# METABOLICALLY ENGINEERING *ASPERGILLUS NIDULANS* FOR CLIENT PROTEIN PRODUCTION

By

JORGE DAVID LIGHTFOOT

Bachelor of Science in Biology
Southern Illinois University Carbondale
Carbondale, Illinois
2012

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2019

METABOLICALLY ENGINEERING *ASPERGILLUS NIDULANS* FOR CLIENT PROTEIN PRODUCTION

Dissertation Approved:

Dr. Rolf Prade

Dissertation Adviser

Dr. Jeff Hadwiger

Dr. Stephen Marek

Dr. Ed Shaw

Dr. Ramanjulu Sunkar

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor Dr. Rolf Prade, whose guidance and mentorship was invaluable to me. Rolf allowed me the flexibility to pursue risky projects but was also able to keep me grounded with his passion for good science. Rolf's knowledge and standard for a strong genetic background was instrumental in my growth as a scientist. Rolf has given me a great deal of opportunity for personal and professional growth while working for him and I am eternally grateful to have him as a mentor.

I would also like to extend my gratitude to Rae Ross, whose encouragement and support were a constant source of inspiration for me. I can honestly say that I could not have done this without her.

To my friends: I could have graduated in two years if it wasn't for you. Jokes aside, if it wasn't for the emotional support and grounding that you provided I would be lost. It has been a privilege to have known all of you and I hope that this isn't goodbye.

I would be remiss if I did not thank my colleagues and fellow students in the Microbiology and Molecular Genetics Department who acted as a family for me. In particular, Dr. Jerreme Jackson and soon-to-be-doctor Melissa Brewer, both of whom talked me off of many ledges over countless baskets of chips and salsa. I look forward to any opportunities to work with you all again, thanks for the memories!

Oklahoma State University, you have given me an education, friends, and a home. In return I will always stay loyal and true.

Lastly, I would like to thank the community of Stillwater. I never really knew what a hometown felt like until I moved to Oklahoma, and I am eternally grateful for the hospitality and support that I have received.

Name: JORGE LIGHTFOOT

Date of Degree: JULY, 2019

Title of Study: METABOLICALLY ENGINEERING *ASPERGILLUS NIDULANS* FOR CLIENT PROTEIN PRODUCTION

Major: MICROBIOLOGY AND MOLECULAR GENETICS

Abstract: The filamentous fungi, *A. nidulans*, can produce nearly 100 grams per liter of industrially relevant proteins under optimal conditions. However, many of these proteins are degraded or produced alongside other proteins, which drastically reduce their efficacy in a cellulose fermentation reaction.

The aim of this work is to redesign the regulatory genetic circuitry of *Aspergillus nidulans* to efficiently produce client proteins. We have successfully reengineered the cellulase regulatory network to produce cellulases in the presence of the C5-sugar xylose. By replacing expensive substrates with a cheap by-product carbon source we reduce enzyme production costs and lower operational costs by eliminating the need for off-site enzyme production, purification, concentration, transport and dilution.

We also propose a novel mechanism, utilizing RNA interference, to combinatorially silence genes, which degrade or contaminate client proteins. Using dual promoters, we will flank a sequence containing 30 or 40bp complementary sequences for multiple client genes. This will induce double stranded RNA production, in turn loading these individual complementary sequences into the Argonaute complex, silencing the messenger RNA for each target gene.

We have also utilized LC-MS/MS to examine changes in the proteome of our silenced strains. We have seen marked decreases in our target gene sequences as well as the induction of new proteins, acting as a compensation mechanism for the fungus.

Our silenced strains, when transformed to produce client proteins, have also had a marked change in the amount of protein produced, as well as how long it lasts in the media during production. We have continued this work by silencing genes responsible for unwanted amylolytic activity in client protein production.

TABLE OF CONTENTS

Chapter Page

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

# CHAPTER I

## REVIEW OF LITERATURE

### 1.1 Lignocellulose as an energy source

Lignocellulose is a promising renewable energy source for biofuel production (Lynd, van Zyl et al. 2005, Harris, Xu et al. 2014, Kim, Lee et al. 2014). Historically the conversion of lignocellulose to biofuels has been completed in three main steps: pretreatment, enzymatic hydrolysis, and fermentation of the released reducing sugars (Lynd, van Zyl et al. 2005, Lynd, Laser et al. 2008). Due to the rigidity of the structure of cellulose contained in lignocellulose, the enzymatic hydrolysis of this molecule is notoriously slow and expensive. (Selig, Viamajala et al. 2007, Lynd, Laser et al. 2008). Recent attention has been directed towards the use of synergistic fungal proteins to enhance the speed and cost of the enzymatic hydrolysis of cellulose (Kim, Lee et al. 2009, Harris, Welner et al. 2010, Kim, Ko et al. 2013, Kim, Lee et al. 2014).

Lignocellulose, which is composed of cellulose, hemicellulose and lignin, is the most abundant renewable biomass on earth (Kim, Lee et al. 2014). The cellulose fiber is composed of β1, 4- glycosidic bond-linked polymers that are tightly packed into a microfibril structure (Somerville, Bauer et al. 2004, Hall, Bansal et al. 2010). These cellulose microfibrils are surrounded by a matrix of hemicellulose and the aromatic molecule, lignin (Somerville, Bauer et al. 2004, Hall, Bansal et al. 2010). Thus far, hydrolytic cellulases such as endoglucanases, cellobiohydrolases and β- glucosidases, have only had moderate success in hydrolyzing cellulose completely (Garvey, Klose et al. 2013).

Low accessibility to the cellulose for these enzymes impedes efficient hydrolysis, with inaccessible regions remaining intact, even after various chemical and physical pretreatments (Ding and Himmel 2006, Arantes and Saddler 2010). Furthermore, cellulose in its fibril form has a high amount of individuality in its molecular architecture depending on its plant source (Ding and Himmel 2006, Arantes and Saddler 2010).

Microfibrils of cellulose are composed of 30-26 glucan chains, which aggregate laterally using hydrogen bonds in order to produce crystalline structures (Somerville, Bauer et al. 2004, Ding and Himmel 2006). It is likely that the crystal structures of cellulose affect the rate of diffusion of key enzymes and other reactants and therefore play an important role in the accessibility of cellulose, thus affecting its hydrolysis (Arantes and Saddler 2010). A higher degree of microfiber aggregation produces a more compact structure, creating an environment that is impenetrable even by water molecules (Krassing 1993). This type of structure means that only the cellulose molecules on the surface are capable of being hydrolyzed by cellulase enzymes (Krassing 1993). Therefore, if cellulose is only susceptible to enzyme activity at the surface area, then creating an environment that maximizes the amount of surface area of cellulose which would contribute to accessibility by cellulases (Wood, McCRAE et al. 1989, Laureano-Perez, Teymouri et al. 2005). In order to do this, synergistic proteins and treatments have been implemented to reduce the degree of aggregation among fibers and create a larger surface area (Arantes and Saddler 2010).

### 1.2 Processing lignocellulose

Biomass, lignocellulosic polymers are a massive, renewable, and available source for production of biofuels and biochemicals, because they trap about 60% of all sugars produced by plants on earth. Just as it happens in nature, man-made lignocellulosic biomass such as corn stover and sugar cane bagasse pileup along bio refineries and could be broken down enzymatically (Lal

2005, Amorim, Lopes et al. 2011). However, the cost of cellulase cocktails are the bottleneck to the economical production of these second generation biofuels (Phillips, Beeson et al. 2011). Enzymatic conversion of lignocellulose into sugars is a slow and expensive process, largely because cellulose is an insoluble crystalline substance (Himmel and Bayer 2009, Regalbuto 2009, Regalbuto 2011).

For cellulase aided breakdown of cellulose to take place, a single chain must be separated from the crystalline fiber and fitted into an enzyme binding site where catalytic Asp or Glu residues hydrolyze through a general acid/base mechanism the glycosydic bond (Divne, Stahlberg et al. 1994, Stahlberg, Divne et al. 1996). The disconnection of the glucan chain from crystalline cellulose fibers has been proposed to be the bottleneck in enzymatic hydrolysis of cellulose (Himmel and Bayer 2009).

The typical process for converting biomass to biofuel and bio based chemicals is composed of three steps: biomass pretreatment, enzymatic hydrolysis, and fermentation (He, Ding et al. 2017). Since the cellulose and cellulose are tightly packed into the biomass with lignin, this results in low accessibility for enzymes to saccharify the biomass (He, Ding et al. 2017). Ideally the biomass will be pretreated in such a way that the recalcitrant lignin structures are disrupted to expose the cellulose and hemicellulose increasing the accessibility of these important carbohydrate sources to the enzymes to enhance the yield of sugars through fermentation (Wyman, Dale et al. 2005). Various technologies for pretreatment exist such as: physical (milling, chipping, and grinding), chemical (alkaline, acidic, oxidizing chemicals, and organic solvents). (Hendriks and Zeeman 2009) There is also the potential to use these pretreatments in combination. (Hendriks and Zeeman 2009) However, while the methods of pretreatment may vary, the goals remain the same. Pretreatment technology aims to: produce solids that are highly digestible enzymatically, avoid the degradation of the final product, and minimize the production of inhibitory chemicals (such as phenolics).

### 1.3 Fungal enzymes for the degradation of lignocellulose

This recalcitrance towards the degradation of cellulose is abundantly illustrated in the repertoire of cellulose degrading enzymes (cellulases) produced by microorganisms that try to use this polymer as a carbon source. Cellulases belong to the hydrolase class of enzymes responsible for hydrolyzing the 1-4 glycosidic linkages found in cellulose(EC 3.2.1.4). (Srivastava, Srivastava et al. 2018) Most microorganisms produce at least three types of cellulose hydrolytic bond breaking enzymes; cellobiohydrolases, endoglucanases and glucosidases. (Payne, Knott et al. 2015) Endoglucanases initiate hydrolysis by exposing reducing and non-reducing ends, which are acted upon by the cellobiohydrolases to produce cello-oligosaccharides and cellobiose units. Glucosidases then can reduce the cellobioses to release glucose molecules completing the hydrolysis process. (Bhat and Bhat 1997) Fungal species are particularly adept at breaking down biomass in nature and can be of great importance in an industrial setting as well.

Cellulases from fungus are currently the third largest industrial enzyme worldwide and could become the largest volume industrial enzyme if a fermentation product (such as ethanol and butanol) becomes a major transportation fuel. (Wilson 2009) Currently industrial cellulases are almost all produced by aerobic cellulolytic fungi such as *Trichoderma reesei* and *Aspergillus nidulans*. (Ortega, Busto et al. 2001) Filamentous fungi are major cellulase producers and are widely utilized due to their ability to produce large amounts of enzymes cheaply. (Segato, Damásio et al. 2012)

### 1.4 Fungal cell factories

Fungal cell factories (Segato, Damásio et al. 2012) or plant and mammalian cell lines are widely utilized in industry to produce large amounts of enzymes for low-cost applications or low quantities of high valued pharmaceuticals, respectively. When naturally occurring, cells are manipulated to forcibly synthesize a product, they often need to be genetically rewired to redirect

metabolic pathways towards a desired product. For example, the genus *Aspergillus* and *Trichoderma* are the main cell factories utilized in large-scale protein production (Bodie, Bower et al. 1994, Conesa, Punt et al. 2001, Punt, van Biezen et al. 2002, Nevalainen, Te'o et al. 2005, Sims, Gent et al. 2005, Squina, Mort et al. 2009, Fleissner and Dersch 2010, Kuck and Hoff 2010, Damásio, Silva et al. 2011). In order to achieve high yield protein production strains need to be manipulated (Verdoes, Punt et al. 1994, Nieto, Prieto et al. 1999, Meyer, Wu et al. 2011) such as design of strong promoters and secretion signals (Wiebe, Robson et al. 2001, Record, Asther et al. 2003, Meyer, Wu et al. 2011); construction of fusion proteins which allow efficient secretion of the target protein (Joosten, Lokman et al. 2003); construction of protease deficient expression strains (de Vries, Burgers et al. 2004, Punt, Schuren et al. 2008, Yoon, Maruyama et al. 2011); specialized medium development (Swift, Karandikar et al. 2000); and random mutagenesis of proteins along with screening for variants for increased secretion potential (Weenink, Punt et al. 2006).

In *Aspergillii* over-expression, translation and secretion of industrial useful proteins, sometimes exceeds 30 g/liter (van den Hondel, Punt et al. 1992, Segato, Damásio et al. 2012), though often other proteins are poorly secreted with yields below the 1g/liter mark. Over-expression and secretion of recombinant proteins in fungal hosts often results in greatly diminished rates (Nevalainen, Te'o et al. 2005). Thus, over-expressing a given protein is not enough to deliver high extracellular protein yields and only a limited number of studies successfully address the posttranslational modifications and extracellular mechanisms that interfere with secretion and accumulation of extracellular proteins (Jeenes, Mackenzie et al. 1991, Guillemette, van Peij et al. 2007, Wang, Xue et al. 2008, Yoon, Kimura et al. 2009, Fleissner and Dersch 2010, Yoon, Aishan et al. 2010, Guillemette, Ram et al. 2011, Yoon, Maruyama et al. 2011, Segato, Damásio et al. 2012).

### 1.5 Cellulase production in fungi

Cellulases can be produced biologically by fungal fermentation (Yoon, Ang et al. 2014). Commercial cellulases are most commonly produced by *Trichoderma* sp. and *Aspergillus* sp. via submerged fermentation methods (Singhania, Sukumaran et al. 2010). Although these fungi are able to produce a complete cellulose degradation system, cultivation of either system results in deficiencies of one or more particular cellulase components. *Trichoderma reesei* for example does not produce substantial amounts of β-glucosidase, and *Aspergillus niger* does not produce large amounts of endoglucanase and exoglucanase (Ahamed and Vermette 2008, Chandel, Chandrasekhar et al. 2012). Other drawbacks are that submerged fermentation often leads to a low concentration of the end-product as well as the need for further purification of the product for downstream applications (Rodríguez Couto and Sanromán 2005).

Due to the shortcomings with submerged fermentation of fungal cellulases, research has been focused on improving the titers of cellulases alongside with reducing production costs (Yoon, Ang et al. 2014). One such solution has been the use of solid state fermentation (SSF) as an alternative production route for cellulases (Rodríguez Couto and Sanromán 2005). SSF more closely resembles the natural habitat of the fungus and it radically improves the titer compared to submerged fermentation (Hölker, Höfer et al. 2004, Singhania, Patel et al. 2009). For example, when *A. niger* has been cultivated on wheat-bran as a lignocellulosic substrate it can produce a cellulase with activities of 10.81 U per $g^{-1}$(Kumar, Sharma et al. 2011). *T. reesei* cultivation results in cellulase activities of 250-430 U per $g^{-1}$ (Chahal 1985).

The demand of cellulase enzymes are increasing, due to their importance in the emerging biofuels industry, and fungi are capable of producing large titers of these useful enzymes. However, high production costs pose a large problem for this budding field. Additional efforts should be made in order to find a suitable combination of fungal strain, lignocellulosic substrate, and process

conditions in order to optimize cellulase yields in fungus.

### 1.6 RNA interference in fungi

Gene silencing is a generic expression relating epigenetic processes of gene regulation (Pastori, Magistri et al. 2010). The term gene silencing is usually employed to describe the "turning off" of a gene by a mechanism other than genetic modification that is, a gene expressed (turned on) under normal conditions is turned off by cell-specific machineries. Genes are usually regulated at the transcriptional or post-transcriptional level (Watanabe 2011). Transcriptional gene silencing is a consequence of histone modifications, creating heterochromatin type of condition around a gene that makes it unavailable to the transcription machinery (Gonzalez and Li 2012). Post-transcriptional silencing is the product of an mRNA of a particular gene being degraded or prevented from translating.

The destruction of mRNA prevents translation to form a protein and a common mechanism of gene silencing is RNA interference (RNAi) (Valencia-Sanchez, Liu et al. 2006). Thus, RNAi is a biochemical activity within cells that regulates the activity of genes. The RNAi pathway is found in eukaryotes including fungi and primed by Dicer, which cleaves double-stranded RNAs (dsRNA) into short ~20 nucleotides pieces defined as siRNAs (Kim, Lee et al. 2006). Each siRNA is denatured into single-stranded (ss) ssRNAs, the passenger and the guide strand. The passenger strand is degraded, and the guide strand loaded onto the RNA-induced silencing complex (Risc) (Matranga, Tomari et al. 2005, Mah, Buske et al. 2010, Parker 2010). When the guide strand pairs with a complementary mRNAs, cleavage by Argonaute, the catalytic component of Risc (Buker and Motamedi 2011) is induced, which in turn spreads systemically. Thus, destruction of a given gene mRNA, results in erasure of the corresponding protein from the given proteome.

### 1.7 Amylolytic profile of *Aspergillus nidulans*

Amylolytic enzymes such as α-amylases, glucoamylases, and α-glucosidases are usually produced by many filamentous fungi. (Nakamura, Maeda et al. 2006) Production of these enzymes is generally induced by starch, maltose, and malto oligosaccharides. (Kato, Murakoshi et al. 2002)The transcription activator AmyR regulates the induction of most amylolytic genes in *A. nidulans*. (Petersen, Lehmbeck et al. 1999) *A. nidulans* possesses 16 amylolytic genes consisting of: 7 α-glucosidases (*agdA-F*), 7 α-amylases (*amyA-F*), and 2 glucoamylases (*glaA* and *glaB*). (Nakamura, Maeda et al. 2006)

Of the seven α-glucosidase genes, five of them fall into GH family 31 and two of them are categorized as GH family 13. (Nakamura, Maeda et al. 2006) GH31α-glucosidases (*agdA-E*) are typically extracellular enzymes and they possess strong glycosylation activity with the formation of an α-1,6-linkage. (Pazur and French 1952, McCleary, Gibson et al. 1989) The GH13 α-glucosidases (*agdF* and *agdG*) have a broader range of substrates. *agdF* is a homolog of a *Saccharomyces cerevisiae* isomaltose, while *agdG* bares homology to a *Bacillus* sp. α-glucosidase. (NAKAO, NAKAYAMA et al. 1994, Yamamoto, Nakayama et al. 2004)

Of the seven α-amylase genes six of them (*amyA-F*) were homologous to the well characterized Taka-amylase genes of *Aspergillus oryzae*. (Norihiro, Makoto et al. 1989) *amyG* is homologous is a G6-forming α-amylase gene from *Bacillus* sp. (Tsukamoto, Kimura et al. 1988) *A. nidulans*' two glucoamylase genes, *glaA* and *glaB,* are homologous to the *glaA* gene from *A. oryzae* with the exception of their additional starch binding domain. (Yoji, Kozo et al. 1991)

## 1.8 References


Ahamed, A. and P. Vermette (2008). "Enhanced enzyme production from mixed cultures of Trichoderma reesei RUT-C30 and Aspergillus niger LMA grown as fed batch in a stirred tank bioreactor." Biochemical Engineering Journal **42**(1): 41-46.

Amorim, H. V., M. L. Lopes, J. V. de Castro Oliveira, M. S. Buckeridge and G. H. Goldman (2011). "Scientific challenges of bioethanol production in Brazil." Appl Microbiol Biotechnol **91**(5): 1267-1275.

Arantes, V. and J. N. Saddler (2010). "Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis." Biotechnol Biofuels **3**(4): 1-11.

Bhat, M. and S. J. B. a. Bhat (1997). "Cellulose degrading enzymes and their potential industrial applications." **15**(3-4): 583-620.

Bodie, E. A., B. Bower, R. M. Berka and N. S. Dunn-Coleman (1994). "Economically important organic acid and enzyme products." Prog Ind Microbiol **29**: 561-602.

Buker, S. M. and M. R. Motamedi (2011). "Purification of native Argonaute complexes from the fission yeast Schizosaccharomyces pombe." Methods Mol Biol **725**: 1-13.

Chahal, D. S. (1985). "Solid-state fermentation with Trichoderma reesei for cellulase production." Applied and Environmental Microbiology **49**(1): 205-210.

Chandel, A. K., G. Chandrasekhar, M. B. Silva and S. Silvério da Silva (2012). "The realm of cellulases in biorefinery development." Critical Reviews in Biotechnology **32**(3): 187-202.

Conesa, A., P. J. Punt, N. van Luijk and C. A. van den Hondel (2001). "The secretion pathway in filamentous fungi: a biotechnological view." Fungal Genet Biol **33**(3): 155-171.

Damásio, A. R. d. L., T. M. Silva, F. B. d. R. Almeida, F. M. Squina, D. A. Ribeiro, A. F. P. Leme, F. Segato, R. A. Prade, J. A. Jorge, H. F. Terenzi and M. d. L. T. M. Polizeli (2011). "Heterologous expression of an Aspergillus niveus xylanase GH11 in Aspergillus nidulans and its characterization and application." Process Biochemistry **46**(6): 1236-1242.

de Vries, R. P., K. Burgers, P. J. van de Vondervoort, J. C. Frisvad, R. A. Samson and J. Visser (2004). "A new black Aspergillus species, A. vadensis, is a promising host for homologous and heterologous protein production." Appl Environ Microbiol **70**(7): 3954-3959.

Ding, S.-Y. and M. E. Himmel (2006). "The maize primary cell wall microfibril: a new model derived from direct visualization." Journal of Agricultural and Food Chemistry **54**(3): 597-606.

Divne, C., J. Stahlberg, T. Reinikainen, L. Ruohonen, G. Pettersson, J. K. Knowles, T. T. Teeri and T. A. Jones (1994). "The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from Trichoderma reesei." Science **265**(5171): 524-528.

Fleissner, A. and P. Dersch (2010). "Expression and export: recombinant protein production systems for Aspergillus." Appl Microbiol Biotechnol **87**(4): 1255-1270.

Garvey, M., H. Klose, R. Fischer, C. Lambertz and U. Commandeur (2013). "Cellulases for biomass degradation: comparing recombinant cellulase expression platforms." Trends in biotechnology **31**(10): 581-593.

Gonzalez, M. and F. Li (2012). "DNA replication, RNAi and epigenetic inheritance." Epigenetics **7**(1).

Guillemette, T., A. F. Ram, N. D. Carvalho, A. Joubert, P. Simoneau and D. B. Archer (2011). "Methods for investigating the UPR in filamentous fungi." Methods Enzymol **490**: 1-29.

Guillemette, T., N. N. van Peij, T. Goosen, K. Lanthaler, G. D. Robson, C. A. van den Hondel, H. Stam and D. B. Archer (2007). "Genomic analysis of the secretion stress response in the enzyme-producing cell factory Aspergillus niger." BMC genomics **8**: 158.

Hall, M., P. Bansal, J. H. Lee, M. J. Realff and A. S. Bommarius (2010). "Cellulose crystallinity–a key predictor of the enzymatic hydrolysis rate." FEBS journal **277**(6): 1571-1582.

Harris, P. V., D. Welner, K. C. McFarland, E. Re, J. C. Navarro Poulsen, K. Brown, R. Salbo, H. Ding, E. Vlasenko, S. Merino, F. Xu, J. Cherry, S. Larsen and L. Lo Leggio (2010). "Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family." Biochemistry **49**(15): 3305-3316.

Harris, P. V., F. Xu, N. E. Kreel, C. Kang and S. Fukuyama (2014). "New enzyme insights drive advances in commercial ethanol production." Current opinion in chemical biology **19**: 162-170.

He, Y., Y. Ding, C. Ma, J. Di, C. Jiang and A. J. G. C. Li (2017). "One-pot conversion of biomass-derived xylose to furfuralcohol by a chemo-enzymatic sequential acid-catalyzed dehydration and bioreduction." **19**(16): 3844-3850.

Hendriks, A. and G. J. B. t. Zeeman (2009). "Pretreatments to enhance the digestibility of lignocellulosic biomass." **100**(1): 10-18.

Himmel, M. E. and E. A. Bayer (2009). "Lignocellulose conversion to biofuels: current challenges, global perspectives." Curr Opin Biotechnol **20**(3): 316-317.

Hölker, U., M. Höfer and J. Lenz (2004). "Biotechnological advantages of laboratory-scale solid-state fermentation with fungi." Applied Microbiology and Biotechnology **64**(2): 175-186.

Jeenes, D. J., D. A. Mackenzie, I. N. Roberts and D. B. Archer (1991). "Heterologous protein production by filamentous fungi." Biotechnology & genetic engineering reviews **9**: 327-367.

Joosten, V., C. Lokman, C. A. Van Den Hondel and P. J. Punt (2003). "The production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi." Microb Cell Fact **2**(1): 1.

Kato, N., Y. Murakoshi, M. Kato, T. Kobayashi and N. J. C. g. Tsukagoshi (2002). "Isomaltose formed by α-glucosidases triggers amylase induction in Aspergillus nidulans." **42**(1): 43-50.

Kim, E. S., H. J. Lee, W. G. Bang, I. G. Choi and K. H. Kim (2009). "Functional characterization of a bacterial expansin from Bacillus subtilis for enhanced enzymatic hydrolysis of cellulose." Biotechnology and bioengineering **102**(5): 1342-1353.

Kim, I. J., H. J. Ko, T. W. Kim, I. G. Choi and K. H. Kim (2013). "Characteristics of the binding of a bacterial expansin (BsEXLX1) to microcrystalline cellulose." Biotechnology and bioengineering **110**(2): 401-407.

Kim, I. J., H. J. Lee, I. G. Choi and K. H. Kim (2014). "Synergistic proteins for the enhanced enzymatic hydrolysis of cellulose by cellulase." Appl Microbiol Biotechnol **98**(20): 8469-8480.

Kim, K., Y. S. Lee, D. Harris, K. Nakahara and R. W. Carthew (2006). "The RNAi pathway initiated by Dicer-2 in Drosophila." Cold Spring Harb Symp Quant Biol **71**: 39-44.

Krassing, H. (1993). "Cellulose: Structure." Accessibility and Reactivity, Gordon and Breach Science Publichers, Swizerland **41**.

Kuck, U. and B. Hoff (2010). "New tools for the genetic manipulation of filamentous fungi." Appl Microbiol Biotechnol **86**(1): 51-62.

Kumar, S., H. K. Sharma and B. C. Sarkar (2011). "Effect of substrate and fermentation conditions on pectinase and cellulase production by Aspergillus niger NCIM 548 in submerged (SmF) and solid state fermentation (SSF)." Food Science and Biotechnology **20**(5): 1289.

Lal, R. (2005). "World crop residues production and implications of its use as a biofuel." Environ Int **31**(4): 575-584.

Laureano-Perez, L., F. Teymouri, H. Alizadeh and B. E. Dale (2005). "Understanding factors that limit enzymatic hydrolysis of biomass." Applied Biochemistry and Biotechnology **124**(1-3): 1081-1099.

Lynd, L. R., M. S. Laser, D. Bransby, B. E. Dale, B. Davison, R. Hamilton, M. Himmel, M. Keller, J. D. McMillan, J. Sheehan and C. E. Wyman (2008). "How biotech can transform biofuels." Nat Biotechnol **26**(2): 169-172.

Lynd, L. R., W. H. van Zyl, J. E. McBride and M. Laser (2005). "Consolidated bioprocessing of cellulosic biomass: an update." Curr Opin Biotechnol **16**(5): 577-583.

Mah, S. M., C. Buske, R. K. Humphries and F. Kuchenbauer (2010). "miRNA*: a passenger stranded in RNA-induced silencing complex?" Crit Rev Eukaryot Gene Expr **20**(2): 141-148.

Matranga, C., Y. Tomari, C. Shin, D. P. Bartel and P. D. Zamore (2005). "Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes." Cell **123**(4): 607-620.

McCleary, B. V., T. S. Gibson, H. Sheehan, A. Casey, L. Horgan and J. J. C. r. O'Flaherty (1989). "Purification, properties, and industrial significance of transglucosidase from Aspergillus niger." **185**(1): 147-162.

Meyer, V., B. Wu and A. F. Ram (2011). "Aspergillus as a multi-purpose cell factory: current status and perspectives." Biotechnol Lett **33**(3): 469-476.

Nakamura, T., Y. Maeda, N. Tanoue, T. Makita, M. Kato, T. J. B. Kobayashi, biotechnology, and biochemistry (2006). "Expression profile of amylolytic genes in Aspergillus nidulans." 0610020104-0610020104.

NAKAO, M., T. NAKAYAMA, A. KAKUDO, M. INOHARA, M. HARADA, F. OMURA and Y. J. E. j. o. b. SHIBANO (1994). "Structure and expression of a gene coding for thermostable α-glucosidase with a broad substrate specificity from Bacillus sp. SAM1606." **220**(2): 293-300.

Nevalainen, K. M., V. S. Te'o and P. L. Bergquist (2005). "Heterologous protein expression in filamentous fungi." Trends Biotechnol **23**(9): 468-474.

Nieto, A., J. A. Prieto and P. Sanz (1999). "Stable high-copy-number integration of Aspergillus oryzae alpha-AMYLASE cDNA in an industrial baker's yeast strain." Biotechnol Prog **15**(3): 459-466.

Norihiro, T., F. Makoto, N. Hiroki, K. Norie, T. Akio and U. J. G. Shigezo (1989). "Isolation of a cDNA encoding Aspergillus oryzae Taka-amylase A: evidence for multiple related genes." **84**(2): 319-327.

Ortega, N., M. a. D. Busto, M. J. I. b. Perez-Mateos and biodegradation (2001). "Kinetics of cellulose saccharification by Trichoderma reesei cellulases." **47**(1): 7-14.

Parker, J. S. (2010). "How to slice: snapshots of Argonaute in action." Silence **1**(1): 3.

Pastori, C., M. Magistri, S. Napoli, G. M. Carbone and C. V. Catapano (2010). "Small RNA-directed transcriptional control: new insights into mechanisms and therapeutic applications." Cell Cycle **9**(12): 2353-2362.

Payne, C. M., B. C. Knott, H. B. Mayes, H. Hansson, M. E. Himmel, M. Sandgren, J. Stahlberg and G. T. J. C. r. Beckham (2015). "Fungal cellulases." **115**(3): 1308-1448.

Pazur, J. H. and D. J. J. b. C. French (1952). "The action of transglucosidase of Aspergillus oryzae on maltose." **196**(1): 265-272.

Petersen, K., J. Lehmbeck, T. J. M. Christensen and G. G. MGG (1999). "A new transcriptional activator for amylase genes in Aspergillus." **262**(4-5): 668-676.

Phillips, C. M., W. T. Beeson, J. H. Cate and M. A. Marletta (2011). "Cellobiose dehydrogenase and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by Neurospora crassa." ACS Chem Biol **6**(12): 1399-1406.

Punt, P. J., F. H. Schuren, J. Lehmbeck, T. Christensen, C. Hjort and C. A. van den Hondel (2008). "Characterization of the Aspergillus niger prtT, a unique regulator of extracellular protease encoding genes." Fungal Genet Biol **45**(12): 1591-1599.

Punt, P. J., N. van Biezen, A. Conesa, A. Albers, J. Mangnus and C. van den Hondel (2002). "Filamentous fungi as cell factories for heterologous protein production." Trends Biotechnol **20**(5): 200-206.

Record, E., M. Asther, C. Sigoillot, S. Pages, P. J. Punt, M. Delattre, M. Haon, C. A. van den Hondel, J. C. Sigoillot and L. Lesage-Meessen (2003). "Overproduction of the Aspergillus niger feruloyl esterase for pulp bleaching application." Appl Microbiol Biotechnol **62**(4): 349-355.

Regalbuto, J. R. (2009). "Engineering. cellulosic biofuels--got gasoline?" Science **325**(5942): 822-824.

Regalbuto, J. R. (2011). "The sea change in US biofuels' funding: from cellulosic ethanol to green gasoline." Biofuels, Bioproducts and Biorefining **5**(5): 495-504.

Rodríguez Couto, S. and M. A. Sanromán (2005). "Application of solid-state fermentation to ligninolytic enzyme production." Biochemical Engineering Journal **22**(3): 211-219.

Segato, F., A. R. L. Damásio, T. A. Gonçalves, R. C. de Lucas, F. M. Squina, S. R. Decker and R. A. Prade (2012). "High-yield secretion of multiple client proteins in Aspergillus." Enzyme and Microbial Technology(0).

Selig, M. J., S. Viamajala, S. R. Decker, M. P. Tucker, M. E. Himmel and T. B. Vinzant (2007). "Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose." Biotechnology progress **23**(6): 1333-1339.

Sims, A. H., M. E. Gent, K. Lanthaler, N. S. Dunn-Coleman, S. G. Oliver and G. D. Robson (2005). "Transcriptome analysis of recombinant protein secretion by Aspergillus nidulans and the unfolded-protein response in vivo." Appl Environ Microbiol **71**(5): 2737-2747.

Singhania, R. R., A. K. Patel, C. R. Soccol and A. Pandey (2009). "Recent advances in solid-state fermentation." Biochemical Engineering Journal **44**(1): 13-18.

Singhania, R. R., R. K. Sukumaran, A. K. Patel, C. Larroche, A. J. E. Pandey and M. Technology (2010). "Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases." **46**(7): 541-549.

Somerville, C., S. Bauer, G. Brininstool, M. Facette, T. Hamann, J. Milne, E. Osborne, A. Paredez, S. Persson and T. Raab (2004). "Toward a systems approach to understanding plant cell walls." Science **306**(5705): 2206-2211.

Squina, F. M., A. J. Mort, S. R. Decker and R. A. Prade (2009). "Xylan decomposition by Aspergillus clavatus endo-xylanase." Protein expression and purification **68**(1): 65-71.

Srivastava, N., M. Srivastava, P. Mishra, V. K. Gupta, G. Molina, S. Rodriguez-Couto, A. Manikanta, P. J. R. Ramteke and S. E. Reviews (2018). "Applications of fungal cellulases in biofuel production: advances and limitations." **82**: 2379-2386.

Stahlberg, J., C. Divne, A. Koivula, K. Piens, M. Claeyssens, T. T. Teeri and T. A. Jones (1996). "Activity Studies and Crystal Structures of Catalytically Deficient Mutants of Cellobiohydrolase I fromTrichoderma reesei." Journal of Molecular Biology **264**(2): 337-349.

Swift, R. J., A. Karandikar, A. M. Griffen, P. J. Punt, C. A. van den Hondel, G. D. Robson, A. P. Trinci and M. G. Wiebe (2000). "The Effect of organic nitrogen sources on recombinant glucoamylase production by Aspergillus niger in chemostat culture." Fungal Genet Biol **31**(2): 125-133.

Tsukamoto, A., K. Kimura, Y. Ishii, T. Takano, K. J. B. Yamane and b. r. communications (1988). "Nucleotide sequence of the maltohexaose-producing amylase gene from an alkalophilic Bacillus sp.# 707 and structural similarity to liquefying type α-amylases." **151**(1): 25-31.

Valencia-Sanchez, M. A., J. Liu, G. J. Hannon and R. Parker (2006). "Control of translation and mRNA degradation by miRNAs and siRNAs." Genes Dev **20**(5): 515-524.

van den Hondel, C. A., P. J. Punt and R. F. van Gorcom (1992). "Production of extracellular proteins by the filamentous fungus Aspergillus." Antonie van Leeuwenhoek **61**(2): 153-160.

Verdoes, J. C., P. J. Punt, A. H. Stouthamer and C. A. van den Hondel (1994). "The effect of multiple copies of the upstream region on expression of the Aspergillus niger glucoamylase-encoding gene." Gene **145**(2): 179-187.

Wang, Y., W. Xue, A. H. Sims, C. Zhao, A. Wang, G. Tang, J. Qin and H. Wang (2008). "Isolation of four pepsin-like protease genes from Aspergillus niger and analysis of the effect of disruptions on heterologous laccase expression." Fungal genetics and biology **45**(1): 17-27.

Watanabe, Y. (2011). "Overview of plant RNAi." Methods Mol Biol **744**: 1-11.

Weenink, X. O., P. J. Punt, C. A. van den Hondel and A. F. Ram (2006). "A new method for screening and isolation of hypersecretion mutants in Aspergillus niger." Appl Microbiol Biotechnol **69**(6): 711-717.

Wiebe, M. G., G. D. Robson, J. Shuster and A. P. Trinci (2001). "Evolution of a recombinant (gucoamylase-producing) strain of Fusarium venenatum A3/5 in chemostat culture." Biotechnol Bioeng **73**(2): 146-156.

Wilson, D. B. J. C. o. i. b. (2009). "Cellulases and biofuels." **20**(3): 295-299.

Wood, T. M., S. I. McCRAE and K. M. Bhat (1989). "The mechanism of fungal cellulase action. Synergism between enzyme components of Penicillium pinophilum cellulase in solubilizing hydrogen bond-ordered cellulose." Biochem. J **260**: 37-43.

Wyman, C. E., B. E. Dale, R. T. Elander, M. Holtzapple, M. R. Ladisch and Y. J. B. t. Lee (2005). "Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover." **96**(18): 2026-2032.

Yamamoto, K., A. Nakayama, Y. Yamamoto and S. J. E. j. o. b. Tabata (2004). "Val216 decides the substrate specificity of α-glucosidase in Saccharomyces cerevisiae." **271**(16): 3414-3420.

Yoji, H., T. Kozo, K. Katsuhiko, G. Katsuya, K. Chieko, T. Gakuzo and H. J. G. Shodo (1991). "Nucleotide sequence and expression of the glucoamylase-encoding gene (glaA) from Aspergillus oryzae." **108**(1): 145-150.

Yoon, J., T. Aishan, J. Maruyama and K. Kitamoto (2010). "Enhanced production and secretion of heterologous proteins by the filamentous fungus Aspergillus oryzae via disruption of vacuolar protein sorting receptor gene Aovps10." Appl Environ Microbiol **76**(17): 5718-5727.

Yoon, J., S. Kimura, J. Maruyama and K. Kitamoto (2009). "Construction of quintuple protease gene disruptant for heterologous protein production in Aspergillus oryzae." Appl Microbiol Biotechnol **82**(4): 691-701.

Yoon, J., J. Maruyama and K. Kitamoto (2011). "Disruption of ten protease genes in the filamentous fungus Aspergillus oryzae highly improves production of heterologous proteins." Appl Microbiol Biotechnol **89**(3): 747-759.

Yoon, L. W., T. N. Ang, G. C. Ngoh, A. S. M. J. B. Chua and Bioenergy (2014). "Fungal solid-state fermentation and various methods of enhancement in cellulase production." **67**: 319-338.

.

# CHAPTER II

# CELLULASE PRODUCTION WITH PRETREATED HEMICELLULOSE C5-SUGAR LIQUORS

## 2.1 Introduction

Lignocellulosic biomass (LCB) is the single most abundant renewable hydrocarbon resource on earth (Bar-On, Phillips et al. 2018). The runner-up hydrocarbon resource, which is non-renewable, is petroleum. Petroleum currently provisions the world-market of starter chemicals for everything from low-cost, cents per gallon products (gasoline and diesel) all the way to high-end substrates such as the primers for plastics, polymers and fibers (Jadidzadeh and Serletis 2018). Two thirds of LCB is composed of hemicellulose (C5-sugars) and cellulose (C6-sugars), the hydrocarbon substrates for fermentation processes that produce low-cost high-volume as well high-cost low-volume chemicals (Lynd, Laser et al. 2008, Ellila, Fonseca et al. 2017, Sheldon 2018). Deconstruction of the LCB sugar moiety has been achieved with a combination of cellulolytic enzymes such as endoglucanases, cellobiohydrolases and β-glucosidases (Segato, Damasio et al. 2014). Enzymatic hydrolysis has had only moderate success in breaking the crystalline cellulose molecule (Garvey, Klose et al. 2013). Low accessibility to cellulose molecules (recalcitrance) by enzymes hinders hydrolytic activity with water inaccessible regions remaining unaffected (Ding and Himmel 2006, Arantes and Saddler 2010, Ding, Liu et al. 2012).

To overcome this natural physical resistance of LCB's towards an enzyme driven digestion process, several pretreatment technologies have been developed, focused in disrupting the intermolecular hydrogen bonds that make LCBs recalcitrant (Ciesielski, Matthews et al. 2013, Bhutto, Qureshi et al. 2017, Chen, Liu et al. 2017). The bottom line on LCB pretreatments is that no matter what method or combinations thereof there is always partial decomposition of the hemicellulosic fraction, which contains an abundance of C5-sugars (xylose) (Lynd, Laser et al. 2008, Arantes and Saddler 2010).

For large-scale production of enzymes that breakdown LCBs, fungi have traditionally been used as cell factories to manufacture cellulases, xylanases and other auxiliary activities (Punt, van Biezen et al. 2002, Nevalainen, Te'o et al. 2005, Demain and Vaishnav 2009, Ding, Liu et al. 2012, Bischof, Ramoni et al. 2016, Zhang, Zhang et al. 2018). Filamentous fungi such as *Trichoderma* and *Aspergillus* are able to use a broad range of sugars such as hexoses (C6) and pentoses (C5) as a carbon source and are used as common industrial cellulase and xylanase producers (Singhania, Sukumaran et al. 2010).

While fungi have been genetically engineered to secrete economically adequate yields of enzymes, the operational costs of synthesizing them continue to be inadequate, largely because they demand an expensive carbon source to cultivate a vegetative tissue necessary to synthesize client proteins as well as the added costs of making them on distant sites, purification, concentration, conditioning and delivery to biomass processing sites (Rana, Eckard et al. 2014, Klein-Marcuschamer and Blanch 2015, Kuhad, Deswal et al. 2016, van Rijn, Nieves et al. 2018).

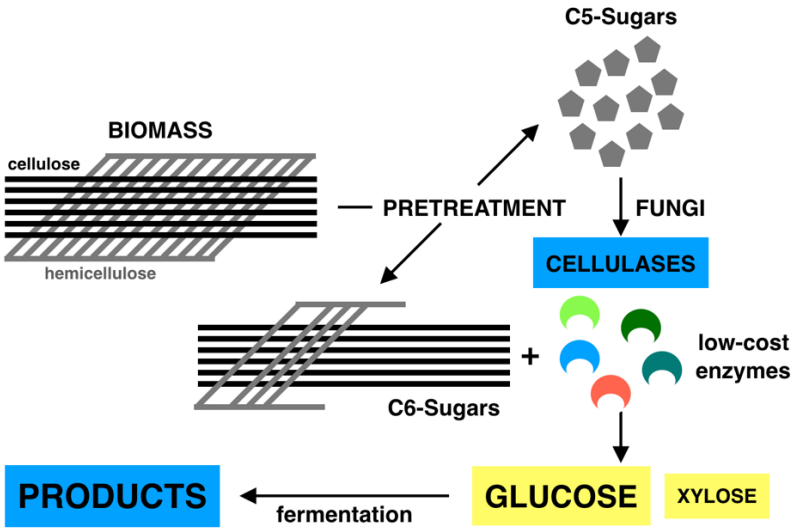



**Figure 2.1** Proposed schematic of total on-site biomass degradation.

BIOMASS main hydrocarbon components are **cellulose** and **hemicellulose** (~60%). PRETREATMENT technologies make cellulose (**C6-sugars**) accessible to enzymatic hydrolysis but compromise integrity of hemicellulose, rendering **C5-sugars** which could be used to make **low-cost enzymes** that degrade cellulose (**C6-sugars**) generating **GLUCOSE** that is converted into fermentation PRODUCTS.

Xylose found in C5-hydrolysates the byproducts of LCB pretreatments is a cheap carbon source that could be used to make enzymes. Using the pre-hydrolysate fraction as the raw material for the production of cellulases with filamentous fungi opens the prospect for low-cost enzyme production. The problem with low-cost on-site enzyme production is that while most fungi grow well with the by-product xylose as a carbon source, they are not prepared to synthesize large quantities of cellulases in the presence of these C5-sugars (Kiesenhofer, Mach et al. 2018).

The research reported here resolves this problem by redesigning the *Aspergillus nidulans* native cellulase gene regulatory circuit, switching the induction mechanism from cellulose to xylose. The strains constructed in this study grow well in C5-hydrolysates simultaneously producing and secreting large amounts of cellulases. We tested two cellobiohydrolases, two endoglucanases and one ß-glucosidase.

Replacing expensive substrates by a cheap by-product carbon source (C5-hydrolysates) directly derived from LCB pretreatment processes does not only reduce enzyme production costs, but also lowers operational costs such as off-site enzyme production, purification, concentration, transport and dilution (Arantes and Saddler 2010, Johnson 2016).

## 2.2 Materials and Methods

### *2.2.1 Chemicals and specialty chemicals*

General chemicals, cellulosic and hemicellulosic substrates were purchased from the best source possible, Sigma Aldrich (St Louis, MO) and Megazyme (Ireland, UK). Phosphoric acid swollen cellulose (PASC) was prepared according to (Schulein 1997). Briefly, 5 g of avicel was mixed with 150 mL of ice cold ortho-phosphoric acid and the suspension stirred for at least 60 mins. 100 mL of ice-cold acetone was stirred in and the slurry transferred to a Buchner filter with a sintered disc number 3, washed three times with 100 mL of ice-cold acetone and once with 500 mL of ice-cold water. The washed slurry was transferred to a 50 mL Falcon tube, filled with deionized water (50 mL) and stored in a refrigerator until further use.

Wheat straw was harvested in 2015 from a local farmer in Rhineland Palatinate (Bad Kreuznach, Germany). The composition was determined according to the method suggested by

the National Renewable Energy Laboratory (NREL) for measurement of structural carbohydrates and lignin (Sluiter, Hames et al. 2008). The wheat straw had 37.1 % (w/w) cellulose, 22.3 % (w/w) hemicellulose, 16.8 % (w/w) lignin, 9 % (w/w) extractives and 4.3 % (w/w) ash. HPLC analytics were done with the Metacarb 87H column (300 mm x 7.8 mm) purchased from Agilent Inc. (Santa Clara, CA, USA). All used chemicals were purchased from VWR International (Radnor, PA, USA).

### *2.2.2 Production of the xylose-containing liquefied wheat straw hydrolysate*

The PPTB, pentosan containing pre-treated biomass liquor was prepared by diluted acid hydrolysis of wheat straw in a 100-l stainless steel reactor. The vessel was heated with direct steam injection until the desired temperature was reached. In a previous study, the optimized treatment process parameters for high xylose and low-by-product concentration were estimated (Gasser, Ballmann et al. 2014). Briefly, dried wheat straw (15 % v/w, dry matter content) and diluted nitric acid (0.45 % v/v) was heated up at 160 °C for 30 minutes. After the pretreatment the pentose-rich liquor was separated from the solid biomass. The pre-hydrolysate solution was concentrated in a rotary evaporator at 75 °C and 110 mbar to enhance the storability of the pre-hydrolysate. The concentrated solution contained 162 g/l xylose, 29.4 g/l glucose and, 19.7 g/l arabinose. Pretreatment by-products such as furfural and 5-HMF were removed through the evaporation process. The concentrated hydrolysate was stored at -20 °C.

### *2.2.3 Strains and spore (conidia) production*

Standard *A. nidulans* minimal medium (MM) and general cultivation techniques were used throughout this work and are based on the work by Guido Pontecorvo (Pontecorvo, Roper et al. 1953, Pontecorvo 1969) and John Clutterbuck (Clutterbuck 1992). All strains constructed in

this work are were derived from *A. nidulans* A773 (*wA3*, *pyrG89*, *pyroA4*) purchased from the Fungal Genetics Stock Center (FGSC, St. Louis, MO). All gene models and promoters were from *Aspergillus nidulans* FGSC4 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000149205.2) and analyzed using the AspGD database (http://aspgd.org (Cerqueira, Arnaud et al. 2014)) Primers and Gibson Assembly hybrid primers were designed using the NEB Builder Assembly Tool (http://nebuilder.neb.com).

Three types of strains were constructed in this study; First the resident CbhC (AN0494) promoter (*cbhCp*) was replaced with four xylanase promoters (*xynABCEp*) in such a way that recombinant strains induce the production of cellobiohydrolase by xylose, second a $XlnR_{(ORF)}$ overexpression strain (PFIX7) was constructed by *pabaA* ectopic integration of a *gpdAp*::$XlnR_{(ORF)}$ DNA fragment, and third**,** xylose induced client protein constructs were randomly introduced into a XlnR overexpressing strain (PFIX7). For a detailed description of DNA fragment fusion construction strategy, genomic data and genetic validation of genetic modifications refer to the supplementary information

In all types of strain constructions, a linear hybrid recombinant DNA fragment was synthesized using Gibson Assembly Technology, GAT (Gibson 2011, Gibson 2014) using hybrid primers, Gibson Assembly Master Mix (New England Biolabs, US) and Phusion DNA Polymerase (New England Biolabs, US). DNA fragment size and DNA sequence verified hybrid DNA fragments were transformed into A773 or PFIX7 protoplasts. In the case of promoter replacements, a single gene replacement event at the *cbh1* locus was selected for each *xyn(p)* promoter replacement by uracil/uridine sufficiency and by diagnostic PCR showing single integration (replacement) into the *cbh1* locus. For the XlnR overexpression the hybrid DNA fragment was integrated into the *pabaA* locus by a double crossover event disrupting it. Recombinants with a single gene replacement event were searched with diagnostic PCR and the resulting strain PFIX7 tested for XlnR over-expression.

For the client protein xylose induced strains we created plasmids carrying the *pUC18$_{UP}$*::*pyroA*: *xynCp*::**CLIENT$_{ORF}$**::*pUC18$_{DWN}$* GAT construct that was transformed into PFI-X7 (XlnR overexpressing) strain and recombinants selected based on the level of client protein production rates. Even though we did not check for multiple integration events in single transformants we screened at least 100 transformants for high secretion levels of client proteins.

*2.2.4 Preparation of total extracellular protein extracts*

Unless otherwise stated, 5 mL of extracellular fluid (medium) harvested from mycelia grown for 24, 36 or 48 h were treated with 3kDa cutoff Nanosep® ultrafiltration Omega™ membrane columns (PALL Corp. USA) and washed with 500 µL of 50 mM ammonium acetate ($NH_4CH_3CO_2$) buffer pH 5 before 10X concentration to a final volume of 50 µL. Samples that were not concentrated (e.g., **Figure 4A** and **B**), 5 mL of extracellular fluid was treated as described above and resuspended to the original volume (5 mL) with 50 mM ammonium acetate buffer pH 5.

*2.2.5. Protein quantification and SDS-polyacrylamide gel electrophoresis*

Total protein content was measured in microtiter dishes using the Bio-Rad assay reagent (Bio-Rad Laboratories, USA), using a procedure based on the Bradford method (Bradford 1976, Marshall and Williams 1992) with bovine serum albumin as standard. Absorption was measured using a UV-Vis 96-well plate reader (Tecan Infinite M200, Männedorf, Switzerland) at 595 nm. Quality of total extracellular protein extracts was validated for integrity by SDS polyacrylamide gel electrophoresis according to Shapiro (Shapiro, Vinuela et al. 1967).

*2.2.6. Liquid chromatography-tandem mass spectrometry*

For LC-MS/MS analysis bands of a fully resolved SDS-PAGE gel (shown in **Figure 3A**) were excised and processed for LC-MS/MS according to (Shevchenko, Wilm et al. 1996) with modifications. Isolated gel bands were reduced with Tris (2-carboxyethyl) phosphine, alkylated by 2-Iodoacetamide, digested for 6-16 h with 8 µg/mL trypsin using ammonium bicarbonate buffer and analyzed by LC-MS/MS using LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific). The LC-MS/MS raw files were used for database Mascot (version 2.2.04, Matrix Science, London UK) searches run on a NCBI Aspergillus nidulans FGSC4 subsets. Searches were validated using Scaffold (version 4.0.7, Proteome Software Inc. Portland, OR) with a protein threshold of 5% FDR and a peptide threshold of 99%.

*2.2.7. Free sugar (reducing end) determinations*

Free sugar determinations were used in two types of experiments: 1) to determine the activity of enzymes that use a non-reducing substrate releasing reducing products (sugars) and 2) to quantitate the amount of reducing sugar accumulated by enzymes added to a cellulose degradations assay (**Figure 5**). In both cases we used the DNS assay developed by Sumner and Graham (Summer and Graham 1921) for detection of reducing sugars. The DNS reducing sugar assay was based on the method described by Miller (Miller 1959) and adapted to a microtiter dish scale. The DNS reagent we used contained 0.75% di-nitrosalicylic acid, 0.5% phenol, 0.5% sodium metabisulfite, and 1.4% sodium hydroxide, 21% sodium and potassium tartarate.

*2.2.8. Determination of enzyme activities*

**Xylanase** and **endoglucanase** activity were determined using beechwood hemicellulose or carboxymethylcellulose (CMC) as a substrate, respectively and activity measured by the release of reducing sugars that react with DNS (Miller 1959). Briefly to 300 µL of 1% beechwood xylan or 1% CMC, 50 mM ammonium acetate buffer 10-50 µL of total extracellular protein extract (treated as described in 2.2) was added and reactions incubated for 10, 20 or 30 mins at 45 $^{0}$C prior to the addition of 300 µL of DNS. Control reactions (blanks that determine the presence of reducing sugars in the starting mixture) contained all the same reagents except that DNS was added prior to the addition of enzyme sample. To determine the amount of reducing sugar produced during the enzyme catalyzed reaction the $ABS^{540nm}$ of the control was subtracted from the enzyme reaction and resulting net gain in $ABS^{540nm}$ converted into enzyme units µMOL/min/µg. protein.

**Cellobiohydrolase** and **β-glucosidase** were assayed using *p*NPC, *p*-nitrophenyl β-D-cellobioside or *p*-nitrophenyl β-D-glucoside (pNPG) (Sigma Aldrich, St. Louis MO)) as a substrate, respectively and activity measured by the release of *p*-nitrophenyl that absorbs at $ABS^{420nm}$ on a TECAN microwell reader. Briefly to 570 µL of 4 mM *p*NPC, 50 mM ammonium acetate buffer 5-10 µL of total extracellular protein extract (treated as described in 2.2) was added and reactions incubated for 5, 10 or 30 mins at 45 $^{0}$C prior to the addition of 60 µL of 2M sodium carbonate. Control reactions contained all the same reagents except that 2M sodium carbonate was added prior to the addition of enzyme sample. To determine the amount of *p*-nitrophenyl produced during the enzyme catalyzed reaction the $ABS^{420nm}$ of the control was subtracted from the enzyme reaction and resulting net gain in $ABS^{420nm}$ converted into enzyme units µMOL/min/µg. protein.

*2.2.9 Production of cellulases with C5 pre-hydrolysates*

Fermentation experiments examining the here constructed strains, PFI-X7, PFI-EA and PFI-BA using pre-hydrolysate were done in shaker flasks. The concentrated pre-hydrolysate was adjusted with water to a 30 g/l xylose-concentration and amended with mineral salts as described in Clutterbuck (Clutterbuck 1992). The inoculum was $1x10^5$ spores/ml medium and fermentations were carried out at 37 °C with 120 rpm for 72 hrs. Samples were taken and the supernatants stored at -20 °C for later analysis.

*2.2.10 Determination of the phenolic content and sugar concentrations*

The total phenolic content was analyzed according to the Folin-Ciocalteau method (Singleton and Rossi 1965). Briefly, properly diluted samples (200 µl) were added to distilled water (800 µl) and mixed with Folin-Ciocalteau regent (500 µl). Sodium carbonate (2.5 ml, 20% w/v) was added after 3 minutes and the samples were incubated in the dark for 30 minutes. The absorbance was measured at 725 nm using a photometer. Vanillin was used as external standard.

The concentrations of glucose, xylose, arabinose, acetic acid, furfural and 5-HMF in the pre-hydrolysate and cultivation samples were determined by HPLC measurements (Agilent 1200 Series). The HPLC was equipped with a pump unit, an autosampler unit, a refractive index detector unit and a computer software-based integration system (LC ChemStation). The MetaCarb 87H column was maintained at 80 °C at the flow rate of 0.5 ml/min with 0.05 M $H_2SO_4$ as the mobile phase. Peaks detected by refractive index were identified and quantified by comparison with the retention times of authentic standards.

*2.2.11. Accumulation of glucose by xylose induced cellulases*

Glucose accumulation assays were carried out with 150 µL of 1% phosphoric acid swollen avicel (PASC), 150 µL of 100 mM ammonium acetate buffer, pH 5 and addition of exactly 1 µg of single or a combination of five cellulases, cellobiohydrolase C, cellobiohydrolase B, endoglucanase A, endoglucanase B and β-glucosidase A, incubated at 45 $^{0}$C for 30 mins. The reaction was stopped by the addition of 300 µL of DNS reagent and samples boiled for 5 mins and 200 µL transferred to microtiter dishes. To determine the amount of glucose produced during the hydrolysis reaction the $ABS^{540nm}$ of the control (no enzyme added) was subtracted from hydrolysis reactions and the resulting net gain in $ABS^{540nm}$ converted into µMOL of glucose.

**2.3 Results**

This work aims to switch the *A. nidulans* natural transcriptional induction regulatory mechanism driven by cellulose signals into a xylose driven induction mechanism and thus allow *A. nidulans* to grow on xylose and simultaneously produce large amounts of cellulases.

In order to figure out which xylanase promoter would induce cellulase production in the presence of xylose we replaced 1kb of upstream *cbhC* (AN0494 ORF) region with ~1kb of four xylanase promoter regions, *xynAp* (AN3613), *xynBp* (AN9365), *xynCp* (AN1818) and *xynEp* (AN7401). In the presence of xylose, *xynCp* showed the best performance in secreting cellobiohydrolase (data not shown). Even though all tested promoters secreted cellobiohydrolase (*cbhC*) at higher levels than wild-type, the total amount of cellobiohydrolase observed in the medium was less than expected and some of the promoters showed interference with pH and strong carbon catabolite repression (data not shown).

*2.3.1 XlnR overexpression and xylose induction*

We thus decided to enhance the expression of client proteins driven by xylose promoters by constructing a *xlnR* (xylanase transcription activator) constitutive overexpression strain. *xlnR* was placed under the control of the *gpdAp* promoter which drives constitutive and strong expression of G3P dehydrogenase (For a detailed description how strains were constructed with gene definition and gene coordinates (see Table S1), strains (Table S2) and primers (Table S3) refer to electronic supplemental information ESI).

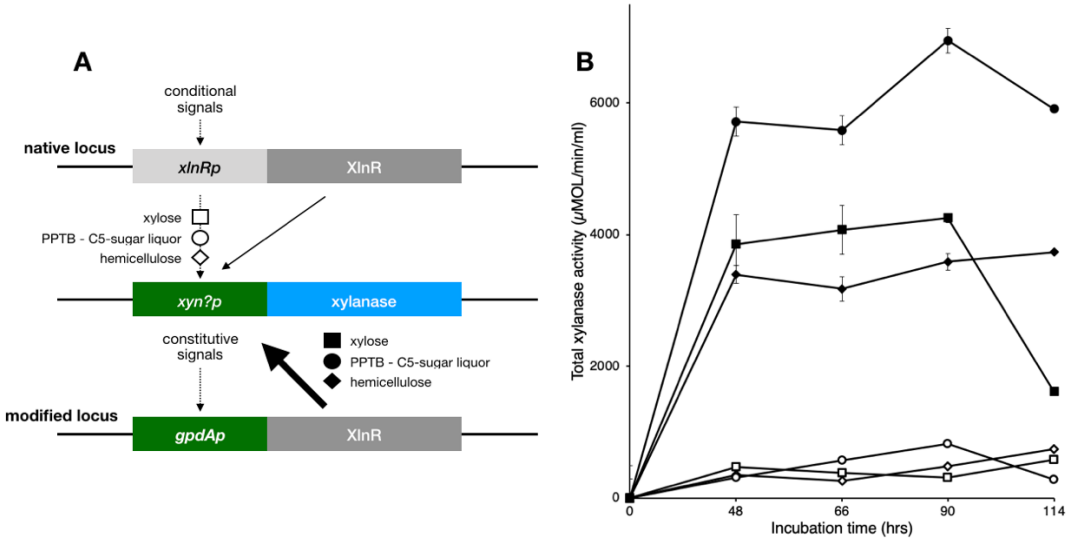



**Figure 2.2** - Constitutive overexpression of XlnR superinduces xylanase production.

**Figure 2.2A-** Shows a schematic of the promoter replacement of XlnR and how over-expression will affect the xylanase genes under the control of their native promoters.

**Figure 2.2B-**Xylanase activity in PFIX7, XlnR over-expression driven by the *gpdAp* promoter (closed symbols) and control (A773) parent strain (open symbols) grown with 1% xylose (squares), 1% hemicellulose (diamonds) or 1% PPTB (circles).

**Figure 2.2** compares xylanase production of PFI-X7, the *gpdAp*::*xlnR* over expressing strain with the reference strain (A773) when growing in media containing 1% xylose, 1% hemicellulose or 2% xylose from PPTB. Vegetative growth rates of PFI-X7 were comparable to A773 (data not shown) in all C5-sugar sources, but PFI-X7 secretes large amounts of xylanases while growing on C5-sugar substrates.

**Table 2.1** Xylanase overexpression and enhanced extracellular protein secretion in PFI-X7

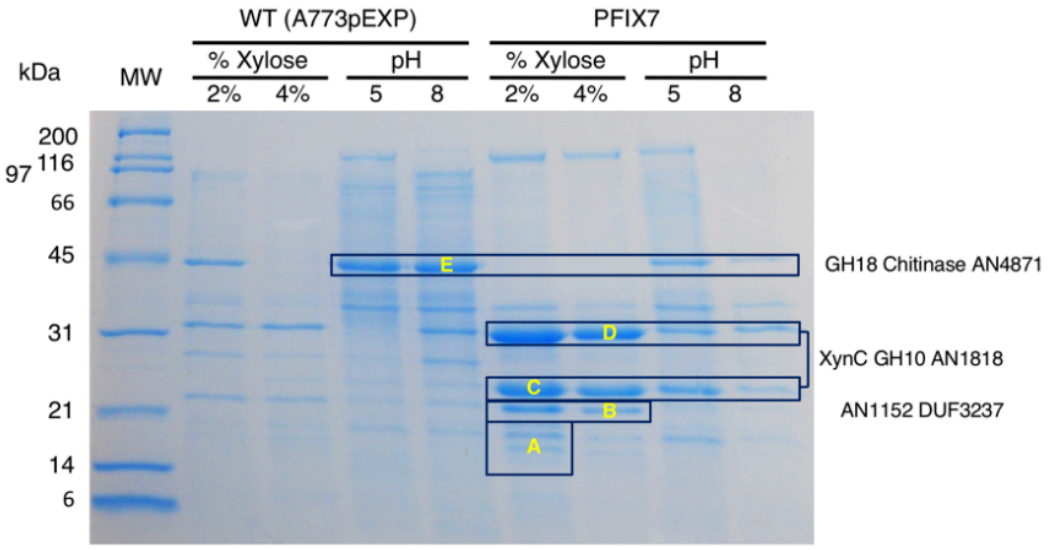
| | **Inducer** | **Xylanase activity** | | **Protein secreted** | | **Cbh1 activity** | |
|---|---|---|---|---|---|---|---|
| | xylose | µmol/µl/min | fold | µg/mL | fold | U(pNPC)/min | fold |
| | | | | | | | |
| WT | 2% | 505+/-70 | - | 259+/-78 | - | 0.63+/-0.29 | - |
| | 4% | 628+/-85 | - | 260+/-39 | - | 0.36+/-0.06 | - |
| | 6% | 593+/-32 | - | 302+/-32 | - | 0.45+/-0.03 | - |
| | | | | | | | |
| PFIX7 | 2% | 14,023+/-4329 | 28X | 1,442+/-349 | 6X | 4.23+/-0.06 | 7X |
| | 4% | 16,248+/-3091 | 26X | 1,176+/-233 | 5X | 4.01+/-0.02 | 11X |
| | 6% | 14,958+/-2746 | 25X | 1,225+/-211 | 4X | 2.59+/-0.01 | 6X |

**Table 2.1** shows xylanase production in A773 and PFI-X7 growing on 2, 4 or 6% xylose. With 4% xylose the reference strain (A773) accumulated 628 U whereas PFI-X7 produced 16,248 U representing a 26-fold increase in xylanase accumulation. Tamayo-Ramos observed a 200-fold increase in xylanase activity of *A. nidulans* strains overexpressing XlnR (*gpdAp*::*xlnR*) growing on hemicellulose and confirmed XlnR driven overactivation by measuring the reporter α-L-rhamnosidase (RhaA) on strains where the *xynAp* and *xynBp* promoters were fused to *rhaA* (Tamayo-Ramos and Orejas 2014).

In addition, as reported by Tamayo-Ramos (Tamayo-Ramos and Orejas 2014) we also observed that the total amount of proteins secreted was enhanced in XlnR over-expressing strains. For example, **Table 2.1** shows that strains growing on 2%, 4% or 6% of xylose secreted 1,442,

1,176 and 1,225 µg/mL total proteins, respectively, much higher than the 259, 260 and 302 µg/mL total proteins, respectively secreted by the reference strain (A773).

**A**




**B**

| protein name | ACC NCBI gi\| | M.W. (kDa) | band/spectral counts | | | | |
|---|---|---|---|---|---|---|---|
| | | | A | B | C | D | E |
| **endo-1,4 β-xylanase C** (AN1818) | 259487165 | 34 | 19 | 6 | **13** | **27** | - |
| superoxide dismutase (AN0241) | 259489541 | 16 | 14 | - | - | - | - |
| phosphatidylglycerol transferase (AN5879) | 259479955 | 18 | 10 | - | - | - | - |
| unnamed protein (AN8692) | 227307486 | 19 | 8 | - | - | - | - |
| **hypothetical protein** (AN1152, DUF3237) | 259488531 | 18 | 3 | **12** | - | - | - |
| endo-1,4 β-xylanase A (AN3613) | 1722899 | 24 | 3 | 9 | - | - | - |
| transaldolase (AN0240) | 259489542 | 36 | - | - | 8 | - | - |
| hypothetical protein (AN6535) | 259480106 | 23 | - | - | 5 | - | - |
| **chitinase** GH18 (AN4871) | 259482266 | 44 | - | - | - | - | **25** |
| *N*-acetylglucosaminidase (AN1502) | 259486825 | 68 | - | - | - | - | 11 |
| hypothetical protein (AN4825) | 259482321 | 97 | - | - | - | - | 9 |
| endo 1,3 β-glucanase, GH81 (AN0472) | 259489279 | 98 | - | - | - | - | 7 |

**Figure 2.3.** Endo-1,4 β-xylanase C (XynC) is the major xylanase secreted by PFIX7, the XlnR overexpression strain when grown on xylose.

**2.3A** SDS-PAGE showing total secreted proteins in WT (A773) and PFIX7 when growing with 2 or 4% xylose in pH5 or pH8. Boxes indicate major proteins present under various conditions and bands identified by letters were excised and analyzed by LC/MS-MS.

**2.3B** Table correlating protein IDs (protein name) with LC/MS-MS spectral counts (abundance) of excised protein bands indicated in Panel A.

**Figure 2.3A** shows protein profiles (SDS-PAGE) of enzymes secreted by A773 (reference strain) and PFI-X7 (XlnR constitutive expression) growing on xylose. **Figure 2.3B** lists the spectral counts (determined by LC-MS/MS) of overexpressed protein bands A, B, C, D and E. Remarkably only three proteins were over-secreted in PFI-X7: a chitinase (GH18, band E), xylanase C (bands C and D) and a protein of unknown function AN1152 (band B). Only small amounts of xylanase A and no other xylanases (B or D) were detected by LC-MS/MS **(Figure 2.3B**). In our experiment, which only examined hyper-secreted proteins of the fungus grown on xylose as the sole carbon source, the XlnR-induced xylanases secreted (PFI-X7) were two versions of xylanase C, namely a full-length version (~34 kDa, band D with CBM1) and a truncated version with a catalytic domain and no CBM1 domain (~22 kDa, band C).

Taken into consideration all of our findings for the overexpression of XlnR in media growing on C5-sugars **(Figures 2.2**, **Table 2.1** and **Figures 2.3A** and **B**), we concluded that overexpressing XlnR (PFI-X7) results in predominant secretion of xylanase C (XynC) when mycelia are grown on xylose. Thus, using the *xynCp* promoter to drive the production of client proteins (cellulases) in a strain that overexpresses XlnR is likely to accumulate large amounts of client proteins.

*2.3.2. Xylose induced production of cellulases*

To test the assumption that XlnR overexpression would drive accumulation of potential client proteins driven by the *xynCp* promoter we constructed a series of strains that overproduce five model cellulase genes, predicted to be necessary to completely convert a cellulose molecule into glucose. Based on the evidence reported by Segato and cols. ((Segato, Damasio et al. 2014) and others cited within), the selected model genes included two cellobiohydrolases (CbhB and CbhC), two endoglucanases (EglA and EglB) and one β-glucosidase (BglA).

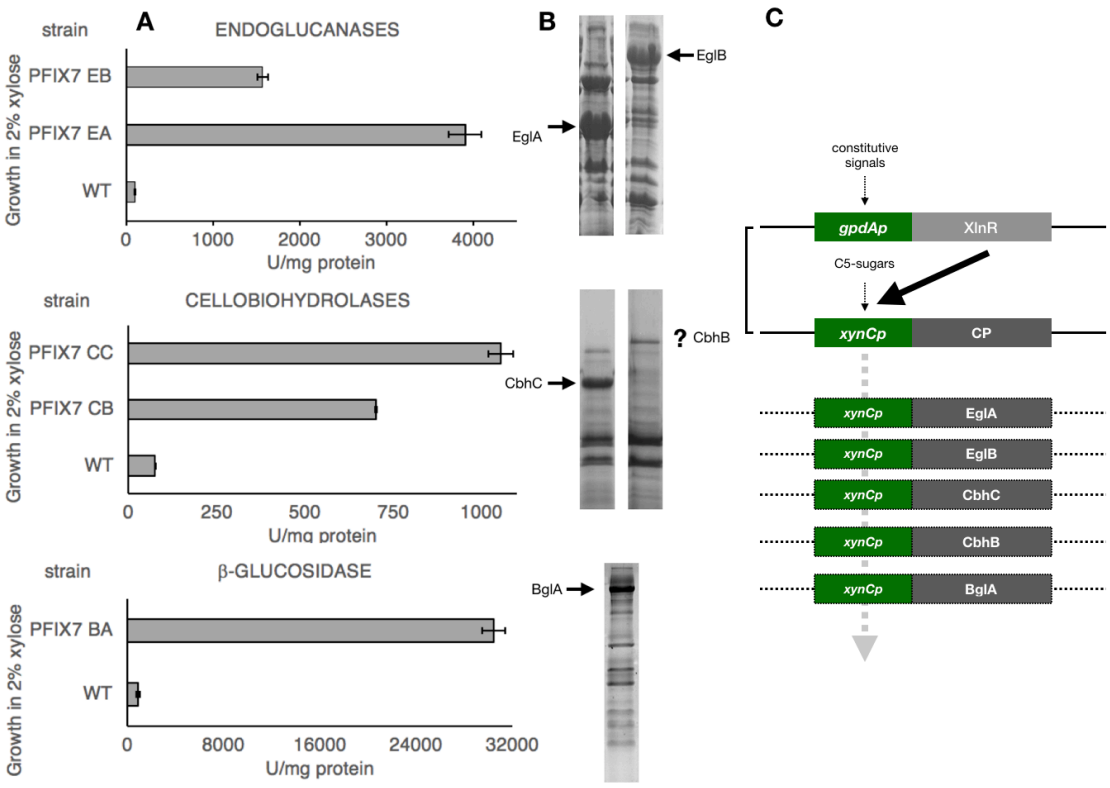



**Figure 2.4.** Client protein hyper accumulation induced by xylose in strains overexpressing XlnR and regulated by the *xynCp* promoter.

Plasmids carrying the *pUC18*$_{UP}$::*pyroA*::*xynCp*::**CLIENT**$_{\mathbf{ORF}}$::*pUC18*$_{DWN}$ Gibson Assembly construct were transformed into PFI-X7 (XlnR overexpressing) strain and recombinants selected based on the level of client protein production rates.

Specific enzyme activity (**Panel A**) and protein accumulation verified by SDS-PAGE (**Panel B**) of five client proteins, two endoglucanases (strain PFIX7-EA, enzyme EglA, strain PFIX7-EB, enzyme EglB), two cellobiohydrolases (strain PFIX7-CC, enzyme CbhC, strain PFIX7-CB, enzyme CbhB) and a β-glucosidase (strain PFIX7-BA, enzyme BglA). **Panel C** shows a schematic of XlnR over-expression, as well as C-5 sugar induction, driving the production of the ectopically integrated xynCp driven cellulases.

**Figure 2.4** shows that total enzyme activity and protein accumulation of the five model genes grown in the presence of 2% xylose. For endoglucanases EglA and EglB we found 3,908+/-190 and 1,570+/-60 enzyme units per milligram total protein, respectively (**Figure 2.4A**). For cellobiohydrolases CbhB and CbhC we found 702+/-3 and 1,054+/-35 enzyme units per milligram total protein, respectively (**Figure 2.4A**). For β-glucosidase BglA we found 30,436+/-964 enzyme units per milligram protein (**Figure 2.4A**). SDS-PAGE of crude unfiltered extracts (**Figure 2.4B**) shows that all of the enzymes over accumulated in the medium. For CbhB we could not unambiguously detect a clear protein band on SDS-PAGE gels despite detecting increased activity (702 U per milligram protein).

The above result is promising because the engineered strains (PFIX7-EA, PFIX7-EB, PFIX7-CB, PFIX7-CB and PFIX7-BA) accumulate large amounts of client proteins relative to the production of cellulases in the reference strain when grown on xylose. The engineered strains (PFIX7-EA, PFIX7-EB, PFIX7-CB, PFIX7-CB and PFIX7-BA) showed a 35-, 40-, 16-, 9- and

14-fold increase in extracellular specific protein accumulation of β-glucosidase, endoglucanase A, endoglucanase B, cellobiohydrolases B and cellobiohydrolase C, respectively.

*2.3.3 Production of xylanases and cellulases with C5 pre-hydrolysates*

Next, we prospect the feasibility of using C5 pre-hydrolysates both as a carbon source and inducer to produce cellulases. Because the C5 pre-hydrolysates are a byproduct of LCB pretreatments they mainly contain xylose, but other sugars and phenols are also present. The C5 pre-hydrolysate, routinely obtained in our laboratories by treating wheat-straw (LCB) with diluted nitric acid at 160 C for 30 minutes and concentrated in a vacuum evaporator contains 162 g/l (76.7%) of xylose, 29.4 g/l (14%) of glucose and 19.7 g/l (9.3%) of arabinose as potential carbon sources.

We tested two media formulations; First, the reference minimal medium contained Clutterbuck salts (Clutterbuck 1992) with xylose as the main carbon source (30 g/l starting concentration) and glucose respectively arabinose (5 g/l respectively 4 g/l). Secondly, the pre-hydrolysate medium with Clutterbuck salts in which the initial C5-sugar concentration was adjusted to 30 g/l of xylose with relational reset of glucose and arabinose levels to 5.6 g/l and 4.2 g/l, respectively.

Three strains were examined for overproduction of enzymes in C5-sugar pre-hydrolysates, **PFI-X7** which due to the overexpression of the XlnR transcription factor naturally over-produces xylanases (Figure 5A), **PFI-EA** which overexpresses endoglucanase A (EglA) and **PFI-BA** overexpressing ß-glucosidase (BglA).

**Table 2.2**: Cellulase and xylanase production in media containing C5-sugars.

| Strain | Enzyme Activity | | | Secreted Protein | Spent sugar | Enzyme Productivity | |
|---|---|---|---|---|---|---|---|
| | activity | total | specific | | | | |
| | U/ml | U | U/g | g/l | % | U/l h | $Y_{P/S}$ |
| **Reference minimal medium** | | | | | | | |
| **PFI-X7** | 2,760±6*[1] | 24,324±3479 | 4,266±238 | 0.68 | 96 | 38,333 | 35,646 |
| **PFI-EA** | 283±26*[2] | 3,191±85 | 350±31 | 0.83 | 96 | 3,930 | 4,598 |
| **PFI-BA** | 155±18*[3] | 1,749±93 | 175±29 | 0.88 | 93 | 2,152 | 2,662 |
| **Pre-hydrolysate medium** | | | | | | | |
| **PFI-X7** | 2,473±51 | 29,222± 859 | 4,667±195 | 0.53 | 80 | 34,347 | 49,420 |
| **PFI-EA** | 328±14 | 4,008±395 | 386±14 | 0.85 | 86 | 4,556 | 6,132 |
| **PFI-BA** | 161±7 | 1,952±133 | 207±11 | 0.78 | 83 | 2,236 | 3,239 |

*[1] xylanase activity; *[2] endoglucanase activity; *[3] beta-glucosidase activity

## 2.4. Discussion

Here we report on a succession of genetic interventions in *Aspergillus nidulans* that redesign the natural regulatory circuitry of cellulase genes in such a way that recombinant strains use C5-sugar liquors to grow a vegetative tissue and simultaneously produce large amounts of cellulases. Five cellulases, two cellobiohydrolases (CbhB and CbhC), two endoglucanases (EglA and EglB) and a β-glucosidase (BglA) completely digest cellulose producing glucose. Cellulase production using high-xylose containing liquors for biomass digestion releasing C6-sugars streamlines the entire biomass degradation process by integrating pretreatment technologies with enzyme production and biomass digestion.

## 2.5. Supplemental Materials

### *2.5.1 Method details*

Three types of strains were constructed in this study; **First** the resident CbhC (AN0494) promoter (*cbhCp*) was replaced with four xylanase promoters (*xynABCEp*) in such a way that

recombinant strains induce the production of cellobiohydrolase by xylose, **second** a $XlnR_{(ORF)}$ overexpression strain (PFIX7) was constructed by *pabaA* ectopic integration of a *gpdAp*::$xlnR_{(ORF)}$ DNA fragment, and **third**, xylose induced client protein constructs were randomly introduced into a XlnR overexpressing strain (PFIX7).

In all types of strain constructions, a linear hybrid recombinant DNA fragment was synthesized using Gibson Assembly Technology, GAT (Gibson, 2011; Gibson, 2014) using hybrid primers, Gibson Assembly Master Mix (New England Biolabs, US) and Phusion DNA Polymerase (New England Biolabs, US). DNA fragment size and DNA sequence verified hybrid DNA fragments were transformed into A773 or PFIX7 protoplasts. In the case of promoter replacements, a single gene replacement event at the cbh1 locus was selected for each *xyn(p)* promoter replacement by uracil/uridine sufficiency and by diagnostic PCR showing single integration (replacement) into the *cbhC* locus. For the XlnR overexpression the hybrid DNA fragment was integrated into the pabaA locus by a double crossover event disrupting it. Recombinants with a single gene replacement event were searched with diagnostic PCR and the resulting strain PFIX7 tested for XlnR over-expression.

For the client protein xylose induced strains we created plasmids carrying the *pUC18UP*::*pyroA*: *xynCp*::C**LIENT**$_{\mathbf{ORF}}$::*pUC18DWN* GAT construct that was transformed into PFI-X7 (XlnR overexpressing) strain and recombinants selected based on the level of client protein production rates. Even though we did not check for multiple integration events in single transformants we screened at least 100 transformants for high secretion levels of client proteins.

*2.5.2 Construction of hemicellulose induced cellobiohydrolase production strains.*

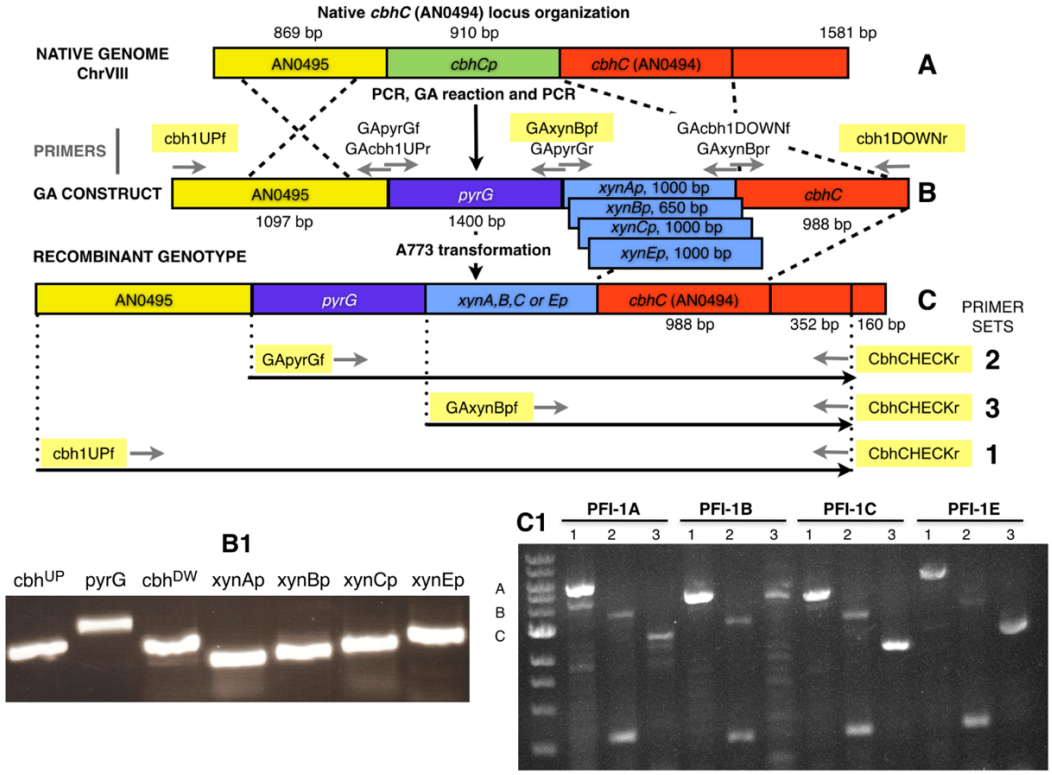



**Figure S2.1.** Construction of xylose induced cellobiohydrolase production strains.

*cbhC* promoter engineering outline. A. Native *cbhC* (AN0494) locus organization on chromosome VIII. **B** and **B1**. Gibson Assembly construct (GA). PCR generated DNA fragments, $cbh^{UP}$ (AN0495), *pyrG* (selectable marker) four *xyn* promoters (see **Table S2.1** for gene data), *xynAp*, *xynBp*, *xynCp* and *xynEp*, and $cbh^{DW}$ (AN0494). PCR fragments were amplified from *A. nidulans* FGSC4 (wild-type) genomic DNA template and GA hybrid primers (see **Table S2.3**). DNA fragments with hybrid ends (shown in **B** and **B1**) were amalgamated together using Gibson Assembly Master Mix (Gibson, 2011; Gibson, 2014). **C** and **C1**. The GA linear hybrid recombinant DNA segment (**B**) was transformed into A773 protoplasts and a single gene replacement (double crossover) event that describe the recombinant genotype shown in **C**

was screened by diagnostic PCR. **C1** shows PCR products generated from genomic DNA of putative transformants, PFI-1A, PFI-1B, PFI-1C and PFI-1E with primer sets **1**, **2** and **3** identifying bands **A**, **B** and **C**, respectively (**C1**).

*2.5.3 Construction of constitutive XlnR over expression strains*

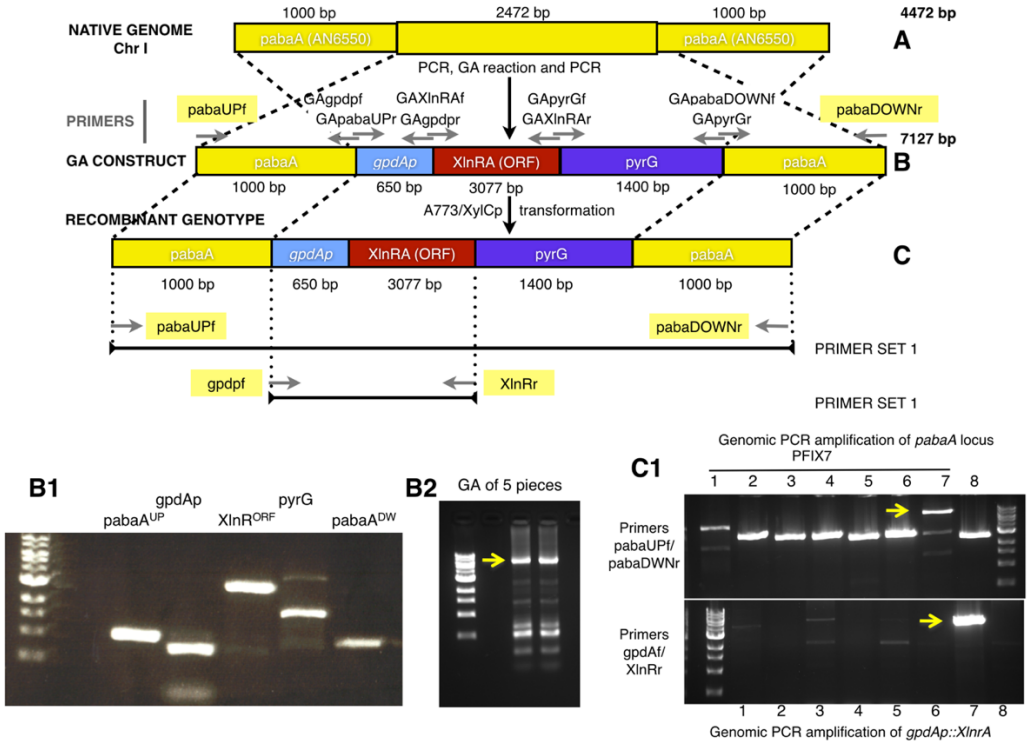



**Figure S2.2** Construction of XLNR overexpression strains.

XlnR transcription factor engineering outline. **A**. Native *pabaA* (AN6550) locus organization on chromosome I. **B**. Proposed GA construct. Five PCR generated DNA fragments, $pabaA^{UP}$, *gpdp*, $xlnR^{ORF}$, *pyrG*, $pabaA^{DW}$ (see **Table S2.1** for gene data). PCR fragments were amplified from *A. nidulans* FGSC4 (wild-type) genomic DNA template and GA

hybrid primers (**Table S2.3**). DNA fragments with hybrid ends (shown in **B1**) were amalgamated together using Gibson Assembly Master Mix (Gibson, 2011; Gibson, 2014), and the 7 kb fused GA DNA fragment amplified with Phusion DNA polymerase (**B2**).

**C** and **C1**. The GA linear hybrid recombinant DNA segment (**B2**) was transformed into A773 protoplasts and a single gene replacement (double crossover) event at the *pabaA* locus was selected among transformants with *pabaA* auxotrophy and *pyrG* sufficiency. Further transformants were screened by diagnostic PCR and **C1** shows PCR products generated from genomic DNA of putative transformants. PFIX7 shows a *pabaA* disruption (in **C1**, primers pabaUPf/pabaDWr and *gpdp*::*xlnRA* fusion, in **C1**, primers gpdAf/XlnRr).

*2.5.4 Construction of xylose induced overproduction of client (cellulase) proteins*

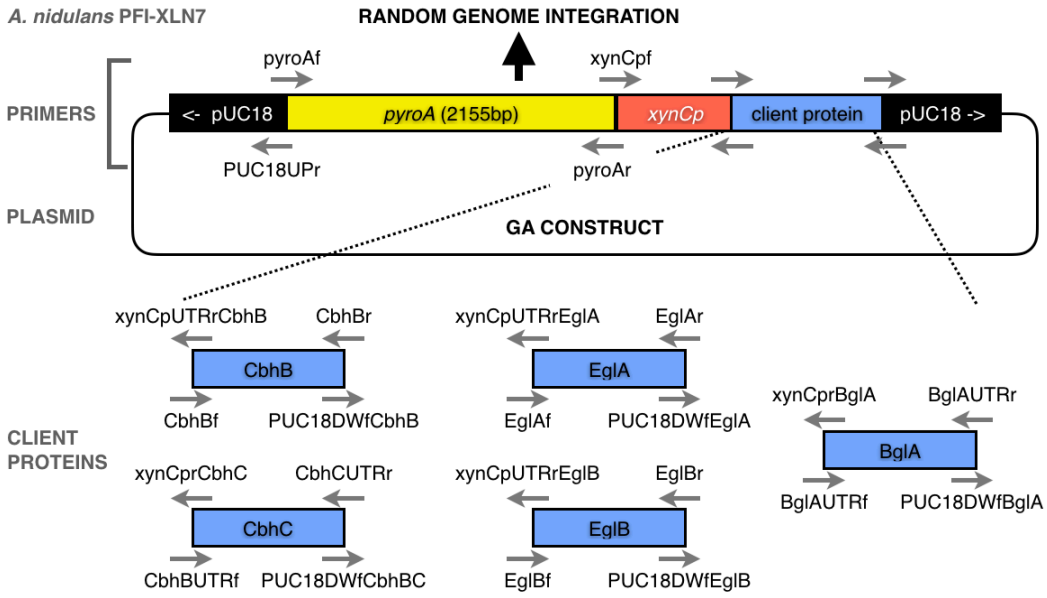


**Figure S2.3** Construction of strains that over secrete client proteins using xylose, hemicellulose or PPTB as a carbon source.

pUC18 based plasmids were constructed using common DNA fragments (*pyroA* and *xynCp*) fused to various client protein ORFs (mRNA), CbhB, CbhC, EglA, EglB and BglA (see **Table S2.1** for ORF/mRNA definitions). All constructs were done using GA technology with hybrid primer sets (see **Table S2.3**) PCR amplifying the pUC18 plasmid from pUC18 template DNA and other hybrid primer sets (see **Table S2.3**) PCR amplifying *pyroA*, *xynCp* and client protein ORFs (see **Table S2.1**) from *A. nidulans* FGSC4 genomic DNA. DNA fragments were fused with Gibson Assembly Master Mix (Gibson, 2011; Gibson, 2014), and plasmids recovered by transformation into DH5 ultracompetent *E. coli* cells.

Positive plasmids were directly transformed into PFIX7 protoplasts and recombinants selected based on complementation of pyridoxine (*pyroA*) requirement. Because multiple integrations could enhance the production of individual client proteins, 100 transformants for each client protein construct were selected based on their ability to secrete and accumulate the specified client proteins. The best preforming strains selected were PFIX7-BA, PFIX7-EA, PFIX7-EB, PFIX7-CB and PFIX7-CC (see **Table 2**).

**Table S2.1** Genes and Gene parts utilized in this work

**Table 1** - Genes and gene parts utilized in this work

| protein name | gene data | | | | | promoter | | ORF | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ACC | locus | name | CAZY | bp | name | bp | name | M.W | ACC |
| endo-1,4-b-xylanase | XM_654330 | AN1818 | *xynC* | GH10 | N.A. | *xynCp* | 692 | N.A. | N.A. | XP_659422 |
| endo-1,4-b-xylanase | XM_654868 | AN2356 | *xynD* | GH10 | N.A. | *xynDp* | 1000 | N.A. | N.A. | XP_659960 |
| endo-1,4-b-xylanase | XM_675578 | AN7401 | *xynE* | GH10 | N.A. | *xynEp* | 1000 | N.A. | N.A. | XP_680670 |
| endo-1,4-b-xylanase | XM_656125 | AN3613 | *xynA* | GH11 | N.A. | *xynAp* | 1000 | N.A. | N.A. | XP_661217 |
| endo-1,4-b-xylanase | XM_677542 | AN9365 | *xynB* | GH11 | N.A. | *xynBp* | 999 | N.A. | N.A. | XP_682634 |
| XlnR transcription factor | XM_675787 | AN7610 | *xlnR* | N.A. | 2628 | N.A. | N.A. | XlnR | 95,645 | XP_680879 |
| G3P dehydrogenase | Z32524.1 | AN8041 | *gpdA* | N.A. | 2481 | *gpdAp* | 705 | N.A. | N.A. | N.A. |
| cellobiohydrolase GH7 | XM_653006 | AN0494 | *cbhC* | GH7 | 1581 | N.A. | N.A. | CbhC | 55,992 | XP_658098 |
| cellobiohydrolase GH6 | XM_657794 | AN5282 | *cbhB* | GH6 | 1353 | N.A. | N.A. | CbhB | 46,921 | XP_662886 |
| endoglucananse | XM_653797 | AN1285 | *eglA* | GH5 | 981 | N.A. | N.A. | EglA | 35,681 | XP_658889 |
| endoglucananse | XM_655930 | AN3418 | *eglB* | GH7 | 1293 | N.A. | N.A. | EglB | 45,799 | XP_661022 |
| beta glucosidase | XM_656614 | AN4102 | *bglA* | GH3 | 2478 | N.A. | N.A. | BglA | 89,090 | XP_661706 |
| *p*-aminobenzoic acid synthase | XM_659062.1 | AN6550 | *pabaA* | N.A. | 2472 | N.A. | N.A. | N.A. | N.A. | CBF70968 |
| pyridoxine biosynthesis protein | XM_675902.1 | AN7725 | *pyroA* | N.A. | 2155 | *pyroAp* | 719 | PyroA | 32,353 | EAA61240 |
| 5'-phosphate decarboxylase | XM_658669.1 | AN6157 | *pyrG* | N.A. | 1400 | *pyrGp* | 477 | PyrG | 30,052 | AAB66359 |

**Table S2.2** *A. nidulans* strains utilized in this work

**Table 2** - *A. nidulans* strains utilized in this work

| Name | genotype | nutritional req. | source |
|---|---|---|---|
| parent strain (FGSC A773) | *wA, pyrG89, pyroA4* | pyro, ura, uri | FGSC |
| PFI-1A (*xAp*::*cbhC*) | *wA, pyrG89, AN0495::pyrG::xynAp::cbhC, pyroA4* | pyro | this study |
| PFI-1B (*xBp*::*cbhC*) | *wA, pyrG89, AN0495::pyrG::xynBp::cbhC, pyroA4* | pyro | this study |
| PFI-1C (*xCp*::*cbhC*) | *wA, pyrG89, AN0495::pyrG::xynCp::cbhC, pyroA4* | pyro | this study |
| PFI-1E (*xEp*::*cbhC*) | *wA, pyrG89, AN0495::pyrG::xynEp::cbhC, pyroA4* | pyro | this study |
| PFI-X7 | *wA, pyrG89, pabaA::gpdp::xlnR::pyrG::pabaA, pyroA4* | pyro, paba | this study |
| PFI-X7P1 | *wA, pyrG89, pabaA::gpdp::xlnR::pabaA, pyroA4* | pyro, paba, ura, uri | this study |
| PFIX7-BA | *wA, pyrG89, pabaA::gpdp::xlnR::pyrG::pabaA, pyroA::xynCp::BglA* | paba | this study |
| PFIX7-EA | *wA, pyrG89, pabaA::gpdp::xlnR::pyrG::pabaA, pyroA::xynCp::EglA* | paba | this study |
| PFIX7-EB | *wA, pyrG89, pabaA::gpdp::xlnR::pyrG::pabaA, pyroA::xynCp::EglB* | paba | this study |
| PFIX7-CB | *wA, pyrG89, pabaA::gpdp::xlnR::pyrG::pabaA, pyroA::xynCp::CbhB* | paba | this study |
| PFIX7-CC | *wA, pyrG89, pabaA::gpdp::xlnR::pyrG::pabaA, pyroA::xynCp::CbhC* | paba | this study |

**ura**, uracil; **uri**, uridine; **pyro**, piridoxine; **paba**, p-aminobenzoic acid;

**Table S2.3** Primers utilized in this work

**Table 3** - Primers utilized in this work

| Primer name | bp | Tm | GC% | Seq |
|---|---|---|---|---|
| **Construction of xylose induced cellobiohydrolase production strains** | | | | |
| cbhUPf | 26 | 57.8 | 42.3 | TAGTTGAAAACGACCGTTTTGTGTCC |
| GAcbhUPr | 40 | 68.7 | 55.0 | GCGTTCTCGAGGAAGTTGCGTGCTGTGGCCTAGAATGTTC |
| GApyrGf | 40 | 68.7 | 55.0 | GAACATTCTAGGCCACAGCACGCAACTTCCTCGAGAACGC |
| GApyrGr | 40 | 65.7 | 45.0 | ACGATGCTTCTACTCACAAGCCCCTTTTAGTCAATACCGT |
| GAcbh1DOWNf | 40 | 67.0 | 45.0 | ACACTTCAACAACCGGCAACATGGCATCTTCATTCCAGTT |
| cbhDOWNr | 27 | 65.5 | 59.3 | TGATTTCCGAGAGGGTGCCTGTGTCCG |
| CbhCHECKr | 30 | 67.6 | 56.7 | ACTCGTCGTCACCGTTGTTGTTGGCCCTGA |
| GAxynA11pf | 40 | 65.7 | 42.3 | ACGGTATTGACTAAAAGGGGTTACTTCTTTCAGAGCCGCT |
| GAxynA11pr | 40 | 65.6 | 55.0 | AACTGGAATGAAGATGCCATTGTGCTGATCCTGTCAGTTG |
| GAxynBpf | 40 | 65.7 | 45.0 | ACGGTATTGACTAAAAGGGGCTTGTGAGTAGAAGCATCGT |
| GAxynBpr | 40 | 67.0 | 45.0 | AACTGGAATGAAGATGCCATGTTGCCGGTTGTTGAAGTGT |
| GAxynC10pf | 40 | 64.4 | 55.0 | ACGGTATTGACTAAAAGGGGAGGTTCTTTGTTTTCACAGC |
| GAxynC10pr | 40 | 64.2 | 45.0 | AACTGGAATGAAGATGCCATTTTGGGTAAGAGTTGAACGA |
| GAxynD10pf | 40 | 67.2 | 45.0 | ACGGTATTGACTAAAAGGGGAAATGTGCGGGTCCGAGATG |
| GAxynD10pr | 40 | 64.1 | 45.0 | AACTGGAATGAAGATGCCATGGTTCTGCTTATGATATGCT |
| GAxynE10pf | 40 | 64.7 | 45.0 | ACGGTATTGACTAAAAGGGGTAAGTAATCAGCCGCATCAA |
| GAxynE10pr | 40 | 65.3 | 59.3 | AACTGGAATGAAGATGCCATCGTCTCTGAGTACTACTGCA |
| **Construction of XLNR overexpreeision strain** | | | | |
| pabAUPf | 30 | 70.1 | 60.0 | ATGGCACCGCTCCTTGGTGATTGGGCAGCA |
| GApabaAUPr | 47 | 67.0 | 46.8 | CAGAAAGAGTCACCGGTCACTTGGCACAGAAATAATGCTGTATCTGC |
| GAgpdApf | 40 | 65.6 | 47.5 | CAGCATTATTTCTGTGCCAAGTGACCGGTGACTCTTTCTG |
| GAgpdApr | 44 | 66.8 | 47.7 | GTCGACATCCTCGCGGAACGGGGAAAAGAAAGAGAAAAGAAAAG |
| GAxlnrAf | 40 | 67.1 | 50.0 | TCTTTTCTCTTTCTTTTCCCCGTTCCGCGAGGATGTCGAC |
| GAxlnrAr | 48 | 67.6 | 43.8 | AGGAAGTTGCGTTCCCAAAAGCAGAAGACAAGGATGCAAGTTGAATTG |
| GApyrGf | 40 | 67.0 | 47.5 | CTTGCATCCTTGTCTTCTGCTTTTGGGAACGCAACTTCCT |
| GApyrGr | 43 | 64.9 | 44.2 | GCAGAATGTCCATACAACAGCCCCTTTTAGTCAATACCGTTAC |
| GApabaADOWNf | 47 | 67.2 | 44.7 | ACGGTATTGACTAAAAGGGGCTGTTGTATGGACATTCTGCCAAAACC |
| pabaDOWNr | 30 | 62.8 | 46.7 | TCATTTCGCGTGCGAAGGCATAAATATGCC |

**Table 3** - continuation

| Primer name | bp | Tm | GC% | Seq |
|---|---|---|---|---|
| **Construction of client protein overexpression and secretion** | | | | |
| Common primers | | | | |
| PUC18UPr | 43 | 71.5 | 60.5 | GCCTCCTGTTCCAGCATGACCTAGAGTCGACCTGCAGGCATGC |
| pyroAf | 40 | 71.1 | 60.0 | TGCCTGCAGGTCGACTCTAGGTCATGCTGGAACAGGAGGC |
| pyroAr | 44 | 64.4 | 40.9 | GCTGTGAAAACAAAGAACCTCAGATGTAAATGTCAAAAGGTCCG |
| XynCpf | 40 | 61.9 | 37.5 | CCTTTTGACATTTACATCTGAGGTTCTTTGTTTTCACAGC |
| BglA primers | | | | |
| XynCrBglA | 40 | 67.8 | 50.0 | AAACGGTCCGGAAAAGGAGGTGCCAGAGCTTTTGATGATG |
| BglAUTRf | 42 | 67.5 | 50.0 | CATCATCAAAAGCTCTGGCACCTCCTTTTCCGGACCGTTTTC |
| BglAUTRr | 40 | 73.2 | 67.5 | GCTCGGTACCCGGGGATCCTTCCCCAGGTGAGTCGGGATG |
| PUC18DWfBglA | 40 | 73.2 | 67.5 | CATCCCGACTCACCTGGGGAAGGATCCCCGGGTACCGAGC |
| CbhC primers | | | | |
| XynCrCbhC | 40 | 63.3 | 42.5 | AGAAGGGTTATATAGGTCTCTGCCAGAGCTTTTGATGATG |
| CbhCUTRf | 45 | 64.5 | 44.4 | CATCATCAAAAGCTCTGGCAGAGACCTATATAACCCTTCTAGGAC |
| CbhCUTRr | 40 | 69.6 | 57.5 | GCTCGGTACCCGGGGATCCTTGCGTACTTTAATGCAGCAG |
| PUC18DWfCbhC | 40 | 69.6 | 57.5 | CTGCTGCATTAAAGTACGCAAGGATCCCCGGGTACCGAGC |
| CbhB primers | | | | |
| XynCpUTRrCbhB | 42 | 64.3 | 40.5 | AACTGGAATGAAGATGCCATTTTGGGTAAGAGTTGAACGATG |
| CbhBf | 41 | 64.4 | 41.5 | TCGTTCAACTCTTACCCAAAATGGCATCTTCATTCCAGTTG |
| CbhBr | 40 | 69.2 | 57.5 | GCTCGGTACCCGGGGATCCTCTGTTAACGCATACTGCATG |
| PUC18DWfCbhB | 40 | 69.2 | 57.5 | CATGCAGTATGCGTTAACAGAGGATCCCCGGGTACCGAGC |
| EglA primers | | | | |
| XynCpUTRrEglA | 42 | 65.2 | 45.2 | AGAAGGACGAGAGACCTCATTTTGGGTAAGAGTTGAACGATG |
| EglAf | 47 | 67.7 | 48.9 | TCGTTCAACTCTTACCCAAAATGAGGTCTCTCGTCCTTCTGTCGTCC |
| EglAr | 40 | 74.7 | 70.0 | GCTCGGTACCCGGGGATCCTTTCGCGTCCCAGCCTGAGCC |
| PUC18DWfEglA | 40 | 74.7 | 70.0 | GGCTCAGGCTGGGACGCGAAAGGATCCCCGGGTACCGAGC |
| EglB primers | | | | |
| XynCpUTRrEglB | 42 | 63.3 | 40.5 | AGAGATAGTAACAGAGCCATTTTGGGTAAGAGTTGAACGATG |
| EglBf | 43 | 63.9 | 41.9 | TCGTTCAACTCTTACCCAAAATGGCTCTGTTACTATCTCTCAG |
| EglBr | 41 | 67.2 | 51.2 | GCTCGGTACCCGGGGATCCTAGGTTTTTGGTGTTTAAAGTG |
| PUC18DWfEglB | 40 | 67.2 | 50.0 | ACTTTAAACACCAAAAACCTAGGATCCCCGGGTACCGAGC |

## 2.6. References


Arantes, V. and J. N. Saddler (2010). "Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis." Biotechnol Biofuels **3**(4): 4.

Bar-On, Y. M., R. Phillips and R. Milo (2018). "The biomass distribution on Earth." Proc Natl Acad Sci U S A **115**(25): 6506-6511.

Bhutto, A. W., K. Qureshi, K. Harijan, R. Abro, T. Abbas, A. A. Bazmi, S. Karim and G. Yu (2017). "Insight into progress in pre-treatment of lignocellulosic biomass." Energy **122**: 724-745.

Bischof, R. H., J. Ramoni and B. Seiboth (2016). "Cellulases and beyond: the first 70 years of the enzyme producer Trichoderma reesei." Microb Cell Fact **15**(1): 106.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**(1–2): 248-254.

Cerqueira, G. C., M. B. Arnaud, D. O. Inglis, M. S. Skrzypek, G. Binkley, M. Simison, S. R. Miyasato, J. Binkley, J. Orvis, P. Shah, F. Wymore, G. Sherlock and J. R. Wortman (2014). "The Aspergillus Genome Database: multispecies curation and incorporation of RNA-Seq data to improve structural gene annotations." Nucleic Acids Res **42**(Database issue): D705-710.

Chen, H., J. Liu, X. Chang, D. Chen, Y. Xue, P. Liu, H. Lin and S. Han (2017). "A review on the pretreatment of lignocellulose for high-value chemicals." Fuel Processing Technology **160**: 196-206.

Ciesielski, P. N., J. F. Matthews, M. P. Tucker, G. T. Beckham, M. F. Crowley, M. E. Himmel and B. S. Donohoe (2013). "3D Electron Tomography of Pretreated Biomass Informs Atomic Modeling of Cellulose Microfibrils." ACS Nano **7**(9): 8011-8019.

Clutterbuck, A. J. (1992). "Sexual and parasexual genetics of Aspergillus species." Biotechnology **23**: 3-18.

Demain, A. L. and P. Vaishnav (2009). "Production of recombinant proteins by microbes and higher organisms." Biotechnol Adv **27**(3): 297-306.

Ding, S.-Y., Y.-S. Liu, Y. Zeng, M. E. Himmel, J. O. Baker and E. A. Bayer (2012). "How Does Plant Cell Wall Nanoscale Architecture Correlate with Enzymatic Digestibility?" Science **338**(6110): 1055.

Ding, S. Y. and M. E. Himmel (2006). "The maize primary cell wall microfibril: a new model derived from direct visualization." J Agric Food Chem **54**(3): 597-606.

Ellila, S., L. Fonseca, C. Uchima, J. Cota, G. H. Goldman, M. Saloheimo, V. Sacon and M. Siika-Aho (2017). "Development of a low-cost cellulase production process using Trichoderma reesei for Brazilian biorefineries." Biotechnol Biofuels **10**: 30.

Garvey, M., H. Klose, R. Fischer, C. Lambertz and U. Commandeur (2013). "Cellulases for biomass degradation: comparing recombinant cellulase expression platforms." Trends Biotechnol **31**(10): 581-593.

Gasser, E., P. Ballmann, S. Dröge, J. Bohn and H. König (2014). "Microbial production of biopolymers from the renewable resource wheat straw." Journal of applied microbiology **117**(4): 1035-1044.

Gibson, D. G. (2011). "Enzymatic assembly of overlapping DNA fragments." Methods Enzymol **498**: 349-361.

Gibson, D. G. (2014). "Programming biological operating systems: genome design, assembly and activation." Nat Methods **11**(5): 521-526.

Jadidzadeh, A. and A. Serletis (2018). "The global crude oil market and biofuel agricultural commodity prices." The Journal of Economic Asymmetries **18**.

Johnson, E. (2016). "Integrated enzyme production lowers the cost of cellulosic ethanol." Biofuels, Bioproducts and Biorefining **10**(2): 164-174.

Kiesenhofer, D. P., R. L. Mach and A. R. Mach-Aigner (2018). "Influence of cis Element Arrangement on Promoter Strength in Trichoderma reesei." Appl Environ Microbiol **84**(1).

Klein-Marcuschamer, D. and H. W. Blanch (2015). "Renewable fuels from biomass: Technical hurdles and economic assessment of biological routes." AIChE Journal **61**(9): 2689-2701.

Kuhad, R. C., D. Deswal, S. Sharma, A. Bhattacharya, K. K. Jain, A. Kaur, B. I. Pletschke, A. Singh and M. Karp (2016). "Revisiting cellulase production and redefining current strategies based on major challenges." Renewable and Sustainable Energy Reviews **55**: 249-272.

Lynd, L. R., M. S. Laser, D. Bransby, B. E. Dale, B. Davison, R. Hamilton, M. Himmel, M. Keller, J. D. McMillan, J. Sheehan and C. E. Wyman (2008). "How biotech can transform biofuels." Nat Biotechnol **26**(2): 169-172.

Marshall, T. and K. M. Williams (1992). "Coomassie blue protein dye-binding assays measure formation of an insoluble protein-dye complex." Anal Biochem **204**(1): 107-109.

Miller, G. L. (1959). "Use of dintirosalicilic acid reagent for determination of reducing sugar." Analytical Chemistry **31**: 426-428.

Nevalainen, K. M., V. S. Te'o and P. L. Bergquist (2005). "Heterologous protein expression in filamentous fungi." Trends Biotechnol **23**(9): 468-474.

Pontecorvo, G. (1969). "Genetic analysis of "somatic" cells in filamentous fungi." Wistar Inst Symp Monogr **9**: 1-8.

Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. D. Macdonald and A. W. Bufton (1953). "The genetics of Aspergillus nidulans." Adv Genet **5**: 141-238.

Punt, P. J., N. van Biezen, A. Conesa, A. Albers, J. Mangnus and C. van den Hondel (2002). "Filamentous fungi as cell factories for heterologous protein production." Trends in Biotechnology **20**(5): 200-206.

Rana, V., A. D. Eckard, P. Teller and B. K. Ahring (2014). "On-site enzymes produced from Trichoderma reesei RUT-C30 and Aspergillus saccharolyticus for hydrolysis of wet exploded corn stover and loblolly pine." Bioresour Technol **154**: 282-289.

Schulein, M. (1997). "Enzymatic properties of cellulases from Humicola insolens." J Biotechnol **57**(1-3): 71-81.

Segato, F., A. R. Damasio, R. C. de Lucas, F. M. Squina and R. A. Prade (2014). "Genomics review of holocellulose deconstruction by aspergilli." Microbiol Mol Biol Rev **78**(4): 588-613.

Shapiro, A. L., E. Vinuela and J. V. Maizel, Jr. (1967). "Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels." Biochem Biophys Res Commun **28**(5): 815-820.

Sheldon, R. A. (2018). "The Road to Biorenewables: Carbohydrates to Commodity Chemicals." ACS Sustainable Chemistry & Engineering **6**(4): 4464-4480.

Shevchenko, A., M. Wilm, O. Vorm and M. Mann (1996). "Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels." Anal Chem **68**(5): 850-858.

Singhania, R. R., R. K. Sukumaran, A. K. Patel, C. Larroche and A. Pandey (2010). "Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases." Enzyme and Microbial Technology **46**(7): 541-549.

Singleton, V. L. and J. A. Rossi (1965). "Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents." American journal of Enology and Viticulture **16**(3): 144-158.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton and D. Crocker (2008). "Determination of structural carbohydrates and lignin in biomass." National Renewable Energy Laboratory(NREL/TP-510-42618).

Summer, J. B. and V. A. Graham (1921). "Dinitrosalicylic aid: a reagent for the estimation of sugar in normal and diabetic urine." J Biol Chem **47**: 5-9.

Tamayo-Ramos, J. and M. Orejas (2014). "Enhanced glycosyl hydrolase production in Aspergillus nidulans using transcription factor engineering approaches." Biotechnology for Biofuels **7**(1): 103.

van Rijn, R., I. U. Nieves, K. T. Shanmugam, L. O. Ingram and W. Vermerris (2018). "Techno-Economic Evaluation of Cellulosic Ethanol Production Based on Pilot Biorefinery Data: a Case Study of Sweet Sorghum Bagasse Processed via L+SScF." BioEnergy Research **11**(2): 414-425.

Zhang, J., G. Zhang, W. Wang, W. Wang and D. Wei (2018). "Enhanced cellulase production in Trichoderma reesei RUT C30 via constitution of minimal transcriptional activators." Microb Cell Fact **17**(1): 75.

# CHAPTER III

## COMBINATORIAL GENE SILENCING USING A NOVEL RNA INTERFERENCE MECHANISM IN *ASPERGILLUS NIDULANS*

### 3.1 Introduction

The filamentous fungus *Aspergillus nidulans* is an important genetic model as well as an industrially relevant enzyme production host. (Segato, Damásio et al. 2012) Fungal cell factories are used extensively in industry to produce large amounts of enzyme to produce large amounts of enzymes when naturally occurring cells are manipulated to forcibly produce a product (heterologously or homologously). (Bodie, Bower et al. 1994, Conesa, Punt et al. 2001, de Vries, Burgers et al. 2004, Segato, Damásio et al. 2012) *Aspergillus* and *Trichoderma* sp. are the main genera used in large-scale protein production. (Bodie, Bower et al. 1994, Conesa, Punt et al. 2001, Punt, van Biezen et al. 2002, Nevalainen, Te'o et al. 2005, Sims, Gent et al. 2005, Squina, Mort et al. 2009, Fleissner and Dersch 2010, Kuck and Hoff 2010, Damásio, Silva et al. 2011) In *Aspergilli* overexpression, translation and secretion of industrially useful proteins can exceed 30 grams per liter. (van den Hondel, Punt et al. 1992) In order to achieve high protein yields strains need to be manipulated (Verdoes, Punt et al. 1994, Nieto, Prieto et al. 1999, Meyer, Wu et al. 2011) such as the design of strong promoters and secretion signals (Wiebe, Robson et al. 2001, Record, Asther et al. 2003, Meyer, Wu et al. 2011); and construction of protease deficient strains. (de Vries, Burgers et al. 2004, Punt, Schuren et al. 2008, Yoon, Maruyama et al. 2011)

Double-stranded RNA (dsRNA) mediated post transcriptional silencing was first discovered as quelling in the fungus *Neurospora crassa* (Romano and Macino 1992). Quelling in *N. crassa* belongs to a broad category of RNA-mediated gene silencing mechanisms typical to RNAi(Mittal, Yadav et al. 2012). Recent advances in genomic sequencing has revealed that mechanisms of RNA silencing are conserved within many fungi. (Nakayashiki, Kadotani et al. 2006) In fact RNA silencing is used as an important genetic tool in various fungal species now, including *A. nidulans*. (Barton and Prade 2008)

RNAi in *A. nidulans* utilizes the DICER protein to degrade double-stranded RNA into 21 base pair siRNA duplexes, which then associate with the Argonaute protein forming the RISC complex. (Bartel 2005, Baulcombe 2007, Jaskiewicz and Filipowicz 2008) The RISC complex uses the guiding strand of the siRNA to target the complementary mRNA sequence and can halt the translation, or even degrade, the mRNA sequence. (Hammond and Keller 2005, Hammond, Bok et al. 2008) For instance, a construct, using inverted repeats of an inducible alcohol dehydrogenase promoter (*alcAp* flanking the *brlAβ*) could silence gene expression in *A. nidulans* using RNAi. (Barton and Prade 2008) The inverted alcAp promoters induce the expression of dsRNA and trigger the RNAi mechanism to silence the target gene, *brlAβ*. (Barton and Prade 2008)

To address the need for further engineering of genetic networks in cell factories, we proposed a serial parallel gene silencing method. Utilizing the conservation of RNA silencing in *A. nidulans*, we prove that entire metabolic pathways can be rewired through a single step genetic intervention by examining pathways involved in the proteolysis of heterologous proteins and the amylolytic pathway. Fungal cell factories secreting large amounts of heterologous client proteins to the extracellular space (Squina, Mort et al. 2009, Damásio, Silva et al. 2011, Santos, Paiva et al. 2011, Wang, Squina et al. 2011, Segato, Damasio et al. 2012) are particularly prone to degrading the protein with various proteases. (Katz, Bernardo et al. 2008, Pena-Montes, Gonzalez

et al. 2008, Wang, Xue et al. 2008, Braaksma, Smilde et al. 2009, Yoon, Kimura et al. 2009, Han, Kim et al. 2010, Matsushita-Morita, Tada et al. 2011, Sriranganadane, Waridel et al. 2011, Ward 2011)

The proteases produced by the host fungus are often times responsible for the failure of the host to accumulate large amounts of these recombinant proteins. Therefore, we propose to genetically reengineer the host, using a novel RNA interference mechanism, to inactivate the expression of these deleterious proteases.

## 3.2 Materials and Methods

### *3.2.1 Aspergillus nidulans strain manipulations*

DNA mediated transformation will be based on the methods described for *A. nidulans* by Yelton (Yelton, Hamer et al. 1984) and modified as follows; a young mycelium grown overnight at 37 $^0$C, 180 rpm in minimal medium with supplements, will be harvested by filtration (Whatman filter paper), washed with 0.6 M $MgSO_4$, suspended in 5 ml DSPS (1.1 M KCl, 0.1 M citric acid, pH 5.8) with 100 mg of lysing enzymes from *Trichoderma harzianum* (Sigma L1412), 100 mg of lysozyme from chicken egg white (Sigma L7651) and 100 mg of albumin bovine fraction V (Sigma A4503). The slurry will be incubated at 30 $^0$C, 100 rpm for 1-2 hours and protoplasts harvested by filtration through a one-layer Miracloth, washed by centrifugation 4,500g, 4 $^0$C, 10 min, twice with 50 ml STC (1.2 M Sorbitol, 50 mM $CaCl_2$, 50 mM TRIS pH 7.5). The final pellet will be suspended in 1 ml STC and stored at 4 $^0$C until further use. In a falcon tube 10 mg of pEXPYR plasmid DNA will be added onto 100 ml STC (final volume) along with additional 150 ml of protoplasts ($\sim 10^8$), incubated at RT for 10-15 minutes prior to the addition of 1 ml of 60% PEG solution (60% PEG4000 in STC). The transforming mixture mixed carefully by swirling and incubated at room temperature for 10-15 minutes, 8 ml of STC will be added and 1 ml poured onto protoplast-recovery (1.2 M sorbitol) and transformant-selection (no

uracil, uridine or 5-FOA) basic medium plates (medium without yeast extract or vitamins). Plates will be incubated at 37 $^{0}$C for one day and then inverted. Transformants will be harvested during a two to three day period, plated and purified through a single spore conidiation cycle (Pontecorvo, Roper et al. 1953, Clutterbuck 1992). Recombinants will be further selected by zeocin resistance (up to 500 ug/ml) and heritable genomic integration validated by PCR amplification of a hybrid pEXPYR-flank and client-insert DNA fragment.

### *3.2.2 Construction of pEXPYR recombinant plasmids*

The starting plasmid will be pEXPYR (Segato, Damásio et al. 2012), a PUC19 based backbone *E. coli* maintaining plasmid, which contains a glucoamylase (*glaA*), maltose inducible, promoter, BbvCI$^{989bp}$ restriction site, glucoamylase signal peptide, NotI$^{1067bp}$ and XbaI$^{1074bp}$ restriction sites, a *A. nige*r *trpC* terminator sequence followed by a zeocin (phleomycin), the *Aspergillus niger*, orotidine 5'-phosphate carboxylase (*pyrG*) and ampicillin resistance genes. Our constructs (ORFs) will be cloned into NotI$^{1067bp}$ and XbaI$^{1074bp}$ restriction sites, validated by re-sequencing and transformed into *A. nidulans* A773 or RF.

### *3.2.3 Construction of pS-ICDS recombinant plasmids*

DNA fragments which contain double inverted promoter repeats, stuffed with ICDS's will be designed "*in silico*" flanked by suitable cloning restriction sites and resynthesized "*in vitro*" in a PUC19 based plasmid. The resynthesized inserts will then be transferred to pS plasmids carrying or the *argB* or *pyrG89* (recyclable) selectable markers and transformed into *A. nidulans* RF (*ΔargB*, *pabaA1*) or A773 (*pyrG89*, *pyroA*), respectively.

*3.2.4 A. nidulans protein secretion strains*

The *A. nidulans* high-expression-secretion vector, pEXPYR that directs proteins towards the extracellular medium (Segato, Damásio et al. 2012) will be used to secrete proteins designed in this study. Briefly, spores will be inoculated in liquid minimal medium supplemented with 0.5 to 15 % of maltose (the inducer), distributed onto dishes (10 ml in 60 mm, 20 ml in 150 mm Petri-dishes and 500 ml onto cafeteria trays) and incubated (stationary) at 37 $^{0}$C for 2-3 days. The mycelial mat will be lifted with spatula and discarded and the medium collected by filtration, centrifuged at 10,000g for 10 minutes prior to concentration by ultra-filtration (10,000 Dalton cutoff Amicon), quantified by the Bradford method (Marshall and Williams 1992), validated for purity by SDS PAGE (Shapiro, Vinuela et al. 1967) and used for biochemical studies. We routinely spin about 2.0 mL to about 20 ul, wash column with 0.5 mL of water and 0.5 mL of ammonium acetate buffer (10-50 mM). Supernatant is ready to be used for PAGE, activity determinations and LC-MS/MS.

*3.2.5 LC-MS/MS methodology*

Samples are analyzed on a hybrid LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to a New Objectives PV-550 nanoelectrospray ion source and an Eksigent NanoLC-2D chromatography system. Peptides are analyzed by trapping on a 2.5 cm ProteoPrepII pre-column (New Objective) and analytical separation on a 75 μm ID fused silica column packed in house with10-cm of Magic C18 AQ, terminated with an integral fused silica emitter pulled in house. Peptides are eluted using a 5-40% ACN/0.1% formic acid gradient performed over 40 min at a flow rate of 300 nL/min. During each one-second full-range FT-MS scan (nominal resolution of 60,000 FWHM, 300 to 2000 m/z), the three most intense ions are analyzed via MS/MS in the linear ion trap. MS/MS settings use a trigger threshold of 1000

counts, monoisotopic precursor selection (MIPS), and rejection of parent ions that had unassigned charge states, were previously identified as contaminants on blank gradient runs, or were previously selected for MS/MS (data dependent acquisition using a dynamic exclusion for 150% of the observed chromatographic peak width). Column performance is monitored using trypsin autolysis fragments (m/z 421.76), and via blank injections between samples to assay for contamination.

*3.2.6 Data analysis*

Centroided ion masses are extracted using the extract_msn.exe utility from Bioworks 3.3.1 and are used for database searching with Mascot v2.2.04 (Matrix Science) and X Tandem v2007.01.01.1 (www.thegpm.org). Peptide and protein identifications are validated using Scaffold v2.2.00 (Proteome Software). Probability thresholds are greater than 99% probability for protein identifications, based upon at least 2 peptides identified with 95% certainty. Proteins that contain similar peptides and cannot be differentiated based on MS/MS analysis alone are grouped to satisfy the principles of parsimony.

*3.2.7 Standard enzyme activity assays*

Enzymatic activity of a variety of glycosyl hydrolases that act on polymeric substrates will be determined by adding 10 ml of enzyme to 50 ml of 1 % (wt/vol) substrate in 100 mM phosphate buffer, pH 6.0 (or as specified) and incubating with agitation at 85 $^{0}$C, or as specified for 30 to 60 minutes. The reaction will be terminated by addition of 60 ml of dinitrosalicyclic acid (DNS) and incubated in a boiling (95 $^{0}$C) water bath for 5 min. The enzymatic release of reducing sugars, which react with DNS will be spectrophotometrically quantified at 575 nm with

a Multimode Infinte M200 Reader (Tecan, SC) and compared with glucose and cellobiose standard curves. This method is partially based on the DNS method described by Miller (Miller 1959). Assays will be carried out on sealed 96-well microtiter plates, or in 96-well-format assembled 8-strip 0.2 ml tubes, with attached hinged caps. Specific activity is defined as U per mg protein at 45 $^{0}$C whereas U is the amount of enzyme that produces one mmole of reducing sugar (glucose or cellobiose) per minute. Other enzyme activities, e.g., protease, glucoamylase and kinase assays will be based on published methods.

**3.3 Results and Discussion**

The overall objective was to develop a parallel gene-silencing tool to enable systems biology, metabolic pathway, and cellular process reengineering, applicable to the proteolytic pathway in *A. nidulans*.

*3.3.1 Construction of a proof of concept silencing vector pSP+G*

We plan to examine the complex extracellular proteolytic pathway that degrades client proteins in the model cell factory *A. nidulans*. While *A. nidulans* is an efficient cell factory, able to secrete large amounts of proteins towards the extracellular space, they also produce many proteases that degrade client proteins. These proteases can be secreted as a result of stress induced stimuli such as pH, nitrogen, carbon, and other metabolite depletion. Notably, the excessive accumulation of extracellular proteins can induce the production of these extracellular proteases. (Ferreira-Nozawa, Silveira et al. 2006, Katz, Bernardo et al. 2008, Braaksma, Smilde et al. 2009, Han, Kim et al. 2010, Ward 2011)

To construct the silencing vector (pSP+G) we mined the *A. nidulans* genome for annotated extracellular proteases from NCBI (Johnson, Zaretskaya et al. 2008). Proteases

containing a signal peptide were deemed to be extracellular. 11 potential proteases were targeted by our silencing vector as well as 3 glucoamylases that were induced by the maltose used in our pEXPYR over-expression and protein secretion system (**Figure 3.1**)

pSP+G has been constructing using two inverted *trpC* promoters in order to induce the formation of double stranded RNA. In between the two *trpC* promoters there is an array of 30bp DNA segments, each complementary to a different gene (See **Figure 3.1**). When this construct is integrated into the host genome the produced dsRNA will be processed by the conserved RNA interference pathway and silence the transcripts of the genes targeted by our construct (**Table 3.1**).

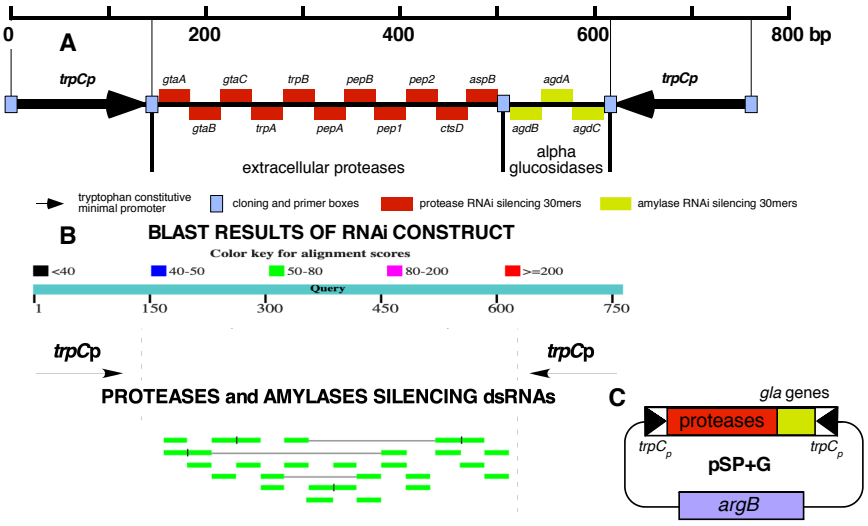



**Figure 3.1** – Construction of a proof of concept silencing vector pSP+G

Panel **A** Lineup of DNA elements; *trpCpfwd* promoter, eleven complementary DNA extracellular protease encoding segments of 30bp each, three glucoamylase encoding 30 bp segments, and the terminal *trpCprev* promoter. Panel **B** BLASTn output of the DNA element lineup, illustrating the

perfect match of each 30bp sequence to its target mRNA. Panel **C** pS-ICDS plasmid; the *trpCp* flanked 30bp segments inserted into plasmid *pDCI*, an *A. nidulans* transformation vector carrying the *argB* gene.

| Position | Protein name | | Accession Number | Chromosome | Description |
|---|---|---|---|---|---|
| 1 | gtaA | AN4809 | XM_657321.1 | III | Putative glutaminase A with a predicted role in glutamate and glutamine metabolism |
| 2 | gtaB | AN4901 | XM_657413.1 | III | Putative glutaminase A with a predicted role in glutamate and glutamine metabolism |
| 3 | gtaC | AN7514 | XM_675691.1 | IV | Putative glutaminase A with a predicted role in glutamate and glutamine metabolism |
| 4 | trpA | AN7159 | XM_659671.1 | IV | Ortholog(s) have tripeptidyl-peptidase activity |
| 5 | trpB | AN3020 | XM_655532.1 | VI | Ortholog(s) have exopeptidase activity |
| 6 | pepA | AN2157 | XM_654669.1 | VII | Putative aspartic endopeptidase |
| 7 | pepB | AN6888 | XM_659400.1 | I | Putative extracellular aspartic protease (aspergillopepsin) |
| 8 | pep1 | AN6686 | XM_659198.1 | I | Has domain(s) with predicted aspartic-type endopeptidase activity and role in proteolysis |
| 9 | pep2 | AN2903 | XM_655415.1 | VI | Aspartic protease |
| 10 | ctsD | AN4422 | XM_656934.1 | III | Putative aspartic-type endopeptidase |
| 11 | aspB | AN8102 | XM_676279.1 | II | Putative pepsin-like aspartic protease; predicted glycosyl phosphatidylinositol (GPI)-anchor |
| 12 | agdB | AN8953 | XM_677130.1 | VII | Putative alpha-glucosidase with a predicted role in maltose metabolism |
| 13 | agdA | AN2017 | XM_654529.1 | VII | Putative alpha-glucosidase with a predicted role in maltose metabolism |
| 14 | agdC | AN0941 | XM_653453.1 | VIII | Protein with alpha-glucosidase activity, predicted role in maltose metabolism |

**Table 3.1**- Genes targeted by pSP+G

The first ten 30bp units of pSP+G are proteases and the final 3 are glucoamylases

*3.3.2 pSP+G targeting of extracellular proteases during heterologous protein expression*

The pSP+G plasmid has been transformed into strain RF (*ΔargB*, *pyrG89*, *pabaA1*), recombinants were then selected and validated for permanent integration into the *A. nidulans* genome. Ten independent transformants were grown on minimal media supplemented with 1% glucose and 2% maltose and protein profiles determined by SDS PAGE (**Figure 3.2**). Figure 2A shows the protein profiles of four S (Silenced) strains compared to the protein profile of the non-silenced control strain (RF). A significant number of proteins are missing in all four S strains and others appear at reduced levels. However, new proteins appear in S strains that are absent in the

RF strains. Appearance of the new proteins might be the result of halted proteolysis or a physiological adaptation induced by the systemic silencing of extracellular proteases. It should be noted that all S strains have identical protein profiles.

When the S strains are transformed with our high yield expression and secretion plasmid pEXPYR-Cbh1, which carries a heterologous cellobiohydrolase known to be degraded by proteases. (Segato, Damásio et al. 2012) Figure 2B shows the protein profiles over a five-day period of Cbh1 accumulation in the medium S strain (S1CBH) and the control strain (RFCBH). Figure 2B shows significant proteolytic protection in the S strain compared to the RF strain, suggesting reduced proteolytic degradation in the medium. However, proteolytic degradation has not been completely halted.

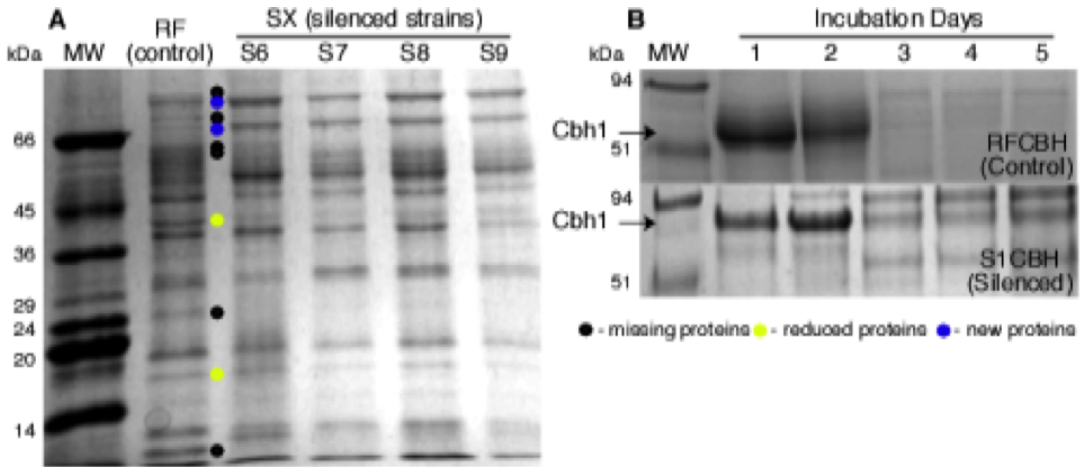



**Figure 3.2 (A)** The pSP+G erases extracellular proteins (**B**) and protects recombinant proteins from proteolytic degradation. Panel **A**- SDS-PAGE of extracellular proteins produced by control (RF) strain and four independent transformants (S6-9). Note that several proteins are missing (denoted by a black dot) in the S6-9 protein extracts, while others appear at reduced levels

(yellow dots) consistently in all four strains. New proteins (blue dots) were detected in the S strains that were not present in the RF strain. Appearance of the new proteins may be the result of halted proteolytic activity or a physiological adaptation induced by the systemic silencing of extracellular proteases. Panel **B**- SDS-PAGE of the extracellular profiles of the non-silenced control (RF) and the silenced (S1CBH) over-producing cellobiohydrolase (Cbh1) strains. Note that Cbh1 is only partially degraded after the second day of incubation while all of the Cbh1 is degraded in the wild type background.

### *3.3.3 pSP+G silencing of glucoamylase activity*

Since there are three 30bp dsRNA fragments complementary to glucoamylases we were able to test for reduced activity in the S (silenced) strains. Table 3.2 shows that glucoamylase activity is significantly reduced in about 50% of the tested S strains when compared to the control strain RF. Thus, even though not all glucoamylase activity was erased, a significant portion of the activity appeared to be silenced. It should be noted that glucoamylase activity reduction is consistent amongst all three strains (46-62%) ruling out the possible transcriptional effects due to varied ectopic genome integration.

**Table 3.2-** Silenced glucoamylase activity

| Extracellular | Silenced strains | | | RF1 |
|---|---|---|---|---|
| Glucoamylase | S1 | S2 | S4 | Control |
| U/mg | 48.98 | 34.55 | 46.72 | 90.69 |
| % reduction | 46% | 62% | 48% | 0% |

Several explanations may account for why the protease and glucoamylase activities are not completely silent. First, the silencing construct does not contain 30-mers from all known proteases and glucoamylases. Second, RNA interference may induce alternate gene sets that complement the inactivated genes. Third, the length of the 30mers may not by the most efficient for the RNAi pathway.

In order to test the first two possibilities, we performed a proteomic analysis of the proteins secreted in a reference strain, a strain containing an empty pEXPYR vector, and a strain expressing Cbh1 as a client protein.

*3.3.4 Proteomics of extracellular client protein production*

Figure 3.3 shows the SDS-PAGE secreted protein profiles of *A. nidulans* strain A773 (*pyrG89, pyroA1),* A773 pEXPYR (A773 with an empty pEXPYR expression/secretion vector), and a Cbh1 expressing and secreting strain (pEXPYR-Cbh1) (Segato, Damásio et al. 2012). Proteins were extracted from the SDS-PAGE lanes and digested with trypsin, analyzed by LC-MS/MS and peptides identified with *A. nidulans* protein database (See methods section). The heterologous Cbh1 protein was not detected because it was not in the Mascot database.

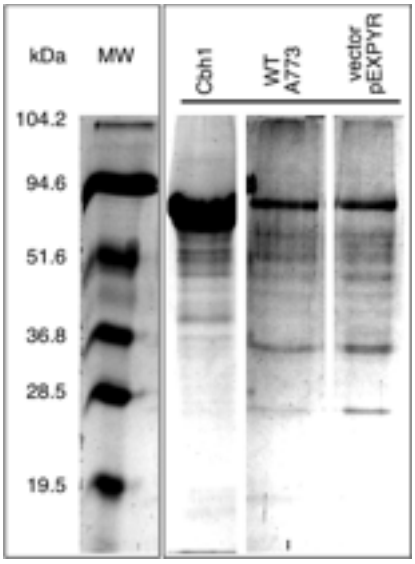



**Figure 3.3** SDS-PAGE extracellular protein profiles of *A. nidulans* strain A773, A773 containing an empty pEXPYR expression/secretion vector, and A773 transformed with pEXPYR-Cbh1.

| Protein Name | LOCUS | MW kDa | # of normalized spectra | | | % of all peptides | | |
|---|---|---|---|---|---|---|---|---|
| | | | Cbh1* | pEXPYR | A773 | Cbh1* | pEXPYR | A773 |
| CARBON CATABOLISM (11) | | | 177 | 507 | 605 | 89% | 44% | 53% |
| STARCH RELATED (5) | | | 164 | 347 | 439 | 82% | 30% | 38% |
| alpha-1,4-glucosidase | AN8953 | 108 | 5 | 297 | 370 | 9% | 30% | 38% |
| alpha-1,4-glucosidase | AN2017 | 110 | 9 | 27 | 28 | | | |
| alpha-1,4-glucosidase | AN0941 | 94 | 4 | 20 | 39 | | | |
| alpha-amylase | AN3402 | 69 | 117 | 1 | 1 | 59% | 0% | 0% |
| alpha-glucoamylase glaB | AN7402 | 71 | 29 | 1 | 1 | 15% | 0% | 0% |
| **OTHER (6)** | | | 14 | 160 | 166 | 7% | 14% | 15% |
| beta-1,3-endoglucanase | AN7950 | 47 | 1 | 60 | 75 | 1% | 5% | 7% |
| 3-beta-glucanosyltransferase | AN7657 | 49 | 9 | 48 | 34 | 4% | 4% | 3% |
| alpha-mannosidase 1B | AN0787 | 56 | 1 | 18 | 9 | 1% | 2% | 1% |
| endoarabinanase | AN8007 | 34 | 1 | 17 | 14 | 1% | 1% | 1% |
| beta-1,4-endoxylanase | AN1818 | 34 | 1 | 12 | 18 | 1% | 1% | 2% |
| polysaccharide deacetylase | AN9380 | 26 | 1 | 5 | 17 | 1% | 0% | 2% |
| PROTEIN CATABOLISM (4) | | | 4 | 123 | 78 | 2% | 11% | 7% |
| alkaline protease | AN5558 | 42 | 1 | 60 | 29 | 1% | 5% | 2% |
| aminopeptidase | AN8445 | 54 | 1 | 27 | 25 | 1% | 2% | 2% |
| dipeptidyl-peptidase | AN2572 | 79 | 1 | 20 | 16 | 1% | 2% | 1% |
| mma-glutamyltranspeptidase | AN10444 | 65 | 1 | 16 | 8 | 1% | 1% | 1% |
| STRESS RESPONSE PROTEINS (3) | | | 3 | 161 | 157 | 2% | 14% | 14% |
| catalase catB | AN9339 | 79 | 1 | 103 | 99 | 1% | 9% | 9% |
| thioredoxin reductase | AN8218 | 42 | 1 | 30 | 31 | 1% | 3% | 3% |
| superoxide dismutase | AN0241 | 16 | 1 | 28 | 27 | 1% | 2% | 2% |
| OTHER ENZYMES (6) | | | 6 | 143 | 170 | 3% | 12% | 15% |
| NADP-G dehydrogenase | AN4376 | 50 | 1 | 41 | 56 | 1% | 4% | 5% |
| methionine synthase methH | AN4443 | 87 | 1 | 28 | 24 | 1% | 2% | 2% |
| phenol oxidase lvoB | AN0231 | 40 | 1 | 21 | 29 | 1% | 2% | 3% |
| exoinulinase | AN5012 | 133 | 1 | 20 | 21 | 1% | 2% | 2% |
| FAD-oxidoreductase | AN10296 | 52 | 1 | 18 | 20 | 1% | 2% | 2% |
| proporphyrinogen III oxidase | AN5130 | 52 | 1 | 16 | 19 | 1% | 1% | 2% |
| UNKNOWN (9) | | | 9 | 229 | 136 | 5% | 20% | 12% |
| unknown | AN5942 | 17 | 1 | 40 | 33 | 1% | 3% | 3% |
| tracellular serine-rich protein | AN2954 | 96 | 1 | 37 | 14 | 1% | 3% | 1% |
| unknown | AN7912 | 42 | 1 | 28 | 9 | 1% | 2% | 1% |
| cell wall organization protein | AN4390 | 41 | 1 | 27 | 13 | 1% | 2% | 1% |
| unknown | AN7181 | 49 | 1 | 22 | 20 | 1% | 2% | 2% |
| unknown | AN7269 | 51 | 1 | 21 | 14 | 1% | 2% | 1% |
| unknown | AN8333 | 19 | 1 | 18 | 14 | 1% | 2% | 1% |
| unknown | AN6535 | 23 | 1 | 18 | 7 | 1% | 2% | 1% |
| unknown | AN8692 | 19 | 1 | 18 | 12 | 1% | 2% | 1% |

**Table 3.3** Proteins secreted by *A. nidulans*

Table 3.3 describes the proteins found by LC-MS/MS. In all three strains (A773, A773 containing an empty pEXPYR vector, and A773 pEXPYR-Cbh1), the most abundant proteins detected by proteomics were starch degrading enzymes. This result is expected since *A. nidulans'* expression and secretion system, pEXPYR, is induced by maltose and secretion is driven by the glucoamylase signal peptide. However, what was not expected is that the Cbh1 expression strain

induced a different set of glucoamylases as compared to A773 and the empty pEXPYR vector control. Hence, these genes were not present in our silencing vector pSP+G which might account for the incomplete inactivation of glucoamylase activity in Table 3.2.

Protein-metabolizing enzymes (proteases) were also analyzed and it was found that four different types of proteases were being expressed: alkaline protease, amino-peptidase, dipeptidyl-peptidase, and gamma-glutamyl-transpeptidase. Using SWISSPROT as the reference data base for Mascot to map peptides, we found that Aspergillopepsin B and carboxypeptidase (*cpdS*) were also expressed in higher amounts during Cbh1 production. This may account for the incomplete inactivation of protease activity illustrated in Figure 3.2B.

### *3.3.5 Discussion*

Proteolysis of client proteins is a significant problem when the protein is recombinant in origin because these proteins often have proteolysis degradation motifs embedded into their amino acid sequence (which is not a problem if they are being expressed homologously). However, when introduced into a fungal hyper-secretion host they are efficiently degraded by resident proteases hampering the accumulation of the client protein in a typical industrial fermentation process. Because physiological conditions are variable and unpredictable in various fermentation procedures, a large variety of proteases maybe activated and secreted. Using our tool, future researchers could inactivate multiple proteases through a single step genetic intervention.

Further, a genetic network-rewiring tool effecting serial gene silencing is a simple but powerful design that has direct implications to all types of eukaryotic cell lines engaged in the production of a specific product. RNAi inactivation of a collection of genes eradicates the requirement of single-gene knockouts, which in most cell line systems becomes difficult after the

third round of knockouts. A genetic network-rewiring tool effecting serial gene silencing enables systems biology to manipulate and redesign gene networks with a single genetic intervention.

### 3.4 References


Bartel, B. (2005). "MicroRNAs directing siRNA biogenesis." Nat Struct Mol Biol **12**(7): 569-571.

Barton, L. M. and R. A. Prade (2008). "Inducible RNA Interference of brlAbeta in Aspergillus nidulans." Eukaryotic cell **7**(11): 2004-2007.

Baulcombe, D. C. (2007). "Molecular biology. Amplified silencing." Science **315**(5809): 199-200.

Bodie, E. A., B. Bower, R. M. Berka and N. S. Dunn-Coleman (1994). "Economically important organic acid and enzyme products." Prog Ind Microbiol **29**: 561-602.

Braaksma, M., A. K. Smilde, M. J. van der Werf and P. J. Punt (2009). "The effect of environmental conditions on extracellular protease activity in controlled fermentations of Aspergillus niger." Microbiology **155**(Pt 10): 3430-3439.

Clutterbuck, A. J. (1992). "Sexual and parasexual genetics of Aspergillus species." Biotechnology (Reading, Mass **23**: 3-18.

Conesa, A., P. J. Punt, N. van Luijk and C. A. van den Hondel (2001). "The secretion pathway in filamentous fungi: a biotechnological view." Fungal Genet Biol **33**(3): 155-171.

Damásio, A. R. d. L., T. M. Silva, F. B. d. R. Almeida, F. M. Squina, D. A. Ribeiro, A. F. P. Leme, F. Segato, R. A. Prade, J. A. Jorge, H. F. Terenzi and M. d. L. T. M. Polizeli (2011).

"Heterologous expression of an Aspergillus niveus xylanase GH11 in Aspergillus nidulans and its characterization and application." Process Biochemistry **46**(6): 1236-1242.

de Vries, R. P., K. Burgers, P. J. van de Vondervoort, J. C. Frisvad, R. A. Samson and J. Visser (2004). "A new black Aspergillus species, A. vadensis, is a promising host for homologous and heterologous protein production." Appl Environ Microbiol **70**(7): 3954-3959.

Ferreira-Nozawa, M. S., H. C. Silveira, C. J. Ono, A. L. Fachin, A. Rossi and N. M. Martinez-Rossi (2006). "The pH signaling transcription factor PacC mediates the growth of Trichophyton rubrum on human nail in vitro." Med Mycol **44**(7): 641-645.

Fleissner, A. and P. Dersch (2010). "Expression and export: recombinant protein production systems for Aspergillus." Appl Microbiol Biotechnol **87**(4): 1255-1270.

Hammond, T., J. Bok, M. Andrewski, Y. Reyes-Dominguez, C. Scazzocchio and N. J. E. c. Keller (2008). "RNA silencing gene truncation in the filamentous fungus Aspergillus nidulans." **7**(2): 339-349.

Hammond, T. M. and N. P. J. G. Keller (2005). "RNA silencing in Aspergillus nidulans is independent of RNA-dependent RNA polymerases." **169**(2): 607-617.

Han, M. J., N. J. Kim, S. Y. Lee and H. N. Chang (2010). "Extracellular proteome of Aspergillus terreus grown on different carbon sources." Curr Genet **56**(4): 369-382.

Jaskiewicz, L. and W. Filipowicz (2008). "Role of Dicer in posttranscriptional RNA silencing." Curr Top Microbiol Immunol **320**: 77-97.

Johnson, M., I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis and T. L. Madden (2008). "NCBI BLAST: a better web interface." Nucleic Acids Res **36**(Web Server issue): W5-9.

Katz, M. E., S. M. Bernardo and B. F. Cheetham (2008). "The interaction of induction, repression and starvation in the regulation of extracellular proteases in Aspergillus nidulans: evidence for a role for CreA in the response to carbon starvation." Curr Genet **54**(1): 47-55.

Kuck, U. and B. Hoff (2010). "New tools for the genetic manipulation of filamentous fungi." Appl Microbiol Biotechnol **86**(1): 51-62.

Marshall, T. and K. M. Williams (1992). "Coomassie blue protein dye-binding assays measure formation of an insoluble protein-dye complex." Anal Biochem **204**(1): 107-109.

Matsushita-Morita, M., S. Tada, S. Suzuki, R. Hattori, J. Marui, I. Furukawa, Y. Yamagata, H. Amano, H. Ishida, M. Takeuchi, Y. Kashiwagi and K. Kusumoto (2011). "Overexpression and characterization of an extracellular leucine aminopeptidase from Aspergillus oryzae." Curr Microbiol **62**(2): 557-564.

Meyer, V., B. Wu and A. F. Ram (2011). "Aspergillus as a multi-purpose cell factory: current status and perspectives." Biotechnol Lett **33**(3): 469-476.

Miller, G. L. (1959). "Use of dintirosalicilic acid reagent for determination of reducing sugar." Analytical Chemistry **31**: 426-428.

Mittal, P., R. Yadav, R. Devi, S. Sharma and A. Goyal (2012). Phenomenal RNA interference: from mechanism to application. Crop Plant, IntechOpen.

Nakayashiki, H., N. Kadotani and S. J. J. o. m. e. Mayama (2006). "Evolution and diversification of RNA silencing proteins in fungi." **63**(1): 127-135.

Nevalainen, K. M., V. S. Te'o and P. L. Bergquist (2005). "Heterologous protein expression in filamentous fungi." Trends Biotechnol **23**(9): 468-474.

Nieto, A., J. A. Prieto and P. Sanz (1999). "Stable high-copy-number integration of Aspergillus oryzae alpha-AMYLASE cDNA in an industrial baker's yeast strain." Biotechnol Prog **15**(3): 459-466.

Pena-Montes, C., A. Gonzalez, D. Castro-Ochoa and A. Farres (2008). "Purification and biochemical characterization of a broad substrate specificity thermostable alkaline protease from Aspergillus nidulans." Appl Microbiol Biotechnol **78**(4): 603-612.

Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. D. McDonald and A. W. J. Bufton (1953). "The genetics of *Aspergillus nidulans*." Adv. Genet. **5**: 141-238.

Punt, P. J., F. H. Schuren, J. Lehmbeck, T. Christensen, C. Hjort and C. A. van den Hondel (2008). "Characterization of the Aspergillus niger prtT, a unique regulator of extracellular protease encoding genes." Fungal Genet Biol **45**(12): 1591-1599.

Punt, P. J., N. van Biezen, A. Conesa, A. Albers, J. Mangnus and C. van den Hondel (2002). "Filamentous fungi as cell factories for heterologous protein production." Trends Biotechnol **20**(5): 200-206.

Record, E., M. Asther, C. Sigoillot, S. Pages, P. J. Punt, M. Delattre, M. Haon, C. A. van den Hondel, J. C. Sigoillot and L. Lesage-Meessen (2003). "Overproduction of the Aspergillus niger feruloyl esterase for pulp bleaching application." Appl Microbiol Biotechnol **62**(4): 349-355.

Romano, N. and G. J. M. m. Macino (1992). "Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences." **6**(22): 3343-3353.

Santos, C. R., J. H. Paiva, A. N. Meza, J. Cota, T. M. Alvarez, R. Ruller, R. A. Prade, F. M. Squina and M. T. Murakami (2011). "Molecular insights into substrate specificity and thermal stability of a bacterial GH5-CBM27 endo-1,4-beta-d-mannanase." J Struct Biol.

Segato, F., A. R. Damasio, T. A. Goncalves, M. T. Murakami, F. M. Squina, M. L. Polizeli, A. Mort and R. A. Prade (2012). "Two structurally discrete GH7-cellobiohydrolases compete for the same cellulosic substrate fiber." Biotechnol Biofuels **5**(1): 21.

Segato, F., A. R. L. Damásio, T. A. Gonçalves, R. C. de Lucas, F. M. Squina, S. R. Decker and R. A. Prade (2012). "High-yield secretion of multiple client proteins in Aspergillus." Enzyme and Microbial Technology(0).

Shapiro, A. L., E. Vinuela and J. V. Maizel, Jr. (1967). "Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels." Biochem Biophys Res Commun **28**(5): 815-820.

Sims, A. H., M. E. Gent, K. Lanthaler, N. S. Dunn-Coleman, S. G. Oliver and G. D. Robson (2005). "Transcriptome analysis of recombinant protein secretion by Aspergillus nidulans and the unfolded-protein response in vivo." Appl Environ Microbiol **71**(5): 2737-2747.

Squina, F. M., A. J. Mort, S. R. Decker and R. A. Prade (2009). "Xylan decomposition by Aspergillus clavatus endo-xylanase." Protein expression and purification **68**(1): 65-71.

Sriranganadane, D., P. Waridel, K. Salamin, M. Feuermann, B. Mignon, P. Staib, J. M. Neuhaus, M. Quadroni and M. Monod (2011). "Identification of novel secreted proteases during extracellular proteolysis by dermatophytes at acidic pH." Proteomics **11**(22): 4422-4433.

van den Hondel, C. A., P. J. Punt and R. F. van Gorcom (1992). "Production of extracellular proteins by the filamentous fungus Aspergillus." Antonie van Leeuwenhoek **61**(2): 153-160.

Verdoes, J. C., P. J. Punt, A. H. Stouthamer and C. A. van den Hondel (1994). "The effect of multiple copies of the upstream region on expression of the Aspergillus niger glucoamylase-encoding gene." Gene **145**(2): 179-187.

Wang, H., F. Squina, F. Segato, A. Mort, D. Lee, K. Pappan and R. Prade (2011). "High-temperature enzymatic breakdown of cellulose." Appl Environ Microbiol **77**(15): 5199-5206.

Wang, Y., W. Xue, A. H. Sims, C. Zhao, A. Wang, G. Tang, J. Qin and H. Wang (2008). "Isolation of four pepsin-like protease genes from Aspergillus niger and analysis of the effect of disruptions on heterologous laccase expression." Fungal genetics and biology **45**(1): 17-27.

Ward, O. P. (2011). "Production of recombinant proteins by filamentous fungi." Biotechnol Adv.

Wiebe, M. G., G. D. Robson, J. Shuster and A. P. Trinci (2001). "Evolution of a recombinant (gucoamylase-producing) strain of Fusarium venenatum A3/5 in chemostat culture." Biotechnol Bioeng **73**(2): 146-156.

Yelton, M. M., J. E. Hamer and W. E. Timberlake (1984). "Transformation of Aspergillus nidulans by using a trpC plasmid." Proceedings of the National Academy of Sciences of the United States of America **81**(5): 1470-1474.

Yoon, J., S. Kimura, J. Maruyama and K. Kitamoto (2009). "Construction of quintuple protease gene disruptant for heterologous protein production in Aspergillus oryzae." Appl Microbiol Biotechnol **82**(4): 691-701.

Yoon, J., J. Maruyama and K. Kitamoto (2011). "Disruption of ten protease genes in the filamentous fungus Aspergillus oryzae highly improves production of heterologous proteins." Appl Microbiol Biotechnol **89**(3): 747-759.

# CHAPTER IV

# SILENCING THE AMYLOLYTIC PATHWAY IN *ASPERGILLUS NIDULANS* WITH A SINGLE-STEP GENETIC INTERVENTION

## 4.1. Introduction

The use of short interfering RNAs to silence gene expression of genes in fugus has proven to be a useful tool for functional genomics. The search for RNA interference (RNAi) components in fungal genomes has revealed that RNA silencing pathways are conserved across various fungal taxa including *Aspergillus nidulans*. (Hammond and Keller 2005, Barton and Prade 2008, Van Den Berg and Maruthachalam 2015). For functional genomics work in fungus, using RNA interference as an alternative to conventional knockouts.

RNAi vectors producing hairpin RNA (hpRNA) and intron containing hairpin RNA (ihpRNA) usually require two steps of direction-oriented cloning of the target gene fragment which can be time consuming. The usefulness of these vectors has therefor been limited to small scale analysis. A less costly alternative is to use a dual promoter system in which sense and antisense transcripts are individually expressed under the control of two opposing RNA polymerase II promoters, thus expressing a double stranded RNA (dsRNA). (Barton and Prade 2008, Van Den Berg and Maruthachalam 2015)

However the main drawback to the of the dual promoter system is that there is a lower RNAi efficiency compared with hpRNA or ihpRNA expressing vectors. (Nguyen, Itoh et al. 2011) In the dual promoter vectors the annealing of the two separately transcribed RNA molecules is required to form the dsRNA necessary for silencing. While in hpRNA and ihpRNA vectors a single strand of RNA is self-folding inverted repeats within a single molecule. (Nakayashiki 2005) The difference in formation of the dsRNA between the two systems is responsible for the change in efficiency. (Van Den Berg and Maruthachalam 2015) In *Magnaporthe oryzae*, the RNAi vector pSilent-dual 1 (pSD1), containing opposing *A. nidulans trpC* and *gpdA* promoters, was shown to induce GFP silencing at moderate levels. (Nguyen, Kadotani et al. 2008) In *Histoplasma capsulatum*, an RNAi vector with opposing promoters triggered only a moderate silencing of a GFP reporter gene with a 35% reduction on average. (Rappleye, Engle et al. 2004)

In our previous work we have established that a single gene can be silenced using an inducible RNA interference vector and that a metabolic network can be partially silenced with a single genetic intervention. An improvement in dual promoter systems is the inducible-RNAi technique, based on the use of inducible promoters, which act as a switch to control the expression of the RNAi construct. The *alcA* promoter, inducible by threonine and repressed by glucose, successfully silenced the *brlAβ* gene of *a. nidulans* when the fungus is grown on inducing medium. (Barton and Prade 2008)

However, RNAi-induced silencing of fungal gene expression at the transcriptional level is found to be incomplete in many other cases. This leaves the space for improvements to be made to this problem. (Jiang, Zhu et al. 2013) For example, enhancing transcription of the dsRNA by the RNAi construct. This can be accomplished by either increasing the copy numbers of the vector or enhancing the expression of the RNAi vector. (Jiang, Zhu et al. 2013) Enhancing

the RNAi expression and using an hpRNA structure, the complete downregulation of alb1 in *Aspergillus fumigatus* was accomplished. (Khalaj, Eslami et al. 2007) Similarly the increased expression of RNA-dependent RNA polymerase enhanced the silencing effect of the *wA* via a vector initiated RNAi in *Aspergillus oryzae.* (Fernandez, Moyer et al. 2012) Overall, RNAi shows incredible promise and high potential as a target dependent tool.

The following experiments outline an attempt to optimize the RNAi silencing pathway in *Aspergillus nidulans* using different promoters to silence a model metabolic pathway. Firstly, promoter activity has been shown to play a role in the efficacy of the RNAi silencing pathway. (Khalaj, Eslami et al. 2007) The *gpdA* promoter in *A.* nidulans have been shown to be very powerful for protein expression so we intend to use it to increase our levels of transcription for our silencing construct. (Punt, Dingemanse et al. 1990) We have also shown previously that the inducible *xynC* promoter is also a powerful tool to regulate expression for our silencing construct. By increasing transcriptional levels of our dsRNA we hope to increase the efficacy of RNAi silencing of our target genes.

Secondly, we have chosen to silence the amylolytic pathway as a model due to the ease of testing enzyme activity. Since previous experiments which targeted proteases did not target all proteases in the genome, an incomplete reduction of protease activity was observed. Conversely there are only 16 genes in the amylolytic pathway in *A. nidulans*, which makes targeting and silencing all of the genes feasible. *A. nidulans* possesses: 7 α-glucosidases (*agdA-F*), 7 α-amylases (*amyA-F*), and 2 glucoamylases (*glaA* and *glaB*). (Nakamura, Maeda et al. 2006) All sixteen genes in the amylolytic pathway have been targeted with 42 bp complementary double stranded RNA in our construct AmSil.

By increasing the expression of RNAi and by targeting a system that is better characterized, we hope to elucidate a more efficient method for silencing multiple genes through a single genetic intervention.

## 4.2. Materials and Methods

### *4.2.1. Strain construction*

Standard *A. nidulans* minimal medium (MM) and general cultivation techniques were used throughout this work and are based on the work by Guido Pontecorvo (Pontecorvo, Roper et al. 1953, Pontecorvo 1969) and John Clutterbuck (Clutterbuck 1992). All strains constructed in this work are were derived from *A. nidulans* A773 (*wA3*, *pyrG89*, *pyroA4*) purchased from the Fungal Genetics Stock Center (FGSC, St. Louis, MO). All gene models and promoters were from *Aspergillus nidulans* FGSC4 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000149205.2) and analyzed using the AspGD database (http://aspgd.org (Cerqueira, Arnaud et al. 2014)) Primers and Gibson Assembly hybrid primers were designed using the NEB Builder Assembly Tool (http://nebuilder.neb.com).

Silencing vectors were assembled into a pUC19 backbone flanked with 1kb homologous sequences to the upstream and downstream regions of the *pabaA*(AN6550) gene in *A. nidulans*. Homologous integration of the construct will result in strains that are unable to synthesize para-aminobenzoic acid. A functional *pyrG* gene (AN6157) is included to complement the *pyrG89* mutation in *A. nidulans* A773. An inverted promoter system flanks a silencing construct (AmSil) constituted of 42mers complementary to all 16 genes in the amylolytic pathway of *A. nidulans*. AmSil is synthesized by IDT's gBlock synthetic gene construction service (IDT, US). All fragments are assembled into a circular vector using Gibson Assembly Technology, GAT (Gibson 2011, Gibson 2014) using hybrid primers, Gibson Assembly Master Mix (New England

Biolabs, US) and Phusion DNA Polymerase (New England Biolabs, US). Resulting vectors are *pUC19::PabaAUpstream::promoter_forward::AmSil::promoter_reverse::pyrG::pabaADownstream*. Two silencing constructs were generated, differing only in their promoters. A silencing vector with dual *gpdA* promoters, *gpdAp/gpdAp* (Primer sequencing in Table 4.1), was successfully assembled, as well as a hybrid *gpdAp/xynCp* vector (Primer sequences in Table 4.2). DNA fragment size and DNA sequence verified hybrid DNA fragments were transformed into A773 protoplasts according to methods by Szewczyk et al. (Szewczyk, Nayak et al. 2006)

**Table 4.1**- gpdAp/gpdAp silencing construct primer sequences

| **gpdAp/gpdAp silencing construct primers** | | |
|---|---|---|
| paba_Up_fwd | ATGGCACCGCTCCTTGGTGATTGG | 3'Tm=76.9 3'Ta(annealing temp)=72.0 |
| paba_Up_rev | agagtcaccggtcacTTGGCACAGAAATAATGCTGTATCTGCCATTC | 3'Tm=74.4 3'Ta(annealing temp)=72.0 |
| gpdAp_fwd | ttatttctgtgccaaGTGACCGGTGACTCTTTCTGGCAT | 3'Tm=71.3 3'Ta(annealing temp)=71.3 |
| gpdAp_rev | cggccgcaatgcaatGGGAAAAGAAAGAGAAAAGAAAAGAGCAG | 3'Tm=68.3 3'Ta(annealing temp)=71.3 |
| AmSil_fwd | tctctttcttttcccATTGCATTGCGGCCGCCGACTGTAG | 3'Tm=79.4 3'Ta(annealing temp)=72.0 |
| AmSil_rev | tctctttcttttcccAATGCAATGCGGCCGCACGGTGTT | 3'Tm=80.9 3'Ta(annealing temp)=72.0 |
| gpdAp_RC_fwd | cggccgcattgcattGGGAAAAGAAAGAGAAAAGAAAAGAGCAG | 3'Tm=68.3 3'Ta(annealing temp)=71.3 |
| gpdAp_RC_rev | gttgcgttcccaaaaGTGACCGGTGACTCTTTCTGGCAT | 3'Tm=71.3 3'Ta(annealing temp)=71.3 |
| pyrG_fwd | agagtcaccggtcacTTTTGGGAACGCAACTTCCTCGAG | 3'Tm=72.5 3'Ta(annealing temp)=71.7 |
| pyrG_rev | atgtccatacaacagCCCCTTTTAGTCAATACCGTTACACATTTC | 3'Tm=68.7 3'Ta(annealing temp)=71.7 |
| paba_Down_fwd | attgactaaaaggggCTGTTGTATGGACATTCTGCCAAAACC | 3'Tm=70.4 3'Ta(annealing temp)=72.0 |
| paba_Down_rev | TCATTTCGCGTGCGAAGGCATAAA | 3'Tm=74.2 3'Ta(annealing temp)=72.0 |

**Table 4.2**- gpdAp/xynCp silencing construct primer sequences

| xynCp/xynCp silencing construct primers | | |
|---|---|---|
| Puc_rev | aaggagcggtgccatCTAGAGTCGACCTGCAGGCATGCAAGCTTG | 3'Tm=78.5 3'Ta(annealing temp)=72.0 |
| Paba_Up_fwd | gcaggtcgactctagATGGCACCGCTCCTTGGTGATTGG | 3'Tm=76.9 3'Ta(annealing temp)=72.0 |
| Paba_Up_rev | tagtgtcacctaaatTTGGCACAGAAATAATGCTGTATCTGCCATTC | 3'Tm=74.4 3'Ta(annealing temp)=72.0 |
| gpdA_Fwd | atttctgtgccaaGTGACCGGTGACTCTTTCTGGCAT | 3'Tm=69.1 3'Ta(annealing temp)=72.0 |
| gpdA_Rev | gccgcaatgcaatGGGAAAAGAAAGAGAAAAGAAAAGAGCAG | 3'Tm=80.3 3'Ta(annealing temp)=72.0 |
| AmSil_fwd | gcagacaccgcgctcATTGCATTGCGGCCGCCGACTGTAG | 3'Tm=79.4 3'Ta(annealing temp)=72.0 |
| AmSil_rev | caactcttacccaaaAATGCAATGCGGCCGCACGGTGTT | 3'Tm=80.9 3'Ta(annealing temp)=72.0 |
| XynCp_fwd | cggccgcattgcattTTTGGGTAAGAGTTGAACGATGGAAG | 3'Tm=68.5 3'Ta(annealing temp)=71.5 |
| XynCp_rev | gttgcgttcccaaaaAGGTTCTTTGTTTTCACAGCATGAATG | 3'Tm=68.5 3'Ta(annealing temp)=71.5 |
| pyrG_fwd | gaaaacaaagaacctTTTTGGGAACGCAACTTCCTCGAG | 3'Tm=72.5 3'Ta(annealing temp)=71.7 |
| pyrG_rev | atgtccatacaacagCCCCTTTTAGTCAATACCGTTACACATTTC | 3'Tm=68.7 3'Ta(annealing temp)=71.7 |
| Paba_Down_fwd | attgactaaaaggggCTGTTGTATGGACATTCTGCCAAAACC | 3'Tm=70.4 3'Ta(annealing temp)=72.0 |
| Paba_Down_rev | gtacccggggatcctTCATTTCGCGTGCGAAGGCATAAA | 3'Tm=74.2 3'Ta(annealing temp)=72.0 |
| Puc_fwd | tcgcacgcgaaatgaAGGATCCCCGGGTACCGAGCTCGA | 3'Tm=78.1 3'Ta(annealing temp)=72.0 |

### *4.2.2 DNA mediated transformation of A. nidulans*

DNA mediated transformations were based on the methods of Szewczyk et al. (Szewczyk, Nayak et al. 2006) $1x10^8$ spores of A773 were grown for 12-14 hours at 30 °C on a gyratory shaker at 120 r.p.m. in YG media (5 g $L^{-1}$ yeast extract and 20 g $L^{-1}$ D-glucose) with the necessary supplements. Young mycelia were filtered through sterile miracloth and washed and resuspended in new YG media. A filter sterilized 2x protoplasting solution (1.1M KCl, 0.1M citric acid, pH 5.8) with VinoFlow CE (Guzman Enterprises, US) is added to mycelia and mixed by swirling at 100 r.p.m. for 2 hours to form protoplasts. Undigested hyphal material is removed

by centrifuging for 10 minutes at 4 °C for 10 minutes with no brake. Protoplasts form a layer on top of the undigested hyphal material. Protoplasts are collected in a sterile centrifuge tube and washed thrice with sterile 0.6M KCl. Protoplasts are then washed and resuspended in 0.6M KCl, 50 mM $CaCl_2$ for transformation.

1 μg of linear or plasmid DNA in TE buffer is added to 100 μl of protoplast suspension and vortexed 6 times for 1 second. 50 μl of 25% PEG solution (25% PEG4000 w/v, 0.6M KCl, 50 mM $CaCl_2$) was added to protoplast and DNA suspension, vortexed 6 times for 1 second, and incubated on ice for 30 minutes. 1 ml of room temperature 25% PEG solution is mixed in by pipetting 5 times and protoplast solution is incubated at room temperature for 30 minutes. Transformation mixture is then plated on selective media plates (containing para-aminobenzoic acid, uracil, and uridine) with 1.2M sorbitol and incubated agar side down at 30 °C over night. Plates are then flipped and incubated at 37 °C for 48 hours. Carbon sources in the selective media were altered to allow for growth of mutants without amylolytic pathway.

Transformants were screened for uracil/uridine sufficiency as well as a *pabaA* deficiency. The DNA fragment was integrated into the *pabaA* locus by a double crossover event disrupting it. Recombinants with a single gene replacement event were searched with diagnostic PCR.

*4.2.3 Preparation of total extracellular protein extracts.*

Mycelia are grown on minimal media with varying carbon sources at a final concentration of 1% (w/v). *xynC* promoters are induced with various xylose containing substrates at 1% (w/v). Unless otherwise stated, 5 mL of extracellular fluid (medium) harvested from mycelia grown for 12, 24, and 36 h were treated with 3kDa cutoff Nanosep® ultrafiltration

Omega™ membrane columns (PALL Corp. USA) and washed with 500 µL of 50 mM ammonium acetate ($NH_4CH_3CO_2$) buffer pH 5 before 10X concentration to a final volume of 50 µL. Samples that were not concentrated 5 mL of extracellular fluid was treated as described above and resuspended to the original volume (5 mL) with 50 mM ammonium acetate buffer pH 5.

*4.2.4 Protein quantification and SDS-polyacrylamide gel electrophoresis.*

Total protein content was measured in microtiter dishes using the Bio-Rad assay reagent (Bio-Rad Laboratories, USA), using a procedure based on the Bradford method (Bradford 1976, Marshall and Williams 1992) with bovine serum albumin as standard. Absorption was measured using a UV-Vis 96-well plate reader (Tecan Infinite M200, Männedorf, Switzerland) at 595 nm. Quality of total extracellular protein extracts was validated for integrity by SDS polyacrylamide gel electrophoresis according to Shapiro (Shapiro, Vinuela et al. 1967).

*4.2.5 Free sugar (reducing end) determinations.*

Free sugar determinations were used in two types of experiments: 1) to determine the activity of enzymes that use a non-reducing substrate releasing reducing products (sugars) and 2) to quantitate the amount of reducing sugar accumulated by enzymes added to a starch degradation assay. In both cases we used the DNS assay developed by Sumner and Graham (Summer and Graham 1921) for detection of reducing sugars. The DNS reducing sugar assay was based on the method described by Miller (Miller 1959) and adapted to a microtiter dish scale. The DNS reagent we used contained 0.75% di-nitrosalicylic acid, 0.5% phenol, 0.5% sodium metabisulfite, and 1.4% sodium hydroxide, 21% sodium and potassium tartarate.

*4.2.6 Determination of enzyme activities.*

**Amylase** and **alpha glucosidase** activities were determined using soluble starch as a substrate, respectively and activity measured by the release of reducing sugars that react with DNS (Miller 1959). Briefly to 300 µL of 1% soluble starch, 50 mM ammonium acetate buffer 10-50 µL of total extracellular protein extract (treated as described in 2.3) was added and reactions incubated for 120 mins at 55 $^{0}$C prior to the addition of 300 µL of DNS. Control reactions (blanks that determine the presence of reducing sugars in the starting mixture) contained all the same reagents except that DNS was added prior to the addition of enzyme sample. To determine the amount of reducing sugar produced during the enzyme catalyzed reaction the $ABS^{540nm}$ of the control was subtracted from the enzyme reaction and resulting net gain in $ABS^{540nm}$ converted into enzyme units µMOL/min/µg. protein.

## 4.3. Results and Discussion

The overall objective was to optimize a parallel gene-silencing tool for systems biology, metabolic pathway, and cellular process engineering. Previously we have applied this method to the proteolytic pathway in *A. nidulans* with limited success, resulting in only partial silencing of the targeted genes. Issues with our previous construct were thought to be due to promoter strength limiting the amount of dsRNA being expressed, and from targeting a select few of the genes allowing the fungus to compensate for loss of activity with other analogous genes. We have addressed these concerns by attempting to use other, stronger, promoters. We have also attempted to silence the more feasible amylolytic pathway, which contains only 16 genes.

*4.3.1 Construction of the amylolytic silencing vector pAmSil*

In order to optimize the use of a dual promoter system to silence genes in *A. nidulans* we have targeted all 16 genes in the amylolytic pathway (Table 4.3) Within the silencing construct AmSil there are 16 42mers, each complementary to a gene in the amylolytic pathway. AmSil is then flanked by two promoters in order to induce the formation of double stranded RNA (dsRNA) which will be processed by the RNAi silencing mechanisms in *A. nidulans*. Two separate constructs were constructed with different pairs of promoters.

First a *gpdAp* and *xynCp* were used to flank the AmSil construct. *xynCp* is an inducible promoter, allowing us to use xylose, xylan, and a pretreated biomass hydrolysate as inducers. (Tamayo, Villanueva et al. 2008) The second promoter set was dual *gpdA* promoters. *gpdAp* is a very strong promoter that has previously been used to express proteins and RNA at very high levels. (Punt, Zegers et al. 1991) AmSil and its two inverted promoters are flanked with a functional *pyrG* sequence and 1000bp of the regions upstream and downstream of *pabaA.* Due to the presence of these homologous sequences the *pabaA* gene will be replaced with our silencing construct upon successful integration into the host genome. (Szewczyk, Nayak et al. 2006) Transformants will be have uracil and uridine sufficiency, and deficient in para-aminobenzoic acid production. AmSil and the surrounding construct are displayed in Figure 4.1.

**Table 4.3 –** Genes targeted by AmSil

| Accession # | | Gene Name | Mode | GH Family |
|---|---|---|---|---|
| XM_654529.1 | AN2017.2 partial mRNA | *agdA* | a-glucosidase | 31 |
| XM_677130.1 | AN8953.2 partial mRNA | *agdB* | a-glucosidase | 31 |
| XM_675522.1 | AN7345.2 partial mRNA | *agdC* | a-glucosidase | 31 |
| XM_675682.1 | AN7505.2 partial mRNA | *agdD* | a-glucosidase | 31 |
| XM_653453.1 | AN0941.2 partial mRNA | *agdE* | a-glucosidase | 31 |
| XM_656028.1 | AN3516.2 partial mRNA | *agdF* | a-glucosidase | 13 |
| XM_657355.1 | AN4843.2 partial mRNA | *agdG* | a-glucosidase | 13 |
| XM_654530.1 | AN2018.2 partial mRNA | *amyA* | a-amylase | 13 |
| XM_655914.1 | AN3402.2 partial mRNA | *amyB* | a-amylase | 13 |
| XM_657019.1 | AN4507.2 partial mRNA | *amyC* | a-amylase | 13 |
| XM_655820.1 | AN3308.2 partial mRNA | *amyD* | a-amylase | 13 |
| XM_658836.1 | AN6324.2 partial mRNA | *amyE* | a-amylase | 13 |
| XM_655900.1 | AN3388.2 partial mRNA | *amyF* | a-amylase | 13 |
| XM_655821.1 | AN3309.2 partial mRNA | *amyG* | a-amylase | 13 |
| XM_677081.1 | AN8904.2 partial mRNA | *glaA* | Glucoamylase | 15 |
| XM_675579.1 | AN7402.2 partial mRNA | *glaB* | Glucoamylase | 15 |

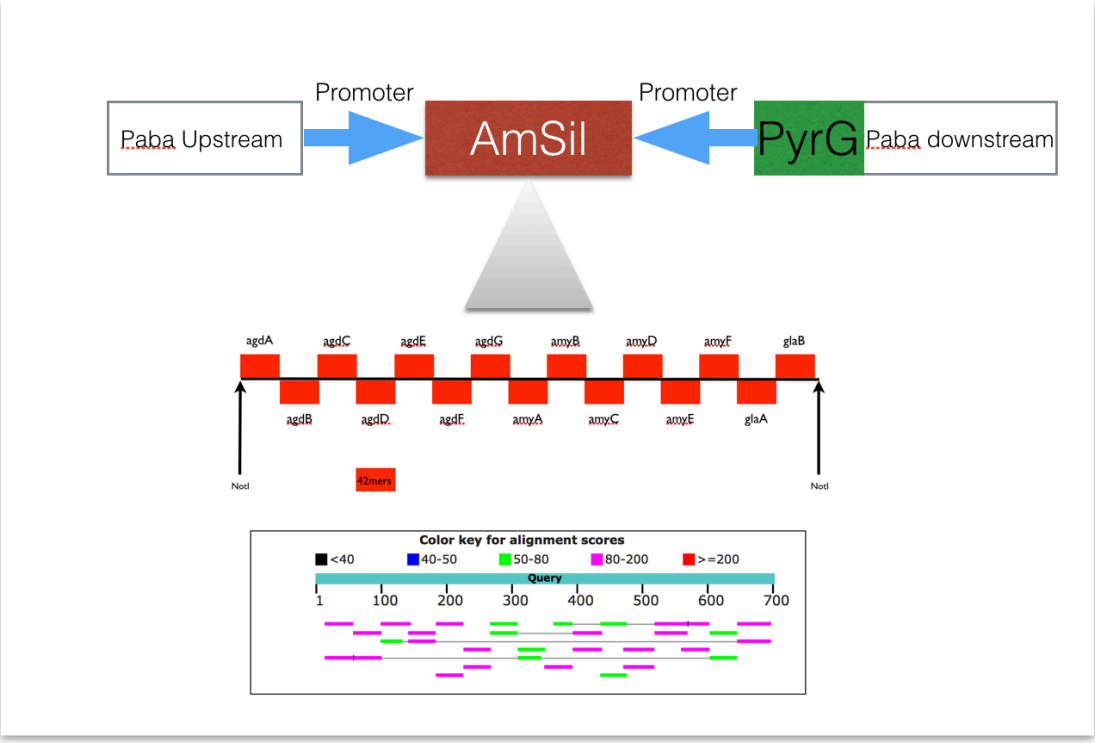



**Figure 4.1** – Construction of pAmSil and BLASTn output of AmSil targets

The lineup of DNA elements in pAmSil. The second promoter sequence is either *gpdAp* or *xynCp*. The AmSil synthetic gBlock sequence was submitted to NCBI BLASTn to ensure that each 42mer correctly targeted its complementary gene in the amylolytic pathway without any off-target effects.

### *4.3.2 Lethal effect of pAmSil gpdAp/gpdAp on A. nidulans*

Viable transformants were never recovered with AmSil containing dual inverted *gpdAp* promoters. The *gpdA* promoter is a highly expressed constitutive promoter in *A. nidulans*. Multiple carbon sources were used in the selection media including 1% glucose, 1% glycerol, 1% yeast extract, 1% casamino acids, and 1% soy peptone. The use of these carbon sources alone or

in combination did not change the transformation efficiency of *A. nidulans* with pAmSil *gpdAp/gpdAp.* The inability to recover viable transformants

*4.3.3 Effect of pAmSil gpdAp/xynCp on the extracellular amylolytic profile*

In order to titrate the amount of RNA being expressed, a construct using the constitutive *gpdA* promoter and the inducible *xynC* promoter was integrated into the *A. nidulans pabaA* locus. One transformant was confirmed via PCR with primers outside of the locus of homologous integration. The transformant and the reference strain were grown on various xylose sources to induce the expression of AmSil and the amylolytic activities of the extracellular space were measured at 12, 24 and 36 hours. Strains were grown on minimal media containing 1% maltose as a positive control as maltose is shown to induce the expression of various amylolytic enzymes under the control of the transcription activator *amyR*. (Tani, Katsuyama et al. 2001, Kato, Murakoshi et al. 2002) Strains were also grown on minimal media containing 1% maltose and 1% glucose as a negative control. The presence of glucose will repress *amyR* activity through the carbon catabolite repressor *creA*. (Tani, Katsuyama et al. 2001)

At 12 hours, the extracellular media was collected and an enzyme activities were measured with 1% soluble starch as a substrate (Figure 4.2). Upon the addition of any of the xylose sources to the media the amylolytic activity of the strain containing pAmSil drops, but this also occurs in the reference strain (A773). The addition of xylose or xylan does not cause a statistically significant change in the amylolytic activity in the silenced strain as compared to the reference strain. This indicates that xylose may be a preferred carbon source to maltose and induce a carbon catabolite repression effect similar to glucose.

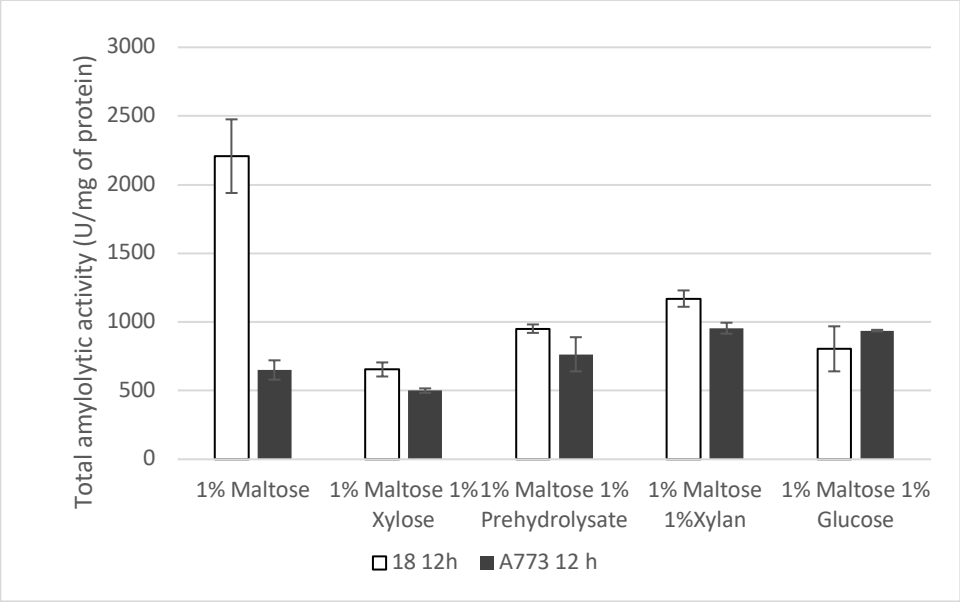



**Figure 4.2**- The enzyme activities on 1% Soluble starch of silenced and non-silenced strains of *A. nidulans* at 12 hours

The carbon sources and *xynCp* inducers are added to glucose-free minimal media. The addition of any xylose source causes a similar silencing effect in the reference strain (closed bars) as it does in the strains containing pAmSil (open bars).

At 24 and 36 hours (Figures 4.3 and 4.4), under xylose induction the amylolytic activity is not completely silenced in the strain containing pAmSil. Though there is a small decrease in the activity of pAmSil compared to the non-induced growth condition it is not statistically significant. The amylolytic activity is also higher than if it had been repressed by glucose. The strain containing pAmSil was a transformant that was recovered on glucose. Recovering on glucose may have allowed for a strain with less expression of AmSil to persist when it would be otherwise lethal.

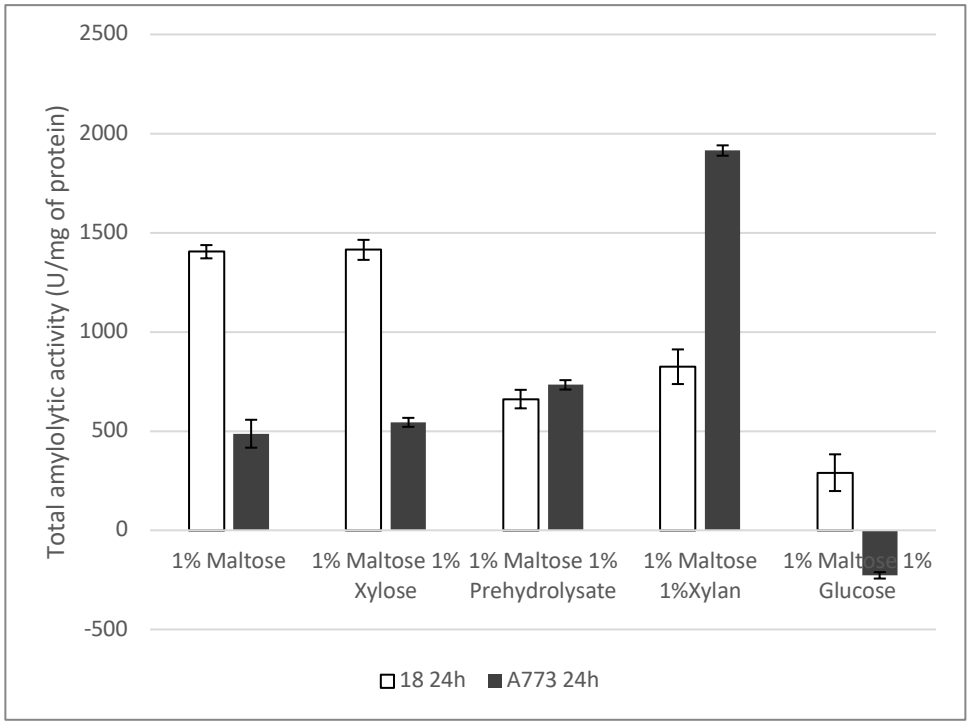



**Figure 4.3-** The enzyme activities on 1% Soluble starch of silenced and non-silenced strains of *A. nidulans* at 24 hours

The carbon sources and *xynCp* inducers are added to glucose-free minimal media. The addition of any xylose source causes a similar silencing effect in the reference strain (closed bars) as it does in the strains containing pAmSil (open bars).

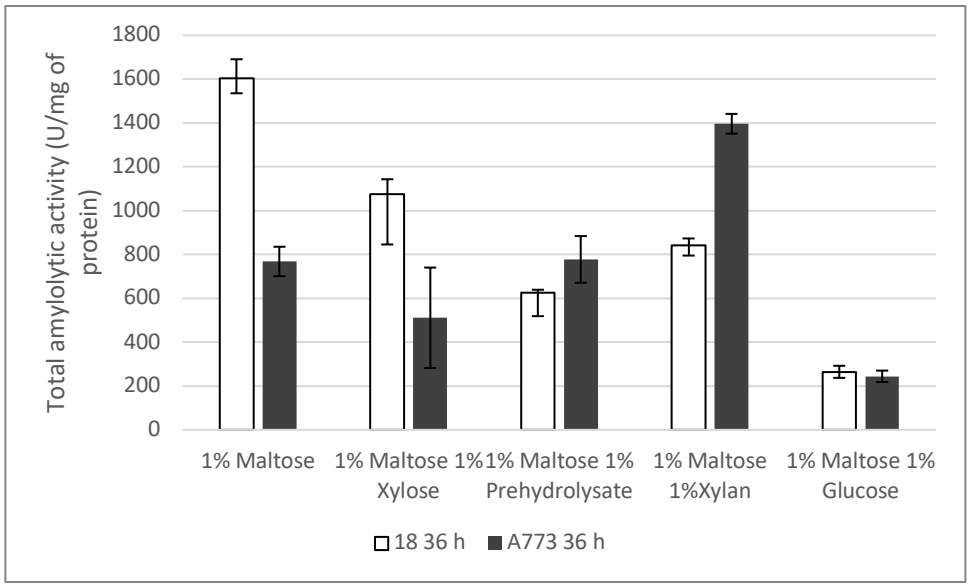



**Figure 4.4**- The enzyme activities on 1% Soluble starch of silenced and non-silenced strains of *A. nidulans* at 36 hours

The carbon sources and *xynCp* inducers are added to glucose-free minimal media. The addition of any xylose source causes a similar silencing effect in the reference strain (closed bars) as it does in the strains containing pAmSil (open bars).

*4.3.4 Discussion*

Using a single-step genetic intervention to reengineer entire metabolic pathways would be an incredibly useful tool for systems biology. Currently our attempts to optimize the dual promoter system to silence multiple genes by RNAi have not provided optimal results. We have been unable to recover viable transformants with our dual *gpdA* promoter system indicating that there may be a lethal consequence to knocking down all of the genes in the amylolytic pathway. Using the *xynC* promoter gives confounded results due to the apparent catabolite repression that occurs upon the addition of xylose sources to minimal media containing maltose.

In order to elucidate the potential lethal effects of pAmSil, a series of vectors can be obtained which serially remove one 42mer from AmSil. In order to elucidate the effects of our silencing vectors on the transcriptome it would also be helpful to perform RNAseq on the pAmSil transformants. Sequencing the short RNA's would also allow us to examine how efficiently our dsRNA is being incorporated into the RNAi pathway in *A. nidulans*.

**4.4. References**

Barton, L. M. and R. A. Prade (2008). "Inducible RNA Interference of brlAbeta in Aspergillus nidulans." Eukaryotic cell **7**(11): 2004-2007.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**(1–2): 248-254.

Cerqueira, G. C., M. B. Arnaud, D. O. Inglis, M. S. Skrzypek, G. Binkley, M. Simison, S. R. Miyasato, J. Binkley, J. Orvis, P. Shah, F. Wymore, G. Sherlock and J. R. Wortman (2014). "The Aspergillus Genome Database: multispecies curation and incorporation of RNA-Seq data to improve structural gene annotations." Nucleic Acids Res **42**(Database issue): D705-710.

Clutterbuck, A. J. (1992). "Sexual and parasexual genetics of Aspergillus species." Biotechnology **23**: 3-18.

Fernandez, E. Q., D. L. Moyer, S. Maiyuran, A. Labaro, H. J. F. g. Brody and biology (2012). "Vector-initiated transitive RNA interference in the filamentous fungus Aspergillus oryzae." **49**(4): 294-301.

Gibson, D. G. (2011). "Enzymatic assembly of overlapping DNA fragments." Methods Enzymol **498**: 349-361.

Gibson, D. G. (2014). "Programming biological operating systems: genome design, assembly and activation." Nat Methods **11**(5): 521-526.

Hammond, T. M. and N. P. J. G. Keller (2005). "RNA silencing in Aspergillus nidulans is independent of RNA-dependent RNA polymerases." **169**(2): 607-617.

Jiang, D., W. Zhu, Y. Wang, C. Sun, K.-Q. Zhang and J. J. B. a. Yang (2013). "Molecular tools for functional genomics in filamentous fungi: recent advances and new strategies." **31**(8): 1562-1574.

Kato, N., Y. Murakoshi, M. Kato, T. Kobayashi and N. J. C. g. Tsukagoshi (2002). "Isomaltose formed by α-glucosidases triggers amylase induction in Aspergillus nidulans." **42**(1): 43-50.

Khalaj, V., H. Eslami, M. Azizi, N. Rovira-Graells and M. J. F. m. l. Bromley (2007). "Efficient downregulation of alb1 gene using an AMA1-based episomal expression of RNAi construct in Aspergillus fumigatus." **270**(2): 250-254.

Marshall, T. and K. M. Williams (1992). "Coomassie blue protein dye-binding assays measure formation of an insoluble protein-dye complex." Anal Biochem **204**(1): 107-109.

Miller, G. L. (1959). "Use of dintirosalicilic acid reagent for determination of reducing sugar." Analytical Chemistry **31**: 426-428.

Nakamura, T., Y. Maeda, N. Tanoue, T. Makita, M. Kato, T. J. B. Kobayashi, biotechnology, and biochemistry (2006). "Expression profile of amylolytic genes in Aspergillus nidulans." 0610020104-0610020104.

Nakayashiki, H. J. F. l. (2005). "RNA silencing in fungi: mechanisms and applications." **579**(26): 5950-5957.

Nguyen, Q. B., K. Itoh, B. Van Vu, Y. Tosa and H. J. M. m. Nakayashiki (2011). "Simultaneous silencing of endo-β-1, 4 xylanase genes reveals their roles in the virulence of Magnaporthe oryzae." **81**(4): 1008-1019.

Nguyen, Q. B., N. Kadotani, S. Kasahara, Y. Tosa, S. Mayama and H. J. M. m. Nakayashiki (2008). "Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, Magnaporthe oryzae, using a high-throughput RNA-silencing system." **68**(6): 1348-1365.

Pontecorvo, G. (1969). "Genetic analysis of "somatic" cells in filamentous fungi." Wistar Inst Symp Monogr **9**: 1-8.

Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. D. Macdonald and A. W. Bufton (1953). "The genetics of Aspergillus nidulans." Adv Genet **5**: 141-238.

Punt, P. J., M. A. Dingemanse, A. Kuyvenhoven, R. D. Soede, P. H. Pouwels and C. A. J. G. van den Hondel (1990). "Functional elements in the promoter region of the Aspergillus nidulans gpdA gene encoding glyceraldehyde-3-phosphate dehydrogenase." **93**(1): 101-109.

Punt, P. J., N. D. Zegers, M. Busscher and P. H. J. J. o. b. Pouwels (1991). "Intracellular and extracellular production of proteins in Aspergillus under the control of expression signals of the highly expressed Aspergillus nidulans gpdA gene." **17**(1): 19-33.

Rappleye, C. A., J. T. Engle and W. E. J. M. m. Goldman (2004). "RNA interference in Histoplasma capsulatum demonstrates a role for α-(1, 3)-glucan in virulence." **53**(1): 153-165.

Shapiro, A. L., E. Vinuela and J. V. Maizel, Jr. (1967). "Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels." Biochem Biophys Res Commun **28**(5): 815-820.

Summer, J. B. and V. A. Graham (1921). "Dinitrosalicylic aid: a reagent for the estimation of sugar in normal and diabetic urine." J Biol Chem **47**: 5-9.

Szewczyk, E., T. Nayak, C. E. Oakley, H. Edgerton, Y. Xiong, N. Taheri-Talesh, S. A. Osmani and B. R. J. N. p. Oakley (2006). "Fusion PCR and gene targeting in Aspergillus nidulans." **1**(6): 3111.

Tamayo, E. N., A. Villanueva, A. A. Hasper, L. H. de Graaff, D. Ramon and M. Orejas (2008). "CreA mediates repression of the regulatory gene xlnR which controls the production of xylanolytic enzymes in Aspergillus nidulans." Fungal Genet Biol **45**(6): 984-993.

Tani, S., Y. Katsuyama, T. Hayashi, H. Suzuki, M. Kato, K. Gomi, T. Kobayashi and N. J. C. g. Tsukagoshi (2001). "Characterization of the amyR gene encoding a transcriptional activator for the amylase genes in Aspergillus nidulans." **39**(1): 10-15.

Van Den Berg, M. A. and K. Maruthachalam (2015). Genetic Transformation Systems in Fungi, Volume 2, Springer.

# REFERENCES


Ahamed, A. and P. Vermette (2008). "Enhanced enzyme production from mixed cultures of Trichoderma reesei RUT-C30 and Aspergillus niger LMA grown as fed batch in a stirred tank bioreactor." Biochemical Engineering Journal **42**(1): 41-46.

Amorim, H. V., M. L. Lopes, J. V. de Castro Oliveira, M. S. Buckeridge and G. H. Goldman (2011). "Scientific challenges of bioethanol production in Brazil." Appl Microbiol Biotechnol **91**(5): 1267-1275.

Arantes, V. and J. N. Saddler (2010). "Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis." Biotechnol Biofuels **3**(4): 1-11.

Arantes, V. and J. N. Saddler (2010). "Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis." Biotechnol Biofuels **3**(4): 4.

Bar-On, Y. M., R. Phillips and R. Milo (2018). "The biomass distribution on Earth." Proc Natl Acad Sci U S A **115**(25): 6506-6511.

Bartel, B. (2005). "MicroRNAs directing siRNA biogenesis." Nat Struct Mol Biol **12**(7): 569-571.

Barton, L. M. and R. A. Prade (2008). "Inducible RNA Interference of brlAbeta in Aspergillus nidulans." Eukaryotic cell **7**(11): 2004-2007.

Ahamed, A. and P. Vermette (2008). "Enhanced enzyme production from mixed cultures of Trichoderma reesei RUT-C30 and Aspergillus niger LMA grown as fed batch in a stirred tank bioreactor." Biochemical Engineering Journal **42**(1): 41-46.

Amorim, H. V., M. L. Lopes, J. V. de Castro Oliveira, M. S. Buckeridge and G. H. Goldman (2011). "Scientific challenges of bioethanol production in Brazil." Appl Microbiol Biotechnol **91**(5): 1267-1275.

Arantes, V. and J. N. Saddler (2010). "Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis." Biotechnol Biofuels **3**(4): 1-11.

Arantes, V. and J. N. Saddler (2010). "Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis." Biotechnol Biofuels **3**(4): 4.

Bar-On, Y. M., R. Phillips and R. Milo (2018). "The biomass distribution on Earth." Proc Natl Acad Sci U S A **115**(25): 6506-6511.

Bartel, B. (2005). "MicroRNAs directing siRNA biogenesis." Nat Struct Mol Biol **12**(7): 569-571.

Barton, L. M. and R. A. Prade (2008). "Inducible RNA Interference of brlAbeta in Aspergillus nidulans." Eukaryotic cell **7**(11): 2004-2007.

Baulcombe, D. C. (2007). "Molecular biology. Amplified silencing." Science **315**(5809): 199-200.

Bhat, M. and S. J. B. a. Bhat (1997). "Cellulose degrading enzymes and their potential industrial applications." **15**(3-4): 583-620.

Bhutto, A. W., K. Qureshi, K. Harijan, R. Abro, T. Abbas, A. A. Bazmi, S. Karim and G. Yu (2017). "Insight into progress in pre-treatment of lignocellulosic biomass." Energy **122**: 724-745.

Bischof, R. H., J. Ramoni and B. Seiboth (2016). "Cellulases and beyond: the first 70 years of the enzyme producer Trichoderma reesei." Microb Cell Fact **15**(1): 106.

Bodie, E. A., B. Bower, R. M. Berka and N. S. Dunn-Coleman (1994). "Economically important organic acid and enzyme products." Prog Ind Microbiol **29**: 561-602.

Braaksma, M., A. K. Smilde, M. J. van der Werf and P. J. Punt (2009). "The effect of environmental conditions on extracellular protease activity in controlled fermentations of Aspergillus niger." Microbiology **155**(Pt 10): 3430-3439.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**(1–2): 248-254.

Buker, S. M. and M. R. Motamedi (2011). "Purification of native Argonaute complexes from the fission yeast Schizosaccharomyces pombe." Methods Mol Biol **725**: 1-13.

Cerqueira, G. C., M. B. Arnaud, D. O. Inglis, M. S. Skrzypek, G. Binkley, M. Simison, S. R. Miyasato, J. Binkley, J. Orvis, P. Shah, F. Wymore, G. Sherlock and J. R. Wortman (2014). "The Aspergillus Genome Database: multispecies curation and incorporation of RNA-Seq data to improve structural gene annotations." Nucleic Acids Res **42**(Database issue): D705-710.

Chahal, D. S. (1985). "Solid-state fermentation with Trichoderma reesei for cellulase production." Applied and Environmental Microbiology **49**(1): 205-210.

Chandel, A. K., G. Chandrasekhar, M. B. Silva and S. Silvério da Silva (2012). "The realm of cellulases in biorefinery development." Critical Reviews in Biotechnology **32**(3): 187-202.

Chen, H., J. Liu, X. Chang, D. Chen, Y. Xue, P. Liu, H. Lin and S. Han (2017). "A review on the pretreatment of lignocellulose for high-value chemicals." Fuel Processing Technology **160**: 196-206.

Ciesielski, P. N., J. F. Matthews, M. P. Tucker, G. T. Beckham, M. F. Crowley, M. E. Himmel and B. S. Donohoe (2013). "3D Electron Tomography of Pretreated Biomass Informs Atomic Modeling of Cellulose Microfibrils." ACS Nano **7**(9): 8011-8019.

Clutterbuck, A. J. (1992). "Sexual and parasexual genetics of Aspergillus species." Biotechnology **23**: 3-18.

Clutterbuck, A. J. (1992). "Sexual and parasexual genetics of Aspergillus species." Biotechnology (Reading, Mass **23**: 3-18.

Conesa, A., P. J. Punt, N. van Luijk and C. A. van den Hondel (2001). "The secretion pathway in filamentous fungi: a biotechnological view." Fungal Genet Biol **33**(3): 155-171.

Damásio, A. R. d. L., T. M. Silva, F. B. d. R. Almeida, F. M. Squina, D. A. Ribeiro, A. F. P. Leme, F. Segato, R. A. Prade, J. A. Jorge, H. F. Terenzi and M. d. L. T. M. Polizeli (2011). "Heterologous expression of an Aspergillus niveus xylanase GH11 in Aspergillus nidulans and its characterization and application." Process Biochemistry **46**(6): 1236-1242.

de Vries, R. P., K. Burgers, P. J. van de Vondervoort, J. C. Frisvad, R. A. Samson and J. Visser (2004). "A new black Aspergillus species, A. vadensis, is a promising host for homologous and heterologous protein production." Appl Environ Microbiol **70**(7): 3954-3959.

Demain, A. L. and P. Vaishnav (2009). "Production of recombinant proteins by microbes and higher organisms." Biotechnol Adv **27**(3): 297-306.

Ding, S.-Y. and M. E. Himmel (2006). "The maize primary cell wall microfibril: a new model derived from direct visualization." Journal of Agricultural and Food Chemistry **54**(3): 597-606.

Ding, S.-Y., Y.-S. Liu, Y. Zeng, M. E. Himmel, J. O. Baker and E. A. Bayer (2012). "How Does Plant Cell Wall Nanoscale Architecture Correlate with Enzymatic Digestibility?" Science **338**(6110): 1055.

Ding, S. Y. and M. E. Himmel (2006). "The maize primary cell wall microfibril: a new model derived from direct visualization." J Agric Food Chem **54**(3): 597-606.

Divne, C., J. Stahlberg, T. Reinikainen, L. Ruohonen, G. Pettersson, J. K. Knowles, T. T. Teeri and T. A. Jones (1994). "The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from Trichoderma reesei." Science **265**(5171): 524-528.

Ellila, S., L. Fonseca, C. Uchima, J. Cota, G. H. Goldman, M. Saloheimo, V. Sacon and M. Siika-Aho (2017). "Development of a low-cost cellulase production process using Trichoderma reesei for Brazilian biorefineries." Biotechnol Biofuels **10**: 30.

Fernandez, E. Q., D. L. Moyer, S. Maiyuran, A. Labaro, H. J. F. g. Brody and biology (2012). "Vector-initiated transitive RNA interference in the filamentous fungus Aspergillus oryzae." **49**(4): 294-301.

Ferreira-Nozawa, M. S., H. C. Silveira, C. J. Ono, A. L. Fachin, A. Rossi and N. M. Martinez-Rossi (2006). "The pH signaling transcription factor PacC mediates the growth of Trichophyton rubrum on human nail in vitro." Med Mycol **44**(7): 641-645.

Fleissner, A. and P. Dersch (2010). "Expression and export: recombinant protein production systems for Aspergillus." Appl Microbiol Biotechnol **87**(4): 1255-1270.

Garvey, M., H. Klose, R. Fischer, C. Lambertz and U. Commandeur (2013). "Cellulases for biomass degradation: comparing recombinant cellulase expression platforms." Trends Biotechnol **31**(10): 581-593.

Garvey, M., H. Klose, R. Fischer, C. Lambertz and U. Commandeur (2013). "Cellulases for biomass degradation: comparing recombinant cellulase expression platforms." Trends in biotechnology **31**(10): 581-593.

Gasser, E., P. Ballmann, S. Dröge, J. Bohn and H. König (2014). "Microbial production of biopolymers from the renewable resource wheat straw." Journal of applied microbiology **117**(4): 1035-1044.

Gibson, D. G. (2011). "Enzymatic assembly of overlapping DNA fragments." Methods Enzymol **498**: 349-361.

Gibson, D. G. (2014). "Programming biological operating systems: genome design, assembly and activation." Nat Methods **11**(5): 521-526.

Gonzalez, M. and F. Li (2012). "DNA replication, RNAi and epigenetic inheritance." Epigenetics **7**(1).

Guillemette, T., A. F. Ram, N. D. Carvalho, A. Joubert, P. Simoneau and D. B. Archer (2011). "Methods for investigating the UPR in filamentous fungi." Methods Enzymol **490**: 1-29.

Guillemette, T., N. N. van Peij, T. Goosen, K. Lanthaler, G. D. Robson, C. A. van den Hondel, H. Stam and D. B. Archer (2007). "Genomic analysis of the secretion stress response in the enzyme-producing cell factory Aspergillus niger." BMC genomics **8**: 158.

Hall, M., P. Bansal, J. H. Lee, M. J. Realff and A. S. Bommarius (2010). "Cellulose crystallinity–a key predictor of the enzymatic hydrolysis rate." FEBS journal **277**(6): 1571-1582.

Hammond, T., J. Bok, M. Andrewski, Y. Reyes-Dominguez, C. Scazzocchio and N. J. E. c. Keller (2008). "RNA silencing gene truncation in the filamentous fungus Aspergillus nidulans." **7**(2): 339-349.

Hammond, T. M. and N. P. J. G. Keller (2005). "RNA silencing in Aspergillus nidulans is independent of RNA-dependent RNA polymerases." **169**(2): 607-617.

Han, M. J., N. J. Kim, S. Y. Lee and H. N. Chang (2010). "Extracellular proteome of Aspergillus terreus grown on different carbon sources." Curr Genet **56**(4): 369-382.

Harris, P. V., D. Welner, K. C. McFarland, E. Re, J. C. Navarro Poulsen, K. Brown, R. Salbo, H. Ding, E. Vlasenko, S. Merino, F. Xu, J. Cherry, S. Larsen and L. Lo Leggio (2010). "Stimulation of lignocellulosic biomass hydrolysis by proteins of glycoside hydrolase family 61: structure and function of a large, enigmatic family." Biochemistry **49**(15): 3305-3316.

Harris, P. V., F. Xu, N. E. Kreel, C. Kang and S. Fukuyama (2014). "New enzyme insights drive advances in commercial ethanol production." Current opinion in chemical biology **19**: 162-170.

He, Y., Y. Ding, C. Ma, J. Di, C. Jiang and A. J. G. C. Li (2017). "One-pot conversion of biomass-derived xylose to furfuralcohol by a chemo-enzymatic sequential acid-catalyzed dehydration and bioreduction." **19**(16): 3844-3850.

Hendriks, A. and G. J. B. t. Zeeman (2009). "Pretreatments to enhance the digestibility of lignocellulosic biomass." **100**(1): 10-18.

Himmel, M. E. and E. A. Bayer (2009). "Lignocellulose conversion to biofuels: current challenges, global perspectives." Curr Opin Biotechnol **20**(3): 316-317.

Hölker, U., M. Höfer and J. Lenz (2004). "Biotechnological advantages of laboratory-scale solid-state fermentation with fungi." Applied Microbiology and Biotechnology **64**(2): 175-186.

Jadidzadeh, A. and A. Serletis (2018). "The global crude oil market and biofuel agricultural commodity prices." The Journal of Economic Asymmetries **18**.

Jaskiewicz, L. and W. Filipowicz (2008). "Role of Dicer in posttranscriptional RNA silencing." Curr Top Microbiol Immunol **320**: 77-97.

Jeenes, D. J., D. A. Mackenzie, I. N. Roberts and D. B. Archer (1991). "Heterologous protein production by filamentous fungi." Biotechnology & genetic engineering reviews **9**: 327-367.

Jiang, D., W. Zhu, Y. Wang, C. Sun, K.-Q. Zhang and J. J. B. a. Yang (2013). "Molecular tools for functional genomics in filamentous fungi: recent advances and new strategies." **31**(8): 1562-1574.

Johnson, E. (2016). "Integrated enzyme production lowers the cost of cellulosic ethanol." Biofuels, Bioproducts and Biorefining **10**(2): 164-174.

Johnson, M., I. Zaretskaya, Y. Raytselis, Y. Merezhuk, S. McGinnis and T. L. Madden (2008). "NCBI BLAST: a better web interface." Nucleic Acids Res **36**(Web Server issue): W5-9.

Joosten, V., C. Lokman, C. A. Van Den Hondel and P. J. Punt (2003). "The production of antibody fragments and antibody fusion proteins by yeasts and filamentous fungi." Microb Cell Fact **2**(1): 1.

Kato, N., Y. Murakoshi, M. Kato, T. Kobayashi and N. J. C. g. Tsukagoshi (2002). "Isomaltose formed by α-glucosidases triggers amylase induction in Aspergillus nidulans." **42**(1): 43-50.

Katz, M. E., S. M. Bernardo and B. F. Cheetham (2008). "The interaction of induction, repression and starvation in the regulation of extracellular proteases in Aspergillus nidulans: evidence for a role for CreA in the response to carbon starvation." Curr Genet **54**(1): 47-55.

Khalaj, V., H. Eslami, M. Azizi, N. Rovira-Graells and M. J. F. m. l. Bromley (2007). "Efficient downregulation of alb1 gene using an AMA1-based episomal expression of RNAi construct in Aspergillus fumigatus." **270**(2): 250-254.

Kiesenhofer, D. P., R. L. Mach and A. R. Mach-Aigner (2018). "Influence of cis Element Arrangement on Promoter Strength in Trichoderma reesei." Appl Environ Microbiol **84**(1).

Kim, E. S., H. J. Lee, W. G. Bang, I. G. Choi and K. H. Kim (2009). "Functional characterization of a bacterial expansin from Bacillus subtilis for enhanced enzymatic hydrolysis of cellulose." Biotechnology and bioengineering **102**(5): 1342-1353.

Kim, I. J., H. J. Ko, T. W. Kim, I. G. Choi and K. H. Kim (2013). "Characteristics of the binding of a bacterial expansin (BsEXLX1) to microcrystalline cellulose." Biotechnology and bioengineering **110**(2): 401-407.

Kim, I. J., H. J. Lee, I. G. Choi and K. H. Kim (2014). "Synergistic proteins for the enhanced enzymatic hydrolysis of cellulose by cellulase." Appl Microbiol Biotechnol **98**(20): 8469-8480.

Kim, K., Y. S. Lee, D. Harris, K. Nakahara and R. W. Carthew (2006). "The RNAi pathway initiated by Dicer-2 in Drosophila." Cold Spring Harb Symp Quant Biol **71**: 39-44.

Klein-Marcuschamer, D. and H. W. Blanch (2015). "Renewable fuels from biomass: Technical hurdles and economic assessment of biological routes." AIChE Journal **61**(9): 2689-2701.

Krassing, H. (1993). "Cellulose: Structure." Accessibility and Reactivity, Gordon and Breach Science Publichers, Swizerland **41**.

Kuck, U. and B. Hoff (2010). "New tools for the genetic manipulation of filamentous fungi." Appl Microbiol Biotechnol **86**(1): 51-62.

Kuhad, R. C., D. Deswal, S. Sharma, A. Bhattacharya, K. K. Jain, A. Kaur, B. I. Pletschke, A. Singh and M. Karp (2016). "Revisiting cellulase production and redefining current strategies based on major challenges." Renewable and Sustainable Energy Reviews **55**: 249-272.

Kumar, S., H. K. Sharma and B. C. Sarkar (2011). "Effect of substrate and fermentation conditions on pectinase and cellulase production by Aspergillus niger NCIM 548 in submerged (SmF) and solid state fermentation (SSF)." Food Science and Biotechnology **20**(5): 1289.

Lal, R. (2005). "World crop residues production and implications of its use as a biofuel." Environ Int **31**(4): 575-584.

Laureano-Perez, L., F. Teymouri, H. Alizadeh and B. E. Dale (2005). "Understanding factors that limit enzymatic hydrolysis of biomass." Applied Biochemistry and Biotechnology **124**(1-3): 1081-1099.

Lynd, L. R., M. S. Laser, D. Bransby, B. E. Dale, B. Davison, R. Hamilton, M. Himmel, M. Keller, J. D. McMillan, J. Sheehan and C. E. Wyman (2008). "How biotech can transform biofuels." Nat Biotechnol **26**(2): 169-172.

Lynd, L. R., W. H. van Zyl, J. E. McBride and M. Laser (2005). "Consolidated bioprocessing of cellulosic biomass: an update." Curr Opin Biotechnol **16**(5): 577-583.

Mah, S. M., C. Buske, R. K. Humphries and F. Kuchenbauer (2010). "miRNA*: a passenger stranded in RNA-induced silencing complex?" Crit Rev Eukaryot Gene Expr **20**(2): 141-148.

Marshall, T. and K. M. Williams (1992). "Coomassie blue protein dye-binding assays measure formation of an insoluble protein-dye complex." Anal Biochem **204**(1): 107-109.

Matranga, C., Y. Tomari, C. Shin, D. P. Bartel and P. D. Zamore (2005). "Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes." Cell **123**(4): 607-620.

Matsushita-Morita, M., S. Tada, S. Suzuki, R. Hattori, J. Marui, I. Furukawa, Y. Yamagata, H. Amano, H. Ishida, M. Takeuchi, Y. Kashiwagi and K. Kusumoto (2011). "Overexpression and characterization of an extracellular leucine aminopeptidase from Aspergillus oryzae." Curr Microbiol **62**(2): 557-564.

McCleary, B. V., T. S. Gibson, H. Sheehan, A. Casey, L. Horgan and J. J. C. r. O'Flaherty (1989). "Purification, properties, and industrial significance of transglucosidase from Aspergillus niger." **185**(1): 147-162.

Meyer, V., B. Wu and A. F. Ram (2011). "Aspergillus as a multi-purpose cell factory: current status and perspectives." Biotechnol Lett **33**(3): 469-476.

Miller, G. L. (1959). "Use of dintirosalicilic acid reagent for determination of reducing sugar." Analytical Chemistry **31**: 426-428.

Mittal, P., R. Yadav, R. Devi, S. Sharma and A. Goyal (2012). Phenomenal RNA interference: from mechanism to application. Crop Plant, IntechOpen.

Nakamura, T., Y. Maeda, N. Tanoue, T. Makita, M. Kato, T. J. B. Kobayashi, biotechnology, and biochemistry (2006). "Expression profile of amylolytic genes in Aspergillus nidulans." 0610020104-0610020104.

NAKAO, M., T. NAKAYAMA, A. KAKUDO, M. INOHARA, M. HARADA, F. OMURA and Y. J. E. j. o. b. SHIBANO (1994). "Structure and expression of a gene coding for thermostable α-glucosidase with a broad substrate specificity from Bacillus sp. SAM1606." **220**(2): 293-300.

Nakayashiki, H., N. Kadotani and S. J. J. o. m. e. Mayama (2006). "Evolution and diversification of RNA silencing proteins in fungi." **63**(1): 127-135.

Nakayashiki, H. J. F. l. (2005). "RNA silencing in fungi: mechanisms and applications." **579**(26): 5950-5957.

Nevalainen, K. M., V. S. Te'o and P. L. Bergquist (2005). "Heterologous protein expression in filamentous fungi." Trends Biotechnol **23**(9): 468-474.

Nguyen, Q. B., K. Itoh, B. Van Vu, Y. Tosa and H. J. M. m. Nakayashiki (2011). "Simultaneous silencing of endo-β-1, 4 xylanase genes reveals their roles in the virulence of Magnaporthe oryzae." **81**(4): 1008-1019.

Nguyen, Q. B., N. Kadotani, S. Kasahara, Y. Tosa, S. Mayama and H. J. M. m. Nakayashiki (2008). "Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, Magnaporthe oryzae, using a high-throughput RNA-silencing system." **68**(6): 1348-1365.

Nieto, A., J. A. Prieto and P. Sanz (1999). "Stable high-copy-number integration of Aspergillus oryzae alpha-AMYLASE cDNA in an industrial baker's yeast strain." Biotechnol Prog **15**(3): 459-466.

Norihiro, T., F. Makoto, N. Hiroki, K. Norie, T. Akio and U. J. G. Shigezo (1989). "Isolation of a cDNA encoding Aspergillus oryzae Taka-amylase A: evidence for multiple related genes." **84**(2): 319-327.

Ortega, N., M. a. D. Busto, M. J. I. b. Perez-Mateos and biodegradation (2001). "Kinetics of cellulose saccharification by Trichoderma reesei cellulases." **47**(1): 7-14.

Parker, J. S. (2010). "How to slice: snapshots of Argonaute in action." Silence **1**(1): 3.

Pastori, C., M. Magistri, S. Napoli, G. M. Carbone and C. V. Catapano (2010). "Small RNA-directed transcriptional control: new insights into mechanisms and therapeutic applications." Cell Cycle **9**(12): 2353-2362.

Payne, C. M., B. C. Knott, H. B. Mayes, H. Hansson, M. E. Himmel, M. Sandgren, J. Stahlberg and G. T. J. C. r. Beckham (2015). "Fungal cellulases." **115**(3): 1308-1448.

Pazur, J. H. and D. J. J. b. C. French (1952). "The action of transglucosidase of Aspergillus oryzae on maltose." **196**(1): 265-272.

Pena-Montes, C., A. Gonzalez, D. Castro-Ochoa and A. Farres (2008). "Purification and biochemical characterization of a broad substrate specificity thermostable alkaline protease from Aspergillus nidulans." Appl Microbiol Biotechnol **78**(4): 603-612.

Petersen, K., J. Lehmbeck, T. J. M. Christensen and G. G. MGG (1999). "A new transcriptional activator for amylase genes in Aspergillus." **262**(4-5): 668-676.

Phillips, C. M., W. T. Beeson, J. H. Cate and M. A. Marletta (2011). "Cellobiose dehydrogenase and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by Neurospora crassa." ACS Chem Biol **6**(12): 1399-1406.

Pontecorvo, G. (1969). "Genetic analysis of "somatic" cells in filamentous fungi." Wistar Inst Symp Monogr **9**: 1-8.

Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. D. Macdonald and A. W. Bufton (1953). "The genetics of Aspergillus nidulans." Adv Genet **5**: 141-238.

Pontecorvo, G., J. A. Roper, L. M. Hemmons, K. D. McDonald and A. W. J. Bufton (1953). "The genetics of *Aspergillus nidulans*." Adv. Genet. **5**: 141-238.

Punt, P. J., M. A. Dingemanse, A. Kuyvenhoven, R. D. Soede, P. H. Pouwels and C. A. J. G. van den Hondel (1990). "Functional elements in the promoter region of the Aspergillus nidulans gpdA gene encoding glyceraldehyde-3-phosphate dehydrogenase." **93**(1): 101-109.

Punt, P. J., F. H. Schuren, J. Lehmbeck, T. Christensen, C. Hjort and C. A. van den Hondel (2008). "Characterization of the Aspergillus niger prtT, a unique regulator of extracellular protease encoding genes." Fungal Genet Biol **45**(12): 1591-1599.

Punt, P. J., N. van Biezen, A. Conesa, A. Albers, J. Mangnus and C. van den Hondel (2002). "Filamentous fungi as cell factories for heterologous protein production." Trends in Biotechnology **20**(5): 200-206.

Punt, P. J., N. van Biezen, A. Conesa, A. Albers, J. Mangnus and C. van den Hondel (2002). "Filamentous fungi as cell factories for heterologous protein production." Trends Biotechnol **20**(5): 200-206.

Punt, P. J., N. D. Zegers, M. Busscher and P. H. J. J. o. b. Pouwels (1991). "Intracellular and extracellular production of proteins in Aspergillus under the control of expression signals of the highly expressed Aspergillus nidulans gpdA gene." **17**(1): 19-33.

Rana, V., A. D. Eckard, P. Teller and B. K. Ahring (2014). "On-site enzymes produced from Trichoderma reesei RUT-C30 and Aspergillus saccharolyticus for hydrolysis of wet exploded corn stover and loblolly pine." Bioresour Technol **154**: 282-289.

Rappleye, C. A., J. T. Engle and W. E. J. M. m. Goldman (2004). "RNA interference in Histoplasma capsulatum demonstrates a role for α-(1, 3)-glucan in virulence." **53**(1): 153-165.

Record, E., M. Asther, C. Sigoillot, S. Pages, P. J. Punt, M. Delattre, M. Haon, C. A. van den Hondel, J. C. Sigoillot and L. Lesage-Meessen (2003). "Overproduction of the Aspergillus niger feruloyl esterase for pulp bleaching application." Appl Microbiol Biotechnol **62**(4): 349-355.

Regalbuto, J. R. (2009). "Engineering. cellulosic biofuels--got gasoline?" Science **325**(5942): 822-824.

Regalbuto, J. R. (2011). "The sea change in US biofuels' funding: from cellulosic ethanol to green gasoline." Biofuels, Bioproducts and Biorefining **5**(5): 495-504.

Rodríguez Couto, S. and M. A. Sanromán (2005). "Application of solid-state fermentation to ligninolytic enzyme production." Biochemical Engineering Journal **22**(3): 211-219.

Romano, N. and G. J. M. m. Macino (1992). "Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences." **6**(22): 3343-3353.

Santos, C. R., J. H. Paiva, A. N. Meza, J. Cota, T. M. Alvarez, R. Ruller, R. A. Prade, F. M. Squina and M. T. Murakami (2011). "Molecular insights into substrate specificity and thermal stability of a bacterial GH5-CBM27 endo-1,4-beta-d-mannanase." J Struct Biol.

Schulein, M. (1997). "Enzymatic properties of cellulases from Humicola insolens." J Biotechnol **57**(1-3): 71-81.

Segato, F., A. R. Damasio, R. C. de Lucas, F. M. Squina and R. A. Prade (2014). "Genomics review of holocellulose deconstruction by aspergilli." Microbiol Mol Biol Rev **78**(4): 588-613.

Segato, F., A. R. Damasio, T. A. Goncalves, M. T. Murakami, F. M. Squina, M. L. Polizeli, A. Mort and R. A. Prade (2012). "Two structurally discrete GH7-cellobiohydrolases compete for the same cellulosic substrate fiber." Biotechnol Biofuels **5**(1): 21.

Segato, F., A. R. L. Damásio, T. A. Gonçalves, R. C. de Lucas, F. M. Squina, S. R. Decker and R. A. Prade (2012). "High-yield secretion of multiple client proteins in Aspergillus." Enzyme and Microbial Technology(0).

Selig, M. J., S. Viamajala, S. R. Decker, M. P. Tucker, M. E. Himmel and T. B. Vinzant (2007). "Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose." Biotechnology progress **23**(6): 1333-1339.

Shapiro, A. L., E. Vinuela and J. V. Maizel, Jr. (1967). "Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels." Biochem Biophys Res Commun **28**(5): 815-820.

Sheldon, R. A. (2018). "The Road to Biorenewables: Carbohydrates to Commodity Chemicals." ACS Sustainable Chemistry & Engineering **6**(4): 4464-4480.

Shevchenko, A., M. Wilm, O. Vorm and M. Mann (1996). "Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels." Anal Chem **68**(5): 850-858.

Sims, A. H., M. E. Gent, K. Lanthaler, N. S. Dunn-Coleman, S. G. Oliver and G. D. Robson (2005). "Transcriptome analysis of recombinant protein secretion by Aspergillus nidulans and the unfolded-protein response in vivo." Appl Environ Microbiol **71**(5): 2737-2747.

Singhania, R. R., A. K. Patel, C. R. Soccol and A. Pandey (2009). "Recent advances in solid-state fermentation." Biochemical Engineering Journal **44**(1): 13-18.

Singhania, R. R., R. K. Sukumaran, A. K. Patel, C. Larroche and A. Pandey (2010). "Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases." Enzyme and Microbial Technology **46**(7): 541-549.

Singhania, R. R., R. K. Sukumaran, A. K. Patel, C. Larroche, A. J. E. Pandey and M. Technology (2010). "Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases." **46**(7): 541-549.

Singleton, V. L. and J. A. Rossi (1965). "Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents." American journal of Enology and Viticulture **16**(3): 144-158.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton and D. Crocker (2008). "Determination of structural carbohydrates and lignin in biomass." National Renewable Energy Laboratory(NREL/TP-510-42618).

Somerville, C., S. Bauer, G. Brininstool, M. Facette, T. Hamann, J. Milne, E. Osborne, A. Paredez, S. Persson and T. Raab (2004). "Toward a systems approach to understanding plant cell walls." Science **306**(5705): 2206-2211.

Squina, F. M., A. J. Mort, S. R. Decker and R. A. Prade (2009). "Xylan decomposition by Aspergillus clavatus endo-xylanase." Protein expression and purification **68**(1): 65-71.

Sriranganadane, D., P. Waridel, K. Salamin, M. Feuermann, B. Mignon, P. Staib, J. M. Neuhaus, M. Quadroni and M. Monod (2011). "Identification of novel secreted proteases during extracellular proteolysis by dermatophytes at acidic pH." Proteomics **11**(22): 4422-4433.

Srivastava, N., M. Srivastava, P. Mishra, V. K. Gupta, G. Molina, S. Rodriguez-Couto, A. Manikanta, P. J. R. Ramteke and S. E. Reviews (2018). "Applications of fungal cellulases in biofuel production: advances and limitations." **82**: 2379-2386.

Stahlberg, J., C. Divne, A. Koivula, K. Piens, M. Claeyssens, T. T. Teeri and T. A. Jones (1996). "Activity Studies and Crystal Structures of Catalytically Deficient Mutants of Cellobiohydrolase I fromTrichoderma reesei." Journal of Molecular Biology **264**(2): 337-349.

Summer, J. B. and V. A. Graham (1921). "Dinitrosalicylic aid: a reagent for the estimation of sugar in normal and diabetic urine." J Biol Chem **47**: 5-9.

Swift, R. J., A. Karandikar, A. M. Griffen, P. J. Punt, C. A. van den Hondel, G. D. Robson, A. P. Trinci and M. G. Wiebe (2000). "The Effect of organic nitrogen sources on recombinant glucoamylase production by Aspergillus niger in chemostat culture." Fungal Genet Biol **31**(2): 125-133.

Szewczyk, E., T. Nayak, C. E. Oakley, H. Edgerton, Y. Xiong, N. Taheri-Talesh, S. A. Osmani and B. R. J. N. p. Oakley (2006). "Fusion PCR and gene targeting in Aspergillus nidulans." **1**(6): 3111.

Tamayo, E. N., A. Villanueva, A. A. Hasper, L. H. de Graaff, D. Ramon and M. Orejas (2008). "CreA mediates repression of the regulatory gene xlnR which controls the production of xylanolytic enzymes in Aspergillus nidulans." Fungal Genet Biol **45**(6): 984-993.

Tamayo-Ramos, J. and M. Orejas (2014). "Enhanced glycosyl hydrolase production in Aspergillus nidulans using transcription factor engineering approaches." Biotechnology for Biofuels **7**(1): 103.

Tani, S., Y. Katsuyama, T. Hayashi, H. Suzuki, M. Kato, K. Gomi, T. Kobayashi and N. J. C. g. Tsukagoshi (2001). "Characterization of the amyR gene encoding a transcriptional activator for the amylase genes in Aspergillus nidulans." **39**(1): 10-15.

Tsukamoto, A., K. Kimura, Y. Ishii, T. Takano, K. J. B. Yamane and b. r. communications (1988). "Nucleotide sequence of the maltohexaose-producing amylase gene from an alkalophilic Bacillus sp.# 707 and structural similarity to liquefying type α-amylases." **151**(1): 25-31.

Valencia-Sanchez, M. A., J. Liu, G. J. Hannon and R. Parker (2006). "Control of translation and mRNA degradation by miRNAs and siRNAs." Genes Dev **20**(5): 515-524.

Van Den Berg, M. A. and K. Maruthachalam (2015). Genetic Transformation Systems in Fungi, Volume 2, Springer.

van den Hondel, C. A., P. J. Punt and R. F. van Gorcom (1992). "Production of extracellular proteins by the filamentous fungus Aspergillus." Antonie van Leeuwenhoek **61**(2): 153-160.

van Rijn, R., I. U. Nieves, K. T. Shanmugam, L. O. Ingram and W. Vermerris (2018). "Techno-Economic Evaluation of Cellulosic Ethanol Production Based on Pilot Biorefinery Data: a Case Study of Sweet Sorghum Bagasse Processed via L+SScF." BioEnergy Research **11**(2): 414-425.

Verdoes, J. C., P. J. Punt, A. H. Stouthamer and C. A. van den Hondel (1994). "The effect of multiple copies of the upstream region on expression of the Aspergillus niger glucoamylase-encoding gene." Gene **145**(2): 179-187.

Wang, H., F. Squina, F. Segato, A. Mort, D. Lee, K. Pappan and R. Prade (2011). "High-temperature enzymatic breakdown of cellulose." Appl Environ Microbiol **77**(15): 5199-5206.

Wang, Y., W. Xue, A. H. Sims, C. Zhao, A. Wang, G. Tang, J. Qin and H. Wang (2008). "Isolation of four pepsin-like protease genes from Aspergillus niger and analysis of the effect of disruptions on heterologous laccase expression." Fungal genetics and biology **45**(1): 17-27.

Ward, O. P. (2011). "Production of recombinant proteins by filamentous fungi." Biotechnol Adv.

Watanabe, Y. (2011). "Overview of plant RNAi." Methods Mol Biol **744**: 1-11.

Weenink, X. O., P. J. Punt, C. A. van den Hondel and A. F. Ram (2006). "A new method for screening and isolation of hypersecretion mutants in Aspergillus niger." Appl Microbiol Biotechnol **69**(6): 711-717.

Wiebe, M. G., G. D. Robson, J. Shuster and A. P. Trinci (2001). "Evolution of a recombinant (gucoamylase-producing) strain of Fusarium venenatum A3/5 in chemostat culture." Biotechnol Bioeng **73**(2): 146-156.

Wilson, D. B. J. C. o. i. b. (2009). "Cellulases and biofuels." **20**(3): 295-299.

Wood, T. M., S. I. McCRAE and K. M. Bhat (1989). "The mechanism of fungal cellulase action. Synergism between enzyme components of Penicillium pinophilum cellulase in solubilizing hydrogen bond-ordered cellulose." Biochem. J **260**: 37-43.

Wyman, C. E., B. E. Dale, R. T. Elander, M. Holtzapple, M. R. Ladisch and Y. J. B. t. Lee (2005). "Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover." **96**(18): 2026-2032.

Yamamoto, K., A. Nakayama, Y. Yamamoto and S. J. E. j. o. b. Tabata (2004). "Val216 decides the substrate specificity of α-glucosidase in Saccharomyces cerevisiae." **271**(16): 3414-3420.

Yelton, M. M., J. E. Hamer and W. E. Timberlake (1984). "Transformation of Aspergillus nidulans by using a trpC plasmid." Proceedings of the National Academy of Sciences of the United States of America **81**(5): 1470-1474.

Yoji, H., T. Kozo, K. Katsuhiko, G. Katsuya, K. Chieko, T. Gakuzo and H. J. G. Shodo (1991). "Nucleotide sequence and expression of the glucoamylase-encoding gene (glaA) from Aspergillus oryzae." **108**(1): 145-150.

Yoon, J., T. Aishan, J. Maruyama and K. Kitamoto (2010). "Enhanced production and secretion of heterologous proteins by the filamentous fungus Aspergillus oryzae via disruption of vacuolar protein sorting receptor gene Aovps10." Appl Environ Microbiol **76**(17): 5718-5727.

Yoon, J., S. Kimura, J. Maruyama and K. Kitamoto (2009). "Construction of quintuple protease gene disruptant for heterologous protein production in Aspergillus oryzae." Appl Microbiol Biotechnol **82**(4): 691-701.

Yoon, J., J. Maruyama and K. Kitamoto (2011). "Disruption of ten protease genes in the filamentous fungus Aspergillus oryzae highly improves production of heterologous proteins." Appl Microbiol Biotechnol **89**(3): 747-759.

Yoon, L. W., T. N. Ang, G. C. Ngoh, A. S. M. J. B. Chua and Bioenergy (2014). "Fungal solid-state fermentation and various methods of enhancement in cellulase production." **67**: 319-338.

Zhang, J., G. Zhang, W. Wang, W. Wang and D. Wei (2018). "Enhanced cellulase production in Trichoderma reesei RUT C30 via constitution of minimal transcriptional activators." Microb Cell Fact **17**(1): 75

VITA

Jorge D Lightfoot

Candidate for the Degree of

Doctor of Philosophy

Dissertation: METABOLICALLY ENGINEERING *ASPERGILLUS NIDULANS* FOR CLIENT PROTEIN PRODUCTION

Major Field: Microbiology and Molecular Genetics

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Microbiology and Molecular Genetics at Oklahoma State University, Stillwater, Oklahoma in July, 2019.

Completed the requirements for the Bachelor of Science in Biology at Southern Illinois University Carbondale, Carbondale, Illinois in 2012.

Experience:

Research Assistant in the Laboratory of Dr. Rolf Prade, Microbiology and Molecular Genetics Department, Oklahoma State University 2013-2019

Undergraduate Research Assistant in the Laboratory of Dr. Vjollca Konjufca, Microbiology, Southern Illinois University 2010-2012