Summary

Utilization of renewable materials from non-feedstock sources is an important research area within white biotechnology these days (Martínez et al. 2009). Cellulose makes up one of the most abundant renewable materials, present in all kinds of plant biomass (Pauly and Keegstra 2010). However, to be able to utilize the cellulose as feedstock, it needs to be separated from lignin which cements the cellulose and hemi-cellulose fibers (Hammel and Cullen 2008). This process currently requires multiple harsh treatments after which the left-over lignin is usually burnt at the mill (Harris and DeBolt 2010; Martínez et al. 2009; Pauly and Keegstra 2010). White-rot fungi produce a range of enzymes like e.g. peroxidases that can contribute to a more environmentally friendly and mild removal of lignin, allowing utilization of lignin for other high-value applications (Martínez et al. 2009). Unfortunately, these slow growing fungi only produce limited amounts of lignolytic enzymes, making them unsuitable for industrial applications. Lignolytic peroxidases can be produced by *Aspergillus niger*, and its production was found to be improved by heme supplementation, suggesting a limiting effect of this co-factor during heterologous expression (Conesa et al. 2002a). This result was also observed in other *Aspergilli* (Andersen et al. 1992; Elrod et al. 1997). Unfortunately the supplementation with heme is unpractical and too expensive to be economically feasible in large scale production processes (Elrod et al. 1997). The research described in this thesis explores fungal heme biosynthesis and its regulation with the final aim to increase the available intracellular heme for peroxidase production.

In **Chapter 1** a detailed introduction is given with respect to cellulose utilization and lignin removal through peroxidases and briefly provides insight into difficulties during heterologous protein production like heme availability. In order to identify the heme biosynthesis pathway and its potential bottlenecks in *A. niger*, a literature study was conducted. The results are described in **Chapter 2**, providing a global understanding about the pathway itself and the potential bottlenecks for overproduction of peroxidases. The pathway is highly conserved and, with a few exceptions, present in all organisms. Organisms that do not contain a (complete) pathway are heme auxothophs (e.g. *Caenorhabditis elegans*, (Rao et al. 2005)) or have abandoned their need for iron entirely (e.g. *Borrelia burgdorferi*, the bacterium that causes Lyme disease in humans (Posey and Gherardini 2000). Regulation of heme biosynthesis, however, is much less conserved than the pathway itself. However, common factors like feedback inhibition and regulation by

heme, iron and oxygen appear to be involved in regulation of the heme biosynthesis pathway in most organisms.

A. niger is a widely used fungus within biotechnology for its capability to produce large amounts of proteins. Therefore it is also an interesting organism for the production of lignolytic enzymes such as peroxidases and laccases. However, a high level of heterologous protein production is not guaranteed as we show in **Chapter 3**. Despite using an expression vector based on the strongly inducible glucoamylase promoter, the production of the desired peroxidases remained (almost) undetectable. As the potential cause of low peroxidase production in our follow-up research we have investigated heme biosynthesis in *A. niger*.

Heme has an essential function but accumulation of heme or its intermediates is also toxic requiring an intricate regulation of heme biosynthesis. In **Chapter 4** we have analysed several compounds that could affect heme biosynthesis. Not surprisingly, iron was found to have the most dominant effect, Also 5'-aminolevulinic acid (ALA) and possibly heme itself control heme biosynthesis mostly via modulating expression of *hemA* (coding for 5'-aminolevulinic acid synthase (ALAS). Through deletion of *hemA* we demonstrated that heme is essential in *A. niger*. We also showed that heme biosynthesis is linked with the utilization of nitrate through the necessity of siroheme, which is synthesized from the same intermediate, uroporphyrinogen III, as heme is. However, unlike the situation in *S. cerevisiae* (Gollub et al. 1977) the involvement of siroheme in sulfur metabolism was not observed in *A. niger* as no methionine auxotrophy was observed for Δ*hemA*. Through deletion of *hemF* and *hemH* encoding coproporphyrinogen III oxidase and ferrochelatase respectively as described in **Chapter 5**, the possibility to utilize exogenous heme for cellular processes was verified. Our experiments also demonstrated that *A. niger* is capable to transport intact heme molecules into the cell. Overexpression of the three selected heme biosynthetic genes (*hemA, hemF, hemH*) under control of the glucoamylase promoter revealed that ferrochelatase encoded by *hemH*, forms a limiting step for heme overproduction. Only in a *hemH* overexpression strain, a moderate increase in heme content was observed as well as accumulation of the intermediate protoporphyrin IX. This latter finding further indicates that the limitation in the pathway is not solved by overexpression of *hemH* alone and that (post)-translational regulation could play a crucial role. Furthermore, *hemH* is not the only potential bottleneck to achieve higher levels of heme. Overexpression of *hemA* led to accumulation of Uroporphyrinogen III and coproporphyrinogen III indicating that the activity of coproporphyrinogen III oxidase (encoded by *hemF*) is not sufficient to deal with higher levels of heme intermediates arising from *hemA* overexpression. The overexpression of *hemF* confirmed the limitation

on *hemH*, as PPIX levels initially increased. However, after prolonged times, the PPIX concentration decreased to WT levels and additionally a depletion of the porphyrin pool was observed, suggesting upregulation of siroheme synthesis as a mechanism to prevent accumulation of the final heme intermediates.

In **Chapter 6** an alternative approach has been examined to enhance heme biosynthesis. Like in many other organisms iron is a major player in the regulation of heme biosynthesis in *A. niger* (**Chapter 4**). Its uptake is balanced through the interaction of two regulatory factors, HapX and SreA, as was shown in *A. nidulans* and *A. fumigatus* and disruption of this balance leads to increased heme synthesis(Hortschansky et al. 2007; Schrettl et al. 2008). A Δ*sreA* strain in *A. niger* showed many phenotypic similarities to the *A. fumigatus* Δ*sreA* strain, but did not result in an increased heme content. Analysis of siderophore production (supported by genome analysis) demonstrated that deletion of sreA in *A. niger* did result in increased siderophore synthesis and increased iron uptake, but no increased heme levels were observed in the *A. niger* Δ*sreA* despite upregulation of the heme biosynthesis pathway and accumulation of intermediates like observed earlier in the *hemA* and *hemF* overexpression strains, further strengthening the limitation of ferrochelatase for overproduction of heme in *A. niger*.