G, were also extracted from several strains of *H. lenormandii*, and *H. jaklitschii* sp. nov. They seem specific for these species or closely related taxa [102].

In the comprehensive review from Stadler et al. [103], it is mentioned that *H. fuscum* and its allies have greenish, olivaceaous or isabelline pigment colors due to daldinins (*H. fuscum* chemotype). *H. rubiginosum* and allies are typically orange, orange brown, or yellowish green from the presence of mitorubrin (*H. fragiforme* chemotype), rubiginosin (*H. rubiginosum* chemotype), or hypomiltin (*H. hypomiltum*/ *perforatum* chemotype). *H. macrocarpum* contains different olivaceous to purplish brown pigments named macrocarpones.

Anyway many members of this genus show very diverse colors and hues: vinaceous (strains of *Hypoxylon rubiginosum*), bright orange (*H. petriniae*), deep orange or sienna (*H. subticinense*), orange–red (*H. rutilum*), amber, ochreous yellow or greenish-yellow (*H. perforatum*), lilaceaous gray to purple (*H.* cf. *vogesiacum*) [104]. These different colors clearly reflect the presence of several different metabolites bound to be identified through additional studies [105].

The diversity and complexity of secondary metabolites in this subfamily (*Xylariaceae*: *Hypoxyloideae*) has been demonstrated through several studies. In 2010, Laessoe et al. [106] identified the yellowish lepraric acid, and derivatives from *Hypoxylon aeruginosum*, but also from *Chlorostroma subcubisporum* and *C. cyaninum* sp. nov. (species close to *Thuemenella* sp.). Similar substituted chromones were also known from lichenized ascomycetes or plants (see review by Ellis [107]).

The production of specific azaphilones like cohaerin A, multiformin A, and sassafrins D recently allowed to place some strains formerly identified as *Hypoxylon sp.* in a new genus, namely *Annulohypoxylon* [108–110]. Additional studies of *A. cohaerens* further established two subtypes, one of which contains cohaerin variants A and B [109], while the other contains the cohaerins C-F [111]. Additional cohaerins G-K in yellow hues were obtained from this strain showing evidence of the coexistance of several cohaerin's structural variations, produced by the fungus *A. cohaerens*, through the combination of single minor changes [112]. Various *Entonaema spp.* (Pyrenomycetes) as *Entonaema splendens* also contain mitorubrins variants such as entonaemins, rubiginosins, or hypomiltin [113, 114]. Sassafrins A–D, four new azaphilones, were also extracted from the stromata of *Creosphaeria sassafras*. Their apparently unique occurrence in *C. sassafras* supports the status of this fungus as a member of a distinct genus within the *Xylariaceae*, coinciding with molecular and morphological traits [108].

Up to date the genus *Daldinia* includes about 20 angiosperm associated species. Some of them have recently been clustered with the genera *Xylaria* and *Hypoxylon* [115]. They develop a wide range of hues from yellow to purple, essentially due to the pigments produced in their stromatal structures but also during the mycelial growth. The main metabolites detected strongly differ according to the culture conditions [116, 117]. However, all the experimented species (*D. bambusicola*, *D. caldariorum*, *D. childiae*, *D. clavata*, *D. concentrica*, *D. eschscholzii*, *D. fissa*, *D. grandis*, *D. lloydi*, *D. loculata*, *D. cfr loculata*, *D. petriniae*, *D. singularis*, *D. sp. "Scania"*) produce the yellow BNT (1,1′-Binaphthalene-4,4′-5,5′-tetrol) and daldinol in their stromata (Fig. 9). The yellowish daldinal A, daldinal B, and daldinal C were also produced by *D. concentrica* and *D. eschscholzii*. The colored 8-methoxy-1-napthol and 2-Hydroxy-5-methylchromone were found from the liquid cultures of all strains. The yellow azaphilones and benzophenones found in *D. childiae* were lacking in species with purple stromatal colors. Most cultures of *Daldinia spp*. then produced naphthalene and chromane derivatives, differing from allied genera by the absence of mellein. Stromata of *Daldinia spp.* did not produce mitorubrin but generally contained binaphthyls.

2.8 Species of the Chaetomiaceae and Sordariaceae Families Producing Pigments

Chaetomiaceae and especially the genus *Chaetomium* is a commoun fungal genus from soil and environment. *Chaetomium cupreum* amongst other exhibit antagonistic activities against many fungi. Ketomium®, a commercial product, has been developed from this species, and is being widely used as broad spectrum bio-fungicide for disease control in various crops [118]. *C. cupreum* abundantly produces a red pigment identified as oosporein (6,6′-tetrahydroxy-2,2′ dimethyl-5,5′-bi-p-benzoquinone), known to have antifungal effects against *Rhizoctonia solani*, *Botrytis cinerea*, *Pytium ultimum* and many pathogenic fungi. More recently three new azaphilones named rotiorinols A – C (red), two new stereoisomers,

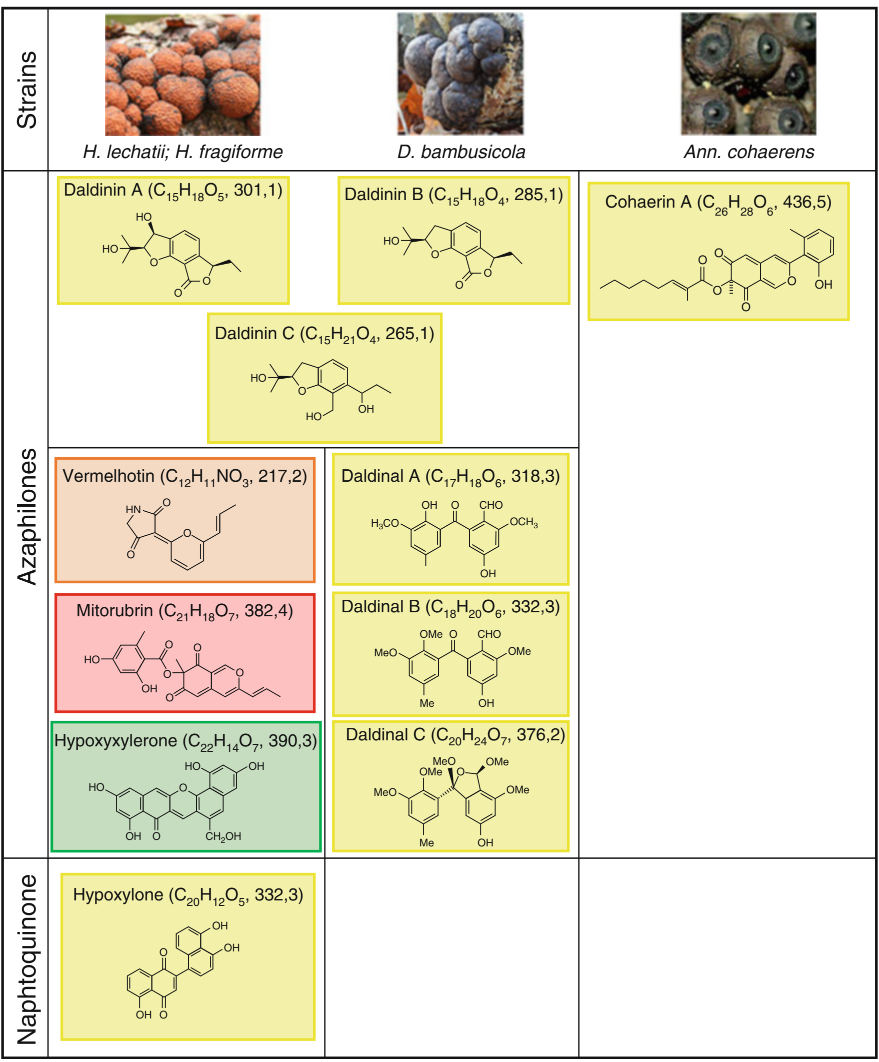

Fig. 9 Main azaphilone and naphthoquinone pigments produced by *Hypoxylon*, *Daldania* and *Annulohypoxylon* species

(−)-rotiorin (red) and epi-isochromophilone II (yellow), and a known compound, rubrorotiorin (red), were isolated from *Chaetomium cupreum* [119]. This rubrorotiorin is also known from *Penicillium hirayame* [120]. Most of these compounds are active mycotoxins. *C. globosum* (*C. globosum* var. *flavo-viridae*) is also producing yellow azaphilones named chaetoviridins (A, B, C, D) [121]. Chaetoviridin A is clearly involved in the induction of chlamydospores-like cells and also inhibits the growth of other fungi. Other strains of *C. globosum*, moreover,

produce the cytochalasan alkaloids chaetoglobosins A–G, J, Q, R, T, U arising from a mixed polyketide-amino acid biosynthetic pathway, and with different hues (F is a pale yellow compound also found in *Chaetomium subaffine*). Strains also produce chaetoglobin A–B, chaetomugilins A–F, I, M, seco-chaetomugilins A–D, and three new nitrogenous azaphilones; 4′-epi-N-2-hydroxyethyl-azachaetoviridin A (dark red), N-2-butyric-azochaetoviridin E (orange), and isochromophilone XIII (orange) [122–124]. *C. globosum* additionally synthesizes a purple pigment called cochliodinol [125].

In the same family, the thermophilic genus *Thielavia zopf* (*Chaetomiaceae*, Sordariales) (anamorphic genus in the human pathogenic *Myceliophthora* sp.) is also widespread as plant endophytes. Many species are characterized by darkly pigmented ascospores containing melanin and/or by pigments exuded in the culture media. *T. intermedia* or *T. rapa-nuiensis* sp. nov. f. i. excrete orange-yellow pigments. *T. terrestris* colonies are yellow to orange in reverse and sometimes exude a diffusible reddish brown pigment [126, 127]. Several nor-spiro-azaphilones (thielavialides A – D), and a bis-spiroazaphilone (thielavialides E), have been identified from the cultures, but they appear as unpigmented [128]. The human pathogenic *Achaetomium* sp., is known to produce the orange hydroxyanthraquinone pigment, parietin [129]. Such agents, sometimes causing osteomyelitis or fatal cerebral mycosis are generally difficult to isolate, identify, and also treat effectively. The pigments production in this family is not the first skill to be studied (Fig. 10).

Neurospora crassa is a well-known *Sordariaceae* originating from tropical or subtropical countries. It produces several yellow-orange carotenoids identified as phytoene, ß-carotens, γ-carotene, lycopene, neurosporen, neurosporaxanthin or spirilloxanthin. Overaccumulation of carotenoids has extensively been studied in this genus, and overexpression has frequently been generated by photoinduction [130–132].

3 Natural Carotenoid Pigments Produced by Filamentous Fungi

For several decades, carotenoids have been commercially produced by chemical synthesis or sold as plant extracts or oleoresins, e.g., of tomato and red pepper. Some unicellular green algae, under appropriate conditions, become red due to the accumulation of high concentrations of "secondary" carotenoids. Two examples, *Dunaliella* spp. and *Haematococcus pluvialis*, are cultured extensively as sources of β-carotene and (3S,3‘S)-astaxanthin, respectively.

Nonphotosynthetic microorganisms – fungi, bacteria, yeasts – are also often strongly pigmented by carotenoids, so commercial production by these organisms is an attractive prospect. Fermentation processes for pigment production at commercial scale are now in use in the food industry, such as the production of β-carotene from the fungus *Blakeslea trispora*, in Europe. Efforts have been made to reduce the production costs so that pigments produced by fermentation can be competitive with

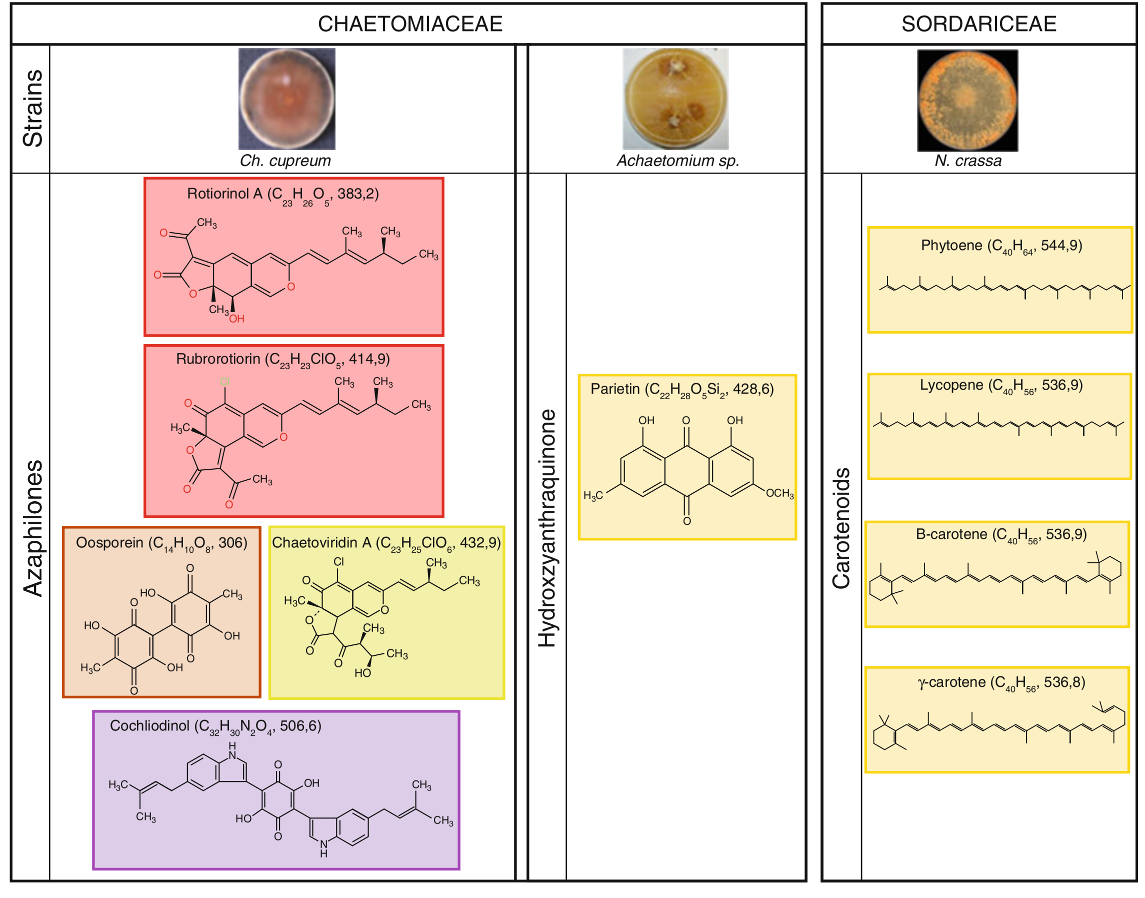

Fig. 10 Main azaphilone, hydroxyanthraquinone and carotenoid pigments produced by *Chaetomium*, *Achaetomium* and *Neurospora* species

synthetic pigments or with those extracted from natural sources, i.e., plant or microalgal. There is scope for innovations to improve the economics of carotenoid production by isolating new microorganisms, creating better ones, or improving the processes. The fungal carotenoid products may be used as color additives in many industries, including food and feed, and are now under consideration for use as health supplements.

3.1 β-Carotene

β-Carotene (Fig. 11) is produced on a large scale by chemical synthesis, and also from plant sources such as red palm oil, as well as by fermentation and from microalgae.

3.1.1 *Blakeslea trispora*

Blakeslea trispora is a commensal microorganism associated with tropical plants. The fungus exists in (+) and (−) mating types; the (+) type synthesizes trisporic acid, which is both a metabolite of β-carotene and a hormonal stimulator of its biosynthesis. On mating the two types in a specific ratio, the (−) type is stimulated by trisporic acid to synthesize large amounts of β-carotene [133, 134]. The production process proceeds essentially in two stages. Glucose and corn steep liquor can be used as carbon and nitrogen sources. Whey, a by-product of cheese manufacture, has also been considered, with strains adapted to metabolize lactose. In the initial fermentation process, seed cultures are produced from the original strain cultures and subsequently used in an aerobic submerged batch fermentation to produce a biomass rich in β-carotene. In the second stage, the recovery process, the biomass is isolated and transformed into a form suitable for isolating the β-carotene, which is then extracted with ethyl acetate, suitably purified and concentrated, and crystallized. The final product is either used as crystalline β-carotene (purity >96 %) or is formulated as a 30 % suspension of micronized crystals in vegetable oil. The production process is subject to Good Manufacturing Practices (GMP) procedures and adequate control

β-carotene

lycopene

Fig. 11 Chemical structures of the carotenoids ß-carotene and lycopene

of hygiene and raw materials. The biomass and the final crystalline product comply with an adequate chemical and microbiological specification and the final crystalline product also complies with the JECFA (Joint FAO/WHO Expert Committee on Food Additives) and European Union (EU) specifications as set out in Directive 95/45/EC for coloring materials in food.

The first β-carotene product from *B. trispora* was launched in 1995. The mold has shown no pathogenicity or toxicity in standard pathogenicity tests in mice, by analysis of extracts of several fermentation mashes for fungal toxins, and by enzyme immunoassays of the final product, the β-carotene crystals, for four mycotoxins. HPLC analysis, stability tests and microbiological tests showed that the β-carotene obtained by co-fermentation of *Blakeslea trispora* complies with the EU specification for β-carotene (E 160 aii), listed in Directive 95/45/EC, including the proportions of *cis* and *trans* isomers, and is free of mycotoxins or other toxic metabolites and free of genotoxic activity. In a 28-day feeding study in rats with the β-carotene manufactured in the EU no adverse findings were noted at a dose of 5 % in the diet, the highest dose level used. The EU Scientific Committee considered that "β-carotene produced by co-fermentation of *Blakeslea trispora* is equivalent to the chemically synthesized material used as food colorant and is therefore acceptable for use as a coloring agent for foodstuffs."

There are now other industrial productions of β-carotene from *B. trispora* in Russia, Ukraine, and Spain. The process has been developed to yield up to 170 mg of β-carotene/g dry mass or about 17 g/L. *Blakeslea trispora* is now also used for the production of lycopene (see Sect. 3.2.).

3.1.2 *Phycomyces blakesleeanus*

Another fungus, *Phycomyces blakesleeanus*, is also a potential source of various chemicals including β-carotene [135]. The carotene content of the wild type grown under standard conditions is modest, about 0.05 mg per g dry mass, but some mutants accumulate up to 10 mg/g. As with *Blakeslea trispora*, sexual stimulation of carotene biosynthesis is essential and can increase yields to 35 mg/g. The most productive strains of *Phycomyces* achieve their full carotenogenic potential on solid substrates or in liquid media without agitation. *Blakeslea trispora* is however more appropriate for production in usual fermentors.

3.1.3 *Mucor circinelloides*

Mucor circinelloides wild type is yellow because it accumulates β-carotene as the main carotenoid. The basic features of carotenoid biosynthesis, including photoinduction by blue light [136], are similar in *Phycomyces* and *Mucor. M. circinelloides* is a dimorphic fungus that grows either as yeast cells or in a mycelium form, and research is now focused on yeast-like mutants that could be useful in a biotechnological production.

Table 2 Percentage of geometrical isomers in "lycopene" from various sources

Source	(all-*trans*)	(5-*cis*)	(9-*cis*)	(13-*cis*)	Others
Chemical synthesis	>70	<25	<1	<1	<3
Tomato	94–96	3–5	0–1	1	<1
Blakeslea trispora	≥ 90	(mixed *cis* isomers) 1–5			

3.2 Lycopene

Lycopene (Fig. 11) is produced on a large scale by chemical synthesis, and from tomato extracts, in addition to production by fermentation. As with β-carotene, the various preparations differ in the composition of geometric isomers (Table 2). Lycopene is an intermediate in the biosynthesis of all dicyclic carotenoids, including β-carotene. In principle, blocking the cyclization reaction and the cyclase enzyme by mutation or inhibition should lead to the accumulation of lycopene. This strategy is employed for the commercial production of lycopene.

3.2.1 *Blakeslea trispora*

A commercial process for lycopene production by *Blakeslea trispora* is now established. Imidazole or pyridine is added to the culture broth to inhibit the enzyme lycopene cyclase. The product, predominantly (all-trans)-lycopene, is formulated into a 20 % or 5 % suspension in sunflower oil, together with α-tocopherol at 1 % of the lycopene level. Also available is an α-tocopherol-containing 10 % or 20 % lycopene cold-water-dispersible (CWD) product. Lycopene oil suspension is intended for use as a food ingredient and in dietary supplements [137]. The proposed level of use for lycopene in food supplements is 20 mg per day.

Approval for the use of lycopene from *B. trispora* was sought under regulation No 258/97/EC of the European Parliament and the Council concerning novel foods and novel food ingredients. The European Food Safety Authority (EFSA) was also asked to evaluate this product for use as a food color. The conclusions were that the lycopene from *B. trispora* is considered to be equivalent to natural dietary lycopene. The toxicity data on lycopene from *B. trispora* and on lycopene from tomatoes do not give indications for concern. In its opinion of January 30, 2008, the EFSA derived a low numerical Acceptable Daily Intake (ADI) of 0.5 mg/kg body weight/day for lycopene from all sources, and the risk assessor also concluded that with the uses and actual use levels presented by the applicants from industry, the intake of lycopene from natural sources and as a food coloring would be expected to remain within this ADI. The main concern is that the proposed use levels of lycopene from *B. trispora* as a food ingredient may result in a substantial increase in the daily intake of lycopene compared to the intakes solely from natural dietary sources. Additionally, the use of lycopene as a health supplement is also becoming very popular. Data from the Framingham Offspring Study – an epidemiological analysis that indicates correlation and not causation – recently reported that increased intakes of lycopene are associated with a reduction in the incidence of cardiovascular disease and coronary heart disease.

3.2.2 *Fusarium sporotrichioides*

The fungus *Fusarium sporotrichioides* has been genetically modified to manufacture lycopene from the cheap corn-fiber material, the “leftovers” of making ethanol. By use of sequential, directional cloning of multiple DNA sequences, the isoprenoid pathway of the fungus was redirected toward the synthesis of carotenoids via carotenoid biosynthesis genes introduced from the bacterium *Erwinia uredovora*. Cultures in laboratory flasks produced 0.5 mg lycopene per g dry mass within 6 days and improvements are predicted.

4 Natural Pigments Produced by Marine-Derived Filamentous Fungi

As their terrestrial counterparts, marine and marine-derived fungi are also able to produce colored compounds. They are therefore able to exhibit bright colors, from yellow to black, mainly belonging to polyketides. Indeed, several papers report that polyketides seem to dominate marine natural products of fungal origin [138]. It is also widespread that the colored molecules identified from terrestrial fungi can often be isolated from the same species living in a marine environment. For instance, catenarin, emodin, erythroglaucin, physcion, questin, and rubrocristin or physcion anthrone are produced by marine derived *Aspergillus* and/or *Eurotium* species, as well as by their terrestrial counterparts. Anyway, fungi from marine ecological niches are today considered as promising novel sources of chemically diverse pigments, and the literature abundantly reports the interest for marine organisms with respect to the production of new molecules and, among them, new pigments [139, 140]. Indeed, many marine ecological niches are still unexplored and it seems plausible that unique features of marine environments can be the inducers of unique substances synthesized by marine or marine-derived microorganisms [141]. The potential of marine derived microorganisms to produce unique and original molecules could therefore come from specific metabolic or genetic adaptation appearing to meet very specific combinations of physico-chemical parameters (high salinity, low O_2 penetration, low temperature, limited light access, and high pressure). Two status lead to particular behaviors and products: either, the challenge of facing unusual living conditions (exogenous fungi), or, the use of specific procedures naturally adapted to the marine niches (for instance marine organisms’ fungal endophytes) (i.e., indigenous micromycetes, naturally selected by aquatic environments). For now, the highest diversity of marine fungi seems to be found in tropical regions, mainly in tropical mangroves, which are extensively studied because of their high richness in organic matters [142, 143]. These biotopes seem favorable to the development of a high diversity of heterotrophic microorganisms. Many genera producing pigments have then been isolated either from water, sediments, decaying plants, or from living organisms as invertebrates, plants (endophytes) or algae. Anyway, in unusual biotopes (sometimes extreme), the fungal species with pigmented cell walls (in the spores and/or mycelium), are clearly able to tolerate dehydration-hydration cycles or high solar radiations, better than the moniliaceous

fungi, whose cells are devoid of pigments. These aromatic compounds, as melanin, sporopollenin (brown product of oxydative polymerization of β-carotene) or cycloleucomelone (terphenylquinone), often show significant antioxidant activities, and are bound to protect the biological structures, giving them an excellent durability and a high potential for survival in hostile environments [144, 145].

5 The Coding Genes and Biosynthetic Pathways of Pigments in Filamentous Fungi

Biosynthesis of polyketide secondary metabolites has been subjected to more intensive studies among other classes of secondary metabolite pathways in fungi [146]. The majority of genes required for the production of these metabolites are mostly organized in gene clusters, which often are silent or barely expressed under laboratory conditions, making discovery and analysis difficult. Fortunately, the genome sequences of several filamentous fungi are now publicly available and greatly facilitate the establishment of links between genes and metabolites. In the last decade, whole genome sequencing of various fungi has revealed that these microorganisms have immense biosynthetic potential surpassing by far the chemical diversity observed in laboratory culture [147]. For example, the genome of many *Aspergilli* are found to encode for a combined 30 to 80 PKS, non-ribosomal peptide synthetases and polyketide non-ribosomal peptide synthetases hybrids, which far exceed the total number of known polyketides and non-ribosomal peptides [147]. From these, the fungal PKS are of considerable interest due to their interesting enzymology and the polyketide structural diversity [37]. One of the earlier major advances in identification of fungal polyketide secondary metabolite gene clusters is the development of degenerate primed Polymerase Chain Reaction (PCR), based on conserved ketosynthase domain of PKS [148].

5.1 Biosynthesis of Fungal Azaphilone Pigments

Besides the fungal polyketide-derived secondary metabolites, azaphilones are a class of fungal metabolites characterized by a highly oxygenated pyrano-quinone bicyclic core [111, 149–154]. Azaphilone compounds exhibit a wide range of interesting biological activities, such as antimicrobial, antifungal, antiviral, antioxidant, cytotoxic, nematicidal and anti-inflammatory activities [8]. Many of these effects may be explained by the reactions of azaphilones with amino groups, such as those found in amino acids, proteins and nucleic acids. According to Osmanova et al. [155], different azaphilone compounds occur in fungi belonging to 23 genera from 13 families: these azaphilone compounds can be classified into ten different structural groups and the largest group (azaphilones with a lactone ring) includes 68 substances, e.g., the yellow pigment ankaflavin [155]. In total, over 370 fungal azaphilone metabolites have been described in the literature [8]. Considering the variety of azaphilone

compounds that occurs, one finds that some fungal species are able to produce a huge variety of diverse types, whereas others are able to produce only one or two different types of azaphilones. An example of the first kind is *Penicillium* spp. of the *Trichocomaceae* family, which produces over 40 azaphilone compounds from five types: azaphilones with a lactone ring; azaphilones with an aliphatic side chain; azaphilones with *o*-orsellinic acid; azaphilones with an ergostane skeleton and *o*-orsellinic acid; and bicyclic spiro-azaphilones. In contrast, *Phomopsis euphorbiae* contains only one type of azaphilone, namely an azaphilone with an aliphatic side chain. Some of these fungal azaphilone compounds absorb visible light and are colored, namely azaphilone pigments, e.g., ankaflavin (yellow), monascin (yellow), monascorubrin (orange), rubropunctatin (orange), monascorubramine (purple), and rubropunctamine (purple), while others are colorless. Azaphilone pigments are responsible for the bright yellow, red, or green colors of fruiting bodies or mycelia of numerous species of ascomyceteous including genera *Monascus*, *Penicillium*, *Talaromyces* and *Aspergillus*. The color of azaphilone pigments depends on their chemical structure.

Biosynthetically, azaphilone pigments as well as most pigments produced by fungi are polyketide based and involve complex pathways. It is known that the biosynthesis of azaphilone pigments uses both the polyketide pathway and the fatty acid synthesis pathway [8, 156]. In fact, the hexaketide chromophore of azaphilone pigments is derived from the condensation of acetate and malonate by polyketide synthases, while the side chain of these azaphilone pigments arises from a medium-chain fatty acid synthesized via the fatty acid synthetic pathway. The polyketide pathway assembles the main polyketide (hexaketide) chain of the azaphilone pigments from acetic acid (the starter unit) and five malonic acid molecules (the chain extender unit) in a conventional way to generate the hexaketide chromophore structure. The fatty acid synthesis pathway produces a medium-chain fatty acid (octanoic or hexanoic acid) that is then bound to the chromophore by a transesterification reaction in order to form the azaphilone pigment (Fig. 12) [156, 157]. In the literature, biosynthetic pathways are suggested for the following azaphilones: monascorubrin and monascoflavin; mitorubrin and rubropunctatin [149]; ascochitine [150]; ochrephilone [150]; citrinin [150, 156]; monascusones A and B, monascin [158] and sassafrin D [108, 109].

5.2 Biosynthesis of Fungal Hydroxyanthraquinone Pigments

Besides intensively investigated fungal azaphilone pigments, hydroxyanthraquinone compounds have been considered among the most abundant fungal natural products giving color to spores, sclerotia, sexual bodies and other developmental structures [159] as exemplified in case of pigments produced by *Curvularia lunata* [17]. Anthraquinones are a class of chemical compounds of the quinone family that differ in the nature and positions of substituent groups [17]. Anthraquinoid derivatives are derivatives of the basic structure 9,10-anthracenedione or also called 9,10-dioxoanthracene, i.e., a tricyclic aromatic organic compound with formula

Fig. 12 Possible biosynthetic pathways to azaphilone metabolites, monascusone B and monascin, produced from the fungus *Monascus kaoliang* via fatty acid synthesized pathway proposed by Jongrungruangchok et al. 2004 (Adapted from Gao et al. [8])

$C_{14}H_8O_2$ and whose ketone groups are on the central ring. In general, for each anthraquinoid derivative there are eight possible hydrogens that can be substituted. The term "hydroxyanthraquinone" usually refers to derivatives of 9,10-hydroxyanthraquinone, i.e., derivatives of 9,10-anthraquinone where any number *n* of hydrogen atoms have been replaced by *n* hydroxyl groups. In this case the number *n* of hydroxyl group is indicated by a multiplier prefix (mono-, di-, tri-, up to octa-). The hydroxyanthraquinones absorb visible light and are colored, whereas strictly 9,10-anthraquinones are colorless. It appears that the color of the hydroxyanthraquinone pigments depends on the position and number of the hydroxyl substituents on the different rings. About 700 anthraquinone derivatives were identified from plants, lichens and fungi; 43 have already been described from fungal cultures [17, 32]. These molecules present a great interest in the field of dyeing molecules: they decline a widerange of nuances in the shades of brown, purple, red, orange to yellow, highly requested in cosmetics, clothes dyeing and foodstuff industries [3, 4, 17, 160].

From their structures, hydroxyanthraquinone pigments, are relatively stable (like the well heat stable hydroxyanthraquinone, carminic acid from insects) and have a superior brightness compared to azo-pigments [3, 4, 17]. Then, they possess good light-fastness properties, which often makes metallization unnecessary, even if hydroxyanthraquinone derivatives can easily form coordination complexes with several cations. In textile industry, hydroxyanthraquinone are, for example, considered as « reactive dyes » as they form a covalent bond with the fibers, usually cotton, although they are used to a small extent on wool and nylon. Therefore, they made it possible to achieve extremely high washfastness properties by relatively simple dyeing methods. A marked advantage of reactive dyes over direct dyes is that, their chemical structures are often much simpler, their absorption spectra show narrower absorption bands, and the dyeing are brighter [161].

Hydroxyanthraquinone pigments are another interesting set of secondary fungal metabolites and exhibit a wide range of interesting biological activities, such as antioxidant, antimicrobial, antifungal, antiviral, and cytotoxic activities [3, 4, 17, 32, 160]. Numerous hydroxyanthraquinone structures have been described, particularly from members of the *Trichocomaceae* (*Aspergillus* spp., *Emericella* spp., *Paecilomyces* spp., and *Eurotium* spp.), *Pleosporaceae* (Fusarium spp.), and *Nectriaceae* (*Alternaria* spp., *Curvularia* spp., and *Drechslera* spp.) families. Gessler et al. [32] and Hanson et al. [162] explained that anthraquinones are formed via the polyketide pathway and regulated by non reducing polyketide synthases, i.e., multienzymes complexes including acyl carrier protein, transacylase, ketosyntase, malonyl-CoA transacylase, methyltransferases and reductases, ensuring the condensation of acetyl-CoA (starter unit) and malonyl-CoA (extender unit), and producing a unstable β-polyketide chain (containing a free carboxyl group) precursor of different aromatic structures like the hydroxyanthraquinone pigments (Fig. 13).

Fungal hydroxyanthraquinones that are synthesized following this acetate-malonate pathway show a characteristic substitution pattern, i.e., they show substitution on both aromatic rings, and more particularly, at least one hydroxyl group in position R1 and one hydroxyl or methoxyl group in position R8, examples being emodin (yellow), physcion (yellow), dermolutein (yellow), chrysophanol (red), erythroglaucin (red), dermocybin (red), dermorubin (red), tritisporin (reddish brown), cynodontin (bronze) and helminthosporin (maroon), which are produced by *Aspergillus* spp., *Eurotium* spp., *Fusarium* spp., *Trichoderma* spp., *Curvularia lunata* and *Drechslera* spp. According to a practical hydroxyanthraquinone classification [17] based on the position of the functional groups added on the 9,10-anthraquinone backbone, the main hydroxyanthraquinone pigments of fungal origin are classified in the "group A1" (compounds which show substitution on both aromatic rings, and at least two hydroxyl groups in both R1 and R8 positions), or into the "group A2" of hydroxyanthraquinone pigments (compounds which show substitution on both aromatic rings and at least two hydroxyl groups in R1 and R6 positions and one methoxyl group in R8 position). According to this polyketide pathway, the biosynthetic relationships show that the yellow hydroxyanthraquinone pigments (e.g., emodin, physcion and dermolutein) exist in the beginning of the synthesis pathway, whereas the red hydroxyanthraquinone ones, like dermorubin

Fig. 13 Scheme of the cyclization of the ß-polyketide chain during the synthesis of anthraquinones in fungi (Based on Gessler et al. [32]). *I, acetyl-CoA; II, malonyl-Coa; III, ß-polyketide chain; IV-anthraquinone (R1-R8 lateral substituents); and ACP, acyl carrier protein*

and dermocybin, are more complicated in structure, and occur in the latter part of the biosynthesis pathway [17]. More recently, Bringmann et al. [163] revealed that the pigment chrysophanol is shown to be formed, according to an organism-specific route, by a third folding mode involving a remarkable cyclization of a bicyclic diketo precursor, thus establishing the first example of multiple convergence in polyketide biosynthesis. A complete knowledge about the biosynthetic pathway of hydroxyanthraquinone pigments is not yet available. The genomic approaches of selection of potential hydroxyanthraquinone pigment producers may not be useful at this point, when none of the fungal hydroxyanthraquinone pigment producers are fully genome sequenced yet. The problem of annoting correct gene sequences should not to be overlooked, especially due to the variation in the domain of the polyketide synthases involved in the biosynthesis of these fungal hydroxyanthraquinone pigments.

5.3 Biosynthesis of Fungal Naphthoquinone Pigments

Naphthoquinone pigments are produced, at least, by a broad range of *Fusarium* species. Few reports have been published on the red naththoquinone, aurofusarin, and bikaverin biosynthetic pathways. The recent sequencing of *Fusarium* genomes has revealed the large number and diversity of secondary metabolic gene clusters [164, 165]. For example, the genome of *F. fujikuroi* was recently sequenced and partially elucidated, showing that less than half of the putative produced secondary metabolites are known [166]. Examples of discovered secondary metabolites, which could be already linked to the corresponding biosynthetic gene cluster, are the putative carcinogen, fusarin C [167], the histone deacetylase inhibitor, apicidin F [48], the PKS-derived fujikurins [168], the perithecial pigments fusarubins [62], as well as the antiprotozoal mycelial pigment bikaverin [54]. Concerning the bikaverin biosynthesis pathway, the responsible non-reducing PKS-encoding gene PKS4 (FFUJ_06742), later re-named to BIK1 (BIKaverin polyketide synthase), was first described in *F. fujikuroi* by Linnemannstöns et al. [169]. Later, the complete bikaverin gene cluster was characterized by Wiemann et al. [54]. In addition to the PKS-encoding gene, five genes downstream of BIK1 were identified as part of the bikaverin gene cluster. The five genes encode a putative FAD-dependent monooxygenase (BIK2; FFUJ_06743), a putative *O*-methyltransferase (BIK3, FFUJ_06744), a putative NmrA-like transcriptional regulator (BIK4, FFUJ_06745), a putative Zn(II)2Cys6 fungal-type transcription factor (BIK5, FFUJ_06746) and a putative major facilitator superfamily (MFS) transporter (BIK6, FFUJ_06747) [54, 165, 170]. Except for BIK4, gene expression of all BIK genes in *F. fujikuroi* is strictly regulated by nitrogen availability and pH [54–56, 169]. In *F. verticillioides*, the same conditions are conducive for BIK gene cluster expression [171]. Pre-bikaverin (red) has been recognized as the first pathway intermediate and product of the biosynthetic gene BIK1. This intermediate, first described by Ma et al. [172], was identified in *F. fujikuroi* by constitutive overexpression of BIK1 in a DDBIK2/BIK3 double mutant background [54]. The condensation of 8 malonyl-CoA molecules and one acetyl-CoA molecule, catalyzed by the biosynthetic gene BIK1, resulted in the formation of the pre-bikaverin in *F. fujikuroi* (Fig. 14). More recently, the putative biosynthetic pathway for bikaverin synthesis in *F. fujikuroi* was confirmed by Arndt et al. [170]. The structure of a new bikaverin intermediate, oxo-pre-bikaverin (red), was identified by NMR and on the basis of HPLC–HRMS and HPLC–UV measurements. The downregulation of the involved cluster genes was identified by overexpression of BIK2 and BIK3 in the DDBIK2/BIK3 + OE:BIK1 mutant [170]. Neither bikaverin nor the new intermediate oxo-pre-bikaverin showed cytotoxic effects in Hep G2-Cells [170].

The aurofusarin biosynthetic pathway starts with the condensation of one acetyl-CoA molecule with six malonyl-CoA molecules, which is catalyzed by the aurofusarin polyketide synthase, AUR1 (= PKS12), resulting in the formation of the naphthopyrone YWA1 in *F. graminearum*. The tailoring enzymes modify this compound and the pathway ends with the formation of aurofusarin via the intermediate red compounds, nor-rubrofusarin and rubrofusarin (Fig. 15) [57, 58].

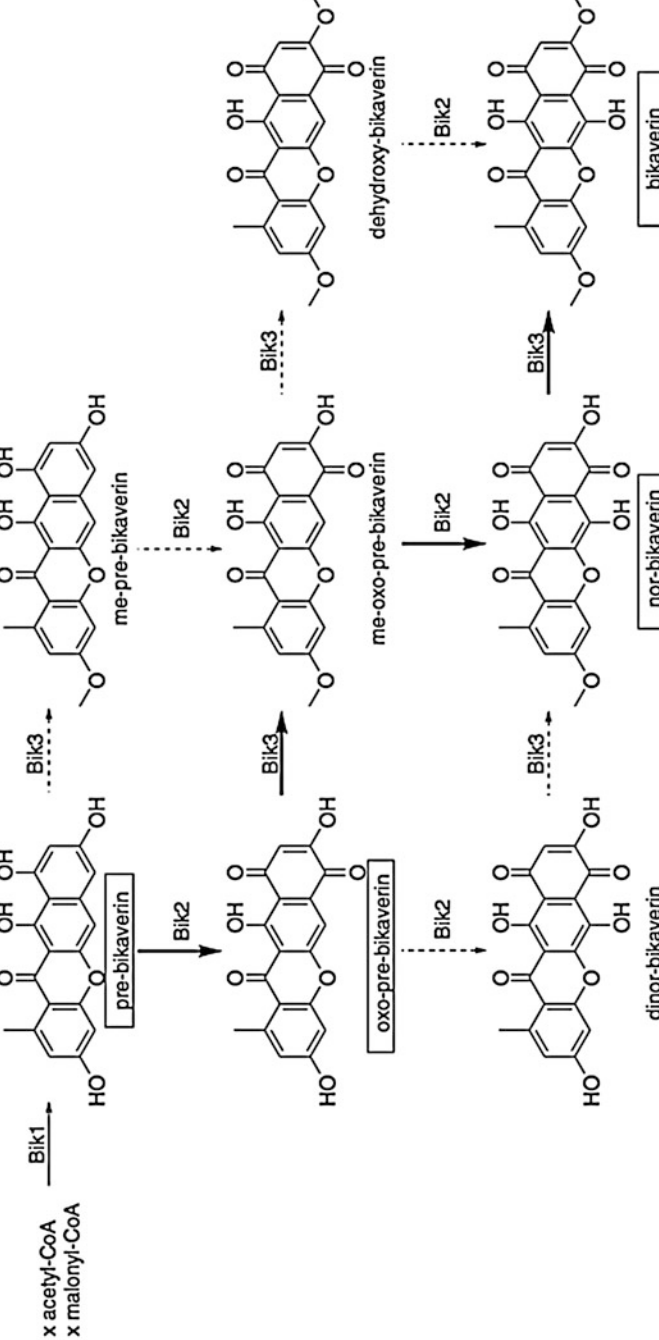

Fig. 14 Modele for biosynthesis of naphthoquinone pigment, bikaverin, in *Fusarium fujikuroi* (Based on Arndt et al. [170])

Fig. 15 The biosynthetic pathway for the dimeric naphthoquinone pigment, aurofusarin, in *Fusarium graminearum* reveals a close link between the naphthoquinones and naphthopyrones (Based on Frandsen et al. [57])

Moreover, it has been shown that aurofusarin production was the lowest under acidic conditions at pH 4 and 5, while better yields were obtained at pH 6-8 [71].

The recent study by Brown et al. [75] showed that the gene cluster associated with the PKS gene, PGL1, required for the violet perithecial pigment, that accumulates in the walls of sexual fruiting bodies in both *F. verticillioides* and *F. graminearum* [60, 73], consists of the same 6 PKS genes (i.e., PGL1 and the adjacent genes PGL2–PGL6). In contrast, the putative gene cluster includes only 3 PKS genes in *F. solani*, and interestingly, the pigment accumulating in the perithecial walls of *F. solani* is red and results from the activity of a different PKS [59]. A homolog of the PGL cluster was also identified in *F. fujikuroi*, and this fungus has been reported to produce particular red fusarubins pigments like 8-*O*-methylfusarubin as the main product [62]. Naphthoquinone pigment related to fusarubin was initially isolated from the fungus *F. solani* [74]. Functional analysis of genes in the cluster demonstrated that the *F. fujikuroi* homologs of PGL1, PGL2 and PGL3 are required for the production of fusarubin pigments, and that the PGL1 homolog is required for perithecial pigmentation [62] (Fig. 16). Although the functional characterization of most of the cluster in *F. fujikuroi* provides strong evidence that fusarubins (red pigment) are precursors of the perithecial pigment (violet hue), the

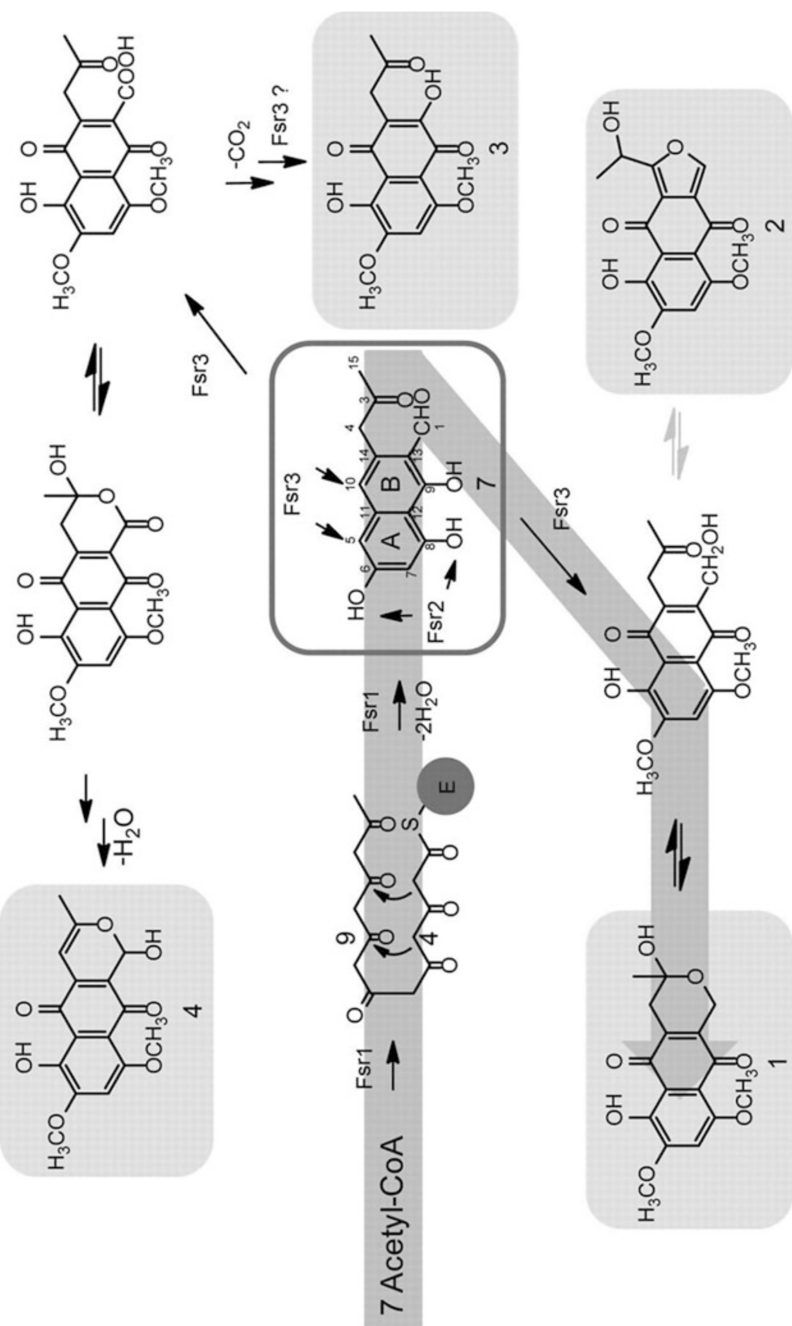

Fig. 16 (continued)

structure of the violet perithecial pigment has yet to be determined [62, 75]. Thus, studies are in progress to determine the relationship between fusarubin pigments and the violet perithecial one. A comparative genomic study of *F. oxysporum*, *F. graminearum and F. verticillioides* identified 46 potential secondary metabolite biosynthesis gene clusters, of which 87 % include a polyketide synthase gene [164].

The genomic studies by Brown et al. [75], Ma et al. [164], and Hansen et al. [165] shown that the *F. oxysporum* genome contains the BIK gene cluster (i.e., PKS16 (BIK1), PKS17, 18, 19, 20 and 21) involved in the red mycelial bikaverin pigment synthesis, which is consistent with the bikaverin production, reported previously in this fungus [69, 70] and the PGL1 (= PKS3) gene involved in the synthesis of the uncharacterized violet perithecial pigment, although no report on a violet perithecial pigment production by *F. oxysporum* has yet been described in the literature.

5.4 Biosynthesis of Fungal Carotenoids

Carotenoids are the most diverse and widespread pigments found in nature. They are a wide group of isoprenoids synthesized by all photosynthetic organisms and also by some non-photosynthetic bacteria, yeasts, and fungi. The central C40 backbone, made up of eight isoprene units, forms a polyene chain of conjugated double bonds and establishes an extended *pi*-electron system that accounts for its ability to absorb both ultraviolet (UV) radiation and visible light. The number of conjugated double bonds within this basic backbone, as well as cyclic and oxygenic modifications, yields a variety of carotenoids whose colors range from yellow to reddish brown. More than 800 carotenoid structures have been isolated from different natural sources [173].

The biosynthesis of carotenoids has been studied for many years in many organisms including fungi. Hundred of genes involved in the pathway have now been isolated from bacteria, plants, algae, and fungi; and some of these have recently been used in biotechnological research. The productivity of carotenoid can be improved by designing an efficient pathway by selecting genes from different organisms, however neither industry nor consumers did observe a surge or even the appearance of "engineered" carotenoids on the market, thus being a quite disappointing situation after so many millions euros invested, and decades after the beginning of research in this field (i.e., carotenogenic gene cluster from the bacteria *Erwinia herbicola* was described in 1991). This situation seems to change now with the appearance of carotenoids from Genetic Modified Organism (GMO) yeasts such as *Yarrowia lipolytica* (developed by Microbia Inc., now DSM Nutri-

Fig. 16 Biosynthetic pathway of fusarubins in *F. fujikuroi* (Based on Studt et al. [62]). Structures highlighted in *gray* were identified by NMR, mass spectrometry, and UV data in the liquid culture of wild-type *F. fujikuroi*. The aldehyde identified as the first intermediate in this biosynthetic pathway is *boxed*. The *solid dark gray arrow* indicates the main route of naphthoquinone formation. 6-*O*-demethylfusarubinaldehyde (compound 7); 8-*O*-methylfusarubin (compound 1); 8-*O*-methylnectriafurone (compound 2); 8-*O*-methyl-13-hydroxynorjavanicin (compound 3); 8-*O*-methylanhydrofusarubinlactol (compound 4)

tional Products) or *Kluyveromyces marxianus*, with the incorporation of an algal gene in the latter [174], new strains that try to compete with highly effective production of β-carotene or lycopene using the fungus *Blakeslea trispora*.

Although not essential, carotenoids play significant roles in some groups of fungi, particularly the zygomycetes. β-Carotene is the precursor of trisporoids, a group of compounds involved in the sexual regulation of Mortierellales and Mucorales [175]. Carotenoids are formed in fungi via the mevalonate pathway, which starts at acetyl-CoA and proceeds through mevalonate to isopentenyl-pyrophosphate (IPP), the general precursor of all isoprenoids. Eight molecules of IPP are subsequently condensed to form colorless carotenoids via several dehydrogenation reactions (Fig. 17). Additional modifications bringing color to the molecules then occur through cyclization, hydroxylation, cleavage, etc.

6 Biotechnological Approaches to Improve Fungal Pigment Production

The past decade was a period of great improvement for pigment productions and the knowledge about the different ways to increase the yields have been greatly extended.

The five major fronts currently ongoing are

- Overall analysis of gene expression i.e., genomics, proteomics, metabolomics. This is to better understand the production pathways and general metabolisms as well as the genes and the molecules involved. More than 100 fungal genomes have already been sequenced, among them several *Aspergillii*, (*A. fumigatus*, *A. nidulans*, *A. oryzae*, *A. niger*, *A. terreus*, *A. clavatus*), *Penicillii* (*P. chrysogenum*, *P. digitatum*, *Talaromyces marneiffei*) and *Saccharomycetes* (*Saccharomyces cerevisiae*, *Debaromyces hansenii*, *Kluyveromyces lactis*, *Candida albicans*...). Many others are in progress, considering humans, animals or plants pathogens, or strains useful in an industrial context.
- Development of alternative hosts that have already been given GRAS (Generally Recognized as Safe) status by the Food and Drug Agency (FDA) in the USA to be used in food industry (*P. roquefortii*, *Aspergillus oryzae*, *A. sojae*, *A. japonicus*, *Mortierella vinaceae*, *M. alpina*, *Fusarium monoliforme*, *F. veneratum*...) [176–178].
- Molecular techniques to improve expression and secretion of non-fungal proteins for the biosynthesis of unusual metabolites in filamentous fungi
- Molecular techniques to carry out metabolic engineering to modify and improve particular biosynthetic pathways. Further metabolic engineering to optimize already existing or exogenous biosynthetic pathways.
- Extensive use of Design Of Experiment (DOE) to improve the conditions of pigments production, combining the main physicochemical parameters: temperature, oxygen, carbon, nitrogen and other nutrient sources, pH regulation, light exposure and physiological stage of the fungi.

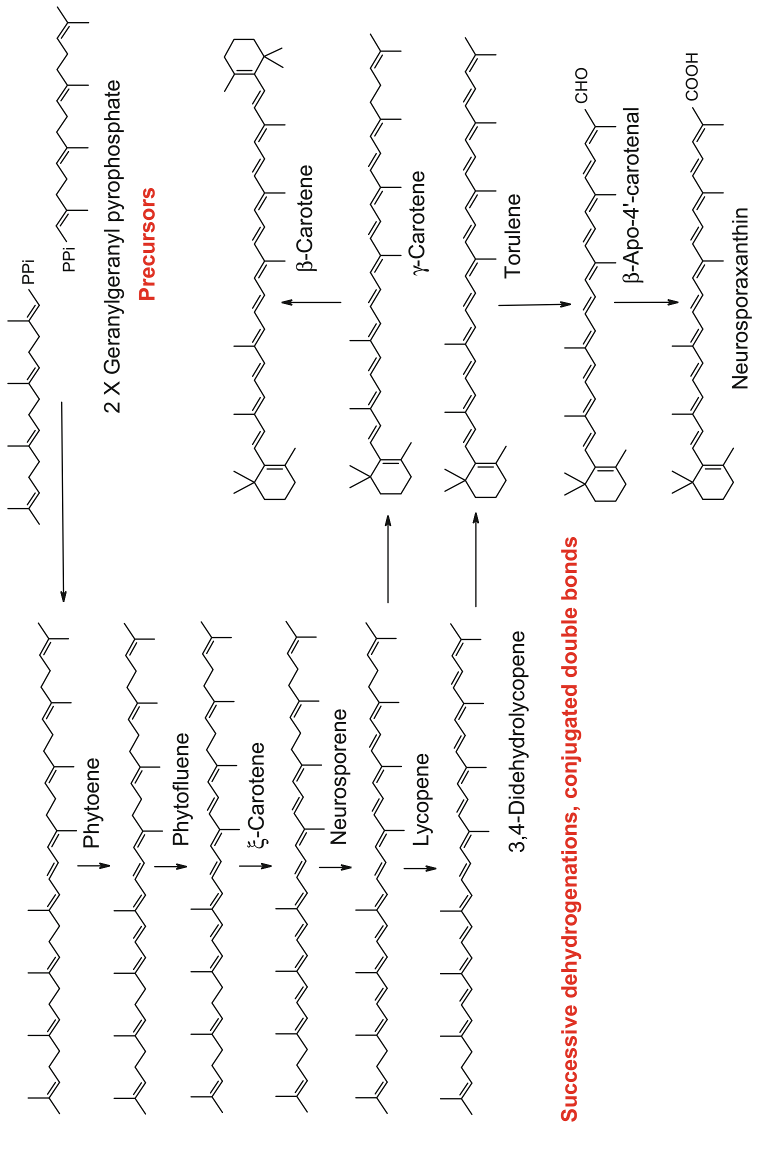

Fig. 17 Biosynthesis of carotenoids in the ascomycete *Gibberella zeae* – anamorph *Fusarium graminearum* (Adapted from Jin et al. [173])

Today the high productivity of petrochemical-based industry make it difficult for the biotechnologies using microorganisms as platform cell factories to spread. The most important reasons are the higher production costs for the process themselves as well as some drastical changes in basic equipments of technical platforms. Some long ago studied bacteria as *Escherichia coli*, *Corynebacterium glutamicum*, *Bacillus subtilis*, or fungi as *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium chrysogenum*, *Saccharomyces cerevisiae* are already operated in industry for enzymes, nutraceuticals or pharmaceuticals.

Indeed, the numerous years of research done on the selected strains led to high robustness and remarkable tolerances against various stresses under industrial conditions, which is the guarantee of stable and efficient productions.

6.1 Genetic Manipulation in the Future

Chen and Nielsen [179] published recently a highly interesting paper reviewing the potential future developments for bio-based productions using whole-cell factories. With concrete examples, they reviewed the main tools for metabolic pathways control and strain engineering acceleration and then focused on the development of powerful computational algorithms, omic-based techniques (metabolomic, proteomic, fluxomic, transcriptomic, genomic) combined with modeling refinement, to enable the reduction in development time and, thus, become attractive to industry leaders. Specifically for metabolic optimization and control, a range of computational pathway prediction algorithms has already been generated. Some of them provide a systematic framework for metabolic pathways (re)design by changing existing pathways through introduction of gene knockouts or overexpressions (OptKnock, OptGene, OptForce or FSEOF). Some others can aid in identifying possible pathways from first principles, based on known enzyme reactions (DESHARKY) or based on possible biotransformations of functional groups by known chemistry (BNICE). As an example, their use allowed to identify more than 10,000 possible pathways for the synthesis of 1,4-butane-diol from common central metabolites. Besides predicting a wide range of possible routes, a prioritization scoring algorithm based on binding covalence, chemical similarity, hermosdynamic favourability and pathway distance, is actually developed to rank the possibilities based on discriminative criteria [180]. Moreover, the recently published web server RetroPath [181] offers a way to retrieve reactions varying in number from the large numbers of reactions found using BNICE to the small numbers of reactions that are presented in the KEGG database. This fully integrative approach will furnish a fast and global analysis contributing to a rapid optimization of the production.

In this field, *Saccharomyces cerevisiae* is one of the most studied strain and it can be considered as an example for production improvement [182–184]. Many of them come from genetic manipulations (insertions, deletions, mutations) directly repressing or promoting the molecules involved in the producing pathways. Unfortunately, the negative aspect of GMOs can be easily concealed, thinking about the

actual progress that is changing industrial waste into useful compounds. The use of renewable biomass and processing of industrial waste are becoming more important due to the increasing pollution of the natural environment and growing concerns about climate change. Many efforts have been exerted to extend the carbon substrate range and to improve carbon utilization efficiency or to extend the physicochemical conditions of production by microorganisms. This is clearly illustrated with the example of xylose isomerase overexpression through the engineering of the pentose phosphate pathway [183] or the improvement of succinate production in *S. cerevisiae* [185].

Cost-effective production of dyestuffs could, for instance, come from complete and fast utilization of lignocellulosic biomass. Xylose is the main pentose and second most abundant sugar in lignocellulosic feedstocks. The engineering of the pentose-phosphate pathway enables a rapid xylose utilization and ethanol production. Based on the fact that succinic acid is an important precursor for the synthesis of high-value-added products, *S. cerevisiae*, as many acidophile fungi, is a valuable platform for acidic productions.

Through a regulation of biotin and urea levels and under optimal supplemental CO_2 conditions in a bioreactor, coupled with engineering strategy, the succinate titer was successfully improved from 6.17 ± 0.34 g/L to 12.97 ± 0.42 g/L at low pH value. Directed mutagenesis has also been developed in the study of Çakar et al. [186], where different culture batches were submitted to oxidative, freezing–thawing, high-temperature, and ethanol stresses before selecting the most tolerant mutants to the environmental conditions generated.

6.2 Cost-Effective Process

As the medium components can represent up to 73 % of the total production cost, by-products of agroindustrial origin have been proposed as low-cost alternative substrates for microbial metabolite production [187]. A wide range of industrial waste, such as fruit pulp, pea pod powder, whey, molasses, corn steep liquor, bran, straw, stem, stalk, leaves, husk, peel, legumes, bagasse, spent grains, mainly composed of cellulose, lignin and residual sugars, can be considered as potential carbon, nitrogen and mineral sources for the microbial production of pigments [188]. One goal is to supply low cost raw material, coupled with the objective of controlling environment pollution. The environmental concern is due to the presence of phenolic and other toxic compounds in these residues (refractory compounds), which may cause deterioration (pollution, saturation) of the environment, when the waste is discarded. These refractory compounds are very difficult to deteriorate in waters and soils, even by microorganisms, and their nitrogen and carbon contents are considered as immobilized. Fungi, as well as actinomycetes, belong to the class of microorganism having the widest enzymatic potential due to a great variety of constitutive and adaptative enzymes, able to recycle the immobilized elements, as key factors of depollution.

6.3 Clean Opportunities for the Future

Clean label is a first response of insistent demand from the consumers for products certified as “natural.” Indeed, their use in industrial processes adds more value to the product. However, taking plant culture as an example, the genetic engineering of producing strains does not seem to be the most suitable way to progress towards natural products synthesized from sustainable process. Some European certification organizations as Ecocert, QualiFrance, Certipaq-Aclave, SGS-ICS, and Agrocert Certisud (for organic certifications), have now a strong impact on the market. This gives the possibility to manufacturers to sell the dyes at a higher price compared to their conventional counterparts. These labels provide a composition based on at least 95 % of natural or organic ingredients and they influence the production process that should be more respectful towards the environment.

Thus, many studies deal with searching the paths to naturally foster the productions of suitable compounds, acting on physicochemical parameters of the growths with wild strains. This needs, in the following years, to increase knowledge about the microbial and metabolic biodiversity naturally occurring in our environments. Reducing or suppressing heavy extraction process would also be a beneficial way for several reasons: first, it alleviates the need to use large amount of solvents, whilst from an economic point of view, it reduces production time and eliminates the cost of an extraction process, thus making dye production more economically and friendly viable.

7 Methods for Extraction and Purification of the Colored Compounds

Filamentous fungi produce a mixture of various metabolites such as pigments, fatty acids, proteins, and other cellular metabolites. Thus, the extraction and isolation of the pigmented molecules of interest are necessary steps before proceeding to any further utilization of these metabolites in commercial products. Pigments can be store within the biomass, excreted in the fermentation broth or both, suggesting that extraction methods need to be developed specifically regarding where the pigments are located.

7.1 Pretreatments of Biomass Before Extraction

Fungal pigments can be extracted from both biomass and fermentative broth. Proceeding to pretreatments of the fungal material before applying the extraction protocol itself can improve the overall extraction effectiveness. The biomass is generally separated from the broth either by centrifugation or Büchner filtration [42]. The biomass and/or the broth are then frozen-dried. It renders the biomass easier to grind

and allows longer conservation without losing bioactivity of the pigments. Grinding is a common pre-treatment in extraction techniques and results in smaller biomass particles. The smaller the size of every particles, the better the diffusion of the solvent. Consequently, the overall extraction efficiency increases [189]. However, extended pre-treatment of the biomass, could result in denaturation and/or degradation of the pigmented molecules. Shearing forces, high temperatures and pressures when proceeding to grinding or drying processes, can drastically affect the final chemical profile of the pigments. Oxidation and enzyme browning are the main risks encountered in physical pre-treatment of biomass due to prolonged contact with oxygen, and the release of hydrolases from the cells after crushing and grinding [190]. Moreover, during physical treatment, coloured molecules can also react with other cell components, resulting in reducing their final extractability and bioactivity. Thus, the conditions of the pre-treatment of biomass are rather crucial and should remain relatively quick and gentle to ensure the efficiency of the further chemical extraction, as well as the maintenance of the bioactivity of the biomolecules.

7.2 Conventional Extraction Methods Using Organic Solvents

Despite progress done in extraction methods, fungal pigmented molecules are generally extracted by extended contact with one or a mixture of organic solvents, such as in maceration or Soxhlet techniques. The main organic solvents in use are ethanol, methanol, acetone, ethyl acetate, and hexane [42, 191–193]. The efficiency of such process relies on the polarity of the compound to extract. Velmurugan et al. [42] have used a mixture of ethanol and water (1:1, v/v) on the unfiltered fermentation broth of *Isaria farinosa* (formerly *Paecilomyces farinosa*) to extract extracellular red pigments, before separating the filtrate and processing to further analysis. Similarly, extracellular pigments from *Trichoderma harzianium* were extracted with ethyl acetate [194]. These methods can be optimized by adapting the side parameters, i.e., the extraction time, the pre-treatment applied, the pH, the temperature or pressure used, as well as the presence of other salts (NaCl, etc). The afore mentioned organic solvents are still widely used for pigments extraction due to their relative efficiency, ease of use, and their easy application at industrial scale. Only one industrial process using organic solvent extraction of fungal hydroxyanthraquinones has been reported and patented by the company, ASCOLOR BIOTECH (now NATURAL RED™), for the production of the pigment Arpink Red™. The process involves a filtration and centrifugation pre-treatment for the removal of the biomass. The pH of the supernatant is then dropped to 2.5–3.0 to precipitate the hydroxyanthraquinones. The dissolution of the precipitate in ethanol is performed, followed by the evaporation of the alcoholic solvent. The fungal pigments are then recovered as a deep-red powder, ready to be used for further application [16]. However, the numerous extraction cycles, the relatively low extraction efficiencies and selectivity, as well as the large volumes of solvent and water used, generally render such methods unsustainable and costly for industrial scale up.

7.3 Alternative Greener Extraction Processes

Over the last two decades and with the emergence of the concept of green chemistry, tremendous progress has been made regarding extraction and separation technologies. The trend is to develop extraction techniques involving cheap and environmental-friendly solvents that can be used under milder conditions with good final efficiency in quicker processes (ideally automatized). Most of them have been tested on fungal material for the extraction of all sorts of biomolecules, such as fatty acids, polyphenols and carotenoids [185, 195–198].

7.3.1 Microwave-Assisted Extraction

Microwave-assisted extractions (MAE) rely on the use of microwave energy to enhance the partitioning of compounds of interest from the biomass matrix into the solvent. The applications of microwaves allow a synergetic effect of both heat and mass transfers occurring in the same direction (from the inside to the outside), while in conventional extraction systems, they are happening in opposite directions [199]. The efficiency of MAE for extraction of molecules is directly linked to the polarity of the solvent, its ionic conduction and its ability to absorb microwave energy [200]. Thus, this method is more suitable for the extraction of compounds showing medium to high polarity [201]. This explains why MAE has been successfully applied on polyphenolic compound extractions such as phenolic acids [202], curcumin [203], and saffron polyphenols [204]. Hemwimol et al. [205] concluded that coupling MAE with appropriate solvent increased the yield of anthraquinones extracted from *Morinda citrifolia* as well as reduced significantly the extraction time, when compared to Soxhlet extraction or maceration [205]. In Hemwimol work, MAE took 15 min to be completed, while 4 h and 3 days were needed with Soxhlet and maceration techniques, respectively, to reach the same efficiency [205]. Thus, MAE is a very promising technique that has been further optimized for limiting side oxidations by applying nitrogen-controlled atmosphere instead of air, or using MAE under vacuum conditions to protect heat sensitive molecules. The major advantages of MAE are the significantly reduced extraction time, and solvent volumes requirements, as well as the improved extraction efficiency, making this technique interesting for biotechnological applications. However, its possible usages at industrial scales remain limited due to heterogeneous heat propagation at bigger scale, along with cost and maintenance of the material.

7.3.2 Ultrasound-Assisted Extraction

Ultrasound-assisted extraction (UAE) is one of the more straightforward method to use as it requires very limited apparatus and is low-cost [201]. UAE involves the application of acoustic waves in the KHz range (>20 kHz) propagated in the heated extraction solvent [189]. Propagation of the sound waves induces successive expansion and compression cycles of air bubbles within the solvent, resulting in their collapsing. This physical phenomenon disrupts cells membrane, facilitating both the release of molecules of interest and diffusion of the solvent within the sample matrix. UAE has been widely used for enhancing the extraction of bioactive pigmented

phenolic molecules from various parts of plants, and was also showed to induce less degradation on phenolic molecules compared to other more intrusive processes such as solid-liquid solvent extraction or MAE. Barreara-Vasquez et al. [206] observed better and quicker extraction of anthraquinones from plant material when performing UAE compared to the conventional Soxhelt method. However, the recurrent issue encountered in UAE is the nonuniformity of the propagation of both the soundwaves and the heat [199]. This heterogeneity is due to the different phases present in the system, which have different heat and mass transfer capacities as well as different compressibilities. These variations can negatively impact on the overall effectiveness of this extraction technique. Nevertheless, UAE has showed good potential for extraction application and is widely used as paired step with MAE and solvent extraction [205, 206].

7.3.3 Pressurized Fluid Extraction

Pressurized fluid extractions (PLE) involve the use of both high pressure and elevated temperatures on the system sample/solvent, where the solvent is near to its critical stage under the conditions of extraction. The high pressures (100–150 bars) allow the solvent to remain liquid despite the elevation of the temperatures [201]. Additionally, higher temperatures (in the range of 100 °C) enhance the extraction capacities and diffusion of the solvent. Thus, the main advantage of PLE is a significant reduction of solvent volumes used and a shortening of extraction cycles. A wide variety of techniques have been derived from PLE and are still based on the same principles of reaching solvent near critical stage. PLE, also going by its trade name, ASE (accelerated solvent extraction) patented by DIONEX, is a relatively recent method and have promising potentialities. Water becomes a very valuable solvent under high pressure and temperature, and the technique is then called subcritical water extraction (SWE). The water is heated up to 200 °C where it chemical properties change and become similar to those characteristic of organic solvents. CO_2 can also be used as an efficient extraction solvent when compressed to its supercritical state, and is classified amongst the super- and subcritical fluid extraction (SFE) techniques. The advantages of this latter are the absence of light and oxygen during the extraction cycles, which prevent any oxidation and other physical degradation due to the elevated pressure and temperatures. Such process showed great potential regarding preservation of bioactivities of oxygen sensitive chemicals like polyphenols and other pigmented molecules. Borges et al. [207] have concluded that the use of supercritical CO_2 for the extraction of red carminic acid from cochineal insects showed improved yields compared to conventional methods. Moreover, it was said to increase the extraction selectivity for the pigmented hydroxyanthraquinones, and reduce the side extraction of proteins [207]. Such selectivity for pigmented hydroxyanthraquinone is highly desirable as some proteins are of concern regarding allergenic reactions in carminic acid containing products [17]. Nevertheless, few studies have been done on using this technique on fungal biomass for pigments extraction, despite the promising results observed on polyphenols from plants and microalgae [208], as well as hydroxyanthraquinones

from insects. PLE allow working quicker, under milder extraction conditions, using more environmental friendly solvent (water, ethanol, methanol or mixtures) at relatively low volumes. However, the complexity of the apparatus needed, as well as the utilisation of high pressure and temperature, remain difficult and costly for any industrial applications yet.

7.3.4 Enzyme-Assisted Extraction

Enzyme-assisted extraction (EAE) is a relatively recent method that was shown as a promising new extraction technique, using enzymatic specific catalytic activities in aqueous solutions [209]. The main types of enzymes used are pectinases, cellulases and hemicellulases, which when applied to the sample matrix, act on the cells membranes components and hydrolyze them. This results in increasing the membrane permeability to solvents and thus, in improving yields of extraction of metabolites. Enzyme catalytic activity can also be used for improving solubility of metabolites in the extraction solvent. Hynninen et al. [191] have used glucosidase for the isolation of hydroxyanthraquinone aglycones from the fungus *Dermocybe sanguinea*. Such enzyme hydrolizes *O*-glycosyl linkages of glucoside and aglycone anthraquinones (mainly emodin- and dermocybin-1-β-D-glucopyranoside), enhancing their solubility in organic solvent (acetone). Indeed, after enzymatic treatment, 94 % of the total pigments were yielded in the organic fraction. The different enzymes can be obtained from bacteria, fungi, yeasts or plants, and most of them are commercially available. The major advantages of EAE are the reduction in solvent volumes as well as the shortening of extraction times and a better preservation of the bioactivity of the product due to the usage of milder conditions. The next trend regarding EAE is the engineering of the enzymes for developing tailor-made extraction methods. However, the cost of the enzyme, the difficulty to recycle them, and the loss of catalytic efficiency with larger raw material volumes currently limit industrial application of such method.

7.3.5 Ionic Liquid–Assisted Extraction

Ionic liquids (IL) have been described as novel type of solvents with promising potential for the development of new extraction methods offering milder, greener, and more efficient processes. Over the last decade, they have been widely applied in various fields of chemistry, and they showed great potential in separation technologies. IL are organic molten salts, showing boiling points below 100°C. They are generally made of a bulky organic cation, such as alkyl- ammonium or phosphonium, dialkylimidazolium, *N*-alkyl-pyrrolidinium or pyridinium, paired with an organic or inorganic anion, such as bromide, chloride, tetrafluoroborate or hexafluorophosphate for the main in use [198, 210]. Low vapour pressure and non-volatile nature, non-flammability, thermal and chemical stability, high solubility, as well as recyclability are some of the unique properties of IL, which classify them as green solvents. Moreover, their properties are tuneable and depend on the couple cation/anion, and can be then optimized accordingly to the molecule to extract. Various types of natural compounds were successfully extracted with IL such as polyphenols [196] or dyes from chilli powder [211]. More recently, focus have been done on

using IL coupled with other techniques such as aqueous two-phases system (ATPS), microwave assisted extraction and ultrasound assisted extraction, which was shown to improve the extraction capacities of the IL themselves. Yan et al. [133] concluded that the extraction of hydroxyanthraquinone pigments (e.g., aloe-emodin, rhein, emodin, chrysophanol and physcion) from plant materials yielded better results when performing an ultrasound emulsification of IL for microextraction. Indeed, the dispersion of water insoluble droplets within the sample mixture increases the surface contact of the IL with the hydroxyanthraquinones, which are trapped in the IL- droplets and, later on, separated from the aqueous phase by centrifugation. Similarly, Tan et al. [212] have successfully isolated aloe anthraquinones using microemulsion of IL. Recently, similar techniques coupling IL with ultrasound, microemulsion or ATPS were performed with good results on filamentous fungi biomass and/or culture broth. Ventura et al. [213] succeeded in isolated anthraquinones from 14 days old fermentation broth of *Penicillium purpurogenum*. Similarly, Shen et al. [214] obtained good results when using hydrophobic IL microemulsion extraction of red *Monascus*-pigments from 7 days old fermentative broth.

7.4 Purification Methods

The different extraction methods previously described present more or less selectivity for the pigmented molecules of interest. The desire to use these pigments for cosmetic and food applications requires further purification steps in order for them to be allowed by the different food and drugs regulation agencies, such as the FDA (USA) or the EFSA (Europe). Some pigments – producing species are known to have a paired biosynthesis pathways of pigments and mycotoxins, such as in *Monascus* species where the hepato-nephrotoxic compounds, citrinin, is produced along with the azaphilone pigments [215]. The more commonly used purification techniques are based on solid phase extraction such as column chromatography, cation exchange, and thin layer chromatography (TLC). Column chromatography was used for the generation of sequential elution of pigments that were previously extracted, and is usually followed by a TLC to separate the different compounds present in the pigmented extract. Red pigments from the yeast *Rhodotorula glutinis* were efficiently fractionated on a magnesium oxide-Hyflo Super cell using acetic acid:ethyl ether (1:2, w/w) as elution solvent mixture [216]. The following TLC used petroleum ether:acetone (80:20, v/v) as the mobile phase for the separation of the different chemical species. Three types of β-carotenoid compounds, β-carotene, torulene and torularhodin, respectively, were purified. A sequential silica gel column chromatography using a gradient elution of methylene chloride:methanol (100:0 to 50:50, v/v), followed by TCL using petroleum ether:acetone (3:2, v/v) was performed on pigmented fractions from the ascomycete fungus *Shiraia bambusicola* and yielded 15.5 mg, 42.3 mg, 21.5 mg and 19.6 mg of perylenequinone derivatives hypocrellin A, B, C and D respectively, which are toxic dark red pigments showing interesting anti-cancer activity [217]. Adsorption column chromatography using

neutral alumina washed with hexane allowed the elution of β-carotene from the filamentous fungus, *Mucor azygosporus* [218]. The pigment was recovered at 94 % in the first fraction. Silica column showed promising potential as it can perform high purification but need to be paired with another separating steps such as TLC. The main disadvantages of this method are the large volumes of solvents required and the time it requires [219]. TLC show limitations as an effective technique due to the large number of plates that are needed for obtaining enough purified pigment in the end. Moreover, TLC imposes an additional step: scrapping the silica gel to recover the purified pigment, which tend to reduce the overall pigment extraction yield. Cation exchange column is another method, which showed good yields for the purification of pigments from *Fusarium graminearum*, with methanol and methylene chloride (50:50, v/v) used as the mobile phase. Styrene-based strong sulfonic acid columns showed the best purification yields. Then, the main advantages of resins chromatography are the use of reduced amounts of solvent, the quick purification cycles (app. 30 min), and the general good efficiency of the process. However, due to the nature of the resin, isomeric transformation of the pigments may occur.

Thus, there are several options available for developing efficient and selective extraction and purification processes of fungal pigments. However, the limiting steps remain linked to the scale-up to industrial applications, associated with the desire of using greener methods. Although great efforts have been done to develop more sustainable extraction protocols (PFE, MAE, UAE, etc), the purification steps still involve chlorine-based solvent (methylene chloride) and other solvents (acetonitrile, acetic acid, petroleum ether, ethyl ether and hexane), classified as "usable" or "undesirable" according to the guide for solvent selection, produced by Ghandi [210], for the design of green extraction method. Then, improvements of these techniques are highly desirable to keep developing more environmental-friendly processes with economic potentialities.

8 Industrial-Scale Applications of Fungal Pigments and Perspectives

8.1 Applications as Natural Food Colorants and Dietary Supplements

Since the food company DSM^{TM} has gained the EU approval for food use of fungal originated β-carotene, produced from the fermentation of *Blakeslea trispora* in 2000, industrial interest on fungal metabolites has been revived, and new investigations have been ongoing to develop cost effective fungal colorants ever since [3, 215]. Moreover, the conclusions of the Southampton study, leading to the obligation for food companies to apply a label mentioning that "*azo-dyes (*i.e., *synthetic dyes) may have an advert effect on activity and attention in children*" have driven all the interests towards bacterial and fungal based pigments for food use. Moreover, the value of international coloring market was estimated to $1.5 billion in 2007 according to Leatherhead Food International (LFI), with the natural colorant (from all natural sources, plants and

microbial origins) representing 31 % of the global market, and the synthetic and nature-identical pigments (i. e. chemically similar to natural pigment but synthetically produced) encountering for 40 % and 29 %, respectively [220]. The growth of the natural pigment market has been blooming from 2007 to 2011, and is now overtaking the synthetical color market for the first time, with 39 % of the global food color market against 37 % for the synthetic food dyes [221]. Such market evolutions render fungal pigments an economically valuable niche with great potentialities, where further investigations are needed.

Nowadays, four fungal strains are used for the production of yellow to red pigments at industrial scales: *Blakeslea trispora*, *Penicillium oxalicum*, *Monascus* sp., and *Ashbya gossypi* (Table 3). The β-carotene from *Blakeslea trispora* has been authorized in the food market and classified under the same E number that of plants originated β-carotene, i.e., E160a(ii) [222]. Industries based in Russia, Ukraine and Spain are now producing this pigment with a yield of up to 17 g per liter of culture [223]. Similarly, lycopene from *Blakeslea trispora*, produced by the company Vitatene S.A., has been approved as food colorant by the European Commission in 2006, and labelled with the E-number 160d(iii) [224]. It is used to color

Table 3 Fungal production of pigments (already in use as natural food colorants or with high potential in this field)

Molecule	Colour	Microorganism	Status[a]
Ankaflavin (azaphilone)	Yellow	*Monascus* sp. (fungus)	IP
Anthraquinones	Red and other hues	*Penicillium oxalicum* (+ other fungi)	IP
Azaphilones	Red	*Talaromyces atroroseus* (fungus)	DS
Azaphilones	Red	*Penicillium purpurogenum* (fungus)	DS
Azulenes	Blue	*Lactarius* sp. (fungus)	RP
β-carotene	Yellow-orange	*Blakeslea trispora* (fungus)	IP
β-carotene	Yellow-orange	*Fusarium sporotrichioides* (fungus)	RP
β-carotene	Yellow-orange	*Mucor circinelloides* (fungus)	DS
β-carotene	Yellow-orange	*Neurospora crassa* (fungus)	RP
β-carotene	Yellow-orange	*Phycomyces blakesleeanus* (fungus)	RP
Lycopene	Red	*Blakeslea trispora* (fungus)	IP
Lycopene	Red	*Fusarium sporotrichioides* (fungus)	RP
Monascorubramin (azaphilone)	Red	*Monascus* sp. (fungus)	IP
Naphthoquinones	Deep blood-red	*Cordyceps unilateralis* (fungus)	RP
Riboflavin	Yellow	*Ashbya gossypi* (fungus)	IP
Rubropunctatin (azaphilone)	Orange	*Monascus* sp. (fungus)	IP
Unknown	Red	*Paecilomyces sinclairii* (fungus)	RP

[a]Industrial production (IP), development stage (DS), research project (RP)

non-alcoholic flavored drinks, fine bakeries, dairy-based product, mustards and condiments, as well as soups and sauces. The recommended use level for lycopene from *B. trispora* is 15 to 30 mg/kg depending on the food matrix. Additionally, fungal lycopene has been recognized to be nutritionally equivalent to plant-based lycopene (tomatoes, watermelons, etc) according to an EFSA reports in 2008 [225]. Interestingly, it also has been decided that both β-carotene and lycopene from the fungus *B. trispora* should be only labelled as "β-carotene" and "lycopene," respectively, with no further details on the plant, fungal or synthetic origin.

Riboflavin (vitamin B2 – E101) is a water-soluble vitamin, also commonly used as a yellow pigment, industrially produced by fermentation of a fungal strain, *Ashbya gossypi* [215]. Another strain, *Eremothecium ashbyii*, showed industrial potentialities regarding yields; however, its genetic instability renders it not suitable for cost effective industrial production [226]. Riboflavin is commonly used in cereal-based products, juices and yogurts; however the concentration to be used is limited due to bitterness aftertaste [227]. In the course of the 51st meeting of the Joint FAO/WHO Expert Committee on Food Additive (JECFA) in 1999 (Geneva), riboflavin produced by genetically modified bacterial strain of *Bacillus subtilis*, built to overexpress the riboflavin biosynthetic pathway, has been concluded as safe and nutritionally equivalent as synthetic vitamin B2, and was included in the same ADI group of 0–0.5 mg/kg of body weight for synthetic riboflavin.

Monascus-like pigments, in particular ankaflavin, have been used to color meat, salami, sausages [228, 229], and fish (e.g., to enhance pink coloration in salmon), as well as coloring sauces (ketchup). Food applications of *Monascus*-like pigments have been widely ongoing for centuries in Asia, but are still not allowed in the EU and the United States [215]. Numerous companies, such as Nestlé (Switzerland) (Patent US4145254 A) and The Quaker Oats (USA) (Patent US4418080 and US4418081) have already filled patents for the production and use of *Monascus* red pigments in food applications, and they have been accepted in 1979 and 1983, respectively.

Similarly, red anthraquinones produced by *Penicillium oxalicum* and sold by the Czech company, ASCOLOR BIOTECH (now NATURAL RED™), have been used in meat, dairy and confectionery products, as well as in alcoholic and non-alcoholic drinks, with recommended dosage levels of 100 mg/kg, 150 mg/kg, 300 mg/kg, 200 mg and 100 mg/kg respectively.

Interestingly, the abovementioned fungal pigments do not only display coloring properties, but demonstrate a wide palette of bioactivities from carcinogenesis-preventing roles due to antioxidative, anti-free radical and apoptosis inducing activities, especially regarding lycopene and β-carotene, to antimicrobial and antifungal activities. Similarly, *Monascus*-like red pigments (monascorubramin and rubropunctatin) were demonstrated to enhance the organoleptic properties of food (taste and consistency), as well as lowering low density lipoproteins (LDL) cholesterol and increasing high density lipoproteins (HDL) cholesterol due to the presence of a statin-like molecule (monacoline K), reducing salt intake, and preventing gastric and digestive disorder [227, 230]. Furthermore, food supplements of Red Yeast Rice, i.e., *Monascus* sp. pigments, like "SuperSmart" food supplement are sold

specifically for the benefits of monacoline K on cholesterol. Interestingly, EU regulations forbid any usage of *Monascus* sp. pigments in food or textile industries, however, these food supplements can be relatively easily found in parapharmacies.

In cheesemaking industries, *Fusarium domesticum* is used as the inoculating strain for the production of Saint Nectaire and Reblochon cheeses. The organoleptic enhancing abilities of this fungus have been known for a long time, as it was called "Anticollanti" before its clear characterization, and was used for its ability to favorize the drying of the cheese surface, and consequently reducing its stickiness [231]. Thus, fungal pigments are unique by showing very interesting and complex profiles: along with being used for their coloring properties, they also could be used as organoleptic properties enhancer, as well as physiologically and health valuable diet supplements.

Numerous other pigments from various fungal strains are currently under investigation: three other pigments (two red azaphilones from *Talaromyces atroroseus* and *Penicillium purpurogenum*, and one β-carotene from *Mucor circinelloides*) are at development stages (Table 3). The next trend now for research projects are focused on developing mycotoxin-free deep red hues producers. Some species such as *Fusarium*, *Cordyceps* and *Paecilomyces* sp. are more specifically considered as promising strains (Table 3).

8.2 Applications in Pharmaceutical and Cosmetic Products

Natural bioactive molecules with clinical properties have been widely investigated by pharmaceutical companies, and many microbial secondary metabolites have been used over since as antibiotics, diuretic, anticancer, estrogenic or immune-modulatory compounds. Some examples of pigmented metabolites from fungal strains are under further clinical studies at laboratory scale, such as norsolorinic acid produced from *Aspergillus nidulans*, which is characterised by its anti-proliferative activity in human breast adenocarcinoma MCF-7 cells [232]. Similarly, red anthraquinones molecules, shiraiarin and hypocrellin D, synthesized by the fungal strain *Shriaia bambusicola*, were found to significantly inhibit the growth of tumor cell lines [217, 233], and thus, are potential candidate for future antitumor and anticancer drugs. However, further clinical and toxicity tests are required before considering any commercialisation of these fungal pigments as drugs.

Cosmetic industries are highly interested in inserting biomolecules and natural ingredients for their products. Indeed, the public demands of "*green label*," and organic cosmetic counts for a growing part of the global cosmetic market, and companies are really looking for new type of natural ingredient to use as alternative to conventional synthetic compounds. Some firms are already producing and selling fungal pigments as natural colorant for cosmetic, such as NINGXIA R.D. (natural pigment co. of ningxia light industry design institute), which is manufacturing red pigments from *Monascus* sp. specifically for cosmetic purposes. Another Asian company, KANEBO Ltd (Japan), has been granted a patent for using *Monascus*-like pigments in lipsticks. LEVER HINDUSTAN Ltd (Indian branch of Unilever),

UNILEVER and l'OREAL S.A had their patents accepted in 2001, 2002 and 2004 for using *Monascus*-like pigments in skin conditioning and in skincare products giving tan coloration. It is worth noticing that many companies are selling those pigments on the Asian market, as the spread of fungal pigments containing cosmetic products is still limited in Europe and the United State. Despite the facts that numerous patents have been filled and accepted, the actual launches on the market remain highly restricted by allowances from Institutes for Public Health Surveillance (leads by the ANSES in Europe). Indeed, before authorizing any products, clinical tests looking at allergic, dermatologic and endocrine responses are required. Moreover, in the case of cosmetic products, both the ingredient to be added –even more if used for specific bioactivity-, i.e., fungal pigment, and the final cosmetic product would have to be authorized by the appropriate authority. These regulation elements are one of the major points slowing down the use of fungal pigments. Thus, so far, only *Monascus*-like pigments have found actual economic applications; however it does pave the way of dyes and bioingredients market to new types of fungal strains and pigments, such as colorants extracted from *Penicillium*, *Fusarium*, *Emericella*, and *Cordyceps* for instance.

8.3 Applications in Textiles and Paint Industries

As regulations on textiles and material colorants are not as strict as in foodstuff, many more microorganism-originated pigments are in use in textiles, clothing, paints, and polymers. Bacterial pigments, prodigiosin (red) and violacein (purple) from *Serratia marcescens* and *Chromobacterium violaceum*, respectively, were used to dye various types of fabrics like acrylic fiber, silk, cotton, polyester, and polyester microfiber [234]. Similarly, fungal colorants are promising, as these pigments display high colorfastness as well as high staining capability, suggesting that a minimal amount of pigment is required for proper standard staining results. Such properties underpin interesting cost-effectiveness. Moreover, synthetic textile colorants are reported as potential carcinogen due to their content in dioxins, such as polychlorinated dibenzo-p-dioxins (PCCD) and polychlorinated dibenzofurans (PCDF) [235], which strengthen the trend to develop eco-friendly and non-toxic colorant, especially regarding infant and children clothes and toys. Then, economically speaking, in 2008, Mapari et al. [19] have patented the used of *Monascus*-like pigments from various strains of nonproducing mycotoxins *Penicillium* (19 *Penicillium* strains) on textile, cotton, wool, silk, leather, paper, paint, polymer, plastic, inks, and tablet. Additionally, red anthraquinones from *Fusarium oxysporum* and from *Dermocybe sanguinea*, as well as yellow pigments from *Trichoderma virens*, bright olive pigments from *Alternaria alternate*, and melanin pigments from *Curvularia lunata* were demonstrated as potential dye on cellulosic matrix, such as wool and silk with good colorfastness and rub fastness [236–238]. Red dyeing capabilities of other fungal strains, such as *Isaria* spp., *Emericella* spp., *Penicillium* spp., *Monascus* spp., and *Fusarium* spp. were concluded as good natural alternate for leather dyeing, and would be less polluting compared to conventional dyes [42]. Moreover, staining

fabrics (silk) with yellow pigments from *Thermocymes* sp. and *Trichoderma* sp. were shown to have antibacterial and/or antifungal activities, and were concluded to be good candidates for producing valuable textiles for hospital/medical uses, such as bed linens, bandages or suture threads [238, 239]. Thus, even when applied for textile dyeing, fungal pigments can enrich the properties of the fabrics with biological activities.

8.4 Limits and Further Opportunities for Industrial Use of Fungal Pigments

The feasibility of developing a wide range of fungal pigments at industrial scale should be considered through the six following main elements: the biological aspect of the strains metabolite production, the health and safety concerns about the putative mycotoxins coproduction, all the legislation and regulations process to do before any actual launch on the market, the trends of the global colorant market along with the cost-effectiveness of such fungal pigments production, and in the end, all the technical and logistical hurdles when designing and running an industrial plant.

Regarding the biological aspect, the main issues could come from the stability of the strains and their pigment production over time, especially if the strains have been previously genetically engineered. However, the use of microorganisms show significant advantages such as fast growth compared to plants, with a complete independency from seasons or weather. Indeed, even cochineal farms production is climate-dependent due to the close interdependency between the cochineal insect and the plants it lives on (Barbary fig), rendering final prices of plants and cochineal-based pigments relatively versatile.

Fungal secondary metabolite commercialization is limited, yet due to health and safety concerns about mycotoxins coproduction with the pigments, or the pigments being toxic themselves. However, similar toxic worries on synthetic dyes and their potential harm on human health are more and more arising. Recently, several research studies, such as the one from Southampton University (2010), show that azo-dyes in food and other synthetic dyes in fabrics should be avoided. Such observations make the public more aware on what sort of additives are added to their food, clothes and cosmetics and drive the demands for "organic" and greener ingredients. To address the health concerns, pigments have to go through a long allowance process in order to be attributed an European number (E number) with an acceptable dietary intake (ADI) before being sold in Europe, for instance. Toxicological and clinical tests are long and require different levels of tests depending on the final application (food, textiles, cosmetics). However, it is worth noticing that numerous patents for the use of fungal pigments (*Monascus*-like pigments) in food and cosmetics have been filled by companies such as Nestlé, Unilever, The Quaker Oat, and l'Oréal, and have all been accepted. Moreover, these pigments have been used for centuries in Asia, which gives good hopes they will be allowed in Europe and in the United States at some point. Then, fungal pigments of *P. oxalicum*

(Natural Red^{TM}) and β-carotene and lycopene from *Blakesla trispora* have been authorized in Europe. So despites a long process, examples attest that positive progresses are made towards the acceptance of fungal colorant on the EU and US markets.

Concerning the global colorant market, all the last elements are encouraging the development of natural dyes from fungal origins. Such market evolution is coherent with the public demands of more eco-friendly products. Economically speaking, the use of microorganisms has an enormous advantage, which is the complete independence toward the variability of prices of petroleum-based raw materials, rendering the price of the feedstock less variable, reducing significantly its impact on the final pigment price. Moreover, the use of agroindustrial wastes instead is a cheaper and more economically stable feedstocks. When considering the development of biorefineries for fungal pigments biosynthesis, two main hurdles arise: first, the initial investment to design and size the plant, and secondly the impossibility, yet, to set up a continuous fermentation process (the bioreactor would need to be drained, cleaned and refilled for every fermentation batch). Additionally, time and carbon source need to be “wasted” to initiate the strain growth and start the secondary metabolism. However, such fermentation process are little energy demanding and, thus, represent a promising alternative.

9 Conclusion

As an example within the whole industry, the trends in the food and beverage markets as well as in the cosmetic and textile markets push for more natural, organic, and clean label products, so the need for more and more natural ingredients is increasing. The formulation of recipes containing natural colors has steadily increased over recent years. Fungal colorants are constituents of commercial products available for the industry as a natural choice among many natural sources. They are either based on alternative production techniques of well-known pigments (e.g., β-carotene, lycopene, riboflavin) or specific molecules so far not biosynthesized by other organisms such as higher plants (e.g., *Monascus* and *Monascus*-like azaphilones).

The use of natural colors in functional, pharmaceutical, cosmetic, textile, beverage, food, and crossover applications requires an understanding of a variety of attributes and concepts, including heat stability, light stability, in addition to being able to provide exciting color hues. Fungal colorants already proved to exert such properties, and novel compounds from fungal biomass produced by applying biotechnological processes should render possible applicable and optimized solutions to the whole industry.

It can be concluded that despite some safety and regulation difficulties, fungal pigmented secondary metabolites stand for promising colorant alternatives with real cost-effective potentialities in the end. However, it would be advisable to bear in mind that an industrial biorefinery cannot be built similarly to conventional chemical

process-based plants. Indeed, new management techniques would have to be developed in order to render such industries competitive.

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Yeast Diversity and Flavor Compounds

17

Francisco Carrau, Eduardo Boido, and Eduardo Dellacassa

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Abstract

Yeast had participated with humans in food fermentation since the production of wine and bread, more than 10,000 years of shared history. It is well understood that fungi diversity is still underestimated and that we are far from understanding its importance and potential impact in biotechnology. Flavor compounds as

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"secondary metabolism" are very sensitive to fermentation conditions and mixed cultures, and although we had experience an exponential development of molecular biology in the last 30 years, metabolomics is still in its infancy. It was demonstrated in recent years that increase strain and species yeast diversity in a fermentation system increases sensory complexity and chemical aroma compound diversity in the final fermented product. Flavor compounds had many key functions for yeast, such as for survival and dispersion strategies, pheromone and defense mechanisms, and "quorum sensing" mechanisms for cell communication. Humans had taken advantage of many of these functions to increase taste and food sensory pleasure for a more exigent consumer, a phenomenon called "yeast domestication." We focus this chapter mainly in the recent discussed yeast synthetic pathways for the formation of phenylpropanoid and terpenoid aroma compounds.

In addition, we will emphasize the current knowledge that grape and wine microbiology research has contributed to understand how complex natural and inoculated yeast flora can affect flavor quality. The flavor phenotype concept and how to screen natural flora and develop consortia starters to innovate in food biotechnology are discussed.

Keywords
Aroma • Wine • Beer • Biotechnology • Terpenoids • Benzenoids

1 Introduction

The grape and wine sector was pioneer in developing sensory analysis with a scientific basis in the earliest 40s [1, 2]. Sensory analysis is widely applied in wine research to describe the effect of factors such as grape variety or processing properties of wine and to study the relationship between chemical and sensory characteristics. Today we are convinced once again that wine research has contributed significantly to understanding how complex natural microbial flora can affect fermentation behavior of commercial inoculated yeasts, as well as how to conduct spontaneous fermentations [3, 4]. In the last three decades, the challenge of developing analytical chemical techniques in order to determine metabolite concentrations under 1 mg was successful and increased our knowledge of yeast and fermentation metabolism, nutrient effects, mixed cultures, and physical conditions such as pH, temperature, redox situation, osmotic stress, etc.

Wine and beer microbiologists are now promoting the use of increase diversity for fermentations, as the limited number of commercial yeast strains used throughout the world is thought to result in products with relatively uniform style, compromising consumer demand due to lack of flavor diversity [5, 6]. Yeast diversity interactions and their effects on flavor quality will demand further studies to understand mixed strain fermentation strategies.

1.1 Yeast Diversity and Foods: Flavor Traits Matter Most

In food biotechnology, the “flavor phenotype” must be considered a fundamental property when developing yeast selection methods [7]. Functional traits are what matter most in a given ecosystem when general biology mechanisms are considered. In contrast, volatile compounds are found in foods under milligram concentration levels and more than 2000 volatile compounds can be identified by the human olfactory system in their over 400 sensory receptors that are present [8, 9]. Interestingly, since flavor traits are not necessarily essential for cell survival, underlying genetic pathways are less affected by positive selection pressures contributing to increased whole genome variability during microbial evolution [10, 11] that could enrich flavor diversity within yeast species. In addition, wine compounds that can affect flavor are more affected by polygenic features than are traditional enological traits, such as alcohol tolerance, low acetic acidity, or good fermentation rate at low temperature [11–14]. These concepts are the milestone for managing and influencing the food fermentation system as a whole (in a holistic way) to impact on quality or increase differentiation of our products from the massive market. Table 1 shows the

Table 1 Aroma threshold of important flavor compounds produced by yeasts during fermentation

	References[a]	Aroma threshold ($\mu g L^{-1}$)	Descriptor
Isoamyl acetate	[15]	2	Banana, pear
Ethyl acetate	[15]	5000	Fruity, solvent
Ethyl hexanoate	[15]	14	Apple, fruit
Ethyl octanoate	[16]	70	Fruity, fatty
Ethyl decanoate	[17]	200	Pleasant
2-Methyl-1-propanol	[16]	1000	Solvent-like
2-Methyl-1-butanol	[16]	30,000	Malted
3-Methyl-1-butanol	[16]	1000	Whiskey, malt, burned
β-Phenylethyl alcohol	[17]	14,000	Floral, honey
1-Propanol	[18]	306,000	Fresh, alcohol
Isobutyric acid	[17]	200,000	Cheesy/rancid
Isovaleric acid	[18]	33	Sweat, acid, rancid
Butanoic acid	[15]	240	Rancid, cheese, sweat
Hexanoic acid	[17]	3000	Cheese, rancid, fatty
Octanoic acid	[17]	500	Rancid, harsh, cheese, fatty acid
Decanoic acid	[17]	15,000	Fatty, unpleasant
γ-Butyrolactone	[19]	50,000	Pleasant, creamy, caramel
Ethyl 4-hydroxybutanoate	[20]	4000	Caramel
β-Phenylethyl acetate	[17]	250	Pleasant, floral
3-Methylthio-1-propanol	[17]	500	Boiled potato, rubber

[a]References indicate the medium in which the threshold values were calculated

known threshold values of many of the compounds discussed in this review. Threshold values could be determined by a sensory panel of tasters adding each compound individually to a simulated hydroalcoholic solution or in a prepared medium with the same beverage that has been evaluated. Odor-active values (OAV) could be easily calculated for a given beverage determining compound concentration divided by the threshold value. Although this is considered an approach to see, the impact of each compound is difficult to obtain conclusive results for the overall flavor of a beverage if a sensory analysis is not performed. Although most of the studied compounds showed significant chemical and OAV differences between strains, some of them may not contribute to the sensory characteristics of a wine at the studied concentrations.

2 Esters, Higher Alcohols, Acids, Lactones, and Sulfur Aroma Compounds

Research on the chemical identification of aroma compounds in wine derived from the metabolic activity of yeasts has been widely reported in the literature during the last decades [21–24]. From these studies, it can be concluded that various fermentation products, including ethyl and acetate esters, higher alcohols, fatty acids, lactones, and sulfur compounds (see Fig. 1), are especially important for the sensory perception of different wine types [24–28]. While this research provides important information on the sensory significance of yeast volatile compounds, more targeted research is required on the sensory impact of these compounds when combined in a

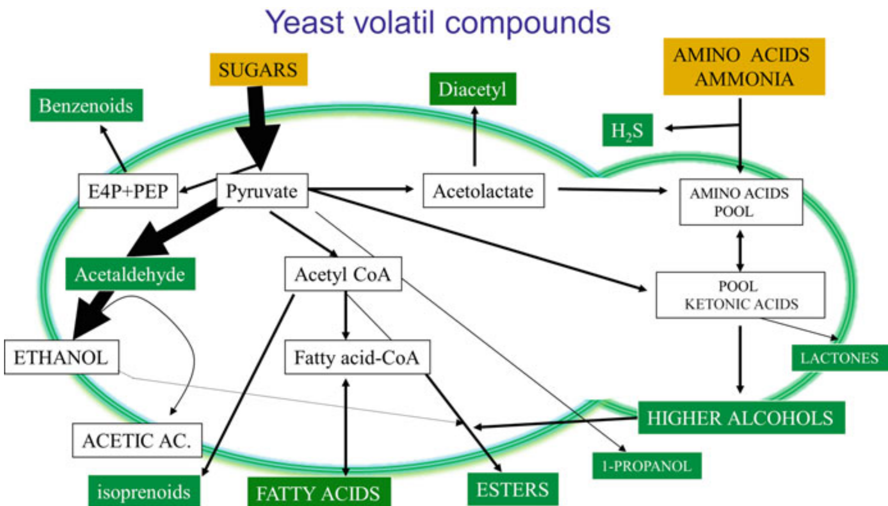

Fig. 1 Yeast volatile compounds that are known to contribute to grape wine and beer final flavor. Different strains will synthesized different concentrations of these compounds

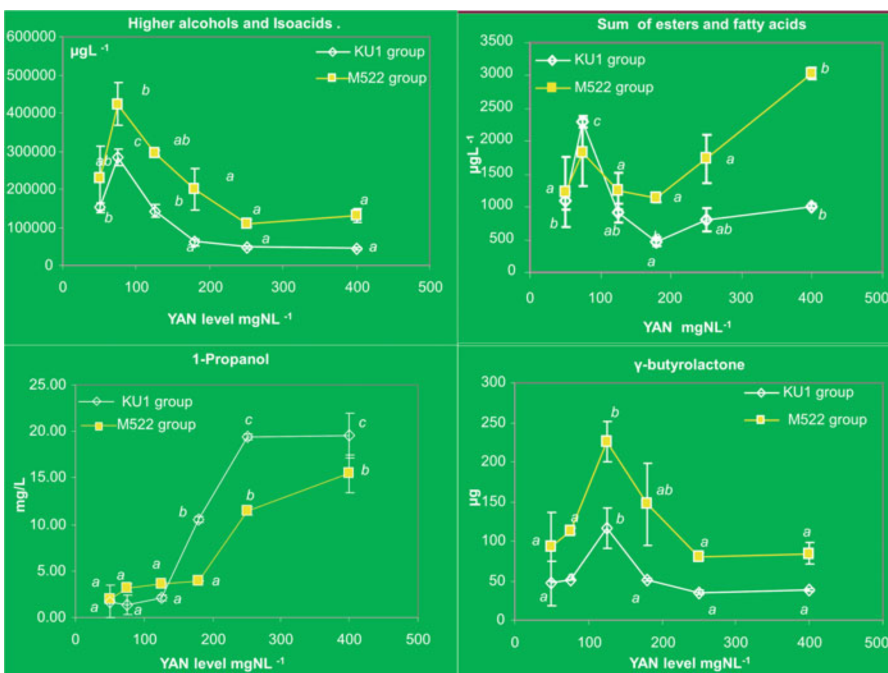

Fig. 2 Aroma compounds produced at different YAN levels by two different yeast groups, KU1 group considered low nitrogen demand strains (*white curves*) and M522 group considered high nitrogen demand strains (*yellow curves*) (Modified from Carrau et al. [24])

complex food matrix such as wine and beer and how they contribute to flavor quality [29].

It is well established that *Saccharomyces cerevisiae* produces different concentrations of aroma compounds as a function of fermentation conditions and must treatments, for example, temperature, grape variety, micronutrients, vitamins and nitrogen composition of the must, size of inoculum, redox situation, etc. However, limited information about non-*Saccharomyces* has been reported. Yeast assimilable nitrogen (YAN) level and amino acid profile is a key nutrient to the majority of these aroma compounds.

In Fig. 2 we showed the comparison between two groups of *Saccharomyces cerevisiae* that behaves differently in relation to YAN levels, KU1 the low nitrogen demand group (KU1 group) and M522 the high nitrogen demand group (M522 group). Flavor compounds accumulation in a model synthetic medium is shown at five YAN levels.

The nitrogen-dependent common trend for the production of isoacids and higher alcohols suggests that their metabolism and production may be coordinated and dependent on the NAD/NADH balance of the cell [30–32]. On the other hand, it was suggested that the significantly higher production of higher alcohols and isoacids by the M522 group could reflect a less efficient usage of nitrogen resulting in an

increase of carbon flux related to branched-chain amino acid metabolism by this strain [24]. KU1 group, which produces less higher alcohols and isoacids at all nitrogen concentrations tested, when compared to M522 group, might regulate more effectively the carbon flux at any given nitrogen level – resulting in less quantities of cell “carbon metabolic wastes” (Ribereau-Gayon et al. 2000).

In contrast 1-propanol is known to be formed by the condensation of pyruvic acid and acetyl CoA [33], and the increase with assimilable nitrogen is an opposite behavior with the other higher alcohols [24]. It was proposed that strains with low nitrogen demand produced relatively higher concentrations of 1-propanol at higher nitrogen concentrations but relatively less at higher nitrogen levels (see Fig. 2). The relative concentration of 1-propanol produced in response to nitrogen is generally reversed to ethyl acetate with respect to each of high and low nitrogen demand strains and would be an interesting topic for further research [24].

Yeasts synthesize fatty acids by the hydrolysis of the acyl-CoA derivatives and esters by esterification of activated fatty acids and alcohols. The behavior of KU1 group strains, in which higher concentrations of these compounds are produced when nitrogen availability is low, contradicts the concept raised in many reports that stated that increase in ester production is directly related to the increase of nitrogen in the must. This contradictory behavior of KU1 group strains could also explain why several studies did not observe a consistent correlation between YAN grape musts and ester and fatty acid concentration [21, 22]. More interestingly, the profiles of fatty acids and esters obtained with the KU1 group are quite similar to the higher alcohols and isoacids profiles as it is shown in Fig. 2, the opposite situation for the high nitrogen demand strains.

Limited information about the production of γ-butyrolactone and ethyl 4-hydroxybutanoate by yeast is found in the literature. The profile of γ-butyrolactone production also resembles those for higher alcohols and isoacids. The relation of γ-butyrolactone with ethyl 4-hydroxybutanoate production was previously proposed [34].

Despite of the large number of papers describing the effects of fermentation conditions on hydrogen sulfide formation [35, 36], only a few studies have investigated the relationship between fermentation management and formation of volatile sulfur compounds (VSCs), in particular, mercaptans, sulfides, and disulfides, which can potentially affect wine aroma [37]. The strategy used for the aroma impact evaluation of VSCs was based on the characterization of the possible odorant zones determined by gas chromatography–olfactometry (GC-O). This analytical technique represents the best way to screen the odor-active compounds in a complex matrix by using the human nose as the detector for molecules eluting from the gas chromatography column.

There is an extremely wide variation in the odor threshold values reported for these compounds, and certain sulfur compounds are known to contribute positively when they are present in sub- or peri-threshold concentrations, but they can be responsible for off-flavors at higher concentrations [38, 39]. Such occurrences highlight the importance of understanding and managing the yeast ecology of fermentation. The selection of yeast with a low propensity to produce sulfur

derivatives can minimize the production of off-flavors, and limited assimilable nitrogen in screening synthetic mediums could be a key tool for this purpose [5]. We suggest the reader for further information on esters, higher alcohols, acids, and sulfur aroma compounds, the excellent reviews made by other authors [7, 14, 29]. In this review, we will emphasize the terpenoid and phenylpropanoid aroma compounds synthesized by yeast; both chemical groups are being normally associated and found in fruits as free and bound (glycosylated) aroma compounds.

3 Yeast Enzymatic Hydrolysis of Bound Aroma Compounds

In several grape varieties, the dominating aroma compounds found are bound to sugars, such as the volatile group of benzenoid/phenylpropanoid and the isoprenoids (monoterpenes), that substantially contribute to wine aroma during fermentation or barrel aging [40]. β-Phenylethyl alcohol, benzyl alcohol, linalool, nerol, geraniol, and citronellol have been identified in grape must in their bound form and contribute with floral or fruity flavors if they are hydrolyzed [41–44]. Many of these compounds can represent 10–90 % of the total hydrolyzed volatile fraction of grapes such as Chardonnay [45], Cabernet Sauvignon, Merlot [46], Tannat [41], Pinot Noir [42], or aromatic varieties such as Muscats [47].

The hydrolysis of monoglucosides requires the action of a β-glucosidase, while hydrolysis of disaccharide glycosides requires the sequential activity of an appropriate exo-glycosidase to remove the outermost sugar residue, followed by a β-glucosidase to remove the remaining glucose (Scheme 1) [48]. It was also shown that an endo-glycosidase alone is capable of hydrolyzing this linkage thus liberating disaccharide and aglycon [49, 50]. Plant and microbial glycosidases have been reported and reviewed by different authors [51, 52].

Grapes have reasonable β-glucosidase activity [53, 54], but low α-rhamnosidase, α-arabinosidase, and β-xylosidase activities have been detected [54]. However,

Scheme 1 Hydrolysis of disaccharide glycosides by sequential action of an appropriate exo-glycosidase, followed by a β-glucosidase to remove the remaining glucose

β-glycosidase activity of grape juice is virtually absent, as its low pH and the presence of glucose inhibit the enzymatic activity [49, 53–57].

3.1 Yeast Glycosidase Activity During Wine Fermentation

Numerous reports have shown that several yeasts involved in vinification processes display β-glycosidase activity and that this activity tends to be greater in non-*Saccharomyces* strains than in *S. cerevisiae* [58–62]. Nevertheless, in recent work, it has been demonstrated that permeabilized cells of *Saccharomyces* species can exhibit β-glycosidase activity as high as non-*Saccharomyces* species, suggesting that the transport of glycosidic precursors is a limiting factor in the aromatic release and could orientate breeding programs for the construction of new interspecific wine yeasts [63]. One large screen of strains belonging to 20 species of yeasts, including *Debaryomyces castellii*, *D. hansenii*, *D. polymorphus*, *Kloeckera apiculata (Hanseniaspora uvarum),* and *Hansenula anomala*, showed β-glucosidase activity [61]. Furthermore, these indigenous species of non-*Saccharomyces* yeasts may impart special characteristics to the wines [64, 65]. Therefore, in order to enhance the sensorial attributes of the wines, it is important to explore the potential of wild yeasts isolated from enological ecosystems producing β-glucosidases. One strain identified as a *Rhodotorula mucillaginosa*, isolated from grapes of cultivars typical of Irpinia region and used in winemaking conditions showed an increase of the free terpene fraction [66]. Some native *Saccharomyces* and non-*Saccharomyces* strains were identified that show glycosidase activity in an esculin solid medium [67]. This technique directly correlates with the glycosyl–glucose (GG) index determination method that measures their enzymatic hydrolysis of glycosylated compounds in grape must [68]. The glycosidic activity related to *S. cerevisiae* has been proven in Riesling and Chardonnay musts [69, 70] and is weakly sensitive to the presence of sugar, but its action is very reduced due to must and wine pH [57, 71, 72]. On the other hand, several non-*Saccharomyces* yeasts have been shown to possess glycosidic activities, but are strongly inhibited by glucose concentration [73–75]. More promising findings come from work with *Debaryomyces* and *Candida* strains [61, 76, 77]. Studies with isolates obtained from enological ecosystems in the Utiel Requena Spanish region found that *Wickerhamomyces anomalus* and *P. membranifaciens* were the most interesting species because the glycosidase activity had a high degree of tolerance to glucose and ethanol high levels [78]. *Metschnikowia pulcherrima* was selected from fermenting grape must for its high β-glucosidase activity [67]. The isolation, purification, and partial characterization of the main enzyme it produced have contributed to a better understanding for the development of immobilized enzyme methods of potential application in wine production [79]. However, many β-glucosidases from non-*Saccharomyces* species are not active also at low pH [3], as shown for *M. pulcherrima* [79], *H. uvarum* [80], *Z. bailii* [81], and *P. pastoris* [82]. The activity of these enzymes in must and wine

(normal pH of about 3.0 to 3.8) has not been extensively characterized, and therefore, their effects on wine flavor are still unclear [83, 84]. The isolation and characterization of an extracellular β-glucosidase enzyme from *Issatchenkia terricola* that was selected for its activity at low pH above 3.0 [85] proved to be very active for white Muscat wine production in the presence of glucose (100 gL^{-1}), ethanol (18 %), and metabisulfite (60 mgL^{-1}). These results support the expectation that further studies of the wide yeast diversity that are still unknown will allow identifying better designed glucosidase enzymes by nature.

4 Biosynthesis and Biotransformation of Terpenoids by Yeast

The identification of monoterpenoids, compounds with strong sensory perception and widely diffused in plants such as *V. vinifera* varieties, had helped to elucidate the basic flavor chemistry of these compounds [21, 23, 86]. Monoterpenols, particularly linalool, geraniol, and nerol, are responsible for the characteristic floral aroma in grapes and wines of *V. vinifera* cultivars such as Muscat varieties, Gewürztraminer, and Riesling [47]. Acid-catalyzed rearrangements during wine processing and aging can also result in changes in concentration and formation of new compounds that were not present in the original grapes [87, 88]. In nonaromatic grape varieties, these compounds usually occur at concentrations lower than their odor thresholds. The question therefore arises as to which chemical and/or biological processes are responsible for the increase of monoterpene alcohols during fermentation of low monoterpene grape varieties. In this review, the contribution of several chemical and biological pathways to the formation of monoterpenes during alcoholic fermentation of wine is discussed by showing results that will make more understandable some of the events of isoprenoid micrometabolism in fungi.

Terpenoids are the most numerous and structurally diverse group of natural products [89–92], including hemi-, mono-, sesqui-, di-, tri-, and tetra-terpenes. It has been estimated that there are more than 30,000 isoprenoid compounds in plants [93]. They play numerous vital roles in basic plant processes, including respiration, photosynthesis, growth, development, reproduction, defense, and adaptation to environmental conditions [91, 94–96]. Terpenoids are biosynthesized by condensation of the isopentenyl diphosphate 5-carbon unit (IPP) and its isomer dimethylallyl diphosphate (DMAPP) in a head-to-tail or head-to-head fashion. In higher plants, there are two isoprenoid biosynthetic pathways which result in the precursors IPP and DMAPP. Over the course of evolution, plants have maintained the well-known eukaryotic mevalonic acid (MVA) pathway [97] in the cytosol (the classical pathway) and acquired the later discovered prokaryotic 2-*C*-methyl-D-erythritol 4-phosphate (MEP) (alternative pathway) [98–100] from the endosymbiotic ancestor of plastid.

4.1 Biotransformation of Terpenoids by Yeasts During Fermentation

The demonstration that exogenous monoterpenes can undergo biotransformation provides further evidence for the existence of a terpene metabolic pathway in yeasts [101]. The transformation of free terpenes by different yeasts, especially non-*Saccharomyces* species, has been reported [102]. This might be important in "low input winemaking" techniques, in which spontaneous fermentations are promoted and more yeast diversity is developed in the process [3, 5, 103–105].

The biotransformation and formation of some terpenes by *S. cerevisiae* in grape musts was proposed previously. In winemaking conditions, *S. cerevisiae* can biotransform geraniol to citronellol [106]. Biotransformation of the monoterpenes linalool, α-terpineol, nerol, and geraniol by *S. cerevisiae* was shown in model fermentations [107]. However, the mechanisms are currently ill-defined; furthermore, only one cyclase gene related to sterol metabolism in yeast, lanosterol synthase, has been reported [108].

4.2 Yeast Biosynthesis of Monoterpenes and Sesquiterpenes

Lynen proposed in 1964 [109] that terpene and sterol biosynthesis are related. Anaerobic conditions were suggested to inhibit several essential steps in ergosterol biosynthesis, including squalene epoxidation and the oxidative demethylation/dehydrogenation of lanosterol, essential steps for the formation of ergosterol. Some researchers suggested that such inhibition of sterol biosynthesis could stimulate terpene formation by fungi due to the accumulation of sterol precursor compounds [110]. In yeast and animals, only the MVA pathway is present and no monoterpene synthases have been reported. Monoterpenes have only rarely been reported from fungi. A chlorinated monoterpene was isolated from the fermentation broth of the mangrove endophytic fungus *Tryblidiopycnis* sp. [111]. *Esteya vermicola*, an endoparasitic fungus of pine wood nematode, has also been reported to produce monoterpenes as a mechanism to predate the nematode [112]. Production and export of monoterpenes by the fungus *Ceratocystis moniliformis* has also been reported [113]. Different volatile sesquiterpenes have been reported as cell–cell signaling molecules in *Candida* species: farnesol for *Candida albicans* and nerolidol for *Candida parapsilosis* [114, 115]. A yeast strain of *Rhodotorula glutinis* has been shown to produce the sesquiterpene nerolidol [116]. Farnesol is a volatile sesquiterpene widely distributed in many plant essential oils, in animals, and in microbes. It is naturally produced in trace amounts and plays essential roles in signal transduction, quorum sensing, and apoptosis induction. Farnesol is produced by dephosphorylation of FPP by phosphatases, pyrophosphatases, or specific sesquiterpene synthases [117].

In yeast, there is a unique enzyme, Erg20p, that synthesizes FPP from isoprene precursors [118]. Deletion of *ERG20* is lethal since mutants are unable to synthesize ergosterol, an essential component of cell membranes required to maintain

membrane permeability and fluidity [119]. In an *erg20* point mutant context (K197E), the tight binding of GPP to the farnesyl diphosphate synthase catalytic site is relaxed, and therefore, GPP is freely available as a precursor for the synthesis of the monoterpenes geraniol and linalool [120]. However, as fungi do not typically produce monoterpenes, research on the synthesis pathways has been focused on polyterpenoids, such as triterpenoids, carotenoids, and ubiquinone [121].

Knowledge on the formation of terpenoids by native yeasts until recently was limited to the production of trace concentrations by *S. cerevisiae* [122] and a small number of non-*Saccharomyces* species, such as *Kluyveromyces lactis* [123], *Torulaspora delbrueckii* [124], *Hanseniaspora uvarum* [122], *Metschnikowia pulcherrima*, *Candida stellata* [125], and *Ambrosiozyma monospora* [126]. Although recently some interesting efforts have been made to produce sesquiterpenes by engineered yeasts, there is limited literature in relation to natural sesquiterpene production by yeasts. A metabolically engineered *Saccharomyces cerevisiae* strain that expressed a plant sesquiterpene *epi*-cedrol synthase using FPP [127], production of the antimalarial drug precursor artemisinic acid [128], and accumulation of higher levels of FPP for commercial synthesis of these kinds of compounds [129] were reported. However, information on monoterpene formation by yeasts is still limited. *S. cerevisiae* M522 was used to evaluate the factors affecting terpene production, where it was demonstrated that, strikingly, the YAN and oxygen content of the fermentation medium influences monoterpene formation. High YAN concentration of the medium (400 compared with 180 mg NL^{-1}), which stimulates fermentation rate, but not biomass yield, stimulates monoterpene, but not sesquiterpene (nerolidol and farnesol) formation [122]. In addition, microaerobic compared with anaerobic conditions favored terpene accumulation in the ferment. To explain these results, based on blast searches performed on the *Saccharomyces* genome database, the authors hypothesized that monoterpenes might not be derived only from the sterol pathway, as sesquiterpenes appear to be, but by an alternative pathway. This latter pathway, which involves the conversion of leucine to mevalonic acid (Fig. 3) is located in the mitochondrion [122], and this fact could explain the non-coordinated synthesis of the two terpene groups (monoterpenes and sesquiterpenes). Assimilable nitrogen, as well as oxygen, is known to regulate mevalonic acid and sterol formation, and hence the concentration of intermediates, such as geranyl pyrophosphate, which can act as a terpene precursor.

Recently, this pathway interconnecting leucine catabolism and isoprenoid metabolism has been studied in fungi [133, 134]. Although the precise enzymatic basis for the link between sterol biosynthesis and leucine catabolism is still lacking, there is strong genetic evidence reported for the fungus, *Aspergillus nidulans* [135] that supports the existence of such a metabolic link. The pivotal reaction required for both leucine catabolism and isoprenoid metabolism is catalyzed by the enzyme 3-methylcrotonyl-CoA carboxylase (MCCase; EC 6.4.1.4), which has been described in some bacterial species and is present in the mitochondria of mammals and plants [136, 137]. The existence of a putative MCCase in yeast mitochondria was proposed [122], *HFA1* a gene with unknown function (see Fig. 4). On the other hand, geranyl diphosphate synthase (GPPase), the other key enzyme for

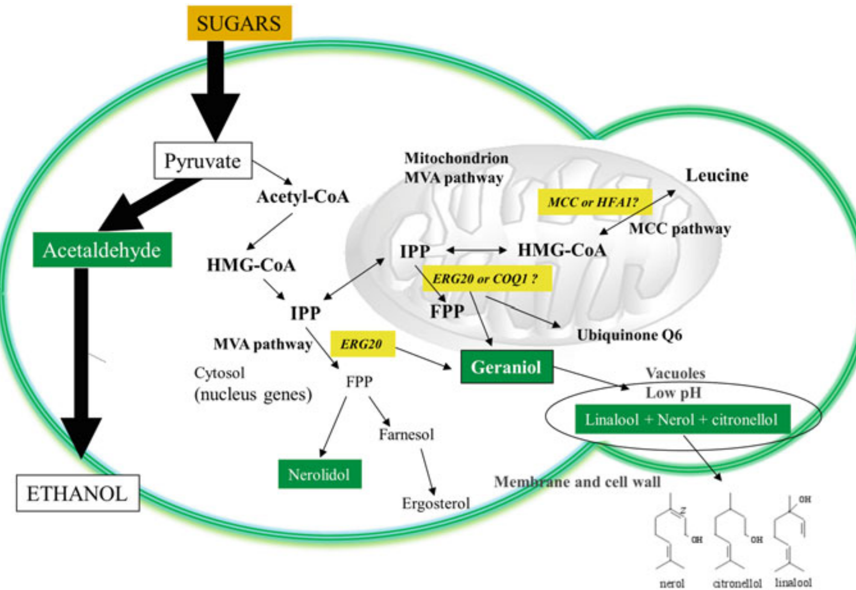

Fig. 3 Biosynthetic pathways of isoprenoids in yeast. These separate pathways (in mitochondria and in cytosol) would explain the differences found between monoterpene formation and sesquiterpene metabolism. This model is in agreement with the following facts: the existence of isozymes in *S. cerevisiae* of 3-hydroxy-3-methylglutaryl reductase *HMGR1* and *HMGR2* [130], the increase production of geraniol by *erg20* mutants [119], and the proposed compartmentation of the MVA pathway for the production of carotenoids and sterols in fungal cells [131, 132]. Although no putative genes were identified for the formation of linalool and α-terpineol in yeast, the low pH of the vacuoles might explain the formation of these compounds in wine fermentation. The key compounds or genes are indicated. *MCC* methylcrotonyl CoA, *FPP* farnesyl diphosphate, *HMGR* 3-hydroxy 3-methylglutaryl CoA reductase, *IPP* isopentenyl diphosphate, *MVA* mevalonic acid, *HFA1* unknown function mitochondrion gene with high homology to the *Aspergillus MCC, COQ1* mitochondrial hexaprenyl pyrophosphate synthetase homologue to plant GPP synthase

monoterpene synthesis that produces geranyl diphosphate from the IPP pool, although not previously described in yeast, might be present, according to a putative gene identified in these studies *COQ1*. Overexpression of this gene in *S. cerevisiae* significantly increases the formation of linalool and the sesquiterpene nerolidol [138]. At exponential growth conditions, *COQ1* gene participates in the isoprenoids biosynthesis fulfilling not only a geranyl pyrophosphate synthase function but also a nerolidol synthase activity. The tight binding of GPP to the farnesyl diphosphate synthase (FPPS) catalytic site might explain why generally in animals and microorganisms no GPP is released and made available for the biosynthesis of C-10 byproducts [139]. However, the mechanism of formation of monoterpenes in natural wine yeasts and in the yeast mutants *erg20* gene that encodes the FPPS [140] is still not well understood. Increase concentration of linalool due to overexpression of COQ1, in the *Saccharomyces cerevisiae* genome, argues in favor of the hypothesis that some of the monoterpenes detected in the ferments could be formed in the

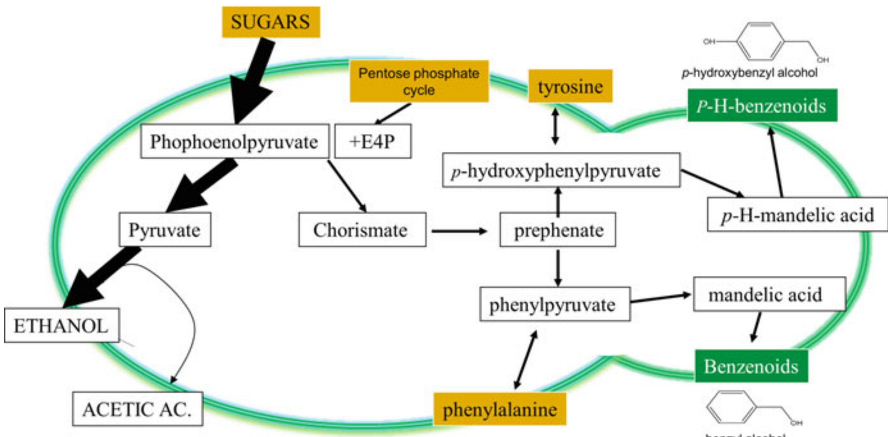

Fig. 4 Proposed pathways for the formation of benzenoids (benzyl alcohol, benzaldehyde) and p-hydroxybenzenoids (*p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde) in *Hanseniaspora vineae* through the chorismate pathway, with phenylpyruvate and *p*-hydroxyphenylpyruvate as intermediates

mitochondrion. Figure 3 shows the behavior of the *erg20* mutants that are blocked in the FPP synthase in the cytosol, resulting in an excess of IPP that could enter the mitochondrion, as was shown in plant plastids and cytosol [141]. Yeast mutants in *COQ1* gene are currently under study to understand this hypothesis. The last step of the formation of free monoterpenes is dephosphorylation. Although the enzyme activity involved in GPP dephosphorylation has not yet been identified in yeast, it was shown that the genes encoding the diacylglycerol phosphate phosphatases (LPP1 and DPP1) accept isoprenoid pyrophosphates as substrates in vitro in *Saccharomyces cerevisiae* [142]. However, the expression of a plant geraniol synthase (GES) in the *erg20* mutant strain of *S. cerevisiae* resulted in a tenfold increase in geraniol production [139], which confirms that GPP dephosphorylation is indeed a limiting step in terpenoid formation in yeast. An endogenous isoprenoid phosphatase activity, apart from LPP1 and DPP1, could explain the formation of geraniol in this mutant. However, the formation of some of the geraniol and probably all the linalool was proposed to take place in the vacuoles due to low pH effect [139]. When an *erg20* mutant was tested, the levels of the monoterpenes geraniol and linalool increased almost 10 times, compared to the wild-type BY4743 genetic context. Linalool levels in BY4743 were around 2 μgL^{-1}, while in K197E background, linalool production was around 200 μgL^{-1}. However, when this *erg20* mutant strain K197E was transformed with a plasmid overexpressing Coq1p and cultivated in a chemically defined medium that mimics grape juice, the levels of linalool reached 760 μgL^{-1} [138]. To the best of our knowledge, this is the highest level of linalool produced by *S. cerevisiae* up to now. On the other hand, several natural strains of *Hanseniaspora vineae* were recently studied and characterized in relation to monoterpene and sesquiterpene accumulation in the same synthetic medium used

previously for *S. cerevisiae* studies, and we had found a wide variability between strains (from 10 to 61 μgL^{-1} of each of these groups) with concentration levels up to 54 μgL^{-1} of monoterpenes and 61 μgL^{-1} for sesquiterpenes in the same strain [143], tenfold higher concentrations compared to natural *S. cerevisiae* strains.

In summary, some strains of yeast, *Saccharomyces* and non-*Saccharomyces*, might contribute to the floral aroma of grape wine and other fermented beverages, by de novo synthesis of monoterpenes, and this effect could be augmented by higher juice nitrogen in combination with microaerobic fermentation.

5 Biosynthesis of Phenylpropanoids by Yeast

The significance of volatile aryl alkyl alcohols in plants was recently well reviewed [144], where the volatile group of benzenoid/phenylpropanoid-related compounds (intermediates and end products) substantially contributes to plant fitness including essential metabolites such as hormones, cofactors, defense compounds, and attractants for pollinators and seed dispersers [144]. Benzyl alcohol is a widely used compound in the cosmetic, pharmaceutical, and fragrance industries [145]. It plays an important role in fungi lignin biodegradation processes, supplying together with other aromatic alcohols, hydrogen peroxide to the ligninolytic system [146]. Although it has not been thoroughly studied yet, benzyl alcohol could also participate in fungi cell–cell interactions, as quorum sensing molecules such as tyrosol, farnesol, or β-phenylethanol [24, 114, 147].

The formation of benzyl alcohol by fungi is limited to some Basidiomycetes [148], and until recently only one study in a synthetic medium was found for some non-*Saccharomyces* species showing the formation of this volatile compound: *Kloeckera apiculata (Hanseniaspora uvarum)*, *Candida stellata*, *Schizosaccharomyces*, *and Zygosaccharomyces* [149]. Furthermore, although the biosynthetic pathway is not elucidated for *p*-hydroxybenzoate in yeast, it is proved to be an intermediate of ubiquinone Q6 synthesis in *Saccharomyces* [150].

Free and bound β-phenylethyl alcohol and benzyl alcohol have been identified in grape must and wine and contribute with floral or fruity flavors [41–44].

We had recently showed that benzyl alcohol, benzaldehyde, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzyl alcohol can be synthesized de novo in absence of grape-derived precursors by the wine yeast *Hanseniaspora vineae*. Although little is known about the complete metabolic pathways in plants leading to the formation of volatile benzenoids, it is known that benzyl alcohol is formed within phenylpropanoid synthesis by *PAL* enzyme (phenylalanine ammonia lyase) [144]. This enzyme catalyzes the conversion of phenylalanine to trans-cinnamic acid [151] which is subsequently converted into benzyl alcohol and other derived compounds. This enzyme is the first of the phenylpropanoid metabolism in plants and has been found in some Basidiomycota and Ascomycota fungi such as *Neurospora*, *Aspergillus*, *and Botrytis* [152]. To our knowledge, this enzyme is rarely found in yeast. Although it has been reported for the Basidiomycota yeast, *Rhodotorula graminis* [153], it has not been found in the subphylum Saccharomycotina.

Genomic analysis of *H. vineae* indicates that the phenylalanine ammonia lyase (*PAL*) and tyrosine ammonia lyase (*TAL*) pathways, used by plants and some fungi to generate benzyl alcohols from aromatic amino acids through cinnamic acids, are absent in *H. vineae* genome-sequenced strains. Consequently, an alternative pathway derived from chorismate through phenylpyruvate and *p*-hydroxyphenylpyruvate through mandelate as intermediate was proposed [154]. Feeding experiments with the aromatic amino acids and the confirmation that *H. vineae* and *S. cerevisiae* are unable to synthesize cinnamic acids in our experimental conditions had supported the pathway presented in Fig. 4.

As we have sequenced the genomes of two *H. vineae* strains [155], using comparative genomics with *S. cerevisiae* data, we had confirmed the existence of genes needed by this yeast species to synthesize benzenoids. In Fig. 4, the mandelate pathway for the synthesis of benzenoid compounds is shown, which will also be used by *S. cerevisiae* for the synthesis of ubiquinone Q6 precursor, the 4-hydroxybenzoate. These results obtained for *H. vineae* will contribute to understand three putative pathways: the peroxisome β-oxidative pathway recently proved in plants, the cytosol non-oxidative pathway CoA-dependent or independent, also proposed for plants but not proved yet [144], and the mandelate pathway proposed here as the main route from sugars to benzenoids when cinnamic acids are not present or synthesize in the medium.

6 Mixed Cultures and Development of Consortia Strategies to Increase Flavor Diversity

Indigenous yeasts present in grape musts at the onset of wine fermentation can be divided broadly into two groups, i.e., the wine yeast *Saccharomyces cerevisiae* and the non-*Saccharomyces* (NS) yeasts. Several studies have shown that NS yeast strains can be detected throughout fermentation [156] and that their dominance during early stages can influence the final composition of wine [157].

Although a large population of active yeast cells is typically used for inoculation, many studies have shown that indigenous strains are not completely suppressed and can develop to a significant extent during early stages of juice fermentation [158, 159]. Indigenous NS yeasts are found predominantly on grapes, and to lesser extent on cellar equipment [160]. NS yeasts are present in highest numbers in grape must prior inoculation with commercial *S. cerevisiae*. In what is termed "spontaneous fermentation," there is a sequence of dominance by various NS grape must yeasts, followed by *S. cerevisiae* which can then complete fermentation [3]. Indigenous yeasts have been reported to contribute either positively or negatively to overall sensory characteristics of wine. Such results could be explained, in part, by the diversity of NS yeasts in grape must and the limited number of studies in different laboratories with consistent methodologies for controlling available nutrients [161]. Competition for nutrients during winemaking process may affect the development of a sequential inoculation process, and the addition of some vitamins and/or YAN may be needed to end the fermentation [161].

However, a number of studies have shown that the presence of NS yeast is associated with increased wine quality and complexity [4, 67, 159, 162–168]. Even though *S. cerevisiae* is responsible for the majority of the ethanol in wine, the presence of NS yeasts may have a significant effect on the production of aroma compounds including esters, higher alcohols, acids, monoterpenes, and benzenoids [23].

We have previously seen and recently demonstrated that during white Chardonnay wine production, inoculation of *Hanseniaspora vineae* increased spontaneous *S. cerevisiae* strain diversity and some key aroma compounds such as benzyl alcohol and β-phenylethyl acetate, compared to *Saccharomyces* single strain fermentations [4, 163, 169]. Further studies with different combinations of mixed cultures are needed to understand the cooperation or competition mechanisms between different species and how these processes improve flavor accumulation in the final fermented beverage.

7 Nutrient Limitations for Discrimination of Flavor Phenotypes

A lack of recognition of the importance of defining the nitrogen content of media in relation to aroma compounds has produced considerable discrepancies and misunderstandings in the literature. The importance of an appropriate YAN level for yeasts characterization in relation to aroma compounds was studied to understand flavor profiles changes due to the main nutrient of industrial juices [24]. Moreover, many commercial yeasts produce undesirable off-flavors, such as hydrogen sulfide, and high concentration of higher alcohols, depending on the concentration of assimilable nitrogen present in the grape must [36]. In the late seventies, these fermentation problems were partially solved by the addition of ammonium salts to deficient musts which increased fermentation rate but also the sensory desirability of the wines [35, 36, 170, 171]. As a consequence, the routine addition of ammoniacal nitrogen to musts to correct nitrogen limitation is widely used by winemakers.

Initial studies have attempted to relate the yeast nitrogen demand concept with the profile of aroma compounds in wines. Paradoxically, various studies reported to characterize the yeast aroma compounds of wines made with various yeast strains had not considered the importance of nitrogen level of the grape must or the fermentation medium utilized [70, 172–188]. Moreover, many studies were conducted under conditions of nitrogen excess, above 300 mg N L^{-1} [24]. This experimental approach has proved to be unsuitable since *S. cerevisiae* yeast strains can produce very different profiles of fermentation rate and aromatic compounds under industrial conditions of lower initial nitrogen level, usually of around 140 mgN L^{-1} [169, 189, 190].

From the chemical point of view, only a few studies have investigated the yeast aroma compounds of wines prepared under defined conditions of YAN contents similar to winemaking conditions [36, 169, 191–198].

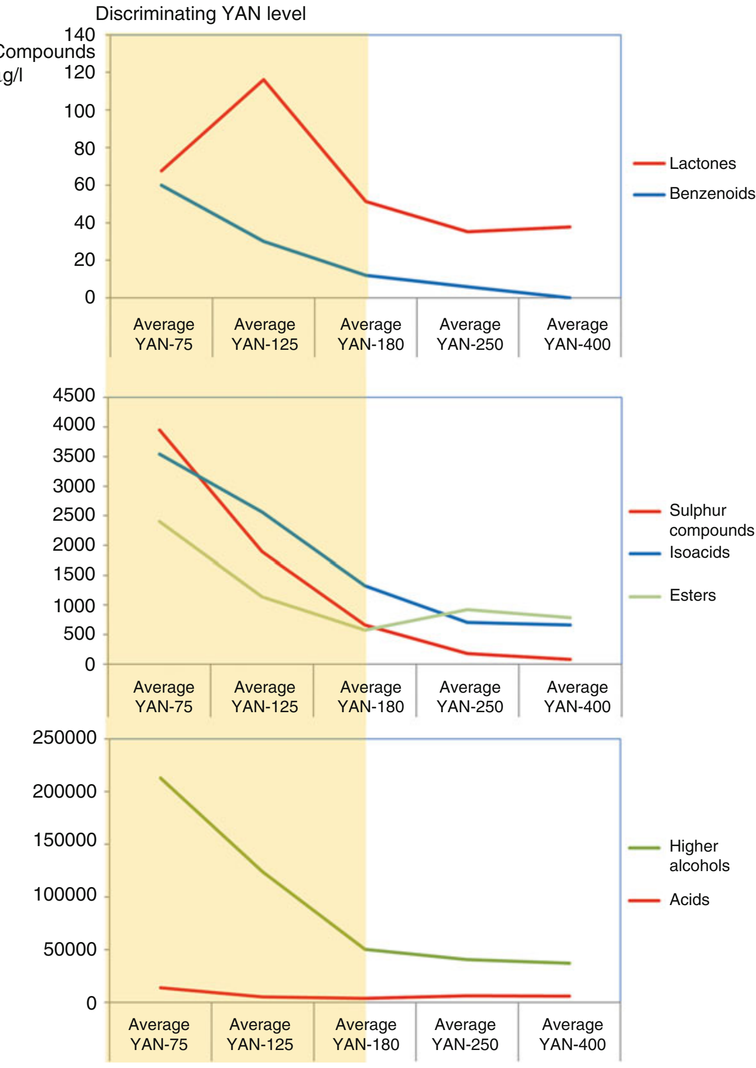

Fig. 5 Aroma profile of *Saccharomyces cerevisiae* strains considered of low nitrogen demand for wine fermentation. Fermentations were carried out in a chemical-defined medium with five YAN levels at 20 C. Clearly with YAN levels above 180 mgNL^{-1}, the production of flavor aroma compounds was decreased significantly making screening methods not ideal in terms of discriminating flavor potential of new yeast strains

However, some interesting changes on the behavior for aroma compounds in two model strains may contribute to chemical yeast discrimination if an appropriate nitrogen level is used in a chemical-defined medium. In Fig. 5, we show how with a limited YAN medium of 100 mgN L^{-1} we can obtain the highest flavor compound production of many of the main group of aromas such as higher alcohols, isoacids,

benzenoids, esters, lactones, and sulfur compounds (left side of the graphs). As it was discussed above due to a limited YAN screening medium, we had identified *H. vineae* as a high producer of benzenoid, compounds that their synthesis is significantly inhibited when diammonium phosphate was added [154]. In contrast, when the medium utilized contains high YAN levels, screenings of natural flora give just only 12 % of different strain species with detectable levels of benzyl acetate production [199].

In addition, as it was reported previously, an improved discrimination of fermentation kinetics capacities between strains was also obtained at low YAN level in a synthetic medium (78 $mgNL^{-1}$) compared to high YAN levels (390 $mgNL^{-1}$) [189]. Increase variations of growth and fermentation kinetics obviously result in increased variation of the flavor secondary metabolism. These results are in agreement with the first gene expression analysis made with an industrial strain [200], where, at low nitrogen level (53 $mgNL^{-1}$) compared to high nitrogen level (400 $mgNL^{-1}$), cultures display greater expression of genes involved in translation and in oxidative carbon metabolism, suggesting that respiration is more nitrogen-conserving than fermentation [200], and this phenomenon may contribute to increase some flavors from secondary metabolism.

8 Genetic Engineering Techniques for Flavors

Examples of yeast genetic modification for increasing concentration or flavor diversity are scarce [201–203]. Moreover, subsequent formal sensory analysis evaluation of the final product is exceptional [203]. Besides the consumer's controversial perception of genetically modified organisms, the current limitation of use of these strains at real production conditions is also the lack of stability or cell vigor of these strains at industrial level [204]. Usually when yeast strains were genetically engineered, they may be applied in a simple or sterilized system for the production of a single valued compound, such as for the pharmaceutical industry. Sterilization methods of food substrates for fermentation are assumed to affect many aroma precursors such as amino acids, vitamins, and other carbon and sulfur compounds that are important for the final product characteristics. Other breeding strategies or generation of interspecific wine yeast hybrids have successfully improved wine flavor by reducing off-flavor production and enhancing volatile thiol release in *Saccharomyces* [205, 206]. However, still very limited information is reported about how these engineered strains behave in mixed culture fermentations as naturally happens in the majority of the food fermentation industries.

9 Conclusions

The role of yeasts biodiversity associated with grapes and the contribution to aroma and flavor compounds of wine is clearly demonstrated from the large number of studies that have been conducted worldwide. In particular, the diversity associated to

Saccharomyces and non-*Saccharomyces* yeasts has only recently become appreciated, and this diversity can directly impact wine aroma or flavor or can interact with grape components to enhance or mask varietal characters.

The more diverse the yeast flora, the more diverse the spectrum of end products of the wine will be. This may be positive, depending upon the desired wine aroma profile. These results open a new field within yeast micrometabolism quantification where flavor compounds are accumulated in a fermented beverage. A better understanding of terpenoid and benzenoid biosynthetic pathways in yeast could help in the interpretation of more complex systems, such as mixed cultures.

Consequently, the application of metabolic footprinting techniques with GC-MS analysis would make possible the evaluation of yeast flavor metabolome, including terpenoids and benzenoids at very low concentration (sub- and perithresholds) from microbial or different raw material origin. Yeast strain selections by using the metabolome is expected to be a more sensitive tool to discriminate quality starters when compared to either the transcriptome or proteome analysis. Future research must be focused on the exploration of some key yeast mutants to better understand yeast benzenoid and terpenoid metabolic pathways and its potential development in food fermentation processes combining nutrients and mixed cultures interactions.

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Immobilized Yeast Cells and Secondary Metabolites

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Abstract

The use of immobilized cell technology (ICT) is viewed as a promising biotechnological tool to achieve high volumetric productivities of yeast fermentation in bioindustry of alcoholic beverages. During this process a huge number of organic compounds are being formed as yeast secondary metabolites, among which volatile compounds, such as higher alcohols, esters, and vicinal diketones, are the most important flavoring compounds. The objective of this chapter is to summarize the knowledge on the origin of the flavor-active and nonvolatile compounds synthesized by yeast and to describe how the composition of the medium, culture strain, process conditions (temperature, aeration, etc.), bioreactor design, and other critical parameters influence the metabolic activities of yeast cultures. Despite the technological and economic advantages provided by ICT, commercialization of this technology experienced only limited success, mainly due to unpredictable effect of immobilization on yeast physiology. This chapter is an attempt to rationalize and make some conclusions about the impact of cell immobilization on yeast metabolism collected from empirical experiences in production of alcoholic beverages. The knowledge addressing this issue may be of particular benefit to the nascent bioflavor industry.

Keywords

Yeast • Bioproduction • Fermentation • Higher alcohols • Esters • Carbonyl compounds • Fatty acids

1 Introduction

The process of ethanol fermentation catalyzed by yeast has a long history in the production of alcoholic drinks and in bioethanol production since the 1970s. Particularly, *Saccharomyces cerevisiae* (brewer's yeast) is an economically attractive biocatalyst due to its availability, ease of handling and disposal, low cost, safety for food and pharmaceutical applications, and a high catalytic capability for a variety of substrates. Namely, yeasts have the capacity to catalyze a wide range of stereoselective biochemical reactions in the production of pure compounds, which are of increasing importance in the fine chemical and pharmaceutical industries in particular. A variety of flavor-active compounds is being produced via yeast metabolism, and the profile of flavor-active compounds largely depends on yeast strain and substrate composition. For example, many pure flavoring compounds can be obtained by asymmetric reductions

of ketones mediated by yeast cells by using glucose as a C-source. However, in contrast to bioethanol and beverage production by yeast fermentation, which are already well-developed industries, production of pure biocompounds has not yet been considered suitable for large-scale processes, mainly due to several constraints such as low concentration of reagents, toxicity of the substrates used or the aroma compounds produced, and complexity of downstream processing, since the reactions are generally performed in a batch process. Furthermore, the process is accompanied by formation of undesired by-products due to the complex pathways involved. Another reason is that physiological changes that yeast goes through during fermentation have not yet been completely resolved despite extensive research including genome-wide expression profiling done so far [1–5]. For the development of economically competitive microbial processes, agro-industrial residues with negligible or even no cost have become popular as substrates for flavor production by microorganisms [6, 7]. Another application area for yeast catalytic activity is in environmental protection since baker's yeast is the whole-cell system, which has been used for reduction of volatile organic compounds present in the environment as pollutants, such as aldehydes and ketones.

Immobilization procedures confine cells to a specific region of space in order to preserve their biocatalytic activity and enable reuse. The technology of immobilizing yeast for alcoholic fermentation has received great attention since the 1970s. It has been put in practice for bioethanol production, production of alcohol-free or low-alcohol beer, the secondary fermentation for sparkling wine, and batch winemaking of white wines, while primary beer fermentation, continuous brewing, and wine production are still under scrutiny.

Carriers that have been proposed for yeast immobilization include inorganic, organic, or natural materials, such as porous mineral rocks, cellulosic agro-industrial products and wastes, hydrocolloid polysaccharides (Ca-alginate, k-carrageenan) and proteins, etc. Porous supports have high specific surface area due to a complex structure, including pores and tubes with sizes down to nano- and microscales, where cells are located mainly inside the pores (Fig. 1a). Fibers, particularly nanofibers produced by electrospinning, are becoming a promising alternative to conventional porous materials [10]. Some microorganisms have been immobilized jointly, either on an inert support, for example, *S. cerevisiae* and *Candida shehatae* yeasts [11], *S. cerevisiae* and *Lactobacillus plantarum* [12], *Saccharomyces bayanus* and *Leuconostoc oenos* [13], *Saccharomyces cerevisiae* and *Oenococcus oeni* [14], or simply by spontaneous co-immobilization without the need for an external support or a chemical binder; this kind of couple is, for example, the flor yeast *S. cerevisiae* and the filamentous fungus *Penicillium chrysogenum*, with nonliving hyphae encapsulating the viable yeast cells [9, 15, 16] as shown in Fig. 1b. Food-grade supports, such as wheat [17, 18], potato [19, 20], corn [21], gluten, and spent grains (a brewing by-product) [22], delignified cellulosic material (having a tubular structure) [23], and pieces or skins of fruits (such as apple, quince, grape, raisins, figs) [24–30] have become popular for research, as they are easily accepted by consumers. Besides, the use of alcohol-resistant and cryotolerant yeasts immobilized on these supports enables fermentations at low-temperature producing wines and beers with excellent taste and aroma. Furthermore, fruits as supports are rich in fiber, trace minerals,

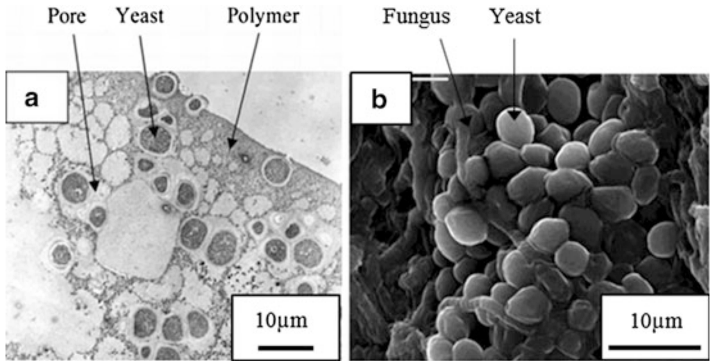

Fig. 1 Immobilized yeast (**a**) within porous polymeric capsules [8] (TEM image of immobilized yeast 5 mm-sized capsule prepared by phase separation of poly(m-phenylene isophthalamide) solution by using starch to position the microorganisms in the capsule pores and to form spaces around the microorganisms; (**b**) by linking to fungus hyphae [9] (SEM image of biocapsules 3–5 mm in diameter obtained from *S. cerevisiae strain G1 (ATCC: MYA-2451)* and *Penicillium chrysogenum strain H3*)

antioxidant polyphenols, proteins, sugars, and volatile compounds that provide a fruity aroma and taste. At present, the only carriers reported to have been used at full scale are porous glass and DEAE-cellulose with additions of titanium dioxide and polystyrene, kieselguhr filters filled with yeast and kieselguhr, silicon carbide tubular matrix, and hydrogel (k-carrageenan, chitosan, and alginate) beads but with less success. The combination of immobilization and freeze-drying has been applied successfully for some of the above biocatalysts, allowing the supply of preserved and marketable ready-to-use immobilized cells to breweries and wineries. Different mechanisms of yeast immobilization onto a carrier have been identified: cell-carrier adhesion, cell-cell attachment (leading to a multilayer yeast immobilization), cell adsorption (accumulation) inside natural shelters (carrier's surface roughness), and flocculation. Actually, immobilization of microbial cells by porous supports usually involves a combination of those, rather than only one mechanism.

1.1 Technological Considerations

The advantages of immobilized cell technology (ICT) in fermentation processes can be summarized as the following:

– Continuous processing. Since continuous fermenters may work in uninterrupted operation for weeks or months, this kind of processing has the economic benefits. Generally, a higher efficiency of the process is achievable and subsequently higher productivities and lower operating costs. Even more, improvements in the product quality have been attained. Such a process can be industrialized and the products have commercial value. The continuous process has been so far industrially applied in beer maturation and alcohol-free beer production.

- Fermentation at extremely low temperatures. Low-temperature fermentation (below 15 °C) by cold-sensitive yeasts has been recognized as a valuable tool to improve the quality of fermented products such as wine and beer. However, at low temperatures the problem arises from the low fermentation rate of sugars, especially of maltose (the sugar involved in processes such as beer, bread, whisky, vodka, fuel and potable alcohol production, etc.), which fermentation rate is markedly below that of glucose [31]. Solid carries can have a significant promotional effect on the rate of alcoholic fermentation in brewing.
- The increased resistance to inhibiting substances (e.g., ethanol tolerance) is ascribed to immobilized yeast in comparison to free suspended cells. This phenomenon is connected either to changes in the composition and organization of the cell wall and plasma membrane of the immobilized cells (increased levels of DNA, structural carbohydrates, glycogen, and fatty acids) [32, 33] or to some protective effect of the immobilization support.
- High productivities due to high cell concentrations within the reactor can be realized. Thus, the maximum yeast concentration, e.g., in a packed bed reactor using immobilized cells, can be up to ten times larger than at the end of a conventional batch fermentation. High yields are achievable even at extremely low temperatures (0–5 °C) by using cryotolerant and ethanol-resistant immobilized yeast cells.
- Possibility of the biocatalyst reuse; in this respect, regeneration of the carrier should be considered. A longer active life of biocatalysts is preferred in industrial productions, particularly when the production is halted, for example, when there is a need to preserve biocatalysts from the end of a winemaking season until the next year. The immobilized biocatalysts can be easily stored and reactivated while keeping their activity after storage for 6 months or even longer.
- Cell separation is facilitated.
- Shorter fermentation time. ICT dramatically reduces fermentation time, especially the maturation time (secondary fermentation) compared with traditional processes. An increased rate of fermentation has been explained by the higher activity of immobilized cells as compared with that of free cells due to a reduction of the activation energy.
- Better sensory characteristics of special-type wines have been ascribed to fruit pieces as support for yeast immobilization.

Disadvantages of ICT. The immobilized biocatalysts have not yet been commercialized due to the following reasons:

- Modified yeast metabolism due to internal and external mass transfer limitations (resulting in different ATP content and altered activity of key enzymes), specific microenvironment (created by the immobilization matrix), cell aging, and continuous mode of operation [34–37].
- A consequence of altered yeast physiology is unbalanced flavor and difficulty in maintaining the traditional character of the product. In general, beverages produced by yeast fermentation technology, such as beer and wine, are complex

aqueous solutions containing CO_2, ethanol, inorganic salts, and as much as about 800 organic compounds. The well-balanced aroma and flavor of the final product is the primary goal, perhaps even more important than an efficient fermentation and high yield.

- The composition of the solid matrix may also interfere in the flavor profile.
- Frequent replacement of biocatalyst is required since accumulation of dead biomass in the biocatalyst occurs due to the relatively short life span of cells.
- High capital costs (due to additional costs of a carrier) and complex and unstable operation often make a process economically unfeasible.
- Engineering problems linked with ICT are excess biomass and problems with CO_2 removal, optimization of operating conditions, clogging, and channeling of the reactor.
- Consumers may be wary of any deviation from the traditional way of production.

This chapter gives an overview on the most important secondary metabolites (higher alcohols, esters, carbonyl compounds, organic acids, and others) produced by yeast during fermentation and presented in Fig. 2. A special focus will be on the impact of ICT on synthesis of these compounds. When judging the metabolic activity of immobilized cells, process parameters (temperature, pH, oxygen, substrate, and product concentration gradients) have to be regarded as well. However, since the information

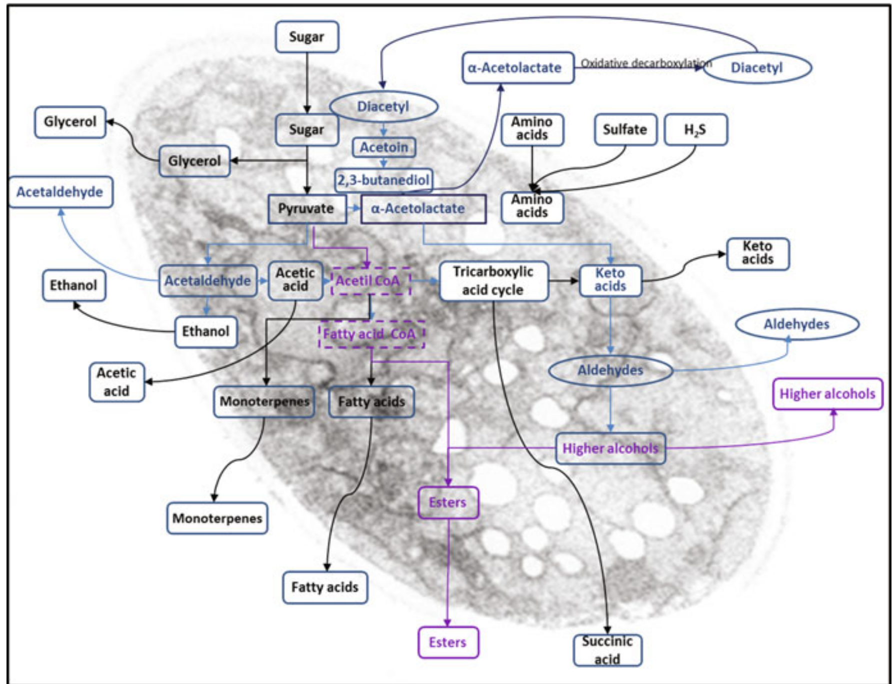

Fig. 2 Schematic presentation of the major flavor groups formation during fermentation

concerning physiological conditions of immobilized yeast is rather complex due to different matrices and variable system configurations, the impact of cell immobilization on synthesis mechanism of any metabolite is difficult to predict. This chapter is an attempt to rationalize and make some conclusions about the impact of cell immobilization on yeast metabolism obtained from different experiments.

2 Higher Alcohols

2.1 Biosynthetic Pathway of Higher Alcohols

"Higher alcohols" (also called "fusel alcohols") are compounds, which have more than two carbon atoms and a higher boiling point than ethanol [38]. They are produced by yeast cells as by-products during beer fermentation. They represent the major fraction of the volatile aroma compounds, but their contribution to beer aroma is usually not very pronounced, due to their relatively high flavor threshold values (10–600 mg/l) [34]. More than 40 higher alcohols in beer have been identified [39]. Table 1 gives the most important compounds, which can be classified into aliphatic (1-propanol, isobutanol (2-methyl-1-propanol), 2-methylbutanol (or active amyl alcohol), and 3-methylbutanol (or isoamyl alcohol)) and aromatic (2-phenylethanol, tyrosol, tryptophol (indole-3-ethanol)) higher alcohols. Aliphatic higher alcohols contribute to the "alcoholic" or "solvent" aroma of beer, and they produce a warm mouthfeel. The aromatic alcohol 2-phenylethanol has a sweet rose-like aroma and has a positive contribution to the beer aroma. Isoamyl alcohol and

Table 1 Major higher alcohols in beer [40–47]

Compound	Flavor threshold (mg/l)	Aroma or taste	Concentration range (mg/l) Bottom fermentation	Concentration range (mg/l) Top fermentation
1-Propanol	600–800	Alcoholic	7–19	20–45
Isobutanol (2-methyl-1-propanol)	100–200	Alcoholic	4–20	10–24
2-Methyl-1-butanol (amyl alcohol)	50–70	Banana, solvent-like	9–25	80–140
3-Methyl-1-butanol (isoamyl alcohol)	50–65	Alcoholic, fusel, banana	25–75	80–140
2-Phenylethanol	5–125	Roses, sweetish, perfume	4–42	8–50
Tyrosol	10–200	Bitter, chemical	6–15	7–22
Tryptophol	10–200	Almonds, solvent	0.5–14	2–12

2-phenylethanol can be found around their flavor threshold concentrations in lager beer and can significantly contribute to the flavor of these beers. The latter compound can mask the sweetcorn-like flavor of dimethyl sulfide (DMS) [48]. The aromas of tyrosol and tryptophol are undesirable, but they are only present above their thresholds in some top-fermented beers. Besides their own contribution to the overall beer aroma, higher alcohols are precursors of the flavor-intensive esters.

Higher alcohols are synthesized via the catabolic and anabolic pathway (synthesis from wort carbohydrates via pyruvate) [49–52]. In the catabolic Ehrlich pathway, the yeast uses the amino acids of the wort to produce the corresponding α-keto (2-oxo) acid via a transamination reaction. Isobutanol, amyl alcohol, and isoamyl alcohol are produced via this route from the superpathway of the branched-chain amino acids leucine, isoleucine, and valine biosynthesis (Fig. 3). The branched-chain amino acids are first deaminated to the corresponding α-keto acids (α-ketoisocapric acid from leucine, α-ketoisovaleric acid from valine, and α-keto-β-methylvaleric acid from leucine). The transamination reaction is mediated by the branched-chain amino acid transaminase (Bat1p) and aminotransferase (Bat2p) [53–56]. The excess oxoacids are subsequently decarboxylated into aldehydes by pyruvate decarboxylases (Pdc1p, Pdc5p, and Pdc6p) and a 2-oxo-acid decarboxylase Aro10p [57, 58]. There are significant differences in the way each α-keto acid is subsequently decarboxylated. In the valine degradation pathway toward the production of isobutanol, any of the pyruvate decarboxylases can mediate the decarboxylation of 2-keto-isovalerate [59]. Leucine degradation toward active 3-methyl-1-butanol is mediated by major decarboxylase Kid1p [60], and in the isoleucine degradation pathway toward 2-methyl-1-butanol, any of the decarboxylases Pdc1p, Pdc5p, Pdc6p, Kid1p, or Aro10p can perform the decarboxylation reaction [61]. Next, the aldehydes are reduced to higher alcohols by alcohol dehydrogenases Adh1-7p or the formaldehyde dehydrogenase Sfa1p [52, 62, 63]. This last reduction step also regenerates NAD^+. An outsider in this pathway is 1-propanol, which is derived from threonine via oxidative deamination.

Likewise, tryptophol, tyrosol, and phenylethanol are also produced via the Ehrlich pathway from the degradation of the aromatic amino acids tryptophan, tyrosine, and phenylalanine, respectively (Fig. 4). The transamination reactions are performed using the aromatic amino acid aminotransferases Aro8p and Aro9p [64, 65]. The decarboxylation and reduction of phenylalanine to 2-phenylethanol and of tryptophan to indole-3-ethanol tryptophol have been studied in more detail than the degradation of tyrosine to tyrosol [66]. The decarboxylases Pdc1p, Pdc5p, Pdc6p, and Aro10p and the alcohol dehydrogenases Ahd4p, Adh5p, and Sfa1p are involved in these reactions.

In the anabolic pathway (Fig. 3), the higher alcohols are synthesized from α-keto acids during the synthesis of amino acids from the carbohydrate source [67, 68]. The pathway choice depends on the individual higher alcohol and on the level of available amino acids available. The importance of the anabolic pathway decreases as the number of carbon atoms in the alcohol increases [50] and increases in the later stage of fermentation as wort amino acids are depleted [69].

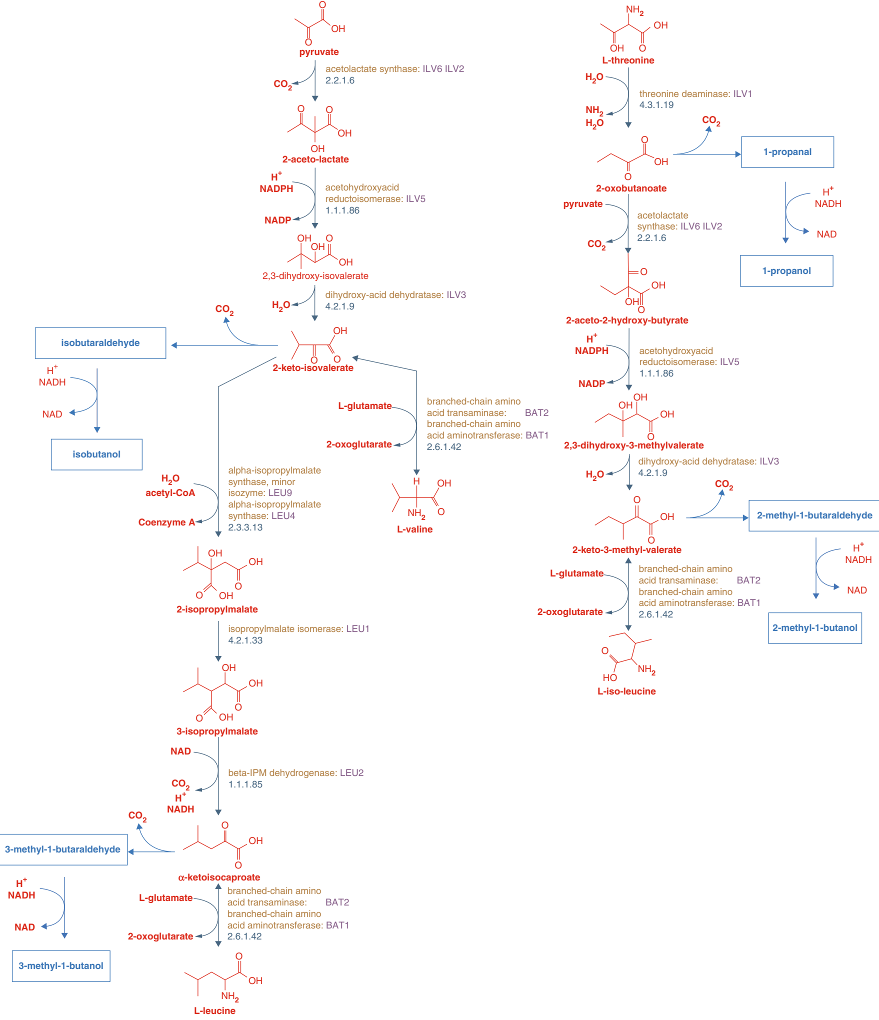

Fig. 3 Biosynthetic routes for the synthesis of the higher alcohols 1-propanol, 2-methyl-1-propanol (isobutanol), 2-methyl-1-butanol, and 3-methyl-1-butanol with indication of the enzymes and their coding genes (Adapted from "pathways.yeastgenome.org")

2.2 Influence of Process Variables on Bioproduction of Higher Alcohols

Conditions that promote yeast cell growth such as high levels of nutrients (amino acids, oxygen, lipids, zinc, etc.) and increased temperature and agitation stimulate the production of higher alcohols [70–74]. The synthesis of aromatic alcohols is especially sensitive to temperature changes. The higher alcohol (1-propanol, isobutanol, amyl and isoamyl alcohol) concentration was increased when the

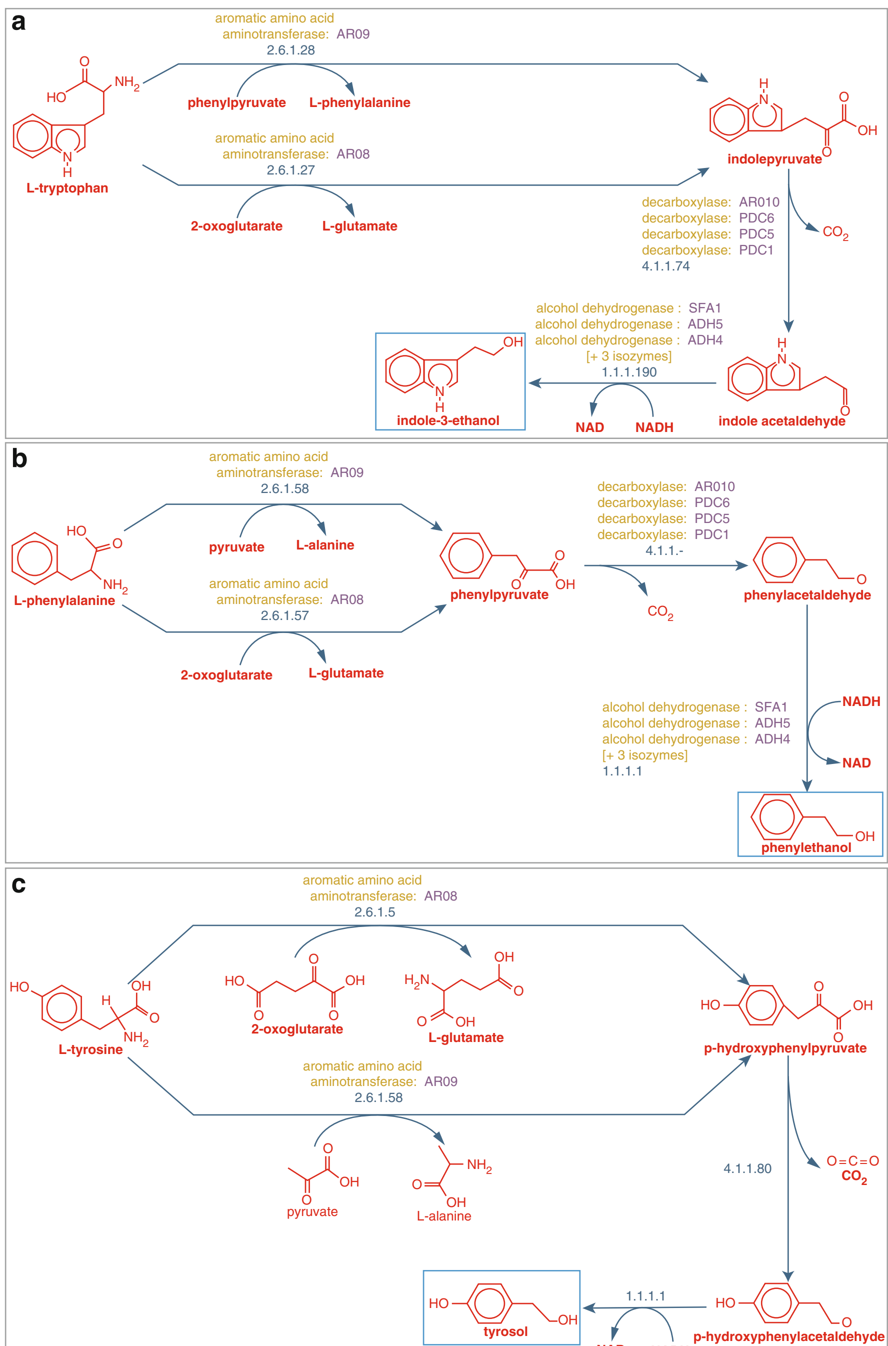

Fig. 4 (continued)

temperature was increased from 7 °C to 15 °C during continuous fermentation using a bubble column reactor with yeast cells immobilized on spent grains [75]. Reduction in the total higher alcohol concentration was also observed due to temperature decrease during repeated discontinuous fermentation with yeast cells immobilized on spent grains [76]. On the other hand, conditions that restrict yeast growth, such as lower temperature and higher pressure, reduce the extent of higher alcohol production. Higher pressures can reduce the extent of cell growth and, therefore, the production of higher alcohols [73]. The yeast strain, fermentation conditions, and wort composition have all significant effects on the pattern and concentrations of synthesized higher alcohols [69]. The amino acid composition has a major effect on the formation of higher alcohols: supplementation of wort with valine, isoleucine, and leucine induces the formation of isobutanol, amyl alcohol, and isoamyl alcohol, respectively [68, 77]. The overexpression of the branched-chain amino acid transferase genes *BAT1* and *BAT2* resulted in an increased production of isoamyl alcohol and isobutanol [78]. The addition of fatty acids and sterols also increased the higher alcohol content of the beer [79].

2.3 Effect of Immobilized Cell Technology on Bioproduction of Higher Alcohols

The obtained higher alcohol concentration in beer that was produced using ICT has been reported to be lower, comparable, or higher than beers produced with suspended cells (Table 2). A decrease has been attributed to the limited cellular growth in immobilized cell systems, leading to a poor nitrogen removal [86, 88, 93, 97, 98], in contrast to rapid yeast growth, which leads to enhanced anabolic production of amino acid precursors with concomitant overflow of higher alcohols, oxoacids, organic acids, and vicinal diketones [99]. A higher specific growth rate of brewers' yeast encapsulated in alginate/chitosan beads with a liquid core compared to free cells was reported, which resulted in slightly higher levels of higher alcohols and esters [100]. Yeast growth control by aeration has been employed to increase the level of higher alcohols. For example, Kirin Brewery (Japan) developed a two-stage immobilized fermentation system where the first reactor was a stirred aerated reactor with suspended yeast cells simulating the yeast growth in the beginning of a conventional batch fermentation and where most of amino acids were consumed with a sufficient amount of higher alcohols produced [101, 102]. In the second reactor, immobilized cells fermented anaerobically the wort further with production of ethanol and esters but no higher alcohols. In an ICT system with limited yeast growth, higher alcohol levels were mostly comparable to free cell batch fermentation, and some of the alcohols (i.e., propanol) were even present at a higher

Fig. 4 Ehrlich pathway of aromatic acid degradation for the production of (**a**) tryptophol (indole-3-ethanol), (**b**) 2-phenylethanol, and (**c**) tyrosol with indication of the enzymes and their coding genes (Adapted from "pathways.yeastgenome.org")

Table 2 Higher alcohol production in immobilized cell and free cell systems (without reference to a footnote, the data correspond to the analysis of green beer) (Adapted from [80])

Compound	Carrier	Immobilized cells	Free cells	Reference
1-Propanol	Porous glass beads	15.5[a]/17.2[b]	–	[81]
	Diatomaceous earth	12.7	–	[82]
	DEAE-cellulose beads	10.9	–	[82]
	Ca-alginate beads	11.6	10.4	[83]
	Ca-pectate beads	31.8[c]/31.2[d]	23.0	[84]
	Silicon carbide rod	14.1	12.4	[85]
	Aspen wood chips	9.8[e]/7.5[f]	8.0	[86]
	Beech wood chips	19.8	14.0	[87]
	Spent grains	6.4	6.1	[88]
	k-carrageenan	20.0	–	[89]
	Ceramic	15.7-50.5[g] (24.9[h])	10.0	[90]
	hydroxylapatite	14.8[i]/20.4[j]	10.2	[91]
	Ceramic chamotte	15	17.5	[92]
		15.6[s]/18.0[t]/32.4[u]	–	[75]
		32.5[j]	9.9[j]	[93]
		5.6[q]	26.2[q]	[94]
		0.0[r]	0.0[r]	[94]
		55.9[q]	44.1[q]	
		2.4[r]	10.6[r]	
Isobutanol	Porous glass beads	9.4[a]/12.1[b]	–	[81]
	DEAE-cellulose	11.0	9.7	[83]
	Ca-pectate beads	10.5[k]/18.9[l]/21.3[m]	10.4[k]/19.6[l]/	[86]
	Silicon carbide rod	29.0[c]/32.3[d]	20.6[m]	[84]
	Polyvinyl alcohol	13.4	24.0	[88]
	Lentikats®	14.3	19.6	[89]
	Aspen wood chips	31.7[n]/29.5[o]	–	[95]
	Beech wood chips	7.5-10.8[g] (8.4[h])	30.1	[90]
	k-carrageenan	8.0[i]/9.2[j]	8.2	[91]
	Spent grains	11.1[j]	6.5	[93]
	Ceramic	10.1	7.8[j]	[92]
	hydroxylapatite	11.7[s]/12.0[t]/18.6[u]	12.5	[75]
	Ceramic chamotte	0.45[q]	–	[94]
		1.7[r]	14.3[q]	[94]
		18.3[q]	11.0[r]	
		10.7[r]	25.5[q]	
			32.4[r]	
3-Methyl-1-butanol	Porous glass beads	35.8[a]/42.2[b]	–	[81]
	Diatomaceous earth	33.9	–	[82]
	DEAE-cellulose	32.8	–	[82]
	Ca-alginate beads	35.7	36.5	[83]
	Silicon carbide rod	60.4[c]/58.4[d]	58.4	[84]
	Aspen wood chips	48.4	60.3	[85]
	Beech wood chips	31.0[e]/38.0[f]	62.0	[86]
	k-carrageenan	51.2	–	[89]
		30.0-59.5[g] (47.0[h])	51.0	[90]
		32.5[i]/29.3[j]	29.7	[91]
		47.4[j]	46.7[j]	[93]
2-Methyl-1-butanol	Porous glass beads	13.7[a]/15.8[b]	–	[81]
	Beech wood chips	12.1[c]/12.6[j]	11.5	[91]

(*continued*)

Table 2 (continued)

Compound	Carrier	Immobilized cells	Free cells	Reference
	DEAE-cellulose beads Ceramic hydroxylapatite Ceramic chamotte	16.2[c]/16.1[d] 12.7[q] 0[r] 23.6[q] 12.3[r]	12.1 25.1[q] 8.1[r] 34.5[q] 21.1[r]	[84] [94] [94]
Phenylethanol	Polyvinyl alcohol Lentikats® Ca-alginate beads Ca-pectate beads	4.1[n]/4.2[o] +[p] 10.1	4.1 +[p] 14.6	[95] [96] [88]

[a]Average over day 0–138; [b]average over day 378–442; [c]day 8; [d]day 10; [e]packed bed reactor; [f]fluidized bed reactor; [g]range; [h]average; [i]maturation with immobilized cells; [j]conventional maturation; [k]8 °C; [l]15 °C; [m]20 °C; [n]recycled CO_2; [o]gas bottle CO_2; [p]no significant difference ($p < 0.05$); [q]ale strain; [r]lager strain; [s]7 °C; [t]10 °C; [u]15 °C

concentration [90]. The high propanol production was linked to the relatively high 2,3-pentanedione concentration and to 2-ketobutyrate-mediated processes. It was hypothesized that the overproduction was the result of a more active 2-ketobutyrate pathway. A high propanol production has also been reported for other ICT systems (Table 2) (e.g., [88, 98, 103]).

Mass transfer limitations of amino acids have also an influence on higher alcohol synthesis. In a fluidized bed reactor, it was observed that the free amino nitrogen (FAN) uptake by entrapped yeast cells increased linearly with the superficial velocity of the wort in the reactor [98, 104]. This mass transfer effect has also been demonstrated in a gas-lift bioreactor [105]. Amino acids of group III amino acids (e.g., tryptophan) were absorbed slower by immobilized yeast cells, where group I and II amino acids were removed at a rate equal to that of free cells [98]. Beer production in a fluidized bed reactor, where yeast cells were immobilized in alginate beads, resulted in lower free amino nitrogen (FAN) levels than in a packed bed reactor, and higher apparent fluid velocities through the reactors resulted in lower FAN levels [106].

Table 2 gives examples of reported higher alcohol levels in ICT systems compared to free cell batch fermentation. These data show that flavor formation is dependent on the bioreactor system and used carrier material. Since fermentation conditions and yeast strains differ for the reported data, it is not possible to evaluate the influence of the carrier material rigorously. In some studies, the effect of the carrier material on the formation of flavor products was evaluated. For example, Smogrovicova et al. compared PVA Lentikats with Ca-alginate carriers [95] and DEAE-cellulose, Ca-pectate, and k-carrageenan beads [88] in a gas-lift bioreactor (Table 2). Higher ethanol evolution was comparable for yeast cells entrapped in PVA Lentikats and Ca-alginate carriers. This was also the case for cells entrapped in Ca-pectate and k-carrageenan beads. However, the behavior of cells adsorbed on DEAE-cellulose was similar to that of free cells but significantly different from entrapped cells. Virkajärvi [107] observed that the carrier material (i.e., porous glass beads, Celite, DEAE-cellulose-based carrier) had an effect on the higher alcohol

concentration, but the effect varied with the yeast strain used [107]. It was hypothesized that the immobilization method (surface adsorption or adsorption onto a porous material) and direct effects of the carrier (e.g., differences in adsorption of wort components onto the carrier) could explain some of the observed effects.

3 Esters

3.1 Biosynthetic Pathway of Esters

Esters are very important flavor compounds in beer since they have a significant effect on the fruity/flowery aromas of beers due to their low flavor threshold despite their presence in low concentrations (Table 3) [108, 110, 111]. The major esters can be subdivided into two categories: (1) acetate esters and (2) medium-chain (C6–C10) fatty acid (MCFA) ethyl esters. Major members of the acetate esters (the acid group is acetate, the alcohol group ethanol or a complex alcohol derived from amino acid metabolism) group are ethyl acetate, isoamyl acetate, and 2-phenyl acetate (Table 3). Isoamyl acetate (banana flavor) is the most influential acetate ester present in most beers. MCFA ethyl ester group (the alcohol group is ethanol, the acid group is a medium-chain fatty acid) includes ethyl hexanoate (ethyl caproate) and ethyl octanoate (ethyl caprylate) (Table 3). Esters are desirable components of beer when present in appropriate quantities and proportions but can become unpleasant (bitter, over fruity taste) when in excess.

Esters are produced by yeast mainly during the growth phase but also during the stationary phase [47]. They are formed by the intracellular reaction between a fatty acyl-coenzyme A and an alcohol:

$$\begin{array}{ccccccc} \mathrm{R'OH} & + & \mathrm{RCO-SCoA} & \rightarrow & \mathrm{RCOOR'} & + & \mathrm{CoASH} \\ \text{An alcohol} & & \text{acetyl}-\text{CoA} & & \text{an acetic ester} & & \text{Coenzyme A} \end{array} \quad (1)$$

This reaction is catalyzed by alcohol acyltransferases (AATases; or ester synthetases) of the yeast [112, 113]. Since acetyl-CoA is also a central molecule in the synthesis of lipids and sterols, ester synthesis is linked to fatty acid metabolism. The majority

Table 3 Major esters in beer [39, 42, 47, 51, 108, 109]

Compound	Flavor threshold (mg/l)	Aroma	Concentration range (mg/l)
Ethyl acetate	25–30	Fruity, solvent-like	8–32
Isoamyl acetate	1.2–2	Banana, pear	0.3–3.8
2-Phenylethyl acetate	0.2–3.8	Roses, honey, apple, sweetish	0.10–0.73
Ethyl hexanoate (ethyl caproate)	0.2–0.21	Apple-like, aniseed	0.05–0.3
Ethyl octanoate (ethyl caprylate)	0.9–1.0	Apple-like, fruity	0.04–0.53

of acetyl-CoA is formed by oxidative decarboxylation of pyruvate, while most of the other acyl-CoAs are derived from the acylation of free CoA, catalyzed by acyl-CoA synthase.

Several enzymes are involved in the synthesis of acetate esters, and the most studied and best characterized are AATases I and II that are encoded by *ATF1*, *ATF2*, and *Lg-ATF1* [113–120]. *ATF1* and *ATF2* are both present in both ale (*S. cerevisiae*) and lager strains (*S. pastorianus*), but *Lg-ATF1* is only present in lager strains [115, 117]. This additional gene expression in lager yeast enhances acetate ester production and ultimately this beer's aroma profile [109]. Alcohol acetyltransferases (AAT) have been initially localized in the plasma membrane [121], and it is strongly inhibited by unsaturated fatty acids, ergosterol, heavy metal ions, and sulfhydryl reagents [122]. Both cytosolic and membrane-bound AAT have been described [123, 124] and shown that the ester-synthesizing activity of AAT is dependent on its positioning within the yeast cell, where acetate ester formation varied directly with the level of cytosolic AAT activity [125]. Acetate ester synthesis rates are dependent on AAT activity, and ester synthesis is modulated by a repression-induction of enzyme synthesis or processing, the regulation of which is presumably linked to lipid metabolism [126]. The presumed association of AATase with the membrane was not supported by a hydrophobicity analysis that indicated that Atf1p and Atf2p did not contain a membrane-spanning region [127]. Atf1p has been localized inside lipid sphere-like organelles in the cytoplasm of the yeast cell [128]. The localization of Atf1p in these lipid particles may indicate that Atf1p has a specific role in the lipid and/or sterol metabolism that takes place in these particles.

To investigate and compare the roles of the known *S. cerevisiae* AATs, Atf1p, Atf2p, and Lg-Atf1p, in volatile ester production, the respective genes were either deleted or overexpressed in a laboratory and industrial brewing strains [78, 115–118, 120, 129–131]. The strong impact of the expression levels of the *ATF* genes on acetate ester production was demonstrated [115, 116, 129]. Overexpression of *ATF1* and *ATF2* in a wine yeast resulted in increased levels of ethyl acetate, isoamyl acetate, 2-phenyl ethyl acetate, and ethyl hexanoate in the produced wine [78, 130]. Analysis of beer fermentation products confirmed that the expression levels of *ATF1* and *ATF2* greatly affect the production of ethyl acetate and isoamyl acetate and that Atf1p and Atf2p are also responsible for the formation of a broad range of less volatile esters, such as propyl acetate, isobutyl acetate, pentyl acetate, hexyl acetate, heptyl acetate, octyl acetate, and phenyl ethyl acetate. Atf2p seemed to play only a minor role compared to Atf1p [131]. The *atf1*Δ*atf2*Δ double deletion strain did not form any isoamyl acetate, showing that together, Atf1p and Atf2p are responsible for the total cellular isoamyl AAT activity. Later, it was confirmed that the maximum expression levels of *ATF1* and *ATF2* were directly correlated to the final acetate ester concentration in the beer [132]. However, the double deletion strain still produced considerable amounts of certain other esters, such as ethyl acetate (50 % of the wild-type strain), propyl acetate (50 %), and isobutyl acetate (40 %), which provides evidence for the existence of additional, as-yet-unknown ester synthases in the yeast proteome [131].

Overexpression of *CAT2*, which encodes the major mitochondrial and peroxisomal carnitine acetyltransferase that catalyzes the reversible reaction between carnitine and acetyl-CoA to form acetylcarnitine and free CoA, resulted in a reduction in ester concentrations, especially in ethyl acetate and isoamyl acetate [133]. It was hypothesized that overproduction of Cat2p favors the formation of acetylcarnitine and CoA and therefore limits the precursor for ester production.

Ester production can be altered by changing the synthesis rate of certain fusel alcohols. Hirata et al. [134] increased the isoamyl acetate levels by introducing extra copies of the *LEU4* gene in the *S. cerevisiae* genome. A comparable *S. pastorianus* mutant has been isolated [135]. The mutants have an altered regulation pattern of amino acid metabolism and produce more isoamyl acetate and phenylethyl acetate. Isoamyl acetate is synthesized from isoamyl alcohol and acetyl coenzyme A by AAT and is hydrolyzed by esterases at the same time in *S. cerevisiae*. To study the effect of balancing both enzyme activities, yeast strains with different numbers of copies of *ATF1* gene and isoamyl acetate-hydrolyzing esterase gene *IAH1* (isoamyl acetate-hydrolyzing esterase) have been constructed and used in small-scale sake brewing [136]. Fermentation profiles as well as components of the resulting sake were largely alike. However, the amount of isoamyl acetate in the sake increased with increasing ratio of AAT/Iah1p esterase activity, which indicates that the balance of these two enzyme activities is important for isoamyl acetate accumulation in sake mash.

MCFA ethyl esters are synthesized by other enzymes than Atf1p and Atf2p since the double deletion of *ATF1* and *ATF2* did not influence the produced amount of ethyl esters [131]. The involvement of another enzyme (called ethanol hexanoyl transferase) was previously suggested to be involved in ethyl ester synthesis [137]. The majority of the MCFA ethyl esters are catalyzed by two acyl-CoA: ethanol *O*-acyltransferases (AEATases), i.e., Eeb1p (ethyl ester biosynthesis) and Eht1p (ethanol hexanoyl transferase) [138]. The evaluation of *EEB1* and *EHT1* gene deletion upon ethyl ester formation indicated that Eeb1p is the most important enzyme for ethyl ester synthesis, while Eht1p plays a minor role [138]. The double deletion of *EHT1* and *EEB1* causes a pronounced drop in the production of all MCFA ethyl esters, while the production of ethyl hexanoate was practically eliminated [138]. The additional deletion of *YMR210w* (a putative acyltransferase with similarity to Eeb1p and Eht1p) in the *eht1Δeeb1Δ* strain produced a further drop in ethyl octanoate and ethyl decanoate concentration. Overexpression of *EHT1* and *EEB1* did not result in a significant increase in MCFA ethyl ester production [138]. Since it was shown that Eht1p and Eeb1p contain also esterase activity besides AEATase activity in vitro, it was suggested that both enzyme activities control the ester synthesis. In contrast, *EHT1* overexpression in a wine yeast strain resulted in a small increase of all esters [78]. The Eht1p enzyme was further characterized in vitro [139]. It was functional as an acyltransferase and, unexpectedly, was optimally active toward octanoyl-CoA. Eht1p was also revealed to be active as a thioesterase but was not able to hydrolyze p-nitrophenyl acyl esters. Recently, an elevated production of ethyl caproate was reported in Chinese liquor using a recombinant

yeast overexpressing *EHT1* with deleted *FAA1* encoding for acyl-CoA synthetases [140].

3.2 Influence of Process Variables on Bioproduction of Esters

Ester formation is highly dependent on the yeast strain used [118, 141, 142] and on some fermentation conditions such as temperature [71, 111, 143–145], specific growth rate [143], pitching rate [143, 146, 147], top pressure [47, 118], and wort gravity [132, 148, 149]. Additionally, the composition of the wort can influence the ester production rate, i.e., the concentration of assimilable nitrogen compounds [144, 150, 151], carbon sources [152–155], dissolved oxygen [144, 156–158], and fatty acids [159, 160]. The carbohydrate source has also an influence on the ester synthesis. Maltose produces less esters compared to glucose and fructose [154]. The main factor controlling ester biosynthesis is the expression level of the *ATF1* gene [130, 161]. *ATF1* gene expression is repressed by oxygen and unsaturated fatty acids [162, 163].

Acetate ester formation in brewer's yeast is controlled mainly by the expression level of the AATase-encoding genes [118]. Additionally, changes in the availability of the two substrates for ester production, higher alcohols and acyl-CoA, also influence ester synthesis rates. Any factor that influences the expression of the ester synthase genes and/or the concentrations of substrates will affect ester production accordingly. Perhaps the most convenient and selective way to reduce ester production is applying tank overpressure, if necessary in combination with (slightly) lower fermentation temperatures, low wort free amino nitrogen (FAN) and glucose levels, and elevated wort aeration or wort lipid concentration [118]. Enhancing ester production is slightly more complicated. If it is possible, overpressure or wort aeration can be reduced. Otherwise, worts rich in glucose and nitrogen combined with higher fermentation temperatures and lower pitching rates or application of the drauflassen technique may prove helpful.

3.3 Effect of Immobilized Cell Technology on Bioproduction of Esters

The low ester concentrations, which were obtained in some ICT processes (Table 4), could be related to the low cellular metabolic activities in these systems [97, 164]. It has also been reported that for some systems, ester synthesis is increased upon cell immobilization (Table 4). This could be attributed to mass transfer limitations of oxygen (low oxygen concentrations in the immobilization matrix) causing reduced cellular growth, so that the cellular acetyl-CoA pool can be more available for ester synthesis instead of channeling to fatty acid biosynthesis [164]. A reduction of cellular total fatty acid content upon yeast immobilization on stainless steel fiber cloth supports this explanation [37]. During the fermentation of alcohol-free beer in

Table 4 Ester production in immobilized cell and free cell systems (without reference to a footnote, the data correspond to the analysis of green beer) (Adapted from [80])

Compound	Carrier	Immobilized cells	Free cells	Reference
Ethyl acetate	Porous glass beads	23.4[a]/15.6[b]	–	[81]
	Diatomaceous earth	24.4	–	[82]
	DEAE-cellulose beads	27.8	–	[82]
	Ca-alginate beads	33.3	16.8	[83]
	Ca-pectate beads	27.9[c]/28.5[d]	16.0	[84]
	Silicon carbide rod	14.3	16.7	[85]
	Polyvinyl alcohol Lentikats®	11.0[e]/8.5[f]	19.0	[86]
	Aspen wood chips	18.8	15.2	[87]
	Beech wood chips	11.6	16.8	[88]
	Spent grains	31.6	–	[89]
	k-carrageenan	10.1[g]/5.4[h]	5.2	[95]
	Ceramic hydroxylapatite	20.1–39.8[i] (26.4[j])	21.5	[90]
	Ceramic chamotte	21.4[k]/24.5[l]	17.2	[91]
		17.9	17.2	[92]
		103.9[q]/48.2[r]/	–	[75]
		52.9[s]	26.4[l]	[93]
		11.3[l]	26.9[o]	[94]
		18.1[o]	3.54[o]	[94]
		0.0[o]	33.6[p]	
		42.5[p]	29.1[p]	
		10.5[p]		
Isoamyl acetate	Porous glass beads	0.7[a]/0.6[b]	–	[81]
	Diatomaceous earth	1.2	–	[82]
	DEAE-cellulose	1.0	–	[82]
	Ca-alginate beads	2.8	1.3	[83]
	Ca-pectate beads	0.38[c]/0.25[d]	1.16	[84]
	Silicon carbide rod	0.66	1.36	[85]
	Polyvinyl alcohol Lentikats®	0.06[e]/0.05[f]	2.0	[86]
	Aspen wood chips	1.25	0.85	[87]
	Beech wood chips	++[m]	+	[96]
	Spent grains	3.19	2.11	[88]
	k-carrageenan	1.3	–	[89]
	Ceramic hydroxylapatite	2.09[g]/0.98[h]	1.06	[95]
	Ceramic chamotte	0.3–1.9[i] (1.0[j])	1.5	[90]
		1.0[k]/0.6[l]	1.0	[91]
		0.8[q]/0.4[r]/1.0[s]	–	[75]
		<0.01[l]	0.08[l]	[93]
		0.0[o]	1.6[o]	[94]
		0.0[o]	0.0[o]	[94]
		3.2[p]	1.3[p]	
		0.0[p]	0.3[p]	
Ethyl hexanoate	Porous glass beads	0.1[a]/0.1[b]	–	[81]
	Polyvinyl alcohol Lentikats®	0.54[g]/0.13[h]	0.11	[95]
	DEAE-cellulose	0.08[c]/0.01[d]	0.14	[84]
	Ca-pectate beads	1.89	1.39	[88]
	Aspen wood chips	0.1–0.5[i] (0.2[j])	0.4	[90]
	Beech wood chips	0.1[k]/0.1[l]	0.1	[91]
	Ceramic hydroxylapatite	0.1[o]	0.7[o]	[94]
	Ceramic chamotte	0.0[o]	0.02[o]	[94]
		0.7[p]	0.5[p]	
		0.1[p]	0.2[p]	

(*continued*)

Table 4 (continued)

Compound	Carrier	Immobilized cells	Free cells	Reference
Ethyl octanoate	Polyvinyl alcohol Lentikats®	0.04[g]/0.01[h]	0.01	[95]
	Ca-pectate beads	0.21	0.59	[88]
	Aspen wood chips	0.2–1.2[i] (0.6[j])	1.4	[90]
	Spent grains	0.2[q]/0.2[r]/0.3[s]	–	[75]
	Ceramic hydroxylapatite	4.2[o]	14.0[o]	[94]
	Ceramic chamotte	0.0[o]	0.1[o]	[94]
		13.2[p]	9.5[p]	
		0.3[p]	1.2[p]	

[a]Average over day 0–138; [b]average over day 378–442; [c]day 8; [d]day 10; [e]Packed bed reactor; [f]fluidized bed reactor; [g]recycled CO_2; [h]gas bottle CO_2; [i]range; [j]average; [k]maturation with immobilized cells; [l]conventional maturation; [m]compound detected in amount significantly higher than free cells ($p < 0.05$); [n]no significant difference ($p < 0.05$); [o]ale strain; [p]lager strain; [q]7 °C; [r]10 °C; [s]15 °C

a packed bed reactor with surface-attached cells on DEAE-cellulose beads, a simultaneous increase in the activity of alcohol acetyl transferase and formation of ethyl acetate and isoamyl acetate were observed [165]. Additionally, the amount of unsaturated fatty acids in wort decreased significantly, which was attributed to the anaerobic conditions and the absence of substantial levels of unsaturated fatty acids that limit cell growth and stimulate the formation of acetate esters. Esters concentration increased by 22 % upon cell immobilization on stainless steel fiber cloth [37]. Additionally, the expression level of *AFT1* was significantly increased in the immobilized cells, resulting in a twofold increase of isoamyl acetate formation. Possibly, the microenvironment created by cell immobilization activates the cAMP/PKA/Sch9p pathway, resulting in an induction of *ATF1* expression leading to enhanced ester concentrations in the final fermentation product. Encapsulation of brewing yeast in alginate/chitosan beads showed a higher degree of fermentation and higher specific growth rate and produced slightly higher levels of esters [100].

4 Carbonyl Compounds

4.1 Biosynthetic Pathway of Carbonyl Compounds

Carbonyl compounds (aldehydes and ketones) contain a functional group composed of a carbon atom double bonded to an oxygen atom. Aldehydes have different flavor characteristics ranging from “green-leaf-like” to “apple-like” to “citrus-like” to “nutty,” depending on the chemical structure, thus contributing to an overall flavor of a number of foods and beverages. In alcoholic beverages among all aldehydes, acetaldehyde is the major component (>90 %) giving different flavors depending on concentration: from a pleasant fruity aroma at low levels to a pungent “green grass-like” odor and “overripe apple” notes at high levels [166]. Higher concentrations of acetaldehyde are also unfavorable because it can bind catechins and other phenolics

[167]. Acetaldehyde concentrations in fermented beverages are usually in the range 13–40 mg/l, and its flavor threshold in cider and apple wines is approximately 30 mg/L. When present in excess, acetaldehyde can be easily masked by the addition of SO_2 [168]. Acetaldehyde is synthesized during fermentative glycolysis by decarboxylation of pyruvate (see Fig. 2). It is mostly formed during the active growth phase of yeast and accumulated when the rate of carbon dissimilation is at its maximum. After this stage acetaldehyde formation decreases, and some of the acetaldehyde that was previously excreted is again absorbed and further reduced to ethanol. Thus, its concentration falls to a low level at the end of fermentation and then slowly increases over time [34, 118]. Other short-chain aliphatic aldehydes (3-methyl butanal, 2-methyl butanal, hexanal, heptanal, etc.) are formed by reduction of keto acids (oxo acids) both via the anabolic process from carbon source and the catabolic pathway from exogenous amino acids. Aldehydes and keto acids are being (1) excreted in the medium and (2) biochemically converted to higher alcohols (see Fig. 2). The biochemical reactions described above are determined by activities of various enzymatic systems (isoenzymes of alcohol dehydrogenase, aldehyde dehydrogenase, and aldo-keto reductases), but the exact mechanism of enzyme action has been the subject of extensive investigation and is still under scrutiny [169–173].

Vicinal diketones (VDK) are produced via the ILV (branched-chain amino acids isoleucine, leucine, and valine) pathway during fermentation. Among them, diacetyl (2,3-butanedione) and 2,3-pentanedione, characterized by a buttery and sweetish aroma, are important due to their low sensory threshold values in beverages [38]. For example, in beer diacetyl has a very low taste threshold of approximately 0.15 mg/L [108]. Diacetyl is sensorily more important than 2,3-pentanedione. These two compounds are formed by chemical oxidative decarboxylation of two acids, α-acetolactate and α-acetohydroxybutyrate, respectively, leaked from the valine and isoleucine biosynthetic pathway to the extracellular environment, respectively. VDK are subsequently re-assimilated by the yeast where they are being degraded by the activity of different alcohol dehydrogenase systems to acetoin and further to 2,3-butanediol and 2,3-pentanedione to 2,3-pentanediol (Fig. 2). These reactions occur at the end of the primary beer fermentation, but at the end of this process, their concentrations are still too high giving unpleasant green-feel-like taste. That's why secondary fermentation (maturation) is needed to reduce diacetyl to an acceptable level. Sufficient yeast cells in suspension are necessary to obtain an efficient reduction [80]. Acetoin is also being excreted into the extracellular surrounding. Acetoin formation depends on the yeast strain used, and it is generally known that non-*Saccharomyces* yeasts produce more of it. The amount of acetoin in wines ranges from 2 to 25 mg/L [174].

4.2 Influence of Process Variables on Bioproduction of Carbonyl Compounds

Fermentation parameters that stimulate cell growth may increase α-acetolactate concentration (which is a precursor of diacetyl) and consequently also the level of

diacetyl. In general, yeast strain, medium composition, and fermentation conditions influence the amount and profile of α-acetohydroxyacids (which are intermediates in the synthesis of valine and isoleucine) and therefore the level of VDK [175–177]. For example, it has been noticed for brewing that worts (substrate medium) deficient in valine result in elevated diacetyl levels and worts deficient in leucine result in increased 2,3-pentanedione concentrations [178, 179]. During the period of valine uptake from wort, the intracellular valine concentration increases causing inhibition of the enzyme responsible for α-acetolactate synthesis and total diacetyl formation becomes reduced [180].

Acetaldehyde accumulation has been more pronounced at high initial sugar concentrations, especially in case of must fermentation during wine production [20]. Apart from substrate composition, oxygen supply has a large influence on the profile of flavor-active compounds and thus on carbonyl compounds as well. Namely, aeration stimulates cell growth, and since acetaldehyde is mostly formed during the active growth phase of yeast, its content can be controlled by proper oxygen supply. Higher temperatures and a longer residence time promote aldehyde reduction to higher alcohols, which was also confirmed for ICT [17, 20, 24, 30, 181], but there are numerous exceptions to this rule [18, 21, 182]. Sipsas et al. [182] confirmed that during batch fermentations, not only both the bioreactor system (packed bed versus multistage fixed bed tower) and the fermentation temperature (5–30 °C) affected significantly the concentration of acetaldehyde, but a strong interaction between the two factors was also observed.

4.3 Effect of Immobilized Cell Technology on Bioproduction of Carbonyl Compounds

It is hard to make any solid conclusion about the impact of immobilization itself on the amount of carbonyl compounds during fermentation since the concentration profile of carbonyl compounds is tightly related to fermentation conditions and yeast strain. Continuous fermentations with immobilized cell systems often end up with increased level of acetaldehyde in comparison to batch free cell systems, but this is connected with over-aeration and high volumetric oxygen mass transfer coefficients of such systems, the technological parameters which stimulate acetaldehyde formation [183]. Regarding the influence of immobilization itself on carbonyl reduction kinetics, different conclusions have been made. Thus, in some cases increased acetaldehyde accumulation (in comparison to free cell systems) was attributed to highly active glyceropyruvic fermentation at the beginning of the process and under hyperosmotic conditions of fermentation of a must (substrate medium in winemaking) containing a high concentration of sugars [9]. Thus, acetaldehyde concentrations obtained by ICT can be very high, for example, up to 106 mg/L in wines produced using quince pieces as support material [184]. On the contrary, lower amounts of acetaldehyde were also reported for wines produced with cells immobilized onto fruit pieces than with free cells but still above the orthonasal perception threshold (10 mg/L) [26, 29, 30]. One explanation for the

improved capacity of yeast to reduce acetaldehyde can be an increased alcohol dehydrogenase activity in immobilized yeast due to a more efficient cofactors NADH and NADPH regeneration ascribed to a higher glucose flux in cells [185]. It should be stressed that different support-based yeast biocatalysts gave different results concerning acetaldehyde levels, even when all other conditions were kept the same (temperature, substrate composition, bioreactor design) [25]. Even more, one immobilized cell biocatalyst can behave inconsistently regarding the profile of flavor-active compounds during repeated batch fermentations and/or after its storage.

Increased levels of diacetyl have been frequently detected as a consequence of cell immobilization. This has been explained by the slow decarboxylation rate of α-acetolactate into diacetyl, which is considered to be the rate-limiting step in traditional batch processes, and the greater barrier in the immobilized reactor toward diffusion of diacetyl from medium to immobilized yeast cells also considered a classical drawback of immobilized systems. Another possible explanation is an increased expression of acetohydroxy acid synthetase gene during growth of the yeast cells in the carrier [186]. It is the biomass concentration that affects the final level of diacetyl. Thus, as a result of the intense biomass growth, high levels of diacetyl (above its flavor threshold of 0.07–0.15 ppm) have been obtained during primary fermentation of wort [92, 183, 187]. Increased levels of diacetyl have also been interpreted as a consequence of the alteration of the amino acid metabolism of the immobilized cells; particularly, lower amino acid uptake by yeast entrapped inside polymer matrix [88, 188]. On the other hand, under continuous operation mode and optimal conditions, it is possible to reduce diacetyl level below its threshold, due to increased mass transfer limitations accompanying the high biomass density achievable in a gas-lift bioreactor with gel microbeads as yeast carriers [189, 190]. Continuous secondary fermentation with immobilized cells usually ends with acceptable or even very low levels of diacetyl [191, 192].

5 Carboxylic Acids

5.1 Biosynthetic Pathway of Carboxylic Acids

5.1.1 Short-Chain Fatty Acids

Numerous short- and medium-chain fatty acids are present in alcoholic beverages. These can be introduced to the system with fermentation substrates, but yeast activity also has a significant impact on organic acid composition and concentration. Organic acids are important biochemical intermediates in several biochemical reactions and have a direct role in maintaining redox balance within the cell. Their concentrations are directly affected by environmental conditions and can influence positively or negatively the organoleptic properties of beverages. Short-chain fatty acids found in beverages include acetic, citric, lactic, malic, pyruvic, succinic, tartaric, and 2-oxoglutaric acids [193, 194]. The concentrations of many of these can be altered by yeast during fermentation. Grape juice, for example, contains

naturally high concentrations of citric and lactic acid, but yeast fermentation is required to contribute organic acids such as succinic and ketoglutaric acids to wines [195, 196]. Concentrations of organic acids during fermentation are dynamic, and yeast in some cases will remove organic acids to some extent through normal metabolic activity. This occurs, for example, with malic acid, the concentration of which may be reduced as much as 45 % during fermentation through yeast activity [197]. Coote and Kirsop [198] noted an increase in wort pyruvic acid during early fermentation. This increase was, however, followed by stabilization and subsequent lowering of concentration toward the end of the fermentation [198].

In addition to their contribution to beverage sourness, these organic acids can impart bitter, astringent, vinegar, salty, and “goaty” flavors [108] and are often deemed to be off-flavors at higher concentrations. However, certain organic acids at appropriate concentrations are considered to be positive or even essential to the flavor profile of certain beverages. Succinate and malate are, for example, important flavor components in sake [199]. Acetic acid can make a positive contribution to wine flavor and aroma at appropriate concentrations (0.2–0.7 g l^{-1}), though at higher levels, they impart a distinct vinegar note to wines [200]. Organic acids may also influence the physical properties of a beverage. Oxalic acid can bind with calcium ions to form insoluble calcium oxalate crystals, which reduce clarity and can in some cases promote excessive foaming by acting as nucleation sites for CO_2 [201]. During fermentation, organic acids are produced during amino acid catabolism and under fermentative conditions due to an inability of the yeast cell to further process them in the absence of oxygen [202].

5.1.2 Acetic Acid

The acetic acid content of wine is the main determinant of volatile acidity, which is an important characteristic of a wine style. Concentrations up to 0.8 g l^{-1} acetic acid can contribute positively to the perceived complexity of a wine’s aroma profile. Higher concentrations introduce vinegar notes and may prevent sale [203]. Control of acetic acid concentration is therefore a necessity in winemaking. There are various routes through which acetic acid may enter wine. In particular, acetic and lactic acid bacteria can produce this acid in the grape or during fermentation. *Botrytis*-infected grapes are particularly susceptible [204]. Production of acetic acid by wine yeast is limited, and rather, certain strains are known to remove acetic acid from wine. As this ability is restricted to a small number of strains, excessive acetic acid content is a relatively common problem in winemaking. To reduce concentrations, such wines are typically blended with wine of low volatile acidity or undergo reverse osmosis [205].

5.2 Influence of Process Variables on Bioproduction of Carboxylic Acids

A number of factors are known to influence short-chain organic acid concentrations during fermentation. The situation is complex as different conditions have different

effects depending on the biochemical pathway of the organic acid in question. It is clear, however, that the yeast strain plays a critical role in determining concentrations. The aforementioned uptake of malic acid is highly variable among strains of *S. cerevisiae*, but strains of other species such as *Schizosaccharomyces pombe* and *S. malidevorans* can effect complete degradation of this acid [197]. Holgate [206] noted that under identical conditions, malic acid could be either increased or decreased depending on the *S. cerevisiae* strain utilized. Malic acid production appears to be relatively rare among *S. cerevisiae* species, and this characteristic is seen more often with the cryotolerant yeast *S. uvarum* [207]. Likewise, *S. uvarum* and the related species *S. bayanus* are known to produce high concentrations of succinic acid, while this property is highly variable in *S. cerevisiae* [207, 208]. Environmental conditions may also influence production. Coote and Kirsop [198] observed that cessation of pyruvic acid production during brewery fermentation coincided with loss of assimilable nitrogen from the wort. In contrast, α-ketoglutaric acid is known to accumulate in response to nitrogen deficiency [209] as well as glutamic acid availability [198]. Malic acid can also increase under low-nitrogen conditions as well as at low pH and under starvation conditions [210]. Succinic acid concentration in wine is influenced by a range of conditions including temperature, must composition, nitrogen availability, acidity, and SO_2 content [211, 212]. Acetic acid concentration can vary greatly depending on the nitrogen content of the must. The lowest concentrations are observed around 200–250 mg l^{-1} and are higher outside of this range [213]. Outside of optimal fermentation temperatures, the production of acetic acid diminishes, presumably due to a reduction in yeast growth rate [214], but generally a lowered fermentation temperature results in a larger acetic acid production [17, 88]. The provision of asparagine or aspartic acid to growth media results in acetic acid accumulation [198]. Clarification of wine must has been observed to reduce acetic acid production and fermentation rate simultaneously [215].

5.3 Effect of Immobilized Cell Technology on Bioproduction of Carboxylic Acids

As described above, immobilized cell technology offers numerous benefits for various fermentation industries. Improvements in cell tolerance, fermentation performance, and productivity should, however, not come at the expense of product quality. In this respect, it is important that flavor profiles of fermented beverages at least match or indeed improve upon those produced through traditional processes. A clear understanding of the relationship between immobilization technology and organic acid production is essential if this is to be achieved. Organic acids can have either a positive or negative influence on perceived quality depending on their concentration, and it is essential that strategies are in place to adjust concentrations, either up or down, to the desired levels.

Malolactic fermentation is essential for the creation of many styles of wine and cider [216]. In this process, the organoleptic properties of the fermented beverage are

improved through the conversion of malic acid to carbon dioxide and lactic acid, thereby creating a stable, less acidic, and more pleasant-tasting beverage. As indicated earlier, the malolactic capabilities of fermentative yeast are generally limited, and the process is mediated by bacteria of the genera *Lactobacillus*, *Oenococcus*, and *Pediococcus* [217]. Immobilization of such bacteria has been proposed as a mechanism to exert control on the process. Immobilized cells are expected to be more tolerant to the stressful conditions in wine (ethanol, SO_2, etc.), to be more concentrated (facilitating higher reaction rates), and to be easily recycled for multiple malolactic fermentations [14].

The form of immobilization is critical to ensure successful industrial application. Calcium-alginate-embedded bacterial cells have been used effectively to remove malic acid, but the support material has been found to be relatively weak and the culture also suffered from a lack of the important cofactors NAD and Mn^{2+} [218], indicating a possible problem with mass transfer. Other support materials have also included k-carrageenan [219], calcium pectate [220], diethyl and diethylaminoethyl cellulose [221], and delignified cellulosic material [222]. Recently, cells of *Oenococcus oeni* immobilized in Lentikats were shown to remove as much as 100 % of the malic acid present in wine [223].

Yeast may also be used as immobilized cell catalysts for the purpose of malic acid degradation. Hong et al. [224] have demonstrated the malic acid-degrading ability of the yeast *Issatchenkia orientalis* when immobilized on a mixture of oriental oak charcoal and alginate. It has been suggested that the approach could be simplified by co-immobilizing an ethanolic fermentation organism and a malolactic fermenting organism in a single catalyst. Such a catalyst could simplify the overall winemaking process. Servetas et al. [14] demonstrated the potential of such a catalyst involving cells of *Oenococcus oeni* trapped in delignified cellulosic material and surrounded by a layer of starch, in which cells of an alcohol-tolerant yeast strain were embedded. Wines produced at an experimental scale with this catalyst were reported of better quality than those produced by immobilized yeast alone (higher ester and lower malic acid concentrations). There was, additionally, no evidence of any biological competition between the two different organisms. Simultaneous bioprocessing can be taken a step further by including the abilities of two different species in a single organism. This has been achieved by incorporating genetic material from different species into a fermentative yeast. Williams et al. [225] successfully incorporated an enzyme from *Lactobacillus delbrueckii* that allowed the yeast strain to convert malic to lactic acid, albeit at low rates. Further developments involved cloning yeast with the responsible enzyme (from *Lactococcus lactis* or *Oenococcus oeni*) but also an active malate permease from *Schizosaccharomyces pombe* [226, 227]. The constructed strains could efficiently remove malic acid from red and white wine musts during fermentation. Such approaches are still met with skepticism by the general public [228], even when strains have achieved the GRAS status [227], and therefore more natural approaches to combined primary and malic acid degradation by yeast have been considered. Many yeast strains including species of *Schizosaccharomyces*, *Zygosaccharomyces*, *Candida*, *Pachysolen*, *Hanseniaspora*, and *Issatchenkia* as well as certain strains of *Saccharomyces cerevisiae* have the ability

to degrade appreciable amounts of malic acid [229]. Therefore, there exists considerable potential for combining ethanolic and malolactic fermentation by creating catalysts involving immobilized yeasts with complementary activities or, in the case of *Saccharomyces* species, strains that naturally carry out both processes efficiently. Mating of different *Saccharomyces* yeasts could be used to generate interspecific hybrids with, for example, the aroma profile from a wine yeast with the MLF capability of another member of the *Saccharomyces* group. Novel hybrids may be a simple and natural way to combine diverse phenotypes in one catalyst. Such yeasts have already been shown to have potential for application in winemaking [230] and brewing [231].

Reduction of acetic acid content through the use of immobilized yeast cells has been proposed as a cost-effective and natural solution. Vilela-Moura et al. [232] have found that selected yeast strains (both *Saccharomyces* and non-*Saccharomyces*) are effective at removing acetic acid from wine during a re-fermentation process. One strain of *S. cerevisiae* in particular (S26) was an efficient deacidification agent, and further investigation involved immobilization of this yeast in alginate beads in an effort to optimize the process [233]. Immobilized yeast cells were capable of reducing acetic acid content from unacceptable to acceptable levels without any major detriment to wine quality. A number of studies have determined the impact of yeast immobilization on wine quality, including volatile acidity, and give an indication of the impact of various conditions (species, strain, carrier, etc.) on acetic acid concentrations. Mallouchos et al. [234] showed that the acetic acid content of wine could be reduced almost 30-fold when cells were immobilized on delignified cellulose or gluten pellets compared to free cells. The utilized carrier also influenced the acetic acid content, though the relative effect appeared to be temperature dependent. The same authors noted a threefold to fourfold reduction in acetic acid content of wines after fermentation with yeast immobilized on brewer's spent grain compared to wines fermented in the traditional manner [235]. Kandylis et al. [20] reported low but detectable levels (7 mg l^{-1}) of acetic acid in wines fermented with free cells, but no acetic acid in wines fermented with yeast immobilized on potato pieces. This was despite a general increase in organic acids when cells were immobilized. A similar result was observed when the same strain was immobilized on wheat and fermentations were scale up to 80 l [17]. This reduction in acetic acid content has been observed in various fermentations [17, 20, 234–236] but is not however universal. Oliveira et al. [237] noted either no change or only a modest reduction in acetic acid concentration of fruit wine fermented with Ca-alginate-immobilized cells relative to free cells. The influence of immobilization appeared to be strain dependent in this case. Pereira et al. [238] found that the acetic acid content of mead was unaffected or even increased when cells were immobilized and, again, the strain used was influential. Šmogrovičová and Dömény [88] found no significant difference between beers produced by immobilized cell technology and those produced traditionally with respect to acetic acid. Also, co-immobilization of yeast cells with *Penicillium chrysogenum* had no influence on acetic acid in wine after fermentation [16, 239]. Therefore, it is clear that while the use of an

immobilized cell catalyst to reduce acetic acid to acceptable levels is feasible, the appropriate choice of the strain and carrier is critical to the outcome.

5.4 Summary and Outlook

Immobilized cell technology has been applied at industrial scale with some notable successes. Despite this, ICT is not yet at a stage where it can be considered a mainstream technology. A number of factors have impeded the full exploitation of this technology's potential. In the beverage fermentation industries, an important issue is the difficulty in matching flavor profiles with those produced using traditional technologies. This issue is compounded by the fact that even small adjustments to yeast physiological state may lead to small but perceptible changes in organoleptic profile. A number of studies have addressed this problem, and in many cases partial or complete solutions have been achieved, examples being the use of overpressure or FAN modification to control ester formation [118] or aeration [102] to steer higher alcohol evolution [102]. The accumulated knowledge from studies addressing this issue may be of particular benefit to the nascent bioflavor industry. There is currently a strong market demand for naturally produced flavors for food and cosmetic applications [240], and results from investigations on ICT use for beverages are directly applicable to the bioflavor industry, where ICT may be used to boost concentrations of individual flavors or aromas.

ICT has been sometimes perceived as a relatively inflexible technology, requiring long start-up times and being prone to contamination. One of its main appeals, its suitability for continuous fermentation, may also be to its detriment as this necessitates constant monitoring that may not be suitable for every business, particularly smaller companies with limited personnel. A more modular approach is however possible and its efficacy has been demonstrated. There are a number of solutions including the use of bioreactors that allow the rapid removal and replacement or maintenance of catalysts without any significant effect on the operation. Catalysts may also be removed and stored to facilitate periods of low demand, e.g., in the wine industry with its seasonal requirement for fermentation catalysts.

How organisms respond to immobilization is still not fully understood, and it is expected that a deeper understanding of the changes occurring at the molecular level may be of benefit. Improved stress tolerance, for example, is typical of immobilized cells, and this phenomenon has been observed in diverse systems with different carriers and organisms. However, the reasons for this increased tolerance remain obscure. The use of modern technologies for genome, transcriptome, or metabolome analysis has not been used extensively to study the relevant changes that occur when cells change from a planktonic to sessile mode of life or when cells remain immobile for extended periods. Such knowledge would help to tailor conditions to enhance stress tolerance and fine-tune flavor development for maximum benefit to the system. The choice of organism may also be critical for full realization of ICT's potential. A typical strategy involves application

of traditional catalysts, e.g., brewer's or wine yeast in ICT systems. However, it may be that alternative organisms could be better suited to immobilization. It may be of benefit to select organisms that are not adapted to aqueous systems but rather naturally exist in an immobile state. Likewise, flavor correction might be achieved by taking advantage of the different flavor profiles produced by different organisms. The use of alternative fermentative organisms has already been considered for flavor modification in wine production [241] and could be of value in ICT systems. In particular, organisms with limited fermentative potential but which produce similar flavor profiles as *S. cerevisiae* could be applied for low-alcohol beverages [242]. Likewise, targeted genetic modification has rarely been applied in the production of fermented beverages due to a low level of consumer acceptance as well as regulatory restrictions. However, recent developments in the technology, in particular the advent of gene editing, have reinitiated the debate surrounding the use of modified organisms in food production [243]. If modified organisms could be applied in ICT, many of the problems associated with flavor matching, such as diacetyl overproduction, could be resolved quite easily [244].

While findings of new research are always of value, it may be argued that the full realization of ICT's potential will be achieved through an integrated, multidisciplinary approach involving the concerted application of varied technologies for improved bioreactor design, carrier optimization, selection (or modification) of suitable catalysts, and a more comprehensive understanding of the biological and chemical mechanisms involved. It is hoped that this review of the current knowledge regarding ICT and its impact on flavor is one step toward achieving this goal.

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Aspergillus Lipases: Biotechnological and Industrial Application

19

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Abstract

Lipases are enzymes with remarkable properties and catalytic versatility. These proteins are capable of catalyzing hydrolytic and synthetic reactions, allowing the production of different compounds. *Aspergillus* are important producers of lipases, since they are able to secrete large amounts of these proteins to the extracellular media. Several studies have reported the importance of fermentation parameters as well as genetic engineering of *Aspergillus* strains in order to improve lipase production. Different *Aspergillus* species secrete lipases with interesting characteristics such as thermostability, stability in a wide pH range, stability in organic solvents, and enantioselectivity toward the substrate. The obtainment of lipases with highlighted characteristics for use in industry is the main focus of several studies. Such lipases can be obtained with screening of *Aspergillus* strains, protein engineering, and immobilization of lipases that can frequently improve thermostability and enantioselectivity. Among the applications of lipases from *Aspergillus*, there are studies on the improvement of sensorial properties of different products in the food industry, compatibility with detergents for removal of fat stains from fabrics, and the obtainment of enantiopure pharmaceuticals.

Keywords

Lipases • *Aspergillus* • Lipase properties • Biotechnological applications • Biotechnology • Immobilization • Lipase production

1 Introduction

Biotechnology has shown to be a promising and efficient approach for many industrial fields that, somehow, require catalysts. In this context, the use of enzymes is considered of immense advantage when compared to conventional chemical catalysts. Although the latter can be very efficient and frequently cheaper, the use of these compounds results in several toxic byproducts, requiring many purification steps. This is mainly due to the low selectivity chemical catalysts present for the substrates. Enzymes, on the other hand, act in mild reaction conditions showing, frequently, remarkable selectivity, allowing the obtainment of high-added value products, such as enantiomers applied in pharmaceutical and food industries [1].

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are enzymes that naturally catalyze the hydrolysis of triacylglycerols into di- and monoacylglycerols, fatty acids, and glycerol at an oil–water interface, through interfacial activation [2]. However, in nonaqueous solutions, they are capable of catalyzing synthetic reactions such as esterification and transesterification. This versatile spectrum of reactions added to their chemo-, regio-, and enantioselectivity for substrates makes them a popular choice as industrial biocatalysts [3].

Despite different sources of lipases, microorganisms are considered the most interesting tool for lipase production when compared to animal and plant sources.

Microorganisms grow faster and easier, and the enzyme production is influenced neither by climatic conditions or seasonal changes nor by regulatory or ethical issues related to animal slaughter or tree or plant felling. Extracellular lipase-producing microorganisms are preferred since they facilitate downstream processing, which lowers production costs [4]. Hence, filamentous fungi are the first choice, since these organisms are capable of secreting large amount of proteins to the extracellular media [5]. Among the fungal sources, *Aspergillus* is a very promising candidate for lipase production. This is because there are many species of this genus with immense biochemical versatility added to large capability of protein secretion. The most reported species are *Aspergillus niger* and *A. oryzae*, considered efficient lipase producers. On the other side, *A. nidulans* is one of the few filamentous fungi for which molecular genetic tools have been created, which is of great interest for the development and construction of strains for heterologous enzyme production [6].

Lipases are applied in many different industrial areas due to their catalytic versatility. In food industry, these lipases can be used in bread making, where it can selectively hydrolyze lipids of the dough, resulting in better characteristics of the bread [7]. They can also be used in detergent industries, as part of detergent composition, where it can hydrolyze fats present in stains in fabrics [8]. In pharmaceutical and chemical industries, lipases can be applied to resolve racemic mixtures in order to obtain enantiomeric compounds [9]. Another interesting application of lipases is in biodiesel production, through esterification and transesterification reactions [10, 11].

Although lipases present great potential to be used in many industrial fields, the immobilization of lipases can bring about advantages when compared to free enzymes. This is because immobilized enzymes can be reused for different cycles of reactions and frequently have their characteristics improved such as thermostability and selectivity [12, 13]. Lipases can be immobilized by adsorption, entrapment, covalent bonding, or the combination of these techniques. Each approach presents different advantages and disadvantages, depending on the application process.

This chapter deals with different aspects of lipases from many *Aspergillus* species, considering the great potential of this enzyme in various industrial fields. The production, characteristics, immobilization, and applications of *Aspergillus* lipases were highlighted.

2 *Aspergillus* as a Biotechnological Tool

The *Aspergillus* genus comprises about 350 species that can live in a wide variety of environments such as soil, plant, and animal parasite, which reflects its natural metabolic and nutritional plasticity [14, 15]. This ascomycete is known by its conidial heads and stalks and was first classified by Micheli's (1729) *Nova Plantarum Genera* [14].

The *Aspergillus* genus is among the most economically important fungi and has been used in food and beverage production processes for more than 1,500 years. The genus is widely used in the food and pharmaceutical industry for the production of Oriental foods, various acids, enzymes, and other compounds useful for humans.

The biotechnological and economic importance has encouraged the genetic and physiologic research of *Aspergillus* species aiming for the expansion of its applications and increase in profit.

Nowadays, filamentous fungi are used as cell factories for a wide range of biotechnological products including organic acids, human therapeutics, polysaccharides, biosurfactants, and an array of enzymes, which may be of fungal or non-fungal origin [16, 17]. Today, the genus is employed in the large-scale production of enzymes with commercial added value such as pectinases, lipases, oxidases, proteases, amylases, cellulases, hemicellulases, and others [18]. The easy separation of biomass and rapid growth in low-cost culture media are some of the advantages to the use of *Aspergillus* as producers of enzymes [18, 19]. In addition, compounds produced by *Aspergillus* can be safely used in the industry, which has resulted in the classification of generally regarded as safe (GRAS) status [17].

Among *Aspergillus* species, *Aspergillus nidulans* is largely studied in detail in basic research since the 1960s and offers advantages due to its well-characterized sexual cycle and ease of handling of its genetic system, which provide benefits in the development and construction of strains [16, 20, 21]. This species is one of the few filamentous fungi for which molecular genetic tools have been developed. In comparison with industrial strains, *A. nidulans* shows potential enzymes in its genome and secretome able to supply the needs of basic research [21, 22].

For centuries, filamentous fungi have been known for their capacity to secrete a large amount of proteins. Biotechnological interest in the genus *Aspergillus* has increased concomitantly with molecular biology techniques for production of homologous and heterologous proteins. In the early 1980s, the first protocols were described using *A. nidulans* as a host for recombinant proteins [23].

3 Obtainment and Properties of Lipases from *Aspergillus*

The first description of the effects of lipolytic enzymes action dates from 1849 when the French scientist Claude Bernard and his associates described the action of pancreatic juice in olive oil releasing fatty acids and glycerol. The term lipase was initially assigned to the most representative and potent lipolytic extracts known at that time, which were from pancreatic sources. Over the years, proteins obtained from different origins that were also capable of catalyzing neutral fats or glycerol ester hydrolysis were also called lipases [24]. Since then, a huge variety of lipases are the object of the study, and today this class of enzymes is the most broadly used in biotechnology, mainly because it possesses the ability of catalyzing reactions with an infinity of substrates, even in heterogeneous media, and was available for applications since the beginning of the industrial enzymology [25].

Lipases belong to the class of serine hydrolases and catalyze the hydrolysis of triacylglycerides (or neutral lipids) into di- and monoacylglycerols, glycerol, and free fatty acids at the lipid–water interface. These hydrolytic reactions can be reversed under micro aqueous environment, such as in organic solvents, leading to synthetic reactions such as esterification and transesterification [1, 26, 27]. From the

variety of reactions catalyzed by lipases and its capacity of hydrolyzing water-soluble substrates, the difficulty in determining a simple terminology in this field became known [24]. In a catalytic point of view, lipases are esterases with high activity toward water-insoluble substrates, but a real distinction between those two is the fact that lipases present the phenomenon of interfacial activation [24, 25].

Additionally, lipases catalyze reactions with high specificity, regioselectivity, and enantioselectivity, characteristics that assure them as the most important group of biocatalysts for synthetic organic chemistry [28]. However, it presents what we can call enzyme promiscuity (in all three defined forms: condition, substrate, and catalytic promiscuity [29]), the ability to catalyze alternative reactions that differ from their physiological reaction [25]. Lipases promiscuity may be a useful characteristic when the subject is the enzyme improvement based on divergent evolution of new catalysts [25]. However, this characteristic jointly with the high sequence diversity presented by lipases avoids a classification similar as the one made for carbohydrate-active enzymes [30]. The latter is based mostly on the amino acid primary sequence, so lipases are classified based on topological characteristics [27].

In 1999, Arpigny and Jaeger were the first group to propose a concrete classification of lipases [31]; an extensive classification was made taking into account the amino acid sequence motifs and some fundamental biological properties of 53 bacterial lipases and esterases leading to the creation of 8 families. Only the enzymes belonging to families II and VIII were identified as not being α/β-hydrolase fold proteins [32] wherein these families correspond to SGNH hydrolase and β-lactamase, respectively [27, 31]. Four years before, the ESTHER database was created [33], initially as a cholinesterase (phylogenetically related to proteins which have a similar α/β-fold structure) server [34], and after being revised several times, it is now a widely used database dedicated to proteins with α/β-hydrolase fold, containing more than 30,000 manually curated proteins [35], and it is available online (http://bioweb.ensam.inra.fr/esther).

After many attempts to categorize lipolytic enzymes originated from a broad variety of organisms, including higher and lower vertebrates, invertebrates, fungi, and bacteria, today we know that lipase classification mainly lies in the α/β-hydrolase fold, a class that also contains esterases, acetylcholinesterases, cutinases, carboxylesterases, and epoxide hydrolases [36]. Lipases and those already cited α/β-hydrolase enzymes share not only a common structure but also a common architecture and conserved active site signatures. Based on these characteristics, the Lipase Engineering Database (LED) was developed [37], a resource of fully and consistently annotated superfamilies and homologous families of α/β-hydrolases available online (http://www.led.uni-stuttgart.de/). The curation and annotation process for the LED is supported by DWARF, an in-house data warehouse system for protein families [36, 38].

Lipases and related enzymes possess a catalytic triad commonly formed by Ser, His, and Asp residues; the serine residue usually appears in the conserved pentapeptide Gly-X-Ser-X-Gly [31, 39]. Another common characteristic is the oxyanion hole, formed by a backbone of amides and well-conserved amino acid side chains [39]. Depending on the amino acids involved in forming the oxyanion hole, the

enzymes can be classified into three classes, GGGX, GX, and Y. A complete description of this classification can be found in [38].

In the LED, protein sequences are grouped hierarchically considering that all sequences with high similarity were assigned to a single homologous family that received the name of the organism that originated and represents the family [36]. Homologous families with low, but significant sequence similarity were grouped into a single superfamily. Superfamilies have no significant sequence similarity between each other, but are grouped according to the oxyanion hole classification [37]. In the beginning of this databank, 92 microbial lipases and homologous serine hydrolases from microbial sources were assigned to 32 homologous families and 15 superfamilies [39]. However, today, among 112 homologous families and 38 superfamilies, we can survey 24,783 sequence entries corresponding to 18,585 proteins [27] belonging to 2,174 organisms.

Microbial lipases show a broad spectrum of industrial application, and filamentous fungi are recognized as the best lipase producers and are currently the preferred sources since they produce extracellular lipases. Despite of the 600,000 possible fungi species living on Earth, only approximately 7 % have been catalogued. Among them, the ubiquitous group of the *Aspergillus* genus is present [27, 40], and although it is known as a great producer of enzymes, a lot of information is lacking in terms of its lipase structures. Considering 241 lipase structures were deposited on the Protein Data Bank and that none belong to fungi from *Aspergillus* genus, the best information source available is in the LED, as observed in Table 1.

Aspergillus α/β-hydrolases are distributed in 19 of 38 superfamilies, but according to Gupta et al. [27], only the superfamilies abh02 and abh04 (GXXX type), abh07 and abh24 (GX type), and abh38 (Y type) are really fungi or yeast lipases; therefore, abh24 lacks in *Aspergillus*.

Aspergillus able to produce lipases are found in several habitats, including soils contaminated with oils, waste of vegetable oils, dairy product industries, seeds, and deteriorated food. Furthermore, the rapid development of molecular biology techniques, as well as the availability of more reliable high-throughput screening methods, has increased the utility that lipase offers for organic synthesis [1]. Lipase production by fungi varies according to the strain, the composition of the growth medium, cultivation conditions, temperature, pH, and the kind of carbon and nitrogen sources [41]. Generally lipidic carbon sources seem to be essential to obtain a high lipase yield, although a few authors observed that the presence of fats and oils was not statistically significant for enzyme production [1].

Approximately 90 % of all industrial biocatalysts are produced by submerged fermentation (SmF), frequently using specifically optimized media and genetically manipulated microorganisms. For this purpose, SmF processing can offer several advantages over solid-state fermentation (SSF), but on the other hand, almost all of these enzymes could also be produced in SSF [1]. A quantitative comparison between submerged fermentation (SmF) and SSF is difficult due to the difference in the methods used for determining the lipase activity [41].

Table 2 summarizes some characteristics of production and purification of *Aspergillus* lipases, and shows their basic biochemical properties as pH and temperature

Table 1 Description of *Aspergillus* alpha/beta-hydrolase superfamilies (lipases included highlighted in red) found in Lipase Engineering Database

Organism	Sum of sequences of alpha/beta proteins	Superfamilies (Number of superfamilies)
A. flavus	5	abH23/abH04/abH32/ (3)
A. kawachii	1	abH32/ (1)
A. nomius	1	abH04/ (1)
A. tamarii	1	abH23/ (1)
A. aculeatus	1	abH32/ (1)
A. fumigatus	71	abH23/abH01/abH07/abH15/abH14/abH36/ abH03/abH04/abH02/abH09/abH08/abH22/ abH32/abH27/abH34/abH13/abH19/abH31/ abH38/ (19)
A. fumigatus Af293	19	abH15/abH14/abH36/abH03/abH09/abH22/ abH27/abH34/abH19/ (9)
A. fumigatus CBS 144.89	1	abH27/ (1)
A. nidulans	61	abH23/abH01/abH07/abH14/abH36/abH03/ abH04/abH02/abH09/abH32/abH27/abH34/ abH13/ abH31/ abH38/ (15)
A. oryzae	106	abH23/abH01/abH07/abH14/abH36/abH03/ abH04/abH02/abH09/abH08/abH22/abH32/ abH27/abH34/abH13/abH19/abH31/abH38/ (18)
A. terreus	92	abH23/abH01/abH07/abH14/abH36/abH03/ abH04/abH02/abH09/abH08/abH22/abH32/ abH27/ abH34/abH13/abH19/abH31/abH38/ (18)
A. awamori	1	abH23/ (1)
A. niger	90	abH23/abH01/abH07/abH15/abH14/abH36/ abH03/abH04/abH02/abH09/abH08/abH22/ abH32/abH27/ abH34/abH13/abH19/abH31/ (18)
A. niger CBS 120.149	1	abH27/ (1)
A. parasiticus	2	abH23/abH04/ (2)
A. tumbingensis	2	abH23/abH32/ (2)
A. clavatus	57	abH23/abH01/abH07/abH15/abH14/abH36/ abH03/abH04/abH02/abH09/abH08/abH22/ abH32/ abH27/ abH34/abH13/abH19/abH31/ (18)
A. sojae	1	abH32/ (1)
A. versicolor	1	abH32/ (1)

Table 2 Description of some characteristics of production, purification, and biochemical properties of *Aspergillus* lipases

Organism	Lipidic carbon source	Complementary carbon source	Fermentation technique	Optimum pH	Optimum temperature (°C)	Purification method	Enzyme activity (IU/g[a] or IU/mL[b])	Cultive time (hours)	MW[c]	Reference
A. niger NCIM 1207	Synthetic oil based (SOB) 1 % olive oil	Wheat bran	SmF and SSF	2.5	45–50	Trimellitic anhydride (TMA)-cross-linked deacetylated chitin Sephacryl-100	630[a]		32.2	[49–51]
A. niger MTCC 2594	Gingelly oil cake		SSF	7.0	37		363.6[a]	72		[52, 53]
A. versicolor CJS-98	Jatropha seed cake	Maltose and peptone (2 %),	SSF				1288[a]	96		[54]
A. niger (*NRRL 334*)	Olive mill wastewater (OMW)						0.33[b]	168		[55]
A. oryzae (*NRRL 1988*)	Olive mill wastewater (OMW)						0.34[b]	168		[55]
A. oryzae (NRRL 495)	Olive mill wastewater (OMW)						0.38[b]	168		[55]
A. niger MZKI Al 16	Olive oil		SmF	7.0	45	Mono Q ion exchange chromatography of the precipitated crude enzyme preparation	~4.5[b]	After70	43 65	[56]

A. nidulans FGSC26		Glucose–lactose								[57, 58]
A. terreus	Olive oil	Glucose–casein	SmF	4.0	50	Ammonium sulfate precipitation Q-Sepharose and Sephacryl S-200	13.7[b]	70	37.2	[59]
A. flavus	Coconut oil		SmF			Ammonium sulfate precipitation	1940[b]	120	47	[60]
A. japonicus LAB01	Sunflower oil		SmF	8.5	45	Ammonium sulfate precipitation Superose 12HR 10/30	28.04[b]	96	~25	[61]
A. ibericus MUM 03.49	Olive mill wastewater (OMW)		SmF				8.319[b]			[62]
A. fumigatus Af293				8.5	65	Single-step Ni-NTA affinity purification			38	[63]
A. tamarii MTCC 5152	Gingelly oil cake	Wheat bran	SSF	7.0	50		758[a]	120		[64]
A. nidulans WG312	Olive oil		SmF	6.5	40	Phenyl-Sepharose chromatography and affinity binding on linolenic acid–agarose			29	[65]

(*continued*)

Table 2 (continued)

Organism	Lipidic carbon source	Complementary carbon source	Fermentation technique	Optimum pH	Optimum temperature (°C)	Purification method	Enzyme activity (IU/g[a] or IU/mL[b])	Cultive time (hours)	MW[c]	Reference
A. awamori BTMFW032	Rice bran oil	Soya bean meal	SmF	7.0	40	(NH4)2SO4 precipitation and ion exchange chromatography	495[a]	96	90	[66]
A. carneus	Sunflower oil						12.7[b]	96		[67]
A. carbonarius			SmF							[68]

[a]IU per gram of protein
[b]IU per milliliter of enzyme
[c]Molecular weight
IU or International enzymatic activity is defined as the amount of enzyme capable of releasing 1 μmol of product per min of reaction

optimum. More information about these subjects can be found in the following works [26, 42–48].

4 Immobilization of Lipases Obtained from the Genus *Aspergillus*

Enzymes are highly specific biocatalysts and have been exploited along the years by different industry sectors due to their inherent catalytic properties [69]. However, the desired purposes by industries are often difficult to achieve using the native forms because the enzymes, when used in certain processes, may be unstable or may show neither optimum activity nor optimum selectivity. From an economic viewpoint, several studies have described the techniques for enzyme immobilization as a powerful tool to reduce operating costs of industries [70–73]. The main advantages of using immobilized enzymes as biocatalysts are the possibility to repeat the use of a single batch of enzymes, thus making the process economically feasible, enzyme stabilization due to binding to the support, and no contamination of the product with the enzyme [74, 75]. Immobilization can also drastically affect enzyme properties such as the resistance to proteolytic digestion and denaturants, pH dependence, temperature profile, thermostability, and kinetics [76].

Many supports have been exploited for lipase immobilization. Generally, these techniques involve traditional methods such as adsorption, entrapment, covalent binding, and cross-linking (Table 3) [10]. The following text will describe the characteristics of each technique for lipase immobilization produced by *Aspergillus* sp. strains.

The physical adsorption is one of the simplest methods used for lipase immobilization. This technique commonly involves adsorption of the enzymes through nonspecific forces such as van der Waals forces, hydrogen bonds, and hydrophobic interactions with the support material [77]. Comparing this technique with other immobilization techniques, the adsorption is advantageous since it can be performed under mild conditions and easy operation, it uses low-cost support materials, it does not require chemical additives during adsorption, and the regeneration of supports can be easily done by recycling. However, one disadvantage of the physical adsorption method is associated with weak interactions of the enzyme with the support materials, which cause biocatalyst leaking, and thus it exhibits reduced activity when used repeatedly [71, 77]. Commercial lipases from *A. oryzae* were analyzed in the work of Yücel et al. [78]. The authors investigated the immobilization onto microporous polymeric matrix using two methods: physical adsorption and covalent linking. The results showed that covalent immobilization was able to load more enzymes on support than the adsorption method, but it lost some activity due to the inactivity of active sites of enzymes. Immobilized process by physical adsorption showed stable operation for 5 reuses [78].

Lipase immobilization *via* adsorption was also studied by Silva et al. [12]. The authors used a lipase from *A. niger* and analyzed various properties of both the free and immobilized enzyme on Celite, such as the hydrolytic and esterification

Table 3 Characteristics of most widely used techniques for the lipase immobilization

Technique	Characteristics					Advantages	Disadvantages
	Immobilization process	Interaction	Recovery of enzyme	Regeneration	Immobilization costs		
Adsorption	Easy	Weak	Low	Possible	Low	No conformational change of the biocatalyst; no need to use reagents; reuse of expensive material	Possible desorption of biocatalyst due to weak bonds
Entrapment	Difficult	Strong	High	Impossible	Low	Protection of biocatalyst; enables continuous operation; facilitates cell separation and simplified downstream process	Mass transfer limitation; low enzyme loading; effective for low molecular weight substrates
Covalent binding	Difficult	Strong	Low	Impossible	High	High heat stability; facilitates the enzyme contacts with its substrate; prevents elution of biocatalysts	Limited mobility of enzymes; less effective for the immobilization of cells
Cross-linking	Difficult	Strong	Moderate	Impossible	Moderate	Prevents leakage; decreases desorption; increases the stability of biocatalyst	Might cause alteration in active site; diffusion limitations; loss of enzyme activity

activities, stability, and enantioselectivity. The most important advantage observed with the immobilized lipase was its thermal stability and an improved esterification activity during the reaction of (*R, S*)-ibuprofen with 1-propanol in isooctane. Furthermore, immobilized *A. niger* lipase showed a high activity for esterification process (73 % after 5 days of storage at 40 °C) and a significant operational stability for recycling and reuse of enzyme [12]. Silva et al. [13] also tested the immobilization of lipase from *A. niger* in five types of support *via* adsorption (Accurel EP-100, Amberlite MB-1, Celite, Montmorillonite K10, and silica gel). This lipase was studied for use in the kinetic resolution of (*R*, *S*)-ibuprofen, and the matrix Amberlite MB-1 was found as the best support, with a conversion of 38.2 %, enantiomeric excess of 50.7 %, and enantiomeric ratio (E value) of 19 in 72 h of reaction. Moreover, the immobilized enzyme maintained a high operational stability of at least 80 % after 8 months in storage at 4 °C and could be reused at least 6 times [13].

Entrapment is another classic way to immobilize lipases. This method refers to the capture of enzymes within a polymeric network or microcapsules of polymers that allows the substrate and products to pass through but retains the enzyme [80]. Entrapment method shows important advantages such as reduced enzyme leakage in comparison with the physical adsorption method, and it is simpler to perform than covalent binding. However, the biggest disadvantage of entrapment is the mass transfer limitation, so the lipase is only effective for low molecular weight substrates. The entrapment of lipases can be carried out in different polymers, both natural and synthetic [1, 70, 79]. Encapsulation in a sol–gel matrix of lipase from *A. niger* was studied by Zubiolo et al. [80]. The encapsulated lipase in a sol–gel matrix showed greater thermal stability at temperatures of 45 and 60 °C than the free enzyme. The positive influence of the immobilization process was observed on the thermal stability of the enzyme, since a longer half-life $t_{1/2}$ and lower deactivation constant were obtained with the encapsulated lipase when compared with the free lipase [80]. Osho et al. [81] tested the lipase immobilization produced by *A. niger* ATCC 1015 in matrices of a structural fibrous network of pawpaw (*Carica papaya*) wood and vegetable sponge (*Luffa aegyptiaca*) by entrapment. The authors showed that the immobilization of lipase successfully enhanced its pH stability, especially in slightly acidic environments. A relative activity above 75 % was achieved for the immobilized lipase stored at 4 °C and 50 % at 28 °C until the fourth week of storage for both supports [81].

Covalent binding is the immobilization technique of enzymes that involves the chemical reaction between certain amino acid residues outside the catalytic site (e.g., thiol and amine groups of enzymes) with the support carriers [70]. The immobilized method by covalent binding to the solid carrier has the expected advantage of strong interactions between the enzyme and the carrier during the catalytic process, which makes the enzyme very stable. However, the covalent binding process has a distinct disadvantage where the enzyme is chemically modified [79, 82]. *A. niger* lipase was immobilized by covalent binding on chitosan-coated magnetic nanoparticles. Immobilization enhanced the enzyme stability against changes of pH and temperature, compared to free lipase. Moreover, the storage stability was studied during 50 days, and high stability was observed in the

immobilized derivatives. Finally, immobilized derivatives retained over 80 % of their initial activity after 15 hydrolytic cycles [83]. In the work by Dhand et al. [84], the immobilization of lipase produced by *A. oryzae* was tested using the covalent binding method onto a polyaniline–nanotube-based film, electrophoretically deposited onto indium tin oxide via glutaraldehyde. Immobilized lipase was used for triglyceride detection and showed an improvement in biosensing characteristics, such as linearity, fast response time, and high sensitivity [84].

In the immobilization method by cross-linking, the lipases are chemically immobilized via the formation of intermolecular cross-linkages. The cross-link of enzymes can be achieved by the addition of bi- or multifunctional cross-linking reagents such as glutaraldehyde. An advantage of this technique is that it is usually support-free and involves joining enzymes to each other to form a three-dimensional structure [85, 86]. Despite this great advantage, cross-linking method has disadvantages in the following aspects: (i) using cross-linking reagents that can change the conformation of lipases and potentially lead to significant loss of activity, (ii) performed under relatively harsh conditions, and (iii) low immobilization yields and absence of desirable mechanical properties [77]. A new strategy applying cross-linking method was described by Tudorache et al. [87] for lipase immobilization produced by *A. niger*. The strategy for lipase immobilization was explored leading to cross-linked enzyme aggregate onto magnetic particles using glutaraldehyde as cross-linker. The lipase immobilized by this method showed high operational performance with 61 % glycerol conversion, 55 % glycerol carbonate yield, and 90 % selectivity in glycerol carbonate. Immobilized biocatalyst also proved to be robust when used in conversion of "crude" glycerol extracted from the biodiesel process leading to glycerol carbonate with similar performance as with pure glycerol. Moreover, immobilized lipase showed in the recycling experiments that it is stable for 20 successive reaction cycles [87].

5 Industrial and Biotechnological Applications of Lipases

5.1 Food Industry

For lipase, the food industry is considered one of the most important markets in their application. In 2014, the Novozymes reported lipase represented the second best-selling product for the food industry, which was 26 % of the total company sales. The sectors of dairy and baking goods were the ones which requested larger production of lipase. Data show that Japan and China are the biggest investors in lipase for the food industry [88]. In addition, many industries also use additives such as stabilizers and preservatives [89].

The majority of the food industries search for enzymes (including lipases) that exhibit catalytic specificity, thermostability, high catalytic activity in a wide range of pH and temperature, and structural properties which can be immobilized with high catalytic efficiency [1].

All materials must be appropriate with high purity degree and current Good Manufacturing Practice (cGMP). The pathogenic and toxigenic potential of the production strain is the main target in evaluating enzyme safety.

As previously stated, the lipase is vastly used in the fields of baking and dairy industries. However, we must increase our interest in creating new sources of these lipases to find alternatives. At the same time, the drive to search this genus in the area of food is important to expand knowledge and create new technological processes. All these processes using enzymes of different genres of microorganisms expand the variability of processes considered green, which drastically decreases the use of waste and toxic compounds.

5.1.1 Dairy Products

Lipases used in the industry are mainly for food processing, such as biomaterial modification and breakdown. Most of them are utilized for flavor development in dairy products and processing of other foods, such as baked goods, aroma, surfactants, and others [90].

Lipases have been successfully used as catalyst for the synthesis of ester. The ester produced from short-chain fatty acids is used as flavoring agents in the food industry. Lipases are used in the dairy industry for the hydrolysis of milk fat to modify the fatty acid chain lengths to enhance the flavor of various cheeses. One of the most important features of lipase is the temperature of reaction. To produce specific compounds for food, the industry uses different types of lipase that are incubated at elevated temperatures in order to produce concentrated flavor by lipase catalysis to be used as ingredients in dips, sauces, soups, and snacks. Current applications also include the acceleration of cheese ripening and the lipolysis of butter, fat, and cream [91, 92].

Hérnandez et al. [93] compared three commercial lipases [two from pregastric bovine and one from *A. niger* ("Palatase 20000 L®", Novozymes, Spain)] to develop the characteristic pungent flavor of Idiazabal (sheep's raw milk) cheese. In the experimental productions, all lipases significantly increased the concentration of total free fatty acids (FFAs), both after 90 and 180 days of ripening. Lipase Palatase 20000 L increased primarily the concentration of short-chain FFA, in which C16:0 and C18:1 were the main FFAs. Cheeses made with no lipase had the lowest concentrations of total FFA. Sensory analysis was performed after 90 and 180 days of ripening. A linear correlation was observed between the percentage of short-chain FFA and the score for pungent flavor for all amounts of lipase used in this study. They concluded that lipases are an adequate enzyme to develop the characteristic flavor of Idiazabal cheese and which type of lipase produces different flavor characteristics, both in artisan and industrial fabrications.

Arbige et al. [94] published a patent that accelerated cheese aging and improved cost efficiency and lessened storage space. This patent relates to a novel lipolytic enzyme derived from *A. oryzae* ATCC 20719. According to the authors, the treatment with this enzyme has not formed associated rancidity compounds, which brings an advantageous process compared to the conventional lipolytic enzymes.

Besides free fatty acids, lactones are also important compounds as flavor ingredients during cheese maturation (ripening). Jolly and Kosikowski [95] reported a lactone production from blue cheese using blended lipases of different microorganisms, including genus *Aspergillus*. After 75 days, the concentrations of δ-tetradecalactone and δ-dodecalactone were the highest produced in blue cheese. That represented five times more than the initial maturation. Also, the same group published in that same year a study of rate and quality of flavor development and type and concentration of carbonyls produced in blue cheese after applying selected microbial and animal enzyme preparations directly to cheese curds. Two out of ten microbial lipase preparations (*Aspergillus*) gave good-quality blue cheese flavor within 45–75 days at 5 °C in cheese made from pasteurized milk. Total carbonyls were maximum in 75 days at 5 °C and were highest in cheeses with added microbial lipase. Cheeses without added enzymes had lowest values and differed in the relative distribution of individual carbonyls. The most important compounds generated during this process were 2-heptanone and 2-nonanone, both increased remarkably the flavor of this dairy products.

5.1.2 Bakery Products

Lipases have been used to enhance the flavor content and increase the shelf life, texture, and softness of bakery products by releasing short-chain fatty acids. Nowadays, there is another important reason to use lipase in baking industries, which is to substitute or supplement traditional emulsifiers in wheat lipids. That is achieved by catalytic reaction to degrade polar compounds and produce emulsifying lipids [96, 97].

Rey et al. [98] published a patent regarding methods for preparing dough. They consisted in incorporating a composition of an effective amount of lipase into the dough, which improved one or more properties of the dough or a baked product. This patent also conveys to the use of different types of enzymes, including lipase from genus *Aspergillus* and compositions comprising an effective amount of such lipase for improving one or more properties of a dough and/or a baked product. The lipase has an advantageous effect on the crumb softness of the final baked product. The combination of the enzyme preparation of the invention and shortening can replace emulsifiers such as monoglycerides, which are used as crumb softener.

Siswoyo et al. [99] reported the effects of lipase from *Aspergillus* and amylase for retrogradation of starch in bread and their impact on its rheological properties. The combined addition of lipase and α-amylase increased significantly the loaf volume compared with the control and slightly increased the stability time of the dough. The addition of lipase alone increased the gelatinization enthalpy of the dough starch and the starch–lipid complexes of dough and bread. After 5 days of storage, the retrogradation rate of bread was slowed to 1.73-fold (against control by adding only lipase), while the combined addition of lipase and α-amylase slowed the retrogradation rate to 2.65-fold. These results suggest that lipase retards retrogradation of bread during storage. The addition of lipase suggests a retardation effect of retrogradation, meaning lipase could retard the retrogradation of bread.

Park et al. [100] evaluated chemical, rheological, and bread-making characteristics on quinoa and wheat flours using lipase from *A. niger*. The additions of lipase

(7.5×10^3 U/g) significantly increased the loaf volume of quinoa and wheat bread. Addition of lipase on wheat bread distinctly decreased the firmness of breadcrumbs compared to those without lipase. Also, lipase decreased the viscosity, while the enthalpy change of melting for starch–lipid complexes was slightly higher than the substituted doughs without lipase. However, addition of lipase made the extensible gluten structure that covered starch granules. These improvements might be caused by the effects of mono- and di-acylglycerol as natural emulsifiers, which were increased from lipid hydrolyzed by lipase during bread making.

5.1.3 Fats and Oils

Fats and oils are among the most important compounds present in foods, and their modifications are of great impact in food processing industries. This industry is currently working to figure out ways to produce more using economic and green technologies to cross out residues. As it was described before, lipase can be used for different reactions, such as hydrolysis, esterification, and interesterification of fats and oils, which is important for the variability of fat and oil products with several features on chemical and physical processes and biological activities. The nutritional and biological functions of food lipid are mainly dependent on the chain length and degree of unsaturation of fatty acids [101]. Therefore, the obtainment of modified lipids by the incorporation of a new fatty acid or restructuration to change the positions of fatty acids results in the obtainment of the structured lipids. Structured lipids may provide the most effective means of delivering the desired fatty acids for nutritive or health purposes, such as prevention of coronary diseases. Structured lipids are suited for use as nutraceuticals because their structure can be manipulated to suit specific patient requirements [101–103].

Zhou et al. [102] produced structured lipids using eicosapentaenoic and docosahexaenoic acids in a batch reactor by lipase-catalyzed acidolysis of fish oil with caprylic acid. The following free lipases – lipase AP, *A. niger*; lipase P, *Pseudomonas* sp.; lipase AY, *Candida rugosa*; lipase AK, *Pseudomonas fluorescens*; lipase F, *Rhizopus oryzae*; and lipase D, *Rhizopus delemar* – were screened under selected reaction conditions. Lipase AP was suitable for the production of structured lipids from fish oil. The optimal molar substrate ratio of fish oil to caprylic acid was 1:6 to 1:8. The time course of the reaction at different enzyme loads demonstrated that 10 % incorporation of caprylic acid could be obtained for lipase AK in 5 h with 10 % enzyme load. Lipase AP had an incorporation of caprylic acid similar to acyl migration of caprylic acid from sn-1,3 positions to the sn-2 position and a slightly lower selectivity toward docosahexaenoic acid.

5.2 Pharmaceutical Industry

In the pharmaceutical industry, lipases are very promising for chemical synthesis, what increases interest in these enzymes. Some advantages in this context include mild conditions that avoid isomerization, epimerization, racemization, and rearrangement reactions, enantio- and regioselectivity, reuse of the immobilized

lipase, overexpression of the lipase, economy of the process, and mutagenesis of the lipase for specific functions [41].

One application that has received attention is the use of lipases in obtaining drugs or pharmaceutical ingredients in their active enantiomeric forms with high optical purity, since these enzymes are chiral molecules able to recognize a specific isomer in a racemic mixture [104]. The use of lipases to resolve racemic mixtures is currently exploited for drug production. Indeed, frequently only one enantiomer of a drug is responsible for the specific therapeutic effect. In some cases, milder or fewer side effects are observed when using optically pure drug products compared with those found with the use of racemic mixtures [105].

Following this perspective, many pharmaceutical companies worldwide are focusing their efforts on the preparation of optically active intermediates on a kilogram scale. Biotechnological companies, such as Enzymatix in the UK, are specialized in biotransformations and offer a whole variety of intermediates prepared via lipase-mediated resolution [106].

Houde et al. listed several lipases that are suitable to be used in the synthesis of various enantiopure molecules such as alcohols, amides, carboxylic acids, and esters [105]. These molecules can produce anti-inflammatory drugs (ibuprofen, naproxen), anticancer drugs (Taxol®, spergualin), antiviral drug (lobucavir), antihypertensive drug (captopril), anticholesterol drugs (squalene synthase inhibitor), anti-Alzheimer disease drug ([S]-2-pentanol), and vitamin A [105]. Ibuprofen is an example of the applications of lipases [105]. Commercialized as Advil or Motrin, ibuprofen sold around $290 million worth of Motrin alone, in the USA. The (S)-ibuprofen molecule is 160 times more potent in inhibiting the prostaglandin synthesis than the (R)-ibuprofen [105].

To obtain lipases, various species of *Aspergillus* are being used in the research and production of these enzymes with the potential of subsequent application in the pharmaceutical industry. To illustrate this, in the context of obtaining enantiomerically pure alcohols for use in organic synthesis to achieve enantiopure pharmaceuticals, Carvalho et al. studied the kinetic resolution of (R,S)-2-octanol with octanoic acid in n-hexane by four *Aspergillus* lipases (*A. flavus* AC-8, *A. niger* AC-54, *A. oryzae* AC-122, and *A. terreus* AC-241) [107]. They observed that lipases from *A. niger* and *A. terreus* showed the best results in terms of enantioselectivity ($E = 4.9$ and $E = 4.5$, respectively). These properties make these lipases good candidates for biocatalysis in organic media [1, 107].

Direct esterification of racemic alcohols or carboxylic acids with dry mycelia of strains of *A. oryzae* and *Rhizopus oryzae* often resulted in an efficient kinetic resolution. For example, *A. oryzae* has been used in pure organic solvents for the resolution of (RS)-flurbiprofen, displaying good enantioselectivity toward (R)-flurbiprofen and providing results that are competitive with the data obtained using commercial enzymes [108].

Among the ways to obtain a specific enantiomer, Hu et al. improved the ability of *A. terreus* lipase to separate the racemic ketoprofen vinyl ester into individual enantiomers using hollow self-assembly of alginate–graft–poly(ethylene glycol)/α-cyclodextrins (Alg-g-PEG/α-CD) spheres as enzyme immobilization carriers [109].

Pandey et al. made a survey on major commercial lipases in 1999 revealing that *Aspergillus* lipases were highly selective for short-chain acids and alcohol [110]. A triacylglycerol lipase from *A. oryzae* stands out showing high specificity toward triacylglycerols of middle-chain saturated fatty acids [110].

To complement *Aspergillus* lipase works, Li et al. reported a novel method of synthesis of 1,3-diglyceride (1,3-DG), a healthy natural lipid which is extensively used in food processing and utilized as pharmaceutical intermediate. The synthesis of 1,3-DG via glycerolysis of triglyceride (TG) was catalyzed by whole-cell lipase from a new isolated strain, *A. niger* GZUF36 [111].

Organic esters are used in various industries such as perfumery, flavor, and pharmaceutical intermediates. Tamalampudi et al. developed an enantioselective transesterification reaction by using recombinant *A. oryzae* whole-cell biocatalyst expressing lipase encoding gene from *Candida antarctica* [112]. Pera et al. showed indications that by manipulating the cultivation conditions of *A. niger* MYA135, it is possible to produce lipase extracts with different enzymatic properties, which would allow them to be used in diverse industrial processes [113].

5.3 Chemical Industry

The use of lipases in the oleochemical industry is enormous as it saves energy and minimizes thermal degradation during hydrolysis, esterification, acidolysis, alcoholysis, interesterification, and aminolysis [41]. Some of the industrially important chemicals manufactured from fats and oils by chemical processes could be produced by lipases faster and with better specificity under mild conditions [106]. The chemo-, regio-, and enantiospecific behavior of these enzymes has caused tremendous interest among scientists and industrialists [106].

These enzymes carry out reactions of prochiral substrates and kinetic resolution of racemic mixtures. The functional groups in which lipases act vary greatly: alcohols and chiral or prochiral carboxylic esters, cyanohydrins, chlorohydrins, diols, α- and β-hydroxy acids, amines, diamines, and amino alcohols [114].

Lipases are also used as biocatalysts in the production of useful biodegradable compounds. 1-Butyl oleate is synthesized by direct esterification of butanol and oleic acid to reduce the viscosity of biodiesel in winter use [41]. The mixture of 2-ethyl-1-hexyl esters is obtained in a good yield by enzymatic transesterification from rapeseed oil fatty acids for use as a solvent [41]. Trimethylolpropane esters are also similarly synthesized as lubricants. Lipases can also catalyze ester syntheses, and transesterification reactions in organic solvent systems have opened up the possibility of enzyme-catalyzed synthesis of biodegradable polyesters [41].

One very important application of lipases has been in the organic synthesis of pesticides for the production of optically active compound [110]. Generally, these compounds were produced through the resolution of racemic mixtures of alcohol or carboxylic esters; stereospecific synthesis reactions were also employed [110].

In a research of *Aspergillus* lipases potentially applied in the chemical industry, Ding et al. studied lipases in the field of Knoevenagel condensation [115]. In the

enzyme-catalyzed organic synthesis, they found that several lipases displayed observable activities for Knoevenagel condensation. In particular, the commercially cheap available lipase lipoprotein (LPL) from *A. niger* could efficiently catalyze the Knoevenagel condensation of aromatic aldehydes with various active methylene compounds in good to excellent yields with Z configuration exclusively [115].

Lipases from *A. niger*, *Rhizopus delemar*, *Geotrichum candidum*, and *Penicillium cyclopium* were employed in the synthesis of esters of oleic acid with various primary alcohols, whereas lipases from the species *Rhizopus niveus* and *A. terreus* have been used for the esterification of free fatty acids (FFAs) in solvent-free systems with promising results [116].

Cellulose esters (CEs), from which cellulose acetate (CA) is the more popular, are of great importance because of their abilities for selective absorption and removal of organic compounds or their compatibilities with plastics as a reinforcing material [117]. Božič et al. studied the acetylation efficiency of nanofibrillated cellulose (NFC) with acetic anhydride as acetyl donor using lipase from *A. niger* in a mixture of dimethyl sulfoxide (DMSO) and phosphate buffer solution at ambient conditions and in supercritical carbon dioxide (scCO_2) [117]. This work shows a promising and straightforward method for the surface modifications of nanocellulose with various functionalities and opens up new opportunities for using it as a new absorbent for the selective adsorptions of proteins and the removals of organic compounds [117].

5.4 Detergent Industry

Lipases have their major application in industrial laundry and household detergents [118]. Lipases added to detergents improve the washing performance, since a reduced amount of detergent is required, and save energy because of the lower washing temperature [2, 119]. In addition to lipases, enzymes such as proteases, amylases, and cellulases are added to the detergents to improve their efficiency [120].

For use as additives, the lipases should present low substrate specificity, because they have greater ability to hydrolyze fat of various compositions and tolerate alkaline washing conditions (pH 10), high temperature (50 °C), and the presence of surfactants and other enzymes [2, 121]. Cold wash conditions can also be performed using cold-active lipase [65]. Considering washing conditions are hostile to most enzymes, a continuous screening of lipases is required. Then, suitable lipases for detergent applications from different hosts were isolated such as *Rhizomucor miehei* and *Humicola lanuginosa* [122].

Lipase from *Aspergillus* has also been reported. Lipase from *A. terreus* hydrolyzed both animal and plant oils and was tolerant in a wide range of pH (pH 3–12), and it remained stable under the highest temperature of 65 °C. There was no change on enzyme activity with the addition of detergents, except SDS that stimulates enzymatic activity [123]. The lipase from *A. niger* MTCC 2594 was isolated and showed pH stability between pH 4.0 and 10.0 and temperature stability between 4 °C and 60 °C. Furthermore, in the presence of SDS, Tween 80 and commercial detergents such as Henko and Surf Ultra improved the enzymatic stability [124].

Another enzyme from *Aspergillus* presented excellent performance and possible application in the detergent industry. The *A. carneus* lipase showed temperature and pH optimum of 37 °C and 9.0, respectively. The pH stability was 8.0–10.0 for 24 h, and stability temperature was 70 °C for 5 min. The SDS was a strong inhibitor, while other types of different detergents such as taurocholic acid, hexadecyl trimethyl ammonium bromide, and *n*-octyl-α- and *n*-octyl-β-D-glucopyranosides strongly stimulated enzyme activity [125].

A cold-active lipase from *A. nidulans* hydrolyzed fatty acids of short and middle chain and showed high activity in the range 0–20 °C and pH stability conserved at alkaline pH [66].

5.5 Biofuel Industry

The use of energy sources in the generation of products depends on their burns. During this process, the carbon that was confined in the oil and gas reserves is transferred to the atmosphere in the form of gases, mainly as CO_2, contributing to the increase of the greenhouse effect and global warming. This scenario, coupled with the finite character of fossil fuels, encourages the search for alternative energy sources that will mitigate the negative effects generated in the environment and the economy [126]. For power generation, renewable sources such as biodiesel have been used to replace the fossil fuels [127].

Biodiesel is a mixture of fatty acid methyl esters (FAME) that are obtained by transesterification or esterification of oils from different origins. Out of these two processes, the enzymatic transesterification using lipase is the most advantageous due to the easy byproduct retrieval of this reaction, the glycerol. Currently, the main obstacle to biodiesel production using lipase is the high cost of enzyme production. Consequently, researchers have invested in lipases prospecting in order to lower the cost biodiesel production [128].

Lipase from *Aspergillus* strains has been used in biodiesel production. A genetically modified lipase from *A. oryzae* was used to perform the transesterification of soybean oil for biodiesel. Under optimized conditions assay using response surface methodology, a yield of 93.6 % ±0.014 (w/w) biodiesel was obtained [129].

The fungus *A. nomius* was isolated from soil and exhibited maximum whole-cell lipase, which remains either inside the cell or in the cell wall, and methanolysis activity when waste cooking oil was used as the carbon source. The maximum yield of biodiesel was 95.3 % [130].

6 Conclusions

Lipases belong to a very select group of enzymes that are capable of catalyzing a diverse number of reactions in mild reaction conditions. Although the major area of application of lipases is the detergent industry, these enzymes are used to obtain high-added value compounds in the pharmaceutical industry, mainly enantiomers,

through esterification, transesterification, and hydrolysis reactions. This is a remarkable feature of lipases since several enantiomerically enriched drugs can be obtained, including ibuprofen, ketoprofen, flurbiprofen, and atenolol, among others. The use of enzymes to this purpose is advantageous since the resolution of these compounds is highly difficult due to their identical physicochemical properties. The source of lipase is a major issue, since depending on it, the cost of enzyme production and enzyme characteristics vary. In this context, filamentous fungi are of great interest since they present a very efficient machinery for protein secretion. Among those, *Aspergillus* are highlighted because they produce different types of enzymes, including lipases, carbohydrate-active enzymes, and proteases. Several *Aspergillus* lipases present good potential to be used in food, pharmaceutical, chemical, detergent, and biofuel industries. However, more efforts should be directed to structurally characterize lipases from this genus, what can result in the knowledge of the action mechanism of these enzymes, optimizing their use. In addition, it is of great help for protein engineering in order to improve *Aspergillus* lipase characteristics for optimal industrial use.

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Part III

Biological Activity

Preclinical and Clinical Perspective on Fungal Metabolites and Their Analogs as Anticancer Agents – From Bench to Bedside

20

Sanjeev Banerjee and Shivani B. Paruthy

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Abstract

Emerging viewpoints from contemporary research on terrestrial fungal metabolites provides an insight into their valuable insidious biological activity including cancer therapeutics. Some well-characterized fungal metabolites surprisingly display remarkable antitumor properties at preclinical and clinical trial stage. Although their underlying mechanism of action is still being investigated,

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overwhelming evidence points to their actions operationally targeting core regulatory pathways and enzymes dysregulated during pathogenesis of cancer. Some metabolites have progressed into clinical pipeline, while others preset unique window of opportunity to capitalize as lead compound for future synthesis of anticancer drug of translational relevance. This chapter presents a succinct preclinical and clinical perspective on a few select and structurally diverse fungal metabolites with supportive mechanism-based bioactivity deciphered against tumor cells and with the presumptive notion of their future development as novel synthetic analog. The metabolites included are: Phenylahistin, Palmarumycin CP-1, Rhizoxin, Epoxyquinol B, Fumagillin, Destruxin B, Cotylenin A, Myriocin, Cytochalasin E, Chaetocin, Apicidin, and Galiellalactone. None of these agents are currently being adopted for treatment of cancer, but with some metabolite analog compounds, clinical trials have been conducted to ensure clinical safety and efficacy. However, based on overwhelming precedence of preclinical and clinical anticancer activity, this new class of structurally diverse fungal metabolites may become an important source of anticancer lead molecules for use either as monotherapy or in combination with other drugs in fight against cancer.

Keywords

Fungal metabolites • Phenylahistin • Palmarumycin CP-1 • Rhizoxin • Epoxyquinol- B • Fumagillin • Destruxin-B • Cotylenin-A • Myriocin • Cytochalasin-E • Chaetocin • Apicidin and Galiellalactone

1 Introduction

Cancer continues to besiege as a threat to humankind since antiquity dating from the times of Pharaohs in ancient Egypt, until today. It derives its name from the father of medicine Hippocrates, who used the Greek word "Karkinos" to describe tumors, but modern concept about this disease differs from ancient view. According to World Health Organization (WHO), cancer is defined as an abnormal growth of cells caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites causing significant morbidity and, if untreated, death of the host. Adding further rhetoric dimensions to the complexity of this disease are some additional recognized set of hallmarks compiled and published by Hanahan D and Weinberg RA [1].

Despite advancements in our knowledge during past quarter of a century leading to identification of critical hallmarks associated with this disease, cancer still remain a major cause of morbidity and mortality both in developing and developed countries. In the year 2016 itself, 1,685,210 new cancer cases and 595,690 cancer deaths are projected to occur in the United States [2]. These dismal figures strongly attest we are still lagging behind perception of "magic bullet," pioneered over 100 years

ago by Paul Ehrlich, the father of chemotherapy, and draw attention to invigorate existing anticancer drug discovery and development agenda. Such efforts may ultimately capitalize on attractive drug leads for patients benefit.

Of all currently available anticancer drugs in the clinics, over 60 % of them are either of natural origin or products from modifications of natural product derived compounds. Interest in search for anticancer agents from natural sources started in earnest in the 1950s with the discovery and development of the vinka alkaloids, vinblastine and vincristine, and later taxol from bark of the Pacific yew, *Taxus brevifolia*. Despite advancements relating to understanding pathobiology of cancer over the past few decades, a number of reports emerged in parallel in the literature highlighting the production of secondary metabolites of interest with valuable biological activities by endophytic and terrestrial fungi. Thereafter, over the time period, our knowledge regarding the role of fungal metabolites in context of cancer therapy also advanced leading to confirmation of antitumor activity against a vast majority of cancers including leukemia, lymphoma, and solid tumors. Moreover, information accrued from underlying mechanism of action of some investigated fungal metabolites provided basis to develop novel synthetic analogs harboring potent efficacy and low toxicity with additional therapeutic advantage. This chapter presents a succinct overview within framework of historical origin, chemical structure, mechanism, and antitumor effects of some select promising fungal metabolites, which have been extensively researched relating to tumor prognosis. Included herein are the bioactive compounds: Phenylahistin, Palmarumycin CP-1, Rhizoxin, Epoxyquinol- B, Fumagillin, Destruxin-B, Cotylenin-A, Myriocin, Cytochalasin-E, Chaetocin, Apicidin, and Galiellalactone. Their structure and other available chemical relevant information are presented in Table 1. Figure 1 depicts a generalized overview of fungal metabolite targets within tumor cells. Almost all these metabolite compounds demonstrate potent therapeutic advantage at preclinical stage of investigation, as inferred from inhibition of tumor growth, delaying the tumor progression as well as, an effect on invasion and metastasis of tumor cells. Some metabolites have entered the clinical trial stage for translation as potential candidate drugs traversing from bench to the clinics as future cancer therapeutics. Some of these presumptively promising metabolite compounds are discussed below individually.

2 Phenylahistin

In 1997, Kanoh et al. isolated phenylahistin (PHL), a low MW metabolite from the agar culture medium of terrestrial fungi *Aspergillus ustus* NSC-F038 as part of a screening program aiming to identify new cell cycle inhibitors [3]. This new and novel class of diketopiperazine consist of L-phenylalanine along with a unique isoprenylated dehydrohistidine residue and a quaternary carbon at the 5-position of the imidazole ring that confers a colchicine-like microtubule activity to the molecule (Fig. 1). Although PHL is produced as a racemic mixture, only the (−) enantiomer has been proven to be more cytotoxic and associated with potent antitumor activity which

Table 1 Chemical structure and important chemical information's on select fungal metabolites

Phenylahistin	CAS No: Not available Molecular wt: 350.41 Molecular Formula: $C_{20}H_{22}N_4O_2$	
Palmarumycin-CP1	CAS NO: 159933-90-1 Molecular wt: 316.30 Molecular Formula: $C_{20}H_{12}O_4$	
Rhizoxin	CAS No: 90996-54-6 Molecular wt: 625.749 Molecular Formula: $C_{35}H_{47}NO_9$	

Epoxyquinol B	CAS No: Not available Molecular wt: Not available Molecular Formula: Not available	
Fumagillin	CAS No: 23110-15-8 Molecular wt: 458. 54 Molecular Formula: $C_{26}H_{34}O_7$	
Destruxin-B	CAS No: 2503-26-6 Molecular wt: 593.76 Molecular Formula: $C_{30}H_{51}N_5O_7$	

(*continued*)

Table 1 (continued)

Cotylenin A	CAS No: 12708-37-1 Molecular wt: 622.74 Molecular Formula: $C_{33}H_{50}O_{11}$	
Myriocin	CAS No: 35891-70-4 Molecular wt: 401.54 Molecular Formula: $C_{21}H_{39}NO_6$	
Cytochalasin E	CAS No: 36011-19-5 Molecular wt: 495.56 Molecular Formula: $C_{28}H_{33}\ NO_7$	

Apicidin	CAS No: 183506-66-3 Molecular wt: 623.78 Molecular Formula: $C_{34}H_{49}N_5O_6$	
Chaetocin	CAS No: 28097-03-2 Molecular wt: 696.84 Molecular Formula: $C_{30}H_{28}N_6O_6S_4$	
Galiellalactone	CAS No: 133613-71-5 Molecular wt: 194.2 Molecular Formula: $C_{11}H_{22}O_3$	

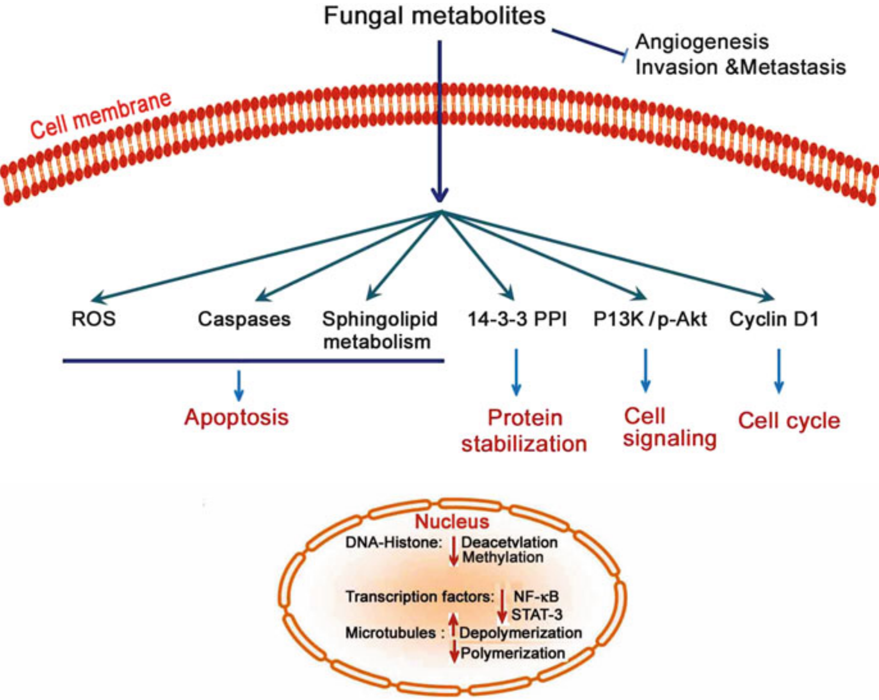

Fig. 1 Molecular targets of fungal metabolites within tumor cells

suggests stereochemistry of the α-carbon of phenylamine residue is important for its activity. Moreover, (−)-PHL specifically inhibits cell cycle activities during G2/M phase by inhibiting tubulin polymerization which are the major constituent of microtubules and an essential component of cytoskeleton system and spindle dynamics within mitotic apparatus of cells [3, 4]. It has been further concluded that (−)-PHL act directly on tubulin, and competitive binding assay using radiolabeled colchicine and vinblastine indicates it recognizes the colchicine-binding site on tubulin [5].

Preliminary investigations towards any predictive antitumor activity of (−)-PHL were initially carried out according to drug evaluation program of the Japanese Foundation for Cancer Research based on the growth inhibition of 38 human tumor cell lines [6]. Further evaluation using seven human tumor cell lines [A431 (dermal), A549 (lung), HeLa (cervical), K562 (leukemia), MCF-7(breast), TE671(CNS), and WiDr (colon)] and a mouse leukemia cell line [P388 (mouse leukemia)], PHL reaffirmed its potent antitumor activity in vitro with IC_{50} values that ranged between 0.18 and 3.7 μM [4]. Additionally, the antitumor activity of (−)-PLH has also been evaluated against P-338 mouse leukemia and Lewis lung carcinoma cells in vivo revealing significant antitumor activity at the highest given dose (100 mg/kg) [4]. Moreover, no toxicity due to phenylahistin has been noted on ex vivo studies

performed on primary cultures of rat or human hepatocytes [7]. Based on foregoing encouraging bioactivity and total synthesis of (−)-PHA, further extensive structural modifications based on structure-activity relationship lead to emergence of a synthetic derivative – NPI-2358 in 2006, which was chosen as a clinical candidate with US adopted name designation as Plinabulin [8]. Plinabulin displays potent cytotoxicity against HT-29 colon cancer cells [IC_{50} value = 15 nM] and inhibits cell cycle progression of HeLa cells in G2/M phase. Initial studies also revealed that Plinabulin induce tubulin depolymerization and permeable at low concentrations through monolayer of human umbilical vein endothelial (HUVEC) cells in culture causing tumor vasculature to collapse earning *status quo* as "vascular disrupting agent" (VDA) [8]. Some recent preclinical in vitro data indicate Plinabulin exhibits growth inhibitory activity and induces apoptotic cell death in multiple myeloma cell lines and tumor cells from patients with multiple myeloma and significantly inhibit tumor growth and prolong survival in an human MM.1S plasmacytoma murine xenograft model bolstering rationale for clinical evaluation of Plinabulin to improve patient outcome in multiple myeloma [9].

Dose escalation of NPI-2358 was conducted in a phase-I trial [NCT00322608 at https://clinicaltrials.gov] enrolling patients with advanced solid tumors and lymphomas who were not candidates for further standard therapy and over 18 years of age or more and were treated with a weekly intravenous infusion of NPI-2358 on days 1, 8, and 15 of 4-week cycles [10]. The study essentially used accelerated dose titration design in which the dose of NPI-2358 was escalated in cohorts from 2 mg/m^2 to a recommended phase II dose (RP2D). In total, 38 patients were enrolled with Plinabulin generally well tolerated without apparent irreversible or cumulative toxicities. Adverse events commonly ascribed to Plinabulin at RP2D included transient hypertension, tumor pain, fatigue, fever and nausea/vomiting. Although no confirmed tumor response have been reported, a notable rate of stable disease (30 % after 2 cycles) was observed with interesting outcomes in several patients. Four patients maintained stable disease for 4 months or more, including a patient with pancreatic adenocarcinoma (4 months), and 1 with hemangiopericytoma [10]. Following this trial data, another clinical trial [NCT00630110] on patients with advanced nonsmall cell lung cancer (NSCLC) with primary outcome endpoint comparing overall survival of patients treated with docetaxel to patients treated with docetaxel + NPI-2358 has been completed, but no results of this study have been posted yet. Currently patient recruitment is ongoing for a randomized phase 3 assessment of a second line chemotherapy with docetaxel plus Plinabulin compared to docetaxel alone in patients with advanced NSCLC with at least one large lung lesion [NCT02504489 at https://clinicaltrials.gov]

3 Palmarumycin CP-1

Palmarumycin was first isolated in 1994 by Krohn and coworkers as part of an screening program to isolate biologically active secondary metabolites from mycelial cultures of an endophytic fungus *Coniothyrium palmarum* [11]. Palmarumycin

CP-1 belongs to a family of unique bioactive fungal metabolites that carry a naphthoquinone spiroketal pharmacophore (Fig. 1). Palmarumycin CP1 displays diverse biological activity including antifungal, antibacterial, and antiproliferative effect although its action against tumor cells remained mostly undermined due to the high electrophilic functionality present in their spiroketal structure making it highly unstable, and issues with aqueous solubility of the compound. At molecular level, an important pharmacological target for therapeutic intervention by Palmarumycin CP-1 is inhibition of the enzyme thioredoxin reductase-1 [12, 13]. With thioredoxin reductase-1 being overexpressed in human tumors and secreted thioredoxin-1-stimulating tumor cell growth, tumor cells' sensitivity to apoptosis induction is compromised [14]. Intriguingly, despite known benchmark inhibitory activity against thioredoxin reductase-1, issues relating to solubility and stability posed a significant challenge to move Palmarumycin CP-1 or its analogs in the clinic, which has now been addressed by total synthesis of a water-soluble prodrug PX-916. This NADPH-dependent prodrug analog also retains its efficacy in irreversibly inhibiting purified thioredoxin reductase-1 most likely by reacting with the selenocysteine-containing catalytic site of the enzyme and subsequently releases the parent compound with half-life of 1 h. Of clinical interest, PX-916 do not inhibit other NADPH-dependent reductases such as human glutathione reductase and cytochrome P_{450} reductase until $\times$ 100-fold high concentration is used; and the major toxicity observed 24 h after 5 daily doses of PX-916 (25 mg/kg iv) is neutropenia and thrombocytopenia, with no evidence of elevation in plasma liver enzymes, and no significant weight loss nor any other gross toxicity being apparent [12]. Of noteworthy, when administered as a single dose to mice bearing MCF-7 breast cancer cells induced xenografts, tumor thioredoxin reductase-1 activity was inhibited up to 60 % and remained inhibited for 48 h [12]. Moreover, repeated administration of the drug for 5 days inhibited the enzyme up to 75 % when evaluated 24 h after the last dose, attesting pharmacological modulation of thioredoxin reductase-1 enzyme in tumor cells and thereby beholding PX-916 as a new therapeutic modality in cancer [12]. An extension of the study by same research group also concluded excellent antitumor activity of PX-916 in other preclinical models against A673 rhabdomyosarcoma, SHP-77 small cell lung cancer, and MCF-7 breast cancer giving the drug either through intraperitoneally (i.p.) or intravenous (i.v.) route. Of interest, with SHP-77 cells, complete tumor regressions were noted in some mice. The most active schedule optimized for PX-916 was every other day administration, and inhibition of tumor growth was seen as long as the drug was given. However, no significant antitumor activity was seen following oral administration at doses that presented i.v. antitumor activity. Another novel palmarumycin analog [8-(furan-3-ylmethoxy)-1oxo-1,4-dihydronaphthalene-4-spiro-2′-naphtho[1″,8″-de] [1′,3′] [dioxin] or SR-7 has been synthesized which blocks mammalian cell cycle transition in G2/M but not in G1 phase [15]. Clearly more studies are warranted before SR-7 or Palmarumycin PX-916 translates into clinics as antitumor therapy for cancer.

4 Rhizoxin

Rhizoxin is a 16-membered macrocyclic polyketide metabolite isolated from the fungus *Rhizopus chinensis* – the virulence factor for rice seedling blight disease which infects and threatens mainly the plant roots. Later, a series of elegant experiments revealed rhizoxin is not made by the fungus but by an endosymbiotic bacterium *Burkholderia* living within the hyphae of its host and providing the fungus with the antimitotic compound rhizoxin. Of interest is the observation that fungal strain without the bacteria is unable to manufacture rhizoxin [16]. In addition to being an important virulence factor for infection to plants by *Rhizopus* sps., rhizoxin has been found and reported very cytotoxic against human and murine tumor cells. Mechanistically, like phenylahistin, rhizoxin also binds efficiently to ß-tubulin and inhibits polymerization of the tubulin molecules, which otherwise is essential for formation of the mitotic apparatus during cell division and therefore classified as an antimitotic agent. Additionally, rhizoxin also has the capability to extensively depolymerize preformed microtubules [17]. However, unlike other fungal metabolite phenylahistine, rhizoxin binding to tubulin is independent from colchicine binding, but instead have a high binding affinity to vinka alkaloids binding site of tubulin and reportedly inhibits microtubule assembly at 10fold lower concentration (10 ng/ml) than vincristine and found more active than vincristine in P-glycoprotein-mediated vincristine-resistant murine and human cell lines (P388/VCR and K562/VCR) [18, 19]. Additionally, rhizoxin demonstrates potent cytotoxicity over an exceptionally broad range of concentrations (10^{-4}M to 10^{-13}M) in vitro in NCI tumor cell screening panel and also found effective against human tumor cell lines resistant to vinca alkaloids Vincristine and Adriamycin in vitro and in vivo [19–21]. Moreover, rhizoxin has also been evaluated in a number of human tumor xenografts and found to exhibit schedule-dependent antitumor effect. It has been concluded that repeated daily drug dosing results in superior antitumor activity compared to single dosing or less frequent repeated dosing schedule [22]. Total synthesis of an enantioselective rhizoxin D has been reported, and its palmitoyl derivative-13-*O*-palmitoyl-rhizoxin (RX-1541) has been found more lipophilic having greater in vivo antitumor activity and increased the length of survival of mouse; these results were inferred on evaluation after intravenous administration of drug to mice bearing subcutaneously inoculated M5076 sarcoma cells [23]. RX-1541 therefore seems to hold promise as a new antitumor drug in future but only after undergoing rigorous toxicity and pharmacological evaluation.

Mainly due to its mitostatic effects, rhizoxin has been the subject of several Phase I and II clinical trials. In the initial Phase-I and subsequent Phase-II studies performed by EORTC (European Organization for Research and Treatment of Cancer), pharmacokinetic profile was characterized by rapid clearance and was undetectable in blood at blood sampling beyond 30 min from drug administration following 5 min bolus infusion schedule, but just modest antitumor activity was noted, possibly due to rapid systemic clearance. To overcome this limitation and to

further exploit the potential for schedule-dependent behavior of rhizoxin, administering rhizoxin as a 72-h continuous intravenous (i.v.) infusion was evaluated [24]. Nineteen patients with advanced solid malignancy were entered into this study. Rhizoxin was administered at doses ranging from 0.2 mg/m^2 i.v. over 12 h to 2.4 mg/m^2 i.v. over 72 h every 3 weeks. The principal dose-limiting toxicities (DLT) were severe neutropenia and mucositis, and the incidence of DLT was unacceptably high at rhizoxin doses above 1.2 mg/m^2, which was determined to be the MTD and recommended dose for Phase-II study. Unfortunately in this trial, no objective antitumor responses were observed, but nonetheless, information obtained can serve as guide for further improvements in dosing schedule and tried with new isolated derivatives that are showing distinct higher effects and therefore still holds promise until clinical trials are planned with new candidate analogs of rhizoxin.

5 Epoxyquinol B

Epoxyquinol B (EPQB) is a highly functionalized pentacyclic compound grouped under epoxyquinoid class of compounds. It was isolated together with closely related epoxyquinol A and C, from an uncharacterized fungus designated as BAUA3564 from soil sample as part of a screening program by Osada and coworkers to identify novel cytotoxic agents of microbial origin. The biosynthetic mechanism of epoxyquinols has attracted considerable interest because of their structural novelty being characterized by a unique pentaketide dimer, about which biosynthetic knowledge is very limited except some indirect evidences suggesting its assembly by a polyketide synthase [25]. Interestingly, EPQB features potent antiangiogenic effects and many of the pathological conditions such as cancer, rheumatoid arthritis, diabetic retinopathy, and other chronic inflammatory diseases are characterized by extensive angiogenesis. Distinct cellular and molecular events have been identified during the various sequential steps of development of pathological angiogenesis including cell migration, proliferation, and formation of capillary tubes by endothelial cells (neovascularization). Additionally, the production and secretion of VEGF by normal and tumor cells also plays a pivotal role in subsequent hyperactivation of downstream signaling pathways of neovascularization, which conceptually can be halted either by inhibiting endothelial cell functioning or VEGF secretion and signaling pathway. Several angiogenesis inhibitors from natural products and chemical synthesis have been developed targeting endothelial cells and signaling pathways, but given that EPQB also inhibits angiogenesis, it may serve as a lead compound towards development of new and novel antiangiogenic and antitumor drug. This stimulated many laboratories to partake synthesis of EPQB from a biosynthetic precursor epoxycyclohexenone aldehyde via an electrocyclization/intermolecular Diels-Alder dimerization cascade reaction and subsequently evaluate their biological potency relevant to generate better efficacious drugs [25–27]. EPQB (10 mg/kg body wt) reportedly reduces the number of blood vessels supplying tumors and tumor volume without toxicity in Renca-cell-bearing Balb/c mice [28].

Several emerging mechanistic studies shed light that EPQB is an unselective covalent kinase inhibitor that inhibits growth factor-induced activation of VEGF receptor-2 (VEGFR2), Epidermal Growth Factor Receptor (EGFR), Fibroblast Growth Factor receptor (FGFR), and Platelet-derived Growth Factor receptor (PDGFR), thus providing an insight into EPQB molecular basis of action and for drug development relating to antiangiogenic therapy during pathophysiological events. Given that EPQB harbors highly reactive moieties two epoxides, it inhibits angiogenesis by covalently binding with nucleophiles, especially cysteine thiol residues of VEGFR2, EGFR, FGFR, and PDGFR [28]. EPQB has been found to inhibit human umbilical endothelial cell (HUVEC) migration stimulated by vascular endothelial growth factor (VEGF) in dose-dependent manner ($ED_{100} = 2.6$ μm) without presenting any significant toxicity [29]. Moreover, in addition to these proteins, EPQB inhibits NF-kB signaling through inhibition of the TGF-β activating kinase-1(TAK1) complex, a factor upstream of IKKβ and NF-kB, and only few inhibitors of TAK-1 have been reported [30]. cDNA microarray analysis reveals that EPQB inhibits the expression of TNF-α-induced genes such as NF-kB, I-kB, ICAM1, VCAM1, and E-selectin. Further, due to its reactive point, EPQB cross-links other target proteins as well mainly through cysteine residues by opening its two epoxides and may potentially inhibit several signal transduction pathways linked with proliferation and migration of tumor cells [31]. Summarizing, the actions of EPQB within tumor cells may be proposed as: (1) dual inhibition of multiple receptor kinases by binding to cysteine residues of receptor kinases and (2) intramolecular cross-linking directly with cysteine residues of multiple targets within signal transduction pathways. Thus, based on literature precedence, interest in EPQB remains elevated because of its potent biological activity.

6 Fumagillin

Fumagillin is a potent natural meroterpenoid metabolite antibiotic that was isolated in 1949 by Elbe and Hanson from soil fungus of the genus *Aspergillus fumigates* [32]. It is widely used in apiculture against nosema disease, and in human medicine, Fumagillin is the most broadly effective antimicrosporidial drug especially in patients with compromised immune system due to AIDS, to relieve symptoms of intestinal microsporidiosis after organ transplant procedures and to treat ocular microsporidial infections [33–37]. NMR and X-ray crystallographic data to characterize the structure of fumagillin revealed the presence of six stereogenic centers, a functionalized *cis* diol and two epoxides (Fig. 1) [38]. This information later prompted to investigate the mechanism of its mode of action until the discovery that fumagillin inhibits angiogenesis – indispensable for tumor growth, invasion, and metastasis in several kinds of cancer by inhibition of the endothelial cells proliferation and migration as mentioned earlier; this led interest to understand structure-activity relationships to limit the size and metastasis of tumors by blocking angiogenesis. Mechanistically, fumagillin acts against and binds to the enzyme methionine aminopeptidase-2 (MetAP-2) [39]. Methionine aminopeptidases (MetAps) are bifunctional cytosolic proteins that play a critical

role in the regulation of posttranslational processing of newly synthesized proteins by removing the amino acid methionine positioned at their amino acid terminal [40]. Overexpression of MetAP-2 plays an important role in the growth of many tumors, and overexpression of MetAP2 has been reported in many tumors such as, colon cancer, B cell lymphoma, and cholangiocarcinoma [41–43]. In lung cancer, patients' survival rate was increasingly favorable in low MetAP-2 expressing patients than in high MetAP-2 expression patients, and compared to normal lung tissue, tumor tissues had significantly higher activity [44]. Crystallography data revealed that fumagillin covalently binds to a histidine moiety (His^{231}) of the enzyme, resulting in opening of the spiroepoxide on the core skeleton of fumagillin causing a 1000-fold decrease in biological activity of MetAP-2 by opening of this cyclohexane ring spiroepoxide [39, 45]. Furthermore, fumagillin arrests endothelial cells in G1 phase of cell cycle along with suppression of cyclin E mRNA expression and protein level resulting in the inhibition of endothelial cell proliferation and motility. Microarray analysis of fumagillin-treated HUVEC shows an upregulation of 71 genes and downregulation of 143 genes that are mostly involved in cell proliferation, migration, adhesion, differentiation, and gene transcription [46]. Low dose of fumagillin treatment to SCID mice bearing colon cancer cells in the subcutis when harvested after 4 weeks of treatment had small tumor mass, fewer pulmonary metastases, and lower microvessel density relative to control group [46]. Suppression of hepatoma growth and metastasis by fumagillin has also been reported [47].

Despite initial overwhelming success in understanding the underlying biological effects of fumagillin in context of cancer therapy, potential toxic and undesirable consequences of fumagillin treatment restricted its therapeutic utility in current form. Common side effect in human clinical trials in which fumagillin was administered orally was gastrointestinal-related cramping, diarrhea, and significant loss of body weight alongside thrombocytopenia, neutropenia, and hyperlipasaemia being most frequent biological adverse events at the highest administered dose of 60 mg which later ceased after the treatment was terminated [34, 35]. Thus, to move fumagillin forward based on the knowledge gained, chemical analogs of fumagillin have been synthesized retaining similar novel mechanism of disrupting tumor vasculature by targeting the enzyme MetAp2. These include TNP-470, PPI-2458, and CKD-732. TNP-470 demonstrate antitumor activity both as monotherapy and in combination with conventional chemotherapy in numerous variety of different tumor types in preclinical models by blocking endothelial cell proliferation in vitro and angiogenesis in vivo and tried in human clinical trials. Unfortunately, one of the trials [NCT00038701 at https://clinicaltrials.gov] with the goal to assess survival and patterns of failure in patients treated with Gemzar-based chemoradiation plus TNP-470 for locally advanced adenocarcinoma of the pancreas had to be terminated because of slow patient accrual. The other trial was a Phase-I study of TNP-470 in the treatment of AIDS-associated Kaposi's sarcoma (AIDS-KS) [NCT00000763 at https://clinicaltrials.gov].This study progressed to completion and in essence concluded with the note that TNP-470 administered as a weekly 1-h infusion to patients with early AIDS-KS is well tolerated at doses up to 70 mg/m^2, which was the highest dose tested, and tumor responses were observed in a substantial number of cases at

various tested dose levels. The clinical researchers propose TNP-470 be further evaluated in patients with AIDS-KS as a single agent and in combination with other biologic response modifiers in early disease or after initial response to cytotoxic chemotherapy [48]. Unpredictably, incidence of central nervous system (CNS) and visual impairment-related adverse effects at optimal therapeutic dose became a limiting factor for its use in clinics, and thus later, PPI-2458 was designed to overcome clinical limitations of TNP-470 with greatly reduced CNS toxicity compared to TNP-470. PPI-2458 shows antitumor activity in a broad range of xenograft tumor models including melanoma, breast cancer, glioblastoma, lung, prostate, and leukemia and lymphoma. A clinical trial [NCT00100347 at https://clinicaltrials.gov] under multi-institutional collaboration was initiated to assess the safety and tolerability of escalating doses of PPI-2458 in subjects with non-Hodgkin's lymphoma (NHL) and solid tumors, but unfortunately the study got prematurely terminated because of nonscientific reasons with no outcome posted. Another semisynthetic analog CKD-732 [6-(4-dimethylaminoehoxy) cinnanoyl-fumagillol] entered into a clinical trial for the treatment of refractory solid cancer, including combination with capecitabine and oxaliplatin for the treatment of metastatic colorectal cancer in patients who had progressed despite being on irinotecan chemotherapy [49, 50].

7 Destruxin B

The Destruxin family of cyclodepsipeptide first isolated and reported by Kodiara (1961) is major small molecule secondary mycotoxin metabolite secreted by the entomopathogenic fungi *Metarhizium anisopliae* [51]. Later, isolation of Destruxin (DxB) has been reported from other pathogenic fungi such as *Alternaria brassicae* and from liquid cultures of *Ophiosphaerella herpotricha* (Fr.) Walker [52]. The structure of DxB has been confirmed comprising of an α-hydroxy acid and five amino acid residues (Fig. 1). A broad spectrum biological effects such as phytoxic, antiviral, insecticidal, antitumor, cytotoxic, and cytostatic effects have been reported, and additional forthcoming information about this metabolite is growing exponentially [53–55]. Largely due to constrain imposed to obtain relatively large amounts of DxB from natural sources needed to investigate their anticancer and biological activity, chemical synthesis of DxB under optimized conditions has provided sufficient amounts of the compound to investigate and evaluate antitumor properties and understand underlying molecular mechanism associated with its action. Of interest, there is good evidence which indicates inhibition by DxB of ubiquitous multisubunit complex proton pump-vacuolar H^+-ATPase (V-ATPase) residing in the endo- and plasma membrane of all eukaryotic and animal cells [56]; this may also be considered as attractive target for cancer therapy because of its potential involvement in tumor invasion, metastasis, and osteopetrosis and may provide basis for development of new drugs for the treatment of bone resorption in osteoporosis and cancer [57–59]. Clearly, there exists supportive experimental evidence to indicate V-ATPase inhibitors overcoming Bcl-xL-mediated chemoresistance through

restoration of caspase-independent apoptotic pathway supporting the possibility of using DxB as a therapeutic adjunct in cancer therapy [60].

Further promising in vitro and in vivo anticancer effect of DxB on human colon and hepatocellular carcinoma have been cited based on inhibition of the Wnt/β-catenin/TCF signaling pathway including invasion and migration of tumor cells coordinated by suppression of MMP-2 and MMP-9 enzymatic activities [61–63]. It is now well accepted that dysregulation of the Wnt pathway plays a critical role in the development of many human cancer including colorectal cancer and hepatocellular cancer, and thus, DxB could be an important pharmacological molecule to antagonize proliferation of tumor cells in many of these cancers expressing aberrant Wnt signaling. Experimentally, in the NOD/SCID mice xenograft model, DxB at dose of 5 mg/kg/day suppresses tumor size and burden significantly without any associated symptoms of toxicity as inferred from food and fluid consumption as well as body weight. Immunohistochemical analysis performed on harvested tumor samples for molecular targets of DxB confirmed diminished expression of β-catenin and associated molecules including cyclin D1 and survivin and downregulation of CD31 indicative of antiangiogenic effect of DxB [61]. There is also good evidence of DxB showing a strong suppressive effect on the production of the hepatitis B surface antigen in human hepatoma cells [64]. In oral cancer and non-Hodgkin lymphoma, apoptotic mechanism including programmed cell death by DxB has been projected resulting in antitumor effect [65, 66]. Finally, although there is good evidence indicating the role of destruxins in cancer therapy, further work is necessary before initiation of clinical therapy.

8 Cotylenin A

Cotylenin A was the main small bioactive effector molecule originally isolated by Sassa (1970) from culture broth of *Cladosporium* sps strain 501-7w along with Cotylenin B-I [67]. Cotylenin A displays cytokinin-like bioactivity in plants, similar to fusicoccin-A. Later, it was established that cotylenin A has a novel structure comprising of a fusicoccin-diterpene glycoside with complex sugar moiety (Fig. 1) [68]. Its stereochemistry was later confirmed by HMBC experiments and X-ray crystallography of its diacetyl-dihydro derivative which supports the observed integral interrelationship between its stereochemical conformation and biological activity [69]. Further investigations with crystallographic data and *in silico* screening analysis, a unique mode of action of cotylenin A in tumor cells came to attention. It acts as a novel stabilizer by making multiple simultaneous contact points with human cancer relevant 14-3-3 PPIs (Protein-protein interactions). PPIs and its partner proteins are reportedly involved in an array of signal transduction and regulatory pathways that lead, among other, to an upregulation of apoptosis regulating genes. Cotylenin A is also potent in stimulating differentiation and therefore evaluated from therapeutically strategic point of view to enhance sensitivity of tumor to many anticancer agents. cDNA microarray of human myeloid leukemia HL-60 cells exposed to cotylenin A revealed significant upregulation in the expression of

21 genes including rapid expression of calcium-binding protein S100P gene that induces differentiation. This gene has been found methylated in pancreatic cancer cells and currently perceived that Cotylenin A may alter the transcriptional activity of S100P promoter and possibly other promoters by its demethylation actions [70].

The differentiation-inducing activity of Cot A in several human and murine myeloid cell lines has been recorded [71–73]. It significantly stimulated both functional and morphological differentiation in primary cultures of 9 out of 12 cases of leukemia cells that were freshly isolated from acute myelogenous leukemia (AML) patients, earmarking Cot A as a candidate agent for inducing differentiation in AML [73]. Further, the therapeutic efficacy of Cot A was also proven in experimental preclinical model of leukemia. Administrating cotylenin A significantly extended survival of SCID (severe combined immunodeficiency) mice inoculated with retinoid-sensitive and retinoid-resistant human acute promyelocytic leukemia (APL) cell line-NB4 without any noticeable adverse effects. This attests that cotylenin A may be useful in therapy for leukemia and some other malignancies, as well [74]. Additionally, a rational combination of cotylenin A and Interferon-α (IFN-α) has also been evaluated for synergism in solid human nonsmall cell lung cancer (NSCLC) preclinical models [75]. The combined treatment induced apoptosis in cancer cells while sparing normal lung epithelial cells and significantly inhibited the growth of human lung cancer cells as xenografts without any apparent adverse effects supporting potential therapeutic advantage of this combination. In an investigated xenograft mouse model, PC14 lung cancer cells were inoculated into athymic nude mice and given a daily subcutaneous (s.c.) injection of 3×10^4 IU of IFNα, and/or s.c. injections every other day of 100 μg of cotylenin A (6.7 mg/kg body weight) at a site distant to the tumors; the first injection was initiated 7 days after the inoculation of tumor cells. As predicted above, significant inhibition in growth of PC14 cells was noted in combination group relative to monotherapy groups as xenografts. An extension of the study also included an arm wherein treatment was continued for 12 days and then stopped, with a follow-up on day 26. All of the untreated mice had a large tumor burden at day 26. On the other hand, >50 % of the treated mice escaped from the disease (13 of 20 mice), and the rest had only a small tumor burden, suggesting and confirming that the therapeutic effects could still persist after the termination of treatment. These results indicate that the combination of cotylenin A and IFNα is more effective therapeutically than treatment with cotylenin A or IFNα alone, and the combined treatment carries a significant therapeutic advantage as antitumor effect.

Cotylenin A plus IFNα also regresses the growth of drug-resistant ovarian carcinoma SK-OV3 and OVCAR-3 cells as xenografts [76]. Furthermore, Rapamycin-a macrolide fungicide with immunosuppressive properties and currently of significant interest having exhibited promising antitumor effect in several types of refractory tumors including breast cancer. It has been found that rapamycin and cotylenin A cooperatively induce growth arrest of breast cancer cell MCF-7 in vitro and tumor cell xenografts in vivo [77]. Further, cotylenin A has also been described to enhance arsenic trioxide (ATO)-induced anticancer activity in experimental human breast cancer even suppressing their invasive behavior [78]. Investigations into synthesis of new analogs are still ongoing, but nothing exciting has come up yet in literature.

9 Myriocin

Myriocin [Synonyms ISP-1, Thermozymocidin] is a novel amino fatty acid [2S-amino-3R,4R-dihydroxy-2-(hydroxymethyl)-14-oxo-6E-eicosenoic acid] anti-fungal antibiotic originally isolated from the culture filtrates and mycelium of the thermophilic Ascomycete, *Myriococcum albomyces* in 1972 [79]. It was later also isolated from extract of the fruiting body of entomopathogenic fungus *Isaria sinclairii* which is native to Asia, mainly China, Korea, and Japan, and from the culture broth of certain other thermophylic fungi such as *Mycelia sterilia* [80]. Myriocin is a potent inhibitor of serine palmitoyltransferase (Ki = 0.28 nM), the enzyme that catalyzes the first committed step in de novo biosynthesis of major class of bioactive sphingolipid with ceramide as the final metabolite turnover. There exists ample evidence implicating members of the sphingolipid family especially sphingosine-1-phosphate (S1P) and sphingosine-1-phosphate receptors 1–5 (S1PR) involvement in the oncogenic transformation, dysregulation of cell proliferation, and resistance to apoptotic cell death as recently reviewed [81, 82]. Accordingly, pharmacological manipulation of sphingolipids content in tumor cells is emerging as a potential target for cancer therapy. Myriocin exerts a strong fungistatic effect against yeast and dermatophytes, antiviral activity (including influenza, hepatitis B, and hepatitis C viruses) and a potent immunosuppressant activity having 10–100-fold more activity than cyclosporin A, but its poor solubility and high toxicity limit its usage in clinics over that of cyclosporin A [83, 84]. Thus, to improve physical characteristic (e.g., solubility) and biological properties including improved activity and toxicity profile of Myriocin, structure-activity guided studies lead to the emergence of an preclinical analog of interest-Fingolimod (FTY-720; trade name Gilenya by Novartis Pharma) that was later approved by US Food and Drug Administration in September 2010 as a new drug for Multiple Sclerosis. Unlike Myriocin, FTY-720 does not interfere with sphingolipid biosynthesis but rapidly converted to FTY720-phosphate (FTY720-P) by sphingosine kinase 2 in vivo and FTY720-P acts as a potent agonist at sphingosine-1-phosphate receptor (S1PR) pathway [85, 86]. Hereafter, FTY-720 emerged as a key player in clinical use and have shown strong preclinical antitumor efficacy in vitro and in vivo across a broad range of malignancies including breast, glioblastoma, prostate, lung, cholangiocarcinoma, gastric, pancreatic, colon, bladder, ovarian, and hematopoietic malignancies either as single agent or in combination with other drugs showing better clinical outcome establishing its value as an potential therapeutic drug [87, 88, 89]. As an example, administration of FTY720 at 10 mg/kg/day reduces the growth of androgen independent prostate CWR22R xenografts in castrated nude mice [90]. Also, FTY-720 enhances the radiosensitivity of prostate cancer cells overexpressing miR-95-microRNA associated with resistance to radiation [91]. Likewise, the combination of FTY720 and radiation affected sphingosine kinase-1 inhibition and tumor suppression in a mouse xenograft model of prostate cancer [92].

Additionally, inhibition of migration and invasion of tumor cells, such as those of prostate, glioblastoma, hepatocellular carcinoma, pancreatic, and cholangiocarcinoma following FTY-720 treatment has been recorded [93–97]. To cite an

example in a preclinical investigative model, tumor cells were implanted into the peritoneal cavity of nude mice followed by FTY-720 treatment. On necropsy after 4 weeks, in control group the tumor cells had extensively colonized the visceral organs and formed multiple metastatic nodules, whereas in the treated mice the number of metastatic nodules was found significantly reduced attesting antimetastatic efficacy of the drug without any overt toxic side effects [98]. Furthermore, FTY-720 has been shown to inhibit angiogenesis and found to reduce the migration of human umbilical vein endothelial cells (HUVEC) [99]. The IC50 values of Fingolimod tested in different tumor cell lines vary between 5 and 20 μM. In general, the anticancer effects of FTY720 are reportedly mainly attributed to its cytotoxicity towards cancer cells through direct mitochondrial damage (caspase-dependent, caspase-independent, or autophagic cell death pathways) [100, 101]. Moreover, in most instances, phosphorylation of FTY720 is not required for its cytotoxic effect, but instead numerous molecular targets have been proposed for the unphosphorylated form of FTY720 including ROS, PP2A, cyclin D1, SphK1, dephosphorylation of Akt, and 14-3-3 proteins acting in concert to suppress cell growth and induce cell death in a variety of cellular settings [88, 102]. An interventional phase-I trial [NCT02490930 at https://clinicaltrials.gov] is currently recruiting patients to evaluate whether Fingolimod can be safely combined with radiation and temozolomide in newly diagnosed high grade glioma patients.

Further to improve clinical activity against a variety of human malignancies, synthetic second generation of FTY-720 compound has been developed – (1) nonimmunosuppressive analogs that lack sphingosine-1 receptor binding capability (OSU-2S and AAL-149) and (2) derivative with enhanced sphingosine kinase inhibition (S-FTY720 vinylphosphonate and (R)-FTY720 methyl ether (ROME) [103–105]. These analogs demonstrate either greater or equal potency like FTY-720. None of these analogs have yet entered into clinical trial.

10 Cytochalasin E

As member of a complex and diverse cytochalasins (Greek cytos, cell; chalasis, relaxation) group, Cytochalasin E is a distinct epoxide-containing cell-permeable mycotoxin isolated as a minor secondary metabolite from the food storage mold *Aspergillus clavatus*. Later, it was confirmed as produced by a range of fungi [*Alternaria chlamydospora, Cochliobolus tuberculatus, Rhinocladiella sps., Rosellinia nectaris and Helminthosporium demantiodeum*] and its production under different fermentation conditions has been reported [106].

Cytochalasin E has been established functioning as a novel inhibitor of angiogenesis and tumor growth [107]. Under experimental conditions, it inhibits capillary endothelial cells proliferation attributable to its distinctive structural group element, the epoxide group. In an in vivo mouse corneal neovascularization model, cytochalasin E inhibits bFGF- and VEGF-induced angiogenesis by approximately 50 % [108]. Cytochalasin E has also been recorded efficacious in inhibiting the growth of Lewis lung tumors in mice by approximately 72 % [107]. Being a cell-permeable

toxin, some other well-characterized biological effect of cytochalasin E has been recorded including depolymerization of actin filaments resulting in impairment of cytokinesis during cell division affecting cell growth and cell migration and cell cycle G2/M phase arrest [109, 110]. These are of considerable clinical interest since it prevents the actively proliferating tumor cells to complete cytokinesis and their sensitivity, especially with different treatment modalities (chemo- and/or radiotherapy), and may elicit synergistic response. A rapid and sustained elevation of intracellular free Ca^{2+} in B lymphocytes by increasing the extracellular Ca^{2+} influx has also been recorded for cytochalasin E [111]. In preclinical evaluation for antiglioma therapy, cytochalasin E has been found to efficiently arrest glioblastoma cell growth using a concentration below 1 μM by inducing cell cycle G2/M phase arrest and apoptosis [112]. Intriguingly, against the SKOV3 ovarian cancer cell line, cytochalasin E has been found less cytotoxic than current clinically approved antineoplastic agents such as doxorubicin, paclitaxel, and vinblastine [113]. Thus, development of novel analogs of cytochalasin E need to be explored for future clinical applications with acceptable tolerable dose in human.

11 Chaetocin

Chaetocin is a natural antimicrobial fungal mycotoxin metabolite originally isolated from fermentation broth of *Chaetomium minutum* and has recently shown promise as an antitumor agent. Structurally, chaetocin belongs to the class of 3–6 epidithioketopiperazines and exists as molecular dimer of two five-membered rings cis fused (Fig. 1). Mechanistic studies reveal chaetocin exerts multiple actions in cancer cells resulting in significant antiproliferative activity against a wide variety of tumor cell lines and therefore attracts much attention in the field of cancer therapeutics.

Published studies attest chaetocin as a drugable epigenetic agent by virtue of its nonselective potential to affect methylation status of Lysine 9 on histone H3 (H3K9) mediated by specific inhibition of the Lysine-specific histone methyltransferase SU (VAR)3-9 [IC50 = 0.8 μM] [114]. Molecular insight reveal histones are methylated at many lysine and arginine residues, and histone lysine methyltransferases (HKMTs) have been envisioned as an important class of targets for epigenetic therapy [115, 116]. Several other targets are also emerging relating to chaetocin action on tumor cells. There are reports that chaetocin inhibits the molecular chaperone heat shock protein 90 (Hsp90) and SUV3-9H1 is a novel client protein of Hsp90 [117]. Additionally, inhibition of SUV3-9H1 by chaetocin reportedly also affects several important steps in metastasis process such as migration and invasion of cells [118, 119].

Recently, it has been reported for hepatocellular carcinoma that cumulative recurrence rate is significantly higher for patients with elevated SUV3-9H1 expression and Histone H3 lysine 9 trimethylation (H3K9me3) [120]. The antitumor efficacy of chaetocin was therefore investigated and validated in vivo by xenograft transplantation of HCC (Huh1/Huh7) cells into NOD/SCID mice. Both tumor initiation and subcutaneous tumor growth were suppressed by chaetocin treatment

in a dose-dependent manner at 8 weeks after transplantation and immunohistochemical staining of tumors revealed chaetocin remarkably reduced H3K9me3 levels, Ki-67, and apoptotic markers compared with control [120]. In acute myeloid leukemia (AML), pharmacological inhibition of SUV3-9H1 by chaetocin induces apoptosis in leukemia cell lines in vitro and primary AML cells ex vivo and delayed leukemia growth in vivo [121–123]. Other researchers have confirmed chaetocin and other HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA, an approved drug for cutaneous T-cell lymphoma) and trichostatin A, generate potent cytotoxicity to leukemia cells derived from patients [121, 124]. Similarly, another small molecule epigenetic inhibitor JQ-1 exhibited synergistic cytotoxicity with chaetocin [121].

One of the molecular entities commonly seen silenced in many malignancies including AML is TSG-SOCS1 expression, and chaetocin by suppressing H3K9 methylation on SOCS1 promotor upregulates SOCS1 expression signifying a novel foresight into AML therapy [125]. Additionally, a close coupling between other Lys9-specific HMTs including G9a [and its related molecule G9a-like protein which exists as G9a/GLP complex] and DIM5 has been reported to be altered by chaetocin. An important biological role of G9a/GLP complex is cell proliferation and upregulation of G9a have been reported in many solid tumors such as breast cancer, lung cancer, colon cancer, and prostate cancer [126–128]. An oncogenic role of this methyltransferase in AML has been suggested and with all pragmatic rationale, the usefulness of chaetocin either as monotherapy in therapeutic regimen or combination with other established drugs merits consideration and attention for beneficial effect.

Chaetocin affects other cell survival-related molecular targets relevant to tumor pathogenicity. It induces apoptosis via caspase-8/caspase-3-mediated pathway in vitro and in vivo as exemplified in a myeloma mouse xenograft model [129, 130]. Additionally, chaetocin has also emerged as a potent inducer of cellular oxidative stress, due in part, by its capacity to compete with thioredoxin (Trx) which is the native substrate of the oxidative stress mitigation enzyme thioredoxin reductase-1 (TrxR1). TrxR1 and/or Trx are known to be upregulated in a variety of human cancers, including lung, colorectal, cervical, hepatic, and pancreatic [131, 132], and Trx overexpression has been linked to aggressive tumor growth and poorer prognosis [133, 134]. As the TrxR1/Trx pathway limits the generation of cellular reactive oxygen species (ROS), it advantageously favors tumor cells with growth and/or survival advantage. Worthily, with redox targeting potential of chaetocin, it imposes within tumor cells ROS stress leading to cell death upholding as a promising candidate in therapeutic strategy of solid tumors [135, 136]. In preclinical setup, redox targeting by chaetocin and its antineoplastic effects have been presented in glioblastoma model wherein ROS-mediated apoptosis by chaetocin results in reduction of tumor burden in glioma xenografts [137]. Another important target for observed anticancer effect of chaetocin relates to inhibition of hypoxia-induced increased production of the angiogenic mediator VEGF by tumor cells, and consequently inhibition of migration and proliferation of the endothelial cells ensues directly; cumulatively this action inhibits tumor growth by reducing angiogenesis at the tumor microvasculature level [138].

12 Apicidin

Apicidin is a novel nonribosomal cyclic tetrapeptide (IUPAC: cyclo(*N*-*O*-methyl-l-tryptophanyl-l-isoleucinyl-d-pipecolinyl-l-2-amino-8-oxodecanoyl) isolated as a metabolite from endophytic fungi (*Fusarium pallidoroseum*) from twigs collected in Costa Rica, at Merck Sharp Laboratory, Rahway, New Jersey [139]. Underlying its effect against several protozoan and parasite sps. is the structure of this compound containing an unusual ethyl ketone site as potential zinc binding group (ZBG), a long alkyl chain, and the cyclic tetrapeptide that interacts with the surface of the HDAC (histone deacetylase) and inhibits both mammalian and protozoan histone deacetylases (HDACs) [140–142]. Apicidin is ranked as a relatively potent HDAC-1 inhibitor (IC_{50} in cervical cancer HeLa cells = 290 nM) and displays good antiproliferative activity against several human cancer cell lines and therefore, considered as promising group of pharmaceutically active compound and cancer treatment agent. Additionally, a succession of multitargeted antineoplastic actions of apicidin in tumor cells has been described. It targets oxidative phosphorylation for apoptosis induction and arrests cancer cell growth through selective induction of p^{21}WAF-1/Cip1 and gelsolin which controls cell cycle and cell morphology, respectively [143]. In human acute promyelocytic leukemia cells (HL-60) and Bcr-Abl-positive leukemic cells, apicidin transiently increases the expression of Fas-Fas ligand resulting in the release of cytochrome c from mitochondria and subsequent activation of caspase-3 and caspase-9 culminating in apoptosis [144]. Deregulated *ras* activation is a common genetic defect in human cancers. Apicidin significantly inhibits H-*ras*-induced invasive phenotype of MCF10A human breast epithelial cells in parallel with specific downregulation of matrix metalloproteinase-2, implying the potential worth of apicidin as inhibitor of invasion and metastasis in cancer therapy [145]. In a related context, CXCR4 activation directs migration towards the specific ligand CXCL12, and CXCR4 activation affects proliferation and migration through ERK, Akt phosphorylation, c-Src phosphorylation, and JAK-STAT pathway. Apicidin indirectly impairs migration-related molecular signaling events by circumventing the activation of STAT-3 and c-Src phosphorylation, and these have shown an effect on migration of human renal, nonsmall cell lung cancer (NSCLC) cells and glioblastoma cells suggesting its possibility in delaying or preventing the metastatic process in solid tumors [146, 147]. Since estrogen receptors play an important role in control of proliferation in breast cancer, apicidin downregulates ERα expression in ER-α-positive human breast cancer cells (MCF-7 cells) along with modulation of cyclin D1 expression and Bax/Bcl2 expression causing G1 phase cell cycle arrest and apoptosis probably in a way associated with ER-α-mediated transcriptional regulation [148]. In pancreatic cancer, MUC-4 has been linked with resistance to gemcitabine therapy and with apicidin reportedly significantly reducing the expression of MUC-4, and its transcription factor hepatocyte nuclear factor 4α appears to be a novel antiproliferative agent against pancreatic cancer cells [149]. In several cervical cancer cells (HeLa, CaSki, and C33A), apicidin upregulates the expression and protein level of hypoxia-inducible factor prolyl 4-hydroxylase PHD2 negating stimulus for angiogenesis and tumor progression [150]. Furthermore,

studies aiming at combination strategies that exploit the unique activity of apicidin as deacetylase inhibitor have been carried out in range of solid tumors and leukemia and lymphomas with common outcome pointing greatly enhanced antitumor activity in combination group. The drugs evaluated in combination screening include docetaxel in breast cancer, proteasome inhibitors in colon cancer, gemcitabine in pancreatic cancer, doxorubicin in hepatocellular carcinoma, imatinib, and TRAIL in leukemia cells and antiviral agents in lymphoma [149, 151–156]. Thus, clearly, the role of apicidin in cancer therapy cannot be underestimated and hopes remain high for its translational output in the future.

13 Galiellalactone

Galiellalactone (GL) is a hexaketide fungal metabolite isolated from strains of *Galiella rufa* (Sarcosomataceae, Ascomycota) in the course of screening for plant growth-regulating compounds and later from two unidentified fungi that was shown by their 18S rDNA sequences as belonging to the Sarcosomataceae family. Sarcosomataceous fungi are known mainly as degraders of wood or as pathogens [157, 158]. GL has not yet been found in any fungus outside the Sarcosomataceae despite thorough screening program, and thus its presence is currently deemed as a chemotaxonomic marker of the Sarcosomataceae family [159]. GL structure has been determined by X-ray crystallography (Fig. 1) [160] and found to contain a reactive α, β-unsaturated lactone which functionally enhances its affinity towards biological nitrogen- and sulfur-nucleophiles including cysteine to produce inactive adducts [161, 162]. GL has also been synthetically produced [163].

GL was initially evaluated for cytotoxicity in few representative human liquid and solid tumor cell lines that included HL-60, L1210, HeLa S3, and COS7 and its potency was found × 10 times higher in suspension cells compared to monolayer cell lines with median IC_{50} values varying in the range between 2.0 and 0.01 μM [164]. GL mechanism of action has been characterized as an inhibitor of signal transducer and activator of transcription-3 (STAT-3) signaling by binding directly to STAT-3 and blocking the binding of STAT-3 to DNA transcriptional elements. STAT-3 is a transcription factor that plays a key role in normal cell growth and is constitutively activated in about 70 % of solid and hematological cancers. Development of potent and selective inhibitors targeting STAT3 is of interest to oncologist since persistently activated STAT-3 also plays a pivotal role in metastasis and angiogenesis and development of therapy resistance [165]. Mass spectrometry analysis of recombinant STAT-3 protein pretreated with GL revealed modifications at three different sites within cysteines – Cys-367, Cys-468 and Cys-542 [166]. Thus, GL being a potential inhibitor of cysteine reactivity covalently binds to one or more cysteine(s) in STAT-3 leading to inhibition of STAT-3 binding to DNA and thus blocks STAT-3 signaling without affecting either tyrosine or serine phosphorylation. In addition, GL has also been shown to inhibit NF-kB and TGF-β signaling, preventing the association of p65 with the importin α3 and inhibiting the binding of the activated Smad2/3 transcription factor to DNA [167, 168]. STAT-3

being a common feature of progression in prostate cancer, GL has been researched in preclinical models as a potential therapeutic candidate against hormone-refractory prostate cancer. Nude mice bearing subcutaneously developing prostate tumor xenograft when subjected to daily intraperitoneal injections of GL for 3 weeks suppresses the xenograft growth of tumor along with reduction noted in the relative mRNA expression of antiapoptotic Bcl-xL and Mcl-1 in vivo [169]. Another recent study mentions GL effectively reduces the growth and metastatic spread of androgen-insensitive prostate tumor cells in a orthotopic xenograft mouse model; mouse that were treated with GL (dose: 5 mg/kg b wt ip daily; and initiated after 3 weeks of cell implantation and continued for up to 6 weeks) had comparatively smaller primary tumors and early metastatic dissemination was significantly reduced compared to the control group, attesting GL capacity to reduce the primary tumor growth and metastasis in vivo primarily by reducing proliferation of primary tumor and apoptosis of tumor cells following treatment with GL [170]. Furthermore, there are reports associating GL inhibiting dose-dependently the growth of stem cell-like ALDH-positive cancer cells, and inducing cell cycle arrest and apoptosis through the ATM/ATR pathway in prostate cancer [171, 172]. Thus, GL could be a promising therapeutic compound and serve as a promising lead structure for development of new and potent analogs against hormone-refractory prostate and other cancers as well, which are associated with poor prognosis and mean survival.

14 Conclusion

Summing up the aforementioned write-up, the findings support a favorable foreseeable premise that fungal metabolites can surge as unique pharmacological compounds with perceived antitumor effects. The metabolites have additional capability of targeting core regulatory pathways in cancer cells, sparing normal cells, and tissues. However, fungal metabolites need to undergo rigorous quality control and dose escalating pharmacological evaluation before human trials become initiated on cancer subjects. In clinical trials, pharmacologically relevant metabolite compound may be utilized as monotherapy, or used as adjuvant agent in cancer therapy. Furthermore, high throughput screening strategies for fungal metabolite library screening need to be standardized since new metabolites are being continuously discovered. Also, the availability of complete genome sequence of fungi producing promising metabolites will facilitate large scale production under experimental condition or cloned for large scale production rather than depending on classical culture medium for their isolation.

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Application of Fungal Metabolites Against Mycotoxins Production 21

Carol Verheecke, Elodie Choque, and Florence Mathieu

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Abstract

Mycotoxins are toxic substances produced by fungi that contaminate various food and feedstuffs. There are about 100 different types of mycotoxins which are produced by a wide range of fungal species. The variety of their toxicity is linked to the diversity of their chemical structure. Amongst them, three biosynthesis origins are mostly studied: the polyketides (e.g., aflatoxins, fumonisins), the terpenes (e.g., trichothecenes), and the ergot alkaloids (e.g., ergotamine). In this chapter we present those biosynthetic origins and focus on the mycotoxins

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threatening human health. Their biosynthesis, producing fungi, toxicity, and regulation are succinctly presented. In the second part of the chapter, we focus our attention on fungal metabolites as a potential source of biocontrol, being antifungal, impacting both fungal growth and mycotoxins production and preventing mycotoxins biosynthesis. We finally conclude on the wide diversity of mycotoxins origins and the need to pursue the discovery of new fungal metabolites to counteract mycotoxins production.

Keywords
Fungal metabolites • Mycotoxins biosynthesis • Polyketides • Terpenes • Alkaloids • Mycotoxin biocontrol

1 Introduction

Among the diversity of fungal metabolites, some are potentially dangerous such as mycotoxins. According to the Collins dictionary, mycotoxins are "any of various toxic substances produced by fungi, some of which may affect food and others of which are alleged to have been used in warfare." The word mycotoxin comes from the ancient Greek word "mykes" which means mushroom and the Latin word "toxicus" which means poison [1]. Bennett and Klich [9] elaborated a more precise definition of mycotoxins:

(i) Low molecular weight molecules;
(ii) Secondary metabolites produced by filamentous fungi;
(iii) Which can cause death or disease to human being or animal at a low concentration.

The first human health disease associated with mycotoxins was the ergotism with major outbreaks during the Middle Ages. In the most severe cases, a leg-necrosis appeared. This illness was the result of eating bread polluted by "ergot." This "ergot" was produced by *Claviceps purpurea* in the rye used for the bread flour [2].

The first outbreak leading to the development of mycotoxins research field happened at the end of 1959; peanuts from Brazil were imported in England to be used as protein supplements in farming feeds. Following that, young turkeys began to die and other animals like pigs developed symptoms. This disease killed 100,000 turkey poults and was called "turkey X disease," with the "X" being for its likeness to a viral-origin illness [3]. A short time after this epidemic, aflatoxins were identified as the source of this intoxication [4]. Indeed, 56 years after, the aflatoxins are still of great concern as they are currently the only mycotoxins validated as human carcinogens by the International Agency for Research on Cancer (Group 1, IARC) [5]. The carcinogenic effect was validated by many cohort studies conducted in China on the incidence of aflatoxins on hepatocellular carcinoma (HCC) occurrence. Blood and/or urine samples were taken from more than 43,000 persons (aflatoxins biomarkers were

quantified). Exposure to aflatoxins led to a 2.4 to 5.5-fold increase of HCC occurrence [6–8].

With the development of the mycotoxins research field, other mycotoxins were identified and currently 300–400 mycotoxins are known, among which 30 have been studied for their toxic and/or disturbing impacts for human and animal [9, 10].

Those mycotoxins are produced mainly by the *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* genera. Those genera are known as common food-borne spoilers. Thus, the *Aspergillus* genus is known to be tolerant of elevated temperatures and reduced water activities [11]. As such, there are only few food commodities spared by *Aspergillus* spp. and it is the predominant food-spoiler in the tropics. *Aspergillus* spp. are known producers of many mycotoxins of great concerns including aflatoxins, ochratoxin A (OTA), and citrinin. These mycotoxins can be detected in a wide variety of food commodities including cereals, grapes, nuts, coffee, cocoa, and spices [12–16]. The *Fusarium* genus is known for its plant pathogenicity and the wide range of associated plant diseases. It is known to be predominant in temperate areas. The *Fusarium* spp. are the major producers of mycotoxins. Among these, trichothecenes (e.g., deoxynivalenol, T2, and HT2), zearalenone, and fumonisins are the most studied. These mycotoxins can be detected in many food commodities including cereals [17]. The *Alternaria* genus is starting to be known as a mycotoxin producer. It is a competitor of *Fusarium* genus in the temperate areas. The *Alternaria* spp. are producing "emergent" mycotoxins such as alternariol and is detected mainly in fruits and vegetables [18]. The last but not least is the *Penicillium* genus: a predominant food-spoiler in the temperate and cold areas. The *Penicillium* spp. are known to produce many mycotoxins including patulin and OTA. These mycotoxins can be detected in a wide range of food commodities including cereals and apples [19, 20]. Due to their diverse chemical structures and origins, mycotoxins are very hard to classify. They can be arranged according to their chemical structure, toxicity, biosynthetic origin, and/or producing fungi. Hereafter, we will summarize the different mycotoxins depending on their biosynthesis pathway and then focus on their toxicity and regulation.

To reduce mycotoxins occurrence all along the agrofood chain (e.g., field, storage, process), two complementary approaches exist. On one hand, solutions for the prevention of mycotoxins production are developed: the management of abiotic (water activity, temperature, CO_2, etc.) and the management of biotic (fungal or bacterial interactions and metabolites) parameters. On the other hand, decontamination techniques are developed: process optimization, chemicals addition (e.g., ammoniation or ozone), sorbents addition (e.g., bentonite), or application of degrading organisms [21, 22].

In this chapter, a section will be focused on fungal metabolites used to prevent mycotoxins production and their modes of action. They can be classified into three types: (i) fungal metabolites with antifungal properties; (ii) fungal metabolites impacting both fungal growth and mycotoxins production, and (iii) metabolites preventing mycotoxins synthesis.

The purpose of this review is first: to highlight the diversity of mycotoxins biosynthesis. And secondly, present the use of fungal metabolites as a potential

preventing methods as biocontrol against fungal growth and/or mycotoxins production.

2 Mycotoxins: Diversity in Their Biosynthesis

There are about 100 different families of mycotoxins which are produced by a wide range of fungal species. The variety of their toxicity is linked to the diversity of their chemical structure and so of their biosynthesis.

2.1 Polyketide-Based Biosynthesis

Polyketide synthases (PKS) are enzymes required for the production of many polyketides in plant, bacteria, and fungi. Among those latter, different fungi were found to be involved in mycotoxins production.

These multimodular enzymes are at least composed of three main domains: the β-ketosynthase (KS), acyl-transferase (AT), and acyl-carrier protein (ACP) domains. The KS module initiates the polyketide production by the condensation of the acetyl-CoA molecule and a malonyl-CoA. The latter is provided thanks to the loading realized by the AT domain. The potentially iterative transfer between the AT and KS module is realized by the ACP domain which will move the molecule in shaping from substrate to product and vice versa. These enzymes can also possess optional domains. The three main optional domains are: β-ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains.

These enzymes are going to be classified according to their reducing functions depending on the absence or the presence of the reduced domains. The highly reducing PKS (HR-PKS) are PKS containing all the main domains (KS, AT, and ACT) and all the additional domains (KR, DH, and ER). The nonreducing PKS (NR-PKS) are PKS containing all the main domains (KS, AT, and ACT) and none of the additional domains. The partially reducing PKS (PR-PKS) also contains the main domains and a part of the additional domains (KR, DH, or ER).

Moreover, apart from being classified by their reducing property, PKS are also divided into three types:

Type I: large enzymes with multiple functional domains only active once during the biosynthesis (bacteria and fungi)

Type II: a complex of several single module proteins with separated enzymatic activities, acting iteratively to produce a polyketide (bacteria)

Type III: a single active site enzyme which acts repeatedly to form the final product; they function as homodimers and do not include an ACT protein domain (mainly in plants)

PKS required for mycotoxins are solely belonging to the type I PKS. Many of the regulated mycotoxins are produced thanks to one or more PKS acting at the first step

Table 1 The main mycotoxins originating from polyketide synthase biosynthesis [23]

Mycotoxins family	PKS type	Structure	Major representative
Aflatoxins	NR-PKS	3 furans and 1 coumarin	Aflatoxin B_1
Citrinin	PR-PKS	Dihydroisocoumarin	Citrinin
Fumonisins	HR-PKS	20 carbons chain coupled with an acid ester and an acetyl amino acid	Fumonisin B_1
Ochratoxins	PR-PKS	Dihydroisocoumarin coupled with an L-phenylalanin	Ochratoxin A
Patulin	PR-PKS	Polyketide lactone	Patulin
Zearalenone	NR and HR-PKS	Acid resorcyclic lactone	Zearalenone

HR highly reducing, *NR* nonreducing, *PR* partially reducing, *PKS* polyketide synthase

of their biosynthesis. For example, aflatoxins are produced thanks to AflC, a NR-PKS required for acetate conversion into the norsolorinic acid (NOR): the first stable precursor of aflatoxin. The different types of PKS and their associated mycotoxin family, the core structure of the produced polyketide, and their major representative are represented in Table 1.

Hereafter, we will describe the main PKS-dependant mycotoxin families. Each time, the PKS involved will be first presented followed by the structure of the polyketides produced, their producing fungi, their toxicity, and their regulation especially in EU.

2.1.1 Aflatoxins

The NR-PKS AflC is needed for aflatoxins production condensation [24] (Fig. 1, step 1). The association of AflA, AflB, and AflC constitutes a complex, called NorS, of 1.4×10^6 Da (partially purified in *A. parasiticus*). The first role of NorS is the synthesis of a hexanoyl primer thanks to the addition of two malonyl CoA units. This primer is then transferred to the acyl carrier or β-ketoacyl synthase domain of AflC [24]

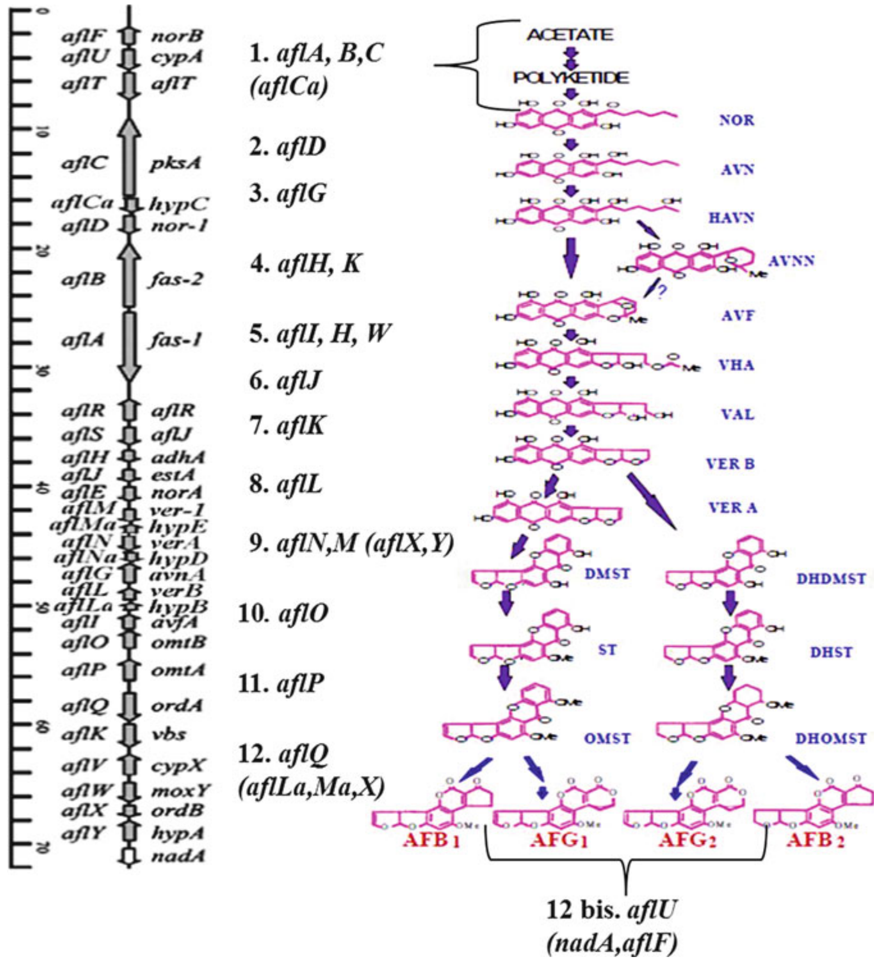


Fig. 1 The genes cluster of the biosynthesis pathway of aflatoxin in *A. parasiticus* [26, 27]. New gene names are labeled on the *left* and old gene names are labeled on the *right* of the cluster. Number *1–12* and their associated genes (predicted genes in brackets) represent the identified genes of aflatoxin biosynthesis. *NOR* norsolorinic acid, *AVN* averantin, *HAVN* 5′-hydroxy-AVN, *AVNN* averufanin, *AVF* averufin, *VHA* versiconal hemiacetal acetate, *VAL* versiconal, *VERB* versicolorin B, *VERA* versicolorin A, *ST* sterigmatocysin, *DMST* demethylST, *DHDMST* dihydroDMST, *DHST* dihydroST, *OMST* *O*-methylST, *DHOMST* dihydro-OMST

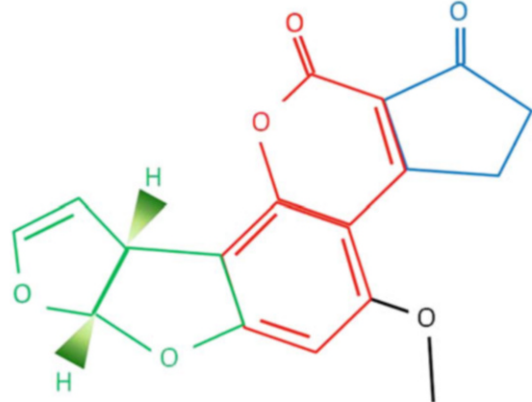


Fig. 2 2D representation of AFB1 structure

and is converted into Noranthrone (NAA) by the iterative addition of seven other Malonyl CoA units. This intermediate is not stable in time and is converted (spontaneously or by AflCa [25]) into NOR: the first stable intermediate of the aflatoxin biosynthesis.

Four aflatoxins are produced by *Aspergillus* spp. Those mycotoxins are divided into two types based on their fluorescence: the "B" aflatoxins (AFB) with a violet-blue fluorescence (445 nm) and the "G" aflatoxins (AFG) with a green fluorescence (455 nm). The AFB are made of AFB1 and AFB2. The chemical structure of AFB1 represented in Fig. 2 is based on a coumarin group (in red) attached to a bisfuran ring (in green) and a pentan group (in blue). AFB1 molecular weight is 312 g.mol^{-1}. Unlike AFB1, the AFB2 structure does not have a double bond in the bisfuran ring. The AFG chemical structure is close to the B's, with the same coumarin and bisfuran ring. The difference is that AFG have a furan group where AFB aflatoxins have a pentan group. The distinction between AFG1 and AFG2 is the same as between AFB1 and AFB2.

Flavi, *Ochraceorosei*, and *Nidulantes* are the three sections of *Aspergillus* producing aflatoxins. Among *Flavi* section, the members biosynthesize AFB and sometimes AFG. The predominant AFB producer is *A. flavus.* The second predominant producers are *A. parasiticus* and *A. nomius* which produce AFB and additionally AFG [28, 29]. AFG production by species belonging to section *Ochraceorosei* and *Nidulantes* are not currently described. Moreover, the production of AFB1 and AFG1 was recently reported by Schmidt-Heydt et al. (2009) in *Fusarium kyushuense* [30].

The entire aflatoxins gene cluster is a 75 kb cluster (29 genes) located in the subtelomeric region of chromosome 3 (represented in Fig. 2) [31]. They encode all the required enzymes that convert malonyl CoA and acetyl CoA into aflatoxins. This pathway is regulated by two specific (AflR, AflS) transcription regulators contained in the cluster. The aflatoxin excretion system is made by the primary metabolism [32]. Both *A. flavus* and *A. parasiticus* have this cluster in the same gene order. There is a slight difference between the two though. *A. flavus* has a deletion in the cluster from 0.8 to 1.5 kb depending on the isolate (5′ ends of *aflF* and *aflU*) and this is the reason why *A. flavus* does not produce AFG [31].

The toxicity of aflatoxins has already been broadly studied [33–35]. Since 2012, those aflatoxins are considered as carcinogen for humans (Group 1 [5]). The main target organ is the liver [33]. Exposure happens through ingestion, inhalation, or intradermal contact. The oral median lethal dose (oral LD50) for AFB1 ranges from 0.3 mg.kg^{-1} bw for rabbits to 18 mg.kg^{-1} bw for rats [36]. For humans, acute exposure (2–6 mg of AFB1 daily during a month) led to clinical symptoms such as hepatitis, bile duct proliferation, edema, anorexia, malaise, reduced kidney function, lethargy, and death [34, 37, 38]. The last case was reported in April 2004 with a maize contamination of up to 46.4 mg.kg^{-1}. This led to 317 cases of aflatoxicosis in children, among which 125 died [39]. The chronic ingestion of aflatoxins can led to increase of: hepatocellular carcinoma occurrence [6–8] (especially for hepatitis B-positives patients) [40], immunodeficiency [41] (particularly for HIV-positive patients) [42], child growth retardation [43] and birth defects [44]. For animals other symptoms can also occur: pulmonary disease and tracheal exudates in horses; and mucus accumulation, pulmonary edema, capillarity fragility and icterus injuries in swine [37].

Faced with those risks, the AFB1 is the mycotoxin which is most regulated worldwide with more than 100 nations having allowance levels for food and feed. Thus, aflatoxins are regulated at 20 μg.kg^{-1} in food in many countries including USA or China, except for EU. They set maximum authorized levels of aflatoxins in various products to reduce consumers' exposure (Table 2). The maximum levels for AFB1 range from 12 μg.kg^{-1} in almonds, pistachios, and apricot kernels (before

Table 2 Maximum levels authorized for aflatoxins in foodstuffs (1881/2006 modified on 6th March 2014) [45]. *AFT* total amount of AFB1 + AFB2 + AFG1 + AFG2; M1 = aflatoxin M1. (−) = no level applied

Mycotoxins	Foodstuffs	Maximum levels (μg.kg^{-1})		
		B1	AFT	M1
Aflatoxins	Dietary foods for special medical purposes	0.1	–	0.025
	Infant milk and follow-on milk	–	–	0.025
	Raw milk, heat-treated milk, and milk for the manufacture of milk-based products	–	–	0.05
	Cereals and food for babies	0.1	–	–
	Groundnuts, nuts, dried fruit, and cereals and derived ingredients	2	4	–
	Tree nuts, dried fruit, and cereals sorted/treated before human consumption, spices (*Capsicum* spp., *Piper* spp., *Pyristica fragnans, Zingiber officinale, Curcuma longa*)	5	10	–
	Dried figs	6	10	–
	Almonds, pistachios, and apricot kernels intended for direct human consumption	8	10	–
	Hazelnuts, Brazil nuts, groundnuts sorted/treated before human consumption	8	15	–
	Almonds, pistachios, and apricot kernels sorted/treated before human consumption	12	15	–

being sorted for human consumption) to 0.1 $\mu g.kg^{-1}$ for baby food and dietary food for medical purposes. For feed materials, aflatoxins are the only mycotoxins with maximum levels in the EU, the maximum levels of AFB1 range from 20 $\mu g.kg^{-1}$ for cattle, sheep, goats, pigs, and poultry to 5 $\mu g.kg^{-1}$ for dairy cattle, calves, lambs, kids, piglets, and young poultry.

2.1.2 Citrinin

The gene *pksCT* of *Monascus purpureus* encodes a PR-PKS without the ER and DH domains. This PR-PKS is essential for the production of citrinin [46]. Its supposed role is the formation of the polyketide dihydroisocoumarin core by the condensation of 1 acetyl CoA and 3 malonyl CoA to produce a tetraketide.

The citrinin (Table 1), a polyketide containing a dihydroisocoumarin moiety, was discovered in 1931 and has a molecular weight of 250.24 $g.mol^{-1}$ [47]. A few is known about the other members of the family, such as dicitrinin A and dicitrinin E, dimers of citrinin, that can be produced by fungi [48].

Citrinin is produced by *Aspergillus*, *Penicillium*, and *Monascus*. For *Penicillium*, 16 species of *Citrina* section (*P. citrinium*), one *Penicillium* section (*P. expansum*), and two *Fasciculata* section (*P. radicicola* and *P. verrucosum*) are citrinin producers [49–51]. For *Aspergillus*, the section *Terrei* contains the citrinin producers with eight known species [52]. For *Monascus*, eight species are producers including *M. aurantiacus, M. purpureus*, and *M. ruber* [53, 54].

The citrinin gene cluster (43 kb) has been recently identified and is represented in Fig. 3. Among the 16 ORF included in this cluster, nine genes have been identified as required for the citrinin biosynthesis. They encode enzymes that convert 1 acetyl CoA and 3 malonyl CoA into citrinin. The putative functions are: a fatty acyl-CoA synthetase (*ctnI*), an oxygenase (*orf3*), dehydrogenases (*ctnE, orf1, ctnH*), and oxidoreductases (*ctnD, orf4*). It also includes potential transcriptional regulator (*ctnA*) and membrane transport protein (*orf5*) [55].

The citrinin is not carcinogenic for humans (Group 3 [56]). The main target organs are the kidneys. The oral LD50 is 56 $mg.kg^{-1}$ bw in turkey poults [57]. For humans, no impact on human health has been confirmed. For animals, it is

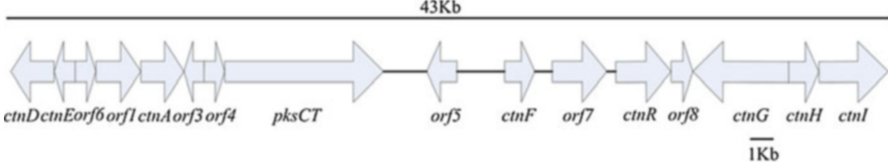


Fig. 3 The 16-ORF cluster of the biosynthesis pathway of citrinin in *M. aurantiacus* [55]. *Arrows* show the genes and their direction of transcription. The designated genes are: *ctnD* oxidoreductase, *ctnE* dehydrogenase, *orf6* hypothetical protein, *orf1* dehydrogenase, *ctnA* transcriptional regulation protein, *orf3* oxygenase, *orf4* oxidoreductase, *pksCT* PKS, *orf5* membrane transport protein, *ctnF* mutase, *orf7* hypothetical protein, *ctnR* WD repeat protein, *orf8* hypothetical protein, *ctnG* carbonic anhydrases, *ctnH* short chain dehydrogenase, *ctnI* acyl-coA synthetase

Table 3 Maximum levels authorized for citrinin in foodstuffs (1881/2006 modified on 6th March 2014) [45]

Mycotoxins	Foodstuffs	Maximum levels (μg.kg^{-1})
Citrinin	Food supplements based on rice fermented with red yeast *Monascus purpureus*	2,000

teratogenic and nephrotoxic [58, 59]. In vitro tests showed genotoxicity [60] and a synergy in nephrotoxicity with another mycotoxin: ochratoxin A [61].

In EU, citrinin is regulated only in food (Table 3). It is only regulated in food supplements based on rice fermented with *M. purpureus* at 2,000 μg.kg^{-1}.

2.1.3 Fumonisins

The HR-PKS Fum1 is required for the first condensation step of fumonisins production [62]. The coding gene *fum1* is the second gene of the cluster (Fig. 5). The first role of Fum1 is to catalyze the carbon-chain assembly. Thanks to the condensation of 1 Acetyl-CoA, 8 Malonyl-CoA, and 2 S-adenosyl methionine (SAM), Fum1 and Fum8 lead to a polyketide-alanine condensation product [63].

Among the 28 fumonisin analogues identified since 1988 [64], only six fumonisins have been widely studied as produced by fungi. Fumonisins are based on a linear chain of 18 carbons as represented in Fig. 4. These six fumonisins are divided into two types: the "B" fumonisins (FB) have a terminal methyl group derived from an amino acid at the R_1 while the "C" fumonisins (FC) have not. Both FB and FC have three main representatives which differ from their R_2 and R_3 composition. The major mycotoxin is fumonisin B_1 (FB1) with a molecular weight of 721 g.mol^{-1}.

The predominant fumonisin producers belong to the *Fusarium* genus with *F. verticillioides* and *F. proliferatum* as the most studied representatives. Nevertheless, *Aspergillus niger* has also been identified as a FB producer and several *Tolypocladium* species are also able to produce fumonisins B_2 and B_4 [65, 66]. The *Fusarium* species producing fumonisins are regrouped in the *Fusarium* (*Gibberella*) *fujikuroi* species complex (FFSC) with the exception of *F. oxysporum* [67, 68]. The FFSC species belong to *Liseola*, *Dlaminia*, and at a smaller scale *Elegans* and *Arthrosporiella* sections [64]. Most of the FFSC species produce mainly FB (99 %) and at a lower scale FC (1 %). However, the opposite ratio of FC over FB also occurs by *F. oxysporum* [67].

The cluster (18 genes) is a 42 kb region sequenced in *F. verticillioides* represented in Fig. 5. The genes included in this cluster encode enzymes that convert acetate and alanine groups into fumonisins [63]. The ratio variation between FB and FC is due to Fum8 function. Indeed, the latter would condensate an alanine with the 18-carbon polyketide for FB producers and a glycine with the 18-carbon polyketide for FC producers [69]. This difference is due to a modification of residues between a valine and an alanine at residue579 of Fum8.

Fumonisin Analog	R_1	R_2	R_3	Molecular Weight
FB_1	CH_3	OH	OH	721
FB_2	CH_3	OH	H	705
FB_3	CH_3	H	OH	705
FC_1	H	OH	OH	707
FC_3	H	H	OH	691
FC_4	H	H	H	675

Fig. 4 Fumonisins B and C chemical structures [67]

The toxicity of fumonisins has been mainly studied for two fumonisins: FB1 and FB2. In 2002, the IARC has confirmed the classification of FB1 as possibly carcinogenic to human (Group 2B [70]). The main targets are the esophagus and the neural tube. The no-observed-adverse-effect-level (NOAEL) in pigs fed with FB1 is lower than 5.0 mg FB1.kg^{-1} bw.day^{-1} [71]. For humans, fumonisins are supposedly linked to esophagus cancer [72, 73]. For animals, the other health impacts include liver toxicity, cancer, leukoencephalomalacia, immunodeficiency, and pulmonary disease in pigs, poultry, calves, equine, and other farm animals [74].

To prevent those risks, the FB1 and FB2 are regulated in EU in food and guidances are provided for feed. The maximum levels for the total amount of FB1 and FB2 (independently of the proportion) are from 200 μg.kg^{-1} for baby foods, infants, and young children to 4,000 μg.kg^{-1} for unprocessed maize (exception of wet milling) (Table 4).

2.1.4 Ochratoxin A

The genes *aoks1* of *A. westerdijkiae*, *otapks PN* of *P. nordicum*, and *AoOTApks-1* of *A. ochraceus* encode two different PR-PKS without the ER and DH domains and a HR-PKS, respectively. Moreover, different putative PKS were identified in *A. carbonarius* [75]. Those encoding PKS are essential for the production of

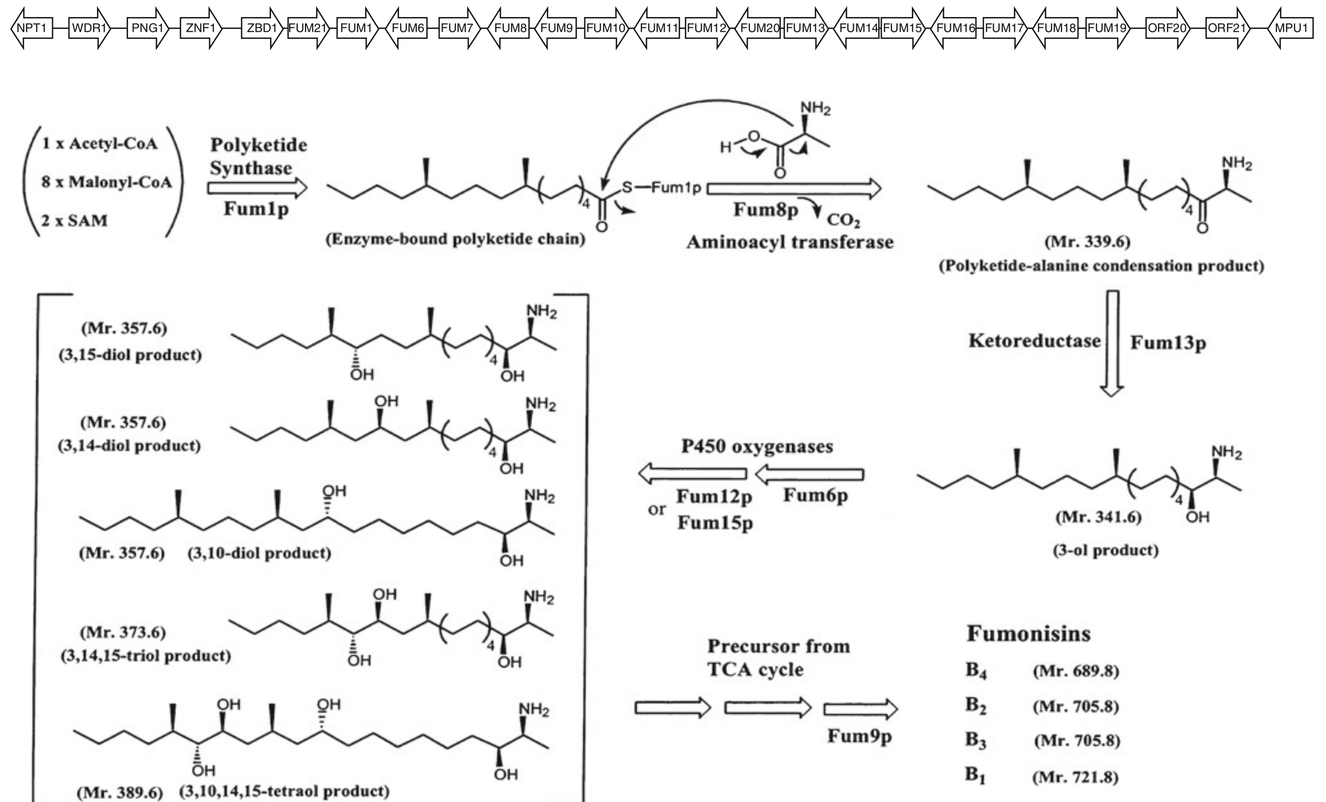


Fig. 5 The 18-genes cluster required for the biosynthesis of fumonisins “B” in *F. verticillioides* [52, 58]. The genes represented in the cluster on the *top* and the action of their encoded enzyme in the scheme *below*. For example, FUM 1 (cluster of the *top*) encodes Fum1p (below scheme). *SAM* S-adenosyl methionine

Table 4 Maximum levels authorized for fumonisins in foodstuffs (1881/2006 modified on 6th March 2014) [45]

Mycotoxins	Foodstuffs	Maximum levels (μg. kg^{-1})
Fumonisins (B_1 + B_2)	Baby foods for infants and young children	200
	Breakfast cereals and snacks	800
	Maize intended for direct human consumption	1,000
	Milling fractions of maize >500 micron/≤ 500 micron	1,400/2,000
	Unprocessed maize (exception of wet milling)	4,000

Fig. 6 Ochratoxin A and B structures

ochratoxins [23, 76, 77]. Their supposed role is the formation of the polyketide dihydroisocoumarin core of the ochratoxins.

Ochratoxins are isocoumarins (Fig. 6). Ochratoxin A (OTA) and ochratoxin B (OTB) are isocoumarins coupled with a l-phenylalanin (R_1) with OTA having a chlorine as R_2 while OTB having an hydrogen as R_2. Ochratoxin α (OTα) and ochratoxin β (OTβ) are isocoumarins coupled with a hydroxide group (R_1). The distinction between OTα and OTβ is the same as between OTA and OTB.

OTA is produced by both *Penicillium* and *Aspergillus* genera while OTB is produced only by *Aspergillus*. The predominant producers are *A. ochraceus*, *A. westerdijkiae*, *A. carbonarius*, and *A. steynii* in warm region [78–81], while *P. verrucosum* and *P. nordicum* are the only OTA producers in cold/temperate area [82, 83]. *Circumdati*, *Flavi*, and *Nigri* are the three sections of *Aspergillus* producing ochratoxins [66].

Unlike many other mycotoxins presented in this chapter, the biosynthesis of OTA in both fungal genera has not yet been elucidated. It is commonly hypothesized that OTα and OTβ are intermediates of OTA and OTB production. Currently, putative PKS have been identified in both fungal genera. Moreover, for *Aspergillus*, two putative p450-type monooxygenase genes, a nonribosomal peptide synthetase (NRPS) were identified as oxygenases and phenylalanine incorporator into ochratoxins, respectively. For *Penicillium*, a NRPS, a putative transport protein and a chlorinating enzyme (OTA chlorination) were also identified [66].

Table 5 Maximum levels authorized for ochratoxin A in foodstuffs (1881/2006 modified on 6th March 2014) [45]

Mycotoxins	Foodstuffs	Maximum levels (μg.kg^{-1})
Ochratoxin A	Dietary foods for special medical purposes, baby foods	0.5
	Wine, grape juice, and wine-based products	2
	All products derived from unprocessed cereals	3
	Unprocessed cereals, roasted coffee	5
	Wheat gluten not sold directly to the consumer	8
	Dried vine fruit, soluble coffee	10
	Spices	15
	Liquorice root, ingredient for herbal infusion	20
	Liquorice extract, in particular beverages and confectionary	80

The toxicity tests on ochratoxins have focused on OTA. It is potentially carcinogenic for humans (Group 2B [84]). The main target organs are the kidneys [85]. The ORAL LD50 of OTA in mice ranges from 46 to 58.3 mg.kg^{-1} bw for mouse to 0.2 mg.kg^{-1} bw for dogs [86]. For humans, it was investigated as the potential origin of the Balkan endemic nephropathy but was disculpate [87]. For animals, OTA is genotoxic, teratogenic, carcinogenic, hepatotoxic, nephrotoxic, and immunotoxic [88–92].

Ochratoxin A is regulated mainly in UE in food, and guidances are provided for feed. The maximum levels for food are 0.5 μg.kg^{-1} for dietary foods for special medical purposes and baby foods and 80 μg.kg^{-1} in liquorice extract, in particular beverages and confectionary (Table 5).

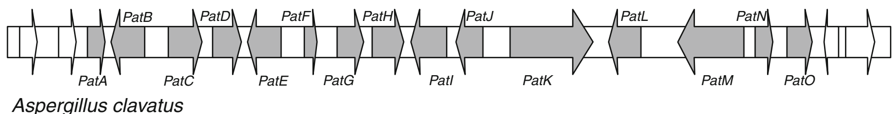
2.1.5 Patulin

The 6-methylsalicylique synthetase (6MSAS), for example, in *P. expansum,* is a PKS without the ER domain [93]. This PR-PKS is essential for patulin production [23]. The role of 6MSAS is to condensate 1 acetyl-CoA and 3 malonyl-CoA to produce the 6-methylsalicylic acid. The latter is the first precursor of the patulin production.

Patulin was discovered in 1943 and had many names (clavacin, expansine, claviformin, clavatin, gigantic acid, or myosin C) [94]. It is a polyketide lactone as represented in Table 1. Its molecular weight is 154.12 g.mol^{-1}.

Patulin is produced by *Penicillium*, *Aspergillus, Paecilomyces* (*P. sturatus*), and *Byssochlamys* (*B. niveai*). Among *Aspergillus*, the *Clavati* section contains patulin producers including *A. clavatus* [95]. Regarding *Penicillium*, most of the producers (11) are belonging to the *Penicillium* section with two exceptions, *P. sclerotigenum* belonging to *Sclerotiora* and *P. paneum* belonging to *Roquefortorum* section [49, 50].

The entire cluster of patulin biosynthesis in *A. clavatus* has been identified and is represented in Fig. 7. It is a 40 kb region encoding the genes necessary for the ten



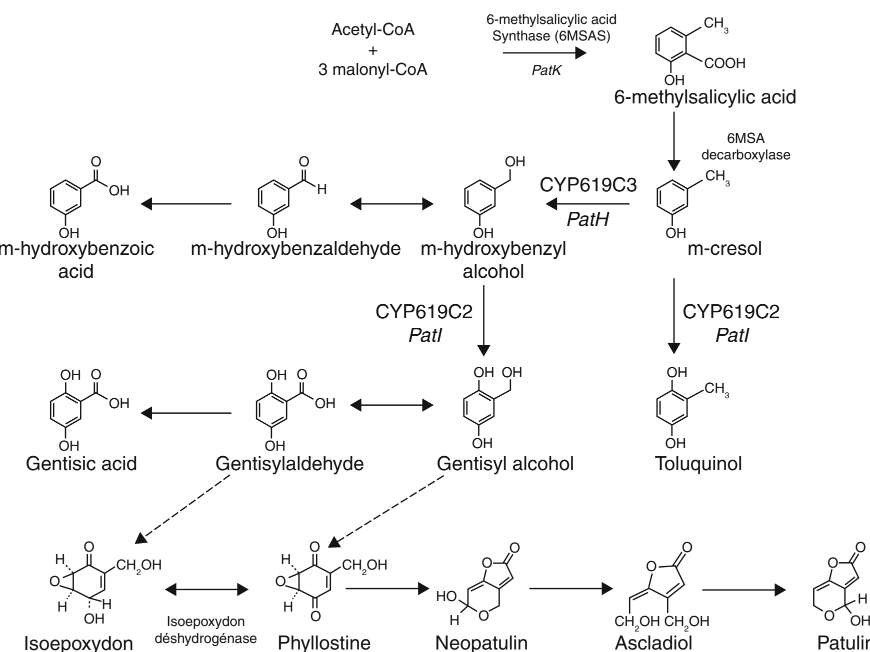


Fig. 7 The putative gene cluster of the patulin in *A. clavatus* [96, 97]. *Arrows* show the genes and their direction of transcription. The designated genes are: *PatA* acetate transporter, *PatB* carboxylesterase, *PatC* MFS transporter, *PatD* Zn-dependent alcohol dehydrogenase, *PatE* GMC oxidoreductase, *PatF* hypothetical protein, *PatG* amido hydroxylase (decarboxylase), *PatH* m-Cresol hydroxylase, *PatI* m-Hydroxybenzyl alcohol hydroxylase, *PatJ* hypothetical protein, *PatK* 6MSAS, *PatL* C6 transcription activator, *PatM* ABC transporter, *PatN* isoepoxydon dehydrogenase, *PatO* isoamyl alcohol oxidase. The genes represented in the cluster on the *top* and the action of their encoded enzyme in the scheme *below*

enzymatic reactions as well as transporters (*PatA, PatC, PatM*) and specific transcription regulator (*PatL*) [96]. The cluster in *Penicillium* sp. remains to be investigated.

Patulin is not carcinogenic for humans (Group 3 [56]). The ORAL LD50 for patulin is 29 mg.kg^{-1} for rats and 55 mg.kg^{-1} for mice [98]. No incidence on humans has been reported. For animals, it is teratogenic and possibly immunotoxic [99–101]. In addition, symptoms such as weight loss, intestinal and gastric problems, neurotoxicity, and nephrotoxicity can occur [98]. The UE has set up patulin regulation for apples and their derivates from 10 to 50 μg.kg^{-1} (Table 6).

Table 6 Maximum levels authorized for patulin in foodstuffs (1881/2006 modified on 6th March 2014) [45]

Mycotoxins	Foodstuffs	Maximum levels (μg. kg^{-1})
Patulin	Apple juice, solid apple products for infants and young children	10
	Solid apple products	25
	Fruit juices and spirit drinks	50

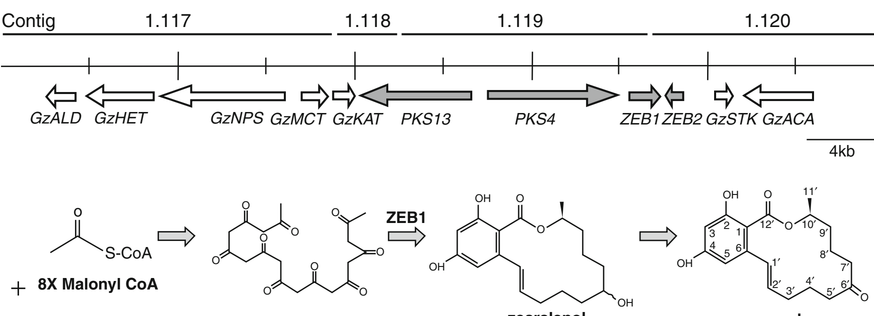


Fig. 8 The supposed genes cluster of the zearalenone in *Fusarium zeae* (the *grey arrow* are validated as needed for ZEA production). *Arrows* show the genes and their direction of transcription. The designated genes are: *GzALD* aldehyde dehydrogenase, *GzHET* heterokaryon incompatibility protein, *GzNPS* nonribosomal peptide synthetase, *GzMCT* monocarboxylate transporter like-protein, *GzKAT* K^+ channel protein, *PKS13* PKS, *PKS4* PKS, *ZEB1* isoamyl alcohol oxidase, *ZEB2* predicted protein, *GzSTK* protein kinase Eg2-like, *GzACA* aldehyde dehydrogenase. The genes represented in the cluster on the *top* and the action of their encoded enzymes in the scheme *below* [102]

2.1.6 Zearalenone

The production of zearalenone (ZEA or ZON) relies on two complementary PKSs. PKS13 is a NR-PKS and PSK4 is a HR-PKS. Nevertheless, it is not known if the two PKS work simultaneously to form the tetraketide backbone and the tetraketide moiety [102]. The ZEA precursors are acetyl CoA and malonylCoA [103].

ZEA was discovered in 1962 [104], it has been differentially named (e.g., F-2, RAL) and is identified as an acid resorcyclic lactone (Table 1). Its molecular weight is 318.36 g.mol^{-1}.

It is only produced by the *Fusarium* genus. Among the producers, *Arthrosporiella* and *Roseum* sections has been identified as producers. The main representative is *F. graminearum* (*Gibberella zeae*) from *Arthrosporiella* section [105].

The ZEA biosynthesis has been partially elucidated. The 50 kb continuous DNA supposed to be the cluster represented in Fig. 8. In addition to the two PKSs, two other genes have been identified as required, a putative isoamyl alcohol oxidase genes (ZEB1) and a putative transcription factor (ZEB2).

The ZEA is not considered as carcinogenic for humans (Group 3 [84]). It is mainly known for its endocrine disruptor capacity due to its close structure to

Table 7 Maximum levels authorized for zearalenone in foodstuffs (1881/2006 modified on 6th March 2014) [45]

Mycotoxins	Foodstuffs	Maximum levels (μg. kg^{-1})
Zearalenone	Cereals-based foods for infants and young children	20
	Bread	50
	Cereals intended for direct human consumption	75
	Unprocessed cereals other than maize	100
	Milling fractions of maize >500 micron/≤500 micron	200/300
	Unprocessed maize (exception of wet milling)	350
	Refined maize oil	400

17 ß-estradiol [106]. The oral LD50 of ZEA in mice is 500 mg.kg^{-1} bw [107]. For humans, there is a presumed link between exposure to ZEA and premature puberty in Puerto-Rico [108]. For animals, it is genotoxic, teratogenic, carcinogenic, hepatotoxic, hematotoxic, immunotoxic, responsible for animal abortion, and infertility [105].

ZEA is regulated mainly in UE in food, and guidances are provided for feed. The maximum levels for food are 20 μg.kg^{-1} for cereals-based foods for infants and young children and up to 350 μg.kg^{-1} for unprocessed maize (with an exception of wet milling) (Table 7).

2.2 Terpene Cyclase-Based Biosynthesis

Another family of mycotoxins takes its biosynthetic origin from terpenes biosynthesis. The terpenes are all produced thanks to the terpene cyclase widely present in plants and fungi. This cyclase uses different diphosphate structures as substrates. Terpenes can be composed of several isoprene units and are classed depending on the diphosphate structure used as substrate by the cyclase. As an example, all the members of the class of sesquiterpenes are generated from farnesyl pyrophosphate as substrate [109].

2.2.1 Trichothecenes

Among the sesquiterpenes, trichothecenes are the main representative and are considered the major class of mycotoxins. They are produced thanks to a terpene cyclase trichodiene synthase (e.g., Tri5). The latter is essential for the cyclization of the farnesyl pyrophosphate which itself induces the production of trichothecenes [110].

There are more than 200 trichothecenes with a common 12–13 epoxytrichothec-9-ene core structure (Fig. 9). They are classified into four groups from A to D, according to their attached radical group [111]. The group at the C-8 position is the differentiating element between groups A and B. The type A has an ester function at C-8, while all type B trichothecenes have a C-8 keto (carbonyl) function. These two

Fig. 9 Structure of the four different types of trichothecenes [111]

types of trichothecenes are the most alarming in terms of occurrence and toxicity. Indeed, for type A trichothecenes the major representatives are T-2 and HT-2, while for type B trichothecenes the major representative is deoxynivalenol (DON) and at a lower rate nivalenol (NIV). The type C have a C-7/C-8 epoxide, while Type D have an additional ring (C-4 and C-15) [112].

Many genera are producers of trichothecenes: *Fusarium, Myrothecium, Spicellum, Stachybotrys, Cephalosporium, Trichoderma*, and *Trichothecium* [113]. *Fusarium* are mainly producers of type A and B, while *Trichoderma*, *Trichothecium, Myrothecium*, and *Stachybotrys* produce the four different types of trichothecenes. There is currently no review precising which of the above producers are precisely producing DON or T-2 and HT-2. Nevertheless, it is known that DON is produced mainly by *F. graminearum*, while T-2 and HT-2 by *F. langsethiae* [114, 115].

The biosynthesis of trichothecenes is atypical. It is the only biosynthesis pathway to be situated in more than one genomic location. Indeed, in *F. graminearum*, three loci have been identified [116]: the core cluster (26 kb) of *TRI* genes [117], the *TRI1-TRI16* locus [118] (both represented in Fig. 10), and the *TRI101* locus [119]. In other *Fusarium* sp. (*F. equiseti*), these loci can be reduced to two: *TRI* cluster (with *TRI1* and *TRI101* included) and *TRI16* locus [120]. They encode enzymes that convert the farnesyl pyrophosphate into the different type of trichothecenes. The differential of trichothecene production is linked to the nonfunction of certain TRI genes in the cluster. Indeed, all the genes (represented in Fig. 10) are necessary for T2 toxin production, while nonfunction of *TRI7*, *TRI13*, and *TRI16* are required for deoxynivalenol (DON) production. This pathway is regulated by two specific (TRI6 and TRI10) transcription regulators with their coding genes located in the cluster [121, 122].

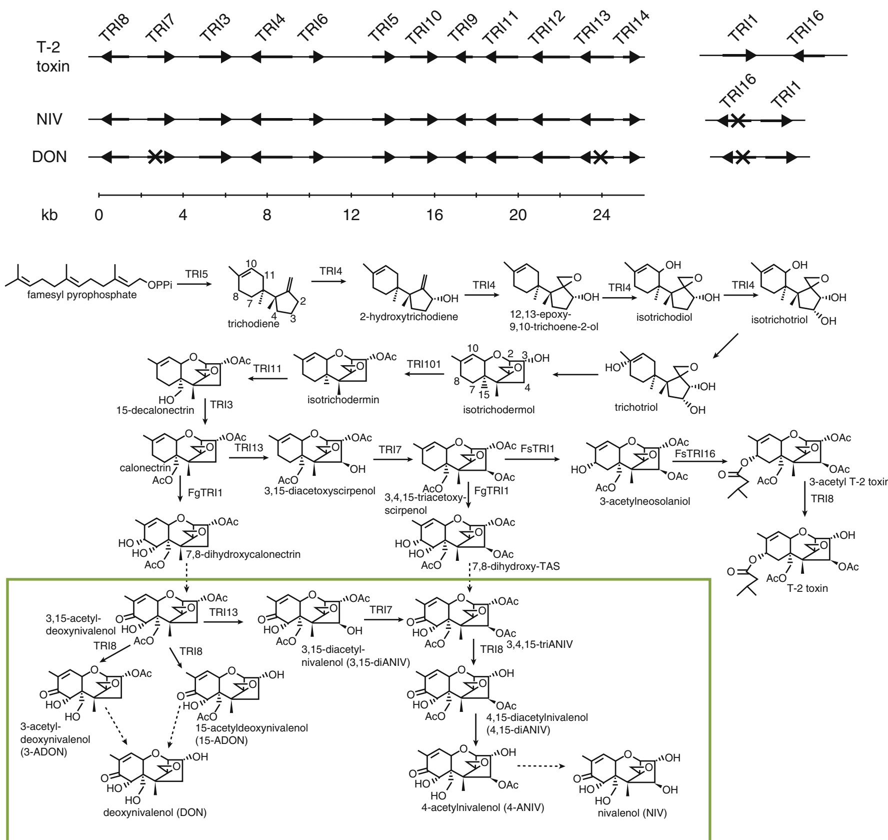


Fig. 10 Representation of the core trichothecenes biosynthesis cluster and *TRI1-TRI16* in *Fusarium*. An X indicates a nonfunction of the represented gene. Below, the proposed trichothecenes biosynthesis in *Fusarium,* type B trichothecenes are in the *green box* [112]. *DON* deoxynivalenol, *NIV* nivalenol

Currently, among more than 200 trichothecenes, only three have been judged as threats to human health by the UE. Those three are the T-2, HT-2, and DON toxins, and they are briefly presented above.

The major representatives for type A trichothecenes are T-2 and HT-2. Those are produced thanks to the trichothecenes pathway with their last common precursor with DON biosynthesis being the calonectrin. T-2 and HT-2 have the 3,4,15-triacetoxyscirpenol as the last common precursor with nivalenol production. T-2 toxin (Group 3 [84]) is teratogenic, hepatotoxic and causes weight loss, decrease in blood cell and leukocyte count, reduction in plasma glucose, and stomach toxicity for animals. There are few studies on the HT-2 toxin, its deacetylated form, which has alleged health impacts. Unfortunately, too little is known on T-2 and HT-2 impacts on human health [123]. T-2 and HT-2 are regulated by the UE in food but

Table 8 Maximum levels authorized for DON, T-2, and HT-2 in foodstuffs (1881/2006 modified on 6th March 2014) [45]

Mycotoxins	Foodstuffs	Maximum levels ($\mu g.kg^{-1}$)
Deoxynivalenol	Baby foods for infants and young children	200
	Cereals, pasta, milling fractions of maize with particle size >500 micron	750
	Unprocessed cereals, milling fractions of maize with particle size ≤500 micron	1,250
	Unprocessed durum wheat, oats, and maize	1,750
T-2 + HT-2	Unprocessed cereals and cereal products	/

no maximum levels for food have been implemented for unprocessed cereals and cereals products (Table 8).

DON is the major representative for type B trichothecenes. It is produced thanks to the trichothecenes pathway with 7,8-dihydroxycalonectin as the first precursor different from NIV and T-2 and HT-2 biosynthesis. DON is not carcinogenic for humans (Group 3 [84]). The symptoms (animals and humans) linked to DON exposure are weight loss, anorexia, nausea, diarrhea, nutritional loss, and immune system modification [124–126]. DON is regulated mainly in UE in food, and guidances are provided for feed. The maximum levels for food are 200 $\mu g.kg^{-1}$ for infants and young children and up to 1,750 $\mu g.kg^{-1}$ for unprocessed durum wheat, oats, and maize (Table 8).

2.3 Alkaloid-Based Biosynthesis

The following family of mycotoxins takes its origin from alkaloids. Alkaloids are widely produced by prokaryotes, plants, animals, and fungi. Among these, the ergots alkaloids are produced by fungi and plants. Among these, some are considered as good medical drugs and are commercialized while others are mycotoxins. All of them are produced thanks to the dimethylallyltryptophan synthase, essential for the conversion of l-tryptophan and dimethylallyl diphosphate into the tetracyclic ergoline ring. As an example, *cpd1* of *Claviceps purpurea* encodes a dimethylallyltryptophan synthase needed for the production of ergotamine.

There is a wide number of ergots alkaloids. All possess the ergoline ring as their main core, represented in Fig. 11a. The ergots alkaloids are divided into three classes: clavine, ergoamides, and ergopeptines alkaloids. Although none of the clavine ergots have been considered as mycotoxins, currently one ergoamide (ergometrine) and five ergopeptines (ergotamine, ergosine, ergocristine, ergocryptine, and ergocornine) have been under monitoring by the UE for potential threats to human health [127]. Among these, ergotamine (Fig. 11b) is the most predominant ergopeptine in ergots.

Fig. 11 Structure of ergot alkaloids (**a**) Structure of the ergoline ring in ergo alkaloids. (**b**) Structure of the ergotamine alkaloid

Ergot alkaloids, apart from being produced by plants, are produced by *Claviceps, Penicillium*, and *Aspergillus* genera. Commonly they are found in sclerotia of *Claviceps. C. purpurea, C. fusiformis, and C. paspali* are the major sources of ergot alkaloids [128].

The biosynthesis of ergots has been characterized at least in *C. purpurea* and *A. fumigatus*. The biosynthesis has been well characterized and is represented in Fig. 12 [129].

Ergot alkaloids are not classified by the IARC. The symptoms (animals and humans) linked to ergots exposure are neurotoxicity, agitation, muscular weakness, shiver, and anorexia [132]. Ergot is regulated mainly in UE in food. The maximum level for food is 0.05 % of ergots in wheat [133].

Hereafter, the second part of this chapter will present the use of fungal metabolites as a potential preventing methods as biocontrol against fungal growth and/or mycotoxins production.

3 Fungal Metabolites: Impact on Mycotoxigenic Fungi and Their Mycotoxins Production

Among the potential methods to prevent mycotoxins production in the agrofood chain, there is the management of biotic parameters. For this, numerous biocontrol agents (BCAs) based on the use of microorganisms were developed as alternatives to phytopharmaceutical inputs. For example, a well-known fungal BCA is AflaSafe® technology. It uses a nonaflatoxigenic *A. flavus* strain to competitively interact with aflatoxigenic fungi at the field, reducing the growth and the production of aflatoxins [134–136]. Bacterial BCAs are also available including bacteria such as *Streptomyces* spp. [137, 138]. Few studies reviewing the use of biocontrol

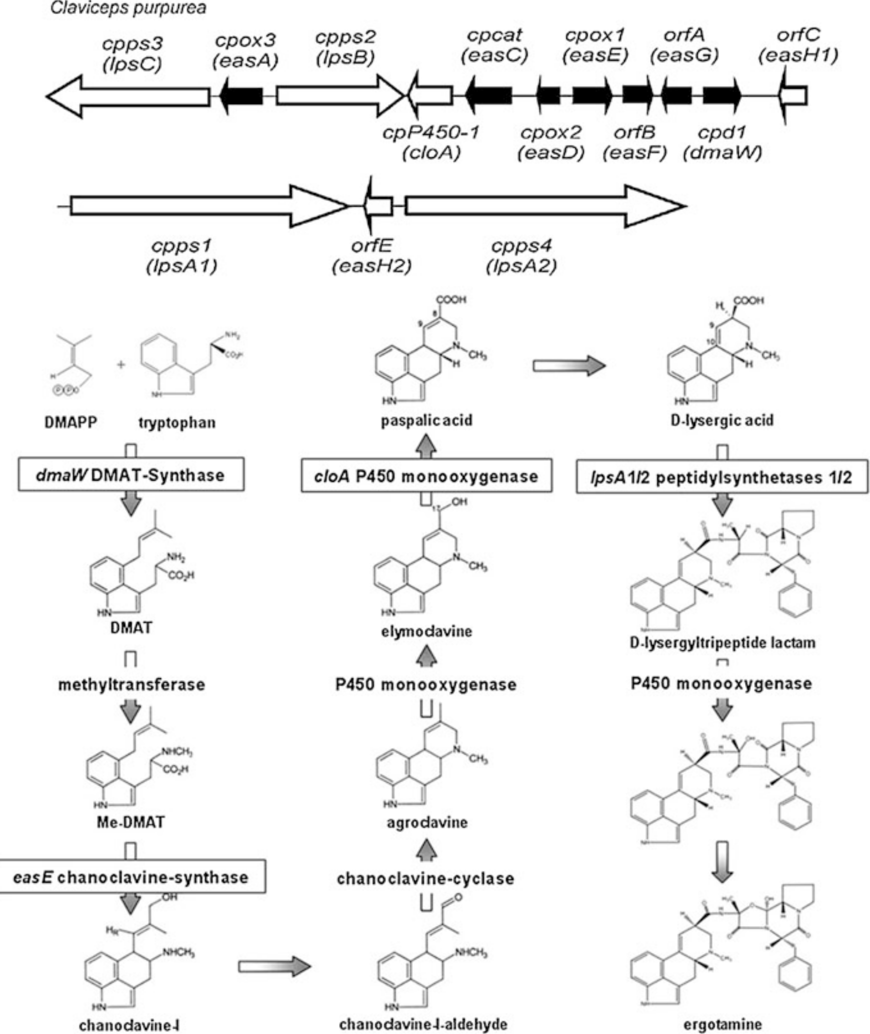


Fig. 12 Representation of ergot alkaloids biosynthesis cluster in *C. purpurea.* [129–131]. *Arrows* show the genes and their direction of transcription. The designated genes are: *cpps3* NRPS, *cpox3* probable NADPH2 dehydrogenase, *cpps2* NRPS, *cpP450-1* CND5p (cytochrome P450), *cpcat* hypothetical catalase, *cpox2* short chain deshydrogenase, *cpox1* isoamyl alcohol oxidase, *orfB* hypothetic protein, *orfA* hypothetic protein, *cpd1* dimethylallyltryptophan synthase, *orfC* phytanoyl-CoA dioxygenase, *cpps1* peptide synthetase, *orfE* phytanoyl-CoA, *cpps4* peptide synthetase

agents to reduce mycotoxin contamination are already available [139–142]. For example, Abbas et al. (2011) reviewed the competitiveness of nonaflatoxigenic strains on maize against production of aflatoxins and cyclopiazonic acid production [139]. Ponsone et al. (2012) reviewed all the epiphytic yeasts considered as

Table 9 List of fungal metabolites able to impact fungal growth (column 3) and/or mycotoxins production (column 4). NE = the impact of the described fungal metabolites has not been directly evaluated on mycotoxins production

Fungal metabolites	Producing fungi	Impact on fungal growth	Impact on mycotoxins production	References
Antifungal compounds				
Peptaibols (atroviridins, neoatroviridins)	*Trichoderma* sp. *F. oxysporum*	*A. niger*	NE	[144, 145]
Pyrrocidines A and B	*Acremonium zeae*	*A. flavus* *F. verticillioides* *F. graminearum*	NE	[146, 147]
Trichodermin	*Trichoderma harzianum*	*A. niger* *F. oxysporum*	NE	[148]
PgAFP, PAF, AFP (antifungal proteins)	*A. giganteus* *A. niger* *P. nalgiovense* *P. chrysogenum* *Gibberella zeae*	 *Aspergillus* sp. *Fusarium* sp. *Penicillium* sp.	NE	[149–151]
Compounds impacting fungal growth and mycotoxins production				
Volatile compounds impacting fungal growth and mycotoxins production				
2-Phenylethanol	*Pichia anomala*	*A. flavus*	Aflatoxins	[152, 153]
2-Phenyl ethyl acetate	*Pichia anomala* *Pichia Kluyveri* *Hanseniaspora uvarum*	*A. ochraceus*	OTA	[154]
Diffusible compounds impacting fungal growth and mycotoxins production				
Lentinans (β-glucan)	*Lentinula edodes* *Trametes versicolor*	*A. flavus*	Aflatoxins	[155–159]
Compounds impacting mycotoxins production				
Ligninolytic enzymes	*Trametes versicolor*	No impact on *A. flavus*	Aflatoxins	[155, 156]

putative biocontrols on berries against OTA production [140]. Future use of BCAs in agrofood chain should be to systematically screen to determine the ecotoxicological properties and putative toxicity of the system (mycotoxigenic fungus/BCAs interaction) [143]. For some active metabolites involved in fungal BCAs, mode of action has already been determined. We will focus on these fungal metabolites (Table 9). They can be classified into three categories: (i) fungal metabolites with antifungal properties; (ii) fungal metabolites impacting both fungal growth and mycotoxins production; and (iii) metabolites preventing mycotoxins synthesis.

3.1 Fungal Metabolites with Antifungal Property Against Mycotoxigenic Fungi

Among the metabolites exerting strong antifungal activity (Table 9), peptaibols, nonribosomal peptides of 10–20 residues, are produced by *Trichoderma* spp. In 2002, seven peptaibols were isolated from a *Trichoderma atroviride* strain (atroviridins A-C and neoatroviridins A-D). These peptaibols showed an antimicrobial activity against filamentous fungi such as *Aspergillus niger* [144]. Recently, Degenkolb et al. (2015) use a HPLC/MS-based peptaibiomics approach to analyze referenced commercial BCAs formulated with *Trichoderma* sp. such as Trichosan® or Vitalin® [145]. The authors show the systematic presence of peptaibols in these commercial BCAs, thus suggesting their implication in fungal inhibition.

Pyrrocidines A and B are polyketide-amino acid-derived antimicrobial compounds produced by *Acremonium zeae* during fermentation [147]. *Ac. zeae*, a preharvest maize contaminant, is known to be antagonistic to various *Aspergillus* spp. and *Fusarium* spp. [146]. Its antagonistic effect is linked to the production of pyrrocidines. Pyrrocidine A seems to be ten times more efficient than pyrrocidine B with minimal inhibitory concentration of 5 μg/ml against *A. flavus* and 10 μg/ml against *F. verticillioides* [146].

Trichodermin is a tricothecene produced by *Trichoderma* spp., a well-known fungal BCA against mycotoxigenic fungi [160]. It was shown that trichodermin has antifungal activity against *A. niger* and *F. oxysporum* [148]. Liu et al. (2012) have shown that antifungal activity of trichodermin in *Trichoderma harzianum* can be enhanced by overexpression of the transporter Thmfs1. This overexpression facilitates trichodermin secretion by the strain, and so trichodermin production increases [148].

Antifungal proteins, presented in Table 9, are produced by various fungi of *Penicillium*, *Aspergillus*, and *Gibberella* genera [151]. For example, *P. chrysogenum* secretes the cysteine-rich protein PAF (*Penicillium* antifungal protein) which inhibits growth of a variety of filamentous fungi [150]. It was determined that this PAF directly impacts conidial germination and hyphal extension by severe changes in cell morphology [150]. Another identified antifungal protein, PgAFP, produced by *P. chrysogenum*, inhibits growth of *A. flavus* by reducing its energy metabolism and increasing stress response [149]. Besides, it appears that 24 h after addition of PgAFP in the culture medium of *A. flavus*, enzymes essential for the biosynthesis of aflatoxins (AflK, AflM) were no longer detectable in hyphae. PgAFP may also prevent mycotoxins production by fungi [149].

3.2 Fungal Compounds Impacting Fungal Growth and Mycotoxins Production

Now are presented some volatile compounds exerting an effect on mycotoxins concentration (Table 9). For example, 2-phenylethanol (2-PE) is a major volatile

compound produced by *Pichia anomala*, a known BCA against aflatoxins production [152]. High level of 2-PE completely inhibits growth of *A. flavus*, while a low level of this volatile compound promotes *A. flavus* growth but suppress aflatoxins production (Table 9) [152, 153]. So, the biocontrol capacity of *Pi. anomala* is attributed to the production of this volatile compound, which affects spores germination, growth, and genes expression in *A. flavus*. *Pi. anomala* was also shown to produce the volatile compound: 2-phenyl ethyl acetate (2- PEA), as well as *Pi. kluyveri* and *Hanseniaspora uvarum* [154]. It was shown that a dose of 48 μg.l^{-1} of 2-PEA allows complete inhibition of *A. ochraceus* growth. Smaller doses could be applied for OTA production inhibition by the producing fungus, but prevention of OTA production by 2-PEA seems to be related to reduction of the fungal biomass [154]. Thus, antifungal effect or mycotoxin production inhibition of these fungal volatile compounds was dose dependent.

Recently the volatile organic compounds (VOCs) of four yeasts (*Cyberlindnera jadinii*, *Candida friedrichii*, *Candida intermedia*, and *Lachancea thermotolerans*) were shown to reduce growth of the OTA producer *A. carbonarius* in grape juice [161]. However, the type of VOCs produced by these yeasts remains undetermined. As *C. friedrichii* shows a significant reduction of the vegetative growth of *A. carbonarius*, further investigation should focus on chemical composition of VOCs produced by this strain.

Another type of fungal metabolites interesting to reduce mycotoxins contamination is lentinans, β-glucan compounds which are sometimes synthetically modified as carboxymethylation or phosphorylation (Table 9) [158, 159]. Lentinan is produced by shiitake mushroom (*Lentinula edodes*) in the culture filtrate which is suggested to inhibit aflatoxins production by *Aspergillus* spp. [158]. Authors suggest that lentinan present in the culture filtrates could stimulate the activation of transcription factors in *Aspergillus* spp. related to antioxidant response and antioxidant enzyme activity. This activation leads to a delay in aflatoxins genes transcription and so to a marked reduction of aflatoxins production. The synthetically modified lentinans show an increased inhibitory potential [159]. For example, the phosphorylated lentinan allows a complete inhibition of aflatoxins production at 50 μg.ml^{-1} while maximum aflatoxins inhibitory activity of lentinan is 200 μg.ml^{-1}. About the carboxymethylated derivative, it acts at the same concentration of lentinan. The author shows that when they increase the concentration of lentinan in the culture medium, its inhibitory activity is reduced. This is not the case for the carboxymethylated lentinan. This difference is due to different modes of action of the lentinan and its carboxymethylated derivative. Indeed, it was shown that lentinan inhibits AflQ transcription, while it is not the case of its derivative. Ma et al. (2014) insist on the fact that chemical modification of this *Lentinula edodes* derivative could improve its impact on prevention of aflatoxins production [159]. Interestingly, lentinan present in the *L. edodes* culture filtrate also enhances biocontrol capacity of the yeast *Cryptococcus laurentii* on *P. expansum* growth and patulin production. Lentinan improves growth of *C. laurentii* and its antioxidant enzymes [157].

3.3 Fungal Metabolites Impacting Mycotoxins Production

Lentinan has also been isolated from liquid filtrates of the basidiomycetes *Trametes versicolor* which inhibits aflatoxins production by *Aspergillus* sp. [156]. The antioxidant capacity and lentinan content of the culture filtrate can vary depending on the *T. versicolo*r strain used for the production of the culture filtrate. Thus, depending on the culture filtrate used, aflatoxins production by *A. parasiticus* is inhibited from 40 % to above 90 % in liquid culture medium. This difference in the aflatoxins inhibition ability of each culture filtrate is linked to its lentinan content and its antioxidant capacity. Some aflatoxins genes (*aflR*, *norA*) transcription are delayed in presence of *T. versicolor* culture filtrates [156].

It was also shown that ligninolytic enzymes present in the liquid filtrates of *T. versicolor* were involved in aflatoxin production inhibition in *A. flavus* [155]. Due to the laccase function of these ligninolytic enzymes, authors cannot define if they inhibit aflatoxins production or degrade produced aflatoxins.

Culture filtrates of *L. edodes* and *T. versicolor*, containing β-glucan and/or ligninolytic enzymes, have the same mode of action concerning inhibition of mycotoxins production. They improve the antioxidant enzymes activity of the mycotoxin producing fungus [156, 158]. This improvement leads to a better oxidative stress response of the mycotoxigenic fungus. As mycotoxins biosynthesis is linked to the oxidative stress suffered by the producing fungus, improvement of its oxidative stress response limits mycotoxins production [158]. So other antioxidant compounds can be used as BCAs against mycotoxins production. For example, ascorbic acid was shown to improve *Pi. caribbica* biocontrol activity against *P. expansum* growth and patulin production on apples [162]. A mix of butylated hydroxyanisol (BHA) and propyl paraben (PP), two chemical antioxidants, were also shown to inhibit OTA production by *A. carbonarius* [163], fumonisin production by *Fusarium* spp., and aflatoxins production by *A. flavus* [164, 165]. Filamentous fungi and edible mushrooms are well-known sources of antioxidant metabolites such as phenolics, flavonoids, glycosides, polysaccharides, tocopherols, ergothioneine, carotenoids, and ascorbic acid [166]. These antioxidant metabolites have to be tested for their putative inhibition properties against mycotoxins production. For example, an *A. niger* crude extract containing naphtho-gamma-pyrones metabolites and presenting an antioxidant capacity equivalent to ascorbic acid is currently tested in our lab for the inhibition of aflatoxins production by *A. flavus* and *A. parasiticus* [167].

3.4 Towards the Elucidation of Metabolites Involved in Mycotoxins Production Inhibition by BCAs

Some information about mechanisms and metabolites responsible for the biocontrol activity of some fungal BCAs are available. For example, control of *A. carbonarius* OTA production by a strain of *Saccharomyces cerevisiae* was studied [168]. Coculture between the two microorganisms shows a strong inhibition of

A. carbonarius growth. The same inhibition of *A. carbonarius* growth is observed when the culture medium is supplemented with the yeast crude supernatant or the yeast autoclaved one. Interestingly, growth of *A. ochraceus* was strongly decreased in presence of yeast crude supernatant while it is only slightly impacted when exposed to autoclaved supernatant. In the latter case, OTA production by *A. ochraceus* was drastically reduced through inhibition of OTA PKS transcription. These results suggest that at least two different compounds produced by *S. cerevisae* were involved in biocontrol activity of the strain. They are still not determined but some have antifungal activity while other directly impacts mycotoxins production [168]. Based on the same approach, our lab tries to elucidate the active compounds involved in T-2 toxin production inhibition by *F. langsethiae* when the yeast *Geotrichum candidum* is used as BCA. It was shown that in vitro coculture of *G. candidum* and *F. langsethiae* led to partial inhibition of both fungal growths but that T-2 toxin production was drastically reduced [169]. The putative mechanisms associated with the biocontrol capacity of different yeast strains (*Cryptoccocus albidus*, *Pi. membranifaciens*, *Cryptoccocus victoriae*) against the patulin-producer *P. expansum* was also partially elucidated [170]. For example, authors have shown that inhibition of pathogen growth is linked to diffusible compounds production by a strain of *C. albidus* while for another strain it is linked to production of VOCs compounds.

4 Conclusion

Mycotoxins are secondary metabolites produced by filamentous fungi which are potentially dangerous for animals and humans. The latter has been confirmed in human illnesses such as ergotism in Middle age or aflatoxicosis in the past century. Nowadays, 300–400 mycotoxins are identified and among these 15 are currently under regulation indicating maximum levels authorized within the EU.

In this chapter, we presented the three different biosynthesis origins of the EU-regulated mycotoxins and their associated toxicities. Within the polyketide family, we highlighted the PKS involved in the known mycotoxins biosynthetic pathways. We started from the aflatoxin biosynthesis (in *Aspergillus* spp.) and continued with citrinin (in *Aspergillus* spp. and *Penicillium* spp.), patulin (in *Aspergillus* spp.), and zearalenone (in *Fusarium* spp.). The following biosynthesis family was the terpenes. Mycotoxins concerned by this family are named trichothecenes. They are first generated by the action of a cyclase enzyme and among these T-2 and HT-2 (Type A – in *Fusarium* spp.) and deoxynivalenol (Type B – in *Fusarium* spp.) are EU regulated. The last biosynthesis family presented in this study was the ergot alkaloids (in *Claviceps* spp.) which requires a dimethylallyltryptophan synthase for it biosynthesis.

Throughout our chapter, the different intermediate precursors were also presented whenever it was possible as well as other products identified from the same biosynthesis (e.g., NIV for DON). Nevertheless, only 15 out of 400 mycotoxins are regulated and were deeply presented in this chapter. Other mycotoxins should be

kept in mind as potential threat to human health. As an example, the *Alternaria* genus is starting to be known as a mycotoxin producer. Its secondary metabolites include different metabolites such as alternariol, alternariol monomethyl ether, tenuazonic acid, tentoxin, and altenuene [127]. Other mycotoxins of concerns include phomopsins produced by *Diaporthe toxica* [169].

To reduce mycotoxins occurrence in the agrofood chain, prevention and decontamination techniques exist. In this chapter, a section was focused on fungal metabolites used to prevent mycotoxins production. Various fungal metabolites can be used as BCAs against mycotoxins production. Those fungal metabolites can be classified into three categories (Table 9). The first category is fungal metabolites with antifungal activity. Among this category we can find peptaibols, pyrrocidines, trichodermin, and antifungal proteins. The second category is fungal metabolites both impacting fungal growth and mycotoxins production. For example, 2-PE, a VOC, acts on a dose-dependent manner: low level of 2-PE produced by *Pi. anomala* inhibits aflatoxins biosynthesis while high level inhibits *A. flavus* growth. Finally, the third category is fungal metabolites inhibiting mycotoxins production. For example, the ligninolytic enzyme is produced by the basidiomycetes *T. versicolor* which improves oxidative stress response of the mycotoxigenic fungus. This induces a limitation of mycotoxins production. Filamentous fungi represent an inexhaustible source of fungal metabolites with a putative biocontrol activity against mycotoxin production. This is also the case for plants and bacterial metabolites. Essentials oils from various plants such as peppermint, eucalyptus, oregano, or thyme are currently used to limit *A. flavus* growth and/or aflatoxins accumulation [170, 171]. For thyme essential oil, it is determined that a phenolic compound named thymol presents antifungal effect against *Aspergillus* spp. and *Fusarium* spp. [172]. Concerning bacterial metabolites, our lab is currently determining the active compounds produced by some actinomycetes strains which impact aflatoxins production by *A. flavus* without impacting its fungal growth [137, 138].

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Antioxidant Activities and Metabolites in Edible Fungi, a Focus on the Almond Mushroom *Agaricus subrufescens*

22

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Abstract

The basidiomycete *Agaricus subrufescens* Peck, also known as the almond mushroom due to its particular flavor, became in a few years one of the most important culinary-medicinal cultivable mushrooms with potentially high added-value products and extended agronomical valorization. As other mushrooms, it produces metabolites of great interest as potential antioxidant defensive agents to reduce the oxidative damage caused by free radicals. The quality of raw mushrooms or extracts and yield in metabolites may vary with the genetic background of the mushrooms and the environmental conditions. This chapter uses *A. subrufescens* as a guideline for illustrating the diversity in radical scavenging activities and metabolites in edible fungi, how it can be studied, and how active molecules might be identified.

Keywords

Agaricus brasiliensis • *Agaricus blazei* • CPC • HPLC • Agaritinal

1 Introduction

Antioxidant supplements, or natural products containing bioactive compounds, may be used to reduce oxidative damage to the human body [1, 2]. Edible mushrooms are good candidates for obtaining such natural products. They are widely recognized as a functional food and a source of various physiologically active compounds for the development of new drugs and nutraceuticals [3, 4]. There have been recent investigations on the antioxidant properties of extracts from various cultivated and wild edible species [5–11]. Antioxidant properties of mushroom polysaccharides are well documented. They improve the activity of antioxidant enzymes, scavenge free radicals, and inhibit lipid peroxidation and they are proposed as valuable functional food additives or sources of therapeutic agents for antioxidant and cancer treatments [8, 12, 13]. On the other hand various phenolic compounds, flavonoids, ascorbic acid [5, 6], and other compounds such as the water-soluble thiol ergothioneine [14, 15] are recognized as antioxidants in mushrooms.

Many studies have compared the content of bioactive compounds or antioxidant activities in different mushroom species [9, 14, 16]. However, in most of them, analyses were done on only few sporophores from one strain of each species, generally collected on a local place in the wild or bought from a local market, sometimes collected at a mushroom farm and rarely cultivated in a research institute under controlled conditions. Consequently in such experiments, what were referred as differences between species could actually be only case studies. Within a species, antioxidant activities and bioactive compounds may vary significantly due to genetic differences between strains, environmental conditions, cultivation techniques, maturity of the harvested mushrooms, and shelf life conditions [17, 18]. In addition comparisons of data from different articles are rendered difficult by the diversity of extraction methods, of assays for antioxidant activities, and of the units the data are expressed.

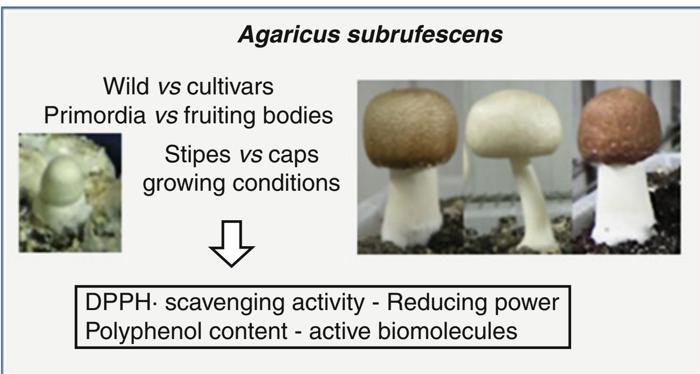

Fig. 1 Factors affecting antioxidant activities in the almond mushroom

Agaricus subrufescens Peck is formerly known in the literature as *Agaricus blazei* Murrill sensu Heinemann (ABM), or *Agaricus brasiliensis* Wasser et al. and is also called Almond Mushroom, Himematsutake and Cogumelo do Sol [19]. The medicinal properties of this culinary-medicinal mushroom are known for more than three decades and several reviews analyzed its importance as functional food or for medicinal purposes [20–24]. Currently, there are few published data for antioxidant activities of *A. subrufescens* not due to polysaccharides [25–31].

In this chapter we describe recent advances in understanding of factors affecting antioxidant activities in mushroom extracts and how to go from activities to the active metabolites, through a focus on the almond mushroom *A. subrufescens* including new data (Fig. 1).

2 The Genetic Background Affects Antioxidant Activities of Mushroom Extracts

2.1 Interspecies Versus Intraspecies Diversity

Finding the best mushrooms for the selection of candidates for either direct use as food additives or for isolation of active molecules is a challenge. For that, many authors compared various edible mushroom species they can obtain locally, but only few of them took into account the intraspecific diversity which could be a significant factor of variation in potentials of antioxidant activities. To evaluate the effect of the genetic background within a species on the expression of a trait, it is necessary to limit the effect of the environment and cultural practices by cultivating different strains or varieties simultaneously under the same conditions. This is convenient with mushrooms that can be cultivated on the same substrate in a cultivation room with climatic managements.

That was what we did with eight strains of *A. subrufescens* cultivated with the methods described in Llarena-Hernadez et al. [32]. Three were Brazilian cultivars

cultivated in the last 10 years, one (CA454) was a supposed subculture of the Brazilian strain at the origin of the modern cultivation of this mushroom, and three were recently isolated strains from France and Spain. The last one was a hybrid between CA454 and a French isolate. An analysis of DNA single sequence repeats (SSR) fingerprinting showed no differences in genetic distances between the three Brazilian cultivars and two clusters were distinguished with the Brazilian cultivars and CA454 on the one hand and the European wild strains on the other hand [28, 32]. These strains had been cultivated under the same conditions in experimental facilities at our institute (INRA) for measuring antioxidant activities in mushrooms collected at (a) the primordium stage and (b) the sporocarp commercial stage, veil closed, considered as stage 3 for the button mushroom *Agaricus bisporus* [33].

Irrespective of the genetic distances, ANOVAs with contrast showed that the group of wild strains did not differ from the group consisting of CA454 and cultivars for scavenging activity and reducing power, measured in caps with the methods previously described in Savoie et al. [34], with $p = 0.312$ and 0.772 respectively (Tables 1 and 2). However, intraspecific variability was observed. When compared to the other strains, the cultivars CA561 and CA565 exhibited the significant highest DPPH scavenging activities in the sporophores and the stipes (Table 1). The highest activity was 2.8-fold the lowest activity. Variations in antioxidant activities with the strains have been documented for the button mushroom. Czapski [35] measured

Table 1 DPPH radical scavenging activities of methanolic extracts from eight *A. subrufescens* strains harvested at the primordium and sporophore stages

	EC_{50} values (mg mushroom mL^{-1})			
Strain[c]	Primordium	Whole sporophore	Cap	Stipe
CA438-A	1.49 c[a] BCD[b]	2.17 b BC	1.72 c BC	2.84 a B
CA487	1.74 b AB	2.06 ab BC	1.88 b BC	2.37 a BC
CA643	1.56 c BC	2.48 b B	2.19 b AB	2.86 a B
Hybrid	1.04 d CD	1.86 b C	1.49 c CD	2.16 a C
CA454	2.12 b A	3.17 a A	2.47 b A	3.49 a A
CA561	0.95 b D	1.38 a D	1.42 a CD	1.35 a D
CA565	1.43 a BCD	1.13 b D	1.13 b D	1.13 b D
CA570	1.00 b CD	1.95 a C	1.75 a BC	2.12 a C

EC_{50} were measured as in Savoie et al. [34]; values are means of 3 replicates

[a]Within a line, values followed by the same lower case letter are not different at $p = 0.05$ by the Duncan's test

[b]Within a column, values followed by the same capital letter are not different at $p = 0.05$ by the Duncan's test

[c]CA561, CA565, CA570 were cultivars of *A. subrufescens* from Brazil purchased by D. Zied in 2007. CA454 is a subculture of the collection strain WC837 in PSUMCC the Pennsylvania State University Mushroom Culture Collection (PSUMCC, USA, PA) provided by D. J. Royse. According to PSUMCC, WC837 is similar to ATCC 76739 which according to ATCC, was originally provided by T. Furumoto, who is known to have discovered *A. subrufescens* in Brazil (São Paulo State) during the 1960–1970 and used by Mizuno et al. [24], at the origin of the modern cultivation of this mushroom. CA438-A, CA487 and CA643 were recently isolated wild European strains of *A. subrufescens*

EC_{50} of radical scavenging activity for ascorbic acid was 0.023 mg mL^{-1}

Table 2 Reducing power of methanolic extracts from eight *A. subrufescens* strains harvested at the primordium and sporophore stages

	Concentration (mg mushroom mL^{-1}) leading to 0.25 absorbance			
Strain	Primordium	Whole sporophore	Cap	Stipe
CA438-A	2.64 c[a] BC[b]	3.87 b CD	2.75 c D	5.50 a BCD
CA487	2.90 c BC	4.30 b BCD	3.18 c CD	5.89 a B
CA643	4.20 c A	6.57 b A	4.29 c AB	9.49 a A
Hybrid	2.65 d BC	4.77 b BC	3.60 c BC	5.69 a BCD
CA454	3.63 c AB	5.33 a B	4.48 b A	5.73 a BC
CA561	2.24 b C	3.23 ab D	2.96 ab CD	3.65 a CD
CA565	3.67 a AB	3.17 b D	2.96 c CD	3.30 b D
CA570	2.85 c BC	4.20 ab BCD	3.70 bc ABC	4.65 a BCD

Reducing power was measured as in Savoie et al. [34]; values are means of 3 replicates
[a]Within a line, values followed by the same lower case letter are not different at $p = 0.05$ by the Duncan's test
[b]Within a column, values followed by the same capital letter are not different at $p = 0.05$ by the Duncan's test
The reducing power measured with ascorbic acid was 0.018 mg mL^{-1}

EC50 values for scavenging of stable DPPH radicals in 50 % methanolic extracts from mushrooms of four strains of *A. bisporus* and observed activities varying from 1.0 to 3.6 times. In a comparison of a white commercial hybrid and two wild strains of *A. bisporus* cultivated under the same conditions and harvested at the same development stage lesser variations in DPPH activities (1.65×) were measured in ethanolic extracts [34]. These magnitudes in intraspecific variations of EC_{50} of DDPH scavenging activities are higher than or similar to those reported in many studies comparing different mushroom species. Tsai et al. [36] have found significant differences, but with a magnitude of only 1.25, in comparison of ethanolic extracts from *A. subrefescens* and two other taxonomically different species, *Boletus edulis* and *Agrocybe cylindracea*. In a comparison of five species in the genus *Agaricus*, the extreme EC_{50} values of DDPH scavenging activity of methanolic extracts were 5.4–15.8 mg mL^{-1} for *Agaricus sylvaticus* and *Agaricus arvensis* respectively [37].

Finally, the studies concluding on ranks of mushroom species for their antioxidant activities without taking into account the intraspecific variability should be considered with precautions. However, in studies where more than 20-fold higher activities are measured in some species than in others [16], one can consider their different potential being characters of the species, even if intraspecific variations due to the variability in genetic backgrounds may exist.

2.2 Selection of Strains with High Antioxidant Potential

Agaricus subrufscens exhibits almost the largest diversity of the genus at the morphological, climatic, and geographical levels [38] and the preliminary data presented above show a potential significant diversity for antioxidant activities.

Rapid ways to rapidly estimate the antioxidant potential of a strain and select in a collection for biotechnological purposes would be useful.

For *A. bisporus*, whereas the main cultivated strains are white hybrids, there are also cream and brown cultivars exhibiting different properties and only a very low percentage of white wild strains have been collected. Comparing white and brown strains, Dubost et al. [39] observed that the white strains they studied contained the least while portabellas (brown) contained the highest content of L-ergothioneine, a naturally occurring antioxidant. Shao et al. [40] measured higher concentrations of ergosterols and antioxidant activities in a brown cultivated strain than in a white hybrid. *Agaricus subrufescens* cultivars are all known to be characterized by a brownish gold color of the cap, which explains that there is no report in the literature on antioxidant capacity in *A. subrufescens* in relation to cap color. In our work using wild strains with different cap colors [28], radical scavenging activity and reducing power of light cream strains (CA438-A, CA487, and the hybrid) were in the same range as those of the brown CA643 and the brownish gold cultivars CA561, CA565, and CA570 (Tables 1 and 2).

To analyze a putative effect of the cap pigmentation on the antioxidant activities, the pileipellis was removed from caps of CA643 and CA438 using a scalpel. The samples were immediately frozen at −80 °C and kept at this temperature until being freeze dried. Actually, no significant difference in DPPH scavenging activity was detected between caps with or without pileipellis (Table 3) either in the light cream strain CA438-A or in the brown strain CA643. The reducing power was reduced when the pileipellis of CA438-A was removed. Removal of the pileipellis by cutting it off produced a yellowish color that could explain this decrease in antioxidant capacity. The antioxidant activity of methanolic extracts of *A. subrufescens* appeared to depend on the strain, and probably without correlation with the cap color.

Radical-scavenging activity in methanolic extracts of sporophores of the eight evaluated strains of *A. subrufescens* ranged among EC_{50} values of 1.13–3.17 mg mushroom mL^{-1}. Some works on the antioxidant properties of *A. subrufescens* cultivars have been made, but data varied greatly depending on the strain, technique of extraction, and method of antioxidant activity measurement. Carvajal et al. [41] determined EC_{50} values of 0.305 mg mL^{-1} of extract for DPPH radical scavenging using a mix of ethanol:water (70:30) for extraction. On the other hand, Soares et al. [30] have observed EC_{50} values of 3.0 – 3.2 mg mL^{-1}, using methanol as

Table 3 Effect of the pileipellis on antioxidant activities of methanolic extracts from a cream (CA438-A) and a brown (CA643) strain of *A. subrufescens*

	Radical scavenging activity		Reducing power (mg mushroom mL^{-1})	
	EC_{50} values (mg mushroom mL^{-1})		Leading to 0.25 absorbance	
Treatment	CA438-A	CA643	CA438-A	CA643
Peeled	1.720 a	2.187 a	3.87 a	4.94 a
Not peeled	1.364 a	2.219 a	2.75 b	4.29 a

Radical scavenging activity and reducing power were measured as in Savoie et al. [34]; values are means of 3 replicates. Within a column, values followed by the same letter are not different at $p = 0.05$ by the Duncan's test

extraction solvent. In the present work we expressed the activities as EC_{50} per mg of dry powder of mushroom used for obtaining the extract, because it takes into account the extraction rate and it is more representative of the use of mushrooms as functional food. It was feasible because no concentration of the extracts was performed due to the high level of activity. This way of data presentation should be used most of the time for favoring comparisons between studies. By a rapid conversion of the published data, we stated that the ranges of antioxidant activity observed herein among the eight strains showed efficient abilities compared to other works.

Over the differences in a trait level between genetically distinct strains, the effect of the genetic background may be stressed by genetic studies of hybrids between strains and evaluation of the heredity of the trait. In the study presented in this chapter, a hybrid (CA454-3 x CA487-100) was provided by E. Huang and P. Callac who crossed homokariotic single spore isolates of the Brazilian strain CA454 and the French strain CA487. It did not differ from its French parent but it had significant higher antioxidant activity (lower EC_{50}) than its Brazilian parent (Table 1). This illustrates that crossing various strains is a putative way to improve the performances of selected strains to be used for the isolation of antioxidant compounds. To date, there is no known study on the improvement of antioxidant activities in mushrooms by breeding programs. This would, however, be feasible taken into account new genomic approaches are developing for cultivated mushrooms, as it is done for plants. Foulongne-Oriol [42] reviewed the used of genetic linkage mapping in fungi. Genetic linkage maps provide foundation for studying genome structure and organization and are highly valuable tools to identify the location of loci controlling important traits of interest. One can expect to improve the mushroom quality and their natural ability to produce selected health-promoting compounds by breeding programs as for other traits. Concerning *A. subrufescens*, intercontinental hybrids between strains with a large diversity of morphological traits [38] and a genetic linkage map have recently been obtained [43]. This opens the door to further breeding programs for improving the antioxidant activities and antioxidant compound contents in cultivated strains.

3 Antioxidant Activities of Mushroom Extracts Vary with the Development Stage and Part of Fruiting Bodies

The choice of the optimal stage of harvest and the part of the fruiting body containing the higher amount of bioactive compounds is also important to improve the use of mushrooms as a functional food. Antioxidant activity had been measured in *A. subrufescens* fruiting bodies with closed caps compared to mushrooms with open caps [29, 30], and compared between caps and stipes [31]. Variations in antioxidant activity during the *A. subrufescens* sporophore ripening were reported. These works concerned the mushroom biology and its use as a nutritional source of antioxidant. Soares et al. [30] found no significant difference in antioxidant properties comparing DPPH radical scavenging activity and reducing power in young (cap closed) and mature (cap opened) sporophores of a Brazilian cultivar.

Mourão et al. [29] analyzed five Brazilian cultivars and measured higher DPPH radical scavenging activity in mushrooms with closed cap compared to aging mushrooms with cap completely turned apart. Nevertheless, no information on the antioxidant activities in the first stage (primordium) of *A. subrufescens* fruiting body development was available in the literature. All these works concerned cultivars which are suspected by Neves et al. [44] to have a common origin, based on genetic studies. To our knowledge, no report has been done about changes in antioxidant activity during the development of various strains of *A. subrufescens* from primordia to fruiting bodies.

In the work described above, all strains but CA565 showed higher antioxidant activities in the primordia (10–15 mm high, collected before gill development) compared to the whole sporophores, although differences were not significant for CA487 ability to scavenge DPPH radicals (Table 1) and for the reducing power of CA561 (Table 2). In contrast to the other strains, CA565 exhibited a higher activity in the whole sporophore whatever the method used. Tsai et al. [45] analyzed ethanol extracts of *A. bisporus* fruiting body at five development stages: pin head, veil intact (tight), veil intact (stretched), veil opened, and gills well exposed. They found the highest radical DPPH scavenging activity and reducing power in the sporophore veil intact stage. Higher antioxidant activities in primordia than sporophores with closed veil we observed here could result from antioxidant mediated defences necessary to protect the cells from the high levels of reactive oxygen species (ROS) generation at this critical stage of mushroom differentiation, as previously proposed for *A. bisporus* [46].

Beyond the development stage of the mushroom, the antioxidant activity can differ depending on the part of the sporophore. Caps of the wild *A. subrufescens* strains, CA454, and the hybrid showed higher DPPH radical scavenging activity compared to stipes whilst similar activity was measured in caps and stipes of cultivars. The reducing power in the caps was higher compared to that found in the stipes in all strains but CA561. Despite some variability between strains and species in various studies [31, 47, 48] the presence of higher antioxidant capacities in the cap compared to the stipe is probably common in edible fungi. This is in agreement with the results of Savoie et al. [34] who found the highest antioxidant activities in the gills of *A. bisporus*, since the gills are organs supporting the differentiation of spores where the redox reactions are important and consequently the antioxidant defences are higher.

In the data presented here, the type of sample showing the highest antioxidant activities was primordium for the hybrid, primordium or cap for the wild strains CA438-A and CA487, and cap for CA565. In strains CA561, CA570, and CA643, the highest radical scavenging activity was measured in the cap, but similar reducing power was found in primordium and cap. The opposite was observed with CA454. *Agaricus subrufescens* is a culinary-medicinal mushroom mainly sold as powder, extracts, or tea. From a scientific point of view, the primordia and the caps without stipe of *A. subrufescens* were the samples the most interesting for antioxidant properties, but processing primordia (weak biomass, time-consuming harvest) or caps (one more step in preparation compared to whole sporophore) seemed not the

best choice for commercial production when antioxidant properties were taken as a whole.

The antioxidant properties of a mushroom collected at the best development stage have to be preserved until its final use as food or for biomolecule extractions. Various postharvest physicochemical treatments and specific storage conditions are applied for limiting the development of microbial contaminations and decreasing the metabolic activity of the mushrooms. They generally have physiological effects leading to increases in the expression of antioxidant systems in mushrooms. Positive effects have been observed in shiitake mushrooms, *Lentinula edodes.* A heat treatment at 121 °C for 30 min increased the ABTS and DPPH radical scavenging activities by 2.0-fold and 2.2-fold compared to the raw sample [18], and postharvest application of UV-C radiation delayed softening and enhanced antioxidant capacity [49]. During the storage of *A. bisporus*, the highest expression of the enzymatic antioxidant system was found in the mushroom stored under modified atmosphere packaging with vacuum cooling treatment [50], or with nitric oxide treatment [51]. Some composite chemical pretreatments using EDTA, citric acid, $CaCl_2$, sorbitol were also efficient for increasing antioxidant enzymes and scavenging ROS, indicating higher defensive potential for mushrooms [52]. Mushrooms exposed to 80 % O_2 exhibited higher DPPH-radical scavenging activity after 6 days of storage [53]. Both in *A. subrufescens* and *A. bisporus*, γ -irradiation did not affect antioxidant properties of methanolic extracts [25, 54]. However, conventional industrial food processing such as freezing and canning were shown to slightly negatively affect the antioxidant activity of seven edible fungi [55]. DPPH free radical scavenging activities of *A. bisporus* were shown to be significantly affected in ethanol extracts by boiling of the mushroom for 30 min [56] whereas activities decreased in methanol extracts of both fresh and frozen mushrooms of *A. subrufuscens* boiled for 20 min [57]. Microwaving had also a detrimental effect [57].

The above selection of studies on postharvest treatment highlights the lack of stability of antioxidant properties in a living material as mushrooms are, but there are some ways to partly stabilize or improve them. However, in reports on antioxidant activities measured in mushrooms from local markets where the storage conditions were not controlled, there is a large risk of misevaluation of the species or strain potential.

4 Changes in Antioxidant Activities of Mushroom Extracts with the Environment

Growing condition is another source of variability in antioxidant activities of mushrooms. The base ingredients of the commercial compost used in the study presented in this chapter were wheat straw and horse manure, whose nutrient content varied with batches. Nine months separated the preparation of the two batches of compost provided by the same company, in two replications of the experiment (expA and expB). The eight studied strains showed significant differences in total mushroom biomass produced until 65 days after casing in the two different crops despite

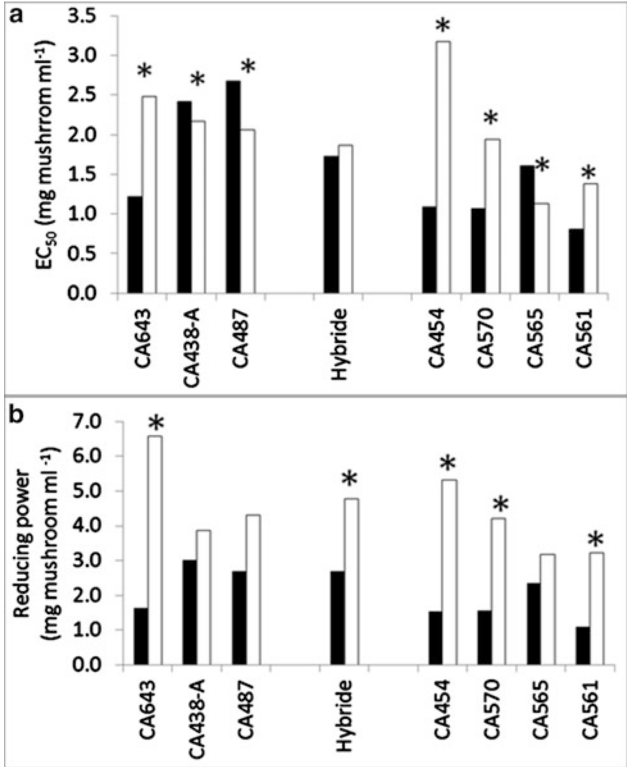

Fig. 2 Comparison of DPPH radical scavenging activity (**a**) and reducing power (**b**) of methanolic extracts from eight *A. subrufescens* strains harvested at the sporophore stages on two different batches of compost. Radical scavenging activity and reducing power were measured as in Savoie et al. [34]; values are means of 3 replicates. *Dark bars* experiment A, *white bars* experiment B. * significant difference between the two experiments at $p < 0.05$, Duncan's test

the control of the climatic conditions. Mushroom yield of wild European strains ranged from 149 to 189 g kg^{-1} substrate in expA, and from 123 to 227 g kg^{-1} in expB, whilst Brazilian cultivars and CA454 produced 7.1 to 28 g kg^{-1} in expA and 13–91 g kg^{-1} in expB. The wild strains were early fruiting compared to the other strains, irrespective of the experiment. Antioxidant capacities varied significantly with the experiment. The DDPH scavenging activity was higher in expA than in expB for CA438-A, CA487, and CA565 whilst it was the opposite for the other strains but the hybrid (Fig. 2). The Pearson coefficient between mushroom yields and DPPH EC$_{50}$ values (r $=$ 0.785 $p = 0.037$ for expA without CA454 (poorly yielding, 7.1 g kg^{-1}), and r $=$ 0.753, $p = 0.031$ for expB, all strains) showed that the more a strain was productive, the more its scavenging activity of DPPH radicals was low. No significant correlation ($p = 0.05$) was observed between mushroom yield and the level of reducing power. A common effect of the experiment was observed; all the strains had higher reducing power in expB, but the differences between experiments

were not significant for CA438-A, CA487, and CA565. As a summary the best batch of substrate for mushroom antioxidant activities depended on the strain. Similarly, Geosel et al. [31] reported antioxidant capacity variations between *A. subrufescens* cultivars and years and suggested that substrate fluctuation may influence the polyphenol biosynthesis pathway of the mushroom. Zied et al. [58] assessed how various cultivation practices (different strains, compost materials, casing layers used for fruiting induction, and cultivation environments) affect the final β-glucan content of the cultivated *A. subrufescens* mushrooms. The factors with the greatest contribution to the variation in β-glucan content were both the strain and the casing layer type (both 35 %), followed by the cultivation environment (16 %), and the type of compost (10 %).

These studies performed with different substrate origins and strains pointed out the importance of the genetic and phenotypic background of the strain and the crossed effect with environmental conditions when the objective is to produce functional food or bioactive components. This crossed effect is probably as significant with the mushroom collected in forest as with the cultivated ones. Consequently it is hazardous to compare data between published works performed on samples coming from different locations.

5 From Activities to Active Metabolites

Antioxidant activity in mushroom extracts is probably the result of the interaction between various metabolites having individually contrasted potential. However many works in mycopharmacy are dedicated to the isolation and identification of a major bioactive molecule in fractions and to the optimization of their production.

5.1 Antioxidant Molecules in Mushrooms

Among the antioxidant compounds in mushrooms, both polysaccharides and phenolic compounds have attracted much attention. In many studies comparing antioxidant activities in alcoholic extracts from different mushrooms, positive correlations were found with the total phenolic content evaluated by means of the Folin–Ciocalteu assay [34, 59–63].

Depending on their composition, all phenolic compounds are not as strong antioxidants as the others. In extracts, the antioxidant effect is affected by interactions between molecules which may result in synergism or antagonism [64]. Several studies measured the contents in individual phenolic compounds in mushroom extracts. Palacios et al. [65] found homogentisic acid to be the most abundant compound in mushrooms, but its concentration did not correlate with the antioxidant efficiency of the mushrooms. On the contrary, the activity of caffeic acid seems to be significant [65]. Finally when only phenolic acids detected by HPLC are taken into account, there is no significant correlation between their content and the antioxidant activity [66]. Actually, other compounds than phenolic acids are present in

mushrooms, react with the Folin-Ciocalteu reagent, and contribute to their antioxidant properties [66]. However, as for antioxidant activity the composition in phenolic compounds in mushrooms might depend on strains, the place the samples come for, the storage conditions of the mushroom, the extraction, and analytical methods.

5.2 Identifying a New Antioxidant Molecule in *A. subrufescens*, Agaritinal

Following is an illustration of a strategy developed to isolate and characterize a new antioxidant compound in *A. subrufescens* extracts and to identify factors affecting its yield of biological production. Powder of *A. subrufescens* sporophores strain CA487 was shaken for 4 h in 70 % ethanol. Ethanol was evaporated and three successive extractions were performed with ethyl acetate. The aqueous residue was lyophilized for drying, the solid residue dissolved with ethyl acetate/butanol/ water (1/4/5; v/v/v) to the concentration of 200 mg mL^{-1} and submitted to Centrifugal Partition Chromatography (CPC) using a Kromaton **CPC** 200 FCPC operating in either descending or ascending mode, with total cell volume of 220 mL. Three fractions (f1–f3) were selected during the ascending mode of the CPC and represented 3, 1.5, and 4.4 %, respectively, of the total dry weight of the extract. Two fractions, f4 and f5, obtained during the descending mode represented the major part of the extract (26–65 %, respectively). The Folin-Ciocalteu method [59] was used to assess the total phenolic content in these fractions and the Oxygen Radical Absorbance Capacity (ORAC) [67] was a measure of their antioxidant activity. The fraction with the highest quantity of phenolic compounds was f3, followed by f2 (Fig. 3). The major part of the antioxidant activity was also found in these two fractions. Far fewer quantities of phenolic compounds were detected in the other fractions, and only f1 had antioxidant activities although about ten times lower than those in f2.

The three fractions with significant antioxidant activities were submitted to analytical HPLC with Prontosil column 120-5-C18 (NC 04 250 × 4.0 mm). Compounds were eluted with a gradient of water TFA 0.005 % (solvent A) and acetonitrile TFA 0.005 % (solvent B) according to the following gradient program (v/v): 0 min 100 % A 0 % B, 50 min 100 % B isocratic for 5 min. Flow rate was 1 mL min^{-1}. Elution material was monitored by an UV detector. Two peaks were detected in fraction f1 and several peaks in fraction f2. Only a major peak (f3-m, Fig. 4) was observed on the chromatogram of fraction f3 which showed the highest polyphenol content with the Folin-Ciocalteu test. Peak wavelengths and retention times were reported in Table 4.

Fraction 3 was analyzed by HPLC-MS and the major compound was purified by preparative HPLC with Microsorb column 100-5-C18 (250 × 21.4 mm). The structure of the purified molecule was determined by ^{1}H NMR from spectra of homonuclear $^{1}H/^{1}H$ correlation experiments (COSY), Heteronuclear Single-Quantum correlation (HSQC), and Heteronuclear Multi-Quata Correlation (HMBC) experiments. The chemical formula obtained was $C_{12}H_{15}O_4N_3$, with

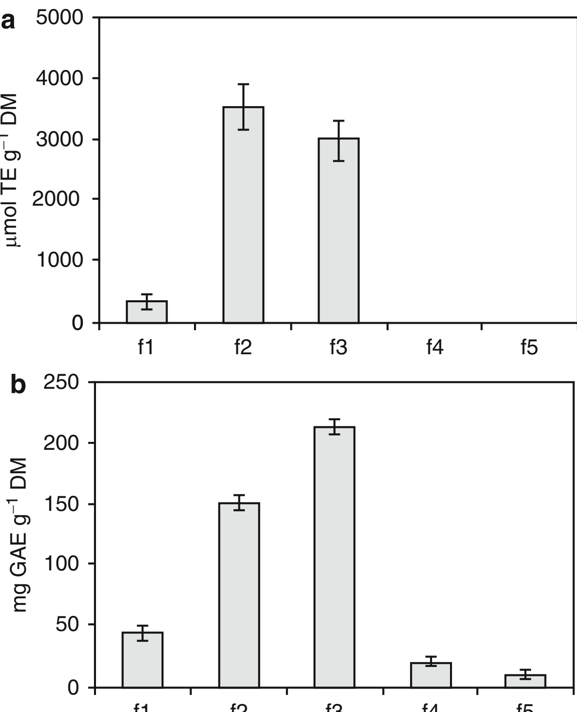

Fig. 3 ORAC values (**a**) and content of polyphenolic compounds (**b**) in the five fractions obtained after CPC of the aqueous extract

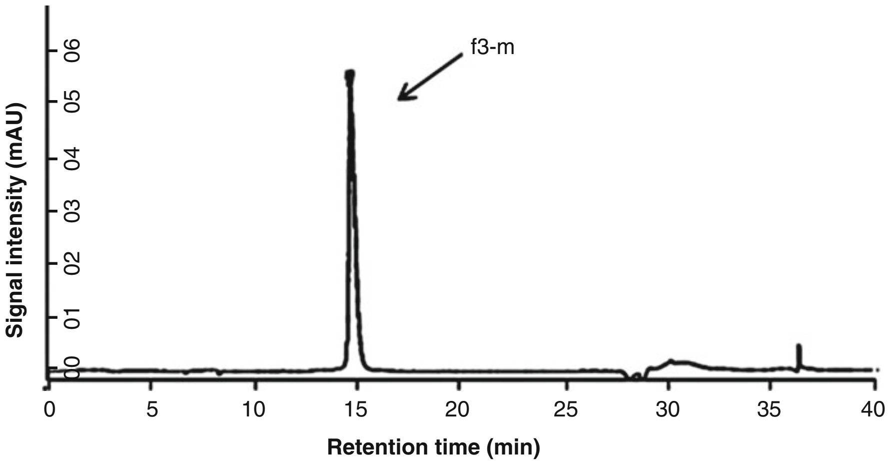

Fig. 4 Chromatogram at 310 nm of the purified f3 aqueous fraction from *A. subrufescens* submitted to analytical HPLC with Prontosil column 120-5-C18 with an elution gradient of water TFA 0.005 % and acetonitrile TFA 0.005 %, showing the major compound, f3-m

Table 4 Peak wavelengths and retention times of the major compounds detected in three aqueous fractions of *A. subrufescens* submitted to analytical HPLC with Prontosil column 120-5-C18 with an elution gradient of water TFA 0.005 % and acetonitrile TFA 0.005 %

Aqueous fractions	Peak wavelengths (nm)	Retention times (min)
f1	383	30.26
	372 (240)[a]	49
f2	260	9.1
	220	10.44
	245	15.03
	258	17.34
	220 (277)	21.18
	281	21.92
f3	312 (232)	16.21

[a]Wavelength of secondary peak

Fig. 5 Agaritinal chemical structure

a molecular mass of 265.265 g mol^{-1}. NMR techniques were used to identify the structure of this compound. In ^{13}C NMR (600 MHz, D_2O), the signature of carbons were at δ 194.3, 174.9, 173.5, 153.7, 132.7, 128.3,111.9, 54.1, 29.5, 25.9. The ^{1}H NMR (600 MHz, D_2O) spectrum gave the following data: δ 9.70 (1H, s); 7.88 (2H, d, J = 8.3Hz); 6.98 (2H, d, J = 8.3Hz); 3.85 (1H,t,J = 6Hz); 2.62 (2H,m); 2.26 (2H,m). Results from 1D and 2D NMR and high-resolution MAS and comparison with the published literature [68] led to the chemical structure of **agaritinal**, β-*N*-(γ-glutamyl)-4-formylphenylhydrazine (Fig. 5). Analytical HPLC of the f3 fraction by comparison with serial dilutions of purified agaritinal revealed that agaritinal contributed only 25 % to the fraction mass, and 1.1 % of the aqueous extract. Considering the mean ORAC value of agaritinal (5186 μmol TE g^{-1}) and the ORAC value measured for f3 (3020 μmol TE g-1), it was estimated that agaritinal contributed to about 40 % of the antioxidant activity of the f3 fraction.

These data allowed linking the antioxidant activity with phenolic compound concentrations in simplification fractions. However, in an attempt to identify active molecules, agaritinal, which is not a phenolic compound, accounted for 40 % of the antioxidant activity of the fraction with the highest antioxidant activity and phenolic content. To our knowledge, this work is the first report on antioxidant properties of *A. subrufescens* related to agaritinal.

In an attempt to characterize the specificity of agaritinal production in *A. subrufescens* we compared the species with two other cultivated mushrooms. *Agaricus subrufescens* CA487 and the commercial *A.bisporus* 30A (France Mycelium) kept in the CGAB collection since 1997, cultivated at our institute, did not differ significantly for their phenolic content and antioxidant activity whilst far less polyphenolic compounds and antioxidant activities were measured in *L. edodes*, strain 4306 (Somycel, France). Very little agaritinal was found in *A. bisporus* and the molecule was not detected in *L. edodes* (Fig. 6). The higher content of phenolic compounds in the wild *A. subrufescens* CA487 compared to the medicinal mushroom *L. edodes* confirmed published data for commercial strains of *A. subrufescens* and *L. edodes* [27]. Besides, the absence of agaritinal in the analyzed sample of *L. edodes* is not surprising, as the molecule is an oxidative form of agaritine which is limited to the genus *Agaricus*. The presence of agaritinal in the Meadow Mushroom *Agaricus campestris* was the first report of the presence of the molecule in a fungus [68]. A study of the distribution of agaritine within the genus *Agaricus* (covering 32 spp.) revealed that the compound is often accompanied by agaritinal, mainly in *A. arvensis*, *A. campestris*, *A. macrosporus*, *A. perrarus,* and *A. subperonatus* [69]. The level of agaritinal could be substantial in some of these mushrooms (above 1 g kg^{-1} fresh weight). Three of these species (*A. arvensis*, *A. macrosporus*, *A. perrarus*) are in the same phylogenetic section (*Arvenses*) as *A. subrufescens* [70]. However the same group from Switzerland reported analyses of samples of *A. subrufescens* from Brazil (cultures and wild isolates), and from cultures in USA showing variations of 1–6 of agaritine concentrations in fruit bodies whilst agaritinal was not detected [71]. The absence of agaritinal is contradictory to our own data and to the abundance of the metabolite in other species of the section *Arvenses*. The differences might be due to the drying condition and storage of the mushrooms before analysis and to the strain origin, but also to the cultivation conditions.

The wild strain CA487 was cultivated under different environmental conditions during the fruiting phase. Treatment 1 (no light, no cold shock) was as follows: the room temperature was set at 23–25 °C with 95–97 % humidity and CO_2 concentration lower than 1200 ppm. In treatment 2, no light but cold shocks for 4 h at 18 °C twice a week were applied; 12 h light/24 h without cold shock characterized treatment 3 while treatment 4 contained both 12 h light/24 h and cold shocks for 4 h at 18 °C twice a week. The different environment conditions had no significant effect on the total phenolic content (Fig. 4a) and antioxidant activity of the strain (Fig. 4b), but marked variations in agaritinal content were observed between treatments and between replicates of treatments 1 and 3 (Fig. 4c). In a published work, agaritine was identified in freeze-dried *A. subrufescens* mushrooms which have been cultivated on sugarcane bagasse and defatted rice bran substrate, but the agaritine peak split into two peaks in ethanol solution but not in methanol solution [72]. We wondered whether the two peaks could represent agaritine and agaritinal.

Controversial conclusions on the effect of agaritine on human health exist in the literature, but *A. subrufescens* is known as a medicinal mushroom and recent works concluded to the safety of the mushroom [73] and the antitumor effect of agaritine

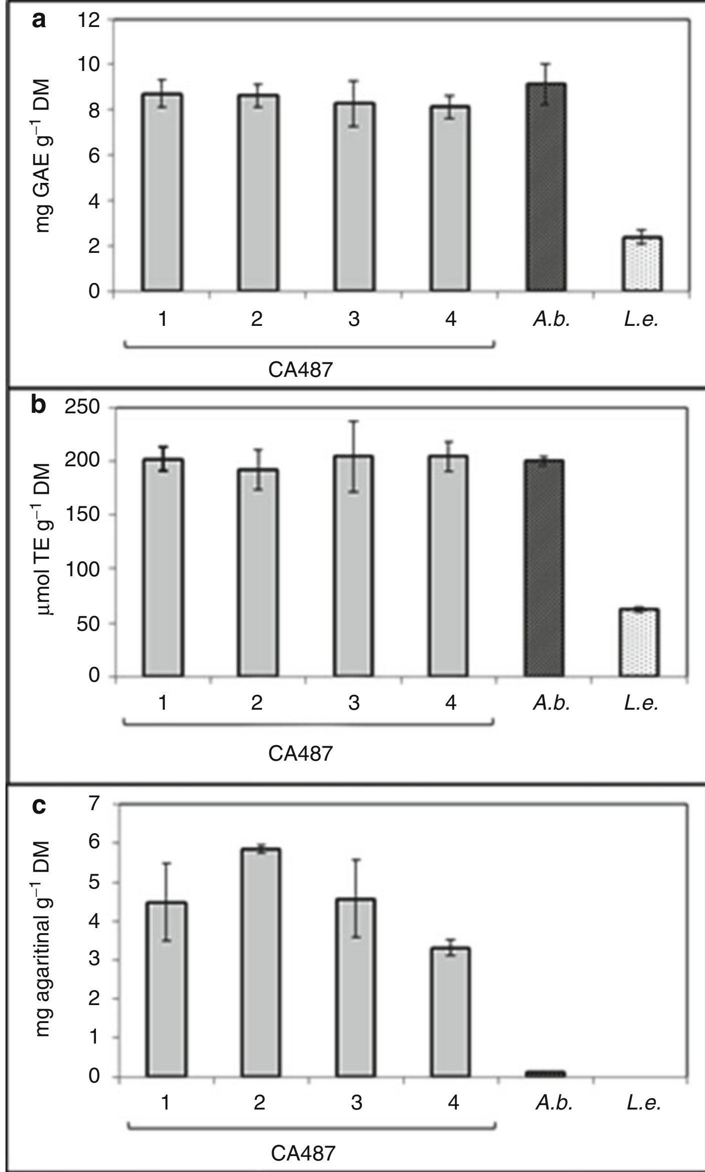

Fig. 6 Total phenolic content measured with Folin-Ciocalteu test (**a**), ORAC antioxidant properties (**b**) and agaritinal measured by analytical HPLC (**c**) in mushrooms. CA487, 1–4 = treatments 1–4 including light and temperature variations applied during the fruiting period of *A. subrufescens*, strain CA487; *A.b A. bisporus*; *L.e. L. edodes*

depending on the concentration used [74]. A putative pharmacological concern of *A. subrufescens* agaritinal might be possible if medicinal properties are identified. It could be an additional specific metabolite as the known blazeispirols, triterpenoid compounds with antioxidant activity and cytotoxic effects against several cancer cell lines, and proposed in the prophylactic and/ or therapeutic treatment of diseases that responds to the modulation of Liver X receptor [75].

5.3 Induction of Antioxidant Enzymes by Mushrooms

The primary function of antioxidant compounds in fungi is to prevent cell damage induced by ROS. Under normal conditions, ROS are cleared from the cell by action of superoxide dismutase (SOD). SOD catalyzes the conversion of O_2-· to H_2O_2 which is then decomposed in the presence of catalase (CAT) into water and oxygen. In addition, glutathione (GSH) and glutathione-related enzymes also play an important role against ROS, which are reduced by GSH in the presence of glutathione peroxidase (GPx) and GSH is regenerated by glutathione reductase (GR).

The oxidative stress can be removed by the induction of these antioxidant enzymes. In a few in vivo studies, generally an aqueous extract of *A. subrufescens* was administered orally to rats or mice. During aging of rats, de Sa-Nakanski et al. showed that *A. subrufescens* was protective mainly to the brain against the oxidative stress by increasing activity of antioxidant enzymes such as SOD and CAT. An improvement in the functionality of mitochondria from brain as evidenced by an increase in the activity of respiratory chain enzymes was also observed [76, 77]. Likewise, Zhou and Chen have observed a protective role of purified polysaccharides from *A. subrufescens* against the oxidative stress in rats suffering from breast cancer by induction of antioxidant enzymes (SOD, CAT, GPx, and GR) in the blood with a strong antitumor activity [78]. Contradictory results were also recorded about the protection by *A. subrufescens* against carbon tetrachloride-induced liver injury as shown by an increase in antioxidant enzymes in serum of animals, positive in a rat model [79] and negative in a mouse model [80]. Polysaccharides appear to be involved in this activity of mushrooms related to the antioxidant enzymes, as has been shown for some fungi such as *L. edodes*, *Ganoderma* sp, *Auricularia* sp, *Grifola frondosa, Hericium erinaceus,* and *Pleurotus abalones* [81]. Many other edible mushrooms were reported to have in vitro and in vivo antioxidant properties due to the presence of various putative bioactive compounds such as polysaccharides, vitamins, carotenoids, micronutrients, and polyphenols [82]. SOD, CAT, and GSH-dependent and recycling enzymes are also present in mushroom cells and they contribute to their antioxidant and detoxicant defences.

6 Conclusions

Antioxidant properties of *A. subrufescens* compounds that are soluble in methanol varied with the strain, development stage, part of the fruiting body studied, and cultivation conditions. All samples showed important radical scavenging activity but primordia and caps were particularly more active. The rapid production of a large biomass easy to transform is of prime importance for commercial valorization of *A. subrufescens* as food or dietary supplement with antioxidant properties. Besides, the antioxidant capacities of the strain should vary little with the batch of commercial substrate. This point might be the most difficult to solve, although strains like CA438-A and CA487 could have this potential, taking into account their short time to fruiting, high yield, and good antioxidant capacities. One of the antioxidants that is not a polysaccharide has been identified as agaritinal. The potential of wild strains can be useful to improve cultivated strains through breeding programs. This set of conclusions was obtained after an analysis of the published works and presentation of new data about a species of mushroom, but it is easily extendable to most of the cultivated mushrooms, as well as the species picked in forest, and is a guide for further improvements of the use of edible mushrooms as sources of new antioxidants.

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Cordycepin: A *Cordyceps* Metabolite with Promising Therapeutic Potential

23

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Abstract

For thousands of years, natural products from medicinal mushroom are being used for the cure of different lethal diseases. Among the huge category of medicinal herbs, the genus Cordyceps is gaining special attention due to its broad spectrum of biological activity. Cordycepin, a nucleoside analogue, is the main bioactive ingredient of Cordyceps and known to mediate a variety of pharmacological effects. Many chemically modified cordycepin derivatives

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have been reported which have shown more potential therapeutic effects. With the advancement in fermentation techniques, it has been possible to produce the higher cordycepin product. The modern techniques enabled the researchers for an easy detection and extraction of cordycepin from fermentation medium. Being a nucleoside analogue, cordycepin can interfere with the DNA/RNA biosynthesis and acts as a potential candidate for the treatment of the dreadful diseases such as cancer. Besides, cordycepin have also been known to modulate a variety of signaling pathways involved in apoptosis, proliferation, metastasis, angiogenesis, and inflammation. This chapter will describe the chemistry, production, detection, and extraction strategies of cordycepin. In addition, variety of therapeutic applications of cordycepin with all possible molecular mechanisms of actions have also been summarized.

Keywords
Cordyceps • Cordycepin • Derivatives • Fermentation • Detection • Extraction • Therapeutic potential

1 Introduction

Although there is an availability of numerous resources to design new therapeutic tools, the natural products are still preferred over the synthetic as they do not have any side effects. About 50 % of prescribed drugs in the USA are either the natural products or their structurally modified compounds [1, 2], which further increases the curiosity about the importance of these natural compounds in medical biology. There have been limited studies about the phytochemistry of the medicinal plants/herbs and their pharmacological potential. Today, the advancement in the research facilities and medical field has enabled us to carry out production, isolation, and identification of bioactive molecules. The modern tools such as ultraviolet, infrared, nuclear magnetic resonance, and mass spectrometry can help to identify an individual compound in a very short period of time.

Medicinal mushrooms have been known for thousands of years to produce a variety of biometabolites, which are being used as a possible therapeutic tool for the treatment of different diseases [3]. Over two third of cancer-related deaths could be prevented or reduced by modifying our diet with eatable mushrooms, as they contain many antioxidants [4, 5]. *Cordyceps militaris* is one of the medicinally important mushrooms, which has remarkable biomedical and pharmacological activities. The name *Cordyceps* has been derived from two Latin words, i.e., cord and ceps meaning club and head, respectively. *Cordyceps militaris* belongs to the Phylum Ascomycota classified in the Order Hypocreales, as spores are produced internally in sacs called ascus [6, 7].

Cordyceps, especially its extract, contains many biologically active compounds, including cordycepin, cordycepic acid, adenosine, exopolysaccharides, vitamins, and enzymes. Among these, cordycepin or 3′-deoxyadenosine (9-(3-deoxy-β-D-ribofuranosyl) adenine), a nucleic acid antibiotic, is the main active constituent

which is most widely studied and have a broad spectrum of biological activity [8]. Cordycepin is known to interfere with various biochemical and molecular processes such as purine biosynthesis, DNA/RNA synthesis, and mTOR (mammalian target of rapamycin) signaling transduction [3, 9]. It is predominantly produced commercially via solid-state fermentation and submerged cultivation of *Cordyceps*. Considerable effort is currently focused on three aspects of cordycepin production: strain screening and improvement, additives, and optimizing fermentation. However, high-efficiency batch fermentation of *C. militaris* is carried out in static culture for more than 30 days, which is too long to achieve high production efficiency and low operational cost and energy consumption [10]. Therefore, various methods have been proposed to extract and analyze bioactive metabolite like cordycepin from liquid culture as well as the fruiting body of *C. militaris*. This chapter reveals about the various production and extraction strategies adopted by the researchers for maximum gain of cordycepin. Furthermore, this chapter will update us about the potential applications of cordycepin as a therapeutic agent.

2 Chemistry of Cordycepin

The chemical formula of cordycepin (9-(3-Deoxy-β-D-ribofuranosyl) adenine) is $C_{10}H_{13}N_5O_3$ and its melting point is 228~231 °C. The structure of cordycepin shows that it has a molecular weight of 251.24 Da. Its UV spectrum reveals strong absorption bands at ≈ 259.0 nm [11]. The NMR spectrum of cordycepin shows singlet at 3.4 ppm, which can be attributed to C-H proton. The $-NH_2$ peak is found to present at 4.6 ppm, whereas the absorption peaks due to different -OH groups are found to be in the range of 8–8.5 ppm. The signals due to R3-CH and -N-C-H protons can be observed at 2.3 and 2.5 ppm, respectively [12]. The structure of cordycepin comprises a purine nucleoside molecule attached to a ribose sugar moiety via a β-N9-glycosidic bond (Fig. 1). Chemical synthesis of cordycepin is mainly achieved by the replacement of deoxyribose ring 3′ CO bond to form 2′, 3′-epoxy deoxyribose structure and region stereo-selective open-loop and direct synthesis of 3-deoxyribose derivatives. In a study, [13] investigated the synthesis of cordycepin monophosphate either via treatment of cordycepin with cyanoethyl phosphate in the presence of *N*, *N*′-dicyclohexylcarbodiimide via enzymatic transference of phosphate from uridine 5′-phosphate to cordycepin [13]. Cordycepin analogue of 2-5A (2–5 linked oligoadenylate) has also been synthesized and found to be a potent antiviral agent with comparison to natural molecule [14, 15]. The synthesis of *N*-acyl-cordycepin derivatives using alkyl chain has also been prepared. The resultant derivatives were not only observed to protect fast oxidation of cordycepin but also enhance its bioavailability and bioactivity [16]. Also, there have been immense possibility toward the formation of cordycepin-based metal complexes due to the presences of electron-donating atoms (N and O) in its structure and can easily donate their lone pairs of electron to the empty d orbital of the metal atoms [11].

Fig. 1 The chemical structure of cordycepin

3 Fermentation Strategy for Cordycepin Production

The medicinal mushrooms are abundant sources of useful natural products with various biological activities. Therefore, the extensive research has been seen in the past few decades on isolation and characterization of bioactive molecules from medicinal mushrooms [17]. Evidences suggested that most of the active contents of the mushrooms are being extracted from their fruiting bodies while fewer parts are derived from mycelium culture [18]. Since there is a huge requirement of medicinal mushroom-based biometabolites, it is necessary to cultivate mycelium biomass artificially for which variety of methods for its cultivation have been proposed by many research groups [19–21]. The *Cordyceps* mycelium can grow on different nutrients containing media, but for commercial fermentation and cultivation, insect larvae (silkworm residue) and various cereal grains had been used in the past. It has been seen consistently that from both insect larvae and cereal grains, fruiting body of fungus can be obtained with almost comparable medicinal properties [22].

There are basically two fermentation techniques by which the cultivation of mycelium biomass of *Cordyceps* can be achieved including surface and submerged fermentation. In surface fermentation, the cultivation of microbial biomass occurs on the surface of liquid or solid substrate. While in submerged fermentation, microorganisms are cultivated in liquid medium aerobically with proper agitation to get the homogenous growth of cells and media components [23]. Some reports are mentioned below describing the cordycepin production using submerged and surface fermentations.

Mao et al. (2005) studied the effects of various carbon sources and carbon/nitrogen ratios on production of cordycepin by submerged cultivation. The highest cordycepin production, i.e., 245.7 ± 4.4 mg L^{-1} on day 18, was obtained with

medium containing 40 g glucose L^{-1}. Further, using central composite design and response surface analysis, cordycepin production and productivity was increased up to 345.4 ± 8.5 and 19.2 ± 0.5 mg L^{-1} day^{-1}, respectively [24]. Similarly, the production conditions of cordycepin using surface culture technique were investigated by Masuda et al. [25]. They reported that under the optimal conditions, the maximum cordycepin concentration in the culture medium reached 640 mg L^{-1}, and the maximum cordycepin productivity was 32 mg L^{-1} day^{-1}. Further, Masuda et al. (2011) studied the effects of adenosine on cordycepin production in a surface liquid culture of the mutant and the wild-type strains [26]. For the mutant strain, the maximum levels of cordycepin production with and without adenosine were 8.6 and 6.7 g L^{-1}, respectively. The effects of nitrogen sources (NH_4^+) on cell growth and cordycepin produced by submerged cultivation of *Cordyceps militaris* were studied by Mao et al. [27]. The authors found that by optimizing the feeding time and feeding amount of $NH4^+$, a maximal cordycepin concentration of 420.5 ± 15.1 mg L^{-1} could be obtained. Similarly, [28] investigated the influence of initial pH value, various nitrogen sources, plant oils, and modes of propagation (shake flask and static culture) on the production of fungal biomass, exopolysaccharide (EPS), adenosine, and cordycepin using *Cordyceps militaris* CCRC 32219 [28]. They employed a Box–Behnken experimental design to optimize the production of cordycepin and achieved up to 2214.5 mg L^{-1} of cordycepin. Effect of ammonium feeding on cordycepin production was also investigated by Leung and Wu [29]. The authors reported that cordycepin production increases nearly fourfold (from 28.5 to 117 1 μg g^{-1}) by the supplementation of 10 mM NH_4Cl. However, at higher concentration, they found its negative effect on mycelium growth. In a study, Das et al. (2009) used mutant of the medicinal mushroom *Cordyceps militaris* for higher cordycepin production [30]. Among all the mutants, G81-3 had the highest cordycepin production of 6.84 g L^{-1} under optimized conditions compared to that of the control of 2.45 g L^{-1} (2.79 times higher). In addition, influences of different additives on the cordycepin production such as glycine and adenosine were also studied by the authors and found that cordycepin production can be increased up to 8.57 g L^{-1}. Xie et al. (2009) optimized fermentation temperature, pH, and medium capacity using Box–Behnken design and showed that highest dry mycelium weight (19.1 g L^{-1}) and cordycepin (1.8 mg g^{-1}) can be obtained at temperature 28 °C, pH 6.2, and medium capacity 57 mL [31]. In another study, [32] explored the effect of inoculation on cordycepin production in surface fermentation using *Cordyceps militaris* [32]. Results showed that cordycepin production increases with increase in inoculum size. The effect of ferrous sulfate addition to production of cordycepin (3′-deoxyadenosine) has also been investigated in submerged cultures of *Cordyceps militaris* in shake flasks [33]. Researchers showed that at a concentration of 1 g L^{-1} of ferrous sulfate addition results in 70 % higher cordycepin production compared to control experiment. The effect of liquid culture conditions on extracellular secretion of cordycepin from *C militaris* was investigated in ref. [34]. They reported the optimal cultural conditions as follows: initial pH 7, cultivation temperature 24 °C, shaking

speed 180 rpm, and cultivation period 9 days. They observed that these culture conditions led to reach cordycepin content of up to 0.537 g L^{-1} in the culture fluid. In another study, Zhang et al. (2013) applied response surface methodology (RSM) to optimize the medium components for the cordycepin production by submerged liquid culture [35]. They also suggested that repeated batch operation could be an efficient method to increase the cordycepin yield. Recently, Kang et al. (2014) studied single-factor design, using Plackett–Burman and central composite design to establish the key factors responsible for cordycepin production. They reported that maximum cordycepin up to 2 g L^{-1} could be achieved with working volume of 700 mL in the 1000 mL glass jar [36]. Similarly, Jiapeng et al. (2014) carried out fermentation to optimize maximum production of cordycepin in static culture using single-factor experiments with Placket–Burman and a central composite design. They demonstrated a maximum cordycepin yield of 7.35 g L^{-1} that can be achieved in a 5 L fermenter under the optimized conditions [37].

4 Analysis Tool for Cordycepin Detection

Nowadays different products of *Cordyceps* are available in the market, as health supplement or neutraceuticals. Hence, it is very important to analyze the presence of cordycepin for its quantitative as well as qualitative analysis [38]. Several techniques such as thin layer chromatography, high performance liquid chromatography (HPLC), and capillary electrophoresis have been reported in the analysis of the cordycepin present in medicinal herb *Cordyceps*. Herein, the development in biochemical analysis of cordycepin is reviewed and discussed.

4.1 Thin Layer Chromatographic Analysis

Thin layer chromatography (TLC) is known to be an easy and versatile method for separation of mixture of chemical components. Kim et al. (2006) developed TLC plates in chloroform/ methanol/water (64:14:1). The spots of separated molecules were stained with 10 % sulfuric acid solution (in ethanol) for visualization [39]. Ma and Wang (2008) established a dual wavelength TLC-scanning method for the determination of nucleosides in the preparation of *Cordyceps sinensis* and analyzed the samples on silica GF254 thin layer plate using 1 % CMC-Na (carboxyl-methyl-cellulose) as adhesive and chloroform-ethyl acetate-isopropanol-water-ammonia (8:2:6:0.5:0.12) as developing agent [40]. Hu and Fang (2008) compared the similarity between chemical components of *Cordyceps sinensis* and solid fermentation of *Cordyceps militaris* by TLC. They showed that solid fermentation of *Cordyceps militaris* and *Cordyceps sinensis* were basically similar in terms of their TLC spots occurred at the corresponding place except for a slight difference in size [41].

4.2 Spectrometry Analysis of Cordycepin

The spectrophotometric detection of cordycepin is based on its color reaction with anthrone. It has been reported that cordycepin reacts with a slightly modified anthrone (0.2 g anthrone in 100 mL 90 % H_2SO_4) reagents at high temperature, which results in the production of a cherry-red color [42]. The reaction was reported negative with adenine.

4.3 HPLC Analysis of Cordycepin

A simple high performance liquid chromatography (HPLC) with UV detection (HPLC–UV) method was proposed for the detection of cordycepin [43–46]. Chang et al. (2005) determined the concentrations of adenosine and cordycepin, 3′ deoxyadenosine in the hot water extract of a cultivated *Antrodia camphorate* by HPLC method. The procedure was carried out on a reversed-phase C-18 column [47]. Meena et al. (2010) compared the cordycepin content in natural and artificial cultured mycelium of *Cordyceps* using reverse phase HPLC [48]. In addition, though UV detection is widely used for chromatographic analysis, MS detection allows more definite identification and quantitative determination of compounds which may not be fully separated. ESI-MS in positive mode is most commonly used in the analysis of nucleosides in *Cordyceps* [49, 50].

4.4 Capillary Electrophoresis

Ling et al. (2002) determined the content of cordycepin by capillary zone electrophoresis in ultrasonic extracted *Cordyceps* for the first time [51]. Similarly, Rao et al. (2006) investigated a modified capillary electrophoresis (CE) procedure with UV detection at 254 nm for determination of cordycepin. They found 20 mM sodium borate buffer with 28.6 % methanol, pH 9.5, separation voltage 20 kV, hydrodynamic injection time 10 s, and temperature 25 °C were the optimal conditions for cordycepin detection [52]. Furthermore, Yang et al. (2009) developed capillary electrophoresis-mass spectrometry (CE-MS) method for the simultaneous analysis of 12 nucleosides and nucleobases including cytosine, adenine, guanine, cytidine, cordycepin, adenosine, hypoxanthine, guanosine, inosine, 2′-deoxyuridine, uridine, and thymidine in natural and cultured *Cordyceps* using 5-chlorocytosine arabinoside as an internal standard (IS). They optimized systematically for achieving good CE resolution and MS response tested compounds and found optimum parameters as follows: 75 % (v/v) methanol containing 0.3 % formic acid with a flow rate of 3 μL min^{-1} as the sheath liquid; the flow rate and temperature of drying gas were 6 L min^{-1} and 350 °C, respectively [53].

5 Extraction Strategy for Cordycepin

Being a biologically active molecule, a large quantity of pure cordycepin is urgently needed for further studies. Several extraction methods have been developed to extract cordycepin from the fermentative fluid and fruiting bodies of *C. militaris*, including ultrasound- or microwave-assisted extraction, pressurized extraction, soxhlet extraction, and reflux extraction. Some of them are discussed as follows.

Kredich and Guarino (1960) gave the first report on cordycepin extraction from liquid culture of *Cordyceps militaris.* They concentrated the fermented broth in an evaporator at 50 °C followed by cold precipitation and removal of impurities. Further, the obtained sample was passed through a column packed with Dowex-I-chloride of 200–400 mesh size [42]. In another study, Wang et al. (2004) compared supersonic water extraction, supersonic ethanol extraction, hydrothermal refluxing extraction, and ethanol thermal refluxing extraction for cordycepin and polysaccharide extraction using an orthogonal design experiment. They showed that hydrothermal refluxing extraction was the best extraction method of cordycepin and polysaccharide and its optimal technological conditions were optimized [54]. Rukachaisirikul et al. (2004) isolated and analyzed nine compounds from fungal mycelium as well as from liquid culture of *Cordyceps militaris*. Out of these, three were 10-membered macrolides, two were cepharosporolides, 2-carboxymethyl-4-(3′-hydroxybutyl) one was, furan, one was cordycepin, and one was pyridine-2, 6-dicarboxylic acid [55]. From dried fruiting bodies of *Cordyceps militaris,* Kim et al. (2006) extracted cordycepin using solvent-solvent extraction method. They extracted aqueous layer of crude fermented broth with hexane, butanol, and ethyl acetate [39]. Similarly, Rao et al. (2010) extracted and purified ten pure compounds, including cordycepin from the fruiting body of *Cordyceps militaris* [56]. Still, all these methods need optimization and were unsuitable for industrial applications. Jiansheng (2008) extracted and purified cordycepin from *Cordyceps militaris* using ion-exchange resin and silica gel column chromatography. They detected cordycepin on HPLC, LC/MS, and CE [57]. In a study, Wei et al. (2009) presented an efficient method of extracting and purifying cordycepin from the waste of the fruiting body production medium. This method included continuous counter-current extraction followed by column chromatography using 732 cation exchange resins. They found under optimized conditions cordycepin extraction yield reached up to 66.0 % [16]. Ni et al. (2009) developed column chromatography extraction (CCE) method for the extraction of cordycepin from the solid waste medium of *Cordyceps militaris*. The dried waste material was imbibed in water for 6 h and transferred to the columns and eluted with water. Eluates were directly separated with macroporous resin DM130 columns followed by purification steps with more than 95 % extraction yield [23]. Song et al. (2007) investigated optimization of cordycepin extraction from cultured *Cordyceps militaris* by HPLC-DAD coupled method. They reported that cordycepin extraction yield reached a peak with ethanol concentration 20.21 %, extraction time 101.88 min, and volume ratio of solvent to sample 33.13 g mL^{-1} [58]. The supercritical fluid extraction (SFE) method was purposed to extract cordycepin and

adenosine from the *Cordyceps kyushuensis* by Ling et al. (2009). They applied orthogonal array design (OAD) test, L_9 $(3)^4$ followed by preparative SFE extraction using high-speed counter-current chromatography (HSCCC). Their results yielded 8.92 mg of cordycepin and 5.94 mg of adenosine with purities of 98.5 % and 99.2 % from 400 mg SFE crude extraction, respectively [59]. Yong et al. (2010) compared six kinds of cordycepin extraction method from *Cordyceps militaris* medium. The authors got a higher extraction rate of cordycepin using microwave extraction method [60]. Zhang and his colleagues (2012) optimized the cordycepin extraction from the fruiting body of *Cordyceps militaris* YCC-01 using water, ethanol, ultrasonic, and synergistic approaches. They found that a synergistic approach was more efficient with cordycepin content of 9.559 mg g^{-1}. Results suggested that the yield was 66.2 % higher than the control group [61]. The microwave-assisted extraction of cordycepin from the cultured mycelium of *Cordyceps militaris* was investigated by Chen et al. (2012). The prepared extract was purified using a cation exchange resin (CER) of LSD-001. They found optimal desorption conditions as follows: 0.2 M of NH_3 combined with 80 % ethanol (v/v), desorption time – 2 h, temperature – 25 °C, and pH – 14 [62]. Yu et al. (2013) investigated the optimal conditions for cordycepin extraction from the waste medium of *Cordyceps militaris* using column chromatography. Initially, they did hot water leaching of *Cordyceps* waste at 70 °C for 8 h with dried feed and water ratio of 1 g: 20 ml followed by separation of cordycepin on macroporus resin XAD16 and polyamide column chromatography [63].

6 Therapeutic Potential of Cordycepin

Our society is facing heavy health burden due to increasing incidences of cancer-related morbidity and mortality. Therefore, to come up with an effective therapeutic strategy to combat cancer is being considered an essential focus of the research and medical field. Among the natural anticancer compounds, cordycepin is considered to be an important molecule in terms of its potent anticancer activity without any potential side effects [64, 65]. The anticancer role of cordycepin has been intensively investigated in a variety of cancers, including glioma and cancers of oral, breast, lung, hepatocellular, bladder, colorectal, testicular, prostate, melanoma, and blood cell. Previous studies demonstrated that cordycepin has potential to modulate multiple signaling pathways involved in cancer cell proliferation, apoptosis, invasion, metastasis, angiogenesis, and cancer immunity (Fig. 2). The reports of anticancer activity and other therapeutic effects of cordycepin along with the molecular mechanisms of actions are summarized in the Tables 1 and 2, respectively.

7 Bioactivity of Some Other *Cordyceps* Constituents

Besides cordycepin, a number of other bioactive compounds including cordycepic acid, ergosterol, and polysaccharides have also been identified from *Cordyceps*. Cordycepic acid, an isomer of quinic acid, is considered to be an active medicinal

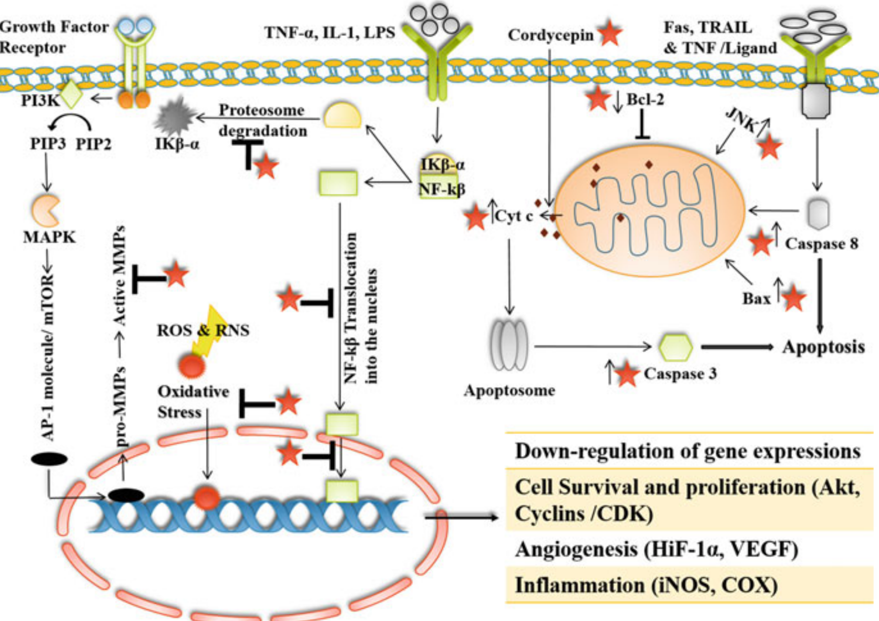

Fig. 2 The variety of signaling pathways modulated by cordycepin in cancer cells. These includes tumor cell proliferation, inflammation, angiogenesis, metastasis, survival, and apoptosis

component with potent anti-inflammatory activity [3]. Polysaccharides are other class of bioactive molecules which vary in the range 3–8 % of the total weight of *Cordyceps*. The exopolysaccharide fraction (EPSF) of *Cordyceps* on the hepatoma (H22) tumor-bearing mice not only inhibited the cancer growth but also significantly improved the immunocytic activity [116–118]. Similarly, a polysaccharide of 210-kDa from *C. sinensis* mycelia has been reported to protect pheochromocytoma (PC12) cells against H_2O_2-induced injuries [119]. A polysaccharide fraction from *C. sinensis* significantly inhibited proliferation of U937 cells by upregulating the levels of interferon (IFN)-gamma, tumor necrosis factor (TNF)-alpha, and interleukin (IL)-1 [120]. In other study, Zhang et al. (2005) demonstrated that exopolysaccharide fraction of *C. sinensis* inhibits the metastasis of melanoma cells and also downregulates the antiapoptotic protein level Bcl-2 into B16 melanoma-bearing mice [121]. The moieties such as glucan and galactosaminoglycan have also been identified from *Cordyceps* and found to suppress the growth of sarcoma 180 solid-type tumors in mice [122]. In addition to antitumor activity, polysaccharides from *Cordyceps* have shown potent hypoglycemic activity in diabetic mice [123, 124]. An antimalarial metabolite, i.e., cordyformamide, a xanthocillin like precursor, was extracted from *Cordyceps* which was found to exhibit toxicity against *Plasmodium falciparum* [125]. Kneifel and his colleagues extracted ophiocordin, an antifungal from submerged cultures of *C. ophioglossoides* [126]. Antifibrotic effects of extracellular biopolymer of *C. militaris* on fibrotic rats were observed by

Table 1 Cordycepin mediated anticancer effects along with mechanism of actions

S. no	Cancer types	Mechanisms of inhibition	References
1	**Glioma** C6 rat glioma cell line SK-N-SH and BE (2)-M17 cells neuroblastoma cell lines	Increased total p53 and phosphorylated p53 protein level, cleaved caspase-7 and poly (ADP-ribose) polymerase (PARP) pathway Apoptosis and autophagy (LC3)	[66] [67]
2	**Oral cancer** OEC-M1 human oral squamous cancer cells KB and HSC3 squamous cell carcinoma cell lines	Cell blebbing, increased cell percentage in subG1 and G2 phase indicating apoptosis	[68, 69]
3	**Breast cancer** MDA-MB-231 and MCF-7 cell lines BRCA1-deficient MCF-7 cell line MCF10A, MCF7, T47D, MDA-MB-435, MDA-MB-231 cell lines MCF-7	Mitochondrial-associated apoptosis, activation of caspase 3 and 9, and autophagosome-associated proteins Inhibit PARP Inhibit RNA synthesis, DNA double strand break Inhibit MMP9, AP-1 protein activation, and MAPK pathways	[70] [71] [72] [73]
4	**Lung cancer** Lewis lung adenocarcinoma	Stimulation of A3 receptor	[74]
5	**Hepatocellular carcinoma** BEL-7402 cells HepG2 cell line Hep3B human cell line	Metabolism-associated protein expression Apoptosis and antiangiogenesis TRAIL-mediated apoptosis, chromatin condensation and accumulation of cells in subG1 phase, inactivation of JUN pathway	[75] [76] [77]
6	**Bladder cancer** 5637 and T-24 cell lines	G2/M-phase arrest, upregulation of p21WAF1 expression, induced phosphorylation of JNK Downregulate MMPTNF and AP-1	[78] [79]
7	**Colorectal cancer** HT-29 (colorectal adenocarcinoma)	G1 and G2/M-phase cell cycle arrest, ROS generation increase, extrinsic and intrinsic apoptotic pathway activation, cleaved PARP expression increased	[80]
8	**Testicular cancer** MA-10 cells (mouse leydig tumor cell line)	DNA fragmentation, increased cell percentages in subG1 phase, extrinsic apoptotic pathway	[81, 82]
9	**Prostate cancer** LNCaP human prostate cancer cell line	Activation of extrinsic and intrinsic apoptotic pathways, autophagy pathway activation (LC3-II level and autophagy flux elevation) Downregulating the activity of TJs and MMPs, possibly in association with suppression of PI3K/Akt pathway G2/M cell cycle arrest, upregulation of CDK inhibitor p21	[83] [84] [85]

(*continued*)

Table 1 (continued)

S. no	Cancer types	Mechanisms of inhibition	References
10	**Melanoma** B16-F1 mouse melanoma cells B16-BL6 mouse melanoma cells	Blocking of ADP-induced platelet aggregation Stimulating adenosine A3 receptors followed by the Wnt signaling pathway, including GSK-3β activation and cyclin D1 inhibition	[86, 87] [86, 87]
11	**Blood cell tumors** Daudi, (Burkitt's lymphoma) Molt-4, (acute lymphoblastic leukemia) Human leukemia Multiple myeloma (MM)	DNA fragmentation, cell cycle arrest ROS generation, mitochondrial dysfunction, activation of executioner caspases, and cleavage of poly (ADP-ribose) polymerase protein Inhibit RNA synthesis, apoptosis Suppressing GSK-3b/b-catenin signaling	[88] [89] [90] [91]

Lipopolysaccharide (LPS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), reduced glutathione (GSH), vitamin C and vitamin E, and elevated levels of malondialdehyde (MDA), serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, and creatinine, inflammation-induced osteoporosis (IMO), serum osteocalcin (OC), homocysteine (HCY), C-terminal cross-linked telopeptides of collagen type I (CTX), RA synovial fibroblasts (RASFs), maleic dialdehyde (MDA), polymorphonuclear cells (PMN), interleukin-1β (IL-1β), and tumor necrosis factor- α (TNF-α), rheumatoid arthritis synovial fibroblasts (RASVs), osteoarthritis (OA), human African trypanosomiasis (HAT), oxygen–glucose deprivation (OGD), malondialdehyde (MDA), superoxide dismutase (SOD), matrix metalloproteinase-3 (MMP-3), amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), *N*-methyl-D-aspartic acid (NMDA), cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-c), levels of phospho–AMP-activated protein kinase (AMPK) and phospho–acetyl-CoA carboxylase (phospho-ACC) phospho–acetyl-CoA carboxylase (phospho-ACC), rat renal interstitial fibroblast (NRK-49 F) cells

significant reduction in aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) along with bilirubin and hydroxyproline content [127]. The vasorelaxant activity of some protein constituents of *Cordyceps* has been found and could play an important role in cardiovascular diseases [128].

8 Conclusions and Future Perspectives

In the last few decades, people have shown faith on mushroom-based products for the treatment of various dreadful diseases. The fruiting body of *Cordyceps* is an excellent reservoir of the therapeutic bio-agents with multidisciplinary mechanism of action. The availability of sophisticated instrumentation has made possible the higher rate of production as well extraction of these bioactive metabolites. Due to the redox behavior, cordycepin can modulate a number of cellular signaling pathways associated with various malignancies. A number of chemical modifications can be

Table 2 An overview of therapeutic potential mediated by cordycepin

S. no	Role/model	Mechanisms	Dose	References
1	**Anti-inflammatory** LPS-stimulated BV2 microglia Wistar rats (IMO) Murine model of acute lung injury Mice (C57BL/6) (lung injury associated) Human OA chondrocytes RASFs	NF-κβ, Akt, and MAPK signaling pathways ↑CTX, MDA, PMN, IL-1β, TNF-α, and nitrate levels in plasma Inhibition of Th2-type responses through the suppression of the p38-MAPK and NF-κβ signaling pathway Block the expression of ICAM-1 and VCAM-1, MCP-1, MIP-1α, MIP-2 and KC, and CXCR2, and ↓TNF Suppress IL-1β-stimulated GAG release, ↑ MMP-1, MMP-13, cathepsin K, cathepsin S, ADAMTS-4 and ADAMTS-5 gene expression, inhibited IL-1β-induced COX-2 and iNOS expression and blocked NO production Inhibit p38/JNK/ AP-1 signaling pathway	7.5 μg/ml 20 mg/kg 10, 20, and 40 mg/kg 2 mg/kg 5–100 μM 50–100 mM	[92] [93] [94] [95] [96] [97]
2	**Antioxidant** Male Sprague–Dawley rats	↑ Activity of SOD, CAT, GPx, GR and GST, GSH, vitamins C and E and↓ levels of MDA, AST, ALT, Urea and creatinine	20 mg/kg	[98]
3	**Neuroprotective** Hippocampal CA1 pyramidal neuron Ischemia mice and (OGD) injury of brain slices Hippocampal brain slices Ischemic mice	↓the frequency of both the spontaneous and evoked action potential (AP) firing ↓the extracellular level of glutamate and aspartate, MDA, ↑ the activity of (SOD) and ↓ MMP-3 Suppresses excitatory synaptic transmission by decreasing the excitatory neurotransmitter release presynaptically, suppressed AMPA and NMDA receptor-mediated responses Improve learning and memory, ↑ number of pyramidal cells, both in hippocampal CA1 and CA3 regions	2, 5, 10, 20, and 100 mg/l 20, 40, 80 μM 20 mg/L 5 and 10 mg/kg	[99] [100] [101] [102]
3	**Cardioprotective** SD rat (rat aortic smooth muscle cells (RASMCs)) Sprague–Dawley rat	Inhibit PDGF-BB–induced migration and proliferation via interfering with adenosine receptor-mediated NOS pathways, suppressed the phosphorylation of p38 MAPK	10 mg/kg 3, 10, 30 mg/kg	[103] [104]

(*continued*)

Table 2 (continued)

S. no	Role/model	Mechanisms	Dose	References
		and Hsp27, ↓ROS, O_2, and H_2O_2 ↑The phosphorylation of Akt/ GSK-3b/p70S6K pathways, ↓Bax and cleaved caspase-3 expression while increasing Bcl-2 expression, Bcl-2/Bax ratio, and heme oxygenase (HO-1) expression		
4	**Antiasthmatic** Mouse model of allergic asthma Calu-3 and 16HBE14o- cells	↓ IL-17A, ↑ IL-10 level ↑ Foxp3, and inhibited RORγt $Na^+-K^+-2Cl^-$ cotransporters and apical CFTR Cl-channels, cAMP- or Ca^{2+}-activated K^+ channel activation	20–40 mg/kg 300 and 10 μM	[105] [106] [107]
5	**Antidiabetes** LPS-stimulated RAW 264.7 cells	Inhibited the production of NO, IL-1β, IL-6, and TNF-α, NF-kβ, ↓11β-HSD1 and PPARγ, ICAM-1, and B7-1/-2	40 μg/ml	[108]
6	**Antihyperlipidemia** 3 T3-L1 preadipocytes Male Syrian golden hamsters, HepG2 cell and abnormal metabolic mice	Intervention in the mTORC1-C/EBPb–PPARg pathway Reduced the accumulation of serum total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-c) and, ↑ the levels of phospho-AMPK and phospho-ACC AMPK activation in HepG2 cells ↑the insulin sensitivity and improved the oral glucose tolerance	100 mg/mL 50 mg/kg, 1 μM and 50 mg/kg	[109] [110]
7	**Antiosteoporosis** Wistar female rats	↓ALP, TRAP activity, and CTX level, ↑ OC	5, 10, 20 mg	[111]
8	**Antidepressant** CUMS mice	Normalized the change of TNF-α, IL-6, and NE levels, by the upregulating BDNF and downregulating 5-HT2AR levels	20, 40 mg/kg	[112]
9	**Renal interstitial fibrosis** NRK-49Fcells	Suppress myofibroblast activation. Inhibit Smad2/3 protein, upregulate HGF expression and the HGF receptor activation	80 μM	[113]

(*continued*)

Table 2 (continued)

S. no	Role/model	Mechanisms	Dose	References
10	**Skin protection** Human dermal fibroblast cells	Inhibition of NF-kβ activation	50–100 μM	[114]
11	**Antiviral** SNU719 cells	↓ Apoptosis, ↑ BCL7A methylation and ↓demethylation, ↓the frequency of Q and F promoter usage, H3K4me3 histone enrichment, ↓ intra and extra cellular copy no of EVB genome, suppressed the transfer of EBV from LCL-EBV-GFP to AGS cells	125 μM	[115]

made in the internal structure of the cordycepin to counter drug resistance development and to increase its pharmaceutical potential. The therapeutic potential of cordycepin may be increased using synergistic approaches with the multiple chemotherapeutic agents [129]. In future, it is essential to characterize the other unknown molecules of *Cordyceps* to understand their structure-function relationship. The scientific community should also focus on nano-biotechnology-mediated targeted drug delivery system not only to reduce the requirement of active doses of drug but also to enhance its bioavailability.

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Production of Cyclosporine A by Submerged Fermentation

24

Tehmina Anjum and Wajiha Iram

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Abstract

Cyclosporin A is a cyclic undecapeptide with a variety of biological activities including immunosuppressive, anti-inflammatory, antifungal, and antiparasitic properties. It is an extremely powerful immunosuppressant and is approved for the use in organ transplantation to prevent graft rejection in kidney, liver, heart, lung, and combined heart–lung transplants. As its role in transplantation surgery increases, the demand on industry for improved yields intensifies. For this reason, this chapter mainly focuses on enhanced production of cyclosporin A from microbes by different techniques.

Keywords

Cyclosporin A • Immunosuppressant • Anti-inflammatory • Antifungal • Antiparasitic • Transplantation

List of Abbreviations

Abu	Aminobutyric acid
Ala	Alanine
EMS	Ethyl methanesulphonate
HPLC	High performance liquid chromatography
MeBmt	Butenyl-methyl-L-threonine
Me(Leu)	Methylleucine
Me(Val)	Methylvaline
NG	Nitrosoguanidine
NMR	Nuclear magnetic resonance spectroscopy
Sar	Sarcosine
UV	Ultra violet

1 Introduction

Fungi fall in those eukaryotic organisms that are known by their usage as the most prolific producers of a number of novel secondary metabolites which can be either serve as lead structures for synthetic modifications or directly use as drugs in various medications [1–7]. The versatility of fungal biosynthesis is demonstrated by the enormous production of enzymes; polysaccharides; vitamins; pigments; polyhydric alcohols; lipids; glycolipids; antibiotics with their derivatives, for instance β-lactam peptide antibiotics, tetracyclines, and the macrolide polyketide erythromycin; aminoglycosides; enzyme inhibitors; hypocholesterolemic agents; immunosuppressants; and antitumor compounds. Of these, certain products are produced at commercial scale while others occupy valuable space in the field of biotechnology [8, 9].

Secondary metabolites are compounds with varied and sophisticated chemical structures, produced by microorganisms. They have a major effect on health, nutrition, and economics of our society. Secondary metabolites have no obvious

function in the growth of the producing organisms and often are produced as a family of structurally related compounds.

A large number of known fungal secondary metabolites have been produce by known filamentous fungi e.g., *Aspergillus oryzae* (Ahlburg) Cohn*, Aspergillus sojae* Sakag & K. Yamada, and *Aspergillus tamarii* Kita used in the food fermentation industry are considered to be safe because they produce no aflatoxins. *A. tamarii* is a morphologically distinct species, also producing kojic and cyclopiazonic acids, among other secondary metabolites. *Aspergillus fumigatus* Fresenius produces many secondary metabolites including fumagillin, fumitremorgin, fumigaclavine, gliotoxin, helvolic acid, verruculogen, and sphingofungins. It has been suggested that the virulence of the strain may be enhanced by these metabolites [10]. The occurrence of indole alkaloids among secondary fungal metabolites was studied in different *Aspergillus* species [11]. Fumigaclavine B was formed by *A. fumigatus*, α-clyclopiazonic acid by the isolates of *A. fumigatus, A. flavus* Johann Heinrich Friedrich, *Aspergillus versicolor* (Vuill.) Tirab, *Aspergillus phoenicis* (Corda) Thom and *A. clavatus* Desm whereas, diketopiperazine alkaloids by *A. flavus, A. fumigatus*, and *Aspergillus ochraceus* G. Wilh [12]. These microbial secondary metabolites are protein in nature several of which possess peculiar medicinal properties among which the immunosuppressant cyclosporine A is an example.

Cyclosporins is a group of nonpolar cyclic oligopeptides with Cyclosporin "A" as the major component possessing immunosuppressive activity [13]. A multifunctional enzyme, Cyclosporin synthetase of the filamentous fungus, *Tolypocladium inflatum* is used for the production of CyA [14]. Dreyfuss et al. [15] first time investigated CyA as an antifungal antibiotic. For the last few decades, CyA has been used as an immunosuppressive agent that was discovered, by employees of Sandoz (now Novartis) in Basle, Switzerland, in January 1972 during the screening test designed by Hartmann F. Stahelin on immune-suppression [16]. It was subsequently approved for clinical use in 1983 by USFDA [17]. Now CyA is widely used in the prevention of rejection of various transplants of heart, kidney, and liver and in treatment of graft-versus-host reactions in bone marrow transplantation. In numerous diseases like Graves disease, uveitis, ulcerative colitis, Crohn disease, primary biliary cirrhosis, chronic active hepatitis, diabetes mellitus, sarcoidosis, myasthenia gravis, systemic lupus erythematosus, dermatomyositis, psoriasis, rheumatoid arthritis, and certain nephropathies where immunological factors might be involved in a pathogenic role, CyA has been found to be effective and proved a substantial role in the respective therapies [18, 19].

In submerged fermentation process, sterilization and process control are rather easier to control; therefore, now a days this is the most preferred method principally used for the large scale production of CyA from *T. inflatum*. Cyclosporin production is also reported in *Cylindrocarpon* spp., *Fusarium* spp., *Tolypocladium geodes*, *Trichoderma virile, Neocosmospora vasinfecta, Isaria* spp.*, Verticellium* spp., *Acremonium* spp., and *Beauveria nivea* [20]. In general, high yield of microbial products like CyA are closely associated with various physical and chemical parameters in fermentation process like the choice of proper organism, medium composition, rate of aeration/agitation, and the control of antifoam/pH etc. These parameters

are however may not provide a suitable rise in the overall product yield in fermentation. Presently, application of induced mutations is the possible way out that is being extensively used in altering industrial organisms for the high production of CyA at commercial scale.

In this chapter, we describe the chemistry and mode of action of CyA, its antifungal activity, different fermentation techniques used for its production, medium optimization, and mutational approach for its enhanced production by fungal species.

2 Chemical Structure of Cyclosporin A

Cyclosporin A (chemical name: Cyclo [[(E)-(2S, 3R, 4R)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoyl]-L-2-aminobutyryl-*N*-methylglycyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanyl-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl]) is a neutral lipophilic cyclic polypeptide. Molecular weight of CyA is 1202 with a molecular formula of $C_{62}H_{111}N_{11}O_{12}$. As shown by acid hydrolysis, it is found to be composed of 11 amino acids, 10 of which are known aliphatic amino acids while 1 is unknown [21]. In further study, Wenger [22] demonstrated that the unknown amino acid of cyclosporin was MeBmt that could be significant for pharmacological activity of CyA and its synthesis in enantiomerically pure form. Moreover, it was illustrated that amino acids i.e., (4R)-4-[(E)-2-butenyl]-4, *N*-dimethyl-L-threonine (MeBmt), L-alpha-aminobutyric acid (Abu), sarcosine (Sar), and *N*-methylvaline (MeVal) are responsible for immunosuppressive properties of CyA.

In CyA, 2S configuration have been found in all amino acids except at position 8 and 3 where alanine residue has 2R configuration and achiral sarcosine respectively. An antiparallel β-pleated sheet confirmation was observed in the backbone amino acid residues having 3 transannular H-bonds at position 1–6 [23]. An open loop is formed by remaining residues (7–11) having only cis amide linkage between two adjacent *N*-methyl leucine residues at position 9 and 10. Lipophilic nature of the molecule is associated with *N*-methylated amino acids at positions 1, 3, 4, 6, 9, 10, and 11. Moreover, the rigidity of the skeleton is due to the hydrogen bond formation by four available amide groups (Fig. 1).

Nuclear magnetic resonance spectroscopy (NMR) of CyA showed to comprise of a compacted sheet in antiparallel orientation joined with four intra molecular hydrogen bonds involving the four non-methylated amide (NH) groups: Abu2 (NH)-Va15 (CO), Va15 (NH)-Abu2(CO), A1a7(NH)-MVall1 (CO), and D-Ala8(NH)-MLeu6 (CO). This tightly folded structure has a highly hydrophobic surface on the outer side due to interior intra-molecular hydrogen bonds [25].

3 Properties

Cyclosporin “A” is a white- to off-white crystalline solid having melting point of 148–151 °C (natural) and 149–150 °C (synthetic) [13, 18].

It is stable in solution at temperatures below 30 °C but is sensitive to light, cold, and oxidization. When heated to decomposition, cyclosporin “A” emits toxic fumes

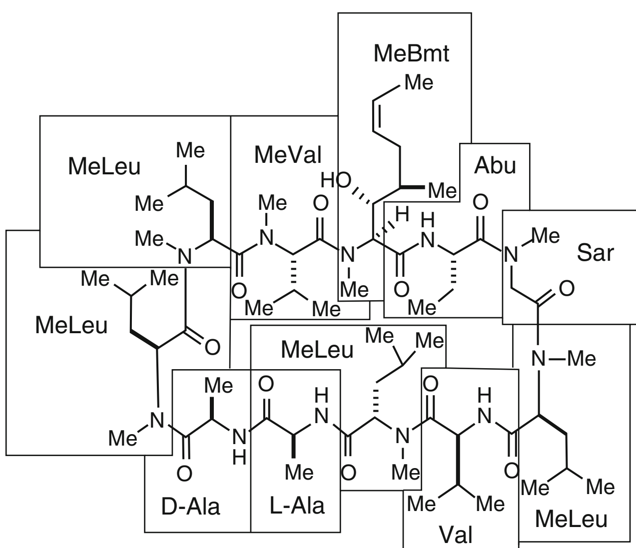

Fig. 1 The chemical structure of CyA [24]

of nitrogen oxides [18]. These are highly lipophilic substances and poorly soluble in water [26]. While they show very good solubility in organic solvents such as methanol, ethanol, acetone, ether, and chloroform. Solubility of cyclosporin “A” was found to be inversely proportional to the temperature [27].

4 Mode of Action

Cyclosporin is assumed to bind to the immunocompetent lymphocytes, especially T-lymphocytes, of cyclophilin (cytosolic protein) [28]. Under normal circumstances, the transcription of interleukin is facilitated by calcineurin, which is inhibited by cyclosporin and cyclophylin complex. Moreover, production of lymphokine and interleukin release is inhibited by this complex resulting in subsequent reduction in the function of effector T-cells [26]. Cytostatic activity is remained unaffected by CyA. However, cyclosporin A prevents the opening of mitochondrial permeability transition (PT) pore which in turn inhibits the release of a potent apoptotic stimulation factor “cytochrome c” [29].

CyA is known to be a human carcinogen [18, 30]. Literature showed that CyA has been found to produce cancer in both laboratory animals and humans. Lymphoma and skin cancer (common malignancies) are associated with long-term exposure to CyA [31]. Literature shows that when CyA is used in combination with prednisolone, it causes chromosomal aberrations in the peripheral blood lymphocytes of kidney transplant patients, sister chromatid exchange in human lymphocyte cell, and in vitro, unscheduled DNA synthesis. It has been documented that patients

undergoing immunosuppressive therapy showed an increased risk of cancer resulting subsequent use of CyA [18]. According to previous literature, risk of developing a malignancy during Cyclosporin “A” treatment is due to immunosuppression rather than genotoxicity [32, 33].

CyA teratogenicity is also not well documented. However, at higher dose (two to five times the normal human dose), CyA causes fetal mortality in rats and rabbits when it was administered during pregnancy [18, 33].

Limited data is available for describing the occupational exposure to CyA. According to literature primary exposure routes to CyA include inhalation, accidental injection, and dermal absorption [30, 34]. However, after treatment with CyA, chronic effects in patients and laboratory animals could lead to a number of serious health effects.

5 Applications

Cyclosporin “A” is used in organ transplantation to prevent graft rejection in kidney, liver, heart, lung, and combined heart–lung transplants. It is used to prevent rejection following bone marrow transplantation and in the prophylaxis of host-versus-graft disease [35]. The pharmacological effects of cyclosporin “A” are rapid when drug is administered in combination with steroids, azathioprin, or mycophenolic acid [36].

Cyclosporin “A” has also been used in number of clinical applications which includes: the reversal of multidrug resistance, antimalarial, herpes virus infection, rheumatoid arthritis, type I diabetes, and also as a potent anti-human immunodeficiency virus 1 (HIV-1) agent [37, 38].

Additionally, it is also use to treat fulminant hepatitis, multiple sclerosis, lupus nephritis, systemic lupus erythematosus, alopecia areata, atopic dermatitis, dermatomyositis, lichen planus, myasthenia gravis, polymyositis, psoriatic arthritis, pulmonary sarcoidosis, and uveitis [39].

Cyclosporin “A” has been investigated as a possible neuroprotective agent in conditions such as traumatic brain injury and has been shown in animal experiments to reduce brain damage associated with injury [29]. Studies indicate that cyclosporin “A” used in combination with antilymphocyte globulin can be used for acquired aplastic anemia [40–42]. It was reported in open studies that cyclosporin is effective for induction of remission in Crohn disease [43].

6 Cyclosporine Metabolism

Cyclosporine is metabolized in liver and the small intestine and is converted into more than 30 metabolites by both phase I and II metabolism. These cyclosporin metabolites in blood stream are responsible for its immunosuppressive activities [44]. The structural characterization of theses metabolites have been done by mass spectrometry. The reaction involved in phase I metabolism of cyclosporin are hydroxylation, demethylation, as well as oxidation and cyclization at amino

acid 1. The enzymes CYP3A4 and CYP3A5 catalyzes phase I metabolic reactions [45–47]. Moreover, cyclosporine also acts as an inhibitor and a substrate of the MDR1 transporter [48] that is found to be located in enterocytes and hepatocytes [49]. These enterocytes and hepatocytes both express CYP3A4 and the efflux transporter MDR1 [50], which maximize the cyclosporin exposure to metabolism by CYP3A4 through repeated cycles of absorption and efflux [51].

7 Cyclosporin Pharmacokinetics

For many years, pharmacokinetics has been extensively used to relate immunosuppressant dose to drug exposure. Its primary method of measurement is drug absorption, distribution, metabolism, routes of excretion, and interactions with other drugs. In blood and serum, the cyclosporin concentration was monitored as a means of reducing the risk of nephrotoxicity or rejection which are associated with inappropriate drug concentrations. In humans, the CyA pharmacokinetics is quite unpredictable [52]. The cyclosporin use become complicated due to high inter and intra patient pharmacokinetic variability [53]. This variability is found to be associated with patient's disease state, the type of organ transplant, the age of the patient, and therapy with other drugs that interact with CyA.

Cyclosporin distribution not only depends upon physicochemical characteristics but also on biological carriers such as lipoproteins and erythrocytes in blood. In the body, cyclosporin distribution have been influenced by cyclophilin which is a binding protein for cyclosporin. Cyclosporin metabolites distribution are found to be different from that of cyclosporin in the body. This drug is mainly eliminated via bile as metabolites. Several metabolites are reported to have less immunosuppressive activity than the parent drug. Some shows renal side effects due to highly variable activity of cyclosporin metabolizing liver enzymes [54].

8 Cyclosporine Chronopharmacokinetics

Previous studies showed that the concentration of cyclosporin is high in morning as compared to evening, while the difference in concentration is not clear. It have been suggested that changes in liver blood flow, microsomal activity differences, different transporter function, or differences in protein binding during night and day may be the possible cause [55]. Heifets et al. [56] conducted a study on five patients and observed high cyclosporine clearance (intravenous administration) in the evening as compared to morning. They hypothesized that this difference may be due to increase activity of the metabolic enzymes in the liver at night. However, Curtis et al. suggested that the metabolic rate remains same throughout the day, and any differences in cyclosporine concentrations are attributable to the fact that patients are more likely to be fasted before the morning dose and fed before the evening dose [57].

9 Therapeutics Side Effects of CyA

Despite its most powerful immunosuppressive effect, one of the most commonly observed adverse effects of cyclosporin "A" is the development of gingival overgrowth [58]. It is also reported to cause less frequently occurring side effects diabetogenic, hyperlipidaemia, abnormal liver enzyme, hypertension, hirsutism, neuropathy, nephrotoxicity, tremor, hyperkalemia [59], gastrointestinal abnormalities, hypomagnesemia, central nervous system disturbances, hyperglycemia, and infection.

10 Cyclosporin Derivatives to Improve Drug Properties

Numerous analogues and derivatives of CyA have been tested in order to improve the drug's therapeutic properties. For example, CyG, a cyclosporin A analogue with L-novaline subtituent at position 2, displays equal immunosuppressive effects as CyA but with less nephrotoxicity [60]. Similarly, $ISA_{TX}247$ is a potent derivative with higher activity and lower nephrotoxicity as compared to CyA [61].

Another derivative SZZ IMM-125, which is a hydroxyethyl derivative of D-serine-8-cyclosporin, was found to be slightly more potent but less nephrotoxic than CyA in both in vitro and in vivo model studies [62].

Several other cyclosporin analogues with high immunosuppressive activity were obtained through chemical modification of side chains at the first and third amino acids [63].

11 Antifungal Activity of Cyclosporin

Antibacterial and antifungal activity of CyA has been examined by Dreyfuss and colleagues [15]. According to their findings, CyA has a very narrow spectrum of antifungal activity while no antibacterial activity was observed. Only deformation and branching of growing hyphal tips was observed during inhibition while the germination of fungal spores or conidia remain unaffected. Strains of some mucorales, ascomycetes, and fungi imperfecti were found to be sensitive to the metabolite. Inhibition of cell wall synthesis (in particular chitin) might be the mode of antifungal action of CyA as hypothesized by Dreyfuss and colleagues after analyzing the taxonomic positions of sensitive organisms. Same narrow spectrum antifungal activity was observed in polyoxin (a chitin blocking antibiotic) when compared with CyA. Polyoxin is an antifungal drug having high specificity and low toxicity to non-fungal hosts that inhibited cell wall synthesis like beta-lactam antibiotics.

Rodriguez et al. [64] studied the in vitro antagonistic activity of *Fusarium oxysporum* strain by dual cultures. They identified that CyA was found to be responsible for antifungal activity of *F. oxysporum* against pathogen. Further, their study showed that in a greenhouse assay, a significant increase in number of

surviving soybean (*Glycine max*) plants was recorded when *S. sclerotiorum* and *F. oxysporum* were inoculated together as compared to plants inoculated with *S. sclerotiorum* alone. CyA also showed antifungal activity against *Aspergillus fresenii* Subram, *A. juponicus* Saito, *A. niger* Tiegh, *Cryptococcus neoformans* (Sanfelice) Vuillemin, *candida* sp., *Trichophyton mentagrophytes* (Robin) Blanchard, *T. tonsurans* Malmsten, *T. violaceum* Sab. apud Bodin, and *Fusarium* sp. [65].

12 Biosynthesis of Cyclosporin A

There are four general mechanisms reported for biosynthesis of peptides, two of which are nucleic acid dependent, i.e., classical ribosomal translation and ribosomal independent bacterial pentaglycine peptide biosynthesis [66–68]. While the other two are nucleic acid independent, namely the enzymatic biosynthesis of short peptides and thiotemplate mechanism involving multienzymes.

CyA is synthesized from its precursor amino acids by cyclosporin synthetase, a single multifunctional enzyme. The enzyme fraction involved in the production of CyA was purified from *Tolypocladium inflatum by* Zocher et al. [69]. The enzyme was found capable of forming covalent enzyme-substrate complexes and catalyzing the ATP-pyrophosphate exchange reactions dependent on the unmethylated constituent amino acids of CyA.

Billich and Zocher [62] isolated an enzyme fraction able to synthesize the undecapeptide CyA from crude extracts of the fungus *Tolypocladium inflatum*. The formation of CyA was monitored by incorporation of the radiolabeled constituent amino acids of CyA or by using S-adenosyl-L-[^{14}C-methyl] methionine.

Similarly, Lawen et al. [70] extracted an enzyme from *Beauveria nivea* which was found to be responsible for in vitro synthesis of cyclosporins. They observed that at sub optimal temperature, a CyA yield of about 50 μg/mL was obtained. The enzyme also produces several naturally occurring congeners of CyA, such as the cyclosporins B, C, D, G, M, O, Q, U, and V and some of the analogues known to be produced by the fungus via precursor directed biosynthesis. Furthermore, Cyclosporins that are not obtainable by the fungus could be prepared by the enzyme system in the presence of the appropriate precursor amino acids as the synthesis of [*N*-methyl-(+)-2-amino-3-hydroxy-4,4-dimethyloctanoic acid]CyA, [L-norvaline, *N*-methyl-L-norvaline]CyA, [L-norvaline, *N*-methyl-L-norvaline] CyA, [L-allo-isoleucine, *N*-methyl-L-allo-isoleucine]CyA, [L-allo-isoleucine] CyA, [D-2-aminobutyric acid]CyA, and [beta-chloro-D-alanine]CyA could be established [71].

In further study, Lawen et al. [72] reported that the cyclosporin synthetase is also capable of introducing β-alanine into position 7 or 8 of the ring instead of the α-alanines present at these positions in CyA. This leads to 34-membered rings in contrast to the 33-membered ring of the cyclic undecapeptide CyA.

In contract to peptide synthetase from prokaryotes (forming, e.g., gramicidin, surfactin, thyrocidine, bacitracin), synthetases present in filamentous fungi such as

enniatin synthetase, delta-(L-alpha-aminoadipyl)- L-cycteinyl-D-valine synthetase, beauvericin synthetase, SDZ 214-1-3 synthetase, and cyclosporin synthetase do not exhibit subunit structure [73–76]. They possess all the catalytic activities necessary for nonribosomal peptide formation.

12.1 Characterization of Cyclosporin Synthetase

This enzyme is able to synthesize cyclosporin A and its congeners using constituent amino acids in their unmethylated form [74]. The methyltransferase activity for methylation of peptide bond is an integral part of the enzyme, contributing to biological activity and stabilization against proteolytic cleavage which was confirmed by sequencing of the entire gene [75]. The correct mass of the enzyme was found to be 1.69 MDa which was determined after sequencing the open reading frame of the corresponding gene. These results were confirmed by electron microscopy of cyclosporin synthetase [77].

At least 40 different reactions steps are catalyzed by the cyclosporin synthetase which includes 11 aminoadenylation reactions, 11 transthiolation reaction, 7 *N*-methylation reactions, 10 elongation reactions, and final cyclization reaction [78].

12.2 Localization of Enzyme Involved in Cyclosporin Biosynthesis

Studies performed on *T. inflatum* revealed the localization and compartmentalization of three key enzymes of cyclosporin biosynthesis (cyclosporin synthetase, alanine racemase, and Bmt-polyketid synthase). Using electron microscopy based immunostaining technology and selective antisera against the key enzymes, the active CySyn and alanine racemase were found to be bound or attached to the outer membrane of vacuoles. In contrast, Bmt-PKS seems to operate in the cytoplasm. At the end of fermentation process, almost all cyclosporin that are produced by the cell is stored in vacuoles. Approximately 75 % of biosynthetic enzymes are detected in the vacuoles which indicates a degradation of inactive enzyme [77].

13 Fungal Production of CyA

CyA was first produced by submerged culturing of aerobic fungi, originally identified as strains of *Trichoderma polysporum* [15] but later identified as belonging to the species of *Tolypocladium inflatum* [14]. CyA formation is also reported from *Cylindrocarpon* spp., *Fusarium* spp., *Tolypocladium geodes* W. Gams, *Trichoderma virile* Pers. ex Gray, *Neocosmospora vasinfecta* Smith, *Isaria* spp., *Verticellium* spp., *Acremonium* spp., *Beauveria nivea* (Rostrup) Arx, and *Aspergillus terreus* [20, 79]. It is evident from the literature that most of secondary metabolites production was carried out by submerged and solid state fermentation.

13.1 Submeregd Fermentation

Generally, the production of commercially important metabolites is done in submerged cultures of filamentous fungi in which fungal growth is in the form of freely suspended mycelia, pellets, and clumps [80]. The pelleted growth is preferred because it produces broth that is relatively less dense, leading to easy mixing and aeration [81]. Under given conditions, specific growth morphology is affected by several factors like fungal strain, the nature of the growth medium, method of culture initiation (e.g., spores, pellets, and dispersed mycelium), and the hydrodynamic regime in the bioreactor [80–82].

In another study, Fattah et al. [83] worked on sequential optimization strategy for production enhancement of CyA by *Tolypocladium inflatum* in a submerged culture based on statistical experimental designs. They design 2-level Plackett-Burman to screen the bioprocess parameters which significantly influence CyA production. Their results showed that sucrose, ammonium sulfate, and soluble starch have significant positive effect on CyA production among 11 tested variables. Moreover, they adopted a response surface methodology which involves a 3-level Box-Behnken design to obtain best process conditions. Thus, a polynomial model was created to correlate the relationship between three variables and CyA yield. In addition, nonlinear optimization algorithm of EXCEL-So/ver were used for evaluation of major media constituents for CyA production, i.e., sucrose 20 %; starch 20 %; and ammonium sulfate 10 %. Their study concluded that predicted optimum yield of CyA was twofold the amount obtained with the basal medium, while the experimental verification of the predicted model resulted in 97 % of the theoretically calculated yield of CyA.

Similarly, El-Enshasy et al. [84] worked on the kinetics of cell growth and production of CyA in shake flasks and bioreactors by *Tolypocladium inflatum* under controlled and uncontrolled pH conditions. They found that in shake flasks, maximum CyA production was 76 mg/L after 226 h. At bioreactor level, significant increase in the cell growth and the drug production level was demonstrated after only 70 h, i.e., 144.72 mg/L and 131.4 mg/L under controlled and uncontrolled pH cultures, respectively. In addition, a significant reduction in both the dry cell mass and the drug concentration was observed after CyA production phase.

13.2 Solid State Fermentation

Solid state fermentation has been successfully used for enzymes and secondary metabolites. In case of *T. inflatum* large intra population variations in colony color and shape were observed on solid media. Thus, colony color can range from white to brownish [85]. The production of pink pigment was found to be associated with cyclosporin production in certain *T. inflatum* strains [86].

Sekar et al. [87] used wheat bran as the solid substrate for CyA production from *Tolypocladium* sp. and recorded ten times more yield than that obtained by submerged fermentation. They used different solvents for the optimization of extraction

of CyA from fermented bran. Furthermore, their results depicted that high drug yield was obtained after hydrolyzing the wheat bran using dilute HCl. In 1998, Sekar and Balaraman [88] optimized various parameters for the optimum production of CyA by solid state fermentation using *Tolypocladium* sp. This study was proved to be economically worthwhile for the production of CyA in bulk quantities.

Solid state fermentation is different from that of submerged fermentation which is carried out at low moisture content with microbial growth on moist solid substrates. As compared to submerged fermentation, solid state fermentation has no systematic study to guide the design and operation of large scale fermentation with proper control. Various modifications in solid state fermentation like the packed bed, rocking/rotating drum, fluidized bed, and stirred tank reactors have been used by Manpreet et al. [89]. Solid state fermentation is affected by various parameters pH, temperature, agitation, and aeration.

In further studies, Khedkar et al. [20] improved the method for the production of CyA by solid state fermentation using *Fusarium solani* (Mart.) Sacc in a bioreactor under optimal fermentation parameters. The product is extracted and further purified by treating with alum and subsequent chromatographic procedures to get pharmaceutically acceptable purity. Their study showed that high efficiency of the alum treatment during the purification process results in higher yields.

14 Optimization of Culture Medium for Cyclosporin Production

Various studies have been reported in past for optimization of culture media for maximum production of CyA. In 1986, Agathos et al. [90] formulated a semi synthetic media containing single carbon source, Bacto-peptone, potassium phosphate, and potassium chloride for CyA production by *Tolypocladium inflatum*. A wide range of carbon sources supported fungal growth and the subsequent CyA production. The highest CyA production among these carbon sources was observed with 2 % sorbose followed by 5 % *myo*-inositol. In order to reach higher volumetric drug production, sequential addition of two carbon sources such as sorbose and maltose was also employed.

It was found that by the external addition of the amino acid constituents of the molecule, the biosynthesis of CyA is heavily influenced. CyA was produced in semisynthetic and synthetic media by *Tolypocladium inflatum* in suspension culture by Lee and Agathos [91]. They found that specific production of CyA was increased by 62 % by the addition of L-valine in semisynthetic media. Similarly, the production was enhanced four times in synthetic media by addition of L-leucine as compared to semisynthetic media. Further results presented that D-valine shows no stimulatory effect on the production of CyA.

Chahal [92] used different fed batch strategies to increase the volumetric production of CyA in a 14 L bioreactor. A nicotinamide adenine dinucleotide (NADH) fluoresensor, interfaced with a computer, was used to monitor the growth of fungus *B. nivea*. In their study, mathematical models were developed to relate the

fluorescence intensity with biomass and fructose concentrations in the bioreactor. Their results concluded that most successful fed-batch fermentation strategy involved a continuous feeding of substrate at the rate of substrate consumption for 48 h, after the fructose concentration in batch mode had dropped down to 5 g/L. The substrate feed rate was then switched to the maintenance level for the next 72 h of fermentation. This strategy gave the highest Cya concentration of 504 mg CyA/l with a yield of 15.14 mg CyA/g mycelial dry weight.

Similarly, Margaritis and Chahal [93] developed a fructose based medium to grow *Beauveria nivea* for the production of CyA. Correspondingly, the highest CyA level of 150–200 mg L^{-1} was recorded by Isaac et al. [94], on Casamino acids medium by *Tolypocladium* strains after 12 days. After that, Lee and Agathos [95] reported the effect of externally supplemented L-valine on the production of the immunosuppressant CyA by *Tolypocladium inflatum* in chemically defined medium. It was observed that in a batch laboratory stirred reactor cultivation, the concentration of intracellular L-valine increased by up to four times between the end of the exponential phase and the beginning of the stationary phase when the medium was supplemented externally with 4 g/L L-valine. The final CyA titre under these conditions was higher as compared to CyA titer attained without L-valine supplementation. In contrast to substantial growth-associated production of CyA in unsupplemented culture, the formation of the immunosuppressant was prolonged during the stationary phase in L-valine supplemented medium. As a result, the conversion yield of CyA on L-valine remained constant during the stationary phase.

Similarly, a feeding strategy for L-valine was also tested in the production of CyA in celite-immobilized cells of *Tolypocladium inflatum* by Chun and Agathos [96]. According to the findings, during exponential growth phase, addition of L-valine to immobilized cells significantly increase the CyA biosynthesis. However, amino acid addition after this phase failed to stimulate CyA production. Later on, the kinetics of submerged fungal growth, consumption of nutrients, and production of CyA was described by a mathematical model in another study by Agathos and Lee [97] in 1993 that highlighted the stimulated effect of L-valine on drug production. The basis of the proposed mathematical model was the emerging mechanistic data and kinetic information of CyA biosynthesis. The findings were based on the hypothesis that L-valine acts like an inducer in the biosynthetic process of the CyA in one of its synthesizing multienzyme and an intermediate of CyA which is unmethylated. The proposed model comprised of two parts, cell growth and substrate consumption was described in first part while the second part addressed the kinetics of CyA biosynthesis. Actually, this kinetic profile of both internal and external variables occupies the major part in the success of this model which not only correctly monitor the optimum concentration level but the time of exogenous L-valine addition for obtaining maximum drug production, suggesting new avenues for improving fermentation process using fungal strains.

Further study on batch fermentation processes by Balakrishnan and Pandey [98] on CyA production showed that the fungal growth and drug production was more prominent in complex media rather than in synthetic media. Under the same conditions, the addition of peptone tremendously increased fungal culture growth

increasing biomass production while casein and acid hydrolyzate supplementation favored CyA production. Balakrishnan and Pandey further demonstrated that in synthetic/semisynthetic media, L-leucine and L-valine act as strong inducers for drug production upto tenfold as compared to an unsupplemented control. Moreover they also found that when sarcosine and L-methionine are added exogenously, drug production was greatly lessened.

Correspondingly, Sallam et al. [79] worked on a local isolate of *Aspergillus terreus* among different microorganisms as a new CyA producing culture. The formation of CyA was investigated under different fermentation conditions. According to their results, relatively higher production of CyA was attained with an addition of glucose in the medium during fermentation carried out using a medium composed of bactopeptone, at pH 5.3, incubated with 2 % standard inoculum of 48 h age, shaken at 200 rpm for 10 days.

In 2006, Balaraman and Mathew [99] conducted a worthwhile study for optimization of medium composition by *Tolypocladium* species in static fermentation for 21 days at 25 +/− 2 °C. CyA extraction was performed by homogenization of fungal cells in methanol. They examined extracted CyA by a chromatographic technique (high performance liquid chromatography-HPLC) as their confirmation step. They optimized the growth medium by glucose 8 %, casein acid hydrolysate 3 %, malt extract 2 %, peptone 1 %, and DL- alpha-amino butyric acid 0.5 % for obtaining the maximum drug production.

In a contemporary study, Kannan and Kalaichelvan [100] investigated change in culture conditions for biomass, protein, and CyA production by *Tolypocladium inflatum*. They observed that pH 6.0 and temperature 24–27 °C significantly promoted the biomass and extracellular proteins. Glucose and yeast extract were more effective carbon and nitrogen sources, respectively, for biomass and extracellular protein production. Synthetic medium supplemented with L-valine promoted 30 % increase of CyA over the medium deficient of L-valine. It was observed that L-valine addition increased CyA production in fermentation.

The extraction kinetics of CyA is greatly influenced by the temperature and this study was done by Margaritis and Ly [101] using the mycelia of *Tolypocladium inflatum*. From fungal mycelia, CyA extraction was performed by using 30 % v/v aqueous methanol in 2-L stirred, baffled vessel at temperature range from 5 °C to 45 °C. A sort of direct relationship was observed between the extraction yield of CyA and temperature. Their results concluded that with increase in temperature, CyA yield also increased, i.e., it found to be maximum at 45 °C, which is 21.3 % higher than the yield obtained at 25 °C. The extraction of CyA from the fungus *T. inflatum* as indicated by activation energy (36.7 KJ/mol) is controlled together by solubilization of CyA and diffusion from the solid phase of mycelia. Additionally, in their study, experimental kinetic data of CyA extraction was fit into a mathematical diffusion model which determine Cyclosporin “A” effective diffusivities at different temperatures.

Furthermore, various other parameters greatly influence solid state fermentation process. These parameters have been sequentially evaluated by Survase et al. [102] which include mainly the choice of solid substrate, hydrolysis of these substrates,

initial moisture content of the medium, salts supplementation, exogenous addition of carbon, and nitrogen sources, as well as the inoculum size and age on CyA production by using *Tolypocladium inflatum* fungus. They found that at initial moisture content of 70 %, maximum CyA production was documented by the addition of combination of hydrolyzed wheat bran flour and coconut oil cake (1:1). Furthermore, supplementation of salts, glycerol (1 % w/w), and ammonium sulfate (1 % w/w) also escalate CyA production per kilogram of substrate. In the same year, a comparison was made between the solid and submerged fermentation processes by Nisha et al. [103], with and without the addition of amino acids. The findings supported solid state fermentation with 40 % increased yield in cyclosporin without any addition of amino acids as compared to the submerged fermentation. However, in solid state fermentation, longer incubation periods were usually observed; increased drug yield was achieved by the exogenous addition of a number of amino acids like L-valine, L-leucine, and L-α-Aminobutyric acid.

In a recent study, Tanseer and Anjum [104] worked on another fungus *Aspergillus terreus* for enhanced production of CyA by the use of various carbon and nitrogen sources in the growth medium. The results of their study illustrated that glucose (10 %) as carbon source and peptone (0.5 %) as nitrogen source improved the CyA yield from selected *Aspergillus terreus* strain (FCBP58). They also concluded that as there was no relationship between CyA production and fungal biomass, the medium modifications are exhausted in increasing overall drug synthesis powers of the fungi. In 2012, Azam et al. [105] explored *Trichoderma harzianum* as a new fungal source for the production of CyA. In the same year, Anjum et al. [106] checked six strains of *Penicillium* for their potential to produce CyA through submerged fermentation. The findings of their study showed that among tested fungal species, CyA production was observed in *P. fellutanum*.

15 Immobilization for CyA Production

The immobilized system has shown to have promise over conventional submerged systems. Foster et al. [107] worked on immobilization of *Tolypocladium inflatum* conidiophores into a porous celite particles for production of CyA. In comparison with free cell cultures originated with spores or mycelia, rapid germination was observed by immobilized cells. Entrapped *Tolypocladium inflatum* produced CyA in low foaming semisynthetic media in an airlift bioreactor with an external circulation loop. Recovery of CyA was carried out by ethyl acetate extraction which was free of endogenously produce microbial products and media contamination.

It has been documented that CyA production has been negatively influenced by L-valine when added at initial stages of immobilized cell growth as compared to free cell cultures; however, cell growth was increased to a certain extent [86]. Significant differences were observed in precursor flow between the immobilized and free cell systems showing the effects of L-valine on CyA biosynthesis by Chun and Agathos [108]. Supplementation of L-valine during or after exponential growth phase act as a stimulator for CyA biosynthesis in

freely suspended cells. This implies an incongruity between primary and secondary metabolic networks apprehensive in CyA biosynthesis within the immobilized state upon external addition of the amino acid.

For immobilization of fungal cell culture, Lee et al. [109] developed a competent immobilization procedure having abridge time and number of steps for sporulation which was applied to an immobilized-cell perfusion bioprocess for unremitting production of CyA. In this technique, fungal cells entrapped in pores of celite beads were cultured in top-driven stirred tank fermentor. Their study demonstrated that because of high density of immobilized cells within the fermentor, productivity of CyA containing free cells within the effluent was terribly high i.e., six- to tenfold over that of batch suspended cell culture. Later on, in this study, subsequent decantor was developed for such a proficient immobilized perfusion bioprocess, which efficiently discrete cell-immobilized beads from effluent while bead loss increased to some extent as the cell loading intensify in the latter part of culture. Furthermore, enduring operation of the method was efficaciously carried out by employing an in-situ immobilization approach. It was found that new immobilized cells were formed by in-situ entrapment of bulk quantities of spores into newly supplemented celite beads in reactor during fermentation.

Moreover, Sallam et al. [110] investigated the CyA formation by immobilized spores and mycelia of *Aspergillus terreus*. They used different immobilizing carriers but further experimentation was carried out by Ca-alginate. Different parameters like biomass weight, pH of the cultivation medium, role of alginate concentration, supplementation of different amino acid precursors, as well as repeated utilization of the immobilized fungus were also studied. Their findings suggested that best CyA productivities were achieved after four repeated cycles with Ca-alginate 3 % (w/v), pH 4.5, and mycelial weight 15 % (w/v). While studying the effect of amino acids on the production of CyA, Sallam et al. noticed marked acceleration in productivity of CyA in the presence of L-valine alone and together with L-leucine mixture.

In a recent study, Survase et al. [102] explored different immobilization matrix like gellan gum, celite beads, and sodium alginate for the production of CyA by immobilized spores and mycelia of *Tolypocladium inflatum* MTCC 557. They also tested the role of the carrier concentration, number of spore inoculated beads, biomass weight, and repeated utilization of the immobilized fungus. The findings of their study showed that both gellan gum [1 % (w/v)] and a mycelial weight [7.5 % (w/v)] supported the utmost production of CyA. The stability of immobilized mycelia beads upto four repetitive cycles indicates its semicontinuous potential for CyA production.

16 Mutational Approach for Strain Improvement

Commercially important metabolites of microbial origin are generally produced in low quantities. So, there is a need to increase the yield of fermentation product in order to maintain competitive economic position for new and existing fermentation. Generally high yield of the fermentation product is associated with proper organism,

medium, aeration, precursor, pH control, etc. [111, 112]. These concerns would not offer an appropriate increase within the overall product yield for fermentation. During this state of affairs, the mutational approach has been extensively used for industrial organisms to improve the overall productivity which is essential for the commercialization of fermentation product [113]. Major objectives of strain improvement program are the yield enhancement of required product, maintenance of desired morphological characteristics, and unwanted cometabolites elimination [114]. Such improved strains thus can rationalize the disbursement of the processes with enhanced productivity and may also possess some specialized fascinating characteristics. A number of cultures that could be screened after mutagenic treatment are mandatory for the successful accomplishment of any strain improvement program.

16.1 Screening for Improved Mutants

Screening is required to detect mutants from hundreds and thousands of individuals exhibiting trait of interest.

A screen can be divided into two types:

1. A direct screen where product is directly analyzed and
2. An indirect, rational or prescreen.

In the second case, a known biochemical or genetic property is assayed that is associated with the product of interest rather than the actual product [115–117].

16.1.1 Random Selection

A direct screen is commonly referred to as a random screen or random selection and can be organized into a multi-level screen. A multi-level screen is a useful screening strategy when improved mutants are rare and the error of testing, or production from, the screen is high [117–120]. Following incubation of the mutagenized conidia, isolated colonies are selected and streaked onto solid medium. After incubation, each colony is inoculated as a single replicate and incubated. Primary screening is designed with a lower resolution to allow the maximum throughput of isolates to be assayed.

The low-resolution screen can be performed with agar plugs or plates, miniature shake flasks, or in tubes [117] and differentiates the high producers from the low producers with the high producers being selected for further analysis in a mid-resolution screen [118]. At this secondary level, the number of replicates is increased and approximately 10 % of the most active producers are selected for assay with the maximum allowable number of replicates in a high-resolution screen at the retest level. The number of replicates is increased to improve accuracy and screening [120]. From this high resolution screen, the top producing strains are then selected as start strains in the next cycle [118, 119, 121].

16.1.2 Rational Selection

A rational screen is a prescreen and is highly effective because it kills those mutants with low productivity [117]. In this way, the application of a prescreen will significantly concentrate the improved mutants of interest within the mutated population thus making the entire screening process more efficient [122].

Swidinsky [123] worked on improvement in Cyclosporin productivity and its relation to growth and glucose metabolism. The regulation of CyA productivity-improved mutants derived from the classical techniques of strain improvement was involved in this research. It focuses on classical methods of mutation, selection of mutant, carbon consumption, growth of producing organism, i.e., *T. inflatum*, activity of enzyme involved in glucose catabolism and their relationship with increase yield of CyA.

Ramana Murthy et al. [124] used solid-state fermentation to produce CyA by growing *Tolypocladium inflatum* strains on moist wheat bran. Among these strains, high CyA yielding strain was selected and its spores were subjected to different mutagenic treatments for enhancement in CyA production. They found that cultivation of mutated strain on wheat bran medium comprised of jawar flour (10 %), millet flour (20 %), Ferric chloride (0.25 %), zinc sulphate (0.15 %), and cobalt chloride (0.05 %) resulted in highest production of CyA under optimum fermentation conditions, i.e., incubation temperature 25 °C, initial moisture content of inoculum (60 %) and bran (70 %), and pH 2. They used solvent extraction for the purification of CyA, followed by column chromatography.

17 Physical Mutagens

Physical mutagens include ultraviolet (UV) light and ionizing radiation such as x-rays and gamma rays, as well as fast neutron exposure. Ultra violet rays are non-ionizing because they have a long wavelength and therefore have less energy. It can induce both base pair substitution and frame shifts mutations. The intra stand cyclobutan pyrimidine dimmer is the predominant DNA lesion produced by UV radiation (254 nm) [125]. Many other lesions have been reported such as hydration across 5–6 double bonds of pyrimidines and dimmers with the amino acid cystein [126].

Fungi manifest two kinds of responses to ultraviolet radiations, i.e., mutation and death, depending upon the doses as well as the wavelength. It has been observed that the shorter wavelengths are more lethal than the longer ones, which are more effective as mutagenic and cause nonlethal mutations. Ultraviolet radiation is absorbed by pyrimidines, particularly thymine. Once the energy is absorbed, the ring structure becomes unstable and sometimes ends up in the formation of thymine–thymine dimmers.

If the thymines are in opposite strands, the chromosomes will break once making an attempt to replicate, however more frequently the thymine dimmers form from adjacent thymines within the same strand. The T: T dimmers do not have traditional

base pairing properties, therefore when DNA tries to replicate, the inappropriate base may be inserted.

17.1 Production Enhancement of CyA by Physical Mutation

Previous studies showed successful use of UV mutagenesis to increase the biosynthetic capabilities of fungi for CyA. Besides the type strain used, the production levels of CyA are dependent on several regulating factor such as inoculum type and size, medium composition and additives, as well as process parameters such as temperature, pH, and partial oxygen pressure. A high density of the spore inoculum was found to be necessary for the development of small pellets, which is preferred morphology for cyclosporin production [15, 94].

Jung and Kyeong [127] worked for making a highly productive fusant of *Tolypocladium inflatum* and also made comparison between wild and fusant strain for the production of CyA by submerged fermentation. They used UV radiation for the development of mutant strains that were dependent on amino acid and nitrogen source for proper functioning and, additionally, established appropriate condition and culturing methodology of fusant strain for prime production of CyA. Among many nitrogen sources, a fine protein source like peptone is required by wild strain for CyA production whereas, a highly productive fusant produces more amounts of CyA than wild strain due to increase proteolytic activity in such a way that it has an ability to use both fine protein source (peptone) and cheap, crude, and natural protein sources like cottonseed meal, soybean meal, cornsteep liquor, and peanut meal. Jung and Kyeong used nutrient media composed of glucose and peptone as carbon and organic nitrogen source with varying concentration of amino acids (L-leucine and L-valine) for the cultivation of wild and fusant strains, L-leucine and L-valine dependent strains. They concluded that wild strain use low concentration of L-leucine and L-valine for the production of CyA while large quantities of these amino acids were required by fusant strain which act as a precursor for target compound resulting in increased CyA production.

Gharavi et al. [128] used UV radiation for higher production of CyA in *Tolypocladium inflatum* (DSM 915). They prepared α-aminobutyric acid dependent auxotroph by UV mutation to enhance the biosynthesis of CyA by altering cell metabolism. Later on, combine strategy of UV mutation and protoplast transformation was used by Lee et al. [129] for higher productivity of CyA by *T. niveum* ATCC 34921. They initially performed random mutagenesis by UV mutation and got ninefold increase in CyA yield, afterwards Vitreoscilla hemoglobin gene VHb (a foreign bacterial gene) was transformed via protoplast regeneration which resulted in 33.5 % increase in CyA production.

In a more recent study, Iram and Anjum [130] used ultraviolet radiation (254 nm) for induction of mutation in *Aspergillus terreus* strains to produce higher level of CyA. In their study, exposure time of parental cultures towards UV light was increased from 5 to 60 min with regular intervals of 5 min. The findings of their results depicted that increased exposure time resulted in decreased number of survivals and complete death in last treatment. Mutants with desired higher drug

production were recorded in very first treatment of 5 min exposure. Maximum CyA production was observed in mutant MU1.3, i.e., 298.4 μg/ml.

18 Chemical Mutagens and Production Enhancement of CyA

Besides the selection of highly productive colonies of wild type strains, attempts were undertaken to increase the strain productivity by mutation using chemical mutagens such as methyl sulphate, epichlorohydrin, or nitrosoguanidine [90]. Agathos and Parekh [131] worked on production enhancement of cyclosporin by *Tolypocladium inflatum* strain using epichlorohydrin treatment. They concluded that new strain arise after treatment with 0.15 M epichlorohydrin showed similar growth rate as that of parent organism but more extensive conidiation with higher production of cyclosporin A.

Classification of chemical mutagens is based on type of modification occurred on DNA after treatment. These chemical reactions include deamination, alkylation, intercalation, or substitution of bases. Once variation has been introduced, strains with the desired characteristic are detected by screening and subsequently selected for commercial use in large-scale fermentation or chosen as the start strain for another round of mutation and selection. All mutation and selection is a form of recycling [116, 132].

The most potent chemical mutagens are alkylating agents, e.g., ethyl methane sulfonate (EMS) and the nitroso compounds such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. The alkylating agent EMS or NG can be use in strains, which are resistant to UV light. Each mutagen induce a characteristic spectrum of mutants, and it is not possible to predict what kind of mutation is essential to boost the production of desired compound. That is why wide range of mutants are generated by several dosages of these mutagens.

EMS is well known for the induction of random mutation through nucleotide substitution in DNA, particularly by guanine alkylation which ultimately produces point mutations. Mutation produced by this chemical can be at the rate of 5×10^{-4} to 5×10^{-2} per gene without any substantial killing. Reaction of ethyl group of EMS with guanine, leads to the formation of an abnormal base *O*-6-ethylguanine. As a result of which, DNA polymerases induce the placement of thymine opposite to *O*-6-ethylguanine instead of cytosine, throughout the DNA replication. Later on by successive rounds of replication, the original G: C base pair can become an A: T pair.

In a research conducted by Iram and Anjum [130], four different concentrations of EMS were used to induce mutation in *Aspergillus terreus* strains for production enhancement of CyA. The number of survivals with greater CyA biosynthesis than parental one was recorded in all treatments. They concluded that treatment with 200 μg/ml of EMS was found the most effective, as all the mutants showed significant increase in immunosuppressant production. The activity ranged between 78.4 and 615 μg/ml. Both UV radiation and EMS are known to increase the yields of CyA by about 33 % and 37.5 %, respectively [133].

19 Isolation and Purification of Cyclosporin A

Cyclosporins are not released in culture media but have to extract from mycelia. In literature, various purification processes have been reported to isolate pharmacopoeial grade CyA.

Conventionally, organic solvent was used to extract fermented biomass, then solvent was evaporated, reextracted and the residue is concentrated. At the end these, residues were analyzed by various chromatographic techniques to separate CyA from other cyclosporins and impurities.

Derk [134] used supercritical CO_2 for extracting cyclosporin from mycelia of *Beauvaria nivea*. Their results showed that about 70–80 % of CyA were extracted from mycelia of tested fungi. Completely dried mycelia showed lower extraction yields as compared to mycelia with 7–29 % moisture content. The addition of methanol showed no effect on cyclosporin extraction. Co-extracted materials, obtained during extraction experiment, were tentatively identified as lipids. Moreover in their study, scanning electron micrographs of mycelial structure were to visualize the physical barriers in cyclosporin removal.

Sekar and Balaraman [88] and Survase et al. [135] used butyl acetate for the extraction of CyA from fermentation broth or fermented solid substrate. Similarly, ethyl acetate was to extract fermented matter by Ramana Murthy et al. [124] with subsequent purification using silica gel and Sephadex LH20 resin. These columns were eluted with hexane:chloroform:methanol (10:9:1) and methanol, respectively. They used NMR and IR techniques for characterization of CyA.

Szanya et al. [136] stated that solid mixture or evaporative residues can be efficiently separated by heating at 80–115 °C prior to chromatography on silica gel. They used mixture of chloroform-dichloromethane–ethanol or chloroform–ethyl acetate–ethanol as eluent. The resultant product was subjected to further chromatography and recrystallization. Another study was reported by Lee and Agathos [91] in which fermentation broth was treated with concentrated NaOH and heated at 60 °C for 30 min for CyA recovery. This mixture was then extracted with equal volume of n-butyl acetate on rotary shaker (250 rpm) for 24 h.

The findings of Ly et al. [137] depicted that acetone with 50 % v/v concentration proved to be best solvent for extracting CyA from mycelia of *T. inflatum* as compared to methanol and isopropanol at room temperature.

20 Analytical Methods for Analysis of Cyclosporin A

Various methods are used for the analysis of cyclosporin A in clinical samples, such as immunoassays, HPLC, liquid chromatography–tandem mass spectrometry [138–140]. For rapid analysis of CyA, immunoassays are used but cross-reactivity of the antibodies with inactive CyA metabolites is its main concern. So, for routine monitoring of CyA in transplant recipients, HPLC-tandem mass spectrometry assay is used as an alternative to immunoassays [141].

In contrast, HPLC is more time consuming but for various researchers it is a method of choice for CyA analysis in fermentation broths. Kreuzig [139] developed a HPLC method, for separation and determination of the closely related cyclosporins, i.e., CyA, CyB, CyC, and CyD present in fermentation broths. They used Nucleosil C8 column with temperature 70 °C and acetonitrile–water–phosphoric acid (70:30:0.01) as a mobile phase. In the later study, HPLC analytical conditions were optimized by George et al. [142] for analysis of different cyclosporins. Their findings depicted that CyA, CyB, and CyC were well separated with a Supelco C8, column (60 °C) using mobile phase [acetonitrile–water (50:50)]. However, Husek [143] developed a simple and reliable HPLC method for analysis of CyA, its congeners, and degradation products.

21 Conclusion

Cyclosporin A is among the most important immune suppressants used. The use of cyclosporin as a transplant medicine has no doubt revolutionized the clinical use since 1970s. It has transformed the medicine field into miracle as it not only improves the rates of acute rejection but also enhance early graft survival. The commercial demand for CyA is increased worldwide due to its numerous novel applications. Recent literature showed that researcher's main concern is focused on the CyA bulk production, its easy purification, and uses in the field of medicine. For this reason, the mechanism of action of cyclosporin is also under thorough investigation. Efforts are being made in the last few years to investigate the use of biologically safe and low-cost sources such as microbial strains to improve drug production. A few dynamic fungal strains were found to produce efficient levels of CyA, however for its commercial production further research for viable organism is the need of hour. In addition, other physical parameters and processes should be opted at commercial scale which could amplify drug yields in turn reducing the cost of production. Other genetic techniques like recombinant DNA, protoplast fusion, RNAi mediated gene silencing, Frame-shift mutation, and DNA manipulation in industrial strain improvement for higher production of Cyclosporin may be the proper way out for its high fold production.

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Streptokinase Production in Yeast Systems 25

Ravi N. Vellanki, Rama R. Baadhe, and Ravichandra Potumarthi

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Abstract

Streptokinase is a fibrinolytic agent widely used in thrombosis. The clinical trials and experimental studies proved that the SK is a safe and inexpensive thrombolytic medicine compared with its homologues such as tissue plasminogen activator (t-PA), urokinase (UK), and other plasminogen activators. Increased risk and prevalence of thrombosis worldwide, demand for SK, low production yields in native strain, high purification, and other antigenicity toxins limit the usage of

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native SK. However, these inadequacies can be overcome by using genetic engineering technology to express SK gene (skc) in microbial host systems. This chapter addresses about the SK structure, mechanism of action, and recombinant SK expression in yeast and fermentation.

Keywords
Streptokinase • *Streptococci* • Yeast • Plasminogen • Plasmin • Fermentation • Heterologous expression

List of Abbreviations

AA	Amino acid
ATCC	American type culture collection
GRAS	Generally regarded as safe organism
H	Hour
HPG/PG	Human plasminogen
HPM/PM	Plasmin
APSAC	Acylated plasminogen-streptokinase activator complex
kDa	Kilo Daltons
L	Liter
Lys	Lysine
mg	Milligram
PEG	Polyethylene glycol
rSK	Recombinant streptokinase
SCU-PA	Single-chain urokinase-type plasminogen activator
SK	Streptokinase
tPA	Tissue-type plasminogen activator
UK	Urokinase

1 Introduction

Streptokinase (SK) is a protein that belongs to a group of hydrolases and is secreted by Lancefield group A, C, and G strains of β-hemolytic streptococci [1, 2]. SK is a single polypeptide chain of 414 amino acids with 47 kDa molecular weight and isoelectric pH 4.7. The enzyme has its maximum activity between pH 7.3 and 7.6 [3, 4]. SK has been widely used in the treatment of acute myocardial infarction for its robust action in liquefying fibrin. Venous thromboembolism (VTE) is the third cause of mortality after myocardial infarction and stroke, and it is the second cause of death in patients with cancer. The clinical trials and experimental studies proved that the SK is a safe and inexpensive thrombolytic medicine compared with its homologues such as tissue plasminogen activator (t-PA), urokinase (UK), and other plasminogen activators [4, 5]. Native SK, purified from hemolytic streptococci, displayed antigenicity which may damage the myocardium and liver due to the hemolysin residues [4, 6]. However, these inadequacies can be overcome by using genetic engineering technology to express SK gene (skc) in native and heterologous bacterial hosts like

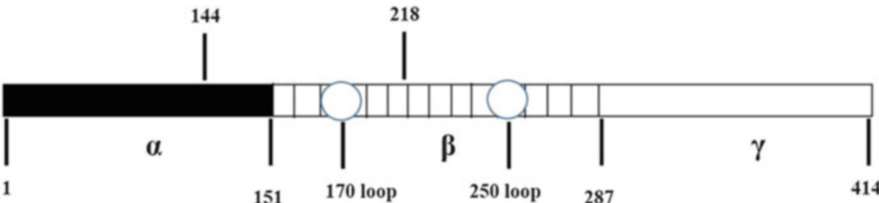

Fig. 1 Structural domains of SK [9–12]

Escherichia coli [2, 7]. Yeast expression systems are also employed as production cells [8]. In this chapter, we discuss about the SK structure, mechanism of action, and recombinant SK production in yeast and fermentation.

2 Structure of SK

Major biophysical techniques revealed structural information of SK [9]. SK contains three structural domains with 414 amino acids: α (AA 1–150), β (AA 151–287), and γ (AA 288–414) [9]. The highly conserved α and γ domains provide maximum contact sites with the plasmin moiety and shows the synergism on plasminogen activation [9–11] (Fig. 1). The β domain provides no direct contact sites with the plasmin active site; however, surface-exposed hairpin loop (250-loop) requires plasminogen docking. The second structural loop (170 loop) function is unknown, but it is responsible for the heterogeneity of the SK and associated with infection and disease in group A of streptococci [9–12].

3 Mechanism of Action

SK activates the fibrinolytic system for the degradation of blood clots (Fig. 2). Fibrinolysis is a common biological phenomenon in mammals for the degradation of blood clots [13]. Since the native SK does not have proteolytic activity, it forms an equimolar complex (SK-HPG) with human plasminogen (HPG). Further conformational changes yield unstable $SK.\ HPG'$ complex which possesses an active site in PG moiety. This nonstable interaction is rapidly converted to $SK * HPM$ complex due to intramolecular cleavage of peptide bond between Arg_{560} and Val_{561} of the $SK.\ HPG'$ complex (Eq. 1).

$$HPG + SK - - \rightarrow SK.HPG - \rightarrow SK.HPG' - \rightarrow SK * HPM \quad (1)$$

$$SK + HPM - \rightarrow SK * HPM \quad (2)$$

$SK * HPM$ can also be formed with the combination SK with HPM (Eq. 2). Simultaneously, after formation of HPM in the complex, *SK* immediately converted other proteolytic form *SK** with 36 kDA molecular weight. The loss of the 9 kDa does not effect the activity of the SK. Further, SK plays an important role in

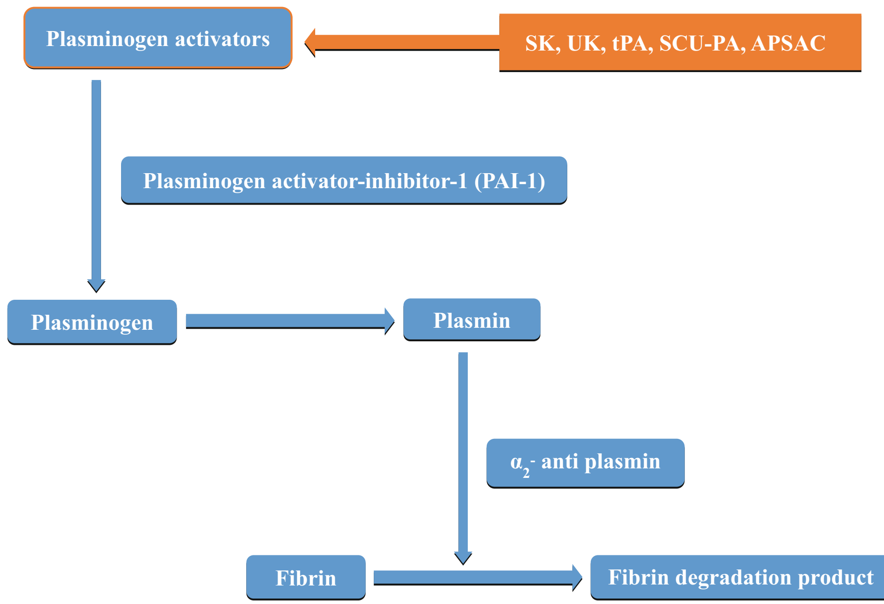

Fig. 2 Schematic representation of the fibrinolytic system. *SK* Streptokinase, *UK* urokinase, *tPA* tissue-type plasminogen activator, *SCU-PA* single-chain urokinase-type plasminogen activator, *APSAC* acylated plasminogen-streptokinase activator complex

modulating the substrate specificity for plasminogen [13]. SK binds to plasminogen through the lysine binding site to trigger conformational activation of plasminogen [14, 15]. SK-HPG activator complex interacts with plasminogen through protein–protein interactions to maximize catalytic turnover [16]. The loss of N-terminal residues (first 59 amino acids) seems to have multiple functional roles in SK [17]. Without these, SK leads to unstable secondary structure and reduces the activity of the remaining SK fragment (i.e., residues 60–414) [18].

4 Production of SK

4.1 Native Microorganism

Streptococci were first identified in 1874 by Billroth in exudates of infected wounds and were later grouped into three categories based upon their ability to hemolyze erythrocytes into α, β, and γ. Serologic distinctions by Lancefield further differentiate the β-hemolytic *streptococci* into groups A to O. Tillet (1933) observed that Lancefield group A β-hemolytic streptococci isolated from patients produced a substance that could be used to dissolve fibrinous exudate. A, C, and G group streptococci are major producers of SK and among them group C is preferred for

its low erythrogenic toxins. *Streptococcus equisimilis* H46A (ATCC 12449) is widely used as the source for the production of SK [1–4].

4.2 Recombinant SK Production in Yeast

The streptokinase gene (skc) cloned from *S. equisimilis* H46A has been used for production in native, several heterologous bacterial and yeast expression systems [7, 8, 19]. A variety of promoter systems like T7, P170, glnAP2, AOX1, and GAP were adapted for clinical grade synthesis of SK [7, 19–22]. Commercial grade production of SK has been reported in *Escherichia coli* through fed-batch and continuous culture system [23, 24]. Nevertheless, the recombinant SK production in *E. coli* has been reported with some complications including plasmid instability, loss of cell viability due to its toxicity towards the expression host and inclusion body formation, and association of endotoxins post purification. To overcome the above limitations, yeast expression systems have been adapted for the production of SK due to their ease of genetic manipulation, access to genomic information resources, and is generally regarded as safe organism (GRAS) due to their extensive utilization in food and beverage industry. The tightly regulated promoters of *P. pastoris*: alcohol oxidase (AOX) and glyceraldehyde 3-phosphate dehydrogenase (GAP) with high specific secretion efficiency for heterologous proteins made downstream processing simple and cost effective [25]. SK was successfully expressed intracellularly and also in secretory mode in *P. pastoris* through AOX1 promoter [8, 26].

The difficulties associated with AOX expression system include high methanol consumption, transient toxicity to host, heat production due to methanol combustion, and hydrogen peroxide formation due to methanol oxidation leading to cell death and also difficulty in bioprocess scale up. Alternate promoter systems like GAP, formaldehyde dehydrogenase (FLD), and isocitrate lyase (ICL1) are applied for protein expression [25, 27, 28]. Of the above promoters, GAP-based promoter system has been applied for constitutive intracellular expression of SK in *P. pastoris*. Statistical optimization indicated that dextrose and peptone are effective and improved the SK expression by 95 % [22]. Another study expressed SK in *S. cerevisiae* by utilizing GAP promoter of *P. pastoris* and reported 110 % increase in activity levels [29].

Degradation of SK during large-scale production could be avoided by a glycosylated form of SK. By expressing the chimeric protein with α-mating factor as signal sequence instead of native signal codon in *P. pastoris*, the secreted glycosylated SK had improved proteolytic stability with activity levels of 3,200 IU m l^{-1} at shake flask level and 3,727 IU m l^{-1} at bioreactor level [26]. However, the secreted proteins expressed in *P. pastoris* and *S. cerevisiae* are often hyperglycosylated [30]. *Schizosaccharomyces pombe* was exploited to evade this limitation. *Sz. pombe* is a good choice over *S. cerevisiae*, since it shows several molecular similarities with higher eukaryotes in splicing mechanism and posttranslational modifications to higher eukaryotes. Chimeric expression of SK with *Sz. Pombe* signal sequence under thiamine-regulated (nmt1) promoter

improved signal sequence processing and secreted mature SK compared to *P. pastoris* [31, 32].

4.3 Fermentation

A detailed batch and fed-batch cultivation methods at bioreactor level are reported for SK production in *E. coli* [23, 24, 33–36]. However, limited studies were reported for SK synthesis by fermentation in yeast. Hagenson et al. [8] have successfully produced SK intracellularly and scaled up the bioprocess using a bioreactor. The fermentor cultures were grown in the batch mode with 2.5 % w/v glycerol-FM21 salts minimal media, and continuous cultures were established using 10 % w/v glycerol feed. The nutrient feed was switched from glycerol to 15 % v/v methanol and maintained for 255 h as sole source of carbon and energy. The amount of streptokinase expressed during continuous fermentation on methanol was determined to be about 77 mg L^{-1} of fermentor broth at an intermediate cell density of 46 g L^{-1} (dry weight).

Adivitiya et al. [37] have generated *P. pastoris* cell systems secreting SK under the control of AOX1 and GAP promoters and adapted them for large-scale cultures. Biomass production was done at 30 °C in batch medium using dextrose as carbon source instead of glycerol until a wet cell weight of 132 g L^{-1} was achieved. The cells were then induced using 100 % methanol at controlled flow rate and cultured in high cell density medium to reach a biomass of 318 g L^{-1}. A volumetric productivity of 57.43 mg $L^{-}1$ h^{-1} and a specific activity of 55,240 IU m g^{-1} partially purified rSK were achieved. Biological and biophysical characterization of the rSK was in agreement with native protein. The constitutive secretory expression under GAP promoter was also attempted; however, expression yields were significantly compromised due to its toxicity towards expression host [37].

At shake flask level, constitutive expression and production of SK in *Pichia pastoris* was achieved with Plackett–Burman design by screening different carbon sources, e.g., dextrose, galactose, fructose, maltose, sucrose, lactose, and glycerol and nitrogen sources, e.g., yeast extract, tryptone, peptone, casamino acids, beef extract, corn steep liquor, and polypeptone. Dextrose and peptone sources have generated maximal rSK expression. Further response surface methodology (RSM) optimization revealed the values for the dextrose, 2.90 %; peptone, 2.49 %; pH, 7.2; and temperature, 30.4. With the combinations of these parameters, *P. pastoris* expressed 2,136.23 IU m L^{-1} more SK than initial levels (2,089 IU m L^{-1}) [22]. Using a baffled flask design and RSM optimization on the *S. cerevisiae expressing SK* revealed yeast extract 3.215, dextrose 2.952, pH 7.42, and temperature 32.45 as optimum conditions. These variables combination improved the SK production 2352.07 IU m L^{-1} [29]. The mature SK produced in both the RSM studies is biologically active and is temperature sensitive.

5 Current Status of Streptokinase-Based Thrombolytic Drugs

Immunogenicity of streptokinase, half-life in circulation, and degradation in circulation by plasmin are some of the limitations of SK in clinical approach. Structurally modified SK has been produced in several ways including genetic mutation, and chemical or enzymatic modification of the native streptokinase. Mutant streptokinase with improved stability has been prepared. Two of the major sites of the proteolytic action of plasmin on streptokinase are Lys59 and Lys386, and variants have been generated that were resistant to plasmin with stability and comparable biologic effectiveness [38]. A mutant streptokinase that lacked the C-terminal 42 amino acids was found to be less immunogenic than the native molecule [39].

Fibrin and activated platelets are the chief constituents of a thrombus/clot and are the main targets for targeted delivery of SK. Conventional and PEGylated liposomes composed of neutral palmitoyl-oleoyl phosphatidyl choline (POPC) have been tested to encapsulate SK. This formulation led to limiting effects of anti-SK antibodies on SK efficacy, prolong systemic circulation, eliminate platelet aggregation and alter biodistribution of SK in favor of the thrombus and therapeutic thrombolysis. The designed liposomes proved controlled release of encapsulated SK close to thrombus vicinity and increased local concentration of SK [40, 41]. Major commercial producers of SK are Aventis Behring GmbH, Germany; BBT Biotech, Germany; Bharat Biotech International, India; Cadila pharmaceuticals, India; Heber Biotec, Havana, Cuba; Shantha biotechnics, India; Dongkook Pharm Co. Ltd, Korea; Kyung Dong Pharm Co. Ltd, Korea; and Pharmacia Upjohn, Sweden with different brands name like Akinase, Durakinase, Heberkinasa, Indikinase, Kabikinase, Myokinase, Shankinase, Streptonase, STPase, Solustrep, etc. [42].

6 Conclusion

SK is as effective as rtPA in treating acute myocardial infarction, and it is certainly more cost effective. Currently available SK with minimum risks is an effective medication for thrombotic disorders. Its adverse effects are very well defined which can be clinically treated. Numerous approaches are developed for the alteration of SK for spreading its half-life in flow, refining plasminogen activation, and diminishing or excluding immunogenicity. Expression of rSK in yeast system is a better choice, since it is a GRAS organism and genetic engineering technologies are well established. Continuous culture fermentation will give better yields over the batch and fed-batch fermentation if used in conjunction with a strong constitutive expression system. Demand for rSK is higher than native SK in the current market than other thrombolytic drugs in developing countries. Subsequently, it can be conceivable to produce a SK at the desired level to compete with tPA.

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Monascus Secondary Metabolites

26

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Abstract

Secondary metabolites of the fungus *Monascus* include pigments, monacolins, and citrinin. This chapter summarizes the biosynthesis of these metabolites, their biological activities, as well as new methods of determination based mainly on chromatography and spectrophotometry. In addition, asexual and sexual reproduction, solid substrates and submerged liquid cultivation conditions, together with the use of this fungus in food biotechnology and condiments are described. Emerging topics such as methods in molecular biology of *Monascus,* based on recent genomic sequencing of *M. purpureus, M. ruber*, and *M. pilosus,* are also discussed.

Keywords

Monascus • Pigment • Citrinin • Monacolin • Red yeast rice

1 Introduction

The fungus *Monascus* has, for centuries, been well known in Asian countries, especially China, Japan, Korea, Indonesia, Philippines, and Thailand, and is mostly associated with the production of *Monascus*-fermented rice (red yeast rice). Red yeast rice is known under various Asian names, i.e., as angkak (Philippinese), hong qu (Chinese), or beni-koji (Japan), and is mostly associated with food colorings with beneficial effects on human health, particularly its positive effects on digestion and against cardiovascular diseases. Medicinal effects of red yeast rice were described in the traditional Chinese Pharmacopoeia from the Ming Dynasty (A.D. 1368–1644), although its culinary uses probably reach back further into history. Dried powder from red yeast rice, or red yeast rice itself, was used for coloring different kinds of foods such as cheese, fish, soya, and meat products. In addition, a red koji (i.e., *Monascus*-fermented substrate, usually rice) is utilized in food fermentations as a source of hydrolytic enzymes and active fungus. In this way, red rice wine, red rice vinegar, fish paste (e.g., bagoong), or fermented tofu (sufu or tofuyo) are produced.

In contrast, in Western countries *Monascus* is mainly associated with food supplements containing red yeast rice, which can be efficient in lowering blood LDL cholesterol and triglyceride levels. It is believed that this effect of red rice is caused mainly by its content of monacolin K, a statin of the same structure as lovastatin (mevinolin), which acts in association with other compounds in red rice (pigments, different monacolins). In the 1990s, *Monascus* was also thought to be a possible source of natural food pigments, a convenient alternative for artificial red and yellow colorants. This period ended with the identification of monascidin A as the mycotoxin citrinin [1]. The complete condemnation of *Monascus* pigments as toxic material, even though some *Monascus* strains are nontoxigenic, is an example of double standards from Western countries, where other fungi associated with food fermentation, e.g., *Penicillium roqueforti,* can also produce mycotoxins under certain conditions, but this has not resulted in a reduction or a ban on blue cheese

production. The attitude of Western countries towards *Monascus* is even more baffling with respect to red rice food supplements. Surprisingly, in this case, the fact that these products may contain varying concentrations of citrinin is often not considered although controversy is possible, through varying contents of monacolin K. The American Food and Drug Administration (FDA) banned several food supplements containing red rice with monacolin K and warned consumers that use of these supplements, especially together with prescribed drugs containing the same compound, may elicit or increase side effects of statins, potentially damaging health [2]. In contrast, the European Food Safety Authority (EFSA) approved the statement: "Monacolin K from red yeast rice contributes to the maintenance of normal blood cholesterol concentrations" [3] which can be shown on *Monascus* food supplements. The basis of the EFSA position comes from proven cause-effect relationships between monacolin K and cholesterol lowering but neglects the fact that monacolin K is identical to lovastatin (mevinolin), which is an efficient (active) compound in approved, prescribed drugs.

In Asian countries, especially in China and Japan, the fungus *Monascus* has been studied intensively. In China, *Monascus* is so respected that a position for it was found in the limited space of the Shenzhou 3 recoverable spaceship (the experiment was performed successfully to obtain mutants with higher monacolin K production) [4]. Unfortunately, not all results of Asian researchers are published in English and therefore they remain hidden from nonnative people. This fact, together with the association of the fungus with exotic fermented meals such as bagoong, red sufu, and red rice wine or brandy give the fungus a mysterious aura. Nevertheless, the genomes of all three most famous *Monascus* species, i.e., *M. purpureus*, *M. ruber*, and *M.pilosus,* were recently sequenced [5–8], enabling a deeper insight into *Monascus* physiology and revealing new approaches to strain improvement.

2 Fungus *Monascus* – Taxonomy, Reproduction, and Metabolism

Originally, the genus *Monascus* was classified in the family Monascaceae (phyllum Eumycota, subphyllum Ascomycotina, class Plectomycetes, order Eurotiales) [9] but based on recent genome sequencing, it seems that the genus is more closely associated with the genus *Aspergillus,* and thus should be reclassified in the Aspergillaceae family [10]. The genus *Monascus* comprises nine species, the most important of which are *M. purpureus*, *M. ruber*, and *M. pilosus*. The most famous species is *M. purpureus (*see Fig. 1*),* which is also known under many synonyms, e.g., *M. anka*, *M. albidus, M. major*, or *M. rubiginosus*.

Monascus species are usually homothallic, teleomorphic fungi that form mycelia composed of branched hyphae. Asexual reproduction also includes formation of single or chained aleurioconidia at the tips of specialized hyphae (see Fig.2). In rare cases, intercallar chlamydoconidia or arthroconidia are developed.

The Western name for the fungus – *Monascus* – is not appropriate because it implies formation of a single ascus (monoascus) during sexual reproduction, which

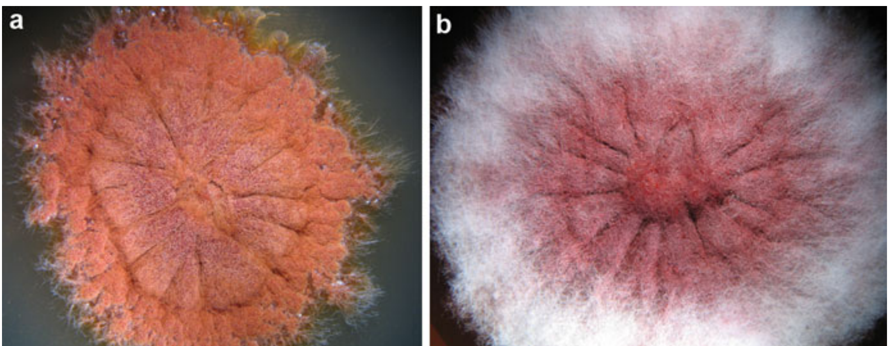
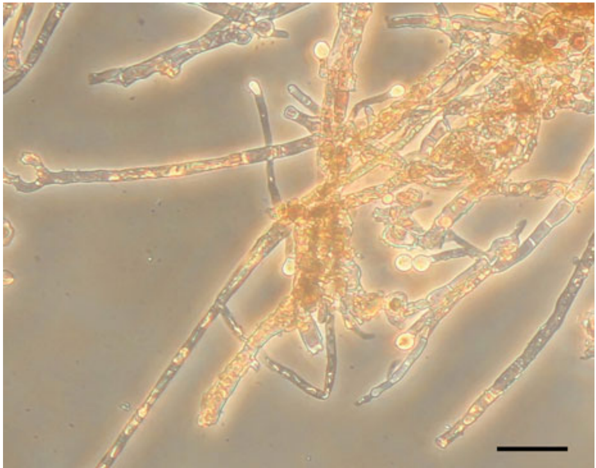


Fig. 1 Colonies of *M. purpureus* grown on Petri dishes containing potato-dextrose agar (**a**) or malt extract agar (**b**). The fungus was cultured for 7 days at 30 °C

Fig. 2 Round aleurioconidia at the tips of specialized hyphae. *M.purpureus* grown on potato-dextrose agar plates. The *bar* represents 30 μm



is only true in rare cases [11]. In fact, one sexual act often results in the formation of multiple croziers at the terminal ends of ascogenous hyphae. The complete chain of events occurring during sexual reproduction, originally proposed by Young (1931) [12], was proven in further studies [11, 13, 14] and remains mostly unchanged. Both male and female sexual organs grow towards each other. The male sexual organ, the antheridium, arises by septation from hyphae tips. The female organ, the ascogonium with trichogyne, arises from protuberant cells beneath the septum. Male nuclei migrate through the trichogyne to the ascogonium but do not fuse with the female nuclei. The ascogonium enlarges and is dotted with sterile hyphae. Ascogenous hyphae inside the ascogonium organize themselves into cells, each containing both nuclei, thus giving rise to asci. It is only within asci that the nuclei fuse and undergo meiotic and mitotic divisions, resulting in eight haploid daughter nuclei, forming the basis of eight ascospores within an ascus. The asci are grouped from 1 to 15, or even

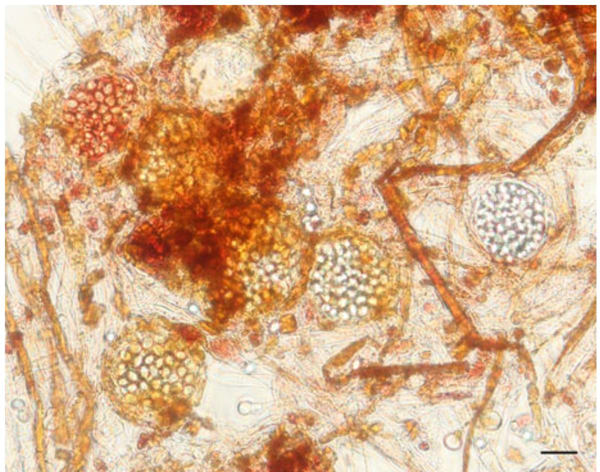


Fig. 3 Cleistothecia containing oval ascospores. *M. purpureus* grown on potato-dextrose agar plates. The *bar* represents 30 μm

more, in a cleistothecium (see Fig. 3) that contains up to 120 or more ascospores. The ascus wall is transparent, dissolving after maturation, and ascospores are released to the cleistothecium. After rupture of the cleistothecium wall, the ascospores are released into the environment.

There is a clear influence of cultivation medium on the formation of spores of all types, the most significant probably being the influence of nitrogen source [15]. In other filamentous fungi, e.g., in Aspergilli [16], there is usually a relationship between mycelium differentiation, followed by sporogenesis, and secondary metabolism. This relationship has never been studied systematically in *Monascus*, but some recent studies [17] with *Monascus* albino mutants (constitutive nonpigment forming) suggest that such a relationship probably exists. Secondary metabolism and reproduction also appear to be regulated by a G-protein signaling pathway [18].

The fungus is aerobic, saprophytic, mesophilic, with respirofermentative metabolism; it can also be characterized as Crabtree negative with limited respiration because it creates ethanol under conditions of glucose excess, even in aerated systems [19]. The fungus produces different lytic enzymes including those with amylolytic, proteolytic, or lipolytic activities that enable growth on different substrates. The species *M. ruber* can degrade cellulose [20]. In addition to ethanol, L-malic and succinic acids can be formed as primary metabolites [21, 22].

2.1 Strain Improvement

Initially, strain improvement methods consisted mainly of random mutagenesis induced by UV irradiation or the action of chemical mutagens. In this way, *Monascus* mutants preferably producing yellow pigments [23] or albino mutants (nonpigment producing) [17] were obtained. Protoplast fusion then became a popular method to strengthen phenotypic traits in the fungi. Intergeneric protoplast

fusion between *Monascus anka* and *Aspergillus oryzae* resulted in better growth and ethanol production by fusants [24]. Monacolin K production was increased by intergeneric protoplast fusion of *M. anka* and *A. terreus* [25]. Recently, different transformation approaches were applied to improve production of pigments and/or monacolins and to decrease/eliminate citrinin production. These methods, including electroporation, biolistic transformation, and *Agrobacterium tumefaciens*-mediated transformation, were reviewed in 2014 [26].

3 Biosynthesis of Secondary Metabolites

The fungus *Monascus* is the most famous for production of pigments and monacolins; however, it can also form other metabolites – citrinin, dimerumic acid, and GABA (γ-aminobutyric acid). Formation of particular metabolites depends on the strain and cultivation conditions.

3.1 Pigments

There are three major pairs of *Monascus* polyketide azaphilone pigments, in which the two analogues differ in length of side chain (see Structure 1) – yellow ones (ankaflavin, monascin), orange ones (rubropunctatin, monascorubrin), and red ones (rubropunctamine, monascorubramine). In addition, there are many minor pigmented compounds that have been isolated recently from *Monascus* strains cultivated under different conditions; for a recent survey see [27, 28]. Some of these novel colored metabolites may originate from *Monascus* strains with defective gene clusters for the formation of major pigments, as in the case of monascusone A, which was produced by a *M. purpureus* strain with a targeted deletion in the FAS gene cluster [7].

The structures of all major pigments were elucidated over the period from 1950 to 1980 [29–34]. In the same period, the pathway for pigment biosynthesis was proposed. Individual steps in this proposed sequence are gradually being confirmed, but there are still some steps that are only hypothesized but not proven.

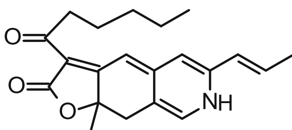
According to current opinion based on transcriptomic analysis [5] which is in agreement with previous chemical views [29, 32] and experimental evidence [6, 7, 35–37], biosynthesis of the orange pigments (rubropunctatin, monascorubrin) requires coordinated activity of fungal polyketide synthase (PKS), in particular nonreducing (NR-) polyketide synthase with a reductive release domain (−R), and fatty acid synthase (FAS). NR-PKS-R ensures formation of the basic hexaketide chromophore structure, which is then methylated and hydroxylated. Subsequently, FAS provides C8 or C10 chains that are necessary for the complete pigment structure (for simplified rubropunctatin biosynthesis, see Scheme 1). Current opinion is that synthesis of the hexaketide backbone starts with the condensation of one acetyl-CoA with five malonyl-CoA subunits, and 3-oxo-octanoic acid or 3-oxo-decanoic acids, which are necessary for rubropunctatin or monascorubrin, respectively, are

Yellow pigments

Monascin $C_{21}H_{26}O_5$, M = 358.43

Ankaflavin $C_{23}H_{30}O_5$, M = 386.48

Orange pigments



Rubropunctatin $C_{21}H_{22}O_5$, M = 354.40

Monascorubrin $C_{23}H_{26}O_5$, M = 382.45

Red pigments

Rubropunctamine $C_{21}H_{23}NO_4$, M = 353.41

Monascorubramine $C_{23}H_{27}NO_4$, M = 381.46

Structure 1 Major *Monascus* pigments

synthesized by FAS and PKS from one acetyl-CoA and two malonyl thioesters. In the case of *Monascus*, NR-PKS-R is a multifunctional polypeptide, composed of catalytic domains consisting of the starter unit ACP transacylase (SAT), β-ketoacyl synthase (KS), acyltransferase (AT), product template (PT), acyl carrier protein (ACP), C-methyltransferase (MT), and an R terminal domain [6]. In general, polyketide synthesis consists of iterative Claisen type condensations from acetyl-CoA and malonyl-CoA subunits, catalyzed by SAT, KS, and ACP catalytic domains of PKS, which result in the generation of a poly-β-ketoacyl thioester intermediate. This is further modified by other catalytic domains, but their actions are not iterative. For biosynthesis of yellow and red pigments, it is believed that the yellow pigments (ankaflavin, monascin) are formed from orange ones by reduction, and red pigments are generated by the reaction of orange ones with compounds containing amino groups through Schiff base formation; for a scheme of red pigment formation see [28, 38]. Surprisingly, mechanisms for biosynthesis of all major *Monascus* pigments were also found in *Penicillium marneffei*, where genes responsible for their biosynthesis were identified and the biosynthetic pathway, which is more or less the same as in *Monascus*, was hypothesized [39].

Scheme 1 Simplified rubropunctatin biosynthesis

PKS- and FAS-encoding genes, which participate in orange pigment biosynthesis, are grouped into PKS-FAS gene clusters that share relatively high homology in *M. purpureus*, *M. ruber*, and *M. pilosus* [5–8]. In general, biosynthetic regulation of fungal secondary metabolites is mediated by a G-protein signaling pathway and a global regulator of secondary metabolism (LaeA). The G-protein is a heterotrimer composed of α, β, and γ subunits and receives signals from the cellular environment by a membrane-bound G-protein receptor. G-protein activation results in regulation of gene expression in three ways, i.e., by cAMP-dependent protein kinase A, mitogen-activated protein kinase (MAPK), or phosphorylation of protein kinase C [40]. LaeA is a master regulator of secondary metabolism and fungal development, the functioning of which was described for Aspergilli. It is located in the nucleus, and its main regulatory functions are attributed to a methyltransferase domain [41, 42]. Regulation of the PKS-FAS gene cluster in *Monascus* has not been described fully. For *M. ruber* M7, inactivation of the *Mga1* gene encoding the G-protein α-subunit induced pigment and citrinin production [43]. In the same strain, signal transfer by

cAMP-dependent protein kinase A was confirmed by addition of varying amounts of cAMP [44]. It was also found that in *M. ruber,* the regulator of the G-protein signaling pathway, MrflbA, controls citrinin and pigment formation, together with growth and reproduction [44]. Overexpression of the *laeA* gene in *M. pilosus* resulted in increased production of pigments and monacolins [45].

3.2 Monacolins

The fungus *Monascus* is also known for production of monacolins, compounds from the statin family that act as inhibitors of 3-hydroxy 3-methyl glutaryl CoA reductase, a key enzyme in cholesterol production in mammals. The fungus can produce different monacolins (monacolin K, L, J; dihydromonacolin L; and others) that share common biosynthetic routes [38]. The most important compound from this group is monacolin K, also known as lovastatin or mevinolin (see Structure 2). Its production was recognized in different fungal species, including *Penicillium citrinum* and *Aspergillus terreus*. For *Monascus*, it was initially isolated from *M. ruber* in 1979 [46]. Currently, monacolin K, under the name lovastatin, is produced by Merck using *A. terreus* fermentation and is the active compound of the drug Mevacor®. Other companies use not only *A. terreus* but also *Monascus* in submerged liquid cultivation for production of monacolin K [47–49].

Although monacolin K production is often associated with health benefits of *M. purpureus* fermented rice, the monacolin K gene cluster has only been described fully for *M. pilosus* [50, 51]. The biosynthetic pathway for monacolin K was proposed based on knowledge gathered for *Aspergillus terreus* [52]. In *A. terreus*, monacolin K is synthesized by lovastatin nonaketide synthase (PKS), through dihydromonacolin K and monacolin L to monacolin J, which is modified by addition of a methylbutyryl side chain synthesized by lovastatin diketide synthase (PKS); for an outline of the pathway, see [52]. Based on homology of lovastatin (monacolin K)

HO O O O O

O OH O HO O

Monacolin K, $C_{24}H_{36}O_5$, M = 404.55   Citrinin, $C_{13}H_{14}O_5$, M = 250.24

Structure 2 Structure of monacolin K and citrinin

gene clusters in *M. pilosus* and *A.terreus*, the biosynthetic pathways in these two species appear to be similar [8]. There are known 27 monacolin compounds [49], including the recently identified unusual aromatic monacolin analogues, monacophenyl and monacophenylone, isolated from rice fermented by *M. purpureus* [53, 54].

3.3 Citrinin

Production of the mycotoxin citrinin, which is produced by many fungal species, e.g., *Penicillium citrinum*, was isolated from *Monascus* in 1995 [1]. Citrinin is a yellow colored aromatic polyketide (Structure 2), and gene clusters for its synthesis were described for *M. purpureus* [55] and *M. aurantiacus* [56], although citrinin was also detected as a product of *M. ruber* [43, 57]. The biosynthetic pathway for citrinin production was originally proposed by Hajjaj (1999) [57], see [8]. Tetraketide is initially formed from acetyl-CoA and three malonyl-CoAs (this step is catalyzed by PKS-CT) and then the pathway proceeds with additional steps such as condensation, methylation, oxidation, reduction, and dehydration, which are all catalyzed by individual domains of PKS-CT.

Citrinin production in *Monascus* appears to be independent of pigment production [58] although its biosynthesis can be regulated by G-protein signal pathway in the same way as pigments production [43]. As citrinin has significant nephrotoxic activity, its production in all *Monascus* products is undesirable. However, disruption of selected genes from the citrinin gene cluster can result in lower or no citrinin production, and in this way it is possible to increase the safety of *Monascus*-fermented products [59, 60].

3.4 Other Metabolites

In addition to the above metabolites, dimerumic acid (DMA) and γ-aminobutyric acid (GABA) were found to be products of *Monascus* fermentation (see Structure 3). DMA is considered to be a breakdown product of the siderophore coprogen B, a secreted iron-chelating compound [61]. In general, siderophores facilitate iron absorption by fungi, and their synthesis comes from the nonproteinogenic amino acid ornithine, catalysed by nonribosomal peptide synthetase [62]. DMA was found to increase iron absorption in *Aspergillus nidulans* [62], and acts as an antioxidant, which is the reason it contributes to the beneficial health effects of *Monascus*-fermented products [63].

GABA is ubiquitously present in fungi, being formed by decarboxylation of L-glutamic acid, and probably influences sporulation [64, 65]. Human consumption of GABA is associated with antihypertensive, liver-protective, and tranquilizing effects.

Dimerumic acid, $C_{22}H_{37}N_4O_5$, M = 485.56

Gamma-aminobutyric acid, $C_4H_9NO_2$, M = 103.12

Structure 3 Structures of DMA and GABA

4 Biological Activity of *Monascus* Metabolites

4.1 *Monascus* Pigments

The predominant *Monascus* yellow and orange pigments (see Structure 1) are azaphilones, which can react, more or less readily, in dependence on pH, with compounds containing amino groups (amino acids, peptides, proteins, and nucleotides). Orange pigments are particularly reactive. These reactions, in which oxygen in the pyrane ring is exchanged for nitrogen, result in the formation of vinylogous γ-pyridones [38, 66, 67] and may be responsible for a spectrum of biological activities.

In red yeast rice, yellow and red pigments are usually detectable while orange ones can be obtained after submerged liquid cultivation (SLC) under certain conditions (see below). The red rice is considered to be a folk medicine in Asian countries, being especially effective against indigestion, dysentery, blood circulatory problems, spleen disfunction, and also externally against skin diseases and muscle bruising.

Antimicrobial activities of orange pigments from SLC were observed several times against selected G^+ and G^- bacteria, yeasts, and fungi [67–70]. Antimicrobial effects of yellow pigments were similar but weaker compared to orange [67, 69]. Derivatives of red pigments with different amino acids exhibited antimicrobial activities against bacteria and fungi [71] and the mechanism of action, as suggested by SEM microphotographs, was pigment adsorption onto the cell wall of the microorganisms. However, in previous tests [67], antimicrobial activity of red pigments was not observed although this might be explained by differing water solubilities of red pigment derivatives and the effect might also be dose dependent.

Monascus pigments, as well as whole red yeast rice, were also tested for different biological activities including antitumor, antiproliferative, anti-inflammatory,

anticholesterolemic, and other effects; for recent reviews see [72–75]. Antitumor effects were observed in different tests for all types of *Monascus* pigments. Most frequently, beneficial effects are attributed to yellow pigments, monascin and ankaflavin, which exhibit anti-inflammatory effects and are often tested for the treatment of metabolic syndromes [76–78].

Dried red yeast rice or its extracts were tested for toxicity on mice and rats, in varying doses (1–5 $g.kg^{-1}$ of body weight) and for varying times [79–81] with negative results. The Ames test for mutagenicity was also performed with red rice but with negative results [80]. Nevertheless, embryotoxicity and teratogenicity of orange, yellow, and glycine derivatives of red pigments were proven at a dose of 100 μg of extract per chicken embryo [69]. Contrary results may arise through different test methodologies and especially through different doses of the active components. Despite this, it seems probable that the use of red yeast rice as a coloring agent and condiment is without risk provided that the red yeast rice is citrinin free or contains citrinin in amounts not exceeding allowed limits (see below).

4.2 Monacolin K (Lovastatin, Mevinolin)

Lovastatin, which is of the same structure as monacolin K, was the first compound from the statin group that was approved in 1987 by the FDA as a cholesterol-lowering drug. Its mechanism of action consists of competitive inhibition of HMG-CoA reductase, a key enzyme in cholesterol de novo synthesis in mammals. Inhibition occurs through structure analogy between an HMG-CoA intermediate and lovastatin in the form of a β-hydroxy acid (for detailed scheme of inhibition mechanism, see [52]). The effect of lovastatin to decrease serum cholesterol is significant within a few days.

As monacolin K can be formed by some *Monascus* strains, it can be found in red yeast rice, and as a result it is not only consumed as a prescribed drug but also in the form of a food supplement. Regular consumption of red yeast rice supplements containing monacolin K can result in an effect comparable with prescribed drug use [79, 82–84]. However, there are two significant concerns regarding the consumption of red yeast rice for medical purposes. The first is possible contamination of the supplements with the nephrotoxic mycotoxin citrinin, and the second is varying concentrations of monacolin K (and other monacolins) in the supplements. In general, production of food supplements is not governed by good manufacturing practice, a regime ensuring standard product quality during drug production, and therefore *Monascus* food supplements cannot be considered as safe as drugs containing the pure compound. Nevertheless, there are opposing attitudes between US and European authorities toward *Monascus* food supplements, and these were described in the Introduction.

It is also important to note that it may be dangerous to consume prescribed statin drugs simultaneously with *Monascus* food supplements because of possible increased serious side effects associated with statins. These include risks of liver injury, memory loss, potential for muscle damage, and a risk of diabetes; see FDA

warning from 2012 [85]. For these reasons, *Monascus* food supplements should not be taken without medical supervision.

In addition to anticholesterolemic effects, red yeast rice containing monacolin K seems to have other beneficial effects on human health, including a slowing of Alzheimers disease [74], reduction in aortic aneurysms [86], and others. Monacolin K produced by *Monascus* can also influence animal health. Impaired digestion was observed in cattle consuming *M. ruber*-contaminated silage [87]. In contrast, feeding hens *Monascus*-fermented products improved their performance and resulted in decreased egg cholesterol and an increase in yolk color [88].

4.3 Citrinin

Citrinin can not only be produced by some *Monascus* strains but also by some *Aspergillus* and *Penicillium* species, and as a result, can be detected in different foods and feeds, especially kernels, often together with other mycotoxins, ochratoxin A or patulin. Citrinin consumption affects kidneys and livers of different mammals, including humans, and seems to be a probable cause of endemic Balkan nephropathy. No mutagenic activity was observed in the Ames test, with or without metabolic activation, but after preculture of citrinin with hepatocytes, a mutagenic product was formed from citrinin [89]. This risk of a citrinin genotoxic effect after metabolic biotransformation has been proven recently [90] and according to EFSA scientific opinion [91], this phenomenon requires further study because the genotoxic effect of citrinin is distinct at lower concentrations than nephrotoxicity or acute toxicity. Because of scientific uncertainty, and because the effect of citrinin is not restricted to potential kidney damage, EFSA has refused to recommend a general citrinin limit in food or feed. However, some Asian countries have introduced their own limits: 50 μg/kg, South Korea [92]; or 200 μg/kg, Japan [93]. Despite the EFSA opinion, according to EU Commission Regulation No.212/2014, an upper limit of citrinin in red yeast rice food supplements sold in Europe is 2000 μg/kg.

Citrinin also acts against microbial growth of different bacteria (both G^+ and G^-) [94, 95]. The antibacterial effect of citrinin against *Bacillus subtilis* was even chosen as a selection marker for screening *M. purpureus* non-citrinin-producing mutants [96], raising doubts about whether extracts of red yeast rice containing only pigments can really exhibit antimicrobial activity (see Sect. 4.1) or whether this activity should be attributed to citrinin contamination.

5 Determination of *Monascus* Secondary Metabolites

Three main groups of compounds, consisting of monacolins, citrinin, and pigments, are frequently analyzed in *Monascus*-fermented solid or liquid media. All compounds share some similar physicochemical properties, enabling simultaneous analysis for most of them. Nevertheless, most published works describe only selected

metabolites of interest and only exceptionally is a simultaneous analysis of all *Monascus* metabolites carried out.

5.1 Extraction of Metabolites from *Monascus*-Fermented Substrates

With few exceptions where substances are analyzed directly from solid matrices, the first step in analysis of *Monascus* metabolites is extraction into a liquid solvent. As the secondary metabolites are mostly water insoluble, extraction agents are frequently organic solvents such as methanol, ethanol, acetonitrile, *n*-hexane, benzene, etc. Micro emulsion extraction for pigments was also introduced [97]. Various extraction reagents, their ratios, and differing formats of the extraction process can lead to different extraction efficiencies for the various *Monascus* metabolites [98, 99]. Therefore, the choice of extraction procedure is a critical part of a successful, sensitive, and reliable analytical quantification. In addition to the significance of a particular solvent, the presence of water or acid might contribute to higher recovery rates based on wetting (moisturizing) effects on the extracted material and disruption of metabolite-sample interactions, respectively [100–102]. Wu et al. [99] provide a comparative study of the effects of various solvents, temperatures, and times on the extraction yield of citrinin, lactone and acidic form of monacolin K, and yellow pigments monascin and ankaflavin. Monacolins and citrinin were extracted with the highest recoveries using 75 % and 50 % ethanol, yellow pigments using 75 % ethanol or ethyl acetate; however, ethyl acetate gave poor results for citrinin and monacolin.

Extraction methods such as vortexing, ultrasonication, mixing, and shaking and sample to solvent ratios, times, and temperatures of extraction all contribute to the final extraction efficiency [99, 102, 103]. The extraction procedure is routinely followed by centrifugation and/or extracts filtration prior to analysis.

A huge variety of metabolites of *Monascus* together with products of side reactions with substances from the medium yield a large number of chemicals that are extracted together; effective separation and identification of target compounds is therefore laborious [104] and some authors have introduced cleanup steps prior to analysis. Cleanup procedures incorporated into the extraction technology are predominantly liquid to liquid [105] or solid phase extraction [106]. However, during cleanup, losses in desired compounds can also occur [102], leading to lower method sensitivities.

5.2 Citrinin Analysis

Citrinin is water insoluble with a conjugated, planar structure, natural fluorescence, and UV adsorption that enables its detection by fluorimetric or spectroscopic methods [100]. Citrinin is acidic with spectral characteristics strongly dependent on pH and solvents [107, 108]. Only the protonated neutral form of citrinin is

fluorescent, therefore higher analytical sensitivity can be achieved by lowering the pH to zero and the use of pure methanol instead of acetonitrile [108]. For citrinin detection, a broad range of methodologies can be found in the literature, including surface enhanced Raman spectroscopy – SERS [109], analysis based on an amperometric biosensor [110], a number of various immunoassays, microsphere-based flow cytometric immunoassay (MFCI) [111], competitive indirect enzyme immunoassay [112], a semi-quantitative fluorimetric technique [113], thin layer chromatography (TLC) [114], and high performance liquid chromatography (HPLC). For a survey of detection limits of these techniques, see [91]. The most commonly used methodology for separation and quantification of citrinin is HPLC, with UV [22] or fluorimetric detection [98, 102, 103, 115, 116]. HPLC followed by mass spectrophotometric detection and quantification of citrinin [101, 102, 107, 115, 117, 118] is also very popular, especially for its direct identification of metabolites and significantly lower LOD and LOQ comparing to fluorescence or UV detection [102]. Although HPLC-MS provides a very sensitive and accurate analytical method for citrinin analysis, it requires expensive equipment that is not generally available in most laboratories. Therefore, for routine analysis of citrinin, UV or FL detection is used, although the sensitivity of UV detection is significantly lower than that of fluorescence and is insufficient for some applications [101]. On the other hand, UV detection is more universal across the whole scale of *Monascus* metabolites.

The preferred system for HPLC separation of citrinin consists of reverse phase material such as a C18 (octadecyl carbon chain bonded silica – ODS) stationary phase. The pH and composition of mobile phases are variables that affect binding interactions and hence the retention time and elution order of citrinin. As a mobile phase, methanol, water, acetonitrile, and an acidifier are usually used in isocratic or preferably gradient mode.

5.3 Monacolin K (Monacolins) Analysis

Within the monacolin group, monacolin K is the most well-known and abundant (75–90 % of total monacolin content) substance [117, 119]. Analysis of monacolins in *Monascus*-fermented products is mostly based on HPLC in a similar configuration as for citrinin except for fluorescence detection, where monacolins can be quantified and identified by mass spectrometry or by their absorption properties in UV light [106, 116, 118, 119]. This similarity with citrinin analysis allows simultaneous assays for citrinin and monacolins [98, 117, 120]. However, the UV detection of citrinin gives poor LOD and LOQ, hence sequential detection comprising fluorescence and UV detectors in series are not unusual [99].

As an alternative to HPLC, micellar electrokinetic capillary chromatography, providing separation within 2 min followed by DAD detection, was introduced by Nigovic [121] for monacolin and citrinin.

In the fermentation broth, monacolin K exists in two forms as a lactone or in an acidic form, having different retention properties and showing as two separate peaks. An acidic or alkaline environment during extraction of monacolins influences the

ratio of forms [106, 122] and in addition, extraction with methanol under acidic conditions can lead to the formation of a methyl ester and the occurrence of a third separated peak [122]. In reality, many more chromatographic peaks can be linked to monacolins, because most of the substances originating from *Monascus*-fermented products and belonging to the monacolin family, whether in hydroxy acid or lactone forms, have absorption spectra showing typical “mountain-like” profiles with a maxima close to 237 nm [123]. Such similar absorption spectra provides insufficient specificity for proper identification, therefore further identification employing mass spectrometry is commonly used [98, 119, 122, 123].

In general, HPLC separation of monacolins is carried out on a reverse phase stationary matrix such as a C18 column with isocratic or gradient elution combining water, organic solvents, or an acid. Li et al. [123] found methanol-water and acetonitrile-water elution systems unsatisfactory and suggested increasing symmetry and resolution by decreasing the pH of the mobile phase. This is usually achieved by addition of phosphoric acid, acetic acid, trifluoracetic acid, or formic acid at concentrations of 0.05–0.1 %.

5.4 *Monascus* Pigment Analysis

As the name itself suggests, *Monascus* pigments are substances with specific spectral properties, whereupon they are generally divided into three groups, yellow, orange, and red pigments. More than 50 different pigments have already been described [116] so each group consists of a number of structurally diverse chemicals, which are often analyzed together by colorimetry or spectrophotometric techniques. Within the huge number of *Monascus* pigments, only six of them predominate (see Structure 1) and these are most frequently quantified. The degree of yellow color is therefore ascribed to the presence of monascin and ankaflavin, orange to rubropunctatin and monascorubrin, and red to rubropunctamine and monascorubramine. UV–VIS spectrometry is a traditional and well-established methodology for characterization and quantification of pigment production by *Monascus*. Direct absorbance measurements of centrifuged or/and filtered fermentation broths or extracts at particular wavelengths, namely 410 nm for yellow, 470 for orange, and 510 nm for red pigments (or 400 nm, 460 nm, and 500 nm) compared with nonfermented extracts or broths, yield absorbance values as OD (optical density), AU (absorbance units), or units per ml or g [18, 98, 124, 125]. To reflect the representation of each color component, a ratio of absorbances at a single wavelength can be used.

Typical features of absorption spectra of all the six pigments are curves with multiple maxima and valleys with absorption in the UV as well as in the visible spectra; complete spectra were published, e.g., in refs. [107, 126]. Colorimetry or/and image analysis enables precise characterization of pigment colors by assessment of lightness and hue angle values for redness, blueness, greenness, and yellowness. Whereas *Monascus* pigments are generally used as a food colorant, such characteristics are used for evaluation of color, stability, and changes [127, 128].

Similar to spectrometry, TLC is a common method for pigment analysis. Individual spots are clearly visible owing to their color and respective substances are characterized by their retention factors (Rf). TLC plates for pigment separation are most frequently coated with silica gel as a solid adsorbent but various combinations of developing agents have been described. 2D TLC was used by Teng and Feldheim [126] for a better separation of *anka* pigments and their nitrogen analogues. In the first dimension, ankaflavin and monascin were effectively separated by *n*-hexane: ethylacetate (7:3, v/v) but red and orange remained close to each other. In a second dimension *n*-hexane: acetone (2:1, v/v) was applied to separate monascorubrin and rubropunctatin from monascorubramine and rubropunctamine.

The review [28] summarizes some applications of HPLC in *Monascus* pigment isolation, purification, and analysis. As well as with previous metabolites, the most common stationary matrix is based on C18 and mobile phases are various combinations of organic solvents and water, in some cases acidified with trifluoracetic acid or formic acid. Detection is performed mostly by photodiode array detector (PDA) or refractive index detector (RID).

Advanced technologies such as nuclear magnetic resonance (NMR), IR-spectroscopy, mass spectrometry, and their combination provide a better insight into structure and chemical compositions of *Monascus* pigments [129–132] and have enabled characterization of a whole range of newly discovered azaphilones.

5.5 Simultaneous Analysis of *Monascus* Metabolites

Some efforts have been made to find simultaneous analytical procedures for the most prevalent metabolites citrinin, monacolins, and yellow, orange, and red pigments. The experimental arrangement is, however, complicated by different contents of analytes in natural samples. Wu et al. [99] developed a combined methodology for citrinin, monacolin K, ankaflavin, and monascin based on synchronous UV and fluorescence detection and HPLC separation by isocratic elution with a mobile phase consisting of 0.05 % trifluoracetic acid in acetonitrile:water (62.5:37.5 v/v) on a C18 column with peak identification by mass spectrometry.

6 Cultivation Conditions

6.1 Solid Substrate Cultivation (SSC)

Fungal cultivation on a solid substrate is a classical process to prepare traditional *Monascus*-fermented products. SSC mimics the natural habitat of filamentous fungi, which are cultivated on the surface of concentrated water-insoluble substrates with low water activity, such as rice. SSC is the original process developed in far eastern countries to manufacture traditional products such as koji, miso, soybean sauce, or sake [133]. Originally, red yeast rice was produced in covered bamboo trays placed

in the fermentation room. Nonglutinous rice was used, and this was agitated by hand and moistened by addition of water, if necessary [134].

Nowadays, the process of red yeast rice manufacturing consists of several steps. The rice is washed to remove surface impurities and soaked in fresh water to soften. Steaming is performed to eliminate interfering microorganisms, then cooling, followed by blending with the *Monascus* strain. Cultivation usually lasts for 7 days and rice kernels are incubated in a temperature controlled chamber on rotary perforated beds for continuous aeration with a ploughing mixer to remove excess heat and to ensure grain separation. The temperature should be maintained at 30–35 °C until the core of the rice becomes deep red colored. The rice is dried at a temperature of around 50 °C. Sterilization is carried out to denature any *Monascus* in the food product, followed by grinding to obtain a rice powder, inspection, and packaging [48, 135, 136].

Monascus cultivation conditions on the solid substrate may vary depending on the intended use of the final product, i.e., food coloring (red yeast rice), starter culture (koji), or food supplement [137]. The production of red yeast rice for food coloring is the most practiced cultivation of *Monascus.* The aim is to obtain pigments that are used as food colorants and food condiments for foodstuffs such as processed fish, meat, soya products, or sausages, in order to enrich their visual or nutritional value. As described previously, red yeast rice has various designations depending on the country in which the fermented product is applied. In China, the product is called hong qu (*Monascus* red rice), in Japan Beni-Koji, in the Philippines Ang-Khak, and in Germany Rotschimmelreis.

Monascus has been grown on many types of solid substrates of different chemical compositions, such as jackfruit seed, cassava, corn, wheat, hardy yam, adlay, or breadfruit, while rice kernels remain the most common substrate for the production of *Monascus*-fermented products [76, 118, 138]. During stationary phase of growth, *Monascus* produces compounds enhancing the sensory and nutritional value of the fermented product, such as pigments, organic acids, and esters. The quality and quantity of these agents are mostly influenced by the following factors: type of solid substrate, water activity, oxygen access, cultivation temperature, and predominantly the *Monascus* strain and any genetic modification [28, 82]. The temperature is usually set between 25 °C and 30 °C. Water activity (a_w) seems to be a critical parameter for the fermentation process. When a_w is high, fungal glucoamylase activity is elevated, causing a rapid release of glucose from starch and resulting in the production of ethanol instead of pigments. During production of *Monascus*-fermented products, water content should be adjusted to 40–50 % and maintained by occasional moistening of the substrate to enhance fungal growth. It is also suggested to initiate the fermentation process at a lower water content (25–30 %) to keep glucoamylase activity low and to increase pigment yield [139]. In the course of culturing, it is necessary to ensure oxygen access by rubbing, stirring, or turning the fermented substrate, because the accumulation of CO_2 in the substrate will inhibit pigment formation [140]. Fermentation of the substrate should proceed in the dark to obtain the maximum yield of pigments, whereas exposure to white light decreases yield to a minimum [141, 142].

Monascus cultivation of cooked kernels or legumes is also utilized for the production of red koji, functioning as a starter culture. The koji mould-based starter helps to release hydrolytic enzymes such as amylases, proteases, and lipases which hydrolyze starches, proteins, or fats into their nutritional precursors glucose, peptides, amino acids, and fatty acids [143]. The decomposition of complex compounds provides nutrition for yeast and bacterial cultures, which are subsequently used in further fermentation stages for the production of traditional Asian food products such as red rice wine, soybean-related food products, Chinese spirits, liqueurs, and others. The production process takes place in a shallow tray fermenter where fermentation is carried out for about 3–4 days.

Another use of *Monascus* cultivation is based on fermentation of cereals such as rice, millet, or wheat for the production of monacolin K. This agent is used as a food supplement or functional food to lower blood cholesterol levels, particularly in humans. The procedure takes place in special fermentation bottles and lasts for about 20 days; monacolin levels are significantly higher after 3 weeks of fermentation compared to 2 weeks. Addition of soybean powder, glycerol, sodium nitrate, or peptone results in higher production [144].

In comparison to liquid submerged cultivation, solid state fermentation has several advantages. These include issues such as: agricultural or food wastes can be used as fermentation substrates; a smaller reaction volume is involved, with low capital investment due to highly concentrated medium; easy technology and low volume of effluent water; higher product yield and easy product recovery; low risk of contamination by other microorganisms because of low a_w and a complex substrate [133, 145, 146].

6.2 Submerged Liquid Cultivation (SLC)

Secondary metabolite formation is influenced by the composition of the cultivation medium. An excess of easily utilized substrate, e.g., glucose at a concentration higher than 30 $g.l^{-1}$, can result in repression of respiratory metabolism and the substrate is processed by glycolysis to ethanol [19]. Both pigment and monacolin K formation are repressed by excess glucose [147]. The nitrogen source, in combination with initial pH of the cultivation medium, represents key factors that affect pigment formation, biomass growth, and sporulation (see Fig. 4). Organic nitrogen sources such as peptone or yeast extract stimulate growth and conidia formation. In a medium containing organic nitrogen sources, free amino acids are available to react with orange pigments forming red ones; the reactions are supported by a neutral pH in the medium [148]. Nitrates limit growth but stimulate ascospores, conidia, and pigment formation; the pH of the medium increases supporting the production of red pigments, but amino group-containing reactants in the cultivation medium are limited. Therefore, the use of nitrates results in a yellow-orange colored cultivation medium. Use of ammonium ions results in a decreased pH, conidia and ascospore formations are limited, but pigment biosynthesis is supported. A low pH prevents nucleophilic addition of oxygen to orange pigments with an amino group, resulting again in a yellow-orange color [149].

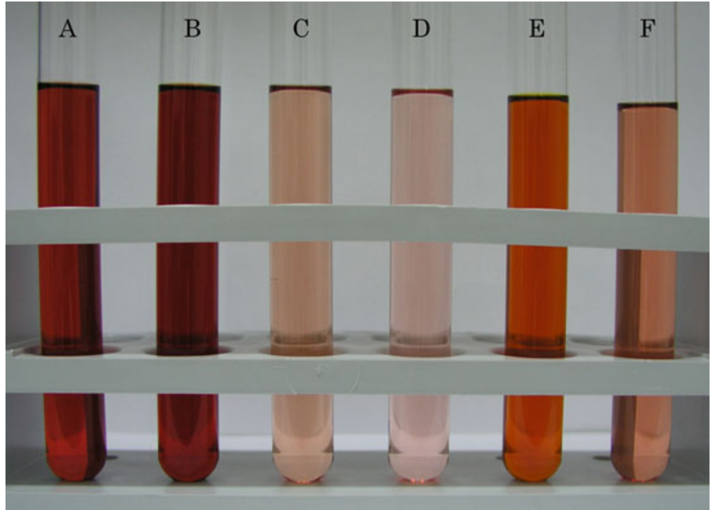


Fig. 4 Ethanol extracts from *M. purpureus* mycelium obtained after 7 days from submerged liquid cultivation at 30 °C, in media differing in nitrogen source: *A* peptone, *B* $NaNO_3$, *C* yeast extract, *D* sodium glutamate, *E* $(NH_4)_2SO_4$, *F* urea

As the conditions for biomass and secondary metabolite formation differ, tests were carried out to separate growth from secondary metabolite production, especially pigment formation, during a two-step cultivation process [150, 151]. Specific pigment production (per biomass amount) increased when ethanol was used as a substrate in the second stage of cultivation [150]. A slightly alkaline pH (about 8.5) in the cultivation medium in this second stage supported the formation of water-soluble red pigment complexes [151].

Currently, statistical approaches like Plackett-Burman designs or response surface methodologies are often applied in the design of SSC and SLC experiments, resulting in the optimization of medium composition and culture conditions. These approaches facilitate the identification of optimal conditions for particular *Monascus* strains [152–154], but they are usually strain specific and not optimal for all strains. As an example, it was found that monacolin K production in *M. purpureus* MTTC 410 was positively influenced by replacing glucose with maltose, the use of peptone as a nitrogen source, a cultivation temperature of 28 °C, and a pH of 5 [155].

7 *Monascus* in Food

7.1 Food Coloring

Traditionally, *Monascus* fermented red yeast rice is used both as a colorant and as a condiment because it influences not only color but also flavor of foods. Usually, a dried powder of the red rice is added directly to selected foods in small amounts or

a red rice ethanol extract can be added. However, the use of *Monascus* pigments is limited to pH-neutral and non-heat-processed foods because the pigments can be degraded at low pH, at higher temperatures, and in full sunlight. Currently, it is assumed that about 1500 t of red yeast rice is produced in China each year, and these are used for coloring 20 different kinds of foodstuffs including cheese, fish, meat products, and beverages [48].

In addition to traditional uses, *Monascus* pigments were tested for coloring processed meat products such as sausages or pate [156, 157]. These tests were conducted with the aim of replacing nitrite salts in meat products with a healthier alternative. However, nitrite salts are not only added for color but mainly as a preservative to prevent possible deadly poisoning due to bacterial toxins. Although the *Monascus*-colored meat products had an attractive color and smell, it is questionable whether pigment addition can prevent growth of toxinogenic bacteria such as *Clostridium botulinum*. *Monascus* pigments exhibit antimicrobial activity against G^+ and G^- bacteria, even against *Staphylococcus aureus* [71], but they were not tested against *C. botulinum*.

7.2 *Monascus* Fermented Foods

Monascus can be used as a starter in different food fermentations. In Japan, this type of microbial starter, which serves both as a source of active microorganisms and many types of hydrolytic enzymes, is called koji. Koji is a term that was adopted into the English language and can often be found in professional literature. However, it should not be forgotten that koji is a generic name and can be used in association with any microorganism, although mostly it is used in association with *Aspergillus oryzae* grown on rice and subsequently for sake production. For *Monascus* koji, the correct term is beni-koji; the Chinese equivalent of Japanese koji is qu (hong qu for *Monascus*). *Monascus* can form not only saccharifying enzymes such as α-amylase and glucoamylase, enabling the breakdown of starch, but also proteases, lipases, and esterifying enzymes [143, 158]. It is believed that mainly esterifying enzymes contribute to the final flavor of the products.

Monascus koji or qu can be used for the production of rice wine and rice vinegar. In China, cereal vinegars have a long tradition of preparation and one of the most famous vinegars is Fujian *Monascus* vinegar, which is produced using *Monascus* rice qu as a saccharifying and alcohol-forming agent [143]. In subsequent steps acetic acid bacteria and salt are added and the process continues with ripening, aging, sterilization, and bottling.

In many Asian countries, fermented tofu is prepared and in some special types of this fermented tofu (sufu), *Monascus* can be used. For example, in Japan, Okinawa Prefecture, fermented tofu called tofuyo is produced [159]. In the preparation of this product, *Bacillus, Aspergillus*, and *Monascus* strains are used. The working procedure for tofuyo consists of preparing dehydrated tofu of the right consistency (not too hard not too soft), which is inoculated with *Bacillus* and then soaked with awamori (indigenous Okinawa distillate from fermented indica long grain rice).

Red and yellow koji are separately prepared using *Monascus* and *Aspergillus* strains respectively, and then mixed with salt and awamori. This mixture is used for ripening awamori-soaked tofu, and as a result, tofuyo, which can be served and directly consumed at tea time with awamori, is produced. Tofoyo can be considered as a functional food because it contains compounds that are beneficial for human health, e.g., peptides inhibiting angiotensin converting enzymes (ACE) or antioxidants [159, 160].

Monascus can also be used for fermentation of fish, resulting in fish sauces or pastes. The most famous of these products is probably bagoong, produced in the Philippines from different kinds of fish or krill. Scientific notes on *Monascus* fish fermentations are rare; however, *Monascus* fermentation of Mackarel mince has been described, resulting in muscle protein hydrolysis and accumulation of free amino acids that enhance the flavor of the product [161]. In addition, growth of contaminating bacteria was inhibited and the product was of an attractive color [161, 162].

8 Conclusion

Monascus secondary metabolites appear to have great potential, both in medicine and the food industry. Despite the publication boom over the last 3 years, the fungus still remains under investigated in comparison with organisms like *Penicillium* or *Aspergillus*, and there are many aspects of its physiology that remain unclear. For example, why does the fungus produce secondary metabolites? From the point of view of the fungus, both citrinin and monacolin K might be considered "biological weapons" that are produced to gain an advantage over other microorganisms living in the same environment. Citrinin is active against bacteria while monacolin K may inhibit ergosterol synthesis in fungal cell walls in the same way as it inhibits cholesterol biosynthesis in mammals. But why does the fungus produce pigments? Do they play a regulatory role in sexual reproduction and conidiation or do they have other unknown functions? Hopefully, future investigations will answer these questions.

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Fungal Protease Inhibitors

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Jerica Sabotič and Janko Kos

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Abstract

Fungi constitute an enormous unexplored pool of protease inhibitors. Only a handful of fungal protease inhibitors have been exhaustively characterized, but

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they reveal great versatility and many unique features and novel types of inhibitory mechanisms. Small molecule and protein inhibitors of all catalytic classes of proteases have been identified in fungi, those that target serine proteases predominating. As important regulators of proteases, the function and potential applications of protease inhibitors are intimately connected with those of proteases they inhibit. In this chapter, both small molecule and protein protease inhibitors from fungi are described, including their biochemical characteristics, inhibitory mechanisms, and biological functions together with their potential for application in the fields of biotechnology, crop protection, and medicine.

Keywords
Peptidase • Protease • Protease inhibitor • Fungi • Regulation • Mycospin • Mycocypin • E-64

List of Abbreviations

ACE	Angiotensin I-converting enzyme
AFLEI	*Aspergillus flavus* elastase inhibitor
AFUEI	*Aspergillus fumigatus* elastase inhibitor
BIR	Baculoviral inhibitor of apoptosis protein repeat
CVPI	*Coriolus versicolor* pepsin inhibitor
E-64	L-*N*-*trans*-Epoxysuccinyl-leucylamido(4-guanidino)butane
GLPIA2	*Ganoderma lucidum* proteinase A inhibitor 2
IAP	Inhibitor of apoptosis
LeSPI	*Lentinula edodes* serine protease inhibitor
PDB	Protein Data Bank
POIA	*Pleurotus ostreatus* proteinase A inhibitor

1 Introduction

Protease inhibitors are important regulators of proteases that fulfill very important physiological roles in many life and death processes. Since their value is intimately connected with that of the proteases they inhibit, an overview of proteolytic enzymes is first provided, together with their classification and relevance.

Proteases occur in all living organisms and are essential for nutrient acquisition and make growth and proliferation possible. Extracellular proteases hydrolyze proteins into smaller peptides and amino acids that can be absorbed by cells as nutrients, an indispensable step in nitrogen metabolism, especially for wood-degrading fungi [1, 2]. Furthermore, they perform important regulatory functions in many biological processes, including DNA replication, regulation of gene expression, transport of proteins, the cell cycle, cell growth and differentiation, heat shock response, SOS response to DNA damage, and responses to oxidative stress, misfolded proteins, senescence, and programmed cell death. They achieve this by regulating the activity, localization, and fate of many proteins, modulating protein-protein interactions, and making significant contributions to the generation, transduction, and amplification of

molecular signals. Furthermore, in multicellular organisms, proteases carry out additional critical tasks in the extracellular environment, where they participate in tissue morphogenesis and remodeling, sexual and asexual reproduction, germination, hormone signaling, and defense responses against pathogens and parasites [2–5]. In pathogenic species, proteases also act as virulence factors, since they are vital for the acquisition of nutrients for growth and proliferation through host tissue degradation and for evasion of host immune responses [5–7].

Proteases, also called peptidases or proteolytic enzymes, are hydrolytic enzymes that catalyze the hydrolysis of peptide bonds. Protease is the narrower term, encompassing peptidases that act on protein substrates. Peptidases that cleave peptide bonds at the termini of polypeptide chains are termed exopeptidases and are further classified into those cleaving N-terminal peptide bonds, i.e., aminopeptidases, and those that cleave C-terminal peptide bonds, i.e., carboxypeptidases. Those that act on a single terminal amino acid residue are termed monoaminopeptidases or monocarboxypeptidases, and those that act on small peptides are termed dipeptidyl or tripeptidyl exopeptidases. Peptidases that cleave peptide bonds within the polypeptide chain are termed endopeptidases. The main classification of proteases is based on their mechanism of catalysis. According to the nucleophilic residue that attacks the substrate peptide bond, peptidases are classified into serine, cysteine, aspartic, glutamic, threonine, and metallopeptidases. In serine, threonine, and cysteine peptidases, nucleophilic attack is mediated by an oxygen or sulfur atom of the side chain of the amino acid in the active site (Ser, Thr, Cys). In the case of metallo-, aspartic, and glutamic peptidases, a water molecule, bound in the active site, acts as the nucleophile [8]. A seventh class of proteolytic enzymes was recently described that utilizes asparagine as the nucleophile for peptide bond cleavage and are not peptidases or hydrolytic enzymes but are termed asparagine peptide lyases [9]. The International Union of Biochemistry and Molecular Biology (IUBMB) enzyme nomenclature system (http://www.chem.qmul.ac.uk/iubmb/enzyme/) classifies peptidases acting on peptide bonds (EC 3.4) based on the position of the cleaved peptide bond and the catalytic class. For example, EC 3.4.16 are serine-type carboxypeptidases, and EC 3.4.22 are cysteine-type endopeptidases. The most comprehensive classification of proteolytic enzymes is in the MEROPS database (http://merops.sanger.ac.uk) where peptidases are classified into families based on sequence similarity. Catalytic class is reflected in the family designation, each family being identified by a letter representing the catalytic class (A for aspartic, C for cysteine, G for glutamic, M for metallo, N for asparagine, P for mixed, S for serine, T for threonine, and U for unknown), together with a unique number. Families are further grouped into clans, based on similarity of 3D structures and amino acid sequences around the catalytic amino acids as well as by the arrangement of catalytic residues in the polypeptide chain. There are currently 253 families of proteolytic enzymes grouped into 61 clans in the MEROPS database (release 9.13; August 2015) [8, 10–12].

Since the hydrolysis of a peptide bond is irreversible, regulation of proteolytic activity is vital for the balance of life and death processes. Activity of peptidases is regulated on several levels. A common characteristic of peptidases is that they are

mostly synthesized as inactive pre-pro-polypeptides, where the pre-sequence represents a signal peptide and the pro-sequence is an auto-inhibitory pro-domain which undergoes strictly controlled cleavage to yield an active enzyme. Mechanisms of peptidase regulation further include regulation of gene expression at transcriptional and posttranscriptional levels, blockade by endogenous inhibitors, pH or solute concentrations, spatial and/or temporal compartmentalization by targeting to specific compartments, posttranslational modification such as glycosylation and phosphorylation, cofactor binding, limited proteolysis, S-S bridging, oligomerization, and, finally, degradation [3, 12].

In this chapter, we describe protease inhibitors that constitute the very important mechanism of regulation of peptidase activity. Following a general description of their characteristics, we focus on small molecule and protein protease inhibitors of fungal origin which are listed according to the peptidase class they inhibit. Finally, their biological roles and potential applications are outlined.

2 Protease Inhibitors

Protease inhibitors are very important regulators of proteolytic activity. They form a complex with the target peptidase, preventing cleavage of the substrate peptide bond. They can be small molecule inhibitors or protein inhibitors that act irreversibly or reversibly. The latter type can be competitive or noncompetitive relative to the substrate [12–14].

2.1 Classification

Protease inhibitors can be classified according to their origin (e.g., microbial and plant protease inhibitors), their inhibitory mechanism (e.g., reversible and irreversible protease inhibitors), and their structural similarity (e.g., inhibitors with a beta-trefoil fold). Very often they are classified roughly according to the class of proteases they inhibit (e.g., serine or aspartic protease inhibitors). They are classified, according to their specificity, into those that inhibit different classes of proteases, those that inhibit one class of proteases, one family or a single protease [14, 15]. However, protease inhibitors that target proteases of different catalytic classes or are composed of multiple inhibitor units restrict unambiguous classification.

The most comprehensive classification of protease inhibitors is included in the MEROPS database (http://merops.sanger.ac.uk/inhibitors) which follows a hierarchy similar to that for the classification of proteases. Protein protease inhibitors are grouped into families based on sequence homology and into clans based on similarity of 3D structures. There are currently 79 families of protease inhibitors listed in the MEROPS database (release 9.13; August 2015), those with available structural data being grouped into 39 clans. Of the 79 families, there are 22 that include members of fungal origin (Table 1), and, of the latter, three families include protease inhibitors exclusively of fungal origin (I34, I48, and I85). From a further three families, only

Table 1 Families of protease inhibitors of fungal origin and their distribution [11]

Family[a] (clan[b])	Common name	Families of peptidases inhibited	Counts of inhibitor homologs in fungi	Distribution[c]						
				Bacteria	Archaea	Fungi	Protozoa	Plants	Animals	Viruses
I1 (IA)	Kazal	S1, S8	1	xx	x	x	—	xx	xxxx	—
I2 (IB)	Kunitz-BPTI	S1	1	xx	—	x	x	x	xxxx	x
I4 (ID)	Serpin	S1, S8, C1, C14	9	xxx	xx	x	xx	xxx	xxxx	xx
I8 (IA)		S1, M4	7	x	x	x	x	xx	xxxx	x
I9 (JC)	YIB or I^B2	S8	122	xx	—	xx	—	xxx	x	—
I12 (IF)	Bowman-Birk	S1, S3	1	—	—	x	—	xxx	xx	—
I13 (IG)	Eglin c	S1, S8	2	x	—	x	—	xxx	xx	—
I15 (IM)	Hirustasin	S1	4	x	—	x	x	xx	xxxx	—
I19 (IW)	Pacifastin	S1	1	—	—	x	—	—	xxxx	—
I20 (JO)		S1, S8	1	—	—	x	—	xx	x	—
I25 (IH)	Cystatin	C1, C13, S8, M12	1	x	—	x	xx	xxx	xxxx	xx
I32 (IV)	Survivin	(C14)	145	—	—	xx	x	x	xxxx	xx
I34 (JA)	IA3	A1	5	—	—	x	—	—	—	—
I48 (IC)	Clitocypin	C1, C13	9	—	—	x	—	—	—	—
I51 (JE)	I^C	S10	193	xxx	xx	xx	xx	xxx	xxx	x
I63 (JB)		M43	1	x	—	x	xx	xx	xxxx	x
I66*	Cospin	S1	33	x	—	xx	—	—	—	—
I71*	Falstatin	C1, C2	3	x	—	x	xx	x	xx	—
I78*		S1	13	xx	—	xx	—	x	—	—
I79*	AVR2	C1	1	—	—	x	—	x	x	—
I85 (IC)	Macrocypin	C1, C13, S1	10	—	—	x	—	—	—	—
I87*	HflC	M41	25	xxxx	xx	xx	xx	xx	xxx	—

[a]**Families in bold** include protease inhibitors identified at the protein level that are exclusively of fungal origin. *Underlined families* include protease inhibitors identified only at the genetic level, as homologs in sequenced genomes

[b]Families unassigned to a clan are marked with an asterisk

[c]x denotes the number of sequence homologs found in each group of organisms: x less than 10, xx 11–200, xxx 201–1000, and xxxx more than 1000

fungal representatives have been identified at the protein level (I66, I78, and I79). The astonishing diversity of protease inhibitors of fungal origin is indicated by the fact that 17 families have been included into 15 clans and another five families have not yet been assigned to a clan [11, 14, 16]. Families of serine protease inhibitors predominate, followed by a few families of cysteine and metalloprotease inhibitors. There is only one family that encompasses aspartic protease inhibitors (I34).

There is no settled classification of small molecule protease inhibitors, but, like protein protease inhibitors, they can be classified according to their mechanism of inhibition (reversible or irreversible), according to their specificity (broad-spectrum class-specific inhibitors or inhibitors specific for one protease), or according to their structural similarity (e.g., amino acid derivatives or vinyl sulfones). Small molecule protease inhibitors are produced naturally by microorganisms. Various protease inhibitors have been isolated from fungi, several from cultures of *Actinobacteria* and predominantly from different species of the genus *Streptomyces*, while many more have been synthesized and derivatized in vitro. An alphabetical list of small molecule protease inhibitors is included in the MEROPS database that provides basic information and references [11].

2.2 Mechanism of Inhibition

Two general mechanisms of protease inhibition are recognized: irreversible, "trapping" reactions and reversible tight-binding reactions.

The irreversible trapping mechanism is specific to proteolytically active endopeptidases, since it depends upon cleavage of an internal peptide bond in the inhibitor molecule that triggers a conformational change. These inhibitors are also called suicide inhibitors, since their conformation is not reformed. They function as guardians, protecting cells and tissues from unwanted proteolytic activity. Only three families utilize this type of irreversible inhibitory mechanism: I4 (serpins), I39 (α_2-macroglobulin), and I50 (viral caspase inhibitors) [14, 17]. Serpins (family I4) inhibit serine and cysteine proteases that cleave an appropriate peptide bond in the large reactive center loop. Cleavage triggers a dramatic conformational change, the N-terminal part of the loop being inserted into a β-sheet of the inhibitor. The enzyme molecule is carried, attached as an acyl-enzyme intermediate, to the opposite pole of the inhibitor. Formation of the covalent serpin-protease complex deforms the enzyme active site and renders the protease inactive [14, 17–19]. Similarly, inhibitors of family I50 form an irreversible complex with the protease after cleavage of a caspase-sensitive bond in the reactive site loop leading to a conformational change, stabilizing the acyl-enzyme complex [14]. In another example, the large inhibitor α_2-macroglobulin encloses the protease after it has cleaved a peptide bond in the bait region. Any catalytic class of protease can be trapped, but without inactivation of the protease. Access to the active site is prevented for protein substrates, while small substrates can still be hydrolyzed [14, 17].

Many protease inhibitors utilize the tight-binding, reversible mechanism of protease inhibition, which involves a high-affinity interaction with the protease

active site. Reversible protease inhibitors block access to the protease active site by binding directly to it in a substrate-like manner or, indirectly, around the active site. The latter can be achieved without influence to the catalytic mechanism (e.g., in the inhibition of cysteine proteases by cystatins of family I25 or by clitocypin of family I48). On the other hand, the metalloprotease inhibitors of family I35 (tissue inhibitors of metalloproteases) interfere with the catalytic machinery of the protease by chelating the catalytic metal ion [11, 14, 17, 20]. The mechanism of protease inhibition by protein protease inhibitors described in most detail is the "standard" or "Laskowski mechanism." These inhibitors possess a stabilized loop that mimics the substrate and binds into the active site. Due to the conformational stability, the specific peptide bond in the reactive site loop is cleaved but not released from the active site, remaining in the complex in equilibrium with the intact form of the inhibitor. The cleavage occurs between the P1 and P1' residues, and consecutive residues in the N-terminal direction of the cleaved peptide bond are labeled P2, P3, and P4, while on the carboxyl side the residues are labeled P2', P3', P4', etc. Correspondingly, in the protease active site, the residues binding the substrate (or inhibitor) are termed subsites and labeled S4-S3-S2-S1-S1'-S2'-S3'-S4' [21]. This "standard" type of mechanism has been conclusively demonstrated only for serine protease inhibitors. Additionally, some of these inhibitors also bind proteases outside the active site, at sites termed exosites, usually through secondary binding, which extends the contact surface area between the inhibitor and protease and influences the affinity and specificity of the interaction. Protease inhibitors of fungal origin belong to several families that utilize the reversible tight-binding mechanism of inhibition, including I1, I2, I8, I12, I13, I15, I19, I20, I25, I48, I66, and I85. Some inhibitors can inhibit different families of proteases, which, in some inhibitors, is achieved through the same reactive site and by others through different reactive sites and mechanisms [14, 17, 20, 22].

Small molecule protease inhibitors utilize similar principles for protease inhibition by competing for the substrate binding site either by direct competition or by deformation of the protease active site. Their potency is increased through interaction with the catalytic machinery and their specificity through interaction with substrate binding sites on either side of the active site [11, 17].

3 Small Molecule Protease Inhibitors from Fungi

Most small molecule protease inhibitors show broad inhibitory specificity and inhibit all proteases of a catalytic class and sometimes even those of different catalytic classes. For this reason, they are often used in protease inhibitor cocktails. Many were originally isolated from bacteria belonging to various *Streptomyces* species, including bestatin (inhibitor of serine and metalloproteases), pepstatin A (inhibitor of aspartic proteases), phosphoramidon (inhibitor of metalloproteases), and leupeptin (inhibitor of cysteine and serine proteases) [12].

Several natural small molecule inhibitors were originally isolated from fungal species (Fig. 1). The most known and widely used is E-64 (L-*N*-

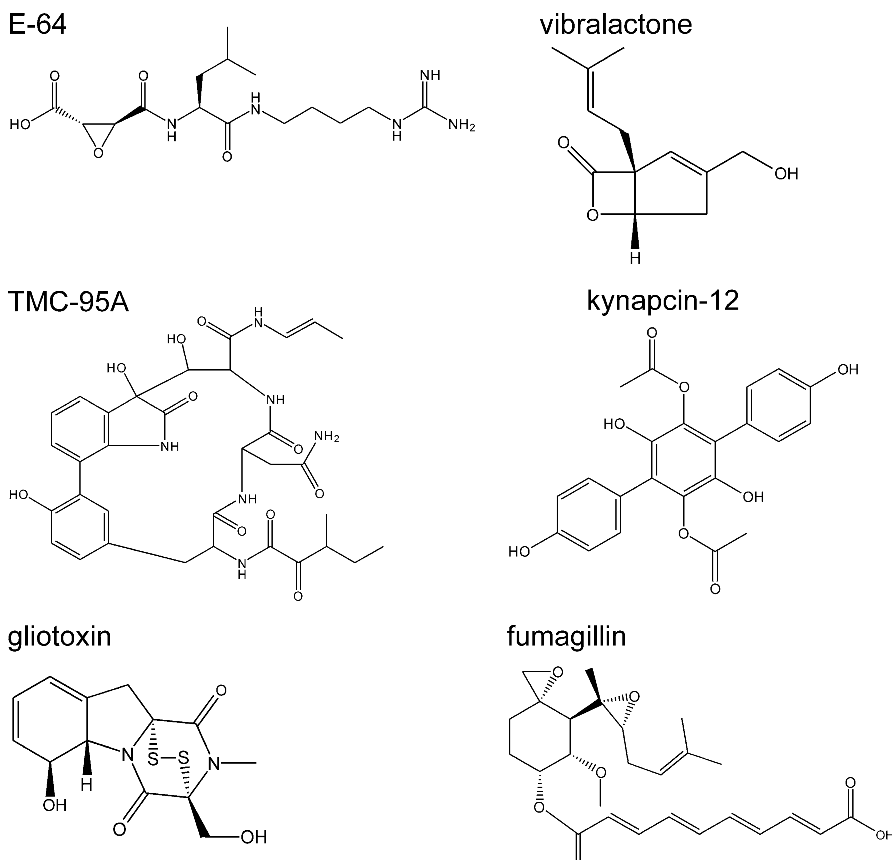

Fig. 1 Structures of small molecule protease inhibitors from fungi. E-64, TMC-95A, gliotoxin, vibralactone, kynapcin-12, and fumagillin

trans-epoxysuccinyl-leucylamido(4-guanidino)butane), an irreversible inhibitor of cysteine proteases from clan CA, that was originally isolated from *Aspergillus japonicus* [23]. Irreversible inhibition is achieved by S-alkylation of the catalytic cysteine, which results in opening of the epoxide ring [11, 24]. Several other cysteine protease inhibitors have been isolated from different ascomycete fungi, including kojistatin A from *Aspergillus oryzae* [25], paecilopeptin from *Paecilomyces carneus* [26], prohisin from *Cephalosporium* sp. [27], and thysanone from *Thysanophora penicilloides* [28]. In addition, many derivatives have been synthesized based on their structures with the aim of improving selectivity for specific proteases.

A few natural proteasome inhibitors have been isolated from fungi, including TMC-95 and gliotoxin (Fig. 1). Four diastereomers of TMC-95 were isolated from the culture broth of a saprophytic ascomycete *Apiospora montagnei*, and two of

them inhibited, noncovalently and selectively, chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide-hydrolyzing activities of the 20S proteasome, with IC50 values at low nanomolar levels [29]. Various types of derivatives were subsequently designed and synthesized in vitro [30–35]. Gliotoxin is an important secondary metabolite of the epipolythiodioxopiperazine class isolated from the opportunistic pathogen *Aspergillus fumigatus*. It shows a range of biological activities, including antimicrobial, antiviral, antiparasitic, immunosuppressing, and apoptosis inducing. It inactivates enzymes such as alcohol dehydrogenase, creatine kinase, and farnesyl-transferase and is an effective noncompetitive inhibitor of the chymotrypsin-like activity of the 20S proteasome [36–39].

A serine protease inhibitor, vibralactone (Fig. 1), was isolated from cultures of a basidiomycete *Boreostereum vibrans* as a lipase inhibitor [40, 41]. This bicyclic β-lactone inhibits the caseinolytic ClpP protease (family S14), an important virulence factor of *Listeria monocytogenes* [42]. From fruiting bodies of the basidiomycete *Polyozellus multiplex*, a serine protease inhibitor specific for prolyl endopeptidase (family S9), polyozellin, was isolated [43]. Several dibenzofuranyl derivatives of polyozellin, called kynapcins (Fig. 1), were subsequently isolated from the same fungus and were also inhibitory to prolyl endopeptidase [44–46].

An irreversible inhibitor specific for methionyl aminopeptidase fumagillin (Fig. 1) was first isolated from *Aspergillus fumigatus* [47]. It has been widely used in apiculture and human medicine to treat fungal infections, and, based on the specific inhibitory profiles, this inhibitor and its derivatives are considered as angiogenesis inhibitors in the treatment of cancer [48–50].

4 Protein Protease Inhibitors from Fungi

Very few protein protease inhibitors from fungi have been thoroughly characterized at the protein level (Table 2). Nevertheless, they exhibit great versatility, many unique features, and novel inhibitory mechanisms that distinguish them from protease inhibitors from other sources [12, 51]. Information on characterized protease inhibitors is provided in this section classified according to the protease catalytic class they inhibit.

4.1 Serine Protease Inhibitors

Several families of serine protease inhibitors have been identified in fungal genomes at the genetic level alone (I1, I2, I12, I13, I15, I19, and I20) (Table 1). Not many serine protease inhibitors have been isolated and characterized, and those with only limited knowledge of primary structures are not yet included in the MEROPS database. More thoroughly characterized serine protease inhibitors from fungi are arranged based on the MEROPS family and described below.

Inhibitory activity against serine proteases trypsin and thrombin (both family S1) has been detected in crude protein extracts of various species of ascomycetes [125]

Table 2 Protein protease inhibitors from fungi

Protease family inhibited	Inhibitor name	Origin	MEROPS family	3D structure[a] (PDB code)	Mw (kDa)	Mechanism of inhibition	Biological function[b]	References
S1, S8	Celpin	*Piromyces* sp.	I4	Nd	59 kDa	Irreversible trapping	Protection	[52]
S8	I^B2	*S. cerevisiae*	I9	Nd	8.5 kDa	Reversible tight-binding	Chaperone, regulation	[53–61]
S8	POIA1	*P. ostreatus*	I9	1ITP, 1V5I	8.3 kDa	Reversible tight-binding	Chaperone, regulation	[62–67]
S10	I^C	*S. cerevisiae*	I51	1WPX	24.4 kDa	Reversible tight-binding	Regulation	[68–76]
S1	Cospin	*C. cinerea*	I66	3N0K	16.7 kDa	Reversible tight-binding	Defense	[77, 78]
S1	Cnispin	*C. nebularis*	I66	Nd	16.4 kDa	Reversible tight-binding	Defense, regulation	[77, 79, 80]
S1	LeSPI	*L. edodes*	I66	Nd	16 kDa	Reversible tight-binding	Regulation or defense	[81]
S1	AFLEI	*A. flavus*	I78	Nd	7.5 kDa	Reversible tight-binding	Unknown	[82–85]
S1	AFUEI	*A. fumigatus*	I78	3W0D, 3W0E	7.5 kDa	Reversible tight-binding	Unknown	[85–87]
C1, C13	Clitocypin	*C. nebularis*	I48	3H6R, 3H6S	17 kDa	Reversible tight-binding	Defense, regulation	[20, 80, 88–92]
C1, C13	Macrocypin 1, 3	*M. procera*	I85	3H6Q	19 kDa	Reversible tight-binding	Defense, regulation	[20, 91–93]

C1, S1	Macrocypin 4	*M. procera*	I85	Nd	18.7 kDa	Reversible tight-binding	Defense, regulation	[20, 91–93]
C1	Avr2	*C. fulvum*	I79	Nd	8.3 kDa	Unknown	Virulence	[94–97]
A1	IA3	*S. cerevisiae*	I34	1DPJ, 1G0V, 1DP5	7.6 kDa	Induced folding tight-binding	Protection, regulation	[55, 98–107]
S1	Trypsin inhibitors	*A. biennis*, *M. procera*, *A. mellea*, *A. phalloides*	Unclassified	Nd	~20 kDa	Reversible tight-binding	Unknown	[108, 109]
S1	PfTI	*P. floridanus*	Unclassified	Nd	38 kDa	Reversible tight-binding	Unknown	[110]
S7	Plectasin	*P. nigrella*	Unclassified	1ZFU, 3E7R, 3E7U	4.4 kDa	Reversible tight-binding	Defense	[111–113]
M2	Peptide ACE inhibitors	*P. cornucopiae*, *T. giganteum*, *H. marmoreus*, *L. tricolor*, *G. frondosa*, *P. cystidiosus*, *G. lingzhi*	Unclassified	Nd	0.2–2 kDa	Reversible tight-binding	Nd	[114–121]
A1	GLPIA2	*G. lucidum*	Unclassified	Nd	15 kDa	Reversible	Unknown	[122]
A1	CVPI	*C. versicolor*	Unclassified	Nd	23 kDa	Reversible	Unknown	[123]
A1	PepA inhibitor	*Penicillium* sp.	Unclassified	Nd	1.6 kDa	Reversible tight-binding	Defense	[124]

[a]Nd, not determined

[b]Biological functions are listed as *protection* for protection against unwanted proteolysis, *regulation* for regulatory function in endogenous proteolytic system, *defense* for defensive function targeting exogenous proteases, *chaperone* for assisting in cognate protease folding, and *virulence* for protease inhibitors that are virulence factors targeting exogenous proteases

and basidiomycetes [126–128], showing that fungi constitute a rich source of serine protease inhibitors. Trypsin inhibitors isolated from fruiting bodies of basidiomycetes *Abortiporus biennis* [109], *Pleurotus floridanus* [129], *Macrolepiota procera*, *Armillaria mellea*, and *Amanita phalloides* [108] were partially characterized. With the exception of that from *P. floridanus*, they are all heterogeneous small acidic proteins with molecular masses around 20 kDa and isoelectric points around pH 4. The molecular mass of the inhibitor from *P. floridanus* is 38 kDa. They all exhibit exceptional thermal stability as well as resistance to exposure to extremes of pH [108, 109, 129]. Similarly, inhibitors of proteinase K (family S8), isolated from the mycelium of white rot basidiomycete *Trametes versicolor*, also exhibit high thermal stability. They are heterogeneous proteins with molecular masses between 14 and 20 kDa and similarly acidic isoelectric points [130].

A peptide antibiotic, plectasin, a fungal defensin isolated from a saprophytic ascomycete *Pseudoplectania nigrella* [112], was shown to inhibit the dengue virus serine protease NS2B-NS3pro (family S7) and, consequently, viral replication in vitro [113]. Plectasin is synthesized as a 95-amino acid peptide with an N-terminal signal sequence (1–23), a propeptide (24–55), and a 40-amino acid mature C-terminal domain (56–95) composed of an α-helix and two β-strands stabilized by three disulfide bonds (Fig. 2). It shows strong antibacterial activity against gram-positive bacteria and high thermal and pH stability, as well as resistance to proteolytic digestion by papain (family C1) and pepsin (family A1) [111–113].

4.1.1 Family I4: Serpins

Serpins (serine protease inhibitors) are the largest and most widely distributed superfamily of protease inhibitors. They are present in all multicellular organisms and more sporadically in primitive unicellular eukaryotes and prokaryotes. In multicellular organisms, they play important roles in many highly regulated physiological processes, including fibrinolysis, blood coagulation, immune responses, and inflammation; viral serpins are required as virulence factors. Most serpins inhibit serine proteases (families S1 and S8), some inhibit cysteine proteases (families C1 and C14), and, rarely, some fulfill other non-inhibitory functions. They are suicide protease inhibitors that utilize a unique and extensive conformational change to inhibit proteases (described in Sect. 2.2) [131–133].

Only one fungal serpin, from an anaerobic fungus *Piromyces* sp., has been characterized to date. Called celpin, it is a component of the cellulosome, the high-molecular-mass complex specialized in degradation of crystalline cellulose. Celpin presumably protects the cellulosome from proteolytic degradation by proteinases in the immediate environment that are secreted by the host's digestive system, competing microbiota or the plant cell substrate [52]. Celpin contains all features of functional serpins and, as inferred from deduced amino acid sequence data, a conserved set of structural features involved in the inhibition process. The serpin fold consists of three C-terminal β-sheets (A, B, and C), an N-terminal helical domain composed of eight or nine α-helices (hA-hI), and the reactive center loop in an exposed conformation above the serpin scaffold. Celpin shares 30 % sequence

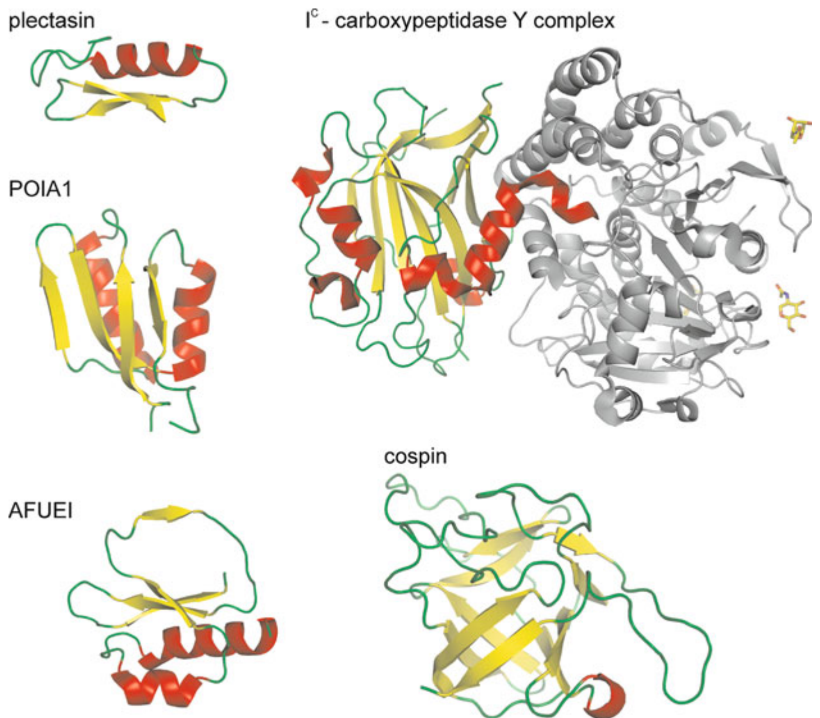

Fig. 2 Structures of fungal serine protease inhibitors. Ribbon diagrams of plectasin (PDB code 3E7U), POIA1 of family I9 (PDB code 1ITP), I^C of family I51 in complex with carboxypeptidase Y (PDB code 1WPX), cospin of family I66 (PDB code 3N0K), and AFUEI of family I78 (PDB code 3W0E) are shown. Secondary structure is indicated by colors: α-helix in *red*, β-strand in *yellow,* and loop in *green*; the protease is shown in *gray.*

identity and 50 % sequence similarity to vertebrate and bacterial serpins [52, 131, 133]. A serpin was also found to be a cellulosome component in the anaerobic thermophilic bacterium *Clostridium thermocellum*. As a broad inhibitor of subtilisin-like proteases, it probably plays a key role in protecting the cellulosome from protease attack [134].

4.1.2 Family I9: Subtilisin Propeptide-Like Inhibitors

The first member of family I9 was isolated from *Saccharomyces cerevisiae* as an endogenous inhibitor I^B of peptidase B [55], a subtilisin-like protease (family S8) now named cerevisin. It was then established that there are two similar inhibitors of yeast vacuolar proteinase B (cerevisin) in *S. cerevisiae*, namely, I^B1 and I^B2, that are 8.5 kDa proteins consisting of 74 amino acids differing only in one amino acid residue [57–59]. The predominant I^B2 inhibitor is not essential for protection of the cell against unwanted peptidase B activity in the cytoplasm [59]. Like the bacterial subtilisin BPN’ propeptide, it probably acts as an intramolecular chaperone assisting folding of the peptidase and, on peptidase activation, is gradually degraded, making it a temporary inhibitor. The C-terminal region of the protein is critical for the

inhibitory interaction with the cognate proteinase [53, 54, 56]. In addition to its function as a proteinase B inhibitor, I^B2 is also a component of a protein complex with thioredoxin required for effective vacuole inheritance in yeast [60, 61].

Two similar acid- and heat-stable isomeric endogenous inhibitors that specifically inhibit an intracellular subtilisin-like proteinase A (family S8) were isolated from fruiting bodies of the oyster mushroom (*Pleurotus ostreatus*) and designated POIA1 (Fig. 2) and POIA2. Their molecular masses are approximately 8.3 kDa, and they consist of 76 amino acids differing in only two residues [62]. Like I^B2, the C-terminal region harbors the inhibitory reactive site and also determines the strength of inhibition and resistance to proteolysis [64]. POIA1 is highly stable as it is, in contrast to the propeptide of the bacterial subtilisin BPN', a structured protein, while folding of the bacterial subtilisin propeptide is induced on binding to the protease. Stabilization of the POIA1 structure by internal hydrophobic residues is important for stable protease inhibition [63, 67]. The high structural stability of POIA1 makes it an effective intramolecular chaperone for the bacterial subtilisin BPN' [65, 66].

4.1.3 Family I51: Carboxypeptidase Y Inhibitors

Family I51 includes inhibitors of carboxypeptidase Y that belong to the phosphatidylethanolamine-binding protein (PEBP) family. The first representative was isolated from yeast *S. cerevisiae*. It is a cytoplasmic N-acetylated small protein (24.4 kDa) with isoelectric point of pH 6.6 that specifically inhibits endogenous vacuolar carboxypeptidase Y (family S10). The N-terminal acetyl group is essential for protease inhibition [70, 75, 135, 136]. The inhibitor I^C binds to carboxypeptidase Y through multiple binding sites, and the N-terminal acetyl group binds to the active site, resulting in complete inhibition of the protease. A secondary binding site contributes to the tight and stable inhibitor-protease interaction. The I^C protein consists of a major β-type domain and an N-terminal helical segment, both binding sites being located on the latter (Fig. 2). A novel type of "tight-binding" inhibitory mechanism and mode of interaction was revealed by solving the structure of I^C inhibitor in complex with carboxypeptidase Y. In contrast to the standard mechanism, the inhibitor binds, in a non-substrate-like manner, through a posttranslationally modified N-terminal. Additional binding at an exosite strengthens the interaction. In addition to the binding sites for carboxypeptidase Y, I^C possesses a ligand-binding site corresponding to the binding site for the phosphate group of phospholipid in phosphatidylethanolamine-binding proteins. The phospholipid probably binds via both the ligand-binding site and the secondary protease-binding site [74, 76].

A dual role was indicated for I^C by high-affinity binding to membranes containing anionic phospholipids (e.g., phosphatidylserine) and by highly specific inhibitory activity against the vacuolar carboxypeptidase Y. Determination of its protein-binding partners suggested that I^C acts as a bridge between cell signaling, through GTPase regulation, and intermediate metabolism in yeast. It is localized in the cytoplasm during logarithmic growth phase and is translocated to the vacuole in the stationary phase. Its transcription is elevated in the stationary growth phase and

in response to environmental stress, including oxidative stress and heat shock [68, 69, 71–73].

4.1.4 Family I66: Mycospins

Trypsin-specific inhibitors – LeSPI from *Lentinula edodes* [81], cnispin from *Clitocybe nebularis* [79], and cospin from *Coprinopsis cinerea* [78] – were isolated from fungal fruiting bodies and constitute the MEROPS family I66. These fungal serine protease inhibitors are collectively called mycospins. They are small proteins (~16 kDa) with isoelectric points around pH 5; they resist exposure to extreme pH conditions. They are all very strong and specific inhibitors of the trypsin (family S1), while their inhibition of chymotrypsin (family S1) is weaker; other serine proteases are not inhibited [78, 79, 81]. The crystal structure of cospin revealed the β-trefoil fold of family I66 (Fig. 2), and classification to clan IC, together with Kunitz protease inhibitors from plants (family I3) and mycocypins (families I48 and I85), has been proposed [20, 78]. The β-trefoil fold is composed of 12 β-strands that form three structurally similar repeats (α, β, and γ) folded together in pseudo-3-fold symmetry. The β-strands are connected with 11 loops of differing length and residue composition. Mycospins show a surprising plasticity of utilization of the loops, since different loops are recruited for trypsin inhibition in cnispin (β11-β12) and cospin (β2-β3). Moreover, most serine protease inhibitors from plants with the β-trefoil fold utilize the β4-5β loop for inhibition of trypsin or chymotrypsin, depending on the P1 residue. Arg or Lys as P1 residues confer trypsin specificity, while chymotrypsin inhibitors generally employ a Phe, Tyr, or Leu as P1 residue. Nevertheless, they are all tight-binding inhibitors that inhibit trypsin (and other S1 family proteases) through the binding loop that binds to the protease active site in a substrate-like manner. The very low dissociation constants generate a stable enzyme-inhibitor complex. The conformation of the binding loop depends on stabilization by a hydrogen bond network of scaffolding residues, which also accounts for the longevity of the complex. The β2-β3 loop of cospin is better optimized for trypsin inhibition, the complex persisting in vitro for over a month at 37°C, while cnispin is degraded within 24 h. Nevertheless, the loops β2-β3 and β11-β12 of both cnispin and cospin can be recruited for trypsin and/or chymotrypsin inhibition, depending on the introduced P1 residues [20, 77].

A dual biological role has been proposed for cnispin and cospin in defense against predators and parasites and in regulation of endogenous serine proteases involved in fruiting body development and/or resource recycling [78–80]. Cnispin and cospin are expressed abundantly in fruiting bodies, while expression is lower in vegetative mycelium. Strong and highly specific inhibition of trypsin, strong entomotoxicity against *Drosophila melanogaster* mediated by protease inhibition, and absence of trypsin-like protease genes in many saprophytic fungal genomes suggest that, in vivo, mycospins are directed mainly toward exogenous proteases, supporting a defensive activity for mycospins as their primary biological role. Mycospins are widely distributed in the subphylum *Agaricomycotina* of *Basidiomycota* that comprises most mushroom-forming fungi [78, 79, 137].

4.1.5 Family I78: *Aspergillus* Elastase Inhibitors

Inhibitors of the serine protease elastase (family S1) have been identified in several strains of *Aspergillus flavus* and *Aspergillus fumigatus* but none in *Aspergillus niger* [85]. Isolation of elastase inhibitors from *A. flavus* (AFLEI) and *A. fumigatus* (AFUEI) showed them to be identical, non-glycosylated proteins composed of 68 amino acids with molecular mass of 7.5 kDa and isoelectric point pH 7.4. They are both strong inhibitors of elastases from *A. fumigatus* and *A. flavus*, and they inhibit human leukocyte elastase, but not porcine pancreas elastase [82, 84, 86]. AFUEI is a wedge-shaped protein composed of an extended binding loop containing a β-strand and a scaffold protein core containing two α-helices and a β-sheet (Fig. 2). Structural similarity to the potato I family (MEROPS family I13) enabled modeling of the inhibitory mechanism and identified family I78 as a probable member of clan IG. The binding loop region shows much higher sequence similarity (56 % sequence identity) than the core region (12 % sequence identity), indicating that the mechanism of inhibition is similar in both families. The disulfide bond between the α-helix and the β-strand in the core scaffold contributes significantly to the inhibitory activity, since it makes the inhibitor more compact and rigid than potato I family inhibitors. Furthermore, the hydrogen bond network that stabilizes the binding loop in its correct orientation enables the tight-binding mechanism of inhibition, in this case also called the clogged gutter mechanism. Determination of the P1 residue to be Met45 indicated that chymotrypsin-like, but not trypsin-like, proteases will be inhibited by AFUEI, which was confirmed experimentally [87]. Both inhibitors are secreted showing their highest levels at 4–7-day-old culture. The same *Aspergillus* strains also secrete elastases, which represent important virulence factors of aspergillosis [84–86].

A smaller elastase inhibitor, asnidin, with a molecular mass of 4.2 kDa, was isolated from *Aspergillus nidulans*. It resists heat treatment and displays stability over a wide pH range. Like AFLEI and AFUEI, asnidin strongly inhibits *A. fumigatus* and *A. flavus* elastases and human leukocyte elastase but not chymotrypsin [138].

4.2 Cysteine Protease Inhibitors

Inhibitors of cysteine proteases are apparently less widely distributed in fungi than serine protease inhibitors. Only a few families have been identified, of which family I25 (cystatins) and I71 (falstatins) were identified at the genomic level only.

Cysteine protease inhibitor Pit2, specific for a set of plant apoplastic cysteine proteases, was identified in the plant pathogenic basidiomycete *Ustilago maydis*. These proteases are crucial components of defense activation in maize, and Pit2 was shown to be essential for fungal virulence. Sequence similarity with orthologs in the related smut species *Sporisorium reilianum* and *Ustilago hordei* revealed a conserved 14-amino acid-long motif that comprises the inhibitory reactive site that, by itself, also inhibits cysteine proteases. Flanking regions around the central inhibitory motif (amino acids 44–57) of the 118-amino acid-long protein confer specificity for selected cysteine proteases [139].

There are only three MEROPS families comprising fungal cysteine protease inhibitors (I48, I79, and I85) that have been identified at the protein level. They are described below. Fungal homologs of survivins or BIR-domain proteins, which belong to family I32 of caspase (family C14) inhibitors, do not function as protease inhibitors but are essential for equal chromosome segregation and cytokinesis and act indirectly as inhibitors of apoptosis-like cell death in yeast. Unlike mammalian inhibitor-of-apoptosis proteins (IAPs) that utilize BIR domain for direct inhibition of caspases, thereby inhibiting apoptosis, yeast homolog Bir1p in *S. cerevisiae* does not interact with the yeast caspase but nevertheless exhibits antiapoptotic activity [140–142].

4.2.1 Families I48 and I85: Mycocypins

Mycocypins, fungal inhibitors of cysteine proteases, comprise two MEROPS families that include clitocypin isolated from fruiting bodies of *Clitocybe nebularis* (family I48) and macrocypins isolated from fruiting bodies of *Macrolepiota procera* (family I85). Mycocypins are small proteins with molecular masses of 16–19 kDa and isoelectric points around pH 4.8. They exhibit resistance to exposure to high temperatures and extreme pH, this apparent stability being mediated by their ability to unfold reversibly. They are inhibitors of papain-like cysteine proteases (family C1) of animal and plant origin. They very strongly inhibit papain and human cysteine cathepsins L, V, K, and S with endopeptidase activities, while cathepsins B and H, which exhibit both endopeptidase and exopeptidase activities, are not or weakly inhibited by mycocypins. Through a second inhibitory reactive site, clitocypin and macrocypins 1 and 3 also inhibit legumain/asparaginyl endopeptidase (family C13), and macrocypin 4 inhibits trypsin (family S1) [88, 89, 93]. The crystal structures of clitocypin and macrocypin 1 show the β-trefoil fold of both mycocypin families (Fig. 3), allocating them to clan IC, together with Kunitz serine protease inhibitors from plants (family I3). Structural and mutagenesis studies showed a

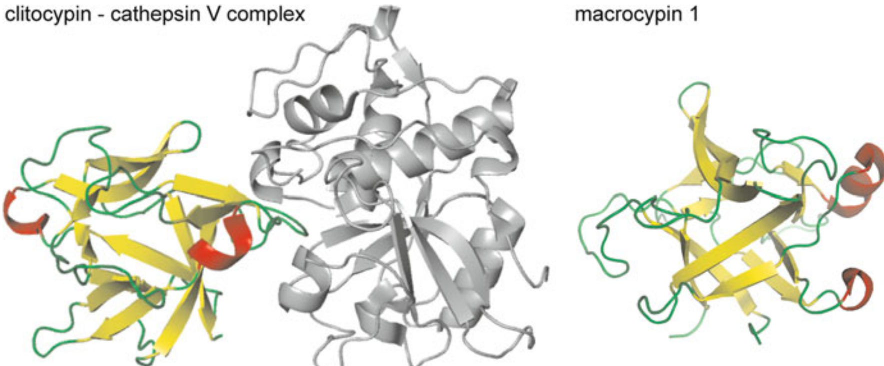

Fig. 3 Structures of fungal cysteine protease inhibitors. Ribbon diagrams of family I48 clitocypin in complex with cathepsin V (PDB code 3H6S) and family I85 macrocypin 1 (PDB code 3H6Q) are shown. The secondary structure of the protease inhibitors is indicated by colors: α-helix in *red*, β-strand in *yellow*, and loop in *green*; the protease is shown in *gray*.

unique tight-binding mechanism of inhibition of papain-like cysteine proteases by mycocypins. The two loops (β1-β2 and β3-β4) bind either side of the protease active site, thereby occluding the catalytic cysteine in the middle and preventing approach of substrate. In addition, a peptide bond flip between two conserved Gly residues in clitocypin enables direct contact with the cysteine in the active site. The interaction is stabilized by several hydrogen bonds formed directly between the inhibitor and protease surface residues or mediated through solvent molecules [20, 92]. For inhibition of legumain/asparaginyl endopeptidase (family C13) or trypsin (family S1) by the second inhibitory reactive site in loop β5-β6, the tight-binding mechanism of inhibition has been proposed, the inhibitor binding to the protease active site in a substrate-like manner. Clitocypin and macrocypins 1 and 3 inhibit legumain by the β5-β6 loop with the conserved Asn residue. Replacement of the P1 Asn residue with Lys in macrocypin 4 turned the legumain inhibitor into a trypsin inhibitor [20, 92]. In addition to the suitable P1 residue, the loop conformation also appears to be important, as engineering a trypsin inhibitor by simple P1 residue replacement (N70K) did not turn clitocypin from a legumain to a trypsin inhibitor [92].

Mycocypins exhibit great genetic diversity and are encoded by gene families in both fungal species. Very high sequence variability is observed for the macrocypin family in *M. procera*, where macrocypin sequences have been grouped into five groups (macrocypins 1–5) with 75–86 % sequence identity between groups and more than 90 % sequence identity within groups. Despite very similar biochemical properties, macrocypins show very low sequence identity with clitocypin – between 17 % and 21 % [89, 90, 93]. The variability is also reflected in regulation of expression as clitocypin is at the protein level uniformly expressed in the fungal fruiting body and in vegetative mycelium, and the protein is not secreted. On the other hand, studies of the regulation of macrocypin expression have revealed tissue-specific expression during fruiting body development in the model mushroom *C. cinerea*. Expression patterns at the promoter level in this mushroom were congruent with that in the origin mushroom at the protein level but not at the mRNA level. Differences in spatial and temporal expression regulation indicate different developmental or defensive roles for individual mycocypins. A primarily defensive function against various fungal antagonists was proposed for mycocypins in which they would target exogenous proteases including mycoviral proteases or digestive proteases of nematodes, insects, mites, and slugs [89–91, 93, 137]. Clitocypin-encoding genes were identified in a few other mushroom species belonging to subphylum *Agaricomycotina* of *Basidiomycota*, and there was no similarity found to macrocypin sequences. Either these protease inhibitors are not widespread or their sequence diversity hinders identification of proteins with similar biochemical functions [137].

4.2.2 Family I79: Avr2

Avr2 protein constitutes the I79 family of cysteine protease inhibitors. It is a small, cysteine-rich protein that is secreted into the tomato apoplast by the phytopathogenic ascomycete *Cladosporium fulvum* (syn. *Passalora fulva*) during the infection that leads to leaf mold of tomato. The mature protein consists of 58 amino acid residues,

eight of which are cysteines that form four disulfide bonds and are important for its stability [11, 97, 143]. Avr2 is an important virulence factor of *C. fulvum* and inhibits a set of papain-like cysteine proteases (family C1) that constitute the essential basal host defense. Several cysteine proteases are secreted into the apoplast by the host, but Avr2 selectively targets cysteine proteases PIP1 and RCR3, while others are only weakly (TDI-65, aleurain, glycinain) or not (cathepsin B) inhibited. The mechanism of inhibition is unknown, but physical interaction and inhibition of proteolytic activity are established, and binding outside the protease active site was proposed [94–97]. A similar approach of apoplast cysteine protease inhibition is employed by the non-fungal distant relative phytopathogenic oomycete *Phytophtora infestans*, with the modification that two cystatin-like cysteine protease inhibitors (family I25) are employed [143–145].

4.3 Metalloprotease Inhibitors

In the MEROPS database, only members of families of metalloprotease inhibitors that have been identified as homologs in a few ascomycete genomes are listed. They include families I8, I63, and I87 (Table 1). In addition, metalloprotease inhibitory activity against different matrix metalloproteinases (family M10), collagenase (family M9), and thermolysin (family M4) has been detected in extracts of fungal fruiting bodies and in mycelia from wood-degrading fungi prepared in organic solvents or buffers. Inhibitory substances were, however, not isolated [146, 147].

Specific inhibition of thermolysin family metalloproteinases (family M4) from *Aspergillus fumigatus* by the cognate propeptide has been demonstrated. The propeptide did not however inhibit *Aspergillus flavus* metalloproteinase. The endogenous regulatory role of protease inhibitors in keeping extracellular enzymes inactive until secretion consists of inhibiting mature enzymes by their respective propeptides [148].

Several angiotensin I-converting enzyme (ACE, family M2) inhibitors have been isolated from mushrooms. ACE is a dipeptidyl carboxypeptidase playing a crucial role in the renin-angiotensin system for blood pressure control. Its fungal inhibitors are mostly peptides ranging from di- to tripeptides isolated from fruiting bodies of *Tricholoma giganteum* [119] and *Ganoderma lingzhi* [121] and from penta- and hexa-peptides from *Grifola frondosa* [114], *Pholiota adiposa* [149], *Agaricus bisporus* [150], and *Pleurotus cystidiosus* [118] to longer oligopeptides with molecular masses ranging from 560 to 2040 Da from *Grifola frondosa* [120], *Pleurotus cornucopiae* [116], and *Hypsizygus marmoreus* [117]. These are mostly competitive ACE inhibitors that bind to the protease active site. Proteins with ACE inhibitory activity have also been isolated from water extracts of *Ganoderma lucidum* mycelia [151] and *Leucopaxillus tricolor* fruiting bodies [115], although their mechanism of action has not been elucidated. Isolation and characterization of ACE inhibitors from edible mushrooms are driven by their potential use as nutraceutical bioactive compounds for antihypertension effects.

4.4 Aspartic Protease Inhibitors

Only a few aspartic protease inhibitors have been identified in fungi, and only one family (family I34) of aspartic protease inhibitors with fungal members is included in the MEROPS database [11].

An aspartic protease inhibitor GLPIA2 was isolated from submerged fermentation of *Ganoderma lucidum*. The 15 kDa inhibitor inhibited saccharopepsin, also named yeast proteinase A, and pepsin (both family A1) [122].

A secreted pepsin inhibitor CVPI was isolated from cultured basidiomycete *Coriolus versicolor* (syn. *Trametes versicolor*). It is a monomeric inhibitor with a molecular mass of 23 kDa which exhibits high thermal and pH stability. It is a reversible inhibitor of pepsin, but the mechanism of action is not known [123].

An oligopeptide inhibitor of fungal aspartic protease PepA was isolated from the culture of *Penicillium* sp. It is a competitive tight-binding inhibitor with a molecular mass of 1585 Da that binds to the protease active site. Its expression is associated with fungal growth and exhibits antifungal activity against *Aspergillus fumigatus* and *Aspergillus niger* by inhibiting mycelial growth and spore germination [124].

4.4.1 Family I34: Saccharopepsin Inhibitor IA3

Inhibitor IA3 or saccharopepsin inhibitor from *Saccharomyces cerevisiae* is the only member of family I34 and is a highly specific endogenous inhibitor of the vacuolar aspartic protease saccharopepsin or yeast proteinase A (family A1). It is a competitive inhibitor with molecular mass 7.6 kDa consisting of 68 amino acids. Inhibition of the cognate protease is most effective at the yeast intracellular pH of around 6.5, while at pH 3, the pH optimum for saccharopepsin activity, only partial inhibition occurs. This, together with the high selectivity and specificity and its cytoplasmic localization, indicates a probable role in protection against unwanted proteolysis by vacuolar proteinase A leaked into the cytoplasm [55, 98, 101, 105, 107].

Structural studies have shown a unique mechanism of inhibition of saccharopepsin by IA3 inhibitor. The 68-amino acid protein is unstructured in solution. Upon contact with saccharopepsin, the N-terminal half of the molecule, namely, residues 2–32, adopts an amphipathic α-helix conformation that completely covers the active site cleft (Fig. 4). The 36 residues of the C-terminal half of the molecule remain unstructured [99, 102, 103]. The exclusive selectivity for saccharopepsin inhibition and its induced folding has been explained in terms of a few key residues strategically positioned in the inhibitor and enzyme molecules. Numerous interactions and an extensive hydrogen bond network along the whole length of the helix keep the inhibitor tightly bound and prevent access of substrates to the active site. This unique interaction presents a valuable tool for studies of protein folding, enzyme-inhibitor interactions, and aspartic protease inhibitor design [102–104, 106, 152].

Fig. 4 Structure of yeast aspartic proteinase A inhibitor IA3 in complex with saccharopepsin (PDB code 1DPJ). The inhibitor's α-helix is shown in *red* and the protease in *gray*.

5 Biological Functions of Fungal Protease Inhibitors

The biological function of a protease inhibitor is intimately connected with that of the protease it inhibits. In general, these proteases are either endogenous or exogenous.

For endogenous proteases, protease inhibitors act either as guardians and protect from unwanted proteolysis by misplaced proteases that have escaped other regulatory mechanisms or as chaperones that assist the folding of proteases. Examples of fungal protease inhibitors acting as chaperones are family I9 protease inhibitors, including the I^B2 inhibitor of cerevisin from *S. cerevisiae* and the POIA1 inhibitor of the subtilisin-like proteinase A from *P. ostreatus*. Examples of guarding protease inhibitors in fungi that protect from unwanted proteolysis are the family I34 inhibitor IA3 specific for *S. cerevisiae* vacuolar saccharopepsin and the family I4 inhibitors serpins, with the single fungal representative celpin from *Piromyces* sp. Proteases targeted by celpin are probably both endogenous and exogenous.

Inhibitors of exogenous proteases act either as defense molecules, protecting the source organism from proteases of parasites, pathogens, and predators, or as virulence factors, enabling evasion of host immune responses and effective invasion of host tissues. Examples of defense protease inhibitors include the serine and cysteine protease inhibitors abundant in fungal fruiting bodies from families I66 (cnispin

from *C. nebularis* and cospin from *C. cinerea*), I48 (clitocypin from *C. nebularis*), and I85 (macrocypins from *M. procera*) that mainly target exogenous proteases. In some plant pathogenic fungi, protease inhibitors are important virulence factors, including, for example, cysteine protease inhibitor Pit2 from *U. maydis* (unclassified) and Avr2 from *C. fulvum* (family I79).

6 Potential Applications of Fungal Protease Inhibitors

Protease inhibitors have found numerous applications in the fields of biotechnology, agriculture, and medicine [12]. Like their biological function, potential applications of protease inhibitors are intimately connected with their target proteases. They can be used to control deregulated proteolytic activity in various diseases, to control important microbial virulence factors, to inhibit digestive proteases of predators and parasites, or to inhibit viral proteases to prevent viral polyprotein processing in the fields of medicine and agriculture. They are invaluable tools in the fields of biotechnology and life sciences research.

6.1 Applications in Biotechnology

Fungal protease inhibitors are valuable research tools, and small molecule protease inhibitors are routinely used as buffer additives for preparation of protein extracts of cells or tissues to prevent proteolytic degradation during downstream analytical or purification procedures. Of the fungal small molecule inhibitors, broad-spectrum cysteine protease inhibitor E-64 is commonly included in protease inhibitor cocktails [12].

Protease inhibitors that act as intramolecular chaperones can be coexpressed as fusion partners to aid with proper protease folding in addition to protection of the recombinant protein during expression and purification processes. An example is the POIA1 serine protease inhibitor from *P. ostreatus* (family I9) that enables proper refolding of the fused subtilisin protease from inclusion bodies in a bacterial expression system [66].

Reversible protease inhibitors can be used as ligands in affinity chromatography for isolating various proteases. Based on the target protease to be purified, broad-spectrum or very specific protease inhibitors can be selected for immobilization to the solid support; however, the strength of inhibition must be considered as very weak (ineffective binding) or very strong (ineffective elution) binding that prevents effective protease purification. Advantages of using fungal protease inhibitors include (i) unique inhibitory profiles that differ from those of protease inhibitors from microbial, plant, or animal sources, (ii) resistance to proteolytic degradation, and (iii) stability on exposure to extreme thermal and pH conditions that may be used for effective immobilization to the solid support and for allowing several cycles of elution steps, which usually include extreme changes in pH and/or ionic strength, without losing their inhibitory activity. Fungal serine protease inhibitor cnispin

(family I66) and cysteine protease inhibitor macrocypin 1 (family I85) have been used as ligands in affinity chromatography for isolation of proteases from various sources [153].

6.2 Applications in Crop Protection

Herbivorous crop pests such as insects, slugs, and mites depend on effective food protein digestion for normal growth and development. Different proteolytic classes predominate in digestion for different groups of organisms; e.g., in beetles and mites, cysteine digestive proteases predominate, while in flies, serine digestive proteases are dominant. Furthermore, for many phytopathogenic bacteria, fungal and virus proteases are essential virulence factors, enabling evasion of host defenses and invasion of tissues. Endogenous protease inhibitors in plants thus constitute an important plant defense strategy against herbivorous, parasitic, and pathogenic organisms [154–156]. Since there is a strong selection pressure to evade plant defenses, pests develop resistance to endogenous plant protease inhibitors [157, 158]. The search for novel protease inhibitors with potential protective function is thus integral to continuous development of environmentally friendly pest management.

Protease inhibitors from fungi display unique characteristics that distinguish them from their plant counterparts and offer several advantages for their potential use as biopesticides. They are exceptionally stable proteins, resisting exposure to extreme temperature and pH conditions and to proteolytic degradation. Further, they could confer a more durable resistance than plant counterparts, since plant pests have not coevolved with these substances, so they are less likely to possess mechanisms for adaptation to fungal proteins. Moreover, protease inhibitors originating from fungi that are generally recognized as safe or from edible mushrooms are more readily acceptable for use in crops intended for human consumption. Finally, their protein nature makes them incapable of accumulating in soil or organisms and therefore convenient for biopesticide usage.

Fungal cysteine protease inhibitors mycocypins (families I48 and I85) display entomotoxic effects against the major potato pest Colorado potato beetle. Clitocypin and macrocypins exhibit antinutritional effects that affect growth and development of Colorado potato beetle larvae when applied to the diet as recombinant proteins heterologously expressed in *Escherichia coli* or as transgenes expressed in potato. The underlying mode of action is inhibition of a specific set of digestive cysteine proteases, intestains. Moreover, no changes in transcription levels of known adaptation-related digestive enzymes have been observed in larval guts, as is regularly observed for other dietary cysteine protease inhibitors from various sources [159, 160].

In addition to dealing with herbivorous pests, protease inhibitors offer concurrent protection against viral, bacterial, and fungal pathogens as well as parasitic nematodes. In the latter, digestive proteases are targeted, and for other pathogens, proteases that constitute important virulence factors are targeted. They are secreted by

fungal and bacterial pathogens to aid in host tissue invasion or manipulation of defense signaling, and, for viral pathogens, proteases are essential for polyprotein processing and replication [12, 155].

In addition to biotic stress, protease inhibitors can provide protection against abiotic stress. Proteases are implicated in plant responses to abiotic stresses involving a dehydration component including drought, freezing, and increased salt concentration to help reduce protein aggregation, degradation, and extensive changes in metabolism [12, 161, 162]. Fungal protease inhibitors have not yet been considered for this purpose.

6.3 Applications in Human and Veterinary Medicine

Proteases play essential metabolic and regulatory roles in many biological processes, and their deregulation often leads to disease. Protease inhibitors are extensively studied as promising therapeutic drugs for many different types of diseases targeting a variety of deregulated proteases, including viral polyprotein processing proteases; secreted bacterial, fungal, and parasite proteases that enable a pathogen's colonization of host tissues and evasion of host defenses; and those involved in cancer, autoimmune, neurodegenerative, inflammatory, and cardiovascular diseases. However, since disease-associated proteases are similar to those involved in normal physiological processes, unselective protease inhibitors could cause severe side effects in treated patients. Therefore, high selectivity and specificity are paramount for a clinically suitable therapeutic protease inhibitor [12, 163–167].

Elastase inhibitors isolated from *Aspergillus* species (family I78, Sect. 4.1.5) have been evaluated as antifungal agents for the prevention and treatment of aspergillosis by inhibiting *Aspergillus* elastases that are the major pathogenic factors involved in degradation of lung tissue. The inhibitor AFLEI has been shown to inhibit pathological changes in rat lung tissue caused by *Aspergillus* elastase, including bleeding, inflammatory cell infiltration, and effusion of fibrin-like substance [83].

Epoxysuccinyl peptide E-64 and its synthetic derivatives have been studied as protective agents in cancer, autoimmune, neurodegenerative, cardiovascular diseases, osteoporosis, muscular dystrophy, diabetes, and others. Derivatives designed to selectively target specific cysteine proteases have been shown to reduce tumor growth, invasion, and angiogenesis of many cancer types [12].

Proteasome inhibitors have been evaluated as anticancer agents due to their preferential antiproliferative and proapoptotic activity on cancer cells [168–170]. Structurally different proteasome inhibitors have been isolated from fungi (Sect. 3).

Several peptide and protein inhibitors of the metalloprotease angiotensin I-converting enzyme have been identified in fungi (Sect. 4.3), and several showed antihypertensive effect in spontaneously hypertensive rats, indicating their potential value as hypertension therapeutics [115–117, 119].

7 Conclusions

Fungi constitute a largely unexplored source of both small molecule and protein protease inhibitors. Those identified so far have unique structures and new types of inhibitory mechanisms, broadening our understanding of enzyme-inhibitor interactions. Their incredible natural diversity, together with considerable possibilities for optimizing additional specificity through derivatization or mutagenesis, provides an impressive starting point for the development of protease inhibitors tailored to many different applications in medicine, agriculture, and biotechnology.

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Ergot Alkaloids: Chemistry, Biosynthesis, Bioactivity, and Methods of Analysis 28

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Abstract

Ergot alkaloids are indole derivatives produced by a wide range of fungi, being considered medically important because of their significant effect on the central nervous system of mammals, due to their structural similarity to neurotransmitters. They are also considered mycotoxins due to the severe toxic effects of ergot-contaminated grains on human and animal health. This chapter summarizes different aspects of ergot alkaloids concerning their chemistry, biosynthesis, and bioactivity, discussing the pharmacological activity as well as some important aspects related to their toxicity, occurrence, and regulations. Finally, an overview of analytical methods for the determination of ergot alkaloids is included, whereby high-performance liquid chromatography coupled to fluorescence or mass spectrometer detection are the most widely used methods, although other techniques such as capillary electrophoresis or immunoassays have also been reported.

Keywords

Ergot alkaloids • Ergot alkaloid chemistry • Fungi • Biosynthesis • Pharmaceutical properties • Toxicology • Mycotoxins • LC–MS analysis

List of Abbreviations

5-HT	5-Hydroxytryptamine
AA	Amino acid
AdoMet	Adenosylmethionine
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
ARfD	Acute reference dose
BGE	Background electrolyte
CE	Capillary electrophoresis
CMC	Critical micellar concentration
CONTAM Panel	Panel on Contaminants in the Food Chain
CPE	Cloud point extraction
CZE	Capillary zone electrophoresis
DMA	Dimethylallyl
DMAPP	Dimethylallyl diphosphate
DMAT	Dimethylallyltryptophan
DMATS	Dimethylallyltryptophan synthase
d-SPE	Dispersive solid phase extraction

EA	Ergot alkaloid
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
EU	European Union
FAD	Flavin adenine dinucleotide
FLD	Fluorescence detection
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
IT	Ion trap
LC	Liquid chromatography
LD_{50}	Lethal dose 50 %
LLE	Liquid–liquid extraction
LSA	Lysergic acid amide
LSD	Lysergic acid diethylamide
MIP	Molecularly imprinted polymer
MS/MS	Tandem mass spectrometry
MS	Mass spectrometry
MT	Methyltransferase
NIR	Near infrared
pCEC	Pressurized capillary electrochromatography
PSA	Primary secondary amine
Q-TOF	Quadrupole time of flight
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
RIA	Radioimmunoassay
SCX	Strong cation exchange
SPE	solid phase extraction
TDI	Tolerable daily intake
TLC	Thin layer chromatography
TOF	Time of flight

1 Introduction

Ergot alkaloids (EAs) are nitrogen-containing natural products belonging to indole alkaloids. They are secondary metabolites produced by a wide range of fungi of the families *Clavicipitaceae* (e.g., *Claviceps*) and *Trichocomaceae* (including *Aspergillus* and *Penicillium*), which parasitize the seed heads of living plants at the time of flowering. Fungal infections are most common in rye and triticale that have open florets, but wheat and other small grains are also potential hosts together with grasses infected with endophytes. The fungal *hyphae* invade the ovule of the host grass and colonize the whole ovary. Around 3–4 weeks after infection, the wintering body of the fungus becomes visible and replaces the developing grain or seed. These

alkaloid-containing wintering bodies, named sclerotia or ergot (derived from old French word “argot,” meaning cock’s spur since grains colonized with *Claviceps* often resemble the spurs on the legs of a rooster), are dark, crescent shaped, and protruding from the regular grains and represent the final stage of the disease [1]. The term ergot refers also to the common name for this disease of cereals and grains caused by these fungi. The sclerotia are harvested together with the cereals or grass and can contaminate cereal-based food and feed products with EAs, being especially important in seasons with heavy rainfall and wet soils [2]. EAs have also been identified in plants of the families Convolvulaceae, Poaceae, and Polygalaceae, in which recently investigations suggest that these compounds are produced by plant-associated fungi [3].

This family of indole derivatives with diverse structures is chemically very complex, showing different biological and pharmacological activities. They can be classified as micotoxins, which have been responsible for historic episodes of mass poisoning in the Middle Ages due to the consumption of grains, flour, or bread contaminated with EAs. Historic events associated with ergot poisoning include the first Crusade (1095), the cause of symptoms associated with witchcraft surrounding the Salem witch trials (1690s), and the interrupted Russian campaign (1720-22) under Peter the Great against the Ottoman Empire [4]. Ergot poisoning in humans and animals is known as ergotism. This disease, one of the oldest known, may cause strange hallucinations, the feeling of itchy and burning skin, gangrene, loss of hands and feet, and even death.

In modern times, the cause of the disease is well understood and improvements in agricultural practices and milling techniques (grading, sieving, and sorting) have removed the risk of severe epidemic outbreaks of ergotism. Mechanical means and other conventional techniques of industrial grain processing like dockage removing, separators, air screens, density separators, color sorting, and their combinations can significantly reduce EA levels in grain. Cleaning procedures become less reliable when the intact ergot sclerotia break into smaller fragments during transport or when dry climatic conditions produce fungal sclerotia which are similar in size to the grain [2]. Also, food processing, such as baking/pancake preparation with EA-contaminated flours, can produce a reduction of EA levels in the final product, as well as the effect of cooking and drying of some products like noodles or spaghetti. A small part of the EA loss is due to leaking into the cooking water, implying that EAs are to some extent heat sensitive, but depending on the EA content of the raw material, significant amounts of EAs may still remain in the final product [5].

In relation to animals, consumption of feedstuffs contaminated with EAs has a broad impact on many different physiological mechanisms that alters the homeostasis of livestock. These alterations on homeostasis cause an increased sensitivity in livestock to environment perturbations, which involve a reduced production and economic losses in livestock producers around the world [6]. Sclerotia can be removed from cereal grains by standard seed-cleaning techniques. Since the EAs are heat sensitive, they may be reduced

during compound feed manufacture, where pellets generally leave the die at temperatures ranging from 60 °C to 95 °C [5]. Other strategies to reduce the risk of ergot infection in most cereal crops include changes in crop rotation, deeper plowing, application of fungicides, breeding for disease resistance, and crossing of natural rye with hybrid rye [1].

Following a request from the European Commission, in 2012, the Panel on Contaminants in the Food Chain (CONTAM Panel) was asked to deliver a scientific opinion on the risks to human and animal health related to the presence of EAs in food and feed [5]. Since the publication in 2005 of the *European Food Safety Authority* (EFSA) opinion on EAs in feed [7], no relevant information was identified that would alter the previous risk assessment. Estimates of exposure based on example diets and levels of EAs in cereal grains reported in Europe would suggest that under normal conditions the risk of toxicosis in livestock is low. Furthermore, the risk of ergotism in livestock as a result of consuming contaminated cereal grains, or compound feeds manufactured from them, is reduced where appropriate seed cleaning is carried out. In relation to humans, the CONTAM Panel performed estimates of both chronic and acute exposure for various age groups across European countries, concluding that while the available data do not indicate a concern for any population subgroup, the dietary exposure estimates are related to a limited number of food groups and a possible unknown contribution from other foods cannot be discounted. As recommendation, they suggest that efforts should continue to collect analytical data on occurrence of EAs in relevant food and feed commodities.

On the other hand, EAs present important applications in medicine, being included with both natural and semisynthetic origins, in different formulations. Their therapeutic potential was already recognized in the Middle Ages, using *Claviceps* sclerotia by midwives in support of childbirth or to induce abortion, according to medieval texts [1]. Their broad physiological effects are mainly based on their interactions with neurotransmitter receptors on the cells. Together with their traditional uses (prolactin inhibition, Parkinsonism, cerebrovascular insufficiency, venous insufficiency, thrombosis, migraine, uterine stimulation), new therapeutic applications have emerged (e.g., against schizophrenia, among others). EAs are also of social relevance because the semisynthetic alkaloid, lysergic acid diethylamide (LSD), is an illicit drug considered one of the most potent hallucinogen. Thus, over the years, EAs and derivatives have been synthesized by artificial parasitic cultivation on rye and saprophytic growth techniques [8, 9]. Today this uneconomic method has been replaced by submerged fermentation. Even after a century of research on EAs, the search still continues for new, more potent, and more selective EA derivatives.

Considering all of the abovementioned aspects, the developments in instrumental techniques in the last decades have led to separate and measure individual ergot compounds and their isomers, being of special interest in the monitoring and regulation of the contamination of cereal-based foods. There is a requirement therefore to measure EA in ergot sclerotia, infected cereals, forage grasses, processed foods, pharmaceutical preparations, illicit preparations, and body fluids and organs [10].

2 Chemistry

In 1920, Stoll isolated the first pure EA, ergotamine [11], and since then, more than 80 different EAs have been isolated, mainly from *Claviceps* spp. (over 70 EAs).

Natural EAs share a common tetracyclic ergoline ring system methylated on nitrogen N6 and substituted on C8 (Fig. 1). Most EAs have a double bond in position C8, C9 ($\Delta^{8,9}$-ergolenes) or in position C9, C10 ($\Delta^{9,10}$-ergolenes), with asymmetric centers at C5-C10 or C5-C8, respectively.

The configurations resulting from those centers of chirality are depicted in Fig. 2. The hydrogen at C5 has always β-configuration and only EA synthesized or prepared by isomerization of natural EAs can have α-configuration at C5. It reflects the derivation of these alkaloids from l-tryptophan (the amino acid precursor of the indole ring). The hydrogen at C10 (not existing in $\Delta^{9,10}$-ergolenes) can have α-configuration (*trans*-position relative to the hydrogen atom on C5) or β-configuration (*cis*-position relative to the hydrogen atom on C5). The stereochemistry C5-C10 has been represented by the use of roman number, I for *trans*-position and II for *cis*-position. However, this nomenclature has sometimes been misused: as example agroclavine-I that has C5-C10 *cis*-position [12]. $\Delta^{9,10}$-Ergolenes undergo epimerization, with respect to the center of symmetry at C8, resulting in rotating isomers: the left rotating (*8R* configuration) or β-$\Delta^{9,10}$-ergolenes and the right rotating (*8S* configuration) or α-$\Delta^{9,10}$-isoergolenes epimers [2, 13].

EAs are classified into four biogenetically related classes based on the substitutions at C8 and the structure of D-ring (Fig. 1) in the tetracyclic ergoline ring system [13–15]: clavine-type alkaloids, simple lysergic acid derivatives or ergoamides, ergopeptines, and ergopeptams.

2.1 Clavine-Type Alkaloids

Clavine-type alkaloids or clavines consist merely of the ergoline ring or its tricyclic precursors. They have been isolated from various fungal strains, especially in the family *Trichocomaceae*. Some of these metabolites are primary products in EA

Fig. 1 Tetracyclic ergoline ring system

d-lysergic acid (*5R*,*8R*)

d-isolysergic acid (*5R*,*8S*)

d-dihydrolysergic acid I (*5R*,*8R*,*10S*)

d-dihydrolysergic acid II (*5R*,*8R*,*10R*)

Fig. 2 Stereochemistry of lysergic acid and dihydrolysergic acid

biosynthetic pathway and they can be precursors of other EAs [12, 16]. According to their structures, clavine-type alkaloids can be classified into six different groups: 6,7-secoergolenes, 6,7-secoergolines, $\Delta^{8,9}$-ergolenes, $\Delta^{9,10}$-ergolenes, ergolines, and alkaloids with modified ergoline structure [13]. Representative structures of clavine-type alkaloids are given in Fig. 3.

6,7-Secoergolenes and 6,7-secoergolines are tricyclic seco derivatives and show a structure in which the D-ring of ergoline system is not closed. 6,7-Secoergolenes have a double bond in position C8, C9, while 6,7-secoergolines have a saturated D-ring. Some important naturally occurring representatives of 6,7-secoergolenes are chanoclavine-I and its two isomers, chanoclavine-II and isochanoclavine-I. Dihydrochanoclavine-I and isodihydrochanoclavine-I are representatives of 6,7-secoergolines.

Clavine metabolites with a closed D-ring include $\Delta^{8,9}$-ergolenes (e.g., agroclavine and elymoclavine) that contain a double bond in position C8-C9, $\Delta^{9,10}$-ergolenes (e.g., lysergol and penniclavine) with a double bond in position C9-C10, and ergolines (e.g., festuclavine and fumigaclavine A) possessing a saturated D-ring.

Finally, alkaloids with modified ergoline structure have been also found in nature, but very few of them are produced by *Claviceps* spp. Paspaclavine, cycloclavine, and rugulovasine A are some of EAs included in this group.

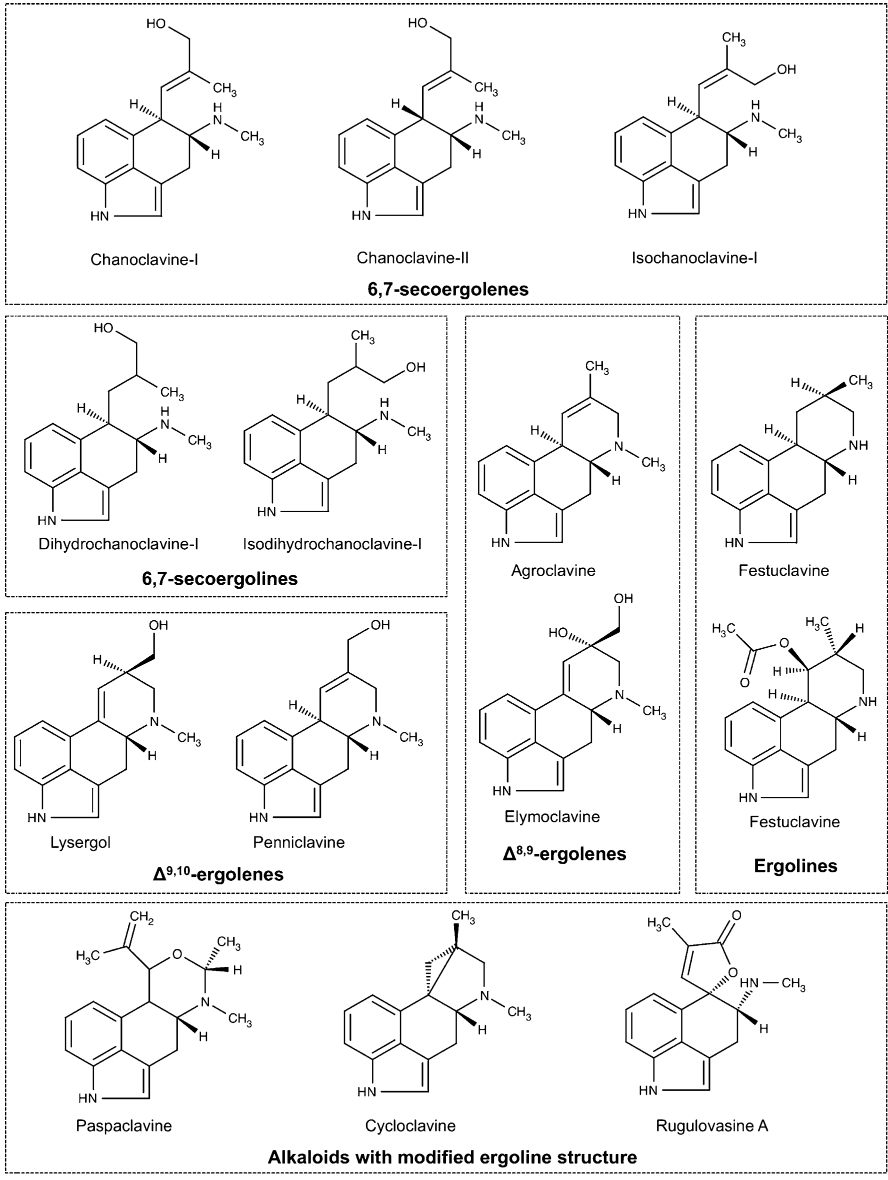

Fig. 3 Representative structures of clavine-type alkaloids

2.2 Simple Lysergic Acid Derivatives or Ergoamides

Ergoamides are primary or secondary carbon acid amides of D-lysergic acid. In this group, paspalic acid and its derivatives (e.g., 10-hydroxy-*trans*-paspalamide and

	R1	R2	R3
Methylergometrine	$-CH_2CH_3$	-H	-H
Ergometrine	$-CH_3$	-H	-H
Methysergide	$-CH_2CH_3$	-H	$-CH_3$

Ergometrinine Ergine 10-hydroxy-*trans*-paspalamide

Fig. 4 Representative structures of ergoamides

10-hydroxy-*cis*-paspalamide) are also included, as D-lysergic acid is biosynthesized from the isomerization of paspalic acid.

The first simple lysergic acid derivative identified was ergometrine (also called ergonovine or ergobasine), and in its structure, D-lysergic acid is amidated with 2-aminopropanol. Ergometrine and its semisynthetic derivatives, methylergometrine (D-lysergic acid amidated with 2-aminobutanol) and methysergide (D-lysergic acid amidated with 2-aminobutanol and methylated on nitrogen N1), are the most important ergoamides.

Ergoamide derivatives of lysergic acid (*8R*-epimers) are indicated by the suffix -*ine* (e.g., ergometrine); those that are derivatives of isolysergic acid (*8S*-epimers) are indicated by the suffix -*inine* (e.g., ergometrinine). Representative structures of ergoamides are given in Fig. 4.

2.3 Ergopeptines

Ergopeptines or ergopeptides are D-lysergic acid peptides containing lysergic acid and three amino acids in their structure (Fig. 5). They are the most widely spread natural peptide-type EAs.

The cyclic part of the tripeptide results from the reaction of an α-hydroxyamino acid adjacent to lysergic acid with the carboxyl group of proline. Ergopeptines have l-proline at AA3 and the variability of ergopeptines is therefore given by the nature of AA1 and AA2. However, other configurations at AA3 have been described, such

D-lysergic acid derivatives D-isolysergic acid derivatives

	R1			
	$-CH_3$	$-CH_2CH_3$	$-CH(CH_3)_2$	$-CH(CH_3)CH_2CH_3$
R2	Ergotamine group	Ergoxine group	Ergotoxine group	Ergoannine group
$-CH(CH_3)_2$	Ergovaline/ergovalinine	Ergonine/ergoninine	Ergocornine/ergocorninine	
$-CH_2CH(CH_3)_2$	α-Ergosine/ergosinine	α-Ergoptine/ergoptinine	α-Ergocryptine/Ergocryptinine	β-α-ergoannine
$-CH(CH_3)CH_2CH_3$	β-Ergosine/ergosinine	β-Ergoptine/ergoptinine	β-Ergocryptine/Ergocryptinine	
$-CH_2Ph$	Ergotamine/ergotaminine	Ergostine/ergostinine	Ergocristine/ergocristinine	
$-CH_2CH_3$	Ergobine	Ergobutine	ergobutinine	
$-CH_3$	Ergoalanine			

Fig. 5 Representative structures of ergopeptines

as ergobalansine and its corresponding epimer, ergobalansinine, where l-proline is substituted by l-alanine [17].

Ergopeptines can be classified into four subgroups based on the different amino acids AA1: ergotamines (AA1: alanine), ergoxines (AA1: α-aminobutyric acid), ergotoxines (AA1: valine), and ergoannines (AA1: isoleucine). Ergopeptines also undergo epimerization, and the isomers derived from D-lysergic acid and D-isolysergic acid are characterized by the suffix *-ine* and *-inine,* respectively. This EA class also includes derivatives with saturated D-ring (e.g., dihydroergotamine, dihydrocristine, or dihydroergosine) and they are subgrouped correspondingly.

2.4 Ergopeptams

The ergopeptams are tripeptidic non-cyclol EAs. Their structure is similar to that of ergopeptines except that l-proline at AA3 is exchanged by d-proline, and the tripeptide chain is a non-cyclol lactam (Fig. 6). It was suggested that ergopeptams are formed as a result of competitive epimerization at the last stage of the cyclopeptide biosynthesis [8].

The first ergopeptam isolated was ergocristam. This class of peptide-type alkaloids was originally found in small amounts in certain strains accompanying the ergopeptines. In addition, the probability of the existence of ergopeptams decreases with the decreasing volume of radical R1, which is explained by its high lability [8]. Later, ergopeptams have been found to predominate in some infected wild grasses from Norway [18].

D-lysergic acid derivatives

D-isolysergic acid derivatives

	R1			
	$-CH_3$	$-CH_2CH_3$	$-CH(CH_3)_2$	$-CH(CH_3)CH_2CH_3$
R2	**Ergotamam group**	**Ergoxam group**	**Ergotoxam group**	**Ergoannam group**
$-CH(CH_3)_2$	Ergovalam	Ergonam	Ergocornam	
$-CH_2CH(CH_3)_2$	α-Ergosam	α-Ergoptam	α-Ergocryptam	α−β−ergoanam
$-CH(CH_3)CH_2CH_3$	β−Ergosam	β−Ergoptam	β-Ergocryptam	
$-CH_2Ph$	Ergotamama	Ergostam	Ergocristam	

Fig. 6 Representative structures of ergopeptams

Similar to ergopeptines, ergopeptams can be classified into four groups based on the AA1 type: ergotamams (AA1: alanine), ergoxams (AA1: α-aminobutyric acid), ergotoxams (AA1: valine), and ergoannams (AA1: isoleucine). Figure 6 shows the representative structures of ergopeptams. No isomers derived from D-isolysergic acid have been reported for ergopeptams, which is related to its high lability because these compounds readily decompose into simpler derivatives in the presence of bases [8]. However, the established nomenclature suggests that these hypothetical *8S*-isomers are characterized by the suffix *-inam* and should be included into the corresponding groups.

2.5 Physicochemical Properties

The variability of EA compounds involves a wide range of physicochemical properties, although most EAs appear as colorless crystals that are readily soluble in various organic solvents, like acetonitrile, methanol, or organic/buffer mixtures [5, 19], and insoluble or only slightly soluble in water [9]. Moreover, EAs are neutral at higher pH values and positively charged at N6 in acidic solutions.

The most important characteristic of $\Delta^{9,10}$-ergolenes is their rapid epimerization with respect to the center of symmetry at C8, resulting in the right rotating (*8S*) and left rotating (*8R*) isomers. These EAs can epimerize from R to S forms and vice versa, especially in aqueous acidic or alkaline solutions [8], via enolization at C8 (Fig. 7), as C9-C10 double bond permits to form a large conjugated π-electron system [20, 21].

The ratio of epimerization depends mainly on the nature of the amide substituent [22]; however, this epimerization is enhanced through exposure to strong light,

8R-epimer

8S-epimer

Fig. 7 Epimerization scheme of ergot alkaloids (Adapted from Ref. [21])

prolonged storage, or contact with some solvents at high or low pH. The C8 epimers show different physicochemical properties, such as basicity and solubility [22]. Moreover, protonated pKa values, given by the N6 nitrogen, showed differences between both C8-epimers. They ranged between 5.5 (ergocristine) and 6.0 (ergometrine) for *8R*-epimer, while pKa values of *8S*-epimers ranged between 4.8 (ergocorninine) and 6.2 (ergometrinine) [11, 23].

$\Delta^{9,10}$-Ergolenes show natural fluorescence with excitation wavelengths of 254, 313, 325, or 366 nm and emission wavelength of 445 nm [24]. On the contrary, the rest of EAs without $\Delta^{9,10}$ double bond do not show native fluorescence, but they show ultraviolet absorption at 280 nm [13].

2.6 Stability

Most EAs melt with decomposition at high temperatures. Moreover, EAs are known to show high sensitivity to light, which leads not only to epimerization but also degradation [8, 22]. $\Delta^{9,10}$-Ergolenes add one molecule of water to C10 carbon of the lysergic acid moiety upon illumination, especially on irradiation with UV light, and acid catalysis. The reaction leads to a mixture of two diastereomers called lumi-derivatives: lumi-I-derivatives (e.g., lumi-ergotamine-I) or 10-α-hydroxy derivatives, with hydroxyl group being *trans* to the hydrogen atom in position 5 and lumi-II-derivatives (e.g., lumi-ergotamine-I) that have a *cis*-junction of rings C and D [22]. These lumi-derivatives are characterized by loss of fluorescence properties and lack of biological activity [25].

In addition, other degradation products called aci-derivatives are formed by acid-catalyzed isomerization of the 2′-carbon of the tricyclic peptide moiety of the ergopeptines [26, 27]. However, the aci-derivatives are formed at much slower rates than those for C8-epimers [27].

Stability of EAs is also affected by the epimerization process during storage, handling, and analysis. In order to minimize changes in the natural ratio of epimers and to improve the understanding of the factors affecting the epimerization, some studies have tried to explain the process using complex simulations [28]. However,

the epimerization process is not yet well understood [10]. Some authors proposed different recommendations to avoid significant epimerization. EA standards are best stored below −20 °C in non-protic solvents or in the form of thin dry films, but they also have shown to be stable, in terms of epimerization and degradation, in chloroform at room temperature [21], over a period in excess of 12 months [29]. Solutions of EA standards in ethanol containing tartaric acid, ethylene glycol, 2-propanediol, and tartaric acid are also recommended [30].

The lability of EAs to decomposition must be taken into account during the technological treatments and the chemical and analytical handling of EAs in the development of stable medicinal forms, and in the storage of raw materials, semiproducts, parent substances, and ready-to-use medicinal preparations [8].

3 Biosynthesis

As it was shown in the previous sections, EAs are a large group of metabolites, showing very different structures. However, all of them share the first biosynthetic steps, consisting of tetracyclic ergoline ring system formation, except the simplest tricyclic compounds as 6,7-secoergolenes and 6,7-secoergolines. Biosynthesis of EAs has been investigated in detail for many years [1, 3, 31, 32], and although most of the biosynthetic pathways have been elucidated from the 1950s, some step remains largely unelucidated [33].

The variability of EAs and biosynthetic pathway depends largely on the EA producer fungi. As example, *Clavicipitaceae* typically produces either lysergic acid-derived EAs or dihydroergot alkaloids and several members of the *Trichocomaceae* produce alkaloids derived from festuclavine.

3.1 Ergot Alkaloid Producers

EAs are produced mainly by two orders of fungi, *Eurotiales* and *Hypocreales*, belonging to the phylum *Ascomycota*. Within the *Hypocreales*, EAs are associated exclusively with *Clavicipitaceae* family, although not all the members of the *Clavicipitaceae* produce EAs [31]. The fungal genera so far known to produce EAs in *Clavicipitaceae* family are *Claviceps* spp. [34, 35], *Epichloë* spp. (including their close relatives, the *Neotyphodium* spp.), *Atkinsonella* spp., *Balansia* spp., *Periglandula* spp. [36, 37], and *Metarhizium* spp. [36]. Within the *Eurotiales* order, the genera *Aspergillus* [38] and *Penicillium* [39] in *Trichocomaceae* family are also EA producers. Recently, it was demonstrated that *Onygenales* order belonging also to phylum *Ascomycota*, in particular *Arthroderma* genera (*Trichophyton*), is also related with EA production [40]. Moreover, EAs producing fungi occupy different ecological niches. *Claviceps* spp. are plant parasites and biotrophic symbionts, while *Aspergillus fumigatus* is an opportunistic pathogen of mammals [31, 41].

It was reported that three families of plants, i.e., Convolvulaceae [42, 43], Poaceae [44], and Polygalaceae [45], also produce EAs. For a long time, it was

believed that horizontal gene transfer from fungi to higher plants had taken place during the evolutionary process. However, further investigations revealed that, at least in Poaceae and Convolvulaceae, the plant-associated fungi are likely responsible for EA production, since the treatment of these plants with fungicides led to elimination of associated fungus and simultaneous loss of alkaloids from the plant [3, 41, 46]. Fungi and plants form mutualistic symbiosis that consists of production of bioactive EAs by fungi to protect the host plant from insect, vertebrate herbivores and root nematodes, enhancements of drought tolerance and nutrient status, and improved growth, particularly of the root, while the fungi benefit from protected niche, nutrition, and dissemination from the plant [3, 41, 47]. The fungal symbionts are vertically transmitted through seed of the host plant, though the mechanism of how the fungi spread in the respective host plant remains unclear [41, 44, 48].

Claviceps purpurea is the most important of all the EA producers and it is otherwise known as the ergot fungus of rye and related grasses. In *C. purpurea*, EAs are found in the sclerotia, and their spectra vary strongly between different *C. purpurea* strains [49], although the main produced compounds are ergocristine, ergotamine, ergocornine, α- and β-ergocryptine, ergometrine, ergosine, ergocristinine, ergotaminine, ergocorninine, α- and β-ergocryptinine, ergometrinine, and ergosinine [5, 50–53]. Moreover, *C. purpurea* is morphologically a highly variable species with respect to sclerotial length and shape, color of the stomata, and conidial size and shape [54, 55].

Other important members of *Claviceps* spp. are *C. africana*, in which dihydroergosine is the principal EA found in its sclerotia [56]; *C. fusiformis*, related to agroclavine, elymoclavine, chanoclavine, penniclavine, and setoclavine production [57], but no D-lysergic acid derivatives [35, 58]; and *C. gigantea* [59], *C. paspali*, and *C. hirtella*, which are also mainly clavine producers [13].

On the other hand, *A. fumigatus* and *Penicillium* strains, including *P. roqueforti*, *P. verrucosum*, and *P. commune*, are fungi also associated to the production to clavines. *Aspergillus* has been related to the production of fumigaclavines A, B, and C, while *Penicillium* has been related to the production of fumigaclavines A and B, but not C [3].

3.2 Ergoline Ring Formation

The biosynthesis of the ergoline ring begins with the prenylation of l-tryptophan at position C4 with dimethylallyl diphosphate (DMAPP) as prenyl donor. This reaction is catalyzed by the prenyltransferase 4-dimethylallyltryptophan synthase (DMATS), also called FgaPT2 in *A. fumigatus* [33, 60–62], leading to the formation of 4-l-dimethylallyltryptophan (DMAT). The DMAT-forming reaction delivers the carbon skeleton of the ergoline ring system. After this first step, all enzymatic steps concern modifications and rearrangements leading to formation of rings C and D. The next step involves a *N*-methylation of the amino nitrogen of DMAT in the presence of S-adenosylmethionine (AdoMet) and is catalyzed by a 4-dimethylallyltryptophan

N-methyltransferase EasF (FgaMT in *A. fumigatus*). The result is the formation of 4-dimethylallyl-l-abrine (4-DMA-l-abrine).

Chanoclavine-I was the next detected intermediate found in the biosynthesis of EAs [41, 63, 64]. Its accumulation is observed in many EA producers, and in some cases at relatively high concentrations [65]. Chanoclavine-I was obtained by decarboxylation and closure of ring C. This step would include at least three reactions, i.e., decarboxylation, cyclization, and hydroxylation [3], and the enzymes flavin adenine dinucleotide (FAD)-dependent oxidoreductase EasE (also called ccsA and FgaOx1 in *A. fumigatus*) and the catalase EasC (known as FgaCat in *A. fumigatus*) are needed [66, 67].

The next step is the oxidation of the hydroxyl group of chanoclavine-I to yield chanoclavine-I aldehyde. This reaction is catalyzed by the short-chain dehydrogenase/reductase (SDR) EasD (FgaDH in *A. fumigatus*). Chanoclavine-I aldehyde is the last shared intermediate and represents the branch point of the biosynthetic pathway of several fungi to produce agroclavine and festuclavine (and its *8S*-stereoisomer, pyroclavine) [41, 65]. Agroclavine is usually the key intermediate in the formation of more complex EAs in *Clavicipitaceae* fungi, while festuclavine and pyroclavine are the substrates in *Trichocomaceae* fungi. The branch point is mainly controlled by the old yellow enzyme EasA (also called FgaOx3 in *A. fumigatus* and $FgaOx3_{pc}$ in *P. commune*) [68–70], and the divergence between fungi depends on the activities of different versions of this enzyme. The version of EasA (FgaOx3) found in *A. fumigatus* reduces the C8-C9 double bond in chanoclavine-I aldehyde to give the cyclized iminium intermediate in ring D formation [41, 71]. On the other hand, the versions of EasA found in most EA producers in the *Clavicipitaceae* do not permanently reduce the double bond; instead, these enzymes promote isomerization around the double bond. Finally, for the formation of agroclavine in the *Clavicipitaceae* or festuclavine (and its *8S*-stereoisomer pyroclavine) in the *Trichocomaceae*, enzyme EasG (also called FgaFS in *A. fumigatus* and $FgaFS_{pc}$ in *P. commune*) is required to reduce the iminium ion [65, 72]. Moreover, versions of EasG found in *A. fumigatus* and *P. commune* differ in the proportion of the stereoisomers (festuclavine and pyroclavine). The formation of festuclavine was significantly higher in *A. fumigatus*, while *P. commune* produced higher concentration of pyroclavine [70].

Further investigation demonstrated that EasA was not necessary for the conversion of chanoclavine-I aldehyde to agroclavine in *C. purpurea*, at least in vitro experiments, and EasG was sufficient for the formation of agroclavine via a nonenzymatic adduct with reduced glutathione [73].

The mentioned steps of ergoline ring formation are shown in Fig. 8.

3.3 Fumigaclavine Formation

Festuclavine and its *8S*-stereoisomer pyroclavine are the main substrates in the formation of fumigaclavines in *Trichocomaceae* fungi (Fig. 8). Both metabolites lead the formation of isomers *8R* and *8S* fumigaclavine B via a hydroxylation in

Fig. 8 Ergoline biosynthetic pathway (Adapted from Ref. [33, 65])

A. fumigatus and *P. commune*, respectively. This reaction is probably catalyzed by the monooxygenase FgaP450-2 in *A. fumigatus* and its analogue FgaP450-2_{PC} in *P. commune* [3]. The next step is the formation of fumigaclavine A catalyzed by the acetyltransferase FgaAT (FgaAT$_{PC}$) in the presence of acetyl-CoA [74]. Finally, fumigaclavine C is obtained by catalysis of the prenyltransferase FgaPT1 only in *A. fumigatus* [33]. Figure 9 shows the fumigaclavine biosynthetic pathway.

3.4 D-Lysergic Acid Formation

Although the formation of D-lysergic acid remains largely unelucidated, two important intermediates have been identified: elymoclavine and paspalic acid. It was

Fig. 9 Fumiclavine biosynthetic pathway (Adapted from Ref. [33])

Fig. 10 D-Lysergic acid biosynthetic pathway

proposed that the conversion of agroclavine to paspalic acid via elymoclavine involves two oxidation steps (2-electron and 4-electron oxidation) [31]. The C8-linked methyl group can be oxidized by the action of a cytochrome P450 monooxygenase CloA [75]. The different isoforms of CloA determine the level of oxidation, i.e., CloA of *C. fusiformis* catalyzes the 2-electron oxidation of agroclavine to elymoclavine, whereas CloA of *C. purpurea* and many other *Clavicipitaceae* catalyze a 6-electron oxidation of agroclavine to paspalic acid [35, 65]. Subsequently, paspalic acid is isomerized enzymatic or spontaneously to D-lysergic acid [76], which serves as the acyl component of ergoamides, ergopeptines, and ergopeptams. The biosynthetic pathway of D-lysergic acid is show in Fig. 10.

3.5 Formation of Ergoamides, Ergopeptines, and Ergopeptams

Most EA-producing *Clavicipitaceae* fungi produce more complex metabolites such ergoamides, ergopeptines, or ergopeptams. The diversity among them arises via an interesting combinatorial system involving two different pairs of peptide synthetases [65].

Ergopeptams are intermediate metabolites in the biosynthetic pathway of ergopeptines. Their formation is controlled by a nonribosomal peptide synthetase (NRPS) enzyme complex, which contains d-lysergyl peptide synthetases 1 and

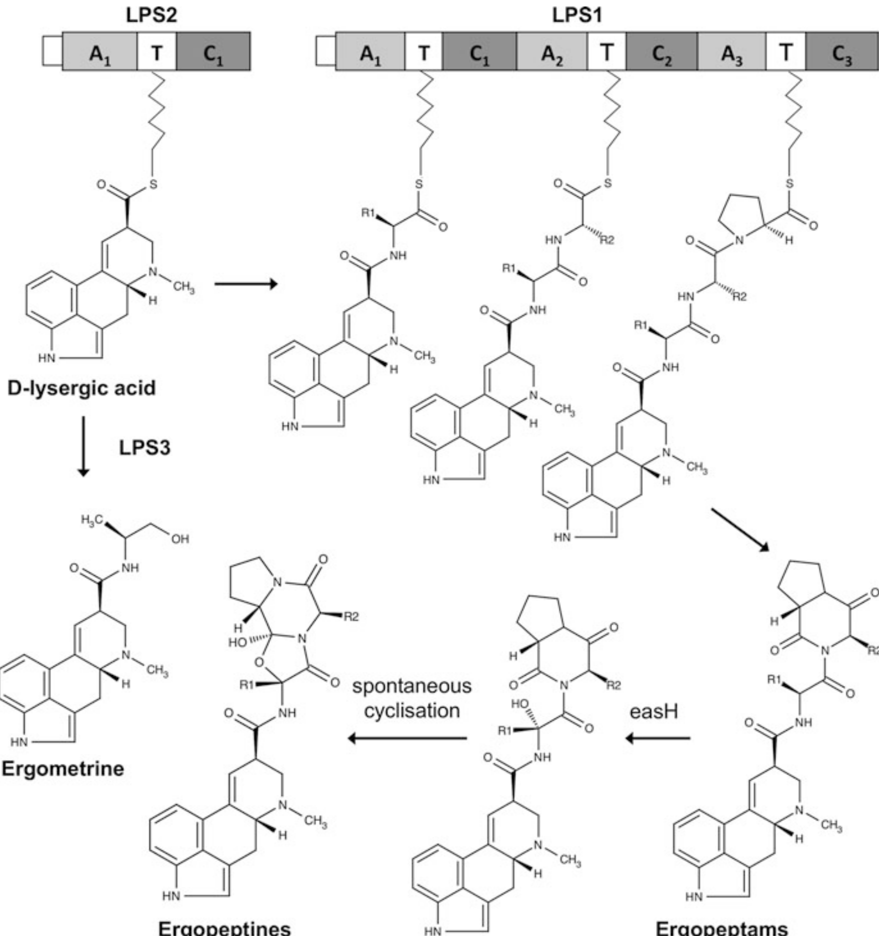

Fig. 11 Ergoamide, ergopeptine, or ergopeptam biosynthetic pathway (Adapted from Ref. [3, 33])

2 (LPS1 and LPS2) [33, 41]. Firstly, D-lysergic acid is activated by LPS2 and subsequently LPS1 catalyzes its progressive elongation to the d-lysergyl mono-, di-, and tripeptide thioester intermediates, to get finally the d-lysergyl tripeptide lactam or ergopeptam [65, 77]. The next step is catalyzed by mono-oxygenase easH yielding an intermediate, which undergoes spontaneous cyclization that leads to the formation of ergopeptines [3].

Ergoamides, such as ergometrine, are also formed from D-lysergic acid. Ergometrine arises by interaction of LPS2 and LPS3 (a monomodular peptide synthetase that recognizes and activates l-Alanine) enzymes [78]. Figure 11 shows the biosynthetic pathway of ergoamides, ergopeptams, and ergopeptines.

Dihydroergot alkaloids, such as dihydroergosine, can also arise from festuclavine in *C. africana* and *C. gigantea* (Fig. 12). The CloA present in these fungi catalyzes

Fig. 12 Dihydroergot alkaloid biosynthetic pathway in *C. africana* and *C. gigantean* (Adapted from Ref. [65])

the oxidation of festuclavine to dihydrolysergol in *C. gigantea* or to dihydrolysergic acid via dihydrolysergol in *C. africana*. Dihydrolysergic acid can follow the same biosynthetic pathway as that of other members of the *Clavicipitaceae* to produce dihydroergosine via LPS1, LPS2, and easH [4, 65, 79, 80].

4 Bioactivity

EAs are particularly important for possessing a potent bioactivity. However, it is necessary to distinguish their valuable pharmacological properties and their toxic effects.

4.1 Pharmacological Activity

EAs have been reported to produce several effects including direct peripheral effects as uterotonic action or vasoconstriction, indirect peripheral effects as serotonin antagonism or adrenergic blockade, and central nervous effect as induction of hypothermia and emesis or control of the secretion of pituitary hormones [1, 81].

The effects of EAs are mainly responses mediated by neurotransmitters as noradrenaline, serotonin, or dopamine (5-hydroxytryptamine, 5-HT). The structure of these neurotransmitters fits well onto the D-lysergic acid ring structure (Fig. 13) [1]. Natural EAs possess the above-described effects to a greater or lesser degree, depending of the substituents attached to the carboxyl group at C8 of D-lysergic acid ring system that define agonistic or antagonistic mode (or a dual role as partial-agonist and antagonist) and the intensity of the interaction with receptors for these neurotransmitters [1, 61, 82, 83]. Peptide ergot alkaloids usually have high affinity for α-adrenergic receptors, while derivatives of D-lysergic acid amidated with small amino alcohols show high affinity for serotonin receptors [61]. Moreover, the biological activity of EAs depends largely on their configuration and epimers *8R*

Fig. 13 Structural analogy between the tetracyclic ergoline system and dopamine, noradrenaline, and serotonin neurotransmitters (Adapted from Ref. [61])

and *8S* differ in biological properties; *8R*-isomers are biologically active, whereas the *8S*-isomers are inactive [2].

The uterine contraction is the most known pharmacological effect. EAs as ergotamine and ergometrine were officially used for the first time in obstetrics to treat postpartum hemorrhage and to accelerate uterine involution in the puerperium [8, 81]. Later, it was demonstrated that although all natural EAs have qualitatively the same effects on uterus, ergometrine is most active and less toxic than ergotamine. So, ergometrine and its semisynthetic derivative methylergometrine replaced other EAs in obstetric applications [9]. Other important direct peripheral effect of EAs is their vasoconstrictor effect. The best-known drug of this type is ergotamine that has been widely used as migraine treatment due to its tonifying effect on the smooth muscle of the blood vessels [81]. However, methysergide, a semisynthetic EA and a serotonin antagonist, unlike ergotamine, has also been used in the treatment of migraine [84].

The indirect peripheral (humoral) effects are manifested in an adrenaline and noradrenaline antagonism, as well as serotonin antagonism. EAs are used in internal medicine as sympathetic agents due to their adrenolytic effect. EAs also present very diverse effects on the central nervous system, such as the reduction of the activity of the vasomotor center and the stimulation of sympathetic structures of the diencephalon, particularly the hypothalamus [81, 85].

The most important effects of natural ergopeptines are mainly their vasoconstrictive and sympatholytic–adrenolytic actions due to their high affinity for adrenergic

receptors [1, 86]. However, slight modifications in the chemical structures produce changes in their biological activity. Dihydroergopeptines such as dihydroergotamine have an increased adrenolytic effect and reduced vasoconstrictive effect; thus, it is preferentially used for the treatment of migraine instead of ergotamine [1, 87–89]. The EAs are administered separately and in numerous complex compositions [8] as dihydroergotoxin, a mixture of three dihydroergopeptines (dihydroergocornine, dihydroergocristine, and dihydroergocryptine) that is used for the treatment of diseases associated with circulatory problems as high blood pressure and cerebral dysfunctions [1, 90, 91].

Clavine-type alkaloids have much less adrenolytic activity and show strong anti-serotoninergic action due to elevated affinity for serotonin (5-HT) receptors [61]. EAs, especially clavine-type alkaloids, possess activity inhibiting the growth of certain mammary tumors in animals and also in humans by blocking the release of prolactin from the anterior pituitary gland. In particular, clavine-type alkaloids with a C8 methyl group as festuclavine and agroclavine were shown to exhibit some inhibitory activity against cell proliferation in the L5178y mouse lymphoma system [92–94]. Recently, six EAs (agroclavine, ergosterol, ergocornine, ergotamine, dihydroergocristine, and 1-propylagroclavine tartrate) were investigated for their inhibitory activity toward a panel of cell lines of different tumor origins (ovarian carcinoma, brain tumor, prostate cancer, lung cancer, melanoma, colon cancer, renal carcinoma, breast cancer, or leukemia). 1-Propylagroclavine tartrate showed the strongest effect on tumor cells, especially against leukemia cell lines [95].

Serotonin agonist in the brain is also thought to be a key factor in hallucinogenic activities [31]. However, none of the naturally occurring EAs have typical hallucinogenic properties; such properties are confined to a number of semisynthetic derivatives of lysergic acid, as LSD [96], which has been used for psychedelic recreation [31].

Many EAs, including ergocryptine, produce a more or less pronounced dopaminergic effect. Bromination of ergocryptine in the 2-position (2-bromo-ergocryptine) strongly increases dopamine agonist activity. 2-Bromo-ergocryptine is semisynthetic derivative that is used for treatment of hyperprolactinaemia. Bromocriptine was also used for treatment of advanced breast cancer [97] and Parkinson's disease due to its high affinity to dopaminergic receptors [98].

Limited information is available on the metabolism of EAs. They are rapidly cleared from the blood and the tissues with a high first pass effect in the liver [99]. In contrast, their physiological effect persists for a longer period of time [2].

Despite the beneficial pharmacological properties, natural and semisynthetic EAs also possess serious and unpredictable side effects and high instability, reducing their medical applications and being replaced by synthetic analogs. As example, ergocristine, ergocryptine, and ergocornine, despite having a similar activity spectrum to ergotamine, present some toxic effects, which prevents them from achieving the same clinical significance [81].

4.2 Toxicity

Intoxications induced by EAs have been known for many centuries. The most severe and frequent epidemic of ergotism took place during the Middle Ages in Europe, where the disease was called Holy Fire or St. Anthony's Fire. It was caused by eating rye bread contaminated with *C. purpurea*, resulting in gangrene of limbs, disturbances in the function of the central nervous system, and ultimately death [100].

There are two symptomatic forms of ergotism: gangrenous and convulsive. The two distinct types of ergotism may be considered as acute and chronic varieties. The gangrenous form is caused by the extreme vasoconstrive properties of some EAs, which results in restriction of the blood flow to parts of the body (ischemia) [31, 101]. As a result, tingling effects are felt in fingers and toes followed in many cases by dry gangrene of the limbs and eventually loss of the limbs [102, 103]. In the convulsive form, tingling is followed by neurotoxic symptoms such as hallucinations, delirium, and epileptic-type seizures [104].

Outbreaks of ergotism tended to happen after cold, wet winters followed by warm spring weather, and arose mainly in areas where rye was commonly eaten. Also, some outbreaks were caused by other types of grain contaminated with ergot [101]. The gangrenous type was mostly seen in France and other European countries west of the Rhine and the convulsive one in Germany and Scandinavia [100, 101]. The symptoms of EAs poisoning vary, probably depending on the particular profiles of alkaloids present in the contaminated food. Clavines are thought to contribute substantially to convulsive ergotism, while the ergopeptines are known to produce similar symptoms and also to cause gangrenous ergotism [31, 101]. Moreover, it was proposed that a deficiency in vitamin A could be a causative factor inducing convulsive ergotism [105] and that EAs present at high concentrations in ergots could cause convulsive ergotism at a circulating concentration insufficient to produce peripheral ischemia [101].

In the late twentieth century, human poisoning from ergot was reported in France [106], India [107], and Ethiopia [108]; and the last recorded outbreak of gangrenous ergotism that occurred in the Arsi Zone (Ethiopia 2001) was attributed to the ingestion of barley containing ergotized wild oats [109]. While nowadays human poisoning from ergot has become of less concern, mainly owing to cleaning procedures at mills, EA contamination remains an important veterinary problem [2, 110]. There are numerous reports of poisoning of farm animals by ergot-contaminated feed [111] and by endophyte-infected grasses [112, 113]. Moreover, gangrenous ergotism has also been reported among free-living moose and roe deer in Norway [102, 114].

The toxic side effects of EAs have been studied in more detail, especially with respect to biological functions beyond the receptor interactions. In vivo studies regarding acute toxicity lead to different LD_{50} (lethal dose, 50 %) values depending on the used animal species, application form, and ergot alkaloid. Griffith et al. [115] reported a series of LD_{50} values (ranging between 0.9 and 275 mg/kg body weight) determined for several naturally occurring and semisynthetic EAs by subcutaneous and oral exposure in mouse, rat, and rabbit, demonstrating that rabbit is the most

susceptible (LD_{50} values between 0.9 and 3.2 mg/kg). Sublethal acute exposure to EAs induces signs of neurotoxicity in mammals, including restlessness, miosis or mydriasis, muscular weakness, tremor, and rigidity. Moreover, tail gangrene was observed in rats after intraperitoneal injection of ergotoxin (ergocristine + ergocryptine + ergocornine). Recently, a case study of spontaneous tail necrosis in a rabbit colony has been reported, concluding that in order to avoid symptoms such as tail lesions and necrosis in younger rabbits, the mean EA content in such feeding must be controlled and kept as low as possible. However, controlled feeding trials under various conditions are necessary, to fully confirm that dietary EAs at concentration levels of around 500 μg/kg may act as a causative agent of mycotoxicosis in rabbits [116].

For in vitro experiments, only limited data are available for the single substances and their toxic effects on human cells [117]. Most data consist of receptor interaction analysis for single substances in dopamine overexpressing cells or tumor cells [118, 119]. Further experiments indicated a different toxic potential for peptide ergot alkaloids and lysergic acid amide alkaloids revealing that the cytotoxicity of EAs in human cell lines obviously depends on the type of alkaloids [120]. Mulac et al. described the apoptotic effect of some ergopeptines (especially ergocristine) [117]. Despite their lack of bioactivity, the *8S*-epimers are considered to be mainly responsible for this effect, since they are preferentially accumulated in hepatic cell lines [121]. Therefore, it is important to consider both epimers when the EA contamination level has to be determined. Recently, it was demonstrated that ergometrine and its corresponding epimer ergometrinine exhibit cytotoxicity on animal smooth muscle cells, showing a positive correlation with alkaloid concentration [122].

EAs have a number of well-established effects on the reproductive process including prevention of pregnancy by interfering with implantation, embryotoxicity, developmental effects, and inhibition of lactation [115]. Several studies reported the effect of EAs reducing livestock reproductive performance with particular emphasis on the female gender [123, 124]. This is due to both direct and indirect effects of EA exposure through regional vasoconstriction and corresponding decreases in blood flow to reproductive tissues, decreases in dry matter intake, and/or increased body temperature [6]. Moreover, EAs inhibit milk production in humans, laboratory animals, and livestock animals [105, 125–127], effect linked by several authors to the decrease of prolactin (a protein hormone secreted mainly by the anterior pituitary gland) [5, 128]. However, later it was reported that decreased serum prolactin in lactating animals did not directly equate to decreased milk production [124, 129, 130]. 2-Bromo-ergocryptine, besides its indications in the treatment of Parkinson's disease, prolactinoma, and hyperprolactinemia, has been described to inhibit lactation [131].

The high toxicity of EAs has led to frame these compounds as mycotoxins, and the interest in assessing the extent of the mycotoxins issue has increased in the last years. Despite improvements in agriculture practices and grain cleaning, generally it is only possible to remove up to 82 % of ergot by mechanical means with conventional grain cleaning equipment such as sieves and separators used during the

harvesting process. So, different studies have demonstrated that EAs can still be present in cereal-based food and feed, sometimes in excessive amounts.

In this sense, De Saeger's group at the University of Ghent carried out a large survey, in which 1,065 samples of cereals and cereal products intended for human consumption and animal feeding in Europe were analyzed. This study included rye, wheat, and multigrain-based food as well as rye, wheat, and triticale-based feed; and it was shown that 59 % of analyzed samples were contaminated with EAs at total levels ranging from 1 to 12,340 μg/kg [132]. Incidence of positive samples and the obtained alkaloid contents were in line with other published data. Storm et al. detected rye flour samples from Danish mills containing an average of 46 μg/kg of EAs with a maximum content of 234 μg/kg [133]; Crews et al. detected EAs in 25 of 28 samples, including all of 11 rye crispbreads that had up to 340 μg/kg [134]; and Müller et al. found EAs in 92 % of analyzed rye product samples with a maximum content of 739.7 μg/kg [19]. Reinhold et al. analyzed 500 food samples from Germany, and approximately 50 % were positive with a highest concentration of 1,063 μg/kg [135], whereas Masloff et al. reported twice higher maximum total EA content in surveys conducted in rye samples in Germany [136] and a maximum of 4,700 μg/kg was detected in Canadian wheat samples [137]. In most surveys, ergocryptine, ergocristine, and ergotamine including their C8-isomers were the most common EAs. Moreover, the main compound co-occurred with its corresponding *8S*-epimers and in most cases the *8S*-epimers had a higher maximum concentration compared with the main compounds [132].

Recently, Bryła et al. tested 65 samples, detecting EAs in 83 % of the tested rye grain, 94 % of rye flour, and 100 % of rye bran and flake samples. Measurable levels of alkaloids were found in the majority of the analyzed samples, particularly in rye flour, where a relatively high mass fraction of 1,215.5 μg/kg was found. Ergotamine, ergocornine, and ergosine were the most commonly found alkaloids, whereas ergometrinine and ergometrine were the least commonly found ones [138].

Regarding cereal-based infant foods, EAs also have been detected in 25 % of samples including oat, barley, soy and rice, and mixed-grain infant cereals from the Canadian retail marketplace. The incidence and overall mean level of EAs was highest in the barley-based samples (56 %, 18 μg/kg) [139].

Therefore, ergot infections of cereals are a severe problem of food security and consequently European Commission (EC), assisted by the EFSA, has established recommendations and directives that limit the maximum amount of ergot (i.e., sclerotia) that may be present in feed and food. So far, no regulatory limits for sclerotia have been set in the European Union (EU) for grain intended for human consumption. However, for intervention grain, a maximum level of 500 mg/kg has been set for ergot. For all feed containing unground cereals, the European Union Directive 2002/32/EC sets a maximum content of ergot of 1,000 mg/kg, whereas the maximum permissible level in the USA and Canada is 300 mg ergot per kg grain [2], and in Australia and New Zealand, a maximum level of 500 mg/kg of ergot sclerotia in cereal grains is applied [5].

There are currently no legislated limits for total EAs in food or feed; however, it is likely that limits for EAs will be included in future mycotoxin legislation [10]. Some

countries have set guideline limits for EAs in cereals by deriving a limiting value for the maximum EA level from the maximum amount of sclerotia that may be present [140]. In this way, Germany and Switzerland have set limits for EAs in cereals for human consumption of 400–500 μg/kg and 100 μg/kg, respectively [140]. In Canada, the guideline limits for the total EA content in feed for poultry, swine, and chicks are 100, 600, and 9,000 μg/kg, respectively, whereas the guideline limits in animal feed in Uruguay is 450 μg/kg [5]. However, at the present, no country has established limits for individual EAs in food or feed.

According to EFSA, physical techniques to determine the contamination rate are often inaccurate as size, weight, and composition of the sclerotia may vary considerably [7]. In addition, sorting is impossible in processed feed materials, and there are significant variations of the total EA content within the sclerotia [141] and differences in the pattern of produced EAs. Hence, EFSA suggested replacing the physical methods by chemical analysis [7].

At present, the data on the toxicological properties of individual EAs are too limited to select individual marker toxins for monitoring the extent of contamination [7]. For that reason, EFSA has stated that more data on the variability of the EA patterns in European food and feed should be collected and that validated analytical methods for the quantification of EAs should be developed. In this way, the basic information needed for scientific risk assessment can be obtained and limits can be set for total and individual EAs via legislative regulations [104].

Recently, the European Commission has issued a recommendation to its member states to perform the monitoring on the presence of EAs in cereals and cereal products intended for animal feeding and in compound feed [142]. The European Commission has also requested the establishment of a relationship between the presence of EAs and the amount of sclerotia present, focusing the monitoring on the six main EAs, i.e., ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, and ergocornine and their related epimers [142].

On the other hand, the interaction with neurotransmitter receptors could result in acute as well as longer-term effects, so EFSA has also established an acute reference dose (ARfD) of 1 μg/kg body weight and a tolerable daily intake (TDI) of 0.6 μg/kg body weight per day for EAs [5].

5 Determination of Ergot Alkaloids

Wide range of analytical methods has been proposed for determination of EAs from several matrices in pharmaceutical, forensic, and food areas. High-performance liquid chromatography (HPLC) and fluorescence detection (FLD) or tandem mass spectrometry (MS/MS) are the most widely used methods; however, other minor techniques including capillary electrophoresis (CE) or immunoassays are also available.

Regardless the analytical technique chosen for determination, a sample treatment is usually mandatory in order to remove interferences and pre-concentrate the analytes. Most of the reported analytical methods involve a liquid extraction

followed by a clean-up by means of liquid–liquid extraction (LLE) or solid phase extraction (SPE) using different sorbents. Moreover, in order to compensate for the matrix effect, most methodologies include matrix-matched calibration.

Another aspect to be highlighted is the fact that during sample preparation and analysis, it is difficult to control the epimerization degree of EAs and both epimeric forms can interconvert. Attempts have been made to avoid this epimerization. Otherwise, it is necessary to determine both epimers and, alternatively, specify the EA content as a sum of both epimers for each EA [5].

The EFSA scientific opinion on EAs in food and feed [5] as well as a several reviews published during the last decade present a comprehensive overview of the different methodologies proposed for the determination of EAs, including sample preparation [2, 10, 24]. Thus, in the next sections, only most recent or relevant contributions in this field will be commented.

5.1 Capillary Electrophoresis

CE offers some advantages over liquid chromatography (LC), such as high efficiency, reduced analysis time, and low sample and reagent consumption, demonstrating its great potential for a wide range of compounds. However, depending on the analytes, sensitivity in CE needs to be improved, especially when UV detection is used.

CE analysis has been rarely applied for determination of EAs. Most of these works were developed in the 1990s, when CE was emerging as a promising analytical technique. Thus, Fanali et al. resolved for the first time a mixture of EA enantiomer derivatives (dl-terguride, dl-lisuride, dl-nicergoline, dl-isolysergic acid hydrazide and dl-1-methyl-10α-methoxy-dihydro-lysergol (dl-meluol), ergotamine, ergotaminine, ergometrine, and ergometrinine) using capillary zone electrophoresis (CZE), studying the effect of cyclodextrins as a chiral additive in the background electrolyte (BGE), on the migration time and the resolution [143]. CZE-UV was also used for simultaneous determination of ergotamine and caffeine in pharmaceutical dosage tablet formulations [144], ergovaline in the seeds of *Festuca arundinacea* (tall fescue) infected with fungus *Acremonium coenophialum* [145]. Also, Frach et al. proposed CE with laser-induced fluorescence (LIF) as an alternative to UV detection, improving limits of detection about 30-fold. Cyclodextrins, urea, and poly (vinyl alcohol) were included in the BGE, achieving the separation of ergometrinine, ergometrine, ergocorninine, ergocryptine, ergocornine, ergosine, ergocristinine, ergocristine, and ergotamine and their determination in sclerotia [146]. Cyclodextrins were also used as BGE modifiers in CZE for the determination of lisuride enantiomers, a chiral compound derived from EA and used for treatment of Parkinson's disease. The method was used for enantiomeric purity checking of commercial lisuride pharmaceuticals, in order to determine the concentration of undesirable l-enantiomer [147].

Later on, CZE was also investigated for the separation of lysergic, isolysergic, and paspalic acid in pharmaceuticals. The method provided a detailed study

describing the possibilities of CZE-UV as well as of mass spectrometry (MS) using quadrupole-time-of-flight (Q-TOF) as detection for the determination of these compounds. BGEs and detection conditions were carefully optimized regarding selectivity and analysis time as well as MS compatibility. The method was applied to the determination of these compounds in samples obtained from different stages of the manufacturing process such as fermentation broth, solution for precipitation, raffinate, or the raw product [148].

In these methods, sample treatment was based mainly on solid–liquid extraction using mixtures of different solvents or by simple dissolution in the case of pharmaceuticals. However, recently a new sample treatment based on cloud point extraction (CPE) prior to CE-UV for determination of ergotamine and ergometrine in cereal samples has been proposed. CPE is one of the nonpolluting phase separation techniques using surfactant at concentrations higher than its critical micellar concentration (CMC). Analytes are extracted from aqueous solutions into micelles. Afterward, the change on the experimental conditions that promotes the phase separation leads to a surfactant-rich phase with concentrated analytes on the one hand and aqueous solution saturated with surfactant monomers on the other. With CPE, a preconcentration factor of 22 of total EAs was achieved. This method was applied to the determination of EA in commercial flour samples, grain samples, and one cereal-based product for infant feeding [149].

5.2 Liquid Chromatography: Fluorescence Detection

The first attempts for chromatographic separation of EAs were carried out by normal phase HPLC and UV detection at different wavelengths [24]. Nowadays, reverse phase-based chromatography is the mode of choice used for the separation of EAs, mainly using C8 and C18-sorbent, because of limited separation provided by the normal phase procedures. Separation can be achieved with both isocratic and gradient mobile phases, and most methods use solvent systems of methanol–water or acetonitrile/water mixtures with added ammonium hydroxide, ammonium carbonate, ammonium carbamate, or triethylamine to provide alkaline pH conditions [5, 10].

Currently, FLD has replaced UV detection since most EAs possess native fluorescence, allowing increasing sensitivity and selectivity [8]. $\Delta^{9,10}$-Ergolenes can be effectively detected with an excitation and detection wavelength of 310 nm and 410 nm, respectively, while $\Delta^{8,9}$-EAs and EA with a saturated D-ring show maximal fluorescence with excitation at 272 nm and emission wavelength at 371 nm [31]. HPLC–FLD provides sufficient chromatographic resolution for the determination of major EAs, according to EFSA, and their corresponding epimers, with typical run times around 40–45 min [150, 151]. However, some compounds, such as α- and β-ergocryptine and similarly α- and β-ergocryptinine, have been reported as single compounds if they co-elute [10].

In the last decade, different applications of HPLC–FLD for the determination of EAs in food and feed have been reported, as well as clinical applications, usually after SPE.

Examples of food and feed analysis comprise a method for the determination of ergocornine, α-ergocryptine, ergocristine, ergometrine, and ergotamine and their C8-isomers in rye flour [133] and ergometrine, ergotamine, ergocristine, α-ergocryptine, and ergocornine analysis of cereals for animal feed [152], where extraction was carried out by liquid extraction under acidic conditions, followed by strong cation exchange (SCX) SPE [133, 152]. Also, 12 main EAs in rye and rye products were determined by HPLC–FLD, where extraction under basic conditions was followed by SPE using basic alumina cartridges [19, 150]. Finally, Köppen et al. reported a HPLC–FLD method to quantitate 12 priority EAs in rye flour and wheat germ oil. In this case, acidic and alkaline conditions were avoided during extraction, enabling minimized epimerization. Moreover, an improved SPE method using SCX material neutralized with sodium (Na + -SCX) was proposed, where EAs (in their protonated form) were eluted from the column by forming ion pairs with sodium hexanesulfonate, delaying epimerization for over 96 h [151]. Recently, a QuEChERS-based extraction has been proposed as sample treatment for the determination of ergovaline in tall fescue seed and straw followed by HPLC–FLD determination. This sample treatment (quick, easy, cheap, effective, rugged, and safe) is developed in two different steps: (i) an extraction/ partitioning step and (ii) a clean-up based on dispersive SPE (d-SPE). In this work, 14 extraction solvents were tested and ammonium carbonate/acetonitrile (50/50, v/v) gave the highest and most consistent recovery (91–101 %), with no necessity of clean-up, eliminating the need for halogenated/chlorinated solvents. QuEChERS procedure was also compared with SPE using Ergosil, a chemically modified silica gel designed for the analysis of ergopeptine alkaloids, obtaining good agreement [153].

In addition to food analysis, Beaulieu et al. used HPLC–FLD to evaluate the diversity and distribution of EAs in seeds and seedlings and variation in alkaloid distribution among different morning glories. The compounds determined were ergobalansine, chanoclavine, lysergol, and ergometrine. In addition, cycloclavine, festuclavine, ergine, and lysergic acid α-hydroxyethylamide could be detected. Identification was confirmed by LC–MS. Before analysis, plant tissues were dried for 3 days at 40 °C and pulverized with 3-mm diam silica beads. The resulting fine powder was soaked in methanol for 3 days at 4 °C with daily vortexing to extract EAs [42]. Also, Nakamichi et al. measured methylergometrine (a postnatal uterotonic drug) in human breast milk using HPLC–FLD. Samples were diluted with McIlvaine buffer containing 5 % EDTA, and after centrifugation, the supernatant was loaded onto a mixed mode cation exchange (MCX) SPE cartridge. Recoveries from 93.5 % to 103.0 % were obtained [154].

5.3 Liquid Chromatography: Mass Spectrometry

Although HPLC–FLD is still a significant technique for the determination of EAs, LC–MS and, more recently, UHPLC–MS are becoming more and more relevant for

the determination of mycotoxins, due to the MS capacity of an unambiguous compound identification, especially when high-resolution MS (HRMS) is used. Most of these methods use acidified acetonitrile/water mixtures as mobile phase in reverse mode, and equipment capable to perform MS/MS such as triple quadrupole or, less frequently, ion trap (IT) are commonly employed.

Due to the lack of available standards, most of the reported LC–MS/MS methods have been developed for the determination of the six major EAs (ergometrine, ergosine, ergotamine, ergocornine, ergocryptine, and ergocristine) and their corresponding -inine epimers (ergometrinine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, and ergocorninine). De Saeger's group determined these compounds in different food and feed samples by a method involving extraction under alkaline conditions and subsequent clean-up by a liquid–liquid partitioning procedure prior to LC–MS/MS analysis. The optimized sample clean-up and a careful selection of the sample solvent allowed minimizing the epimerization of the ergot alkaloids during analysis [132, 155]. This was also the methodology chosen for the in vitro binding efficacy study of a clay-based mycotoxin binder toward EAs [156]. Moreover, the same group reported the first molecularly imprinted polymer (MIP) toward these compounds and its application in SPE for clean-up of barley samples before LC–MS/MS determination. Metergoline was used as template in the production of suspension polymerized beads used as selective sorbent, obtaining recoveries between 65 % and 79 % [157].

These six major EAs and four of their respective epimers were determined by UHPLC–MS/MS in rye and wheat. In this case, the analytes were extracted with acetonitrile–ammonium carbonate solution and the extract was clean-up with a commercial SPE column (Mycosep 150 Ergot) [104]. EAs were also determined in rye-based food products and ergot sclerotia isolated from rye grains by LC–IT–MS. In this case, neutral alumina-based SPE was selected for clean-up, avoiding the problems of matrix ions that may easily degrade the performance of the IT [138]. LC–IT–MS was also used for the determination of ergovaline in infected tall fescue, after liquid extraction [158].

Concerning ionization sources, electrospray ionization (ESI) has been preferred above other ionization techniques, such as atmospheric pressure photoionization (APPI) or atmospheric pressure chemical ionization (APCI), for determination of mycotoxins. In all these techniques, the sample is ionized at atmospheric pressure before entering the mass spectrometer, but in different ways: in ESI the ionization is achieved by application of a voltage to the spray tip; in APPI by reaction of aerosol droplets with photons produced by a UV lamp; and in APCI by gas-phase ion–molecule reactions. In a very recent paper, ESI and APPI have been compared as ionization sources in the LC–MS/MS determination of lysergic acid amide (LSA) and ergometrine in grass samples, after extraction with methanol [159]. The conclusion of this study was that the performance of APPI and ESI methods was comparable.

One of the main drawbacks of MS detection is matrix effect (signal suppression/enhancement) due to matrix component. In an interesting study, the effect of sample treatment, chromatographic separation, and ionization technique on the

matrix effect in EA determination was studied [160]. Thus, LLE, d-SPE using primary secondary amine (PSA), and SPE with different sorbents, such as SCX, MycoSep Ergot multifunctional, and MIP, were compared. For all the procedures tested, no clear signal enhancement was noted, although, for the later eluting ergot alkaloids, MycoSep and SCX cartridges minimized signal suppression. ESI and APCI were also compared; signal suppression was observed in the ESI mode for almost all analytes (with ergometrine being the most susceptible) with no significant difference between ESI+ and ESI-. On the other hand, the use of APCI resulted in a very high signal enhancement for most of the EA. In the same study, LC and UHPLC were compared, concluding that UHPLC was more preferred for the later eluting compounds, as matrix effects were minimized. Other interesting conclusion of this study is that matrix effect varied significantly not only between grain types but also to a lesser extent within one grain type. This fact must be considered when selecting an appropriate blank sample for preparation of a matrix-matched calibration [160].

Multi-mycotoxin determination including EAs by LC–MS/MS has also been reported. For instance, 22 mycotoxins (including ergotamine and ergocornine) were determined in wheat, barley, oats, rye, and maize grain [161] and 26 mycotoxins (including aflatoxins, ochratoxins, fumonisins, trichothecenes, and EAs) in corn, rice, wheat, almond, peanut, and pistachio products using ^{13}C-isotope-labeled internal standards for some of the mycotoxins and liquid extraction with acetonitrile/water [162]. In another work, 63 fungal and bacterial metabolites (including 11 EAs) were determined in commercial poultry feed from Nigeria. The samples were extracted in just one step with acidified acetonitrile, and no further clean-up was required [163]. QuEChERS were the method chosen for the simultaneous determination of 56 mycotoxins (including 12 EAs) in 343 samples of animal feed (non-fermented or fermented feeding stuffs, feeding stuff supplements, and complex compound feeds) by UHPLC–MS/MS, with the aim of estimating absolute mycotoxin concentrations and animal exposure to mycotoxins. In this case, a Q-Trap mass spectrometer was used, operating in both ESI+ and ESI- mode. Among other conclusions, a fairly high co-occurrence was noticed for deoxynivalenol and EAs [164]. The same MS (Q-Trap) and a simple "dilute and shoot" treatment were proposed for the LC–MS/MS determination of 295 fungal and bacterial metabolites (including several EAs). This method was validated in four different food matrices: apple puree for infants, hazelnuts, maize, and green pepper [165]. Other interesting studies involving liquid extraction and subsequent semiquantitative determination of mycotoxins (including EAs) in different samples also with LC–Q-Trap–MS/MS have been reported by Sulyok's group; those studies comprise analysis of foods infected by molds (concluding that EAs occurred in all samples of dark bread/pastries at low ppb [166]), analysis of grain grown in exceptional climatic conditions [167], and grain dust from Norwegian grain elevators and compound feed mills [168].

HRMS, as Orbitrap or time-of-flight (TOF) MS analyzers, is becoming more and more popular for identification purposes in natural product analysis. In this sense, a QuEChERS-based extraction and UHPLC-Orbitrap MS with APCI have been

proposed in the determination of four different groups of mycotoxins (including four EAs) in cereal-based products [169]. Moreover, HRMS has been used not only for the determination of major EAs but also for the identification of less studied or novel EA derivatives. In this regard, recently Arroyo-Manzanares et al. developed a method based on HRMS and IT-MS technology for the study of the fragmentation pattern of EAs and established a simple strategy for the identification of novel ergot alkaloid derivatives [170]. With this approach, besides the six most common ergot alkaloids and their corresponding epimers, 11 EA derivatives, for which commerical standards were not available, were identified. The same authors investigated the suitability of a Q-TOF-MS instrument based on the TripleTOF technology to provide simultaneously a quantitative analysis of common ergot alkaloids and the screening, detection, and identification of unexpected or novel EAs in rye samples [171]. On the other hand, Paulke et al. also used LC–HR–MS/MS for the identification of EAs in different "legal highs" derived from *Argyreia nervosa*, concluding that LSA/iso-LSA and ergometrine are the main ergot alkaloids present in these products, although a variety of additional EA could be identified, contributing to the pharmacological effects of these drugs [172, 173].

5.4 Immunological Methods

Immunological methods rely on the specificity of binding between antibodies and antigens. Radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) have been a rapid and inexpensive alternative for EAs determination. However, these methods are less specific and less accurate than HPLC–FLD or LC–MS methods [10].

ELISA has largely replaced RIA, since it has the advantage of not using radioisotopes, avoiding the associated disposal problems, with no sacrifice in sensitivity. Early assays used polyclonal antibodies, which recognized peptide EAs having a phenylalanine moiety, such as ergotamine, ergosine, and ergocristine [24, 174]. Nevertheless, these polyclonal antibodies were replaced by monoclonal antibodies [56, 175, 176] that provided the potential advantage of being more specific for the target hapten, since specific anti-target antibody producing hybridoma cell lines could be selected [13]. Monoclonal antibodies recognized a much wider range of EAs, any with an ergoline ring, since they hinder the antibody binding to the lysergic acid ring structure. However, many peptide EAs with large groups attached to the lysergic acid (ergocryptine, ergocristine, ergocornine, and ergotamine) are not amenable to ELISA [10, 24]. Molloy et al. compared monoclonal and polyclonal antibodies for determination of dihydroergosine in sorghum ergot and both assays were capable of detecting dihydroergosine concentrations above 0.01 mg/kg [56].

ELISA methods have frequently been used to determine the total concentration of EAs produced by endophytic fungi in fescue grass forage [175, 177, 178]. Sample preparation was based mainly on drying and grinding before diluting with phosphate-buffered saline with Tween 20. This was allowed to stand and the liquid portion of the sample was analyzed by ELISA [176]. Different commercial ELISA

kits are available, providing LOD around 2 μg/kg [5]. These kits have been applied for the determination of total EAs in tall fescue [179] and in urine samples from lambs fed with tall fescue [180]. However, ELISA methods are not specific for individual EA and gave only an estimation of total EA content. Thus, when quantification of individual EA is required, analysis by HPLC–FLD or LC–MS is mandatory.

Immunological methods have been widely used to determine LSD in biological fluids, and commercial assays are also available [181]. Unfortunately, they are subject to cross-reactivity with structurally related and unrelated compounds potentially yielding false-positive results. So, the best practice following a positive LSD involves confirmation with MS [182].

5.5 Miscellaneous

Besides the liquid separation techniques and immunoassays previously commented, other techniques have been reported for EA determination.

Regarding separation techniques, gas chromatography (GC) was used for the analysis of low molecular weight clavine-type alkaloids and simple lysergic acid derivatives lacking hydrophilic functional groups [183–185], and it has mainly been applied in pharmaceutical and forensic areas. Nevertheless, this technique was not very useful for the determination of peptide EAs because of their high molar mass, low vapor tension, and heat instability, decomposing in a hot injector (225–300 °C) [13, 186]. Thus, most of the applications of GC–MS were published during the 1990s and concern the monitoring of LSD, proposing several derivatization strategies in order to improve volatility and stability and to reduce the peak tailing [187–190]. In food analysis, GC–MS was used for confirmation of identity of the alkaloids in grain foods [191], while Franzmann et al. investigated a method based on the determination of ricinoleic acid (as a characteristic components of ergot) by GC–FLD to estimate the distribution of EAs (determined by HPLC–FLD) in different milling fractions [53].

Thin layer chromatography (TLC) has also been proposed for the determination of naturally occurring EAs in extracts of ergots, grasses, grains, and feeds [24], although this technique has been clearly replaced by LC. TLC on silica gel was used to identify EAs (agroclavine-I and epoxyagroclavine I and their N–N dimers, such as dimer of epoxyagroclavine I and the mixed dimer of epoxyagroclavine I and agroclavine-I) in *Penicillium* fungi [37], and different metabolites produced by *Penicillium* fungi isolated from cheese-making and meat-processing plants included EAs (festuclavine and its isomers pyroclavin, costaclavin, and epicostaclavin) [192]. EAs were detected by UV absorbance after spraying the plates with Ehrlich's reagent, while dimers were detected by luminescence in UV light at 366 nm and positive staining by Dragendorff's reagent.

Another chromatographic method, such as supercritical fluid chromatography with UV detection at 280 nm or electron impact MS, has also been applied to the identification of a number of clavine alkaloids from *Claviceps purpurea* [193].

Also, Stahl et al. proposed the use of hybrid techniques such as capillary size exclusion chromatography performed under pressurized capillary electrochromatography (pCEC) on-line with MS for the separation of a crude extract of ergot fungus (*secalis cornuti*). This set up was compared with other one- and two-dimensional configurations of capillary HPLC [194].

Spectroscopic techniques have also been applied to the detection and quantification of EA. Thus, early methods for EA determination were based on colorimetric measures, where EAs reacted with p-dimethylaminobenzaldehyde under acid conditions, yielding an intensely colored blue solution, which could be measured at 580 nm. Other color reactions like addition of ferric chloride or sodium nitrite and combinations were also proposed [24]. Near-infrared (NIR) spectroscopy was also proposed to determine total EA content on tall fescue [195], and more recently, a method based on NIR hyperspectral imaging and multivariate image analysis has been reported for quantification of ergot bodies in cereals [196]. This method was intended for use in cereal conveyor belt systems at an industrial level.

6 Conclusion

Since the times of Holy Fire, EAs have been an interesting family of compounds, which have roused the interest of the scientific community. These compounds have, on the one hand, valuable pharmacological properties based on their interactions with neurotransmitter receptors on the cells. However, on the other hand, some natural and semisynthetic EAs also possess serious and unpredictable side effects. So, they are considered toxic compounds and have been framed as mycotoxins. Regarding their determination, a wide range of methods have been proposed in pharmaceutical, forensic, and food areas, being those based on liquid chromatography the most popular ones. LC–MS/MS deserves a special mention, as the recent advances in this technique have allowed not only an accurate quantification of major EAs in complex matrices but also the elucidation and identification of novel EA, not described before. EAs are still a challenge, and the elucidation of their biosynthesis pathway is still of great interest, especially because of their broad range of pharmaceutical uses.

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Lanostanoids from Fungi as Potential Medicinal Agents

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Abstract

Lanostanes are a group of tetracyclic triterpenoids derived from lanosterol. They have relevant biological and pharmacological properties, such as cytotoxicity, immunomodulation, and anti-inflammation. Some of them also have interesting effects on metabolism and anti-infectious properties. This review will compile chemical data, biological effects, and mechanisms on the most relevant lanostanoids isolated from fungi, such as those from *Ganoderma lucidum*, *Poria cocos*, *Laetiporus sulphureus*, *Inonotus obliquus*, *Antrodia camphorata*, *Daedalea dickinsii*, and other.

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List of Abbreviations

ABCB	ATP-binding cassette
AIF	Apoptosis-inducing factor
AKT	Protein kinase B
AMPK	AMP-activated kinase
C/EBPα	CCAAT-enhancer-binding protein-α
CCAAT	Cytosine-cytosine-adenosine-adenosine-thymidine
cdk4	Cyclin D kinase 4
COX	Cyclooxygenase
CYP3A4	Cytochrome P450 3A4
DBD	DNA-binding domain
DMBA	7,12-Dimethylbenz[a]anthracene
EBV-EA	Epstein-Barr virus early antigen
ERK Erk	Extracellular-regulated kinase
FXR	Farnesoid X receptor
GLUT4	Glucose transporter type 4
GPDH	Glycerol-3-phosphate dehydrogenase
HIV	Human immunodeficiency virus
HO-1	Heme-oxygenase-1
hPXR	Human pregnane X receptor
IC_{50}	Inhibitory concentration 50
IFN-γ	Interferon-γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRS-1	Insulin receptor substrate-1
IκBα	Inhibitor of κBα
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LBD	Ligand-binding domain
LPS	Lipopolysaccharide
MDD	Mean day of death
MIC	Minimum inhibitory concentration
MMP-9	Matrix metalloproteinase-9
NF-κB	Nuclear factor-κB
NK	Natural killer
NO	Nitric oxide
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLA_2	Phospholipase A_2
PARP	Poly(ADP-ribose)-polymerase

PPAR-γ	Peroxisome proliferator-activated receptor-γ
RXR	Retinoid X receptor
SREBP-1c	Sterol regulatory element-binding protein-1c
STAT3	Signal transducer and activator of transcription 3
T2DM	Type 2 diabetes mellitus
TNFα	Tumor necrosis factor-α
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
uPA	Urokinase-type plasminogen activator

1 Introduction

Lanostanes are a group of tetracyclic triterpenoids derived from lanosterol. They have a tetracyclic skeleton with 30 carbons and include a gem-dimethyl group in C4. There are other compounds with similar structures, such as dammaranes, tirucallanes, euphanes, and cucurbitanes, but they are usually found in the plant kingdom. In the case of fungi, the compounds known as lanostanes are the most common of the possible triterpenes found in different fungi species [1–3].

Lanosterol is the previous metabolite synthetized by cyclization of squalene-2,3-epoxide (**1**). This compound suffers a multiple cyclization to give a chair-boat-chair-boat conformation in the carbonium ion intermediate metabolite (**2**), which after proton elimination is transformed to protosterol (**3**), and a later backbone rearrangement leads to give the metabolites derived from lanostane (**4**) and cycloartane (**5**), the former intermediates in the metabolism of lanosterols (Fig. 1). Different ways of cyclization and rearrangements give other structural compounds related to lanostanes, such as tirucallanes and euphanes as the principal groups [1, 4, 5].

In this chapter, we cover the reports on the lanostanes and closely related compounds obtained from fungi, with some relevant pharmacological interest. In addition to lanostane (**4**) derivatives, other common lanostane-related compounds are the members of C-31 group, called eburicanes (**6**) and their seco-derivatives, nor-3,4-*seco*-lanostanes (**7**) and nor-3,4-*seco*-eburicanes (**8**), respectively [2–5].

The presence of tetracyclic triterpenes in nature is less notorious than that of pentacyclic derivatives in the kingdom Plantae. However, the presence of lanostanoids (tetracyclic triterpenes) in fungi is more relevant than that of its pentacyclic derivatives. The Polyporaceae (Basidiomycetes class) is the family in which the largest number of lanostanes has been isolated. In addition, only a small number of tetracyclic triterpenes (fusidanes and protostanes) have been described in the parent class Ascomycetes [3].

Different complete reviews on lanostanoids have been published in the last decade. Some of them are included in the complete revision of triterpenes published by Connolly and Hill, who have systematically reviewed the triterpenoids isolated since 1989–2015 [6–29]. However, other more specific studies were carried out focusing only in this kind of triterpenes, such as the reviews of Giner-Larza et al. [2] on lanostanoids and the reviews of Ríos et al. [30] and Popović et al. [31].

Fig. 1 Principal groups of lanostanoids

Since the year 1990, the most relevant species of fungi studied were *Albatrellus flettii* [32], *Antrodia camphorata* [33–37], *A. cinnamomea* [38], *Ascotricha* sp. [39], *Ascotricha amphitricha* [40, 41], *Astraeus hygrometricus* [42], *A. odoratus* [43], *A. pteridis* [44], *Coriolellus malicola* [45], *Daedalea dickinsii* [46], *Elfvingia applanata* [47], *Fomes officinalis* [48–50], *Fomitopsis nigra* [51], *F. pinicola* [52–54], *F. rosea* [55], *F. spraguei* [56], *Fuscoporia oblique* [57], *Ganoderma applanatum* [58–60], *G. colossum* [61, 62], *G. concinna* [63], *G. lucidum* [64–84], *G. hainanense* [85], *G. lingzhi* [86], *G. orbiforme* [87], *G. resinaceum* [88],

G. sinense [89–92], *G. theaecolum* [93], *Hebeloma senescens* [94], *H. versipelle* [95], *Hypocrella* sp. [96], *Inonotus obliquus* [97–105], *Jahnoporus hirtus* [32], *Laetiporus sulphureus* [106], *L. versisporus* [107], *Naematoloma fasciculare* [108, 109], *Phellinus gilvus* [110], *P. punctatus* [101], *Piptoporus betulinus* [111], *Poria cocos* [112–124], *Scleroderma citrinum* [125], *Spongiporus leucomallellus* [80], *Stropharia aeruginosa* [126, 127], *Tomophagus cattienensis* [128], and *Tyromyces fissilis* [129, 130].

2 Pharmacological Interest of Lanostanoids

The species with the highest number of isolated lanostanoids are *Ganoderma lucidum*, *Inonotus obliquus*, and *Poria cocos*. With respect to their potentiality as medicinal agents, their effects on metabolic disorders such as diabetes mellitus and hyperlipidemias can be of high interest as well as the relative pharmacological potency of some of them. Other compounds have relevant anti-inflammatory and anticancer activities, with high interest because of the mechanisms implicated. Their role as anti-infectious agents depends on the effects of some specific compounds.

2.1 Lanostanoids in Diabetes Mellitus and its Associated Undesirable Effects

Type 2 Diabetes Mellitus (T2DM) is a metabolic disease characterized by a persistent increase in blood glucose above normal values (hyperglycemia) due to a progressive insulin secretory defect on the background of insulin resistance [131]. Some negative effects of chronic hyperglycemia involve damage in eyes, kidneys, nerves, heart, and blood vessels. They are associated with other cardiovascular risk factors such as hypertension, overweight, and dyslipidemia [132]. Development of T2DM can be prevented or at least delayed in patients with impaired glucose tolerance by implementing lifestyle changes [133] or the use of therapeutic agents [134–136]. Some mushrooms have been used and studied for their potential hypoglycemic effect: it is the case of the edible mushroom maitake (*Grifola frondosa*). However, in this case the active compounds were identified as polysaccharides [137]. In the case of *Phellinus gilvus*, the hypoglycemic activity of the crude extract and four isolated lanostanoids were tested, but the results were not promising [110]. The studies focused on specific targets to avoid the negative effects of diabetes are of high interest, such as the enzymes aldose reductase and α-glucosidase, and the glucose transporter type 4 (GLUT4).

2.1.1 Effect on Aldose Reductase

Aldose reductase catalyzes the reduction of glucose to sorbitol, the first step in polyol pathway of glucose metabolism [138]. Aldose reductase is involved in the pathogenesis of diabetic complications. Its inhibitors could prevent eye and nerve damage in people with diabetes [139]. In this case, some lanostanes from fungi have

shown capacity for inhibiting aldose reductase, avoiding the metabolism of glucose to sorbitol, which is increased in chronic hyperglycemia. The accumulation of sorbitol in lens capsule is in part responsible of diabetic cataract formation, together the osmotic imbalance, alteration of balance sodium/potassium, and the glutathione decrease [139].

Fatmawati et al. [67] isolated ganoderic acid Df (**9**), from the fruiting body of *Ganoderma lucidum*, which was assayed as a potential inhibitor of human aldose reductase and showed in vitro activity with an inhibitory concentration 50 (IC_{50}) of 22.8 μM. In this report, they demonstrated that the presence of a carboxyl group in the side chain could be essential for eliciting inhibitory activity because its methyl ester was less active ($IC_{50} > 200$ μM) [67].

To ratify this hypothesis, the same authors screened 17 lanostane-type triterpenoids, previously isolated from the same source in order to establish a structure-activity relationship. However, only two compounds had relevant effects: ganoderic acid C2 (**10**) and ganoderenic acid A (**11**), which showed IC_{50} values of 43.8 and 119.2 μM, respectively. Both compounds showed less activity than the previous isolated compound.

After the evaluation of the results of the active compounds in vitro on human recombinant aldose reductase, they established that the OH substituent at C-11 is an important feature and the carboxylic group in the side chain (C-26) is essential for the recognition of aldose reductase inhibitory activity. In addition, a double bond between C-20 and C-22 improves the inhibitory activity. Hydroxyl groups in C-3, C-7, and C-15 are also important for the inhibitory activity. The authors conclude that the basic structure for future design of active molecules could be that represented in Fig. 2 [68]. They arrived to this idea after comparing the effects of pairs of compounds. However, the most relevant compounds are **9** and **10** and none of them

Ganoderic acid Df (**9**)

Ganoderic acid C2 (**10**)

Ganoderenic acid A (**11**)

Basic chemical structure for having aldose reductase inhibitory activity

Fig. 2 Active lanostanoids as aldose reductase inhibitors

have C-20 and C-22 double bond. Only, the free carboxylic acid at C-26 seems to be essential for activity (Fig. 2).

2.1.2 Effect on α-Glucosidase

The enzyme α-glucosidase breaks down starch and disaccharides to glucose. It is located in the brush border of the small intestine. A specific mechanism for treating diabetes is the inhibition of this enzyme, as acarbose does [131]. Different natural products have been studied in this sense; however, the number of compounds with high interest is reduced. Ying et al. [97] screened eight compounds isolated from the submerged culture of chaga mushroom, *Inonotus obliquus*, and compared their activity versus the standard inhibitor acarbose. Four of them are lanostanoids, and the higher activity was obtained with inotolactone A (**12**) and inotolactone B (**13**), both with IC_{50} values of 0.24 μM, whereas acarbose gave a value of 0.46 μM. These values seem to be of interest for studying their potentiality for the treatment of diabetes. The active lanostanes (**12** and **13**) were the unique with an α,β-dimethyl, α,β-unsaturated δ-lactone side-chain moiety, data that could be of interest for future studies of these compounds such as α-glucosidase inhibitors.

In a similar study, Fatmawati et al. [86] screened 19 lanostane-type triterpenoids isolated from the fruiting body of *Ganoderma lingzhi* in order to establish a chemical structure-inhibitory activity relationship. However, none of them showed remarkable activity: the most active of them had an IC_{50} value of 110.1 μM (ganoderol B, **14**). The rest of the active compounds showed IC_{50} values between 207.8 μM (ganoderiol F, **15**) and 540.3 μM, whereas 12 compounds had IC_{50} values >600 μM. Lanostane **9** showed similar limited effects than in the aldose reductase test, with a modest activity (IC_{50} of 218.8 μM). As in the case commented above, the authors established a chemical structure-activity relationship, with similar results: a hydroxyl group in C-3 enhances the inhibitory activity and the double bond at the side chain is necessary for inhibition (C-23/C-24 for ganoderma alcohols or C-20/C-22 for ganoderma acids). In the case of ganoderma acids, the presence of a hydroxyl at C-11 enhances the inhibitory activity. However, as in the case of aldose reductase, these features did not have a great relevance in the activity or potency and a clear relationship cannot be established, especially when data are compared with values obtained for compounds **12** and **13** (Fig. 3).

2.1.3 Effect on Glucose Transporter Type 4

Insulin regulates and stimulates the transport of glucose into peripheral tissues through the insulin-regulated GLUT4 found primarily in adipose tissues and striated muscle. Insulin facilitates glucose uptake through the stimulation, translocation, and redistribution of GLUT4 from specific intracellular compartments to plasma [140].

In traditional Chinese medicine, *Poria cocos* is used to treat hyperglycemic disorders. However, the number of studies in these subjects is quite limited. In order to investigate potential antidiabetic agents, Huang et al. [114] studied the effect on glucose uptake and the possible mechanism of six lanostane-type triterpenoids isolated from *Poria cocos*. Among them, pachymic acid (**16**) had the best effect on glucose uptake in 3T3-L1 adipocytes. In fact, **16** induced an increase in

Inotolactone A (**12**)

Inotolactone B (**13**)

Ganoderol B (**14**)

Ganoderiol F (**15**)

Fig. 3 Active lanostanoids as α-glucosidase inhibitors

Pachymic acid (**16**)

Fig. 4 Pachymic acid increases GLUT4 expression

GLUT4 expression at both the mRNA and protein levels, without affecting GLUT1, which facilitates the transport of glucose across the plasma membranes of mammalian cells. This transporter mediates basal glucose transport in various tissues [141], whereas GLUT4 is expressed in insulin-responsive tissues, including adipose and skeletal muscle cells [141, 142]. Moreover, **16** also increased the phosphorylation of insulin receptor substrate (IRS)-1, protein kinase B (AKT), and AMP-activated kinase (AMPK). These effects are completely blocked by phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and AMPK inhibitors, demonstrating that **16** exerts its effects through these kinases. Finally, **16** also induces the triglyceride accumulation and inhibits lipolysis in differentiated adipocytes (3 T3-L1 cells). In conclusion, the hypoglycemic activity of *P. cocos* is due to the insulin-like activity of its lanostanes, especially compound **16**, which stimulates glucose uptake, GLUT4

gene expression, and translocation and promotes triglyceride accumulation in adipocytes (Fig. 4).

2.1.4 Promotion of Adipocyte Differentiation

Dehydrotrametenolic acid (**17**), isolated from *Poria cocos*, induces adipose conversion, activates peroxisome proliferator-activated receptor (PPAR)-γ in vitro, and reduces hyperglycemia in animal models of noninsulin-dependent diabetes mellitus [143]. This lanostane acts as an insulin sensitizer in vivo and promotes adipocyte differentiation in vitro. Two possible mechanisms for the induction of adipose conversion are suggested, one is the activation of PPARγ and another is the activation of retinoid X receptor (RXR), both of which act as insulin sensitizers.

PPARγ plays an important role in lipid metabolism but also in other pathways such as inflammation, immunity, and glucose homeostasis. It is induced during differentiation of preadipocytes into adipocytes and can directly modulate the expression of genes involved in glucose homeostasis, such as GLUT4 [144]. The conclusion of Sato's study [143] indicates that **17** acts as an insulin sensitizer by activating PPARγ after binding; however, it is not clear if **17** binds directly to PPARγ.

2.2 Lanostanoids in Prevention and Treatment of Hyperlipidemias

Different mechanisms have been described for treatment of hyperlipidemias. In the case of lanostanes, different authors have studied three principal mechanisms. The adipocyte differentiation and adipogenesis inhibition could be relevant ways for decreasing hyperlipidemias. On the other hand, lanostanoids can be of interest for searching new therapeutic agents for the treatment of cholestasis and dyslipidemias as farnesoid X receptor agonists.

2.2.1 Effects on Adipocyte Differentiation and Adipogenesis Inhibition

As commented above, lanostane **17** promotes adipocyte differentiation in vitro [143], affecting both adipose conversion and insulin sensitization through PPARγ. This nuclear receptor is involved in the adipocyte differentiation and stimulates the expression of several genes critical to adipogenesis. Lanostane-type triterpenes, such as **17**, but **16** also, promote adipose conversion of ST 13 preadipose cells in vitro [143].

In this same way, Lee et al. [71, 72] isolated a series of new lanostane triterpenes from the fruiting bodies of *Ganoderma lucidum* and studied their effect on triglyceride accumulation during the differentiation of 3 T3-L1 preadipocytes. This effect is considered as an indicator of adipocyte differentiation. Of them, *t*-butyl lucidenate B (**18**) at 80 μM reduced the triglyceride accumulation by 72% with respect to the nontreated group. In addition, **18** suppressed the glycerol-3-phosphate dehydrogenase (GPDH) activity in the cells and the gene expressions of PPARγ,

CCAAT-enhancer-binding protein-α (C/EBPα), and sterol regulatory element-binding protein-1c (SREBP-1c) in a dose-dependent manner during differentiation [71]. They also tested 18 isolated compounds from the same source, and butyl lucidenate N (**19**) showed the highest inhibition of lipid droplet formation (56 % at 40 μg/mL). It reduced in a dose-dependent manner the accumulation of lipid droplets without toxicity against the cells, whereas other compounds showed toxicity at concentrations higher than 40 μg/mL. Lanostane **19** also suppressed GPDH activity in a similar way to lipid accumulation. This cytosolic enzyme plays a central role in the triglyceride synthesis, and for that reason, the effect of **19** on GPDH activity could justify the adipocyte differentiation in 3 T3-L1 cells [72].

In a complementary study, the same authors [73] tested four lanostane triterpenes bearing a butyl ester side chain, such as the active compounds from the previous research, as potential inhibitors of adipogenesis. They demonstrated inhibitory effects on adipogenesis in 3T3-L1 cells and determined the mechanism of action behind this effect on two of the lanostanes studied (**19** and butyl ganoderate A (**20**). Both of them reduced the mRNA and protein expression levels of SREBP-1c with respect to the untreated control and suppressed the mRNA expression levels of fatty acid synthase and acetyl-CoA carboxylase [73]. In summary, lanostane bearing a butyl ester side chain showed remarkable inhibition on adipogenesis in 3T3-L1 cells, indicating that the ester side chain influences the inhibitory potential of lanostanes during adipogenesis. Moreover, these results demonstrated that this inhibition is mediated in part through downregulation of the adipogenic transcription factor SREBP-1c and its target genes, such as fatty acid synthase and acetyl-CoA carboxylase [71–73] (Fig. 5).

Dehydrotrametenolic acid (**17**)

t-Butyl lucidenate B (**18**)

Butyl lucidenate N (**19**)

Butyl ganoderate A (**20**)

Fig. 5 Lanostanoids as promoters of adipocyte differentiation

2.2.2 Lanostanoids as Farnesoid X Receptor Agonist

The farnesoid X receptor (FXR) is a member of the nuclear hormone receptor superfamily. Like other nuclear receptors, it comprises a variable modular region, a conserved DNA-binding domain (DBD) and a ligand-binding domain (LBD). As a part of its physiological functions, FXR can act as a monomer stimulating the expression of GLUT4, as a homodimer, or preferentially with its partner the nuclear receptor 9-*cis*- RXR, forming an FXR/RXR heterodimer. Upon ligand binding, the receptor connects to DNA, which results either in an upregulation or repression of gene transcription [69, 145]. FXR is expressed in the liver, intestine, kidney, and adipose tissue and is considered a key gene involved in the maintenance of cholesterol and bile acid homeostasis. Its ligands are currently under clinical investigation for the treatment of cholestasis, dyslipidemic disorders, and conditions of insulin resistance in T2DM and nonalcoholic steatohepatitis [146]. In this way, Grienke et al. [69] carried out a virtual screening of 25 *Ganoderma* lanostanes and obtained positive results of some of them as putative FXR ligands. The *in silico* data were then pharmacologically investigated and demonstrated that five lanostanes out of 25 secondary metabolites from *G. lucidum*, dose-dependently induced FXR in the low micromolar range in a reporter gene assay. They were **15**, lucidumol A (**21**), ganoderic acid TR (**22**), and ganodermanontriol (**23**). After additional pharmacophore profiling and molecular docking studies, the first structure-activity relationship of the investigated lanostanes was established. Data of the putative binding mode by molecular docking studies revealed crucial hydrogen bond interactions between the contemplated structure and Arg331 in the nuclear receptor backbone [69] (Fig. 6).

Lucidumol A (**21**)

Ganoderic acid TR (**22**)

Ganodermanontriol (**23**)

Fig. 6 Lanostanoids as farnesoid X receptor agonists

2.3 Anti-inflammatory Properties

Extracellular phospholipase A_2 (PLA_2) is a key anti-inflammatory target since it plays a pivotal role on chronic autoimmune diseases such as psoriasis, rheumatoid arthritis, and uveitis, among others. Its pathogenic mechanism involves phospholipid hydrolysis, which directly damages cellular membranes and its participation in the synthesis of the eicosanoid-precursor arachidonic acid, responsible for inflammatory response [147]. PLA_2 inactivation requires a bulk tetracyclic structure and a side alkyl carboxylic chain that blocks the catalytic site of the enzyme. The lanostanes isolated from different fungi have been reported as PLA_2 inhibitors in in vitro and in vivo models. In this sense, the hydroalcoholic extract of *Poria cocos* was demonstrated to be active in vivo against acute, chronic, and delayed hypersensitivity tests in mice [2, 124]. The inhibition of PLA_2 activity is involved in the mechanism of action of these lanostanes. Two lanostane derivatives were described as responsible for the anti-inflammatory effects: **16** and dehydrotumulosic acid (**24**) [123]. A study on the anti-PLA_2 activity of triterpenoids against three different forms of PLA_2 was performed by Jain et al. [148] in which they established that lanostanoids have a novel pharmacophore that interacts with the catalytic site of the enzyme. These results were compared with those of the related lanostane derivatives from *Ganoderma lucidum* and demonstrated that ganoderic acids presented different results depending on the substitution pattern of the rings. So, ganoderic acids R (**25**) and S (**26**) were active against pig pancreas PLA_2, while ganoderic acid T (**27**) was able to inhibit all the enzymes regardless of the origin.

Other in vitro studies have gone more in depth in the mechanism of action of lanostane-type triterpenoids form *Poria cocos* in different cell lines. In this sense, Li et al. [149] recently described that **16** attenuates lipopolysaccharide (LPS)-induced pro-inflammatory cytokine secretion, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6, in H9c2 cardiomyocytes. Furthermore, in this same cells, **16** treatment also prevented the expression of caspases 3, 8, and 9 and the phosphorylations of extracellular-regulated kinase (Erk)1/2 and p38, therefore preventing the apoptotic response in these cells.

Ganoderic acids from *Ganoderma lucidum* also have anti-inflammatory properties. They have been studied in an acute model of edema in mice and observed that their anti-inflammatory activity was comparable of even better than that of the anti-inflammatory drug indomethacin [78]. The mechanism of action of these ganoderic acids has been studied in vitro by different authors in RAW 264.7 macrophages. As these studies highlight, ganoderic acids inhibit LPS-induced nitric oxide (NO) production, through the inhibition of the inducible nitric oxide synthase (iNOS) [64, 66]. This anti-inflammatory effect was accompanied by a reduction in LPS-induced TNF-α and IL-6 secretion [64] and the inhibition of cyclooxygenase (COX)-2 [64, 66]. Choi et al. [64] postulate that this anti-inflammatory effect generated by ganoderic acid occurs through the induction of heme-oxygenase (HO)-1 expression via the PI3K/AKT-nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway, since the inhibition of this pathway abrogated the anti-inflammatory effect of the lanostane triterpenoids. The active compounds were **15**,

19, butyl lucidenate E2 (**28**), butyl lucidenate D2 (**29**), butyl lucidenate P (**30**), butyl lucidenate Q (**31**), methyl ganoderate J (**32**), butyl lucidenate N (**33**), methyl ganoderate H (**34**), lucidumol B (**35**), ganodermanondiol (**36**), methyl lucidenate N (**37**), and methyl lucidenate A (**38**). Of them, the best activity as inhibitor of NO production was **19** with an IC_{50} value of 4.5 μM.

As a final remark on the anti-inflammatory properties of lanostanes, it is worth mentioning that other studies have been carried out to study lanostanes triterpenes acids in other species, such as *Fomitopsis pinicola*: fomitopinic acids A (**39**) and B (**40**), and their glycosides named fomitosides A-J [54], *Piptoporus betulinus*: polyporenic acids A (**41**) and C (**42**) and their derivatives [111] and *Antrodia camphorate* nonidentified compounds [37], and all of them describe similar mechanisms of action for these type of compounds: inhibition of COX-1 and COX-2 in a prostaglandin biosynthesis assay in vitro [54] and inhibition of reactive oxygen species production in peripheral human neutrophils and mononuclear cells [37] (Fig. 7).

2.4 Potentiality as Anticancer Agents

Before 2013, the majority of lanostanoids isolated from fungi with potential anticancer properties were reviewed by Popović et al. [31]. For that reason, we included here the most recent studies as well as the most relevant compounds described until 2015. There are two principal groups of lanostanoids studied as potential anticancer agents. They are the lanostanes isolated from different species of *Ganoderma* and those isolated from *Poria cocos*.

2.4.1 Lanostanoids Isolated from Species of *Ganoderma*

There is currently a great interest in screening for products that enhance the immune system and with antitumor properties. Compounds in *Ganoderma* spp., in particular in *G. lucidum*, are standing out as important chemotherapeutic candidates. The bioactive compounds in *G. lucidum* are polysaccharides, which stimulate the immune system, and ganoderic acids, a group of lanostane triterpenes, which have demonstrated cytotoxicity against different cancer cells [30]. In this sense, Tang et al. [79] demonstrated that ganoderic acid T (**27**) exerts a dose-dependent cytotoxic activity on several human carcinoma cell lines, being less toxic to normal human cell lines. These authors described the mechanism of action of this cytotoxic effect in the cell line 95-D, and they found that **27** markedly inhibited proliferation by inducing apoptosis and cell cycle arrest at G_1 phase through a mechanism that involved the reduction of mitochondria membrane potential and release of cytochrome C. Moreover, they observed an increase in the expression of pro-apoptotic p53, a decrease in Bcl-2/Bax ratio and caspase-3 activation. Their results demonstrate that **27** induces apoptosis in metastatic lung tumor cells through a mechanism mediated by mitochondrial dysfunction and p53 expression. In this same study, this anticarcinogenic effect was demonstrated in vivo, because **27** (25 mg/kg) suppressed the growth of human solid tumor in athymic mice.

Dehydrotumulosic acid (**24**)

Ganoderic acid R(**25**)

Ganoderic acid S (**26**)

Ganoderic acid T (**27**)

Butyl lucidenate E2 (**28**)

Butyl lucidenate D2 (**29**)

Butyl lucidenate P (**30**)

Butyl lucidenate Q (**31**)

Methyl ganoderate J (**32**)

Butyl lucidenate N (**33**)

Fig. 7 (continued)

Methyl ganoderate H (**34**)

Lucidumol B (**35**)

Ganodermanondiol (**36**)

Methyl lucidenate N (**37**)

Methyl lucidenate A (**38**)

Fomitopinic acid A (**39**)

Fomitopinic acid B (**40**)

Polyporenic acid A (**41**)

Polyporenic acid B (**42**)

Fig. 7 Lanostanoids with anti-inflammatory activity

The results of Tang et al. [79] are consistent with those published one year after by Wang et al. [76], who demonstrate that ganoderic acid Me (**43**) purified from *G. lucidum* mycelia is effective in increasing the immune function, inhibiting tumor growth and metastasis in an in vivo model of Lewis lung carcinoma in C57BL/6 mice. The increase in the immune function was evidenced by an increase in the expression of T helper type 1 cytokines, such as IL-2 and interferon (IFN)-γ, and an increase in the cytotoxic activity of natural killer (NK) cells, induced by **43**. These authors also describe an increase in the protein expression of nuclear factor (NF)-κB in this model; however, other authors have described an opposite regulation of NF-κB, because they describe that this transcription factor is inhibited by ganoderic acids in vitro [75, 150]. In particular, Jiang et al. [75] studied the effect of different ganoderic acids in growth and invasive behavior of breast cancer cells and found that ganoderic acids A (**44**) and H (**45**) suppressed both events in MDA-MB-231 cells. These authors postulate that these lanostane-type triterpenes act through the inhibition of transcription factors AP-1 and NF-κB, therefore resulting in the downregulation of cyclin D kinase 4 (cdk4) and the suppression of secretion of urokinase-type plasminogen activator (uPA), respectively.

Lanostane **44** has also been studied as a sensitizer to chemopreventive treatments, such as cisplatin, in HepG2 cells [150]. As demonstrated by the authors, **44** inhibits both constitutively active and IL-6-induced activation of signal transducer and activator of transcription 3 (STAT3) activity through a mechanism that involves the inhibition of Janus kinase (JAK)-1 and JAK2. This inhibition enhances the sensitivity to cell death induction caused by cisplatin, reinforcing the idea that this lanostane-type triterpene could be a good candidate for combined chemotherapy. Moreover, Liu et al. [151] in a recent study demonstrated that ganoderenic acid B (**46**) have an ATP-binding cassette (ABCB)1-mediated multidrug resistance reversal properties. These authors demonstrated that **46** reversed ABCB1-mediated multidrug resistance of HepG2/ADM cells to doxorubicin, vincristine, and paclitaxel, enhancing intracellular accumulation of rhodamine-123 through inhibition of its efflux, and reversed the resistance of ABCB1-overexpressing MCF-7/ADR cells to doxorubicin.

Other studies carried out by other authors also demonstrate the in vivo anticarcinogenic effect of other compounds isolated from *G. lucidum*, such as lucidenic acids. In particular, Akihisa et al. [78] demonstrated that 20-hydroxylucidenic acid N (**47**) at a dose of 85 nM (topical application) suppresses the incidence of papilloma-bearing mice and the average number of papillomas per mouse in a two-stage carcinogenesis mouse-skin model.

From the species *Ganoderma sinense*, six compounds have been isolated and tested as selective cytotoxic compounds and for their ability to induce human pregnane X receptor (hPXR)-mediated cytochrome P450 3A4 (CYP3A4) expression. Of them, ganoderic acid Jc (**48**) showed selective inhibitory activity against MCF-7 cells with an IC_{50} value of 8.30 μM, whereas ganoderiol E (**49**) had an IC_{50} value of 6.35 μM against HL-60 cells. The other compounds had no activity at concentrations of 40 μM and higher [89]. As inductor of hPXR-mediated CYP3A4 expression, the active compounds were ganodermatetraol (**50**), ganolucitade F (**51**),

ganolucidic acids B (**52**), and C (**53**). Altogether, the studies carried out on the different compounds found in *G. lucidum* and *G. sinense* give a scientific rationale to its use in traditional oriental medicine.

2.4.2 Lanostanes from *Poria cocos*

The alcoholic extract of *Poria cocos* is rich in lanostane triterpenoids that exert antitumor properties in vitro such as DNA polymerase inhibition [115, 121] and inhibition of DNA topoisomerase II [121], showing in vivo anticancer effects in different experimental models, such as skin tumor formation in mouse following initiation with 7,12-dimethylbenz[a]anthracene (DMBA) [78, 117] and Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells [78, 117, 122]. In vitro, lanostanoids from *P. cocos* have demonstrated cytotoxic activity in different cancer cell lines such as human leukemia HL-60 cells [113, 117, 122], melanoma CRL1579 [117] human lung cancer cell line A549, and human prostate cancer cell line DU145 [118, 152], among others. A few studies have gone in depth in the determination of the antitumor mechanism of action of these compounds. Ling et al. [152] studied the mechanism by which polyporenic acid C (**42**) reduces cell proliferation of human lung cancer A549 cells. From their results, it can be concluded that **42** induces apoptosis via activation of caspase-8, which in turn cleaves caspase-3 and poly (ADP-ribose)-polymerase (PARP) with no disruption of the mitochondrial membrane potential, which suggests that the induction of apoptosis which suggests that the induction of apoptosis is mediated by the death receptor. Moreover, they describe an enhanced p53 activation and a suppression of PI3-kinase/Akt signaling pathway.

More recently, Kikuchi et al. [113] described a selective mechanism of action of a different lanostane-type triterpene: poricotriol A (**54**), which varied depending on the cell line. This way, in HL-60 cells, **48** activated caspases 3, 8, and 9 and increased the ratio of Bax/Bcl-2 an apoptosis induction via mitochondrial and death receptor pathways. On the other hand, this compound did not activate these caspases in A549 cells. Instead, the induction of apoptosis occurred via the translocation of apoptosis-inducing factor (AIF) from mitochondria and an increase of the ratio of Bax/Bcl-2. Moreover, **54** showed selective toxicity in lung cancer cells, being only weakly toxic to normal lung cells (WI-38).

Dehydrotrametenolic acid (**17**) inhibits the growth of H-ras transformed rat2 cells with a similar mechanism as **54** in HL-60 cells [153]. It arrests cell cycle in G_2/M phase inducing apoptosis through caspase-3 activation, PARP degradation, chromosomal DNA fragmentation, and Lamin A/C degradation.

Finally, the mechanism of action of pachymic acid (**16**) has also been described in human-derived MDA-MB-231 and MCF-7 breast carcinoma cells [154]). Treatment reduced matrix metalloproteinase-9 (MMP-9) secretion because of the downregulation of its mRNA expression, therefore inhibiting the invasiveness of this cancer cells. According to their results, in this case, the target of **16** is NF-κB signaling pathway, since a decrease in the degradation of inhibitor of κBα (IκBα) and in p65 nuclear translocation was observed, together without altering the phosphorylation states of mitogen-activated protein kinases, such as c-Jun N-terminal kinase (JNK), ERK, and p38.

2.4.3 Other Lanostanoid-Type Triterpenes with Anticancer Properties

The identification of new lanostane-type triterpenes from different mushroom species is generating a huge body of scientific papers. Some of these studies include a screening of antitumor activity of the identified lanostanoids. Among the studied species, we find lanostane-type triterpenes with anticancer activity in *Naematoloma fasciculare* [108, 109], *Antrodia* spp. [33, 35, 155], *Astraeus* spp. [43, 44], *Fomitopsis nigra* [51], *Hebeloma versipelle* [95], *Inonotus obliquus* [98, 102–105, 156], *Daedalea dickisii* [46], and *Scleroderma citrinum* [125].

Moreover, Kikuchi et al. [157] have recently synthesized cyanogenated and oxygenated lanostane-type triterpenes and evaluated their cytotoxic activities against leukemia (HL60), lung (A549), stomach (AZ521), and breast (SK-BR-3) cancer cell lines. Of the 35 assayed compounds, one natural triterpene, and ten semisynthetic triterpenes exhibited potent (IC_{50} values in the range of 1.4–9.9 μM) cytotoxic activity against one or more cell lines. Two lanostane-type triterpenes with a cyanoenone functionality induced apoptosis in HL60 cells via both the mitochondrial and the death receptor-mediated pathways. In addition, in vivo activity was also evaluated on EBV-EA activation induced with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells, and seven natural triterpenes and ten semisynthetic triterpenes exhibited inhibitory effects.

Some of the above-mentioned studies not only screen for the anticancer activity but also try to elucidate the mechanism of action, and it should be pointed out that the majority of the lanostane-type triterpenoids analyzed share a common mechanism: they induce cell cycle arrest followed by apoptosis, PARP cleavage, caspase-3 activation, and DNA fragmentation [35, 46, 51, 98, 103] (Fig. 8).

2.5 Lanostanoids as Anti-infectious Agents

2.5.1 Antibacterial Properties

Different fungi showed anti-infectious properties, and in some cases, lanostanoids have been reported as the active principles. Some isolated compounds did not show antimicrobial activity, such as cattienoids A-C isolated from fruiting bodies of *Tomophagus cattienensis* [128], whereas other showed modest effects, such as compounds isolated from *Fomitopsis pinicola* [52] or *Ganoderma lucidum* [65]. In the first case, the activity of five lanostanes was assayed against *Bacillus cereus* and obtained minimum inhibitory concentration (MIC) values comprised between 16 and 64 μg/mL. Of them, pinicolic acid (**55**) showed the best activity (16 μg/mL), and it was not due to its cytotoxicity. It is worth highlighting that a 16-*O*-acetylation decreases the activity, from 32 μg/mL (**43**) to 128 μg/mL (16α-acetyl-polyporenic acid C, **56**), and increases the cytotoxicity [52]. From *G. lucidum*, the authors isolated seven compounds but only tested two of them. Only 12β-acetoxy-3β,7β-dihydroxy-11,15,23-trioxolanost-8-en-26-oic acid butyl ester (**57**) was active against *S. aureus* and *B. subtilis*, with MIC values of 68.5 and 123.8 μM, respectively [65].

Fig. 8 (continued)

Ganolucidic acid C (**53**) Poricotriol A (**54**)

Fig. 8 Lanostanoids from *Ganoderma* species and *Poria cocos* with antitumoral activity

Other species, such as *Poria cocos*, have been studied for their anti-infectious properties [112, 158]. In a test of 58 samples, the extract of *P. cocos* inhibited both bacterial (*Acinetobacter baumannii* and *Staphylococcus aureus*) and fungal (*Aspergillus fumigatus*) strains [112]. No active compounds have been described; nevertheless, some lanostanes present in this fungus were previously described as antimicrobial [158].

An antibacterial bioassay-guided fractionation led to the isolation and identification of an antibacterial lanostane, 3,11-dioxolanosta-8,24(Z)-diene-26-oic acid (**58**) from *Jahnoporus hirtus*, which had MIC values of 40 and 32 μg/mL against *Bacillus cereus* and *Enterococcus faecalis*, respectively. However, this potency seems to have no interest as antimicrobial agent [32]. *Fomitopsis rosea* is another potential source of antimicrobial lanostanoids, and five compounds showed antibacterial but no antifungal activity. However, the authors did not establish the potency, only the zone of inhibition was determined. The following lanostanes were tested: **42**, 3α-oxepanoquercinic acid C (**59**), and 3α-carboxyacetoxyquercinic acid C (**60**), but its activity was not relevant [55] (Fig. 9).

Other investigations were focused to specific diseases, such as tuberculosis. In this case, Stanikunaite et al. [44] investigated the lanostanes from the mushroom *Astraeus pteridis* as antituberculosis agents. They tested five compounds, but only two showed moderate activity against *Mycobacterium tuberculosis*, giving MIC values of 34 μg/mL for 3-*epi*-astrapteridiol (**61**), 58.0 μg/mL for 3-*epi*-astrahygrol (**62**), and 64 μg/mL for astrahygrone (**63**).

Similar values were obtained from the edible mushroom *Astraeus odoratus* by Arpha et al. [43] who isolated four lanostanes, and two of them, astraodoric acids A (**64**) and B (**65**), exhibited moderate antibacterial activity, with MICs values of 50 and 25 μg/mL, respectively, whereas 3-αOH derivatives were inactive. A compound isolated by Kanokmedhakul et al. [125] from *Scleroderma citrinum* and identified as (20 S,22 S,23E)-22-*O*-acetyl-25-hydroxylanosta-8,23(E)-dien-3-one had no activity against *M. tuberculosis*.

The results of higher interest were obtained with 3-*epi*-ganoderic acid T (**66**) and its 3-isomer **27** both isolated from cultures of the mushroom fungus *Ganoderma orbiforme* BCC 22324, which gave an IC_{50} value of 1.3 and 10 μM against *Mycobacterium tuberculosis* H37Ra. Of ten lanostanes assayed, only two

Pinicolic acid (**55**)

Polyporenic acid C (**56**)

12β-acetoxy-3β,7β-dihydroxy-11,15,23-
-trioxolanost-8-en-26-oic acid butyl ester (**57**)

3,11-dioxolanosta-8,24(Z)-diene-26-oic acid (**58**)

3α-oxepanoquecinic acid (**59**)

3α-carboxyacetoxyquercinic acid C (**60**)

Fig. 9 Lanostanoids with antibacterial properties

demonstrated relevant activity. These were the per-acetylated compounds, whereas partial or nonacetylated derivatives had no activity [87] (Fig. 10).

2.5.2 Antifungal Properties

Ascosteroside (**67**), isolated from cultured broth of *Ascotricha amphitricha*, inhibited the growth of *Saccharomyces cerevisiae* and *Candida albicans*, but not its methyl ester [40]. It is active also against other yeasts (*C. tropicalis*, *C. glabrata*) and filamentous fungi (*Trichophyton mentagrophytes*, *Aspergillus nidulans*) but shows no activity against bacteria (*S. aureus*, *E. coli*). This lanostane at 5 μg/mL gave similar in vitro inhibition zone that fluconazole at 25 μg/mL. Lanostane **17** also had effect in vivo in *C. albicans* infected mice, and at 30 mg/kg/day it increases the survival time in infected mice, with a mean day of death (MDD) of 16.3 days versus 10.2 days for the control group [41].

Astrakurkurol (**68**) and astrakurkurone (**69**) isolated from *Astraeus hygrometricus* also had in vitro activity against *C. albicans*, comparable to standard antifungal

Fig. 10 Lanostanoids with antituberculosis properties

clotrimazole [42]. Both lanostanes showed similar inhibition zone at the assayed doses (0.2–10 μg/disk), with an inhibition range of 25 mm (10 μg/disk) to 9 mm (0.2 μg/disk) for **68** and 29 mm (10 μg/disk) to 10 mm (0.25 μg/disk), whereas the reference drug had an inhibition zone of 15 mm at 20 μg/disk for **69** [42] (Fig. 11).

2.5.3 Antiparasitic Properties

Both **68** and **69** were tested as potential leishmanicidal agents, but only **69** showed relevant effects. Certainly, **69** significantly inhibited the growth of *Leishmania donovani* promastigotes in vitro. At a concentration of 10 μg/mL, **69** inhibited its growth by 68 % (2nd day), 91 % (4th day), and 95 % (6th day) versus control (dimethyl sulfoxide). The reference drug, miltefosine, at the same dose only inhibited by 49 % on 6th day of culture, a value significantly lower than that of **69** [42].

HOH$_2$C O H$_3$CO HO OH O COOH OH H Ascosteroside (**67**)

O OEt HO H Astrakurkurol (**68**)

O O HO H Astrakurkurone (**69**)

Fig. 11 Lanostanoids with antiparasitic and antifungal properties

2.5.4 Antiviral Properties

The anti-human immunodeficiency virus (HIV) has been also tested for different lanostanoids from fungi: five compounds isolated from the spores of *Ganoderma lucidum* showed anti-HIV properties: ganoderic acid β (**70**), **35**, **36**, **23**, and ganolucidic acid A (**71**). They showed significant anti-HIV-1 protease activity with IC_{50} value range of 20–90 μM [82]. Ten years later, El Dine et al. [62] isolated a series of lanostane triterpenes from the mushroom *Ganoderma colossum*, which were tested as potential HIV-1 protease inhibitor, and four of them gave IC_{50} values under 14 μg/mL. Colossolactone G (**72**) showed the highest potency (5 μg/mL), followed by schisanlactone A (**73**, 8 μg/mL) and colossolactone V (**74**, 9 μg/mL), whereas colossolactone VII (**75**, 13.8 μg/mL) was the less potent of these compounds. Other lanostanes showed moderate or no activity. Sato et al. [90] also tested a series of lanostanoids isolated from the fruiting body of *Ganoderma sinense*, and four of them showed modest inhibitory effects on HIV-1 protease, with IC_{50} values of 20–40 μM. The active compounds were **15** (22 μM), (**47**) (25 μM), ganoderic acid GS-2 (**76**, 30 μM), and 20(21)-dehydrolucidenic acid N (**77**, 48 μM). Values of other analyzed compounds were higher than 50 μM [90]. A previous and limited relationship between the chemical structure and the activity was established. In the case of 24(25) unsaturated ganoderic acids, the 3-oxo derivatives had more inhibitory effects than the 3-hydroxy compounds. However, in the case of lucidenic acids, the 3-hydroxy compounds showed higher inhibitory action than the 3-oxo derivatives. Finally, in the case of ganoderma alcohols, the 24 (25) unsaturated compounds were more active than the 24-hydroxy derivatives [90] (Fig. 12).

Ganoderic acid β (**70**)

Ganolucidic acid A (**71**)

Colossolactone G (**72**)

Schisanlactone A (**73**)

Colossolactone V (**74**)

Colossolactone VII (**75**)

Ganoderic acid GS-2 (**76**)

20(21)-dehydrolucidenic acid N (**77**)

Fig. 12 Lanostanoids anti-human immunodeficiency virus

Ganoderic acid XL_1 (**78**)

Ganoderenic acid AM_1 (**79**)

Ganoderesin C (**80**)

Lucidone B (**81**)

Fig. 13 Lanostanoids with hepatoprotective properties

2.6 Other Properties of Interest

Some authors [86] have studied the possible hepatoprotective effect of lanostanoids isolated from *Ganoderma theaecolum*. They tested the activities of ten lanostanoids on a human hepatic cell (HL-7702) injury model induced by DL-galactosamine using a colorimetric assay, and the results were compared with a hepatoprotective drug used in clinic, bicyclol. Six of the ten assayed compounds had significant activity at a concentration of 10 μM, with a survival rate from 55 % to 80 % versus 55 % for the positive control. Active compounds were **10**, **46**, ganoderic acid XL_1 (**78**), ganoderenic acid AM_1 (**79**), ganoderesin C (**80**), and lucidone B (**81**) (Fig. 13).

3 Future Perspectives and Conclusions

Lanostanes constitute a group of tetracyclic triterpenes common in fungi. Some of them have peculiarities in their chemical structures, which confer a clear difference with lanostanoids isolated in plant kingdom. These characteristics give lanostanes from fungi a clear interest and potentiality as medicinal agents. The majority of studies focus on their cytotoxic effects and their possible interest as anticancer agents, as in the case of lanostanes from *Ganoderma lucidum*, which have been widely studied. Different compounds and mechanisms of action have been proposed

for them, such as induction of apoptosis (30), the cell cycle arrest, modification of the transporting or expression of proteins, and inhibition of transcription factors implicated in the tumorigenic process. However, the added value of these triterpenoids relies in their selective inhibition of topo II activity without affecting topo I, and their ability to enhance chemosensitivity to other anticancer drugs, such as vinblastine among others, reducing their IC_{50} values and their nondesirable side effects [30].

Fungi lanostanes have also risen interest for their anti-inflammatory activity, since some compounds have a specific anti-inflammatory mechanism, such as lanostanes from *Poria cocos* as anti-PLA_2 inhibitors, and ganoderic acids from *Ganoderma lucidum* as inhibitors of NO production, through the inhibition of iNOS, as well as inhibition of TNF-α and IL-6 secretion, and COX-2 and HO-1 expression, all of them via the PI3K/AKT-Nrf2 pathway [64, 66].

The third interesting activity of lanostanes is their potential effects on metabolic disorders such as diabetes mellitus and hyperlipidemias. In this case, lanostanes can act by different mechanisms, such as the inhibition of aldose reductase and α-glucosidase activities, avoiding the damage caused by sorbitol, the formation of cataracts in chronic diabetic patients (aldose reductase inhibition) and reducing the production of glucose from starch, a mechanism that implies an increase in GLUT4 expression without affecting GLUT1. In this sense, the hypoglycemic activity of some lanostanes (especially pachymic acid, **17**, from *P. cocos*) is due to their insulin-like activity, which stimulates glucose uptake, GLUT4 gene expression, and translocation and promotes triglyceride accumulation in adipocytes. In addition, the promotion of adipocyte differentiation, adipogenesis inhibition, and FXR agonist properties give some lanostanes high interest for clinical investigation in the treatment of cholestasis, dyslipidemic disorders, and conditions of insulin resistance in hypercholesterolemia and T2DM [69, 140–146].

Finally, the potential as anti-infectious agents seems to be of reduced interest as can be observed when antimicrobial or antifungal values are analyzed. Some compounds can be of interest for future research, such as ascosteroside (**67**) as antifungal or colossolactone G (**72**) as antiviral.

Taken together, the studies on lanostanes from fungi have a high interest for future research, especially for their novelty; the effects on metabolic disorders, such as metabolic syndrome, can be relevant.

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Kombucha Tea: Metabolites

30

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Abstract
Kombucha, fermented black tea with symbiotic association of bacteria and yeast, has been claimed by its drinkers for several health benefits. Health benefits of kombucha tea are directly associated with the composition and the concentration of the biomolecules present in it. Being a product fermented by bacteria and yeast association, kombucha has very complex composition which has a range of components from tea plant, bacteria, yeast, and compounds produced during fermentation process. The compounds responsible for the claimed benefits of kombucha have not been explored due to its complexity. This chapter focuses on the metabolites of kombucha which have been reported.

Keywords
Acetic acid bacteria • Cellulose pellicle • Fermentation • Fermented tea • Kombucha • Kombucha tea • *Medusomyces gisevii* • Symbiotic culture • Tea beverage • Tea fungus • Tea fermentation • Yeast

1 Introduction

Kombucha tea is a slightly sweet, slightly acidic refreshing beverage consumed worldwide, obtained by the fermentation of sugared tea by a symbiotic association of bacteria and yeasts, forming "tea fungus" [1]. The tea fungus broth is composed of two portions, a floating cellulosic pellicle layer and the sour liquid broth (Fig. 1). This refreshing beverage tasting like sparkling apple cider is often produced in the home by fermentation using a tea fungus passed from home to home. Black tea and white sugar are the best substrates for the preparation of kombucha, although green tea can also be used. Tea fungus is a best example of biofilm made by symbiotic association of acetic acid bacteria and yeasts. The association of Kombucha with human was reported to be since BC, but the exact details about the origin are unclear. Details about the invention of tea fungus are also missing in the history. Kombucha tea is prepared by inoculating the tea fungus culture into cooled sugared tea decoction along with some amount of previous batch of fermented tea and allowing fermenting in dark for 7–14 days. During fermentation, the pH reduces drastically due to production of organic acids from added sugar due to yeast and bacterial metabolism. Tea polyphenols undergo degradation or transformation by the enzymes of bacteria and yeast which was evident by changes in color of the black tea during the course of fermentation. Various enzymes have been reported to be active in kombucha tea. Tea fungus is basically cellulose network where bacteria and yeast cells are attached which finally appears as a jelly membrane. The thickness of this biofilm is due to the deposition of cellulose as layer by layer during fermentation time. Reports about the first use of tea fungus, its formation for the first time, and inventor details are missing in the history. Kombucha has been reported continuously by scientific community and users for its health benefits. Composition and the concentration of the metabolites available in kombucha after the required time

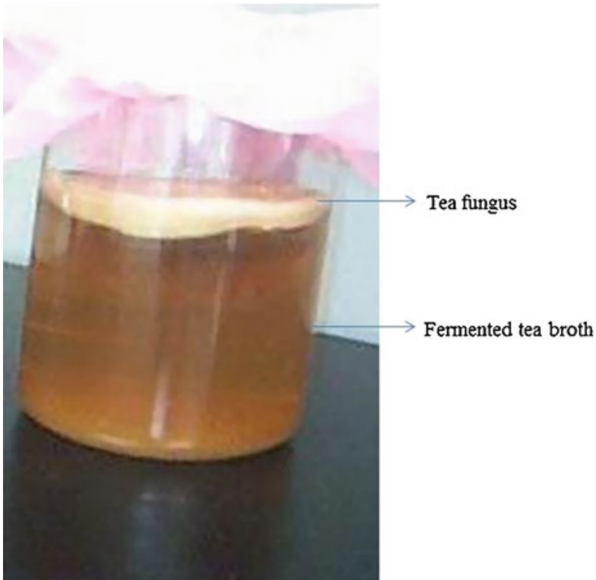


Fig. 1 Kombucha black tea having fermented broth and tea fungus (Reproduced with prior permission, Jayabalan et al. [2])

of fermentation would be the sole reason for the health benefits claimed. Kombucha has a range of metabolites originated from tea plant, bacterial metabolism, yeast metabolism, sugar, and the biotransformed compounds produced during fermentation which makes it very complex to study even by the state-of-the-art instruments [2]. It is surprised to see in the literature that there were very less attempts taken to reveal the complex composition of kombucha tea. As an initiative to achieve this, the present chapter focussed to review the metabolites already reported in the literature.

2 Kombucha Tea Preparation

Kombucha tea is traditionally prepared by freshly making sugared tea decoction and inoculating the portion of tea fungus and previously fermented kombucha. The preparation will be covered with paper towel or cheese cloth and will be kept for fermentation in dark at room temperature for 7–14 days. After fermentation, the fermented beverage will be separated from the newly formed tea fungus and filtered through cheese cloth. The filtered beverage will be refrigerated and consumed whenever required. The traditional preparation was subjected to different modifications based on the taste of kombucha drinkers. The modification lies in the amount of sugar, amount and types of tea substrate, time taken for preparing the tea decoction, period of fermentation, amount of inoculum, and fermentation temperature [2].

3 Beneficial Effects of Kombucha Tea

Kombucha tea has been claimed to have several beneficial effects on human health by the kombucha drinkers from all over the world. Except few, reported effects on human health are yet to be studied scientifically. Reported effects of kombucha from tea drinkers' testimony and Russian researchers are to [3]:

Detoxify the blood
Reduce cholesterol level
Reduce atherosclerosis by regeneration of cell walls
Reduce blood pressure
Reduce inflammatory problems
Alleviate arthritis, rheumatism, and gout symptoms
Promote liver functions
Normalize intestinal activity, balance intestinal flora, and cure hemorrhoids
Reduce obesity and regulate appetite
Prevent/heal bladder infection and reduce kidney calcification
Stimulate glandular systems
Protect against diabetes
Increase body resistance to cancer
Have an antibiotic effect against bacteria, viruses, and yeasts
Enhance the immune system and stimulate interferon production
Relieve bronchitis and asthma
Reduce menstrual disorders and menopausal hot flashes
Improve hair, skin, and nail health
Reduce an alcoholic's craving for alcohol
Reduce stress and nervous disturbances and insomnia
Relieve headaches
Improve eyesight
Counteract aging
Enhance general metabolism

4 Biochemical Composition of Kombucha Tea

Several scientific investigations published in the last 15 years have reported the presence of several metabolites in kombucha tea. The composition of kombucha tea, what is known today, is given in Fig. 2. All the analysis carried out to explore the biochemical composition was done in static mode. The biochemical composition of tea fungus and kombucha tea was not similar in all the reports. This might be due to the fact that the microbial composition of tea fungus varies with region and country. Hence, the metabolites produced by the bacteria and yeasts also vary which reflects in the chemical composition of kombucha tea. The difference in composition may also be due to the differences in amount of sugar and tea substrate, differences in the

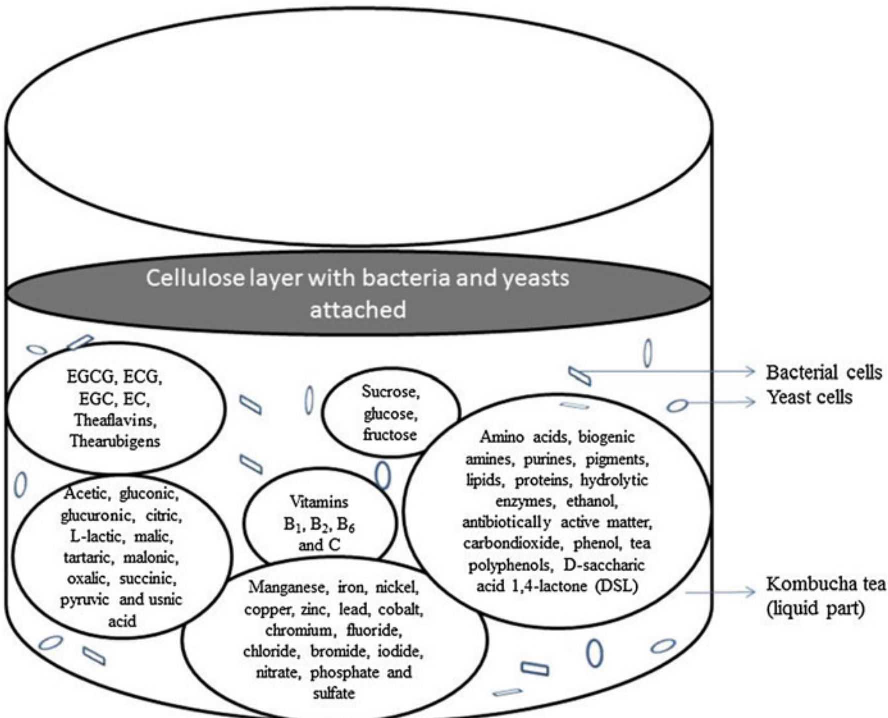


Fig. 2 Biochemical composition of kombucha tea

amount of tea fungus and kombucha tea used as inoculum, and difference in fermentation time. However, uniform trends of change in some property have been discussed by most of the researchers. For example, reduction in pH, increase in content of organic acids, and initial increase and intermittent decrease in concentration of bacteria and yeast cells in tea broth were observed by many researchers around world irrespective of the above mentioned differences. Few reports have also revealed the trend of increase in antioxidant activity throughout the fermentation time. Considering the contents of the biomolecules present in kombucha, an intermittent increase and decrease during fermentation time was observed for most of the compounds studied. Table 1 lists out the concentration of metabolites measured in kombucha tea [2].

4.1 Sugar

Kombucha drinkers use only table sugar (sucrose) for the preparation and hence the scientific analysis. One molecule of alpha-D-glucose and beta-D-fructose linked by an alpha-1,4-glycosidic bond make one molecule of a disaccharide, sucrose.

Table 1 Predominant components in kombucha tea at the end of the fermentation on sugared black tea infusion (Reproduced with prior permission, Jayabalan et al. [2])

Component	Component content (g/L)	Initial sucrose (%)	Black tea	Fermentation temperature (°C)	Fermentation time (days)
Acetic acid	8	10	2 bags	24 ± 3	60
	4.69	10	12 g/L	24 ± 3	18
Glucuronic acid	0.0031	5	1.5 g/L	28	21
	0.0026	7	1.5 g/L	28	21
	0.0034	10	1.5 g/L	28	21
	1.71	10	12 g/L	24 ± 3	18
Gluconic acid	39	10	2 bags	24 ± 3	60
Glucose	179.5	7	1.5 g/L	28	21
	24.59	7	1.5 g/L	28	21
	12	10	2 bags	24 ± 3	60
Fructose	76.9	7	1.5 g/L	28	21
	5.40	7	1.5 g/L	28	21
	55	10	2 bags	24 ± 3	60
Remaining sucrose	192.8	7	1.5 g/L	28	21
	11	10	2 bags	24 ± 3	60
	2.09	7	1.5 g/L	28	21

Hydrolysis of alpha-1,4-glycosidic bond releases equimolar mixture of glucose and fructose. During kombucha fermentation, the yeast cells from the initial inoculum hydrolyse sucrose to glucose and fructose by producing invertase or sucrase enzyme (beta-fructofuranosidase, EC 3.2.1.26). Due to its broad range in acidic pH (3.5–5.5), invertase activity is not inhibited by acids produced during kombucha fermentation, and hence the added sucrose is continuously hydrolysed to glucose and fructose.

$$\text{Sucrose } (C_{22}H_{12}O_{11}) \xrightarrow{\text{Yeast Invertase}} \text{Glucose } (C_6H_{12}O_6) + \text{Fructose } (C_6H_{12}O_6)$$

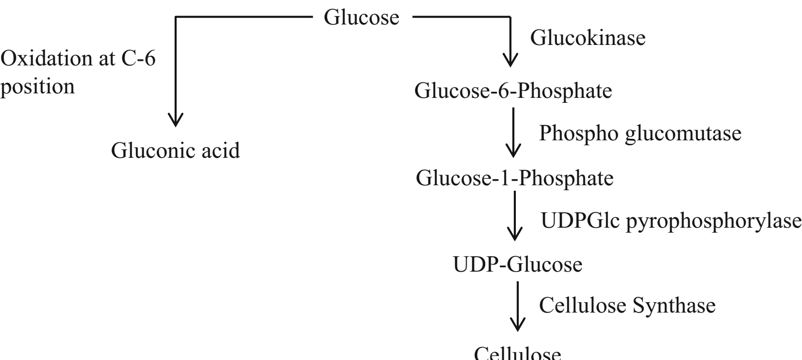
Yeast cells consume most of the fructose released by invertase action through glycolysis and convert them to ethanol and carbondioxide. The produced ethanol is rapidly oxidized to acetic acid by acetic acid bacteria present in the consortium. Acetic acid is the predominant organic acid produced during fermentation and the main reason for the pH decrease. Acetic acid bacteria also oxidizes glucose to gluconic acid [2].

$$\text{Fructose } (C_6H_{12}O_6) \xrightarrow{\text{Yeast Glycolysis}} \text{Ethanol } (CH_3CH_2OH) + \text{Caron dioxide } (CO_2)$$

4.2 Cellulose

Cellulose is a homopolysaccharide composed of beta-D-glucose monomers linked by beta 1,4-glycosidic bond. Cellulose is the predominant material found in tea fungus and is produced by aerobic acetic acid bacteria found in its consortium. Acetic acid bacteria found in the air–liquid interface of the vessel used to produce kombucha produces cellulose biofilm in direct contact with oxygen to protect themselves from the high stressful growth conditions due to the presence of high concentration of acetic acid or ethanol [4]. Biofilm production by acetic acid bacteria is reported to be through cell–cell communication via quorum-sensing signaling [5]. Synthesis of cellulose involves the synthesis of uridine diphosphoglucose (UDPGlc) by UDPGlc pyrophosphorylase which were later polymerized into long and unbranched chains through beta-1,4-glycosidic bond by cellulose synthase enzyme. However, the conversion of glucose to UDPGlc requires two more additional steps which converts initial glucose molecules to glucose-6-phosphate by glucose kinase and then to glucose-1-phosphate by phosphoglucomutase (Scheme 1). It is also possible to produce cellulose through fructose by its conversion to glucose-6-phosphate through successive actions of fructose kinase and phosphoglucose isomerase enzymes. But fructose may not be available to acetic acid bacteria due to the action of yeast cells. Only part of the cellulose would be available for cellulose synthesis since glucose is also oxidized to gluconic acid by acetic acid bacteria. Both gluconic acid production and cellulose synthesis requires the presence of oxygen and this is the reason why cellulose layer formation occurs only at the air–liquid interface of the kombucha fermentation vessel [2].

Bacterial cellulose prepared from pellicles of *A. xylinum* (*Gluconacetobacter xylinus*) is a unique biopolymer in terms of its molecular structure, mechanical strength, and chemical stability [11]. A similar cellulose network floating on the surface of various fruit juices fermented by a symbiotic culture composed of *A. xylinum* and yeasts and named “nata” is consumed in Philippines as a delicacy.



Scheme 1 Formation of gluconic acid and cellulose from cellulose

In Brazil, this cellulose network is used for the treatment of skin burns and other dermal injuries and is produced by a pure culture of *A. xylinum* grown on a medium composed mainly of sucrose and tea xanthines [6]. Caffeine and related compounds (theophylline and theobromine) are identified as activators for cellulose production in *A. xylinum* [7]. In ancient days, this cellulose biofilm has been used for the treatment of wounds. Microbial cellulose synthesized in abundance by *Acetobacter xylinum* shows vast potential as a novel wound healing system. The high mechanical strength and remarkable physical properties result from the unique nanostructure of the never-dried membrane [8].

4.3 Organic Acids

Kombucha was reported to have several acetic, gluconic, glucuronic, citric, L-lactic, malic, tartaric, malonic, oxalic, succinic, pyruvic, and usnic acids. Most of these acids are having origin of the tea substrate used to prepare kombucha tea. About 0.5–0.6 % of dry weight of fresh tea shoot consists of organic acids. Among the organic acids present in kombucha tea citric, malic, tartaric, oxalic, and succinic acids are reported to be present in fresh tea shoots [9]. Acetic, gluconic, glucuronic, L-lactic, malonic, pyruvic, and usnic acids present in kombucha tea are produced by the action of microbes on sugar during fermentation time. Acetic acid is the predominant organic acid and is produced by acetic acid bacteria through oxidation of ethanol. It is the main reason for the decrease in pH. Due to increased concentration of organic acids produced during the fermentation process by bacteria and yeasts in the tea fungus consortium, the pH value decreased from 5.0 to 3.0. Apparently the fermentation broth possessed some buffer capacity. During the fermentation process, carbon dioxide is released at first slowly and much faster after 2–3 days. The obtained water solution of carbon dioxide dissociates and produces the amphiprotic hydrocarbonate anion (HCO_3^-), which easily reacts with hydrogen ions (H^+) from organic acids, preventing further changes in the H^+ concentration and contributing to a buffer character of the system. This will be the valid reason for slight decrease in pH after 3 days [2].

Acetic acid was reported even when the sugar source was molasses. L-lactic and citric acid is not characteristic compound for traditional kombucha beverage. L-lactic acid was detected in traditional kombucha beverage and even when molasses and green tea was used as sugar source and tea substrate, respectively. Citric acid was detected in very small amount when black tea and green tea was used as tea substrate [2]. Oxidation of first carbon of glucose gives gluconic acid and at sixth carbon gives glucuronic acid (Fig. 3). Glucuronic acid is therapeutically important due to the detoxification action inside human body. Conjugation of glucuronic acid with undesirable compounds results in decreased toxicity due to the increased solubility of them that further facilitates transport and elimination from the body. Glucuronidation is aided by UDP glucuronosyltransferases enzyme [10]. Acetic acid bacteria convert glucose to gluconic acid and ethanol to acetic acid by oxidation.

Fig. 3 Structure of D-gluconic and D-glucuronic acid

D-gluconic acid	D-glucuronic acid
COOH	CHO
H — C — OH	H — C — OH
HO — C — H	HO — C — H
H — C — OH	H — C — OH
H — C — OH	H — C — OH
CH_2OH	COOH

4.4 Total Phenolic Compounds

Total phenolic compounds were progressively increased with fermentation time. Phenolic compounds are considered as high-level antioxidants because of their ability to scavenge free radical and active oxygen species such as singlet oxygen, superoxide free radicals, and hydroxyl radicals. Complex phenolic compounds in green tea, black tea, and waste tea might be subjected to degradation in acidic environment of kombucha and by the enzymes liberated by bacteria and yeast in tea fungus consortium. So, there are many chances for the enzymes liberated by bacteria and yeast during kombucha fermentation which be the reason for the degradation of complex polyphenols to small molecules which in turn results in the increase of total phenolic compounds [2].

4.5 Tea Polyphenols

Source of polyphenols in kombucha tea is the tea substrate, black tea, or green tea. The amount of polyphenols present depends on the variety or the grade of tea substrate, the amount used, brewing time given to prepare decoction, and time of fermentation. Total phenol content of tea decoction increases with time during kombucha fermentation. Gallic acid, epicatechin isomers (-)-epigallocatechin-3-gallate, (-)-epigallocatechin, (-)-epicatechin-3-gallate, (-)-epicatechin, theaflavin isomers, and thearubigins were detected and quantified during fermentation period (Fig. 4a, b, c). Theaflavin isomers (theaflavin-3-gallate, theaflavin 3′-gallate, and theaflavin 3,3′-digallate) were not detected in kombucha tea. Highly complexed polyphenols like EGCG and ECG undergo degradation and get converted to EGC and EC, respectively, which was evident through the quantification of polyphenols. The color of finally fermented kombucha tea is lighter than the initial tea decoction. This suggests that the compounds responsible for color, thearubigins undergo degradation in acidic environment of kombucha or by the enzymes liberated by bacteria and yeasts. Loss in initial color might be also due to the microbial or enzymatic transformation of thearubigins to less colored compounds [2].

a

(-)-Epicatechin (EC)

(-)-Epigallocatechin (EGC)

(-)-Epicatechin-3-gallate (ECG)

(-)-Epigallocatechin-3-gallate (EGCG)

b

Theaflavin [TF_1]: $R_1 = R_2 = OH$
Theaflavin-3-gallate [TF_2A]: R_1=Galloyl: R_2=OH
Theaflavin-3'-gallate [TF_2B]: R_1=OH; R_2=Galloyl
Theaflavin-3-3'-digallate [TF_s]: $R_1 = R_2$Galloyl

c

R – Galloyl or other groups

Fig. 4 Structure of epicatechin isomers (**a**), theaflavin isomers (**b**), and thearubigins (**c**)

5 Microbial Composition of Kombucha

The name "Kombucha" usually denotes the beverage prepared through fermentation or the inoculum (tea fungus) used to ferment. Tea fungus or *Medusomyces gisevii* is a symbiotic association of bacteria and yeast in cellulose biofilm (Fig. 5a, b). The name "tea fungus" is wrongly given to this association by local people due to its resemblance with the statically grown fungus mat or with the upper portion of the mushroom. Bacteria belong to acetic acid producers and yeasts belong to osmophilic group. Cellulose is the metabolite produced by acetic acid bacteria when it is aerobically grown and microbial cells present near the cellulose fibers are trapped inside it. Composition of microbes present in tea fungus is not similar throughout the world. The composition of metabolites of kombucha tea is depending on the

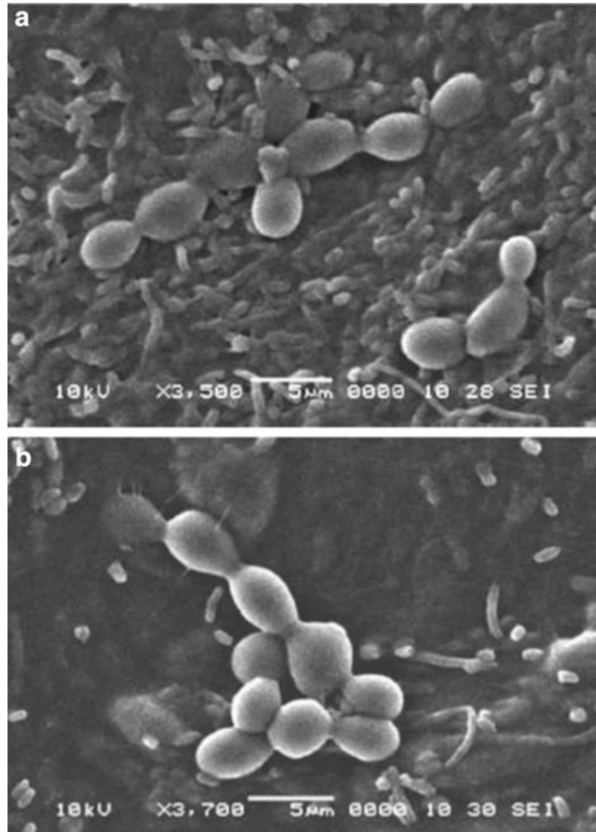


Fig. 5 Scanning electron microscope image of the consortia of yeasts and bacteria in a portion of tea fungus (magnification 4A = 3500× and 4B = 3700×) (Reproduced with prior permission, Jayabalan et al. [2])

metabolism of the microbes present in this symbiotic association. Hence, the composition of metabolites of kombucha is not same everywhere. Bacteria belong to the genus *Acetobacter* and *Gluconobacter*. Among *Acetobacter*, *A. xylinum, A. pasteurianus, A. aceti, A. intermedium* sp. nov., and *Acetobacter nitrogenifigens* sp. nov are reported. *Gluconobacter oxydans, Gluconoacetobacter* sp. A4, and *Gluconoacetobacter kombuchae* sp. nov. are found to be present among Gluconobacter genus. Presence of *Lactobacillus* species were reported very recently. Yeasts in tea fungus includes the genus *Brettanomyces/Dekkera, Candida, Kloeckera, Mycotorula, Mycoderma, Pichia, Saccharomyces, Schizosaccharomyces, Torulospora,* and *Zygosaccharomyces.* Genus *Brettanomyces* includes *Brettanomyces intermedius, B. bruxellensis,* and *B. claussenii*. The reported species in the genus *Candida* includes *Candida famata*, *C. guilliermondii, C. obutsa*, *C. famata*, *C. stellate*, *C. guilliermondi, C. colleculosa, C. kefyr,* and *C. krusei. Saccharomyces* genus includes *Saccharomyces cerevisiae* and *Saccharomyces bisporus*. *Schizosacchromyces* genus was found to have *Schizosaccharomyces pombe* and *Zygosaccharomyces* was identified as *Zygosaccharomyces rouxii*,

Zygosaccharomyces bailii, and *Zygosaccharomyces kombuchaensis* sp. n. Apart from these yeast species, *Sacchromyccoides ludwigii* and *Schizosaccharomyces pombe* were also reported. The following yeast species were also reported: *Torula*, *Torulopsis*, *Torulaspora delbrueckii*, *Mycoderma*, *Pichia*, *Pichia membranefaciens*, *Kloeckera apiculata*, and *Kluyveromyces africanus*. It is reported that viable count of acetic acid bacteria and yeast reached maximum after 6 days of fermentation and continued to decrease in latter period of fermentation. The decreased number of bacteria and yeast during latter period of fermentation was likely caused by acid shock (low pH), which influenced the multiplication of bacteria and yeast [2]. Chen and Liu [1] reported that anaerobic and starved environment created could also be the reason for the decrease in microbial content during the fermentation period. Carbon dioxide generated as a result of alcohol fermentation by yeasts accumulated in the interface between the pellicle and broth. This separates the pellicle from the broth and creates an anaerobic and starved environment due to block of transfer of nutrients from broth to pellicle and transfer of oxygen from the surface of the pellicle to broth. There are controversial statements existing in literature about the concentration of viable microbial cells in tea broth and cellulose pellicle.

6 Other Minor Metabolites

Yeast cells produce ethanol as a fermentative product from fructose through glycolysis and by the action of pyruvate dehydrogenase and alcohol dehydrogenase during kombucha fermentation. Ethanol was detected only in very less concentrations (0.55 %) due to its oxidation to acetic acid by acetic acid bacteria. Water soluble vitamins B1, B6, B12, and C are reported to be present in kombucha prepared with traditional substrates, sugar, and black tea. Yeasts are responsible for the biosynthesis of B vitamins. Presence of manganese, iron, nickel, copper, zinc, lead, cobalt, and chromium was determined in kombucha beverage. Essential minerals like copper, iron, manganese, nickel, and zinc were increased during fermentation period. Due to the inclusion of cobalt in vitamin B_{12}, it is not increased. Presence of anionic minerals like fluoride, chloride, bromide, iodide, nitrate, phosphate, and sulphate is also proved. The content of d-saccharic acid 1,4 lactone (DSL) increases during fermentation period up to 8th day and found to be decreased after that. Likewise, the protein content of kombucha beverage also increases up to 12th day of fermentation and started to decrease after that. It may be due to the decrease in content of extracellular proteins secreted by the bacteria and yeasts [2].

7 Factors Influencing the Presence and Concentration of Kombucha Metabolites

Presence of different metabolites and their concentration in Kombucha from different regions cannot be similar due to the following reasons:

i. Changes in the microbial composition of tea fungus consortium
ii. Changes in the variety of tea substrates
iii. Changes in the amount of sugar, tea, inoculum, and temperature
iv. Changes in the fermentation time

8 Conclusion

Beneficial effects reported for kombucha drinking are based on the presence and concentration of polyphenols, organic acids, and other micronutrients produced during fermentation. Tea polyphenols are important in cancer prevention and other metabolites are essential for the beneficial effects of kombucha tea. It is expected that there will be an influence of microorganisms present in kombucha on the concentration of kombucha metabolites. Changes in the concentration of metabolites during fermentation period and the detailed composition of kombucha tea have not been studied well. Studies on biotransformation of components by acidic environment and enzymes of microbes during kombucha fermentation will be interesting and provide details of therapeutic benefits of kombucha tea.

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