

Monitoring and control of protein production in fungi

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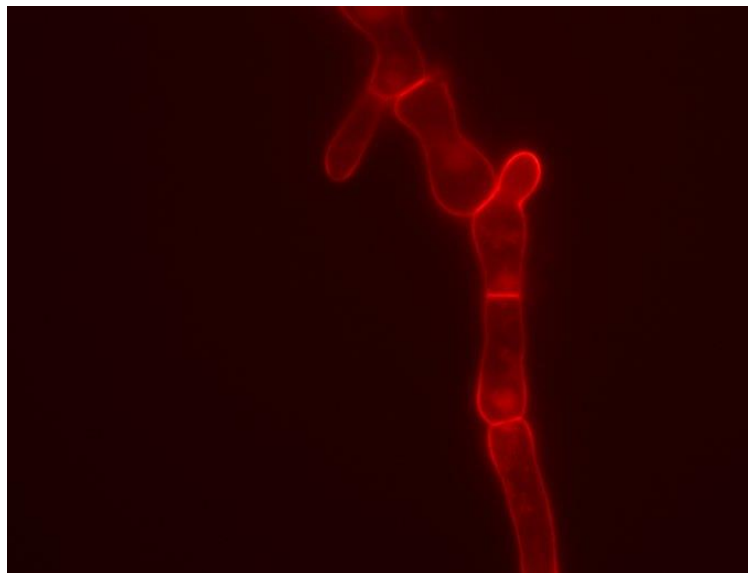
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Monitoring and control of protein production in fungi



Ph.D. Thesis

Martin Schalén

January 2015

Acknowledgements

My Ph.D. was performed between June 2011 and January 2015 at DTU Systems Biology. During this period, I have been fortunate to meet many new friends, talented students and researchers without whom this work would not have been possible. In the following lines I would like to take the opportunity to thank all of those who have been there for me also during the hard times, and encouraged me to pursue the task. It should be emphasized that this has been invaluable to me, and I am extremely grateful to all of you!

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Martin Schalén

January 2015

Summary

The work presented in this Ph.D. thesis describes the utilization of fluorescent proteins to investigate, compare and optimize protein expression and secretion in three different hosts; *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Aspergillus niger*. The following questions were addressed:

- How is protein production affected on a single cell level due to environmental stress factors?
- How can we improve heterologous protein production in filamentous fungi, and how does production in *Aspergillus nidulans* compare to protein production in the industrially exploited *Aspergillus niger*?

In Chapter **one** a solid background to recombinant protein production and the eukaryotic secretory pathway is given. Industrial products from yeasts and Aspergilli are presented, demonstrating the importance of recombinant protein production in these hosts. Since secretion of recombinant proteins is often preferred, the secretory pathway is described in detail. Differences between yeasts and Aspergilli are highlighted when appropriate. In addition, genetic manipulations of the secretory pathway in yeasts and Aspergilli are discussed, with a focus on how to improve recombinant protein production in these hosts. This field has been under a lot of investigation during the last few decades, and results show that alterations of the secretory pathway may have different effects depending on the host and also depending on what product is being produced. The chapter provides a foundation for later chapters, where the secretory pathway and molecular mechanisms is discussed based on experimental work.

Chapter **two** describes different reporter systems utilized in order to facilitate investigations within yeasts and Aspergilli. Significant focus lies on reporter systems based on fluorescent proteins in order to be able to

monitor or quantify different processes within a cell. The chapter will also give an insight to how the secretory pathway and recombinant protein production can be investigated by utilizing fluorescent proteins.

S. cerevisiae is one of the most commonly used eukaryotic model organisms. Advantages include highly developed molecular tools as well as an ease of performing physiological characterizations with a high reproducibility. Furthermore, it can be used for production of pharmaceutical proteins as well as for bio-ethanol production. In chapter **three** the impact of various environmental stress elements on the production of heterologous proteins in *S. cerevisiae* is investigated. A fluorescent reporter strain, producing an intracellular protein linked to tagRFP from the glycolytic *PGK1* promoter is constructed. This strain is used to monitor the level of production in each cell when exposed to environmental stress. The cells are grown in shake flasks as well as bioreactors and protein levels are analyzed by flow cytometry. It is demonstrated that the fluorescent reporter can be used to study the effects on stress elements on a population basis. Production of the protein was affected when cells were exposed to lower pH values, ethanol stress, increased osmotic pressure and increased glucose availability. A shift in fluorescence distribution is seen when cells are exposed to 1%, 3% and 5% ethanol, and this demonstrates that the strain is sensitive to small changes in the environment. Furthermore, increased levels of NaCl might be a way to improve protein levels. This effect is either due to inductive effects on the promoter, or other physiological changes in the cell. As the glycolytic *PGK1* promoter was used, it was not surprising that increasing levels of glucose led to higher production levels of the protein. In addition, it is shown that a binomial distribution occurs when cells are growing on ethanol after the diauxic shift. Two subpopulations occur, and this may be due to some cells having a higher sensitivity to ethanol, or some cells entering the stationary phase earlier than others. This study provides a more detailed knowledge of how protein production is affected due to various environmental stress factors. In the longer perspective, the approach could potentially be used for improving industrial processes where these stress factors are likely to occur.

In chapter **four**, a protein secretion reporter strain in *A. nidulans* is developed (Figure 1). The strain secretes mRFP through a carrier fusion to the well secreted glucoamylase from *A. niger*. Secretion of mRFP is verified through fluorescence measurements, microscopy and SDS-PAGE. The protein localizes primarily to the plasma membrane, septa and hyphal tips. The reporter strain is used to overexpress 14 genes within the secretory pathway, with the aim of investigating how protein secretion is affected. Genes were chosen based on other studies, and are from different compartments within the pathway. Several of the chosen genes have an impact on growth and protein secretion. An increase of protein secretion with 25% is seen when overexpressing the Rab GTPase RabD, a protein involved in transport between the Golgi and the plasma membrane. Other genes show substantial negative effects on protein secretion. Furthermore, single cell protein distribution is investigated by microscopy, and it is demonstrated that the secretory cargo localizes more to the plasma membrane when *rabD* is overexpressed. The study demonstrates the effect of secretory pathway engineering, and shows how a secretion reporter can be used in order to investigate the effect in the engineered strains. The study gives a deeper insight to recombinant protein secretion in Aspergilli, and the results are one step towards providing a detailed overview on the secretory pathway. The study can be used as a starting point for engineering Aspergilli cell factories in order to improve strains for recombinant protein secretion.

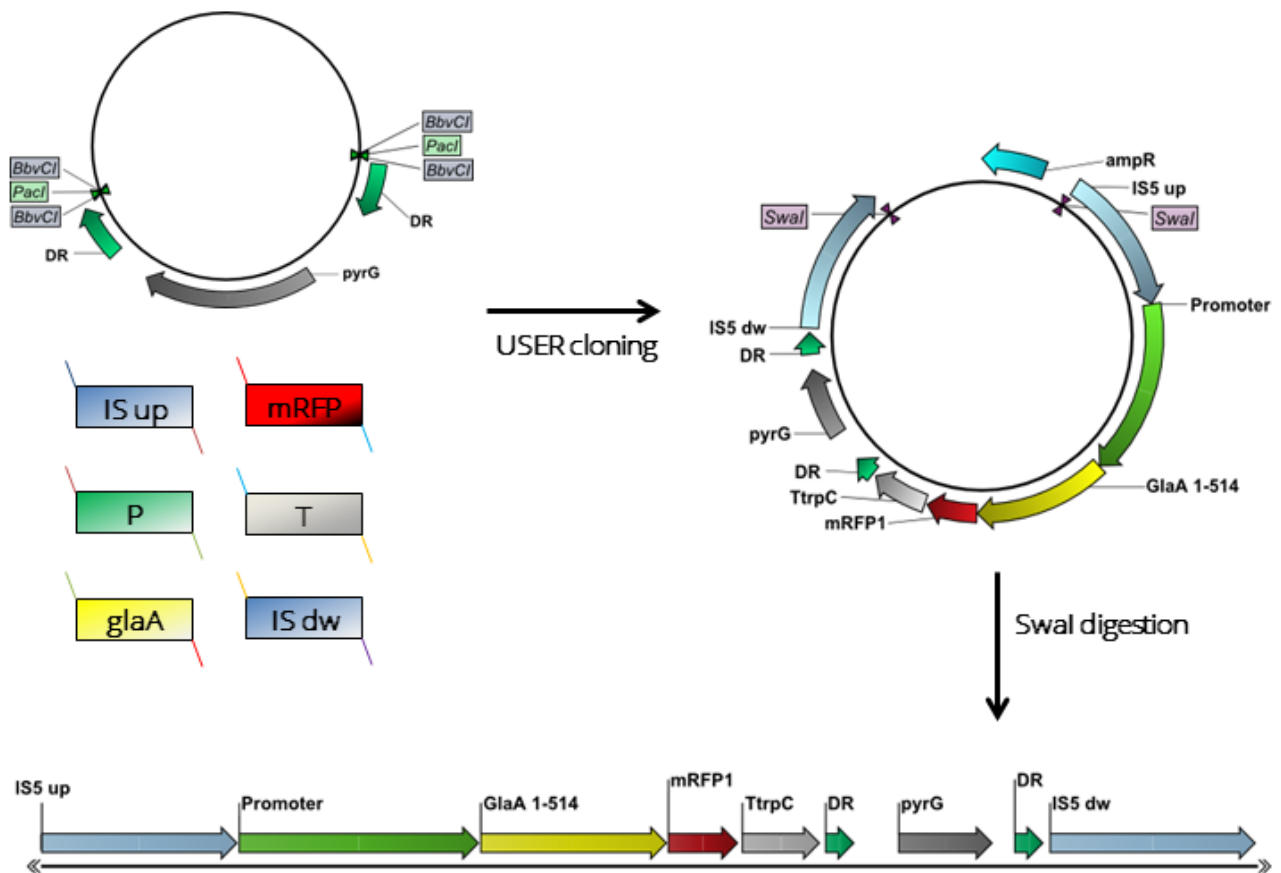


Figure 1. Plasmid construction for secretion reporter strain in *Aspergillus nidulans*. IS = Insertion Site, P = promoter, glaA = glucoamylase, T = terminator, DR = Direct Repeat

In Chapter **five** two commonly used promoters, the constitutive *PgpdA* and the maltose inducible *PglaA*, are compared in protein secreting *A. nidulans*. The strains are cultivated in 2 L bioreactors, and results show that *PglaA* is more effective in secreting the model protein. *PglaA* results in secretion of the model protein at a later stage in the bioprocess compared to the *gpdA* promoter, but final protein titers are higher with *PglaA*. Finally, an *A. niger* strain secreting the model protein from *PglaA* is constructed. It is seen that this strain results in secretion of more protein compared to *A. nidulans*, however the characteristics of the promoter are similar between the species. The study demonstrates how choice of promoter affects protein

secretion, and this is important for the future construction of efficient cell factories in Aspergilli. Furthermore, *A. nidulans* is a potential producer of heterologous proteins. If not in an industrial setting, it can be used for its advantages within molecular biology in order to develop novel tools for protein secretion in filamentous fungi.

Dansk sammenfatning

Denne Ph.D. afhandling beskriver arbejdet med at anvende fluorescerende proteiner til at undersøge, sammenligne og optimere protein ekspression og sekretion i tre forskellige værtsorganismer; *Saccharomyces cerevisiae*, *Aspergillus nidulans* og *Aspergillus niger*. De følgende spørgsmål blev undersøgt:

- Hvordan påvirker stressfaktorer fra det omgivende miljø protein produktionen i den enkelte celle i en population?
- Hvordan kan vi forbedre heterolog proteinproduktion i filamentøse svampe og hvordan er proteinproduktionen i *Aspergillus nidulans* sammenlignet med proteinproduktionen i den industrielt brugte *Aspergillus niger*?

I kapitel **et** gives en grundig gennemgang af rekombinant proteinproduktion og sekretionsvejen i eukaryoter. Industrielle produkter fra gær og Aspergilli bliver introduceret for at vise vigtigheden af rekombinant proteinproduktion i disse værter. Eftersom sekretionen af rekombinante proteiner ofte er foretrukket, beskrives sekretionsvejen i detaljer. Forskelle mellem gær og Aspergilli bliver fremhævet, når det er relevant. Derudover, diskuteres hvordan sekretionsvejen kan manipuleres for at forbedre proteinproduktionen i disse værter. I de sidste årtier har der været meget fokus på dette felt og resultater viser, at ændringer i sekretionsvejen kan have forskellige effekter afhængig af værten og også af, hvad der bliver produceret. Dette kapitel giver et fundament for senere kapitler, hvor sekretionsvejen og molekylærmekanismer bliver diskuteret på grundlag af eksperimentelt arbejde.

Kapitel **to** beskriver forskellige reportersystemer anvendt for at muliggøre studier i gær og Aspergilli. En væsentlig fokus ligger på reportersystemer baseret på fluorescensproteiner, der gør det muligt at følge eller kvantificere forskellige processer i cellen. Kapitlet vil også give indsigt i hvordan sekretionsvejen og produktionen af rekombinante proteiner kan studeres ved at anvende fluorescerende proteiner.

S. cerevisiae er en af de mest anvendte eukaryotisk modelorganisme. Nogle af fordelene inkluderer et højt udviklede molekylærværktøjskasse, samt gode muligheder for at foretage fysiologiske karakteriseringer med høj reproducerbarhed. Ydermere, kan den anvendes til produktionen af farmaceutiske proteiner og bioethanol. I kapitel **tre** studeres hvordan forskellige stresserelementer i det omgivende miljø påvirker produktionen af heterologe proteiner i *S. cerevisiae*. En fluorescerende reporter stamme, der producerer et intracellulært protein som er koblet til tagRFP under kontrollen af *PGK1* promoteren blev konstrueret. Denne stamme bruges til at følge produktionsniveauet i hver celle, når de udsættes for stress fra miljøet. Cellerne dyrkes i rystekolber og bioreaktorer og proteinniveauet analyseres med hjælp af flow cytometry. Det bliver demonstreret, at den fluorescerende stamme kan anvendes til at studere effekten af stresserelementer på en population. Proteinproduktionen blev påvirket, når cellerne blev udsat for lave pH værdier, ethanol stress, øgede osmotisk tryk og øgede glukose koncentrationer. En ændring i fordelingen af fluorescens blev observeret, når cellerne blev udsat for 1%, 3% og 5% ethanol, og dette viser, at stammen er i stand til at registrere små ændringer i miljøet. Ydermere, kan øgede niveauer af NaCl være en måde at forbedre proteinproduktionen. Denne effekt skyldes enten inducerende effekter på promoteren eller andre fysiologiske ændringer i cellen. Da den glykolytisk *PGK1* promoter blev anvendt, var det ikke overraskende at øgede mængder af glukose førte til højere produktionsniveau. Derudover bliver det vist, at to subpopulationer opstår, når celler vokser på ethanol efter glukosen er blevet opbrugt. Dette kan skyldes, at nogle celler er mere sensitive over for ethanol, eller at nogle celler går i den stationære fase tidligere end andre. Dette studium giver et mere detaljerede viden om, hvordan proteinproduktionen påvirkes af

forskellige stressfaktorer i miljøet. I et længere perspektiv kan denne fremgangsmåde bruges for at forbedre industrielle processer, hvor lignende faktorer opstår.

I kapitel **fire**, udvikles en reporter stamme for protein sekretion i *A. nidulans* (Figure 1, summary). Stammen udskiller mRFP via en fusion til glucoamylase fra *A. niger*. Sekretion af mRFP blev verificeret vha. fluorescens målinger, mikroskopi og SDS-PAGE. Proteinet lokaliserer hovedsageligt til plasmamembranen, septa og hyfespids. 14 gener i sekretionsvejen blev overudtrykt i reporter stammen med det mål at undersøge, hvordan protein sekretionen påvirkes. Mange af de valgte gener påvirkede vækst og protein sekretion. En 25% forøgelse af protein sekretion blev observeret, når genet kodende for Rab GTPase, *rabD* blev overudtrykt. RabD er involveret i transporten af proteiner mellem Golgi og plasmamembranen. Overudtryk af andre gener havde en betydelig negativ effekt på protein sekretion. Ydermere, blev protein fordelingen i enkelt celler undersøgt ved brug af mikroskopi, og det blev demonstreret at mRFP i højere grad lokaliserede til plasmamembranen, når *rabD* var overudtrykt. Dette studium giver et dybere indsigt i rekombinant protein sekretion i Aspergilli, og resultaterne er et trin mod at give en detaljerede oversigt over sekretionsvejen. Studiet kan bruges som et udgangspunkt for at udvikle cellefabrikker i Aspergilli for at forbedre stammer for rekombinant protein sekretion.

I kapitel **fem** sammenlignes to almindeligt brugte promotorer, den konstitutive *PgpdA* og den maltose inducerede *PglaA*, for protein sekretion i *A. nidulans*. Stammerne kultiveres i 2 L bioreaktorer, og resultaterne viser, at *PglaA* er mere effektiv til at udskille modelproteinet. Modelproteinet udskilles senere med *PglaA* end med *PgpdA*, men den endelige protein koncentration er højere med *PglaA*. Afslutningsvis konstrueres en *A. niger* stamme, som udskiller modelproteinet under kontrol af *PglaA*. Denne stamme udskiller mere protein end *A. nidulans*, dog har promoteren lignende karakteristika mellem arterne. Dette studium demonstrerer, hvordan valget af promoter påvirker protein sekretion, og dette er vigtigt for at

udvikle effektive cellefabrikker i Aspergilli. Ydermere, er *A. nidulans* en potentiel vært for produktionen af heterologe proteiner. Hvis ikke i industrien, så har den mange fordele i molekylærbiologien for at udvikle nye værktøj for protein sekretion i filamentøse svampe.

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Chapter 1 – Protein secretion in fungal cell factories

Introduction

Genetic engineering has revolutionized industrial biotechnology. The possibility to insert foreign DNA into microorganisms has made it possible to create tailor-made cell factories that can produce valuable compounds such as biochemicals, biopharmaceuticals and enzymes. This enables mass production of compounds to relatively low costs, giving possibilities to treat diseases in less developed countries where the cost of e.g. one vaccination may be higher than the annual health expenditures per capita (Weinacker et al. 2014). The first recombinant biopharmaceutical to be approved by the FDA was insulin that was produced in *E. coli* (Ferrer-Miralles et al. 2009). Since then, the biopharmaceutical market has grown rapidly, and sales are now exceeding 100 billion USD (Nielsen 2013). Approximately 25% of approved pharmaceuticals are biopharmaceuticals, and out of these almost 20% were produced in the yeast *Saccharomyces cerevisiae* in 2008 (30% in *Escherichia coli*, 40% in mammalian cells and 10% in hybridomas) (Ferrer-Miralles et al. 2009; Martínez et al. 2012). Insulin is the most produced biopharmaceutical in *S. cerevisiae* (Nielsen 2013). Other important products produced by *S. cerevisiae* are virus like particles for vaccination against human papillomavirus, hepatitis B surface antigen, and granulocyte-macrophage colony stimulating factor used for recovery after chemotherapy (Demain & Vaishnav 2009; Gerngross 2004; Ferrer-Miralles et al. 2009). Monoclonal antibodies, hormones (e.g. insulin) and growth factors are the three top sales categories in the US for biological drugs (Aggarwal 2012).

Although *E. coli* is a preferred protein production host for a number of reasons, including ease of manipulation and high growth rate, it cannot perform some modifications that may be essential for biological activity of a protein. Other hosts, such as yeasts and mammalian cells may then be the most appropriate choice. However, each host has specific drawbacks, and the choice of host is dependent on desired traits of the product. For example, genetic engineering of *S. cerevisiae* is relatively straight forward, but products are often hyperglycosylated, which may affect half-life and immunogenicity of the protein. On

the contrary, mammalian expression systems often produce products with high quality, but as they grow slower and require expensive media, costs are generally higher.

Filamentous fungi are well-known for secreting high amounts of enzymes to the extracellular environment. However, this trait has been difficult to benefit from when it comes to production of recombinant proteins of non-fungal origin. As an example, mammalian cell production of monoclonal antibodies has been reported to be as high as 20 grams/L. In comparison, *A. niger* has produced Trataztmab at 0,9 g/L (Nevalainen & Peterson 2014). Productivity of heterologous proteins are generally low in filamentous fungi, and it may well be that the production of biopharmaceuticals is better suited for other hosts that are already well-developed and has advantages such as those mentioned above. In Table 1 examples of filamentous fungi cell factories are given.

Table 1. Heterologous protein production in filamentous fungi. Ss = signal sequence.

Organism	Compound	Donor organism	Expression strategy	Cultivation	Titer	Reference
A. awamori	Glucoamylase	A. niger	<i>gpdA</i> promoter		4,6 g/L	(Radzio & Kück 1997)
A. niger	Human interleukin 6	Human	GlaA carrier, <i>nidulans gpdA</i> promoter	Stirred tank	150 mg/L	(Punt et al. 2002)
A. nidulans	Interferon-a 2	Human	A. niger <i>AphA</i> gene ss and promoter	10 mL stirred cultures	200 U/mgdw	(MacRae et al. 1993)
A. oryzae	Lactoferrin	Human	GlaA fusion		2 g/L	
A. niger	Lysozyme	Hen egg white	<i>glaA</i> promoter	15 L stirred tank, batch	70 mg/L	(Gyamerah et al. 2002)
A. nidulans/A. niger	Peroxidase	Pleurotus eryngii	<i>alcA</i> promoter <i>nidulans</i> ,	1 L shake flask	460 U/L	(Eibes et al. 2009)
A. awamori	Llama variable heavy-chain antibody	Llama	A. awamori <i>exIA</i> promoter and ss	50 mL working volume shake flask	7,5 mg/L	(Joosten et al. 2005)
A. niger	lipase	Thermomyces lanuginosus	TAKA amylase from <i>Aspergillus Oryzae</i>	2 L stirred tank, batch	5,7 KU/g dw	(Prathumpai et al. 2004)
A. niger	Green fluorescent protein	Aequoria victoria	<i>glaA</i> promoter, GlaA fusion	100 mL working volume SF	N/A	(Gordon et al. 2000)
A. niger	alpha1-proteinase inhibitor	Human	GlaA fusion, <i>gpdA</i> promoter	SF	12 mg/L	(Karnaukhova et al. 2007)
A. niger	Monoclonal antibody Trastazumab	Human	GlaA fusion, <i>glaA</i> promoter	SF, fermentation (8 L working volume)	0,9 g/L	(Ward et al. 2004)

While yields of non-fungal heterologous proteins are low, enzymes are preferably produced in filamentous fungi. Filamentous fungi account for approximately 50% of the total enzyme production (Lubertozzi & Keasling 2009). Filamentous fungi secrete high amounts of different enzymes, and with recombinant technologies production has been further improved. For example, plant phytase (animal feed) production was improved 1000 times by recombinant approaches. The biggest markets for industrial enzyme production are the food and feed market, and important products include proteases, amylase, glucoamylase, lipases and cellulases (Demain & Vaishnav 2009). Important markets for industrial enzymes include detergents, textiles and the leather industry (Sharma et al. 2009). The enzymes may be either

homologous to the production host, or heterologously expressed. Roughly 65% of the enzymes are homologous, whereas 35% are heterologous. The donor organism for enzyme production in filamentous fungi is however often of a fungal origin (AMFEP). The donor organism may not be used in industry for various reasons, such as difficulties with genetic engineering or unattained GRAS (Generally Regarded as Safe) status. Table 2 lists some industrial enzymes produced in fungal hosts, the donor organism and the application of the product.

Table 2. Industrial enzyme production in various species of filamentous fungi (AMFEP, 2015).

Organism	Product	Donor organism	Application
T. reesei	Alpha-amylase	Aspergillus spp.	Starch hydrolysis (bio-ethanol production)
A. niger	Asparaginase	Aspergillus spp.	Food processing to reduce acrylamide formation
A. niger	Carboxypeptidase	Aspergillus spp.	Various, e.g. cheese ripening
A. niger	Glucoamylase	Aspergillus spp.	Starch hydrolysis (bio-ethanol production)
A. niger	Glucose oxidase	Aspergillus spp. / Penicillium spp.	Baking
A. niger	Phospholipase A-2	Pig (pancreas)	Baking, cheese making (De Maria et al. 2007)
A. niger	Catalase	Aspergillus spp.	Cheese making, prevents oxidation of food
A. oryzae	Lactase	Aspergillus spp.	Lactose-free products
A. niger	Peroxidase	Marasmius spp.	Lignin degradation
A. niger	Phytase	Aspergillus spp.	Animal feed
A. oryzae	Phytase	Peniophora spp.	Animal feed
T. reesei	Pectinase	Aspergillus spp.	Juice production
A. oryzae	Protease	Rhizomucor spp.	Various, including cheese making
A. niger	Xylanase	Talaromyces spp.	Food additive, pulp and paper industry

Heterologous expression of proteins and metabolites is the preferred way to meet the demands of a global market that is rapidly growing. The establishment of suitable expression systems, genetic engineering of host strains for beneficial alterations in physiology and improved bioprocessing techniques has been the focus for improving cell factories. The possibility of analyzing cell factories in a systems biology manner, i.e. the analysis of the -omes (transcriptome, proteome, metabolome, fluxome etc.) in combination with mathematical modelling, has led to more rational target selection for improvement of strains. As shown

above, the establishment of one main host is impossible, due to the diversity of products and traits of the strains. Although ongoing research is improving production of e.g. biopharmaceuticals in filamentous fungi, available production systems indicate that enzymes are more suited to be produced in filamentous fungi, whereas biopharmaceutical cell factories may be more relevant in other hosts, such as yeasts, mammalian cells and *E. coli*.

To lower production costs it is preferred that proteins are secreted to the extra-cellular space. This facilitates down-stream processing of the final product, since recovery does not include breakage of the cells. The core pathway of protein secretion is similar between yeasts and higher eukaryotes, and generally secretion is regarded as the limiting factor in protein production. Therefore, a lot of attention has been focused towards improving the secretory and folding capacity of the specific host. The secretory pathway (Figure 1) begins with translocation of the nascent protein to the Endoplasmic Reticulum (ER). Here, the protein is initially glycosylated and accurate folding is ensured by the help of chaperones. Correctly folded proteins leave the ER in vesicles budding off from the ER membrane and travels to the Golgi. In the Golgi, further modifications occur, such as cleavage of the protein at specific sites, and additional glycosylations, which may allow final maturation of the protein. The proteins leave the Golgi in vesicles destined for the plasma membrane, where exocytosis occurs. However, the Golgi acts as a sorting station, and the protein may be transported to other compartments as well. For example, transport to the vacuole for degradation will decrease the product yield and is therefore unwanted.

When overexpressing heterologous genes in order to produce large amount of proteins the cell homeostasis is disturbed. This triggers responses in the cell in order to cope with the increased burden. These responses include the Unfolded Protein Response (UPR) and Endoplasmic Reticulum Associated Degradation (ERAD). UPR ensures an increased folding capacity in the ER, as well as upregulation of a large set of genes to cope with the secretory stress. ERAD occurs if UPR is not sufficient to deal with the stress, and proteins are targeted for degradation in order to relieve the stress.

In each of the steps in the secretory pathway specific mechanisms ensure that transport and processing of the cargo occurs in a correct manner. The fusion of vesicles to the correct compartment is a highly specific process, which is regulated by proteins attached to vesicles as well as the acceptor compartment.

In the following sections, the secretory pathway will be explained more in depth. Since *S. cerevisiae* is an extensively used model organism, a lot of knowledge on the secretory pathway stems from this unicellular fungus. However, the secretory pathway is relatively well conserved among eukaryotes, and when appropriate differences between filamentous fungal species and yeast will be highlighted. Furthermore, findings relating to improving fungal cell factories will be discussed.

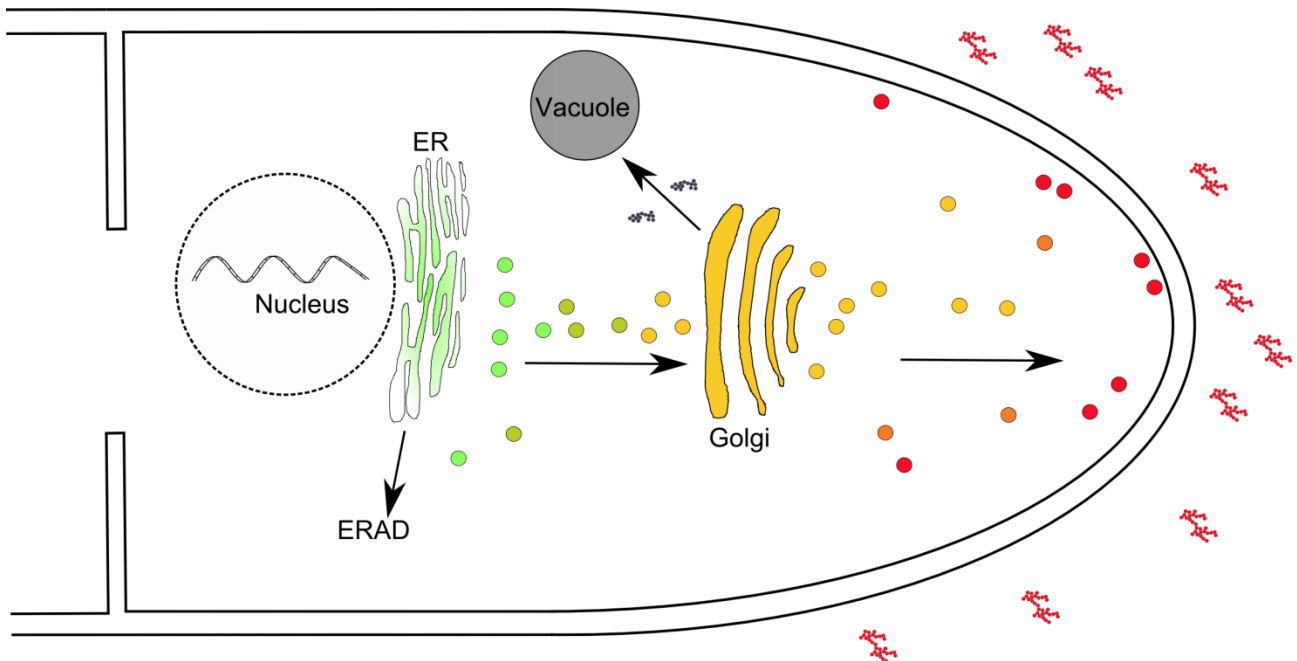


Figure 1. Protein secretion pathway in a schematic fungal hyphae. Proteins enter the Endoplasmic Reticulum (ER) where glycosylation and folding occurs. An overload of the folding capacity in the ER may result in the Unfolded Protein Response (UPR) or Endoplasmic Reticulum Associated Degradation (ERAD). Correctly folded proteins exit the ER in vesicles destined for the Golgi. In the Golgi, further protein maturation occurs, as well as additional glycosylation. Proteins may be transported from the Golgi to the vacuole for degradation. Proteins aimed for secretion exit the Golgi in vesicles bound for the plasma membrane, where exocytosis occurs. For visualization the Golgi is stacked. However, in *A. nidulans*, Golgi is not stacked (Peñalva et al. 2012). See the text for further details.

Secretory pathway

Translocation to ER

Proteins enter the secretory pathway (reviewed and discussed in (Hou, Keith E J Tyo, et al. 2012; Fleissner & Dersch 2010; Conesa et al. 2001; Delic et al. 2013; Young & Robinson 2014; Delic et al. 2014)) through translocation into the endoplasmatic reticulum (ER), which is the first compartment in the secretory pathway. Depending on the signal peptide (~15-50 amino acids in the N-terminal of the protein), translocation to the ER is performed during translation (cotranslational translocation) or afterwards (also called SRP-dependent or SRP-independent pathway, SRP for Signal Recognition Particle). Less hydrophobic signal sequences are more prone to the SRP-independent pathway, whereas more hydrophobic signal peptides can follow both routes. In the SRP-dependent pathway, an SRP binds the ribosomal-bound peptide once the N-terminal signal peptide is synthesized. Translation is paused and the SRP/ribosome/peptide complex is targeted to the SRP receptor, located in the membrane of the ER. When the complex has docked at the SRP receptor translation continues and the peptide is transported through the sec61 complex into the ER lumen during translation. Ribosomal binding of the sec61 complex is an evolutionary conserved, essential feature (Gogala et al. 2014). SRP-dependent translocation delivers secretory as well as membrane proteins to the ER (Denks et al. 2014). In the SRP-independent pathway, targeting to the ER occurs posttranslationally. In this case the unfolded protein is bound by chaperones and targeted to the ER-membrane. Upon interaction with the sec61 and sec62/63 complexes located in the ER membrane, the signal peptide is dragged through the membrane by the luminal ER chaperone BiP (Kar2p in yeast), which binds more and more of the prepeptide in the ER.

In filamentous fungi, a common strategy to ensure translocation and further on secretion of a protein is to fuse it with a well secreted protein at the N-terminal end (Gouka et al. 1997b). The most common carrier protein in *Aspergilli* is the glucoamylase (GlaA) from *Aspergillus niger*, although other proteins have also been used in this regard, for example α -amylases and signal peptides of laccases and lipases (Fleissner & Dersch 2010). It is believed that a well secreted protein will aid in translocating and transporting the

heterologous proteins through the secretory pathway. Recently, the effect of different signal peptides was studied in a secretion GFP reporter in *A. oryzae* (Ogino et al. 2014). This study demonstrated that using multiple signal sequences or combining signal sequences is a way to improve secretion of GFP and possibly other heterologous proteins.

In yeasts, the *S. cerevisiae* leader sequence of α -mating factor is commonly used. Engineering of the α -mating factor signal peptide have shown that protein secretion with this leader can be further improved (Lin-Cereghino et al. 2013). Synthetic leaders are also commonly used, and a recent study demonstrated that a synthetic leader was superior to the α -mating factor leader when secreting α -amylase in *S. cerevisiae* (Liu et al. 2012).

ER processing

The ER accounts for a wide range of modifications and quality controls of translated proteins. The most important ones are processing of the signal sequence, disulfide bond formation, glycosylation, folding, ER-associated degradation (ERAD) of unfolded or misfolded proteins and Unfolded Protein Response (UPR) (Idiris et al. 2010; Carvalho et al. 2011; Ellgaard & Helenius 2003). If the protein is not folded correctly, it will not pass the ER quality control system and thus not continue its journey through the secretory pathway. After signal peptide cleavage by Signal Peptidase Complex, correct folding is ensured and assisted by chaperones and foldases such as BiP (*S. cerevisiae* Kar2p), PDI, calnexin and calreticulin (not present in yeast) (Ellgaard & Helenius 2003). Whether a protein will interact with the calnexin or BiP chaperone (or both) depends on the location of N-linked glycan structures and which of the chaperones that bind the nascent protein first (Molinari 2000).

Glycoproteins are initially glycosylated during translocation. N-linked glycan structures are added to the nascent protein by oligosaccharyltransferase and in order to promote correct folding the glycan core structure is trimmed by glucosidase I and glucosidase II by removal of glucose. Monoglucosylated glycan cores are recognized and bound by calreticulin and calnexin, which interact with erp57p to form disulfide

bonds. Glucosidase II cleaves off the final glucose molecule, and correctly folded proteins will continue to exit the ER. However, if the protein is incorrectly folded, UDP-Glc:glucosyltransferase will add a new glucose to the glycan structure and the protein can once again be bound by calreticulin/calnexin. Only correctly folded proteins are allowed to exit this cycle (Ellgaard & Helenius 2003). Homologs of Erp57p and UDP-Glc-glucosyltransferase does not exist in *S. cerevisiae*, but can be found in Aspergilli (Conesa et al. 2001).

Chaperones assist in protein folding by preventing protein-protein interactions, while foldases catalyze slow reactions such as disulfide bridge formation (Conesa et al. 2001). When proteins are improperly folded they display features (including exposure of hydrophobic regions, aggregation tendencies, improper glycosylation) that are recognized by the chaperones. As the protein is bound by different chaperones and other “helper proteins” it is retained in the ER until proper folding is achieved. BiP (*S. cerevisiae* Kar2p), an hsp70 (Heat Shock Protein) molecular chaperone, binds to hydrophobic amino acids that are exposed on the proteins surface (Blond-Elguindi et al. 1993). The protein is retained in the ER by BiP binding until proper folding is achieved. A conformational change in BiP occurs through the hydrolyzation of ATP to ADP, and this releases the protein from being bound to BiP. The hydrolysis of ATP to ADP is regulated by DNAJ co-chaperones and proteins such as Lhs1p and Sls1p/Sil1p (Idiris et al. 2010). The sensing of unfolded proteins is universal, i.e. it applies to homologous as well as heterologously expressed proteins, as it is physical characteristics that are recognized, rather than amino acid sequences (Ellgaard & Helenius 2003).

As *bipA* and *pdiA* plays key roles in ER translocation and folding, the expression of these genes has been analyzed in protein producing strains. Subsequent overexpression has generated various results, which suggests that the effects are protein specific. Punt et al. analyzed expression of the *bipA* gene when expressing proteins from *A. niger*, and the gene expression was increased with increased protein expression (Punt et al. 1998). Although expression was increased in protein expressing strains, *bipA* overexpression in these strains did not result in increased protein secretion. A 2 fold increase in *bipA* levels

was seen when expressing hen egg-white lysozyme in *A. niger*, as well as increased levels of *pdiA* (Ngiam et al. 2000). However, attempts to overexpress *pdiA* in these strains did not generate more protein production. Successful chaperone overexpression includes a study where *bipA* overexpression increased protein production in *A. awamori* by approximately 2-fold (Lombraña et al. 2004), and *pdiA* overexpression that increased secretion of thaumatin 5-fold (Moralejo et al. 2001).

In *S. cerevisiae*, BiP overexpression has improved human erythropoietin expression 5-fold (Robinson et al. 1994), and bovine prochymosin production was increased 26 times (Harmsen et al. 1996). Also in yeast results have been seen to be protein specific. Plant thaumatin production was not increased by upregulation of BiP (Harmsen et al. 1996). Interestingly, overexpression of *PDI* resulted in increase of β -glucosidase secretion in *S. cerevisiae*, although β -glucosidase does not contain any disulfide bonds (Smith et al. 2004). This suggests that PDI may also have chaperone-like activity. Synergistic effects of multiple chaperone/foldase overexpressions may also be encountered. When a single-chain antibody fragment was expressed, overexpression of BiP or PDI alone increased titers by approximately 2-fold. However, when both proteins were overexpressed, the titers increased 8-fold (Shusta et al. 1998).

The Unfolded Protein Response (UPR)

A number of studies have shown that a major bottleneck for protein secretion is ER stress, and a high flux of proteins through the ER induces stress in the host cell (Guillemette et al. 2007; Kwon et al. 2012; Sims et al. 2005; Travers et al. 2000). The Unfolded Protein Response (UPR) (see Figure 2) is a response in the host cell to counteract this stress. Although somewhat different in yeasts and filamentous fungi, the main event of the UPR is the cleavage of an unconventional intron in the mRNA of the transcription factor HacA/Hac1/XBP1 (filamentous fungi/yeast/metazoans). When the intron is cleaved off, the gene can be translated to the corresponding protein, which binds to specific sequences (UPR elements, UPRE) in the promoter of other genes (Mulder et al. 2006). The result is an up - or down - regulation of secretion related genes in order to cope with the increased secretion stress (Saloheimo et al. 2003; Rügsegger et al. 2001;

Walter & Ron 2011). This increases the folding and secretion capacity of the cell, and maintains homeostasis in the folding environment.

The UPR is initiated when Ire1p (*S. cerevisiae*), an ER transmembrane kinase/RNase, forms a dimer which activates its cytoplasmic endoribonuclease domain. Initial studies suggested that BiP was bound to Ire1p, and that the presence of unfolded proteins would cause BiP to dissociate from Ire1p and bind unfolded proteins instead, allowing Ire1p to dimerize. However, recent results in *S. cerevisiae* suggest that Ire1p senses and binds to unfolded proteins, which would cause its activation (Pincus et al. 2010; Walter & Ron 2011). Upon activation, an unconventional intron from the transcription factor *hacA/hac1/XBP1* (filamentous fungi/yeast/metazoans) is spliced by Ire1p. In yeast the intron is 252 nucleotides, in mouse it is 26 nucleotides and it is around 20 nucleotides in filamentous fungi (Saloheimo et al. 2003; Chapman & Walter 1997). Furthermore, the induced form of the *HacA* gene in *A. nidulans* and *T. reesei* is truncated at the 5' end. The truncated form of *hacA* is more efficiently translated compared to the full length mRNA (Saloheimo et al. 2003). After cleavage by Ire1p the mRNA is ligated by Rlg1p (Sidrauski et al. 1996). The splicing of *hac1* mRNA relieves a translational block caused by hairpin loop formation in the secondary mRNA structure and the mRNA can be translated to its corresponding protein (Saloheimo et al. 2003; Mulder & Nikolaev 2009). The translated protein then binds specific sequences in the promoter of other genes, called UPR Elements. This causes an up – or – downregulation of those genes, allowing the cells to cope with the increased protein load. In yeast, as many as 381 genes have been shown to be upregulated by the UPR. The genes are involved in several different processes, such as translocation, glycosylation, protein folding, protein degradation and vesicle trafficking (Travers et al. 2000). In order to investigate genes affected in the UPR response, strains with constitutively activated *hacA/hac1* has been generated, providing detailed understanding of the UPR through transcriptome analysis (Carvalho et al. 2012). Through comparison of several different transcriptome analysis performed under secretion stress in filamentous

fungi, Kwon et al. (2012) have pinpointed approximately 30 genes that are believed to be key regulatory genes in the secretory pathway of *A. niger*.

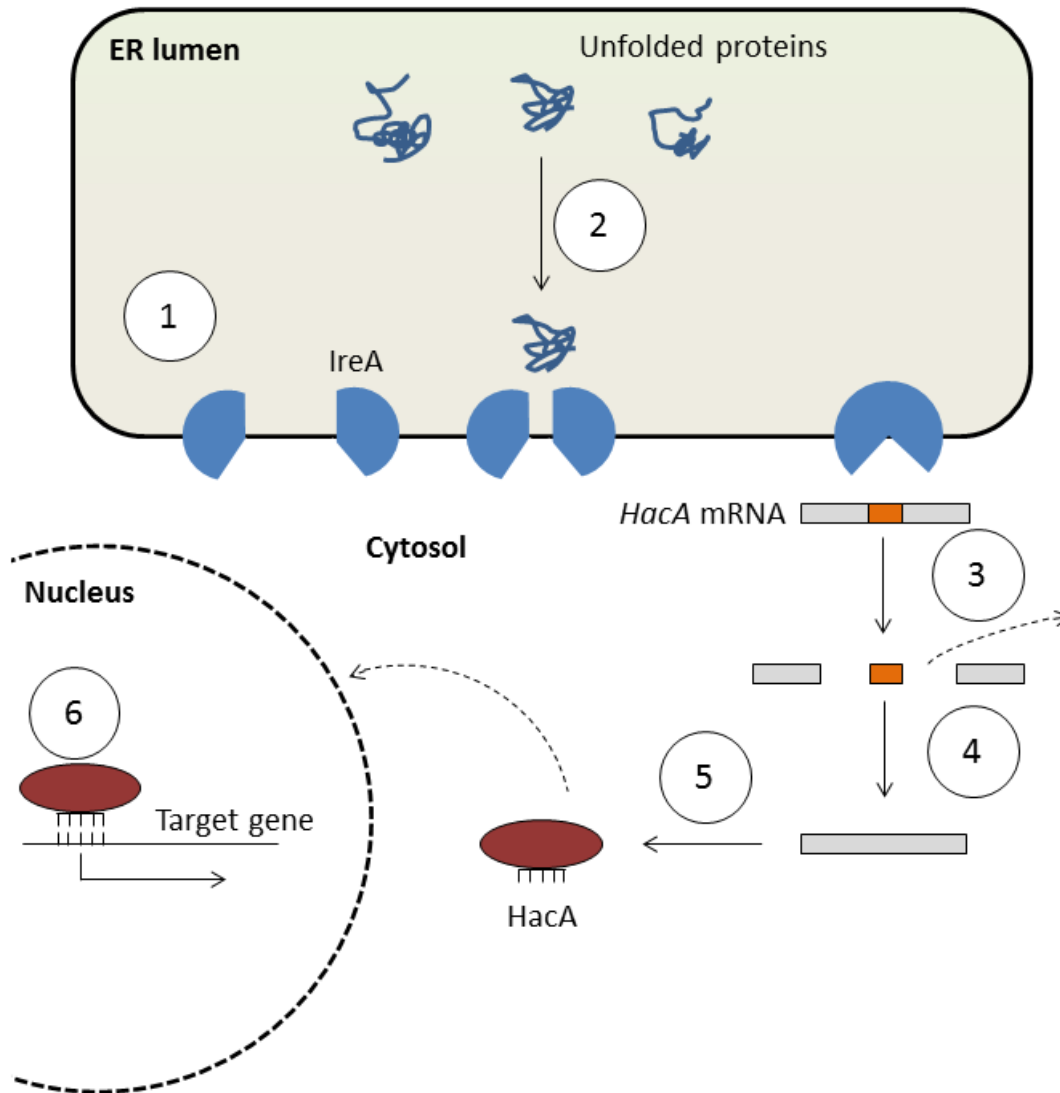


Figure 2. The Unfolded Protein Response (here filamentous fungi are depicted, but the mechanism is conserved between species) is initiated when IreA (1) senses the presence of unfolded proteins, causing the membrane protein to dimerize (2). This activates the cytoplasmic ribonuclease domain, which cuts an unconventional intron from the *hacA* mRNA (3). The mRNA is ligated (4) and the removal of the intron allows for translation of the gene (5), since no hairpin loop in the mRNA secondary structure prevents the translation. The HacA protein then binds (6) to genes containing UPR elements, consisting of specific stretches of bases. The result is an activation or deactivation of the genes. See text for further details.

Constitutive activation of *hacA* increased production of *Trametes versicolor* laccase and bovine preprochymosin in *A. awamori* by 7-fold and 2.8-fold respectively (Valkonen, Ward, et al. 2003). Constitutive activation of *HacA* is performed by expressing the spliced version of *hacA* and thereby activating the unfolded protein response. In another study, Valkonen et al. investigated the effects of disrupting and activating the UPR in *S. cerevisiae*. The results were that disruption of UPR led to decreased production of two model proteins, whereas activation of UPR increased production of one of the model proteins, but not the other (Valkonen, Penttila, et al. 2003).

ER Associated Protein Degradation (ERAD)

If proteins are terminally misfolded and UPR alone is not sufficient to counteract the stress they are targeted to the ER Associated Degradation pathway (ERAD) (Nishikawa et al. 2005). ERAD is thus another way for the cell to maintain homeostasis in the folding environment. Proteins marked for degradation cause retro-translocation to the cytosol from the ER via either the Sec61 channel or the Der1p channel (Ye et al. 2004). In the cytosol, the proteins are ubiquitinated allowing recognition and degradation by the 26S proteasome (Meusser et al. 2005). Carvalho et al. deleted *derA* in a protein secreting *A. niger*, and showed that this caused a decrease in intracellular protein degradation (Carvalho et al. 2011). That shows that engineering ERAD to decrease protein degradation might be a way to increase protein production.

ER-Golgi anterograde transport

Once the protein is modified correctly, ensured by the sophisticated quality control mechanism in the ER, proteins exit the ER in vesicles destined for the Golgi apparatus (Bonifacino & Glick 2004). The anterograde (forward) transport from the ER to Golgi is realized by vesicles coated with COPII (coat protein complex). These vesicles travel from the membrane of the ER, from which they bud off, through the ER-Golgi intermediate compartment (ERGIC) to the Golgi (Ellgaard & Helenius 2003). Several signals initiate the binding of proteins that are ready for trafficking to the Golgi by the COPII vesicles.

COPII vesicle formation (Figure 3) and cargo recognition occurs by a series of events starting with GDP-bound GTPase Sar1p, being converted to Sar1p-GTP by Sec12p. Sar1p-GTP then recruits Sec23p-Sec24p complex by binding to Sec23p (results in the so called pre-budding complex), whereas proteins that are to be transported binds to Sec24p or other proteins, such as Emp24p, Erv14p, Erv25p, Erv26p and Erv29p (Hou, Keith E J Tyo, et al. 2012). The pre-budding complex interacts with Sec13p-Sec31p, which polymerizes onto Sec23p-Sec24p. Several pre-budding complexes are in this way crosslinked, and more protein binds to the pre-budding-complexes at the ER Exit Sites (ERES) (Bonifacino & Glick 2004). The Sar1p, Sec23p-Sec24p Sec13p-Sec31p are some of the main constituents of the COPII vesicles, although there are other proteins involved as well. It is believed that Sec24p binds most of the secretory proteins from the ER, while Sar1p might be involved in signal recognition by modulating Sec24p (Bonifacino & Glick 2004). COPII vesicles are the main transporter for secretory proteins between the ER and Golgi (Dancourt & Barlowe 2010).

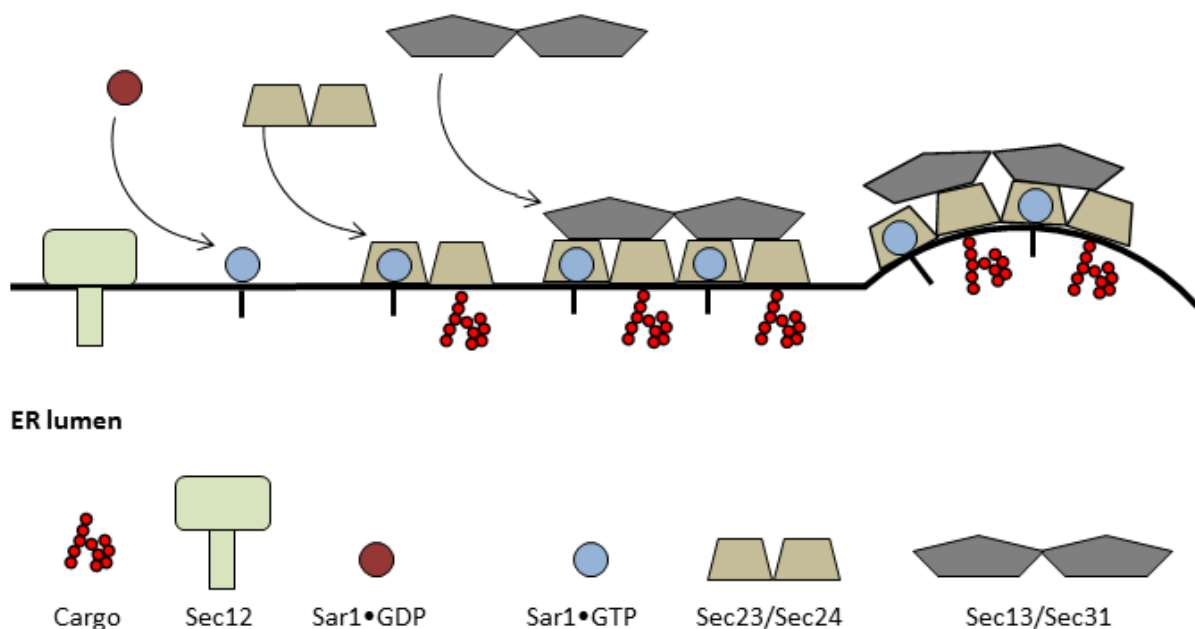


Figure 3. COPII vesicle formation starts with Sar1•GDP being converted to Sar1•GTP. Sec23-Sec24 binds to Sar1•GTP and interacts with cargo for transport. This is called the pre-budding complex. Sec13-Sec31 binds multiple pre-budding complexes that are crosslinked, and the vesicle is formed. See text for further details.

Golgi processing

In the Golgi the protein is glycosylated further. Mannosylation of N – and O-linked glycosylation occurs, and this is important for the exocytosis as well as therapeutic effect of the protein (Hou, Keith E J Tyo, et al. 2012; Dimitrov 2012). Cleavage of the protein also occurs in the Golgi, for instance by Kex2 proteases (Gouka et al. 1997a). This cleavage might induce catalytic activity or allow final maturation of the protein. Kex2 cleavage is often utilized when fusing a protein to be secreted with a well-secreted carrier protein. A Kex2 site between the two proteins assures cleavage of the carrier protein from the protein of interest (James et al. 2012). Proteins are also transported in a retrograde manner to the ER in COPI vesicles, mainly if they need to be further modified (i.e. the protein managed to “escape” the ER although not properly modified) or if it contains an ER retention motif (HDEL/KDEL). Retrograde membrane trafficking also ensures homeostasis in the organelles, as proteins and vesicle constituents are transported back to their respective donor compartments (Bonifacino & Glick 2004).

Post-golgi trafficking

After Golgi processing the protein can move several ways from the trans-golgi network, which acts as a sorting station (Gu F. 2001). If the protein has in some way escaped the ER incorrectly folded or with a motif for ER retention, it is transported back to the ER in COPI vesicles. Other transport possibilities are to vacuoles, secretory vesicles and the plasma membrane (Herrmann & Spang 2008; Hou, Keith E J Tyo, et al. 2012; Gu F. 2001). The sorting of proteins from the Golgi is achieved by coated vesicles, clathrins, in a similar manner as for ER-Golgi anterograde and retrograde transport (Gu F. 2001). Clathrins were among the first vesicles to be discovered, and has been shown to be involved in mainly post-Golgi sorting of proteins (Bonifacino & Glick 2004). Sorting of proteins occurs in collaboration with Adaptor Protein Complexes, which targets vesicles to different compartments (Gu F. 2001). In yeast, the default route for protein delivery to vacuoles is via the CPY (carboxypeptidase) pathway, a pathway similar to that of transport of lysosomal enzymes in mammals (Hou, Keith E J Tyo, et al. 2012). The proteins are sorted to endosomes with AP-1, and are transported to the late endosomes and further to the vacuole. An

alternative vacuolar route in yeast is the ALP pathway, in which AP-3 complexes signals for traffic directly to the vacuole.

In *A. oryzae*, deletion of a vacuolar protein sorting gene, *Vps10*, enhanced production of recombinant chymosin and human lysozyme by 3 and 2.2 fold respectively (Yoon et al. 2010).

Rab GTPases and SNAREs – directing and promoting vesicle fusion

Fusion of vesicles (see Figure 4) with the acceptor compartments is performed by a group of proteins called SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein). *S. cerevisiae* has 25 different SNARE proteins, where some are specialized in one intracellular fusion step, whereas others are more promiscuous (Jahn & Scheller 2006). SNAREs catalyze the fusion event of the vesicle and the target membrane, with specific SNARE proteins present on each of the membranes. This has led to the classification of SNAREs in to v-SNAREs (vesicle SNAREs) and t-SNAREs (target SNAREs) (Hong 2005).

Another type of classification exists, where v-SNAREs are typically R-SNAREs and t-SNAREs are Q-SNAREs.

Since v-SNAREs are present in both anterograde and retrograde traffic, other factors are required in order to ensure specificity of donor (vesicle) and acceptor compartment in fusion (Bonifacino & Glick 2004). The process of recognition by physical contact is called tethering, membrane attachment or docking (Jahn et al. 2003). A key to ensure correct membrane fusion are Rab GTPases (Ypt proteins in *S. cerevisiae*). In *S. cerevisiae*, 11 genes encode Ypt proteins, and each GTPase/Ypt protein functions in a specific step in the secretory and endocytic pathway. For example, Sec4p (RabD in *A. nidulans*, SrgA in *A. niger*) functions in the transport from golgi to the plasma membrane and Ypt1p (RabO in *A. nidulans*, SrgB in *A. niger*) is involved in ER-Golgi transport (Segev 2001). Rab GTPases are physically attached to both donor and acceptor compartments. They are termed molecular switches, as they cycle between an inactive GDP-bound state and an active GTP-bound state (Hutagalung & Novick 2011; Pfeffer & Aivazian 2004). Membrane bound, inactive GTPases are activated by guanine nucleotide exchange factors (GEFs). GEFs activate the GTPase by GDP-GTP exchange, and the GTPase can thereby interact with and activate tethering factors to ensure

facilitated fusion between the two membranes (Bröcker et al. 2010). After fusion, GTP is cleaved to GDP by a GTPase activating protein (GAP) and the GTPase can be recycled (Hutagalung & Novick 2011; Jahn et al. 2003).

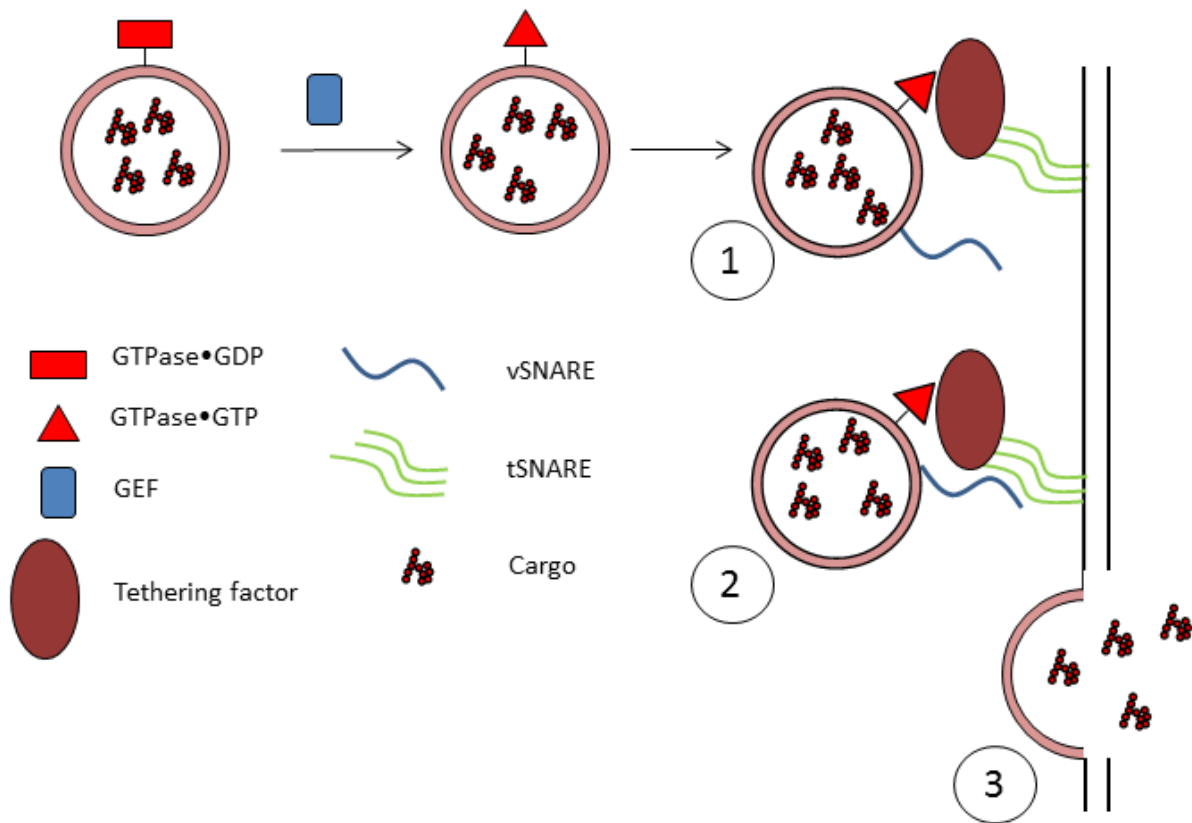


Figure 4. In vesicle fusion specificity is ensured by Rab GTPases. The Rab GTPase is activated by the guanine nucleotide exchange factor by GDP-GTP exchange. The activated Rab GTPase can bind to the tethering factor (1), allowing SNARE complexes to form (docking, 2) and membrane fusion occurs (3).

The deletion of the *SEC4* gene is lethal in yeast, whereas *A. Niger* survives without the ortholog *SrgA*, however with some altered secretion characteristics (Punt et al. 2001). In *A. fumigatus* the deletion of *SrgA* affected the sexual development and filamentous growth. Furthermore, a large heterogeneity between colonies was observed. This difference was not observed in *A. niger*, and might suggest partly different roles of the protein in the two species (Powers-Fletcher et al. 2013). Another discrepancy between yeast and filamentous fungi is the *SSO1* and *SSO2* genes in *S. cerevisiae*, which gene products are t-SNAREs in the plasma membrane. In *A. nidulans* and *A. fumigatus* only one such protein exists. However, *A. niger* and *T.*

reesei have two, also illustrating the differences within the filamentous fungi species (Shoji et al. 2008). Further differences and similarities in vesicular trafficking between *A. niger* and *S. cerevisiae* have been shown by Kwon et al. (2014). They deleted 7 different genes in the secretion pathway of *A. niger*. Deletion of the GEF for *A. niger srgA, secB*, was not lethal as is the case in *S. cerevisiae*. Furthermore, several genes (*SecA, SecH* and *SsoA*) were essential, which is in line with results obtained in *S. cerevisiae* (Kwon et al. 2014).

Deletion of the Rab protein YptA and the fusion factor NsfA in *T. reesei* and *A. niger var. awamori* showed that the genes had high conservation between yeast, filamentous fungi and mammalian cells (Saloheimo et al. 2004).

Engineering of SNARE proteins have been shown to improve protein secretion in *S. cerevisiae*. Overexpression of two Sec1/Munc18 (SM) proteins, Sly1p and Sec1p, increased secretion of α -amylase. SM proteins stimulate SNARE assembly and disassembly. Sly1p, functional in ER-golgi transport, increased secretion of α -amylase only, whereas Sec1p, functional in golgi to plasma membrane transport, also increased the secretion of insulin (Hou, Keith Tyo, et al. 2012).

Kuratsu et al. have analyzed the SNARE localization in *A. oryzae*. They identified 21 SNARE proteins, and through fluorescent tagging and subsequent microscopic analysis they concluded that the proteins had similar subcellular distributions as the orthologs in *S. cerevisiae*. The main difference was SNAREs localizing to septa (Kuratsu et al. 2007).

Proteases

One major problem when utilizing filamentous fungi as cell factories are proteases degrading the product of interest. In *A. niger*, more than 150 protease encoding genes have been identified (Braaksma & Punt 2008). The problem with proteases have been addressed by creating protease deficient strains (Homborgh et al. 1997), and the most successful discovery was a transcription factor regulating extracellular protease activity in *A. niger*, designated *prtT*. A protease deficient *A. niger* mutant, AB1.13, was obtained after UV

mutagenesis, and the location of the mutation was later identified by to be within the *pvtT* gene (Punt et al. 2008). Orthologs of *pvtT* has been identified in several *Aspergilli*, but no ortholog has been found in *A. nidulans*. Expression of four out of seven investigated protease genes was shown to be regulated by *pvtT* and studies have shown that a *pvtT* deletion strain had approximately 20% of the protease activity compared to the wild type at pH 4,5 (Braaksma & Punt 2008).

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Chapter 2 - Reporter systems in fungi

Introduction

In molecular biotechnology the utilization of reporter systems is a valuable tool to study and monitor cellular processes. Essentially, a reporter gene can be described as a gene that is introduced into a biological system and generates a measurable signal or phenotype upon its expression. The possibilities for creating specific reporter systems are endless, and especially the discovery of fluorescent proteins conferred considerable advantages in molecular and cell biology. The discovery and development of the green fluorescent protein (GFP) was awarded the Nobel Prize in chemistry in 2008, and fluorescent proteins are nowadays commonly used in most laboratories studying cell or molecular biology.

Reporter systems are often used for measuring promoter strength by coupling a reporter gene to the promoter of interest (Ghim et al., 2010). Promoter strength can be measured by qPCR, but a reporter system for screening facilitates the process since mRNA purification and cDNA construction is avoided. Although still commonly used, promoter activity is only one out of many reporter system applications today. For example, studying intracellular pH changes, cellular redox balance, protein secretion, Unfolded Protein Response (UPR) and protein localization are some examples of how reporter systems can be applied. This chapter will provide an overview of some reporter genes and describe the use of fluorescent reporter systems for monitoring and quantifying recombinant protein production in common fungal hosts.

Examples of reporter systems

LacZ

The first *lacZ* gene fusion was published in 1980 (Ghim et al., 2010). In *Escherichia coli*, the product of the *lacZ* gene, β -galactosidase, can convert lactose into glucose and galactose that can enter glycolysis. However, in molecular biology it has been extensively used due to a further advantageous characteristic. *LacZ* is a popular reporter gene as it can hydrolyze X-gal (5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside). The hydrolysis of X-gal liberates indole, which dimerizes into an insoluble, blue-colored product (Juers et al., 2012). Therefore, cells expressing the *lacZ* gene will appear blue when plated on agar plates prepared with X-gal. Cells can also be lysed and the enzymatic activity can be measured in order to

quantify for example promoter strength. The lysing of cells is however a time-consuming drawback of applications of the lacZ reporter system. The lacZ gene has been extensively used for studying expression in both yeast and filamentous fungi (Lubertozzi and Keasling, 2006; Partow et al., 2010).

Luciferase

Luciferases are a group of enzymes that generate light: bioluminescence. They can be isolated from insects, marine organisms and prokaryotes, where the most commonly used is firefly luciferase (Li et al., 2013; Ozawa et al., 2013). Luciferases act on their substrate, luciferins, by oxidation. After oxidation, luciferins will emit light that can be detected by e.g. a sensitive charge-coupled device (CDD) and an image can be created based on the light intensity (Li et al., 2013). Advantages include a high signal-to-noise ratio and therefore a high sensitivity, high correlation between amount of protein and signal output, and the fact that no light is required in order to produce the luminescence (Ghim et al., 2010; Ozawa et al., 2013). However, fluorescent assays are generally much brighter due to the possibility of exciting proteins at a high rate, and costly substrates need to be added for the luciferase to emit luminescence (Fan and Wood, 2007; Ghim et al., 2010)

Fluorescent proteins

Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* was the first fluorescent protein (FP) to be discovered, in 1962. GFP forms a cylinder (β -barrel) structure composed of 11 β -sheets, and the fluorophore responsible for fluorescence emission is synthesized by the autocatalytic cyclisation of internal residues, and is packed inside the β -barrel (Craggs, 2009; Tsien, 1998). The *A. victoria* wild-type GFP absorbs light at 395 nm with a smaller absorption peak at 475 nm, and excitation leads to green fluorescence emission at approximately 505 nm. GFP was cloned in 1992, and shortly thereafter also shown to be efficiently expressed with fluorescence emission in other hosts (Chalfie et al., 1994; Prasher et al., 1992). Chalfie et al. (1994) concluded that it can be used to monitor gene expression and protein localization in living cells since it does not require any cofactors or exogenous substrates. Since then, many mutant FP variants with various characteristics have been developed (Shaner et al., 2005). New variants may be acquired through small changes. For example, one amino acid substitution changes green

fluorescence emission to blue fluorescence emission (Sample et al., 2009). Other characteristics require more effort, such as the monomerization of dsRed, which was made possible through mutagenesis and required 33 mutations (Campbell et al., 2002). Additional colors, ranging from blue to red, as well as stable and non-stable variants have been developed. FPs are divided into seven classes; blue, cyan, green, yellow, orange, red and far-red. Nowadays the question is not whether there is a fluorescent protein that suits a certain application, but rather how to find and choose the correct one. Most FP monomers have a size of 25-30 kDa, and although fluorescence spectra and physical properties such as half-life and maturation time may differ substantially, the biochemical fluorescence mechanism is similar between the different variants (Verkhusha and Lukyanov, 2004). Obviously, emission and excitation spectra are important factors to consider when choosing a suitable FP. Other important factors include pKa, maturation time, photostability, toxicity and brightness. Table 1 lists some fluorescent proteins and their characteristics.

Table 1. Advantages and disadvantages of commonly used fluorescent proteins. Adapted from (Chudakov et al., 2010).

Protein	Emission color	Excitation peak, nm	Emission peak, nm	pKa	Brightness	Maturation	Photostability	pH stability
tagBFP	Blue	402	457	4,5	+	+	+	++
ECFP	Cyan	434	477	4,7				+
tagCFP	Cyan	458	480	4,7		+		+
EGFP	Green	489	509	5,9			+	
tagGFP2	Green	483	506	5,0		+	+	+
tagYFP	Yellow	508	524	5,5		+		+
EYFP	Yellow	514	527	6,5	+			-
Venus	Yellow	515	528	6,0	+	+	-	
mOrange	Orange	548	562	6,5	+		-	-
tagRFP	Red	555	584	<4,0	+			+
mStrawberry	Red	574	596	<4,5			-	+
mCherry	Red	587	610	<4,5			+	+
mKate2	Far-red	588	633	5,4		+	+	
mPlum	Far-red	590	649	<4,5	-		-	+

Besides using FPs for tagging or monitoring expression, they may also be used to monitor protein-protein interactions and distances between proteins. Förster Resonance Energy Transfer (FRET) and Bimolecular Fluorescence Complementation (BiFC) are two methods for investigating interactions between proteins. In FRET, a donor FP is excited, and the emission wavelength of that donor is the absorption wavelength of an acceptor FP. If the two FP proteins are in close proximity (<10 nm) FRET will occur and this can be used to determine distance between two proteins or the interaction of the proteins (Sinha et al., 2014). Two commonly used FPs for FRET are CFP and YFP (Tamura and Hamachi, 2014).

BiFC is based on complementation of two non-fluorescent parts of an FP. An FP is split in two domains, and when the two halves are in close proximity, they will form an intact fluorescence molecule. Thus, protein interaction can be studied by fusing probable interaction partners with one domain each. If fluorescence occurs, that is an indication of protein interaction. The BiFC system has been shown to be irreversible, which limits its use in analyzing dynamic interactions. However, this trait is an advantage when studying weak interactions (Kodama and Hu, 2012). BiFC does not require as high protein levels as in FRET, and the distance between the interacting partners can be greater than for FRET. One advantage with FRET over BiFC, is the possibility to study real-time detection of complexes. This is not possible in BiFC due to the maturation time of the FP once the two halves complement each other (Kerppola, 2010).

Properties, developments and use of fluorescent proteins

Depending on the application, FPs with various characteristics may be chosen. For example, long-time imaging would require an FP that does not bleach quickly, quantification of proteins present in low levels requires high brightness, multiple labelling requires emission spectra that do not overlap, and so forth. In order to not interfere with the normal function of a tagged protein it is important to use monomeric FPs. For this reason, most FP variants are monomerized by reducing aggregation tendencies through protein engineering or mutagenesis. To be able to measure changes that occur through transcriptional changes,

rapid turnover of the reporter is desirable. This was demonstrated by generating a destabilized green fluorescent protein (Li et al., 1998). Furthermore, it is important that folding occurs quickly and without misfolding. This was addressed by the development of superfolderGFP (Pédelacq et al., 2006).

FPs are sensitive to the surrounding environment, such as pH and redox changes. Emission and excitation spectra may be altered at different conditions, which causes problems in live-cell imaging where pH of may differ between cellular compartments. For such applications, pH stable proteins should be used when possible (Okumoto et al., 2012; Shaner et al., 2005). For quantification purposes, for example when measuring fluorescence in the supernatant in order to quantify secretion of a protein, pH should be kept stable by using buffers for shake flask growth or automatic pH control in bioreactors. Although the sensitivity of fluorescent proteins to different environments may be unwanted in many applications, it can be utilized to measure different intracellular states when there is a clear quantitative relationship for the environmental factor and the fluorescence of the FP. This has been used to measure intracellular pH as well as monitoring intracellular redox conditions. (Delic et al., 2010; Llopis et al., 1998; Naciri and Al-Rubeai, 2006). An NADH dependent biosensor was recently constructed in *S. cerevisiae*. In this study GFP was fused to the *GPD2* promoter. The promoter is induced during excess of NADH, which resulted in increased fluorescence of the reporter strain under such conditions (Knudsen et al., 2014). This shows that there are several possible ways to design biosensors that respond to intracellular conditions.

Fluorescent proteins to study recombinant protein production and bio-processing

FPs have had a dramatic impact on the possibilities for studying cell physiology, cell development and recombinant protein production. Combined with sophisticated microscopy methods, the localization and interactions of proteins involved in development and protein secretion have been studied, leading to more detailed knowledge of important cell factories.

Direct assessment of promoters and signal peptides have increased knowledge and discovery of efficient elements for high-level production of recombinant products. Furthermore, FPs have been of importance in the development and functional demonstration of improved cloning systems.

The construction of fluorescent reporters enables online monitoring of bioprocesses, and also to study the cellular behavior on a detailed scale that would not have been possible without FPs.

The following sections will provide a selection of fluorescent reporters used in biotechnological important cell factories in regard to cellular development, cell signaling, recombinant protein production and bioprocess developments.

Secretion of fluorescent proteins – testing promoters and signal sequences

To secrete heterologous proteins in filamentous fungi fusion to a well secreted homologous protein is often employed (see chapter 1). In order to study the localization of secretory proteins in filamentous fungi, a GFP was fused to Glucoamylase (GlaA) in *A. niger* (Gordon et al., 2000). Fluorescence was seen to accumulate especially at hyphal tips, but was also present at cell wall and septa. This was the first study to utilize an FP to investigate protein secretion in filamentous fungi. Apical secretion of glucoamylase had previously been visualized by immunogold labeling (Wosten et al., 1991). The same GFP expressing strain was used to study the effect when inducing various secretion-blocking treatments. It was seen that cold shock led to the protein being trapped in the ER. Enlightened ER was also seen when treating cells with the ER-Golgi transport inhibitor Brefeldin A, however prolonged exposure led to vacuolar transport of the protein (Khalaj et al., 2001).

Systems expressing and secreting fluorescent proteins can be used to study the effect of promoters, signal sequences and genetic manipulations on the secretory pathway. However, secretory expression of GFP in *S. cerevisiae* is not straight forward, as attempts with various signal sequences and fusions for GFP secretion have failed (Li et al., 2002). The use of viral preprotoxin signal sequences led to secretion of GFP in *Candida glabrata*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*, however in low

levels (Eiden-Plach et al., 2004). In *S. pombe* several different signal peptides were tested to secrete GFP with various results. The highest production levels were 10 mg/L (Kjaerulff and Jensen, 2005). When utilizing a 2 μ plasmid, *Gal1-10* promoter and a synthetic prepro leader sequence in *S. cerevisiae*, production levels of 6 mg/L was reported (Huang and Shusta, 2006). This study also reported on the cell surface display of GFP by fusing it to the Aga2p mating agglutinin and co-overexpressing Aga1p, a cell surface anchoring protein. Proteins displayed on the cell surface must pass the secretory pathway. Therefore, cell surface display of FPs enables sorting of cells with high secretory capacity, as high fluorescence correlates to a high flux through the secretory pathway and thereby selection of high-producing clones is possible through Fluorescence Automated Cell Sorting (FACS). Other techniques to sort cells with high secretory capacity involves capturing the secreted product in the vicinity of the cell (Kumar and Borth, 2012; Mattanovich and Borth, 2006). Recently, a study on the secretory processing of a superfolder GFP, msGFP, in *S. cerevisiae* and *P. pastoris* showed that a hybrid signal sequence could alter posttranslational translocation to a more efficient cotranslational translocation. Furthermore, truncation of a vacuolar protein sorting gene, *VPS10*, prohibited vacuolar transport of the msGFP (Fitzgerald and Glick, 2014).

By expressing an intracellular GFP from the *glaA* promoter in *A. niger*, an in-depth analysis of the promoter was possible. The strain was tested on different media and through fluorescence measurements media components effect on transcription could be measured (Ganzlin and Rinas, 2008). Using promoters and signal peptides from fungal hosts to secrete proteins in *Kluyveromyces lactis* was shown to be efficient when GFP was secreted from the *T. reesei cellobiohydrolase (cbh1)* promoter and signal sequence (Madhavan and Sukumaran, 2014). Finally, several signal peptides were evaluated in GFP-secreting *A. oryzae*. The study demonstrated the positive effect of combining different signal sequences in order to enhance secretion of recombinant proteins (Ogino et al., 2014).

Studying protein localization and gene function with FPs

Several studies have investigated the localization of genes, and the effect on vesicle trafficking when genes involved in the secretory machinery have been deleted. These studies may help in understanding the high secretion capacity of filamentous fungi in comparison with for example *S. cerevisiae*. One study investigated vacuole formation in *A. oryzae* by tagging the SNARE protein *Aovam3* (Shoji et al., 2006). Later on a systematic analysis on the total set of SNAREs in *A. oryzae* was performed by tagging all SNAREs with FPs. The authors found many similarities with *S. cerevisiae* SNARE distribution, but some differences were also seen such as SNAREs localizing to septa (Kuratsu et al., 2007). In *A. niger*, the Rab GTPase *SrgC* was shown to have a role in maintaining Golgi structures. A mannose transporter localizing to Golgi bodies was tagged with YFP and shown to be functional by complementing a deletion strain. Fluorescence in the Golgi structures disappeared when *srgC* was disrupted (Carvalho et al., 2011). Another Rab GTPase, *SrgA*, was investigated in *A. fumigatus* and shown to accumulate at hyphal tips as well as in conidiophores. Interestingly, the effect of *srgA* deletion was unclear, as phenotypic heterogeneity of individual colonies was seen. The study concluded however that the *srgA* gene is involved in asexual development and filamentous growth (Powers-Fletcher et al., 2013).

In *A. niger*, the deletion of seven secretion-related genes have been investigated. *SncA* is a v-SNARE protein involved in fusion of golgi-derived vesicles with the plasma membrane. A vesicle reporter strain, expressing a GFP-tagged *sncA* gene was created, and the secretion related genes were deleted in the reporter strain. Similarities as well as differences to *S. cerevisiae* were seen (Kwon et al., 2014). The same vesicle reporter strain was used in another study, where the importance of the GTPase *RacA* was investigated. Deletion of *racA*, a gene involved in polarity maintenance, led to a hyperbranching phenotype. Protein secretion did not differ in the two strains, and fluorescence measurements showed that fluorescence intensity at hyphal tips was lower in the deletion strain with more hyphal tips. This indicates that the total amount of vesicles

was similar between the two strains, but in the deletion strain the vesicles were distributed to more hyphal tips, which lowered the fluorescence intensity (Kwon et al., 2013).

Evaluation of vector systems and gene copy number

When expressing pathways for bio-synthesis of chemicals and pharmaceuticals, it is important to have relatively high expression of all necessary products in order to avoid bottlenecks in the production. This may be a problem when utilizing plasmid based systems for expression of multiple proteins as the copy numbers of each plasmid may vary, which affects protein titers for bio-synthesis. This problem was recently addressed, when a vector set for stable genome integration of multiple genes was presented. In order to test the vector set, three FPs (cyan, yellow, red) were co-expressed and intracellular fluorescence was measured with flow cytometry. Compared to plasmid based systems, the simultaneous expression of all three FPs were much higher in the described integration system (Jensen et al., 2013).

The effect of gene copy number on protein secretion in *P. pastoris* was studied by secreting GFP with the α -factor preprosequence. Results demonstrated that additional copies of the gene resulted in increased secretion up to three copies. A plateau was reached at four and five copies, whereas secretion was decreased at six copies. The lowered secretion was due to increased stress and induced UPR, and this shows that GFP-tagging of proteins could be a plausible way to optimize expression in various hosts (Liu et al., 2014).

Reporters for studying the Unfolded Protein Response (UPR)

The Unfolded Protein Response (UPR) (see chapter 1) consists of several steps and thus it can be studied in different ways. Tagging of any gene that is upregulated during UPR can be one way to create a UPR reporter. However, it is important that the chosen gene does not have too high background expression, since it may be hard to distinguish between stressed and unstressed cells. A second option is to create a transcriptional reporter. This can be done by adding UPR elements (UPRE) to a minimal promoter which controls the expression of an FP. When UPR occurs, the HacA transcription factor binds the UPRE and

increases transcription of the FP. This has been demonstrated in yeast as well as mammalian cells. A further approach has been to exchange the first exon in the *hacA* mRNA with an FP. Since translation of *hacA* can only be performed once the intron is spliced, this approach functions as a splicing reporter of *hacA* (Lajoie et al., 2014, 2012; Pincus et al., 2010; Rügsegger et al., 2001).

UPR can also be monitored by studying the Ire1 protein, which dimerizes before the ribonuclease domain for cleavage of *hacA* is activated. FRET of FP-tagged Ire1 has been demonstrated in yeast (Pincus et al., 2010). Another plausible way to monitor Ire1 dimerization could be to utilize the BiFC system. However, there are no studies available so far to demonstrate this theory.

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Chapter 3 - Response of *Saccharomyces cerevisiae* to environmental and stress conditions

Abstract

Microbial cultures are inherently phenotypically heterogeneous and thus single cells respond differently to changes in the environment. This can confer advantages; allowing faster adaptation to change, but also means that not all cells perform optimally. Typically applied methods for quantification of cellular performance provide a measure of the mean of the culture, which masks information on variability, relevant for large scale bioprocess design. We have thus created a fluorescent reporter for recombinant protein production in *Saccharomyces cerevisiae* which expresses cytoplasmic human Carbonyl Reductase I from a strong constitutive promoter (*PGK1*), and was linked to tagRFP. Expression was monitored by flow cytometry. Shake flask cultivations were performed to investigate the influence of pH, temperature, osmotic stress, glucose and ethanol concentrations. It was observed that osmotic stress (0.5 M NaCl) may increase protein production. Furthermore, pH was seen to affect product distributions when cells were in the same growth phase. Exposure to ethanol was shown to affect product formation. As ethanol concentration increased, the production levels decreased. This demonstrated the robustness of the reporter system, as incremental addition of ethanol resulted in a shift of the whole population to a lower state of fluorescence, which was detectable at 1%, 3% and 5% ethanol. High glucose concentration (100 g/L) and low pH were further investigated in submerged cultivations in controlled bioreactors. Population dynamics and heterogeneity varied dependent on the conditions. Subpopulations developed over time, particularly after the diauxic shift, when a subpopulation of lower producing cells was seen to emerge. The fluorescent reporter for protein production was shown to be a sensitive tool that can be used to study the impact of isolated environmental changes in culture surroundings.

Keywords: fluorescent reporter, stress, heterogeneity, *S. cerevisiae*

Introduction

Despite being genetically identical, a clonal microbial cell culture is not homogenous. Phenotypic heterogeneity occurs as the single cells have different ages and histories and thus respond differently to environmental factors, such as substrate availability, pH, temperature or inhibitor concentrations (Avery, 2006; Lidstrom and Konopka, 2010), dependent on their physiological and metabolic state. It has been suggested that this benefits the population as a whole, enabling adaptation to changes in the process environment and exposure to stress inducing conditions (Avery, 2006). In industrial biotechnology applications, this may also have unwanted effects, as not all cells are performing optimally at the same time. Such effects are hard to detect as typically applied methods for quantification of cellular performance provide a measure of the mean of the cell culture, leaving out important information on the degree of variability at the single cell level. Understanding the underlying factors of population variability and how it is affected by environmental conditions is important to improve our knowledge on microbial cell factories, and the role of variation in the overall performance of a culture in large scale bioprocesses.

Differences in population distribution can arise from stochastic variation in translation and transcription and from different cell age and cell cycle stage (Avery, 2006; Lidstrom and Konopka, 2010; Müller et al., 2010). It has been shown that budding in *Saccharomyces cerevisiae* is asymmetric. This means that mother and daughter cells are not physiologically the same, despite being genetically identical (Shcheprova et al., 2008). "Age factors" remain in the mother cell, whereas the daughter cell does not inherit these factors. This means that any microbial culture will exhibit an inherent phenotypic variation which may be manifested as differences in the active metabolic pathways and growth characteristics of the individual cells.

Another source of single cell differences within a microbial culture is the existence of microenvironments in large-scale bioreactors, which means that individual cells are constantly cycling through, and adapting to, different environments in the dimensions of space and time. This is an important consideration in the

scale-up and design of bioprocesses (Lara et al., 2006; Müller et al., 2010; Neubauer and Junne, 2010). At high cell densities, or with large volumes, mixing can often be insufficient, leading to zones with different substrate concentrations, dissolved oxygen tensions, pH and even temperature (Enfors et al., 2001; Lara et al., 2006; Neubauer and Junne, 2010; Schweder et al., 1999). Cells travelling through these zones need to constantly adapt to new conditions, which have been suggested to lead to cellular stress (Schweder et al., 1999) but also to have beneficial features such as increased robustness of the fermentation process (Enfors et al., 2001). Environmental stress factors (for example osmotic and heat stress) can induce different cell responses, depending on the physiological state of an individual cell. These have been described elsewhere (Gasch et al., 2000), including single cell investigations (Avery, 2006). Plesset et al. (Plesset et al., 1987) showed that heat shock resistance is cell cycle dependent in *S. cerevisiae*, while up to a 1500-fold variation in heat shock protein *HSP104* promoter transcription has been demonstrated in different subpopulations of the yeast (Attfield et al., 2001).

Many products of industrial biotechnology are heterologous and variability in heterologous gene expression can also occur in microbial cell populations, specifically when overexpressing genes for protein or metabolite production. This can be avoided to some extent with the development of more stable expression systems, based on chromosomal integration rather than plasmid based systems which can cause variability in gene copy number. Recently, several systems have been developed where multiple copies of genes (or several different genes) can be integrated in the genome of *S. cerevisiae* in an efficient and stable way, using several markers (Jensen et al., 2013; Kuijpers et al., 2013). These are promising tools for creating more efficient cell factories, where a larger proportion of cells are contributing optimally to product formation. The effect of stressors on the level of heterologous protein expression at the single cell level has not, to our knowledge, been shown, although a thorough characterization of the transcriptional response to different stress conditions has been performed (Gasch et al. 2000).

The focus of this study is the characterization of heterologous protein expression in single cells in response to various environmental changes and stress factors. A fluorescent reporter for recombinant protein production in *S. cerevisiae* has been created. The reporter expresses cytoplasmic human Carbonyl Reductase I (hCBRI) from a strong constitutive promoter, *PGK1*. This model protein was selected in order to study a recombinant protein of non-microbial origin, which is of relevance for industrial applications, and as hCBRI is a small protein, it is unlikely to impose a stress on cell physiology. The construct is integrated as a single copy in the genome. A fluorescent protein, tagRFP, is linked to the hCBRI in the C-terminal end providing a way to quantify the amount of recombinant protein produced at a single cell resolution using flow cytometry. Our aim was to characterize the influence of defined environmental stress phenomena, such as may be encountered in large scale yeast cultivations and to examine the influence on population heterogeneity and recombinant protein expression at the single cell level. Two cultivation systems were used: a batch set-up in shake flasks, representing a somewhat uncontrolled and thus dynamic process environment with respect to time, and a batch set-up in bioreactors where processing parameters were controlled automatically over the time course of the cultivations.

Materials & methods

Chemicals

Restriction enzymes and buffers were purchased from New England Biolabs. Uracil containing primers were ordered from Integrated DNA technologies.

Strains and plasmids

Plasmids were propagated in *E. coli* strain DH5 α . The *Saccharomyces cerevisiae* strain used was CEN.PK113-5D (*MATa MAL2-8c SUC2 ura3-52*) and was provided by Dr. Peter Kötter, der Johan Wolfgang Goethe-Universität, Frankfurt am Main, Germany. Plasmids containing human Carbonyl Reductase 1 (*hCBRI*) and *tagRFP* were codon optimized for *S. cerevisiae* and synthesized by GeneArt, Life Technologies. Yeast

integrative plasmid pXI-1 was constructed in our lab previously (Mikkelsen et al., 2012). Plasmid pSP-G1, used for the amplification of *PGK1* promoter, was a kind gift from Jens Nielsen, Chalmers University of Technology, Gothenburg.

Cloning

PGK1 promoter, *hCBR1* and *tagRFP* were amplified with primers containing Uracil tails suitable for USER cloning (Nour-Eldin et al., 2006). Primers used in this study are listed in Table 1. *PGK1* promoter was amplified from pSP-G1 with primers PGK1fw_USER and PGK1rv_U1. *hCBR1* gene was PCR amplified with forward primer hCBR1fw_U1 and reverse primer hCBR1rv_U3. Amplification of *TagRFP* was performed with forward primer TagRFPfw_U3 and reverse primer TagRFPrv. The amplified fragments, namely *PGK* promoter, *hCBR1* and *tagRFP* were purified from a 1% agarose gel and cloned into plasmid pXI-1 with USER cloning. The USER-treated mix was transformed into *Escherichia coli* and the transformants were selected on Luria-Bertani medium with ampicillin (100 µg/mL). Several colonies were inoculated in approximately 4 mL liquid Luria-Bertani medium supplemented with ampicillin (100 µg/mL) and the next day the plasmids were purified and analyzed with restriction analysis. Positive candidates were sequenced, and one (referred to as pXI-1-P-CR-tRFP) was selected to be transformed in to *S. cerevisiae*.

Table 1. Primers used for construction of the *S. cerevisiae* reporter strain. Uracil is for USER-cloning and has been marked in red.

Primer name	Sequence
PGK1fw_USER	CGTGCGAUUGGAAGTACCTTCAAAGAATGG
PGK1rv_U1	ACGTATCGCUTTGTGTTTTATATTTGTTGTAAAAAGTAG
hCBR1fw_U1	AGCGATACGUATGTCATCTGGTATTCATGTTGC
hCBR1rv_U3	AGAGCCACUCCATTGTTCTGACTCTTTTTTCGG
TagRFPfw_U3	AGTGGCTCUATGGTCAGTAAGGGTGAAGAATTGATCAAAGAA
TagRFPrv	CACGCGAUTTACAAGTCCTCTTCAGAAATAAGCTTTTGTTCGTTCAACTTGTGAC CCAACCTTAGATGGCA

Yeast transformation

Plasmid pXI-1-P-CR-tRFP was linearized by digestion with NOTI enzyme for 2 hours at 37°C. The cassette containing the insertion sites, *PGK1* promoter, *hCBR1*, *tagRFP*, *CYC1* terminator and *URA3* marker was purified from a 1% agarose gel, transformed into *S. cerevisiae* CEN.PK 113-5D and plated on SC-Ura plates. Transformation was performed as described by Gietz & Schiestl (Gietz and Schiestl, 2007). Integration in chromosome XI site 1 was verified by PCR on genomic DNA and by fluorescence microscopy.

Media preparation

Minimal media contained (per liter) 7.5 g $(\text{NH}_4)_2\text{SO}_4$, 14.4 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 2 mL trace metal solution (3 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 4.5 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 4.5 g $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.84 g $\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.3 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.4 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1 g H_3BO_3 , 0.1 g KI and 15 g Na_2EDTA was suspended in 1000 mL dH_2O , pH was adjusted to pH 4), 50 μL Sigma 204 antifoam and 1 mL vitamin solution (25 mg d-biotin was dissolved in 10 mL 0.1 M NaOH and 400 mL dH_2O . pH was adjusted to 6.5, and the following vitamins were added: 500 mg Ca-Pantothenat, 500 mg Thiamin-HCl, 500 mg Pyridoxin-HCl, 500 mg Nicotinic acid and 100 mg p-aminobenzoic acid. pH was adjusted to 6.5 and 12.5 g m-Inositol was added. pH was adjusted to 6.5, and the volume was adjusted to 500 mL.). The medium and carbon source were autoclaved separately and mixed afterwards. Vitamins were added to each cultivation through sterile filtration.

Shake flasks

Shake flask cultivations were performed in 500 mL shake flasks with a working volume of 100 mL. Standard cultivation conditions employed a defined medium (Verduyn et al., 1992) supplemented with 10 g/L glucose and grown at 30°C with stirring at 150 rpm. The flasks were inoculated from a pre-culture at OD 0.0005 unless stated otherwise and grown overnight. For the pH experiments cells were grown in the relevant pH from the beginning of the cultivation. For other stressors, they were applied in early exponential phase the day after, and sampling started approximately 30 minutes after the addition of the stressor (see below for more details). All experiments were carried out in triplicate.

Response to stress factors in shake flasks

Elevated temperature

To investigate the effect of increased temperature cells were grown over night at standard conditions, and then moved to a 35°C incubator at an OD of 0.2.

Osmotic stress

In order to investigate the influence of osmotic stress, cells were grown over night at standard conditions with 10 g/L glucose. When the cultures reached an OD of approximately 0.25 they were supplemented with NaCl from a 10 mL concentrated stock solution (1.46 g NaCl and 2.92 g NaCl in 10 mL dH₂O) which was added to the flasks to give either 0,25 or 0,5 M.

Glucose availability

The effect of increased glucose concentration was tested with 50 and 100 g/L glucose. Cells were grown over night at standard conditions to OD 0.25, and 11 ml (5.5 g glucose in 11 mL dH₂O) or 16 mL (11 g glucose in 16 mL dH₂O) of concentrated sterile glucose solutions was added to the shake flasks..

Different pH

Three different pH levels (4, 5, 6.5) were investigated. Cells were grown at the pH of investigation from the start of the cultivation. pH4 was inoculated with OD 0.001.

Ethanol stress

Cells were cultured overnight to OD 0.2 and spiked with ethanol to give 1%, 3% and 5% (v/v) in the flasks, 1, 3 and 5 ml of 99% ethanol was added respectively.

Bioreactors

Cultivations were performed in 1 L fully instrumented and automatically controlled BIOSTAT® Q Plus fermenters (Sartorius Stedim Biotech S.A, Germany), with a working volume of 800 mL. Temperature was controlled at 30°C and pH was controlled by automatic addition of 2 M NaOH and 1 M HCl. Stirring rate was set to 750 rpm, and the bioreactors were sparged with atmospheric air at 1 volume per volume per minute (vvm).

Sampling

At regular timepoints, samples were taken for OD, HPLC and flow cytometry. OD was measured at 600 nm with a Shimadzu UV mini-1240 spectrophotometer (Shimadzu, Japan). OD was analyzed in the linear range between 0.2 and 0.8. HPLC samples were filtered through a 0.45 µm Q-Max® Ca-Plus Syringe Filter and stored at -20°C, and thawed prior to analysis. HPLC was performed in a Bio-Rad Aminex HPX-87H column coupled to a RI detector. The solvent used was 5 mM H₂SO₄ with a flow velocity of 0.6 ml min⁻¹ at 60°C. For flow cytometry samples 500 µL cell culture was added to 500 µL 30% glycerol and stored in -80°C until analyzed.

Flow cytometry

Flow cytometry was performed in a Becton Dickinson FACS Aria III. Excitation and emission wavelengths were 561 and 610 nm respectively. A total of 10000 events were measured per sample. Fcs files were analyzed with Flowing software, developed by Perttu Terho, Turku Centre for Biotechnology, Finland. Prior to analysis, samples were centrifuged and cells were washed 1 time in PBS buffer at neutral pH, and then resuspended in 1 mL PBS buffer.

Results and discussion

Verification of fluorescent reporter

To have a bright fluorescent reporter, *tagRFP* was codon optimized for *S. cerevisiae* and synthesized. TagRFP is a monomeric red fluorescent protein, with bright fluorescence (three times brighter than mcherry) (Merzlyak et al., 2007). By using a previously developed yeast expression system (Mikkelsen et al., 2012) the fluorescent protein was fused with a recombinant human protein; Carbonyl Reductase 1 (hCBR1), using USER cloning (Nour-Eldin et al., 2006). The construct was expressed from a strong constitutive promoter often used for overexpression of heterologous products in yeast, PGK1. The plasmids were verified by sequencing, linearized with NOTI restriction enzyme and transformed in to *S. cerevisiae* CEN.PK 113-5D. Transformation was verified by PCR on genomic DNA. Fluorescence was also verified with fluorescence microscopy and flow cytometry before proceeding with the stress inducing experiment.

Verification tests showed a clear fluorescence in the constructed strain compared to no fluorescence in the parental strain (data not shown). The reporter strain thus expresses cytoplasmic tagRFP-fused hCBR1, and is a good model system to study protein production on a single cell level with flow cytometry.

Growth response to environmental and stress factors

The effect of environmental and stress inducing conditions relevant for submerged cultivation and large scale bioprocessing were tested in batch cultures of *S. cerevisiae* with respect to growth, metabolite profile and single cell dynamics. The first round of batch cultivations was performed in shake flasks (Figure 1). The factors tested were glucose concentration (10, 50, 100 g/L), ethanol concentration (1, 3, 5% (v/v)), osmotic stress with NaCl (0.25, 0.5 M), temperature (30, 35°C) and pH stress (4, 5, 6.5). The standard cultivation conditions used for reference were 30°C and a start pH 6.5, with 10 g/L glucose, the reference strain was CEN.PK 113-7D. In the test cultivations, only one parameter was altered with each experiment compared to the standard process. Shaking rate (150rpm) and temperature could be controlled for these cultivations, however, as the culture grew, pH and oxygen availability changed as well as the concentration of substrates and products in the medium.

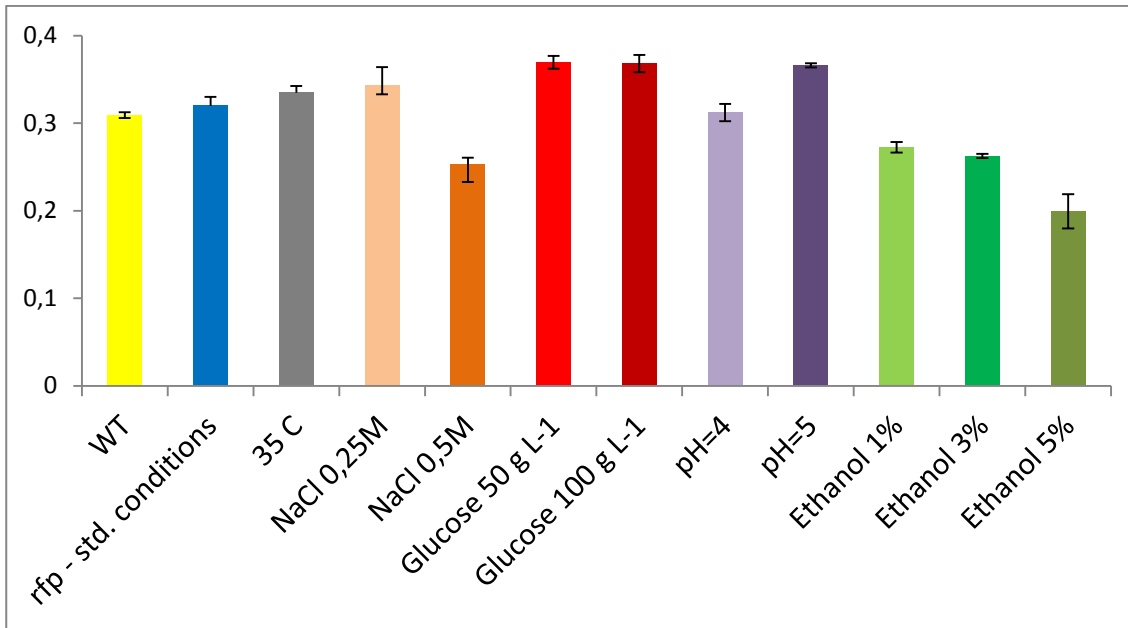


Figure 1. Growth rates (h⁻¹) in shake flask cultivations. Cultivations were performed in triplicate, mean values are plotted and error bars correspond to one standard deviation.

The growth rate for the reference strain and standard conditions in shake flasks was 0.31 h⁻¹. The reporter strain expressing the cytoplasmic human Carbonyl Reductase fused to tagRFP showed no change in growth rate (0.32 h⁻¹). As the growth profiles for the reference strain and the reporter are very similar (data not shown), it was evident that expression of the protein was not in itself causing stress to the cells. Growth rates differed significantly from the standard conditions (10 g/L glucose, pH 6.5) in some cases when an environmental stress was applied (Figure 1). The highest growth rates were obtained with high glucose concentrations of 100 g/L (0.37 h⁻¹) and 50 g/L (0.37 h⁻¹) or when the start pH was decreased to 5 (0.37 h⁻¹), whereas 5% ethanol inhibited growth to the highest degree (0.20 h⁻¹), followed by 0.5 M NaCl (0.25 h⁻¹).

Production and heterogeneity in response to environmental and stress factors

For the shake flask cultivations, three samples were taken to investigate cellular responses to the different conditions; one sample in early exponential phase (approximately 30 minutes after switching to the stress factor investigated), one in late exponential phase during growth on glucose and one sample after the diauxic shift and during growth on ethanol. Figure 2 shows the fluorescence distributions for each of the three time points for each condition tested (one representative replicate shown), and gives an overview of

the population dynamics for the shake flask cultivations. It should be noted a good reproducibility for all experiments was obtained: minor subpopulations, skewed distributions and shift in fluorescence were pronounced in both biological duplicates that were analyzed by flow cytometry, in all conditions tested, demonstrating high robustness of the system.

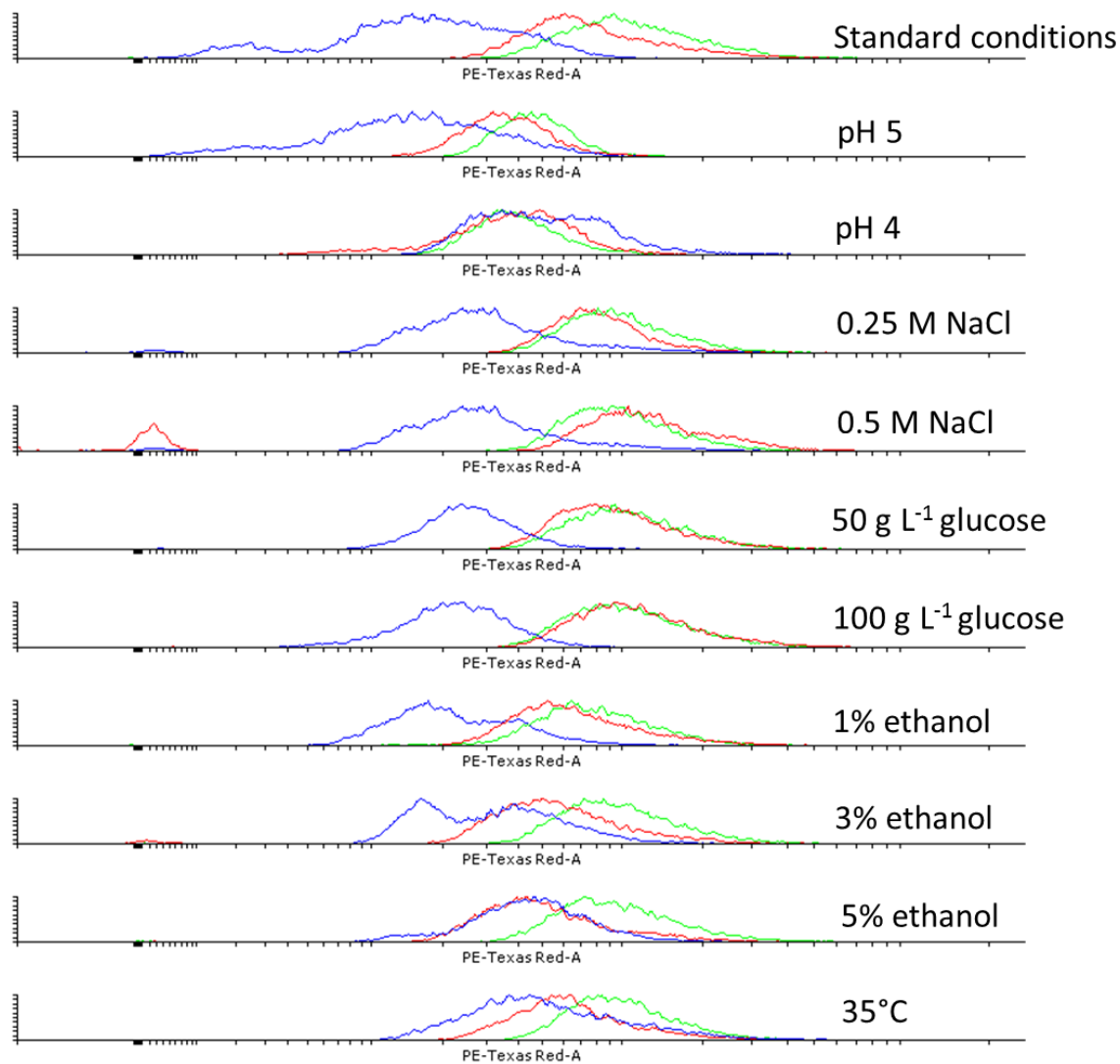


Figure 2. Fluorescence distributions for the different environmental conditions investigated in shake flasks. Cell count is shown on x-axis and red fluorescence intensity on y-axis. Green line = early exponential phase, red line = late exponential phase, blue line = ethanol phase.

In the standard conditions, it can be seen that the cells have a higher level of fluorescence in the start, and fluorescence decreases over time. A lower fluorescence could be expected when glucose is exhausted due to the characteristics of the promoter; *PGK1* is constitutively expressed but has a higher expression when glucose is present (Partow et al., 2010). However in batch cultivations in bioreactors (Figures 3 to 6), fluorescence was actually seen to increase late in the cultivation even while glucose concentration was decreasing. The shake flask mode of cultivation is somewhat uncontrolled, and thus represents a dynamic environment over time, with unfavourable conditions at later stages in the cultivation, such as lower pH and increasing ethanol and glycerol concentrations. This could contribute to the reduced protein production over time. These conditions also result in a more heterogeneous population being observed in the final time sample and there was a pronounced subpopulation formed which had lower production levels. This could suggest that a part of the population was more adapted to growth on ethanol whereas other cells entered stationary phase earlier. Age of the cells also influences the behavior of the cell culture (Allen et al., 2006) and the response to environmental changes at the single cell level. It is not only the proportion of live/dead cells that is influential in overall cell culture performance, but the history of cells, for example in terms of number of cell divisions (Ku et al., 2009), and the subsequent bud scars which can weaken the cell walls over time. Such heterogeneity can confer an advantage to the population as a whole, providing a survival mechanism (Levy et al., 2012) as there will be some individual cells in the population capable of multiplying and thus outcompeting the cells with unfavorable phenotypes.

Response to changes in pH

Lower pH resulted in lower fluorescence levels (Figure 2). When compared to pH 6.5, both pH 4 and pH 5 gave lower protein production. In Figure 2 it can also be seen that pH 6.5 resulted in a more heterogeneous population during exponential growth than the lower pH values investigated. At pH 4 an ethanol utilizing subpopulation was seen to emerge as the population divided in two. At pH 5, as growth rate was higher, ethanol was absent in the last sample, with a larger proportion of cells in stationary phase and thus exhibiting lower fluorescence.

Response to osmotic stress

The addition of 0.25 M NaCl did not alter growth, whereas fluorescence was comparable to standard conditions. Interestingly, exposing the culture to 0.5 M NaCl decreased the growth rate from 0.32 h⁻¹ (reference) to 0.25 h⁻¹, whereas fluorescence increased in late exponential phase. It can be speculated that the decrease in growth rate was concomitant with increased carbon flow towards protein synthesis, although this link was not seen in all cases where growth rate was reduced. It appears the reporter is somewhat decoupled from growth, and other physiological factors are more important for single cell product formation. In the growth phase on ethanol (last sample), the cultures exposed to 0.5 M NaCl show the highest coefficient of variance of all samples, showing a heterogeneous response. Thus, growth, heterogeneity and production levels were affected by the addition of 0.5 M NaCl. The response was seen throughout the cultivation, which contradicts the findings from Gasch et al (Gasch et al., 2000), where osmotic stress was suggested to give a highly transient response, at least on the transcriptional level. It is worth noting however that the previous study used sorbitol (1M) to provoke osmotic stress. The response to osmotic stress has been shown to have a positive influence of protein production in several other species (Dragosits et al., 2010), and our findings further support this theory. However, it should also be noted that in *Pichia pastoris* osmotic stress has been shown to induce the transcription of phosphoglycerate kinase, the enzyme under control of the endogenous *PGK1* promoter (Dragosits et al., 2010). Whether the reason for this is promoter characteristics or the locus of the promoter is unknown.

Ethanol stress

As expected an increasing ethanol concentration resulted in decreasing growth rates (Figure 1). Higher ethanol concentrations led to lower fluorescence levels in late exponential phase. The response on the single cell level can be seen clearly by comparing late exponential samples at varying ethanol concentrations. The whole population is moving towards a lower production state at increasing ethanol concentrations (Figure 2). Thus, there is a difference in response to ethanol stress during glucose growth and during ethanol growth, where subpopulations are formed as discussed above. It is worth noting the sensitivity of the fluorescent reporter, which is demonstrated in the ethanol stress experiment. It can be

clearly seen that the differences between early exponential phase and late exponential phase increases with higher ethanol concentrations. Thus, ethanol concentration was clearly seen to affect the fluorescence levels, and our fluorescent reporter can be a robust tool to measure the response of different stressors on protein production on the population level. One might argue that the differences observed are due to differences in growth phase but it is unlikely since the addition of ethanol decreases growth, and the cultures with higher ethanol concentration have more glucose available than the ones with lower ethanol concentrations at the sampling time. Although the *PGK1* promoter has been shown to respond to glucose concentrations, it is evident that this is not always the deciding factor when the cell culture is subjected to stress.

Increased glucose concentration

Increasing the glucose concentration led to higher growth rates, and due to higher glucose availability, fluorescence increased in late exponential phase. As mentioned before, the promoter *PGK1* is constitutively expressed during growth, however it is also induced by glucose (Partow et al., 2010). Thus, higher levels of glucose present in the later phase of the cultivation drives the expression of the heterologous protein and results in the observed increased fluorescence. The cultures accumulated higher levels of ethanol (approximately 33 g/L) and glycerol (app. 9 g/L) compared to standard conditions (2.5 and 2.8 g/L respectively), as higher glucose levels induce osmotic stress and overflow metabolism. It is interesting, that although the fluorescence levels in 100 g/L glucose were high, the last sample have low fluorescence. The cells are in ethanol growth phase, and it is possible that a high level of ethanol is stressing the cells in the later phase of the cultivation so that production levels are dropping.

Response to elevated temperature

Growth was not affected at the increased temperatures tested. The fluorescence levels were slightly lower than at standard conditions. Previous studies (Gasch et al., 2000), demonstrated a massive genomic response after heat shock, but it should be noted much larger temperature shifts were performed than in this study (25°C to 37°C compared to 30°C to 35°C).

Heterogeneity in response to different environmental conditions is an important factor that may be neglected in process optimization strategies. In the shake flask studies, a reporter system has been evaluated which makes it possible to investigate and compare culture response in *S. cerevisiae*. Since expression is driven by the *PGK1* promoter the response shows how this promoter is affected by different conditions. However, the reporter also gives insight in how sensitive protein production might be to different environmental stimuli, and results show that it is not solely glucose availability that is important for the production, although the promoter has been shown to be induced by glucose.

Results show the diauxic shift is a highly heterogeneous process, with widespread differences in the population. Change in pH also results in a different profile of production when looking at the histogram distributions, even when cells were in the same growth phase (Figure 2). An increased response of the *PGK1* promoter to increasing levels of osmotic pressure have been observed in *Pichia pastoris* (Dragosits et al., 2010). This study has tested two different levels of osmotic pressure (0.25 M and 0.5 M NaCl) and has shown that this response was not triggered at lower levels of osmotic pressure (Figure 2). The response resulting in an increased production was only seen with 0.5 M NaCl.

Increased glucose availability gave expected results in exponential phase, with more glucose resulting in higher fluorescence levels. It was possible to observe the differences between 50 g/L and 100 g/L, with a slight shift to increased fluorescence for 100 g/L glucose. It was also evident that ethanol levels increased the heterogeneity in the ethanol consumption phase for 100 g/L compared to 50 g/L.

The initial screening experiments were performed in shake flasks, which is a more dynamic environment compared to controlled bioreactor experiments. However, the reproducibility of the replicates, and the differences that were possible to distinguish between conditions, shows that the system is a useful tool to observe differences between the environmental conditions tested.

Reactor experiments

Based on the results from the experiments in shake flasks, two factors were chosen for further investigation in controlled batch cultivations in bioreactors, to follow the population heterogeneity in a

more controlled environment (aeration and pH were kept constant and off gas was removed). As pH 4 and glucose concentration showed the greatest deviation from standard condition results and gave interesting profiles on the single cell level in shake flasks, these were selected for a more detailed analysis. These factors are also relevant in industrial applications of *S. cerevisiae*, as they are amongst the operating parameters that can be altered dependent on the particular strain applied and product of interest.

Cells were grown with 10 g/L glucose at pH 4 and pH 5 (standard pH for bioreactor cultivations), and 100 g/L glucose at pH5 (Figure 3). The growth rate was unchanged in the three conditions (0.33 h^{-1} for pH4 and pH5, 0.34 for glucose 100 g/L). As the growth rates were unchanged, more relevant comparisons can be made between the conditions, since the effect of overall growth dynamics possibly influencing the results in shake flasks are reduced. Figure 3a shows growth and glucose concentration profile for one representative bioreactor cultivation with 10 g/L glucose at pH 5. Triplicate experiments were performed with a standard deviation on the mean of less than 5% for the replicates. Exponential phase started after around 20 hours, and glucose was depleted around 30 hours. After this, ethanol growth occurred, and at the last sample the ethanol was at low levels.

At pH4 (Figure 3b) and 100 g/L glucose (Figure 3c) similar growth profile trends to the pH5 conditions were obtained, though with a slightly longer lag phase at pH4. Glucose was depleted later when 100g/l was present, and the higher glucose availability resulted in higher ethanol production (approximately 35 g/L compared to 3.5 g/L for standard conditions). The diauxic shift can be seen in all the processes, though the level of growth after the shift is dependent on the amount of ethanol available.

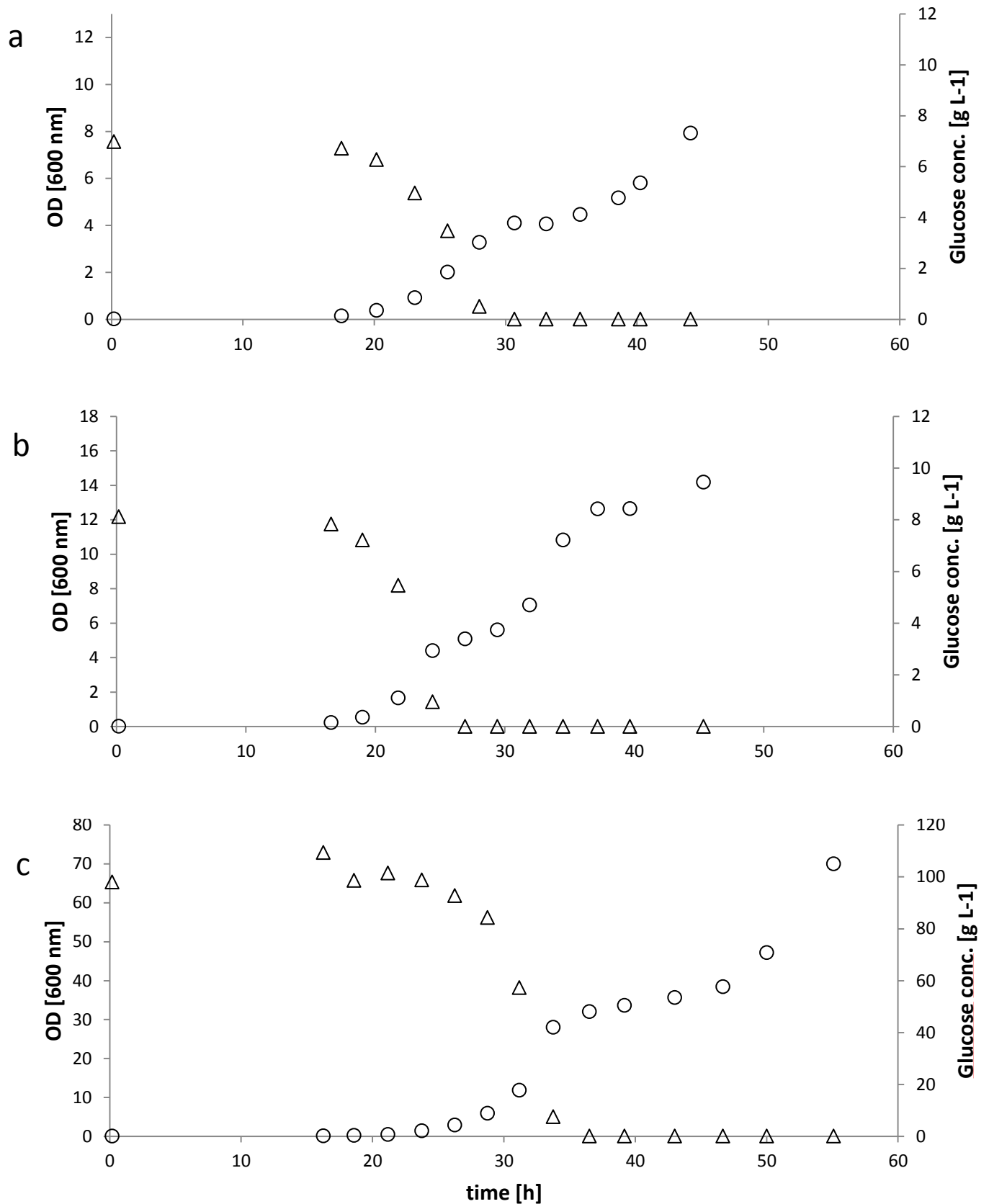


Figure 3. Growth (Optical Density) and glucose concentrations over time for the bioreactor processes at pH5, 10 g/L glucose (3a), pH4, 10 g/L glucose (3b) and pH5, 100 g/L glucose (3c). One representative process is shown for each condition, where triplicate processes were carried out (pH 5 and 100 g/L glucose with a standard deviation on the mean of less than 5%, pH4 with a standard deviation on the mean of less than 10%). Circles: OD, triangles: Glucose concentration.

To investigate heterogeneity in the populations and the expression of the fluorescence of single cells, samples were removed frequently from the cultures for flow cytometry. Mean fluorescence gradually increased during the cultivations until glucose was depleted (Figure 4-6). This would be expected as the reporter protein must be synthesized and matured before the fluorescence was detected and fluorescence then increased while glucose was available. The fluorescence increase is due to the whole population reaching a higher state of fluorescence, i.e. no subpopulations are emerging in this process. Rather, the population behaves in a homogeneous way. However, as noted also in the shake flask experiments, a subpopulation of lower producing cells was seen to emerge after the diauxic shift. This suggested that the population enters the diauxic shift in a heterogeneous manner, but it could also suggest that the lower producing subpopulation is more prone to ethanol stress than the higher producing subpopulation.

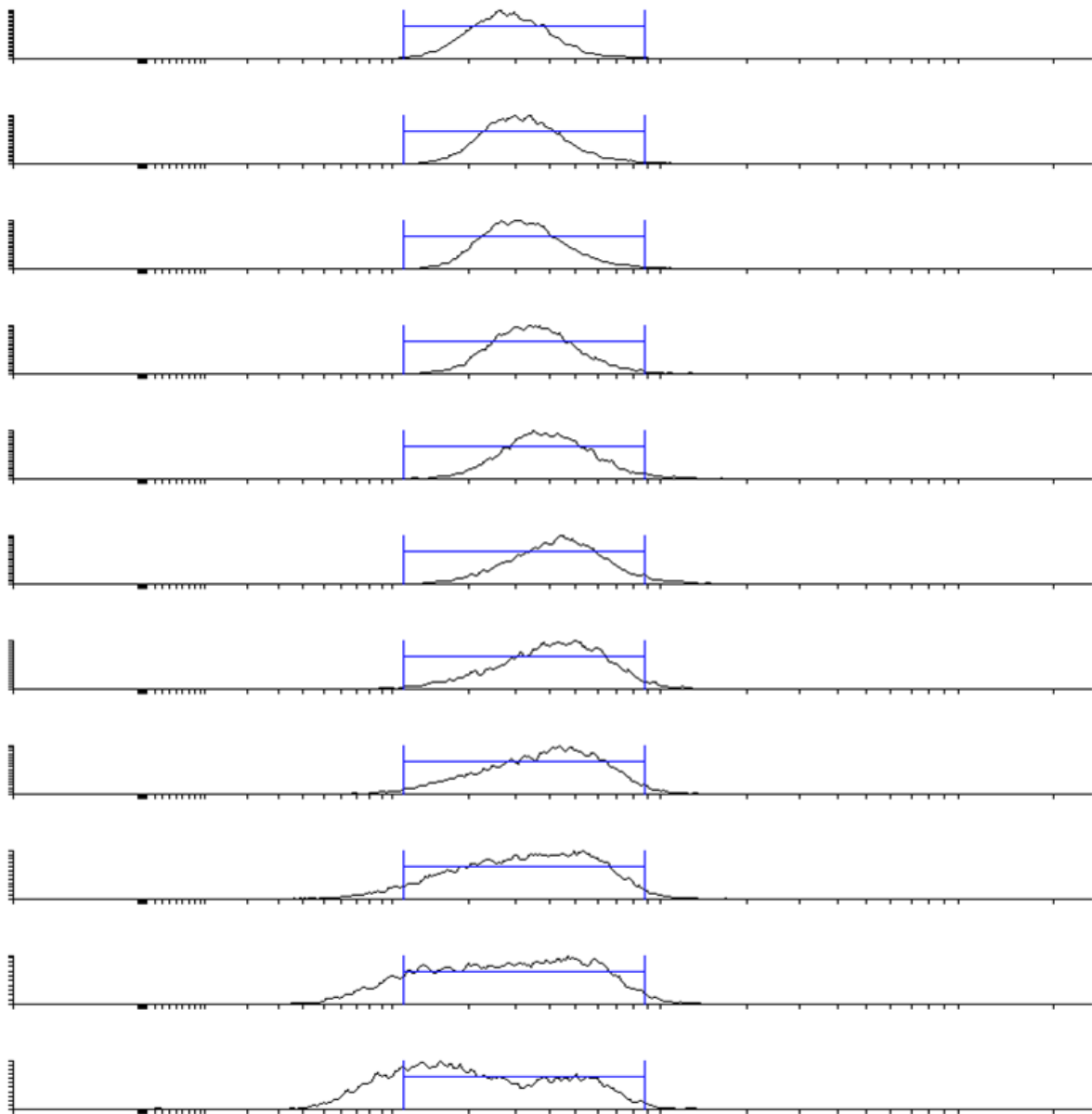


Figure 4. Fluorescence distribution histograms for each sample-point in the bioreactor cultivation at pH 5, with 10 g/L glucose. The top histogram shows the first sample after the zero time point, and then each consecutive histogram represents the following time points of the cultivation (Figure 3a). Cell count is shown on x-axis and red fluorescence intensity on y-axis. The blue bar represents the peak width in the first sample.

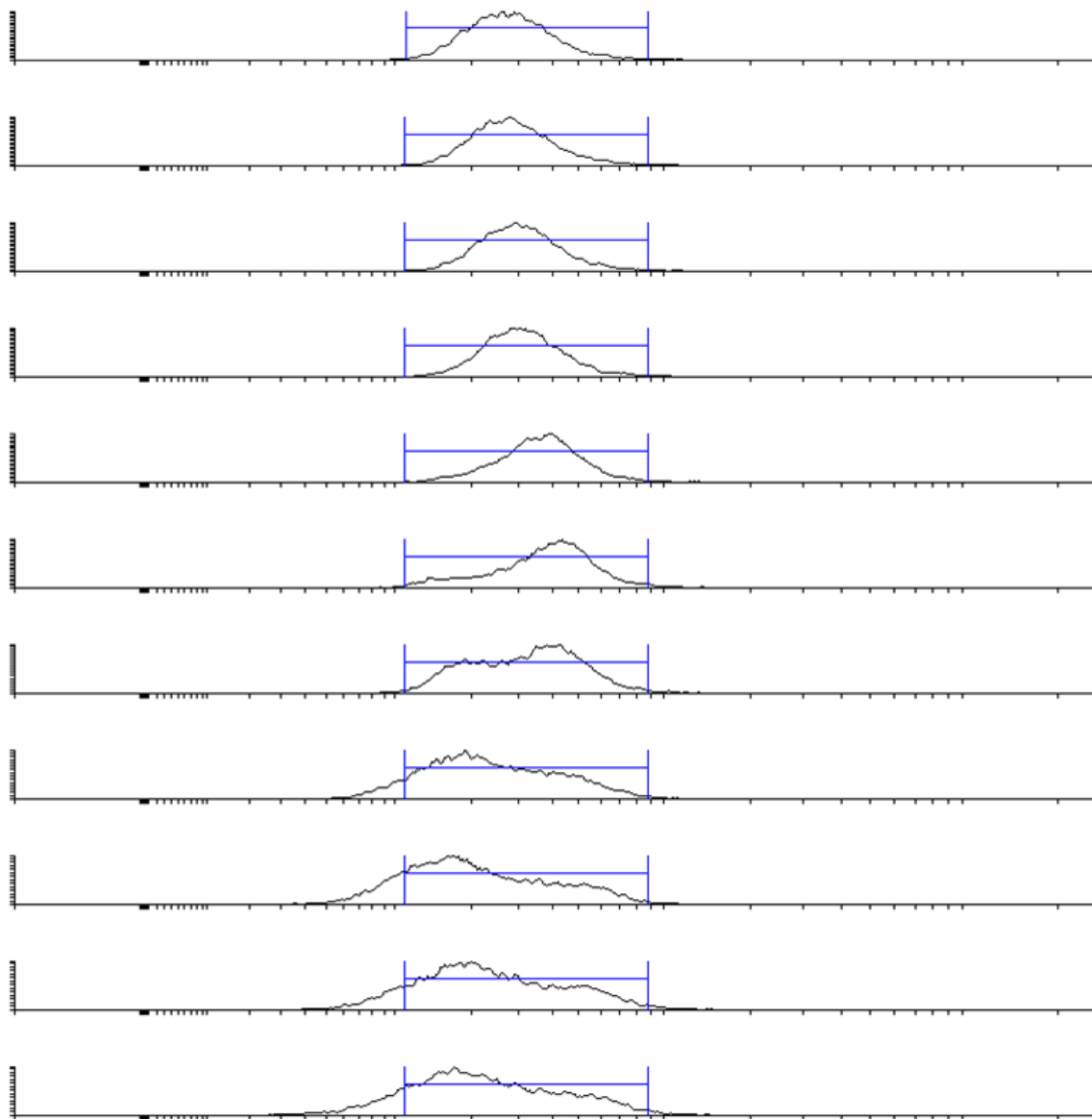


Figure 5. Fluorescence distribution histograms for each sample-point in the bioreactor cultivation at pH 4, with 10 g/L glucose. The top histogram shows the first sample after the zero time point, and then each consecutive histogram represents the following time points of the cultivation (Figure 3b). Cell count is shown on x-axis and red fluorescence intensity on y-axis. The blue bar represents the peak width in the first sample of standard conditions (Figure 4).

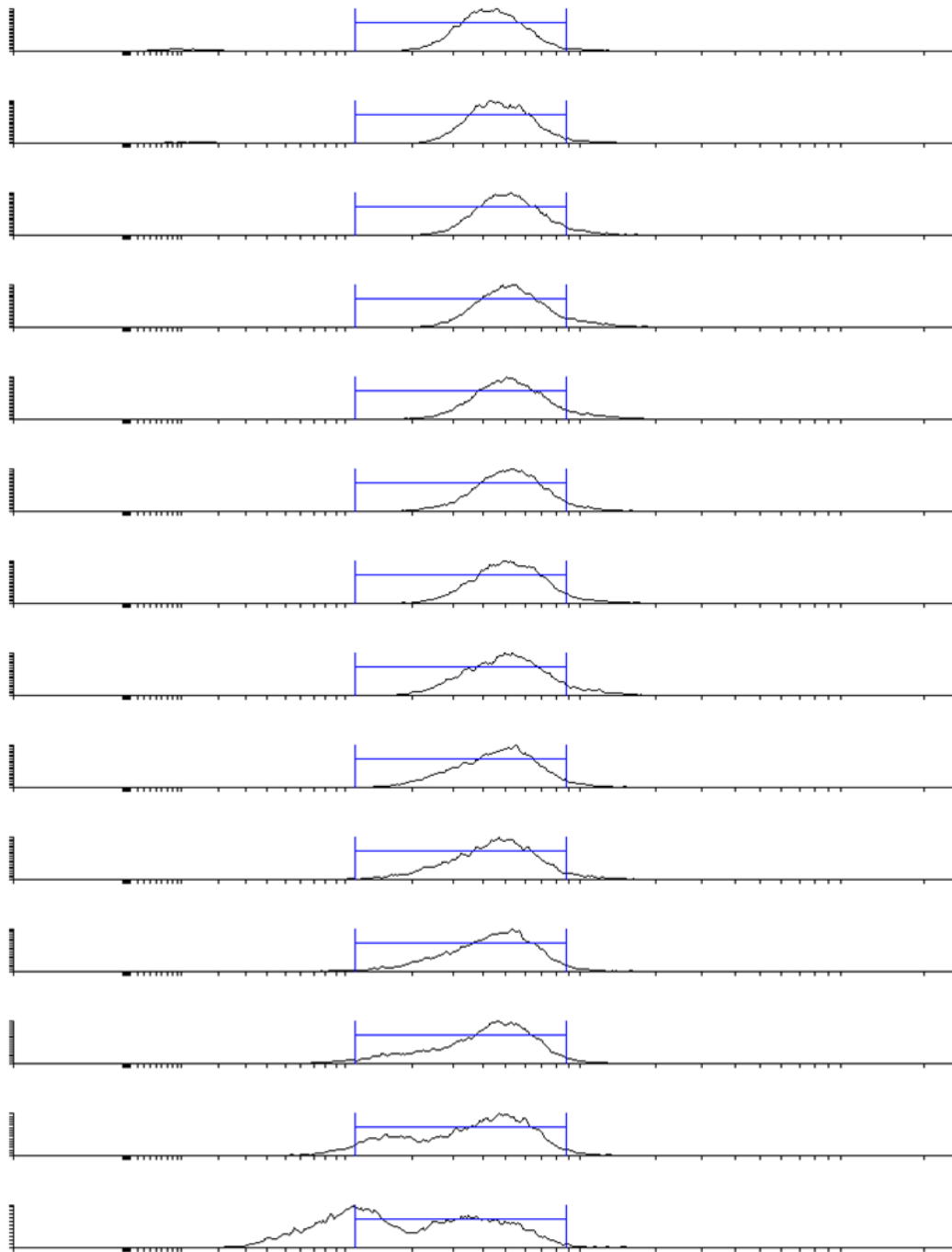


Figure 6. Fluorescence distribution histograms for each sample-point in bioreactor grown at pH 5, with 100 g/L glucose. The top histogram shows the first sample after the zero time point, and then each consecutive histogram represents the following time points of the cultivation (Figure 3c). Cell count is shown on x-axis and red fluorescence intensity on y-axis. The blue bar represents the peak width in the first sample of standard conditions (Figure 4).

Fluorescence levels were higher for cultures supplemented with 100 g/L glucose and production levels increase at a higher rate compared to the other conditions. This is probably due to glucose induction of the *PGK1* promoter.

The coefficient of variation (CV) measures the spreading of a distribution, normalized to the mean value. The CV (data not shown) was at similar levels in the high glucose cultivation compared to standard conditions, suggesting that the glucose concentration did not affect the spreading of the data in these experiments. At pH 4 the distribution was more heterogeneous. This implies that a lower pH might give a more pronounced heterogeneity in protein production with *S. cerevisiae*. Due to the binomial distribution after the diauxic shift, with one high and one low protein producing population, the CV values increase at the later part of the cultivations, for all conditions.

This work verifies the application of fluorescent reporters for the improved assessment of a microbial process. Screening in shake flasks showed a clear microbial culture response in relation to different environmental stresses, and culture variability was visualized with the aid of flow cytometry. This screening enabled quantification of the heterogeneous response as well as assessing the physiological impact of the environmental condition examined. Bioreactor experiments allowed more detailed characterization of the cultures, and subpopulations of cells were seen to emerge when the yeast culture switched from growth on glucose to ethanol after the diauxic shift. Entering diauxic shift was a heterogeneous process for each of the conditions studied, where two populations emerged although ethanol was still being consumed and cells were actively growing. This can be related to several factors, such as susceptibility to ethanol, age differences among individual cells or some cells entering stationary phase earlier than others.

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Chapter 4 - Effect of secretory pathway gene overexpression on cellular physiology and secretion of a fluorescent reporter protein in *Aspergillus nidulans*

Abstract

In order to study recombinant protein secretion in filamentous fungi, we constructed an *Aspergillus nidulans* secretion reporter strain. The strain secretes mRFP fused to glucoamylase, with proteolytic processing of a KEX2 site in the Golgi. Fourteen secretion related genes were overexpressed with a tet-ON promoter in the reporter strain and macromorphology, physiology and protein secretion was monitored when the secretory genes were induced. Results show that several of the chosen genes have an effect on growth, morphology and protein secretion. Several overexpressions resulted in decreased secretion of the model protein, whereas the overexpression of the Rab GTPase RabD resulted in a 25% increase in secretion. This study shows how protein secretion can be affected by targeted manipulations within the secretory pathway. Furthermore, fluorescence microscopy revealed alterations of protein localization in the induced strains, demonstrating the effect of the investigated genes.

Introduction

Filamentous fungi have a naturally high protein secretion capacity. Therefore, they are interesting hosts for production of industrially relevant enzymes and therapeutic proteins. Approximately 50% of industrial enzymes are produced in filamentous fungi, with production levels reported to be as high as tens of grams per liter (Lubertozzi and Keasling, 2009). Production levels with proteins of non-fungal origin are often disappointingly low, typically in the milligram per liter range. The reasons for this phenomenon are relatively poorly understood, but it seems that the limitations are at the post-transcriptional level with bottlenecks occurring due to compartmentalisation or at stages in the processing of the protein for secretion (Gouka et al., 1997).

Several studies have attempted to shed light on the extraordinary secretion capacity of filamentous fungi, primarily at the transcriptomic level (Carvalho et al., 2012; Guillemette et al., 2007; Kwon et al., 2012; Sims et al., 2005). These studies have led to the identification of genes that play major roles in the different stages of protein secretion such as translocation, folding, cargo transport and exocytosis. In combination with gene deletion studies, the functionality and importance of some secretion related genes have been characterized in more depth. For example, the *Aspergillus niger* Rab GTPase *argA* (*SEC4* in *Saccharomyces cerevisiae*, *rabD* in *A. nidulans*) has been shown to have a role in protein secretion, but is not required for survival (Punt et al., 2001). Recently, Kwon et al (2014) created *in vivo* reporter strains to study the trafficking and dynamics of secretory vesicles in *A. niger* and highlighted gene-specific differences between the secretory pathways of *S. cerevisiae* and *A. niger*.

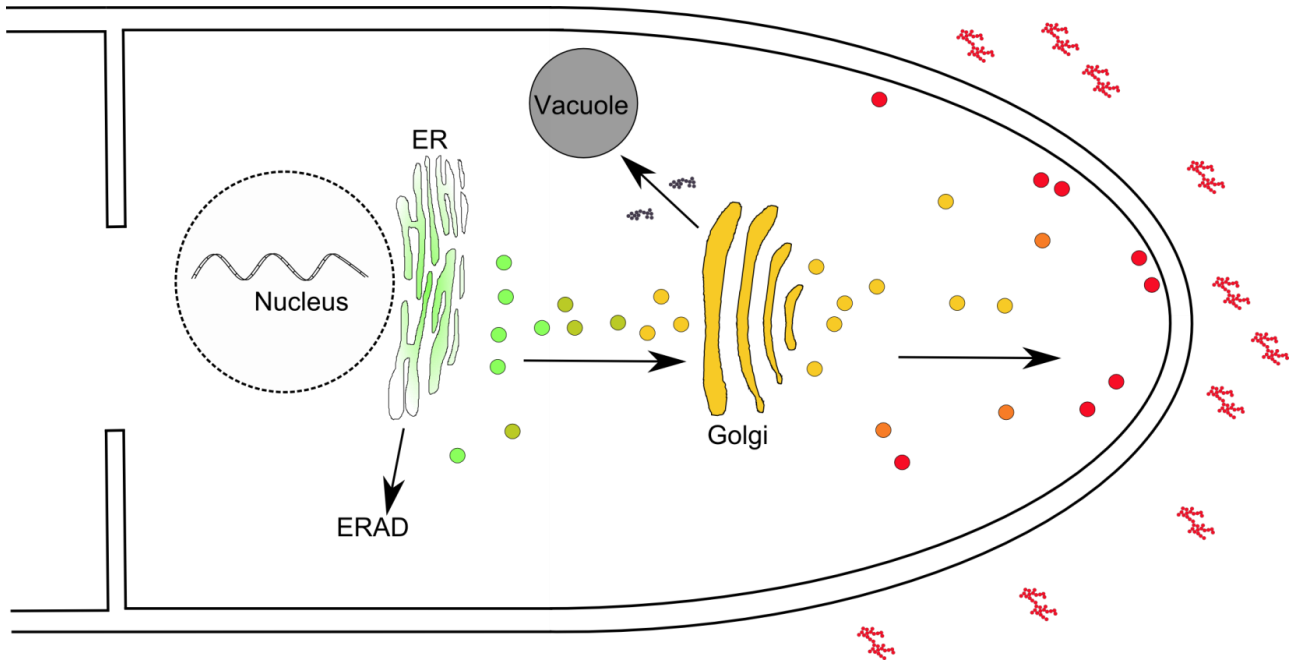
Transport through the secretory pathway begins with translocation of the protein to the ER, where the protein is glycosylated, phosphorylation occurs and disulfide bridges are formed. After passing a sophisticated quality control mechanism, the cargo is transported in vesicles from the ER to the Golgi apparatus. The vesicles bud off from the ER membrane and tether to the Golgi with the aid of soluble N-

ethylmaleimide-sensitive (NSF) factor receptor (SNARE) that mediates vesicle docking and fusion (Kuratsu et al., 2007). After further modifications in the Golgi apparatus, such as glycosylation and peptide processing, the secretory cargo leaves the Golgi in vesicles bound for the plasma membrane, where exocytosis occurs. The secretory pathway in yeast and filamentous fungi is described in detail in several reviews (Conesa et al., 2001; Delic et al., 2014, 2013; Fleissner and Dersch, 2010; Gouka et al., 1997; Hou et al., 2012b; Punt et al., 2002).

Typically, studies on the secretory pathway in filamentous fungi involve the deletion of genes to investigate the role or effect of that gene product, whereas the strategy of using overexpression of genes in filamentous fungi is not as frequent as in *S. cerevisiae*. A recent example of engineering the secretory pathway in *S. cerevisiae* is the overexpression of two Sec1/Munc18 (SM) proteins involved in different transport steps (Hou et al., 2012a). SM proteins assist in SNARE complex formation for vesicle fusion. Overexpression of *SEC1* was shown to cause increased secretion of insulin and α -amylase, whereas overexpression of *SLY1* only increased the secretion of α -amylase. The study showed that engineering single genes in the secretion pathway may be an efficient strategy to improve protein secretion, but also that results depend on characteristics of the protein to be secreted.

A common approach for secreting heterologous proteins in filamentous fungi is fusion of the heterologous protein to a known, well-secreted, native protein and this strategy has been extensively used for studying the process of protein secretion (Gordon et al., 2000; Khalaj et al., 2001; Masai et al., 2003). Gordon et al. (2000) employed this technique in order to study protein secretion *in vivo*. GFP was fused to glucoamylase, and protein secretion was shown to localize to the hyphal tips. Reporter strains expressing fluorescent proteins are interesting as they give several possibilities of analysis, for example microscopy for single cell studies and fluorescence measurements for quantitative studies.

In the current study, the effects of manipulating the secretion pathway in *A. nidulans* have been characterised using a fluorescent reporter in *A. nidulans*. The reporter strain created utilizes mRFP fused to the first 514 amino acids of glucoamylase (glaA₁₋₅₁₄) from *A. niger* as a carrier protein. This reporter strain has been used as the background strain for construction of 14 strains that overexpress different genes known to have roles in the secretion pathway. The strains have been constructed in a manner that allows overexpression of the secretory genes to be induced by doxycycline, making it possible to study the effect of a variable overexpression of the relevant gene (Meyer et al., 2011). An overview presenting the selected genes in relation to their localisation in the fungal hyphal compartments is shown in Figure 1. The genes have been selected on the basis of existing knowledge from studies investigating the effect of protein overexpression on the transcriptome of filamentous fungi and *Saccharomyces cerevisiae* (Arvas et al., 2006; Carvalho et al., 2012; Guillemette et al., 2007; Kwon et al., 2012; Sims et al., 2005). Some of the chosen genes are part of complex structures, such as COPII vesicles, whereas others have targeted modes of action, such as fusion of vesicles to the plasma membrane. Importantly, the selection of genes was chosen from several parts of the secretory pathway, covering different compartments and processes (translocation to ER, transport to Golgi, intra-Golgi transport, Golgi to plasma membrane transport and vesicle fusion at the plasma membrane) in order to understand how transport of the secretory cargo through the cell can be improved.



Process	Gene	Strain	Comment	Transcript increase in protein expressing strain
Translocation to ER	<i>AN0834/Sc SEC63</i>	NID1600	Subunit in translocation complex (forms complex with SEC62, SEC66 and SEC72 in <i>S. cerevisiae</i>)	2,02 ¹
	<i>AN10354/Sc SEC11</i>	NID1608	Catalytic subunit of Signal Peptidase Complex (SPC) involved in cleavage of signal sequence in ER	1,84 ¹
Transport ER-->golgi	<i>AN7679/Sc ERV41</i>	NID1596	Localized to COPII vesicles, forms complex with AN2738/ERV46	2,12 ¹
	<i>AN2738/Sc ERV46</i>	NID1597	Localized to COPII vesicles, forms complex with AN7679/ERV41	2,12 ¹
	<i>AN10724/Sc YIP3</i>	NID1598	Localized to COPII vesicles	1,67 ¹
	<i>AN11900/Sc BOS1</i>	NID1599	v-SNARE	1,91 ¹
	<i>AN7302/Sc EMP47</i>	NID1601	Membrane component of COPII vesicles	1,69 ¹
	<i>nsfA</i>	NID1606	Putative secretory protein	1,20/1,24 ² (protein overexpression/DTT treatment)
	<i>AN6307/An02g04250</i>	NID1609	Similar to ER protein P58, <i>Rattus norvegicus</i>	1,77 ¹
Transport golgi-->PM	<i>rabD/An srgA</i>	NID1602	Rab GTPase	1,70/1,38 ² (protein overexpression/DTT treatment)
	<i>rabE/An srgE</i>	NID1603	Rab GTPase	1,49/2,27 ² (protein overexpression/DTT treatment)
	<i>AN4759/Sc SEC2</i>	NID1607	Rab GEF for rabD	-
PM membrane fusion	<i>synA</i>	NID1604	v-SNARE	-
	<i>ssmA</i>	NID1605	t-SNARE	1,08/1,22 ² (protein overexpression/DTT treatment)

Figure 1. Protein secretion pathway in Aspergilli and genes overexpressed in the secretory pathway of *A. nidulans*. For visualization the Golgi is stacked, although this is not the case in *A. nidulans* (Peñalva et al., 2012). AN: *Aspergillus nidulans*, An: *Aspergillus niger*, Sc: *Saccharomyces cerevisiae*. 1: (Kwon et al., 2012), 2: (Sims et al., 2005).

Materials and methods

Strains

The *A. nidulans* strains used in this study are listed in Table 1. The *A. nidulans* strain IBT 29539 (*argB2*, *pyrG89*, *veA1*, *nkuAΔ*) (referred to as NID1) was used as parental strain for construction of mRFP secreting strain (Nielsen et al., 2008). Plasmids were propagated in *E. coli* strain DH5α.

Table 1. Strains used in this study. AN: Aspergillus nidulans, An: Aspergillus niger, AF: Aspergillus fumigatus, Sc: Saccharomyces cerevisiae.

Name	Genotype	Gene(s) affected	Description	Source
NID1	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i>	<i>nkuAΔ</i> for efficient gene targeting	Parental strain used to construct mRFP secreting strain	IBT collection #29539 (Nielsen et al., 2008)
NID3	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuA-trS::AFpyrG</i>	Transient small repeat in <i>nkuA</i>	Reference strain	IBT collection #28738 (Nielsen et al., 2008)
NID912	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i> , <i>IS1::PgpdA::RFP::TtrpC::pyrG</i>	Intracellular mRFP expression	Negative control	Our lab
NID1439	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i> , <i>IS1::PgpdA-ASNGlaA-mRFP-TtrpC::AFpyrG</i>	mRFP secretion	Strain secreting mRFP	This study
NID1595	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i> , <i>IS1::PgpdA-ASNGlaA-mRFP-TtrpC</i>	mRFP secretion	Strain secreting mRFP, parental strain for NID1596-NID1609	This study
NID1596	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i> , <i>IS5::PgpdA-ASNGlaA-mRFP-TtrpC</i> , <i>IS1::PtetON-AN7679-TtrpC::AFpyrG</i>	mRFP secretion, AN7679 overexpressed	Sc <i>ERV41</i> ortholog	This study
NID1597	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i> , <i>IS5::PgpdA-ASNGlaA-mRFP-TtrpC</i> , <i>IS1::PtetON-AN2738-TtrpC::AFpyrG</i>	mRFP secretion, AN2738 overexpressed	Sc <i>ERV46</i> ortholog	This study
NID1598	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i> , <i>IS5::PgpdA-ASNGlaA-mRFP-TtrpC</i> , <i>IS1::PtetON-AN10724-TtrpC::AFpyrG</i>	mRFP secretion, AN10724 overexpressed	Sc <i>YIP3</i> ortholog	This study
NID1599	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i> , <i>IS5::PgpdA-ASNGlaA-mRFP-TtrpC</i> , <i>IS1::PtetON-AN11900-TtrpC::AFpyrG</i>	mRFP secretion, AN11900 overexpressed	Sc <i>BOS1</i> ortholog	This study
NID1600	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i> , <i>IS5::PgpdA-ASNGlaA-mRFP-TtrpC</i> , <i>IS1::PtetON-AN0834-TtrpC::AFpyrG</i>	mRFP secretion, AN0834 overexpressed	Sc <i>SEC63</i> ortholog	This study
NID1601	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i> , <i>IS5::PgpdA-ASNGlaA-mRFP-TtrpC</i> , <i>IS1::PtetON-AN7302-TtrpC::AFpyrG</i>	mRFP secretion, AN7302 overexpressed	Sc <i>EMP47</i> ortholog	This study
NID1602	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuAΔ</i> , <i>IS5::PgpdA-ASNGlaA-mRFP-TtrpC</i> , <i>IS1::PtetON-ANRabD-TtrpC::AFpyrG</i>	mRFP secretion, AN <i>rabD</i>	AN <i>rabD</i>	This study

		overexpressed		
NID1603	<i>argB2, pyrG89, veA1, nkuAΔ, IS5::Pgpda-ASNGlaA-mRFP-TtrpC, IS1::PtetON-ANRabE-TtrpC::AFpyrG</i>	mRFP secretion, ANrabE overexpressed	AN rabE	This study
NID1604	<i>argB2, pyrG89, veA1, nkuAΔ, IS5::Pgpda-ASNGlaA-mRFP-TtrpC, IS1::PtetON-ANSynA-TtrpC::AFpyrG</i>	mRFP secretion, ANsynA overexpressed	AN synA	This study
NID1605	<i>argB2, pyrG89, veA1, nkuAΔ, IS5::Pgpda-ASNGlaA-mRFP-TtrpC, IS1::PtetON-ANSsoA-TtrpC::AFpyrG</i>	mRFP secretion, ANssoA overexpressed	AN ssoA	This study
NID1606	<i>argB2, pyrG89, veA1, nkuAΔ, IS5::Pgpda-ASNGlaA-mRFP-TtrpC, IS1::PtetON-ANNsfA-TtrpC::AFpyrG</i>	mRFP secretion, ANnsfA overexpressed	AN nsfA	This study
NID1607	<i>argB2, pyrG89, veA1, nkuAΔ, IS5::Pgpda-ASNGlaA-mRFP-TtrpC, IS1::PtetON-AN4759-TtrpC::AFpyrG</i>	mRFP secretion, AN4759 overexpressed	Sc SEC2 ortholog	This study
NID1608	<i>argB2, pyrG89, veA1, nkuAΔ, IS5::Pgpda-ASNGlaA-mRFP-TtrpC, IS1::PtetON-AN10354-TtrpC::AFpyrG</i>	mRFP secretion, AN10354 overexpressed	S.c. SEC11 ortholog	This study
NID1609	<i>argB2, pyrG89, veA1, nkuAΔ, IS5::Pgpda-ASNGlaA-mRFP-TtrpC, IS1::PtetON-AN6307-TtrpC::AFpyrG</i>	mRFP secretion, AN6307 overexpressed	An02g04250 ortholog	This study

Media and culture conditions

Minimal medium (MM) (per Liter): 50 mL nitrate salts solution, 1 mL trace element solution, 0,001% thiamine, 10 g D-glucose.

Complex medium (CM) (per Liter): 2 g yeast extract, 3 g tryptone, 20 mL mineral mix solution, 10 g D-glucose, 0,1 M MES Buffer, pH 5,5.

20x nitrate salts solution (per Liter): 120 g NaNO₃, 10,4 g KCl, 10,4 g MgSO₄•7H₂O, 30,4 g KH₂PO₄.

50x mineral mix (per Liter): 26 g KCl, 26 g MgSO₄•7H₂O, 26 g KH₂PO₄, 50 mL trace element solution.

20x Trace element solution (per Liter): 0,4 g CuSO₄•5H₂O, 0,04 g Na₂B₄O₇•10H₂O, 0,8 g FeSO₄•7H₂O, 0,8 g MnSO₄•2H₂O, 0,8 g Na₂MoO₄•2H₂O, 8 g ZnSO₄•7H₂O.

Plates and media were supplemented with doxycycline, L-arginine (0,7 g/L), Uracil (10 mM), Uridine (10 mM), sucrose (171,15 g/L) or 5-fluoroorotic acid (5-FOA, 1.3 mg/mL) when necessary. For shake flask cultivations spores were harvested in distilled water and filtered through a sterile miracloth. 500 mL shake flasks (without baffles) were inoculated with 10⁷ spores/mL, in a working volume of 100 mL, at 30°C and 150 rpm.

Molecular cloning

All PCR reactions were performed using the PfuX7 polymerase (Nørholm, 2010) in 35 reaction cycles with 60°C annealing temperature and an extension time of 30s/kb. All fragments relating to *A. nidulans* were amplified from *A. nidulans* NID1 gDNA. *A. niger* ATCC 1015 gDNA was used as template for amplification of glucoamylase (*glaA*) encoding gene. The plasmid pWJ1350 was used as template for amplification of *mRFP*. Primers, synthesized by Integrated DNA Technologies, are presented in supplementary table 1. Restriction enzymes and buffers were from New England Biolabs.

A list of all plasmids used in this study is presented in supplementary table 2. The plasmids for expressing *glaA*₁₋₅₁₄ (aa 1-514 of *glaA*) fused mRFP (Toews et al., 2004) in *A. nidulans* from the *A. nidulans* *gpdA* promoter was constructed by fusing 6 individual DNA fragments with the vector backbone pU2002 (Hansen et al., 2011). The resulting plasmid was named pMAS1. To ensure proteolytic cleavage of the glucoamylase from the mRFP a KEX2 (Lys-Arg) proteolytic site was inserted between the glucoamylase and the mRFP protein. For purification, a C-terminal 6•His-tag was added to the mRFP. All plasmids were prepared for USER cloning by digesting with respective restriction and nicking enzymes, and the cloning procedure was as described in Nour-Eldin et al. (Nour-Eldin et al., 2006).

To construct the plasmids for overexpression of secretion related genes, plasmid pU2311-1-ccdB was used. It contains the tetON promoter (Meyer et al., 2011) which is induced by addition of doxycycline, *ampicillin* gene for selection in *E. coli*, *A. fumigatus* *pyrG* (*AFpyrG*) for selection in *A. nidulans* and up – and downstream targeting sequences for integration in IS1 (Hansen et al., 2011). The secretion related genes were amplified from genomic DNA of *A. nidulans*. The constructed plasmids were named pMAS2-pMAS15.

Genetic transformation

Protoplastation and transformation of *A. nidulans* were performed as described in Nielsen et al. (Nielsen et al., 2006) using *AFpyrG* as a selectable marker. Transformants were verified with PCR by using spores as the source of DNA. In order to liberate the DNA from the cells, the PCR mix with the spores was subjected to 20 minutes at 98°C at the start of the PCR program. Then, a touchdown PCR program with annealing temperatures from 65 to 58°C was performed. The spores were transferred to the PCR mix by gently

touching a colony with the pipette tip and transferring the spores to two vials with the same reaction mix, ensuring that one of the reactions would have the correct amount of spores for DNA amplification.

The *A. nidulans* strain secreting mRFP was constructed by transforming NID1 with the linearized cassette from plasmid pMAS1 that integrates into a locus that has been previously used in our lab for high production of small metabolites. The cassette was liberated from the plasmid by treatment with *Swa*I restriction enzyme for 2 hours at 25°C. The transformation mix was plated on MM+Arg and transformants were verified by spore PCR. The constructed strain (NID1439) was streaked out on MM+Arg+Ura+Uri+5-FOA in order to regenerate the marker by Direct Repeat recombination generating strain NID1595.

In order to construct secretion-related mutants the transformation cassette was liberated from pMAS2-15 by treatment with *Swa*I. The linearized cassette was transformed into NID1595, and the transformants were verified for integration of the secretion related gene into integration site 1 (IS1, (Hansen et al., 2011)).

The constructed strains were named NID1596-NID1609.

Cell dry weight determination

Cell dry weight was determined by filtering of cell culture through a pre-dried and weighed filter (Advantec). The filter was dried and weighed again, and the dry weight was determined by calculating the amount of dry cell weight per liter of cell culture.

Fluorescence measurement

Fluorescence of culture filtrates were measured in a Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek Instruments) using excitation/emission 584/607 nm. A 96-well microtiter plate (PS microplate, Greiner bio-one) was used and 200 µL samples were loaded in triplicates. Background fluorescence was corrected by subtraction of values derived from a negative control.

SDS-PAGE

SDS-PAGE was performed on Novex NuPAGE 4-12% Bis-Tris gel (Life Technologies) according to the instructions of the manufacturer. The ladder used was Novex Sharp Pre-stained Protein Standard (Life Technologies).

His purification

Purification of His-tagged mRFP was performed with His SpinTrap kit (GE Healthcare).

Microscopy

MM agar slides were prepared by pipetting 1 ml agar containing MM. MM agar slides were inoculated with spores and grown at 30°C in petri dishes until analysis. Live cell images were captured with a cooled Evolution QEi monochrome digital camera (Media Cybernetics Inc.) mounted on a Nikon Eclipse E1000 microscope (Nikon). Images were captured using a Plan-Fluor x100, 1.30 numerical aperture objective lens. The illumination source was a 103-watt mercury arc lamp (Osram). The fluorophore RFP was visualised using a band pass RFP filter (EX545/30, EM620/60 combination filter; Nikon). Each slide was scanned manually, and representative images were captured to document the morphological phenotype and fluorescence pattern of each strain. Red colour was added to each image where a fluorescence signal was obtained using image processing in ImageJ.

Results and Discussion

Evaluation of reporter strain

To be able to compare the characteristics of different secretory pathway mutants an *A. nidulans* strain secreting the model protein mRFP was constructed. A *glaA*₁₋₅₁₄-fused mRFP construct was integrated in the genome of NID1, and verified by spore PCR. To release the mRFP model protein from the glucoamylase, a KEX2 site (Lys-Arg) for proteolytic processing in the Golgi was inserted between the fused proteins. This reporter strain, NID1439, was screened for protein secretion by microscopy, liquid cultures and SDS-PAGE (Figure 2). Microscopy showed fluorescence predominantly localizing to hyphal tips, plasma membrane and septa, as expected for a protein in the process of secretion (Gordon et al., 2000; Hayakawa et al., 2011). This finding was backed by fluorescence measurements of the supernatant from 48 h liquid cultures which confirmed that the protein was secreted. Fluorescence was around 4000 units, whereas for the control strain expressing an intracellular mRFP (NID912), no fluorescence was detected in the supernatant. Due to

fluorescence at intracellular structures there is a possibility that some of the secretory cargo is trapped inside the cell. Finally, proteins bearing His-tag was purified and subsequent SDS-PAGE of the cell culture supernatant and the his-purified protein demonstrated that mRFP (28 kDa band) was secreted from the cells, and inherently that it was efficiently cleaved from the glucoamylase gene, since no band corresponding to glucoamylase fused mRFP (approximately 85 kDa) was seen. A very faint band at around 60 kDa can be seen, and this corresponds to the size of the glucoamylase cleaved off from the mRFP peptide.

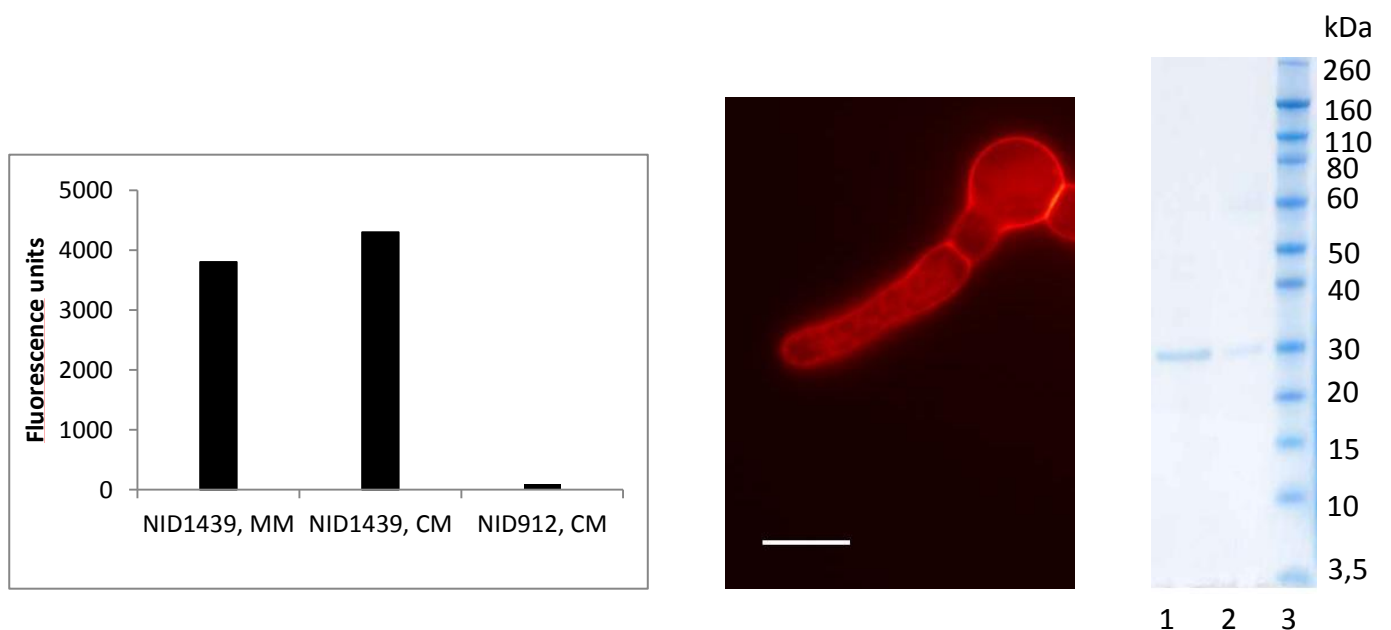


Figure 2. Validation of the secretion reporter. Left: Liquid cultures of NID1439 grown for 48 hours at 30°C without shaking. Middle: Fluorescence microscopy of NID1439. Fluorescence localizes to plasma membrane, septa and ER-like structures. Right: SDS-PAGE of His-purified (1) mRFP, cell culture supernatant from CM (2) and ladder (3). Scale bar: 10 μ m.

Construction of secretory mutants & initial observations

The auxotrophy for uridine and uracil was regenerated by plating NID1439 on MM+5-FOA, thus generating strain NID1595 where the *AFpyrG* had looped out by direct-repeat recombination. Fourteen genes (see Figure 1) were chosen for overexpression using the doxycycline inducible tet-ON promoter. The genes were transformed in to NID1595, resulting in strains NID1596-NID1609, see table 1. Each of the strains were rigorously verified (by spore PCR) for integration of the secretory genes in IS1. The integration site has been

characterized in an earlier study (Hansen et al., 2011). All strains were plated on MM and MM supplemented with DOX (1 µg/mL) to study growth and any morphological effect of overexpressing the secretory genes. None of the strains had an altered growth when no DOX was present, however upon induction by DOX nine out of 14 strains demonstrated decreased radial growth (NID1597-98, 1600, 1602-07) (Figure 3). These nine strains individually overexpress genes in different compartments, so no general trends in decreased growth relating to an alteration of a specific compartment could be seen. Interestingly, limited growth on agar plates did not correlate with reduced protein secretion in submerged cultivations. Furthermore, altered morphology when DOX is added demonstrates that it is indeed overexpression of the specific secretion related gene causing the effect. If integration has occurred at additional sites and this would affect the strain, the effect would be seen also when DOX is not added to the plates.

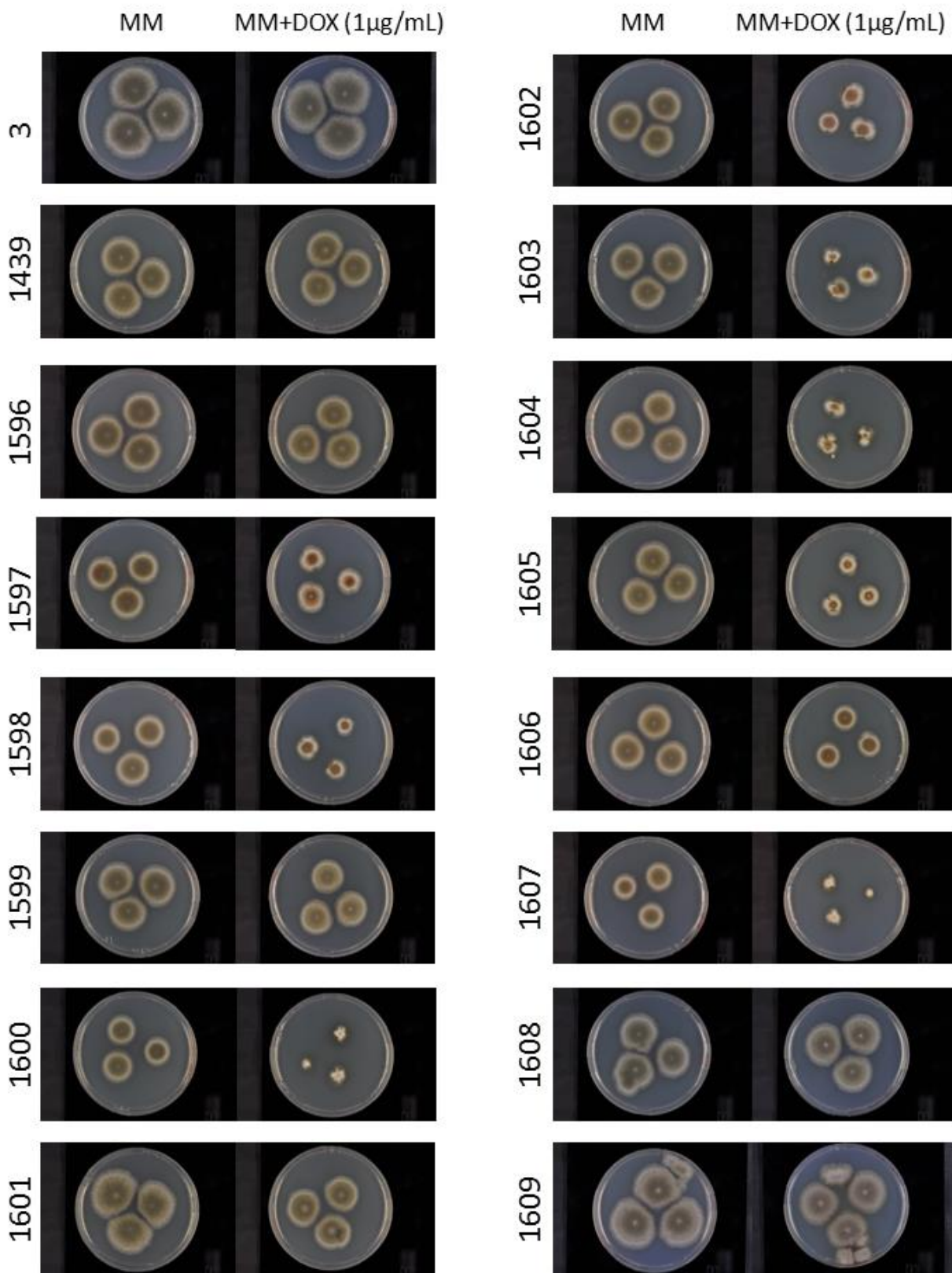


Figure 3. Growth on MM and MM+DOX (1 μ g/mL). Numbers refer to the strain identification numbers in Table 1.

Protein secretion and macromorphology

With the aim of investigating how overexpression of the selected genes affected the secretion of the model protein, submerged cultivations were used as a basis for providing quantitative measurements of cellular physiology parameters. The strains were cultivated in shake flasks and fluorescence levels and cell dry weight were measured over time. The medium was supplemented with MES buffer in order to avoid pH related effects on the fluorescent signal. Figure 4 shows how the maximum mRFP fluorescence change due to induction of the individual secretion related genes (DOX concentration 10 µg/mL). Secretion of mRFP was slightly decreased in the control strain when DOX was added. However, as total biomass (measured as maximum dry weight) was comparable with and without DOX, it was assumed that DOX had no significant negative effect on growth (Meyer et al., 2011). Furthermore, the maximum fluorescence was reached at the same time with and without DOX. As we wanted to examine the effect on secretory production of a recombinant protein by engineering the secretory pathway, we decided to use the maximum fluorescence values reached in each cultivation, rather than collecting samples after a certain time point. The maximum fluorescence levels were in general reached at similar time points, however differences within a few hours occurred between strains.

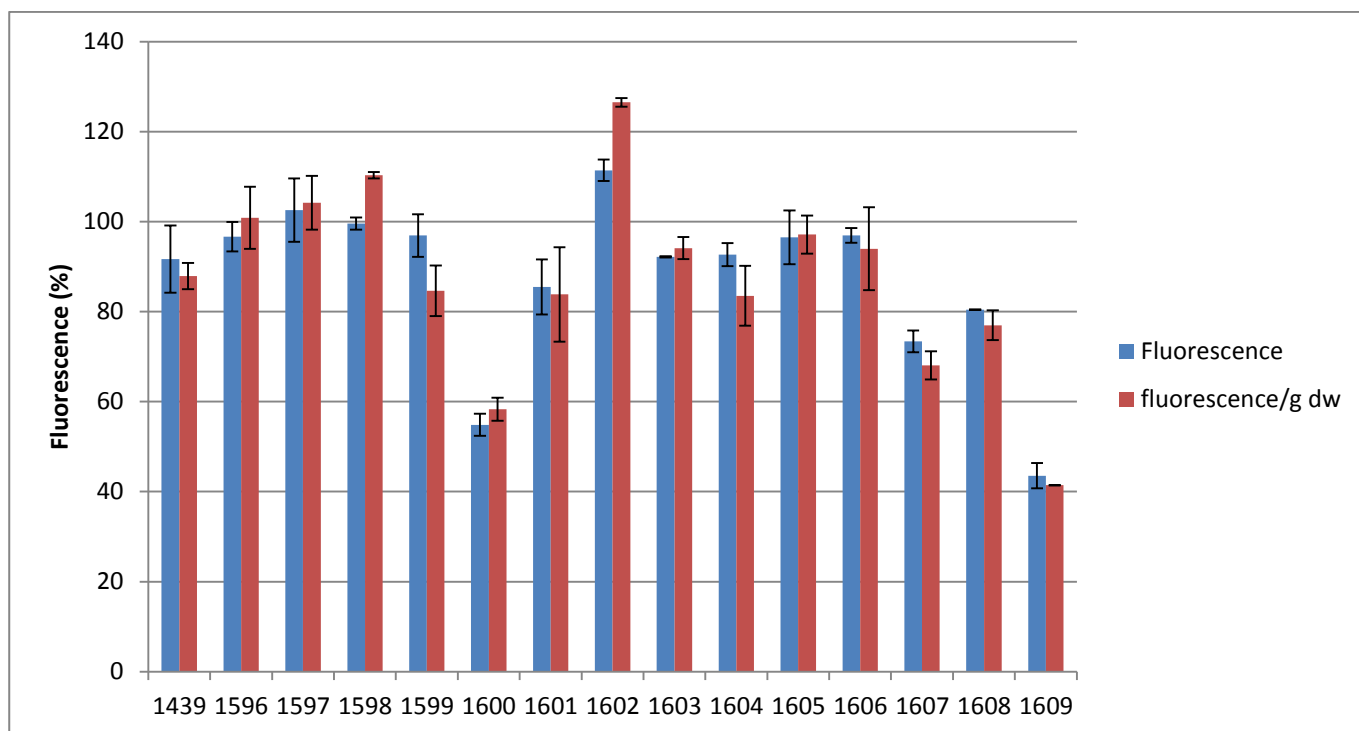


Figure 4. Protein secretion as measured by fluorescence in culture supernatant in shake flask cultivations at 150 rpm and 30 °C. Shake flasks were performed in duplicate, and the bars depict the maximum fluorescence level in the strain when DOX (10 µg/mL) was added compared to non-induced conditions (\pm one standard error). Blue bars: Fluorescence, Red bars: Fluorescence per dry weight. Numbers refer to the strain identification numbers in Table 1.

To increase the flux of proteins to ER *A. nidulans* *Sec63* (AN0834) and the ortholog of *S. cerevisiae* *SEC11* (AN10354) were overexpressed (generating strains NID1600 and NID1608, respectively). Overexpression of *SEC63* resulted in significantly lower fluorescence, approximately 40% decrease, whereas *SEC11* overexpression resulted in a slight decrease in secretion. Moreover, intracellular fluorescence in ER-like structures was seen in NID1600 induced with DOX (Figure 5) and this strain exhibited a substantially decreased radial growth on solid MM+DOX, indicating a stress response when the gene was overexpressed. However, decreased radial growth on plates does not per se relate to a decreased secretory capacity. NID1609 was the strain secreting the least mRFP of all strains investigated having approximately 40% of the secretory capacity compared to non-induced conditions, which is not reflected on solid media, as growth is stronger than e.g. NID1602 on MM+DOX, albeit with a different morphology. In NID1609, an ortholog of *A. niger* An02g04250 (AN6307) was overexpressed. An02g04250 is similar to the ER chaperone P58 in *Rattus*

norvegicus, a rat homolog of human ERGIC-53 (Kwon et al., 2012). In humans this protein is involved in glycoprotein sorting between the ER and the Golgi (Velloso et al., 2002). A possible explanation for the decreased secretion in NID1600 might be an overload of the ER which can result in Endoplasmatic Reticulum Associated Degradation (ERAD) (Carvalho et al., 2011).

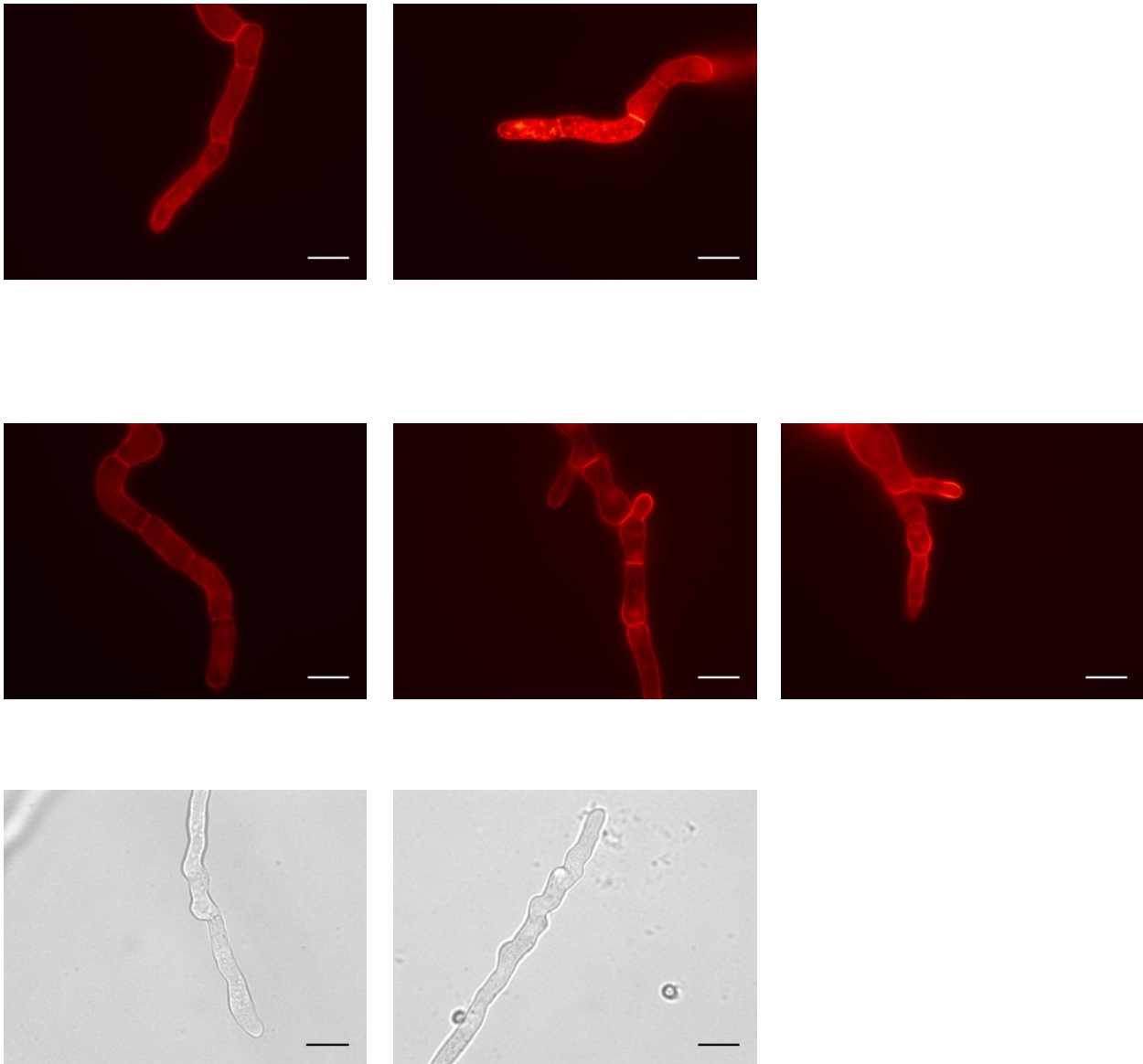


Figure 5. Microscopy of NID1600 and NID1602. Spores were inoculated on MM and MM+DOX (1 $\mu\text{g}/\text{mL}$) slides and incubated at 30°C in petri dishes. Top: NID1600 non-induced (left) and induced (right). ER-like structures are more pronounced when the secretory gene is induced. Middle: NID1602 non-induced (left) and induced (middle and right). Fluorescence increases at plasma membrane when *rabD* is overexpressed. Bottom: Stunted growth of NID1602 induced with DOX (left and right). Scale bar: 10 μm .

Genes involved in ER to golgi transport

In *S. cerevisiae*, *ERV41* and *ERV46* are localized to COPII vesicles where they form a complex. The overexpression of the *ERV41* ortholog, AN7679, in *A. nidulans* (NID1596) resulted in markedly different phenotypic behaviour on plates compared to *ERV46* (AN2738) overexpression (NID1597). NID1597 showed decreased radial growth on MM+DOX, whereas NID1596 was not affected. Based on mRFP secretion in liquid cultures, it does not seem plausible that overexpression of either of the two proteins has a major effect on protein secretion. Overexpression of the *EMP47* ortholog AN7302 (NID1601) did not affect growth on plates. However, secretion of mRFP was negatively affected in submerged cultivations.

In *S. cerevisiae*, results have shown that the expression levels of *ERV41* and *ERV46* are interdependent. *Erv46p* levels are lowered in an *erv41Δ* strain, and the *Erv41p* was not detected in an *erv46Δ* strain. Furthermore, the same study showed that overexpression of both proteins on 2μ plasmids did not result in higher expression of any of the proteins compared to a single overexpression of *ERV46*. Unaffected secretion in NID1596-1597 is in line with results from this study. Lastly, results have shown that expression of *Erv41p* is highly dependent on *Erv46p*, whereas *Erv46p* levels depends less on *Erv41p* (Otte et al., 2001). We therefore speculate that it is possible that the phenotypic effect in strain NID1597 is due to the fact that overexpression of *Erv46p* results in concomitant increasing levels of *Erv41p*. Since the two proteins forms a complex, this causes a phenotypic effect due to higher levels of formed complexes. In NID1596, overexpression of *ERV41* might not result in increased *Erv46p* levels, thus resulting in a “normal” phenotype.

Recently, the *S. cerevisiae* *EMP47* ortholog *AoEmp47* was deleted and overexpressed in protein producing strains of *A. oryzae*. It was seen that deletion of *AoEmp47* improved heterologous protein production, whereas overexpression decreased secretion (Hoang et al., 2014). The reason for the decreased secretion upon overexpression of *AoEmp47* was that the protein is involved in retention of heterologous proteins in

the ER. Our data with overexpression of *EMP47* ortholog in *A. nidulans* shows similar results as the control strain. Thus, there was no positive effect on secretion when overexpressing *EMP47*.

Overexpression of the rab GTPASE rabD significantly improves protein secretion

Overexpression of the rab GTPase *rabD* (NID1602) increased mRFP secretion by approximately 25% in submerged cultivations (fluorescence units/g dw) (Figure 6). Hyphae of NID1602 appeared swollen compared to the reference strain, possibly due to stunted growth (Figure 5), and on solid media decreased radial growth was seen on agar plates. The maximum dry weight in shake flask cultivations reached slightly lower levels when DOX was added to the media, however growth was not reduced to the same extent as on plates. Furthermore, fluorescence microscopy revealed different fluorescence distribution of the secretory protein than for the non-induced/WT condition (see Figure 5). Fluorescence was more distributed towards the hyphal tip and the plasma membrane.

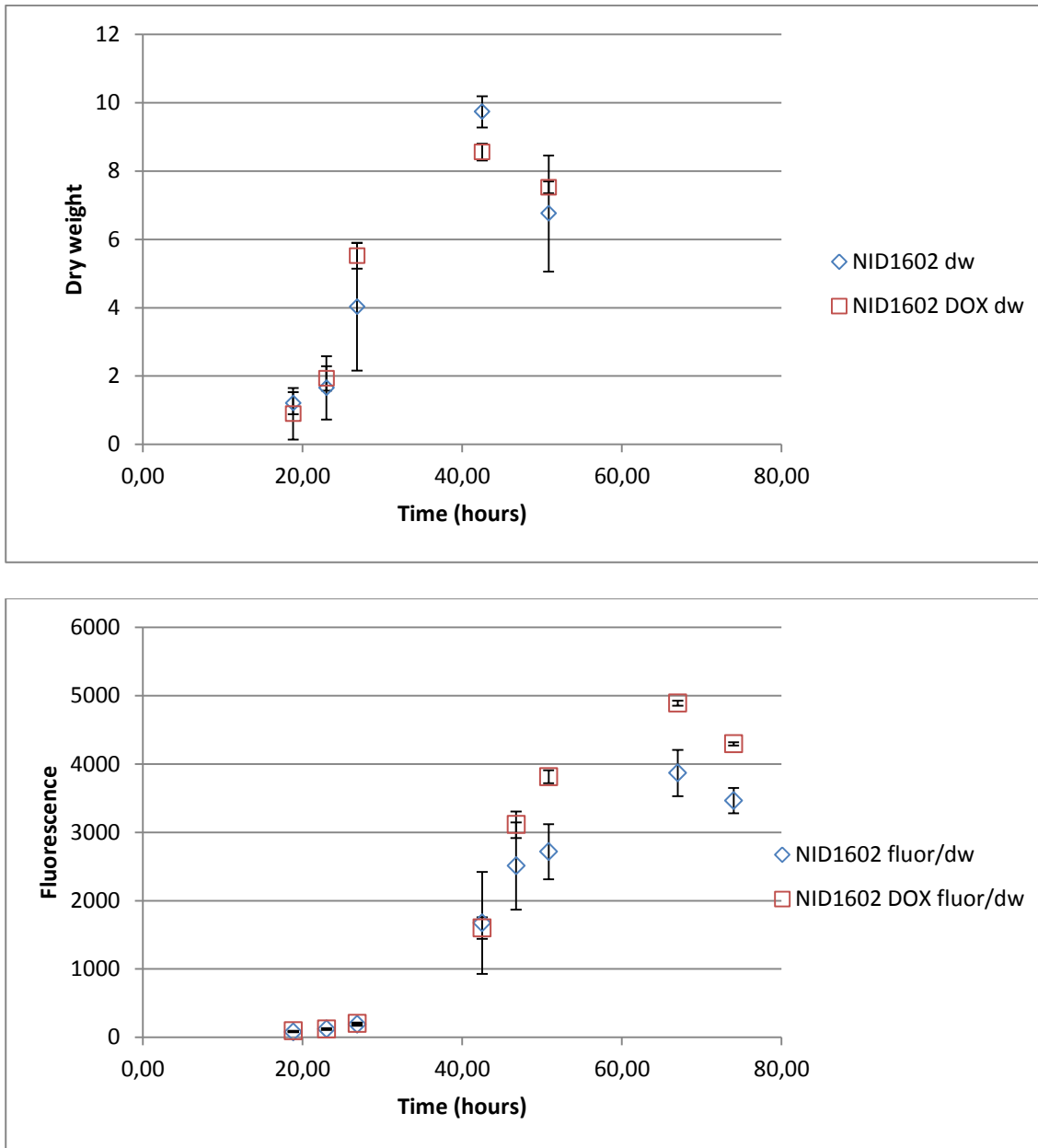


Figure 6. Dry weight measurements (above) and fluorescence per g dw (below) over time in NID 1602 cultivated in 150 rpm at 30°C.

The *rabD* guanine nucleotide exchange factor AN4759 (*S.c. SEC2*) (NID1607) was chosen for overexpression to test whether it would have similar effects as the overexpression of *rabD*, since it functions as an activator of *rabD*. However, overexpressing AN4759 (NID1607) resulted in decreased protein secretion by approximately 30%.

RabD is involved in vesicle transport from the golgi to the plasma membrane, and the *A. niger* homolog *SrgA* has previously been found to influence protein secretion and morphology in *A. niger*. A deletion mutant showed decreased protein secretion as well as increased hyphal diameter during growth on glucose (Punt et al., 2001). Unlike the situation in *S. cerevisiae*, it is not an essential gene for survival. In *A. fumigatus*, *srgA* deletion showed that the gene is involved in asexual growth and filamentous development. The deletion mutant also demonstrated increased susceptibility to Brefeldin A treatment, which inhibits vesicular trafficking in the cell (Powers-Fletcher et al., 2013). In *A. fumigatus* *SrgA* localizes to the hyphal tip (Powers-Fletcher et al., 2013), and it can be speculated that the increased fluorescence in NID1602 hyphal tips was a result of more efficient transport of the secretory cargo towards the plasma membrane, which was also demonstrated in the fluorescence microscopy.

Exocytosis

Overexpression of the t-SNARE protein *SsoA* resulted in unchanged secretion of the model protein in this study. *S. cerevisiae* has two *SSO* genes, whereas *A. nidulans* has one. This suggests that there might be different roles of the proteins in the species. In *S. cerevisiae* overexpression of *SSO1* or *SSO2* has been shown to improve production of heterologous and homologous products (Ruohonen et al., 1997).

General discussion & conclusion

Due to recent genome sequencing of filamentous fungal species, a cellular response to recombinant protein production is well documented and important proteins in this process well known. Nevertheless, reasons for the high secretion capacity of filamentous fungi are still relatively unknown, although some insights can be gained from studies on other microbial hosts, such as *S. cerevisiae*. As mentioned previously there has been a lot of attention towards accurate protein folding in fungal cells. Results are contradictory, and to some extent protein specific, indicating the complexity of the secretory pathway. In order to make use of available data and study the cellular response of manipulating the secretory pathway, this study has investigated processes that are involved in transport from or to different compartments. Results demonstrate that engineering the pathway leads to different secretion profiles for the fungal strains constructed, as well as differences in growth and morphology of the strains. As secretion modifications are

likely to alter the transport of intracellular endogenous proteins it was not surprising that several of the modifications resulted in altered morphology (Peñalva et al., 2012). The tet-ON promoter have previously been characterized by Meyer et al., and is an interesting tool for manipulation of genes that are important for the maintenance of cellular functions (Meyer et al., 2011). The promoter was therefore well suited for our study, as strain construction was facilitated by silencing the gene of interest to promote normal growth on transformation plates.

There are several reasons to why some of the genes overexpressed resulted in unchanged protein secretion in the constructed strains. In this study, one single copy of *glaA*₁₋₅₁₄-fused mRFP was integrated, and this may not result in high enough throughput to saturate the system. It has previously been shown that increased gene copy number may result in increased secretion (Verdoes et al., 1994). Thus, if the system is not saturated, overexpression of genes involved in translocation to the ER might not result in increased secretion of the model protein. Furthermore, protein dependent factors cannot be overlooked. For example, a more complex protein where folding is more difficult and stressful to the cell may lead to other bottlenecks within the secretory pathway than the mRFP protein.

This study demonstrates the possibility of significantly increasing cellular recombinant protein secretion with approximately 25% by overexpressing the Rab GTPase *rabD*. Other targets, including genes from different compartments of the secretory pathway resulted in no significant change in protein secretion, or in significantly lowered protein titres. The overexpression of *AN6307* (*S.c. SEC63* ortholog), the *A. niger* *An02g04250* ortholog *AN6307* and the *rabD* GEF *AN4759* (*S.c. SEC2* ortholog) resulted in substantially lowered titres of the recombinant protein. It may well be, that increased expression of these genes during protein secretion is a way for the cell to slow down secretion in order to cope with the increased protein load, similarly to for the observation for the gene *emp47* in other studies (Hoang et al., 2014). It is therefore not always obvious if upregulation of such genes will improve or decrease protein secretion.

An important factor for optimizing a protein cell factory is to relieve bottlenecks within the specific system that is being studied. The upregulation of *rabD* significantly boosted the secretion of the model protein, and it is possible that the bottlenecks for this strain now lie downstream of this gene, in the exocytosis step, or that overexpression of upstream genes will result in improved secretion due to the absence of the *rabD* bottleneck. Therefore, sequential overexpressions/deletions of a well-known system might be necessary in order to reach the full secretion potential of the host.

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Supplementary material

Supplementary Table 1. Primers used in this study.

Primer name	Sequence	Amplified fragment
MS56	GGGTTTAAU GTTTGCCATGCTGCAACTGT	IS5up
MS57	ACGAAGTCUTCGCTGAGTGTGAGTCTGACTTC	
MS65	GGCATTAAU GCATGGCAATCAAGTCCCTG	IS5dw
MS66	GGTCTTAAU AGGGAACGGTTGTGATGG	
MS60	AGACTTCGUATTCCTTGTATCTCTACACACAGG	PgpdA
MS61	AGTAGACTUCGCGGTAGTGATGTCTGCTC	
MS59	AAGTCTACUATGTCGTTCCGATCTCTACTCG	glaA 1-514
MS62	ATGCGCTUGGTCGAGGTCACGCTGCC	
MS67	AAGCGCAUGGCCTCCTCCGAGGAC	mRFP1
MS68	ATTACACTUTTATCAATGATGATGATGATGATGGGCGCCGGTGGAGT G	
MS63	AAGTGTA AUGGATCCACTTAACGTTACTGAAATC	TtrpC
MS64	GGA CTTAAUGGGCGCTTACACAGTACACGA	
MS90	AGAGCGAU ATGAACGGGTTTCGCAGCA	AN7679
MS91	TCTGCGAU CTA CTCTCTCCA ACTTCCCA	
MS92	AGAGCGAU ATGGCGGCCAAATCACG	AN2738
MS93	TCTGCGAU TCAGTTCGAATGCAATTTCTAA	
MS94	AGAGCGAU ATGAGCTCAATCCAGATTCCC	AN10724
MS95	TCTGCGAU CTAGACCGCCTCCTCGGAA	
MS96	AGAGCGAU ATGGTATGAGGCTCCCCTATAGA	AN11900
MS97	TCTGCGAU CTATCTCAAGAAGTGCAACTACTGCC	
MS98	AGAGCGAU ATGTCTACGGATTATACCTACGATGA	AN0834
MS99	TCTGCGAU TTAGTCGTCAACATCGGTTTCG	
MS100	AGAGCGAU ATGAAGGTTCCAGCATTGTTTT	AN7302
MS101	TCTGCGAU TCATAGGAATTTTTTAGGCATGTTG	
MS102	AGAGCGAU ATGGCTGGCACTAGAAACTATGAC	AN6974
MS103	TCTGCGAU TCAACAACACTTTCTCCGG	
MS104	AGAGCGAU ATGGCTAACGACGAGTATGATGT	

MS105	TCTGCGAU TTAACAGCATCCACCCTTGTTCC	AN0347
MS106	AGAGCGAU ATGTCTGAGCAACCGTACGATC	AN8769
MS107	TCTGCGAU TTAGCGCGTGGTTGTAACAAC	
MS108	AGAGCGAU ATGAGTGTATGTCCGCCGG	AN3416
MS109	TCTGCGAU TTATCTCGTCTGCGTAACAGCA	
MS110	AGAGCGAU ATGTTCAATCGCAATAATTACTCG	AN3098
MS111	TCTGCGAU CTAAACTGCCCTTCTAGTAGTCAATC	
MS112	AGAGCGAU ATGGCCGAGTGAGTCTTCAAC	AN4759
MS113	TCTGCGAU TCAAGTCATGGCATCGGC	
MS114	AGAGCGAU ATGCTATCCTTCCTATCCTCAAATC	AN10354
MS115	TCTGCGAU TTAAGTCCCTTTGCAGTATGACCA	
MS116	AGAGCGAU ATGTTGTTGCCGAGGTTCTCG	AN6307
MS117	TCTGCGAU TCAGGCAAGCAGATACAGAATAACAG	
X12	CCTAGCTCATTCTCAGTCCGTC	Integration site 5 check primers
X13	GATTGCATGGTTGGATCTGGATG	
J43	GGACAACGGGAAGAGGCTCAG	Integration site 1 check primers
J44	GGAGAGGGAGAGAAGAAGAAGGG	

Supplementary Table 2. Plasmids used in this study.

Plasmid name	Description	Source
pU2002	Vector containing <i>AFPyrG</i> , Direct Repeats and ampicillin gene for selection in <i>E.coli</i> . Used for construction of secretory reporter plasmid	Hansen et al., 2011
pWJ1350	Plasmid for amplification of mRFP	Our lab
pMAS1	Plasmid with <i>PgpdA</i> , UP and DOWN sequences for integration in IS5, <i>glucoamylase</i> (1-514) fused <i>mRFP</i> separated by KEX2 site and <i>AFPyrG</i> marker with Direct Repeats. Used for construction of the secretion reporter strain NID1439	This work
pU2311-1-ccdB	Plasmid with Tet-on promoter and sequences for integration in IS1. Used for construction of pMAS2-pMAS15	Our lab
pMAS2	Tet-ON promoter expressing AN7679	This work
pMAS3	Tet-ON promoter expressing AN2738	This work
pMAS4	Tet-ON promoter expressing AN10724	This work
pMAS5	Tet-ON promoter expressing AN11900	This work
pMAS6	Tet-ON promoter expressing AN0834	This work
pMAS7	Tet-ON promoter expressing AN7302	This work
pMAS8	Tet-ON promoter expressing ANrabD	This work
pMAS9	Tet-ON promoter expressing ANrabE	This work
pMAS10	Tet-ON promoter expressing ANsynA	This work
pMAS11	Tet-ON promoter expressing ANssoA	This work
pMAS12	Tet-ON promoter expressing ANnsfA	This work
pMAS13	Tet-ON promoter expressing AN4759	This work
pMAS14	Tet-ON promoter expressing AN10354	This work
pMAS15	Tet-ON promoter expressing AN6307	This work

**Chapter 5 – Promoter comparison of recombinant protein secretion in
Aspergillus nidulans and *Aspergillus niger***

Introduction

Aspergillus species are widely used today for the production of many industrially important enzymes. Around 60% of the industrial enzymes produced in filamentous fungi are products from *Aspergilli* (AMFEP). The most common species used for production purposes are *Aspergillus niger* and *Aspergillus oryzae*. They have been shown to have excellent secretion capacities, especially for proteins of fungal origin. Enzymes that are typically produced in *Aspergillus* species include glucoamylase, glucose oxidase, laccase, lactase, phytase and proteases (AMFEP). Overexpression of the gene of interest has led to higher production, either in the natural producer or in commonly used cell factories such as *A. niger*. For production of heterologous proteins of non-fungal origin yields are often 10-100 fold lower compared to fungal proteins (g/L range compared to µg/L range) (Nevalainen and Peterson, 2014). This is due to insufficient protein translocation, folding, transport and exocytosis of the foreign protein (Ward, 2011). Codon usage has also been seen to play a role in production of recombinant proteins in *Aspergillus* (Ward, 2011). Furthermore, proteolytic degradation of foreign proteins is a common problem, due to the degradation of the product (Hombergh et al., 1997; Punt et al., 2008).

One of the first steps towards a high protein titer is ensuring a high level of the protein encoding gene. For this reason, several promoters have been tested and utilized for various applications. For example a tight, inducible promoter is of interest when production has severe effects on growth. By inducing production at a late stage, biomass formation can be separated from product formation, thus avoiding growth defects.

Two commonly used promoters in *Aspergillus* protein production are the constitutively active glyceraldehyde-3-phosphate dehydrogenase promoter (*PgpdA*) from *Aspergillus nidulans* and the starch/maltose inducible and xylose repressed glucoamylase promoter (*PglaA*) from *A. niger*. The *glaA* promoter has been used for monitoring expression with reporters such as fluorescent proteins (Ganzlin and Rinas, 2008; Gordon et al., 2000) and for production of various recombinant proteins (Gouka et al., 1997; James et al., 2012; Lubertozzi and Keasling, 2009; Punt et al., 2002; Wiebe et al., 2001). It is induced by

starch and byproducts of starch degradation, such as maltose and even glucose to some extent (Ganzlin and Rinas, 2008). *PgpdA* is a strong constitutive promoter, that has also been extensively used in expression systems for protein production (Fleissner and Dersch, 2010; Gouka et al., 1997; Punt et al., 1991; Ward, 2011).

Promoter strength can be measured by RT-PCR, or by using intracellular reporter constructs like lacZ, luciferase or fluorescent proteins. However, it is not implicit that the strongest promoter will result in the highest secretion of recombinant proteins, since several other factors play a role. These factors include translocation, protein folding, maturation and glycosylation, cargo transport through the cell and subsequent exocytosis of the protein (Conesa et al., 2001). A high flux of proteins through the ER is likely to result in ER stress and intracellular degradation of the product. Thus, there is a necessity of testing different promoters for optimization of recombinant protein secretion (Carvalho et al., 2012; Geysens et al., 2009).

In this study, we have created strains that secrete mRFP by using glucoamylase as a carrier protein. We compared protein secretion in *A. nidulans* using the *glaA* promoter (*PglaA*) of *A. niger* and the *gpdA* (*PgpdA*) promoter of *A. nidulans*. Furthermore, an *A. niger* strain expressing *glaA*-fused mRFP from *PglaA* was constructed. The strains were grown in batch cultivations, and the promoters were compared for their efficiency on recombinant protein production and secretion. The set-up allowed us to compare protein secretion of *A. nidulans* and *A. niger* allowing an assessment of the potential of these species as hosts for protein production.

Materials & methods

Strains

The strains used in this study are listed in table 1. The *A. nidulans* strain IBT 29539 (*argB2*, *pyrG89*, *veA1*, *nkuA*Δ) (referred to as NID1) and *A. niger* ATCC1015 (WT) were used as host strains for construction of mRFP secreting strains. Plasmids were propagated in *E. coli* strain DH5α.

Table 1. Strains and plasmids used in this study.

Strain	Genotype	Source
NID1	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuA</i> Δ	IBT collection #29539
NID3	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuA</i> - <i>trS</i> :: <i>AFpyrG</i>	IBT collection #28738
NID912	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuA</i> Δ, IS1::Pgp <i>dA</i> ::RFP::TtrpC::pyr <i>G</i>	Our lab
NID1439	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuA</i> Δ, IS5::Pgp <i>dA</i> -ASNGlaA-mRFP-TtrpC::AFpyr <i>G</i>	This study
NID1714	<i>argB2</i> , <i>pyrG89</i> , <i>veA1</i> , <i>nkuA</i> Δ, IS5::Pgl <i>aA</i> -ASNGlaA-mRFP-TtrpC::AFpyr <i>G</i>	This study
NIG1	Wild type	<i>A. niger</i> ATCC1015
NIG77	<i>AlbA</i> ::Pgl <i>aA</i> -ASNGlaA(1-514)-mRFP-TtrpC:: <i>hph</i>	This study
Plasmid	Description	Source
pU2002	Recipient plasmid for construction of secretion cassette in <i>A. nidulans</i>	(Hansen et al., 2011)
pDHU2	Recipient plasmid for construction of pMAS17	Our lab
pMAS17	Recipient plasmid for construction of pMAS18	This study
pMAS1	Plasmid for mRFP secretion from <i>Pgp<i>dA</i></i> in <i>A. nidulans</i>	This study
pMAS16	Plasmid for mRFP secretion from <i>Pgl<i>aA</i></i> in <i>A. nidulans</i>	This study
pMAS18	Plasmid for mRFP secretion from <i>Pgl<i>aA</i></i> in <i>A. niger</i>	This study
pWJ1350	<i>mRFP</i> gene	Our lab

Media

Minimal medium (MM) (per Liter): 50 mL nitrate salts solution, 1 mL trace element solution, 0,001% thiamine, 10 g D-glucose

Complex medium (CM) (per Liter): 2 g yeast extract, 3 g tryptone, 20 mL mineral mix solution, 10 g D-glucose, 5 g/L maltose

20x nitrate salts solution (per Liter): 120 g NaNO₃, 10,4 g KCl, 10,4 g MgSO₄•7H₂O, 30,4 g KH₂PO₄

50x mineral mix (per Liter): 26 g KCl, 26 g MgSO₄•7H₂O, 26 g KH₂PO₄, 50 mL trace element solution

20x Trace element solution (per Liter): 0,4 g CuSO₄•5H₂O, 0,04 g Na₂B₄O₇•10H₂O, 0,8 g FeSO₄•7H₂O, 0,8 g MnSO₄•2H₂O, 0,8 g Na₂MoO₄•2H₂O, 8 g ZnSO₄•7H₂O

Plates and media were supplemented with L-arginine (0,7 g/L), sucrose (171,15 g/L) or hygromycin (100 µg/mL) when necessary. Transformation media (TM) was as described for MM except for 171,15 g/L sucrose as carbon source.

Molecular cloning

Primers were synthesized by Integrated DNA Technologies, and restriction enzymes and buffers were from New England Biolabs. All PCR reactions were performed using the PfuX7 polymerase (Nørholm, 2010) in 35 reaction cycles with 60°C annealing temperature and an extension time of 30 s/kb. All fragments relating to *A. nidulans* were amplified from *A. nidulans* NID1 gDNA. *A. niger* ATCC 1015 gDNA was used as template for amplification of *glucoamylase* (*glaA*) promoter and carrier protein (aa 1-514, GlaA₁₋₅₁₄). The plasmid pWJ1350 was used as template for amplification of *mRFP*. A list of all primers is presented in table S1.

All plasmid constructions were performed with USER cloning (Nour-Eldin et al., 2006). A list of plasmids used in this study is presented in supplementary table 1. The plasmids for expressing glaA₁₋₅₁₄ fused mRFP in *A. nidulans* (expressed either from the *A. niger* maltose/starch inducible *glaA* promoter or the constitutive *A. nidulans* *gpdA* promoter) was constructed by fusing 6 individual DNA fragments with the vector backbone pU2002 (Hansen et al., 2011), generating plasmids pMAS1 and pMAS16. To ensure

proteolytic cleavage of the glucoamylase from the mRFP a KEX2 (Lys-Arg) proteolytic site was inserted between the glucoamylase and the mRFP protein.

The plasmids with *glaA*₁₋₅₁₄-mRFP for expression in *A. niger* were constructed from plasmid pDHU2, which was kindly provided by Dorte Holm. pDHU2 contains targeting sequences for the *alba* locus in *A. niger*, an *A. nidulans gpdA* promoter, the ampicillin resistance gene for selection in *E. coli*, the *hph* genetic marker for hygromycin selection of *A. niger* transformants and an AsiSI/Nb.BtsI restriction site for USER cloning. Integration in the *alba* locus makes it possible to identify integration of the cassette in the locus based on the color of the spores; *alba* disruption results in white spores.

The *gpdA* promoter was exchanged for the *glaA* promoter in pDHU2 by amplifying two fragments of the vector excluding the promoter and combining these two fragments with the *glaA* promoter by USER fusion, generating plasmid pMAS17. The USER tail for the *glaA* promoter reverse primer was constructed to regenerate the site for USER cloning in pMAS17. *GlaA*₁₋₅₁₄ and *mRFP* was cloned into pMAS17, generating pMAS18.

All plasmids were prepared for USER cloning by digesting with respective restriction and nicking enzymes, and the cloning procedure was as described in (Nour-Eldin et al., 2006).

Genetic transformation

Protoplastation and transformation of *A. nidulans* were performed as described in (Nielsen et al., 2006) using *Aspergillus fumigatus purG* (*AFpyrG*) as a selectable marker. Transformants were verified with PCR by using spores and mycelium as DNA template. In order to liberate the DNA from the cells, the PCR mix with the spores was subjected to 20 minutes at 98°C at the start of the PCR program. Then, a touchdown PCR running with annealing temperatures from 65°C to 58°C, and 4 minutes extension time was performed. The spores were transferred to the PCR mix by gently touching a colony with the pipette tip and transferring the spores to two vials with the same reaction mix, ensuring that one of the reactions would have the correct amount of spores for DNA amplification.

The *A. nidulans* strains secreting mRFP from the two chosen promoters was constructed by transforming NID1 with the linearized cassette from plasmid pMAS1 and pMAS16 respectively. The cassette was liberated from the plasmid by treatment with *Swa*I restriction enzyme for 2 hours at 25°C. The transformation mix was plated on MM+Arg and transformants were verified by spore PCR. The strains generated were named NID1439 (*Pgpda*) and NID1714 (*PglaA*).

A. niger transformants were analyzed with spore PCR. pMAS18 was digested with restriction enzyme *Swa*I to linearize the cassette, and the cassette were transformed into *A. niger*, generating strain NIG77. The transformation mix was plated on transformation media supplemented with hygromycin (100 µg/mL).

Preparation of inoculum

Conidia were propagated on MM plates with the appropriate selection and incubated for 5 to 7 days at 30°C (for *A. niger* strains) and 37°C (for *A. nidulans* strains). Conidia were harvested with 0.9% NaCl solution and filtered through a miracloth to a sterile falcon tube. The spore concentration was determined using a Neubauer cell counting chamber.

Cultivations

Batch cultivations were performed in Biostat B bioreactor (B. Braun Biotech) with a working volume of 2 L. The bioreactors were equipped with two six-bladed Rushton turbine impellers, pH electrode, thermosensor, sparger, and sampling outlet. All cultivations were conducted in duplicates. A spore concentration of $1 \cdot 10^9$ spores/L were inoculated in CM. In cultivations with *A. nidulans* strains, 4 mM L-arginine was added to the medium before sterilization. Aeration was initially set to 0.1 volumes of air per volume of fluid per minute (vvm), stirring rate to 100 rpm and pH was set to 3.0. After germination, aeration was ramped up to 1 vvm, agitation was ramped up to 800, and the pH was ramped up to 5. The temperature was maintained at 30°C and pH was adjusted and controlled with 2M NaOH and 2M H₂SO₄.

Cell dry weight determination

Cell dry weight was determined by filtering 5 mL of cell culture through a pre-dried and weighed filter (Advantec). The filter was dried and weighed again, and the dry weight was determined by calculating the amount of dry cell weight per liter of cell culture.

HPLC analysis

For quantification of glucose and maltose, culture samples were taken and immediately filtered through a 0.45 µm nitrocellulose syringe filter (PTFE membrane). The filtrate was frozen and kept at -20 °C. Glucose and maltose concentrations were detected and quantified by refractive index (RI) using an Aminex HPX-87H cation-exchange column (Biorad) eluted at 35 °C with 5 mM H₂SO₄ at a flow rate of 0.6 mL/min. The glucose and maltose concentrations were determined from a standard curve.

Fluorescence measurement

Fluorescence of culture filtrates were measured in a Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek Instruments) using excitation/emission 584/607 nm. A 96-well microtiter plate (PS microplate, Greiner bio-one) was used and 200 µL samples were loaded in triplicates. Background fluorescence was corrected by subtraction of values derived from a negative control.

SDS-PAGE

SDS-PAGE was performed on Novex NuPAGE 4-12% Bis-Tris gel (Life Technologies) according to the instructions of the manufacturer. The ladder used was Novex Sharp Pre-stained Protein Standard (Life Technologies).

Protein upconcentration

Amicon Ultra centrifugal filters (0,5 mL, 10 kDa cut-off value, Millipore) were used to upconcentrate the cell culture supernatant.

Results & discussion

Evaluation of reporter strains

The use of carrier proteins has been successfully applied in production and secretion of many recombinant proteins, such as hIL-6, hen egg white lysozyme, bovine prochymosin, human lactoferrin and reporter proteins as for example GFP in several *Aspergillus* species (Gordon et al., 2000; Gouka et al., 1997; Punt et al., 2002; Ward, 2011). The beneficial effects of using carrier proteins are most likely due to enhanced translocation of the secretory cargo, improved folding and also prevention of degradation by proteases (Fleissner and Dersch, 2010; Ward, 2011).

In order to evaluate promoter efficiency for secretion of heterologous proteins in *Aspergillus*, strains secreting a model protein (mRFP) were constructed in this study. Secretion of the model protein was ensured by fusing the *mRFP* gene to the *glucoamylase (glaA)* gene from *A. niger*. Between the carrier protein and the recombinant protein, a KEX2 site (Lys-Arg) was inserted to ensure cleavage in the golgi. First, the ability of the mutant strains to secrete the fused *glaA*-mRFP was evaluated by fluorescence measurements and SDS-PAGE analysis (Figure 1). Fluorescence measurements based on the supernatant fraction obtained from shake flask cultivations showed that mRFP was secreted into the media. An *A. nidulans* mutant strain producing mRFP without the carrier protein (NID912, previous work at our lab) was analyzed as a control and no fluorescence could be detected in the supernatant. This showed that no efficient secretion of mRFP occurs and that the carrier protein transports mRFP into the secretory pathway. Moreover, the SDS-PAGE analysis showed that cleavage of the *GlaA*-mRFP protein fusion was efficient, as individual bands for glucoamylase (60 kDa) and mRFP (28 kDa) were observed, but no band corresponding to a protein fusion was visible. Thus, it was concluded that the *GlaA*-mRFP reporter strains successfully cleaved off the carrier protein glucoamylase at the KEX2 cleavage site and thus secreted mRFP.

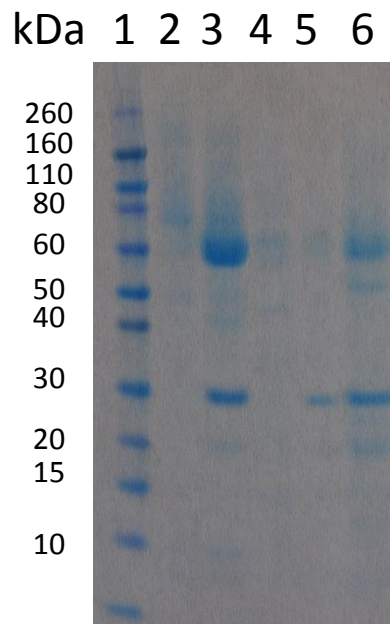


Figure 1. SDS-PAGE of *A. nidulans* and *A. niger* grown for 40 hours in shake flasks. The supernatant was upconcentrated and 6 μ L was loaded in each well. 1=Ladder, 2=NIG1, 3=NIG77, 4=NID3, 5=NID1714, 6=NID1439.

Physiological characterization of recombinant protein secreting *Aspergillus nidulans*

To compare the effect of the *glaA* promoter and the *gpdA* promoter in *Aspergillus nidulans* on heterologous protein secretion, NID3, NID1439 and NID1714 were grown in controlled batch cultivations with complex medium supplemented with 10 g/L glucose and 5 g/L maltose. Figures 2-6 show the time course of the cultivations with biomass, glucose, maltose and fluorescence profiles over time.

For the reference strain, NID3 (Figure 2), growth proceeded after a lag-phase of approximately 15 hours. Analysis of the substrates revealed that glucose and maltose were consumed simultaneously. The maximum biomass concentration was approximately 9 g/L for both replicates. A slight offset in the start of exponential growth was seen between the replicates, but similar profiles in general demonstrate the reproducibility.

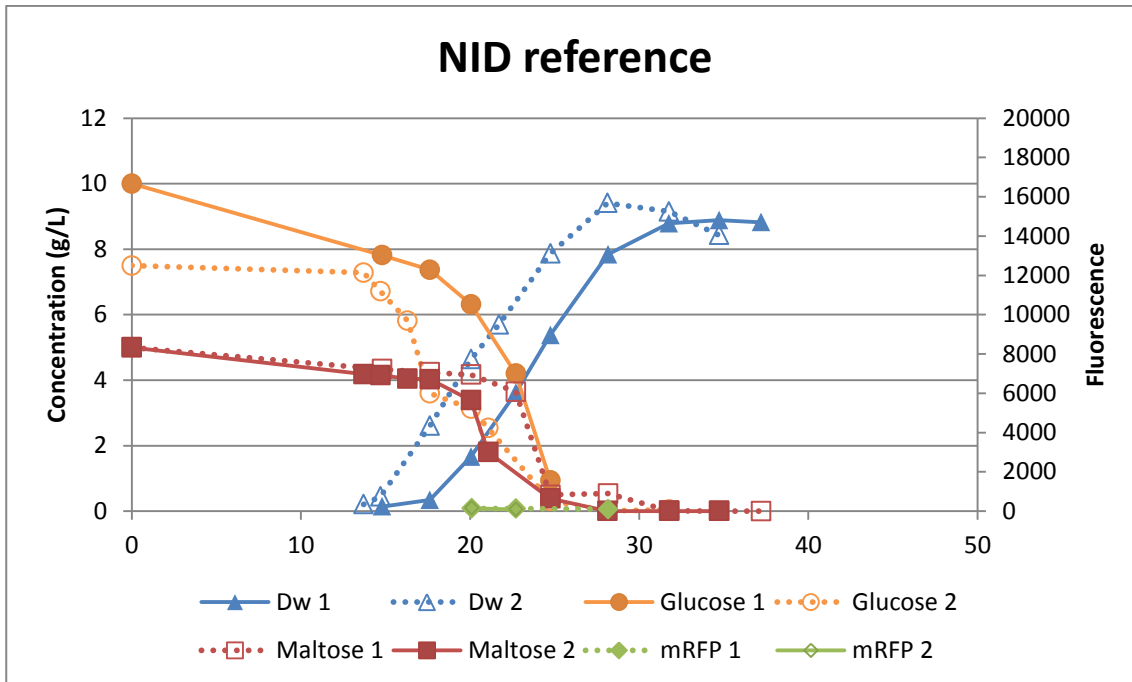


Figure 2. Batch cultivations of *A. nidulans* reference strain in CM supplemented with 10 g/L glucose and 5 g/L maltose. Duplicate processes are plotted.

The *A. nidulans* strain secreting mRFP under control of the *gpdA* promoter (NID1439, Figure 3) exhibited a longer lag phase than the reference strain, approximately 22 hours. After exponential growth initiated, it took approximately 15 hours until the maximum dry weight (dw) was reached, similar to the reference strain. Biomass levels were slightly lower than for the reference strain, reaching approximately 8 g/L. Another difference in relation to the reference process was that when maltose was digested there was still a high level of glucose left. In the reference process most of the glucose had been depleted when maltose levels reached zero. Fluorescence levels increased steadily in a growth related manner.

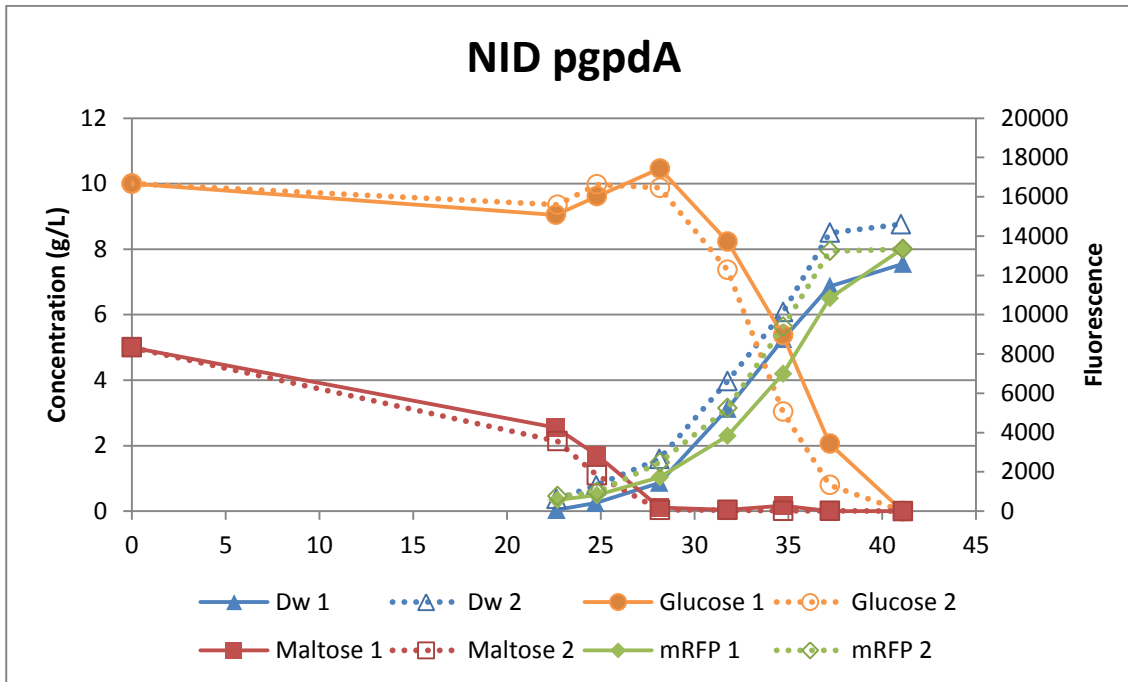


Figure 3. Batch cultivations of *A. nidulans* secreting mRFP under control of the *gpdA* promoter. CM supplemented with 10 g/L glucose and 5 g/L maltose was used. Duplicate processes are plotted.

When expressing *glaA*-mRFP driven by the *A. niger glaA* promoter (NID1714, Figure 4), production was initiated later than with the *gpdA* promoter. Biomass levels had reached approximately 2 g/L when fluorescence started to increase. The lag-phase was around 22 hours and thus very similar to the process with NID1439. Maximum biomass was lower than for the reference strain, similar to NID1439, reaching 8 g/L in both replicates. The growth rate did not vary between NID1439 and NID1714. CO₂-based growth rates were 0,21 h⁻¹ in both cases. Interestingly, fluorescence reached higher levels with the *glaA* promoter. However, there was a fluorescence drop for one of the replicates after approximately 50 hours.

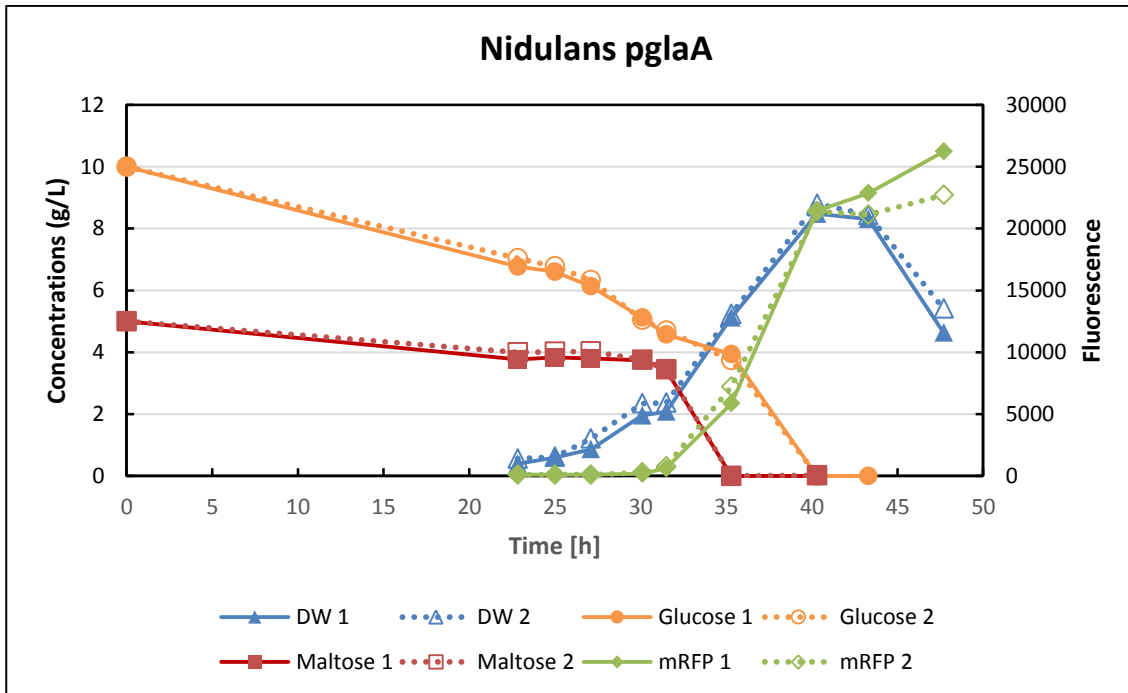


Figure 4. Batch cultivations of *A. nidulans* secreting mRFP driven by the *glaA* promoter. CM supplemented with 10 g/L glucose and 5 g/L maltose was used. Duplicate processes are plotted.

The use of the *glaA* promoter led to secretion of more protein in *A. nidulans* than the *gpdA* promoter. Interestingly, the *glaA* driven production was initiated later than when the *gpdA* promoter was used. One would expect to see expression from *glaA* occurring earlier, as both glucose and maltose are known to induce expression from the *glaA* promoter, at least in the concentrations used in this study (Ganzlin and Rinas, 2008). However, it should be noted that the strain reports on protein secretion rather than promoter activity. Thus, it may well be that expression was high, but that secretion for some reason was delayed until later. One plausible explanation for higher secretion in NID1714 is that glucoamylase have been shown to be highly expressed close to glucose depletion, with a subsequent boost of glucoamylase yields (Pedersen, 2011). This boost in production can be seen in Figure 4, where fluorescence levels increase at 45 hours. A boost of enzyme production when the carbon source is being depleted is probably a way for the fungus to search for more food, that can be degraded by enzymes such as glucoamylase (Nevalainen and Peterson, 2014).

In NID1439 mRFP increased in a growth related manner. This was not unexpected, as the *gpdA* promoter is the promoter of the glyceraldehyde-3-phosphate dehydrogenase enzyme, which has a role in glycolysis. Thus, when glucose was exhausted, *gpdA* promoter activity may be lower.

Physiological characterization of recombinant protein secreting *Aspergillus niger*

As can be seen in Figure 5, the *A. niger* reference strain (NIG1) had a lag-phase of approximately 10 hours. Maltose was depleted after 18 hours; at this time point there was approximately half of the glucose left. The maximum (measured) dry weight was reached after 25 hours, 6 g/L, which was substantially lower than for the *A. nidulans* reference strain.

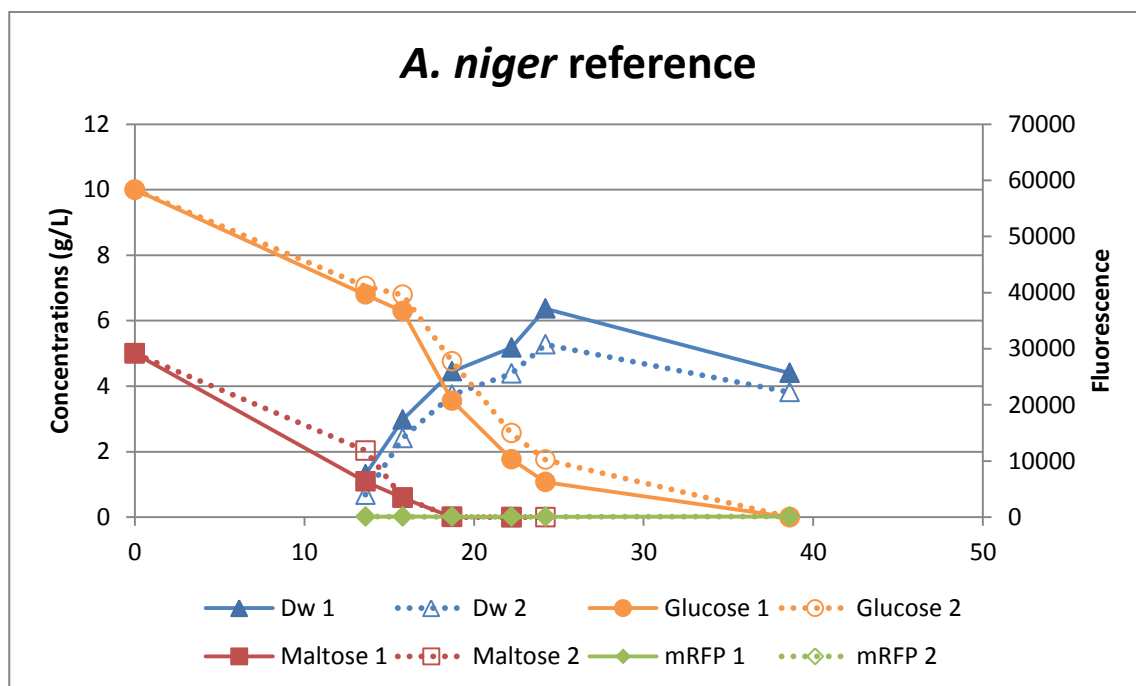


Figure 5. Bioprocess of the *A. niger* reference strain grown on CM supplemented with 10 g/L glucose and 5 g/L maltose.

Duplicate processes are plotted.

In order to compare protein secretion between *A. nidulans* and *A. niger*, a strain secreting mRFP with the *glaA* carrier protein was constructed also in *A. niger*. The cassette was integrated in the *alba* locus, allowing detection of correct transformants as spores will be light coloured when *alba* is disturbed. In Figure 6 growth, substrate and fluorescence profiles over time are presented. The maximum dw reached was 6 g/L and the lag phase was around 10 hours, similar to the reference strain. Glucose and maltose consumption

were similar to the reference as well, with maltose being depleted earlier than glucose. As was the case with NID1714, secretion in NIG77 also occurred after growth was initiated. Thus, the characteristics of the promoter were comparable between the fungi. Fluorescence is much higher than for the *A. nidulans* strains, reaching around 60000 units after 40 hours. The bioprocess profile of the protein secreting *A. niger* strain resembles that of the reference strain in terms of growth and substrate utilization.

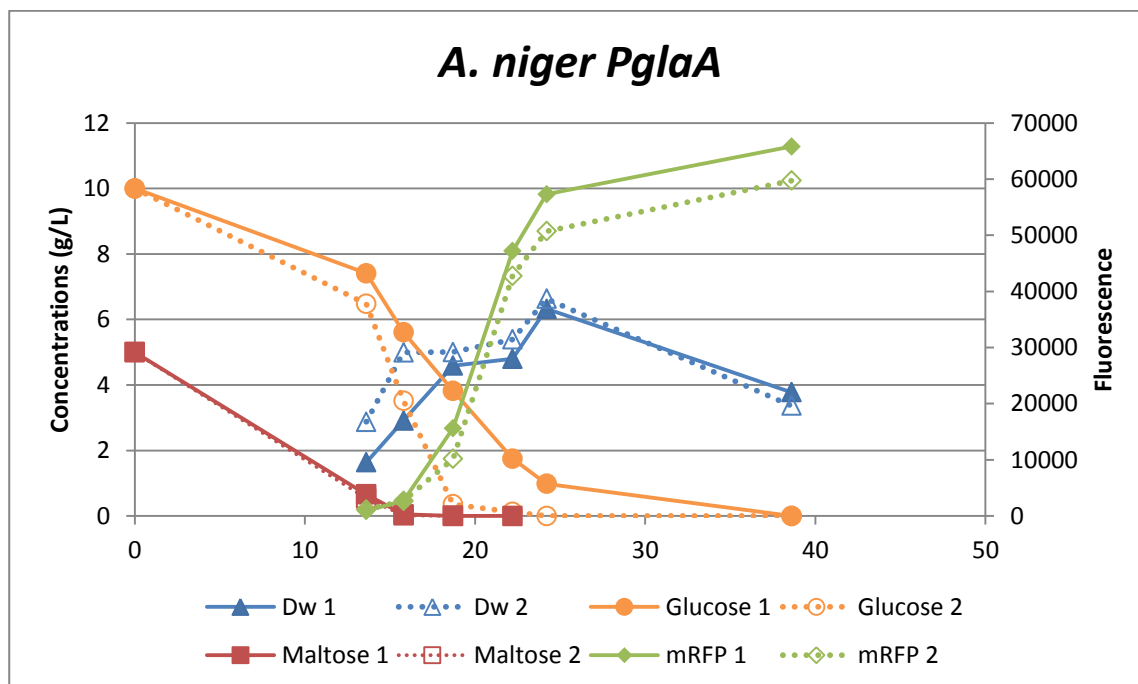


Figure 6. Bioprocess of the *A. niger* strain secreting mRFP under control of the *glaA* promoter. The strain was grown on CM supplemented with 10 g/L glucose and 5 g/L maltose. Duplicate processes are plotted.

Aspergillus niger is established as a protein production cell factory, and there are several industrial processes utilizing this species due to its well-known ability to secrete high amounts of proteins to the extracellular space (Pel et al., 2007). Early research on fungal genetics often utilized *Aspergillus nidulans* as a model organism, due to its well-known genetics. Although omics technologies have developed drastically over the last couple of decades, and an increasing number of filamentous fungal strains are being sequenced, the main differences in relation to secretion and production efficiency are relatively unknown. Therefore, it is interesting to determine if trends are similar between the fungi, and also examine the

differences in secretion and protein production. For this reason, we chose to construct an mRFP-secreting *A. niger* strain and perform a similar physiological characterization as for *A. nidulans*. We chose to use the *glaA* promoter, as this was shown to be most effective for protein secretion in *A. nidulans*. This enabled a comparison of the two hosts secreting recombinant proteins from the strong *glaA* promoter.

Ganzlin et al. characterized the glucoamylase promoter in *A. niger* by using an intracellular GFP reporter strain (Ganzlin and Rinas, 2008). They screened a variety of carbon sources and concluded that starch and its hydrolytic products acted as inducers of the glucoamylase promoter, whereas xylose repressed the activity of the promoter. Although *PglaA* was partly regulated by carbon catabolite repression in a CreA-dependent manner (Verdoes et al., 1994), glucose at levels of 10 g/L induced expression of the promoter, although to a lesser extent than maltose and other starch degradation products such as maltodextrin and maltotriose (Ganzlin and Rinas, 2008).

The *glaA* promoter generated higher levels of secreted proteins compared to the *gpdA* promoter in *A. nidulans*. In *A. nidulans* we have decided to use an *nkuAΔ* strain in order to promote Homologous Recombination (HR) over Non-Homologous End-Joining (NHEJ). This facilitates strain construction and verification. In our lab we have not detected NHEJ to occur in *nkuAΔ* background when using homologous flanks of 2000 bp (Nielsen, 2014). For this reason, we assume that only one single copy of the construct was integrated in *A. nidulans*. Thus, since the only thing differing in the two strains constructed is the promoter, the *glaA* promoter was shown to be more efficient in secretion of the model protein tested in this set-up. Interestingly, the *gpdA* promoter led to secretion of the model protein at a slightly earlier stage than the *glaA* promoter. Final protein titers were however higher when the model protein was expressed from the *glaA* promoter. The use of inducible promoters provide a means of separating growth and product formation, allowing high cell concentrations to be obtained before product formation is initiated. Since product formation occurs when there is a high level of biomass, more cells contribute to product formation

thus leading to higher yields of the product. If product formation hampers growth, inducible promoters are a way to avoid this effect and thereby reach higher biomass levels earlier, with more production of the protein as a result.

When comparing protein secretion in *A. nidulans* and *A. niger* several factors may play a role for the differences in secretion. For example, the site of integration has an effect on transcription of the gene (Verdoes et al., 1995). In *A. nidulans*, the *glaA*-mRFP cassette is integrated in the same locus, thus leaving out such variations. Obviously, the same approach is not possible for *A. niger*, and thus it is an uncontrolled factor that may play a role in the interpretation of the secretion potential.

It has also been shown that genetic markers may cause a variation in promoter activity. Dramatic differences in expression from single-copy transformants of *A. nidulans* were seen depending on the marker used (Lubertozzi and Keasling, 2006). In *A. nidulans* the *AFpyrG* marker was used, whereas hygromycin resistance was used to generate the *A. niger* strain. This may have an effect on the expression of the construct in the different strains.

Furthermore, the highest expression for each promoter and gene pair tested in the study by Lubertozzi and Keasling (2006) was achieved in single-copy transformants. This implies that multiple copies do not increase expression in Aspergilli. However, this may also be disputed, as other studies have showed that increasing the copy number does indeed increase expression of a gene in Aspergilli (Verdoes et al., 1994).

Although the relation between copy numbers and expression is unclear, it is still a factor that may affect our interpretations of the results, since a WT background was used for cloning in *A. niger*, possibly resulting in multiple insertions of the expression cassette.

As mentioned previously *A. niger* has been a preferred production host for enzymes and organic acids in the industry and is well-characterized in this regard. On the contrary, *A. nidulans* has a well-developed toolbox for genetic engineering compared to *A. niger*, but is not currently used for industrial purposes. Thus

depending on the objective of a study, one of the hosts may be preferred over the other. When developing novel tools, or implementing new strategies, *A. nidulans* may be preferred due to the advanced genetic tools and the advantage conferred due to the possibility of performing sexual crossings. For these reasons, cell biology and gene regulation are oftentimes studied in *A. nidulans*. As demonstrated in this study, similarities in terms of for example promoter characteristics show that *A. nidulans* may be a good model organism in order to study recombinant protein production. It is not unlikely that further studies with protein production in *A. nidulans* reveals that this species is well suited also for large scale production of specific proteins. In order to go more in depth of a comparison between the two species more detailed studies are needed. This includes production of various proteins and even more detailed analysis in terms of growth, substrate utilization, intracellular retention of secretory cargo and more. Table 2 presents some advantages and disadvantages of utilizing *A. nidulans* and *A. niger* for biotechnological purposes.

Table 2. Advantages of using *A. nidulans* and *A. niger* in various biotechnological aspects. A + sign represents an advantage of one of the species, but does not imply that it cannot be performed in the other species (unless stated with a – sign).

Advantage	<i>Aspergillus niger</i>	<i>Aspergillus nidulans</i>
Auxotrophy markers		+
Heterologous enzyme production	+	
Sexual crossing	-	+
Studying cell biology/gene regulation		+
Citric acid production	+	
pH tolerance	+	
Developing novel molecular tools		+

In conclusion, *PglaA* resulted in a higher secretion of the fluorescent reporter protein compared to *PgpdA*. As *A. niger* is a common cell factory for production of enzymes, and has a well-known high secretory

capacity, it was not surprising to find that it was also more efficient in secreting the fluorescent reporter protein compared to *A. nidulans*, although the reasons cannot be fully interpreted due to the argumentation above. Considering difficulties with secreting fluorescent proteins in various yeasts (Li et al., 2002), *A. nidulans* could be seen as a plausible alternative for production of recombinant proteins. Interestingly, the secretion of mRFP had similar profiles when utilizing the same promoter in *A. nidulans* and *A. niger*, demonstrating that *A. nidulans* may be a good model organism in terms of generating novel tools for protein production within filamentous fungi.

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Supplementary material

Table S1. Primers used in this study.

Primer name	Sequence	Amplified fragment
MS56	GGGTTTAAU GTTTGCCATGCTGCAACTGT	<i>IS5up</i>
MS57	ACGAAGTCUTCGCTGAGTGTGAGTCTGACTTC	
MS65	GGCATTAAU GCATGGCAATCAAGTCCCTG	<i>IS5dw</i>
MS66	GGTCTTAAU AGGGAACGGTTGTCGATGG	
MS60	AGACTTCGUATTCCTTGTATCTCTACACACAGG	<i>PgpdA</i>
MS61	AGTAGACTUCGCGGTAGTGATGTCTGCTC	
MS59	AAGTCTACUATGTCGTTCCGATCTCTACTCG	<i>glaA 1-514</i>
MS62	ATGCGCTUGGTCGAGGTCACGCTGCC	
MS67	AAGCGCAUGGCCTCCTCCGAGGAC	<i>mRFP1</i>
MS68	ATTACACTUTTATCAATGATGATGATGATGATGGGCGCCGGTGGAGTG	
MS63	AAGTGTAUUGGATCCACTTAACGTTACTGAAATC	<i>TtrpC</i>
MS64	GGACTTAAUUGGCGCTTACACAGTACACGA	
MS58	AGACTTCGUCTTCGGATCAGCGATCATCAG	<i>PglaA</i> + <i>glaA 1-514</i> for nidulans (combined with MS67)
MS127	AGACTTCGUATCCCCACTACCGATTAAGAC	Part of pDHU2 for changing promoter to <i>PglaA</i>
MS128	AGTAGACTUATATTGGGCTCACACGAGATGG	
MS131	AGTGAGAGCGAUCGAGACAC	Part of pDHU2 for changing promoter to <i>PglaA</i>
MS132	ACGAAGTCUCTCCCTCGTATTCGCCTCCT	
MS129	AAGTCTACUCTTCGGATCAGCGATCATCAG	<i>PglaA</i> for cloning in pDHU2
MS130	ATCGCTCTCACUGCTGCTGAGGTGTAATGATGCTGG	
MS133	ACTCAGCATGTTGGTTATATATTCGAG	Check primers for integration in <i>alba</i>
MS134	TGGTTCATCGCTTGCAGGTC	
X12	CCTAGCTCATTCCCTCAGTCCGTC	Integration site 5 check primers
X13	GATTGCATGGTTGGATCTGGATG	

Chapter 6 - Conclusions and future perspectives

In this Ph.D. thesis recombinant protein production has been investigated in three species; *Saccharomyces cerevisiae*, *Aspergillus nidulans* and *Aspergillus niger*.

The influence of various environmental conditions on *S. cerevisiae* protein production was investigated in shake flasks and bioreactors. In order to be able to measure single cell protein levels a fluorescent reporter protein, tagRFP, was integrated in the genome of *S. cerevisiae*. It was found that small changes in the extracellular environment had an influence on protein production. Positive as well as negative effects on protein production were demonstrated using flow cytometry studies allowing measurement of fluorescence in single cells. Glucose concentration, pH, osmotic stress and ethanol concentration were investigated. Previously, the influence of environmental stress on cellular physiology has been addressed, but the effects on recombinant protein production at a single cell level have to our knowledge not been investigated before. In addition, culture heterogeneity during growth on ethanol was demonstrated. A binomial fluorescence distribution was seen, with one population of cells maintaining a higher fluorescence level during ethanol growth. Further experiments could be performed to investigate the reasons for this. Culture age may be a factor here and this could be monitored by staining bud scars and performing multichannel fluorescence cytometry thus including an indicator for cell age as well as obtaining measurements for recombinant protein production. Furthermore, recombinant protein secretion could be examined in a similar manner by constructing a strain that transports the recombinant protein to the surface of the cell. In this case, it could be interesting to investigate the effect of gene copy number on secretion and heterogeneity. Finally, it could be relevant to construct a UPR-reporter in such a strain, and investigate the correlation between the UPR and efficiency in secretion of the model protein.

As filamentous fungi are important enzyme producers, and potentially could be used for production of recombinant proteins, a fluorescent protein secretion reporter was constructed in *A. nidulans*. Fourteen different genes in the secretory pathway were overexpressed and the effect on secretion of the reporter

protein was measured. It was seen that overexpression of the Rab GTPase RabD, involved in protein transport between the Golgi and the plasma membrane, increased protein secretion with approximately 25%. A difference in protein localization was demonstrated with fluorescence microscopy; the protein localized more towards hyphal tips and the plasma membrane when RabD was overexpressed. The study demonstrates how engineering the secretory pathway of filamentous fungi can aid in improving production of heterologous proteins. Previous studies in filamentous fungi have typically focused on a few genes and their impact on protein production. Our work shows the possibility of screening the secretory pathway for genes that could be beneficial for protein secretion. In the longer perspective this could potentially be a way to develop more efficient cell factories in filamentous fungi. Further studies could be performed to investigate whether secretion could be improved further by using the RabD overexpressing strain for overexpression of other secretion related genes. Another suggestion would be to investigate the effect of RabD overexpression on secretion of other proteins. Are the effects protein specific or are they valid also in the production of larger, more complex proteins?

In chapter five, the *gpdA* promoter of *A. nidulans* was compared with the *A. niger glaA* promoter. Results showed that *PglaA* resulted in higher secretion of the model protein compared to *PgpdA* in *A. nidulans*. It was also seen that secretion occurred later in the bioprocess during *PglaA* driven secretion. To compare secretion in *A. nidulans* with *A. niger*, a *PglaA* driven mRFP secretion reporter was constructed also in *A. niger*. *A. niger* protein production resulted in more secretion of the model protein, however the reasons for this needs to be investigated further. Interestingly, the characteristics of the promoter were similar between the species, and this shows that *A. nidulans* could be a potential candidate for studying recombinant protein production within filamentous fungi. Typically, promoters are studied by intracellular reporter constructs, providing a measurement of promoter activity. To construct efficient cell factories it is however important to consider what effects the promoter has on secretion of proteins. This study clearly demonstrates that promoter choice plays a major role for the secretion of heterologous proteins in filamentous fungi. The study compares two of the most commonly used promoters for production of

proteins in filamentous fungi, and the results show a clear advantage of using *PglaA*. Therefore a thorough investigation of several promoters would be interesting. Optimally, transcriptomic data could be used to find highly expressed genes during specific conditions. Additionally, the effect of various signal peptides and recombinant proteins could be screened in order to find novel candidates for secretion of recombinant proteins.