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Mini-Review

Mini-Review: The Distinct Carbohydrate Active Enzyme Secretome of *Rhizopus* spp. Represents Fitness for Mycelium Remodeling and Solid-State Plant Food Fermentation

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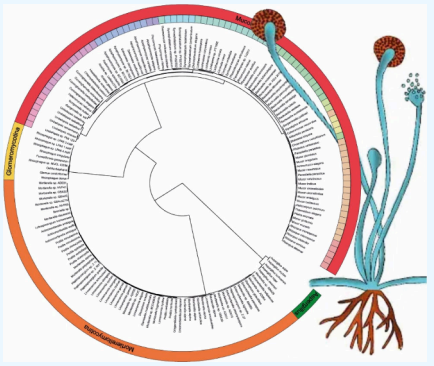
ABSTRACT: *Rhizopus* is a genus of filamentous fungi belonging to the Mucoromycota division. *Rhizopus* species produce a white, dense mycelium, which is used to create tempeh, a solid-state fermented Asian soybean product, that is gaining renewed attention as a proteinaceous plant food. The profile of carbohydrate-active enzymes (CAZymes) of a fungus or group of fungi, particularly the secretome CAZymes profile, reflects adaptation to different lifestyles and habitats, and has a significant impact on fermentative capacity. This review examines the CAZymes profiles of *Rhizopus* species focusing on their implication for carbohydrate utilization and solid-state fermentation of plant materials. Through comprehensive genomic assessments and comparisons with other filamentous fungi, we particularly highlight how the unique CAZymes secretome profile is closely correlated with the taxonomy and ecological niches of *Rhizopus* species. We discuss how the CAZymes secretome capacity of *Rhizopus* species differs from other fungi and summarize the current state of knowledge regarding the specific CAZymes involved in the modification of carbohydrates in the fungal cell wall and in plant cell walls. We foresee that advanced genomic and proteomic technologies will be used to expand the biotechnology applications of *Rhizopus* spp.



INTRODUCTION

Filamentous fungi encode an armament of carbohydrate active enzymes (CAZymes), and notably the CAZymes secretome reflects the fungal speciation related to the evolved habitat adaptation and substrate utilization. In Southeast Asia, *Rhizopus* fungi, notably *Rhizopus oligosporus*, are utilized in the production of tempeh, a solid-state fermented food product, usually based on soybeans. Tempeh is a compact, white, cake-shaped product having a characteristic nutty flavor, mushroom-like texture, and high nutritional value. The fungal mycelium constitutes an important part of the tempeh, and the mycelium obviously forms as a result of the efficient fermentation that leaves the soybeans relatively firm; yet the fungal fermentation is a result of selective fungal digestion of the plant substrate by the fungus during its growth and mycelium formation. Tempeh fermentation has thus been shown to not only improve the digestibility of soybeans but also to increase the bioavailability of essential nutrients, making it a source of protein and a relevant sustainable plant-based food product.[1]

The specific CAZymes encoded and produced by *Rhizopus* species govern the mycelial growth and its impact on the substrate plant matrix. The understanding of the enzymology of *Rhizopus* spp. in relation to solid-state fermentation is also important for the development of new plant-based foods to provide alternatives to current animal-based protein-rich foods. Here, we examine the phylogeny, taxonomy, and ecological niches of *Rhizopus* spp. in relation to CAZymes profile, discuss the function of specific CAZymes of *Rhizopus* spp., and compare the CAZymes composition to that of other filamentous fungi. We also provide an integrative view of the relationships between the encoded CAZyme repertoire and substrate utilization in relation to solid-state plant food fermentation and new applications of *Rhizopus* species.

PHYLOGENY AND ECOLOGY OF *RHIZOPUS* SPECIES

Rhizopus species are part of the Mucoromycota phylum, a sister group to Dikarya, which is divided into three subphyla: Glomeromycotina, Mortierellomycotina, and Mucoromycotina. Glomeromycotina includes arbuscular mycorrhizal fungi that establish a symbiosis with plants and play an essential role in improving the host plant nutrient uptake. Mortierellomycotina species are soil fungi acting as root endophytes (that will

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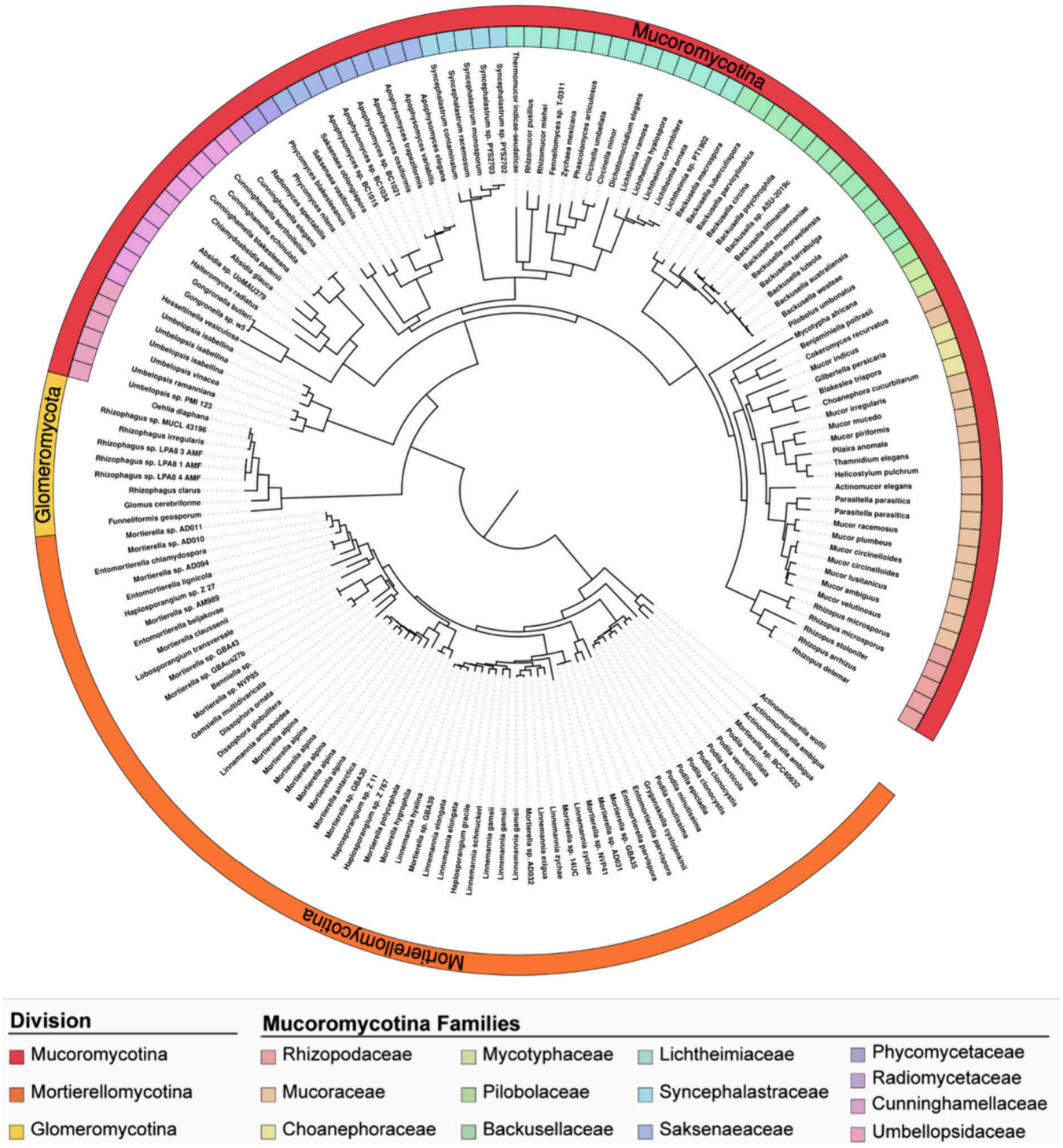

Figure 1. Genome-based maximum likelihood rooted phylogenomic tree of Mucoromycota based on 126 single-copy protein coding orthologues obtained from a BUSCO quality assessment of 161 genomes. Subdivisions and Mucoromycotina families are indicated by boxes of different colors. (Using the BUSCO phylogenomics pipeline, the single-copy orthologs (n = 126) present in 99.5% of the 291 selected genomes out of the 323 available assemblies from Mucoromycota species at the National Center for Biotechnological Information (NCBI) were retrieved to build a superalignment and a Maximum-Likelihood tree with 1000 bootstraps. All unique species were kept and those species presenting a branch length less than 0.01 were considered as the same organism (n = 161).)

have part of the fungal hyphae inside of the host plant root matrix without damaging the plant growth) and as saprobes (decomposers that feed on decaying plant material). In this classification, *Rhizopus* species are included within the Mucoromycotina subphylum, whose members are characterized by being fast growing, early colonizers and preferring easily accessible sugars.[2]

In 2016, by comparing 192 orthologous protein-coding genes from 46 taxa including 14 from the Mucoromycota division, Spatafora et al.[2] proved that the genus *Rhizopus* is a well-defined monophyletic group. Shortly thereafter, in 2018,

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using a similar approach, Gryganskyi and collaborators[3] confirmed the monophyly of *Rhizopus* as a genus that can be divided into three different clades: *Rhizopus microsporus*, *Rhizopus stolonifer*, and a clade containing both *Rhizopus oryzae* and *Rhizopus delemar*. In addition, *R. microsporus* was shown to be the basal species. In the same study[3] it was moreover highlighted that the genome size of *Rhizopus* can vary widely between species and between isolates of the same species. The average genome size observed in the *R. stolonifer* and *R. oryzae/delemar* clades was 44.5 Mb, whereas the *R. microsporus* clade had the smallest, 16 Mb, and the largest, 75 Mb, assembled genomes. These variations seem to be driven by changes in transposable elements copy numbers and notably by genome duplications and may have practical implications for applications of *Rhizopus* species in terms of the potency of the catalytic machinery even in same species - but a systematic analysis of this phenomenon is lacking. To examine the taxonomy of the *Rhizopus* clades it is relevant to analyze the whole Mucoromycota phylum using bioinformatics: we retrieved all the 323 available assemblies from Mucoromycota species from the National Center for Biotechnological Information (NCBI) and filtered them using BUSCO (5.5.0) with the mucoromycota_odb10 lineage to keep only the high-quality genomes (n = 291). BUSCO is a tool for assessing the quality of genome assemblies that uses data sets of single copy orthologs among various lineages to estimate the completeness and redundancy of genomic data. In addition to this quality evaluation, the obtained near-universal single-copy genes can be used as markers for genome scale phylogeny.[4] The resulting phylogenetic tree (Figure 1) clearly depicts the classification of the three Mucoromycota subphyla, i.e., the Mortierellomycotina, Glomeromycotina, and Mucoromycotina. The tree also shows the monophyly of *Rhizopus* and the subdivision level and the organization of the Mucoromycotina families in accord with the family structure proposed in recent taxonomic studies.[2,3]

Regarding the habitat, *Rhizopus* species are ubiquitous, thermotolerant molds found widely on organic substrates, notably on decaying fruits and plant debris. Their ability to sporulate quickly under suitable conditions allows them to dominate in ecological niches characterized by transient resources. It is important to note that certain *Rhizopus* species, such as *R. oryzae*, are opportunistic pathogens that can infect immunosuppressed individuals to cause various infections, including mucormycosis.[3] *R. oryzae* is also described as *R. arrhizus* and the recommended name is still being discussed. Although it is mainly *R. microsporus* species that are associated with tempeh, indeed strains of *R. oryzae* (or *R. arrhizus*; we will use the name *R. oryzae* in the present treatise), *R. delemar* as well as *R. microsporus* can be isolated from tempeh produced using traditional starters. However, during the last 25 years mainly strains of *R. microsporus* are used as a commercial tempeh starter culture.

PRACTICAL APPLICATIONS OF *RHIZOPUS* SPP.

As mentioned above, *Rhizopus* species are mainly known for their widespread use in the fermentation of traditional Asian foods such as tempeh, but *Rhizopus* is also used for industrial manufacture of enzymes and metabolites such as carboxylic acids. During solid-state fermentation, the fermenting organism produces enzymes that degrade the carbohydrates and proteins present in the substrate or plant matrix to obtain the energy and the building blocks needed for their growth. In solid-state food fermentation, including tempeh production, the CAZymes are particularly important, because they are responsible for both the catabolism of plant storage polysaccharides and cell wall polysaccharide structures and the building and restructuring of the fungal mycelium, all of which have a direct impact on the visual appearance, texture, mouthfeel, taste, and nutritional quality of the resulting product.[1]

CAZymes are divided into 5 different classes according to their amino acid sequence and protein fold of the catalytic module (http://www.cazy.org):[5] Glycoside hydrolases (GH), glycosyltransferases (GT), carbohydrate esterases (CE), polysaccharide lyases (PL), and auxiliary activities (AA). CAZymes may also contain noncatalytic carbohydrate binding modules (CBMs) that bind to the substrate and increase enzyme−substrate proximity. Although CAZymes within a family share sequence similarity and thus have closely related catalytic mechanisms, the CAZymes within a family may present different substrate specificities. The activity of CAZymes on the structural and storage polysaccharides present in the plant material affect the texture and consistency of the original substrate, for example, a particularly saccharifying CAZyme profile can liquify the plant food matrix. The available set of CAZymes will thus determine the mycelial growth and overall texture and other macroscopic effects of the fungal fermentation on a specific substrate, and in turn also impart the release of metabolites and formation of flavor compounds that contribute to the unique taste profiles of fermented foods.[1,6]

Beyond tempeh, *Rhizopus* spp. are used in a range of other processes. Notably, *Rhizopus* spp. play a key role in the industrial production of fumaric and lactic acid, important in the food and pharmaceutical industry for their preservative and flavor-enhancing properties. In addition, enzymes such as lipases and amylases from *R. microsporus* are industrially produced.[6] *Rhizopus* species are also known for their ability to produce bioactive compounds through fermentation. These bioactive compounds, including isoflavones and bioactive peptides, have numerous potential applications in pharmaceuticals and as health supplements. The versatility of *Rhizopus* in bioprocessing is further exemplified by its application in waste management, where it contributes to the biodegradation of organic wastes, including fruit and vegetable processing residues, transforming them into valuable products like compost or animal feed.[6]

THE UNIQUE CAZymes PROFILE OF *RHIZOPUS* SPP.

The CAZymes profile of *Rhizopus* species has long been considered distinct to that of other fungal species. In 2011, Battaglia and colleagues[7] published the first genome-based annotation of *R. delemar* strain 99−880 reporting the CAZyme profile and noted a significantly lower count of predicted GHs (namely 116) in *R. delemar* strain 99−880 compared to other filamentous fungi such as *Aspergillus oryzae* (that has 293), but also noted similar numbers of GTs (130) in *R. delemar* and *A. oryzae*, that encode 115 GTs, and typical numbers of PLs (6), CEs (41), and CBMs (24) in *R. delemar*. In 2020 the CAZyme profiles of several *Mucor* species were found by Lebreton et al. to have a similar pattern of fewer GHs (average around 77) but an elevated number of GTs (approximately 110).[8] A comparison of the two studies suggests that *R. delemar* encodes more CAZymes across all classes than the examined

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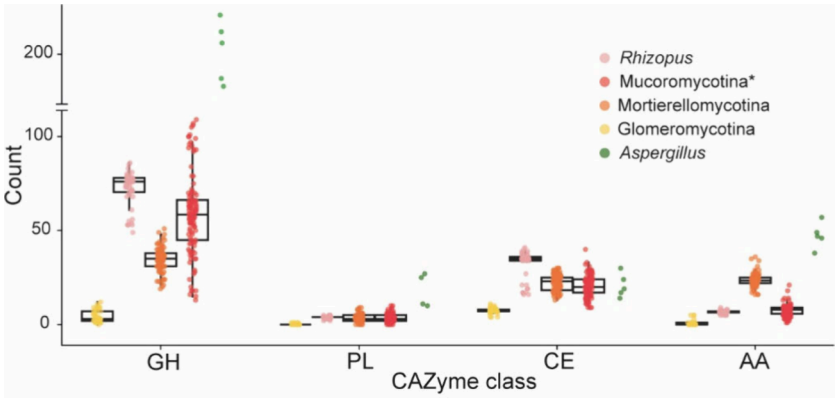

Figure 2. Count of carbohydrate active enzymes classes for *Rhizopus* (n = 55), Mucoromycotina (n = 112), Mortierellomycotina (n = 86), and Glomeromycotina (n = 41). GH (glycoside hydrolases), PL (polysaccharide lyases), CE (carbohydrate esterases), AA (auxiliary activities). The boxplots show the median, Q1, Q3. *Mucoromycotina excluding *Rhizopus* for comparison. Data are based on the predicted proteins for the 291 genomes resulting from the BUSCO quality assessment (used for Figure 1), using the bioinformatic pipeline described by Barret et al.[9] the protein sequences were predicted, and secretion and CAZymes were annotated (as GTs are usually considered to be intracellular, they are absent from this analysis).

Mucor species, emphasizing the distinctive CAZyme profile of *Rhizopus* species also when compared to more closely related organisms such as *Mucor* species.

Prediction of the proteins encoded by the 291 genomes resulting from the BUSCO quality assessment used in Figure 1, shows that *Rhizopus* species have capacity to secrete the highest number of GHs and CEs, namely averages of 72 and 33, respectively within the Mucoromycota (Figure 2). In contrast, the number of PLs in the Rhizopus secretome is low (average of 4), only presenting higher counts than members of Glomeromycotina (which in our analysis have capacity to produce only 1 PL); it can thus be inferred that PLs represent the less abundant CAZyme class of *Rhizopus* species. In general, the analyzed Glomeromycotina species present the lowest number of predicted CAZymes in all classes, with the number of predicted GHs being particularly low averaging less than 10 (Figure 2). Additionally, the predicted number of AAs in the secretome of the Glomeromycotina species is lower than in the Mucoromycotina and *Rhizopus* species (*Rhizopus* spp. has 7 on average), and lower than in Mortierellomycotina (24).

In comparison, the five selected *Aspergillus* species used in the analysis, and widely used in fermentation processes, have much higher counts in all CAZyme classes, except the CEs (Figure 2). The predicted number of CEs (21) in the *Aspergillus* secretomes were thus found to be comparable to those found in the Mortierellomycotina and Mucoromycotina subphyla, excluding *Rhizopus*, which has slightly higher average values of CEs than members of the other subphyla (Figure 2). These findings of the secretome counts align well with the complete CAZyme annotation for *R. delemar*, further emphasizing the distinctive nature of the *Rhizopus* CAZyme set.[7]

CAZymes SECRETOME PROFILE-BASED CATEGORIZATION

The secretome CAZyme composition determines the substrate utilization of the organism, and fungal secretomes of CAZymes can therefore be categorized according to the substrate utilization, reflecting the preferred habitat of the organism in question. Expanding upon the approach we used previously for *Aspergillus* and *Penicillium* categorization,[9] we analyzed the CAZyme secretome profiles within the whole Mucoromycota division (n = 161) and compared them with those of five *Aspergillus* species relevant for fermentation processes. A presence-absence matrix was constructed for the CUPP groups for each organism (CUPP groups are based on identifying conserved unique peptide patterns of enzyme proteins using a similarity assessment algorithm);[9] the resulting profiles were subsequently compared using a Yule-dissimilarity scoring algorithm to derive an enzyme profile relatedness (EPR) mapping, which is visualized in a dendrogram map in Figure 3.

This EPR mapping confirms a taxonomical categorization which is congruent to that obtained with BUSCO-based phylogenetic trees (Figure 1). Notably, this mapping corroborates that the taxonomical separation within the Mucoromycotina families agrees with the fungal phylogeny, distinctly positioning *Rhizopus* species apart from other groups. This CAZymes based categorization of the *Rhizopus* clade resembles the characteristics of the original phylogeny, i.e. the monophyly with *R. microsporus* as the basal species, which is sister to a clade containing *R. stolonifer* and a clade containing both *R. delemar* and *R. oryzae*.[3]

The EPR mapping thus supports the proper separation of the *Rhizopus* genera in terms of CAZyme secretome even at the species level. (The findings were validated by multidimensional scaling (MDS) analysis showing distinct clusters for different Mucoromycotina families according to their CAZyme secretome profiles, which also corresponds to taxonomic organization).

It is therefore evident that the CAZymes profile of *Rhizopus* is distinct from other fungi within the same subphylum, confirming that CAZyme profiles are inextricably linked to taxonomy, evolution, and ecological specialization.

In addition, the counts of the 10 most represented CAZymes in the secretome of *Rhizopus* (Figure 4) illustrate how *Rhizopus* spp. have the unique capacity to secrete a relatively abundant number of enzymes belonging to CAZyme families CE4 and GH18, respectively. The CE4 family harbors several different types of esterase activities, including acetyl esterase (EC 3.1.1.6), acetylxylan esterase (EC 3.1.1.72), LPS deacetylase (EC 3.5.1.-), poly-β-1,6-N-acetylglucosamine deacetylase (EC 3.5.1.-), peptidoglycan N-acetylglucosamine deacetylase (EC 3.5.1.104), and not least chitin oligosacchar-

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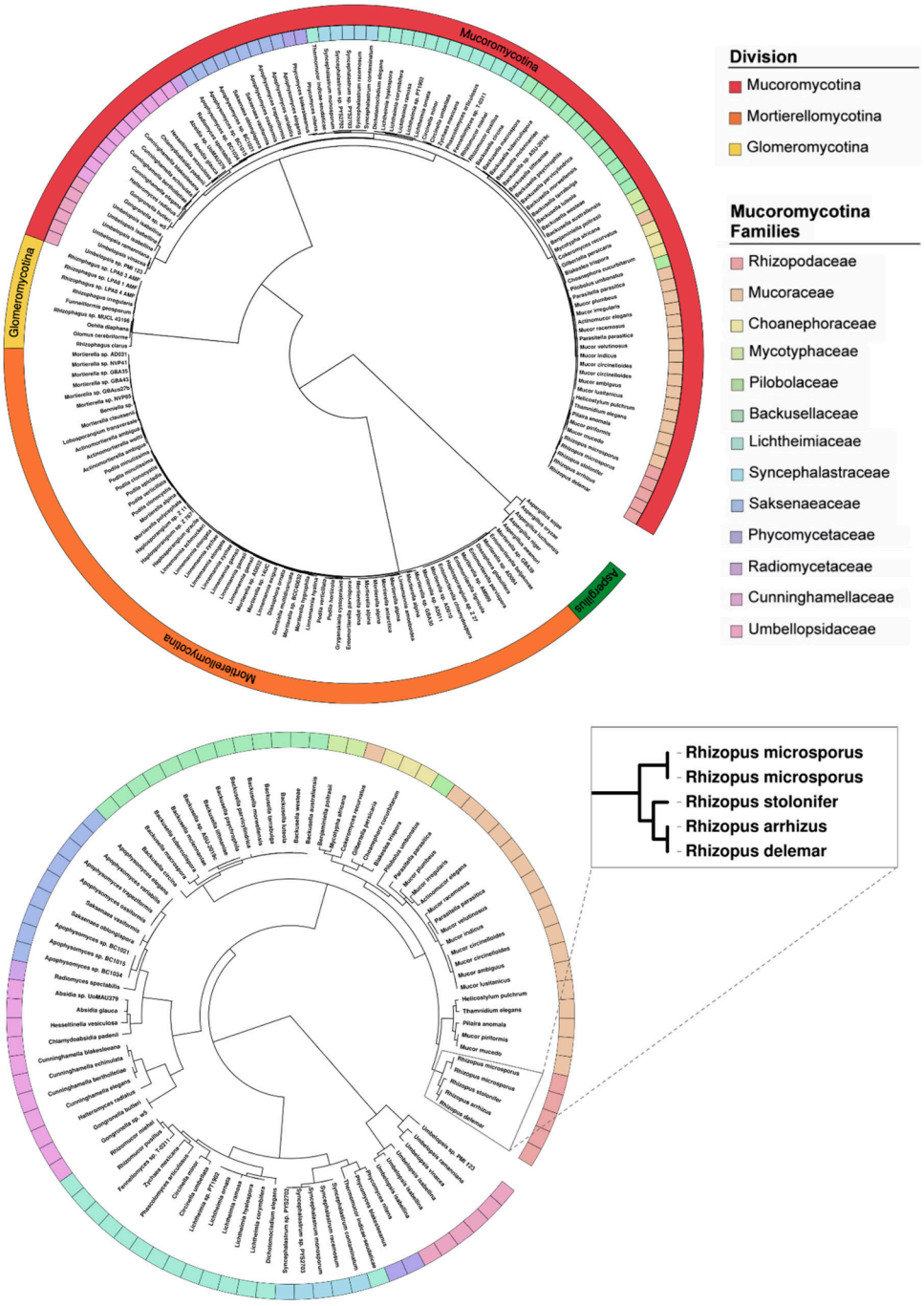

Figure 3. Circular dendrogram representing the carbohydrate active enzyme secretome enzyme profile relatedness of Mucoromycota divisions (top) and Mucoromycotina families (bottom). The distances are based on presence-absence observations of CUPP groups from the prediction of the CAZyme secretomes using the Yule-dissimilarity scoring algorithm. Subdivisions and Mucoromycotina families are indicated by boxes of different colors.

ide deacetylase (EC 3.1.1.-), and chitin deacetylase (EC 3.5.1.41) (www.cazy.org).[5] The GH18 family also harbors several different EC numbers, first and foremost different chitin modifying activities: chitin exo-β-1,4-N-acetylglucosaminidase (EC 3.2.1.-), di-N-acetylchitobiase/reducing-end exohexosaminidase (EC 3.2.1.-), chitinase (EC 3.2.1.14), β-N-acetylhexosaminidase (EC 3.2.1.52), chitosanase (EC 3.2.1.132), chitin exo-β-1,4-N-acetylglucobiosaminidase (EC 3.2.1.200), [reducing end] exochitinase (EC 3.2.1.201) (www.cazy.org).[5] These dominant CAZymes families in *Rhizopus* spp. can thus be concluded to harbor enzymes involved in the modification of chitin and this includes enzymes catalyzing remodeling of the fungal cell wall, to be discussed below.

The data (Figure 4) also show that *Rhizopus* spp. have a high capacity for secreting a high number of family GH28 and CE8 enzymes. The GH28 family harbors a range of activities specific for different types of pectin including both homogalacturonan and rhamnogalacturonan structural moieties, i.e., xylogalacturonan hydrolase (EC 3.2.1.-), endopolygalacturonase (EC 3.2.1.15), galacturonan α-1,4-galacturonidase (EC 3.2.1.67), exopoly-α-digalacturonosidase (EC 3.2.1.82), rhamnogalacturonase (EC 3.2.1.171), rhamnogalac-

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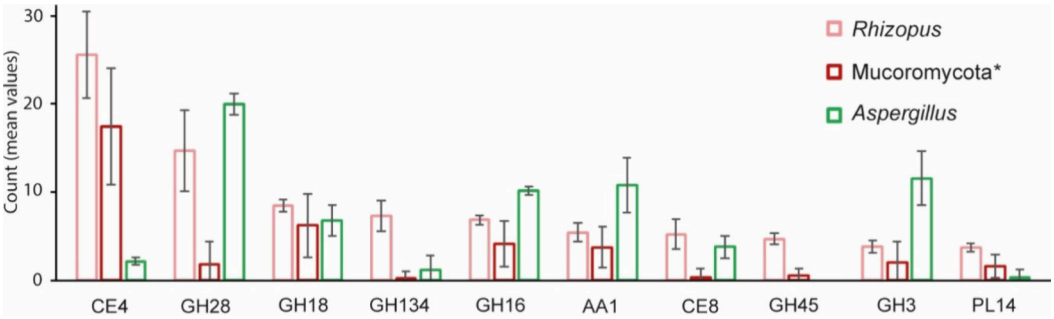

Figure 4. Mean values with standard deviation of the ten most abundant carbohydrate active enzymes in *Rhizopus* species (n = 55) compared with the same families in Mucoromycota (n = 239) and *Aspergillus* (n = 5). *Mucoromycota excluding *Rhizopus* for comparison.

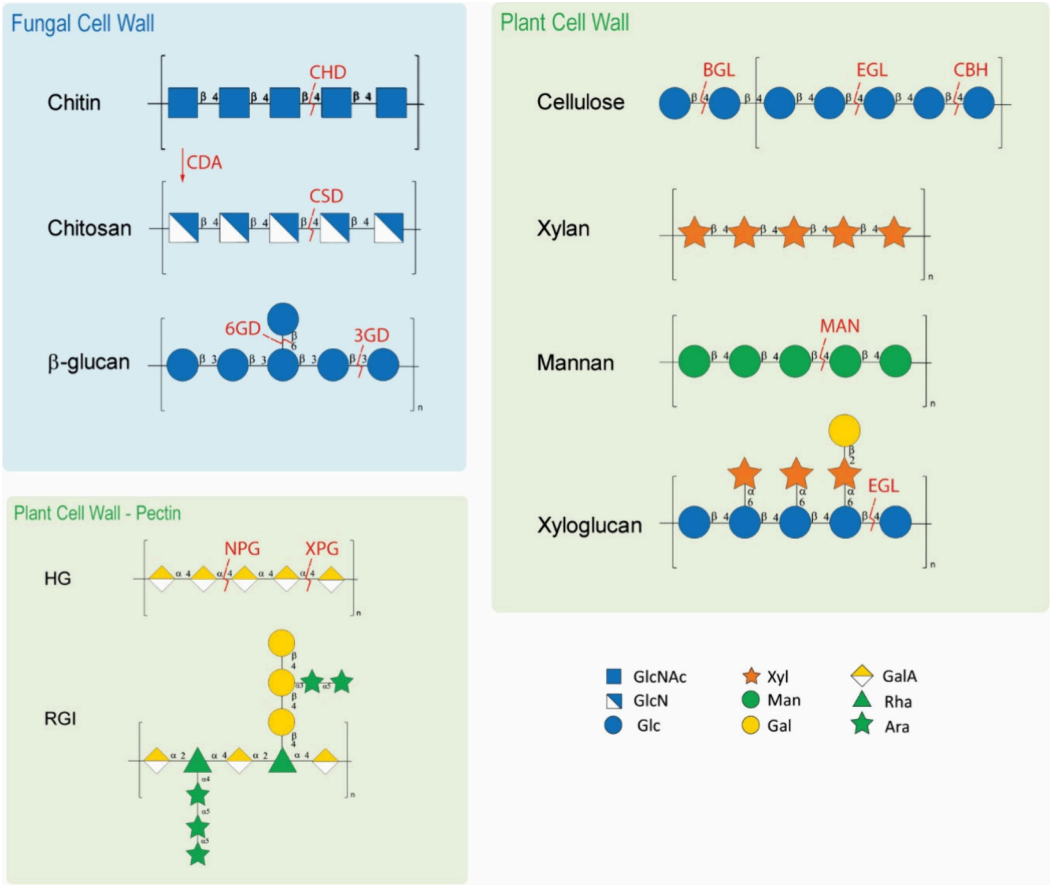

Figure 5. Schematic structure representing polysaccharides and carbohydrate active enzymes involved in the processing of the fungal and plant cell wall. Glc, Glucose; GlcN, Glucosamine; GlcNAc, *N*-acetyl-glucosamine; Gal, Galactose; Man, Mannose; Xyl, Xylose; Ara, Arabinose; Rha, Rhamnose; GalA, Galacturonic acid. The enzyme names in red font are CHD, Chitinase; CDA, chitin deacetylase; CSD, chitosanase; 6GD, 1,6-β-glucosidase; 3GD, 1,3-β-glucosidase; NPG, endopolygalacturonase; XPG, exopolygalacturonase; BGL, β-glucosidase; EGL, β-1,4-endoglucanase; CBH, cellobiohydrolase; MAN, β-1,4-endomannanase; HG, Homogalacturonan; RGI, Rhamnogalacturonan I.

turonan α-1,2-galacturonohydrolase (EC 3.2.1.173). The CE8 family is synonymous with pectin methyl esterase (EC 3.1.1.11), mostly of bacterial and fungal origin, although two enzymes from land plants (Embryophyta) have also been categorized in this family. In conclusion, the GH28 and CE8 families thus hold enzymes implicated in the degradation of pectin. Further, the counts affirm that *Rhizopus* spp. also have higher capacity than other Mucoromycota fungi to secrete enzymes of families GH134 and GH16, acting on hemicellulose. The CAZyme family GH16 harbors a lot of different enzyme activities that attack a broad range of substrates—the family has nearly 40,000 entries, and more than 20 different enzyme activities distributed in more than 25 subfamilies in the CAZY database (www.cazy.org). In contrast, interestingly, the GH134 family is known to harbor only endo-β-1,4-mannanase activity.[5]

Finally, the *Rhizopus* spp. have relatively high capacity to produce enzymes of the families AA1, GH45, and GH3 as well (Figure 4), indicating a high ability to modify cellulose and possibly lignin. Interestingly, the potential to secrete enzymes belonging to family PL14, which harbors endo-β-1,4-glucuronan lyase and different alginate lyases, is also higher for *Rhizopus* than for members of the other Mucoromycota subphyla and the selected *Aspergillus* spp. (Figure 4). It is

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especially noteworthy that the PL14 count for *Rhizopus* is higher than the number of secreted PL14 enzymes encoded by the *Aspergillus* spp. The putative ability to produce and secrete endo-β-1,4-glucuronan lyases concurs with the ability to modify glucuronan in fungal cell walls.

Genome sequencing analyses have previously revealed that fungal genomes of *Aspergillus* spp. often contain multiple copies of genes encoding carbohydrate-active enzymes with similar activity. This phenomenon, usually referred to as multigenecity, may represent advanced evolutionary fitness adaptation of the fungus to allow fast substrate utilization at different environmental reaction conditions or even small differences in substrate attack preferences as the copies typically have minor sequence variations.[10] The extent and implications of CAZymes multigenecity in *Rhizopus* spp. is less studied. Interestingly, the genomes in the current analysis show that, for example, the type strains of *R. delemar* and *R. stolonifer* are predicted to contain 18 and 20 copies of GH28, respectively, but all the gene sequences are not completely identical. It is tempting to speculate that the multiple copies may indicate advanced evolutionary adaptation to different environmental conditions such as temperature or pH or that the multigenecity is an example of biological plasticity that allows the fungus, via its enzymes, to accommodate for small variations in the substrates, e.g., methoxylation, acetylation or xylose-substitutions on the homogalacturonan backbone of pectin as observed for pectin degrading enzymes of *Aspergillus* spp.[10]

THE FUNGAL CELL WALL AND ITS ENZYMATIC MODIFICATION

The fungal cell wall aids in maintaining cellular morphology and provides protection. The structure of the fungal cell wall can be divided into two parts: the relatively conserved inner cell wall mainly composed of chitin and β-glucans, and the outer cell wall with a more variable composition across fungi consisting of different structural polysaccharides, proteins, and pigments.[11] Chitin is a homopolymer of β-1,4-linked N-acetylglucosamine (Figure 5), produced by chitin synthases, that establish intramolecular hydrogen bonds forming antiparallel assemblies of the polysaccharide chains in a similar way to that of the structure of cellulose. Postsynthetic variable levels of deacetylation of chitin, produced by chitin deacetylases, can form chitosan resulting in changes in the biological functions of the polymer. Further modifications or degradation of these polymers can happen through the action of chitinases (EC 3.2.1.14) or chitosanases (EC 3.2.1.132) that catalyze the endohydrolysis of β-(1,4) linked N-acetylglucosamine moieties in chitin or the hydrolysis of β-(1,4) linked glucosamine in partially acetylated chitosan, respectively. Fungal β-glucans are also structural polymers of D-glucose formed by mainly β-1,3 and β-1,6 linkages, creating a structure with variable degrees of branching (Figure 5);[11] in addition, the fungal cell wall may contain linear β-(1,4)-polyglucuronic acid (glucuronan). While the presence of chitin, chitosan, and glucans is relatively conserved in the fungal kingdom, the proportion of these components can vary drastically between lineages.

A recent study by Mélida et al.[12] showed that chitin represented 34% of the cell wall polysaccharides of *R. oryzae* and that the β-1,3-glucan content was lower than 3−4% of the total cell wall glycans, strikingly different to the chitin and glucan levels found in other fungi, e.g., *Aspergillus fumigatus* with 7−15% and 60−85%, respectively. The same study also revealed the presence of polysaccharides with high fucose content and lower proportions of other sugars glucuronic acid, mannose, and galactose, supporting the existence of the so-called "mucorans", namely the fungal equivalent of algal fucoidans.

From Figure 4 it is evident that CE4 is the most represented CAZyme predicted to be encoded by the *Rhizopus* genomes, and GH18 counts are also relatively high (Figure 4). Members of family CE4 are enzymes involved in chitin deacetylation,[12,13] and GH18 signifies chitin-degrading activities. Whereas the degree of acetylation in chitin is high (Figure 5), with the N-acetyl groups being the reason for the ability of chitin to crystallize, the N-deacetylation, catalyzed by CE4 enzymes, will gradually render the chitin more soluble, to give chitosan.

R. delemar has been shown to be capable of growing on chitin and chitosan, as well as on cell wall fractions of *Saccharomyces cerevisiae*, *Aspergillus niger* and *Agaricus bisporus*, but not on *R. oryzae* cell wall fractions.[7] However, *Rhizopus* spp. are *not* particularly well-known for growing on chitin-rich substrates such as marine crustaceans or insect exoskeletons or having this type of decaying material as habitat. Hence, the CAZymes secretome profile analysis suggests that CE4 and GH18 play important roles in the remodeling of the fast-growing mycelium (which contains chitin) characteristic of *Rhizopus* species. Although it cannot be excluded that the high potential to produce these enzymes indicates an antagonistic relationship with other organisms, the enzyme profile with CE4 and GH18 concurs with *Rhizopus* species and other zygomycetes having higher chitosan/chitin ratios than other fungi classes, supporting the hypothesis that these fungi have a highly dynamic fungal cell wall with varying levels of acetylation during the different developmental stages.[12]

In 2009 Ma and collaborators[13] performed whole genome sequencing of *R. delemar* 99−880. The highly repetitive nature of the genome together with the high proportion of paralogous genes suggested an ancient whole-genome duplication event followed by massive gene loss. The retained expanded gene families include, among others, gene families responsible for the biosynthesis of the fungal cell wall, involving both chitin synthases and chitin deacetylases. Nonrandom expansion of the chitinase GH18 family of *R. oryzae* is known and phylogenetic analysis of the different GH18s shows correlation with chitinases that are associated with mycoparasitic fungi and may be involved in fungal−fungal interactions in Basidiomycetes.[14] The further annotation of the CAZyme profile for *R. delemar* done by Battaglia et al.[7] showed a large set of chitin deacetylases of the CE4 family, with a total of 34 CE4 models in the genome, most of which were predicted to be glycophosphatidyl-inositol anchored proteins. In the same study five out of six protein predictions were annotated for family GH3 as exo-1,3-β-glucosidases, and none of them were predicted to be β-glucosidases involved in cellulose degradation. The modest amounts of β-1,3-glucans in the cell wall of *Rhizopus* together with the high amount of 1,3-β-glucosidases found in its genomes have been suggested to signify that the 1,3-β-glucosidases play a role in the degradation of other fungal cell walls rich β-1,3-glucans, for example those from Ascomycetes and Basidiomycetes.

Detailed enzymatic characterization of the CAZymes involved in the modification of the fungal cell wall of *Rhizopus* is limited. An active chitin deacetylase enzyme was purified

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from *R. stolonifer*, and the enzyme was reported to be heavily glycosylated with the carbohydrate content representing 53% of the weight of the protein.[15] In another study, three different chitin deacetylases from a cDNA library of *Rhizopus circinans* were isolated. The isolated enzymes were reported to have the typical features of chitin deacetylases in other fungi, with conserved domains, catalytic residues, and levels of glycosylation similar to those reported for *e.g. Mucor rouxii*. Only one of the proteins isolated was shown to be active, with optimal activity at 37 °C in a pH range of 5.5−6, and having high pH and thermal stability. The enzyme was strongly inhibited by Cu^{2+} and deglycosylation resulted in a complete loss of enzyme activity.[16] Two secreted chitinases (chi1 and chi2) were purified from *R. oligosporus* and cloned for characterization. The enzymes presented a pH optimum in the 3.5−4.0 range and relatively high thermal stability. Both enzymes showed five distinct domains in the protein sequence including a signal peptide in the N-terminal, the catalytic domain followed by a serine-threonine rich domain consistent with the observed glycosylations, and a chitin binding domain. The fifth domain consists of a variable region and a prosequence removed from the C-terminal during the maturation of the chitinases. The same group also found an intracellular chitinase (chi3) lacking a secretory signal sequence expressed in *R. oligosporus* during hyphal growth.[17] This enzyme was not glycosylated, had an optimal pH of approximately 6, and presented sequence similarity to chitinases from bacteria and mycoparasitic fungi. Furthermore, chi3 exhibited higher affinity than chi1 for small chitooligomers, similarly to what was shown for the bacterial-type Chit42 and the fungal-type Chit33 from *Trichoderma harzianum*. Additionally, one secreted chitinase from *R. microsporus* has been shown to exhibit antifungal activities by being able to degrade the cell wall of *Fusarium solani* in vitro.[18]

Two main, intertwined theories can be used to explain the functional and evolutionary significance of the particular chitin-modifying and β-1,3-glucan modifying CAZymes composition of *Rhizopus* spp.: 1. The expansions in the chitin modifying and chitin deacetylase encoding genes signify the characteristic composition and fast fungal cell wall remodeling capabilities of Mucoromycotina and *Rhizopus* spp., which would result in the distinct rapid growth of these species; 2. *Rhizopus* species use the chitinolytic and glucanase machinery primarily for rapid cell wall modification and competition with other species, rather than substrate conversion.

UTILIZATION OF THE FUNGAL CELL WALL MATERIAL AND ENZYMES OF *RHIZOPUS* SPP.

Rhizopus species have indeed been used as a source of chitin and chitosan fractions useful for industrial and agricultural processes due to the high content of these polymers in the fungal cell wall of these species.[6] Conversion of chitin and chitosan from the sources to desirable oligosaccharides is slowly drifting from the traditionally used chemical processes toward enzymatic modification of the polymers for a more sustainable and higher quality product. While considerable attention has been paid to *Rhizopus* species to produce lipases and amylases, the main efforts in the production of fungal cell wall CAZymes appear to be focused on *Bacillus, Aspergillus,* and *Trichoderma* species.[6] Notably, chitin and chitosan modifying CAZymes from the large and diverse set present in *Rhizopus* species could be of value to drive a broader valorization of mycelial material resulting from large scale industrial enzyme production processes.

THE PLANT CELL WALL AND ITS ENZYMATIC MODIFICATION AS SUBSTRATE FOR FUNGAL GROWTH

Polysaccharides constitute the primary structural components of plant cell walls and account for up to 70% of plant biomass. Although the composition of the plant cell wall varies significantly between types of plants, three major types of polysaccharides can be distinguished in dicots: cellulose, hemicelluloses, and pectic polysaccharides. The decomposition of these polysaccharides in nature is primarily facilitated by CAZymes. The enzymes employed by *Rhizopus* for plant cell wall degradation are explored below with a focus on the different plant cell wall polysaccharides and the most represented CAZymes in *Rhizopus* species.[19]

Cellulose Degradation. Cellulose is the most abundant polysaccharide in plant cell walls, it consists of a linear polymer of β-1,4-linked D-glucose units (Figure 5). These chains interact through hydrogen bonds forming tightly packed microfibrils with different levels of crystallinity, which are structurally supported by hemicelluloses. Degradation of cellulose requires the action of three different enzymatic activities that span through many different GH families, β-1,4-endoglucanases (EGL, EC 3.2.1.4), cellobiohydrolases (CBH, EC 3.2.1.91), and β-glucosidases (BGL, EC 3.2.1.21) (Figure 5).[19]

Rhizopus species are typically primary colonizers with a preference for easily accessible carbohydrates. In accordance with this, the cellulolytic repertoire of *R. delemar* has been shown to be limited, although it encodes for β-1,4-endoglucanases from the GH45 family and cellobiohydrolases from family GH3.[7] *R. delemar* has been reported to possess 21 cellulolytic enzymes, less than half of that reported for *A. oryzae*, with none of them predicted to be β-glucosidases.

Our analysis confirms the presence of GH45 in the *Rhizopus* spp. secretome, versus the low counts in Mucoromycota (Figure 4). Moriya and collaborators (2003)[20] isolated and cloned three cellulases from *R. oryzae*, RCE1, RCE2, and RCE3. The sequence from the enzymes presented three domains consisting of a signal peptide, followed by cellulose binding domains, and a catalytic domain belonging to GH45. All three enzymes showed higher activity against soluble cellulose than crystalline cellulose and confirmed EGL activity with temperature optima between 50 and 55 °C and an optimal pH in the 5−7.7 range. In a later study a secreted GH45 EGL having activity on cellulose from *R. stolonifer* with a similar domain structure as that reported for the RCE1 and RCE2 of *R. oryzae* mentioned above, was found.[21] The presence of GH3 family members was also confirmed in our analysis, and GH3 was indeed among the ten most represented CAZymes in *Rhizopus* (Figure 4). A study by Wilde et al. (2012)[22] identified five and four BGLs from the GH3 and GH5 families, respectively, in the transcriptome data from *R. oryzae*. They expressed these enzymes in *Saccharomyces cerevisiae* and detected activity for four out of the five GH3 members. The presence of these enzymes corroborates the idea that GH3 exo-1,3-β-glucosidases are critical for fungal cell wall modification and growth and at the same time explains the ability of *Rhizopus* spp. to use cellulose as a carbon source for growth.

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Hemicellulose Degradation. Hemicellulose is a term used for a range of plant cell wall polysaccharides that are classified according to the monomer forming the backbone of the polymer, such as xylan (β-1,4-linked D-xylose), mannan (β-1,4-linked D-mannose), or xyloglucan (β-1,4-linked D-glucose). These polymers may be linear or substituted (or branched) with D-galactose, D-xylose, L-arabinose, and D-glucuronic acid (Figure 5).[19]

One of the most prevalent groups of CAZymes in the secretome of *Rhizopus* is the glycoside hydrolases belonging to family GH16. These enzymes are typically involved in the breakdown of complex "hemicellulose" glucans, abundant in the cell walls of dicots (fruits and vegetables) as well as cereals. Battaglia et al. (2011)[7] reported that *R. delemar* presented the lowest number of putative enzymes involved in β-1,3−1,4-glucan degradation. The count of GH16 in *Rhizopus* spp. is similar to that reported for Mucoromycota and lower than for *Aspergillus* (Figure 4).

Interestingly, CAZymes from the GH134 family involved in the degradation of mannan, are overrepresented in *Rhizopus* compared to both Mucoromycota and *Aspergilli*. Battaglia and collaborators (2011)[7] noted the absence of genes encoding for β-endomannanase (EC 3.2.1.78) in the genome of *R. delemar*. However, an enzyme from the relatively recently described GH134 family from *R. microsporus* has been characterized and structurally solved showing maximal activity at pH 5 and 50 °C and a high specific activity toward the substituted mannan of locust bean gum.[23]

Pectin Degradation. Pectin is an important polysaccharide component in the primary cell wall and a key component of the matrix between plant cells ("middle lamella"). Pectin consists of a backbone of mainly α-1,4-linked galacturonic acid interrupted by rhamnogalacturonan I, that consists of a backbone of alternating moieties of galacturonic acid and rhamnose, and extended side chains rich in arabinose and galactose (Figure 5).[19] The genome of *R. delemar* has been predicted to present an expanded family of polygalacturonases of family GH28. This family contains endo and exo polygalacturonases for the degradation of homogalacturonan, i.e., the major backbone polysaccharide in the pectin component of the plant cell walls and particularly present in fruits. Fifteen genes encode for unique enzymes in the genome of *R. delemar*, 12 of which correspond to endopolygalacturonases and 3 to exopolygalacturonases.[24] The presence of such a large family of genes for pectin degradation and the high sequence similarity between the predicted proteins could be explained by a whole genome duplication event, however, the reason for the maintenance of the whole set remains unclear. While only 6 out of the 15 genes could be detected as actively transcribed by isolation of cDNA of the fungi grown on multiple carbon sources, all the heterologously expressed proteins were active on polygalacturonic acid despite the large differences in activity, 200-fold in the extreme case. The pH and temperature optima range from 4 to 5 and from 30 to 40 °C, respectively. The low sequence similarity in the upstream and downstream regions of the genes for most of the sequences, together with the differences in transcriptional activation depending on the source carbon, could point toward a phasic expression of the pectinolytic repertoire for an enhanced efficiency of the degradation.[24]

As already discussed above, both *Rhizopus* and *Aspergillus* present high counts of GH28 members in the secretome, although *Rhizopus* show high variability in the numbers (Figure 4). The Mucoromycota has notably lower GH28 mean counts (Figure 4), pointing toward a lesser emphasis on pectin degradation, highlighting the specialization of *Rhizopus*. The fact that family CE8 is equivalent to pectin methyl esterase activity and the CE8 count in *Rhizopus* is among the top ten highest counts (Figure 4), higher than Mucoromycota but like that of *Aspergillus*, corroborates a profound pectin degrading ability of the *Rhizopus* CAZymes. A recent study reported that three CE8 genes were upregulated when growing *R. delemar* at pH 4 compared to pH 7,[25] but no CE8 CAZyme has been characterized for *Rhizopus* species.

Other Polysaccharides. *Rhizopus* presents similar levels of PL14 compared to Mucoromycota and PL14 is among the 10 most represented CAZymes in *Rhizopus* genomes, with higher levels than those observed for *Aspergilli* (Figure 4). PL14s are usually considered as being alginate lyases catalyzing the degradation of alginate, a polysaccharide found in brown algae. Although at this point it cannot be excluded that *Rhizopus* spp. encode PL14 alginate lyases, it is more likely that the PL14 members represent endo-β-1,4-glucuronan lyase activities, which would concur with the CAZymes machinery of *Rhizopus* representing a profound ability to restructure fungal cell wall glucuronan, indicating the mycelial cell wall building capacity and/or antagonism or opportunistic mycoparasitic capacity against other fungi. The latter would concur with the idea that fungal cell wall glucuronan might be a target carbon-source for many types of fungi.[11]

Finally, AA1 is a family of oxidases, notably laccase, that use diphenols as donors and oxygen as the acceptor. This enzyme family has been related to modification of lignin and oxidation of many different phenolic substances. The numbers of AA1 in *Rhizopus* are similar to those found in Mucoromycota, but lower than those in common *Aspergilli*. The function of these enzymes in *Rhizopus* is still to be determined.

CONCLUSIONS AND FUTURE OUTLOOK

Rhizopus species possess the capacity to produce a distinct set of CAZymes which enable these fungi to produce a characteristic, white, dense mycelium and desirable non-saccharified, solid-state fermented plant food products. The detailed analysis of the CAZyme secretome profile of *Rhizopus* spp., including the prevalence of chitin-modifying, GH3 β-glucosidase, and potentially glucuronan-degrading enzymes, suggests that a high proportion of the CAZyme machinery is used for mycelial cell wall modification and restructuring, supporting the characteristic fast and extensive mycelium formation of *Rhizopus* spp. The unique prevalence of fungal cell wall remodeling enzymes may also aid in the growth competition against other fungi. Research on *Rhizopus* species integrating genomic, proteomic, and metabolomic data is warranted to provide a holistic view of the enzymatic processes. The exploration of the ecological roles and interactions of *Rhizopus* during conversion of various substrates can yield insights into their adaptive mechanisms and potential for biotechnological applications. For instance, understanding the competitive and symbiotic relationships established by *Rhizopus* in its natural habitats could inform the development of coculturing techniques that improve fermentation processes or waste decomposition. Likewise, the development and integration of novel bioinformatics tools improving the understanding of the CAZyme profiles could help identify novel complementary enzyme functionalities and lead to breakthroughs in the industrial applications of these

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fungi. Genetic modification of *Rhizopus* strains is notoriously difficult because foreign DNA rarely integrates seamlessly into the host genome. However, genetic tools such as CRISPR-Cas9 and programmable nucleases have recently been used in a few studies on *Rhizopus* spp. (mainly to investigate mucormycosis pathogenesis). Although no genetic CAZymes manipulation in *Rhizopus* spp. has been reported yet, the use of novel genetic engineering techniques in these organisms may provide new insights into the CAZymes as well. Such new understanding may in turn facilitate rational enhancement of specific CAZyme activities to improve the nutritional and sensory profiles of fermented food products. The ability of *Rhizopus* spp. to transform substrates into a variety of valuable products is very much a result of the particular profile of the secreted carbohydrate active enzymes, and it is deemed to have potential beyond current uses in contributing to new sustainable practices and products in the circular economy. Collaboration between researchers and industry will be key to translating such research findings into practical applications that exploit the unique capabilities of *Rhizopus* enzymes.

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Notes

The authors declare no competing financial interest.

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Kristian Barrett. Kristian is Assistant Professor in Enzyme Bioinformatics at DTU. His research focuses on categorizing, discovering, and understanding carbohydrate-active enzymes, as well as developing bioinformatics tools. Kristian has advanced protein sequence analysis through peptide-based functional annotation of carbohydrate-active enzymes via conserved unique peptide patterns (CUPP).

Marlene Vuillemin. Marlene has a PhD degree from the National Institute of Applied Sciences of Toulouse, France. Her research focuses on carbohydrate-active enzymes, particularly glycoside hydrolases for transglycosylation reactions and synthesis of tailor-made oligosaccharides. Her work also involves exploration of the uncharacterized enzyme sequence space to provide new knowledge about the functional role of carbohydrate-active enzymes.

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