Fungal Research Symposium

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Department of Plant and Environmental Sciences,
University of Copenhagen

Program and Abstracts

Organizers:

Henrik H. De Fine Licht, University of Copenhagen
Jacob Heilmann-Clausen, University of Copenhagen
Jonathan Z. Shik, University of Copenhagen
Mette Lübeck, Aalborg University
Uffe Hasbro Mortensen, Danish Technical University

CARLSBERG FOUNDATION

Program

8.55 – 9.00 Brief welcome and practical announcements

Session ”Fungal biodiversity and role in ecosystem functioning”

9.00	Jacob Heilman-Clausen	The Danish fungal atlas – mapping fungal biodiversity with citizen scientists
9.30	Michael Cowled	Dual culture of *Penicillium expansum* and *Monilinia fructigena* reveal insights into the decomposition of apples in Nature
9.45	Uffe Hasbro Mortensen	Novel Insights into Fungal Specialized Metabolite Biosynthesis
10.00	Carolina Nogueira	The pangenome behind the multifunctionality of *Metarhizium brunneum*
10.15	Lene Lange	Fungal secretome composition is an integrated part of fungal speciation and organismal evolution

10.30 – 11.00 Break and posters

Session ”Evolution and genetics of fungal interactions”

11.00	Andi Wilson	Structure and number of mating pheromone genes is closely linked to sexual reproductive strategy in *Huntiella*
11.15	Suzanne Schmidt	*Termitomyces* fungi cultivated by termites contain a consistent set of secondary metabolite gene clusters encoding biochemistry involved in signalling and defence
11.30	Michał Kochanowski	Does the fungal parasite *Rickia wasmannii* (Laboulbeniales) change the task performance and immune response of *Myrmica scabrinodis* ants?
11.45	Jonathan Z. Shik	Cellular mechanisms of nutritional regulation in a leafcutter ant-fungus farming system
12.00	Sam Edwards	Zombie-flies: Is symbiosis at the heart of behavioural manipulation by an insect-destroying fungus?

12.15 – 13.15 Lunch and posters

Session ”Cool tools and Fungal Product Discovery”

13.15	Miia Mäkelä *Invited*	Characterization of *Aspergillus niger* sugar transportome
13.45	Adrian Gadar	Expanding the repertoire of fungal heterologous hosts for the expression of natural products
14.00	Andreas Vestergaard	CRI-SPA as a tool for guiding cell factory engineering in *Saccharomyces cerevisiae*
14.15	Katherina Garcia Vanegas	A versatile high–throughput friendly system for construction and validation of fungal cell factories

14.30 – 15.00 Break and posters

Session “Biosustainabillity and Use of Fungi in industry”

15.00	José Arnau *Invited*	Fungal cell factories to manufacture recombinant proteins to help the World
15.30	Jens Laurids Sørensen	Building a fungal battery for storing renewable energy
15.45	Pablo Cruz-Morales	Learning Chemistry from fungi to make sustainable prodcuts
16.00	Mihaela Bejenari	Heterologous production of type I fungal polyketides in *Yarrowia lipolytica* for sustainable energy storage

16.15 – 17.00 Poster session, informal networking

Oral presentations

The Danish fungal atlas – mapping fungal biodiversity with citizen scientists

Jacob Heilman-Clausen - Globe Institute, University of Copenhagen

Dual culture of *Penicillium expansum* and *Monilinia fructigena* reveal insights into the decomposition of apples in Nature

Michael Cowled - Technical University of Denmark

Apple rot can occur naturally following the growing season or any time during the picking and handling process. From rotten apples obtained from an apple orchard in Denmark, several fungi have been isolated and taxonomically identified. The initial microbial contaminant was observed to be *Monilinia fructigena*, and pervades as a monoculture until a later stage in the apple decomposition lifecycle, at which point a second microbial invader – *Pencillium expansum*, a producer of the mycotoxin patulin – can become established. However, in rarer circumstances, *P. expansum* can also be observed to be the initial primary contaminant. Other more minor microbial isolates include *Talaromyces minioluteus*, *T. leteus, P. thymicola,* and *P. polonicum*. Metabolic profiling of 3-4 strains of each of the major species isolated was conducted. This suite of microbial metabolites was then used as a basis for metabolomics analyses of rotten apple extracts to identify and semi-quantitatively determine the microbial compounds present naturally. To gain insight into the interplay of secondary metabolites derived from fungi contaminating apples in nature, the co-culture of *M. fructigena* and *P. expansum* was investigated and was of particular interest, given their notability as the primary sources of contamination in the lifecycle of a decomposing apple. The co-culture was investigated on a variety of media, including the natural apple substrate, and apple puree agar, in order to determine the possible chemical interactions most in line with that which occurs in Nature. Here, MALDI imaging was also used as a tool to understand the spatial distribution of metabolites across the interaction of the two fungi.

Novel Insights into Fungal Specialized Metabolite Biosynthesis

Uffe Hasbro Mortensen – DTU

Fungi produce a vast amounts of specialized metabolites, SMs, of which only the tip of the iceberg has been characterized and many compounds and exciting biochemistry await discovery. To uncover new SM gene functions we use strategies based on gene deletion in the host, pathway reconstitution in a cell factory, and/or feeding experiments using labeled precursors. Using these strategies, we have recently investigated SM pathways in Aspergillus californicus, which has never previously bee genetically engineered, and in A. niger, and discovered and characterized pathways with unusual fungal SM biochemistry. In the case of A. californicus, dissection of an SM pathway identified a novel ring-closure mechanism for production of 2-pyridones. For A. niger, we revisited yanuthone production and determined that yanuthone X2 appears to originate from a precursor from the shikimate pathway. Previously, we have shown that yanuthone D is derived from the polyketide 6-MSA and that both types of yanuthones share a number of biosynthetic steps. Hence, the yanuthone SM pathway is complex as precursors from two different sources of the primary metabolism are able to enter the same biosynthetic pathway to expand the repertoire of products that this pathway is able to produce.

The pangenome behind the multifuntionality of *Metarhizium brunneum*

Carolina Nogueira – Department of Plant and Environmental Sciences, University of Copenhagen

Metahrizium is an ascomycete fungal genus that present a multifunctional lifestyle. Though being able to survive as a saprophyte, insect pathogen, and plant-root symbiont, the ability to maintain each of these lifestyles varies between and within species. *M. brunneum* has a cosmopolitan distribution and is able to adapt well to all these lifestyles. The species is a generalist insect pathogen that infect insects from at least seven different orders. In addition, it can also establish symbiotic relationship with plant-roots, being able to survive as an endophyte and to induce plant resistance against pathogens. Despite having a variable and versatile lifestyle, the genome-wide genetic diversity within the species is not known. In order to increase the understanding of *M. brunneum* genetic diversity we are generating a reference pan-genome based on six isolates sampled in different regions and habitats. These isolates exhibit different phenotypic traits such as growth rate, virulence against *Tenebrio molitor* larvae, and ability to colonize wheat roots. Our preliminary data gives a hint on the high genetic diversity within the species. Unlike the two reference genomes of *M. brunneum* already available, which do not differ in genome size, our genomes have a variation of up to 9 Mb in length. We also found evidence for the existence of accessory chromosomes in some isolates, a feature never described for *M. brunneum* before. The species pan-genome comprises more than 86,000 genes, which have been assigned to 11,835 different orthogroups. The core orthogroups represent ± 67% of the pan-genome. A phylogeny based on the core genes shows a very consistent and defined division within the species into three clades. Therefore, the next steps will focus on investigating these three groups of *M. brunneum* based on gene functions.

Fungal secretome composition is an integrated part of fungal speciation and organismal evolution

Lene Lange – LL-BioEconomy

The strongest characteristics of the fungal life-form are their invasive power and efficiency in dissemination of spores/conidia. Most prominent mechanisms of fungal genome development are gene-copying and gene-loss (plus HGT). The basis for the invasive power of fungi is its digestive secretome, composed of the enzymes needed for degrading/mobilizing its substrate. The digestive secretome can be analyzed across taxonomies by the Enzyme Profile Relatedness method (EPR), based on functional annotation (by CUPP, Conserved Unique Peptide Patterns). EPR clusters secretomes, who share having (or share not-having) the same type of enzymes (same protein-family, with same function). The output of EPR is a secretome-composition relatedness dendrogram. A stunning congruence was found between the EPR dendrogram of all genome- sequenced species of Aspergillus and Penicillium and the organismal phylogenetic tree of these two genera. Recently we have documented that a similar matching congruence between EPR dendrogram and organismal phylogenetic tree is found by analyzing all sequenced species of Fusarium sensu lato. The interpretation of these findings is, that the fungal digestive secretome reflects and impacts fungal fitness as it determines efficiency in substrate mobilization/growth; hereby providing basis for speciation. Based on such similar results for these three large ascomycetous genera we hypothesize, that secretome composition is an integrated part of speciation also in the other fungal phyla. Next question: Can we find other genome patterns, (impacting e.g., spore-dissemination efficiency or profile of bioactive metabolites); patterns leading to clustering dendrograms, matching organismal phylogeny? Maybe the most prominent character of fungi is their rich/diversified interaction-profile?

Structure and number of mating pheromone genes is closely linked to sexual reproductive strategy in *Huntiella*

Andi Wilson - Forestry & Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

Huntiella species exhibit different mating behaviours, providing an opportunity to investigate mechanisms of transition between reproductive strategies. Comparative genomics tools were used to investigate the differences between heterothallism and unisexuality across the genus. Sexual strategy was shown to be closely linked to the structure of the mating pheromone genes. Heterothallic species harboured multiple a-factor pheromone genes, each with numerous repeats, while unisexual species had fewer copies with fewer repeats. Heterothallic species also possessed more mature α-factor repeats than unisexual species. These differences suggest that unisexual species do not rely on the same partner recognition system as heterothallic fungi.

Termitomyces fungi cultivated by termites contain a consistent set of secondary metabolite gene clusters encoding biochemistry involved in signalling and defence

Suzanne Schmidt – Department of Biology, University of Copenhagen

The use of compounds produced by hosts or symbionts for defence against antagonists has been identified in many organisms, including fungus-farming termites (Macrotermitinae). The obligate mutualistic fungus *Termitomyces* plays an essential role in the symbiosis through plant biomass decomposition and as the main food source for these termites. However, several secondary metabolites have been isolated from different species of this fungal genus, suggesting that *Termitomyces* may also aid in antimicrobial defence. To explore the biochemical potential encoded for by diverse *Termitomyces* species, we comparatively analysed genomes of 39 isolates of Termitomyces (21 species) spanning five genera (10 species) of termite hosts. We used fungiSMASH for initial detecting of biosynthetic gene clusters involved in the production of secondary metabolites. This resulted in the detection of more than 50 distinct biosynthetic gene cluster families (GCFs), of which seven were present in all 21 species of *Termitomyces* and 14 were present in all genomes within a subset of species. The 22 most abundant GCFs were subsequently used for a codon-based phylogenetic analyses to characterise their evolutionary histories. We identified eight BGCs that showed consistent positive selection in the same gene across the phylogeny of *Termitomyces* and seven that exhibited species-specific episodic positive selection events across genes within gene clusters. We suggest that gene clusters under strong positive selection could be due to arms race dynamics with target pathogens, indicating potential defensive roles. In contrast, conserved gene clusters are more likely serving communication or signalling functions. Further, the gene cluster profiles are seemingly driven, in part, by both the *Termitomyces* species and termite host the isolate originates from. This comprehensive study demonstrates the vast non-random unknown chemical potential of *Termitomyces*.

Does the fungal parasite *Rickia wasmannii* (Laboulbeniales) change the task performance and immune response of *Myrmica scabrinodis* ants?

Michał Kochanowski - Warsaw University

The order Laboulbeniales (Ascomycota) is a group of fungi with a very peculiar biology. They grow as the ectoparasites of arthropods, not developing mycelium, but anchoring to the host's cuticle by a melanised holdfast. One of the Laboulbeniales representatives, *Rickia wasmannii* and its host - ants belonging to the genus *Myrmica* are a model system for studying host-parasite interactions. While *R. wasmannii* can occur on its host cuticle in a high density, it does not penetrate the cuticle nor feed on the nutrients from the ant. Previous studies have shown that *R. wasmannii* affects ants' fitness by decreasing the lifespan of workers and that workers from infected colonies are smaller. It also changes ants' physiology (e.g. cuticular carbohydrates profile) and behaviour (e.g. grooming frequency, aggression, interactions with social parasites). However, other effects of the parasite on the host remain unknown. In our research, we were interested in how infection affects task performance and immune activity in different casts of ants, estimated by phenoloxidase measurement. Our study showed that the more intensely infested the colony, the faster the workers will switch their tasks from intranidal to forager. We have not found any difference in the immune response of workers depending on infection status, but we did show that the immune system of infected queens is more active than uninfected ones. The cost of activating the immune system by the fungus might lead to a reduced fecundity. Testing that hypothesis might be a goal for further research.

Cellular mechanisms of nutritional regulation in a leafcutter ant-fungus farming system

Jonathan Z. Shik - Department of Biology, University of Copenhagen

Nutritional rewards provide striking examples of how mutualisms can guide trait evolution (e.g. beltian bodies, elaiosomes on plant seeds, hemipteran honeydew). Nutritional rewards have also evolved in a lineage of *Leucoagaricus* fungi that are farmed for food by attine ants. These swollen hyphal cells are signatures of crop domestication and serve as multimodal hubs of a nutritional, enzymatic, and signaling complexity we are just beginning to grasp. I will discuss ongoing research from my group that uses a variety of nutritional approaches to understand how these traits evolved, how they form, and how they serve as lynchpins of symbiotic stability. Key questions include:

1) Do free-ranging foragers in tropical rainforests select plant resources that target the nutritional needs of their cultivar?
2) Does the fungal cultivar provide feedback signals when ant farmers provision it with suboptimal nutrients?
3) What are the cellular and molecular mechanisms by which nutrients are metabolized by the fungal cultivar and shunted to its nutritional reward structures?

This research provides a uniquely integrative nutritional framework for testing hypotheses about how symbionts interact with special focus on fungal physiology.

Zombie-flies: Is symbiosis at the heart of behavioural manipulation by an insect-destroying fungus?

Sam Edwards – Department of Plant and Environmental Sciences, University of Copenhagen

Certain insect pathogens can manipulate the behaviour of their hosts to increase their chance of transmission. The specialist fungal pathogen *Entomophthora muscae* turns their housefly hosts into so-called 'zombie-flies' after six days of infection. There are three stereotypical and sequentially observed manipulated behaviours: (1) summiting, whereby the fly climbs to an elevated position, (2) proboscis affixation, where the fly 'glues' itself to the substrate surface, and (3) wing raising, where the wings raise to not obstruct the fungal spores that will be shot out of the abdomen. We are using comparative transcriptomics to assess how this pernicious hijacker provokes these striking moribund displays and how the host responds. Preliminary analyses have uncovered a handful of candidate genes, including two secreted effector proteins. Moreover, we have found an overwhelming amount of RNA reads that correspond to a known mycoviral symbiont. Iflaviruses are known to have a role in insect manipulation, e.g. bodyguard behaviour in ladybirds. This supports the idea of a possible manipulation role of our virus, potentially redefining what we know about host-fungus interactions. I will present our insights into the genetic underpinnings of the co-evolutionary processes of this tri-Kingdom interaction leading to the extended phenotypic response of zombie-flies.

Characterization of *Aspergillus niger* sugar transportome

Miia Mäkelä – University of Helsinki

Filamentous fungi have a crucial role in the degradation and modification of plant biomass, contributing significantly to terrestrial carbon cycling and the development of a bio-based economy. Key aspects of the fungal conversion process include extracellular enzymatic degradation of plant biomass, uptake of the released sugars, intracellular catabolism of these sugars, and its regulatory system. In contrast to extensive studies on the other three aspects, knowledge on sugar transport remains largely limited. However, the significant biological role and biotechnological potential of fungal sugar transporters have been recognized.

To systematically investigate the sugar transportome of *Aspergillus niger*, one of the most studied plant biomass degrading filamentous fungi and a well-established biotechnology workhorse for enzyme and metabolite production, we have identified 90 candidate sugar transporters in its genome. These transporters are predicted to exhibit diverse and overlapping sugar specificities, and different affinities. To determine the physiological functions of these candidates, we are utilizing CRISPR/Cas9 methodology to generate multiple sugar transporter deletion strains of *A. niger*. To facilitate the characterization of the in vitro functions of the *A. niger* transporters, we use an engineered Saccharomyces cerevisiae strain that is deficient in hexose and disaccharide transporters, and disaccharide hydrolases. This strain is further engineered to obtain *S. cerevisiae* platform strains that, in addition to hexoses, are able to metabolize other plant biomass derived sugars, including pentoses, polyols and disaccharides. The comprehensive physiological and functional data obtained from this research will provide valuable insights into the role of individual transporters within the context of plant biomass conversion by *A. niger*. Additionally, it is expected to identify novel target genes for the rational engineering of industrial fungi, thus facilitating their biotechnological applications.

Expanding the repertoire of fungal heterologous hosts for the expression of natural products

Adrian Gadar - DTU biosustain

Natural products (NP) are a chemically diverse group of molecules produced by living organisms under specific environmental conditions. NP are characterized by complex chemical structures which confers them biological activities that have been widely exploited to develop medicines. In fact, NP are one of the major sources of drugs, and most of the current known antibiotic and anticancer drugs in use are derived from NP, mostly from bacteria and plants. Nevertheless, genomic-based predictions have shown that fungal chemical repertoires are largely unexplored and are rich Non ribosomal peptides and polyketides (e.g., penicillin, cyclosporine, and statins). The current methods for detection and extraction of NP derived from Filamentous Fungi are limited to species that can be cultivated under laboratory conditions and that produce an enough amount of the NP of interest so that they can be detected by current analytical methods. Moreover, it is very frequent that the expected NP derived from filamentous fungi is not produced because its biosynthetic gene cluster is cryptic, and the cultivation conditions are not optimal to enhance its expression. In addition, the lack of genetic engineering tools complicates the access to their NP expression potential. Hence, Filamentous fungi remain as an underexploited source of NP when compared to plants or bacteria-derived NP. One of the most promising strategies to exploit fungal chemical diversity is to transfer the biosynthetic gene clusters driving the synthesis of NPs into more amenable cell factories such as the filamentous fungi *Aspergillus* spp. and the widely known yeast *S. cerevisiae*. Nevertheless, host suitability for NRPSs and PKSs depend in its ability to properly produce very large proteins, to provide the needed precursors and to tolerate the potentially toxic products. Therefore, to select a suitable host for PKS and NRPS expression a systematic exploration and comparison of hosts from different taxonomic groups is much needed. While Aspergillus spp. and S. cerevisiae offer great advantages as cell factories for the expression of NP, there is not guarantee that they are the nest choice for polyketide and/or non-ribosomal peptides production. Therefore, we postulate that alternative fungal hosts that grow as yeast and have native NRPS and PKS systems are better hosts. We will develop and test non-conventional hosts such as the basidiomycete *R. toruloides, Knufia petricola, Phaffia Rhodozyma, Hortaea werneckii*, or the recently discovered black yeasts from the genus *Exophiala*, as NRPS and PKS-specialized cell factories and compare their performance with conventional hosts.

CRI-SPA as a tool for guiding cell factory engineering in *Saccharomyces cerevisiae*

Andreas Vestergaard - DTU Bioengineering

Saccharomyces cerevisiae represents a commonly used cell factory chassis and has been used for the production of a wide variety of small molecules including terpenes, polyketides, non-ribosomal peptides etc. As a model organism, *S. cerevisiae* has been the target extensive research to understand gene function and regulation governing cellular metabolism. Metabolic engineering strategies guided by genome scale models and flux balance analyses provide a path towards improving *S. cerevisiae* as a host for small molecules. However, as our understanding of gene function and cellular metabolism is incomplete, even in model organisms such as S. cerevisiae, gene targets for cell factory improvement are likely to be overlooked in such approaches. We have recently developed a method CRI-SPA, which combines CRISPR-Cas9 induced genetic engineering with Selective Ploidy Ablation (SPA) to allow for the transfer of a metabolic pathway from a donor strain to a recipient strain library. We have so far applied CRI-SPA to transfer the metabolic pathways necessary for the production of the small molecules: betaxanthin, bikaverin and aspulvinone E, to the genome-wide gene deletion library in *S. cerevisiae*. The biosynthesis of all three metabolites in *S. cerevisiae* is associated with visible phenotypes, allowing for high-throughput screens for gene deletions, which positively and negatively affect accumulation of each metabolite. Our hope is that betaxanthin, bikaverin and aspulvinone E may each serve as proxies for production of other small molecules sharing similar substrates or classes of pathway enzymes, and it thus, based on CRI-SPA screen data, will be possible to devise superior metabolic engineering strategies for the production of these valuable small molecules in *S. cerevisiae*.

A versatile high–throughput friendly system for construction and validation of fungal cell factories

Katherina Garcia Vanegas - DTU

Filamentous fungi are industrially important because of their large production of metabolites and enzymes. However, compared to cell factories based on Escherichia coli and yeasts where high-throughput strain construction tools are highly developed, most strain construction work on filamentous fungi is still mostly done manually and, hence, in low throughput. One reason for this technology gap is a lack of fungal genetic tools that are compatible with an automated setup. However, recent developments in in vivo DNA assembly and CRISPR based gene-editing techniques are setting the stage for implementing high throughput fungal cell factory engineering. To this end, we have established a flexible platform, DIVERSIFY, for multi-species heterologous gene expression using methods that do not involve E. coli cloning steps. Specifically, relevant gene-expression cassettes compatible with the platform can quickly be assembled from libraries of parts, directly into the fungal strain by in vivo recombination. All individual parts for gene-expression cassette construction are made by PCR that are co-transformed into a given host. Importantly, the key parts of our expression cassettes are compatible with a string of DIVERSIFY strains that have all been pre-engineered to harbor a common landing platform, which is designed to accept the common gene-targeting expression cassette. Together the system makes it possible to produce a large number of gene-expression cassettes that can be easily introduced and tested for production efficiency in a number of different production hosts in parallel to increase the experimental success rate. We are currently adapting these technologies to fit into an automated workflow that will allow us to construct and validate 100-1000 in defined strains for heterologous protein production in a time frame of two weeks.

Fungal cell factories to manufacture recombinant proteins to help the World

José Arnau – 21st Bio

Biosustainability, severe climate changes and food shortage are some of the main challenges the World is facing right now. The next food revolution -where protein for human consumption is manufactured in a fermenter and not in an animal- is arriving as your door shortly. New food ingredients and biomaterials produced by precision fermentation in a more sustainable and climate friendly fashion are already entering the market. We need the technology to provide very high yields of protein for very different proteins with very different requirements for the (fungal) cell factory. We use “advanced” fungal cell factories (Aspergillus) developed in the last 40+ years. Not only the protein yields but also product safety and stability are critical. One more property that is essential is the scalability. Your lab test should be predictable of what will happen at manufacturing scale. Many companies believe upscaling is a short time effort… and failed e.g., after more than 10 years of trying. Our setup as a company is to help innovators scale up their protein wonder to enable them to reach the market, thereby helping the World become more sustainable. This is not done “just” developing a great strain and a fantastic manufacturing process but also driving the regulatory approval process.

Building a fungal battery for storing renewable energy

Jens Laurids Sørensen - Aalborg University

The fungal kingdom is full of colorful pigments with a quinone structure, which are used as protective agents against oxidative stress and competing microorganisms. Besides their natural biological role, quinones are gaining increased interest as promising electrolytes in organic redox flow batteries (RFBs) that can be used to store energy from solar and wind power plants. However, the current quinones used in RFBs have been chemically synthesized from crude oil, which is not aligned with the sustainable thinking behind renewable energy. Here I will present our work in developing a RFB based on the bibenzoquinone phoenicin, which is produced by several *Penicillium* species. The wildtype *P. atrosanguineum* strain produced approximately 3 g/L phoenicin in a week, which was then used to generate a RFB with a cell voltage of 0.86 V and an initial capacity of 11.75 Ah/L. The electrochemical properties of phoenicin are similar to the published petro-quinones, which demonstrates that fungal biosynthesized quinones provide a sustainable solution for energy storage.

Learning chemistry from fungi to make sustainable prodcuts

Pablo Cruz-Morales - Novo Nordisk Center for Biosustainability, DTU biosustain

We need sustainable alternatives to make the products that we use and power our activities. Nature's chemistry is the result of millions of years of natural selection, as a result it covers a huge chemical space beyond the reach of organic chemistry. We are cataloging the fungal natural products chemical and genetic space. We are using this information to program fungal hosts to make new molecules using sustainable and inexpensive feedstocks. We believe that many products currently derived from petroleum can be made using fungal specialized metabolism. Our goal is to develop platforms for heterologous production of chemicals using fungal biosynthetic pathways in fungal hosts. We have developed a bioinformatics toolkit and mined thousands of fungal genomes for biosynthetic gene clusters (BGCs). We have catalogued tens of thousands of PKSs and NRPSs. We use tandem mass spectrometry to assess the chemical repertoire deployed by the strains in our collection. And we have found the BGCs for new and known molecules. We have obtained a genetic and chemical catalog of bioparts, and we are using it for design and assembly of biosynthetic pathways to produce new chemicals. In this talk I will present our workflow: from genomes to heterologous products, and examples of the application of our approach to make fuels, materials and pesticides

Heterologous production of type I fungal polyketides in *Yarrowia lipolytica* for sustainable energy storage

Mihaela Bejenari - Aalborg University Esbjerg

Fungal polyketides are a large group of secondary metabolites, valuable due to their diverse spectrum of pharmacological activities. Simultaneously, several fungal polyketides possess electroactive properties, which make them good electrolyte candidates for redox-flow batteries (RFB). However, polyketide production in fungi is associated with several challenges: reduced production efficiency and increased costs, which hinder polyketide use for RFBs. To tackle these aspects, we switched from fungi to the yeast *Yarrowia lipolytica*, an easily cultivable heterologous host. As an oleaginous yeast, *Y. lipolytica* displays a high flux of acetyl- and malonyl-CoA precursors used in fatty acid biosynthesis. Likewise, acetyl- and malonyl-CoA are the building blocks of fungal polyketides, and we explored the possibility of redirecting this flux toward efficient polyketide production. Despite its promising prospect, *Y. lipolytica* has so far only been used for heterologous production of simple type III polyketides from plants. We, therefore, examined the potential for more complex polyketide production in *Y. lipolytica* by targeting fungal polyketides derived from type I PKSs. We employed a CRISPR-Cas9-mediated genome editing method to investigate the production of the model polyketide 6-MSA and achieved a titer of 299 mg/L in the initial trials. Subsequently, we used the same markerless gene integration system to produce a complex polyketide, bostrycoidin, suitable for RFB application. We expressed in *Y. lipolytica* the genes fsr1, fsr2, and fsr3, together with the activating co-enzyme phosphopantetheinyl transferase, FsPPT1, all being responsible for bostrycoidin biosynthesis in *Fusarium solani*, and obtained a 29 mg/mL titer in the initial unoptimized shake flask cultures. Currently, we are working on a more promising fermentation set-up in bioreactors, but the work demonstrates the potential of *Yarrowia lipolytica* as a production workhorse for complex fungal polyketides, which can subsequently be used for sustainable energy storage.

Poster presentations

P1

Fungal cutinase discovery from compost sample by enrichment in shake flasks using PET powder as an inducer

Dalila Mortera Aguirre and Marjeta Klinkon Breckling – Aalborg University

Despite of recent efforts towards plastic waste management, the issues with the residues of the material across the ecosystems persist. Therefore, it is of great importance to develop applications using biocatalysts, for minimizing or remediating the impact of the plastic polymer on the landfills. One of the biggest challenges is finding the enzymes that can perform under optimal conditions, while they are as efficient and of equal quality when produced on industrial scale. The purpose of this research was finding the enzyme cutinase, which is active on plastic polymer substrates. The initial approach was to find fungal strains that can degrade plastic polymers, by performing shake flask liquid media enrichment of a compost soil sample, using PET powder as the inducer. The DNA in the enrichment flasks was analyzed by metagenomics and the sequences with homology to known cutinases were transformed in Aspergillus oryzae by a CRISPR technique. The transformants were fermented in liquid media for recombinant protein expression and the supernatants were screened for enzyme activity, showing promising results on substrates of interest.

P2

Genes for an extended phenotype: Fungal biosynthesis of volatiles in zombie flies entice male flies to mate with female cadavers

Henrik H. De Fine Licht – Department of Plant and Environmental Sciences, University of Copenhagen

To ensure dispersal, many parasites and pathogens behaviourally manipulate infected hosts. In the fungal kingdom, famous examples include so-called zombie ants and zombie flies. Here, we show that the host-specific and behaviourally manipulating pathogenic fungus, *Entomophthora muscae*, generates a chemical blend of volatile sesquiterpenes and alters the level of natural host cuticular hydrocarbons in dead infected female house fly (*Musca domestica*) cadavers. We used a combination of behavioural, chemical (GC-MS), and physiological (GC-EAD) analyses to identify the chemical cues eliciting male mating attraction and how the male antennae respond. We show that healthy male house flies respond to the fungal compounds and are enticed into mating with dead female cadavers. This is advantageous for the fungus as close proximity between host individuals leads to an increased probability of infection. We further use transcriptional profiling (RNAseq) of expressed genes in volatile chemical biosynthesis pathways to verify the fungus *E. muscae* as source of the behaviourally active volatile compounds in fungus-killed cadavers. It is unusual pathogens to rely on both behavioural host manipulation and sexual mimicry, and the *E. muscae*-emitted volatiles represent the evolution of an extended phenotypic trait that exploit male flies' willingness to mate and benefit the fungus by altering the behavioural phenotype of uninfected healthy male host flies.

P3

Metabolomic comparison between three *Monilina* species

Domenico Di Cosmo – Università degli studi di Bari "Aldo Moro"

Monilinia species are among the most devastating fungal pathogens for stone and pome fruit, causing blossom blight and brown rot both in-field and post-harvest. The three main species in Western Europe are *M. fructicola*, *M. fructigena* and *M. laxa*. Although their economic relevance in agriculture, little has been done to characterize the metabolic profiles of these fungi. Furthermore, species misidentification among this genus is incredibly common due to their similarities in appearance, and hence what little is known about the chemistry of these species is equally incoherent and unclear. Our project aims to ascertain a global understanding of the secondary metabolite production of the three *Monilinia* sp. in order to clarify which of the known *Monilinia* compounds are produced by each species and in which way they are involved in the pathogenic process on fruit. To ascertain the metabolome of *Monilinia* sp., five strains of each species were cultivated on four different media (PDA, YES, MEA, Jasmine rice) for 3 days in the dark at 25 °C, followed by cultivation in alternating light (12 h light, 12 dark) at 20 °C. Chemical extracts were collected by taking 5 plugs (5 mm diameter) and extracting with 33% isopropanol/ethyl acetate (0.1% formic acid), followed by HPLC-MS/MS analysis. Using the One Strain Many Compounds (OSMaC) approach, the strains with the highest and most diverse production of secondary metabolites were further cultivated on an extended suite of media to encapsulate the expression of as many of their biosynthetic gene clusters (BGC) as possible. Samples will also be collected for transcriptomic analysis which will be subsequently used to compare the expression patterns in an attempt to link the specific compounds produced on each media to the corresponding BGC of the reference genome (which have also been acquired).

P4

Uncovering intraspecific phenotypic diversity and potential trade-offs of the entomopathogenic fungus *Metarhizium brunneum*

Jinglin Zhao – Department of Plant and Environmental Sciences, University of Copenhagen

Metahrizium is a versatile ascomycete fungal genus that grow as a saprophyte, insect pathogen, and plant-root symbiont, with *M. brunneum* being highly adaptable to all three and serving as a generalist insect pathogen and endophyte capable of inducing plant resistance against pathogens. This study focuses on phenotypical differences among seven isolates of the entomopathogenic fungus *M. brunneum* collected from diverse locations worldwide. We investigate the differences in nutrition preferences, rhizosphere colonization abilities, sporulation on the surface of wheat seeds, and mycelium growth rates in the presence of wheat seed exudate. The results show significant variation among the isolates in all these phenotypic traits, indicating that the phenotypical difference among *M. brunneum* isolates is real and potentially attributable to the source from which they were isolated. These findings provide important insights into the ecology and evolution of *M. brunneum* and have potential applications for the biological control of insect pests.

P5

Novel Insights into Fungal Specialized Metabolite Biosynthesis

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Fungi produce a vast amounts of specialized metabolites, SMs, of which only the tip of the iceberg has been characterized and many compounds and exciting biochemistry await discovery. To uncover new SM gene functions we use strategies based on gene deletion in the host, pathway reconstitution in a cell factory, and/or feeding experiments using labeled precursors. Using these strategies, we have recently investigated SM pathways in *Aspergillus californicus*, which has never previously bee genetically engineered, and in A. niger, and discovered and characterized pathways with unusual fungal SM biochemistry. In the case of A. californicus, dissection of an SM pathway identified a novel ring-closure mechanism for production of 2-pyridones. For A. niger, we revisited yanuthone production and determined that yanuthone X2 appears to originate from a precursor from the shikimate pathway. Previously, we have shown that yanuthone D is derived from the polyketide 6-MSA and that both types of yanuthones share a number of biosynthetic steps. Hence, the yanuthone SM pathway is complex as precursors from two different sources of the primary metabolism are able to enter the same biosynthetic pathway to expand the repertoire of products that this pathway is able to produce.

P6

Production and characterization of carbohydrate active enzymes

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To support the transition to a green economy, novel industrial enzymes are necessary. Enzymes of particular interest in this context are Carbohydrate-Active EnZymes (CAZymes) that are produced by microbes including some commercially available fungal enzymes. Although novel CAZymes targeting specific substrates are still in great demand genome sequences show that there is a vast repertoire of uncharacterized CAZyme genes, which may deliver the desired activities. Hunting down a specific desirable enzyme in the available sequence space is demanding because there are so many potential sequence candidates. Serious screening work for identifying such an activity is therefore substantial and the workload is immense if there is little knowledge available to reduce the sequence space to explore. A strategy to address this challenge is to position CAZyme sequences and activities into a map and use a nearest neighbor principle to elucidate the activities. Unfortunately, there are too few characterized CAZymes to cover the expected enzymatic landscape and many “white areas” remain. To explore the white area, there have been substantial efforts aiming at identifying novel CAZymes via heterologous production in bacterial hosts. However, the success rate of such approaches with eukaryotic enzymes, including fungal targets, has been extremely low. We hypothesize that fungal cell factories are better suited for eukaryotic enzymes, due to a dedicated secretory pathway that offers folding control and post-translational modifications. So far, this strategy has been impaired by lack of specific tools for high-throughput strain engineering, but recently developed tools at DTU-Bioengineering may eliminate this obstacle. The goal of this project is to establish an automated setup that allows heterologous expression and characterization of uncharacterized CAZyme genes in high throughput, with a vision to cover unexplored territory in the eukaryotic CAZyme map for novel enzyme discovery.

P7

Aspergillus oryzae strain engineering for anticancer l-asparaginase production

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Aspergillus oryzae is an important industrial workhorse used for the production of different enzymes due to its efficient protein secretory pathway and low or no levels of mycotoxin production. Interestingly, *A. oryzae* is a natural producer of L-asparaginases. Those enzymes, mainly from bacteria produced in Escherichia coli are commercialized as anticancer enzymes against acute lymphoblastic leukemia (ALL) and for the best of our knowledge it has not been studied at the genetic level in *A. oryzae*. This study aimed to engineer an *A. oryzae* strain for recombinant production of L-asparaginase, characterize the enzyme and assess its anticancer properties in vitro. Initially, six potential L-asparaginase genes were identified and individually deleted in *A. oryzae* using CRISPR/Cas9 technology. The knockout of a candidate gene, here named asp5, resulted in no L-asparaginase activity in plate assays and this mutant was selected for the reintegration of the six different L-asparaginase genes. This process was facilitated as the strain contains a target expression site that harbors an uidA marker gene
resulting in an easy identification of the correct mutants via white or blue color formation in the colonies. A total of 10 mutants constructed with different signal peptides resulted in one L-asparaginase, named zere Asp2, with molecular mass of approximately 75 kDa and 40 kDa before and after N-glycans removal, respectively. A native gel showed that the enzyme is at least dimeric as the molecular mass was substantially higher compared to denaturation conditions. Asp2 showed optimal activity at pH 7-8 and 37°C, which is compatible with human body conditions. When applied to several cancer cell lines, Asp2 has zeen found to be highly efficient against ALL. The results show that the L-asparaginase, Asp2, from A. oryzae has the potential to be further studied as an anticancer drug.

P8

Fungal fermentation for food protein production in upcycled agro-industrial side-streams

Mette Lübeck - Aalborg University

There is an increasing demand for proteins due to the human population rise and increase in living standards. At the same time, animal production has a huge negative climate impact and demand for agricultural land for feed production. This calls for a shift in the dietary pattern towards more sustainable food sources with better utilization of the arable land to sustain feeding the population without further burdening the environment. Due to their efficient biomass degradation apparatus, filamentous fungi are excellent organisms to retrieve nutrients from complex material. Thereby they have the capacity to upcycle agro-industrial side-streams into food proteins. The production of food proteins can be carried out using native strains or engineered strains to produce multiple proteins or to produce specific secreted proteins in highly specialized fungal hosts. Production of proteins is carried out in Solid State Fermentation (SSF) and Submerged Fermentation (SmF) where SmF is based on easily fermentable sugar streams. These sugars can come from 1. generation processes or pre-treated and hydrolyzed 2. generation biomasses, whereas SSF can utilize substrates without pre-treatment. The fermentation substrates are often considered among the most important components in the cost of the fermentation products, which usually can account for almost 50% of the whole production process. Thus, to lower the costs of production for lower value products that need to be produced in high amounts such as food proteins, the search for cheaper sources of fermentation substrate has high priority for the industry. We are working on upcycling of low-cost side-streams from the food- and agro-industry and adapting these streams to specific fungal production hosts as alternative cheap and sustainable fermentation substrates in the production of alternative proteins for the food industry in SSF and SmF.

P9

Development of a molecular toolbox for the oleaginous yeast *Cutaneotrichosporon Oleaginosus*

Mattia Gamberoni - Aalborg University

Oleaginous yeasts are commonly used for the production of biofuels and high-value poly-unsaturated fatty acids. Among these yeasts, the basidiomycete *Cutaneotrichosporon oleaginosus* is an excellent candidate due to its ability to accumulate up to 70% of its dry biomass in lipids and tolerate common fermentation inhibitors such as furfurals, ammonium ions and acetate at a much higher concentration compared to other common oleaginous yeasts. However, introducing exogenous DNA into C. oleaginosus has proven to be challenging due to its thick cell wall and natural predisposition towards non-homologous end joining. In this study, we developed an electroporation protocol using a combination of nourseothricin and hygromycin B supplemented with cefoxitin for selection of transformants. We were able to introduce linear DNA fragments of 9000+ bp, which were randomly integrated into the genome. We used this protocol to create a *C. oleaginosus* strain carrying a tetracycline-induced TetON system for Cre-Lox recombination. The aim is to excise the nourseothricin selection marker from the genome, allowing for multiple subsequent transformations of genes of interest using nourseothricin for selection. This in-vivo selection marker-recycling system is currently being tested. If successful, this research will enable quick genetic modifications of *C. oleaginosus*, unlocking its full potential as a fermentation workhorse.

P10

CRISPR-Cas9 tools for genetic engineering of CUG-clade yeast *Debaryomyces hansenii*

Tomas Strucko - DTU Bioengineering

The robustness of *Debaryomyces hansenii* makes it a promising candidate for cell factory applications. However, currently available genetic techniques did not allow the full potential of *D. hansenii* to be implemented mainly due to limited genetic markers that are compatible with wild-type strains. In addition to this, the non-homologous end-joining (NHEJ) DNA damage repair mechanism poses challenges for precise gene targeting. To address these issues, we have developed a plasmid-based CRISPR-Cas9 method for efficient gene editing in prototrophic strains of *D. hansenii*. Our method utilizes a dominant marker and enables quick assembly of vectors expressing Cas9 and single or multiple sgRNAs. Furthermore, we have created an NHEJ-deficient *D. hansenii* strain that facilitates highly efficient introduction of point mutations and gene deletions. We have also shown that single stranded (90-nt) DNA oligonucleotides can precisely repair DNA break induced by sgRNA-Cas9, achieving 100% efficiency. Our tools have significant potential to advance basic and applied research in *D. hansenii* and can be adapted for gene editing in other non-conventional yeast species, including those in the CUG clade.

P11

CRISPR-Cas9 mediated gene integration of fungal cytochrome P450 monooxygenase for the production of ω-hydroxy fatty acids in *Yarrowia lipolytica*

Anne Kathrine Clausen – Aalborg University

The aim of this project was to produce ω-hydroxy fatty acids which can be used for synthesizing the bio-plastic polyurethane. *Yarrowia lipolytica*, a yeast known to produce high amounts of lipids, was genetically engineered by introducing two foreign genes, FoCYP655C2 and FoCPR, from *Fusarium oxysporum*. FoCYP655C2 expresses a cytochrome P450 monooxygenase, while FoCPR expresses the homologous redox partner. FoCYP655C2 and FoCPR were integrated into the AXP and A08 loci, respectively. Besides modifying the fatty acids, the lipid production was also increased by integrating the gene ACC1 expressing acetyl-CoA carboxylase 1 and DGA1 expressing the diacylglycerol acyltransferase 1 into the XPR2 and D17 loci, respectively. The plasmids were assembled through the Gibson Assembly method, where transformations were performed through the CRISPR-Cas9 mediated gene integration. Furthermore, the lipid production was investigated with glucose as carbon source in shake-flask fermentations. The extracted lipids were derivatized through transesterification and trimethylsilylation before analysis with GC-MS. The results are to be used for the industrial production of polyurethane which is used as a protective coating in the windmill industry. Therefore, this project was made in collaboration with Polytech A/S which can use the microbially produced ω-hydroxy fatty acids for a sustainable synthesis of polyurethane.

P12

Genomic Patterns of adaptation following serial infection of a specialist fungal pathogen in a novel host

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The genus *Metarhizium* comprises a set of diverse insect-pathogenic fungi that exhibit a wide spectrum of host ranges. Within this genus, *M. acridum* is a specialist pathogen that infects orthopteran insects and is currently used in environmentally friendly biological control of locust pests. Although this species exhibits a global distribution across tropical and sub-tropical regions, much of the current genotypic and phenotypic characterization is based on only two isolates. To increase our understanding of how evolutionary factors and genomic diversity drive host colonization, we first expand current knowledge of intra-specific genomic diversity by establishing a reference-quality pangenome of *M. acridum* based on six assembled genomes of isolates from four continents. We find that 7,242 of the 10,177 gene clusters (71%) are shared among all isolates (core genome), and used enrichment analysis to determine the functional differences between the core and accessory regions. Using this foundational genomic dataset, we then selected three isolates displaying divergent genotypic and virulence profiles for a serial passage experiment to investigate how standing genetic variation in a pathogen contributes or constrains the ability to colonize novel hosts. We passaged *M. acridum* through three host environments for five generations: *Locusta migratoria* grasshoppers, representing the natural host; *Tenebrio molitor* beetles, representing a novel host; and sabouraud dextrose agar media representing an experimental control. We observe clear changes in virulence and gene expression following serial passaging, and use our studies to reveal how standing genetic variation and the pan-genomic structure of a specialist pathogen influence genomic patterns of early adaptation.

P13

The interaction between resident bacteria and fungal pathogens in an insect

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Fungi are predominant natural enemies of insects and are a promising strategy for environmentally-safe pest control. Many insects also harbor bacterial associates that can have a major influence on the biology of their host, either as specialized symbionts or as members of the gut microbiota. While interactions between insects and insect pathogenic fungi or insects and their bacterial symbionts have been studied independently, little is known about the tripartite interaction between these players. Using Western Flower Thrips (*Frankliniella occidentalis*), a globally invasive insect pest, we are investigating if and how symbiotic bacteria affect infections by the generalist entomopathogen *Beauveria bassiana*. In vitro assays show that metabolites produced by the bacterial gut symbiont BFo1 (Erwiniaceae) hinder the germination of *B. bassiana* conidia. Also, preliminary in vivo assays indicate that the presence of the gut bacteria delays infection by *B. bassiana* in thrips larvae. We are interested in the mechanisms by which bacteria and fungi interact in the insect, and what factors determine the role of the resident microbiome in the progression of fungal infections. Fungal gene expression profiles upon infection of symbiotic and symbiont free insect hosts in this system are providing insights on the direct and indirect effects that insect symbiotic bacteria can have on a fungal entomopathogen.

P14

Unravelling the secrets of fungicides through *Streptomyces*-pathogen interactions

Kah Yean Lum – Technical University of Denmark

Crop diseases caused by pests and pathogens are of global concern and pose a significant threat to agricultural production and food security worldwide. Among the plant pathogens, fungi and fungi-like oomycetes incite the most economically significant diseases of crops. These phytopathogens cause enormous global yield losses annually, and several fungi also produce toxins that can severely threaten animal and human health. As the impact of climate change on agriculture is becoming more apparent, this will lead to an environmental condition that is extremely favourable for fungal growth and mycotoxins production, hence escalating the problems of plant diseases. Synthetic pesticides have been widely used to chemically control microbial infestation in agricultural fields, however, their extensive use has raised serious concerns to the environment and human health. Biological controls by introducing naturally occurring agents like beneficial microorganisms or natural product extracts directly into a natural ecosystem can potentially mitigate this dilemma without risk to the environment and human/animal health. As Streptomyces are the most important antibiotic producer and co-exist with fungi in nature, we aim to unravel the biotechnological potential of Streptomyces and their secondary metabolites for use in the biocontrol of phytopathogens through advanced chemical ecology and natural product chemistry. Several Streptomyces strains collected from Danish soils are being tested against a panel of fungal pathogens through co-cultivation, and the active strain will be subjected to LC-MS analysis and bioassay-guided fractionation for the identification of antifungal compounds.

P15

Host specificity influence how fungal pathogens navigate nutritional landscapes of their insect hosts

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Nutrition often mediates the outcomes of host-pathogen interactions. These effects can be indirect when a host's immune response is constrained by specific nutritional deficiencies (*e.g.* iron or glutamine), or direct when pathogens colonize the heterogeneous nutritional landscape comprised of host organs and tissues. Here we use nutritional geometry to study these dynamics as ecological niches in two steps. First, we measured the fundamental nutritional niche (FNN) breadth of insect-pathogenic *Metarhizium* fungi in simulated *in vitro* host environments. Second, we measured how these pathogens navigate nutritional landscapes to acquire a realized nutritional niche (RNN) in simulated host environments of semi-solid liquid media to determine nutrient-specific intake. We further compared nutritional niche evolution across three *Metarhizium* species with different levels of host specificity from propagule introduction to onset of spore dispersal. Host-specificity did not influence FNN dimensions for fungal growth, as each fungal pathogen species grew maximally across a broad range of carbohydrates (C), assuming protein (P) was present above a minimal threshold. Fungal pathogens also similarly initiated dispersal behaviors leading to sporulation when either C or P became depleted. In contrast, host specialists and generalists prioritized P and C intake differently. The host specialist *M. acridum* always prioritized C intake, but the generalists *M. anisopliae* and *M. robertsii* prioritized P and C equally, with P:C intake reflecting diet media P:C ratios. In this way, pathogens may span a continuum of dietary specialization corresponding to degrees of host specificity, similar to that used to predict the success of potential invasive species.