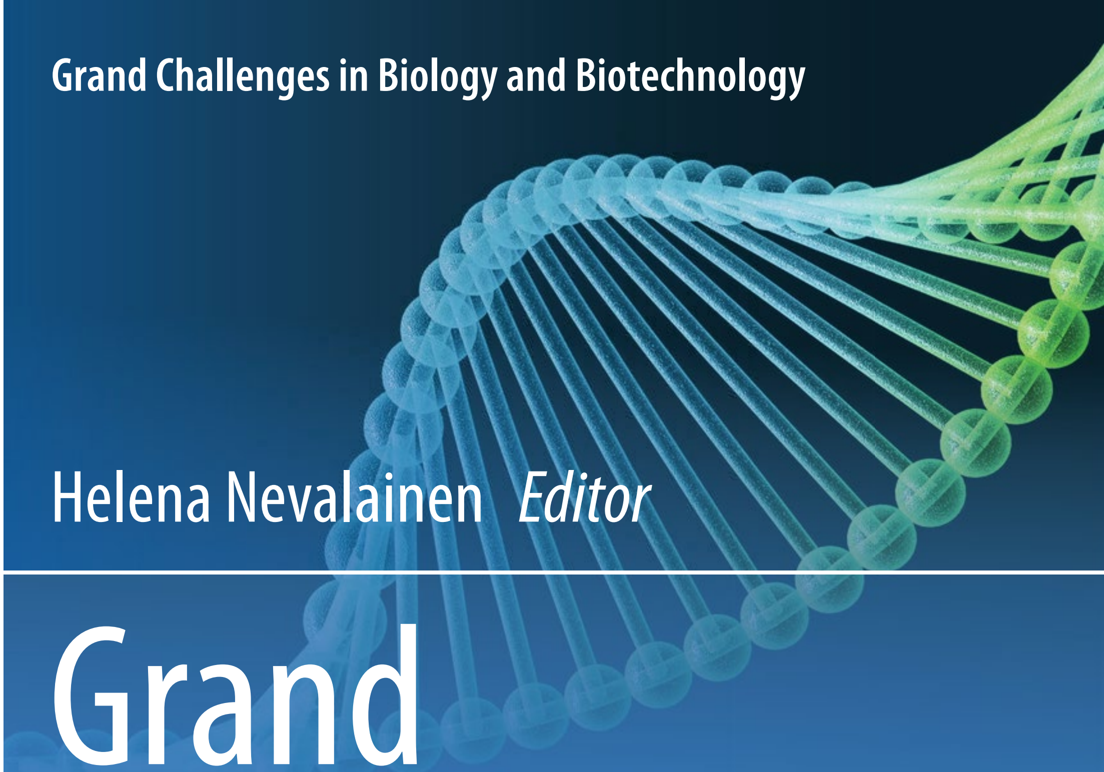
Grand Challenges in Biology and Biotechnology



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Grand Challenges in Fungal Biotechnology

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Grand Challenges in Biology and Biotechnology

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Grand Challenges in Fungal Biotechnology

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Preface

The twenty-first century is facing a number of challenges in the areas of health, developing sustainable bioeconomy, facilitating agricultural production and securing practices that support a cleaner environment. While there are chemical solutions to some of the challenges, finding biobased solutions is becoming increasingly important. Nature has produced organisms that can perform some truly amazing chemistry at a level of sophistication that human chemists simply cannot match. Filamentous fungi that have earned monikers like 'the forgotten kingdom' and 'the highly productive black box' are one such group of organisms of which the full potential is yet to be exploited. Some of their properties, such as the extraordinary capacity to secrete enzymes into the external environment, have been harnessed for the production of industrial enzymes and for purposes such as hydrolysis of cellulosic biomass for biofuel production. Efficient improvement of current strains is pending on the success of key enabling molecular technologies including high-throughput screening and molecular technologies enabling targeted editing of the fungal genome, discussed in this book.

The 18 chapters in the book are organized into four parts. The first part opens the discussion by providing an overview of global challenges and describes some of the approaches taken towards utilizing fungi as food and their capabilities for degradation of environmental pollutants. Hydrolysis of plant and algal biomass is discussed with a view of obtaining valuable products thereof and current challenges in the process. The second part is dedicated to key enabling technologies for fungal strain improvement providing discussion on the importance of understanding the genetic constitution and gene regulation in the organism of interest and advances of high-throughput screening of fungal strains. The third part touches upon the economic potential of fungal biotechnology taking the reader through the laboratory to the market place and explores fungi-based solutions in the area of agriculture. The fourth and final part looks into emerging opportunities in the fungal biotechnology. This part introduces small molecules produced by fungi and highlights the broad scope for new applications of fungal biotechnology on both Earth and the Space.

This book is thought-provoking reading for research scientists and biotechnologists in academia and industry, readers particularly interested in fungal biotechnology and also those working within the broader area of microbial biotechnology. With stand-alone chapters written in an accessible language, the book is also recommended as a reference text for decision-makers in government and non-governmental organizations in their efforts to foster the development of cleaner technologies and global bioeconomy. The solutions that nature has developed to cope with challenging biochemical problems provide inspiration for further innovations in industrial processing technologies, food security and environmental protection amongst other challenges.

Sydney, Australia Helena Nevalainen

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Part I

Fungal Biotechnology and the Global Challenges

Chapter 1
Fungal Biotechnology: Unlocking the Full Potential of Fungi for a More Sustainable World

Lene Lange, Jane W. Agger, and Anne S. Meyer

1 Introduction

Fungi have a paramount presence in the common global food culture heritage and are essential for making bread, beer and wine, as well as products such as tempeh (by *Rhizopus oryzae* or *R. oligosporus*), miso/tamari (species of *Aspergillus*) and red yeast (*Monascus* spp.), widely consumed in Asia. They also provide unique characteristics and taste to a series of cheese products, e.g. blue vein cheese (*Penicillium roquefortii* and other *Penicillium spp.*) and brie and camembert cheeses (*P. candidum, P. camemberti*). The control of infectious diseases through use of penicillin is harnessing the rich fungal metabolome, and so are the many other fungal-derived drugs (e.g. cephalosporin, cyclosporine, statins and strobilurins). As an example, control of hyper-cholesterol in humans may be highlighted, where use of the fungal metabolite statin (and its derivatives) is among the globally most commonly used type of drug. The so-called white biotechnology is an established and growing business. It includes novel production (by filamentous fungi) of technical compounds, such as itaconic, gluconic and citric acid, and is hereby along with production of industrial fungal enzymes dominating the market of fungal biotechnology. The next steps in white biotechnology are already on their way. The producers of these components (often in collaboration with academia) are developing the business by expanding it into a production of platform chemicals and fungal-derived building blocks for materials, many of which could potentially replace petroleum-based chemistry (by drop in products) but also lead to developing materials with new value-adding functionalities. Notably, the residual fungal biomass

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from such commercial-scale fungal productions is in general not upgraded to higher-value products; most common use is as feedstock for biogas production.

Fungal enzymes are considered essential for converting lignocellulose and other types of plant biomass to valuable products (Gupta et al. 2016). Fungal genomes appear an almost inexhaustible source for discovery of new enzymes, when considering how many enzymes are already found, described and brought in use, from tapping into only a minute part of the fungal kingdom. However, use of fungi today goes far beyond these examples and includes employing fungi as cell factories or as production hosts for manufacture of different products and metabolites (Amor et al. 2016; Martins-Santana et al. 2018). The recent literature is abundant with new genomic analyses revealing the huge potential of using fungal biosynthetic gene clusters for production of different natural compounds (e.g. Li et al. 2016; Nielsen et al. 2017). A noteworthy classical case is production of human insulin (in baker's yeast), griseofulvin (an antifungal antibiotic) and an array of enzymes (and blends of enzymes) produced recombinantly in primarily *Aspergillus* spp. and *Trichoderma* spp. (see Fig. 1.1).

In the twentieth century, use of fungal products has moved substantially into a new industrial era. From approximately 1985 onwards, the industrial biotechnology sector developed methods for large-scale production of a wide range of enzymes, making it possible for agroindustrial sectors in particular, such as textile and leather industries, to switch from chemical processing to the milder, more environmentally benign enzyme processing. In parallel, processes for commercially viable fungal production of chemicals were developed. Now the world is at the doorstep of the new bio-based society, where fossil-based products are to be replaced by bio-based products. We have entered the postgenomic era offering new methods for genomic investigations and genetic engineering, hence paving the way for developing sustainable biological solutions to important problems to take over from the environmentally burdensome use of synthetic chemicals.

In this chapter, we provide an overview of the many areas where fungi and fungal products (metabolites and enzyme proteins) can contribute to solving a wide spectrum of the current global societal challenges. However, we go beyond such an overview in offering updated information and visions of the future that new breakthroughs in fungal biotechnology can open up for solving important global challenges. The areas of climate change mitigation and adaptation, where fungi and fungal products could contribute significantly, are very broad. They include many areas of more value adding than production of biofuels for substituting for fossil-based products. Energy is too low in price to be commercially viable to produce as the only value-added product from biorefinery conversion of a given biomass. Notably, we will have less focus on the field of emerging threats from fungi, so excellently reviewed recently by Matthew et al. (2013).

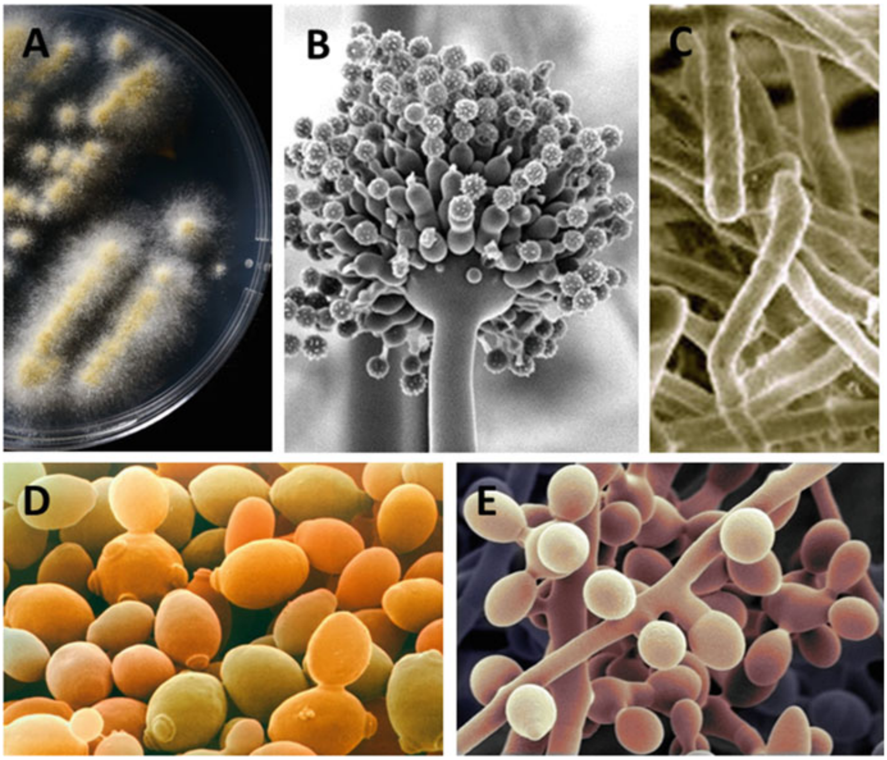

Fig. 1.1 "Fungal Hall of Fame" illustrating the five types of fungi, which are the most important players in industrial lignocellulose biorefinery processing: (**a**) *Aspergillus oryzae*, which along with (**b**) *A. niger* and *A. nidulans*, are the most widely used monocomponent enzyme production organisms. (**c**) *Trichoderma reesei* is, due to its exceptional secretion capacity, the preferred production host for enzyme blends specifically designed for efficient biomass conversion. (**d**) *Saccharomyces cerevisiae*, the organism of choice for production of ethanol from the biomass conversion-derived sugar platform. *Pichia pastoris*, the expression host most often used for producing laboratory-scale volumes of newly discovered enzymes, to facilitate characterization and evaluation of the new enzymes for industrial potentials. (**e**) *Myceliophthora thermophila* (along with another thermophilic fungus, *Thermoascus aurantiacus*), are representing alternatives to production by species of *Aspergillus*. Credits: (**a**) Courtesy of Reinhard Wilting, Novozymes A/S; (**b**) from Read ND, in *Electron Microscopy of Plant Pathogens* (Mendgen K, Lesemann D-E, eds.), Springer-Verlag, Berlin, Germany, 1991, with permission; (**c**) U.S. Department of Energy Office of Science (http://www.jgi.doe.gov/sequencing/why/Treesei.html); (**d**) Sciencephoto.com; (**e**) courtesy of de Vries R, CBS-KNAW, Fungal Biodiversity Centre, The Netherlands (Source: Lange L: Fungal enzymes and yeasts for conversion of plant biomass to bioenergy and high-value products. Microbiol Spectrum 5(1): doi: https://doi.org/10.1128/microbiolspec.FUNK 2016)

2 New Tools and Technologies: Making It Possible to Do More

The new bio-based bioeconomy era is built on a strong foundation of technologies for large-scale, biological (fungal or bacterial) recombinant production of stable proteins. Developing the industrial biotechnology sector to be a competitive business segment has required process conditions and yields to be refined beyond what had been seen as possible just 25–30 years ago. These efforts were made out of necessity in order to match the cost structure of industrial chemical processing of bulk products. However, the core technologies for biological production and bioprocessing are now complemented by a broad set of newer technologies. The development of the field of genomics has been of paramount importance and includes affordable and fast genome sequencing, affordable and efficient production of codon-optimized synthetic genes, provision of shortcuts and robust systems for successful recombinant production of enzyme proteins and, more specifically, optimized strains of, e.g. the yeast expression system (*Pichia pastoris*), for use in research laboratories. Similarly, the development within the field of metabolic engineering has led to advanced production of higher-value products in, e.g. baker's yeast. New gene editing technologies such as CRISP-Cas 9 will add momentum to this field. However, synthetic biology goes beyond this in using more disruptional, molecular technologies for creation, e.g. of new types of cells and new types of functions, not found in nature.

Genome sequence analysis for direct sequence-based annotation of carbohydrate active enzymes and discovery and characterization of new enzyme activities are continuously being refined through a range of different approaches [blast searches, sigP, pFam, HMM, dbCAN, PPR (Busk and Lange 2013), Hotpep (Busk et al. 2017) and, the newest, SACCHARIS (Jones et al. 2018), dbCAN2 (Zhang et al. 2018) and lastly CUPP (conserved unique peptide patterns), an automated, peptide-based functional annotation method (Barrett and Lange 2019)]. The most recent version of the dbCAN2 server (Zhang et al. 2018) integrates other tools for annotation of carbohydrate active enzymes (CAZymes): (1) HMMER for annotated CAZyme domain boundaries determination according to the dbCAN CAZyme domain HMM database, (2) DIAMOND for fast Blast hits in the CAZy database and (3) Hotpep for short conserved motifs in the Peptide Pattern Recognition (PPR) library (Meaning of this list of acronyms appears from the quoted papers).

All these approaches to sequence analysis are tailor-made to be able to derive information and knowledge from genome data. Emphasis, attention and focus over the last decades have been on a rapidly growing number of genome-sequencing projects; notably, the 1000 fungal genome project was made possible by a broad concerted effort from European and American mycologists https://genome.jgi.doe.gov/programs/fungi/1000fungalgenomes.jsf; also sequencing efforts by the Beijing Genomics Institute (https://www.bgi.com/global/) have contributed significantly.

The focus on DNA sequencing has pushed transcriptomics a little out of focus and has almost obliterated the use of cDNA technologies. However, there is likely to be renewed interest and refocus on transcriptome studies (by RNA sequencing) and on proteome studies, especially for eukaryotes (including fungi). Understanding of the transcriptome and proteome opens for insight into functionality and action mechanisms of the proteins. It hereby provides significant input to (1) discovery of new mechanisms and strategies used by the fungi in nature to, e.g. degrade plant materials (e.g. Kuuskeri et al. 2016; Miyauchi et al. 2018); (2) understanding and combatting plant diseases (e.g. Collins et al. 2017); and not least for (3) achieving significant advances in the next-level biotechnological processes, i.e. improved production and use of enzymes and metabolites in bioprocessing.

Research support functions have been established and widely used globally [CAZy (Carbohydrate-Active Enzymes database), JGI (Joint Genome Institute), EBI/EMBL (European Bioinformatics Institute), dbCAN (database for automated carbohydrate-active enzyme annotation), BGI (Beijing Genome Institute)]. Furthermore, as regards research-based culture collections, valuable collections have in recent years been given up due to change in priorities combined with high maintenance costs. However, many have succeeded to modernize their technologies and collections (taking onboard also molecular approaches), thus upholding their position as invaluable assets for both basic and applied research. Another strong trend in methodological approaches, which function as enabler for the new bio-based era, is in a holistic approach aiming for conceptually increased biological understanding. This can be done by integrated use of biological methods along with structural biology methods and with refined chemical/physical methods like NMR, MS and bioimaging.

Additional trends are the move from studying a genome in isolation to studying comparative genomics and, not least, studying interactions between organisms, as well as gene regulation and prediction of gene function. By all such measures, it is possible to convert the overwhelming amount of data into information and even to new biological understanding. Rapid growth of computer power and speed and ease of bioinformatics analysis enable us simultaneously to analyse all published fungal genomes (approx. 2500) on a normal laptop computer. Seen from an applied perspective, development of the biomass conversion processes as a basis for the new bioeconomy has moved ahead quickly and efficiently due to the pre-eminent CAZy database. Furthermore, enzyme performance has been improved through sophisticated and integrated uses of 3D structures, protein engineering, artificial evolution, machine learning and robotics.

This rapid increase in versatility and accessibility of methodological tools and approaches has enabled the overall field of mycology to reach a higher level, not only generating new data but also providing relevant information to enable us to answer conceptually interesting mycological biological questions. In the last decade, we have moved from developing tools and methods to using them in an integrated manner: bringing us “back to biology”. However, unlocking the full potential of the fungal contribution to the new bio-economy will require many more biotechnology breakthroughs. Five general trends are noteworthy (see Table 1.1).

Table 1.1 Five key fungal biotechnology trends in relation to exploitation of fungi and fungal enzymes and metabolites for solving societal challenges

No.	Principle	Goal
I	Combine datasets, e.g. genomic, transcriptomic, proteomic and phenotypic data	Identify genetic basis for organismal and molecular diversity; and gain improved insight into protein function
II	Combine genomics and explorative experimental methodological approaches	Discover new enzyme functions, interactions and mechanisms. Find and understand synergies between exoproteome and exometabolome
III	Expand recombinant production (and use) to complex proteins (e.g. membrane proteins, molecular pumps)	Learn from and harness nature's microbial strategies to solve problems by new transformative uses of biology-based principles
IV	Broaden enzyme classification and functional annotation to more enzyme classes, going beyond carbohydrate active enzymes	Expand options for exploiting enzyme catalysis in new processes enabled by function-targeted (in silico and experimental) discoveries making more types of proteases and lipases available for biorefinery upgrade beyond lignocellulose
V	Elucidate not only the organismal composition but also the molecular function of microbiomes, including not only prokaryotes but also eukaryotes	Expand the knowledge base of biological microbiome strategies and functions, leveraged to, e.g. facilitating development of food and feed ingredients with prebiotic, gut-health-promoting effect

I. Making it possible to combine many types of data sets, not just genomes but also transcriptomic, phenotypic, physiology (anaerobic or aerobic), lifestyle (saprotrophic, biotrophic, etc.), substrate specificity and affinity, and making it possible to predict the composition of the fungal interaction secretome through using state-of-the-art sequence analysis, e.g. the CUPP method (Barrett and Lange 2019).
II. Combining several types of methodological approaches, e.g. experimental enzymology, NMR, mass spectrometry, bioinformatics, chemistry and separation and recovery technologies.
III. Expanding recombinant production capabilities to express enzymes and handle metabolites far beyond the beaten path of core sets of ascomycetous products; even industrial use of the membrane-bound proteins is within reach!
IV. Expanding the types of enzymes, we are able to handle; here, we need to make huge explorative steps, taking in new virgin land within proteases, lipases, esterases and transferases.
V. And not least, enabling us to master understanding the composition, function and use of the complexity of microbiomes often composed of a broad organismal and funcional biodiversity of fungi, protozoa and bacteria.

3 Learning from Nature

The methodological approaches to understanding the role of fungi in nature are legio, and the data generated risk overwhelming us, blocking the view of patterns and new conceptual understanding of essential but complex biological features and integrated interactions. For moving quickly ahead, it is clever and smart to collaborate across different disciplines to learn what nature has developed to perfection in the fungal kingdom over evolutionary time. Such interdisciplinary research may target new high-throughput methods, e.g. in the form of developing new expression platforms, as recently done using *Saccharomyces cerevisiae* for fast assessment of fungal natural products (Harvey et al. 2018). Learning from nature may also involve understanding species diversity by comparing genome sequences (predicted genes), protein function and enzyme profiles against phenotype of fungi (see, e.g. Vesth et al. 2018). Knowledge about fungal physiology in nature combined with use of novel functional classification systems for enzymes can identify hot spots for discovery of specific functions of enzymes and metabolites (see, e.g. Busk and Lange 2013; Agger et al. 2017). Of crucial importance is also detailed reaction analysis of relevant enzymes to provide the decision base for possible practical exploitation in various biorefinery processes (see, e.g. Mosbech et al. 2018; von Freiesleben et al. 2018).

We can provide shortcuts to understanding molecular functions by studying signature species, which have distinct physiological specialization (as done, e.g. in the recent studies by Collins et al. 2017; Kuuskeri et al. 2016; Miyauchi et al. 2018). We can recognize differences and similarities between anaerobic and aerobic life forms and compare ancient zoosporic fungi with the dikaria asco- and basidiomycetes to unravel the basal and extremely diverse part of the fungal tree of life (Fig. 1.2). We can observe patterns of optimized use of CBMs, linkers and LPMOs, in specialized fungi, and we can elucidate the wonders of the sophisticated co-evolution of the fungus-gardening termites and leafcutter ants (see Fig. 1.3); and what is more, we can harness the wealth of enzyme discoveries from such systems, which represent nature's prototype of the yellow and the green biorefinery, termites and ants, respectively. In all of such ways, we can learn and make use of genomics data much better by learning from nature. We can further identify new ways to harness the mechanisms and products used by fungi in nature to help develop a more sustainable future.

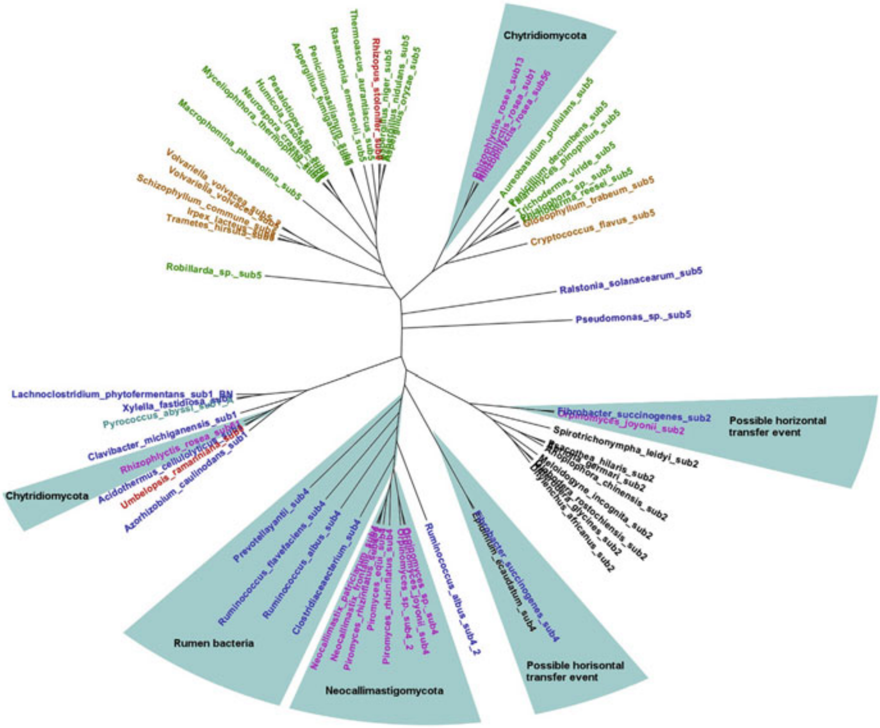

Fig. 1.2 Phylogeny of the cellulose active enzyme, glycohydrolase 5 (GH5) of early lineage fungi. The GH5 (EC 3.2.1.4) 1,4 β-endoglucanases of the chytrid *R. rosea* are embedded in an "all-fungal" clade, containing also ascomycetous, basidiomycetous and zygomycetous GH5 endoglucanases. GH5 endoglucanases of anaerobic rumen fungi (Neocallimastigomycota) are found in a clade dominated by rumen bacterial GH5 proteins. Besides these two major occurrences of early lineage fungal GH5, one copy of *R. rosea* GH5 is found in a predominantly bacterial GH5 clade (to the left) together with one GH5 protein from a zygomycetous species (*Umbelopsis ramanniana*) and an Archaea (*Pyrococcus abyssi*) GH5 protein. Two possible horizontal gene transfer events are indicated (between rumen bacteria and rumen fungi and between rumen bacteria and rumen protozoa). Colour code: purple, early lineage fungi; red, *Zygomycota*; green, *Ascomycota*; orange, *Basidiomycota*; blue, bacteria; turquoise, Archaea; dark green, plants; black, animals (Source: Lange, L., Pilgaard, B., Herbst, F.-A., Busk, P.K., Gleason, F., Pedersen, A.G., 2018. Origin of fungal biomass-degrading enzymes: Evolution, diversity and function of enzymes of early lineage fungi. Fungal Biology Reviews. https://doi.org/10.1016/j.fbr.2018.09.001

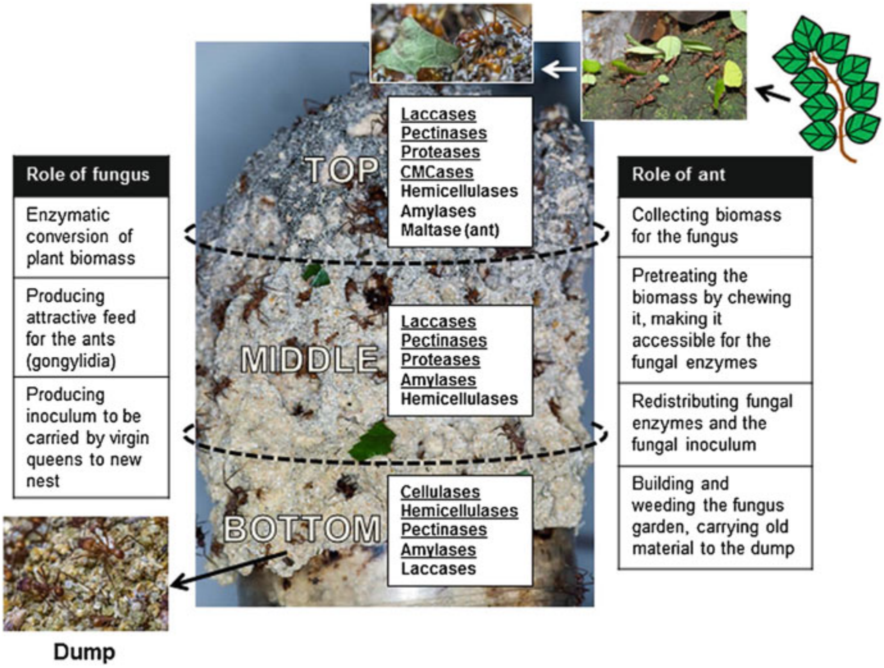

Fig. 1.3 Leafcutter ants are growing and feeding a fungal mycelial colony in their subterraneous nest. Enzyme analysis of the layers of the leafcutter ant's fungal garden has documented that the symbiosis between the leafcutter ant the fungus enables full decomposition of the lignocellulosic biomass (Source: Lange, L and Grell M: The prominent role of fungi and fungal enzymes in the ant–fungus biomass conversion symbiosis. Appl. Microbiol. Biotechnol. 98, 4839–4851, 2014. DOI https://doi.org/10.1007/s00253-014-5708-5)

4 Global Challenges Where Fungi Can Contribute to Solutions

4.1 Climate Change: The Role of Fungi in Mitigation and Adaptation

4.1.1 Fungal Enzymes

Microbial enzymes are essential for developing and producing bio-based substitutes for fossil-based materials, chemicals and fuel. In nature, evolution of efficient biomass-converting enzymes is an integrated part of the evolution of the entire fungal kingdom. The heterotrophic fungal lifestyle relies on plant materials being broken down into smaller building blocks that provide the basis for the fungus to grow and reproduce. Further, through evolution, filamentous fungi have developed the most efficient secretion mechanism to make enzymes available for breaking down the substrate into which fungal hyphae grow. Large-scale production of industrial enzymes is competitive (also when sold as bulk products) due only to efficient secretion of the enzymes, paving the way for low-cost protein recovery.

The biomass conversion needed to make new bio-based products as substitutes for fossil-based products builds on the success of the industrial biotechnology, which is substituting chemical processing in forestry and agroindustries (e.g. textiles, juice and paper and pulp). Development of industrial biotechnology required optimized recombinant production of fungal enzymes at an industrial scale. Species of *Aspergillus* and *Trichoderm*a became the production hosts of choice. Globally only rather few production hosts have been developed for large-scale use. The canonical fungal species in the biological production "Hall of Fame" are few (see Fig. 1.1). Furthermore, sophisticated protein engineering and the integrated use of artificial evolution enabled the production of even more efficient and stable enzymes, which were suitable for use in industrial processes and for global commercialization as bulk products. The new bioeconomy, which will produce bio-based products as substitutes for fossil-based products, builds on such biotechnological methods.

The enzyme part of the classic industrial (white) biotechnology is, basically, the era of one gene, one protein, one product, one solution. However, enzymatic conversion of complex and often recalcitrant biomass (e.g. lignocellulose or keratin) for the new bioeconomy requires not just one enzyme at a time but an entire, carefully designed blend of enzymes (Merino and Cherry 2007). The marketed enzyme blends for biomass conversion are usually produced in one multigene recombinant production host (*Trichoderma* spp.; the Accellerase type of blends (Dupont) and the Cellic-CTec type of blends (Novozymes)). Notably, producing recombinantly one enzyme at a time would be too costly (e.g. in fermentation tank year investments) and would therefore not comply with the cost structure of bulk enzymes. However, optimizing both the blend composition and production technology remains a biotechnology challenge: the enzymes needed for biomass decomposition must be identified, and their combined action must be optimized to constitute "minimal cocktails", i.e. the minimal number, the minimal levels and the optimal combination of the best performing enzyme activities for the purpose (Bunterngsook et al. 2018; Meyer et al. 2009). Pretreatment procedures need to be adjusted to ensure enzyme access to substrate. The blend needs to be designed; the production host needs to be constructed. Fermentation conditions need to be optimized so that a bottleneck is not introduced, by a single enzyme being present in too low amounts, which will limit commercial use of the blend. Furthermore, in the years to come, enzyme blends must be developed for decomposing the entire spectrum of new types of biomasses, residues and sidestreams with valorization potentials, hereby going far beyond the wheat straw, corn stover and wood chips currently exploited.

There are many biotechnology challenges and opportunities for optimizing even further the production of bio-based substitutes for fossil-based products, and new industrially relevant kinetic phenomena, hypotheses and mechanisms underlying concerted enzyme functions for efficient conversion of complex biomass continue to emerge (Kari et al. 2018; Lange et al. 2016a, b; Pierce et al. 2017).

Three examples are chosen to illustrate the new biotech opportunities for further improvement of enzymatic conversion of biomass.

1. The recent discoveries that the lytic polysaccharide monooxygenases (LPMOs, belonging to the auxiliary activity enzymes) play a key role in natural (primarily fungal) degradation of recalcitrant lignocellulosic. This has opened the way for a whole, new approach to improved efficiency in biomass conversion (Bissaro et al. 2018). However, to unlock the full potential of the LPMO technologies is not a trivial matter. Most LPMOs are difficult to express recombinantly, and process efficiency will most likely benefit from including not just one but a number of synergistic LPMOs in the blend. Nor is it straightforward to make an informed choice of which LPMOs to select because the different functions of the many LPMOs often present in efficient fungal biomass degraders are not yet fully elucidated. As an example of LPMO complexity, it has recently been established that in 98% of publicly available fungal genomes, the number of AA9 (LPMO) genes is larger than the sum of the genes encoding for GH6 and GH7 cellulases (Lenfant et al. 2017), and, e.g. a white-rot basidiomycete fungus, *Schizophyllum commune* has 22 AA9 encoding genes (Ohm et al. 2010; Knabe et al. 2010).
2. The importance of the CBM (carbohydrate-binding module) for enzyme function has been extensively studied (see, e.g. review by Shoseyov et al. 2006). New progress within CBM technology has revealed unexpected new functions that do not relate to the original biological roles of the enzymes (Armenta et al. 2017). CBM engineering could lead to improved blends of cellulose-degrading enzymes. This could, for example, be achieved by introducing different types of CBMs in different positions (N- or C terminal, or both) bound to linkers of varying length, but this remains an underexploited field.
3. Construction of integrated and flexible biorefinery conversions that allow recovery, for example, of protein content, first and finally convert only the fibres into a sugar platform for production of chemicals, materials and fuel. Further, through a cascading conversion of the biomass, other components of the plant biomass could be converted into higher-value products such as feed additives from the hemicellulose and functional materials (e.g. binders) from the lignin (Silva et al. 2018).

4.1.2 Significant Reduction of CO_2 Emissions per Ton Food and Feed Produced

Significant reduction of CO_2 emissions of each ton of food or feed is possible by efficiently using all parts of the plant products harvested. This can be achieved by cutting down the huge losses that occur all over the world in food and feed value chains. Currently as much as 35–50% of the total harvest is going to waste globally: protein rots in the field. Industrial sidestreams burden waste water streams instead of being used as raw materials for new food, feed, other materials and energy; upgraded use of the organic part of municipal waste and sludge is not pursued even though technologies have been developed, such as production of organic acids, building blocks for bioplastics, oils, etc. Fungal enzymes are essential for the majority of such upgrading processes and can improve the overall efficiency of our use of biological resources.

4.1.3 Significant Reduction of Methane Emissions

Significant reduction of methane emissions from meat and dairy production must be attempted. So far, efforts directed towards reduction of methane from ruminants have primarily been conducted with the aim of improving feed conversion efficiency. However, at time of writing, the focus is changing and is now also directed towards reducing methane from cows in order to deliver the required and demanded reduction of greenhouse gas emissions from this type of agricultural production. Basically, two different approaches can be pursued (independently or combined) for reducing methane from cattle production: one is through targeted genetic cattle breeding; the other is the feeding strategy approach. For the latter, fungi are key in two different ways: the rumen fungi are part of the healthy and non-methane-producing rumen microbiome; and fungal enzymes could possibly also be of importance in developing new (prebiotic), methane-reducing feed additives for ruminants.

The enzymes of anaerobic, zoosporic rumen fungi have been studied in detail in the past and provided interesting results and insights; this field has recently been revitalized in relation to possible utilization for biogas production (see, e.g. Cheng et al. 2018). However, the understanding of the role of rumen fungi in the rumen microbiome has not yet benefitted significantly from the development of genomics. The majority of the microbiome studies of livestock gut and rumen microflora have included only the bacteria; the fungi (and the protozoans) have been excluded by the sequencing protocol followed, which has presented genomic DNA of prokaryotes, bacteria and archaea only for rumen microbiome sequencing. From earlier studies of rumen fungi, we know that rumen fungi inhabit the small rumen particles (primarily composed of plant cell wall materials, at μm or mm scales); the fungal rhizoids open up the plant cells and thus expose much more of the plant biomass polymers to bacterial decomposition (Nagpal et al. 2009).

The cellulose-degrading enzymes of the rumen fungi, recently reviewed (Lange et al. 2018), form an intrinsic and complex molecular structure, named the fungal cellulosome. In the cellulosome, all the enzymes needed for breaking down plant cellulose polymers are integrated. The catalytic part of the rumen biomass-degrading enzymes has been shown to be of bacterial origin, acquired by the rumen fungi from the rumen bacteria through horizontal gene transfer (Haitjema et al. 2017; Lange et al. 2018). However, surprisingly, the dockerin and the structuring components of the fungal cellulosome were by Haitjema and coworkers found to be of de novo fungal origin and do not resemble any bacterial structure. Further studies and feeding experiments are needed to reveal if methane reduction can be achieved by adding components with prebiotic effect to animal feed, thus stimulating the non-methane-producing part of the microbiome (including the rumen fungi). In addition, the prebiotic feed additive could be further enforced by adding a probiotic element, for example, by enriching the rumen fungal microflora by adding inocula of rumen fungi (Nagpal et al. 2009).

4.1.4 Improved Resource Efficiency Can Free Land for Forestry and Biodiversity

Using fungal enzymes to convert hitherto underutilized biological resources to feed could lead to indirect land use changes and free land for other purposes such as for CO_2 sequestering (by planting more trees) or for providing space for biodiversity to flourish. Currently, around 75% of all arable land in the world is used for production of animal feed. If we succeed in converting especially industrial sidestreams into nutritious animal feed (e.g. upgrading the protein fraction of rapeseed press pulp or sunflower press pulp to protein-rich animal feed), we will be able to free more land for growing forests and lessen the pressure on rainforest to be converted into agricultural land. This will in itself be one of the most efficient routes to follow in order to mitigate climate change. The biotechnology challenges to achieve this are covered above. However, new and improved technologies are not sufficient. We must also change the policies and the incentive structure to ensure climate change mitigation to happen.

4.1.5 Using Misplaced Carbon Resources to Produce Single-Cell Protein

There could also be a possible role for fungi in the new negative emission technologies (NET). The world is facing serious difficulties in keeping global warming under 1.5 °C. This calls not only for a reduction in the greenhouse gas emissions but also that we introduce new technologies which give a net deficit of emissions, i.e. by drawing more emissions out of the atmosphere through carbon-capturing technologies. A highly interesting use of captured methane is achieved by using the methane as a substrate for growing bacteria and thus producing single-cell bacterial protein (see https://www.unibio.dk/). This type of sustainable NET approach, carbon capture and use (CCU), has already been upscaled. The bacterial protein-rich, methane-based animal feed product has been approved in the EU since 1995 according to Commission Directive 95/33/EC. However, new opportunities can be developed that use the resources even more efficiently. It would most likely be feasible and efficient to grow biomass, for example, of fungal baker's yeast, on the residues of the bacterial biomass after the protein has been extracted from the bacterial biomass. Fungi can produce new protein content provided minerals, and C and N sources (plus some accessible sugars to get started) are available. Further, and most importantly, baker's yeast (called yeast cream when used for animal feed) has already been documented to be a highly nutritious feed, for example, for pigs. Large-scale application of yeast biomass from biological production has been used widely in Denmark for more than a decade as feed in Danish pork production.

4.1.6 New Fungal Products Are Instrumental for Climate Change Adaptation

Climate change-induced impact on patterns of rainfall is challenging agriculture in many parts of the world. The latest reporting from IPCC (Intergovernmental Panel on Climate Change) reporting specifically underlines that there is a high risk of reduced agricultural yields, for example, in sub-Saharan Africa, and an IPCC expert group has identified West Africa as a particular "climate change hot spot" to experience significant negative impacts from climate change on crop yields and production (Hoegh-Guldberg et al. 2018; Masson-Delmotte et al. 2018). The need for adaptation to global warming and unpredictable rainfall is pressing, and additional compensating food and feed resources may be needed. However, developing and using new fungus-derived products can play a significant role for both adaptation and compensation. More specifically, new products, similar in effect to the JumpStart product (Novozymes A/S), could be instrumental in also adaption to the occurrence of drought conditions in the critical period after crop seeds have germinated. JumpStart is a fungal inoculant sown together with the seed. The causal agent, a filamentous fungus (a species of *Penicillium*), establishes its mycelium around the roots of the crop seedlings where the mycelium functions as a set of add-on filamentous "roots" and increases water efficiency. Furthermore, the selected types of *Penicillium* species have one more significant specialized characteristic. The fungi impact on the soil around the roots, solubilize the hitherto non-accessible soil resources of phosphorous and thus make phosphorus more accessible for the plant. This de facto leads to increased nutrient efficiency of the plants. Together, these two effects—increased water efficiency and improved nutrient efficiency—act as a plant strengthener and enable the plant to have greater tolerance towards periods of especially "spring" drought.

Based on this very promising example, one additional climate positive effect of fungal inoculants can be hypothesized. Wheat plants have been shown to be highly sensitive to rise in temperature. So far, breeding efforts have not been successful in overcoming this detrimental risk imposed by global warming. It is apparently an inherent characteristic of the wheat plant to be sensitive to higher temperatures especially during flowering, an inherent wheat trait, which is found difficult to change through breeding. Based on the positive effect of *Penicillium* inoculum (described above) experiments could be done to elucidate if plant strengthening fungal inocula (or a combination of fungal and bacterial inocula) could have a positive effect on wheat plants through conferring a higher tolerance of elevated temperatures.

For more details of how to get more out of the harvest, waste significantly less, and thus compensate for decrease in crop yield due to climate change-challenged agriculture, see below (Sect. 4.2).

4.1.7 Understanding the Threat of Loss of Biodiversity Due to Global Warming

In recent years, new epidemic diseases caused by fungi have been spreading within wild populations of bats, toads and snakes; the diseases move and spread to such an extent and speed that they threaten habitat biodiversity, survival of populations and species and may be even be a threat to signature life forms. It is still not clear if such epidemics are due to changes in the fungal pathogens becoming more aggressive through mechanisms such as enzyme mutations or HGT, horizontal gene transfer (Rosewich and Kistler 2000), or are due to climate change possibly weakening animal host defence responses (or a combination of both). Increased molecular understanding of fungal pathogenesis and epidemiological spread of such fungal infections is a necessity for creating a basis for forming a strategy for how these new diseases may be combatted.

In recent years, the most severe fungal epidemic has been the attack by the chytrid *Batrachochytrium dendrobatidis* on toads, which is endangering populations of toads in California, for example. Similarly, the white nose fungal-based syndrome has spread in population of bats, such as those on the east coast of the USA, and most recently, the fungal snake disease has raised alarm and concern in the USA, Australia and Europe (Franklinos et al. 2017). The white nose syndrome has been carefully studied to reveal that the causal agent is the ascomycete fungus *Geomyces destructans* (Lorch et al. 2011). Very recently, another new disease is spreading, invading and weakening populations of wild snakes (named snake fungal disease, SFD). The causal agent of SFD is believed to be the fungus *Ophidiomyces ophiodiicola* (formerly known as *Chrysosporium ophiodiicola*) (Lorch et al. 2015). Also, for this potentially devastating fungal attack on wild snakes, we still do not know, whether it has developed due to changes taking place in the fungal pathogen (e.g. through developing or acquiring more potent proteases, which enables it to also invade intact and healthy snake skin) or whether SFD attacks are due to climate change, making the snakes more susceptible to skin-invading fungi.

Concerted efforts should be invested into understanding these globally most devastating, life-threatening attacks on vertebrates in general. Such increased understanding could help us be prepared for the situation where fungal dermatophyte attacks on humans also become more serious due to global warming (maybe in synergy with chemical pollutants).

4.2 Feeding a Rapidly Growing Global Population

4.2.1 Using the Biological Resources More Efficiently

The most straightforward efforts to enable us to feed the rapidly growing global population is to use a greater part of the bio-resources, which now go to waste. This can be done by applying the set of (fungal-derived) enzymatic biomass conversion technologies developed within the scope of the new bioeconomy for upgrading residues, sidestreams and wastes. The result would be significantly improved resource efficiency, with far more responsible use of global bio-resources where crop residues and industrial sidestreams are seen as new raw materials for producing more animal feed and food. By doing this, one could also focus in an integrated manner on developing a new type of biorefinery products—the much-needed soil improvers. The three most well-known soil improvers already in use are (1) the product "biochar", (2) the residual after biogas production and (3) the organic part of the sludge after waste water treatment (holding a significant amount of phosphorous, upconcentrated in the microbial biomass). Furthermore, fractions of partially degraded lignocellulose, after the free sugars have been used for production of bioethanol, materials or chemicals, may be optimized for use as soil improvers as well. Through employing soil improvers, the fertility of an extensive portion of the world's agricultural land, which currently does not receive sufficient fertilizer, could increase, even in a cost-efficient manner, upgrading available local bioresources.

The best way of communicating the immense potential within this area is by giving concrete examples. An industrial example of improved use of biological resources is found in the dairy sector. Here, use of the leftover products from cheese manufacturing, the whey, has provided basis for a wide range of new higher-value products such as food ingredients for premature babies, pregnant women, infants or athletes. Whey-based products are also used in ice cream, in "light" products as well as in low-fat cheese (see, e.g. Arla Foods, https://www.arla.dk) (see also Nordic Council of Ministers 2017). Another very successful industrial development is the transformation of potato starch production to a modern biorefinery process (see, e.g. http://www.kmc.dk/).

Fungal (and bacterial) enzymes are also cornerstones for such upgraded use of industrial sidestreams from, for example, forestry, textiles, brewery, milling, etc. However, it can also be the fungal organism itself, which is used directly as the new biomass. A striking example of value-added use of an underexploited bio-resource is from Ghana (Adu-Amankwa 2006) where cassava peel is used as substrate on which to grow baker's yeast and in that way produce nutritious, protein-rich animal feed based on the fungal biomass. A similar concept has been developed in Norway to produce protein-rich yeast cream for salmon fed by growing yeast (*Candida utilis*) on wood paste mixed with brown algae; the algae are added to provide an N-supply for the yeast growth (Sharma et al. 2018). The potentials are numerous within the broad field of upgrading sidestreams from processing products from agriculture, fisheries and forestry. In a nutshell, this is the most promising field of all efforts to transform the world towards a more responsible consumption of global bio-resources, lifting the biomass resource from wood paste to protein-rich, fungal-based healthy animal feed. And, basically, the technologies are ready now for local development and upscaling.

4.2.2 Develop, Recover and Refine Local Protein Resources

A significant part of the European meat production is based on imported protein-rich feed products such as soy. This import takes place at the same time as significant local European protein resources are either underexploited or go to waste. Such new local sources of proteins can come not only from plants but also from fungi, algae, insects, invasive animals, etc. The current European focus is on growing more local protein-rich plant crops; however, fungal enzymes also have a role to play in such attempts. A current trend is to consider changing agricultural practices to grow green grass and clover (as feedstock for a green biorefinery) instead of cereals in the northern part of Europe. The green biorefinery provides a shortcut to improved use of the land through longer growing seasons and to reduced pollution from agricultural land due to more active root systems over a larger part of the year. The processing of the green grass and clover is simple—screw press processing—and results in a juice and a pulp fraction. Here again, fungal enzymes can play a role. Besides precipitation of the soluble enzymes present in this juice, an additional 40% protein can be extracted from the green pulp (Dotsenko and Lange 2017) by a simple and low-cost protease enzyme treatment using a broadly acting protease (Savinase from Novozymes A/S; this enzyme is a *Bacillus*-derived subtilisin protease). The search for increased use of alternative local protein resources is moving also beyond protein-rich crops. Sources of new and alternative proteins can also come from macro- or microalgae [extracted by fungal enzymes (Dotsenko and Lange 2017)], from insect larvae (Belghi et al. 2018), from starfish (Mazorra-Manzano et al. 2018; Sørensen and Nørgaard 2016) or from fungal single-cell proteins (Sharma et al. 2018). Examples of production of single-cell fungal proteins are given under Sect. 4.1.5.

4.2.3 Gut-Health-Promoting Food Ingredients and Animal Feed Additives

Genome sequencing of gut microbiomes coupled with the need for improved health and welfare in both man and animal has led to concerted efforts to develop new types of food ingredients and animal feed additives. Gut-health active compounds were, however, known before sequencing of the microbiomes was performed. The most well-known concepts of such first-generation gut-health products were nutritional fibre products from corn, which were especially commonly used in the last century in India, or more specifically products such as XOS and FOS, acronyms for xylan-based oligosaccharides and fructose-based oligosaccharides, respectively. A new generation of such products has recently emerged, which again is partially based on use of fungal enzymes. The evidence-based development of such new gut-health ingredients has been made possible by and coincided and in synergy with sequencing of the gut microbiome.

Feed ingredients with prebiotic action are coming within reach at the same time as the world is experiencing a serious rise in antibiotic resistance believed to be caused by rising use of antibiotics in both industrial livestock production and as drugs for combating infectious diseases in man. Concerted efforts are now being invested in trying to develop feed products, which could strengthen the animal gut flora and reduce the need for (also prophylactic) antibiotic treatments. The results are very encouraging. Short oligosaccharides prepared by enzyme treatment of polysaccharides (using fungal endo-xylanases, Dotsenko et al. 2018) have been shown to have a positive effect on the composition of pig gut flora. Such results have been obtained by in vitro fermentation of pig gut microbiome. Similarly, co-fermented and enzyme-treated seaweed and rapeseed press pulp have been shown in large-scale, on-farm experiments to lead to significant improvement of the pig gut flora (fermentation expert https://fermentationexperts.com/). Such initiatives are put in the ambitious perspective, enabling reduced use of antibiotics in industrial meat production, and as a means towards substituting also for zinc treatment. Overall, the field of prebiotic and probiotic feed ingredients as mode of actions for improved gut health is moving forward (de Lange et al. 2010). Such results may usher into a new era within animal feed, which paves the way for reducing and replacing the use of antibiotics in livestock production, including all types of non-ruminant animals, pigs, chicken and fish, and possibly also suitable for cattle with a prebiotic perspective also for reducing methane emission (see Sect. 4.1.2).

In human health, numerous but still preliminary results from medical studies suggest that a healthy gut flora lowers the risk of lifestyle diseases as well as of a number of other types of serious diseases, not least inflammatory bowel disease. As a consequence, of such highly promising and inspiring results achieved from gut microbiome studies, a series of new gut-health-promoting food ingredients are being developed. The most promising effects seem to come from combining the effect of both prebiotic compounds (e.g. produced by fungal enzymes and favouring the healthy part of the gut microbiome) and a probiotic component that adds live bacteria, such as lactic acid bacteria, to the feed additive. Further improvement may be achieved by administering the probiotic inoculum so that it is accompanied by its favourite substrate and by ensuring presence of enzymes to convert feed substrates into more compounds of prebiotic effect, while even better results may be achieved by including microbial products, which also have additional anti-inflammatory effect.

So far, the fungal role has primarily been the use of fungal-derived enzymes to prepare optimized food and feed additives. However, the next steps (for both feed and food ingredients) are to test whether (a) fungal cell wall-derived products could have an even stronger effect than the products derived from plant cell walls (i.e. notably whether the fungal cell wall has relevant chitin, mixed linked-glucans and beta-glucans, that may be instrumental for giving the prebiotic effect) or (b) whether fungal probiotic inoculum, for ruminants, for example, could have beneficial effects on the level of methane emission (and feed conversion).

4.2.4 Strengthening Human Health Through Fermented Food

Scientific investigations in the last century already supported the conclusion that intake of fermented food as part of your daily staple food could have significant positive effect especially for the health of children. More specifically, such intake could prophylactically increase tolerance (foster robustness) to diarrhoea and save children from dying from diarrhoea if infected (e.g. by using traditional yeast-fermented food in West Africa). Similarly, positive results have been achieved with regard to documented low risk of microbes (e.g. mycotoxin-producing species) contaminating the food fermentations when locally developed cultural traditions for preparing fermented food are followed. A modern trend in western cuisine is also to use more fermented food, including partially adopting the Asian tradition of using fungi for preparing fermented food. Food fermentations often include many different types of fungal yeasts as well as mucoromycotous fungi (such as *Rhizopus oryzae*), ascomycetous fungi (e.g. *Aspergillus*) and/or bacteria such as LAB (lactic acid bacteria). It would be highly interesting to be able to measure possible effects of intake of such modern fungal food fermentations on the human gut flora and to see whether even further improvements could be achieved by adding prebiotic-acting polysaccharide oligomers prepared from fungal cell wall materials to the fermentation. The simplest and cheapest shortcut to improved public health would seem to be a daily intake of even minor amounts of gut-health-stimulating compounds (a good choice could be fungal and/or bacterial fermented seaweeds with your morning yoghurt). Diet amendments could also be a shortcut towards making affordable health improvements in lower income and rural areas by reintroducing locally prepared gut-stimulating food and feed ingredients, made even safer by the use of modern quality control measures to avoid contamination by mycotoxin producing microbes.

4.2.5 Mushrooms and Tasty Climate-Friendly Food from Coffee Grounds

Spent coffee beans are one of the most underutilized biological resources. More than 99% of the biomass is left-behind, when the coffee has been made and the coffee ground is discarded. SME initiatives in several countries in both Europe, Asia and Africa have started to use spent coffee grounds as a new growth substrate, for example, for growing oyster mushroom (*Pleurotus ostreatus*) (see, e.g. Pleissner and Venus 2017; Pauli 2017; and Beyond Coffee, https://www.beyondcoffee.dk). However, even after the mushrooms have been harvested, the greater part of the coffee ground substrate is still left behind. A new project and business trend has been initiated that aims at converting the entire coffee ground biomass into a tasty food ingredient. The basic principle is that the fungal mycelium, which has efficiently invaded the coffee grounds even before the fruiting bodies are harvested, can be regrown and the entire biomass converted into a food ingredient, for example, of