# Chitin in the fungal cell wall: Towards valorization of spent biomass of Aspergillus niger

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# Summary

The use of filamentous fungi for the production of numerous products dates far back into human history. Common examples are fermentation of soy and milk. Their long-lived role throughout human history is still continues on to this day and has expanded to a formidable array of products. Products such as antibiotics, enzymes and preservative acids that are produced on a massive scale. Production on a massive scale means that fungi are cultivated in (an often) liquid medium, inside containers that are called fermenters. These fermenters hold tens of thousands of liters of both fungal biomass and a desired product that these fungi secrete. After the required product is harvested, the post-fermentation biomass is considered as waste; a spent tool that served a means to an end. Because a lot of energy is invested in the assimilation of biomass in order to produce the desired compound, discarding the spent biomass can be considered as a missed opportunity to recover valuable resources.

The Fungal Chitosan (FunChi) project set out to repurpose the spent biomass by investigating its potential for the stimulation of plant growth. A considerable portion of this spent biomass is the fungal cell wall and constitutes up to 80% of the biomass dry weight. Specifically, chitin (and its derivative chitosan) are parts of the cell wall that are of particular interest due to their wide range of applications. Among these applications is the possibility to stimulate the plant immune system Because chitin and chitosan naturally occur in cell walls of many fungi, plants have adapted to respond by activating their immune system to combat an impending infection when they are exposed to these compounds. Thus, cell walls from spent biomass of filamentous fungi can provide a natural plant defense-priming agent that is of natural origin. In order to make the use of fungal cell walls economically feasible, two main issues are to be considered: (i) what is the chitin yield for fungal biomass and can this be increased? (ii) Can the extractability of chitin from the fungal cell wall be enhanced (or be made cheaper) by removing chitin-to-cell wall linkages? The work described in this thesis has taken a fundamental approach to tackle both of these problems individually. To bridge the gap between fundamental science and industry, we have used the organism *Aspergillus niger* that is widely used in laboratory environments for fundamental work, but is also used in industrial settings for the production of numerous enzymes and citric acid.

The yield of chitin and chitosan in the cell wall of the wild type *A. niger* was found to be ~15% of the total biomass dry weight. In the attempt to find candidate strains that have an increased chitin fraction, cell wall mutants with a constitutive state of cell wall stress were screened. When a strain experiences a state of cell wall stress, many cell wall changes can occur, among which is the upregulation of chitin production. A collection of cell wall mutants was screened for an increase in cell wall chitin content. Two main candidates, RD15.4#55 and RD15.8#16, showed an increase in chitin content compared to wild type of ~35% and ~60%, respectively. The causative mutations have been identified for each strain individually (see **Chapter 4** and **Chapter 5),** using a combination of genome sequencing, the parasexual cross, and co-segregation analysis.

Following the abovementioned approach, mutant strain RD15.4#55 was found to have a C-terminal truncation of a previously unknown negative regulator of transcription. A gene deletion of the putative transcriptional repressor was not lethal, and resulted in the same cell wall chitin phenotype as the C-terminally truncated version in RD15.4#55, suggesting full loss of function. No previous studies have reported on this repressor in *A. niger*. Therefore, we named this gene *cwcA* (***c***ell ***w***all ***c***hitin A) after its cell wall chitin phenotype. (**Chapter 4**). Mutant strain RD15.8#16, with ~60% increased cell wall chitin content, was identified to contain an intronic mutation in the Rab GDP dissociation inhibitor A (*gdiA*) that resulted in lower amounts of correctly spliced transcript, in addition to an unspliced intron 2, and two versions of alternatively spliced transcript (**Chapter 5**). We also observed that a full knockout of *gdiA* is lethal. From our results, we deduced that the intronic mutation likely causes a Rab GTPase GTP/GDP cycle imbalance that either directly or indirectly leads to a in chitin deposition in the cell wall.

Next to employing a forward genetic screen, we also considered an existing deletion strain (*kexB*) for cell wall investigation due to its visibly thicker cell walls and prominent hyperbranching phenotype. The latter trait is of additional industrial interest as it reduces viscosity at high biomass densities, which may lead to both increased energy consumption and inconsistent production yields. Notably, a deletion of *kexB* showed strong transcriptional signs of cell wall stress and upregulation of a series of enzymes involved in chitin synthesis. These data were corroborated by a ~20% increase in cell wall chitin content compared to the wild type. However, the increase was found to be irrespective of the hyperbranching phenotype. It was further shown that a deletion of *kexB* showed phenotypic plasticity between pH 5 and pH 6, displaying a wild type like phenotype at pH 5 and a hyperbranching phenotype at pH 6, with a clear transitioning phenotype at pH 5.5 (**Chapter 6**).

In our attempts to enhance the extractability of chitin and chitosan from the cell wall of *A. niger*, we set out to delete the *crh* gene family that is considered to be responsible for facilitating the cross-link of chitin to β-1,3-glucan in the cell wall. Because *A. niger* contains seven *crh* gene family members, the knockout of all genes posed a practical constraint. We solved this by designing a marker free CRISPR/Cas9 gene editing system that allowed the accurate and efficient knockout of all seven gene members of the *crh* family (**Chapter 2**). In addition to showing this system's efficiency, concurrent *crh* gene knockout construction using existing auxotrophic marker selection recycling (*AOpyrG*) showed that these selection markers caused occasional false-positive phenotypes due to locus-dependent-side-effects of poor auxotrophic marker expression (**Chapter 2**).

The impact of deleting the *crh* gene family was, surprisingly, not visible on cell wall integrity; several cell wall disturbing compounds did not show an effect on growth which contrasts yeast literature. Growth rates of both wild type and the sevenfold *crh* mutant were also identical in controlled batch fermentations, as was the case on a transcriptional level. Fractionated cell walls neither showed differences in cell wall composition nor shifts in glucan and chitin (**Chapter 2** and **Chapter 3**). As such the *crh* genes appear dispensable, however we showed that the *crh* genes

are important when other cell wall components, such as galactomannan or α-glucan are absent. Interestingly, as α-glucan is absent in yeast, the discrepancy in effect of deleting the *crh* genes between yeast and *A. niger* may be explained by α-glucan. In line with our results, the lack of effect on the cell wall in absence of all *crh* genes has also been observed for *A. fumigatus*. Taken together, from these results we conclude that the interaction between chitin and α-glucan may play a pivotal role in anchoring chitin to the cell wall in addition to the chitin-β-1,3-glucan crosslinks of α-glucan containing filamentous fungi.

In addition to the results obtained in our research, we discuss alternative approaches to increase chitin production than the ones presented in this thesis. In relation to the FunChi project, the most important results and conclusions are discussed and ideas for future implementation are proposed (**Chapter 7**).