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Tese de Doutoramento em Engenharia Quimica e Biológica

Trabalho realizado sob a orientação da
Doutora Lucília Domingues
e co-orientação da
Professora Merja Penttilä

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Dedico esta tese

aos meus pais

Abstract

Physiological characterization of *Ashbya gossypii* and strain development for recombinant protein production

Ashbya gossypii is a phytopathogenic hemiascomycete belonging to the *Saccharomycetaceae* family. This fungus has attracted attention because of its natural ability to produce riboflavin (vitamin B2) as a detoxifying and protective mechanism being used at an industrial level as a biotechnological important producer of riboflavin. The genome sequence of this filamentous fungus revealed remarkable similarities to that of the budding yeast *Saccharomyces cerevisiae* both at the level of homology and synteny. *A. gossypii* is a very promising experimental system because it has a small genome (the smallest eukaryotic genome known to date) and haploid nuclei. Moreover, efficient gene targeting due to the high homologous recombination efficiency in *A. gossypi*i makes it possible to do one-step gene replacement by PCR-based gene targeting. *A. gossypii* also allows extrachromosomal free replication of plasmids bearing an autonomous replicator. Taking advantage of these unique features, the overhall purpose of this project was to explore the potential of *A. gossypii* as an alternative cell factory organism. As little information was available for this organism, the project began with the physiological characterization of different *A. gossypii* strains. Also, the information in the literature regarding its secretory ability was inexistent and thus two heterologous proteins were used to evaluate this feature. In addition, different strategies were undertaken to improve its secretion ability and a global transcriptome analysis was conducted to identify the bottlenecks on *A. gossypii* secretory pathway.

Colony radial growth rates and specific growth rates of three related *A. gossypii* strains ATCC10895, IMI31268, MUCL29450 and an unrelated strain, CBS109.26, were measured on various carbon and nitrogen sources at pH 4.5 and pH 6.5 to elucidate physiological growth requirements and strain differences. All strains grew on yeast extract or ammonium as nitrogen sources, but not on nitrate. Substantial growth at pH 4.5 was observed only on complex medium. D-Glucose, glycerol and starch were utilised as carbon sources. Ethanol was produced during growth on glycerol. Conversion of xylose into xylitol demonstrates that the xylose reductase is active. Phenotypic differences between related strains were greater than expected. It was shown that *A. gossypii* utilizes ammonium as sole nitrogen source at pH 6.5, facilitating further physiological studies using chemically defined media in the future.

Even though filamentous fungi are excellent producers of a broad spectrum of extracellular enzymes such as amylases, proteases and catalases, little is known about the secretory capacity of *A. gossypii*. To explore the potential of *A. gossypii* as a host for the expression of recombinant proteins and to assess whether protein secretion would be more similar to the closely related *S. cerevisiae* or to other filamentous fungi, endoglucanase I (EGI) and cellobiohydrolase I (CBHI) from the fungus *Trichoderma reesei* were successfully expressed in *A. gossypii* from plasmids containing the two micron sequences from *S. cerevisiae*. The native signal sequences of EGI and CBHI were able to direct the secretion of EGI and CBHI into the culture medium in *A. gossypii*.

Although CBHI activity was not detected using 4-methylumbelliferyl-β-D-lactoside as substrate, the protein was detected by Western blot using monoclonal antibodies. EGI activity was detectable and the specific activity being comparable to that produced by a similar EGI producing *S. cerevisiae* construct. More EGI was secreted than CBHI, or more active protein was produced. Partial characterization of CBHI and EGI expressed in A. gossypii revealed overglycosylation when compared with the native *T. reesei* proteins, but the glycosylation was less extensive than on cellulases expressed in *S. cerevisiae*.

In order to improve the general secretion ability, *A. gossypii* was subject to random mutagenesis with ethyl methane sulfonate (EMS). Selection and screening was carried out in order to identify secretion mutants with improved protein secretion ability. Secreted EGI, amylase and beta glucosidase activities of the parental strain and five key mutants were investigated and used as an indicator for enhanced protein production after the mutagenic treatment. Mutagenesis improved EGI and amylase activity in the culture supernatant of the mutants S436 and S466 by 2 and 3-fold increase respectively, compared to the initial parental strain. At the same time, the mutant S436 also revealed 40% improvement in the beta glucosidase activity. Mutant S397 showed a 2 fold increase in beta glucosidase activity. Overall, mutant S436 seems to be the most promising *A. gossypii* strain since all the activities tested were enhanced when comparing to the parental strain. This means that the general secretion capacity of this mutant was enhanced.

Another attempt to improve the secretion capacity of *A. gossypii* relied on the deletion of *GAS*1 gene, which codes for a β-1,3-glucanosyltransglycosylase involved in cell wall assembly. *GAS1* gene is present as a tandem repeat in *A. gossypii* genome. With this approach, a higher permeability of the cell wall was expected and hence an increase in the protein secretion capacity. However two scenarios where observed. The individual deletion of one copy of the gene severely impaired growth whereas the abolishment of the other copy resulted in similar amounts of EGI secreted into the extracellular medium when compared to the initial recombinant strain. As a result this strategy failed to enhance *A. gossypii* secretory capacity.

In order to understand and determine the limitations encountered along *A. gossypii* secretory pathway, a transcriptomic analysis was carried out in the recombinant EGI producing strain and also under chemical induced stress by dithiotreitol (DTT). Surprisingly, none of the conditions tested were able to induce unfolded protein response (UPR) in *A. gossypii*. EGI production can be expected to have such an effect since the production levels are very low to cause ER stress. However, the translation machinery was down regulated under EGI producing conditions, which can explain the low EGI production levels. Neither DTT, a widely used UPR inducer was able to activate UPR in *A. gossypii*. Instead endoplasmatic reticulum associated degradation (ERAD) was highly induced when the mycelium was treated with DTT. The lack of a strong UPR response when low levels of an heterologous protein is being produced, or during chemically induced stress, strongly suggests that non-UPR mediated bottlenecks might exist in *A. gossypii* that hamper efficient secretion.

Resumo

Caracterização fisiológica do fungo *Ashbya gossypii* e desenvolvimento de estirpes para produção de proteínas recombinantes

Ashbya gossypii é uma hemiascomicete pertencente á família *Saccharomycetaceae.* Este fungo tem atraído atenção devido à capacidade de produzir riboflavina (vitamina B2) naturalmente como um mecanismo de destoxificação e de defesa. Sendo considerado um produtor de riboflavina biotecnologicamente importante e como tal, tem sido usado a nível industrial. A sequência genómica deste fungo filamentoso revelou semelhanças extraordinárias com a levedura *Saccharomyces cerevisiae* quer ao nível da homologia quer da sintenia. Este fungo é um sistema experimental muito promissor porque possui um genoma pequeno (o genoma eucariota mais pequeno conhecido até á data) e núcleos haplóides. Para além disso, a elevada eficiência de recombinação permite a deleção de genes de modo eficiente, sendo possível de ser feito num único passo por técnicas de PCR dirigidas. *A. gossypii* também permite a replicação livre extracromossomal de plasmídeos que possuam um replicador autónomo. Tomando partido destas vantagens únicas, o objetivo geral deste projeto consistiu na avaliação do potencial de *A. gossypii* como uma fábrica celular alternativa. Como havia pouca informação acerca deste organismo, o projeto começou com a caracterização fisiológica de diferentes estirpes de *A. gossypii*. A informação relativa à sua capacidade de secreção era inexistente e portanto, duas proteínas heterólogas foram usadas para avaliar esta capacidade. Para além disso, várias estratégias foram implementadas com o objetivo de melhorar a sua capacidade de secreção e uma análise de transcriptoma foi efetuada para identificar os passos limitantes da via de secreção.

Foram determinadas as taxas de crescimento radial de colónia e taxas específicas de crescimento em várias fontes de carbono e de azoto a pH 4.5 e pH 6.5 de três espécies de *A. gossypii* similares, nomeadamente, ATCC10895, IMI31268, MUCL29450 e de uma espécie mais afastada, CBS 109.26, de modo a elucidar os requisitos fisiológicos de crescimento e as diferenças entre as espécies. Todas as estirpes cresceram em extrato de levedura ou amónio como fonte de azoto, mas não em nitrato. Foi observado crescimento substancial a pH 4.5 apenas em meio complexo. D-glucose, glicerol e amido foram utilizados como fonte de carbono. Durante o crescimento em glicerol, detetou-se produção de etanol. A conversão de xilose em xilitol demonstrou que a xilose redutase está ativa. Diferenças fenotípicas entre as estirpes mais semelhantes foram maiores do que o esperado. Foi mostrado que o *A. gossypii* utiliza amónio como fonte de azoto a pH 6.5, facilitando estudos fisiológicos que no futuro usem meio definido.

Embora os fungos filamentosos sejam excelentes produtores de uma vasta gama de enzimas extracelulares, tais como amilase, protease e catalase, pouco se sabe acerca da capacidade secretória de *A. gossypii*. Com o objetivo de explorar o potencial de *A. gossypii* como um organismo usado para a expressão de proteínas recombinantes e de modo a avaliar se a secreção de proteínas será mais semelhante à *S. cerevisiae* ou a outros fungos filamentoso, a endoglucanase I (EGI) e a celobiohidrolase I (CBHI) do fungo *Trichoderma reesei,* foram expressas com sucesso em *A. gossypii* a partir de plasmídeos que contêm a sequência 2 micra de *S. cerevisiae* sob o promotor *PGK*1 de *S. cerevisiae*. A sequência sinal nativa da EGI e da CBHI direcionou a secreção da EGI e da CBHI para o

meio de cultura em *A. gossypii*. Embora a atividade da CBHI não tenha sido detetada com o substrato 4- methylumbelliferyl-β-D-lactoside, a proteína foi detectada por Western blot realizado com anticorpos monoclonais. A actividade da EGI foi detetada, sendo que a atividade específica é comparável à de uma estirpe de *S. cerevisiae* produtora de EGI. Mais EGI foi secretada em comparação com CBHI ou foi produzida proteína mais ativa. A caracterização parcial da CBHI e da EGI produzidas em *A. gossypii* revelou hiperglicosilação quando comparada com as proteínas nativas de *T. reesei*, mas a glicosilação foi menos extensa do que nas celulases expressas em *S. cerevisiae*.

De modo a melhorar a capacidade geral de secreção, *A. gossypii* foi sujeito a mutagénese aleatória com etil metano sulfonato (EMS). A seleção e o rastreio foram realizados com a finalidade de identificar mutantes com uma capacidade melhorada de secretar proteínas. Foram medidas as atividades das enzimas secretadas, EGI, amilase e beta glucosidaes da estirpe parental e de cinco mutantes chave e usadas como um indicador de aumento de produção de proteínas após o tratamento mutagénico. A mutagénese resultou num aumento de 2 e 3 vezes na atividade da EGI e da amilase no sobrenadante da cultura dos mutantes S436 e S466, comparado com a estirpe parental. Simultaneamente, o mutante S436 também revelou um aumento na atividade de beta glucosidase. O mutante mutante S397 apresentou um aumento de 2 vezes na atividade da beta glucosidase. No geral, o mutante S436 parece ser a estirpe de *A. gossypii* mais promissora uma vez que todas as atividades medidas sofreram um aumento quando comparadas com a estirpe parental. Isto significa que a capacidade geral de secreção deste mutante foi melhorada.

Uma outra tentativa para melhorar a capacidade de secreção do *A. gossypii* baseou-se na deleção do gene *GAS*1, que codifica para uma β-1,3-glucanosyltransglycosylase envolvida na montagem da parede celular. No genoma de *A. gossypii* existem duas cópias do gene *GAS*1. Com esta abordagem esperava-se um aumento na permeabilidade da parede celular e consequentemente um aumento na capacidade de secreção de proteínas. Contudo, foram observados dois cenários. A deleção individual de uma cópia do gene afetou gravemente o crescimento, ao passo que a eliminação da outra cópia resultou em quantidades semelhantes de EGI secretadas para o meio extracelular, quando comparado com a estirpe recombinante inicial. Como resultado, esta estratégia falhou no aumento da capacidade secretora de *A. gossypii*.

De modo a compreender e a determinar as limitações encontradas ao longo da via de secreção de *A. gossypii*, foi realizada uma análise de transcriptoma na estirpe recombinante produtora de EGI e também sob stress quimicamente induzido por ditiotreitol (DTT). Surpreendentemente, nenhuma das condições testadas foi capaz de induzir a "*Unfolded protein response*" (UPR) em *A. gossypii*. Seria de esperar tal efeito por parte da EGI, tendo em conta os baixos níveis de expressão. Contudo, a maquinaria de tradução foi reprimida nas condições de produção de EGI, o que por sua vez ajuda a explicar os baixos níveis de produção de EGI. Nem mesmo o DTT, um indutor de UPR amplamente utilizado, foi capaz de ativar a UPR em *A. gossypii*. Em vez disso, a degradação associada ao reticulo endoplasmático (ERAD) foi altamente induzida quando o micélio foi tratado com DTT. A ausência de uma resposta UPR forte quando uma proteína heteróloga está a ser produzida a baixos níveis ou durante stress quimicamente induzido, sugere que limitações não relacionadas com a UPR possam existir em *A. gossypii* e que estejam a condicionar uma secreção eficiente.

Publications

This thesis is based on the following original articles:

Ribeiro O, Domingues L, Penttilä M, Wiebe M. (2011) Nutritional requirements and strain heterogeneity in *Ashbya gossypii* (*Eremothecium gossypii*). *Journal of Basic Microbiology* 51: 1-8. (Chapter 2)

Ribeiro O, Wiebe M, Ilmén M, Domingues L, Penttilä M. (2010) Expression of *Trichoderma reesei* cellulases CBHI and EGI in *Ashbya gossypii*. *Applied Microbiology and Biotechnology* 87: 1437–1446. (Chapter 3)

Ribeiro O, Wiebe M, Penttilä M, Domingues L. Random and direct mutagenesis to enhance protein secretion in *Ashbya gossypii*. *Submitted*. (Chapter 4)

Ribeiro O, Aguiar T, Arvas M, Wiebe M, Penttilä M, Domingues L. Transcriptome analysis of recombinant *Ashbya gossypii* expressing EGI from *Trichoderma reesei* treated with ditiothreitol. *Submitted*. (Chapter 5)

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Abbreviations

A	adenine (base) or alanine (amino-acid)
AFM	*Ashbya* full medium
AGD	Ashbya Genome Database
AGT	alanine: glyoxylate aminotransferase
AGX1	gene encoding alanine: glyoxylate aminotransferase
AMP	Adenosine monophosphate
AOX	alcohol oxidase promoter
Ar	argon
Arg	arginine
ARS	autonomously replicating sequence
ATP	adenosine triphosphate
BMCC	crystalline cellulose
bp	base pairs
BSA	bovine serum albumin
C	cytosine
CBHI	cellobiohydrolase I
CBM	carbohydrate binding module
CD	catalytic domain
cDNA	complementary DNA
CMC	carboxymethyl cellulose
CreA	carbon catabolite repressor
CSL	corn steep liquor
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
Doa10	ER membrane integrated ubiquitin ligase
DTT	ditiothreitol
DW	dry weight
EGI	endoglucanase I
EMS	ethyl methane sulfonate
ENO1	enolase 1
ER	endoplasmic reticulum

Ero1	thiol oxidase
ERAD	endoplasmic reticulum associated degradation
FIRE	Finding Informative Regulatory Elements
FITC	fluorescein isothiocyanate
FMN	flavin mononucleotide
G	guanine (base) or glycine (amino-acid)
G418	geneticin
GalNAc	N-acetylgalactosamine
GAP	Glyceraldehyde-3-phosphate dehydrogenase
GAS1	β-1,3-glucanosyltransglycosylase
GFP	green fluorescent protein
GH	glycoside hydrolases
GlcNAc	N-acetylglucosamine
GLY	gene encoding threonine aldolase
GMP	guanosine 5’ monophosphate
GO	Gene Ontology
GRAS	generally recognized as safe
GTP	guanosine triphosphate
h	hour(s)
HAC	Basic leucine zipper (bZIP) transcription factor
hCMV	human cytomegalovirus
HPLC	high performance liquid chromatography
HRD1	Ubiquitin-protein ligase
ICL	isocitrate lyase
IPTG	isopropyl-β-D-thiogalactopyranoside
IRE1	Serine-threonine kinase and endoribonuclease
KAR2	member of the HSP70 family of molecular chaperones
kb	kilobase pairs
kDa	kiloDalton
KEX2	Subtilisin-like protease
LB	Luria-Bertani broth medium
LIMMA	Linear Models for Microarrays
min	minutes
MMNG	*N*-methyl-*N′*-nitro-*N*-nitrosoguanidine

mRNA	messenger ribonucleic acid
MU	4- methylumbelliferone
MuLac	4- methylumbelliferyl-β-D-lactoside
MW	molecular weight
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information (*USA*)
NTG	*N*-methyl-*N′*-nitro-*N*-nitrosoguanidine
OCH1	gene encoding an α-1,6-Mannosyltransferase
OD	Optical density
ORF	open reading frame
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PDI	protein disulfide isomerise
PGK	3-phosphoglycerate kinase
pI	isoelectric point
Pro	proline
PRPP	5'-phosphoribosyl-1-pyrophosphate
RMA	Robust Multichip Average
RNA	ribonucleic acid
RT-PCR	reverse transcription-PCR
SCD	synthetic complete medium
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Sec61	essential subunit of complex Sec61p
Sf9	*Spodoptera frugiperda* ovarian insect cells
SHM2	serine hydroxymethyltransferase
SHMT	serine hydroxymethyltransferase
SPK	Spizenkörper
SV40	simian virus 40
TEF	translation elongation factor
THR4	Threonine synthase
tRNA	transference ribonucleic acid
5′UTR	5′ untranslated region
UPR	unfolded protein response

UPRE	unfolded protein response element
UV	ultraviolet radiation
VMA1	vacuolar ATPase subunit
XPR2	gene coding for an alkaline protease

Aims and thesis planning

A. gossypii is a hemiascomycete closely related to the yeast *Saccharomyces cerevisiae*, known for the ability to naturally overproduce riboflavin (vitamin B2), responsible for the yellow colour of the mycelium. With its close ties to yeast and the ease of genetic manipulation in this fungal species, *A. gossypii* is well suited as a model to elucidate the regulatory networks that govern the functional differences between filamentous growth and yeast growth, especially that the *A. gossypii* genome sequence has been completed. Filamentous fungi are well known for the remarkable capacity of secreting large amounts of proteins to the extracellular medium. Taking all this into account, the aim of this work consists in evaluating and developing *A. gossypii* as a host for recombinant protein production.

To achieve that aim, a comparative physiological study was carried out between four *A. gossypii* strains. In the following step, two heterologous proteins, namely EGI and CBHI from *Trichoderma reesei* were cloned and expressed in *A. gossypii*. Thereafter, two strategies were followed in order to enhance the secretory ability of *A. gossypii* by random and directed mutagenesis. In order to determine the bottleneck(s) responsible for the limited secretion ability of *A. gossypii*, a study of the transcriptome was done under recombinant protein production conditions and under chemical stress induced by DTT. Finally, the results obtained were analysed and discussed.

The first chapter corresponds to a general literature review of the main subjects of this work. Chapters 2 to 5 describe the experimental work and the main results attained, and finally, in chapter 6, a summary of the main conclusions and some future perspectives are presented.

The specific aims of this work can be divided on:

- Physiological characterization of four *A. gossypii* strains (chapter 2);
- Expression of EGI and CBHI from *Trichoderma reesei* in *A. gossypii* (chapter 3);
- Improvement of *A. gossypii* secretion capacity by random mutagenesis (chapter 4);
- Transcriptome analysis of *A. gossypii* under recombinant protein production conditions and DTT stress (chapter 5).

Chapter 1

General introduction

1.1 Introduction

Fungi represent one of the major Eukaryotic kingdoms besides the Plantae and Animalia. Around 70 000 fungal species have been described while the total number of species has been estimated at 1.5 million (Hawksworth 2002; Hawksworth 2004; Lutzoni et al. 2004).

Fungi, including both true filamentous fungi and yeasts, have been used since ancient times, in the production of bread, wine, beer, koji, distilled spirits, vinegar, cheese, pickles and other fermented materials (Bennett 1998; Demain 1999). Some fungi are well known pathogens of humans, animals and plants (Cutler et al. 2007; Maor and Shirasu 2005) while others, for example mycorhizal fungi, have beneficial associations with plants and/or participate in nutrient recycling in soil. Several fungi are responsible for food spoilage, wood decay and many fungal spores are known allergens (Meyer et al. 2004). In the past, the presence of microorganisms such as molds and yeasts was revealed by their activities. For example, after Pasteur observed that living organisms could always be seen under the microscope during sugar fermentations, the organisms producing them, mostly yeasts, were called ‘organized ferments’ (Bennett 1998).This phase can be called traditional industrial microbiology.

In the early 20th century, another major phase of industrial microbiology (modern industrial fermentations) began. The golden era of industrial fermentation was marked by the first large scale fermentations dedicated to manufacture of solvents, organic acids, vitamins, enzymes, and other products. Later on, in the mid-century, with the development of processes for the production of penicillin and streptomycin antibiotic fermentations a new field of biochemical (microbiological) engineering as emerged. Microbial products may be very large materials such as proteins, nucleic acids, carbohydrate polymers, or even cells, or they can be smaller molecules which we usually divide into metabolites essential for vegetative growth and those inessential, i.e., primary and secondary metabolites, respectively (Demain and Adrio 2008).

1.1.1 Primary metabolites

During the process of fermentation, filamentous fungi are capable of producing a wide range of primary metabolites (Table 1.1), including organic acids, such as citric, gluconic, fumaric, kojic, itaconic acid and fatty acids (Demain 2000).

The development of citric acid production by manipulating culture conditions, developing submerged processes, and improving product recovery, had a very important role in the development of modern biotechnology. Originally isolated from citrus fruits, since the end of the nineteenth century it was known that citric acid was also made by filamentous fungi (Gómez et al. 1988; McIntyre and McNeil 1997; Papagianni et al. 1999; Roukas 1991). This organic acid is produced via the Embden-Meyerhof pathway and the first step of the tricarboxylic acid cycle. The major control of the process involves the feedback inhibition of phosphofructokinase by citric acid. The commercial process employs the fungus *Aspergillus niger* in media deficient in iron and manganese. There is a widespread use of the compound in the food and beverage industry, cosmetics, detergents, antifoaming, textile treatment, and as a preservative for stored blood.

Other valuable organic acids include acetic, lactic, malic, gluconic, itaconic, tartaric and succinic acids.

Table 1.1 – Examples of some of the most important compound produced by filamentous fungi. (Adrio and Demain 2003; Archer 2000; Bennett 1998; Leathers 2003; Linder et al. 2005; Olempska-Beer et al. 2006; Ooi and Liu 2000; Polizeli et al. 2005; Willke and Vorlop 2001)

Compound	Organism
Acids	
Citric and gluconic acid	*Aspergillus niger*
Itaconic acid	*A. terreus*
Kojic acid	*A. oryzae*
Gibbereillic acid	*Gibberella fujikuroi*
Linolenic acid	*Martierella isabellina*
Enzymes	
α-amylase	*A. niger, A. oryzae*
Chymosin	*A. niger*
Cellulases	*Trichoderma viride, T. reesei*
Glucoamylase	*A. Phoenicis, Rhizopus delemar, A. sp.*
Glucose oxidase	*A. niger, A. oryzae*
Laccase	Trametes vesicolor
Lipases	*A. niger, A. oryzae*
Pectin lyase	T. reesei
Proteases	*A. niger, A. oryzae, R. delemar*
Phytase	*A. niger, A. oryzae*
Rennin	*Mucor miehei*
Xylanases	*T. reesei, T. konignii, A. niger*
Antibiotics	
Cephalosporin C	*Cephalosporium acremonium*

Penicillin N	*Emericellopsis sp.*
Penicillins G and V	*Penicillium chrysogenum*
Griseofulvin	*P. patulum*
Pleuromutilin	*Pleurotus mutilus and P. passeckerianus*
Strobilurins A and B	*Strobilurus sp.*
Secondary metabolites	
Cephalosporin	*Acremonium chrysogenum*
Cyclosporin	*Tolypocladium nivenum*
Ergot alkaloids	*Claviceps purpurea*
Griseofulvin	*P. griseofulvum*
Lovastatin	*Monascus rubber, A. terreus*
Taxol	*Taxomyces andrenae*
Zeranol	*Fusarium graminearum*
Mycotoxins	
Aflatoxins, citrinin, ochratoxin, and sterigmatocystin	*Aspergillus sp.*
Ergot	*Claviceps sp.*
Trichothecenes and zearalanone	*Fusarium sp.*
Citrinin, ochratoxin, and patulin	*Penicillium sp*.
Exopolysaccharides	
Scleroglucan	*Sclerotium rolfsii*
Pullulan	*Aureobasidium pullulans*
Schizophyllan	*Schizophyllum commune*
PSK, PSP	*Tr. versicolor*
Others	
Polyunsaturated faty acid	*Mucor circinelloides*
Panthothenic acid	*F. oxysporum*
Hydrophobin	*T. reesei*
Biomass Food industry	*Agaricus bisporus, F. venentatum (Quorn™)*

1.1.2 Secondary metabolites

In addition, filamentous fungi naturally produce an astonishing array of secondary metabolites, especially as human therapeutics, for example, penicillin, cephalosporin, ergot alkaloids, griseofulvin, lovastatin, taxol and zeranol (Meyer 2008). The immunosuppressant cyclosporins and the anti-hypertensive mevalonins are two of the most important pharmaceuticals produced by filamentous fungi.

The best known of the secondary metabolites are the antibiotics. This remarkable group of compounds forms a heterogeneous group of biologically active molecules with different structures and modes of action. They attack virtually every type of molecule/function such as DNA, RNA, and protein synthesis, membrane function, electron transport, sporulation, germination, and many others. Without any doubt, the turning point in the development of modern industrial microbiology happened with the discovery of the ‘wonder drug’ penicillin in 1929 (Wainwright 1990). Penicillin triggered the search for other secondary metabolites with antibacterial activity, as well as stimulated research on fungal physiology, fermentation

technology, and industrial strain development. So many new antibiotics were discovered during the 1940s and 1950s that it has been called The Golden Age of Antibiotics.

Microbially produced secondary metabolites (Table 1.1) are extremely important to our health and nutrition (Demain and Fang 1995). As a group that includes antibiotics, other medicinals, toxins, pesticides, animal and plant growth factors, they have a tremendous economic importance.

Many secondary metabolites which have antibiotic activity are used for other purposes. These include hypocholesterolemic agents, immunosuppressants, anticancer agents, bioherbicides, bioinsecticides and animal growth promoters. Since 1940, there was a burst of new and potent antibiotic molecules which have been of tremendous importance in medicine, agriculture, and basic research. However, the search for new antibiotics continues in order to combat evolving pathogens and naturally resistant bacteria and fungi, and previously susceptible microbes that have developed resistance; improve pharmacological properties; combat tumors, viruses, and parasites; and discover safer and more potent compounds. From 1990 to 1994, over 1000 new secondary metabolites were characterized from actinomycetes alone (Sanglier et al. 1996). About 6000 antibiotics have been described, 4000 from actinomycetes, and they still are being discovered at a rate of about 500 per year.

Usually, secondary metabolites are produced when growth slows down. They have no function in growth of the producing microorganism, are produced by certain restricted taxonomic groups of organisms, and are usually formed as mixtures of closely related members of a chemical family. In nature, secondary metabolites are important for the organisms that produce them, functioning as: sex hormones, ionophores, competitive weapons against other bacteria, fungi, amoebae, insects and plants; agents of symbiosis and effectors of differentiation (Demain 2000).

One huge success has been the statins, including lovastatin (mevinolin), pravastatin and others produced by fungi (Endo 1985) which act as cholesterol-lowering agents. Lovastatin is produced by *Aspergillus terreus*. Another important enzyme inhibitor is clavulanic acid, an actinomycete β-lactam, which acts as an inhibitor of β-lactamases. Also in commercial use are biopesticides including fungicides (e.g. kasugamycin, polyoxins), bioinsecticides (*Bacillus thuringiensis* crystals, nikkomycin, spinosyns), bioherbicides (bialaphos), ruminant growth promoters (monensin, lasalocid, salinomycin), plant growth regulators (gibberellins), immunosuppressants for organ transplants (cyclosporin A, rapamycin), anabolic agents in farm animals (zearelanone), uterocontractants (ergot alkaloids), and antitumor agents (doxorubicin, daunorubicin, mitomycin, bleomycin) (Demain 1983).

Many of the above compounds were first isolated as poor or toxic antibiotics (e.g. monensin, cyclosporin, rapamycin) or as mycotoxins (ergot alkaloids, gibberellins, zearelanone) before a new application was discovered.

Although the examples cited above do not represent a complete listing of industrially produced fungal-based compounds, it still reflects the metabolic versatility of filamentous fungi and their importance as cell factories in biotechnology.

Although microbes are good in producing a quite large range of valuable compounds, they usually produce them only in amounts that they need for their own benefit; thus they tend not to overproduce their metabolites. Microorganisms are equipped with mechanisms of regulation that avoid excessive production of its metabolites so that it can compete efficiently with its competitors and survive in nature. The main reason for the use of microorganisms to produce compounds that can otherwise be isolated from plants and animals or synthesized by chemists is the ease of increasing production by environmental and genetic manipulation. When the production level is high, it makes it easier the process of purification. The way to overcome this limitation consists in increasing the production by mutagenesis and screening for higher producing microbial strains. At the same time, the cost of production can be reduced with the increase production. Mutation has also served to elucidate the pathways of secondary metabolism, and yield new compounds (Demain 2000).

In the early 1970s, with the birth of recombinant DNA technology the production of primary and secondary metabolites, bioconversions and especially the enzyme industry have experienced a tremendous expansion (Demain 2000).The twentieth century, a golden age of industrial microbiology, yielded a myriad of products made by fermentation processes: solvents, antibiotics, enzymes, vitamins, amino acids, polymers, and many other useful compounds (Demain 1999). A good example is the vitamin B2 (Riboflavin) that was produced by both fermentation and chemical synthesis (Demain 1972). Today, fermentation is the major route. Riboflavin overproducers include two yeast-like molds, *Eremothecium ashbyii* and *Ashbya gossypii*, which synthesize riboflavin in concentrations greater than 20 g per L. A riboflavin-overproducer such as *A. gossypii* makes 40 000 times more vitamin than it needs for its own growth. A new processe using *Candida* species or recombinant *Bacillus subtilis* strains have been developed in recent years which produce 20–30 g riboflavin per L.

Due to their exceptional high capacity to express and secrete proteins, filamentous fungi have become indispensable for the production of enzymes of fungal and non-fungal origin. Jokichi Takamine, in 1894, was probably the first to realize the technical possibilities of enzymes from molds and to introduce these fungal enzymes to industry. Takamine used the

Japanese koji mold, *Aspergillus oryzae*, to produce diastase (Takamine 1894). During the early years of the twentieth century, similar processes were developed for numerous other enzymes. By 1983, there were approximately 30 different classes of enzyme in common commercial use, of which approximately half were of fungal origin. Currently, native or recombinant enzymes are mainly produced by *Aspergillus niger*, *A. oryzae* and *Trichoderma reesei* and also other strains are currently under development (Punt et al. 2002).

1.2 Description of *Ashbya gossypii*

Ashbya gossypii was isolated and characterized for the first time by Nowell in 1916 (Nowell 1916). *A. gossypii* is a filamentous phytopathogenic fungus that has been shown to cause a disease on cotton plant, known as stigmatomycosis, "Internal Boll Rot," or "Cotton Staining (Ashby and Nowell 1926). This lead to incredible annual losses in cotton-growing regions. The seeds are only infected when a mechanical injury occurs since the fungus itself is unable to penetrate the outer cells of the plant. The most common mode of infection is through injection of the spores by insects into the tissue of the plant. The characteristic needle-shaped spores of *A. gossypii* are especially well adapted for this mode of infection. Infection results either in premature dropping of the bolls, or in a drying out of those which remain on the plant (Pearson 1947). The fungus infects the developing lint fibers which become dirty-yellowish in color and mat onto the seeds. This yellow pigment was later identified as being riboflavin. In addition to cotton, other important economic crop-plants such as coffee, citrus fruits, tomatoes, and various leguminous are also prone to be infected by *A. gossypii*. When infection occurs in leguminous plants, the disease is generally termed "Yeast Spot." Adequate control of the *Ashbya* spore transmitting insects has solved this problem (Pridham and Raper 1950).

Of particular biotechnological interest is the fact that *A. gossypii* is a natural overproducer of riboflavin (vitamin B2) and is together with *Candida famata* and *Bacillus subtilis* in use for industrial riboflavin production (Bacher et al. 1983).

Several genetic properties of *A. gossypii* make it a very attractive experimental system, such as one of the smallest eukaryotic genomes, haploid nuclei, growth on defined media and efficient gene targeting since homologous recombination is highly efficient (Steiner et al. 1995), which allows PCR-based gene targeting, (Alberti-Segui et al. 2001; Ayad-Durieux et al. 2000; Knechtle et al. 2003; Steiner et al. 1995; Wendland et al. 2000; Wendland and Philippsen 2001; Wright and Philippsen 1991). The degree of synteny (conservation of gene order) between *A. gossypii* and *Saccharomyces cerevisiae* genomes is high, which facilitates

the assignment of potential functions to *A. gossypii* genes (Altmann-Johl and Philippsen 1996; Wendland and Philippsen 2000), autonomously replicating sequences (ARS) of *S. cerevisiae* are functional in *A. gossypii*, permitting construction of replicative vectors, e.g. for complementation experiments (Steiner et al. 1995; Wright and Philippsen 1991).

1.2.1 Taxonomy

Finding a consensus concerning *A. gossypii* taxonomy it's not a very straightforward task since it appears to possess characteristics of both phycomycetous and ascomycetous fungi. Its anomalous morphology represents a difficulty in establishing a classification and consequently its phylogenetic relationships. In other words, the fact that *A. gossypii* grows as a hyphae shape instead of a yeast form like in Nematospora species, and considering the sporiferous sacs of these organisms as sporangia that are very similar to the spores of Nematospora is somehow strange. Guilliermond (1927) suggested that the organism should be assigned to the Hemiascomycetes but gave to it a new genus name, *Ashbya*. This suggestion was based on his observations of spore formation at the expense of a sporoplasm, the presence of an epiplasm, even numbers of spores, and two mitoses of the nuclei prior to spore formation. He considered the fruiting body of *A. gossypii* as intermediate between a sporangium and an ascus because of its multinucleate nature. Fragoso and Ciferri (1928) considered the organism to be a Saccharomycete. It would thus appear that taxonomists generally agree upon placing the organism in the order Endomycetales of the Hemiascomycetes.

The uni nucleate spores of *A. gossypii* have a haploid genome and are similar in cell wall carbohydrate and di-tyrosine content to *S. cerevisiae* ascospores (Prillinger et al. 1997) also taking into account the phylogenetic studies based on partial rRNA sequences (Kurtzman 1995), it was proposed that *A. gossypii* should be placed within the family *Saccharomycetaceae.*

1.2.2 *A. gossypii* life cycle

A. gossypii was first described as a 'filamentous yeast' in 1950 by Mickelson. *A. gossypii* grows in hyphal filaments for most of its life cycle, and only has single cell stages during spore germination and after fragmentation of the mycelium prior to sporulation. *A. gossypii* spores are needle-shaped being attached to each other by a string (Figure 1.1, f). In contrast to most filamentous fungi, *A. gossypii* exhibits a very simple life cycle, depicted in Figure 1.1. In the initial phase of the germination, the central region of the spore swollens in an isotropic

manner and forms a germ bubble (Figure 1.1, a). This is the only stage in which isotropic growth is observed in *A. gossypii* development (Knechtle et al. 2006). This growth phase can last up to 8 hours, and up to 3 rounds of nuclear divisions can occur. In *A. gossypii* hyphal cells, multiple nuclei reside in one cytoplasm and divide asynchronously without cell division (Gladfelter 2006; Gladfelter et al. 2006b). Next, a first germ tube emerges from the germ bubble perpendicular to the axis of the spore (Figure 1.1, b). A bipolar germination pattern is observed as the second germ tube grows opposite to the first tube (Figure 1.1, c). New hyphal tips are formed by lateral branching as hyphal tubes elongate (Figure 1.1, d). When a hyphal tip has formed, polarized growth can continue incessantly under favorable growth conditions. This generates new mycelium. Following hyphal maturation, branching happens at the hyphal tip in a dichotomous manner, producing Y-shaped hyphal filaments (Ayad-Durieux et al. 2000; Wendland and Philippsen 2000) in a continuous manner (Figure 1.1, e). The life cycle ends with the production of new spores. Sporulation occurs spontaneously on solid medium in the older parts of the mycelium. At this stage, a drastic enlargement of several segments in close proximity to the tip is observed. Spores are formed within these enlarged regions that can be described as sporangia. Hyphal tubes fragment at septal sites and form single-celled sporangia that contain the endospores. Spores are arranged in bundles. In *A. gossypii*, the secondary metabolite riboflavin is only produced when a culture is old and growth has ceased. Once growth has ceased, *A. gossypii* enters the sporulation phase, and it has been observed that riboflavin has a protective effect on spores against ultra violet light (Stahmann et al. 2001). In *A. nidulans*, a link between the production of a secondary metabolite (sterigmatocystin) and sporulation has also been observed (Hicks et al. 1997). *A. gossypii* is always haploid and it is not yet known if these spores are the products of meiotic or mitotic events. These spores are mononucleated and as soon as germination occurs, also nuclear division is happening, leading to hyphae which are filled with hundreds of nuclei, present in one common cytoplasm (Brachat et al. 2003).

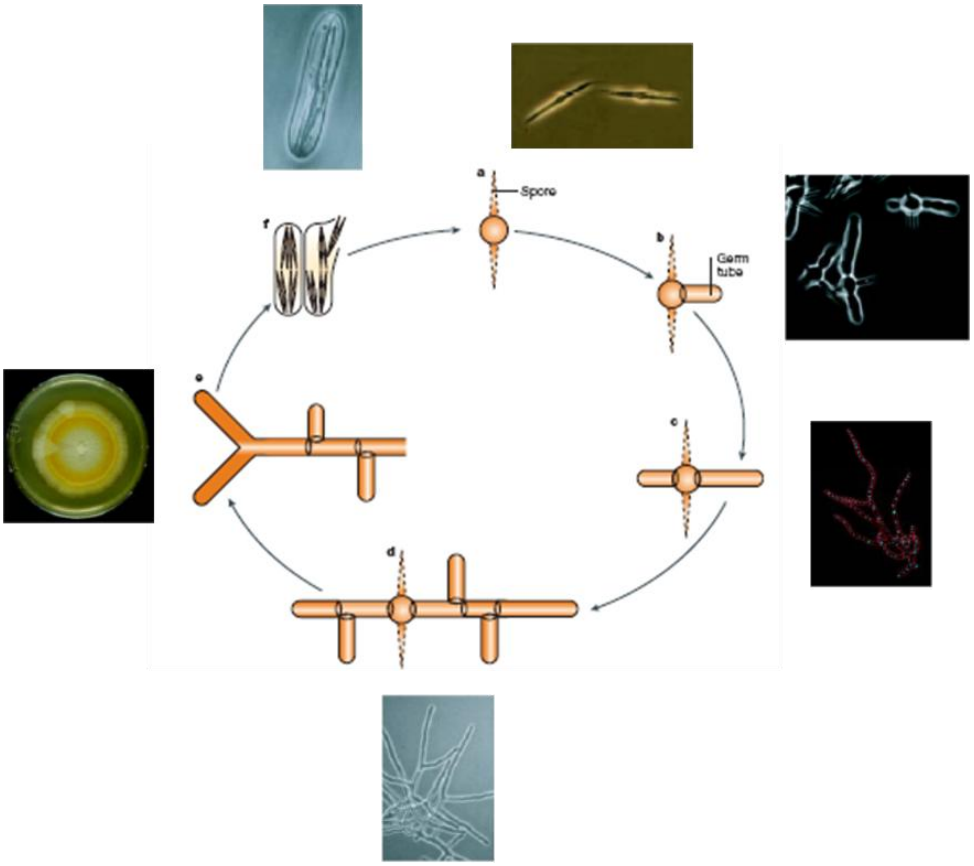


Figure 1.1 – Life cycle of *Ashbya gossypii*. Characteristic growth stages in *A. gossypii* development are (**a**) the isotropic growth phase during germination, (**b**) germ tube formation, (**c**) bipolar branching pattern, (**d**) juvenile mycelium, (**e**) dichotomous tip branching and (**f**) sporulation Riboflavin overproduction is responsible for the yellow pigmentation of the colony. Adapted from (Wendland and Walther 2005).

1.2.3 Genome organization

The genome of *A. gossypii* is available since 2004 (Dietrich 2004) and is one of the best-annotated eukaryotic genome sequences. The *A. gossypii* genome sequence was used to re-analyse the genome annotation of *S. cerevisiae* and, as was concluded in a similar study using the genome sequence of *Kluyveromyces waltii,* provides strong evidence for an ancient genome duplication in the *Saccharomyces* lineage (Brachat et al. 2003; Dietrich 2004; Kellis et al. 2004).

The 9.2 Mb genome of the filamentous fungus *A. gossypii* consists of seven chromosomes that encode for 4718 protein, 194 tRNA genes, at least 60 small RNA genes, and 40-50 copies of rRNA genes. With respect to both, the size and the number of genes, this represents the smallest known genome of a free-living eukaryote. This is much smaller than the estimated sizes for other filamentous fungal genomes that are in the range of 20-50Mb, encoding 9000–13,000 genes (Chavez et al. 2001; Galagan et al. 2003; Kupfer et al. 1997;

Machida 2002; Osiewacz and Ridder 1991). Surprisingly, it is also 30% smaller than the *S. cerevisiae* genome (13Mb including rDNA). Over 95% of the *A. gossypii* open reading frames encode proteins with homology to *S. cerevisiae* proteins (Table 1.2). In addition, 90% of *A. gossypii* genes show both, homology and a particular pattern of synteny (conservation of gene order), with the genome of budding yeast.

Table 1.2 – Comparison of *A. gossypii* and *S. cerevisiae* genomes (Brachat et al. 2003)

Feature	***Ashbya gossypii***	***Saccharomyces cerevisiae***
Genome size	8.8 Mb (+rDNA repeats)	12.1 Mb (+rDNA repeats)
Number of chromosomes	7	16
G-C content	52%	38%
Number of genes	4.718	5.570
Number of introns	221	~250
% of genes with homologues in *S. cerevisiae*	95%	100%
Protein coding- sequences	80%	~70%

One important difference between *S. cerevisiae* and *A. gossypii* is the complete lack of transposons in *A. gossypii*; their absence is likely to have prevented extensive genome rearrangements and explains the long blocks of synteny. The number of protein-coding genes is similar to the 4824 genes found in *Schizosaccharomyces pombe* (Wood et al. 2002), and much less than the 6000 ORFs found in *S. cerevisiae* (Goffeau et al. 1996) suggesting that this may be close to the minimum number of genes needed by a free-living fungus. The presence of only 221 introns in the entire *A. gossypii* genome, many at identical positions in *S. cerevisiae* homologs, contributes to the compact nature of this genome. Still, for 95% of the protein-coding sequences of *A. gossypii*, there are the corresponding homologs in the *S. cerevisiae* genome, the majority (4281 ORFs) at syntenic locations. The annotation of the genome sequence revealed that *A. gossypii* has a gene set very similar to that of *S. cerevisiae*, and this allowed the reconstruction of the evolutionary history of both organisms (Dietrich 2004). *A. gossypii* and *S. cerevisiae* diverged more than 100 million years ago, and their genomes differ substantially in GC content (Table 1.2; 52% for *A. gossypii* and 38% for *S. cerevisiae*) a fact that could reflect the different temperatures in their respective habitats

(Ashby and Nowell 1926). *A. gossypii* shares a common ancestor with *S. cerevisiae* before the duplication of the budding yeast genome (Dietrich 2004; Kellis et al. 2004). A speciation event, probably involving translocations and an accompanying change in chromosome number, generated the precursors of *A. gossypii* and *S. cerevisiae*. At some later time, a genome duplication in the *S. cerevisiae* precursor opened new possibilities for functional divergence not available for the evolution of *A. gossypii*. The duplication event created 5000 twin ORFs in the duplicated *S. cerevisiae* genome. Unlike *S. cerevisiae*, gene duplications are rare in *A. gossypii* and only 50 of their 4700 protein-coding genes are encoded in tandem gene duplications or in triplications (Dietrich 2004).

The Ashbya Genome Database (AGD) is a comprehensive online source of information covering genes from the filamentous fungus *A. gossypii*. The database content is based upon comparative genome annotation between *A. gossypii* and the closely related budding yeast *S. cerevisiae* taking both sequence similarity and synteny into account (Gattiker et al. 2007; Hermida et al. 2005). Comparison of the gene sequences of both organisms, resulted in the discovery of 46 novel ORFs and the identification of 72 putative annotation errors in the *S. cerevisiae* genome (Brachat et al. 2003).

The degree of similarity between *S. cerevisiae* and *A. gossypii* is not reflected by the obvious differences in their biology. Both species display distinct morphologies as well as growth and differentiation properties. *A. gossypii* grows as multinucleated hyphae in the subtropics, preferably on plants (Stahmann et al. 1994) and *S. cerevisiae* proliferates as single cells, is found in nature associated with sugar-containing fruits, and uses trehalose and glycogen as storage compounds (Thevelein 1984). On the other hand, *S. cerevisiae* genes that are absent from *A. gossypii* might be important for growth differences. One such gene is *CTS1,* which encodes a chitinase in yeast (Colman-Lerner et al. 2001). In *S. cerevisiae*, this protein is required for mother–daughter cell separation/cytokinesis. In the *S. cerevisiae* genome two chitinase genes occur, CTS1 and CTS2. Deletion of CTS1 results in a cell separation defect generating chains of non- separated cells (Kuranda and Robbins 1991). *A. gossypii* represents a unique case in that its compact genome contains only a single chitinase gene AgCTS2, which belongs to the family 18 of glycosidic hydrolases. The presence of only one chitinase,in part,maybe explained by the filamentous growth mode of *A. gossypii* in contrast to *S. cerevisiae*, which makes a chitinase activity for mother–daughter cell separation superfluous.

In the case of *Ashbya*, for example, there is no evidence for sexual reproduction although its genome contains the relevant set of genes involved in yeast meiosis and spore development (Brachat et al. 2003). Besides the differences in morphogenesis also significantly different controls of nuclear migration seem to operate in both systems.

Currently, there is no sexuall cycle known, although similar to *Candida albicans*, homologs of meiotic genes can readily be found in the *A. gossypii* genome sequence. In both *C. albicans* and *A. gossypii,* the knowledge of a complete sexual cycle is unknown. *A. gossypii* sequenced reference strain contains three loci, all bearing MATα information (Dietrich 2004; Wendland and Walther 2005). This *A. gossypii* strain is able to sporulate. These spores are uni- nucleate and contain a haploid genome. The nature of these spores and a potentially homothallic life style have not been clarified in *A. gossypii*. The identification of a mating-type locus in *A. gossypii* and the assignment of the wild type as a MATα strain enables a more detailed analysis of whether *A. gossypii* spores are of sexual or asexual origin. Further analyses of the genome sequence revealed, for example, the presence of homologues of sporulation- specific *S. cerevisiae* genes such as the initiator of meiosis genes, *IME1* and *IME2*, which might also indicate the presence of a sexual cycle in *A. gossypii* (Wendland and Walther 2005).

1.3 Review on *A. gossypii* physiology

The composition of the medium used for cultivation of micro-organisms is directly reflected in their physiological phenotype and their fermentation performance. For this reason, the successful development of strains for large scale industrial production of heterologous proteins (Macauley-Patrick et al. 2005; Porro and Mattanovich 2004) and low-value fuels, chemicals and materials (Chotani et al. 2000; Ostergaard et al. 2000) requires that the composition of cultivation media in various steps of strain development to be reconsidered. Generally, microorganisms grow more vigorously in rich media than in mineral media, because rich media contain biosynthetic precursors that can be channeled directly into anabolic pathways, reducing the need to produce biosynthetic precursors and saving metabolic energy.

Herbert (1961) summarised this in the early 60s in this way: ‘‘There are few characteristics of microorganisms which are so directly and markedly affected by the environment as their chemical composition. So much is this the case that it is virtually meaningless to speak of the chemical composition of a microorganism without at the same time specifying the environmental conditions that produced it.’’ One of the most important parameters that influence cell composition and performance is the availability of nutrients. In fact, restriction of specific nutrients is used in many biotechnological processes to induce and optimize microbial product formation. Good examples are the use of phosphate-limited growth conditions to

enhance production of certain antibiotics or that of citric acid by cultivating the culture under Fe^{2+}, Mn^{2+} and/or Zn-limited growth conditions (Calam 1986).

The successful production of a fungal metabolite requires a detailed knowledge of the growth characteristics and the physiology of the fungus in question. Not only does the production of different metabolites require different physiological conditions but also each fungus is unique in its anatomical, morphological and physiological development. Thus, for each fermentation, the precise physiological conditions and the correct stage of development must be established for maximal product formation.

Like all fungi, filamentous fungi are heterotrophic. This means that they require organic compounds as a source of carbon and energy. A few exceptional reports indicate that filamentous fungi can fix carbon dioxide. Mirocha and De Vay (1971) reported that *Fusarium sp*. and *Cephalosporium sp*. not only fix carbon dioxide but also grow on an inorganic salts medium without added carbon. Organic compounds supporting most growth are usually sugars (e.g., D-glucose, D-fructose, sucrose) which are rapidly taken up. Polysaccharides, amino acids, lipids, organic acids, proteins, alcohols and hydrocarbons are also used. A small amount of exogenous carbon may be required to maintain the fungus even when it is not growing. Carter et al. (1971) estimated that at zero growth rate *Apergillus nidulans* consumed 0.029 g of glucose per gram of fungal biomass per hour.

Fungal growth depends on various important factors, namely, inoculum development, sporulation, pH, nitrogen source and carbon source.

The filamentous hemiascomycete *A. gossypii* is a natural producer of riboflavin (Demain 1972) that has been used for industrial riboflavin production. Even though its wide applicability, some basic information regarding biological and physiological characterization of *Ashbya* is missing or unclear. In the following section and in chapter 2, some aspects of *A. gossypii* physiology will be approached. Numerous studies have been carried out to improve riboflavin production, however, a survey of the literature makes it clear that fundamental aspects of *A. gossypii* physiology need a deeper analysis and clarification.

1.3.1 Nitrogen sources

Nitrogen is an essential requirement for growth, and fungi are able to use a wide variety of compounds as nitrogen sources.

Nitrogen may be supplied as ammonia, as nitrate or in organic compounds, such as amino acids or proteins, beet or cane molasses, corn-steep liquor, whey powder, soy flour, yeast extract and others are used as industrial raw materials, rich in nitrogen.

The nitrogen requirements of *A. gossypii* have been investigated quite extensively. Farries and Bell (1930) found that potassium nitrate and ammonium salts were not utilize as nitrogen sources. Their findings were confirmed by Buston et al. (1938) who observed no utilization of the ammonium salts of sulfuric, nitric, hydrochloric, tartaric, lactic, or pyruvic acids in defined media containing inositol and biotin. When these media were supplemented with l-alanine or l-aspartic acid, moderate growth was attained. When peptone or casein were added to the medium, growth was obtained with some of these supplements. With media containing myo-inositol and biotin, *A. gossypii* was capable of utilizing simple mixtures of amino acids, asparagine, or ammonium aspartate as nitrogen sources.

Extensive studies of nitrogen metabolism and its control have been carried out in three fungi, *Neurospora crassa*, *Aspergilus nidulans*, and *S. cerevisiae*. Although certain compounds, particularly ammonia, glutamate, and glutamine, are favored nitrogen sources, these fungi are capable of utilizing many diverse secondary sources, including nitrate, nitrite, purins, proteins, numerous amino acids, acetamide, and even acrylamide. The use of these secondary nitrogen sources invariably requires the synthesis of catabolic enzymes or, in some cases, an activation of previously existing enzymes. De novo synthesis of many of the nitrogen-regulated enzymes requires that two conditions are met. First, there must be a lifting of nitrogen catabolite repression (also called amonium repression); second, in many cases, specific induction of the enzymes of a particular catabolic pathway by a substrate or intermediate of the pathway must also occur. The majority of the available information and evidence concerning the regulation of nitrogen metabolism in the above fungi is derived from genetic analysis of various structural and control mutants (Marzluf 1981). However, a survey in literature reports about nitrogen metabolism regulation in *A. gossypii* show that this information is missing or very scarce. In chapter 2 a comparison between four *A. gossypii* strains will be described taking into account the nitrogen necessities.

1.3.2 Carbon sources

The carbon requirements of *A. gossypii* have been studied by several investigators. Marsh (1926) observed that the organism was unable to attack potato starch in defined medium, however Farries and Bell (1930) have indicated that the organism may attack soluble starch only weakly. Glucose, fructose, and sucrose have been shown to serve as adequate carbon sources. Maltose and cellobiose are assimilated less readily. Rhamnose, arabinose, xylose, galactose, lactose, and inulin, however, do not serve as adequate carbon sources in peptone or defined media (Farries and Bell 1930). Marsh (1926) and Pearson (1947) have reported that the organism is unable to attack cellulose. Nowadays, with the genomic sequence

available, its rather straight forward to explain some of these results, i.e. the fact that *A. gossypii* is not able to grow on cellulose or lactose its simply because the genes coding for the enzymes responsible for the metabolism of these sugars are absent in the genome of this fungus. Stelling-Dekker (1931) has reported that *A. gossypii* is unable to ferment glucose, fructose, mannose, galactose, sucrose, maltose, or lactose, when measured in terms of gas production. Ethyl alcohol was shown to support slight growth when employed as a substrate. Farries and Bell (1930) observed traces of ethyl alcohol in certain media in which *A. gossypii* has been cultivated. According to Mickelson and Schuler (1953) the end products of glucose metabolism of *A. gossypii* are cells and carbon dioxide. Though *A. gossypii* has primarily an aerobic metabolism, it is able also to ferment glucose under anaerobic conditions (Mickelson 1950). Under aerobic conditions the end products of glucose oxidation are primarily carbon dioxide and water. However, small amounts of ethyl alcohol and traces of acetic acid were found when the medium was analyzed during the course of the fermentation. When glucose was added and the reaction allowed to go to completion in phosphate buffer, approximately 2 moles of carbon dioxide resulted from each mole of glucose consumed, which approaches the theoretical yield for an alcohol fermentation. Apparently the *A. gossypii* fermentation is a typical yeast alcoholic fermentation of glucose, even under aerobic conditions, but because of the strong aerobic system present, the alcohol is oxidized completely to carbon dioxide as soon as the glucose is exhausted in the medium. No evidence of a fermentative reaction was noted with suspensions from 24-hour cultures *of A. gossypii* under aerobic conditions (Mickelson 1950).

Mickelson (1950) suggested that a major portion of the glucose oxidized to carbon dioxide proceeded through acetic acid. The strongest supports for a tricarboxylic acid cycle type of mechanism are the apparent participation of α-ketoglutaric and citric acids and the lack of evidence for the C2 to C4 condensation.

In Chapter 2 the assimilation of some C-sources by four *A. gossypii* strains will be presented and discussed in the light of the information available from the *A. gossypii* genome sequencing.

1.3.3 Effect of pH

The pH of the medium is a very important but often neglected environmental factor. It can profoundly influence the parameters under study. The pH of a solution is a measure of the concentration of H^+ ions. Different points on a pH–growth plot may be interpreted in terms of effects on transport of nutrients, nutrient solubilities, enzyme reactions or surface phenomena. Fungi can grow over a wide range of pH. Most tolerate a pH range from 4 to 9 but grow and sporulate maximally near neutral pH (Cochrane 1958). The composition of the medium can

affect the initial pH and the extent and direction of pH drifts during growth of the fungus. Poorly buffered media containing ammonium salts are likely to become more acidic during growth, while media containing nitrate are likely to become alkaline. Minimizing pH drifts during growth is a desirable objective that is often difficult to achieve. The high concentrations of ions such as phosphate that are required to achieve some measure of pH stability often appreciably influence the biological activity being measured (e.g., growth and enzyme activity). The culture pH influences the concentration of dissolved bicarbonate formed from gaseous carbon dioxide. In this way, the prevailing pH and buffer capacity of the culture medium can influence fungal growth and product formation.

Tanner (1949) and later Mickelson and Schuler (1953) have observed that in the case of *A. gossypii*, when the initial pH was between pH 4.5 and 5.5, there was a good and rapid multiplication, but a decrease on the amount of riboflavin produced. Media initially adjusted to pH 4.0 gave little growth or riboflavin. The best yields of riboflavin were obtained when the initial pH of the medium was above pH 5.5, and preferably in the range of pH 6.0 to 7.0. A typical *A. gossypii* riboflavin production is comprised of two rather distinct phases. In the first, glucose is consumed and the medium becomes acid, generally reaching about pH 4.7 with an occasional minimum of pH 4.5. This change occurs within the first 24 to 36 hours. When glucose consumption is substantially complete, there is a gradual rise to an alkaline reaction occasionally reaching pH 8.5. Only negligible amounts of riboflavin appear in the medium before the carbohydrate is metabolized, the bulk of it being formed during the second phase in which a neutral or alkaline reaction develops (Mickelson and Schuler 1953; Tanner 1949).

1.3.4 Riboflavin production

Riboflavin is a water-soluble vitamin produced by plants and many microorganisms. However, humans and animals lack this capability and they must therefore obtain this essential nutrient from the diet or as a supplement. Riboflavin is present in the meat, egg yolk, fortified cereals and green vegetables, in addition to dairy products, which contribute most significantly to riboflavin intake (Cooperman and Lopez 1991). Because of its intense yellow color, riboflavin it is also used in small amounts as a coloring agent (E-101) in foods such as ice cream, yogurt and sauces, and in medical detection tests. Riboflavin deficiency (ariboflavinosis) mainly manifests itself clinically in the mucocutaneous surfaces of the mouth, through the occurrence of cracks at the corners, and inflammation of the lips and tongue (Baku and Dickerson 1996), include sore throat, hyperemia, edema of oral and mucous membranes (Wilson 1983). Riboflavin deficiency is also associated with vision deterioration and growth failure. Furthermore, riboflavin is used as a treatment for Parkinson's disease

(Coimbra and Junqueira 2003), for migraine (Krymchantowski et al. 2002) and malaria (Akompong et al. 2000). To avoid deficiency symptoms, a nutritional requirement of 0.3±1.8 mg day^{-1} for humans and 1±4 $mgkg^{-1}$ diet for animals is recommended (Eggersdorfer and Adam 1996) and sufficient amounts of riboflavin need to be ingested regularly since the body is unable to store the vitamin. When ingested in excess, riboflavin is harmless, because of efficient excretion via urine.

In order to be functional, riboflavin must be biochemically transformed into the flavin coenzymes such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Figure 1.2). This occurs when riboflavin is phosphorylated. These flavin coenzymes act as prosthetic groups for flavoprotein enzymes (Hoppel and Tandler 1990) acting as hydrogen carriers in biological redox reactions involving enzymes such as NADH dehydrogenase (Massey 2000), they also have a very important role in metabolism, being involved in oxidative decarboxylations as well as in the respiratory chain, lipid metabolism, the cytochrome P-450 system, and drug metabolism (Rivlin 2002).

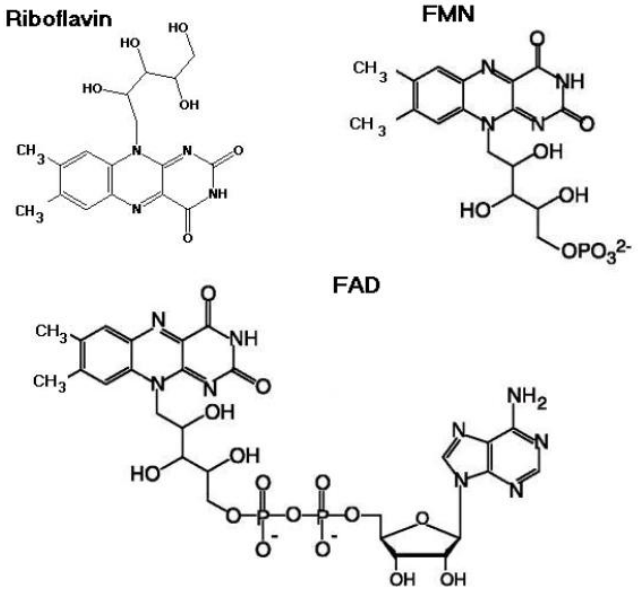


Figure 1.2 – Different forms of riboflavin.

Riboflavin also participates in other, nonredox processes, such as protecting DNA against UV light, light sensing, phototropism, circadian time keeping, and bioluminescence. Besides

having a pivotal biological function as a component of coenzymes, riboflavin appears a promising antitumoral agent, but the underlying molecular mechanism remains unclear (de Souza et al. 2006).

A. gossypii overproduces riboflavin as a detoxifying and protective mechanism during the late growth phase, when the maximum amount of mycelial has been formed (Stahmann et al. 2001). Thus, in terms of riboflavin production, two stages can be differentiated in an *A. gossypii* culture: a trophic phase when riboflavin production is minimal and the growth rate increases, and a productive phase when the growth rate decreases and riboflavin is overproduced (Stahmann et al. 2001). Many physiological and morphological changes occur during the shift from the trophic to the productive phase (Philippsen et al. 2005; Stahmann et al. 2001), but the mechanisms triggering the transition are not fully understood. The productive phase is associated with a characteristic intense yellow color of the mycelia, due to the accumulation of the vitamin in the vacuolar compartment (Forster et al. 1999).

Since the first quantitative report numerous studies have been carried out to overproduce riboflavin (Wickerham et al. 1946). Fermentative production of riboflavin is carried out in submerged culture. Factors such as the microbial strain, carbon source, minerals, and pH affect the fermentative production of riboflavin. Various researchers have optimized these factors to obtain the maximum yield of riboflavin. Different carbon sources like palm oil (Park et al. 2004), corn steep liquor (CSL), glucose (Horiuchi and Hiraga 1999), molasses (Pujari and Chandra 2000) and whey (Ertrk et al. 1998) have been tested for riboflavin production.

In early studies, some defined media were established using Tween 80 and purine, and 145 mg l^{-1} and later 370 mg l^{-1} of riboflavin were produced in these media (Goodman and Ferrera 1954; Smith et al. 1961). Many media were then developed, which contained glucose, vitamins, amino acids and mineral salts. In these markedly improved media, the production of riboflavin reached 1 g l^{-1}. It was found that glycine was an important component and Tween 80 served to prevent mycelium lysis and as stimulator for riboflavin overproduction. Apart from these media, more complex media for industrial application were studied, and using soybean oil as a carbon source and collagenous protein and CSL as nitrogen sources, yields of 5 g l^{-1} were obtained (Hanson 1967). The same amount of riboflavin was produced using other newly developed media, which included 3% bone fat and 2% soybean oil as carbon sources, supplemented with additional soybean oil (Szczesniak et al. 1971). Peptone and gelatin were investigated as complex nitrogen sources (Chiao et al. 1960), and some waste proteins of animal origin, such as skin and bone glues were also tested (Szczesniak et al. 1971b). Skin and bone glues contain several important amino acids for riboflavin production, such as glycine (about 20%) and threonine. These amino acids were found to strongly

stimulate riboflavin synthesis in *A. gossypii* (Chiao et al. 1960; Plaut 1961), and in medium containing 3% skin glue as a nitrogen source, and 4% gelatin lard as a carbon source, to which 2.5% gelatin lard was added, the amount of riboflavin produced was higher than 5.5 g l^{-1} (Szczesniak et al. 1971b).

A. gossypii was the first organism used in industrial riboflavin production. Many efforts have been made to improve riboflavin productivity in *A. gossypii*. Metabolic engineering by gene manipulation has become more readily available since the genome sequencing of *A. gossypii* has been completed (Dietrich et al. 2004). Riboflavin is produced from fatty acids through the glyoxylate cycle, gluconeogenesis, the pentose phosphate pathway and the purine pathways.

On a medium containing plant oil as the carbon source, isocitrate lyase (ICL), which is part of the glyoxylate cycle that catalyzes the cleavage of isocitrate to succinate and glyoxylate, plays a key role in growth and riboflavin production in *A. gossypii* (Schmidt et al. 1996a). Itaconate and oxalate strongly inhibit of ICL activity (Schmidt et al. 1996b) and were used as anti-metabolites for the isolation of a riboflavin overproducing mutant. An itaconate-resistant mutant produced a 25-fold higher yield of riboflavin than the wild type on soybean oil medium and had 15% higher ICL activity. A mutant naturally isolated from spores grown on an oxalate-containing medium produced a 5-fold higher quantity of riboflavin in an optimized medium compared to the wild type (Sugimoto et al. 2011).

The first example of improved riboflavin production in *A. gossypii* by gene manipulation was reported by Monschau et al. (1998). Glycine is one of the most important precursors for riboflavin production in *A. gossypii*. A further example of increased riboflavin formation was shown by overexpression of *AgGLY*1, encoding threonine aldolase (Monschau et al. 1998). A greater increase in riboflavin production was obtained by feeding threonine than by feeding glycine, which was caused by a better uptake of threonine. Therefore, constitutive overexpression of GLY1 supplied glycine in the presence of threonine in *A. gossypii*, and riboflavin production improved. SHM2 encodes cytoplasmic serine hydroxymethyltransferase (SHMT), which catalyzes the conversion of glycine to serine using 5,10- methylene-tetrahydrofolate. Disruption of SHM2 directed a change in carbon flux by blocking the conversion of glycine to serine and produced 10-fold enhancement of riboflavin productivity in a glucose medium (Schlüpen et al. 2003). Similar to overexpression of GLY1, disruption of SHM2 led to accumulation of glycine in *A. gossypii*. Moreover, heterologous expression of yeast alanine: glyoxylate aminotransferase, converting glyoxylate to glycine, slightly enhanced riboflavin production in *A. gossypii* compared to wild type (Kato and Park 2006).

Another precursor for riboflavin biosynthesis is GTP and is supplied de novo through the purine biosynthetic pathway. Metabolic engineering of the purine biosynthetic pathway in *A. gossypii* by constitutive expression of phosphoribosyl pyrophosphate (PRPP)

amidotransferase (ADE4), which catalyzes the first step in the purine biosynthetic pathway, also improved riboflavin production (Jimenez et al. 2005).
Apart from metabolic factors, disruption of VMA1, which encodes vacuolar ATPase subunit A, led to the complete excretion of riboflavin in *A. gossypii* on glucose and soybean media in contrast to wild type, which accumulated riboflavin in its vacuolar compartment (Forster et al. 1999). Therefore, riboflavin production in the medium was improved by VMA1 disruption in *A. gossypii* compared to wild type.

1.4 Recombinant protein production by filamentous fungi

The first attempts to produce foreign proteins in filamentous fungi produced proteins of fungal origin (Fincham 1989). Native proteins such as α-amylase are often produced at very high levels e.g., 15-20 g l^{-1} in submerged bioprocesses; for example, *T. reesei* produces hydrolases in 100 grams per litre quantities (Schuster and Schmoll 2010; Saloheimo and Pakula 2012) and *A. niger* has been shown to excrete 25 g of glucoamylase per litre (Ward et al. 2006) only a few yeast species (*Pichia, Hansenula*) reach the gram-per-liter production level (Werten et al. 1999; Wyss et al. 1999). Thus, the potential of some species of filamentous fungi to secrete large quantities of biologically active proteins is considerable (Jeenes et al. 1991; Verdoes et al. 1995). Production of heterologous proteins by filamentous fungi has been extensively reviewed (e.g. Archer et al. 1994; Archer and Peberdy 1997; Gouka et al. 1997a; Jeenes et al. 1991; Keranen and Penttilä 1995; MacKenzie et al. 1993; Verdoes et al. 1995; Nevalainen et al. 2005; Wang et al. 2005; Meyer et al. 2011; Ward 2011). Nevertheless, some filamentous fungi, *Aspergilli*, in particular, have received considerable attention as viable alternatives in producing heterologous proteins (Punt et al. 2002) since they overcome some of the deficiencies of other recombinant systems such as hyperglycosylation in yeasts, absence of glycosylation, phosphorylation in bacteria, and expensive media requirements for animal cells. Glucoamylase, bovine chymosin, human lactoferrin, hen egg-white lysozyme, human interleukin-6, and thaumatin are some examples of proteins being produced in *Aspergillus* strains.

Starting from the early 1980s, the majority of recombinant proteins produced in yeasts were expressed using *S. cerevisiae* (Hitzeman et al. 1981). The choice was determined by the familiarity of molecular biologists with this yeast, together with the accumulated knowledge about its genetics and physiology. Furthermore, *S. cerevisiae* is an organism generally regarded as safe. Over time, the application of *S. cerevisiae* as a host for

heterologous production has shown a number of limitations such as low production of secreted proteins, limited number of signal sequences available, hyperglycosylation, retention and degradation of the recombinant product and, it being a Crabtree-positive yeast, low biomass yield. Disadvantages such as these have promoted a search for alternative hosts, trying to exploit the great biodiversity existing among fungi, and starting the development of expression systems.

Because of their ability to secrete large amounts of proteins into their culture media, native or recombinant enzymes are currently, mainly produced by *A. niger*, *A. oryzae*,*T. reesei*, *Mucor*, *Penicillium* and *Rhizopus* spp. but other strains are presently under development (Punt et al. 2002). Novozymes has used *Fusarium graminearum* as a host for heterologous proteins.

Preferred host strains are chosen from among those for which successful Generally Recognized as Safe (GRAS) and Food-Additive petitions at the Food and Drug Administration (FDA) have been filed. With the advent of molecular cloning in the mid-1970s, it became possible to produce foreign proteins in new hosts. The development of molecular techniques for the production of recombinant heterologous proteins in filamentous fungi has been laborious and has contrasted markedly with the success achieved in yeasts. The ability to introduce or delete genes remains difficult although some advances in transformation have been reported, e.g., restriction enzyme-mediated integration (Graf et al. 2008) and *Agrobacterium tumefaciens*-Ti plasmid mediated transformation (de Groot et al. 1998; Gouka et al. 1999). Most filamentous fungi are transformed by plasmids that integrate into the fungal genome, suggesting potentially superior long-term stability of the fungal transformants. For these reasons, filamentous fungi have tremendous potential as hosts of recombinant DNA. In recent years, great improvements have been achieved (Wiebe 2003). Several efficient transformation systems have been developed for a large number of fungal species. In addition to DNA-based methods, introduction of RNA-based methods such as antisense RNA, hammerhead ribozymes and RNA interference approaches have been found to be very useful for silencing particular genes in filamentous fungi (Fulci and Macino 2007; Hammond and Keller 2005; Mueller et al. 2006; Yamada et al. 2007). Detailed classical physiological and biochemical knowledge is available for many of the candidate hosts and molecular techniques, including genome sequencing and annotation strategies. These give very good support for the optimization of expression and secretion of recombinant proteins in filamentous fungi.

Productivity and secretion potential of filamentous fungi, which is in the range of 30 – 100 g l^{-1} for homologous enzymes like cellulases and amylases, is considered to be superior to any other system, but unfortunately could not be converted into corresponding yields for many

heterologous recombinant products (Schmidt 2004). As with other industrially exploited microbial hosts, filamentous fungi have gone through intricate strain improvement programs. In some cases, a fungal cell factory used for the expression of selected gene products is a high protein secreting mutant made by traditional random mutagenesis, for which the characteristics have further been modified by genetic engineering (Nevalainen et al. 2005).

The development of molecular tools suitable to manipulate *A. gossypii* opened the door to evaluate its potential as a production host. The transformation method for *A. gossypii* has been established using plasmid DNA carrying an autonomously replicating sequence (ARS) from *S. cerevisiae* (Wright and Philippsen 1991). The ARS elements of *S. cerevisiae* do not work in most fungi (Fincham 1989). *A. gossypii* is also able to integrate recombinant plasmids without the background nonhomologous recombination (Steiner et al. 1995). Other tools, including shuttle vectors, promoter and reporter constructs, as well as several selectable marker genes for dominant selection (antibiotic resistance) and strains auxotrophic for *LEU2* (leucine biosynthesis) and *THR4* (threonine biosynthesis) have been developed (Wendland and Walther 2005).

In chapter 3 evaluation of *A. gossypii* as a recombinant protein producer host will be described.

1.4.1 Strategies to improve heterologous protein production

The levels of heterologous proteins of mammalian, bacterial or plant origin secreted by fungal hosts are often only a fraction of the levels of native products these species are capable of, reaching a few tens of milligrams per liter of culture medium (Gouka et al. 1997a). Several factors that negatively affect the production levels of non-fungal proteins have been reported (Archer and Peberdy 1997; Punt et al. 1994), showing that the production can be limited at any level, i.e., low transcriptional levels, mRNA instability, inefficient translation, secretion, with possible limitations also at the post-translational level (i.e., inefficient translocation, folding, transport, processing, or secretion) (Broekhuijsen et al. 1993; Gouka et al. 1997a; Jeenes et al. 1994) and extracellular degradation (Archer and Peberdy 1997; Archer 2000; Gouka et al. 1997a; Punt et al. 1994; Punt et al. 2002). On the basis of limitations observed for the production of non-fungal proteins, different genetic strategies have been developed to surmount these problems (Archer et al. 1994; Nevalainen et al. 2005; Wang et al. 2005; Meyer 2008). Most of these strategies are similar to those for fungal proteins (Verdoes et al. 1995) and include, the introduction of multicopies of the protein gene (Archer et al. 1994), the use of strong promoters and efficient secretion signals (Gouka et al.

1997a; Moralejo et al. 1999) gene fusion with a gene encoding all or part of a well-expressed and secreted protein (Punt et al. 2002), optimization of the codon usage of the gene of interest according to the preferred codon-usage of the expression host (Roubos and van Peij 2008) and the construction of protease-deficient host strains (Mattern et al. 1992; Punt et al. 2002; Verdoes et al. 1995, Wang et al. 2008).

One of the most obvious reasons for the low yields was the abundant production of secreted proteases by most of the fungal host strains (Mattern et al. 1992; van den Hombergh et al. 1997), leading to proteolytic degradation of the protein secreted. Heterologous proteins are more prone to proteolysis than homologous proteins. Therefore, one solution to this intrinsic limitation was the development of improved protease deficient host strains (Mattern et al. 1992; van den Hombergh et al. 1997). Such strains have largely alleviated the problem of extracellular degradation. However, even in the best protease-deficient strains there are residual levels of protease activity, which for some proteins and under certain conditions might still result in a low protein yield. Various efforts have been made in both strain and fermentation process improvements to overcome this limitation. In addition to the secreted proteases, filamentous fungi may be subject to different degrees of lysis during the fermentation process, the extent of which may vary with other aspects of fungal physiology including morphology, such that intracellular proteases may be released which can attack the recombinant protein. To overcome or alleviate protease degradation problems using protease deficient mutant strains as hosts can be a solution to improve recombinant protein production. However, such strains are often so debilitated that they are not desirable for large-scale bioreactor applications. Bioprocess strategies have also been shown to be of great help (O'Donnell et al. 2001; Wang et al. 2003; Xu et al. 2000).

Another strategy has been to introduce multiple copies of the gene product of interest, expressed under a strong homologous gene promoter into a high-protein-secreting mutant strain. Foreign genes can be incorporated via plasmids into chromosomes of the filamentous fungi. Integration is relatively stable, sometimes as tandem repeats. As many as 100 copies of a gene have been observed. Integration of the incoming genetic material can be targeted to a particular gene locus, often encoding a major endogenous secreted protein such as cellobiohydrolase I in *T. reesei* and glucoamylase in *A. niger*. Potential benefits from integration by gene replacement include decreasing the preloading of protein in the secretory pathway to make room for the recombinant product and expressing the gene of interest from a locus that is naturally strongly expressed (Gouka et al. 1997).

A major improvement in the secretion of heterologous proteins can be achieved with the 'carrier' approach, where the heterologous protein is fused with a fungal protein that will act as a carrier for more efficient secretion. Gene fusion is the first choice in attempting to

produce non-fungal proteins in fungal hosts. The heterologous gene is fused to the 3'end of a highly expressed gene, of which the gene product is efficiently secreted. For example, Ward et al. (2006) expressed a gene-fusion comprising the genes encoding *A. niger* glucoamylase and bovine chymosin. Fusion resulted in higher levels of secreted bovine prochymosin (Ward et al. 1990), porcine pancreatic phospholipase A2 (Roberts et al. 1992), human interleukin-6 (Broekhuijsen et al. 1993; Contreras et al. 1991), hen egg-white lysozyme (Jeenes et al. 1993), and human lactoferrin (Ward et al. 1995). The increase varies from 5 to 1000-fold, depending on the protein, resulting in protein levels varying from 5 mg l^{-1} to 250 mg l^{-1}. Even higher levels, up to 1±2 g l^{-1}, were obtained for chymosin (Dunn-Coleman et al. 1991) and lactoferrin (Ward et al. 1995) when high-level-production strains were subjected to several rounds of mutagenesis. Usually, the *A. niger* or *A. awamori* glucoamylase (*glaA*) genes have been used as carriers. Besides *glaA* gene fusions, fusion constructs were made with *T. reesei* cellobiohydrolase I gene (Nyyssonen and Keranen 1995), resulting in more than 150-fold improvement of the production levels of antibody fragments. A fusion of *A. awamori* α-amylase with prochymosin was also successful (Korman et al. 1990). Shibuya et al. (1992) constructed an *A. oryzae* Taka-amylase/glucoamylase gene fusion to produce a fusion protein with both α-amylase and glucoamylase activities, up to levels of 0.5 g l^{-1}. Baron et al. (1992) showed that the carrier gene does not necessarily needs to be of fungal origin. Fusion of the human lysozyme gene to the 3' end of the bacterial *Streptoalloteichus hindustanus* phleomycin-resistance gene, provided with a synthetic secretion signal, resulted in a 10- to 150- fold increase in lysozyme protein levels in *Tolypocladium geodes*, as compared to non-fused lysozyme. In general, the N-terminal fungal protein is believed to serve as a carrier, improving the translocation of the protein into the endoplasmic reticulum (ER), to aid folding and to protect the heterologous protein from degradation (Gouka et al. 1997a). Within the secretory pathway, in most cases the fusion protein is cleaved, resulting in the secretion of separate proteins. Cleavage occurs either by autocatalytic processing of the heterologous protein (Ward et al. 1990), by an unknown fungal protease (Baron et al. 1992; Nyyssonen et al. 1993; Nyyssonen and Keranen 1995) or by a KEX2-like protease, for which a recognition site had been introduced specifically into the fusion protein (Broekhuijsen et al. 1993; Contreras et al. 1991; Ward et al. 1995).

Fungal promoters that are used for the expression of heterologous genes, can be divided into two groups (Saunders et al. 1989): promoters from housekeeping genes with a high level of expression; and inducible promoters. The most frequently used constitutive promoter is the *gpd*A promoter from *A. nidulans*, which is part of a gene encoding glyceraldehyde-3-phosphatedehydrogenase. This promoter is functional in several species, including *P. chrysogenum* (Kolar et al. 1988), *A. nidulans* (Punt et al. 1991) and *A. niger* (Archer et al.

1990). A disadvantage in using constitutively expressed housekeeping gene promoters is that the promoters are functional during growth and therefore unsuitable for expression of foreign proteins that might be toxic to the host cells. Well-known inducible promoters used in many applications are those of the cellobiohydrolase I (cbh1) from *T. reesei* (Harkki et al. 1991), glucoamylase A (glaA) from *A. niger* (Smith et al. 1990), Taka-amylase (amyA) from *A. oryzae* (Tsuchiya et al. 1992) and xylanase (exlA) from *A. awamori* (Gouka et al. 1996).

The selection of a strong promoter, such as that from cellobiohydrolase I of *T. reesei* and glucoamylase A of *A. niger*, can lead to saturation of the system, for example, with regard to insufficient supply of factors required for promoter regulation. Different fungi and genes seem to behave somewhat differently in this respect: the reported maximum number of the *T. reesei* cbh1 promoters supporting an increased yield of a recombinant product expressed under this promoter is three (Karhunen et al. 1993), whereas in *Aspergillus*, higher copy numbers have been reported to be effective in terms of improving gene product yields (Moralejo et al. 1999). However, regulation of the strong *A. niger* gla A promoter appears more simple, with AmyR as the main positively acting transcription factor and CreA as the major negatively acting factor (Kato 2005).

The constitutive housekeeping gpdA promoter and the *A. nidulans* alcA/ alcR system, inducible by ethanol, ethylamine and ketones (Flipphi et al. 2002), have also been successfully used for expression of heterologous gene products (Punt et al. 2002).

One particular difficulty with the filamentous fungi lies in their morphological form. Filamentous fungi are tip-growing systems which grow by apical elongation with a flow of cytoplasm toward the hyphal tip. With time, older hyphal become more vacuolated, and may become metabolically less active; thus, in a fungal culture in a submerged bioprocess, there is a range of hyphal compartment with different metabolic capability. The formation of these macroscopic morphologies can only descriptively be monitored but not controlled. To overcome this constraint and to optimize the morphology by rational genetic engineering, transcriptomics and functional genomics studies have recently been initiated aiming at the reconstruction of the morphogenetic machinery of *A. niger* (Meyer et al. 2008, 2009). Morphological form has been shown to be an important influence in the formation of a range of metabolites by the filamentous fungi. In the secretion of protein, Wosten et *al.* (1991) indicate that secretion is primarily associated with the apical tip region, since the growing hyphal tips are more porous, making it easier for the exoenzymes to pass through the cell wall (Peberdy 1994; Punt et al. 1994). *In vivo* monitoring of protein secretion on *A. niger*, was studied using a glucoamylase::GFP fusion protein to localise the site of secretion. The GFP fluorescence was mainly observed at the hyphal tip (Gordon et al. 2000; Lee et al. 1998) and showed that this approach is a promising tool to be used in the study of protein secretion.

A structure unique to filamentous fungi, the Spizenkörper (SPK), has been found in the growing hyphal tips. This is an aggregate of vesicles that have been postulated to act as a center for vesicle supply and to direct their transport to the plasma membrane (Gierz and Bartnicki-Garcia 2001). Therefore, factors that increase the number of active tips, such as the properly controlled fungal morphology, may improve the protein yield (Juge et al. 1998; Pluschkell et al. 1996). The apical localization of protein secretion has led to the suggestion of employing morphological mutants displaying an increased apical surface, i.e., hyperbranching mutants, as “supersecretion” strains (Lee et al. 1998). Moreover, hyperbranching strains often grow as compact pellets, which results in low-viscosity cultures and have additional technical advantages in the fermentation process.

However this is not always the case, for example, it was shown that *A. oryzae* hyphal branch frequency had little effect on specific amylase yield although a small increase in the total amount of amylase secreted was achieved from one highlybranched mutant (Bocking et al. 1999). Although an increased production of glucoamylase was measured for *A. oryzae* hyperbranching mutants when grown in stirred batch cultures, this was not the case when other culturing techniques were employed, and no correlation was found between tip density and protein (Bocking et al. 1999). On the other hand, a UV mutagenesis approach to obtain *Trichoderma viride* mutants with an increased production of extracellular cellulase in some cases resulted in the recovery of overproducing strains which had a concomitant hyperbranching phenotype (Farkas et al. 1981). Furthermore, a *Neurospora crassa* mutant in growth polarity, with cAMP-dependent protein kinase conditional mutation displayed an enlarged growth surface area (Bruno et al. 1996) and was also shown to secrete more protein than the wild-type strain (Lee et al. 1998). However, disruption of *myoA*, encoding a myosin I in *A. nidulans*, which also alters polarized growth, had a negative effect on the secretion levels of acid phosphatase (McGoldrick et al. 1995).

In *T.reesei*, secretion of a foreign protein, barley cysteine endopeptidase, has been shown to occur at the apical and subapical regions of the hyphae (Nykänen et al. 1997). On the other hand, cellobiohydrolase I (CBHI), calf chymosin and CBHI-chymosin fusion proteins can be found from secretory vesicles all over the mycelium in *T. reesei*. This suggests that secretion can also take place in the older parts of the mycelium (Nykänen 2002). This shows that further research is needed to establish the applicability of hyperbranching strains for production purposes.

At the post-translational level, glycosylation and protein folding processes largely influence structure and function of heterologous proteins. For some heterologous proteins, glycosylation is essential to obtain the proper conformation and high secretion levels and in such cases low yield of heterologous protein produced could be due to ineffective

glycosylation (Maras et al. 1999). A targeted modification of glycosylation processes might prove to be a powerful tool for use in improving heterologous gene expression in filamentous fungi. In molds, hyperglycosylation does not seem to occur and low-mannose side chains are formed (Elbein et al. 1985; Maras et al. 1999; Salovouri et al. 1987). Protein glycosylation processes in filamentous fungi are, as far as it is currently known, very similar to the mechanisms found in other eukaryotes, particularly *S. cerevisiae.* However, in contrast to *S. cerevisiae* where hypermannosylation frequently occurs, the glycosylation patterns in filamentous fungi are more similar to those found in higher eukaryotes (Maras et al. 1999; Nevalainen et al. 2005). The two main glycosylation processes common to eukaryotes involve *N*- and *O*-glycosylation, whereby oligosaccharides attach to the beta-amide moiety of asparagine residues and mainly to serine and threonine β-hydroxy groups resulting in the build up of the high mannose *N*-glycans characteristic of filamentous fungi and yeasts. *O*-glycosylation in fungi starts in the endoplasmic reticulum, and involves *O*-mannosylations resulting in the sequential build up of *O*-glucosyl. Although *O*-linked glycosylation in yeast is quite different from that in higher eukaryotes, *N*-linked glycosylation is more conserved. For many proteins that have pharmaceutical applications, *N*-glycosylation is necessary for stability and proper folding. In the production of recombinant human therapeutic proteins by filamentous fungi, it is extremely important to obtain the correct glycoform, otherwise an immune response may be induced in the patient being treated, reducing treatment efficacy. Implementation of strategies for increasing glycosylation in *Aspergillus* resulted in an approximately 20-fold increased production of the recombinant protein chymosin (van den Brink et al. 2006). In one case, a poorly used glycosylation site within the chymosin molecule was improved, resulting in much more efficient production of the glycosylated chymosin. In the second case, when the *N*-glycosylation site was located away from the native chymosin attached via a linker, a substantial increase in recombinant protein was observed (Ward 1989). As this was still insufficient for commercial purposes, a classical mutagenesis with nitrosoguanidine was performed, which led to another three- to five-fold improvement in chymosin production (Archer et al. 1994). A more detailed understanding of the mechanisms of post-translational modifications would be useful for optimizing production of heterologous proteins in filamentous fungi.

The availability of genomic data, combined with other methods including proteomics (de Oliveira and de Graaff 2011) and metabolomics, has and will continue to support strain development strategies for production of recombinant proteins through use of molecular methods for industrial fermentations. For example, comparative genomic studies among *Aspergillus* species suggest that *A. oryzae*, is enriched with genes which participate in the degradation of biomass and in primary and secondary metabolism (Kobayashi et al. 2007). Gene arrays, gene deletion and insertion strategies and other emerging molecular techniques

are of widespread application as a means to better exploit the mechanisms of industrial product formation, regulation, and secretion by filamentous fungi (Akao et al. 2002; Bautista et al. 2000; Moralejo et al. 2002; Ngiam et al. 2000; Sims et al. 2004; Zarrin et al. 2005, Gasser et al. 2007, Carvalho et al 2011).

1.4.2 Secretory pathway

The fact that fungi secrete enzymes into the culture medium has a clear advantage in producing proteins that might be toxic if they accumulate inside the cells. Secretion is also beneficial for the purification of the desired products, since there is no need to break the cells and to eliminate all the intracellular proteins, a process that is often tedious, reducing yield of recovery and expensive. Also the protein is not exposed to intracellular host proteases; and the metabolism of the fungus is not disturbed by the foreign protein.

Protein translocation to the secretory system (Figure 1.3) can be achieved by inserting a secretion signal sequence between the promoter and the heterologous gene. This sequence encodes a signal peptide with a length of 13-50 amino acids which targets the protein to the endoplasmic reticulum, and subsequently to the secretion pathway. In spite of lacking consensus sequences, signal sequence structures among different organisms are somewhat conserved and consist of three parts: a stretch of mainly positively charged amino acids at the N-terminus, followed by an uninterrupted region of hydrophobic amino acids, whilst the cleavage site for the signal peptidase, which removes the signal peptide after translocation into the endoplasmic reticulum, is located at the 3' end of the signal sequence (Schatz and Dobberstein 1996). As mentioned before, one special feature of protein secretion in filamentous fungi is that proteins are secreted mainly at the tips of growing hyphae (Punt et al. 1994). The molecular mechanism of protein secretion in fungi is assumed to be similar to the mechanisms found in other organisms (Jeenes et al. 1991) since many heterologous signal sequences from other organisms are functional in fungi.

The fungal secretory pathway can be a limiting factor in heterologous enzyme production. Studies on screening for mutant strains with altered secretion properties using green fluorescent protein as reporter (Gordon et al. 2000), elucidation of the role of secretion-related chaperones and foldases (Conesa et al. 2001; Kasuya et al. 1999; Saloheimo et al. 1999; Wang and Ward 2000), kinetic studies on protein secretion (Pakula et al. 2000, Arvas et al 2012), and the effects of hyphal branch frequency (Bocking et al. 1999) are examples of the work carried out to understand this complex process.

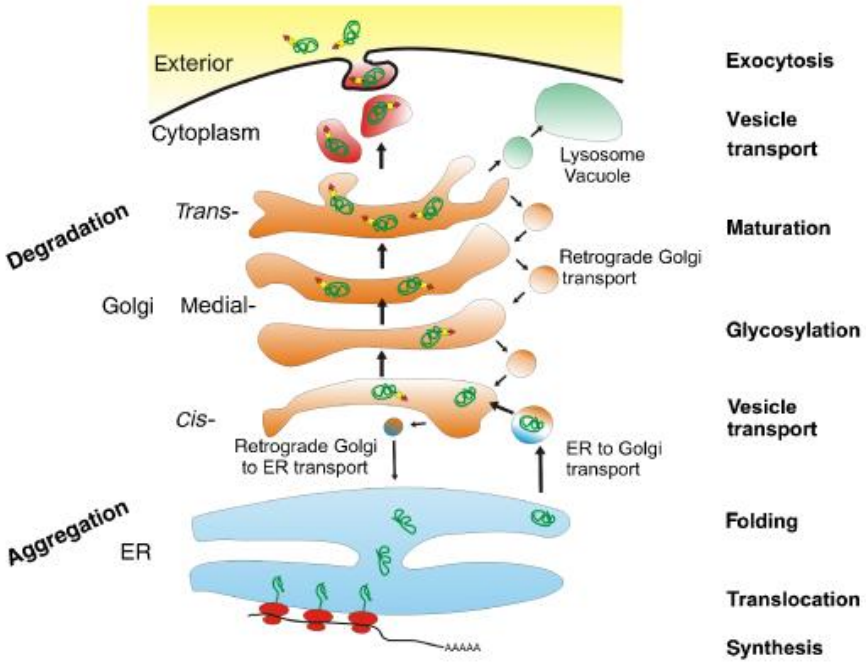


Figure 1.3 – Protein synthesis and secretion in eukaryotic cells. Pathways and key 'metabolic' processes of secreted proteins are indicated, including unproductive steps such as degradation or aggregation (Graf et al. 2009).

Secretory proteins begin their journey to the extracellular medium by entering the endoplasmic reticulum (ER). In the ER proteins are folded and can undergo distinct modifications such as glycosylation, disulfide bridge formation, phosphorylation, and subunit assembly (Peberdy 1994). Along with the protein folding processes, these modifications play a crucial role in determining the structure of many proteins. Folding of secreted proteins occurs in the lumen of the endoplasmic reticulum (ER) and this process is assisted *in vivo* by helper proteins named chaperones and foldases. A well studied ER resident protein is the chaperone protein BiP, encoded by *KAR2*. BiP is the only member of the stress-70 (hsp70) family of proteins that is located in the ER and is involved in several processes such as, protein translocation, protein folding, protein assembly and protein degradation (Bole et al. 1986; Dorner et al. 1987; Hurtley et al. 1989; Machamer et al. 1990). The tertiary structure of many extracellular proteins is stabilized by disulphide bridges. The correct formation of disulphide bridges is ensured by the protein-disulphide isomerase (pdi), an enzyme of the endoplasmic reticulum which catalyses the formation and breakage of disulphide bonds (Xiao et al. 2004). The first fungal protein disulphide isomerase has been cloned from *A. niger* (Jeenes et al. 1996). Proteolytic cleavage is another important type of post-translational modification occurring in the ER. The most common proteolytic modifications are the removal

of signal sequences by signal peptidases after translocation through the ER membrane (Dalbey and Von Heijne 1992) and endoproteolytic processing by endoproteases, such as KEX2. A schematic view of the fungal secretory pathway is given in Figure 1.3. Subsequently, proteins leave the ER packed in transport vesicles and head to the Golgi compartment, where additional modifications can take place such as further glycosylation and peptide processing. Finally, again packed in secretory vesicles, proteins are directed to the plasma membrane from where they are secreted. In some cases, the proteins will not reach the extracellular space, but are targeted to intracellular compartments such as the vacuole, either to become resident proteins or to undergo proteolytic degradation (Conesa et al. 2001).

1.4.3 Unfolded protein response (UPR)

Deficiencies in the protein folding capacity of the endoplasmic reticulum (ER) in eucaryotic cells lead to ER stress and trigger an intracellular signaling pathway, the unfolded protein response (UPR) (Bernales et al. 2006; van Anken 2005). UPR is a regulatory system that maintains the homeostasis of ER functions under stress conditions by detecting the presence of unfolded proteins (Schröder and Kaufman 2005). This causes the overproduction of ER resident chaperones and foldases to increase the protein folding capacity, as well as the up-regulation of ER-associated protein degradation (ERAD) by the proteasome to decrease the unfolded protein load of the ER, induction of phospholipid synthesis, attenuation of general translation and an antioxidant response (Schröder 2005b).

The UPR may be induced by several factors that include the expression of at least some heterologous proteins, especially at high yield, high level expression of some native proteins, or chemicals that adversely affect the folding environment within the ER. The transcriptional regulation of genes by unfolded secretory proteins has been described in *S. cerevisiae* (Travers et al. 2000), *A. niger* (Guillemette et al. 2007), *A. nidulans* (Sims et al. 2004), *P. pastoris* (Graf et al. 2008) and *T. reesei* (Arvas et al. 2006). Each of these studies has revealed that the UPR affects transcription of a larger number of genes than those directly involved in protein secretion. This may be the main reason for the low levels of heterologous proteins secreted by filamentous fungi (Conesa et al. 2001).

UPR leads to increased transcription of genes encoding ER-resident proteins. As overexpression of heterologous proteins often induces a UPR, it seems likely that the folding capacity of the cell cannot cope with the large amount of unfolded proteins in the ER (Cudna and Dickson 2003). To facilitate proper folding of the proteins, helper proteins, called chaperones and foldases, assist in the process of folding. After translocation, proteins are folded through several cycles assisted by ER-chaperones interacting with the hydrophobic

regions of the secretory proteins. Incomplete folding leads to the binding of the proteins by one or more chaperones resulting in ER retention. The retained proteins either have acess to a new folding cycle or are targeted to ERAD. ERAD involves retrotranslocation of aberrant proteins across the ER membrane to the cytoplasm, where they become ubiquinylated and degraded by the 26S proteasome (Brodsky and McCracken 1999; Ellgaard and Helenius 2001). ER stress is sensed by Ire1 (Cox et al. 1993; Mori et al. 1993), a transmembrane kinase/endoribonuclease, which initiates the non-conventional splicing of the mRNA encoding a key transcription activator, Hac1 in yeast (Bertolotti et al. 2000; Credle et al. 2005; Kawahara et al. 1997; Lee et al. 2008; Oikawa et al. 2007; Papa et al. 2003; Zhou et al. 2006). *HAC1* encodes a bZIP-type transcription factor responsible for activating expression of UPR target genes (Cox and Walter 1996; Kimata et al. 2006; Mori et al. 1996; Travers et al. 2000) or XBP-1 in metazoans (Yoshida et al. 2001; Calfon et al. 2002). In the absence of ER stress, ribosomes are stalled on unspliced *HAC1* mRNA. The translational control is imposed by a base pairing interaction between the *HAC1* intron and the *HAC1* 5′ untranslated region (5′UTR). After excision of the intron, tRNA ligase joins the severed exons, lifting the translational block and allowing synthesis of Hac1 from the spliced *HAC1* mRNA to proceed (Ruegsegger et al. 2001). The Ire1p pathway is conserved among eukaryotes, and has been studied extensively in the yeast *S. cerevisiae*. The target genes of the UPR share a common upstream activating sequence in their promoters, the unfolded protein response element (UPRE) that directs their transcription upon induction of the pathway (Mori et al. 1992). Although the UPR exists in all eukaryotic cells, much of the progress in understanding this signaling pathway comes from studies in the yeast *S. cerevisiae*.

Another regulatory responses to protein overexpression and secretion is described by a mechanism named repression under secretion stress (RESS), shown in cultures of *T. reesei* treated with chemical agents such as dithiotreitol (DTT) or brefeldin A, which inhibit protein folding and/or transport (Pakula et al. 2003). Genes subjected to RESS included the cellulase genes *cbh*1, *cbh*2, *egl*1 and *egl*2, as well as the hemicellulase gene *xyn*1, which together encode the major extracellular proteins produced by *T. reesei*, and a hydrophobin gene *hfb*2. Repression of the genes occurred at approximately the same time as the activation of the *hac*1 gene. A similar type of stress response leading to reduced expression levels of glucoamylase in cultures treated with DTT has been reported for *A. niger*. A RESS-type response was also observed in an *Aspergillus* strain expressing an antisense construct of pdiA, leading to synthesis of reduced amounts of the foldase protein disulfide isomerase A (PDIA) (Al-Sheikh et al. 2004).

To improve cell specific productivities the bottleneck for heterologous protein secretion has to be identified and resolved.

1.5 Cellulases

In nature cellulose exists mainly in plant cell walls as an insoluble, highly-ordered crystalline form. Cellulose is composed of D-glucose residues linked by β-1,4-glycosidic bonds to form linear polymers with an average chain length of 10 000 glycosidic residues, or even more. The smallest repetitive unit in cellulose is cellobiose, a disaccharide. Since cellulose is the most abundant renewable biosource on Earth (Zhang and Lynd 2006), enzymatic cellulose degradation constitutes an important step for carbon flow in the biosphere. Enzymatic cellulose degradation has considerable interest due to its evident ecological and industrial importance. Due to the insolubility and physical complexity of cellulose, several different enzymes are needed for its complete solubilization. Naturally, cellulose is degraded by both fungi and bacteria, that produce a set of enzymes which synergistically hydrolyze crystalline cellulose to smaller oligosaccharides and finally to glucose. The cellulolytic capability may also play a significant role in the ability of fungal plant pathogens or antagonistic fungi to attack their target organisms. For efficient degradation of crystalline cellulose, usually both endoglucanases (EGs) (1,4-β-D-glucan 4-glucanohydrolases; EC 3.2.1.4), which have high affinity towards soluble cellulose derivatives and attack these by endoaction (Teeri 1997) and exoglucanases (1,4-β-D-glucan glucanohydrolases; EC 3.2.1.74) which act as exoenzymes and release cellobiose as a main product from crystalline cellulose; are needed (Henrissat 1996; Teeri et al. 1998; Wood and Bhat 1988; Zhang and Lynd 2004). β-Glucosidases (BGL) (β-glucoside glucohydrolases; EC 3.2.1.21) complete the cellulose hydrolysis by cleaving the resulting cellobiose into glucose providing the fungus with an easily utilizable carbon source for growth. The concerted action of three different types of cellulases as a non complexed enzyme system is presented in Figure 1.4.

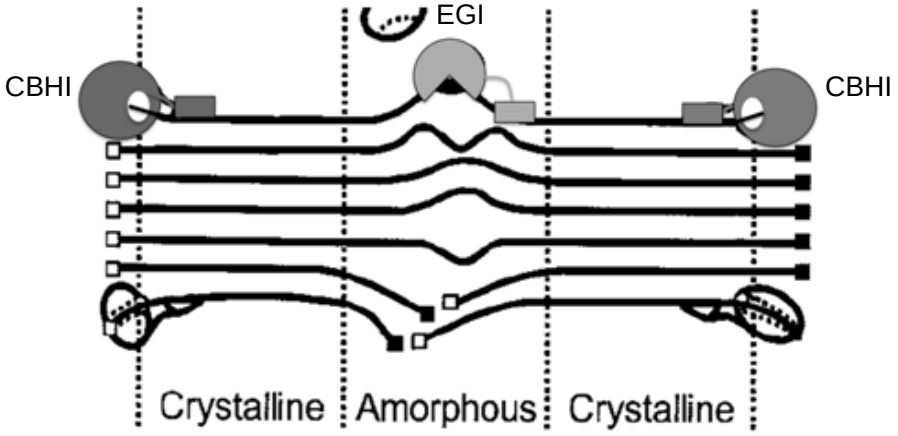


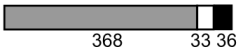
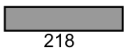
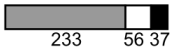
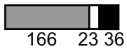
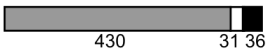
Figure 1.4 – Mechanism of enzymatic hydrolysis of cellulose. The two cellobiohydrolases (CBH) attack the crystalline cellulose at the opposite chain ends and endoglucanase (EG I) in the middle of the more disordered regions of cellulose (Lynd et al. 2002).

Cellulases are *O*-glycoside hydrolases which are a widespread group of enzymes hydrolyzing the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Endoglucanases, often called carboxymethylcellulases (because of the artificial substrate used for their detection), attack randomly at multiple internal sites in the amorphous regions of the cellulose fibre which creates sites for subsequent attack by the cellobiohydrolases (Lynd et al. 1991). Cellobiohydrolase, often called exoglucanase, is the major component of the fungal cellulase system accounting for 40 – 70% of the total cellulase proteins, and can hydrolyze highly crystalline cellulose (Esterbauer et al. 1991).

Most fungal cellulases consist of two domains, a larger catalytic domain (CD) and a smaller carbohydrate binding module (CBM), which is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate-binding activity (Bourne and Henrissat 2001). These domains are joined by a glycosylated linker peptide (Tomme et al. 1998). Only EGIII from *T. reesei* lacks the CBM and linker region (Table 1.3). The catalytic domain contains an active site with the shape of a tunnel or an open cleft in the case of CBHs and EGs respectively (Divne et al. 1993; Divne et al. 1994). The tunnel-shaped topology of CBHI and CBHII explains the way of action of exoglucanase. The presence of the CBM is essential to the degradation of solid crystalline cellulose by mediating binding of the

enzyme to the insoluble substrate, destabilizing the hydrogen bond structure of cellulose, making the polysaccharide chains more accessible to the catalytic domain (Srisodsuk et al. 1993; Tomme et al. 1998). The CBMs of fungal cellulases are invariably small modules, approximately 40 amino acids in size, stabilized by two or three disulphide bridges and belong to the CBM-1 family (Boraston *et al.*, 2004). Three disulphide bridges in the EGI CBM stabilise the structure (Linder et al. 1999).

Table 1.3 – Characteristics of *T. reesei* cellulases (Srisodsuk, 1994; Saloheimo et al., 1997)

Enzyme	Family	Amino acid residues	Molecular mass kDa	isoelectric point (pI)	Structural organization[b]
EGI	7	437	50-55	4.6	
EGII	5	397	48	5.5	
EGIII	12	218	25	7.4	
EGIV	61	326	(37)[a]	-	
EGV	45	225	(23)[a]	2.8-3	
EGVI	unknown*	unknown*	95-105	5.6-6.8	unknown*
CBHI	7	497	59-68	3.5-4.2	
CBHII	6	447	50-58	5.1-6.3	

[a] The molecular mass calculated from the amino acids sequence
[b] ▭, the catalytic domain; □, linker region; ■, CBD
* gene not described

The EGI and CBHI of *T. reesei* have significant homology (45% identity, Penttilä et al. 1986), belong to the same family (Cel7), and both use a retaining mechanism. The active site of EGI is a groove rather than a tunnel (Henriksson et al. 1996), allowing glucan chains to be cleaved randomly to two shorter chains (Kleman-Leyer et al. 1992; Selby 1961; Srisodsuk et al. 1998; Whitaker 1957).

There are five potential *N*-glycosylation sites in EGI from *T. reesei*, and one proposed *O*-glycosylation site on the catalytic domain (Eriksson et al. 2004). The linker region of EGI has been described to be heavily *O*-glycosylated (Eriksson et al. 2004; Hui et al. 2002). *O*-linked

glycosylation has been shown to be important for enzyme stabilization (Neustroev et al. 1993; Williamson et al. 1992) and linker conformation (Receveur et al. 2002). CBHI catalytic domain has four *N*-linked motifs, three of which are glycosylated (Eriksson et al. 2004).

Cellulases can be used in pulp and paper, textile, detergent, food and feed industries, as well as in total hydrolysis of biomass to sugars for production of bioethanol (Bhat and Bhat 1997). The main technological impediment to more widespread utilization of this resource for production of fuels and chemicals is the lack of low-cost technologies to overcome the recalcitrance of the cellulosic structure (van Zyl et al. 2007). Producing biofuels such as ethanol from cellulosic plant material has the potential to meet capacity requirements without competing directly on food production.

1.5.1 Cellulolytic system of *Trichoderma reesei*

One of the most extensively studied cellulolytic organisms is the soft rot fungus *T. reesei* (Kubicek et al. 1993; Penttilä et al. 1991). The extracellular cellulolytic system of *T. reesei* is composed of 60–80% cellobiohydrolases or exogluconases, in which CBHI accounts for about 60% of the total secreted protein (Uusitalo et al. 1991), 20–36% of endogluconases and 1% of β-glucosidases, which all act synergistically in the conversion of cellulose into glucose (Knowles et al. 1987; Muthuvelayudham et al. 2006; Zaldivar et al. 2001).

Even one of the best cellulases producer organisms as *T. reesei* has gone through several steps of improvements and as a result, hypercellulolytic mutant strains that secrete large amounts of cellulases were obtained (Durand et al. 1988b). *T. reesei* has considerable industrial importance. The fungus produces a complete set of cellulases that are able to cleave the β−1,4-glycosidic bonds present in cellulose or cellulose derivatives. Two genes encoding CBHs, *cbh1* (Shoemaker et al. 1983; Teeri et al. 1983) and *cbh2* (Chen et al. 1987; Teeri 1987), four encoding EGs, *egl1* (Penttilä et al. 1986; van Arsdell et al. 1987), *egl2* (Saloheimo et al. 1988), *egl3* (Ward et al. 1993), and *egl5* (Saloheimo et al. 1993; Saloheimo et al. 1994) and one encoding a β-glucosidase (Barnett et al. 1991; Mach et al. 1995) have been identified.

The fungus produces high levels of cellulases on media containing cellulose or complex plant material, and to a variable extent, in the presence of the disaccharides cellobiose or lactose (Bisaria and Mishra 1989; Kubicek et al. 1993). Production is not significant when glucose, fructose, or glycerol is used as the carbon source. A high level of cellulose expression is obtained when sophorose, a molecule consisting of two glucose units linked by

a β–1,2-linkage, is added into the culture (Mandels et al. 1962; Nisizawa et al. 1971). This molecule could be formed from cellooligosaccharides by the transglycosylation activity of EGI (Biely et al. 1991; Claeyssens et al. 1990) or β-glucosidase (Gritzali and Brown Jr. 1979; Vaheri et al. 1979). A disadvantage of using *T. reesei* as a cellulase producer is that its cellulase synthesis is regulated through induction by cellulose-derived compounds and repressed by glucose (Kubicek et al. 1993; Mandels and Reese 1960).

1.5.2 Production of recombinant *T. reesei* cellulases

Current technology for conversion of cellulose to ethanol requires chemical or enzymatic conversion of the substrate to fermentable sugars followed by fermentation by ethanol tolerant micro-organism such as *S. cerevisiae*. The large amounts of enzymes required for enzymatic conversion of cellulose to fermentable sugars impacts severely on the cost effectiveness of this technology. Cellulases from bacterial and fungal sources have been transferred to *S. cerevisiae*, enabling the hydrolysis of cellulosic derivatives (Lynd et al. 2002), or growth on cellobiose (McBride et al. 2005; van Rooyen et al. 2005). However, despite several reports of successful expression of CBH-encoding genes in *S. cerevisiae*, the titres achieved were generally low (Den Haan et al. 2007a).

In order to study the enzymatic properties of individual cellulases from *T. reesei* there are some difficulties due to the contamination of endoglucanases with cellobiohydrolases and vice versa. Therefore, to better understand the *T. reesei* cellulose degradation system, the individual enzymes have been expressed in cellulase non-producing hosts (e.g. *Eschericha coli, S. cerevisiae, and Schizosaccharomyces pombe*). Indeed, expression of *T. reesei* cellulases in these hosts has been evaluated for more than 20 years ago (Adney et al. 2003; Boer et al. 2000; Laymon et al. 1996; Okada et al. 1998a; Okada et al. 1998b; Penttilä et al. 1987; Penttilä et al. 1988; Rose and van Zyl 2002; Saloheimo et al. 1997; Takashima et al. 1998; van Arsdell et al. 1987). However, in some cases, heterologous expression resulted in low yields, proteolytic degradation of the expressed enzymes, formation of inclusion bodies, or high levels of glycosylation. Species of *Aspergillus* have also been used as possible hosts because they produce high amounts of heterologous proteins and possess transcriptional, translational, and post-translational mechanisms similar to those in *T. reesei* (Rose and van Zyl 2002; Takashima et al. 1998). Expression of *T. reesei* cellulases in *E. coli* has only been reported for CBHI and EGIII. It was reported that slight CBHI activity was detectable when its catalytic core was expressed, but that full-length CBHI was not expressed (Adney et al. 2003). Nevertheless there are reports where EGIII was slightly expressed as an active enzyme in the soluble fraction in *E. coli*, while most of the recombinant protein was found in

an inclusion body (Okada et al. 1998a; Okada et al. 1998b; Okada et al. 2000). Several endoglucanases from other Trichoderma species were also expressed in *E. coli*. Kwon and coworkers showed that *T. viride* EG I was expressed in *E. coli* as an inclusion body and that refolding of the recombinant EG I was successful (Kwon et al. 1999). Nakazawa et al. (2008) succeded to express the catalytic domains of EG I and EG II (EG I-CD and EG II-CD) from *T. reesei* III in active form in *E. coli*.

1.5.2.1 Yeast system

Cellulases were among the first heterologous proteins expressed in yeast (Bailey et al. 1993; Penttilä et al. 1987; Penttilä et al. 1988; Zurbriggen et al. 1990) and since then several reports have shown that *S. cerevisiae* can secrete fungal hydrolytic enzymes, including CBHs. Relatively high protein production levels of 1-10% of cellular protein have been reported in *S. cerevisiae* (Schmidt 2004). However, there have also been reports of poor levels of protein secretion (Den Haan et al. 2007). The examples of CBH expressed in yeast include CBHI and CBHII of *T. reese*i (Penttilä et al 1988; den Haan et al. 2007; Heinzelman et al. 2009; Du Plessis et al 2010), and CBHs of other fungi (Hong et al. 2003; den Haan et al 2007; Voutilainen et al. 2010; Takada et al. 1998; Heinzelman et al. 2010). Recently, Ilmen et al. (2011) expressed 14 *cbh*1 and 10 *cbh*2 genes from several ascomycetes, in order to identify which enzymes were more efficiently secreted by *S. cerevisiae*.

The activity of *Talaromyces emersonii* CBHI and its derivative with the *T.reesei* CBM attached to its C-terminus exceeded that of *T.reesei* CBHI by at least two orders of magnitude, yielding 100-200 mg l^{-1} in shake flasks and 300 mg l^{-1} in high cell density conditions. This shows a large improvement compared with a recent report of 5-10 mgl^{-1} *T. emersonii* CBHI (Heinzelman et al 2010). The reason for this difference may result from differences in codon optimization, or strain and construct specific effects or, in the case of *T. aurantiacus*, a difference in the amino acid sequences introduced. The highest CBH level secreted, 1000 mg l^{-1} *Chrysosporium lucknowense* CBH2b, corresponding to 4% of the total cellular protein, was produced in high cell density conditions, exceeded any previous reports on CBH production in *S. cerevisiae*.

It would appear that some CBHs are thus more compatible with high-level expression and production in *S. cerevisiae* than others, although which features lead to incompatibility, marked by low levels of plasmid, mRNA and secreted protein and strong induction of UPR, are difficult to define. Gene or protein specific features and compatibility with the host are important for efficient cellobiohydrolase secretion in yeast.

The endoglucanase I and II genes of *T. reesei* QM6a were successfully cloned and expressed in *S. cerevisiae* under the transcriptional control of the yeast ENO1 promoter and terminator sequences (du Plessis et al. 2010). Random mutagenesis of the egI-bearing plasmid resulted in a two fold increase in extracellular EGI activity. Both endoglucanase genes were co-expressed with the synthetic, codon-optimised cellobiohydrolase gene (s-*cbh*I) from *T. reesei* as well as the β- glucosidase gene (*bgl*1) from *Saccharomycopsis fibuligera* in *S. cerevisiae* (du Plessis et al. 2010*)*. Extracellular endoglucanase activity was lower when co-expressed with s-*cbh*I or *bgl*1. Recombinant strains were able to hydrolyse phosphoric acid swollen cellulose, generating mainly cellotriose, cellobiose and glucose. Cellobiose accumulated, suggesting the β-glucosidase activity was the rate-limiting factor. As a consequence, the recombinant strains were unable to produce enough glucose for growth on amorphous cellulose.

Although CBHI enzymes retain activity when expressed in *S. cerevisiae*, there are results indicating that the activity of the yeast-produced enzymes is impaired in comparison to the native proteins, which in some cases could be due to overglycosylation (Penttilä et al. 1988; Takada et al. 1998; Reinikainen et al. 1992), or misfolding (Boer et al. 2000).

Besides *S. cerevisiae*, other yeast hosts have been used for *T.reesei* cellulases expression. For instance, *T. reesei* EGI was successfully expressed and secreted in *Yarrowia lipolytica* using the signal peptide of EGI under the control of strong *XPR2* promoter. However, the secreted EGI had a higher molecular weight compared to the native enzyme from *T. reesei*, but similar to the recombinant EGI in *S. cerevisiae*. The main band was detected at 66 kDa with faint smears above the band, indicating a hyperglycosylated protein was produced. Endo-H treatment removed hyperglycosylation and altered the molecular weight of the protein from 66 kDa to 55 kDa (Park 2000). The results suggested a difference in the degree of glycosylation between native and recombinant EGI. When *T. reesei* EGI was expressed in *S. cerevisiae*, Van Arsdell et al. (1987) and Penttilä et al. (1987) observed two main bands of about 70 kDa with heterogeneous smears and these bands were retained after endo-H treatment. This observation suggested that there were similarities in hyperglycosylation and differences in the types of glycosylation between *Y. lipolytica* and *S. cerevisiae* in their secretory pathways. In another study by Song et al. (2007), the *Y. lipolytica OCH1* gene that encodes an α-1,6-mannosyltransferase was eliminated. *OCH1* gene plays an important role in the addition of the first mannose to the core oligosaccharide in *Y. lipolytica*, as in other yeasts. Analysis of the structure of the *N*-linked oligosaccharide of the EGI produced by the Yl*och1* mutant shows that the recombinant glycoprotein EGI secreted was less heavily glycosylated than the recombinant EGI from the wild-type strain.

The fission yeast *S. pombe* is the second most frequently used yeast host after *S. cerevisiae*. A high-expression vector for heterologous genes in *S. pombe* that carries a human cytomegalovirus (hCMV) promoter to drive foreign gene expression has been designed by Okada et al. (1998a).The copy number is controlled by the level of gentamicin (G418) through expression of the neomycin-resistance coding gene, which is regulated by the simian virus 40 (SV40) early promoter having a transcriptional activity of one-tenth that of the hCMV promoter (Toyama and Okayama 1990). To evaluate *S. pombe* as an alternative host to *S. cerevisiae* for secretion of a recombinant protein*, T. reesei* cbh2 gene was expressed in *S. pombe* by using the above high-expression vector (Okada et al. 1998a). The recombinant *S. pombe* secreted two molecules of 72 kDa and 70 kDa, probably with different degrees of *N*-glycosylation. The two proteins, however, have the same enzymatic characteristics as the native *T. reesei* CBHII. In comparison to *S. cerevisiae, S. pombe* seems to be similar to *S. cerevisiae* in the extent of glycans that are added to the recombinant protein being produced. Deglycosylation treatments revealed that the recombinant enzymes were overglycosylated and scarcely susceptible to α-mannosidase.

The methylotrophic yeast *Pichia pastoris* has been used to express active *T. reesei* CBHI under the *P. pastoris* alcohol oxidase (*AOX*1) promoter and the glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter. Production of CBHI with the AOX1 promoter gave a good yield, although part of the enzyme expressed was apparently not correctly folded. CBHI expressed in *P. pastoris* was overglycosylated, as compared to the native *T. reesei* protein, but less extensively than CBHI expressed in *S. cerevisiae*. The degradation rate on crystalline substrate (BMCC) was somewhat reduced. The measured pH optimum also closely resembles that of purified *T. reesei* CBHI. Furthermore, the hyperglycosylation did not affect the thermostability of the enzyme monitored with tryptophane fluorescence or activity measurements. On the other hand, CD measurements indicated that the formation of disulfide bridges was an important step in the correct folding of CBHI and might explain the difficulties encountered in heterologous expression of *T. reesei* CBHI (Boer et al. 2000).

1.5.2.2 Fungal system

Takashima et al. (1998) have constructed an expression system of *T*. *reesei* EGI, EGIV, CBHI and CBHII genes using the filamentous fungus *Aspergillus oryzae* as a host. The recombinant cellulases produced by *A*. *oryzae* had molecular masses larger than those of native enzymes produced by *T*. *reesei* but smaller than those of recombinant cellulases produced by yeast.

The endoglucanase I of *T. reesei* QM6a was successfully expressed in *Aspergillus niger* D15 under the transcriptional control of the glyceraldehyde-6-phosphate dehydrogenase (*GPD*) promoter from *A. niger* and the *glaA* terminator of *Aspergillus awamori* (Rose and van Zyl 2002). A significant amount of protein was inactive since the activity obtained was lower than what would be expected when considering the amount of protein present on the SDS-polyacrylamide gel. Similarly, Aho et al. (1996) also obtained only 2% of active EGI heterologously produced in *S. cerevisiae*. The endoglucanase protein was more heterogenous in size when expressed in *A. niger* than when expressed in *S. cerevisiae* (Penttilä et al. 1987). This phenomena, however, is not uncommon with expression of *T. reesei* enzymes in *Aspergillus* and can be ascribed to variable glycosylation patterns (Takashima et al. 1998). Glycosylation, responsible for the heterogenous nature of the protein, could be responsible for the lack of activity, with the sugars covering the active site.

Another ascomycete, *Aspergillus niger var. awamori*, is also known to produce large quantities of heterologous proteins for industry and was used for the expression of *T. reesei* CBHI (Adney et al. 2003; Jeoh T 2008). The purified recombinant enzyme contained six times the amount of *N*-linked glycan than the enzyme purified from a commercial *T. reesei* enzyme preparation. The increased level of *N*-glycosylation of the recombinant CBHI (rCBHI) was thought to result in reduced activity and increased non-productive binding on cellulose. When treated with the *N*-glycosidase PNGaseF, the molecular weight of the recombinant enzyme approached that of the commercial enzyme and the activity on cellulose was improved (Adney et al. 2003). *A. niger var. awamori* expressed enzyme had equivalent thermal stability to *T. reesei* CBHI (Adney et al. 2003).

1.5.2.3 Other expression systems

The use of the baculovirus vector system for heterologous gene expression in insect cells has been widely and successfully implemented in the production of foreign proteins. The *T. reesei* cellobiohydrolase I (CBHI) was expressed with a recombinant baculovirus and high levels of secreted protein were produced in *Spodoptera frugiperda* and *Trichoplusia* in insect cells. Electophoretic analysis indicated that the recombinant CBHI was similar to the apparent molecular weight of the native form and immunoblotting with anti-CBHI monoclonal antibodies confirmed its identity.The rCBHI was easily purified by affinity and hydrophobic interaction chromatography and demonstrated enzymatic activity on soluble substrate (von Ossowski I 1997).

Although efforts have been made to clone *T. reesei* CBHI in other hosts such as *E. coli* (Laymon et al. 1996; Teeri 1997) poor solubility has made the combinant form of CBHI of limited use in structure/function studies.

In sum, various expression systems, including *E. coli*, *Spodoptera frugiperda* (insect cells), *P. pastoris*, *S. cerevisiae*, and *A. niger var. awamori* have been used for the heterologous expression of fungal cellulases (Adney et al. 2003). Successful expression of functional protein has been limited due to stringent co- and post-translational requirements, including formation of disulfide bridges and native-like glycosylation or misfolding. Of these hosts, *A. niger var. awamori* and insect cells were most effective in producing functional enzyme, but show greater extents of glycosylation compared to native enzyme (Adney et al. 2003). Chapter 3 of this thesis describes the expression of *T. reesei* EGI in *A. gossypii*, which is similar to its expression in *S. cerevisiae*.

1.6 References

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Chapter 2

Nutritional requirements and strain heterogeneity in *Ashbya gossypii* (*Eremothecium gossypii*)

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Abstract

Colony radial growth rates and specific growth rates of three related *Ashbya gossypii* strains ATCC10895, IMI31268, MUCL29450 and an unrelated strain, CBS109.26, were measured on various carbon and nitrogen sources at pH 4.5 and pH 6.5 to elucidate physiological growth requirements and strain differences. All strains grew on yeast extract or ammonium as nitrogen sources, but not on nitrate. Substantial growth at pH 4.5 was observed only on complex medium. D-Glucose, glycerol and starch were utilised as carbon sources. Ethanol was produced during growth on glycerol. Conversion of xylose into xylitol demonstrates that the xylose reductase is active. Phenotypic differences between related strains were greater than expected. We demonstrate that *A. gossypii* utilizes ammonium as sole nitrogen source at pH 6.5, facilitating further physiological studies using chemically defined media in the future

2.1 Introduction

The filamentous hemiascomycete *Ashbya gossypii* (Kurtzman 1995; Tanner et al. 1949) was one of four species infecting cotton in the British West Indies, first characterised by Nowell (1915) (as cited in Pridham and Raper 1950). The fungus infects the developing lint fibers which become dirty-yellowish and mat onto the seeds. The yellow pigment was later identified as riboflavin.

A. gossypii is a natural producer of riboflavin (Demain 1972; Wickerham et al. 1946) and is used for industrial riboflavin production (Stahmann et al. 2000). Its genome has been sequenced and annotated (Dietrich et al. 2004; Hermida et al. 2005), revealing an evolutionary relationship with *Sacharomyces cerevisiae*, with remarkable similarities at the synteny level but lacking sequence duplications that are present in *S. cerevisae*. *A. gossypii* has primarily been studied in the context of riboflavin production (Kanamasa et al. 2007; Maeting et al. 2000; Mateos et al. 2006; Park et al. 2007; Schlösser et al. 2001; Schmidt et al. 1996) and as a model to investigate polarized hyphal growth on the molecular level (Ayad-Durieux et al. 2000; Gladfelter et al. 2007; Kohli et al. 2008; Philippsen et al. 2005; Wendland and Philippsen 2001; Wendland and Walther 2005).

A. gossypii as a riboflavin producer represents an example of environmentally-friendly white biotechnology. Other, more novel applications of *A. gossypii* could be envisioned, for which it is useful to clarify its nutritional requirements, reports of which have been incomplete or contradictory and may reflect strain differences. The ability to utilize polymeric carbohydrates, pentoses and glycerol, which are currently readily available from plant biomass and the biodiesel industry (Yazdani and Gonzalez 2007), is of particular interest. Although *A. gossypii* is known to grow on glycerol, its ability to produce ethanol from glycerol, converting it into a useful fuel, has not previously been reported.

In this paper, we demonstrate the similarities and differences of three related *A. gossypii* strains, ATCC10895, MUCL29450, IMI31268 and the more distantly related CBS109.26 and assess their ability to grow in chemically defined medium on various carbon and nitrogen sources, in order to gain insights on *A. gossypii* physiology. ATCC10895 and MUCL29450 were deposited at ATCC and MUCL as the same strain, and both should thus be derived from the parent strain ATCC8717, also deposited as IMI31268. The sequenced strain ATCC10895 and derivatives of it are now the most commonly used *A. gossypii* strains.

2.2 Materials and methods

2.2.1 Strains

The four *Ashbya gossypii* (sometimes also referred as *Eremothecium gossypii*) strains used in this study were ATCC 10895 (kindly provided by Prof. P. Philippsen, University of Basel, the sequenced strain), MUCL29450 (equivalent of ATCC10895, CBS109.51, or NRRL Y-1056), IMI31268 (equivalent of ATCC8717, the parent strain of ATCC10895, isolated from cotton) and CBS109.26, an independent strain of *A. gossypii* isolated from Asclepias fruit in Trinidad and Tobago.

2.2.2 Media

YP medium (10 g l^{-1} yeast extract, 20 g l^{-1} peptone), defined medium (Verduyn et al. 1992) with minor modifications as indicated, synthetic complete (SC) defined medium, containing complete amino acid supplement (Sherman et al. 1986) and yeast nitrogen base (Difco) and *Ashbya* Full Medium (AFM; 10 g l^{-1} peptone, 10 g l^{-1} yeast extract, 1 g l^{-1} myo-inositol plus a carbon source) were used for growth of *A. gossypii*. D-Glucose, glycerol, starch, carboxymethyl cellulose (CMC) (20 g l^{-1}), D-xylose or L-arabinose (10 g l^{-1}) were provided as carbon sources. Ammonium sulphate in Verduyn defined medium was in some cases replaced with potassium nitrate (5 g l^{-1}) or yeast extract (5 g l^{-1}).

When used for submerged growth, SC medium was supplemented with 1g l^{-1} $CaCO_3$ for buffering and 1 g l^{-1} agar to facilitate filamentous growth. For measurement of colony radial growth rate, media were solidified with 20 g l^{-1} agar. The initial pH of agar-solidified defined media was adjusted to 4.5 or 6.5.

2.2.3 Inoculum preparation

Strains MUCL 29450, IMI31268 and CBS 109.26 did not sporulate on any of the media used in this study, nor on a variety of other fungal sporulation media. Therefore, all strains were preserved as mycelium in glycerol (20% v/v) at -80 °C to provide reproducible inoculum for liquid cultures. ATCC10895, which does sporulate, was also stored as spores in glycerol (20% v/v) at -80 °C. Mycelia were prepared by collecting 8-10 day old mycelia from agar-solidified medium, digesting with zymolyase (150 mg ml^{-1}) for 2 h, and washing with a solution of 0.8% (w/v) NaCl, 20% (v/v) glycerol and 0.025% (v/v) Tween 20. Fragmented mycelia were inoculated into YPD in flasks and allowed to grow (200 rpm, 30 °C) for approximately 20 h. The mycelial suspension was diluted with an equal volume of 40% (v/v) glycerol and frozen in

aliquots at -80 °C. Long-term viability of frozen mycelia was not assessed, but mycelia remained viable for at least 2 years.

2.2.4 Culture conditions

Submerged cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml medium and incubated at 30 °C, 200 rpm. Flasks were inoculated with 0.6 ml mycelia which had been stored at -80 °C. Agar-solidified medium was inoculated with 10 µl mycelia at up to three locations per 9 cm diameter Petri dish.

2.2.5 Colony radial growth rate, culture optical density and dry weight

Colony radial growth rates (Kr; (Brancato and Golding 1953; Trinci 1969)) were determined by measuring diameters of colonies growing on agar-solidified medium in 9 cm diameter Petri dishes.

Culture optical density at 600 nm (OD_{600}) was used as a measure of biomass for submerged cultures with dispersed, filamentous growth (Trinci 1972). The relationship between OD_{600} and mycelial biomass was determined by collecting and washing mycelia on glass fibre GF/C filters and drying at 100 °C. One OD_{600} unit corresponded to 1.3 g l^{-1} DW of *A. gossypii*.

2.2.6 Extracellular substrates and metabolite concentrations

Extracellular substrates and metabolites (ethanol, glycerol, D-glucose, D-xylose, L-arabinose and xylitol) were analyzed by HPLC as previously described (Toivari et al. 2010).

The production of starch degrading enzymes on agar-solidified medium was detected by staining the surface with iodine solution (Carlsen et al. 1994) to visualize zones in which starch had been broken down.

2.3 Results

2.3.1 Use of inorganic nitrogen by *A. gossypii*

The Kr of *A. gossypii* ATCC10895, MUCL29450, IMI31268 and CBS109.26 was determined on Verduyn defined medium containing $(NH_4)_2SO_4$, KNO_3, or YE as the nitrogen source or lacking a nitrogen source, with the initial pH adjusted to 4.5 or 6.5. Although all strains showed measurable colony expansion on KNO_3 at pH 6.5, the density of mycelium was similar to that on medium lacking nitrogen and clearly represented only background growth on the nitrogen available in the inocula (Figure 2.1).

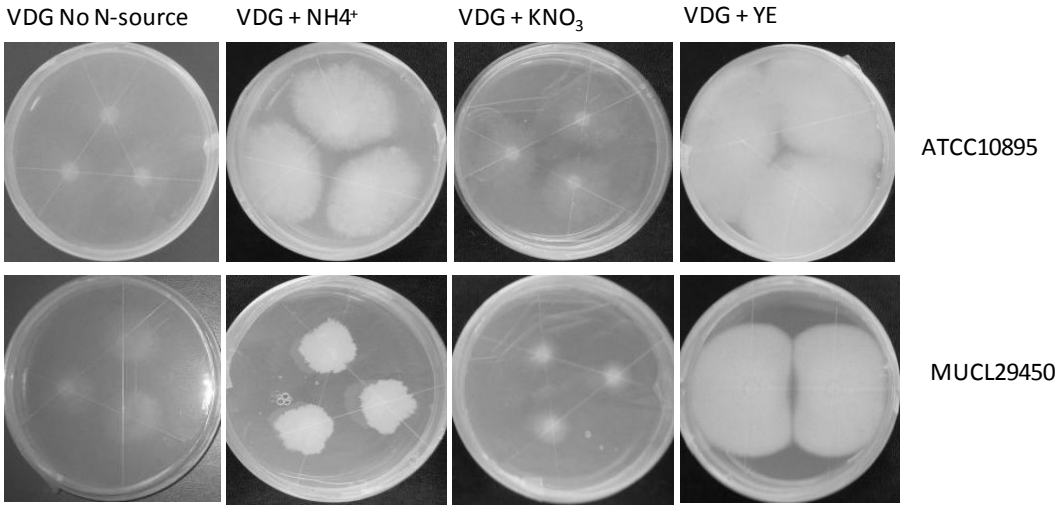

Figure 2.1 – Colonies of *A. gossypii* ATCC10895 and MUCL29450 on chemically defined medium at pH 6.5 containing no nitrogen source, or ammonium, nitrate or YE as nitrogen source. Colonies were incubated for 7 days.

All four *Ashbya* strains in this study grew well on Verduyn defined medium with only ammonium as nitrogen source at pH 6.5 (Figure 2.1), but not at pH 4.5, although they grew at both pH values when YE was provided as the nitrogen source (Table 2.1). Subsequently, experiments with $(NH_4)_2SO_4$ as sole nitrogen source were supplemented with $CaCO_3$ to maintain the pH above 6.0.

The Kr of ATCC10895 was generally lower than that of the parental strain IMI31268, although it was more similar with YE as the nitrogen source (183 ± 4 μm h^{-1} and 195 ± 4 μm h^{-1}, respectively, $p > 0.01$) than with ammonium (165 ± 2 μm h^{-1} and 220 ± 5 μm h^{-1},

respectively, $p < 0.01$, Table 2.1). The Kr of MUCL29450 was significantly lower ($p < 0.05$) than that of ATCC10895 on all media, indicating that the two strains are phenotypically different. The Kr of CBS109.26 was also lower ($p < 0.05$) than that of ATCC10895 with either YE or ammonium as nitrogen source.

Table 2.1 – Colony radial growth rate (Kr, μm h^{-1}) of *A. gossypii* ATCC10895, MUCL29450, IMI31268 and CBS109.26 on Verduyn defined medium containing $(NH_4)_2SO_4$ or YE as nitrogen sources, and with the initial pH adjusted to 4.5 or 6.5. Values in the same row with the same superscript (a to d) did not differ significantly ($p < 0.05$)

pH	Nitrogen source	ATCC10895	MUCL29450	IMI31268	CBS109.26
6.5	YE	183 ± 4^{b}	149 ± 2^{a}	195 ± 4^{c}	154 ± 3^{a}
	$(NH_4)_2SO_4$	165 ± 2^{c}	76 ± 2^{a}	220 ± 5^{d}	134 ± 1^{b}
4.5	YE	152 ± 1^{c}	117 ± 1^{a}	160 ± 1^{d}	137 ± 2^{c}
	$(NH_4)_2SO_4$*	4 ± 1^{a}	3 ± 1^{a}	11 ± 1^{b}	4 ± 1^{a}

* sparse growth

2.3.2 Comparison of strains on different carbon sources

All four strains grew in chemically defined medium with D-glucose, glycerol or starch as carbon sources and $(NH_4)_2SO_4$ as nitrogen source at pH 6.5 (Table 2.2). As on D-glucose, MUCL29450 had a significantly lower Kr ($p < 0.05$) than ATCC10895 or IMI31268 on both glycerol and starch (Table 2.2). The Kr of ATCC10895 and IMI31268 also differed. None of the strains grew on CMC, although background growth, comparable to that on medium lacking a carbon source, was observed.

A. gossypii did not grow as well on starch as on D-glucose or glycerol, but produced more biomass than on CMC or medium lacking the carbon source. When grown on starch-containing AFM medium, zones from which starch had been degraded were visible around ATCC10895 (Kr = 69 ± 3 μm h^{-1}), MUCL29450 (Kr = 68 ± 1 μm h^{-1}) and IMI31268 (Kr = 105 ± 5 μm h^{-1}), indicating the secretion of amylase. Amylase activity has also been observed in culture supernatant of ATCC10895 grown on starch in submerged culture (O. Ribeiro, unpublished result). No clearing zone was observed for CBS109.26 on AFM with starch as the carbon source, although it grew (Kr = 71 ± 2 μm h^{-1}), suggesting it differed in amylase production from the other, more closely related strains.

Table 2.2 – Colony radial growth rate (Kr, μm h^{-1}) of *E. gossypii* ATCC10895, MUCL29450, IMI31268 and CBS109.26 on Verduyn defined medium containing D-glucose, glycerol or starch as carbon sources, and with the initial pH adjusted to 6.5. Values in the same row with the same superscript (a to d) did not differ significantly ($p < 0.05$). Cultures were incubated at 30°C. Data represents the average ± SEM for 12 replicates.

Carbon source	ATCC10895	MUCL29450	IMI31268	CBS109.26
D-glucose	165 ± 2^{e}	76 ± 2^{a}	220 ± 5^{d}	134 ± 1^{b}
Glycerol	99 ± 2^{c}	56 ± 1^{a}	119 ± 3^{c}	116 ± 4^{c}
Starch	115 ± 3^{d}	44 ± 5^{a}	92 ± 2^{b}	52 ± 3^{a}
no C*	41 ± 2^{a}	-	-	-

* sparse growth; - represents no growth

2.3.3 Use of pentose sugars as sole carbon source by *A. gossypii* ATCC10895

A. gossypii ATCC10895 did not grow with either D-xylose or L-arabinose as sole carbon source. When subcultured sequentially from YP or SC medium containing D-xylose or L-arabinose as carbon source to the same medium, growth visibly decreased. Thus, while some yeast and filamentous fungi have improved growth on pentose sugars when they have been previously exposed to the sugars, *A. gossypii* did not. When grown in submerged culture at pH ~6, L-arabinose was not taken up by the mycelium and arabitol was not produced. D-Xylose was converted to xylitol (Figure 2.2) with a yield of 0.99 g xylitol (g D-xylose consumed)$^{-1}$, indicating that the xylose reductase (XR) was active. Although some growth was observed ($\mu = 0.09 \pm 0.004$ h^{-1}, ~3 g DW l^{-1}; Figure 2.2), this resulted from the glycerol present in the inoculum and storage compounds present in the mycelia. In subsequent experiments, mycelial inocula were washed by centrifugation to remove glycerol and reduce the extent of background growth.

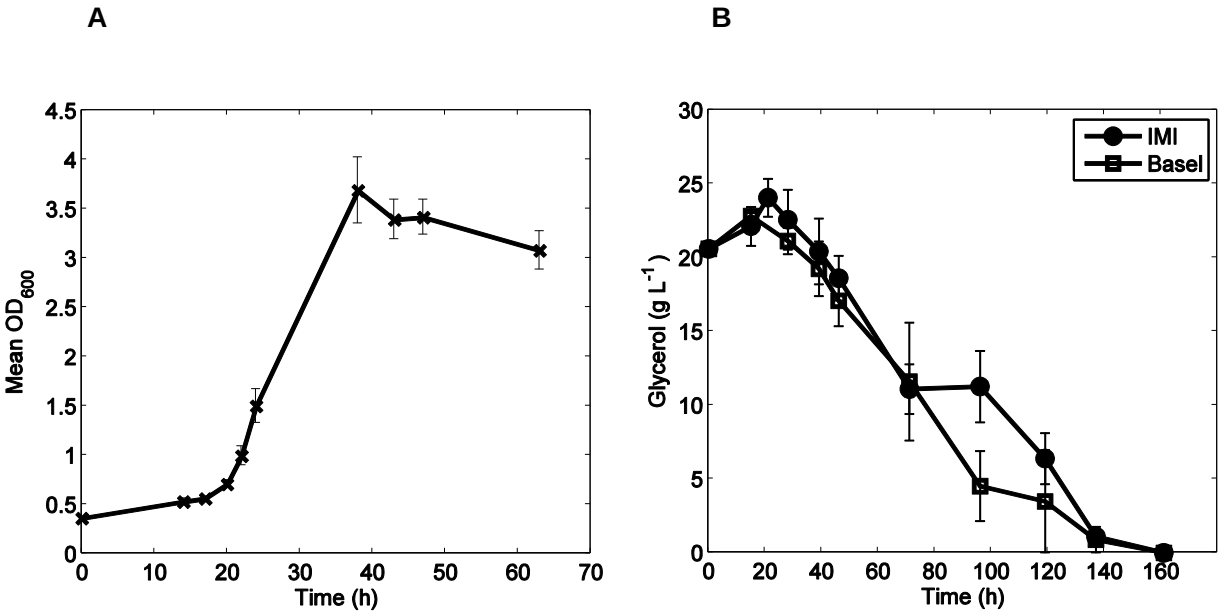

Figure 2.2 – Biomass (A) and xylitol (B) production by *A. gossypii* ATCC10895, and D-xylose consumption (B), in SC medium containing D-xylose (10 g l^{-1}) as carbon source and $(NH_4)_2SO_4$ as nitrogen source buffered with 1 g $CaCO_3$ l^{-1}. Flasks were inoculated with mycelia and filamentous growth was sustained by the addition of 1 g agar l^{-1}. Flasks were incubated at 30°C, 200 rpm. Results are mean ± SEM for 3 cultures.

2.3.4 Growth and metabolite production in submerged cultures

ATCC10895 and MUCL29450 grew at the same specific rate (0.33 ± 0.02 h^{-1}) in YPD in submerged culture. The specific growth rate of both strains was slightly ($p < 0.10$) lower than that of their parent strain, IMI31268 (0.39 ± 0.01 h^{-1}). CBS109.26 (0.22 ± 0.01 h^{-1}) grew at a significantly ($p < 0.05$) slower specific growth rate than the other strains.

ATCC10895, MUCL29450 and CBS109.26 produced small amounts (< 1 g l^{-1}) of ethanol aerobically from D-glucose in YPD during batch cultures in flasks. Ethanol production was also detected when ATCC10895 was grown in bioreactor batch cultures in AFM. Ethanol was produced (2.5 ± 0.05 g l^{-1}) at a yield of 0.13 g (g glycerol consumed)$^{-1}$ when ATCC10895 was grown in YP with glycerol as carbon source, but not in SC with glycerol (Figure 2.3). Specific growth rates of 0.12 ± 0.01 and 0.11 ± 0.003 h^{-1} were observed in YP-glycerol and SC-glycerol media, respectively. The parent strain IMI31268 consumed glycerol at a similar rate to ATCC10895 in SC-glycerol medium (Figure 2.3), but had a specific growth rate of 0.20 ± 0.01 h^{-1}.

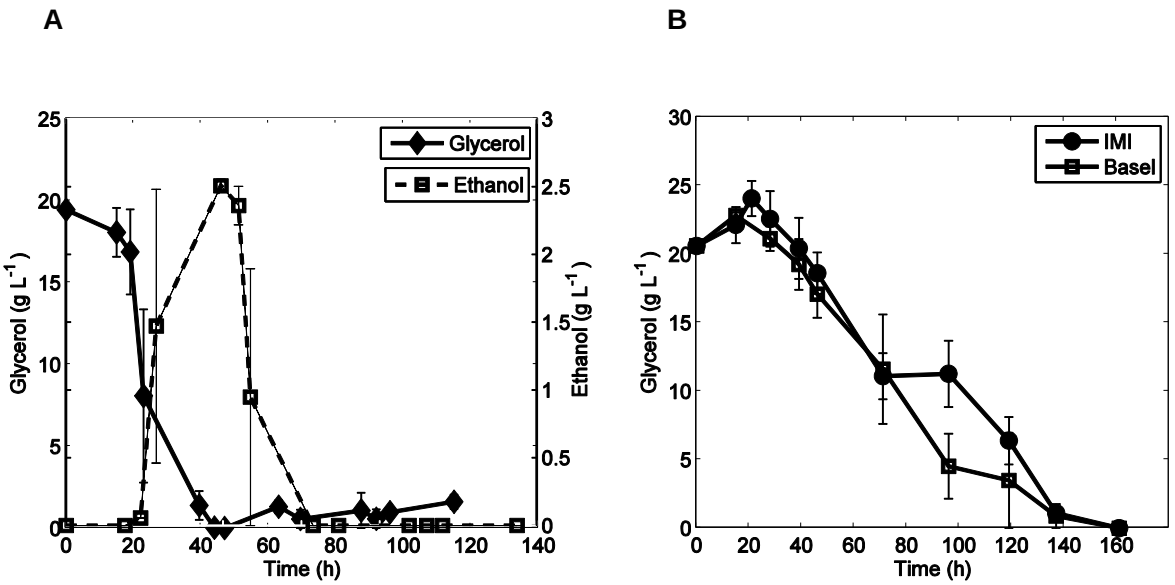

Figure 2.3 – Glycerol consumption and ethanol production of ATCC10895 in YP medium (A) and SC medium (B) with glycerol as carbon source in flask cultures incubated at 30°C, 200 rpm. Error bars represent ± SEM for 3 cultures. Glycerol consumption of IMI31268 in SC medium with glycerol is also shown.

2.4 Discussion

Cultivation media for *A. gossypii* are typically complex (e.g. AFM; (Altmann-Jöhl and Philippsen 1996)) or supplemented with yeast extract or an amino acid (Pridham and Raper 1950). However, here we demonstrate that *A. gossypii* does grow on inorganic nitrogen without supplements. Although Farries and Bell (1930), Buston et al. (1938) and Wright and Philippsen (1991) have indicated that *A. gossypii* (strains Ashby and Nowell and ATCC10895) did not grow or grew poorly on ammonium as nitrogen source, we found that all four strains of *A. gossypii* used here grew well on ammonium as sole nitrogen source when the initial pH was 6.5 (Figure 2.1). Growth was poor on chemically defined medium at pH 4.5 (Table 2.1), and associated with very long lag phases in submerged cultures (Yvonne Nygård, VTT Finland, personal communication), indicating that the lack of growth or limited growth previously reported (Buston et al. 1938; Farries and Bell 1930; Wright and Philippsen 1991) probably reflects low medium pH. Growth on ammonium is improved by buffering to reduce the pH decrease as ammonium is taken into the cells.

Ammonium could be used by *A. gossypii* as sole nitrogen source, but nitrate could not (Figure 2.1). The *A. gossypii* genome contains genes orthologous to those of *S. cerevisiae* for

energy dependent ammonium assimilation (*GLN1* and *GLT1*, Filetici et al. 1996), including gene sequences encoding both glutamine synthetase (GS) (ACR182C; EC 6.3.1.2), and glutamate synthase (GOGAT) (ADR290W; EC 1.4.1.13), confirming that *A. gossypii* has the genetic capacity for ammonium assimilation. Although NAD^+ dependent glutamate dehydrogenase (*S. cerevisiae* homolog GDH2) (AGL040C; EC 1.4.1.2) is present, the $NADP^+$ dependent (*S. cerevisiae* homologs GDH1 or GDH3) form of the enzyme is not, suggesting that like the basidiomycete *Agaricus bisporus* (Baars et al. 1996), *A. gossypii* lacks the glutamate dehydrogenase ammonium assimilation route which is dominant in *S. cerevisiae* (DeLuna et al. 2001). The relative importance of the GS/GOGAT and glutamate dehydrogenase pathways for ammonium assimilation in fungi is species dependent, with the glutamate dehydrogenase pathway dominant in *S. cerevisiae*, *Neurospora crassa*, *Kluyveromyces aerogenes* (Avendano et al. 1997) and *Aspergillus nidulans* (Macheda et al. 1999), but the GS/GOGAT pathway dominant in *A. bisporus* (Baars et al. 1996), *Candida albicans*, and *Schizoccharomyces pombe* (Barel and MacDonald 1993; Holmes et al. 1989; Perysinakis et al. 1995). Even in organisms in which the glutamate dehydrogenase pathway is dominant, such as *A.s nidulans*, the GS/GOGAT pathway is sufficient for ammonium assimilation in mutants lacking NADP-GDH activity (Macheda et al. 1999). In contrast, no sequence orthologous to a nitrate reductase is found in the *A. gossypii* genome, explaining the lack of growth on nitrate observed here and by Farries and Bell (1930).

As a fungus initially isolated as a pathogen of cotton plants, *A. gossypii* might be expected to produce cellulases, but there are no genes encoding putative cellulolytic enzymes in the genome (Dietrich et al. 2004). Early studies of *A. gossypii* (Ashby and Nowell) similarly found no growth on cellulose (Marsh 1926; Pearson 1947). However, Marsh (1926) and Tanner et al. (1949) also reported that *A. gossypii* (Ashby and Nowell) was unable to degrade starch, while Farries and Bell (1930) observed a small amount of degradation. Here we observed growth on starch and clear amylolytic activity, although only one gene (AEL276C) containing a putative amylolytic catalytic domain has been identified in the genome.

Although Farries and Bell (1930) did not observe growth of *A. gossypii* (Ashby and Nowell) on either D-xylose or L-arabinose, growth on these substrates was re-evaluated here since a putative aldose or D-xylose reductase (XR – ACL107c, EC 1.1.1.21), xylitol dehydrogenase (XDH – ABR229c, EC 1.1.1.9) and xylulokinase (XK – AGR324c, EC 2.7.1.17) are present in the *A. gossypii* ATCC10895 genome (manual annotation). Most fungi which utilise D-xylose as a carbon source do so by reducing D-xylose to xylitol with XR, oxidizing xylitol to D-xylulose with XDH and phosphorylating D-xylulose to xylulose-5-phosphate with XK. Although the necessary putative genes are present in the genome, they are not necessarily active, as in *S. cerevisiae*, which does not grow on D-xylose or grows extremely slowly (Attfield and Bell

2006; van Zyl et al. 1989). *A. gossypii* ATCC10895 resembled *S. cerevisiae* in D-xylose metabolism, being able to produce xylitol from D-xylose, but not to grow.

The catabolic pathways of L-arabinose and D-xylose metabolism in fungi share many enzymes (Chiang and Knight 1960; Fonseca et al. 2007; Witteveen et al. 1989). L-Arabinose is reduced by a non-specific aldose reductase (EC 1.1.1.21) to L-arabitol, which is converted to xylitol in two consecutive redox steps catalyzed by L-arabitol 4-dehydrogenase (LAD; EC 1.1.1.12) and L-xylulose reductase (LXR; EC 1.1.1.10). The genes encoding LAD and LXR are not present in the *A. gossypii* genome (manual annotation) so that utilization of L-arabinose by *A. gossypii* was not expected. The lack of arabitol production probably reflects poor arabinose uptake under the growth conditions used here.

Although *A. gossypii* ATCC10895 is frequently described as a wild type, it was isolated as a highly pigmented variant of ATCC8717 (equivalent of IMI31268) and clearly differed from this strain in specific growth rate and in colony radial growth rate on various carbon or nitrogen sources. Kr is specific for specific strains in specific environments (Brancato and Golding 1953; Trinci 1971) and reflects a combination of specific growth rate and branch density. In addition, although MUCL29450 was originally deposited as ATCC10895, it clearly differed phenotypically from the strain sequenced as ATCC10895 and showed greater differences from the parental strain IMI31268 than ATCC10895. MUCL29450 and ATCC10895 were more similar in submerged culture, where the same specific growth rate was observed for both ($0.34 \pm 0.02\ h^{-1}$). Each of these strains has been maintained in independent culture collections for an extensive time, and it should be noted that the parent IMI31268 may also show phenotypic divergence from its equivalent strain ATCC8717.

The specific growth rate of $0.33 \pm 0.02\ h^{-1}$ observed here for ATCC10895 in YPD was comparable to that expected of *S. cerevisiae* in complex medium. It was higher than previously reported specific growth rates of *A. gossypii*, which have been between $0.02\ h^{-1}$ (NRRL1363/ATCC12995; Kalingan and Krishnan 1997; Kalingan 1998) and $0.16\ h^{-1}$ (ATCC10895 and derivatives; Schlosser et al. 2007), including that of an ATCC10895 transformant ($0.09 \pm 0.00\ h^{-1}$) in AFM in flasks (Ribeiro et al. 2010). However, we have observed similar rates in other cultivations (e.g. $0.32\ h^{-1}$ in YPD and $0.40\ h^{-1}$ in AFM, unpublished data). Specific growth rates were slower with glycerol than with D-glucose as carbon source.

Since glycerol is becoming a considerable waste product of biodiesel production, the ability of *A. gossypii* to convert glycerol to ethanol is interesting. Although the yield of ethanol on glycerol was only 0.13 g $(g\ glycerol\ consumed)^{-1}$, it was comparable to that achieved with genetically engineered *S. cerevisae* ($2.4\ g\ l^{-1}$ at a yield of 0.14 g $(g\ glycerol\ consumed)^{-1}$ with a strain overexpressing glycerol dehydrogenase and dihydroxyacetone kinase; (Yu et al.

2010). Thus non-recombinant *A. gossypii* is as efficient at converting glycerol to ethanol as engineered *S. cerevisiae*, but would need further improvement to achieve the yields observed in bacterial systems.

A. gossypii strains grew well on chemically defined medium at high pH (e.g. 6.5), but had greater sensitivity to low pH than many filamentous fungi. D-glucose, glycerol and starch were all utilised as carbon sources and high specific growth rates (comparable to those of *S. cerevisiae*) were achieved in complex medium. Although D-xylose was not used as a carbon source, the presence of active D-xylose reductase was demonstrated.

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Chapter 3

Expression of *Trichoderma reesei* cellulases CBHI and EGI in *Ashbya gossypii*

Adapted from *Applied Microbiology and Biotechnology* (2010) 87:1437–1446

Abstract

To explore the potential of *Ashbya gossypii* as a host for the expression of recombinant proteins and to assess whether protein secretion would be more similar to the closely related *Saccharomyces cerevisiae* or to other filamentous fungi, endoglucanase I (EGI) and cellobiohydrolase I (CBHI) from the fungus *Trichoderma reesei* were successfully expressed in *A. gossypii* from plasmids containing the two micron sequences from *S. cerevisiae*, under the *S. cerevisiae PGK1* promoter. The native signal sequences of EGI and CBHI were able to direct the secretion of EGI and CBHI into the culture medium in *A. gossypii*. Although CBHI activity was not detected using 4-methylumbelliferyl-β-D-lactoside as substrate, the protein was detected by Western blot using monoclonal antibodies. EGI activity was detectable, the specific activity being comparable to that produced by a similar EGI producing *S. cerevisiae* construct. More EGI was secreted than CBHI, or more active protein was produced. Partial characterization of CBHI and EGI expressed in *A. gossypii* revealed overglycosylation when compared with the native *T. reesei* proteins, but the glycosylation was less extensive than on cellulases expressed in *S. cerevisiae*.

3.1 Introduction

The filamentous hemiascomycete *Ashbya gossypii* (Ashby and Nowell 1926) is a biotechnologically important producer of vitamin B2 (riboflavin; Demain 1972; Wickerham et al. 1946). Based on rDNA sequences, *A. gossypii* is more closely related to *Saccharomyces cerevisiae* than *Neurospora crassa* or *Aspergillus nidulans* (Wendland et al. 1999). *A. gossypii* has one of the smallest known eukaryotic genomes (Dietrich et al. 2004), a high homologous recombination efficiency allowing simple gene knockout strategies and precise positioning of gene constructs (Steiner et al. 1995; Wendland et al. 2000), extrachromosomal replication of plasmids bearing an autonomous replicator (Wright and Philippsen 1991), efficient introduction of heterologous DNA, and the fungus lacks the extensive duplication of chromosomal segments observed in *S. cerevisiae*, making it an extremely attractive candidate not only for riboflavin production, but also for the production of other industrial products such as recombinant proteins. However, little is known about the secretion of proteins by *A. gossypii*. Extracellular lipase has been detected, but activity was low in most conditions (Stahmann et al. 1997). An endo-β-1,4-glucanase (celA1) from *Streptomyces halstedii* has previously been expressed in *A. gossypii* with limited success (Althöefer et al. 2001). However, other filamentous fungi which do not secret large amounts of native enzymes/proteins have been found to still be good producers of recombinant proteins (Royer et al. 1995). Since *A. gossypii* is a filamentous fungus, it might be expected to have efficient protein secretion, but its close relationship to *S. cerevisiae* may suggest limited secretion ability. We therefore expressed two cellulase genes from the filamentous fungus *Trichoderma reesei* in *A. gossypii* in order to assess its ability to produce and secrete recombinant proteins.

T. reesei is one of the most extensively studied cellulolytic organisms (Kubicek et al. 1993). It produces three types of enzyme activities needed for degradation of crystalline cellulose into glucose. These are cellobiohydrolases (EC 3.2.1.91), which act as exoenzymes and release cellobiose as a main product from crystalline cellulose, endoglucanases (EC 3.2.1.4), which have high affinity towards soluble cellulose derivatives and attack these by endo-action, and β-glucosidases (EC 3.2.1.21), which hydrolyse cellooligosaccharides and the disaccharide cellobiose into glucose.

T. reesei CBHI and endoglucanase I (EGI) are useful as model proteins for recombinant protein secretion, since they have been extensively studied in a variety of expression systems, as well as in *T. reesei*, and appear to be challenging for other organisms to produce. Expression of CBHI in *Escherichia coli* (Laymon et al. 1996; Teeri 1987) and yeast (Godbole et al. 1999; Penttilä et al. 1988; Reinikainen et al. 1992) has resulted in the production of

either insoluble or low activity enzyme. Whereas *E. coli* produced misfolded CBHI peptide and/or CBHI inclusion bodies from which active enzyme could not be obtained, yeasts (*S. cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica*) produced hyperglycosylated cellulases with low activity on either soluble substrates or amorphous cellulose. Even when *T. reesei* cellulases have been expressed in the efficient protein producing fungus *Aspergillus oryzae*, the activity of both CBHI and CBHII toward Avicel were relatively low when compared with the native enzymes, possibly because of differences in the modes of glycosylation (Takashima et al. 1998). *Aspergillus niger* var. *awamori* (Jeoh et al. 2008) produced functional enzyme, but it was more glycosylated than the native enzyme. Reports of successful expression of *T. reesei* CBHI from non-*T. reesei* hosts have been limited to one example of insect cell (*Spodoptera frugipedra*) production, following baculovirus transfection (von Ossowski et al. 1997).

Heterologous expression of EGI from *T. reesei* has been reported in *S. cerevisiae* and *Y. lipolytica* (Park et al. 2000; Penttilä et al. 1987b; Van Arsdell et al. 1987). The recombinant EGI produced by *S. cerevisiae* was hyperglycosylated and significantly larger than the native enzyme produced by *T. reesei*. There were similarities in the extent of hyperglycosylation, but differences in the types of glycosylation between *Y. lipolytica* and *S. cerevisiae* (Park et al. 2000). Apart from the differences in the extent of asparagine-linked glycosylation, recombinant EGI from *Y. lipolytica* appeared to be processed in a similar manner to the processing of the native enzyme by *T. reesei*. EGI seems to be slightly easier than CBHI to produce in non-*Trichoderma* recombinant hosts.

In this paper, we evaluate the potential of *A. gossypii* as a host for recombinant protein production, using the *T. reesei* cellulases CBHI and EGI as model proteins. These proteins were chosen because they represent a challenge in recombinant protein production and have previously been expressed in several expression platforms. Because of the close genetic relationship between *A. gossypii* and *S. cerevisiae*, the comparison with expression in *S. cerevisiae* was of particular interest.

3.2 Materials and methods

3.2.1 Strains

A. gossypii ATCC10895 was obtained from Prof. P. Philippsen (Basel University) and maintained on agar (20 g l^{-1}) on *Ashbya* full medium (AFM; 10 g l^{-1} tryptone, 10 g l^{-1} yeast extract, 1 g l^{-1} myo-inositol, 20 g l^{-1} glucose). *A. gossypii* EGI (VTT D-101398) and *A. gossypii* CBHI (VTT D-101399) were obtained by transformation of ATCC10895 as described

below. *S. cerevisiae* H81 (α, *his3Δ1 leu2–3,112 ura3–52 trp1–289* Cyh^R) was used as host to obtain comparable transformants of *S. cerevisiae*.

Spores were prepared by collecting 7–10 day-old mycelium from agar solidified plates, digesting the mycelia with zymolyase (150 mg ml^{-1}) for 2 h and washing two times with a solution of 0.8% (w/v) NaCl, 20% (v/v) glycerol, and 0.025% (v/v) Tween 20. Aliquots were suspended in the same solution and were stored at −80°C.

3.2.2 Culture media

LB medium supplemented with 100 μg ampicillin ml^{-1} was used for *E. coli* cultivation. AFM supplemented with 200 μg ml^{-1} G418 was used for the selection and maintenance of *A. gossypii* transformants containing the G418 resistance gene (Dünkler and Wendland 2007; Wendland et al. 2000). For regeneration of protoplasts, AFM with 20 g agar l^{-1} was supplemented with 1 M sorbitol. To detect cellulase-producing strains, agar solidified AFM was supplemented with 0.1% (w/v) barley β-glucan or 0.1% (w/v) carboxymethylcellulose (CMC). Synthetic complete defined medium (SCD) contained complete amino acid supplement (Sherman et al. 1986), yeast nitrogen base (Difco), 20 g l^{-1} glucose, 1 g l^{-1} $CaCo_3$, and 1 g l^{-1} agar.

3.2.3 Plasmid construction

Plasmid pMI519 (Figure 3.1 a) contained *T. reesei egl1* cDNA and plasmid pMI514 (Figure 3.1 b) *T. reesei cbh1* cDNA. Plasmids pMI519 and pMI514 were constructed from plasmids pTTc11 (Penttilä et al. 1987b) and pTTc1 (Teeri et al. 1987), respectively, together with B609 (Ruohonen et al. 1995), using the primers listed in Table 3.1. Both genes were under the control of the *S. cerevisiae PGK1* promoter and terminator obtained from plasmid pAJ401 (Saloheimo et al. 1994). The *kanMX* expression module was derived from a modified pUG6 vector, with the *kan*^{r} gene from the *E. coli* transposon *Tn903* under control of the *TEF* promoter from *A. gossypii* (Güldener et al. 1996; Wach et al. 1994) and the *ADH1* terminator from *S. cerevisiae*, to confer to the transformants resistance to the aminoglycoside antibiotic G418 (Jimenez and Davies 1980; Webster and Dickson 1983).

The control vector pMI516 without a cellulase insert was constructed by digesting pMI514 with *Pac*I–*Asc*I, filling in the ends using T4 polymerase and circularizing the 6.8 kb fragment.

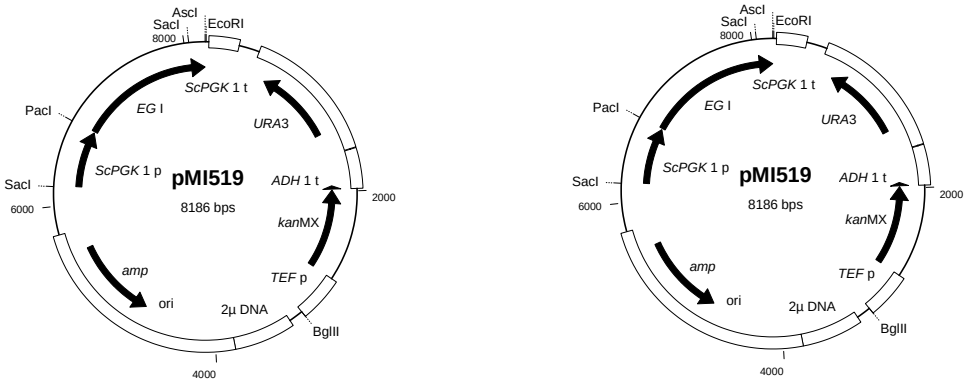


Figure 3.1 – Plasmids for expression of *T. reesei* CBHI or EGI in *A. gossypii*. cDNA was inserted under the control of the PGK promoter and terminator from *S. cerevisiae*.

Table 3.1 – Oligonucleotides used for construction of plasmids and strain screening

Oligonucleotide	Sequence
373 EGL1 WT EcoRI-PacI-ATG	GCGTT*GAATTC**TTAATTAA***ACAATGGCGCCCTCAGTTACACT
374 EGL1 WT TAG-AscI-EcoRI	GCGAT*GAATTC**GGCGCGCC***CTAAAGGCATTGCGAGTAGTAGTCG
379 ScPGK1prom-786 SacI + ApaI	GCGTT*GAGCTC**GGGCCC***TAATTTTTATTTTAGATTCCTGACTTCAAC
380 ScPGK1prom EcoRI-PacI	GCGTT*GAATTC**TTAATTAA***GTAAAAAGTAGATAATTACTTCCTTG
387 CBH1 WT EcoRI-PacI-ATG	GCGTT*GAATTC**TTAATTAAA***CAATGTATCGGAAGTTGGCCGTCATCTC
392 CBH1 WT TAA-AscI-EcoRI	GCGAT*GAATTC**GGCGCGCC***TTACAGGCACTGAGAGTAGTAAGG

The restriction sites used for the construction of plasmids are highlighted in *bold* and *underlined*

3.2.4 *A. gossypii* transformation and screening of transformants

Transformation of *A. gossypii* was carried using protoplasts as described by Penttilä et al. (1987a), and selection was carried out on medium containing G418. Spores from single colonies were collected as described above and stored at −80 °C for further analysis.

Transformants were screened in two ways. Initial screening was carried out by PCR using specific primers (Table 3.1). Template DNA was extracted by a rapid-miniprep method in which a piece of mycelium was transferred to a microfuge tube and lysed with lysis buffer (400 mM Tris–HCl pH 8.0; 60 mM EDTA, pH 8.0; 150 mM NaCl; 1% w/v SDS) at room temperature for 10 min. Proteins were precipitated by adding 150 µl of 3 M potassium acetate pH 4.8, vortexing and centrifuging at 13,000×g for 1 min. The supernatant was transferred to

a clean tube, and the DNA was precipitated by adding an equal amount of isopropanol. The sample was mixed by inverting and centrifuged at 13,000×g for 2 min. The DNA was washed with ethanol 70% (v/v) and air-dried, before being dissolved in 50 μl TE buffer. PCR products were amplified with polymerase DNA2 MGII (Finnzymes) using 5 μl sample DNA with, F5 buffer (Finnzymes), 2 μl 2 mM dNTP mix, 0.2 μl oligo (5 nmol μl^{-1}) EGI, CBHI, or G418-specific primers.

After the initial screening, mycelia from several colonies were grown in test tubes containing 5 ml AFM supplemented with 200 μg ml^{-1} G418 and samples of the culture medium assayed for secreted EGI or CBHI using 4-methylumbelliferyl-β-D-lactoside (MULac, Sigma) as the substrate. Based on these results, two strains, VTT D-101398 (expressing EGI) and VTT D-101399 (expressing CBHI), were selected for further study.

S. cerevisiae H81 was transformed with the same plasmids as *A. gossypii* using the LiAc transformation method (Gietz et al. 1995) and screened in the same way as *A. gossypii* transformants.

3.2.5 Culture conditions

Transformed strains of *A. gossypii* were grown at 30°C or 24°C in flasks (250 ml flasks containing 50 ml medium) at 200 rpm on AFM or SCD medium with 20 g l^{-1} glucose as carbon source and 200 μg ml^{-1} G418. Samples were collected to determine cell concentration (absorbance measured at 600 nm) and to obtain supernatant by filtration through glass fiber GF/C filters or by centrifugation (10 min at 4,000×g and 4°C).

Dry weight was determined by converting OD at 600 nm with a conversion factor obtained from a calibration curve, where one unit OD at 600 nm was found to correspond to 1.3 g l^{-1} DW for *A. gossypii* and 3.9 g l^{-1} DW for *S. cerevisiae* strains.

3.2.6 Cellulase activity on soluble substrates and insoluble substrates

Cellulase activities were determined using soluble MULac as substrate. The reaction consists in adding 50 μl MULAc (4 mM in DMSO) to 50 μl culture supernatant and incubating at room temperature. Reactions were stopped after 30 min incubation by adding 100 μl of 1 M Na_2CO_3, and liberation of 4-methylumbelliferone (MU) was detected by fluorescence measurement (excitation wavelength=355 nm and emission wavelength=460 nm) with a Varian Varioscan spectrofluorometer in a black, flat bottom microtiterplate. MU (Sigma) was used as a standard. Volumetric enzyme activity was measured as micromoles of MU formed

per minute per liter under the assay conditions. Specific activity is given as micromoles MU formed per minute per gram dry weight mycelium. Protein concentrations in the cell-free broth were measured with Bio-Rad protein reagent, using bovine serum albumin as standard.

Cellulase activity was also detected by the presence of a yellow halo after Congo red staining of CMC or β-glucan containing agar solidified medium, according to the method of Teather and Wood (1982) using 0.1% (w/v) CMC or 0.1% (w/v) β-glucan. To detect activity on agar solidified medium, 10 μl of sample was loaded into small wells punctured in the agar and the Petri dishes were incubated at 30°C for 20 h. Supernatants were first filtered through 0.22-μm pore size nylon filters, concentrated, and washed with 50 mM sodium acetate buffer pH 5.0 in 10 kDa Vivaspin concentrators (Sartorius). After 20 h, the Petri dishes were flooded with Congo red (0.1% w/v) for 1 h and washed with 1 M NaCl.

3.2.7 Binding to Avicel

For qualitative analysis of binding, 50 μl culture supernatant containing 0.6 mg of total protein, previously concentrated thirty-fold, were mixed with 12 mg Avicel in 50 mM sodium acetate pH 5.0. After incubation at 4°C overnight with end-over end rotation, the mixtures were centrifuged at 13,000×g for 5 min to sediment the substrate and bound proteins. The cellulose was washed with 50 mM sodium acetate pH 5.0 and then with 50 mM sodium acetate, pH 5.0, containing 1 M NaCl. The cellulose with bound proteins was mixed with 50 μl SDS sample buffer (250 mM Tris–HCl pH 6.8; 5% v/v glycerol; 10 mg ml^{-1} SDS; 2.5 % v/v β-mercaptoethanol; 0.05 mg ml^{-1} bromophenol blue) at 95°C for 3 min, and the supernatant was analyzed by SDS-PAGE in 12% (w/v) gels, followed by Western blot.

3.2.8 Enzymatic deglycosylation

To determine whether the recombinant cellulases were *N*-glycosylated, the proteins were digested with endoglycosidase H (Endo H, Roche) and PNGAseF (New England Biolabs) according to the manufacturer's instructions. In control samples, enzyme was replaced by water. Samples were incubated overnight, separated by 12% (w/v) SDS-PAGE, and visualized by Western blot.

3.2.9 Zymograms

Zymograms were made according to the general procedure of Flint et al. (1994), to visualize enzyme activity and estimate approximate molecular weight. The stacking gel was

4% (w/v) polyacrylamide, and the separating gel contained 12% (w/v) polyacrylamide with 0.1% (w/v) CMC (Sigma) added prior to polymerization. After electrophoresis, the gels were washed in 1% (v/v) Triton X100 (32×200 ml, 20 min per wash), soaked in sodium phosphate buffer (50 mM, pH 6.5) to allow renaturation of the enzymes (2 h at 4°C), and then incubated overnight at 37°C in sodium phosphate buffer (50 mM, pH 6.5). Following incubation, the gels were stained with 0.1% (w/v) Congo red for 1 h, and then destained with sodium chloride (1 M) for a further 1 h.

3.2.10 SDS-PAGE and Western blot analyses

Proteins were separated using denaturing SDS-PAGE with 12% (w/v) gels, as described by Laemmli (1970). Proteins were blotted onto 0.45 micron nitrocellulose membrane (Hybond) and assayed with monoclonal antibodies specific for *T. reesei* CBHI and EGI (Aho et al. 1991). The nitrocellulose membrane was blocked for 1 h in TBS (10 mM Tris–HCl pH 8.0; 150 mM NaCl) containing 5% (w/v) non-fat milk powder and 0.01% (v/v) Tween 20 (Sigma). After washing with TBST (10 mM Tris–HCl pH 8.0; 150 mM NaCl; 0.05% v/v Tween 20), the monoclonal antibody was added. Goat anti-mouse Fc labeled with alkaline phosphatase was used as secondary antibody and BCIP/nitro-blue tetrazolium chloride substrate (Bio-Rad Laboratories, Hercules, CA) was used to visualize CBHI and EGI. The reaction was stopped by washing with distilled water and drying at room temperature. All washes and incubations were conducted at room temperature with gentle shaking.

3.2.11 Nucleotide sequence accession numbers

The nucleotide sequence for EGI is in GenBank database under accession number M15665 and CBHI under the number E00389.

3.3 Results

3.3.1 Transformation and heterologous expression of EGI and CBHI in *A. gossypii*

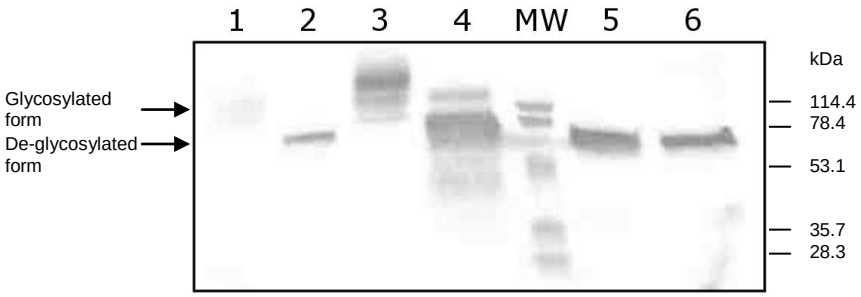
In order to express *T. reesei* cellulases CBHI and EGI in *A. gossypii*, protoplasts were transformed with plasmids pMI514, pMI519, and pMI516, with a transformation efficiency of 70–80 transformants per microgram plasmid DNA.

Transformants with the highest cellulase activity, based on the MULac assay as described in Material and methods, were grown in batch cultures in rich AFM and SCD medium at 30°C or 24°C at 200 rpm. Production of cellulases did not reduce the specific growth rate of the recombinant strains, i.e., expression of EGI (or CBHI) did not appear to impose a metabolic load on the host. *A. gossypii* CBHI had a specific growth rate of 0.12±0.01 h^{-1}, *A. gossypii* EGI of 0.11±0.01 h^{-1} and the negative control of 0.09±0.00 h^{-1} in AFM containing 20 g l^{-1} glucose in flask cultures. Similarly, the production of *T. reesei* EGI did not reduce the specific growth rate of *S. cerevisiae* (Penttilä et al. 1987b).

Western blot analyses (Figures 3.2 and 3.3) using monoclonal antibodies raised against the catalytic domain of the EGI or CBHI protein confirmed that *T. reesei* EGI (Figure 3.3) and CBHI (Figure 3.2) were present in the supernatant. CBHI was only detected by Western blot after removal of *N*-linked glycans, concentrating the protein to a single band (Figure 3.2 a, lane 2 and Figure 3.2 b, lane 4), demonstrating that the amount of secreted protein was very low.

Growth temperature may affect expression level, protein solubility, protein degradation, or cell viability, such that expression at a lower temperature increases the yield of the recombinant protein (Li et al. 2001; Shi et al. 2003). In *A. gossypii*, cellulase expression was not enhanced when the temperature was lowered from 30°C to 24°C (data not shown) either in rich AFM or in SCD medium.

(a)



(b)

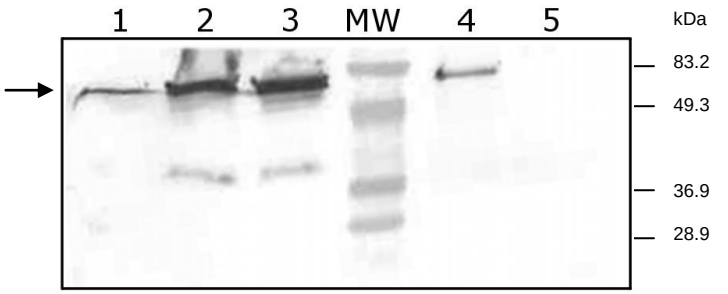


Figure 3.2 – Western blot analysis of recombinant CBHI treated with (a) endoglycosidase H or (b) PNGase F. (a) Lane 1, *A. gossypii* recombinant CBHI before Endo H treatment; lane 2, *A. gossypii* recombinant CBHI after Endo H treatment; lane 3, *S. cerevisiae* recombinant CBHI before Endo H treatment; lane 4, *S. cerevisiae* recombinant CBHI after Endo H; MW molecular weight standard (sizes given at right); lane 5, *T. reesei* CBHI before Endo H treatment; lane 6, *T. reesei* CBHI after Endo H treatment. (b) Lane 1, *T. reesei* CBHI without treatment; lane 2, *T. reesei* CBHI after PNGaseF treatment; lane 3, *T. reesei* CBHI incubated with water instead of PNGaseF; lane 4, *A. gossypii* CBHI after PNGaseF treatment; lane 5, *A. gossypii* CBHI without PNGaseF treatment. Lanes were loaded with 130 μg total protein. Protein bands are indicated with arrows.

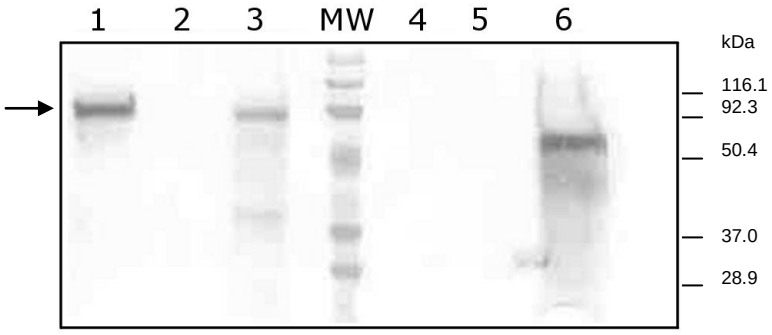


Figure 3.3 – Binding of EGI to Avicel. Six hundred micrograms of total protein was mixed with Avicel as described in Materials and methods. Legend: lane 1, recombinant protein that did not bind to Avicel; lane 2, washing Avicel with sodium acetate buffer 50 mM, pH 5.0 and 1 M NaCl; lane 3, EGI eluted from Avicel; MW, molecular weight standard (sizes given at right); lane 4, supernatant of *T. reesei* EGI after incubation with Avicel; lane 5, washing Avicel with sodium acetate buffer 50 mM, pH 5.0, and 1 M NaCl; lane 6, *T. reesei* EGI eluted from Avicel. Protein bands are indicated with arrows.

3.3.2 Activity of recombinant cellulases produced in *A. gossypii*

Several methods were used to detect and evaluate whether the recombinant EGI and CBHI expressed in *A. gossypii* were in an active form. Secretion of active EGI was demonstrated by Congo red staining after incubation of concentrated sample of culture supernatant in CMC (Figure 3.4 a) or β-glucan (Figure 3.4 b) Petri dishes. The presence of a clearing zone indicated endoglucanase activity due to degradation of the substrate.

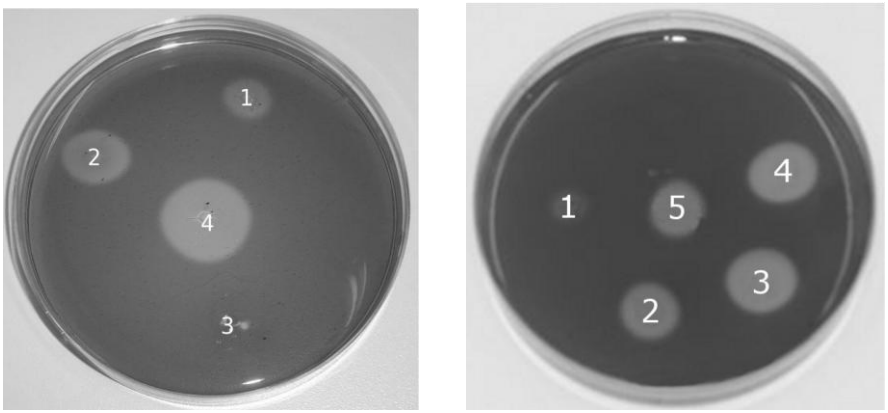


Figure 3.4 – Plate assay for detection of activity towards (a) carboxymethylcellulose (CMC) and (b) β-glucan. Thirty times concentrated protein samples, containing 60–120 μg of total protein were loaded in small wells and stained with Congo red after 20 h incubation at 30°C. (a) Wells contained ***1*** supernatant from *A. gossypii* expressing EGI grown in AFM; ***2*** supernatant from *S. cerevisiae* expressing EGI; ***3*** supernatant from the *A. gossypii* negative control strain; ***4*** native *T. reesei* EGI purified protein (5 μg). Or (b) wells contained ***1*** supernatant from *A. gossypii* expressing EGI grown in SCD medium; ***2*** supernatant from *A. gossypii* expressing EGI grown in AFM; ***3*** and ***4*** supernatant from two clones of *S. cerevisiae* expressing EGI; ***5*** native *T. reesei* EGI purified protein (5 μg).

Zymograms containing 0.1% (w/v) CMC in the resolving gel confirmed that EGI secreted by *A. gossypii* was active against CMC (Figure 3.5, lane 5–6). Cellulases have a bimodal nature, with a cellulose binding domain (CBD) as well as an active site. We confirmed that the carbohydrate binding module was also present in the *A. gossypii* recombinant protein by incubation of recombinant EGI with Avicel. The protein was eluted with SDS-PAGE sample buffer and detected by Western blot analysis (Figure 3.3, lane 3), confirming that both the cellulose binding domain and the active catalytic domain were present. CBHI activity was not detected in the plate assay or in zymograms containing 0.1% (w/v) Avicel, confirming that less CBHI was secreted than EGI or that the secreted protein was not active.

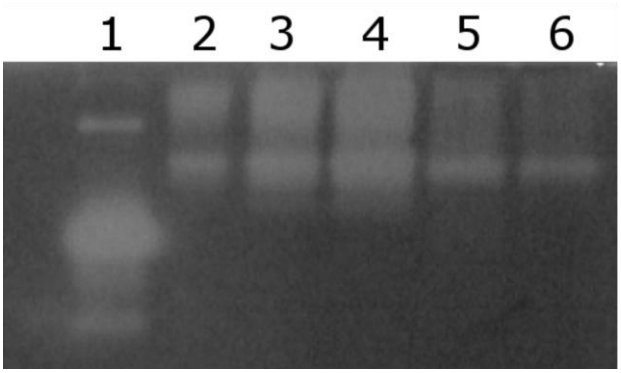


Figure 3.5 – Zymogram containing 0.1% (w/v) CMC incorporated in the SDS-PAGE gel, stained with Congo red, and washed with 1 M NaCl. Legend: lane 1, 80 ng of purified *T. reesei* EGI; lane 2, *S. cerevisiae* recombinant EGI, 70 h after inoculation three times concentrated (1.2 μg total protein); lane 3, *S. cerevisiae* recombinant EGI 15 times concentrated (6 μg total protein); lane 4, *S. cerevisiae* recombinant EGI 30 times concentrated (12 μg total protein); lane 5, supernatant from a culture of *A. gossypii* expressing EGI, 62 h after inoculation (11 μg total protein); and lane 6, supernatant from an *A. gossypii* EGI producing culture, 43 h after inoculation (6 μg total protein). *A. gossypii* samples were concentrated thirty-fold.

Heterologous expression of CBHI and EGI was also analyzed by measuring the activity on the soluble substrate MULac (Figure 3.6). EGI activity in *A. gossypii* supernatant was maximal (400 μmol min^{-1} l^{-1}, 1.3 nmol min^{-1} μg^{-1} secreted protein) after 50 h growth in batch culture (Figure 3.6 a), at the end of the decelerating growth phase. In *S. cerevisiae*, maximal activity of 1,000 μmol min^{-1} l^{-1} (2.2 nmol min^{-1} μg^{-1} secreted protein) was detected after 60 h (Figure 3.6 b), also as the cells entered stationary phase. Specific EGI production (activity per gram biomass) for both organisms was highest (200–450 μmol min^{-1} g^{-1} dry weight) during exponential growth, as expected for proteins produced under the *PGK* promoter (Figure 3.6 c). CBHI activity was not detectable in *A. gossypii* cultures with the MULac assay (Figure 3.6 a), but *S. cerevisiae* produced a maximum of 348 μmol min^{-1} l^{-1} (1 nmol min^{-1} μg^{-1} secreted protein; Figure 3.6 b). Although protease activity was negligible in the culture supernatant of *A. gossypii* cultures (data not shown), EGI appeared to be degraded during the stationary phase (Figure 3.6), probably when cell lysis occurred, and intracellular proteases were released into the culture medium, as has sometimes been observed for recombinant proteins in other fungi (Archer et al. 1992; Broekhuijsen et al. 1993; Roberts et al. 1992; van den Hombergh et al. 1997).

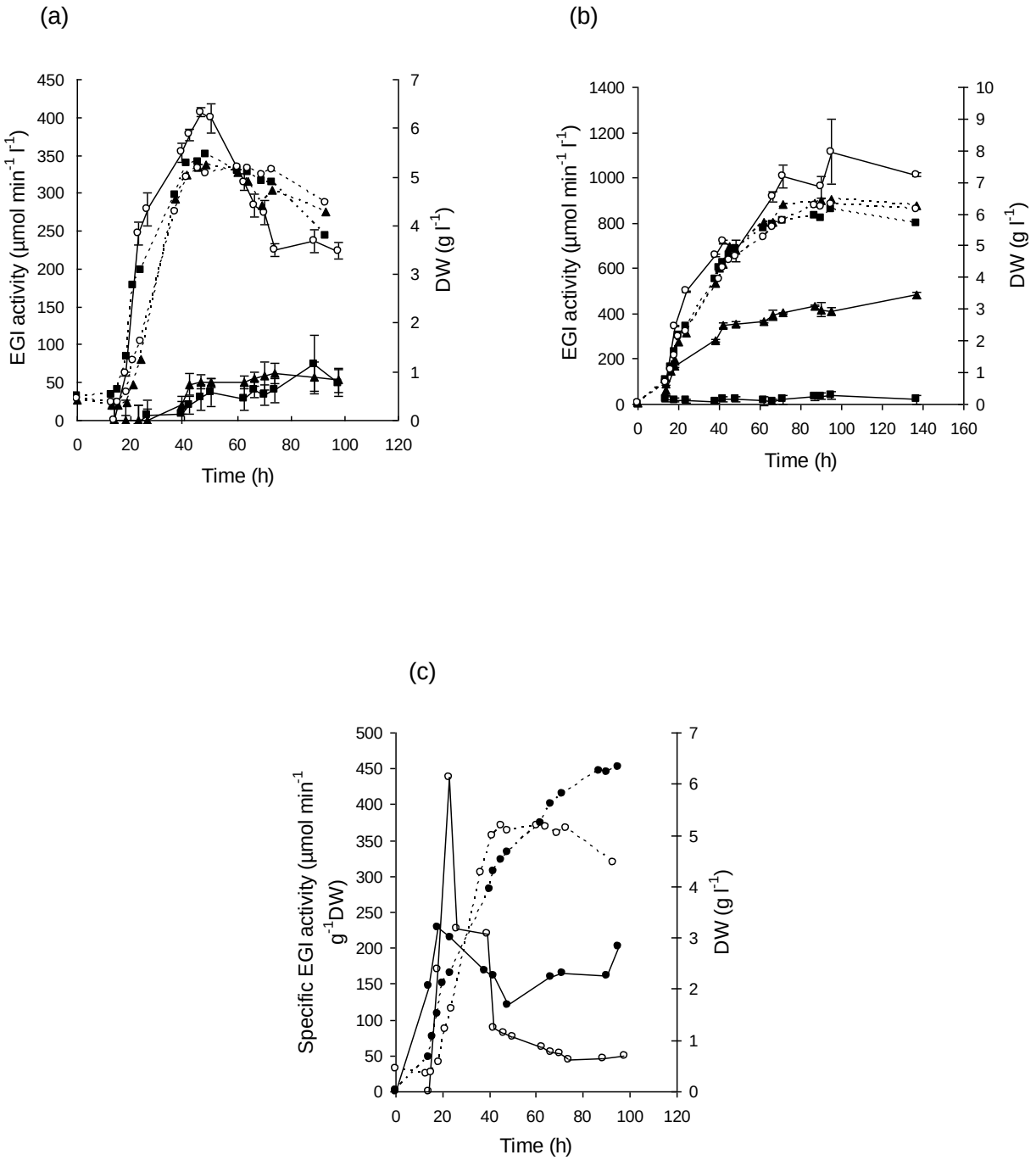


Figure 3.6 – Average volumetric cellulose activities and dry weight (right axis) from supernatant of *A. gossypii* (a) and *S. cerevisiae* (b) growing in flasks in AFM at 30°C, 200 rpm. Activities were measured with the MULAc assay, described in Materials and methods. Data represent average ± standard error of the mean obtained from three independent cultures. Strains were transformed with plasmids with genes for (solid triangle) CBHI, (open circle) EGI, or (solid square) an empty vector as the negative control. Dashed lines show dry weight (grams per liter) for the corresponding strain. (c) *A. gossypii* (open circle)

and *S. cerevisiae* (solid circle) EGI specific activity per gram biomass during growth, represented as dry weight (dashed line).

3.3.3 Partial characterisation of recombinant cellulases secreted by *A. gossypii*

Zymogram analysis revealed that EGI secreted by *A. gossypii* had a higher molecular weight than the native enzyme from *T. reesei* (Figure 3.5, lane 1), but was similar to the recombinant EGI in *S. cerevisiae* (Figure 3.5, lane 2–4).

Even though N-terminal sequence was not evaluated, the 20 amino acids composing the signal peptide sequence, being too small, would not account for the band shift observed in the zymogram (Figure 3.5) or Western blot analysis (Figures 3.2 and 3.3). The higher molecular weight indicated that the protein secreted by *A. gossypii* was probably glycosylated, as shown in Figures 3.2 and 3.3, in which endoglycosidase treatment was used to remove glycans attached to the protein. Comparing lanes 2 and 5 of Figure 3.5, *S. cerevisiae* appeared to produce more of the most highly glycosylated EGI than *A. gossypii* produced. After treating EGI from *A. gossypii* with PNGaseF (Plummer and Tarentino 1991), a minor band shift occurred (data not shown). PNGaseF was used to deglycosylate EGI produced in *A. gossypii* because it is known that EGI has a variety of glycosylation sites and that these have different susceptibilities to Endo H treatment. For example, Endo H is not able to completely deglycosylate recombinant EGI produced by *S. cerevisiae* (Penttilä et al. 1988; Van Arsdell et al. 1987). The reduction in molecular weight of *A. gossypii* produced EGI following enzymatic treatment was strong evidence that the recombinant enzyme was glycosylated (Trimble and Maley 1984; Maley et al. 1989), but not as extensively as EGI produced in *S. cerevisiae*.

Although only small amounts of CBHI were produced by *A. gossypii*, Western blot analysis after glycan removal, demonstrated that the CBHI produced by *A. gossypii* was also hyperglycosylated (Figure 3.2 a, lane 2 and Figure 3.2 b lane 4).

Hyperglycosylation was not as extensive as that observed in CBHI produced by *S. cerevisiae*, since both PNGaseF (Figure 3.2 b) and Endo H were able to deglycosylate the *A. gossypii* recombinant protein, resulting in a protein of similar molecular weight to the native *T. reesei* CBHI. In contrast, Endo H was only able to partially deglycosylate recombinant CBHI produced by *S. cerevisiae* (Figure 3.2 a, lane 4).

3.4 Discussion

The filamentous fungus *A. gossypii* has been used in the industrial production of the vitamin riboflavin. The experimental tools available for use in *A. gossypii* together with its small genome and the regularity of filamentous growth and hyphal branching make it suitable for extensive study of polar (filamentous) growth. In this study, we take advantage of the same distinctive features to express and secrete heterologous cellulases under the *S. cerevisiae PGK1* promoter with *T. reesei* native signal peptides in *A. gossypii*. The transformation method used here was efficient, compared with that described by Kato and Park (2005), in which only ten to 20 transformants per microgram of plasmid DNA were obtained. Plasmids containing the 2-μm replication origin were also used. Plasmids containing autonomously replicating sequence (ARS) such as the 2-micron plasmid replication origin and chromosomal replication origin (ARS1) from *S. cerevisiae* replicate freely in *A. gossypii* in contrast to lack of replication in other filamentous ascomycetes (Wright and Philippsen 1991), resulting in maintenance of higher copy number of the recombinant DNA than is generally obtained by integration.

Several aspects should be taken into account in recombinant proteins production, namely the promoter, signal sequence, culture conditions, and host genetic background. The most commonly used promoters in *A. gossypii* are AgGPD (Jiménez et al. 2005) and AgTEF (Kanamasa et al. 2007; Kato and Park 2005). Recently, Dünkler and Wendland (2007) reported the use of *S. cerevisiae* and *A. gossypii MET3* promoters (regulated by methionine), contributing to the molecular toolbox available for *A. gossypii*. To our knowledge, this is the first time that the *S. cerevisiae PGK1* promoter has been used in *A. gossypii*. The PGK promoter is growth-related, and thus, protein production would be unlikely to be affected by riboflavin production, which occurs primarily after substrate depletion (Karos et al. 2004). The recombinant EGI and CBHI, carrying the heterologous, native signal sequences, entered the secretory pathway of *A. gossypii*, and functional EGI was found in the extracellular medium. EGI activity was detectable against both soluble and insoluble substrates. This was comparable to the endoglucanase activity against CMC illustrated by Althöefer et al. (2001) for *A. gossypii* expressing the endo-β-1,4-glucanase from *S. halstedii*. *A. gossypii* was not able to secrete CBHI protein to the same extent as EGI. CBHI production is always lower than other cellulases, including CBHII (Penttilä et al. 1988), possibly because the formation of disulfide bridges is important for correct folding (Boer et al. 2000). These results demonstrated the protein secretion by *A. gossypii* is comparable to *S. cerevisiae* and that a filamentous growth form is not sufficient to ensure high levels of protein secretion.

Both CBHs and EGs are glycoproteins containing heterogeneous *N*- and/or *O*-linked glycans (Kubicek 1992) which may result in multiple protein bands in Western analysis. Glycosylation plays a role in the secretion of these cellulolytic enzymes, providing not only the optimal distance between the core and CBD but protecting the linker peptide from proteolytic attack (Clarke 1997; Srisodsuk et al. 1993). Similar to what has been observed in other host microorganisms, both EGI and CBHI were glycosylated by *A. gossypii*. The glycosylation was less extensive than in *S. cerevisiae*. A limitation in the extent of glycosylation is an advantage in the production of heterologous proteins whose properties may be adversely affected by extensive glycosylation. *S. cerevisiae*, in particular, forms high-mannose glycoproteins (Dean 1999; Gemmill and Trimble 1999). Filamentous fungi also have mannose rich glycans, but of more limited size than *S. cerevisiae* (Deshpande et al. 2008; Maras et al. 1997; Stals et al. 2004). In addition, filamentous fungi are known to secrete different glycosidases to the extracellular medium that may trim protein-linked glycans, adding heterogeneity to the glycan structure (Maras et al. 1999). Although *A. gossypii* is more closely related to *S. cerevisiae* than to other filamentous fungi, it appears to be able to limit the extent of protein glycosylation. Further analysis would be required to unveil the glycosylation pattern and degree of glycosylation performed by *A. gossypii*.

Although high levels of protein secretion are generally associated with filamentous growth, *A. gossypii* was more similar to the closely related yeast *S. cerevisiae* than to other filamentous fungi in its ability to produce and secrete EGI, demonstrating that filamentous growth alone is not sufficient to ensure good protein secretion. In comparison with the yeast *S. cerevisiae*, *A. gossypii* showed similar specific production of EGI, but less of CBHI. The expression of recombinant cellulases in *A. gossypii* provides opportunity for additional insights on the relationship between protein secretion and the filamentous growth form, while, as with *S. cerevisiae*, the low levels of protein secretion observed here do not necessarily limit the future development of *A. gossypii* as a heterologous protein production host.

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Chapter 4

Random and direct mutagenesis to enhance protein secretion in *Ashbya gossypii*

Abstract

To improve the general secretion ability of the biotechnologically relevant fungus *Ashbya gossypii*, random mutagenesis with ethyl methane sulfonate (EMS) was performed. The selection and screening strategy followed revealed mutants with improved secretion of EGI, amylase and/or β-glucosidase activities. One mutant, S436, presented 1.4 to 2 fold increases in all extracellular enzymatic activities measured, when compared to the parent strain, pointing to a global improvement in protein secretion. Four other mutants showed 2 to 3 fold improvements in only one (S397, B390) or two (S466, S436) of the measured activities.

A targeted genetic approach was also followed. Two homologs of the *GAS1* gene, which encodes β-1,3-glucanosyltransglycosylase involved in cell wall assembly, were deleted from the *A. gossypii* genome. Higher permeability of the cell wall was expected to increase the protein secretion capacity. However, secreted EGI activity did not increase in the Δ*AgGAS1a* single deletion strain, while *AgGAS1b* deletion resulted in strains exhibiting severe retardation of growth and, thus, secretion could not be tested in the Δ*AgGAS1b* single deletion and Δ*AgGAS1a* Δ*AgGAS1b* double deletion strains.

4.1 Introduction

Because of their natural ability to secrete high amounts of extracellular proteins, filamentous fungi have been extensively exploited for the production of homologous and heterologous proteins. Although homologous protein production can reach the level of hundreds of grams per liter, the production levels of heterologous proteins can be several orders of magnitude lower (Cherry and Fidantsef, 2003; Punt et al., 2002). In the search to further improve the properties of fungi as protein producers, many strategies have been employed and optimized, including signal sequence optimization, co-expression of chaperones and foldases, genetic modifications which improve secretion capabilities, such as the knockout of specific genes, reduction of proteolytic activity (Chow et al., 1992), or optimization of fermentation conditions, the use of strong promoters and mutagenesis, among others (Jeenes et al., 1991; Archer et al., 1994; Punt et al., 1994; Gouka et al., 1997; Nakari-Setala et al., 2009; Nemoto et al., 2009; Meyer and Punt, 2010). Improvement of secretion of extracellular enzymes by the application of random mutagenesis and screening has been successfully carried out with various filamentous fungi (Bailey and Nevalainen, 1981; Mattern et al., 1992). Many of the high-secreting mutants are used currently for industrial production of fungal enzymes and applied as expression hosts for recombinant gene products (Mäntylä et al., 1998).

It is a routine practice to develop mutants through random mutagenesis. Strain improvement by random mutagenesis is a successful method, but it is mainly a trial-and-error process, involving laborious procedures. Moreover, in many cases improved performance is a black box, and the underlying mechanism is not easily identified (Baker, 1991). The main effect of mutagenic agents (X-rays, UV-rays, nitrous acid, dimethyl sulphonate, ethyl methane sulfonate (EMS) and acridine mustards) relies on creating a lesion or a modification of the base sequence of the DNA molecule. If this lesion is not repaired a mutation is caused (Devehand and Gwynne, 1991). Mutagenic procedures can be optimized in terms of type of mutagen, dose and temperature. The nature of mutagenic agent is such that changes are not directed exclusively at loci which will generate beneficial change, requiring the screening of large numbers of strains for the desired phenotypes. Different methods have been used to introduce random mutations in different microorganisms (Takahashi et al., 2001), including mutagenesis using nitrous acid, hydroxylamine, ultraviolet radiation (UV), transposons, and EMS (Walton et al., 1991). EMS is an alkylating agent that induces point mutations by A-T transition to G-C (French et al., 2006). This mutagenic agent has been used to increase glucose oxidase activity (Fiedurek and Gromada, 1997), phytase (Chelius and Wodzinski, 1994), pectinase (Minjares-Carranco et al., 1997), catalase (Fiedurek and Gromada, 1997),

lipase (Mala et al., 2001), laccase (Weenink et al., 2006) and citric acid production in *Aspergillus niger* (Khattab and Bazaraa, 2005; Lotfy et al., 2007), as well as to improve recombinant strains of *Saccharomyces cerevisiae* (Lussier et al., 1997) and *Trichoderma viride* (Mandels et al., 1971), among others.

Secretion of a heterologous protein often represents a major bottleneck. Once a protein has been released out of the ER–Golgi system, it has to cross the cell wall. Mutations in genes involved in the construction and in the maintenance of the cell wall, such as *PMR1*, *SEC14*, *ERD1*, *MNN9* and *MNN10*, have in some cases been demonstrated to lead to supersecreting mutants in *S. cerevisiae* and other yeasts (Bartkeviciute and Sasnauskas, 2004). In addition, the deletion of the cell wall cross-linking enzyme glycophospholipid-anchored surface (Gas1) protein led to an almost seven-fold increase in the level of human insulin-like growth factor 1 (hIGF1) in *S. cerevisiae* (Vai et al., 2000). In *Pichia pastoris* deletion of the *GAS1* homolog resulted in a supersecreting phenotype for some heterologous proteins, like a yeast lipase, but not for others, like human trypsinogen (Marx et al., 2006). More recently, deletion of the *Zygosaccharomyces bailii GAS1* homolog almost doubled the amount of enzymatic activity found in the medium in the case of the *Candida rugosa* lipase CRL1 and of the *Yarrowia lipolytica* protease XPR2, while for human IL-1b secretion disruption had no relevant effect (Passolunghi et al., 2010).

Ashbya gossypii is a filamentous hemiascomycete (Tanner et al., 1949; Kurtzman, 1995); with a natural capacity to produce riboflavin (vitamin B2) (Wickerham et al., 1946; Demain, 1972), which is used for industrial riboflavin production.

The potential of *A. gossypii* as a host for recombinant protein production was previously evaluated by expressing cellobiohydrolase I (CBHI) and endoglucanase I (EGI) from *Trichoderma reesei* (Ribeiro et al., 2010). However, the secretion levels obtained were very low, particularly in the case of CBHI (Ribeiro et al., 2010). Here, we describe the improvement of the general secretion capability of *A. gossypii*. Two approaches were used: random mutagenesis with EMS and direct mutagenesis by individual deletion of both copies of the *GAS1* homolog (open reading frames *AGL351W*, *AgGAS1a*, and *AGL352W*, *AgGAS1b*) from the genome.

4.2 Material and methods

4.2.1 Strains

Recombinant *A. gossypii* VTT D-101398 strain previously constructed (Ribeiro et al. 2010) was used throughout this study. *A. gossypii* ATCC10895 (kindly provided by Prof. P. Philippsen) was used for gene deletion experiments.

4.2.2 Media

Ashbya Full Medium (AFM; 10 g l^{-1} peptone, 10 g l^{-1} yeast extract, 1 g l^{-1} myo-inositol plus a carbon source) was used for growth of *A. gossypii*. Starch (10 g l^{-1}), sucrose (20 g l^{-1}), carboxymethyl cellulose (CMC) (1 g l^{-1}) or tributyrin (10 ml l^{-1}) were provided as carbon sources for mutants screening. Media were solidified with agar (20 g l^{-1}).

4.2.3 Mutagenesis and selection

Mutations were induced in *A. gossypii* VTT D-101398 (recombinant strain expressing EGI from *T. reesei*, (Ribeiro et al., 2010) with EMS. Several concentrations of EMS and different incubation periods were tested to determine a treatment suitable for 90 - 99% killing. Based on the results, mutagenesis was subsequently carried out by diluting a spore suspension of *A. gossypii* ten times in sodium phosphate buffer and incubating with 5% (v/v) of EMS for 90 minutes at room temperature with shaking. Mutagenized spores were diluted in 0.9% NaCl and plated on agar-solidified AFM medium containing 0.1% CMC plus G418 (200 µg ml^{-1}) in order to keep the plasmid containing the recombinant EGI. A control was treated with water instead of EMS. Colonies appeared after 3 days.

For rapid screening of EGI production, mutants and the control strain of *A. gossypii* were incubated for 48 h on agar medium containing 0.1% (w/v) CMC as the sole carbon source and then flooded with 1% (w/v) Congo red solution according to the method of (Teather and Wood, 1982). From this procedure, zones of CMC hydrolysis were clearly visible indicating cellulase production and its activity on the substrate (CMC). Mutants were selected on the basis of clearing zone size after the Congo red treatment. Before flooding the plate with Congo red, colonies were transferred to agar-solidified AFM plus G418, with glucose as carbon source, in order to store the putative mutants. Selected mutants and *A. gossypii* VTT D-101398 were further assessed for cell growth and production of extracellular enzymes in AFM containing either CMC 0.1% (w/v), starch (10 g l^{-1}) or trybutyrin (10 ml l^{-1}).

4.2.4 Culture conditions

Spores were prepared by collecting 8-10 day old mycelia from agar-solidified medium, digesting with zymolyase (75 mg ml^{-1}) for 2 h, and washing with a solution of 0.8% (w/v) NaCl, 20% (v/v) glycerol and 0.025% (v/v) Tween 20.

Submerged cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml AFM and a carbon source, and incubated at 30 °C, 200 r.p.m. Flasks were inoculated with spores which had been stored at -80 °C.

4.2.5 Enzymatic assays

Endoglucanase I (EGI) activity was assayed as described previously (Ribeiro et al. 2010) by using 4- methylumbelliferyl-β-D-lactoside (MULac, Sigma) as substrate.

β -glucosidase activity was assayed according to the method described by Bailey and Nevalainen (1981) with 1 mM 4-nitrophenyl-β-D-glucopyranoside (Sigma) as substrate.

α-amylase The production of starch degrading enzymes on agar-solidified medium was detected by staining the surface with iodine solution (Carlsen 1994) to visualize zones in which starch had been broken down. Amylase activity by mutants exhibiting the largest hydrolysis haloes were then tested in liquid cultures. Alpha-amylase was measured by incubating 0.5 ml of supernatant at 25°C for 3-4 minutes to achieve temperature equilibration, after which 0.5 ml starch solution (at 25°C) was added. After 3 minutes incubation, 1 ml dinitrosalicylic acid color reagent was added. Samples were incubated in a boiling water bath for 5 minutes. After cooling to room temperature, 1 ml of water was added, samples were mixed and absorbance at 540 nm read versus a blank. Maltose release was determined from a standard curve. One unit released from soluble starch one micromole of reducing groups (calculated as maltose) per minute at 25°C and pH 6.9 under the specified conditions.

Lipase was detected by growing the fungus on agar solidified trybutyrin AFM. Degradation of this compound gives rise to clear zones surrounding the lipolytic colonies in the otherwise turbid culture medium.

Protein content The protein concentration present in the supernatant was determined by the Bradford assay using bovine serum albumin (BSA) (Sigma) as the standard.

4.2.6 Protein content

The protein concentration present in the supernatant was determined by the Bradford assay using bovine serum albumin (BSA) (Sigma) as the standard.

4.2.7 *GAS1* gene deletion from *A. gossypii* genome

In the A. gossypii genome, the *GAS1* gene is present as a tandem gene duplication, namely *AgGAS1a* (*AGL351W*) and *AgGAS1b* (*AGL352W*). In this study, *A. gossypii* strains were created, in which the copies were individually deleted using the PCR-based, one-step gene targeting approach (Wach, 1996; Wendland et al., 2000). A third strain in which both copies were deleted was also constructed, but grew too slowly to be characterized. Briefly, the deletion cassettes, containing the *NATPS* marker that confers nourseothricin/clonNAT resistance, were amplified by PCR from pUC19NATPS (lower case letters in the primer sequence), using the primers 351FW, 351RV, 352FW and 352RV shown in table 4.1. The primers were designed to have 66 bp and 67 bp flanking regions corresponding to the 5' and 3' flanking regions of *AgGAS1a* and *AgGAS1b*, repectively. Phusion® High-Fidelity DNA Polymerase (Finnzymes) was used and the PCR conditions were 98 ºC for 30 s, 30 cycles of 98 ºC for 10 s, 50 ºC for 30 s, 72 ºC for 1 min and a final step of 72 ºC for 10 minutes. The deletion modules were amplified, purified using the PCR purification Kit from Qiagen, and used to transform *A. gossypii* ATCC 10895 spores by electroporation, as previously described (Wendland et al., 2000). Transformants were selected on AFM agar containing 200 µg ml^{-1} clonNAT (WERNER BioAgent).

The *A. gossypii* strain with the *AgGAS1a* (*AGL351W*) copy deleted was named *A. gossypii* NQ89. Since this strain was clonNAT sensitive, the cassette used for the deletion of the second copy of *AgGAS1* conferred resistance to phleomycin (*ble*^{r}). Plasmid pUGGEN3 was created by amplification of the GEN3 module from pGEN3 with the primers GEN3_FW and GEN3_XhoI_RV (Table 4.1), which was digested with *Bgl*II and *Xho*I and ligated to pUG66 digested with the same enzymes. Using primers ScTEFt_EcoRI_FW and ScTEFp_NcoI_RV (Table 4.1), all of plasmid pUGGEN3, with the exception of the *kan*^{r} gene, was amplified with the Phusion® High-Fidelity DNA Polymerase (Finnzymes) as described above. The *ble*^{r} gene was obtained by PCR from plasmid pUG66 with the primers Ble_FW_and Ble_EcoRI_RV (Table 4.1). Both PCR fragments were digested with *EcoR*I e *Nco*I and ligation was carried out with T4 DNA Ligase (Promega) according to the manufacturer instructions. The resulting plasmid was named pUGBLE3.

Table 4.1 – Oligonucleotides used in this study

Primer name	Sequence
351FW	5’GTTCAGTGGACACGCATTCAAACAATCGTGAGGTTTGCGGAGC TTTGAATTTGGTTGAAAATCGTAgttacggtattttactatgg 3’
351RV	5’GTACATTTCTTTAAGCTAAATGTGTCAAAGCTTGAGGCGACAA TA GAGTGCTCAAGACATAGCTATGcaacagtgttccttaatcaagg 3’
352FW	5’TAAGTTGGGGCCTTGCGCATATTCGGAAGGTAACAGCAGTCGC TATATTTGTCAATCGCTATGTTATTCAATAAGCTAGCcctgcagaaccgtt acggta 3’
352RV	5’AAAGAACATAGAGCTTCGCCTTATAGAGTAATATAGAAGAGGC TGCATGAAAATCTATTTTTAAATGAAGATTAGCCCGAcctgcagccaaa cagt gtt 3’
GEN3_FW	5’ GCTAGGGATAACAGGGTAAT 3’
GEN3_XhoI_RV	5’ CCGCTCGAGGATCTGATGAGGCCGTCT 3’
ScTEFt_EcoRI_FW	5’ CGGAATTCTCGATGAGTTTTTCTAAGAG 3’
ScTEFp_NcoI_RV	5’ TCTATTCCATGGTAATTATAGTTCGTTGACCG 3’
Ble_FW	5’GCAGGTCGACAACCCTTAAT 3’
Ble_EcoRI_RV	5’CGGAATTCTCATGAGATGCCTGCAAG 3’
352BleFW	5’TAAGTTGGGGCCTTGCGCATATTCGGAAGGTAACAGCAGTCGC TATATTTGTCAATCGCTATGTTATTCAATAAGCTAGCgcaggtcgacaac ccttaat 3’
352BleRV	5’AAAGAACATAGAGCTTCGCCTTATAGAGTAATATAGAAGAGGC TGCATGAAAATCTATTTTTAAATGAAGATTAGCCCGAgcataggccact agtggatc 3’
V6FW_351	5’ CTAGTGGCATGTCTCGAG 3’
V4FW_351	5’ GCAGGTGTGGTTGACGAAATGCAG 3’
V5RV_351	5’ GCAGATGAAGTGCACATCTTACTGC 3‘
V2FW_352	5’ CAACGATCCCGTCTGTCATTG 3’
V3RV_352	5’ GTAGACGTTGAAGCCGTAGAAGTCAG 3’
Ver352_RV	5’ GCTGGACTTTGGTCATAGCC 3’
V1RV_NATPS	5’GATTCGTCGTCCGATTCGTC 3’

The deletion cassette containing the resistance module for phleomycin (lower case letters) used for the elimination of the second copy of the *AgGAS1b* (*AGL352W*) gene was obtained by PCR from the plasmid pUGBLE3 with the primers 352BleFW and 352BleRV (Table 4.1).

The deletion modules were used to transform *A. gossypii* NQ89 spores by electroporation as previously described (Wendland et al., 2000). Transformants were selected on AFM agar containing 10 µg/ml phleomycin (InvivoGen).

The resulting mutants grew very slowly and did not sporulate and as a consequence, it was not possible to obtain individual homokaryotic spores to proceed with the characterization of the mutant with both *AgGAS1* gene copies deleted.

4.2.8 Confirmation of *GAS1* – homolog genes deletion from *A. gossypii* genome

Correct integration of the gene disruption cassette on the *AgGAS1a* (*AGL351W*) region, was verified by analytical PCR with primers V6FW_351 and V5RV_351 that anneal outside the deleted gene, and with primers V4FW_351 and V5RV_351 that anneal inside the deleted region (Table 4.1). With the first set of primers an amplicon of 1975 bp is expected in the parental strain and of 1618 bp in the mutant strain, whereas with the second set, it is only expected to obtain an amplicon of 1355 bp in the parental strain. Transformation of multinucleate spores potentially leads to heterokaryotic cells, which contain a mixture of transformed and parental nuclei, and do not necessarily display an apparent phenotype. Clonal selection of homokaryotic mutants was achieved by isolating and growing single germinating spores that carry haploid nuclei. For subsequent analysis, homokaryotic mycelium was obtained by isolating and growing single spores.

The disruption of the *AgGAS1b* (*AGL352W*) homologue was confirmed by PCR using the primers V2FW_352 and V3RV_352 (Table 4.1) which anneal inside the deleted region and therefore an 877 bp amplicon is expected only in the parent strain and in a heterokaryotic strain. With the set of primers that anneal outside the deleted region, V2FW_352 and Ver352_RV (Table 4.1), a PCR product with 1692 bp in case the disruption cassette has been integrated in the right locus or 1935 bp in the parent strain and both fragments are expected in heterokaryotic strains. An 835 bp amplicon is expected only in the strains where integration of the deletion module has occurred since the primers V2FW_352 and V1RV_NATPS (Table 4.1) anneal outside the deleted region and in the middle of the NATPS module, respectively.

In order to evaluate the production of EGI by the Δ*AgGAS1a* strain, one isolate was transformed with an EGI expression plasmid, or with the empty vector, pMI516 (Ribeiro et al., 2010) as negative control. The expression plasmid pMI519 containing EGI from *T. reesei* (Ribeiro et al., 2010) was modified by replacing the kanMX module, where *kan*^{r} gene is flanked by the *A. gossypii TEF1* promoter and *S. cerevisiae ADH1* terminator, with the GEN3 module, in which the *kan*^{r} gene is under the *S. cerevisiae TEF2* promoter and terminator. The kanMx cassette together with the *URA3* selection marker were excised from pMI519 with *Bgl*II

and substituted with the GEN3 module obtained from pGEN3 (Wendland et al 2000) by digestion with *Bgl*II. This plasmid is referred to as modified pMI519.

Transformation of *A. gossypii* was performed by electroporation as previously described (Wendland et al., 2000).

4.3 Results

4.3.1 EMS mutagenesis of recombinant *A. gossypii*

Since *A. gossypii* spores cluster together in groups of 8 needle shape spores which are difficult to separate, treatment with EMS resulted in highly variable numbers of colonies per plate after treatment. Between 46-100% lethality was obtained when using 5-20% (v/v) EMS during 1 and 2 h of incubation at room temperature. One hundred per cent mortality was recorded after 3 h of EMS treatment. Average killing rates of 50-90% were obtained after treatment with 5% (v/v) EMS for 90 minutes, which were considered to be suitable for mutant screening.

4.3.2 Mutants screening

4.3.2.1 Characterization of putative *A. gossypii* mutants with improved secretion capacity

Primary selection of hyperproducing mutants was done based on the diameter of clearing zone surrounding the colony on the screening media containing 0.1% CMC supplemented with G418 (200 µg ml^{-1}). In addition to CMC, mutants were also screened on starch and tributyrin agar plates for α-amylase and lipase activity, respectively. Screening on medium containing CMC gave a fairly reliable indication of increased cellulolytic activity, whereas screening on starch and tributyrin plates was less conclusive (data not shown). After mutagenesis and screening, colonies showing improved protein production, on the basis of the clearing zones were selected and enzyme production was determined in submerged cultures. The most promising strains were further analysed.

In the primary selection, approximately 1000 mutants were isolated from screening plates. Of these, 41 mutants were grown in liquid culture for enzymatic activity measurement. Comparison was made at several times after inoculation, to take into account differences in lag phase or specific growth rate on EGI production. From these 41 mutants (Figure 4.1), 5

were selected, which showed increase in both EGI and β-glucosidase activity, only EGI activity, or only β-glucosidase activity. EGI, α-amylase, and β-glucosidase activity was measured for these strains (Figure 4.2). There were no significant ($p > 0.05$) differences in the specific growth rate of the mutants when compared with the parent strain. The specific growth rate varied between 0.09-0.16 h^{-1} in AFM, which was in agreement with values reported for the recombinant *A. gossypii* VTT D-101398 (Ribeiro et al., 2010).

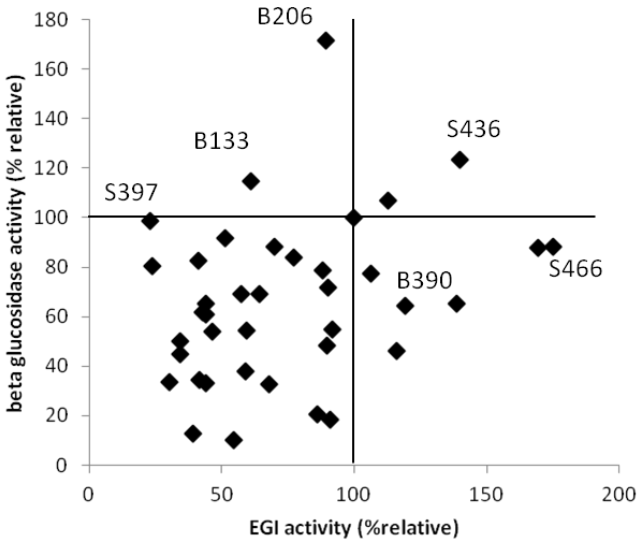

Figure 4.1 – EGI and β-glucosidase activity from *A. gossypii* in small scale batch cultures. Activities are expressed relative to those of the parent strain (U mg^{-1} protein for beta glucosidase and nkat mg^{-1} protein for EGI activity).

Three mutants showed enhanced EGI activity ($p<0.05$), with mutant B390 showing nearly a 3-fold increase and mutants S466 and S436 a 2 fold increase compared to the parent in flask culture after 63 h of growth (Figure 4.2). Of these, mutant S436 also had increased α-amylase and β-glucosidase activities, as compared to the parent strain (Figure 4.2). Mutant S466 also had increased amylase activity, but mutant B390 only showed increased EGI activity. The mutant with highest β-glucosidase activity was S397, which produced 2 fold ($p<0.05$) more compared to the non-mutageneized strain. Thus, four mutants were isolated with improved extracellular enzyme activity when compared to the parent strain.

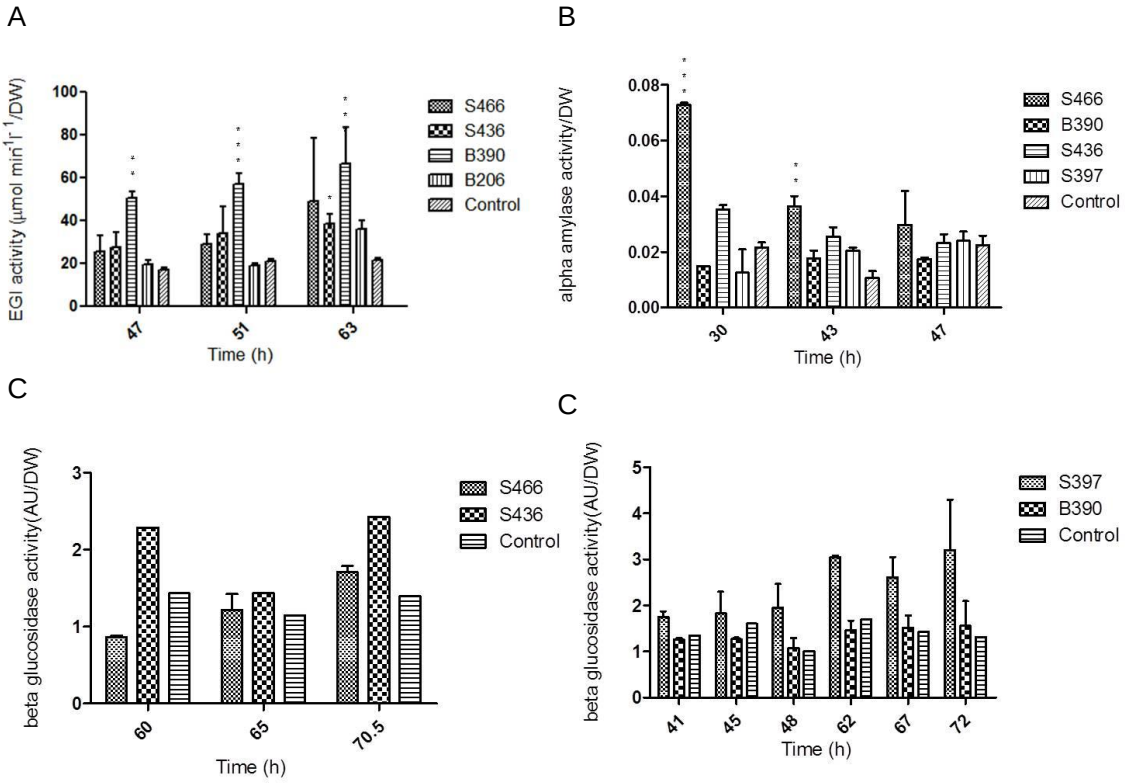

Figure 4.2 – Secreted enzyme activity from *A. gossypii* mutants obtained by random mutagenesis. Production of (A) EGI, (B) α-amylase and (C) ß-glucosidase by *A. gossypii* EMS mutants, compared to the parent strain (control, VTT D-101398) in batch culture. Values represent the standard error of the mean of two independent experiments with triplicates (n = 6). Activities were analyzed with ANOVA using Graphpad from Prism5 and significant ($p < 0.05$) differences are indicated by asterix (*).

4.3.2.2 Effect of *AgGAS1* gene deletion on recombinant protein production

For *A. gossypii* ATCC10895 *GAS1a* and *GAS1b* inactivation, the entire ORFs *AGL351W* and *AGL352W* were replaced with the NATPS cassette flanked by 66 bp and 67 bp upstream and downstream of *AGL351W* and *AGL352W* sequences, to enable homologous recombination and genomic insertion using a PCR strategy (Wendland et al., 2000). As can be seen in figure 4.3, *AgGAS1a* deletion was analysed in three transformants. The expected size fragments (Figure 4.3 B) or no amplification (Figure 4.3 C), were obtained. The mutants did not show any visible difference to the parent strain when grown on solid or in liquid AFM with glucose. Elimination of the *AgGAS1b* was also accomplished (Figure 4.3 D) as can be seen by the products obtained by PCR, using primers designed to confirm this. Either no

amplification was obtained in the mutant using primers that anneal inside the abolished region, or different fragment sizes with primers that anneal outside the deleted sequence or inside the resistance module for clonNAT.

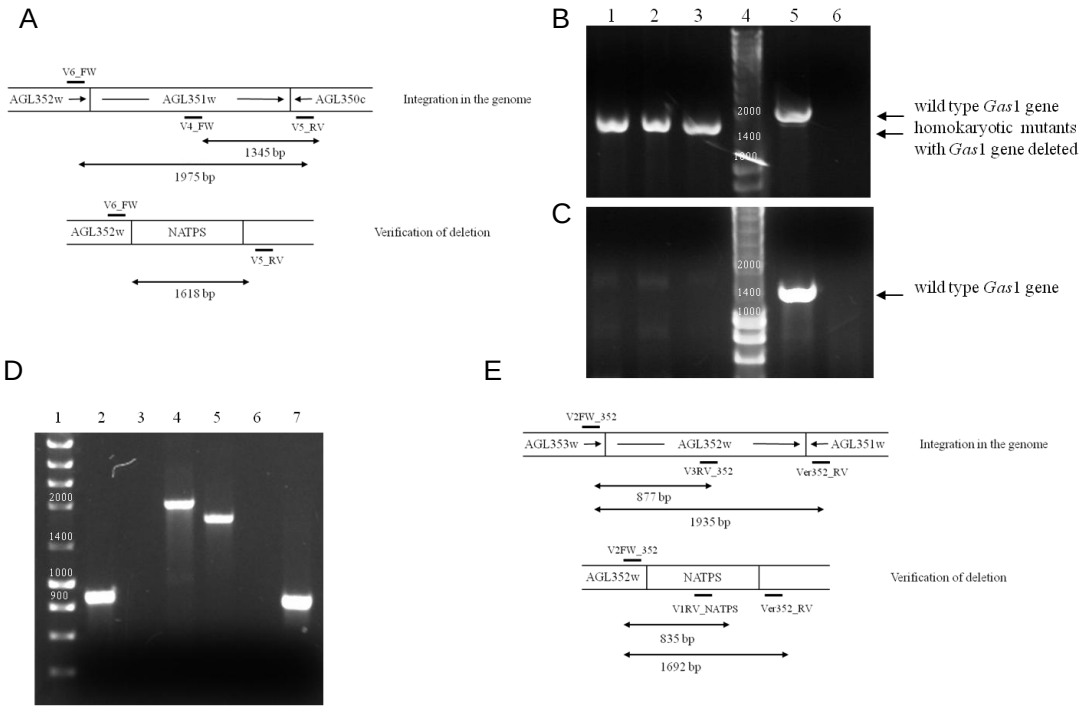

Figure 4.3 – PCR confirmation of *GAS*1 deletion in *A. gossypii*. (A) Schematic representation with the location of the primers used for verification of *AgGAS1a* gene deletion, with the corresponding amplicon size. (B) PCR made with primers V6FW_351 and V5RV_351 and using as template: lanes 1, 2 and 3 gDNA from homokaryotic transformants (predicted fragment size of 1618 bp); lane 5 parental gDNA (predicted fragment size of 1975 bp). Lane 4 molecular marker (NZYTech) and lane 6 negative control. (C) PCR made with primers V4FW_351 and V5RV_351 and using as template: lanes 1, 2 and 3 gDNA from homokaryotic transformants (no amplification); and lane 5 parental gDNA (predicted fragment size of 1345 bp). Lane 4 molecular marker (NZYTech) and lane 6 negative control. (D) Lane 1 molecular marker (NZYTech). Lanes 2, 4 and 6 parental gDNA amplified with primers V2FW_352 and V3RV_352 (predicted fragment size of 877 bp for the parent and no amplification in the deleted strain), V2FW_352 and Ver352_RV (predicted fragment size of 1935 bp for the parent and 1692 bp when the deletion cassette has been integrated in the genome), V2FW_352 and V1RV_NATPS (predicted fragment size of 835 bp for the deleted strain and no amplification in the parental strain), respectively. Lanes 3, 5 and 7 correspond to gDNA from a

homokaryotic Δ*AgGAS1b* mutant amplified with the same sets of primers used with the parental strain. (E) Schematic representation with the location of the primers used for verification of *AgGAS1b* deletion, with the corresponding amplicon size.

Nevertheless, the resulting mutant presented an extremely slow growth rate (Figure 4.4) which was unable to produce new mycelium after 8 days of incubation at 30 ºC on solid AFM. As a consequence, this mutant was not further characterized.

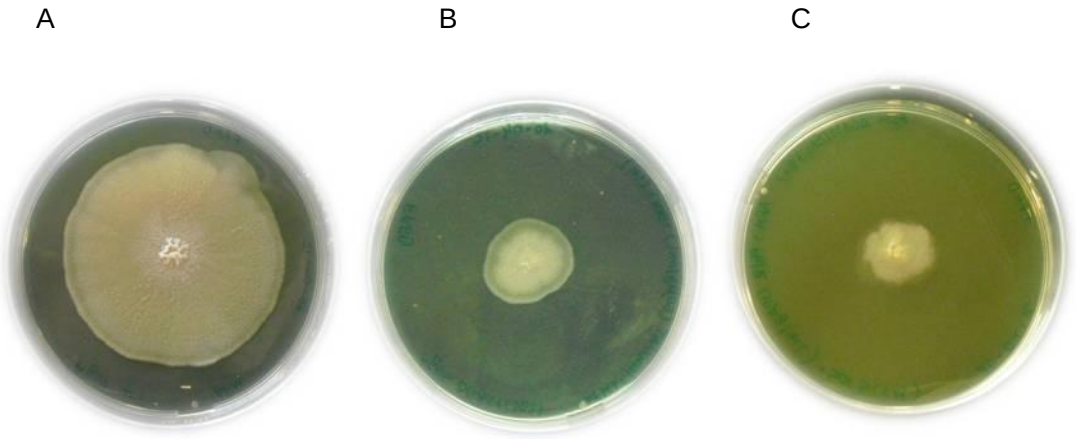

Figure 4.4 – Colonies of *A. gossypii* after 8 days incubation at 30 °C on AFM. (A) parental strain (ATCC10895), (B) homokaryotic Δ*AgGAS1b* (*AGL352W*) mutant and (C) heterokaryotic Δ*AgGAS1a* Δ*AgGAS1b* double mutant.

The *S. cerevisiae ADH1* terminator has been reported to display autonomous replicating sequence activity in *A. gossypii* (Knechtle et al., 2003). In this study, the modification of the plasmid pMI519, which also included the removal of the *S. cerevisiae ADH1* terminator sequence present, resulted in a 2 fold increase in the extracellular EGI activity, when compared to the strain expressing EGI from the original pMI519 plasmid (VTT D-101398 strain) (Figure 4.5). Therefore, the modified pMI519 plasmid was chosen to be introduced in *A. gossypi* NQ89 strain to assess the effect of the Δ*AgGAS1a* deletion in the expression of *T. reesei* EGI. Two *A. gossypii* Δ*AgGAS1a* EGI transformants and the negative control containing the empty vector were grown in 250 ml flasks in AFM supplemented with G418, in

order to evaluate total extracellular EGI protein production. The amount of total protein secreted into the culture medium was very similar between the mutant (61 – 440 mg ml^{-1}) and the parent VTT D-101398 strain (66 – 377 mg ml^{-1}). EGI activity, measured with the fluorogenic substrate MuLac from the two Δ*AgGAS1a* transformants and from the parent EGI strain containing the modified vector did not differ significantly ($p > 0.05$).

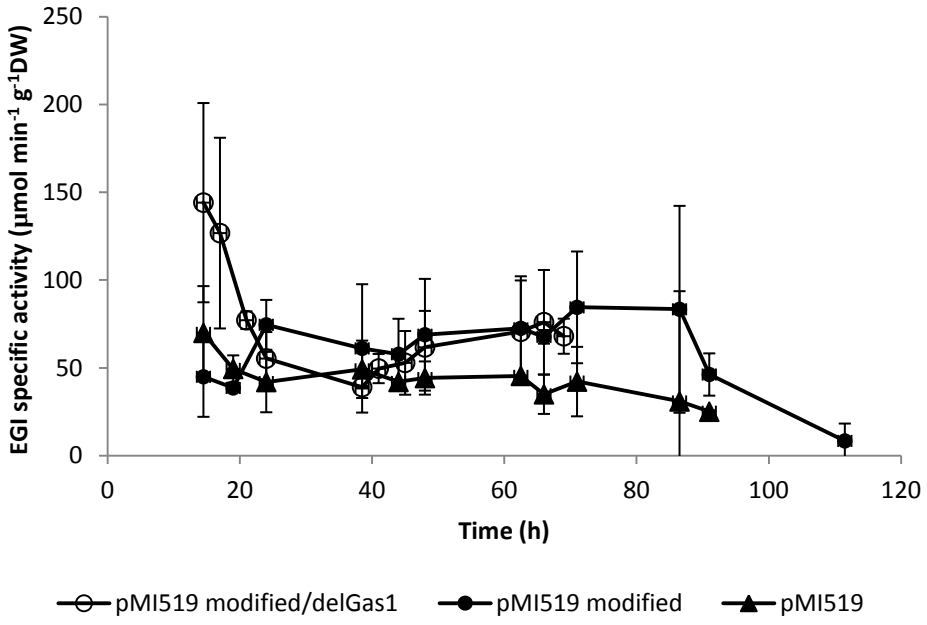

Figure 4.5 – Average EGI activity from supernatant of recombinant *A. gossypii* growing in flasks in AFM with glucose as carbon source at 30 ºC, 200 r.p.m. Activities were measured with the MULAc assay, described in materials and methods. Data represent average ± standard error of the mean obtained from three independent cultures. (○) Δ*AgGAS1a* strain expressing EGI from the modified pMI519 plasmid, (●) parent strain expressing EGI from the modified pMI519 plasmid and (▲) parent strain expressing EGI from the original pMI519 plasmid.

4.4 Discussion

Several mutants of *A. gossypii* have been generated using UV radiation and NTG mutagenesis with the goal of increasing riboflavin production (Park et al., 2007; Tajima et al., 2009). Although there are numerous studies on the application of EMS to induce mutagenesis in other microorganisms, at the moment, this is the first time that *A. gossypii* secretion mutants have been produced by means of EMS mutagenesis. Targeted genetic modification, by deletion of the gene for the cell wall modifying enzyme encoded by *GAS1* was less successful. *GAS1* was chosen as a target for strain improvement because its deletion has successfully improved secretion in several other yeast or filamentous fungi.

Cell wall defective strains deleted in the *GAS1* gene of *S. cerevisiae* (Gaynor et al., 1999), *Pichia pastoris* (Marx et al., 2006), *T. reesei* (Kruszewska et al., 1999) and *Zygosaccharomyces bailii* (Passolunghi et al., 2010), have been reported as super-secreting. Deletion of *GAS1* led to an almost seven-fold increase in the level of human insulin-like growth factor 1 (hIGF1) in *S. cerevisiae* (Vai et al., 2000). Similarly, secreted enzymatic activity was almost doubled when *GAS1* was deleted in *Z. bailii* producing *C. rugosa* lipase CRL1 or *Y. lipolytica* protease XPR2 (Passolunghi et al., 2010). However, the deletion of *AgGAS1a* gene from *A. gossypii* genome did not cause any effect on the capacity of *A. gossypii* to secrete EGI or α-amylase (Figure 4.5). It is worth noting that also in *P. pastoris* the disruption of *GAS1* had no effect on the secretion of human trypsinogen or human serum albumin, although the amount of *Rhizopus oryzae* lipase released from the cells was doubled (Marx et al., 2006). Similarly, *GAS1* deletion in *Z. bailii* did not increase IL-1b secretion, although secretion of protease could be increased (Passolunghi et al., 2010).

Since phylogenetically *A. gossypii* is closely related to *S. cerevisiae* and both show similar protein secretion characteristics (Ribeiro et al., 2010), deletion of *GAS1* would be expected to result in similar improvements in secretion. However, unlike in *S. cerevisiae,* there are two tandem copies of the *GAS1* gene in the *A. gossypii* genome. Here we have only tested the secretion ability of the mutant *AgGAS1a* with one copy deleted (*AGL351W*), since the double mutant showed limitations in terms of growth. There may still be sufficient protein in the wall to explain the lack of improved secretion. However, since improvements in Δ*GAS1* strains have been protein dependent and EGI is not a readily secreted protein, this may contribute to the lack of improvement.

EMS treatment was effective in generating five *A. gossypii* mutants with improved extracellular enzymatic activity (Figure 4.1), even though only 50-90% of killing was obtained after 90 minutes of incubation with EMS (5%). One of these mutants appeared to be improved in general secretion. Three mutants had enhanced EGI activity (Figure 4.2). The increased

EGI, α-amylase and β-glucosidase activities from mutant S436, as compared to the parent strain, indicated that the mutation(s) affected the secretory pathway in general. Mutant S466 also showed a 3-fold increased α-amylase activity, whereas mutant B390 had only increased EGI activity. Thus, mutant B390 may have a mutation in the plasmid containing EGI or at the level of specific transcription factors. Additionally, two mutants were isolated which did not have improved EGI production, but which produced more β-glucosidase (S397). As with B390 mutant, these mutations may relate to the specific gene or to the transcription factors associated with them.

Mutant S436, with 1.4 to 2 fold improved secreted activity (Figure 4.2), would be useful for further development of *A. gossypii* protein production. Two fold improvements in secreted hydrolytic activity are generally observed for other mutants obtained by random mutagenesis. For example, Chand et al. (2005) reported 2.0 and 1.3 fold increased FPase and endoglucanase production, respectively, using successive treatments of different mutagens (EtBr and NNMG followed by UV) in spores of *Trichoderma* sp. Dillon et al., (2006) reported 1.5 fold more cellulase productivity in *Penicillium echinulatum* by three repeated mutagenic treatment steps of UV. *T. viride* TL-124, was subjected to successive mutagenic treatments with UV irradiation, low energy ion beam implantation, atmospheric pressure non-equilibrium discharge plasma (APNEDP), and N-methyl N'-nitro N-nitrosoguanidine, to obtain mutants with 2 to 2.6 fold increased cellulase production relative to the wild type strain (Xu et al., 2011). *Aspergillus sp*. has been improved for cellulase production by sequential treatments of γ–irradiation, ultraviolet treatment and N-methyl-N'-nitro-N-nitrosoguanidine to obtain improved mutants which produced 2 to 3 fold increased cellulolityc activity, when compared to the wild type strain (Van Hanh et al., 2009). In this regard, the range of improvement obtained here is similar to what has been obtained in other microorganisms, although secretion is low compared to eg. *Trichoderma*.

The low secretory ability of *A. gossypii* made screening for secretory mutants difficult. The difficulty of rapidly screening for hypercellulase producing mutants of *Neurospora* and *Trichoderma* has previously been noted (Adsul et al., 2007). For *A. gossypii*, the haloes in agar plate assays were small and affected by the depth of agar and size of fungal colony. Germination time following mutagenesis was variable and thus colonies of different sizes needed to be compared. It was also not practical to restrict colony expansion by inclusion of agents such as Tween 20 or Triton X100, which have been used with *T. reesei* (Nakari-Setala et al., 2009), because they inhibited *A. gossypi* spores germination. Nonetheless, this screening method did allow direct selection of hyperproducing mutants of the target protein from the colonies after mutagenesis, even though false positives were also isolated. There were no significative differences in the growth of both parent and mutant strains. This

indicates that the enhancement of enzyme production by the mutant strain is not due to an increase in growth but to the enhancement in production and/or protein secretion.

The major output of this study was one mutant with overall improved secretion capability obtained by random mutagenesis. Targeted deletion of *AgGAS1a* had no significant effect on the overall *A. gossypii* secretion ability and the deletion of *AgGAS1b* led to sporulation and cellular growth defects.

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Chapter 5

Transcriptome analysis of recombinant *Ashbya gossypii* expressing EGI from *Trichoderma reesei* treated with dithiothreitol

Abstract

The secretory pathway of the hemiascomycete *Ashbya gossypii* was examined in a genome-wide transcriptional analysis of protein secretion-related stress using NimbleGen arrays, to determine the responses occurring in *A. gossypii*. Stress was induced either by chemical treatment with dithiothreitol (DTT), or by expressing EGI from *Trichoderma reesei*. DTT had a more pronounced effect than EGI production. A much smaller number of genes were significantly differentially expressed by inducing stress with the secretion of a recombinant protein than by the addition of a secretion blocker. In the EGI recombinant strain, the fungus responded by down regulating genes involved in translation, in agreement with the low EGI production levels observed in *A. gossypii*.

Most of the up-regulated genes after DTT treatment are related to the ERAD (ER-associated degradation) system including cytosolic chaperones, vesicle transport proteins, and ER-associated degradation proteins. On the other hand, glycosylation related genes were down regulated. Surprisingly, neither treatment activated a conventional UPR in *A. gossypii*, since the expression levels of several well-known UPR target genes remained unaffected in both situations. In addition, a consensus UPRE motif, as described in other fungi was not found upstream of up regulated genes, which further explains the lack of a conventional UPR in *A. gossypii* under ER stress. Together, these results indicate that *A. gossypii* may have an alternative protein quality control system.

5.1 Introduction

Ashbya gossypii is a filamentous fungus with recognized biotechnological interest, known for its ability to naturally produce riboflavin. Due to its small genome (Dietrich et al. 2004), efficient gene targeting (Wendland et al. 2000) and easy propagation of plasmids, it has also been exploited as a host for the production of heterologous proteins (Wright and Philippsen 1991 ; Ribeiro et al 2010). *A. gossypii*, like many other filamentous fungi, is well equipped for protein secretion, with 3 - 4 % of its genome predicted to correspond to secreted proteins (Aguiar et al. 2011, in preparation). However, the secretion of the recombinant proteins EGI (endoglucanase I) and CBHI (cellobiohydrolase I) from *Trichoderma reesei* has been rather low (Ribeiro et al. 2010). Several steps occurring during the secretion pathway in filamentous fungi have been identified as potential bottlenecks for heterologous protein production (Gouka et al. 1997; Sims et al. 2005; Resina et al. 2009; Yoon et al. 2010). Problems can occur at translation initiation or elongation, translocation into the endoplasmic reticulum (ER), folding, transport, processing, or secretion (Gouka et al. 1997). In eukaryotic cells, newly synthesised proteins are folded, assembled and subject to post-translational modifications in the ER. The ER provides an oxidizing environment in which protein folding is assisted by a number of molecular chaperones and folding enzymes and ultimately subjected to a strict quality control (Gething and Sambrook 1992; Helenius et al. 1992; Ellgaard et al. 1999). Protein folding in the ER can be compromised by several endogenous and exogenous factors like changing environmental conditions including pH, osmolarity, oxidative stress (Holtz et al. 2006; Yan et al. 2008), oxygen availability, nutrient limitation (Kaufman et al. 2002), temperature (Kasuya et al. 1999; Matsumoto et al. 2005), cell differentiation, pathogenic infections, and expression of heterologous proteins (Rutkowski and Kaufman 2004). However, problems with proper folding may also occur under normal physiological conditions in response to a change in the demand for secretion. This ultimately results in the accumulation of unfolded proteins within the ER, since only correctly folded proteins transit the secretory pathway (Ellgaard and Helenius 2001). Proteins that fail to fold properly, usually accumulate in the ER leading to the induction of the unfolded protein response (UPR) (Cox et al. 1993; Sidrauski et al. 1998; Kaufman 1999; Welihinda et al. 1999; Mori 2000), and if UPR is not sufficient to relieve stress, they are eventually targeted to destruction by ER-associated degradation (ERAD) (Nishikawa et al. 2005). The UPR is a complex intracellular signaling pathway that increases the transcriptional activity of a number of genes involved in protein folding, secretion, degradation (Travers et al. 2000; Martinez and Chrispeels 2003; Malhotra and Kaufman 2007; Hetz and Glimcher 2009) glycosylation and transport (Mori 2000; Travers et al. 2000). UPR appears to be intimately linked to the ERAD pathway (Ng et al. 2000; Travers et al. 2000), in which misfolded proteins in the ER lumen are retrotranslocated (dislocated) through the translocon to the cytoplasm, and are ubiquitinated and degraded by the proteasome (Bonifacino and Weissman 1998).

Protein “quality control” is an essential process in monitoring protein folding, ultimately targeting misfolded proteins for degradation via the ubiquitin-proteasome system. Distinct protein quality control systems appear to exist in various cellular compartments, including the nucleus, mitochondria, cytoplasm (Metzger and Michaelis 2009) and ER, with the best characterized system being ER quality control (Gardner et al. 2005; Sayeed and Ng 2005; Nakatsukasa and Brodsky 2008; Radke et al. 2008).

The mechanisms of recognition and targeting of misfolded proteins for destruction are conserved from yeasts to mammals, allowing the cells to cope with the presence of abnormal proteins and their harmful effects. However, not all the processes described in yeast and mammalian systems have been established in filamentous fungi (van Anken E 2005a; van Anken E 2005b). Apart from the work of Jacobs et al. (2009) and Carvalho et al. (2010) ERAD-related genes have not been extensively studied in filamentous fungi.

UPR reduces overall translation speed and enforces ERAD via the transmembrane kinase/nuclease (Ire1) signaling cascade (Schroder and Kaufman 2005). Accumulation of unfolded proteins in the ER lumen results in the dissociation of the ER chaperone *BiP* (encoded by *KAR2*) from Ire1p leading to Ire1p dimerization and thereby the activation of its kinase and endoribonuclease functions (Shamu and Walter 1996; Sidrauski and Walter 1997; Oikawa et al. 2009). In *S. cerevisiae*, Ire1p is responsible for excising a 252-nt intron in *HAC1* messenger RNA (mRNA), enabling its translation into an active protein and migration into the nucleus where it binds to the unfolded protein response element (UPRE; CANCNTG, (Mori et al. 1998)) in target genes coding for chaperones and foldases as well as other components of the secretory pathway (Sidrauski and Walter 1997; Sidrauski et al. 1998; Gonzalez et al. 1999; Travers et al. 2000). Thus, splicing of the intron activates *HAC1* as a transcription factor, which in turn controls the expression of genes involved in UPR (Ruegsegger et al. 2001; Mulder et al. 2004; Mulder et al. 2006). Although the pathway is much more elaborate in higher eukaryotes, the central activation step of the transcriptional activator of the UPR by unconventional splicing of its mRNA has been conserved (Zhang 2004). ERAD causes proteolytic digestion of misfolded protein in the cytosolic proteasome (Hirsch et al. 2009). The UPR-induced *BiP* and protein disulfide isomerase (PDI) play important roles in ERAD by preventing misfolded protein aggregation (Nishikawa et al. 2001) and delivering ERAD substrates to the retrotranslocation machinery (Plemper and Wolf 1999).

Glycosylation is also an important factor in protein folding, and the processing of glycans is indicative of the folding state of the protein (Kleizen and Braakman 2004; Lederkremer 2009). If the protein fails to achieve correct conformation, the removal of 1,2 α-mannose units by a specific 1,2 α-mannosidase (mns1) targets the substrate to degradation by ERAD (Gonzalez et al. 1999; Tremblay and Herscovics 1999). When marked for degradation, proteins are retrotranslocated through the Sec61p translocon (Schafer and Wolf 2009) and/or through Der1p retrotranslocation channel (Ye et al. 2001; Goder et al. 2008). The Hrd1 complex is involved in the ubiquitination of substrates that contain misfolded luminal domains (Bordallo et al. 1998; Deak and Wolf 2001). In

S. cerevisiae, Hrd3p regulates the activity and stability of Hrd1p (Plemper et al. 1999b; Gardner et al. 2000). Together with Sec61p, Hrd1–Hrd3 complex mediates the transfer to the cytosol of proteins targeted for degradation (Figure 5.1) (Plemper and Wolf 1999). Misfolded membrane proteins accumulated in the cytosol find their way to the proteasome via the trimeric Cdc48 complex after polyubiquitylation by the ER membrane integrated ubiquitin ligase Doa10 (Figure 5.1) (Sommer and Wolf 1997; Plemper and Wolf 1999; Kostova and Wolf 2003; Vembar and Brodsky 2008; Hirsch et al. 2009).

Previous attempts to characterize the genes associated with recombinant protein secretion in filamentous fungi have often relied on inducing secretion stress by secretion blockers such as dithiothreitol (DTT), brefeldin A, and tunicamycin. These methods have identified genes involved in protein folding, such as *tigA* (van Gemeren et al. 11997) and *cypB* (Derkx and Madrid 2001). Secretion blockers have also been used in combination with green fluorescent protein fusions to visualize unfolded protein and locate potential bottlenecks (Gordon et al. 2000).

This study aims at uncovering transcriptional responses happening in *A. gossypii*, when exposed to secretion stress. The present study was undertaken to identify possible bottlenecks of recombinant glycoprotein production in *A. gossypii*. To our knowledge this is the first report that unveils the events taking place at the transcription level in *A. gossypii,* under recombinant protein production conditions and also under ER chemically induced stress with DTT.

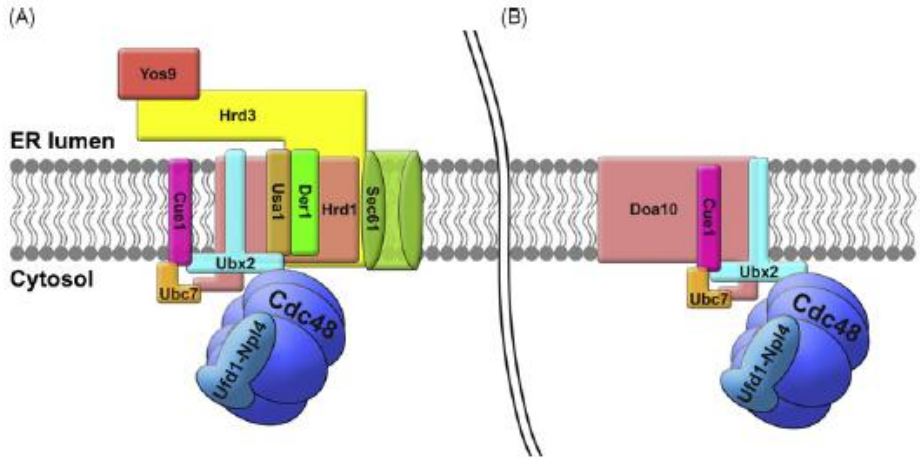

Figure 5.1 – The Hrd1 and Doa10 E3 complexes. (A) Schematic representation of the Hrd1 complex. (B) Schematic representation of the Doa10 complex (Xie and Ng 2010).

5.2 Materials and methods

5.2.1 Strains and batch cultivations

The *A. gossypii* EGI producing strain and its corresponding empty vector control strain were generated as described in Ribeiro et al (2010). Stock cultures were maintained as spores suspended in 20% (v/v) glycerol, 0.8% (w/v) NaCl with ~0.025% (v/v) Tween 20 at -80°C.

Pre-cultures to inoculate bioreactors, were grown in 250 ml Erlenmeyer flasks containing 50 ml AFM plus 200 µg ml^{-1}G418. *A. gossyppii* pre-cultures were allowed to grow for 14-16 h at 30ºC, 200 rpm.

For larger scale batch cultivations a Biostat® CT bioreactor, maximum working volume 2.5 l (B. Braun Biotech International, Sartorius AG, Germany) was used at a working volume of 1.5 l or 2 l for the DTT cultures. The medium was AFM supplemented with 200 µg ml^{-1}G418. Bioreactors were inoculated with 25 – 30 ml pre-culture. Cultures were maintained at 30°C, 500 rpm, with 1.0 volume gas (volume culture)$^{-1}$ min^{-1} (vvm). pH was controlled at pH 6.0 by the addition of sterile 1 M KOH or 1 M H_3PO_4. Polypropylene glycol (mixed molecular weight, Wiebe et al. 2001) was added to control foam production. Gas concentration (CO_2, O_2, N_2 and Ar) was analysed continuously in an Omnistar quadrupole mass spectrometer (Balzers AG, Liechtenstein), calibrated with 3% CO_2 in Ar.

Duplicate samples for the DNA microarray experiments were taken at several times and in the DTT stressed cultures samples were collected 30min, 1h, 2h and 4h after 10 mM DTT addition. Mycelium was separated from the supernatant by filtration through GF/B glass fiber filter (Whatman). Biomass was washed with 9 g NaCl l^{-1} (2 × volume) and frozen as fast as possible in liquid nitrogen.

Biomass from bioreactor cultures was measured by filtering culture samples through pre-dried and pre-weighted Whatman GF/B glass fiber filters. The harvested biomass was washed with at least 20 ml of deionised water and dried to constant weight. Aliquots of the supernatant were stored frozen at -20°C for subsequent analysis.

5.2.2 Analysis of growth and protein production in the bioreactor cultures

Protein concentrations in the cell-free broth were measured with a Coomassie (Bradford) protein assay kit (Thermo scientific), using bovine serum albumin (BSA) as standard.

Samples of the culture medium were assayed for secreted EGI using 4-methylumbelliferyl-β-D-lactoside (MULac, Sigma) as the substrate. The reaction consists in

adding 50 µl MULAc (4 mM in DMSO) to 50 µl culture supernatant and incubating at room temperature. Reactions were stopped after 30 minutes incubation by adding 100 µl of 1 M Na_2CO_3, and liberation of 4-methylumbelliferone (MU) was detected by fluorescence measurement (excitation wavelength = 355 nm and emission wavelength = 460 nm) with a Varian Varioscan spectrofluorometer in a black, flat bottom microtiterplate. MU (Sigma) was used as a standard. Specific enzyme activity was defined as nmol of MU formed per minute per g dry weight under the assay conditions.

Metabolite production and sugar consumption was analyzed by HPLC (Ribeiro et al 2010).

5.2.3 Microarray sample preparation and data acquisition

Total RNA was isolated using the the RNeasy® Plant Mini kit (QIAGEN), according to the protocol for isolation of total RNA from filamentous fungi. Quality and purity of total RNA were analyzed according to the Roche NimbleGen guidelines(www.nimblegen.com). RNA concentration and purity were determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Quality of total RNA was analyzed using an Agilent Bioanalyzer 2100 and RNA 6000 Nano Kit (Agilent Technologies). The total RNA was stored at -80°C until further processing. Ten micrograms of each total RNA sample was used for reverse transcription and synthesis of cDNA using the SuperScript® II Double-Stranded cDNA Synthesis Kit (Invitrogen), as recommended by NimbleGen(www.nimblegen.com). The cDNA was quantified in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and its quality was assessed using an Agilent Bioanalyzer 2100 and RNA 6000 Nano Kit (Agilent Technologies). Each cDNA sample was random-primer labeled with Cy3-nonamers according to manufacturer standard protocol using NimbleGen One-Color DNA Labeling Kit (Roche NimbleGen). By random assignment, four micrograms of each Cy3-labeled cDNA sample was applied to custom-made *A. gossypii* gene expression 12x135K arrays, designed and manufactured by Roche NimbleGen (WI, USA). Each slide contained 12 independent arrays, each containing four replicates of 33,364 probes covering 4767 ORFs of *A. gossypii* (7 probes/target ORF). Following NimbleGen recommendations(www.nimblegen.com), the arrays were then hybridized, washed, dried and scanned using a NimbleGen MS 200 Microarray Scanner (Roche NimbleGen). The NimbleScan V2.5.26 software (Roche NimbleGen) was used to extract from the scanned images fluorescence intensity signals for each probe.

5.2.4 Analysis of transcriptome data

The raw array data obtained from NimbleScan was preprocessed with the RMA (Robust Multichip Average) method (Irizarry et al. 2003). Array data quality was controlled with

arrayQualityMetrics (Kauffmann et al. 2009) and sample wise Principal Component Analysis of raw, RMA preprocessed and repeat averaged data. LIMMA (Linear Models for Microarrays) (Smyth 2004) was subsequently used to select significantly changing genes with a cut-off of p-value < 0.01 (which corresponds to a false-discovery rate of 1% in this analysis) and fold-change > 1.5. For each gene its Pearson correlation with EGI Activity (µmol $min^{-1}L^{-1}$) was calculated. The false discovery rate of these correlations was estimated from the Q-value (Storey J 2004) using the R package 'qvalue', and found to be 4.4% for absolute correlation > 0.7.

Each gene's repeat averaged expression values over all the samples i.e. expression profiles were clustered with R-package 'Mfuzz' (Futschik 2005) with parameter *m* set to 1.35 and number of clusters to 9. Parameters were selected based on visual evaluation of cluster profiles. Genes with cluster membership > 0.7 were assigned to a co-expression cluster for further analysis.

A. gossypii gene mappings to *S. cerevisiae* genes from Ashbya Genome Database were used to map the array results of differential gene expression, gene expression correlation with EGI Activity (µmol $min^{-1}L^{-1}$) and co-expression clusters to *S. cerevisiae*. Gene Ontology (GO) class analyses were then carried out using *S. cerevisiae* GO annotation. For differential expression of genes GO analysis was carried out using R-package 'GSA' (Efron and Tibshirani 2007) and for gene expression correlation and co-expression clusters with R-package 'GOstats' (Falcon and Gentleman 2007). REVIGO (Supek et al. 2011) was used to summarize the GO terms lists.

Promoter analysis was carried out with a tool that allows Finding Informative Regulatory Elements (FIRE) (Elemento et al. 2007) with default settings using the *A. gossypii* genome. Co-expression clusters and clusters of differentially expressed genes were used as groups of genes from which to find common promoter elements. Genes which were not assigned to any cluster were assigned to group number 0 for this analysis. As promoter we used 1500 bases backwards starting from the protein coding region of each gene. Found promoter elements were mapped with FIRE to known promoter elements described in (Gasch et al. 2004) in order to annotate them.

5.3 Results and discussion

5.3.1 Overview of the transcriptional analysis under secretion stress

To gain insight into the secretory pathway of *A. gossypii*, a global transcriptome analysis was performed using NimbleGen microarray data from a recombinant EGI producing *A. gossypii* strain and a reference strain treated for 30min, 1h and 4h with the chemical agent DTT, known to disrupt protein folding in the ER and as a consequence to induce UPR. DTT is a strong reducing agent that prevents disulfide bond formation. A strain producing recombinant EGI, endoglucanase I from *T. reeesei*, was chosen to assess ER stress under conditions of heterologous protein production.

The overall variations in gene expression levels between cells expressing EGI and those which do not, were extremely small or nonexistent. DTT treatment caused relatively large changes in gene expression. Similarly, Sims et al. (2005) have previously reported that a much smaller number of genes were significantly differentially expressed by inducing the UPR with the secretion of a recombinant protein than by the addition of a secretion blocker.

DTT treatment exerted a gradual effect in the gene expression along time (Table 5.1; Figure 5.2 and Figure 5.3). When comparing the expression levels between the time point immediately before adding DTT (time point defined as zero) with 30min after the addition of DTT 128 genes were up-regulated, 733 genes after 1h and 922 genes after 4h of treatment. In total 43 genes which were up regulated after 30min continued to be up regulated after 4h. The number of genes down-regulated was 189 genes after 30min., 449 genes after 1h and 767 genes after 4h of DTT treatment. In total, 140 genes were down-regulated at all time points (Figure 5.2). Hence, the effect of DTT was initially more inducing than repressing.

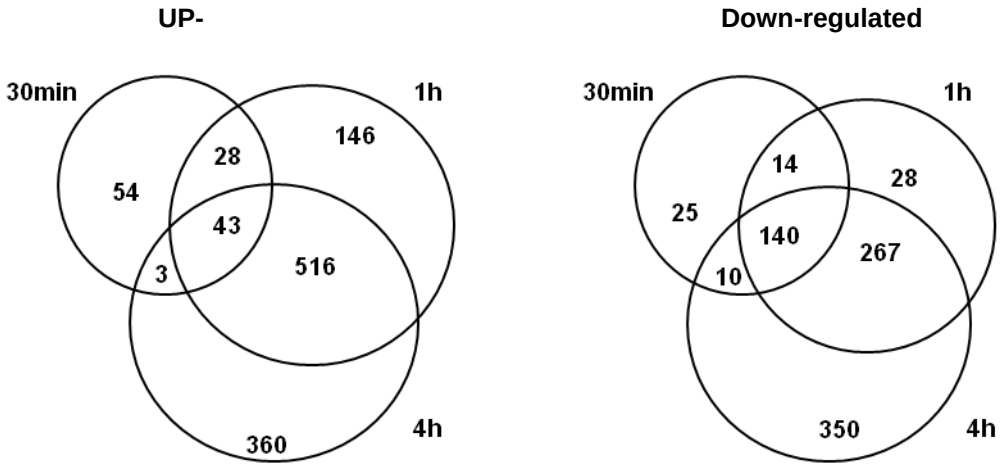

Figure 5.2 – Overall variation of gene expression after DTT treatment of *A. gossypii* for 30min, 1h or 4h. The data represented corresponds to outcome of analysis with LIMMA (p-value < 0.01 and fold change > log2(1.5)).

Table 5.1 – Microarrays statistics. Data refers to the outcome from LIMMA analysis (p-value < 0.01 and fold change > log2(1.5))

	DTT 30min	**DTT 1h**	**DTT 4h**
UP	128 (2.7 %)	733 (15.4 %)	922 (19.3 %)
DOWN	189 (4.0 %)	449 (9.4 %)	767 (16.1 %)
Unchanged	4450 (93.3 %)	3585 (75.2 %)	3078 (64.6 %)

After 30 min from DTT addition a clear response was detectable similarly to what has been observed in *S. cerevisiae* (Travers et al. 2000), *Candida albicans* (Wimalasena et al. 2008; Wimalasena TT 2008), *Aspergilus niger* (Guillemette et al. 2007) *Yarrowia lipolytica* (Oh et al. 2010) and *Trichoderma reesei* (Arvas et al. 2006). In *S. cerevisiae*, the induction of target gene expression was complete after 15 min (Travers et al 2000), while in another study, it occurred within 30 min of DTT exposure (Gasch et al. 2000; Pakula et al. 2003). However, in *A. nidulans,* a transcriptional response consistent with blocking secretion was not seen until 1 h after the addition of 20 mM DTT, and this response was sustained at 2 and 4h (Sims et al. 2005).

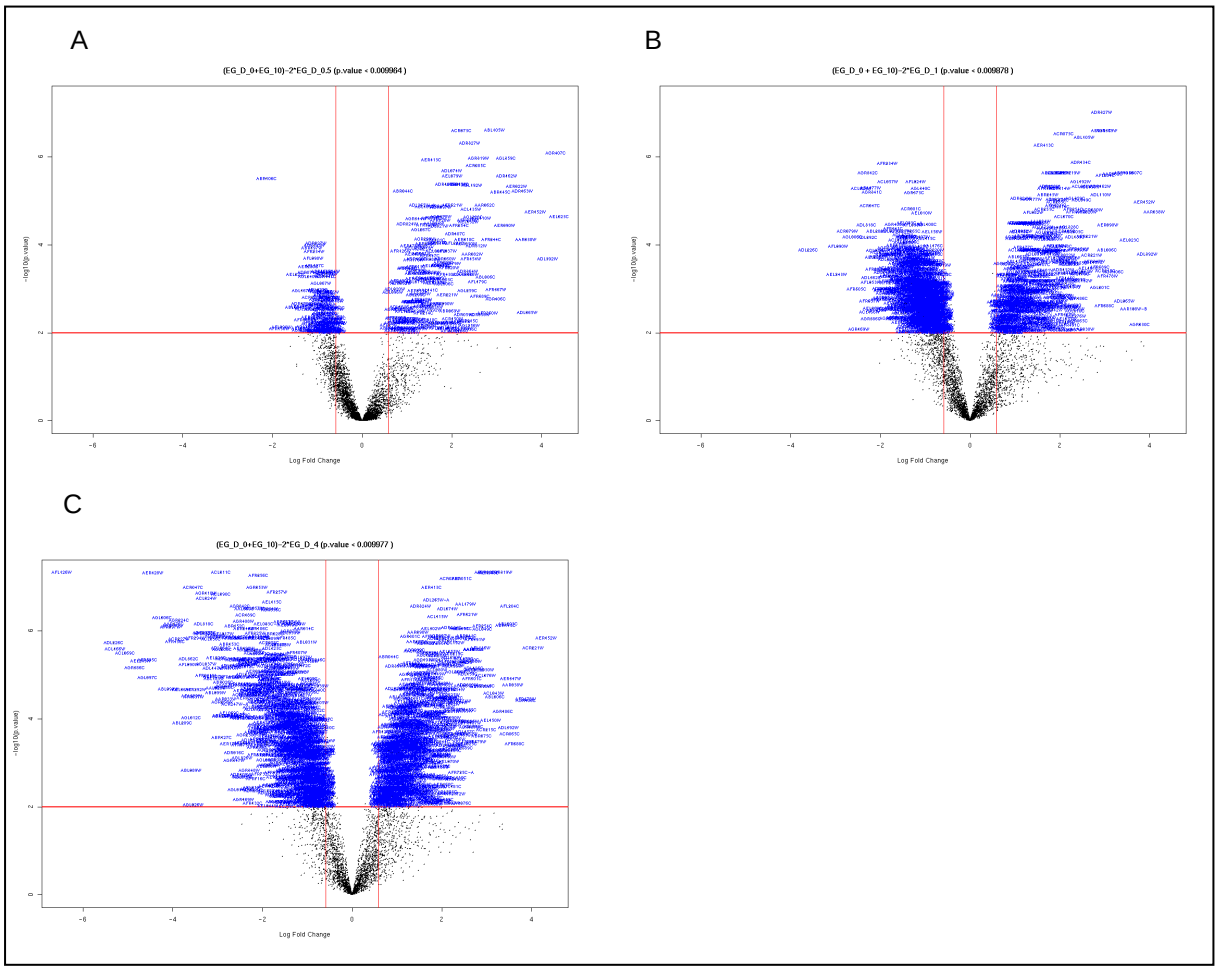

Figure 5.3 – Volcano plots by contrast obtained after LIMMA analysis, with the differentially expressed genes (p-value < 0.001). After DTT addition. A - 30min, B -1h, C - 4h. Negative fold changes represent DTT up-regulated genes and positive fold changes DTT down-regulated genes.

5.3.2 Effect of of recombinant protein production (EGI) on *A. gossypii* transcriptional profile

No genes were differentially expressed in cells producing EGI, relative to non-producing cells, when analysed using standard criteria as for the DTT treatment. However, 21 genes were significantly differently expressed (correlation cut-off to 0.7, qvalue 0.044) when the correlation of EGI production and gene expression was taken into account. The correlation analysis takes into account the variation in replicates of single conditions (unlike LIMMA) and was thus expected to be more sensitive.

Tables 5.2 and 5.3 present the lists of genes that were up-regulated or down-regulated in the recombinant *A. gossypii* strain, respectively, when the expression analysis was done taking into account EGI production.

Table 5.2 – Genes up-regulated in the *A. gossypii* EGI producing strain, with the corresponding *S. cerevisiae* homologues, clustered by GO class (p-value < 0.01)

GO ID	Biological process	*S. cerevisiae* ORF	*A. gossypii* ORF
GO:0006865	aminoacid transport	CAN1, ALP1; PUT4	AFR668W; AFR156W
GO:0034220	ion transmembrane transport	FRE1; CTR1; FTR1	AGL114C; AAR030W; AEL294C
GO:0055085	transmembrane transport	PHO84; FRE1; CTR1; FTR1; ENA1, ENA2, ENA5; CAN1, ALP1	AFR442C; AGL114C; AAR030w; AEL294C; AGL097C; AFR668W
GO:0006811	ion transport	PHO84; MCH1; FRE1; CTR1; FTR1; ENA1, ENA2, ENA5	AFR442C; AFR595W; AGL114C; AEL294C; AAR030W; AGL097C

The transcriptional response of the EGI producing strain revealed that the majority of the up regulated genes encode transporter proteins that may be essential for the maintenance of cellular ion homeostasis. In contrast to the up-regulation of ion transporters in *A. gossypii*, in *Arabidopsis thaliana* (Martinez and Chrispeels 2003) and *A. niger* (Guillemette et al. 2007) many genes encoding membrane proteins and transporter proteins are repressed when recombinant proteins are produced. Previous studies showed that both calcium (Bonilla and Cunningham 2003) and zinc (Ellis et al. 2004) are required for ER function in yeast and that their deficiency induces the UPR. Since several ion transporters have increased rather than reduced expression in *A. gossypii,* ion deficiency would be less likely to act as a UPR switch during EGI production.

Valkonen and co-workers suggested that the optimal extent of UPR induction is different for different proteins. Furthermore, they were unable to detect improved production of *T. reesei* EGI protein in a *S. cerevisiae* strain overexpressing Hac1p. The reason for this lack of improvement could be explained by the fact that EGI production was not very dependent on the level of the UPR pathway components. To support this suggestion, *HAC1* deletion affected production of EGI less than it affected production of α-amylase. This may be at least partially because different proteins may have secretion problems at different levels of the secretory pathway. On the other hand, UPR induction by DTT would have been expected, since it is well documented in other microorganisms.

Table 5.3 – Genes down-regulated in the *A. gossypii* EGI producing strain, with the corresponding *S. cerevisiae* homologues, clustered by GO class (p-value < 0.01)

GO ID	Biological process	*S. cerevisiae* ORF	*A. gossypii* ORF
GO:0006417	regulation of translation	SSB1, SSB2; GCN20; RPG1	ABL174C; AEL032W; ABL065W
GO:0010608	posttranscriptional regulation of gene expression	SSB1, SSB2; GCN20; RPG1	ABL174C; AEL032W; ABL065W
GO:0032268	regulation of cellular protein metabolic process	SSB1, SSB2; GCN20; RPG1	ABL174C; AEL032W; ABL065W
GO:0006448	regulation of translational elongation	SSB1, SSB2; GCN20	ABL174C; AEL032W
GO:0006412	translation	SSB1, SSB2; RPS28A, RPS28B; RPG1	ABL174C; AGR261W; ABL065W

The predicted proteins encoded by the down-regulated genes as a result of EGI production are related to translation process (Table 5.3). When *S. cerevisiae* cells were treated with DTT (Payne et al. 2008), a down-regulation of proteins involved in ribosomal biogenesis was reported. Transcriptional down-regulation of ribosomal proteins during ER stress conditions was also observed in *S. cerevisiae* by Travers et al. (2000). The up-regulation of ribosomal biogenesis, RNA metabolism, translation, and organelle biosynthesis is specific for *HAC1* overexpression in *P. pastoris* and not observed with DTT treatment, while the latter leads specifically to the up-regulation of genes related to chemical stimulus, and the down-regulation in the groups electron transport and respiration, so that these reactions have to be regarded as specific for the treatment with a reducing agent rather than UPR regulated.

5.3.3 Effect of DTT on *A. gossypii* growth and transcription profile

The addition of DTT at the culture time point 10h caused a significant and immediate reduction in the specific growth rate of the culture (Figure 5.4), which was expected to cause wide-ranging effects on the gene expression profile. Transcription of genes encoding ribosomal proteins is directly correlated with growth rate (Hayes et al. 2002; Lim et al. 2003), and these were seen to be down-regulated 1h after the addition of DTT to the cultivation (Cluster 1 in Figure 5.5 and Table 5.4). A similar response has been observed in *A. nidulans*, where most of the ribosomal encoding genes were down-regulated after 1h of DTT exposure (Sims et al. 2004; Sims et al. 2005), and *A. niger* (Guillemette et al. 2007). Also, genes involved in filamentous growth (Table 5.5), glycosylation and lipoprotein biosynthesis (Cluster 1 in Figure 5.5, Table 5.4 and 5.5), required for cell wall biosynthesis, were already down-regulated 30min after DTT addition, which also helps to explain the observed reduction in the specific growth rate.

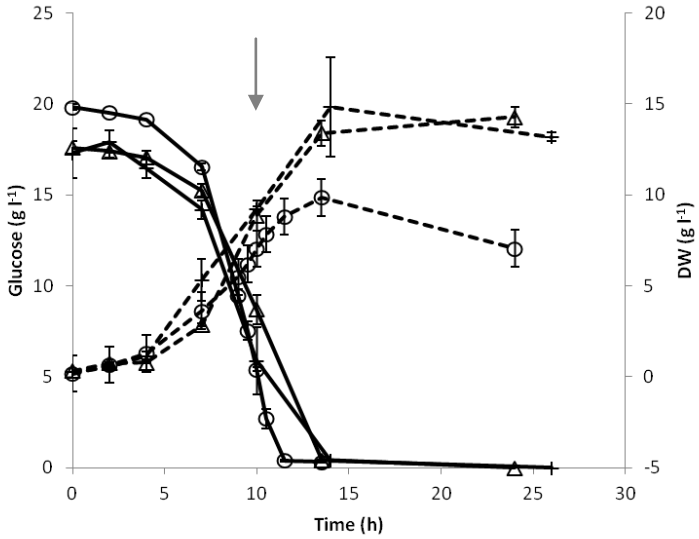

Figure 5.4 – Glucose consumption and growth curves of the *A. gossypii* batch cultures in AFM supplemented with G418, at 30 ºC. Solid lines correspond to glucose and dash lines to DW: (○) DTT, (Δ) EGI and (+) empty vector. Values represent the average and the standard deviation of two independent bioreactor cultures. Arrow indicates the culture time point where DTT was added.

5.3.3.1 Biological functions regulated by DTT in *A. gossypii*

As can be observed in Figure 5.5, the co-expression clusters with different expression profiles between recombinant EGI production with and without DTT treatment are clusters 1, 2, 6, 7 and 9. The genes included in clusters 1 and 6 were down-regulated by DTT, whereas the genes included in clusters 2, 7 and 9 were up-regulated by DTT. The GO classes overrepresented in each of these clusters are indicated in Table 5.4. Briefly, cluster 1 was statistically enriched for the GO terms anion transport, glycosylation, lipoprotein biosynthesis and translational elongation; cluster 2 for the GO categories of growth, organelle organization, regulation of biological processes, mitotic cell cycle, among others; cluster 6 in reproduction and peptide transport functions, cluster 7 was enriched in the response to stress, cell division and proteasome assembly categories and finally, cluster 9 was up-regulated in the terms of protein transport, cellular membrane fusion and autophagy.

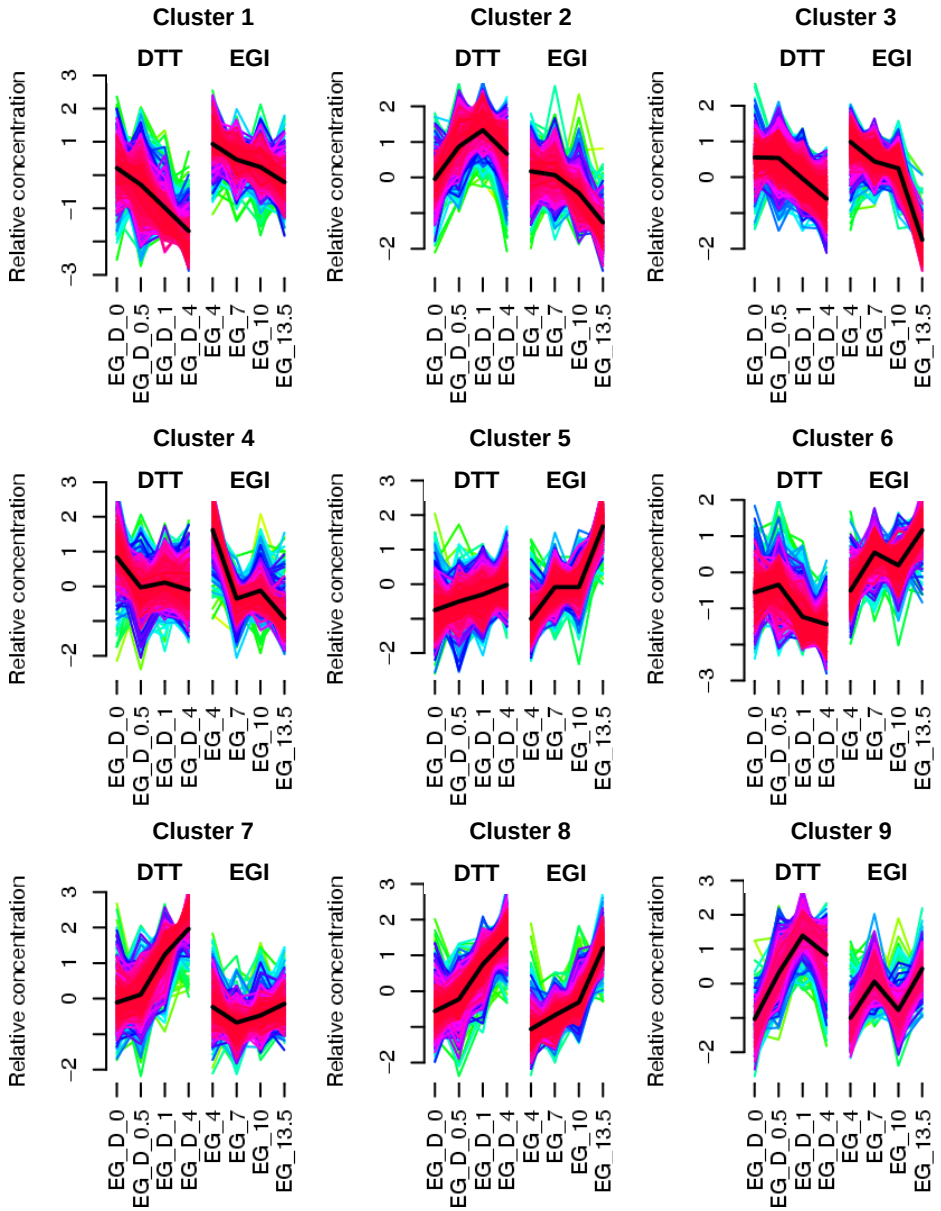

Figure 5.5 – Co-expression clusters with different expression profiles between recombinant EGI production with and without DTT treatment. Legend: EG_D_0 – 10h fermentation time sample, immediately before DTT addition; EG_D_0.5 – 10h30min, EG_D_1 – 11h, and EG_D_4 – 14h fermentation time samples from recombinant EGI producing strain treated with DTT for 30 min, 1h and 4h, respectively; EG_4 – 4h, EG_7 – 7h, EG_10 – 10h, and EG_13.5 – 13h30min fermentation time samples from recombinant EGI producing strain.

Table 5.4 – GO enrichment in the co-expression clusters with different expression profiles between recombinant EGI production with DTT treatment and recombinant EGI production alone ($p < 0.01$)

Cluster	GO class
C1	GO:0006820 anion transport GO:0042273 ribosomal large subunit biogenesis GO:0070085 glycosylation GO:0043412 macromolecule modification GO:0006879 cellular iron ion homeostasis GO:0006505 GPI anchor metabolic process GO:0009100 glycoprotein metabolic process GO:0000101 sulfur amino acid transport GO:0006811 ion transport GO:0006486 protein glycosylation GO:0042158 lipoprotein biosynthetic process GO:0042157 lipoprotein metabolic process GO:0006414 translational elongation
C2	GO:0000003 reproduction GO:0000746 conjugation GO:0006996 organelle organization GO:0019236 response to pheromone GO:0040007 growth GO:0051704 multi-organism process GO:0065007 biological regulation GO:0007117 budding cell bud growth GO:0033365 protein localization to organelle GO:0000278 mitotic cell cycle GO:0007049 cell cycle GO:0006368 transcription elongation from RNA polymerase II promoter GO:0030029 actin filament-based process GO:0007059 chromosome segregation GO:0015931 nucleobase-containing compound transport GO:0051641 cellular localization GO:0033036 macromolecule localization GO:0010467 gene expression GO:0030447 filamentous growth GO:0006428 isoleucyl-tRNA aminoacylation GO:0006508 proteolysis GO:0055085 transmembrane transport GO:0042176 regulation of protein catabolic process GO:0009059 macromolecule biosynthetic process GO:0016049 cell growth GO:0045896 regulation of transcription during mitosis GO:0006997 nucleus organization GO:0034645 cellular macromolecule biosynthetic process
C6	GO:0000003 reproduction GO:0000746 conjugation GO:0015833 peptide transport GO:0019236 response to pheromone GO:0030476 ascospore wall assembly GO:0000398 nuclear mRNA splicing, via spliceosome GO:0000279 M phase GO:0006310 DNA recombination GO:0006259 DNA metabolic process GO:0031110 regulation of microtubule polymerization or depolymerization

Table 5.4 – continued

Cluster	GO class
C7	GO:0006897 endocytosis GO:0009607 response to biotic stimulus GO:0032846 positive regulation of homeostatic process GO:0043248 proteasome assembly GO:0051301 cell division GO:0009102 biotin biosynthetic process GO:0046130 purine ribonucleoside catabolic process GO:0046700 heterocycle catabolic process GO:0010324 membrane invagination GO:0032212 positive regulation of telomere maintenance via telomerase
C9	GO:0007034 vacuolar transport GO:0032502 developmental process GO:0050789 regulation of biological process GO:0050896 response to stimulus GO:0065007 biological regulation GO:0006944 cellular membrane fusion GO:0051301 cell division GO:0051716 cellular response to stimulus GO:0006384 transcription initiation from RNA polymerase III promoter GO:0007154 cell communication GO:0000910 cytokinesis GO:0006914 autophagy GO:0016192 vesicle-mediated transport GO:0033036 macromolecule localization GO:0051641 cellular localization GO:0015031 protein transport GO:0006624 vacuolar protein processing GO:0006259 DNA metabolic process GO:0006352 transcription initiation, DNA-dependent GO:0007033 vacuole organization GO:0006996organelle organization

As expected, DTT treatment caused wide-ranging effects on the gene expression profile, with a generally more inducing than repressing effect. Genes involved in the response to stress, transcription, protein unfolding, proteolysis, endosome-to-Golgi vesicle-mediated transport, secretion and DNA repair were induced by DTT (Table 5.4 and 5.5), indicating the activation of a secretion stress response by DTT. Conversely, genes involved in filamentous growth, protein translation, ion, aminoacid and vitamin transport, protein glycosylation, lipoprotein biosynthesis and purine base biosynthesis were repressed by DTT (Table 5.4 and 5.5).

Table 5.5 – GO gene enrichment of gene clusters differentially regulated after 30min, 1h and 4h of DTT treatment, in comparison to time point 0h (p-value < 0.001). Highlighted classes are overrepresented at different DTT exposure times

	DTT (30min)	DTT (1h)	DTT (4h)
Upregulated	GO:0006597 spermine biosynthetic process GO:0044419 interspecies interaction between organisms GO:0015805 S-adenosylmethionine transport GO:0007089 traversing start control point of mitotic cell cycle GO:0043170 macromolecule metabolic process GO:0019255 glucose 1-phosphate metabolic process GO:0000354 cis assembly of pre-catalytic spliceosome **GO:0009057 macromolecule catabolic process** **GO:0043335 protein unfolding** GO:0051645 Golgi localization GO:0006272 leading strand elongation GO:0044260 cellular macromolecule metabolic process GO:0007070 negative regulation of transcription from RNA polymerase II promoter during mitosis **GO:0006508 proteolysis** GO:0006352 transcription initiation, DNA-dependent GO:0031098 stress-activated protein kinase signaling cascade **GO:0051348 negative regulation of transferase activity** **GO:0005992 trehalose biosynthetic process**	**GO:0043335 protein unfolding** GO:0007121 bipolar cellular bud site selection **GO:0034498 early endosome to Golgi transport** GO:0022406 membrane docking **GO:0005992 trehalose biosynthetic process** GO:0006793 phosphorus metabolic process GO:0006282 regulation of DNA repair GO:0015755 fructose transport GO:0008105 asymmetric protein localization GO:0016192 vesicle-mediated transport GO:0006378 mRNA polyadenylation GO:0051641 cellular localization GO:0016998 cell wall macromolecule catabolic process **GO:0046903 secretion** GO:0033036 macromolecule localization GO:0051181 cofactor transport **GO:0009311 oligosaccharide metabolic process** **GO:0009057 macromolecule catabolic process** GO:0045834 positive regulation of lipid metabolic process GO:0006366 transcription from RNA polymerase II promoter **GO:0043086 negative regulation of catalytic activity** **GO:0051336 regulation of hydrolase activity** **GO:0051338 regulation of transferase activity** **GO:0006508 proteolysis** GO:0048583 regulation of response to stimulus **GO:0051093 negative regulation of developmental process**	GO:0006307 DNA dealkylation involved in DNA repair GO:0015976 carbon utilization GO:0019954 asexual reproduction GO:0050896 response to stimulus **GO:0051348 negative regulation of transferase activity** **GO:0034498 early endosome to Golgi transport** GO:0006081 cellular aldehyde metabolic process GO:0034356 NAD biosynthesis via nicotinamide riboside salvage pathway **GO:0005992 trehalose biosynthetic process** **GO:0043335 protein unfolding** GO:0000395 nuclear mRNA 5'-splice site recognition GO:0008105 asymmetric protein localization **GO:0046903 secretion** GO:0006814 sodium ion transport **GO:0051093 negative regulation of developmental process** **GO:0009311 oligosaccharide metabolic process** GO:0033015 tetrapyrrole catabolic process GO:0006314 intron homing **GO:0043086 negative regulation of catalytic activity** **GO:0051336 regulation of hydrolase activity** **GO:0051338 regulation of transferase activity** GO:0046475 glycerophospholipid catabolic process

Table 5.5 – *Continued*

	DTT (30min)	DTT (1h)	DTT (4h)
Downregulated	**GO:0015888 thiamine transport** GO:0030447 filamentous growth **GO:0046688 response to copper ion** GO:0046938 phytochelatin biosynthetic process GO:0070783 growth of unicellular organism as a thread of attached cells GO:0006629 lipid metabolic process GO:0000917 barrier septum assembly GO:0009896 positive regulation of catabolic process GO:0005975 carbohydrate metabolic process GO:0042743 hydrogen peroxide metabolic process GO:0009100 glycoprotein metabolic process GO:0019401 alditol biosynthetic process GO:0009167 purine ribonucleoside monophosphate metabolic process **GO:0046463 acylglycerol biosynthetic process** **GO:0015850 organic alcohol transport** GO:0015858 nucleoside transport **GO:0019915 lipid storage** **GO:0015691 cadmium ion transport** GO:0006066 alcohol metabolic process **GO:0051180 vitamin transport** **GO:0009123 nucleoside monophosphate metabolic process** **GO:0070085 glycosylation** **GO:0018904 organic ether metabolic process** GO:0042158 lipoprotein biosynthetic process GO:0009113 purine base biosynthetic process GO:0042157 lipoprotein metabolic process GO:0000290 deadenylation-dependent decapping of nuclear-transcribed mRNA GO:0009112 nucleobase metabolic process GO:0006662 glycerol ether metabolic process GO:0044262 cellular carbohydrate metabolic process **GO:0006491 N-glycan processing** GO:0015937 coenzyme A biosynthetic process **GO:0034654 nucleobase-containing compound biosynthetic process**	GO:0006189 'de novo' IMP biosynthetic process **GO:0015691 cadmium ion transport** **O:0046688 response to copper ion** GO:0016337 cell-cell adhesion GO:0033212 iron assimilation GO:0006797 polyphosphate metabolic process **GO:0006491 N-glycan processing** GO:0043043 peptide biosynthetic process GO:0006541 glutamine metabolic process **GO:0046463 acylglycerol biosynthetic process** **GO:0015850 organic alcohol transport** **GO:0015888 thiamine transport** GO:0000461 endonucleolytic cleavage to generate mature 3'-end of SSU-rRNA from (SSU-rRNA, 5.8S rRNA, LSU-rRNA) **GO:0019915 lipid storage** **GO:0018904 organic ether metabolic process** **GO:0051180 vitamin transport** **GO:0009123 nucleoside monophosphate metabolic process** GO:0046036 CTP metabolic process **GO:0034654 nucleobase-containing compound biosynthetic process** GO:0006662 glycerol ether metabolic process	GO:0006168 adenine salvage **GO:0015691 cadmium ion transport** **GO:0046688 response to copper ion** GO:0019637 organophosphate metabolic process GO:0006797 polyphosphate metabolic process GO:0051691 cellular oligosaccharide metabolic process GO:0000373 Group II intron splicing GO:0019432 triglyceride biosynthetic process GO:0006057 mannoprotein biosynthetic process **GO:0015888 thiamine transport** GO:0044249 cellular biosynthetic process GO:0006450 regulation of translational fidelity **GO:0051180 vitamin transport** GO:0009100 glycoprotein metabolic process **GO:0019915 lipid storage** GO:0000135 septin checkpoint GO:0019538 protein metabolic process GO:0033967 box C/D snoRNA metabolic process GO:0009116 nucleoside metabolic process **GO:0009123 nucleoside monophosphate metabolic process** **GO:0070085 glycosylation** GO:0007532 regulation of mating-type specific transcription, DNA-dependent GO:0031506 cell wall glycoprotein biosynthetic process GO:0042278 purine nucleoside metabolic process

5.3.4 Comparison of *A. gossypii* secretion stress to previously described secretion stress responses

We analyzed the breadth of the secretion stress response in *Ashbya* using gene expression analysis with Nimblegen arrays. Gene ontology analysis indicated that patterns of similarly down-regulated GO groups under DTT stress could be observed for filamentous growth, protein translation, ion, aminoacid and vitamin transport, protein glycosylation, lipoprotein biosynthesis

and purine base biosynthesis (Table 5.4 and 5.5). The most significant up-regulated DTT GO categories were related to response to stress, transcription, protein unfolding, proteolysis, endosome-to-Golgi vesicle-mediated transport, secretion and DNA repair. Equivalent functions are regulated by the UPR in yeasts and filamentous fungi (Schröder and Kaufman, 2005; Travers et al., 2000; Kimata et al., 2005; Sims et al., 2005; Guillemette et al., 2007, Wimalasena et al. 2008).

Contrary to *T. reesei*, *P. pastoris* and *S. cerevisiae*, in *A. gossypii* there were no variations in the expression levels of genes belonging to the core metabolism (TCA cycle – *CIT1*; glycolysis – *PFK1* and aminoacid biosynthesis - *GLT1*, *ARO4*). This shows that DTT treatment has some impact on decreasing anabolic processes. In *P. pastoris*, levels of *GLT1* (glutamate biosynthesis) were strongly reduced, while *ARG1* (arginine) transcription was enhanced during UPR induction (Gasser et al. 2007). In *T. reesei* it was reported enhanced expression levels of some of its putative target genes related to amino acid biosynthesis, among them *GLT1*, *ARG1*, *ARO4* (Arvas et al. 2006). Although it might be speculated that a lower concentration of the transcriptional activator of amino acid biosynthetic genes in response to amino acid starvation *GCN*4 can lead to lower transcription of amino acid biosynthesis genes, in *A. gossypii* there is no variation in the expression level in the aminoacid biosynthesis. In *P. pastoris*, the pathways for aromatic amino acids (with *ARO4* as marker) and arginine biosynthesis (*ARG1*) tend to be down-regulated.

Furthermore, general stress response markers (*MSN2* and *SNZ*) were up-regulated in *A. gossypii* when DTT was added to the culture and ribosomal components (*RPL3, RPS23*) were down-regulated, as has been observed in *P. pastoris* (Graf et al. 2008). Most significantly, a fraction of genes that are correlated to general stress response appears to be induced in *S. cerevisiae* but remains unchanged to down-regulated in *P. pastoris* along with Msn2. Remarkably, the oppositional regulation of the two general stress markers *GCN4* and *MSN2* was noticed. In *A. gossypii*, *GCN4* behaved the same way as in *S. cerevisiae*, being down-regulated, while in *P. pastoris* it has been shown to be up-regulated (Gasser et al. 2007). Induction of *GCN4* transcription in response to DTT treatment or heterologous protein expression was reported for *T. reesei* (Arvas et al. 2006). Patil and coworkers (Patil et al. 2004) described activation of Gcn4p by ER stress and suggested that it acts as an essential transcription factor for the UPR together with or downstream of Hac1p.

Both glutathione (*GLR1*) and thioredoxin (*TRR1*, *TSA1*) related genes did not show any variation in the expression levels in the DTT treated strain compared to the control strain in *A. gossypii.* Overexpression of these genes was reported in a *HAC1*-overexpressing *P. pastoris* strain, being reduced at lower temperatures. It was not surprising that in *A. gossypii* the redox switch was not turned on as the expression of *GCN4* was repressed under DTT stress.

Moreover, in *A. gossypii*, DTT stress lead to a decreased transcription of genes coding for the large and small subunits of the mitochondrial ribosomes (*MRPS*, *RSM* and *MRPL* families), whereas in *P. pastoris* the variation resulted in an increase of transcription (Graf et al. 2008).

Prominent members of the mitochondrial inner membrane electron transport chain such as the ubiquinol cytochrome-c reductase complex (*CO,*) are repressed in *A. gossypii* upon DTT treatment. Additionally, cytochrome c (*CYC1*) and cytochrome c1 (*CYT1*) are only under DTT-dependent repression. The down-regulation of genes belonging to 'electron transport' and 'cellular respiration' can easily be explained by the strong reducing capacities of DTT.

Regarding lipid metabolism, the results are not very clear since the variation in gene expression is variable. On the other hand, a down-regulation of the major cell wall constituents (β-1,3 glucanases *EXG1*, cell wall mannoproteins *CCW12* and *TPI1*) was observed. *CWP2* and genes coding for proteins required for the transport of cell wall components to the cell surface (*SBE22*) were up-regulated. Taken together, these results indicate a significant remodeling process regarding the *A. gossypii* cell envelope during DTT stress conditions.

When the ER stress is extensive or sustained, and the function of ER cannot be restored, it leads to the removal of the affected cells by apoptosis. Accumulating data now indicate that ER stress is also a potent trigger of autophagy. DTT and tunicamycin at concentrations that trigger UPR, up-regulate the expression of ATG genes as well as the activation of *ATG1* kinase and ATG-dependent autophagy, but in an *IRE1*- and *HAC1*-independent manner (Bernales et al. 2006; Yorimitsu et al. 2006). This too was observed in *A. gossypii*, with several autophagy genes, *ATG1* kinase included, being induced after 1h of DTT treatment.

5.3.4.1 Conventional UPR was not activated in *A. gossypii* under the secretion stress conditions tested

As in previous transcriptomics work with yeast and other filamentous fungi, the induction of UPR was either accomplished by addition of DTT or recombinant EGI production. Surprisingly, contrary to what has been commonly observed in *S. cerevisiae* (Travers et al. 2000), *P. pastoris* (Graf et al. 2008), *T. reesei (*Arvas et al. 2006*), A. niger (*Guillemette et al. 2007*)* and *A. nidulans* (Sims et al. 2004; Sims et al. 2005), the expression levels of UPR activated genes, such as *HAC1*, *BIP1, IRE1*, and *PDI1* (Mulder et al 2004; Saloheimo et al. 2003) were not altered in *A. gossypii*, either as a result of recombinant protein production condition or by treatment with 10 mM DTT, suggesting that the UPR was not functional in *A. gossypii* and that other mechanisms were activated to balance ER stress. Moreover, the *A. gossypii* homologue of Ero1p (thiol oxidase), an

essential luminal protein involved in electron transfer during the formation of disulfide bonds in *S. cerevisiae*, was down-regulated 1h after DTT addition. Conversely, in *A. niger, S. cerevisiae* and *P. pastoris*, the respective homolog was induced under all ER-associated stress conditions tested (Travers et al. 2000; Gasser et al. 2007; Guillemette et al. 2007). The *A. gossypii* homolog of *S. cerevisiae* FLX1 (protein required for transport of FAD) was also down-regulated either under EGI production and DTT treatment conditions, which may have contributed to the lack of Ero1 induction.

The UPR, a regulation circuit of high relevance for heterologous protein production in eukaryotic cells (Valkonen et al. 2003a), has been shown to be differentially regulated in *P. pastoris* (Gasser et al. 2007) compared to *S. cerevisiae* (Travers et al. 2000), which is the typical model species for hemiascomycete yeasts. However, most of the folding/secretion related genes showed similar regulation patterns in both yeasts, whereas only genes associated with the general stress response were differentially regulated (Gasser et al. 2007).

The *A. gossypii* response to secretion stress was, therefore, very different from what was expected, as in several other studies (Travers et al. 2000; Sims et al. 2005; Gasser et al. 2007; Guillemette et al. 2007; Graf et al. 2008) the production of recombinant proteins or the chemically induced ER stress have been described to affect the cell behavior by inducing the defense mechanism UPR. A somehow similar situation was reported in *P. pastoris* by Resina et al (2007), where the basal *KAR2* or *PDI1* mRNA levels measured did not change when comparing the *HAC1*-overexpressing strain with the control strain. In *S. cerevisiae* the constitutive overexpression of the *HAC1* gene leads to up-regulation of the synthesis of more than 300 genes involved in the UPR, including *KAR2* and *PDI1* (Travers et al. 2000). In particular, these results from *A. gossypii* transcriptomic analysis and from *P. pastoris* overexpressing *S. cerevisiae HAC1* gene, suggests that the host cells can undergo a "pre-conditioning" step which results in a lower expression of UPR-related genes (*PDI1* and *KAR2*) (Resina et al. 2007).

5.3.4.2 UPR regulatory elements were not overrepresented in *A. gossypii* DTT-regulated genes

To support the above mentioned observation, a search for UPR elements (UPRE) in the promoter region of DTT-regulated genes was done using the tool FIRE to find common regulatory DNA motifs (Elemento et al. 2007). Analysis of the promoter regions of several UPR target genes has revealed different cis-acting elements in *S. cerevisiae* and mammalian cells. In *S. cerevisiae*, the *KAR2* UPRE corresponds to CAGCGTG, a partly palindromic sequence separated by a spacer nucleotide (Mori et al. 1998). In *A. gossypii*, an UPRE-like consensus sequence has not been characterized thus far. However, in *T. reesei*, Saloheimo et al. (2004) have shown that the binding specificity of *HAC1* closely resembles the UPRE consensus sequence of yeast genes under UPR

control (GNCAGNGTGNC) (Mori et al. 1998). Analysis of the *T. reesei NSF1* promoter revealed a sequence closely resembling the UPRE sequences of the *T. reesei PDI1* and *BIP1* and yeast promoters (ATCAG TCGTGAC). The consensus *A. niger* UPRE sequence, CAN(G/A)NTGT/GCCT, uncovered by (Mulder et al. 2006) also shows similarities with the *S. cerevisiae* UPRE. Both UPREs contain an E-box like palindrome (CANNTG) around a single spacer nucleotide.

In many cases, Gasch et al. (2004) were unable to detect significant enrichment of the *S. cerevisiae* upstream elements in the orthologous gene groups from other species, particularly in the more distantly related fungi. One possible explanation for this observation is that, although the genes are still coregulated in these species, the cis-regulatory mechanisms that control their expression have evolved.

The outcome of this analysis (Figure 5.6 and 5.7) resulted in the identification of only six motifs common to some different gene clusters, neither of which are known binding sequences for genes involved in the UPR. There were no clear *HAC1* or *GCN4* binding sites identified, though in the clusters 2 and 5 a weak signal for CAGCTC element was observed. This is similar to GACCTC motif, a potential binding sequence for *GCN4*. However, this element was under-represented, supporting the hypothesis that there was no UPR induction by DTT treatment. Gcn4p, as mentioned before, is involved in yeast general amino acid control but also appears to be a key element of the UPR (Patil et al. 2004). Together with Hac1p, Gcn4p drives transcription of a subset of UPR targets.

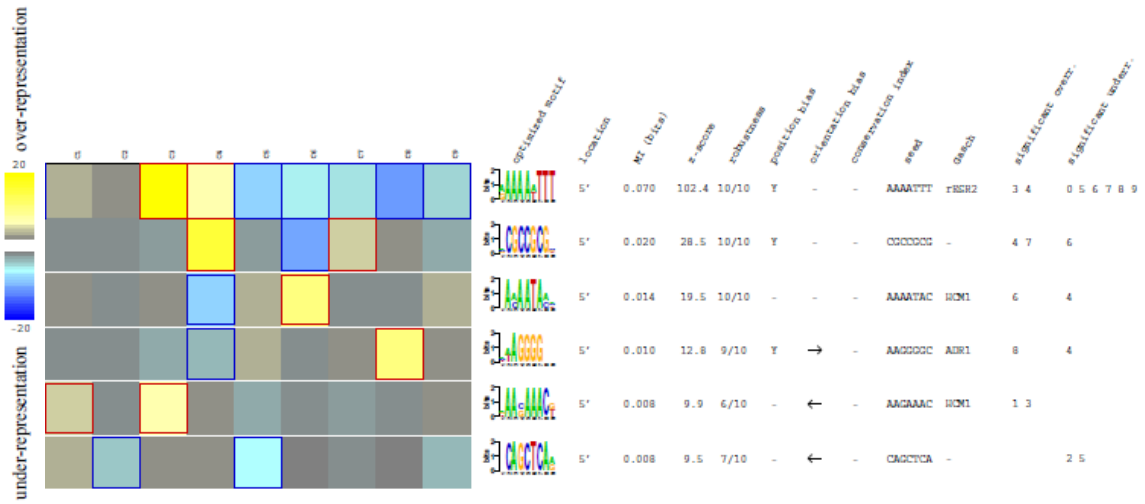

Figure 5.6 – Regulatory DNA elements significantly over- and under-represented ($p<0.05$) in the different co-expression clusters.

Binding sequences similar to those described for the *S. cerevisiae* transcription factors *HCM1* (AAR050C) and *ADR1* (YDR216W) were identified. *HCM1* is a forkhead transcription factor that

drives S-phase specific expression of genes involved in chromosome segregation, spindle dynamics, and budding (Askree et al. 2004; Pramila et al. 2006). ADR1 is a carbon source-responsive zinc-finger transcription factor, required for transcription of the glucose-repressed gene ADH2, of peroxisomal protein genes, and of genes required for ethanol, glycerol, and fatty acid utilization (Denis and Young 1983).

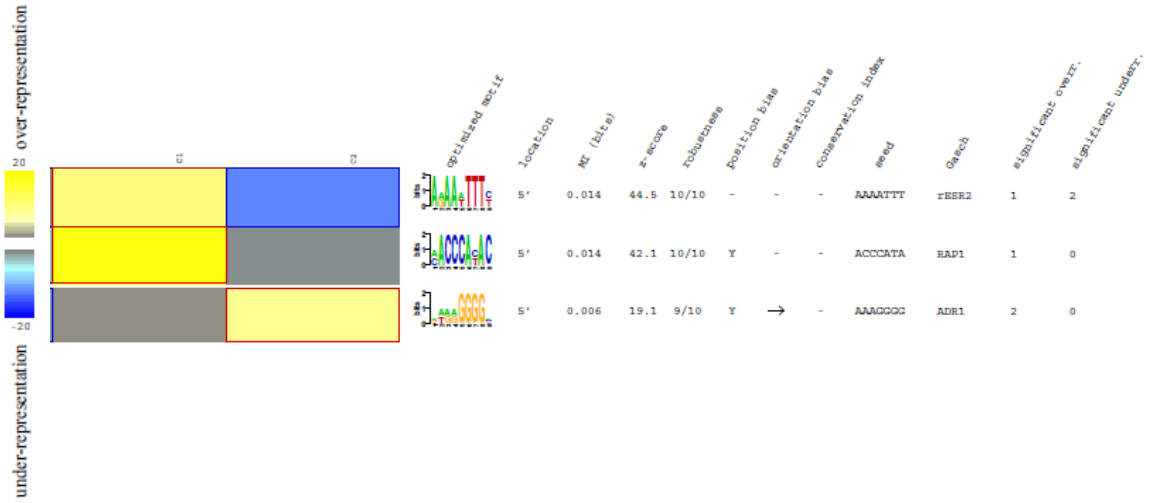

Figure 5.7 – Regulatory DNA elements significantly over- and under-represented ($p<0.05$) in the differentially up- (C1) and down-regulated (C2) genes. Simultaneously at 1h and 4h of DTT exposure.

Binding sequences for *RAP1* (ABL180W) and rESR2 were also found. *RAP1* is a DNA-binding protein involved in either activation or repression of transcription, depending on binding site context. It also binds telomere sequences and plays a role in telomeric position effect (silencing) and telomere structure (Chen et al. 2011). Its binding sequence was overrepresented in the cluster of DTT up-regulated genes (Figure 5.7), where there is an enrichment of genes involved in transcription (Table 5.5). rESR2 motif is involved in stress response, either by activating or repressing many genes involved in protein synthesis. In this case, since it was overrepresented in the cluster of up-regulated genes, probably it is involved in the activation of genes in response to stress induced by DTT.

Table 5.6 – Changes (n fold) in transcript levels of *A. gossypii* secretion-related genes and putative yeast orthologs after treatmen with DTT. Light grey corresponds to the up-regulated genes and dark grey to the down regulated genes.

Function	*S. cerevisiae* gene	*A. gossypii* ORF	Change (fold)[a]		
			0.5h	1h	4h
Translocation					
Subunit of the Ssh1 translocon complex	SSH1	AFR613C	-1.19	-1.02	-1.39
Protein folding					
DnaJ-like chaperone, localizes to the ER membrane	JEM1	ADR124C		0.93	1.09
Molecular chaperone of the ER lumen	LHS1	ADR128C	-1.21		-0.72
Member of a transmembrane complex required for efficient folding of proteins in the ER	ECM1	AFR720W	-1.73	-1.19	-1.17
Member of a transmembrane complex required for efficient folding of proteins in the ER	ECM3	AFR429C		-0.74	-0.85
Member of a transmembrane complex required for efficient folding of proteins in the ER	EMC5	ADL092W		-1.38	-1.43
Putative protein with a suggest a role in folding of ER membrane proteins	YER140W	AFR176W	-1.14	-0.82	-1.31
ATP binding protein, member of the HSP70 family	SSA2	AFR114W			1.01
Nucleotide exchange factor fo the Hsp70 Ssa1 protein, cytosolic homolog of Sil1p	FES1	ADL319W		1.11	1.61
Heat shock protein that cooperates with Ydj1p (Hsp40) and Ssa1p (Hsp70) in protein refolding	HSP104	AGL036C		1.15	1.96
Hsp90 co-chaperone, interacts with the Ssa group of the cytosolic Hsp70 chaperones	STI1	AEL224W			1.19
Cytoplasmic chaperone of the Hsp90 family	HSC82, HSP82	AFL148C			1.08
Co-chaperone that binds to Hsp82p and activates its ATPase activity	AHA1	AGL090W			1.48
Type II HSP40 co-chaperone that interacts with the HSP70 Ssa1 protein	SIS1	AAL008W		1.49	1.88
Type I HSP40 co-chaperone for the HSP70 Ssc1 protein in the mitochodrial matrix	MDJ1	AFR507W	0.59		1.25
Mitochondrial chaperone that cooperates with Ssc1p, prevents misfolded proteins aggregation	HSP78	AEL223C		2.14	3.04
Putative chaperone of the HSP40 (DNAJ) family	APJ1	AER427W			1.50
Small heat shock protein with chaperone activity, suppresses unfolded proteins aggregation	HSP26	ADL397C			2.26
Glycosylation/modification					
ER N/O-linked glycosylation					
Dolichyl-phosphoglucose-dependent alpha-1,2 glucosyltransferase	DIE2	ADL138C		1.07	
UDP-N-acetyl-glucosamine-1-P transferase	ALG7	AFL037W	-1.87	-1.72	-1.81
Mannosyltransferase catalyzes two consecutive steps in the N-linked glycosylation pathway	ALG2	AFL098W	1.10	1.28	
Dolichol-P-Man dependent alpha(1-3) mannosyltransferase	ALG3	AGL299C	-1.18	-1.66	-2.39
Mannosyltransferase involved in N-linked glycosylation	ALG9	ABL185C	-1.38	-0.81	-1.03
Alpha 1,3 glucosyltransferase involved in N-linked protein glycosylation in the ER	ALG6	ACR004W		0.90	1.03
Dolichyl pyrophosphate (Dol-P-P) phosphatase	CAX4 (CWH8)	ACL186W		-0.67	-0.97
Dolichol kinase required for protein N-glycosylation	SEC59	ABR051C	-1.03	-1.30	-2.43
Gamma subunit of the oligosaccharyltransferase complex of the ER lumen	OST3	AER413C	-1.53	-1.64	-1.77
Subunit of the oligosaccharyltransferase complex of the ER lumen	STT3	AFR491W	-2.33	-2.35	-2.72
Alpha glucosidase I, role in assembly of cell wall beta-1,6-glucan and N-linked protein glycosylation	CWH41	AFR483C	-1.28		
Glucosidase II alpha subunit, involved in N-linked protein glycosylation in the ER	ROT2	AAR173C	-1.64	-1.09	-1.21
Glucosidase II beta subunit, involved in N-linked protein glycosylation in the ER	GTB1	AGR178W	-1.74	-1.31	-1.30
Protein O-mannosyltransferase	PMT4	ABL085W		0.80	0.96
ER-localized and oxidants sensitive protein involved in N-glycosylation	EOS1	AER423C		-0.71	-1.09

Table 5.6 - continued

Golgi N/O-linked glycosylation					
Guanosine diphosphatase involved in the transport of GDP-mannose into the Golgi lumen	GDA1	AFR362C	-1.37	-0.92	-0.89
Subunit of the Golgi mannosyltransferase complex	MNN9	AGL259C	-2.34	-1.82	
Alpha1,6-mannosyltransferase of the Golgi mannosyltransferase complex	MNN10	AFR454W	-2.41	-2.41	-2.42
Alpha1,6-mannosyltransferase of the Golgi mannosyltransferase complex	MNN11	AEL142W	-1.31	-1.14	-1.93
Alpha-1,2-mannosyltransferase, responsible for addition of the first alpha-1,2-linked mannose	MNN2	AER018C	-2.27	-2.04	-1.72
Alpha-1,2-mannosyltransferase, responsible for addition of the second alpha-1,2-linked mannose	MNN5	AEL148W	-1.57	-1.12	-1.39
Alpha1,2-mannosyltransferase of the Golgi involved in O- and N-linked protein mannosylation	KRE2, KTR6	AGL267C	-1.30	-1.19	-0.93
Alpha-1,2-mannosyltransferase involved in O- and N-linked protein glycosylation	KTR1	ADL265W-A	-1.33	-1.15	-1.87
Mannosyltransferase involved in protein N-glycosylation in the Golgi	KTR2, YUR1	ACR251C	-1.35	-1.65	-1.90
Putative KRE2/MNT1 mannosyltransferase family protein involved in protein glycosylation	KTR4	AEL243W		2.99	4.72
Glycosylphosphatidylinositol (GPI) anchoring					
UDP-GlcNAc-binding and catalytic subunit of enzyme that mediates the first step in GPI biosynthesis	SPT14	AAL108C		0.97	0.73
Subunit of GPI-GlcNAc transferase involved in synthesis of the first intermediate in GPI biosynthesis	GPI19	AER333C			-1.98
Putative dolichyl-phosphate mannosyltransferase polypeptide 2, regulatory subunit	YIL102C-A	ACR063W-A			-1.05
Protein involved in the inositol acylation of glucosaminyl phosphatidylinositol (GlcN-PI)	GWT1	AFR094C		0.84	1.02
Mannosyltransferase that transfers the second mannose in GPI biosynthesis	GPI18	AER005C		1.28	2.04
ER membrane protein involved in a late step of GPI anchor assembly	GPI11	AAL059W	0.84	1.03	0.75
Subunit of the GPI transamidase complex that adds GPIs to newly synthesized proteins	GPI8	ADR299W		1.05	1.12
Subunit of the GPI transamidase complex that adds GPIs to newly synthesized proteins	GPI16	ADR333C	-1.17	-1.11	-1.23
Subunit of the GPI transamidase complex that adds GPIs to newly synthesized proteins	GPI17	ADR180C	-1.19	-1.26	-1.24
Integral plasma membrane protein involved in the synthesis of the GPI core structure	LAS21	AEL166C	-1.01	-1.07	-1.65
Alpha 1,2-mannosyltransferase involved in GPI biosynthesis	SMP3	AFR395C			-1.24
Vacuolar protein sorting					
Sec1-like protein essential for vacuolar protein sorting (Golgi to endosome)	VPS45	AFL053W		2.23	2.66
Subunit of the vacuole protein sorting complex (Golgi to endosome and endosome to vacuole)	VPS41	AGR149W		1.03	0.80
Subunit of the vacuole protein sorting complex (Golgi to endosome and endosome to vacuole)	VPS16	AGL252W		1.35	1.04
Component of the Golgi-associated retrograde protein complex (endosome to late Golgi)	VPS52	ADR112W		1.23	2.00
Component of the Golgi-associated retrograde protein complex (endosome to late Golgi)	VPS54	ABL093W		1.49	1.77
Nexin-1 homolog involved in the recycling of proteins (endosome to late Golgi)	VPS5, YKR078W	ADR211W		1.27	1.27
Vacuolar sorting protein (cytoplasm to vacuole)	VPS62, YPR157W	AFR324W		0.66	1.60
Vacuolar sorting protein (cytoplasm to vacuole)	VPS64, FAR10	AGL106C	0.88	0.93	0.80
Protein required for sorting proteins to the vacuole	MVP1	ACL014C		1.37	1.07
Htz1p-binding component of the SWR1 complex required for vacuolar protein sorting	VPS72	ADR114C		1.01	1.25
Endosomal protein required for recycling Golgi proteins and sorting ubiquitinated proteins	VPS27	AFL176C		1.44	1.22
Cytoplasmic class E vacuolar protein sorting factor	BRO1 (VPS31)	AGR204W		1.12	1.45

Table 5.6 – continued

Protein degradation					
ER-associated degradation (ERAD)					
Ubiquitin-protein ligase required for ERAD of misfolded proteins	HRD1	ACL019C		1.08	1.44
Protein involved in ERAD; component of the Hrd1p complex	USA1	AER311W		1.47	1.40
Protein of the ER membrane that plays a central role in ERAD; forms HRD complex with Hrd1p	HRD3	ACL160C	-1.42	-0.93	-0.90
ER quality-control lectin	YOS9	AER171W	-1.07	-0.81	
Alpha mannosidase-like protein of the ER required for degradation of glycoproteins	MNL1 (HTM1)	ADL390W			-1.51
Protein proposed to coordinate the assembly of proteins involved in ERAD	UBX2	ADR182W		0.83	0.89
Co-chaperone for Hsp40p that promotes ERAD of integral membrane substrates	HLJ1	AFL190C			0.94
ER membrane protein that recruits the ubiquitin-conjugating enzyme Ubc7p to the ER	CUE1, CUE4	AER279W		-0.95	-1.31
Ubiquitin/proteasome					
Ubiquitin isopeptidase	DOA4, UBP5	AFR007W	0.78	1.41	1.76
ATPases of the 26S proteasome involved in the degradation of ubiquitinated substrates	RPT3	AFR394W	0.79	0.71	
Ubiquitin activating enzyme (E1), involved in ubiquitin-mediated protein degradation	UBA1	AFR433C		1.07	
Ubiquitin-specific protease required for MVB sorting of membrane proteins	UBP2	ACL164C		1.14	1.50
ATPase involved in the transport of ubiquitinated proteins for degradation by the proteasome	CDC48	AFR158W		0.86	0.81
Ubiquitin fusion degradation protein that interacts with Cdc48p and Npl4p	UFD1	AFR662C		1.13	
Putative ubiquitin fusion degradation protein that interacts with Cdc48p and Npl4p	Putative UFD1	ADL320C		1.98	2.49
Protein required for ubiquitin-mediated protein degradation, forms complex with Cdc48p	DOA1	AER448W		1.26	
Ubiquitin regulatory X domain-containing protein that interacts with Cdc48p	SHP1	ABR211C		0.92	1.15
Vesicle trafficking/transport					
Ras-like small GTPase, involved in the ER to Golgi step of the secretory pathway	YPT1	ABR220W		1.09	
Alpha subunit of Type II geranylgeranyltransferase required for vesicular transport (ER to Golgi)	BET4	ACR042C		0.74	0.95
GTPase-activating protein for the Ypt1p, involved in protein trafficking (ER to Golgi)	GYP5, GYL1	AFL161C		0.99	0.76
GTPase-activating protein for the Ypt1p, involved in the regulation of vesicle transport (ER to Golgi)	GYP8	AFR154C		1.26	
ATPase required for ER to Golgi transport	SEC18	AER169C		1.12	
GPI inositol deacylase of the ER that negatively regulates COPII vesicle formation (ER to Golgi)	BST1	AFR521W	-2.00	-1.89	-2.55
Integral membrane component of ER-derived COPII-coated vesicles (ER to Golgi)	EMP47, EMP46	ABR243W	-1.69	-1.73	-1.27
Protein localized to COPII-coated vesicles, forms a complex with Erv46p (ER to Golgi)	ERV41	AER136W		-1.34	-1.67

Tabel 5.6 – Continued

ER chaperone, required for incorporation of amino acid permeases into COPII coated vesicles	SHR3	ABL137W		-1.35	-1.12
Integral membrane protein of the early Golgi and ER, involved in COP II vesicle transport (ER to Golgi)	SVP26	AFR127W			-1.12
Protein member of the p24 family involved in ER to Golgi transport	ERV25	ADR185W		-1.90	
Conserved phosphoesterase domain-containing protein that acts in cargo exit from the ER	TED1	ADL110W	-2.63	-2.90	-2.64
GTPase, Ras-like GTP binding protein involved in the secretory pathway (Golgi to vacuole)	YPT6	AGR257C			-1.05
GTPase-activating protein for the Ypt6p, involved in vesicle mediated protein transport	GYP6	ACR057C		1.49	1.99
Target membrane receptor (t-SNARE) for vesicular intermediates (Golgi to vacuole)	PEP12 (VPS6)	ACR092C		1.04	1.98
Alpha subunit of COPI vesicle coatomer complex (Golgi to ER)	COP1	ADR077C		0.92	
Essential beta'-coat protein of the COPI coatomer (Golgi to ER)	SEC27	AFL118W		0.74	0.70
Delta subunit of the coatomer complex COPI (Golgi to ER)	RET2	AFR274C		0.59	0.66
Peripheral membrane protein required for retrograde traffic (Golgi to ER)	DSL1	AFR652W		1.19	1.34
t-SNARE required for ER membrane fusion and vesicular traffic, part of Dsl1p complex (Golgi to ER)	UFE1	ADR220W		0.84	0.63
Protein of unknown function proposed to be involved in protein secretion, interacts with Dsl1p	SEC39	AER129C	0.81	1.33	1.42
Peripheral membrane protein required for fusion of COPI vesicles with the ER (Golgi to ER)	TIP20	ADL286W		1.23	0.90
v-SNARE binding protein that facilitates specific protein retrieval from a late endosome to the Golgi	BTN2, YPR158W	AFR325W		1.57	2.18
Protein presumably functioning within the endosomal-vacuolar plasma membrane proteins trafficking	RCR1, RCR2	AER006W		1.35	2.53
Non-essential subunit of the exocyst complex (distal secretion)	SEC3	ADR012C		1.23	1.37
Essential 107kDa subunit of the exocyst complex (distal secretion)	SEC5	AGL158C		1.64	2.10
Essential 121kDa subunit of the exocyst complex (distal secretion)	SEC8	ADL317C		1.10	1.44
Essential 113kDa subunit of the exocyst complex (distal secretion)	SEC15	AFR251C		1.48	1.65
Essential 100kDa subunit of the exocyst complex (distal secretion)	SEC10	AGL130C		1.56	1.80
Subunit of the exocyst complex (distal secretion)	EXO70	AFR100W		1.63	2.12
Essential protein dual roles in spliceosome assembly and exocytosis (distal secretion)	EXO84	ADL321W		1.29	1.32
Alpha-adaptin involved in vesicle mediated transport	APL3	ADL302W		1.05	1.05
Guanine nucleotide exchange factor for Arf proteins involved in vesicular transport	SYT1	ABR102W		1.35	1.49

Table 5.6 – Continued

Lipid/inositol metabolism					
Fatty acid metabolism					
Beta-keto-reductase involved in elongation of very long chain fatty acids	IFA38	ADR059C	-2.26		
Enoyl reductase involved in elongation of very long chain fatty acids	TSC13	AER215W	-0.73	-0.68	-0.75
3-hydroxyacyl-CoA dehydratase involved in elongation of very long chain fatty acids	PHS1	AFR090W	-1.80	-2.39	-2.18
Protein involved in positive regulation of unsaturated fatty acid biosynthetic process	SPT23, MGA2	ACR165W			1.42
Acyltransferases that contributes to triglyceride synthesis	LRO1	AFL179C	-2.56	-2.43	-2.25
Phospholipid biosynthesis					
sn-1,2-diacylglycerol ethanolamine- and cholinephosphotranferase	CPT1, EPT1	AGR344W	-1.19	-1.00	-0.79
Phosphatidylserine synthase	CHO1	AER357C	-0.82	-0.84	
Phosphatidylserine decarboxylase	PSD2	AAL131C	0.63	0.76	
Glycerol-3-phosphate/dihydroxyacetone phosphate acyltransferase	GPT2	AGR301C		-0.74	-1.28
1-acyl-sn-gylcerol-3-phosphate acyltransferase	SLC1	AFR592W	-1.53	-1.76	
Polyphosphatidylinositol phosphatase, dephosphorylates multiple phosphatidylinositols	INP53, INP52	AFL228W		1.39	1.01
Phosphatidylinositol 3-phosphatase, functions in phosphatidylinositol dephosphorylation	YMR1	ABR152C		1.65	2.63
Diacylglycerol pyrophosphate phosphatase involved in phospholipid metabolic process	DPP1	AAL084W	-1.59	-1.55	-1.34
Protein with disputed role in the synthesis of inositol phospholipids from inositol	SCS3	AAR165W	-0.68	-0.75	-1.00
Protein with a possible role in phospholipid biosynthesis	OPI10	AEL339C		0.91	1.55
Sphingolipid biosynthesis					
Component of serine palmitoyltransferase, functions in the first step of sphingolipid synthesis	LCB1	ABR145C	-3.08	-3.43	-3.41
Dihydrosphingosine 1-phosphate phosphatase, involved in sphingolipid metabolism	LCB3, YSR3	ACR259W			-1.16
Sphinganine C4-hydroxylase, required for sphingolipid biosyntheis	SUR2	AAL066W			1.53
Phosphatidylinositol:ceramide phosphoinositol transferase, required for sphingolipid synthesis	AUR1	ABR004C		1.01	1.02
Subunit of TORC2, a membrane-associated complex that is involved in sphingolipid metabolism	TSC11	ABR214C		0.84	0.66

[a] values obtained from LIMMA analysis

Well-documented stress reactions in recombinant protein producing yeasts are limited mostly to UPR in endoplasmic reticulum (called UPR-L) and there is a lack of knowledge concerning the impact of other stress responses on heterologous membrane protein expression. Only recently two additional different stress responses induced by misfolded membrane proteins with lesions in a membrane span or a cytosolic domain (called UPR-M/C), and by misfolded cytosolic proteins that do not enter the secretory pathway at all (called UPR-Cyto) have been preliminarily characterized in *S. cerevisiae* (Geiler-Samerotte et al. 2010; Metzger and Michaelis 2009). Transcriptomic

analysis of DTT-stressed *A. gossypii* cells expressing EGI revealed a specific stress response that, according to the list of induced proteins, is similar to recently reported cytosolic UPR-Cyto (Geiler-Samerotte et al. 2011). The majority of significantly UPR-Cyto induced proteins form an interacting set of cytosolic chaperones and co-chaperones. Also in *A. gossypii*, the Hsp70 Ssa1p activator *Sti1*, co-chaperone *Sis1*, cytosolic nucleotide exchange factor *FES1* and interaction partner *HSP104* were up-regulated by DTT (Table 5.6). *HSP82* and *HSC82* (abundant HSP90 family chaperones) along with their activator *AHA1* were up-regulated as well. These studies have led to the proposal that the UPR-Cyto is a specific *HSF1*-mediated module of the eukaryotic heat-shock response. *HSF1* transcript levels in DTT-stressed *A. gossypii* cells were only slightly increased. Moreover, no binding sequence for *HSF1* was overrepresented in the gene clusters analyzed. However, 13% of the differentially up-regulated genes after 4h of DTT exposure have promoters (in *S. cerevisiae* homologs) known to be bound by the heat shock reactive transcription factor Hsf1p encoded by *HSF1* (Teixeira et al. 2006), among which figure *JEM1*, *SSA2*, *STI1*, *SIS1*, *FES1*, *HSP104*, *HSP82*, *AHA1*, *MDJ1*, *HSP78*, *HSP26*. So, all the genes involved in protein folding that were significantly induced by DTT in *A. gossypii* (Table 5.6) seem to have been regulated by *HSF1*, which points to the possibility that an UPR-Cyto may have been activated in *A. gossypii* to cope with secretion stress induced by DTT.

5.3.5 Induction of ERAD by DTT

Quality control represents an essential keystone of the eukaryotic secretory pathway (Ellgaard et al. 1999). This role is performed by the ER that serves as the quality control system of the secretory pathway by keeping proteins that are misfolded in the ER, and ultimately, by targeting them to the ERAD, primarily via retro-translocation to the cytosolic ubiquitin-proteasome system (Werner et al. 1996), the degradation machinery of misfolded ER proteins located in the cytosol (Sommer and Jentsch 1993; Hiller et al. 1996). The ER provides an environment suitable for the folding and initial maturation of secretory proteins. Two interrelated systems responsible for keeping the homeostasis in the ER, termed the UPR and ERAD, govern the flux of proteins through the ER. Both systems are induced by ER perturbation, either via chemical treatment (e.g., with DTT to inhibit disulfide bond formation or tunicamycin to prevent N-linked glycosylation) or by overexpression of certain heterologous proteins (Casagrande et al. 2000; Travers et al. 2001; Kauffman et al. 2002). The yeast ER protein folding machinery consists of three distinct groups of proteins, Hsp70 chaperones (e.g., Kar2p and Lhs1p), along with their co-chaperones, the protein disulfide isomerase family (and accessory proteins such as Ero1p and Erv2p) and ERAD/ER quality control proteins (e.g., Der1p and Cne1p) (Silberstein et al. 1998; Nishikawa et al. 2005).

Calnexin (Cen1p) exhibits efficient chaperone activity for glycosylated and non-glycosylated substrates in mammals (Xu et al. 2004). The ER resident chaperone *CNE1* has been shown to be induced by both DTT and tunicamycin, in *A. niger*. In *A. nidulans,* however, the expression levels were not altered as a result of chymosin expression or DTT addition (Sims et al 2005) despite having a putative UPR element in its promoter sequence. The 2F5 Fab production by *P. pastoris* induces *CNE1*, although not to the same magnitude as in the Hac1p-overproducing strains (Gasser et al. 2007). An interesting finding is that in *A. gossypii* genome, the sequence coding for this protein was not found (Dietrich et al. 2004). Therefore an alternative mechanism of quality control might exist in order to balance this absence.

In addition to the ER membrane embedded Sec63, two well studied J-proteins, Jem1 and Scj1, reside in the ER lumen and enhance the ATPase activity of *KAR2* (Silberstein et al. 1998; Nishikawa et al. 2001). Kar2p is the major Hsp70 chaperone present in the ER lumen and participates in protein translocation and folding, ERAD, and regulation of UPR signaling (Fewell et al. 2001; Taxis et al. 2003; Kimata et al. 2004; Nishikawa et al. 2005; Xu et al. 2005). In *S. cerevisiae*, the chaperone cycle involves products encoded by *KAR2* (encodes a Bip-like chaperone) and *LHS1* (where the ATPase activities of these two Hsp70p chaperones are coordinately regulated) as well as nucleotide exchange activity provided by Sil1p and DNAJ proteins such as Scj1p and Jem1p (Steel et al. 2004). Although *LHS1* has been identified in *A. gossypii* as being a down-regulated gene (Table 5.6), *JEM1* together with *FES1,* the cytosolic homolog of *SIL1,* were up-regulated in the DTT treated cells. Also in *A. niger,* the homolog of the *S. cerevisiae LHS1* gene, was transcriptionally down-regulated under DTT stress induced condition compared to the control (Guillemette et al. 2007). The DnaJ homologs *JEM1* and *SCJ1* were significantly up-regulated in *P. pastoris* strains that either overexpress *HAC1* or were chemically treated with DTT (Graf et al. 2008).

The fundamental problem of ER quality control is the differentiation between folded, actively folding, and misfolded proteins. Misfolded proteins are recognized by having exposed hydrophobic amino acid stretches at their surface, which represent the interaction motifs of chaperones. Usually, this interaction keeps unfolded proteins soluble and helps to acquire a native structure. Exposure of large hydrophobic patches in the cytosol leads to the recruitment of the cytosolic Hsp70 machinery, which was generally induced in *A. gossypii* DTT treated cells, indicating that DTT may have lead to an accumulation of unfolded proteins in the cytosol.

For some time, the role of the Golgi complex has been neglected in the quality control process. (Fewell et al. 2001). Like the ER, the Golgi complex may also make conformation-based sorting decisions leading to disposal of abnormal proteins that were targeted for degradation. Here we

show that several post-ER pathways for protein disposal are up-regulated upon DTT treatment in *A. gossypii* (Table 5.6). Recombinant proteins may be routed from the Golgi either back to the ER for ERAD, or forward, sometimes appearing transiently at the cell surface, but most often routed via the endosomal system for degradation.

Proteins that fail to fold correctly are eventually degraded by the proteasome, however, there are differences in how these are handled prior to degradation. Proteins with misfolded ER-luminal domains use the ERAD-L pathway and proteins with disrupted transmembrane domains use the ERAD-M, membrane pathway (Huyer et al. 2004; Vashist and Ng 2004; Carvalho et al. 2006). For misfolded ER lumenal proteins the retro-translocation complex (RTC) comprises the Hrd1p/Hrd3p ubiquitin ligase complex of the ER membrane, connected to the Derlin Der1 and the Sec61 translocon. The Hrd1p/Hrd3p ligase forms a near stoichiometric membrane complex by binding to Der1p via the linker protein Usa1p (Vashist and Ng 2004; Carvalho et al. 2006; Willer et al. 2008). During DTT stimulus, the transcription levels of *USA1* (AER311W) were increased in *A. gossypii* (Table 5.6). This complex associates through Hrd3p with Yos9p, a substrate recognition protein in the ER lumen. The recognition of these substrates involves luminal chaperones, including the lectin-like proteins Yos9p and Htm1p/Mnl1p (Bhamidipati et al. 2005; Buschhorn et al. 2004; Jakob et al. 2001; Kim et al. 2005; Szathmary et al. 2005), both of which were repressed by DTT (Table 5.6). In our dataset when cells were exposed to DTT, there was a up-regulation of *HRD1* (ACL019C), like in the *P. pastoris* Hac1 strain (Graf et al. 2008). *HRD3* (ACL160C) had the opposite behavior (Table 5.6). However, transcript levels of a putative ortholog of *HRD3* were significantly increased in *A. nidulans* in DTT-induced and chymosin-producing strains, confirming previous observations that the UPR and ERAD are intimately linked (Casagrande et al. 2000; Travers et al. 2000).

Misfolded membrane proteins with lesions in the cytosol find their way to the proteasome after polyubiquitylation by the ER membrane integrated ubiquitin ligase Doa10 (Figure 5.1) (Plemper and Wolf 1999; Kostova and Wolf 2003; Vembar and Brodsky 2008). Thus, the site of lesion is an important determinant for the pathway used. Doa10p is a ubiquitin ligase required for the recognition of membrane proteins misfolded in their cytosolic domains (Huyer et al. 2004; Vashist and Ng 2004) and its transcript levels were slightly increased in *A. gossypii* cells treated with DTT. Substrates with misfolded intramembrane domains define a pathway (ERAD-M) that differs from ERAD-L by being independent of Usa1p and Der1p.

All three pathways converge at the Cdc48p ATPase complex (Cdc48p/Ufd1p/Npl4p), which further delivers the misfolded polyubiquitylated proteins to the proteasome for degradation (Figure 5.1) (Bays et al. 2001; Jarosch et al. 2002; Rabinovich et al. 2002). The Cdc48p/Ufd1p/Npl4p ATPase complex and the adaptor protein Ubx2p, a membrane protein that binds Cdc48p through its cytosolic Ubx domain (Neuber et al. 2005; Schuberth and Buchberger 2005) were up-regulated by DTT in *A. gossypii* (Table 5.6).

Interestingly, several genes belonging to the vesicular protein transport system involved in ER to Golgi, Golgi to ER and distal secretion were induced in the *A. gossypii* strain exposed to DTT. In *S. cerevisiae*, UPR causes up-regulation of genes affecting ER export (such as those encoding COPII components) as well as components of more distal portions of the secretory pathway (Travers et al. 2000). Indeed, Erv29p (AGL340C), which appears to function as a cargo receptor for ER export (Belden and Barlowe 2001), is also needed for efficient degradation of misfolded proteins (Caldwell et al. 2001), apparently by delivering these proteins to the Golgi. From the Golgi, proteins may be retrieved to the ER for ERAD (Vashist et al. 2001). Rer1p, a yeast Golgi membrane protein, appears to function as a receptor that interacts with the transmembrane domain(s) of a number of membrane proteins to retrieve them from Golgi to ER (Sato et al. 2001) for another try at folding or for ERAD. Thus, Rer1p can be considered as one example of a Golgi quality control receptor. In *A. gossypii*, Erv29p (AGL340C) and Rer1p (AAL002W) homologs were up-regulated when cells were treated with DTT. Also, proteins building the COPI coatomer, which are required for retrograde Golgi-to-ER transport, showed increased transcription levels upon ER stress in our experiments (Table 5.6), similarly to what happens in *P. pastoris* (Graf et al. 2008), strengthening the hypothesis that retrograde transport might contribute to quality control system in *A. gossypii*.

Another check point for quality control is located in the Golgi lumen, where a transmembrane sorting receptor, Vps10p, serves as a quality control cargo receptor, recognizing features of misfolded proteins in the Golgi lumen and handing them over to the endosomal system for vacuolar degradation (Hong et al. 1996). This route of control does not seem to occur in *A. gossypii* since down-regulation of Vps10p (AFR018C) after DTT addition was observed.

5.3.6 Glycosylation and ERAD

When in the ER, two modifications are introduced in the protein during the folding process: carbohydrates are added and disulfide bonds between cysteine residues are formed. A glycan consisting of Glc3-Man9-GlcNAc2 is linked to asparagine residues in the consensus sequence Asn-X-Ser/Thr of the protein. A trimmed N-glycosyl structure serves as an indicator that a protein is misfolded, directing it for degradation. A crucial protein factor in the delivery pathway of misfolded lumenal proteins out of the ER to the cytosol for retrograde transport and subsequent ubiquitylation is Yos9p (yeast osteosarcoma 9). Yos9p is a lectin-like protein required for the degradation of misfolded glycoproteins (Buschhorn et al. 2004; Kim et al. 2005; Szathmary et al. 2005). Only terminally misfolded proteins carrying a glycan with a terminal 1,6-linked mannose are allowed to leave the ER for proteasomal degradation (Bhamidipati et al. 2005; Denic et al. 2006; Quan et al. 2008). Structurally, Yos9p is part of the Hrd1 complex through a direct interaction with the large luminal domain of Hrd3p (Figure 5.1) (Carvalho et al. 2006; Denic et al. 2006). The link

between Hdr3p and Yos9p correlates well in *A. gossypii* when cells are subject to ER stress. Both transcripts (*HDR3* and *YOS9*) are down-regulated (Table 5.6).

Surprisingly, treatment of *A. gossypii* cells with DTT led to a major repression of the protein glycosylation pathway,(in particular of the *N*-glycosylation pathway) (Table 5.6), effect that at similar extent has only been described for treatments with tunicamycin. The observed repression was not only at the ER, but also at the Golgi processing level, indicating that a major accumulation of unglycosylated proteins must have occurred.

In yeast the α-1,2-mannosidase Mns1 removes a mannose residue of the middle branch of the Man9-GlcNAc2 carbohydrate tree, resulting in a terminal α-1,3-linked mannose. This leads to an acceleration of ERAD of N-glycosylated substrates (Knop et al. 1996). The best understood mechanism of ERAD substrate recognition is the glycan-dependent pathway of ERAD-L. In addition, the structure of the glycan is critical for recognition. An early candidate for the glycan receptor was a homolog of ER mannosidase I. This conserved protein is called Htm1p/Mnl1p in yeast and EDEM1 (ER degradation-enhancing αmannosidase-like protein) in mammals (Jakob et al. 2001; Nakatsukasa et al. 2001). The *A. gossypii* Htm1p/Mnl1p gene homolog (ADL390w), coding for alpha mannosidase-like protein of the ER required for degradation of glycoproteins is repressed under DTT stress (Table 5.6). Thus the rate of degradation of glycoproteins during *A. gossypii* secretory pathway might have been slower, leading to the accumulation of misfolded proteins.

5.4 Conclusions

The impact of recombinant EGI production and chemical treatment of cells with DTT upon genome-wide expression patterns of *A. gossypii* was studied. Secretion stress was induced in *A. gossypii*, either by chemical treatment of the cells with DTT or by expressing a recombinant EGI protein. Induction of ER stress forced *A. gossypii* cells to adapt to the new situation, by changing gene expression at the transcriptional level. The transcriptional responses to each stress were investigated and some unforeseen results were obtained.

The global analysis of the transcriptome as a result of recombinant EGI protein secretion resulted in a very weak response. Only 21 genes had different expression profile. Genes involved in ion transport and translation were up regulated, whereas the translation machinery was down-regulated in the *A. gossypii* recombinant strain. Folding limitations might be one possible explanation to what has been happening to EGI and as a consequence the protein is degraded.

Previous studies have shown that secretion of recombinant proteins can induce the UPR (Valkonen et al. 2003b; Mulder et al. 2004) and there are both general and recombinant product-specific responses. However, in this study that was not the case. The use of DTT to stress the ER is widespread in investigations of the UPR but DTT has a variety of other effects on the cell. DTT can affect a wide range of genes, some of which may not be closely related to the UPR (Travers et al. 2000; Watson et al. 2000; Martinez and Chrispeels 2003). A transcriptome comparison of two different stresses used to induce the UPR in *A. nidulans* revealed that the expression of some genes was similar under both conditions (DTT treatment and recombinant chymosin secretion), while other genes were only induced in response to one of the stresses (Sims et al. 2005). This suggests that different stresses elicit different responses. ER-associated stress was more strongly induced by chemical treatment of the cells with DTT, than by expressing the recombinant protein, EGI. None of these treatments triggered the endoplasmatic reticulum UPR, as the expression levels of several well-known UPR target genes remained unchanged in *A. gossypii*. The amount of DTT used in this study (10 mM) should have been enough to elicit a response, as happened in several other studies with yeast and filamentous fungus where 10 mM or 5 mM (Wimalasena et al. 2008) was used (Martinez and Chrispeels 2003; Arvas et al. 2006). Only in *A. niger* studies 20 mM DTT was added to the cells (Guillemette et al. 2007).

In yeast, the UPR was thought to be exclusively dependent on Ire1p-mediated splicing of *HAC1* mRNA. However, Schröder et al. (2003) suggested that additional signal transduction pathways from the ER to the nucleus exist in yeast that do not require either *IRE1* or *HAC1* since an UPRE*lacZ* reporter was activated in (Schröder et al. 2003) Δ*ire1* and *Δhac1* mutants in the presence of tunicamycin, but not in the absence of tunicamycin. This might be the case in *A. gossypii*, since the expression levels of these previously defined UPR targets remained unchanged even after DTT treatment.

Clearly, the lack of a strong UPR response when low levels of a heterologous protein is being produced strongly suggests that also non-UPR mediated bottlenecks might exist in *A. gossypii* that hamper efficient secretion. Carvalho et al (2011) have recently shown that, in *A. niger*, the induction of the UPR pathway is dependent on the level of protein expression. Apparently, under relative low-expressing conditions, *A. niger* was capable of dealing with the protein in such a way that the protein did not induce the UPR. Only high levels of recombinant protein in the ER induced ER stress. Moreover, ER stress due to overexpression of membrane proteins can elicit the transcriptional up-regulation of *BIPA* without apparent splicing of the *HAC1* intron in *A. niger* (Dave et al. 2006).

Interestingly, in *A. gossypii* the expression of genes involved in ERAD pathway (*HRD1* and *USA1*) were induced by DTT, suggesting that even though UPR is not activated *A. gossypii* is targeting proteins for proteolytic degradation via the ERAD system. Giving strength to this hypothesis is the up-regulation of the vesicle trafficking inside the cell, such as ER to Golgi, Golgi to vacuole and Golgi back to ER, as some of the ubiquitin/proteasome related genes.

By deleting ERAD components from *A. niger* genome, Carvalho et al (2011) suggested alternative pathways to remove misfolded proteins, such as the presence of proteases directly in the ER, Sec61p- DerA/HrdC-independent transport to the cytosol, or mechanisms of direct targeting of misfolded proteins to the vacuole. Therefore, other mechanisms besides ERAD might be of importance in the clearance of misfolded proteins and help the cells cope with the stress. In the absence of a functional UPR, the ERAD is sufficient to dispose aberrant proteins. Therefore UPR and ERAD are compensatory mechanisms to the same end.

The search for UPRE in *A. gossypii* resulted in the identification of an underrepresented motif which does not correspond to the consensus sequence described for the UPRE, giving support to the lack of a conventional UPR in *A. gossypii* under ER stress.

Despite the unexpected response by the most common UPR targets, some similarities to other studies were found, namely as previously reported in yeast (Travers et al. 2000), our results show that ER stress responses affected multiple ER and secretory pathway functions. As expected, we observe induction of some chaperones and other proteins involved in protein folding. However, these represent only a fraction of this set of target genes and we also found several categories of induced genes with other functions throughout the secretory pathway including vacuolar protein sorting, vesicular transport, ER-associated degradation. The transcription data analysis from *A. gossypii* revealed several striking differences when compared to *S. cerevisiae* or other fungi like *A. niger* or *T. reesei*, such as down regulation of translocation and protein glycosylation under DTT stimulus. This severe down regulation of glycosylation in DTT-stressed *A. gossypii* cells may have lead to the accumulation of unglycosylated proteins. In the present study, we have shown that the lack of an active conventional UPR in *A. gossypii* can be compensated by alternative quality control mechanisms. Moreover, we cannot exclude the possibility that several stress responses may be occurring at the same time and therefore signaling to different reactions.

The fact that *A. gossypii* has one of the smallest eukaryotic genomes, and as a consequence a reduced genetic machinery, might have contributed to the observed differences in quality control during protein folding when compared to other hosts. In adition, *A. gossypii* does not secret as many proteins as other fungi and protein misfolding may be a frequent event in *A. gossypii* therefore, the basal expression of some ER chaperones could be already high and masking the DTT effects. Thus, *A. gossypii* seems to be a more basic organism in what concerns protein quality control. Nevertheless, further systematic studies are needed at the transcriptional level to unravel the complex physiological dynamic responses that take place when *A. gossypii* operates as a host for recombinant protein production.

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Chapter 6

Conclusions and future perspectives

This work consisted in the physiological characterization of some *A. gossypii* strains and in the evaluation of the capacity to produce recombinant proteins and consecutive development of *A. gossypii* as a host for recombinant protein production. The results obtained lead to the following conclusions:

- Based on the colony radial growth rates and specific growth rates, the phenotypic differences between related *A. gossypii* strains, ATCC10895, IMI31268 and MUCL29450 were greater than expected (chapter 2). Special relevance to the fact that ATCC01895 and MUCL29450 although they are the same strain deposited in different culture collections, they show different physiological behaviour. Contrary to previous reports in the literature it was demonstrated that *A. gossypii* utilizes ammonium as sole nitrogen source at pH 6.5. Considerable growth at pH 4.5 was attained only on complex medium. D-Glucose, glycerol and starch were utilised as carbon sources. Production of ethanol during growth on glycerol was reported for the first time. Conversion of xylose into xylitol demonstrated that the xylose reductase is active. The information gathered in chapter 2 can be of use in physiological studies using chemically defined media.

- Endoglucanase I (EGI) and cellobiohydrolase I (CBHI) from the fungus *Trichoderma reesei* were successfully expressed in *A. gossypii* from plasmids containing the two micron sequences from *S. cerevisiae*, under the *S. cerevisiae* PGK1 promoter (chapter 3). Comparing secretion of both proteins, more EGI was secreted than CBHI, or more active protein was produced. EGI activity was detectable with soluble substrate, reaching levels of specific activity comparable to that produced by a similar EGI producing S*. cerevisiae* construct. Partial characterization of CBHI and EGI expressed in *A. gossypii* revealed overglycosylation when compared with the native *T. reesei* proteins, but the glycosylation was less extensive than on cellulases expressed in *S. cerevisiae*. This is represents a clear advantage when using *A. gossypii* as a host for recombinant protein production.

- Exposition of *A. gossypii* to ethyl methane sulfonate (EMS) (chapter 4) lead to the selection of innumerous mutants. Screening was carried out in order to identify secretion mutants with improved protein secretion ability. Secreted EGI, amylase, invertase and beta glucosidase activities of the parental strain and from five key mutants were investigated and

used as an indicator for enhanced protein production after the mutagenic treatment. From these five mutants that were studied in more detail, mutant S436 came out to be most promising since it had an increase in all the activities measured. Thus, the secretion capacity of this mutant might have been improved.

In another attempt to improve the secretion capacity of *A. gossypii*, the *GAS*1 gene which codes for a β-1,3-glucanosyltransglycosylase involved in cell wall assembly, was abolished from *A. gossypii* genome (chapter 4). In this way a higher permeability of the cell wall was expected and hence an increase in the protein secretion capacity. Nevertheless the amount of EGI secreted into the extracellular medium was similar or even lower than the amount of EGI secreted by the original recombinant strain.

- To better understand and determine the bottlenecks regarding recombinant protein production in *A. gossypii*, a global transcriptomic analysis was carried out in the recombinant EGI producing strain and also under chemical induced stress by DTT (chapter 5). The most striking outcome from this study is the fact that UPR is not activated in none of the conditions tested. Nevertheless, the down regulation of the translation related genes under EGI producing conditions, helps to explain the low EGI production levels. Also DTT, a widely used UPR inducer was unable to activate UPR in *A. gossypii*. Instead ERAD was highly induced when the mycelium was treated with DTT. In *A. gossypii* the lack of UPR activation is compensated by ERAD which exerts the quality control function during recombinant protein production.

- Some aspects of *A. gossypii* physiology, such as ammonium and xylose utilization were clarified and comparison between strains was conducted. *A. gossypi* secretory capacity was studied and strategies for strain improvement were implemented, from where one mutant with enhanced secretion arised from. Once the bottlenecks in the secretory pathway have been identified, the design of strategies to improve the secretion capacity of *A. gossypii* in a more efficient way can be facilitated. Thus, the aim of this work was achieved.

The following strategies are proposed as future work:

- The pH control mechanisms have never been studied in *A. gossypii* and this would be of extreme importance in such an organism that has been widely used at the industrial level of production.

- Ethanol production from glycerol could be further explored by metabolic engineering of the fungus.

- The potential of *A. gossypii* as a host for recombinant protein production can still be further investigated. Namely, other model proteins should be tested, in particular, proteins that

have been easily expressed in other micro-organisms, such as beta galactosidase. Also the use of a codon optimized sequence might be of some relevance in terms of improving recombinant protein production by *A. gossypii*.

- The recombinant proteins produced in *A. gossypii* should be purified in order to determine the level and nature of the glycosylation done by *A. gossypii* (task under development).

- The mechanisms behind the lack of a functional UPR in *A. gossypii* should be elucidated in more detail. Namely, the confirmation by real-time of the expression levels of the UPR target genes should be performed. At the same time the identification of the Hac splicing site is of extreme relevance in order to clarify the absence of an active UPR mechanism in *A. gossypii*. In addition, the transcriptome analysis should contribute to the identification of the most promising targets that will lead to a strain with enhanced secretion capacity.