

The importance of fungi and of mycology for a global development of the bioeconomy

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Abstract: The vision of the European common research programme for 2014–2020, called *Horizon 2020*, is to create a smarter, more sustainable and more inclusive society. However, this is a global endeavor, which is important for mycologists all over the world because it includes a special role for fungi and fungal products. After ten years of research on industrial scale conversion of biowaste, the conclusion is that the most efficient and gentle way of converting recalcitrant lignocellulosic materials into high value products for industrial purposes, is through the use of fungal enzymes. Moreover, fungi and fungal products are also instrumental in producing fermented foods, to give storage stability and improved health. Climate change will lead to increasingly severe stress on agricultural production and productivity, and here the solution may very well be that fungi will be brought into use as a new generation of agricultural inoculants to provide more robust, more nutrient efficient, and more drought tolerant crop plants. However, much more knowledge is required in order to be able to fully exploit the potentials of fungi, to deliver what is needed and to address the major global challenges through new biological processes, products, and solutions. This knowledge can be obtained by studying the fungal proteome and metabolome; the biology of fungal RNA and epigenetics; protein expression, homologous as well as heterologous; fungal host/substrate relations; physiology, especially of extremophiles; and, not the least, the extent of global fungal biodiversity. We also need much more knowledge and understanding of how fungi degrade biomass in nature.

The projects in our group in Aalborg University are examples of the basic and applied research going on to increase the understanding of the biology of the fungal secretome and to discover new enzymes and new molecular/bioinformatics tools.

However, we need to put Mycology higher up on global agendas, e.g. by positioning Mycology as a candidate for an OECD Excellency Program. This could pave the way for increased funding of international collaboration, increased global visibility, and higher priority among decision makers all over the world.

Key words:

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INTRODUCTION

Horizon 2020 is a visionary document for the European common research programme 2014–2020 (<http://ec.europa.eu/research/horizon2020>). The vision is to create a smarter, more sustainable and more inclusive society. However, such endeavor is not only European. It is global. Most importantly, for the members of the International Mycological Association (IMA), it includes a special role for fungi and fungal products. Therefore, it is an agenda of special relevance for mycologists all over the world.

The *Horizon 2020* document emphasizes that the most important goals and objectives for common research programmes are to address the major global challenges. Among the challenges of priority is climate change, the need for increased efficiency in resource utilization, and the urgency of developing renewable substitutes for fossils; and

not least to provide for improved human health – combating life-style diseases and ensuring food security for a rapidly growing population. Essential for overcoming much of these challenges is improved use of natural resources; especially biological resources, plant nutrients and water. Regarding the efficient use of bioresources, we can do much better: After harvesting the food and feed, crop residues beyond what is needed to sustain a productive and healthy soil, are left to rot or burned. Further, the potentials of side streams and waste streams from agroindustries often remain unexploited. Also, the organic part of municipality waste is deposited in landfills, burned, or used as combustion feed stock in power plants. However, biomaterials are much too precious for such low value uses. We need more upgraded use of bioresources to both feed the growing population and as a substitute for what we now get from fossil resources.

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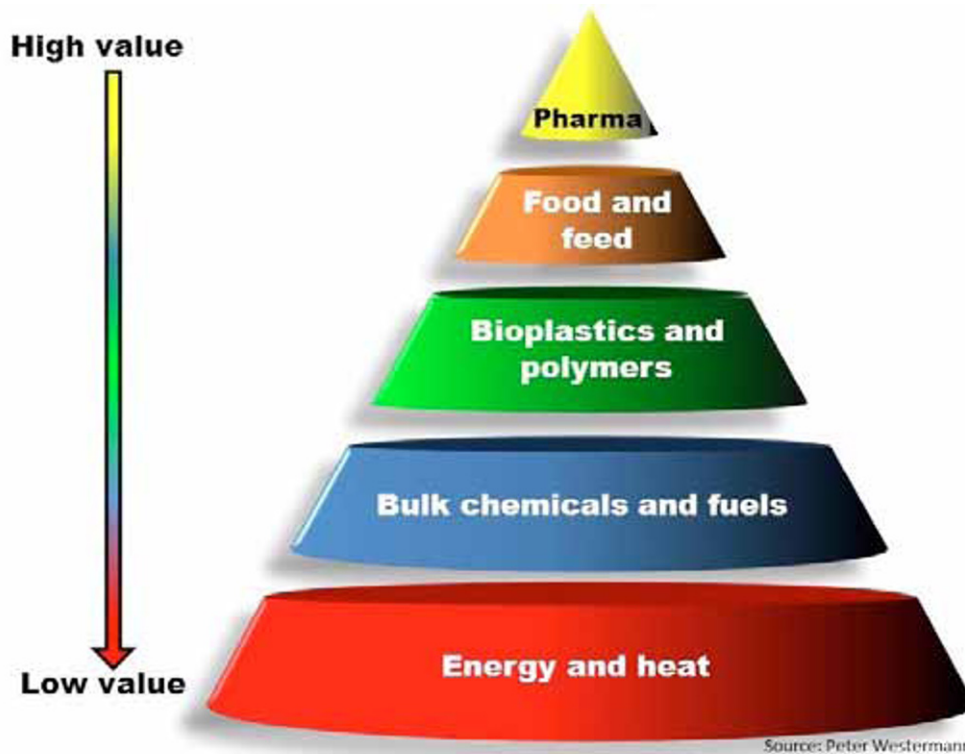


Fig. 1. The Biomass Value Pyramid shows the entire cascade of value adding products which can be produced from agricultural crop residues and other left over bio materials. The lowest value is achieved by burning the biomass and converting it into heat and electricity. Higher value products can be achieved by converting the biomass through treatment with fungal enzymes!

THE POTENTIAL ROLE OF FUNGI

In nature, the breakdown of plant materials is primarily by fungi, by the means of secreted fungal enzymes. Driven by the urge for non-food based bioenergy, industrial scale conversion of biowaste has been researched and developed over the last ten years. After all this research, the conclusion is that the most efficient and gentle way of converting recalcitrant lignocellulosic materials for industrial purposes, is through the use of fungal enzymes (Lange 2010). Through such conversions, the building blocks of the organic materials are kept intact, ready to use in the value cascade (Fig. 1). The enzymatic conversion of biowaste and -sidestreams will provide the basis for an entirely new way for the more efficient use of natural resources, paving the way for a larger bioeconomy sector in a more biobased society:

Plant materials, obtained as crop residues, municipality waste, or from agroindustrial waste streams, will increasingly substitute for fossil carbon from crude oil. Not just substituting fossil energy with bioenergy, but more importantly also substituting the higher value fossil-based materials, such as plastics and chemical building blocks, with biomaterials made from the sugar molecules of the plant cell wall polymers. Thus, the conversion of biowaste is primarily the conversion of plant cell wall materials into higher value products; achieved primarily by a process based on the refined use of fungi and fungal enzymes.

But fungi are providing even more of the solutions for meeting and addressing the various global challenges: Fungi and fungal products are also instrumental in producing fermented foods, to give storage stability and improved

health; and it is a fungus (baker's yeast) that is the production organism of choice for producing insulin for the global population of diabetics. Also, cholesterol lowering drugs (the statins), major immunosuppressant drugs (cyclosporins), the cancer drug Taxol, and penicillins are fungal products.

Climate change will lead to increasingly severe stress on agricultural production and productivity, and here the solution may very well be that fungi will be brought in use as a new generation of agricultural inoculants (e.g. mycorrhizas, endophytes, biocontrol agents) to provide more robust, more nutrient efficient, and more drought-tolerant crop plants.

POSITIONING MYCOLOGY IN THE WORLD

Mycologists have over time delivered so much knowledge about fungi (taxonomy, physiology, genetics, host/substrate relations (including plant pathology and studies of biotrophic interactions), molecular biology, metabolites, enzymes and protein expression) that biological products, biological processes, and biological solutions to important problems are already widespread within many industrial sectors. To mention a few: fungal enzymes are instrumental in laundry detergents at lower temperature and in the less polluting production of both paper and textiles, by replacing chemical processing. Thus, our knowledge and insight into fungal growth and fungal products (proteins as well as metabolites) have made biological processes competitive against chemical processes because they have been developed to be both highly efficient and safe. However, much more knowledge is required in order to be able to fully exploit the potentials of fungi, to deliver

what is needed, and to address the major global challenges through new biological processes, products, and solutions.

Additional basic knowledge about fungi is required across an entire spectrum of research fields: The fungal proteome and metabolome; the biology of fungal RNA and epigenetics; protein expression, homologous as well as heterologous; fungal host/substrate relations; physiology, especially of extremophiles; and not least the extent of global fungal biodiversity. Indeed, many of the new applications of fungi and fungal products will be made possible through “unlocking the magic” of fungi we have not yet discovered – let alone described, characterized, or classified.

We also need much more knowledge and understanding of how fungi degrade biomass in nature, and especially on how they interact with each other and with microorganisms, especially bacteria. In order to achieve all this, we need to train next generation of mycologists to be experts in their fields, mastering both the new and the classical methods. Besides researchers, we also need to train the skilled workers in how to handle biological production at the industrial scale. Last but not least, we need skilled and enthusiastic teachers at all levels who can teach about the fascinating world of the fungi, both the friends and the foes, from kindergartens to graduate schools.

THE WAY FORWARD

As a first step forward, we propose a specific global learning loop for knowledge sharing of relevance to speeding up the application of mycology in addressing issues of global concern.

Most importantly, we see that we need to start to change our mindset as mycologists, taking the importance of fungi and fungal products seriously in our personal research agendas. Not with the objective of making all of us to work in applied mycological research in a traditional sense, but recognizing that we also need blue-sky, curiosity-, biodiversity- and exploration- driven research within mycology – perhaps more than ever before, in order to realize the huge potential.

To this end, mycologists in our research group in Aalborg University, Denmark, located on the AAU Copenhagen Campus, now orient our research projects to have a double focus, to: (1) forward the scientific field in which we are working, by increased understanding of the biology of the fungal secretome (regulation, composition and function); and (2) discover new enzymes and new molecular/bioinformatics tools, thereby contributing to the development of new biological products, biological processes, and biological solutions to important problems. Examples of activities with such a double focus, both basic *and* applied are:

The phylogeny of a fungal cellulase

A comparative study of an endoglucanase belonging to protein family GH45, gave surprising results, which lead to a new enzyme discovery approach: A phylogenetic analysis of the GH45 proteins, from all parts of the fungal kingdom, asco-, basidio-, zygo-, and chytridiomycetes (Kauppinen *et al.* 1999), indicated that distantly related fungi, such as the basidiomycete *Fomes fomentarius* and the ascomycete *Xylaria hypoxylon*, had GH45 cellulases in their secretome

with an extremely high similarity in the amino acid composition of their active site. Strikingly, both these fungi inhabit and decompose very similar substrates (hard wood). A similar pattern can be seen amongst straw decomposing fungi, for example the basidiomycete *Crinipellis scabellia* and the chytrid *Rhizophlyctis rosea*. These two fungi are from two very different parts of the fungal kingdom. Anyway, their GH45 cellulase proteins have an almost identical amino acid composition of their active sites. These observations can tentatively be explained by the following molecular mechanism: Evolution of the fungal GH45 is impacted by gene copying and subsequent gene loss, maintaining the version of the gene which is most suitable for breaking down the cellulose of the substrate of the fungus. This conclusion provided the basis for a new screening approach: select a relevant ecological niche in nature with regard to type of substrate, temperature, and pH; construct a meta-library of the entire microbial (fungal and bacterial) community at such a site; and screen this library for the best enzyme candidates for industrial applications. It also inspired the following hypothesis: evolution of the fungal secretome composition may be interpreted as taking place at the molecular level rather than at the organismal level.

Peptide pattern recognition (PPR)

A new method has been invented for the improved prediction of protein function from protein sequences. It is unique in being non-alignment-based, and permits the comparison of a vast number of sequences with even very low sequence identities. PPR analysis is potent for revealing new protein subfamily groupings, where the subgrouping is correlated with a specific function (Fig. 2). Such new understanding can again be used to understand the biological role of the secreted proteins, interactions between organisms, and interactions between the organism and the substrate. A new subfamily can be described by a list of peptides that is specific to just that subfamily. PPR analysis, moreover, opens the possibility of finding more of a given type of functional proteins belonging to a single subfamily. This can be done by using the conserved peptides for discovering new subfamily members, either by following a bioinformatics approach or by screening biological materials with degenerated primers, constructed based on the list of the identified most conserved peptides (Busk & Lange 2011, 2012).

We analyzed 8138 GH13 proteins represented in the B. Henrissat CAZy database with PPR to generate subfamilies. The subfamily-specific peptide lists were used to predict the function of 541 functionally characterized GH13 proteins. Overall, the function of 85 % of the proteins was correctly predicted (Fig. 2). The figure shows the percentage correct prediction of the enzymatic functions for each of the enzyme classes (new data; P.K. Busk & L. Lange, unpubl.).

Fungal decomposition of specific substrates

Understanding enzymatic degradation of plant cell wall materials is improved by studying in parallel both the plant cell wall composition (by the CoMPP technology, Moller *et al.* 2007) and the fungal secretome enzymes of the fungus responsible for the degradation. The materials under study in a Chinese/Danish research project are duckweed (Cheng & Stomp 2009;

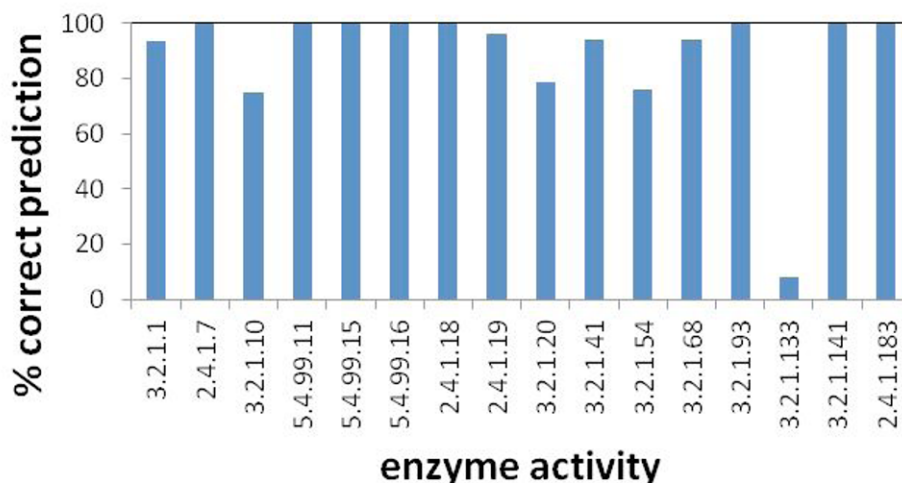


Fig. 2. The Peptide Pattern Recognition, (PPR), generated GH13 protein subfamilies which predicted the enzyme function correctly with 78–100 % accuracy; except for one enzyme class (3.2.1.133) where for so far unknown reasons the PPR subgrouping did not match the function annotations found in the CAZy database.

Fig. 3) and industrial pulp of non-food uses of basic rhizomes such as sweet potato (Zhang *et al.* 2011), cassava, and *Canna edulis*. Using next generation sequencing, the transcriptomes of tropical fungal species, isolated from relevant substrates, are analyzed and novel enzymes are expected to be identified. The secretomes will be further characterized to compare the phylogenetic relationships of the secretome proteins as compared to the phylogenetic relationships of the organisms (L. Bech, Y. Huang, Z. Hai, P.K. Busk, W.G.T. Willats, M.N. Grell, and L. Lange, unpubl.).

Accessory proteins

In 2011 it was discovered that proteins of family GH61 act directly on crystalline cellulose, partially degrading and loosening the structure of the microfibrils, thereby increasing the substrate accessibility for other types of cellulases (Beeson *et al.* 2012, Langston *et al.* 2011, Quinlan *et al.* 2011, Westereng *et al.* 2011). The PPR analysis of all publicly available GH61 sequences resulted in a tentative subgrouping in 16 new subfamilies. We are now studying

the possible correlation of such subfamily groupings with the function of the given GH61 proteins, attempting to answer the following biological questions: What is the function and role of the high number of very different GH61 genes, as is so commonly seen among plant cell wall degrading fungi? We wish to increase understanding of the biological role of these non-hydrolytic accessory proteins in nature; and to provide a basis for choosing which GH61 subfamily proteins should be incorporated into new and improved industrial enzyme blends for conversion of lignocellulosic biomasses into free sugars (M. Lange, P.K. Busk, and L. Lange, unpubl.).

Enzymes from thermophilic fungi

Are the enzymes of thermophilic fungi more thermotolerant than those of mesophilic fungi? We are attempting to answer this fundamental physiological question, and at the same time provide a basis for developing a new type of biomass conversion process which can function at high temperatures, in order to improve the efficiency of the added enzymes and to speed up the biomass conversion (Busk & Lange 2011).

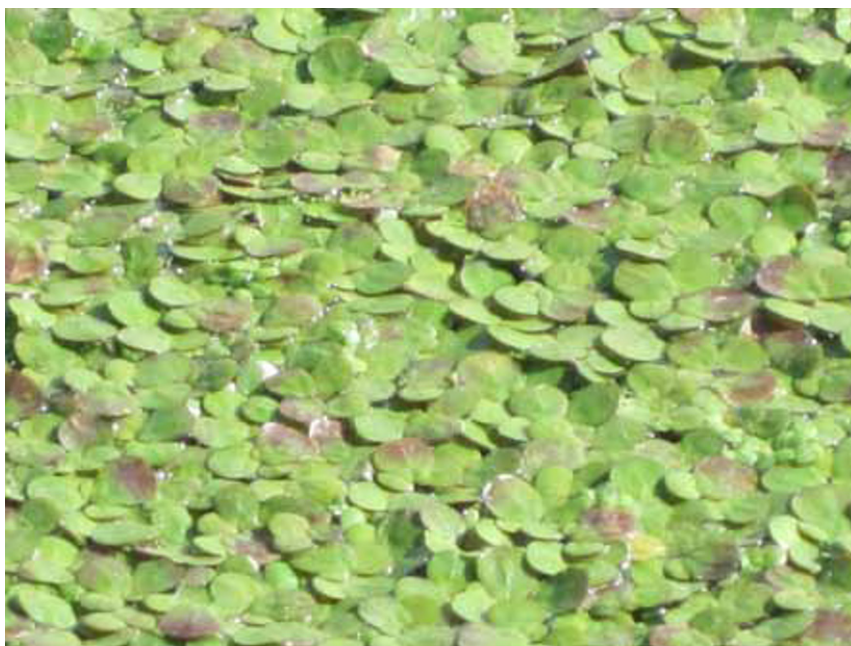


Fig. 3. When grown in swine wastewater, some duckweed species such as the *Spirodela polyrhiza* contains up to 40 % protein, which makes it a valuable animal feed source. Picture by courtesy of Armando Asuncion Salmean.



Fig. 4. Leaf-cutter ant colony established in the laboratory of JJ Boomsma (University of Copenhagen). The ants have built three fungal gardens under plastic beakers. The beaker has been removed from the garden to the upper right. The gradient of biomass decomposition, from top to bottom, is indicated by the green arrow. The dark material on the surface of the garden is newly incorporated leaf fragments. Non-degraded material is removed by the ants from the bottom of the garden and placed in the refuse dump (upright beaker to the lower right) (photo, Morten N. Grell).

A molecular analysis of biomass conversion in the leaf-cutter ant fungal garden

The fungal garden of leaf-cutter ants constitutes a natural biomass conversion system (Fig. 4). Mediated by fungal secreted enzymes, leaf fragments brought into the nest by the ants are converted to food for the ant larvae as well as serve as substrate for fungal growth. In this study, we investigated which enzymes are produced and their relative expression level along the decomposition gradient of the garden structure (Fig. 4), using the DeepSAGE method. DeepSAGE is a global digital transcript-profiling technology, facilitating measurement of rare transcripts (Nielsen *et al.* 2006). The results of the study have given us interesting new molecular insights into a social insect-fungus symbiosis that relies on conversion of a fresh leaves biomass, recalcitrant to degradation (M.N. Grell, K.L. Nielsen, T. Linde, J.J. Boomsma, and L. Lange, unpubl.). Now the question arises: what can we learn from the type of biomass degradation that the fungus growing leaf cutter ants have developed so successfully?

The subgrouping of esterases and their possible function in biomass conversion in nature

At present we focus on a study of additional and so far almost neglected types of enzymes needed for full biomass conversion, more specifically, on the esterases, especially the ferulic acid esterases (X. Tong, P.K. Busk, M.N. Grell, and L. Lange, unpubl.). A feature of plant cell wall polysaccharides is that they are able to cross-link, and that such cross-links can include phenolic groups represented by ferulic acid (feruloyl). The ferulic acid units can be oxidatively cross-linked by cell wall peroxidases into other polysaccharides, proteins and lignin. This cross-linking increases plant resistance to microbial degradation. The enzymes responsible for cleaving the ester-link between the polysaccharide main chain of xyans and either monomeric or dimeric feruloyl are the ferulic acid

esterases (EC 3.1.1.73). The breakage of one or both ester bonds from dehydrodimer cross-links between plant cell wall polymers is essential for optimal action of carbohydrases on substrates such as cellulosic biomass. Subfamily groupings within the field of lipases and esterases are still disputed and unresolved. We attempt to use the PPR method also within these types of enzymes, to provide increased insight in the fungal secretome by achieving function-related subgroupings also of this class of enzymes; and to elucidate further the role also of esterases in biomass conversion.

Studies of secreted enzymes from edible wood-decaying fungi

These studies aim at providing a basis for onsite production of enzyme blends for biomass conversion. Edible basidiomycetes, such as *Pleurotus ostreatus*, are chosen because they do not produce mycotoxins which would prohibit their use as production organisms; and because they have been shown to have the potential to secrete sufficient biomass degrading enzymes, to significantly lower the need for commercial enzyme blends in the production of second generation biofuels. Some even produces secondary metabolites with potential for use in other industries. The combination of these attributes can provide a significant cost reduction of the final products and, most importantly, open for decentralized low-investment use of biorefinery technologies for the production of animal feed, fertilizer, and fuel from crop residues (B. Pilgaard, L. Bech, M. Lange, and L. Lange, unpubl.).

The evolution of obligate insect pathogens, elucidated by studies of their secreted enzymes

In an earlier secretome study of field-collected grain aphids (*Sitobion avenae*) infected with fungi of the order *Entomophthorales* (subphylum Entomophthoromycotina), we identified a number of pathogenesis-related, secreted enzymes (Grell *et al.* 2011). Among these were cuticle

degrading serine proteases and chitinases, involved in fungal penetration of the aphid cuticle, and a number of lipases most likely involved in nutrient acquisition. In a continuation of this study, we are investigating the distribution and variation of selected enzyme-encoding genes within the genera *Entomophthora* and *Pandora*, using fungal genomic DNA originating from field-collected, infected insect host species of dipteran (flies, mosquitoes) or hemipteran (aphid) origin. We anticipate that this study will shed new light on this highly specialized group of entomophthoralean insect pathogenic fungi and their secreted enzymes (M.N. Grell, A.B. Jensen, J. Eilenberg, and L. Lange, unpubl.).

Evidence for a new biomass conversion role of ectomycorrhizal fungi and their use of a chemical mechanism for biomass conversion

The ectomycorrhizal fungus *Paxillus involutus* converts organic matter in plant litter using a trimmed brown-rot mechanism involving both enzymatic activities and Fenton chemistry (Rineau *et al.* 2012). These results could serve as a model for future industrial biomass conversion, combining chemistry and biology to achieve more efficient biomass conversion.

Studies of the cellulases of the aerobic soil chytrids

Pilgaard *et al.* (2011) have provided insight in the roots and origin of the fungal cellulases by studying the cellulases of aerobic soil inhabiting chytrids; and we are also attempting to further elucidate the aerobic chytrid secretome potentials for industrial exploitation of this unique group of fungi, so far almost totally neglected.

CONCLUSION

In order to achieve the goal of more mycological knowledge brought into use, for a more sustainable world of tomorrow, where the bioeconomy becomes an important pillar for our global society, we need fungi to be recognized with heightened visibility. They need to be higher up on global agendas. One way towards that goal could be to position Mycology as a candidate for an OECD Excellency Program. This could pave the way both for increased (national and international) funding of international collaboration, increased global visibility, and hopefully higher priority among decision makers all over the world. We hope you as mycologists, and the IMA as a global institution, will work together towards realizing this vision.

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