Chapter 3

Peroxidase production in A. niger for white biotechnology

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Abstract

To improve the process of lignin removal from woody material in an environmental friendly manner, heterologous expression of laccases and peroxidases in *Aspergillus* spp has been studied. For this study a universal inducible expression vector under the control of the glucoamlylase promotor was constructed for cloning of genes of interest. The expression construct was used for the generation of various peroxidase-producing strains in a protease deficient *Aspergillus niger.* GFP analysis demonstrated the expression construct is fully functional. However, only low peroxidase activity of the producing strains was detected by plate assay, which remained undetectable in liquid cultures. As transcription levels are adequate, the bottleneck is located after translation and processes like UPR, ERAD and cofactor limitation (heme) could be responsible for the low activities and require further research.

Introduction

For the utilization of renewable materials from non-feedstock sources an enormous research effort is going on in the area of white biotechnology these days. One of the most abundant renewable materials is cellulose, present in all kinds of wood. This cellulose is extensively used in the paper and pulp industry, and has demonstrated its value as a carbon/energy source for the production of bio-ethanol. The use of raw cellulose, obtained from wood chips is however, complicated by the presence of also lignin. This aromatic polymer cements cellulose and hemicellulose fibers providing strength and pathogen protection to the plant (Harris and DeBolt 2010).

The current approach to remove lignin involves harsh pretreatments (uncatalysed steam explosion) and chemicals like ammonia fibre/freeze explosion, acid- or base-catalyzed hydrolysis and liquid hot water pretreatment (Harris and DeBolt 2010). However, these methods are costly and environmentally unfriendly. Despite the potential for using lignin in various applications (Harris and DeBolt 2010), the lignin residue is mostly burned for the generation of heat for e.g. distillation processes (Pauly and Keegstra 2010).

Consequently, alternative methods to remove lignin are needed. Several approaches such as decreasing lignin content in plant material by altering the composition or structure of lignin (Harris and DeBolt 2010; Pauly and Keegstra 2010; Simmons et al. 2010) are pursued. However, research on genetically modified lignin mutant plants in extensive field trials is only still limited (Simmons et al. 2010).

White rot fungi efficiently use/degrade lignin due to the plethora of enzymes they produce. These enzymes are usually a composite of laccases, ligninases and peroxidases (Martínez 2002). Unfortunately, white-rot basidiomycetes are slow growers and do not produce high quantities of these enzymes which makes them unsuitable for industrial

applications. Heterologous production of these enzymes in *Escherichia coli* and yeasts regularly leads to intracellular and/or inactive protein requiring purification and *in vitro* refolding of the enzymes (Pérez-Boada et al. 2002; Smith et al. 1990). Although *Pichia pastoris* has recently been used in the successful production of *Coprinus cinereus* peroxidase (CiP) (Kim et al. 2009) the development for more efficient host systems is still needed.

Filamentous fungi like *Aspergillus* spp. are considered as preferred hosts for protein production as they have a high capacity of producing homologous and heterologous proteins (Punt et al. 2002). They have the capacity for the required post-translational modifications (Limongi et al. 1995) and have been successfully used for the production of various peroxidases (Conesa et al. 2002a; Conesa et al. 2000; Eibes et al. 2009; Elrod et al. 1997; Lokman et al. 2003). The production of heterologous proteins however, is often relatively low (Punt et al. 2002) which could be a result of improper folding of the heterologous proteins in the ER resulting in processes like, unfolded protein response (UPR) and ER associated degradation (ERAD) (Carvalho et al. 2011; Guillemette et al. 2007). For the production of heterologous proteins in *A. niger*, earlier studies have also demonstrated the huge impact of extracellular proteases on the production yield. Many of these proteases are controlled through the transcription factor prtT. Mutating this transcription factor positively contributes to protein production (Punt et al. 2008). Cofactor availability, is suggested to be another limiting factor in the production of fungal peroxidases, which require heme as a cofactor (Andersen et al. 1992; Elrod et al. 1997). For the production of active peroxidases, heme is required to be inserted during folding of the protein in the endoplasmic reticulum (ER) (Elrod et al. 1997). Addition of a heme source to growth medium in *A. niger and A. oryzae* (Andersen et al. 1992; Conesa et al. 2000; Elrod et al. 1997), as well as over-expression of the first two genes in the heme biosynthesis pathway of *A. oryzae* (Elrod et al. 1997) have been reported to improve the production of peroxidases. However, the exact reason of heme limitation (e.g. synthesis, transport, incorporation etc.), or the mechanism by which heme supplementation improves peroxidase production is currently not fully understood. In this chapter we describe our attempts on peroxidase production in *A. niger prtT* deletion mutant strains.

Materials and methods

Strains and culture conditions

Aspergillus niger N402 (*cspA1* derivative of ATCC9029 (Bos et al. 1988)) and the *pyrG*$^{-}$ derivative of this strain, AB4.1 (van Hartingsveldt et al. 1987), were used during this study. *Aspergillus* strains were grown on *Aspergillus* minimal medium (MM) (Bennet and Lasure

1991) or on *Aspergillus* complete medium (CM) consisting of minimal medium with addition of 10 g l^{-1} yeast extract and 5 g l^{-1} casamino acids. Growth medium was supplemented with 10 mM uridine when required. MM plates containing acetamide as a sole nitrogen source were made as described by Kelly and Hynes (1985). Conidiospores were obtained by harvesting conidia from a CM plate after 4 – 6 days growth at 30 °C, using 0.9 g l^{-1} (w/v) NaCl solution and stored at 4 °C.
Escherichia coli DH5α or methylation deficient cells when required were used for the amplification of recombinant DNA. Transformation of *E. coli* was performed according to the heat shock protocol as previously described (Inoue et al. 1990).

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Chromosomal DNA of *A. niger* was isolated as described by Kolar *et al.* (1988). Both Southern and Northern analyses were performed according to Sambrook and Russell (2001). α-^{32}P-dCTP-labelled probes were synthesized using Rediprime II DNA Labeling System (Amersham Pharmacia Biotech) according to instructions of the manufacturer. Restriction enzymes were obtained from Invitrogen, New England Biolabs and Fermentas and used according to instructions of the manufacturer. Ligation of DNA fragments was performed using the Rapid DNA ligation Kit (Fermentas). When required, fragments were dephosphorylated using Shrimp Alkaline Phosphatase (Fermentas). Sequencing was performed by Service XS (Leiden, The Netherlands).

Construction of pΔprtT
pΔ*prtT* plasmid was constructed by using N402 genomic DNA as template for PCR. The 5' flanking region of the *prtT* gene was amplified as a 0.8 kb fragment introducing a *Not*I site at the 5'end using primers pPrtT1Fw and pPrtT2rev (Table 3.1). The 3'flanking region of the *prtT* gene was amplified as a 1.4 kb fragment using pPrtT3Fw and pPrtT4rev (Table 3.1). The 5'flanking region of the *prtT* gene was cloned into pUC21 as a *Not*I-*Xba*I fragment, the *Xba*I restriction site being an original restriction site in the amplified DNA. The 3'flanking region of the *prtT* gene was cloned into pUC21 as a *Sst*II-*Sph*I fragment using the original restriction sites present in the amplified fragment. For each flanking region two correct clones were analyzed by sequencing. The 5'prtT-pUC21 vector was subsequently opened with *Xba*I-*Hin*dIII followed by insertion of *Aspergillus oryzae pyrG* derived from pAO4-13 (de Ruiter-Jacobs et al. 1989), as *Xba*I-*Sst*II together with the 3'flanking region as a *Sst*II-*Hin*dIII fragment in a three-way ligation yielding pΔ*prtT*. The plasmid was linearized by *Not*I digestion prior to transformation.

Table 3.1: Primers and PCR product sizes used for cloning of the *prtT* deletion construct, the expression vector and genes of interest. Introduced restriction sites are underlined.

primer name	sequence 5' --> 3'	product size (Kb)	Gene of interest
Construction prtT deletion			
prtT 1 FW	attagaatgcggccgcTTTCCGTTGATAGCCGACAGG	0.8	
prtT 2 rev	TCCATGTCCCTGCAGGTCTT		
prtT 3 FW	CCTTTGTTCGGACCGTTCC	1.4	
prtT 4 Rev	CAGTCAACTCAAATGGCTCGG		
Construction expression cassette			
pGlaA FW 1	tgctctagaACAGGAGCCTCGCAATCGT	2.1	
pGlaA Rev 2	ataagaatgcggccgcTGCTGAGGTGTAATGATGCT		
tTrpC FW 1	ggaattccatatgggcgcgccTCGTTGGTGTCGATGTCAGC	0.7	
tTrpC Rev 2	cacagaattcCCTGTGCATTCTGGGTAAACG		
Cloning of genes of interest			
GFP_fw_Not	ataagaatgcggccgcATGGTGAGCAAGGG	0.7	eGFP
GFP_rev_sgs/nde	ggaattccatatgggcgcgccTTACTTGTACAGCTC		
CiP 1 Fw	ataagaatgcggccgcATGTTAACTGTGACTTGT	1.1	CiP-WT; CiP-Lip; & CiP-AAE
CiP 2 Rev	ggaattccatatgggcgcgccTTAAGGAGCAGGAGCTAA		
MnpA 1 FW	ataagaatgcggccgcATGGCTTTCGCAGCTCTC	1.2	MnPA
MnpA 2 Rev	ggaattccatatgggcgcgccTTAAGCGGGACCGTCGAA		
MnpB 1 FW	ataagaatgcggccgcATGGCTTTCAAGTCTCTC	1.1	MnPB
mnpB 3 rev	ggaattccatatgggcgcgccCTACGAGGCGGGAACAGGCA		

Construction of over expression cassette

The *glaA* promoter (P*glaA)* has been amplified from N402 genomic DNA as a 2.1 kb fragment, introducing an *Xba*I restriction site at the 5'end and an *Not*I restriction site at de 3'end of the promoter prior to the start codon using the primers pGlaA Fw 1 and pGlaA rev 2 (Table 3.1). The *trpC* terminator (T*trpC*) has been amplified as a 0.66 kb fragment, introducing *Nde*I-*Asc*I restriction sites at the 5'end and an *Eco*RI site at the 3'end using the primers pTtrpC fw 1 and pTtrpC rev 2 (Table 3.1). T*trpC* has been cloned into pUC21 as a *Nde*I-*Eco*RI fragment and P*glaA* has been cloned to pUC21 as a *Xba*I-*Not*I fragment. After verification by restriction analysis, the inserts of two clones for P*glaA* and four clones for T*trpC* were verified by sequence analysis (Service XS; Leiden, The Netherlands). Next, P*glaA* was cloned into pUC21-T*trpC* as *Xba*I- *Not*I, resulting in the plasmid pFMM2.1. The selection marker *amdS* from p3SR2 (Corrick et al. 1987) has been inserted into pFMM2.1 as an *Xba*I fragment, resulting in the plasmid pFMM2.2. pFMM2.3 was constructed by

cloning *pyrG** into pUC21-T*trpC* as a *Xho*I fragment followed by insertion of P*glaA* as a *Xba*I-*Not*I fragment (Figure 3.1).

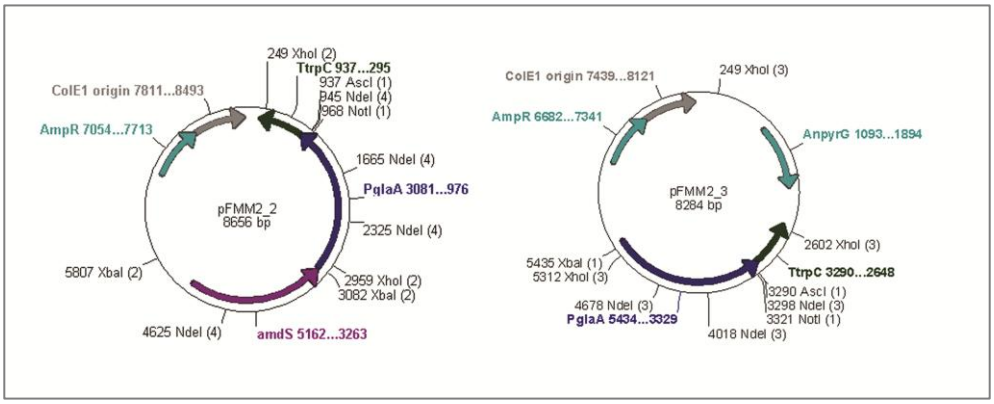

Figure 3.1: representation of the expression vectors pFMM2.2 and pFMM2.3. The vectors consist of a 2.1 kb glucoamylase promoter and the *trpC* Terminator in combination with the selection marker *amdS* for multicopy integration (pFMM2.2) or *pyrG** for targeted integration (pFMM2.3). The introduced *Not*I and the *Asc*I allow for fast and efficient cloning of various genes of interest in both vectors. The respective restriction sites can be introduced prior to the ATG (*Not*I) and directly after the stop codon (*Asc*I) by PCR of the gene of interest.

Genes encoding different peroxidases were selected for over-expression. For two modified versions of the Coprinus peroxidase, CiP-LiP (Smith and Doyle 2006) and CiP-AAE, the genes were kindly provided by A.T. Smith and W. Doyle, University of Sussex, Two genes encoding manganese peroxidases from *Physisporinus rivulosus* (MnPA and MnPB (Hakala et al. 2006)) were obtained by A. Hatakka and K. Hildén, University of Helsinki, Finland. The genes were amplified by PCR (Table 3.1), introducing *Not*I and *Nde*I-*Asc*I restriction sites for efficient cloning. PCR fragments were subsequently cloned into pUC21 as *Not*I-*Nde*I, followed by sequencing. Finally, the peroxidase genes were inserted into pFMM2.2 and pFMM2.3 as *Not*I-*Asc*I.
To test the functionality of the expression cassette eGFP was cloned into pFMM2.3 using a suitable PCR fragment (Table 3.1)

Transformation of A. niger

Transformations were performed according to Meyer *et al.* (2010) with the following modifications. 100 ml cultures inoculated with $1 \cdot 10^8$ conidia were grown at 30 °C at 250 rpm for 12-16 h. 200 mg lysing enzymes (Sigma-Aldrich) were dissolved in 10 ml SMC and set to pH 5.6. Protoplastation was performed at 37 °C and 120 rpm and was verified every 30 minutes by microscopy. 100 µl of protoplasts were mixed with 10 µl of DNA solution (5

to 10 µg), and 25 µl of freshly made PEG buffer. Top-agarose was added to a volume of 40 ml, mixed and poured onto two 15 cm selective transformation plates.

Analysis of transformants

Putative protease deficient Δ*prtT* transformants were prescreened for reduced protease activity on milk-plates (Mattern et al. 1992). GFP production was analyzed by DIC microscopy using a Zeiss Auxioplan 2 (Mannheim, Germany) of O/N cultures, grown in MM containing 1% maltose or 1% glucose at 30 °C. Peroxidase producing strains were screened by means of an plate overlay assay using *o*-anisidine (Conesa et al. 2000). To detect enzyme activity ten-fold increased levels of substrate (25 mM *o*-anisidine) were used. Transformants were inoculated on MM containing 5% maltose to induce the expression cassette. After 48 h of growth at 30 °C, plates are flooded by 1% agarose in phosphate/citrate buffer (McIlvaine 1921) at pH 7.0 for CiP producing strains, or 1% D5-agarose in phosphate/citrate buffer at pH 4.5 for MnP production. Enzyme activity in liquid cultures was determined in 2-9 days as previously described for CiP activity (Elrod et al. 1997) and MnP activity (Wariishi et al. 1992). Samples (15 ml) were concentrated using ultrafiltration (macrosep 30 kDa, PALL Soph tech.). As a control 50 µl of NovoSample NS42041 (Coprinus cinereus peroxidase (EC1.11.1.7; Novozymes)) was used. *Aspergillus niger* strains MGG029[pgpdMnp1.I]**#**13 (Conesa et al. 2002a) and MGG029(pMnp1.I)**#**25 (Conesa et al. 2000) were used as manganese peroxidase control production strains (Kindly provided by P.J. Punt).

Results

Protease deficient Aspergillus niger

A protease deficient strain was constructed by introducing plasmid pΔ*prtT* into *A. niger* stain AB4.1. Putative transformants were selected and tested for reduced protease activity on milk-plates. Nine transformants with reduced protease activity were analyzed by Southern analysis and three proved to be correct deletion strains (data not shown). One of these three strains, designated AF11#56, was used during this study.

Construction of expression cassette and testing its functionality using eGFP

An expression cassette was constructed using the inducible *A. niger* glucoamylase promoter as driver of the efficient transcription of the gene of interest. For efficient termination of transcription the *A. nidulans* TtrpC terminator region was used. Between the promoter and terminator region a *Not*I and an *Asc*I site were introduced respectively.

Using this approach, the gene of interest can be cloned as a *Not*I-*Asc*I fragment. Sites can be incorporated into the gene of interest by PCR. Two variants of the expression vector were constructed containing either the *AmdS* gene as selection marker, to allow selection of multicopy integration of the expression cassette (pFMM2.2) or the *pyrG** gene for targeted integration of in general one copy of the vector (pFMM2.3) (Figure 3.1).
Subsequently eGFP was cloned into the pFMM2.3 vector as a *Not*I-*Asc*I fragment and introduced into *A. niger* strain AB4.1. Correct integration was confirmed by Southern analysis (data not shown). DIC Microscopy showed a clear GFP-signal in maltose grown cultures, confirming the functionality of the new expression vector (Figure 3.2).

Peroxidase producing strains
As previous research had shown considerable limitations for peroxidase production, four different peroxidases were included in our study to test whether they could be produced efficiently The genes were cloned in the expression vectors pFMM2.2 and pFMM2.3 after introducing of *Not*I-*Asc*I restriction sites by PCR (for details see M&M), and introduced into *A. niger* Δ*prtT* strain AF11#56.

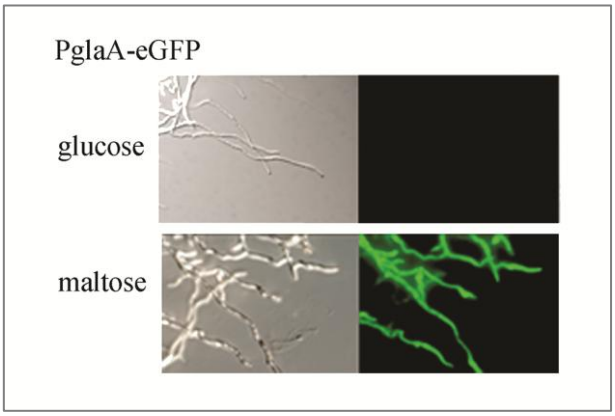

Figure 3.2: validation of functionality of the expression vectors by eGFP. A clear GFP signal is observed under induced (maltose) conditions. No signal is observed under non-inducing conditions (glucose) indicating the induction is specific. O/N cultures have been grown at 30 °C and 250 rpm.

Prescreening transformants obtained with the CiP plasmids on plate using 25 mM *o*-anisidine at optimal pH overnight for each enzyme revealed the presence of positive transformants showing a clear halo (Figure 3.3). However, only a diffuse signal is observed on plates containing MnP transformants (data not shown). Southern analysis of positive transformants confirmed multicopy integration and the absence of a construct in negative transformants (data not shown).

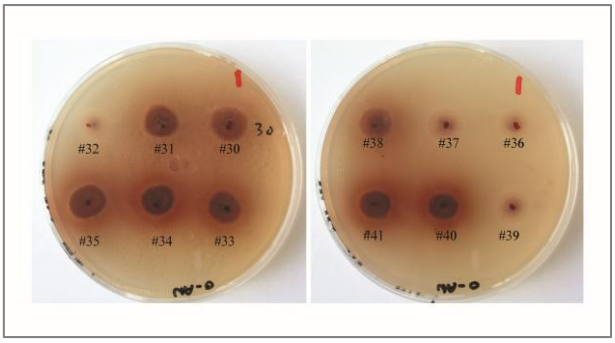

Figure 3.3: example of prescreening of CiP-WT transformants by an *o*-anisidine overlay. Transformants have been inoculated on MM containing 5% maltose and cultivated for 2 days at 30 °C. The plates are subsequently flooded with an overlay containing 25 mM *o*-anisidine. Positive transformants (#30, 31, 33, -35, 38, 40 & 41) develop an orange halo after O/N incubation at 30 °C. Differences in intensities represent enzyme activity levels . Strain #32, 36, 37 & 39, which did not carry introduced peroxidase gene copies do not develop a halo.

Peroxidase expression and activity in liquid cultures

The peroxidase strains were subsequently examined for enzyme production in liquid cultures under inducing conditions. Control strains MGG029[pgpdMnp1.I]**#**13 and MGG029(pMnp1.I)**#**25 show activity in liquid culture, but only when supernatant was concentrated 250 en 62.5 times respectively. In contrast, no measurable active enzyme is present in the supernatant of the various new peroxidase producing strains despite concentrating the samples 1000 times.

To verify successful gene expression, transcription levels of the *cip* and *mnp* genes were analyzed using the *cip*, *mnpA* and *mnpB* PCR products as probes respectively. Northern analysis of the peroxidase producing strains was performed after 48 hours of cultivation in MM containing 5% maltose. A strong and specific expression signal was observed for all peroxidase producing strains (Figure 3.4), excluding that the lack of peroxidase production was due to a limitation at the transcriptional level.

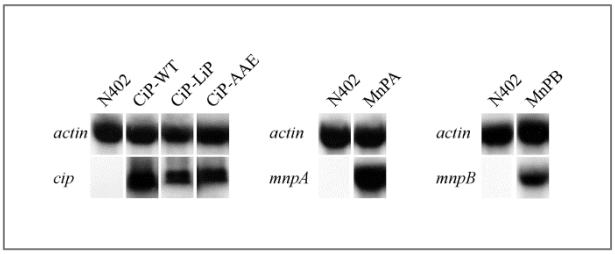

Figure 3.4: Northern analysis of the CiP and MnP production strains. Cultures were cultivated for 48h in MM containing 5% maltose and 0.01 % casaminoacids at 30 °C and 250 rpm before RNA extraction. Labeling with PCR products of A) BR2.1 (CiP-WT); BR2.2 (CiP-LiP) and BR2.3 (CiP AAE) with *cip-wt* PCR product, B)BR2.4 (MnPA) with *mnpA* PCR product or C) BR2.5 (MnPB) with *mnpB* PRC product, indicated a high and specific expression level in the production strains, but no signal in N402. The actin probe is used for loading differences. Differences in expression level among clones may be due to different copy numbers of the construct.

Discussion

Lignin is recalcitrant towards degradation and currently used methods are environmental unfriendly. Multiple approaches are being explored these days, to improve the process of lignin removal in a more environmental friendly manner (Harris and DeBolt 2010; Pauly and Keegstra 2010; Simmons et al. 2010). One of the approaches involves the usage of laccases and peroxidases, originating from basidiomycetes.

The enzymes derived from white-rot fungi have the capacity to efficiently degrade lignin through oxidation of the aromatic compound and free radical release (Leonowicz et al. 2001). These enzymes are unfortunately, not produced in the quantities required for industrial purposes by these fungi. Filamentous fungi like *Aspergillus* spp. have been successfully used for commercial production of peroxidases like CiP. However, the production of other interesting peroxidases like MnP was not equally successful. Given the importance of these oxidative enzymes in white biotechnology, we have evaluated production of various peroxidases in *A. niger.*

One of the bottlenecks of heterologous enzyme production by *A. niger* is extracellular degradation of enzymes by proteases. Previous research (Punt et al. 2008) has shown that the expression of most of the extracellular protease genes of *A. niger* are controlled through the transcription factor PrtT. Further research has shown that mutating of this transcription factor positively contributes to heterologous enzyme production. For this reason a *prtT* deletion strain was constructed to be used for the production of various peroxidases. To allow efficient cloning of a variety of genes encoding oxidative enzymes (laccases and peroxidases), a set of expression vectors were generated using the strong, inducible glucoamylase promoter to drive the expression upon maltose/dextrin induction after generating maximal biomass. Analysis of transformants obtained with CiP, MnPA or

MnPB expression vectors revealed that low levels of active protein were observed when performing an overlay assay. Only with increasing substrate concentrations and extended incubation times activity was observed on plates (Figure 3.3). In liquid cultures, peroxidase production remained undetectable even though strong mRNA signals were observed in Northern analysis (Figure 3.4). These results indicate that processes after transcription are limiting for production of the peroxidases.

As the production strains are constructed in a prtT deletion background, we anticipate that the amount of extracellular proteases is low. Although we cannot exclude that residual proteases are responsible for the degradation of the peroxidases or laccases, other factors are more likely contributing to the lack of peroxidase production (for a review on this see De Weert and Lokman 2010). Processes which could be responsible for low production levels are the quality control processes like the unfolded protein response and subsequently endoplasmic reticulum associated degradation (ERAD). Proteins identified as incorrect folded proteins are retrieved in the ER to be refolded. Accumulation of the unfolded proteins in the ER however, results in ER-stress and this targets the unfolded protein response machinery for degradation through ERAD by the proteasome, thereby reducing the ER-stress but also the efficiency of protein production (Carvalho et al. 2011 and references therein).

Another possibility explanation for the very low production levels could be that secreted peroxidases are retained in the cell wall as has been suggested for laccase producing *A. niger* strains, based on similar results as observed for peroxidases in our studies (Weenink et al. 2006). More specifically, also a limitation in cofactor availability (heme for peroxidases) could be responsible for low production of peroxidases. This has been observed for various peroxidase producing *Aspergillus* spp. (Andersen et al. 1992; Elrod et al. 1997). Addition of a heme source to growth medium improves production of peroxidases (Andersen et al. 1992; Conesa et al. 2000; Elrod et al. 1997), but is not suited for industrial applications as this compound is too costly to implement (Elrod et al. 1997). Furthermore, the exact mechanism on how this addition improves peroxidase production is unclear. Actually also folding processes as described above could play a role in removing peroxidase apo-proteins devoid of their heme cofactor.

Increasing intracellular levels of heme would be an option to relieve the potential heme limitation. However, heme is not only an essential, but also highly reactive molecule. Its synthesis is therefore strictly controlled but can be, albeit limited, enhanced. Over-expression of the first two genes in the heme pathway (*hemA* and *hemB*) in a CiP producing *Aspergillus oryzae* strain increased CiP production 4-fold. Maximum production was not yet achieved as hemin supplementation still improved CiP production (Elrod et al.

1997) in this strain. This could indicate bottlenecks in heme synthesis or heme transport to the ER, but does not exclude other factors.

Obviously also indirect effects of heme can not be excluded as it is also required as a cofactor for many primary functions in the cell (Hamza 2006). The addition of a heme source, or increasing the intracellular concentration of heme, could also affect the efficiency of processes like protein production in general. Next to its function as cofactor, heme (and compounds derived from it) also has a role as a signaling molecule (Hortschansky et al. 2007; Hou et al. 2006). In mammals it has been shown that hemin can act as an inhibitor of the proteasome (Guo et al. 1994). However, since knowledge on heme in *Aspergilli* is limited, further studies on biosynthesis and its regulation are required in order establish its role in peroxidase production in these organisms. Identifying bottlenecks in the synthesis of heme could enlarge our knowledge and provide useful information on how to increase peroxidase production levels in *A. niger.*

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