Chapter 6

Summary and concluding remarks
Fungi are eukaryotic organisms, characteristically highly variable in size, shape and natural habitat. Within the phylum of ascomycete fungi, the aspergilli form a well studied genus with more than 250 separate species. Some of these, such as *Aspergillus fumigatus* and *Aspergillus flavus*, are known to be human pathogens. The filamentous fungus *Aspergillus niger* is a common soil fungus with many industrial applications. It produces a large variety of enzymes with applications in the food and paper industry. This mould, named after the black appearance of its sporulating colonies, is the organism studied in this thesis.

Fitting to their chemotrophic and heterotrophic lifestyle, fungi are able to degrade various polysaccharides; they import the small carbohydrates released to serve as energy and carbon source. Fungi produce various types of extracellular enzymes specialized in hydrolysis of specific carbohydrates. The expression of these enzymes is often tightly regulated, so that only enzymes needed to degrade the carbohydrate at hand are produced and excreted by the fungus.

An example of such a system of carbohydrate degradation by fungi is the hydrolysis of starch, a polymer abundantly present in plant storage organs. Starch consists of amylose, a linear chain of α-(1,4) linked glucose molecules, and amyllopectin, a branched glucan with α-(1,4) and approximately 3-5 % α-(1,6) glycosidic bonds. Fungi produce a mixture of different extracellular enzymes for starch degradation. Alpha-amylases hydrolyze some of the internal α-(1,4) glycosidic bonds, and the resulting maltooligosaccharides are subsequently hydrolyzed into β- and α-glucose by glucoamylases and α-glucosidases, respectively. In aspergilli, the expression of starch-degrading enzymes is induced in the presence of maltose or isomaltose, molecules formed by the partial hydrolysis of starch. This regulation is mediated by the AmyR transcription factor, acting as a positive regulator (Petersen et al., 1999).

Alpha-amylases are ubiquitous enzymes, present in all kingdoms of life. Most α-amylases belong to the family 13 of glycoside hydrolases (GH13), according to the classification of carbohydrate acting enzymes based on sequence similarity (see CAZy website at http://www.cazy.org) (Coutinho and Henrissat, 1999). Also family GH57 contains enzymes with α-amylase activity, but these proteins only occur in prokaryotes. Other types of starch acting enzymes, both from prokaryotes and eukaryotes, are classified in family GH15 (glucoamylases) or in family GH31 (α-glucosidases). Apart from enzymes hydrolyzing α-(1,4) glycosidic bonds, family GH13 also comprises enzymes hydrolyzing α-(1,6) glycosidic bonds, and enzymes with α-glucanotransferase activities. Most of the family GH13 enzymes known are involved in degradation or synthesis of starch, glycogen and similar α-glucan polymers. Some enzymes may be
involved in metabolism of trehalose, a eukaryotic storage compound, and others act on sucrose. The overall sequence similarity of GH13 enzymes with different reaction specificities may be very low, but they share four conserved amino acid regions which together form the active site of the proteins. Three amino acids within these regions are invariable, and are known to be essential for catalysis. Four other amino acids are highly conserved within the family, and play a role in binding and positioning of the substrate (Nakajima et al., 1986).

At the start of this PhD study, the aim of the project was defined as follows: to identify the enzymes from *A. niger* involved in starch degradation, and to assess their individual roles in this process by studying their biochemical properties and the transcriptional regulation of their encoding genes. These studies were greatly facilitated by the availability of the recently completed genome sequence of *A. niger* CBS 513.88. Chapter 2 describes the identification of genes encoding enzymes putatively involved in starch degradation in the genome of *A. niger*, and the effect of maltose on the expression of these genes. Additionally, an *amyR* deletion strain was constructed to study the effect of this transcriptional regulator on the expression of the genes identified. The genome search for family GH13, GH15 and GH31 enzymes yielded a total of 17 novel predicted proteins. Thus, although *A. niger* already represents a rich source for commercial starch acting enzymes, analysis of its genome sequence revealed that it encodes a surprisingly large number of unknown, putatively starch acting enzymes. These enzymes are potentially interesting both from a scientific and from an industrial point of view. Only a small number of these newly identified enzymes were subsequently predicted to act *in vivo* on starch in the extracellular environment. These predictions were based on the presence or absence of a putative signal sequence for secretion in combination with expression data. Apart from the previously described acid amylase (Boel et al., 1990) and AmyA and AmyB (Korman et al., 1990), we identified one extracellular α-amylase designated AmyC. The *amyC* gene is located in close proximity to the *amyR* gene, but its expression was relatively low and not as tightly regulated by AmyR as that of the other α-amylases. This protein possibly acts as a scouting enzyme, producing maltose to initiate the expression of other starch degrading enzymes regulated by AmyR.

Among the α-glucosidases of family GH31, only a few members were clearly regulated by AmyR and expressed in the presence of maltose, suggesting a role in starch degradation. Many other family GH13, GH15 and GH31 enzymes identified in the *A. niger* genome were predicted not to be involved in starch degradation, for a variety of reasons. For example, many of the predicted enzymes from all three families lacked a signal sequence for secretion. These enzymes are therefore thought to function in the
intracellular environment, although it is unclear what their functions in the cell might be. Alternatively, the enzymes might be released into the environment upon autolysis of the mycelium. This hypothesis is supported by the observed upregulation of chitinases during the autolysis phase of *A. niger* (Yamazaki *et al.*, 2007), and the finding of a predicted intracellular laccase in the culture filtrate of the white rot fungus *Trematomyces clypeatus* (Bose *et al.*, 2007). Expression of the intracellular GH13, 15, 31 enzymes in *A. niger* was very poor or not detectable in our experiments, all of which were performed with rather young, submerged cultures. An exception was formed by enzymes involved in glycogen metabolism. The production and degradation of this intracellular storage molecule involves, amongst others, two family GH13 enzymes (glycogen branching and debranching enzyme), and both these enzymes were expressed in our *A. niger* cultures.

Based on a phylogenetic analysis, we identified three novel subgroups of family GH13 enzymes in the genome sequence of *A. niger* (chapter 2), comprising respectively two predicted intracellular α-glucosidases, two predicted intracellular α-amylases, and three extracellular enzymes that were predicted to be anchored on the cell wall with a glycosylphosphatidylinositol (GPI) anchor. The same groups with homologous enzymes were also predicted from the genome sequences of other aspergilli, and partly in other Ascomycetes and Basidiomycetes as well. The characteristics of the predicted intracellular α-amylases and the GPI-anchored enzymes were studied in more detail, as described in chapters 3, 4 and 5 of this thesis.

*Chapters 3 and 4* describe the detailed characterization of two novel family GH13 α-glucanotransferase enzymes from *A. niger*, predicted to be localized in the cell wall. The enzymes, named α-glucanotransferase (Agt) A and B, showed transglycosylation activity on substrates with α-(1,4)-glycosidic bonds. The minimum size of the donor substrate was maltopentaose, and the minimal size for the acceptor substrate was maltose. The enzymes produced new α-(1,4)-glycosidic bonds (EC 2.4.1.25) and their reaction products reached a degree of polymerization (DP) of at least 30. This is the first time that enzymes with such a disproportionating type of reaction are described in fungi. Based on similar sequence properties, a third enzyme predicted from the genome sequence was named AgtC. Although the basic reaction of the two studied Agt enzymes is very similar, a more detailed characterization (chapter 4) revealed significant differences. When tested with a synthetic donor substrate, the specific transglycosylation activity of AgtA was ten times higher than the activity of AgtB. AgtB showed a broad temperature optimum and relatively poor heat stability, compared to AgtA. Both enzymes where able to use nigerose and nigerotriose as acceptor substrates (with α-(1,3) glycosidic bonds), although only to a limited extent compared to
maltooligosaccharide substrates. The enzymatic activity of AgtA and AgtB is similar to that of bacterial amylomaltases, which act on smaller maltooligosaccharides substrates, using maltose as a donor and glucose as acceptor molecule (Kaper et al., 2004). They usually release one glucose molecule for every transglycosylation event, a clear difference with AgtA and B, which do not produce significant amounts of glucose. Most amylomaltases are members of family GH77, although some belong in family GH13.

Despite the similar reaction performed by AgtA/B and bacterial disproportionating enzymes such as amylomaltases, there is no significant overall sequence similarity between these groups of enzymes. There is, however, a specific sequence trait shared between fungal Agt enzymes and family GH77 amylomaltases: one of the 7 highly conserved amino acids in GH13, the equivalent of His143 in A. niger acid amylase, is not present (MacGregor et al., 2001; Kaper et al., 2007). It is possible that the absence of His143 plays a decisive role in the determination of disproportionating activity in the Agt enzymes. Interestingly, the same His residue is missing in the GH13 domain of α-glucan synthase (Ags) enzymes, which are also predicted to act as α-glucanotransferases (Grün et al., 2005). On the other hand, His143 is present in other GH13 enzymes with disproportionating activity, notably Thermotoga maritima amylomaltase (Liebl et al., 1992) and cyclodextrin glucanotransferase (CGTase) enzymes, and is known to be important for catalytic activity in these enzymes (Mattsson et al., 1995; Leemhuis et al., 2004). Alternatively, the absence of the equivalent of His143 might have something to do with the in vivo substrate of Agt and Ags enzymes, which is probably a mixed type of α-glucan with both α-(1,4) and α-(1,3) glycosidic linkages (see below). To investigate the possible role of the equivalent of His143 in Agt and Ags enzymes, the in vivo reaction and physiological role of these enzymes need to be elucidated, combined with biochemical studies of wild type and mutant enzymes, with modified amino residues at this position.

Homologues of AgtA/B were identified in the genome sequences of many Ascomycetes and Basidiomycetes, e.g. in all available Aspergillus genomes, but also in Botrytis cinerea, Neurospora crassa, the fission yeast Schizosaccharomyces pombe and the Basidiomycete Cryptococcus neoformans. Homologues of Agt enzymes may be recognized by their high sequence similarity to fungal α-amylases, the lack of His143 and the presence of a GPI-anchoring signal. The identified homologues of the Agt enzymes in other fungi have all been annotated as α-amylases, but the biochemical characterization of AgtA and AgtB presented in this thesis shows that reannotation of these predicted enzymes as α-glucanotransferases is required.

Expression of the agr genes in A. niger was not induced by maltose and not dependent on the presence of AmyR (chapter 2), suggesting that the proteins are not involved in
the catabolism of starch. Rather, agtA was constitutively expressed, and agtB was co-regulated with its neighbouring gene agsC, one of five genes encoding an α-glucan synthase in A. niger. The latter are membrane bound enzymes responsible for the production of cell wall α-glucan (Hochstenbach et al., 1998). The cell wall of aspergilli contains α-(1,3) glucan and nigeran, with alternating α-(1,3) and α-(1,4) glycosidic bonds, as well as other types of polysaccharides. Many of the agt genes in different fungi are located next to, or in the neighbourhood of, ags genes. This conserved clustering and co-expression of agt and ags genes are strong indications that the two types of enzymes serve in the same physiological process, namely the synthesis of cell wall α-glucan. This hypothesis is in line with the absence of agt homologues in the Saccharomycotina which do not produce cell wall α-glucan. A deletion of an agt homologue (aah3) in S. pombe resulted in a strain with an aberrant cell shape which was hypersensitive towards cell wall degrading enzymes, suggesting that the deletion had an effect on cell wall integrity (Morita et al., 2006). In chapter 3, we have shown that both the knockout of agtA and the overexpression of either AgtA or AgtB in A. niger had a clear negative effect on its susceptibility towards CalcoFluorWhite (CFW), a cell wall disrupting compound (Ram and Klis, 2006). This is a strong indication that cell wall strength is impaired in these strains, caused by the knockout or overexpression of agt genes. However, such an effect of CFW might also be indirect and caused by the disturbance of other cellular functions. The deletion of S. pombe aah3, for example, also caused a defect in vacuolar protein sorting, probably representing an indirect effect of the impaired integrity of the cell wall (Iwaki et al., 2007). More evidence for the function of Agt enzymes might be obtained from the comparative analysis of the cell wall α-glucan components of A. niger wild type, and the ΔagtA and AgtA/B overexpression strains. At present we cannot exclude that AgtB or AgtC partly replace the function of AgtA in the ΔagtA strain. Therefore, also agtA/B/C double and triple knockouts should be constructed in further work, possibly showing even more pronounced effects on cell wall physiology.

In chapter 5, a cluster of predicted intracellular fungal α-amylases was studied in more detail. Phylogenetic analysis of this group of 12 protein sequences from 7 different fungal species demonstrated that they belong to subfamily GH13_5, a subfamily previously thought to contain only bacterial α-amylases (Stam et al., 2006). These proteins show only very little similarity to the fungal α-amylases described previously, which all belong to subfamily GH13_1. We expressed one of these enzymes, AmyD from A. niger, in E. coli and studied its biochemical characteristics. The enzyme showed a low hydrolyzing activity on starch and similar substrates, a broad pH optimum and rather poor temperature stability. The activity or stability of the enzyme was not dependent on Ca²⁺ ions, although analysis of the 3D structures of related bacterial
enzymes revealed the presence of at least three Ca\textsuperscript{2+} ions. A homologue of AmyD, Amy1p from *Histoplasma capsulatum*, was identified in a search for genes involved in biosynthesis of cell wall \(\alpha\)-(1,3) glucan (Marion *et al.*, 2006). A functional knockout of *AMY1* in *H. capsulatum* was unable to produce \(\alpha\)-(1,3) glucan and showed reduced virulence. The exact function of Amy1p in \(\alpha\)-glucan formation in *H. capsulatum* has not been studied, but the suggested involvement of family GH13_5 enzymes with \(\alpha\)-glucan formation is strengthened by the organization of the genes encoding these enzymes, which are often clustered with *agt* and *ags* genes (Fig. 1).

The results described in this thesis suggest that enzymes homologous to *A. niger* AgtA/B and AmyD/E play a role in fungal cell wall \(\alpha\)-glucan formation or maintenance. Cell wall \(\alpha\)-glucan is thought to be produced by \(\alpha\)-glucan synthases (Ags enzymes), but the biochemistry of this process is still largely unknown. Ags enzymes have two predicted catalytic domains; an extracellular domain that resembles family GH13 enzymes, and an intracellular one resembling enzymes of Glycosyltransferase family 5 (GT5) (Hochstenbach *et al.*, 1998). Family GT5 also comprises glycogen synthases, which require a short oligosaccharide as a primer molecule to produce glycogen from UDP- or ADP-glucose (Lomako *et al.*, 2004). A similar oligosaccharide primer might be provided to the GT5 domain of \(\alpha\)-glucan synthases by AmyD, which was shown to release mainly maltotriose when incubated with maltooligosaccharides. The *amyD* and
氨E基因，然而，没有在A. niger中表达。尽管几条ags基因在A. niger中表达了（chapter 2）。这表明AmyD/E的活性对于α-葡聚糖的形成并非必要。在所研究的条件下，或者它们的表达水平太低，从而检测不到。第三种可能性是amyD/E基因在表达，但未被检测到，因为用于微阵列的基因组是A. niger CBS 513.88，而实验是在N402菌株上进行的，后者可能在DNA水平上有一些差异。

已经描述了不同的Ags酶在真菌菌落中的特定生理功能，这也反映了它们的表达模式（Beauvais et al., 2005; Damveld et al., 2005b; Garcia et al., 2006）。AmyD/E的表达也可能会受到类似的调节，如果它们的活性只需与其中的五Ags酶酶特别结合的话。一个令人困惑的问题是关于A. niger中GH13_5酶的底物。AmyD有非常差的水解活性。Ags1酶在S. pombe中，其功能是在细胞质中，通过transglycosylation反应以短的α-(1,4)糖苷键连接两个α-(1,3)骨链。AgtA和AgtB可能通过类似transglycosylation反应将不同α-葡聚糖链连接起来。假设AgtA和AgtB（主要）作用于α-(1,4)连接的寡糖，就像它们在体外一样，那么很难确定这些酶的自然底物。S. pombe孢子细胞壁α-(1,4)连接的葡聚糖可以被碘染色（Garcia et al., 2006）。最近的研究表明，Ags1的过表达导致S. pombe中α-(1,4)连接的葡聚糖过生产（Vos et al., 2007）。另一种研究也报导了在A. niger中，通过物理压力在震荡培养中形成的一种类似淀粉的葡聚糖。该多糖硫酸盐只附着在菌丝上，似乎不是细胞壁的结构成分（Kirimura et al., 1999）。相反，Agt酶可能会作用于α-(1,4)键在nigeran中，但这种类型的葡聚糖是未被研究的。
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present in a limited number of fungal species, while Agt homologues occur widespread. In vitro activity of AgtA/B on nigeran could not be demonstrated, but a more straightforward way to exclude it as a substrate would be to offer well dissolvable nigerooligosaccharides to these enzymes and check for formation of elongated products.

Possibly, Agt enzymes act on short α-(1,4) linked stretches in α-(1,3) glucan, as was also suggested for the Ags GH13 domain. Indeed, early studies report small percentages of α-(1,4) linkages in A. niger α-(1,3) glucan, which could represent α-(1,4) linked stretches similar to those identified in S. pombe α-glucan (Horisberger et al., 1972; Johnston, 1965). However, to perform a crosslinking reaction between α-(1,3) glucan chains with short α-(1,4) stretches would involve an α-(1,3) linked acceptor molecule, which is a poor acceptor substrate for both AgtA and AgtB (chapter 4). With the present knowledge on Aspergillus α-glucan, the exact in vivo reaction of Agt enzymes is uncertain, but this topic might be addressed in future studies by determining the composition of the cell walls of different Agt and Ags mutants.

In contrast to most filamentous Ascomycetes, yeasts like Saccharomyces cerevisiae and Candida albicans do not produce cell wall α-glucan. Other components such as chitin and β-glucan are shared among both yeast-like and filamentous Ascomycetes, and the enzymes involved in e.g. β-glucan synthesis and remodelling are highly homologous between the species. Simultaneous expression of all enzymes involved in α-glucan synthesis in a yeast host might result in α-glucan production, thereby offering a system to study α-glucan synthesis in detail. Such an approach may provide definite proof for the involvement of the different types of enzymes. Additionally, it would offer an experimental model to study the in vivo effects of mutagenesis of specific amino acids in Agt, Ags and family GH13_5 enzymes.

Considering the arguments mentioned above, we hereby propose a highly speculative model for the function of AgtA/B and AmyD/E in the A. niger cell wall (Fig. 2). It is already clear that this model does not provide the full picture, experimental evidence lacking for most points raised. Such a model, however, combines the present knowledge on the topic, and offers a starting-point for future experiments:

In the cell, AmyD/E provides an α-(1,4) linked oligosaccharide primer molecule to the intracellular GT5 domain of Ags. Subsequently, UDP/ADP-glucose is used for elongation of the glucan chain. The growing chain, which might be of the α-(1,3) glucan or nigeran type, is transported over the membrane via the membrane spanning domain of Ags, and subsequently coupled to another α-glucan chain by the GH13 domain of Ags. Agt enzymes assist in the coupling of newly formed α-glucan chains to
existing chains, and allow for growth and branching of the mycelium by keeping the α-glucan component flexible. The stretch of α-(1,4) linkages, possibly originating from the oligosaccharide primer molecule supplied by AmyD/E, might be the main part of the α-glucan available for a coupling reaction. Coregulated agt and ags genes encode for proteins producing or acting on the same type of α-glucan.

Fig. 2 Hypothetical model of α-glucan synthesis in *A. niger* as described in this chapter. AmyD/E is located in the cell, where it may provide a primer molecule to an α-glucan synthase (Ags). Subsequently, the primer is elongated by the GT5 domain of the Ags enzyme using UDP- or ADP-glucose, and the α-glucan produced is transported out of the cell via the Ags transmembrane regions. In the cell wall, the α-glucan chains may be linked to each other or other cell wall components by the action of the GH13 domain of Ags enzymes or by GPI-anchored α-glucanotransferases.

The hypothetical model on α-glucan biosynthesis in fungal cell walls has some resemblance to the system for β-glucan production and maintenance. Fungal β-(1,3) glucan is produced by β-glucan synthases, members of family GT48 and related to plant cellulose synthases, which have been identified in several yeasts and filamentous fungi (Lesage and Bussey, 2006). Like α-glucan synthases, the enzymes comprise several domains, including two cytoplasmic domains and two separate domains with membrane spanning regions for localization. Although β-glucan formation has been studied more
extensively than α-glucan formation, the biochemistry of the synthesis process is still not known exactly. *S. cerevisiae* harbors two similar β-glucan synthases, FKS1 and FKS2, which use UDP-glucose to form a β-(1,3) glucan that is subsequently transported to the outside of the cell. A double knockout for both FKS genes is not viable (Mazur et al., 1995). Additional enzymes involved in β-glucan synthesis are GPI-anchored β-(1,3) glucanosyltransferases, called GAS proteins in *S. cerevisiae*, PHR in *C. albicans* and Gel proteins in *A. fumigatus*. Deletion strains for these enzymes affected virulence of *C. albicans* and *A. fumigatus*, the causative agent of aspergillosis (De Bernardis et al., 1998; Mouyna et al., 2005). The genes encoding the different enzymes involved in β-glucan synthesis are not clustered, as is the case for the *ags*, *agt*, and *amyD/E* genes.

The fungal cell wall is an important structure for the targeting of antifungal drugs; it forms an essential part of the cell, it is easily accessible and it is composed of fungus specific components not produced by mammalian hosts. A relatively novel type of antifungal drugs, the echinocandins, act as inhibitors of the enzymes producing cell wall β-(1,3) glucan and are used against a wide variety of human fungal pathogens (Morris and Villmann, 2006). In contrast to cell wall β-glucan, α-glucan has not been a major target for the development of antifungal drugs, mainly because it does not occur in *C. albicans*, the most frequently encountered fungal pathogen of humans. As for the aspergilli, it was suggested that α-glucan is not important for pathogenicity of *A. fumigatus* (Beauvais et al., 2005; Maubon et al., 2006). However, α-glucans are known to be important or even essential factors for pathogenicity in a number of other fungi, mainly dimorphic species such as *H. capsulatum* (Rapleye et al., 2004), *Blastomyces dermatitidis* (Hogan and Klein, 1994) and *Paracoccidioides brasiliensis* (Borges-Walmsley et al., 2002), but also in the pathogenic Basidiomycete *C. neoformans* (Reese et al., 2007). Although the molecular mechanism for the involvement of α-glucan in virulence is often unknown, it is clear that the α-glucan component of fungal cell walls deserves to be studied as a potential target for antifungal drugs.