

**Expression of Glycoside Hydrolases in**  
***Aspergillus niger***

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## Abstract

Enzymes from filamentous fungi have a key role in degradation of the most abundant biopolymers found in nature, cellulose and hemicelluloses. For this reason, these enzymes are of great interest in the industrial conversion of lignocellulosic substrates into biofuels. The production of plant cell wall degrading enzymes is regulated mainly at the transcriptional level in filamentous fungi but little is known about the signalling pathways and transcription factors (TFs) involved in this regulation in *Aspergillus niger*. RNA-sequencing analysis has been previously carried out to investigate the transcriptional changes that occur when *A. niger* is transferred from the simple carbon source glucose onto the complex lignocellulosic biomass wheat straw. This has highlighted the up-regulation in transcript level of genes encoding some glycosyl hydrolase (GH) enzymes as well as hydrophobic surface interacting proteins (HSIPs) that may be involved in the interface between lignocellulosic biomass and *A. niger*.

Genes encoding the key TFs XlnR, ClrA and ClrB were deleted from *A. niger* and the resulting strains were assessed for growth on glucose and wheat straw, transcription of genes encoding glycosyl hydrolases and saccharification activity. Growth of all mutant strains, based in straw on measurement of pH and assay of glucosamine, was impaired in relation to the wild-type (WT) strain although deletion of *clrA* had less effect than deletion of *xlnR* or *clrB*. Release of sugars from wheat straw was also lowered when culture filtrates from TF deletion strains were compared with WT culture filtrates. Transcript levels of *cbhA*, *bglB*, *eglC* and *xynA* were measured in all strains in glucose and wheat straw media in batch culture with and without pH control. Transcript levels from *cbhA*, *bglB* and *eglC* were lowered in all mutant strains compared to WT although the impact of deleting *clrA* was not pronounced with expression of *eglC* and had no effect on *xynA*. The impact on transcription was not related to changes in pH. In addition to impaired growth on wheat straw, the  $\Delta xlnR$  strain was sensitive to oxidative stress and displayed cell wall defects in the glucose condition suggesting additional roles for XlnR.

Phosphorylation is a key reversible modification that regulates protein function, subcellular localization, complex formation, activation of TFs and cell signalling pathways. A phosphoproteomic study was carried out on both the WT and the  $\Delta xlnR$  deletion strains of *A. niger* in order to identify key regulators of the signalling pathways involved in the breakdown of a lignocellulosic substrate, wheat straw. The analysis consisted of comparing the phosphoproteome profiles of the strains when grown in glucose with the phosphoproteome profile of the same strains when

exposed to wheat straw for 6h, 12h and 24h. The results suggested a difference in the phosphoproteome profiles of the two strains when exposed to both glucose and wheat straw. These data may provide new information on the importance of XlnR in the regulation of expression of GHs but also in controlling the environment to which *A. niger* is exposed depending on the nutrient availability.

To investigate the role of HSIPs in the induction of *A. niger* response to wheat straw, single gene deletion strains for *hfbD*, *hyp1* and *hsbA* as well as the double deletion strain for *hfbD* and *hyp1* have been constructed. The expression of some genes encoding GH enzymes was then followed in these strains using qRT-PCR. The results showed that the transcript levels of the GH genes studied were lowered in the HSIPs deletion strains when compared to the wild-type strain, when the cultures were transferred from glucose medium to wheat straw. These results suggest that HSIPs may have a role in the utilisation of lignocellulosic biomass in *A. niger*. The precise nature of such a role as well as the characterisation of new TFs, such as ClrB, provides new areas of improvement for industrial processes for production of second generation biofuels.

## Publications

**Raulo R.**, Kokolski M. and Archer D. B (2016) The roles of the zinc finger transcription factors XlnR, ClrA and ClrB in the breakdown of lignocellulose by *Aspergillus niger*. Applied Microbiology and Biotechnology doi:10.1186/s13568-016-0177-0.

**Raulo R.**<sup>1</sup>, van Munster J. M<sup>1</sup>, Fetherston R., Kokolski M., and Archer D. B (2015) Role of *Aspergillus niger* Hydrophobic Surface-Interacting Proteins in the Degradation of the Lignocellulosic Substrate Wheat Straw. Manuscript in preparation.

Daly P., van Munster J. M., **Raulo R.** and Archer D. B. 2015 (in press). Transcriptional Regulation and Responses in Filamentous Fungi Exposed to Lignocellulose. In: Silva, R. (ed.) Fungal Biotechnology for Biofuels. Bentham ebooks.

Daly P.<sup>1</sup>, van Munster J. M.<sup>1</sup>, Blythe M. J., Ibbett R., Kokolski M., Gaddipati S., Lindquist E., Singan V. R., Barry K. W., Lipzen A., Ngan C. Y., Petzold C. J., Chan L., Arvas M., **Raulo R.**, Pullan S. T., Delmas S., Waldron P., Tucker G. A., Simmons B. A., Archer D. B. (2015) The response of *Aspergillus niger* to lignocellulose is sequential and varies due to the compositional changes generated by pre-treatments. Submitted to Genome Biology.

Nguyen G.<sup>1</sup>, **Raulo R.**<sup>1</sup>, Porquier A., Calmes B., Iacomini-Vacilescu B., Dumur J., Pelletier S., Renou JP., Kerkoud M., Bataillé-Simoneau N., Champion C., Pigné S., Hamon B., Dias E., Helesbeux JJ., Hudhomme P., Macherel D., Richomme P., Simoneau P., Guillemette T. (2015) The indolic phytoalexin brassinin triggers *Alternaria brassicicola* responses through mitochondria-mediated ROS production. Submitted to Environmental Microbiology.

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## List of abbreviations

|                     |                                                  |
|---------------------|--------------------------------------------------|
| 1G/2G               | First generation/second generation               |
| 2D/3D               | Di-dimensional/Tri-dimensional                   |
| 5'-FOA              | 5-Fluorouracil-6-carboxylic acid monohydrate     |
| <i>A. fumigatus</i> | <i>Aspergillus fumigatus</i>                     |
| <i>A. nidulans</i>  | <i>Aspergillus nidulans</i>                      |
| <i>A. niger</i>     | <i>Aspergillus niger</i>                         |
| <i>A. oryzae</i>    | <i>Aspergillus oryzae</i>                        |
| AA9                 | Auxiliary Activity Family 9                      |
| ABF                 | Arabinofuranosidase                              |
| ACE                 | Activator of cellulases                          |
| ACM                 | <i>Aspergillus</i> complete media                |
| AMM                 | <i>Aspergillus</i> minimal media                 |
| AntiA               | Antimycin A                                      |
| AOX                 | Alternative oxidase                              |
| AraR                | L-arabinose responsive transcriptional activator |
| ATCC                | American Type Culture Collection Manassas (USA)  |
| ATP                 | Adenosine triphosphate                           |
| BiP                 | Binding protein                                  |
| BLAST               | Basic Local Alignment Search Tool                |
| BLR                 | Blue light regulator                             |
| bp                  | Base pair                                        |
| BSA                 | Bovine serum albumin                             |
| bZIP                | Basic leucine zipper                             |
| cAMP                | Cyclic adenosine monophosphate                   |
| CAZy                | Carbohydrate active enzyme                       |
| Cbh                 | Cellobiohydrolase                                |
| CBM                 | Carbohydrate binding module                      |
| CBP                 | Consolidated BioProcessing                       |
| CCR                 | Carbon catabolite repression                     |
| CD                  | Catalytic domain                                 |
| cDNA/gDNA           | Complementary DNA/ Genomic DNA                   |
| CE                  | Carbohydrate esterase                            |
| CreA/CRE1           | Carbon catabolite repressor                      |
| CWI                 | Cell Wall Integrity                              |
| DCF                 | 2',7'-dichlorofluorescein                        |

|                                 |                                               |
|---------------------------------|-----------------------------------------------|
| DEPC                            | Diethyl pyrocarbonate                         |
| DNA                             | Deoxyribonucleic acid                         |
| DP                              | Degree of Polymerisation                      |
| DTT                             | Dithiothreitol                                |
| EDTA                            | Ethylenediaminetetraacetic acid               |
| Egl                             | Endoglucanase                                 |
| ER                              | Endoplasmic Reticulum                         |
| ERAD                            | ER-associated protein degradation             |
| GH                              | Glycosidase Hydrolase                         |
| GlcNAc                          | N-acetylglucosamine                           |
| GPCR                            | G-protein coupled receptor                    |
| GT                              | Glycosyl Transferase                          |
| GTP                             | Guanosine triphosphate                        |
| GTPase                          | GTP hydrolysing enzyme                        |
| <i>H. jecorina</i>              | <i>Hypocrea jecorina</i>                      |
| H <sub>2</sub> DCFDA            | 2',7'-dichlorodihydrofluorescein diacetate    |
| H <sub>2</sub> SO <sub>4</sub>  | Sulphuric Acid                                |
| HCl                             | Hydrochloric acid                             |
| Hfb                             | Hydrophobin                                   |
| HPLC                            | High Performance Liquid Chromatography        |
| HsbA                            | Hydrophobic surface binding protein A         |
| HygB                            | Hygromycin B                                  |
| IRE1                            | Inositol-requiring protein 1                  |
| KEGG                            | Kyoto Encyclopedia of Genes and Genomes       |
| LB                              | Lysogeny Broth                                |
| LME                             | Lignin Modifying Enzyme                       |
| <i>M. grisea</i>                | <i>Magnaporthe grisea</i>                     |
| MAPK                            | Mitogen-activated protein kinase              |
| mRNA                            | Messenger RNA/ ribosomal RNA                  |
| MS                              | Mass spectrometry                             |
| <i>N. crassa</i>                | <i>Neurospora crassa</i>                      |
| Na <sub>2</sub> CO <sub>3</sub> | Sodium carbonate                              |
| NADH                            | Nicotinamide adenine dinucleotide             |
| NaOH                            | Sodium hydroxide                              |
| NCBI                            | National Center for Biotechnology Information |
| NoC                             | No carbon source                              |

|                      |                                                               |
|----------------------|---------------------------------------------------------------|
| NRRL                 | Agriculture Research Service Culture Collection               |
| ORF                  | Open reading frame                                            |
| PBSA                 | PolyButylene Succinate Adipate                                |
| PCR                  | Polymerase chain reaction                                     |
| PDA                  | Potato Dextrose Agar                                          |
| PEG                  | Polyethylene glycol                                           |
| PKA                  | Protein kinase A                                              |
| PKC                  | Protein kinase C                                              |
| PL                   | Polysaccharide lyase                                          |
| qRT-PCR              | Real-time quantitative reverse transcription polymerase chain |
| RNA                  | Ribonucleic acid                                              |
| RPKM                 | Reads per kilobase of exon model per million mapped reads     |
| RT-PCR               | Reverse-transcription PCR                                     |
| <i>S. cerevisiae</i> | <i>Saccharomyces cerevisiae</i>                               |
| <i>S. pombe</i>      | <i>Schizosaccharomyces pombe</i>                              |
| SDS                  | Sodium dodecyl sulphate                                       |
| SSF                  | Solid State Fermentation                                      |
| SWI/ISWI             | Switch/Imitation switch                                       |
| <i>T. reesei</i>     | <i>Trichoderma reesei</i>                                     |
| TAE                  | Tris acetate EDTA                                             |
| TBE                  | Tris borate EDTA                                              |
| TE                   | Tris-EDTA                                                     |
| TEM                  | Transmission electron microscopy                              |
| TF                   | Transcription factor                                          |
| Tris                 | Tris(hydroxymethyl)aminomethane                               |
| Tyr-P                | Tyrosine Phosphorylated                                       |
| UPR                  | Unfolded protein response                                     |
| UV                   | Ultraviolet                                                   |
| Xln                  | Xylanase                                                      |
| XlnR                 | Xylanolytic transcriptional activator                         |
| Xyn                  | Xylanase                                                      |
| XYR1                 | Xylanase regulator 1                                          |
| Zn                   | Zinc                                                          |

## International units

°C: degree Celsius

g, mg,  $\mu$ g, ng : gram, milligram,  
microgram, nanogram

L, mL,  $\mu$ L: litre, millilitre, microlitre

M, mM,  $\mu$ M, nM: molar, millimolar,  
micromolar, nanomolar

Mol, mmol: mol, millimol

## **Chapter 1. General Introduction**

The use of lignocellulosic biomass for production of liquid fuels such as ethanol is an important component of policies for generation of renewable energy, worldwide. The aims are to provide transport fuels from renewable sources, to contribute towards energy security, to reduce greenhouse gas emissions and not to compete with food.

The conversion of lignocellulosic biomass to ethanol requires several steps, including pre-treatment of lignocellulose, enzymatic hydrolysis of sugar-containing polymers and fermentation of sugars to ethanol. Among these different steps of the process, the enzymatic hydrolysis is the most costly step (Agrawal *et al.* 2013) but that could be improved in several ways such as optimizing the enzymatic cocktail to improve the efficiency, increase the yields of production, discovering new enzymes or reducing the costs of production and transport of the enzymes. Doing this, the cost of production could be reduced if the biomass was converted at maximum yields (Klein-Marcuschamer *et al.* 2012).

### 1.1 Background to biofuels

#### 1.1.1 The energetic context

By 2030, the world's requirement for energy is likely to increase by 60 to 75% mainly due to the increase in population but also due to the increase in energy consumption by developing nations. Among the primary energetic resources, oil, mainly used for transport, will remain the main fuel and will represent 35 to 40% of the world energetic consumption. However, the use of fossil fuels leads to emission of greenhouse gas. According to a report of the French Sciences Academy published in 2010 (Pugnet *et al.* 2010), the amount of CO<sub>2</sub> in the atmosphere went from 280 ppm in 1870 to 388 ppm in 2009 and the rate of increase measured since 1970 is 50 fold higher than the average one observed during the past 5000 years. This report highlights the fact that half of these emissions is due to the combustion of fossil fuels and are responsible for global warming as well as for the main climatic disturbances. By the end of the 20<sup>th</sup> century, people became aware of this issue and some industrialized countries now work to reduce their carbon dioxide emissions. Even if we have still not come up with a solution for the actual environmental issues, the establishing of the Kyoto protocol (1998) showed a desire of these countries to reduce CO<sub>2</sub> emissions and find new energetic solutions. In this context, developing new alternative fuels for transport is an important goal for research, with the aim to reduce the dependence on oil and thereby reduce CO<sub>2</sub> emissions. The European

commission set an example by aiming for the replacement by 2020 of 20% of traditional fuels by substitution products such as biofuels (2003/30/EC). The European commission also limits the taxation of biofuels, with a total or partial exemption of excise duties on biofuels (2003/96/EC).

### 1.1.2 Biofuels

Due to photosynthesis, CO<sub>2</sub> is stored in plant biomass. By using this biomass as raw material for production of biofuels, no new CO<sub>2</sub> is released in the carbon cycle and it can therefore contribute to the fight against greenhouse gas emissions. In addition, the use of these fuels in conventional fuel mixtures can lessen greenhouse gas emissions in the transport sector, without major changes in distribution infrastructure or vehicle modification. Assessment of emissions of greenhouse gasses from the production of raw materials to final products of combustion shows that the use of biofuels reduces nett emissions compared to conventional fuels (Graboski *et al.* 1998). However, it is important to note that these new fuels are not the only solution to the problems of emissions.

Two main generations of biofuels can be distinguished. The first generation biofuels (1G) are produced from sugary plants (sugar cane, beet) and starch plants (maize, wheat), and the second generation biofuels (2G) are obtained from sustainable feedstocks such as lignocellulose, the woody parts of plants that were previously considered as waste materials. The 1G biofuels are already used with several units of production in Europe and elsewhere whereas the 2G biofuels are still in development.

#### 1.1.2.1 First generation biofuels

First generation biofuels are produced from the reserve resources of plants, such as sugar, starch, or vegetable oil. The cultivation of these plants for biofuels competes with the cultivation of plants used for food consumption.

#### 1.1.2.2 Second generation biofuels

In contrast, second generation biofuels are produced from sustainable feedstocks such as lignocellulose, the woody parts of plants. An important problem to address in the production of second generation biofuel is to release useful sugars from the raw biomass material. All plants contain hemicellulose and cellulose, which are complex carbohydrates, and lignin which is a polymer of aromatics. The first step in the production of bioethanol is to release useful sugars from lignocellulose by freeing the

sugar molecules from cellulose and hemicellulose using enzymes, steam heating, or other pre-treatments. Secondly, these sugars can then be fermented to produce ethanol in the same way as 1G bioethanol production (**Figure 1.1**). The by-product of this process is lignin. Lignin can be burned as a carbon neutral fuel to produce heat and power for the processing plant and possibly for surrounding homes and businesses.

There are still problems with the current generation of biofuels that need to be solved first before they can be used on a larger scale. For example the different steps in the production of biofuels cost energy and that needs to be reduced. Also the economic cost of producing biofuels is still relatively high (Stephanopoulos 2007).

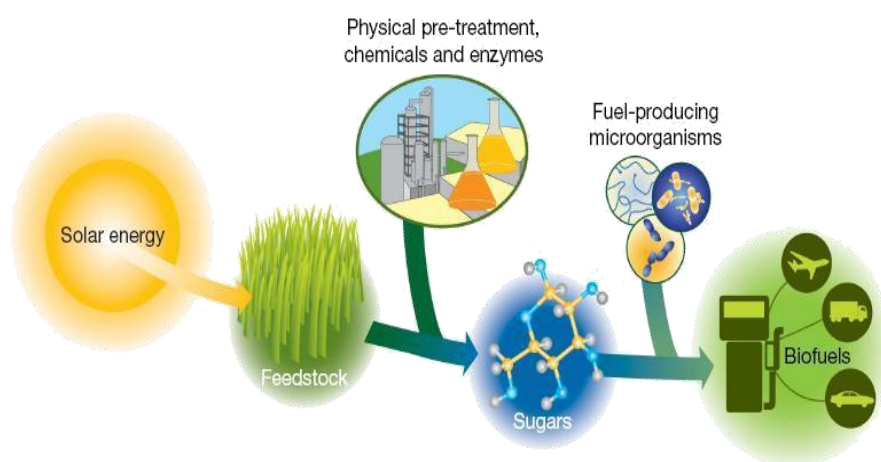


**Figure 1.1:** Main stages of biofuel production. Biomass feedstock is converted to fermentable sugars by pre-processing. Source: <http://uspace.shef.ac.uk/docs/DOC-51908>. Enzymes that can convert lignocellulose to sugars can come from filamentous fungi which are the main sources of enzymes.

### 1.1.2.3 Consolidated bioprocessing (CBP)

Consolidated bioprocessing, or CBP, has recently been a subject of increased research in the production of biofuels as a low cost process. CBP of lignocellulose to bioethanol refers to the combining of all the biological events required for this conversion process (production of saccharolytic enzymes, hydrolysis of the polysaccharides present in pre-treated biomass, fermentation of glucose to ethanol) in one reactor (Lynd *et al.* 2005). *Saccharomyces cerevisiae* is a universally used strain for industrial ethanol production due to its high ethanol productivity and high inherent ethanol/osmo-tolerance. However, it doesn't have the ability to hydrolyse cellulose. A CBP-enabling microorganism must be able to both solubilise a practical

biomass substrate and produce desired products at high yield and titre under industrial conditions. Since microorganisms with these properties have not been found in nature and therefore genetic engineering is required *via* one of the two following strategies. The native strategy: beginning with microbes that have native ability to utilise insoluble components of cellulosic biomass, and the recombinant strategy: beginning with microbes that do not have this ability and thus require heterologous expression of a saccharolytic enzyme system. Although considerable progress has been made in recent years by engineering several hosts to produce a range of different biofuels from lignocellulose (Bokinsky *et al.* 2011; Hyeon *et al.* 2011; Li *et al.* 2012), most studies have a proof of concept character with low titres achieved from model celluloses and currently no commercially viable CBP organism has been reported (Jordan *et al.* 2012).

## 1.2 Lignocellulosic biomass and its degradation

### 1.2.1 Lignocellulosic biomass

Lignocellulosic biomass is a composite material of which cellulose (40%-60%), hemicelluloses (20%-40%) and lignin (10%-25%) are the main components (**Figure 1.2**). Other materials such as pectins and proteins can be found in small quantities (Dashtban *et al.* 2009; Mathew *et al.* 2008). In the lignocellulosic biomass, the component macromolecules are cross-linked together and form a complex and ultra-resistant 3D structure, maintained by hydrogen and covalent bonds. Lignocellulose presents resistance to plant pathogens and gives rigidity to plants. The proportion and the nature of each macromolecule are functions of the individual species and variation in proportions between species can be important (O'Donohue 2008).

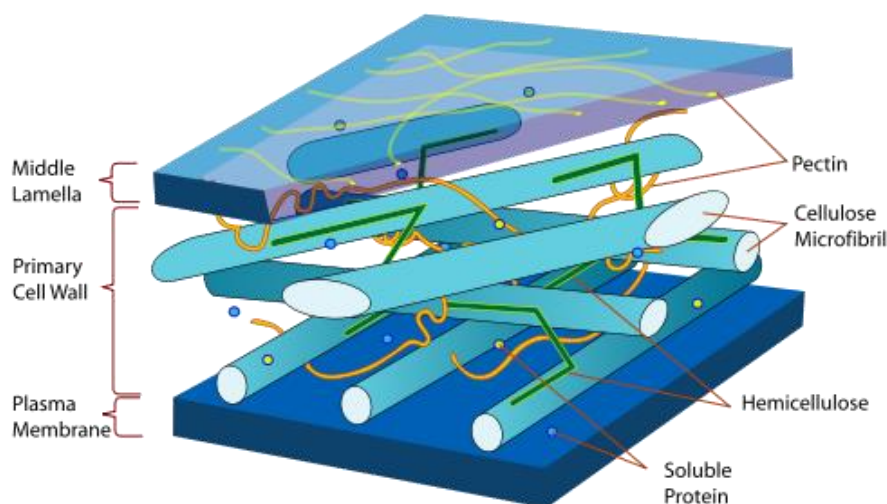


**Figure 1.2:** Plant cell wall diagram (Source: [http://www.wpclipart.com/plants/diagrams/Plant\\_cell\\_wall\\_diagram.png.html](http://www.wpclipart.com/plants/diagrams/Plant_cell_wall_diagram.png.html)).

**Cellulose** is the main component of the lignocellulosic biomass representing, according to the species, between 40 and 60% of the plant cell wall. It is the most abundant biopolymer on earth and is a linear homopolymer made of glucose units linked together by  $\beta$ -1,4 glycosidic bonds within crystalline structures known as microfibrils. These chains can be linked to each other by hydrogen bonds, hydrophobic interactions and van der Waals forces (Dashtban *et al.* 2009). Microfibrils can be stacked on top of each other to create a highly recalcitrant, crystalline structure (Liu *et al.* 2011). Hemicelluloses and lignin act as a glue to bind the cellulose-based structure, and so forming a robust matrix which makes up the plant cell wall (Zhu *et al.* 2010).

**Hemicelluloses** are the second most common polymers in lignocelluloses and are found predominantly in hardwoods and cereals. They consist of a complex heteropolysaccharide, linear or branched with a low degree of polymerization (DP) (between 150 and 200). Hemicelluloses are made of pentoses (C5 sugars such as xylose or arabinose), hexoses (C6 sugars such as glucose, galactose or mannose) and uronic acids (sugars with an acid group instead of a primary alcohol group) (Dashtban *et al.* 2009). Xylose is the most abundant sugar in hemicellulose followed by arabinose (Seiboth *et al.* 2011). The backbone of hemicellulose is made of either xylan, or mannan consisting of  $\beta$ -1,4 linked glucose and mannose units (Juhasz *et al.* 2005). These can be decorated with L-arabinosyl, D-galactosyl, glucuronyl, p-coumaroyl, feruloyl and acetyl groups (Dodd *et al.* 2009; Knob *et al.* 2010). Arabinose

is present in arabinoxylan, arabinogalactan and arabinan (Seiboth *et al.* 2011) (**Figure 1.3**). Hemicellulosic acetyl groups are linked to lignin thus forming a very rigid structure. Hemicelluloses are highly hydrophilic and can easily be degraded with acid or alkaline treatments. Their compositions are very variable depending on the plant type and species (Dodd *et al.* 2009).

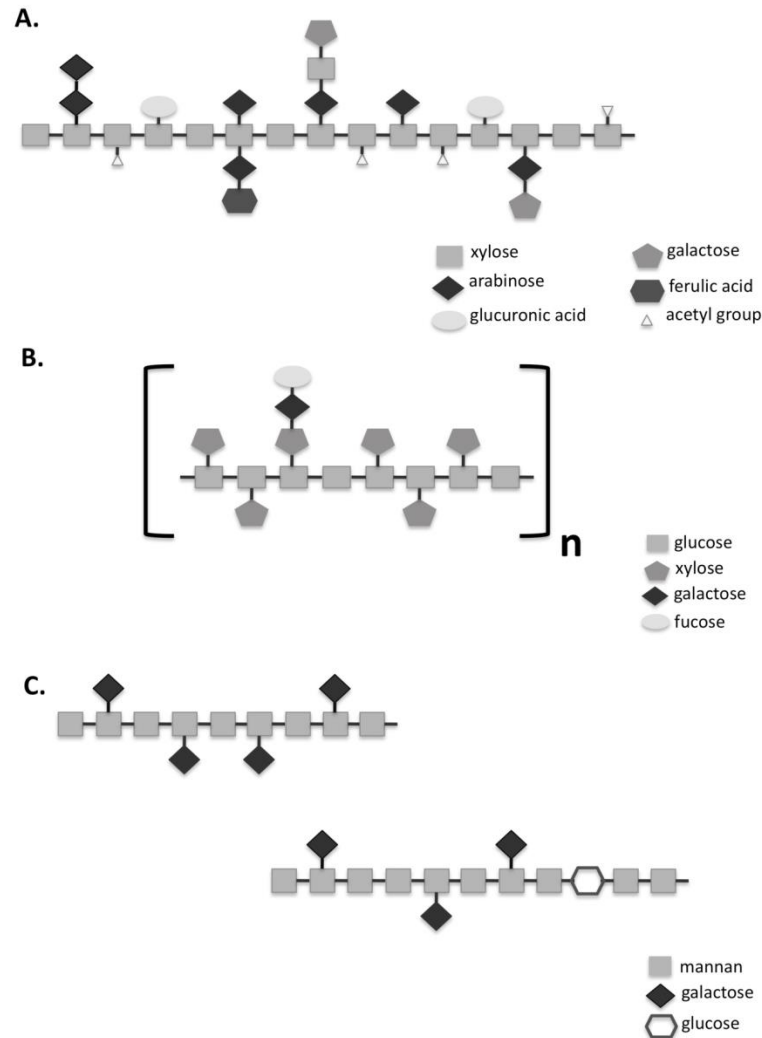


**Figure 1.3:** Schematic representation of the three major hemicellulose structures. **A.** Xylan. **B.** Xyloglucan. **C.** Galactomannan (upper left) and Galactoglucomannan (lower right) (Souza 2013).

**Lignin** is the third major component of lignocellulosic biomass and is the main component of the intercellular space. Its role is to reinforce the wall structure to give it rigidity by cross-linking through hydroxycinnamic acids, such as p-coumaric acid and ferulic acid, to cell wall polysaccharides and also to xylan, thus acting as protection against cell wall degrading enzymes (Dodd *et al.* 2009). Lignin is a reticulated macromolecule principally comprised of three aromatic alcohols: p- coumaryl alcohol,

coniferyl alcohol and sinapyl alcohol (Dashtban *et al.* 2009). It is the most recalcitrant material found in lignocellulosic biomass.

Lignocellulosic biomass for energy production includes plant or animal matter that can be converted into fibres or other industrial chemicals, including biofuels. Industrial biomass can be grown from numerous types of plants, including *Miscanthus*, switchgrass, corn (maize, mainly used in the USA), poplar, willow, sorghum, sugarcane (mainly used in Brazil), bamboo, (Volk 2000) and a variety of tree species, ranging from eucalyptus to oil palm. Biomass can be converted to other usable forms of energy like methane gas or transportation fuels like ethanol and biodiesel. Rotting garbage, and agricultural and human waste, all release methane gas - also called landfill gas or biogas. Crops, such as corn and sugar cane, can be fermented to produce the transportation fuel, ethanol. Biodiesel, a renewable, clean burning diesel replacement, can be produced from left-over food products like vegetable oils and animal fats in a reaction between these lipids and an alcohol to produce fatty acid esters.

An attractive source of lignocellulose, in Europe, is wheat straw (*Triticum* spp.), because it is a co-product of one of the most abundant crops in European countries with a production of more than 132 million tons (Kim *et al.* 2004). Wheat, which typically yield 7.5–8 tonnes of grain per hectare, and straw, which typically yield 3.5–5 tonnes per hectare in the UK can be used for liquid transportation fuels as well as be burned to produce heat or electricity (BIOMASS Energy Centre 2013). Also willow (*Salix* spp.) is already being grown in parts of the UK as a plant biomass source, because the coppicing response of willows allows them to be grown in short rotation coppice cycles. Coppicing reinvigorates the plant and accelerates the growth which leads to higher biomass yields (Karp *et al.* 2008).

Both wheat straw and willow have a different cell wall composition that may require different sets of enzymes to degrade the biomass to usable monosaccharides. Growing *A. niger* on either wheat straw or willow may therefore yield different gene expression patterns on the different substrates in particular for genes coding for proteins involved in the degradation of the plant cell wall. Alternatively, both substrates share many of the same structural molecules albeit in a different ratio and thus should share a core set of hydrolytic enzymes expressed by *A. niger*. However, different ratios of structural components can determine the ease of degradation for hydrolytic enzymes. The difference in the amount of lignin between straw and willow

could for example contribute to the difference in difficulty to degrade one of the substrates compared to the other.

### 1.2.2 Lignocellulose degradation

#### 1.2.2.1 Microorganisms able to degrade lignocellulose

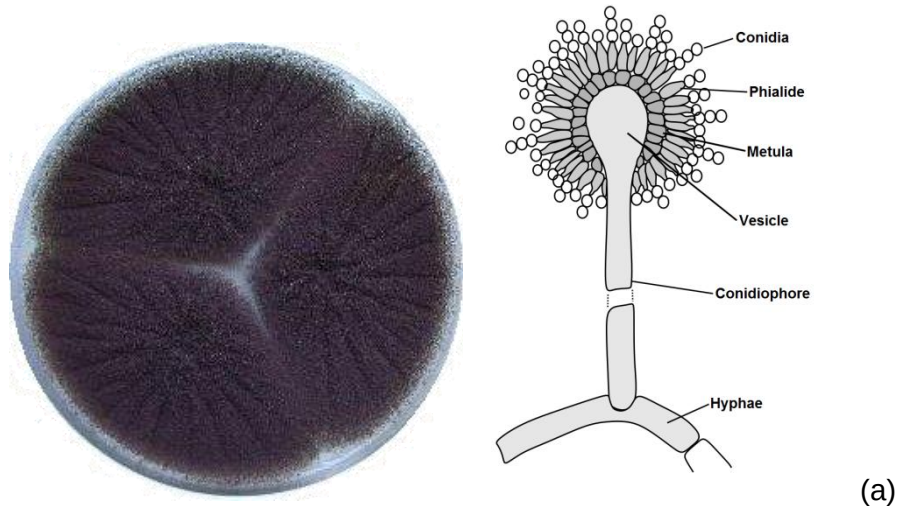
The complex nature of plant cell walls makes the components highly recalcitrant to degradation. Different microorganisms such as fungi and bacteria are able to degrade plant cell walls by producing different types of enzymes to degrade lignocellulose. However, the mechanisms involved in this degradation are still poorly understood. Many of the enzymes known to degrade lignocellulose have a Carbohydrate Binding Module (CBM) and they are described in the Carbohydrate Active EnZymes (CAZy) database ([www.cazy.org](http://www.cazy.org)). CBMs were previously known as cellulose-binding domains after the discovery of several domains binding to cellulose (Gilkes *et al.* 1991). A classification of the CBMs has been made according to the amino acid sequence similarities between the different CBMs (Tomme *et al.* 1995). The CAZy database contains 67 CBM families (April 2013). Three roles are commonly attributed to CBMs: (i) providing a proximity effect (tighter association of the enzyme with the polysaccharide thus keeping entire enzyme and the substrate in proximity), (ii) a targeting function (potentiate enzyme activity against cell walls by targeting polymers that are in close proximity to the substrate of the appended catalytic module), (iii) a disruptive function (disruption of the surface of tightly packed polysaccharides causing the substrate to loosen and become more exposed to the catalytic module for more efficient degradation) (Gao *et al.* 2010; Herve *et al.* 2010; Wang *et al.* 2008b).

In contrast to cellulases in fungi, bacteria produce a self-assembling protein complex that functions as an enzyme called the cellulosome. The enzymes forming the cellulosome usually don't have CBM (Poole *et al.* 1992). However, the protein to which these enzymes are attached (called scaffoldin) does contain a CBM which permits the binding of the complex to the cellulose (Wilson 2011). Both fungi and bacteria are able to produce cellulases whether they are aerobic or anaerobic microorganisms. These enzymes generally belong to the same families but some exceptions exist. Only aerobic fungi produce GH7 cellulases and the bacterial cellulosomes do not seem to have family 6 exocellulases. The anaerobic cellulolytic thermophilic bacterium of the species *Caldicellulosiruptor* has been found to secrete

cellulases containing CBMs. These bacteria are known for their efficient degradation of un-pre-treated plant biomass (Blumer-Schuette *et al.* 2010).

### 1.2.2.2 *Aspergillus niger*

*Aspergillus niger* is a black-spored filamentous ascomycete fungus that is commonly found in nature (**Figure 1.4**) (Aro *et al.* 2005; Baker 2006). Genomes of at least three strains of *A. niger* have been sequenced so far: NRRL 3 (Baker 2006), ATCC 1015 (Andersen *et al.* 2011; Baker 2006) and CBS 513.88 (Pel *et al.* 2007). NRRL 3 and ATCC 1015 are wild-type strains of *A. niger* whereas CBS 513.88 is a mutant strain derived from NRRL 3122, sequenced by DSM.



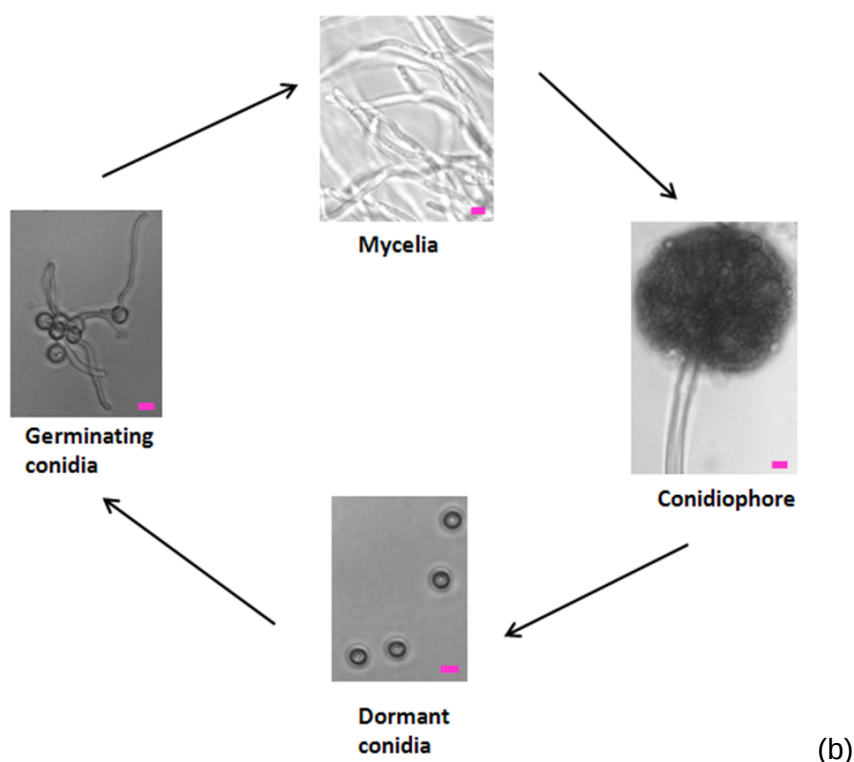


**Figure 1.4:** (a) Macroscopic (Read *et al.* 1991) and microscopic aspect of *A. niger* (adapted from [http://commons.wikimedia.org/wiki/File:Conidiophore\\_Aspergillus\\_niger.svg](http://commons.wikimedia.org/wiki/File:Conidiophore_Aspergillus_niger.svg)) (b). *Aspergillus niger* asexual life cycle. Pictures were taken from cultures of *A. niger* N402 growing in glucose (1% m/v) minimal media. The dormant spores and conidiophore images were taken from conidia washed directly from glucose minimal agar slopes after 6 days, the image for germinating conidia was taken after 10h and the image of mycelium was taken 24h after incubation. ■ = 5μm (Hayer 2013).

*A. niger* is a common member of microbial communities found in the soil (Baker 2006) and it is saprobic in nature - capable of degrading plant biomass. In this way, it contributes to the global re-cycling of carbon (Aro *et al.* 2005; Baker 2006; Lynd *et al.* 2002). It is also a commercially important species because of its ability to produce organic acids, e.g. citric acid and gluconic acid. Citric acid is used in the food industry for the purpose of flavouring and controlling pH of food and beverages. It used to be extracted from citrus fruits, until the potential of *A. niger* to produce vast quantities of citric acid was realised (Hattori *et al.* 2009; Karaffa *et al.* 2003; Punekar *et al.* 1984). Greater than one million metric tons can be produced annually, with a conversion of 95% (by weight) of sugar substrate, making this fermentation process highly efficient on an industrial scale (Baker 2006; Karaffa *et al.* 2003). Gluconic acid is used as a

food additive, regulating the acidity of food products. It is also used in cleaning products and for therapy of iron/calcium deficiencies (Bennett 2010).

*A. niger* is also of major interest to mycologists because it is a fungus that causes spoilage of food. Visible black mould can be seen on common household fruits and vegetables, e.g. grapes, tomatoes and onions (Hasan 1995; Sinha *et al.* 1994). Food spoilage can be caused by the fungus at different stages of its development: both the conidia and the hyphae can cause spoilage. As well as this, the production of mycotoxins (toxic secondary metabolites produced by moulds) can also lead to food spoilage (Klich 2009). Infection of crops like maize by the fungus is a route of entry for mycotoxins into the food chain, since humans can directly consume it. The toxins themselves are resistant to: degradation by the digestive system, high temperatures and they resist decomposition, which means that they remain for long periods in the food chain in meat and dairy products without being destroyed. Ochratoxin A (Abarca *et al.* 1994; Klich 2009) is the mycotoxin produced by *A. niger* in food that has not been stored properly. Approximately 25% of the world's agricultural products are thought to be contaminated with mycotoxins which results in crop losses, costing \$1 billion dollars (Council for Agricultural Science and Technology 2003).

Although *A. niger* is a well-known cause of food spoilage, it is also an opportunistic human pathogen. *A. niger* however, is a species of *Aspergillus* that is thought to be less of a human pathogen than other members of the genus, especially when compared to *Aspergillus fumigatus* which is often responsible for the majority of human infections (Latge 1999; Moss 2002). Since spores can easily be aerosolised, humans are constantly exposed to them, both in outdoor and indoor environments (Klich 2009; Latge 1999). The spores are therefore capable of entering the bodies through breathing (Bennett 2010). Added to this, *A. fumigatus* and other aspergilli are able to grow at 37°C, body temperature, which means that inhalation of a large number of spores can lead to diseases, especially respiratory problems, allergies and aspergillosis (Moss 2002). Allergies can be caused by the presence of either fungal conidia or hyphae; and aspergillosis occurs as a result of direct growth of the fungus inside host tissue. It usually affects immune-compromised patients (e.g. patients recovering from a bone marrow transplant) and leads to either localised or disseminated/systemic disease (Bennett 2010; Klich 2009; Vonberg *et al.* 2006). Manifestation of aspergillosis indicates that the body's immune system is weakened and the use of immune-suppressive drugs in hospitals has created a niche for *Aspergillus* species to grow (Bennett 2010; Klich 2009). This knowledge has demonstrated that new, more effective anti-fungal drugs are required (Bennett 2010).

*A. niger* is also commonly used in industry for enzyme production. As *Trichoderma* spp. are the main industrial species of choice for cellulase production, *A. niger* is therefore used as a model organism to provide new insights into more efficient sustainable approaches for the generation of renewable energy, instead of being used as a direct replacement for other microorganisms as a source for cellulases. *A. niger* is already a source of industrial enzymes, such as amylases (Hernández 2006), proteases (Punt *et al.* 2003) and pectinases (Bai *et al.* 2004). Glucoamylase is used in the production of high fructose/sugar syrups; whilst pectinases are used in the cider and wine industry (Bennett 2010). *A. niger* also produces enzymes that are capable of breaking down substrates encountered in their environment. By understanding the types of hydrolytic enzymes produced and how they are regulated, they can be efficiently exploited for the saccharification of lignocellulosic biomass. Thus, transcriptomic studies of *A. niger* growing in the presence of such substrates become relevant here. *A. niger* is being used as a model fungus in the Nottingham laboratory group, where research projects involve understanding how the fungus responds to the presence of wheat straw as a model lignocellulosic substrate (Delmas *et al.* 2012).

### 1.2.2.3 Enzymatic hydrolysis

The enzymatic hydrolysis is a key step in the production process of 2G biofuels as this step permits the release of fermentable sugars from lignocellulosic biomass (**Figure 1.5** and **Figure 1.6**). The susceptibility of the lignocellulosic substrates towards enzymatic hydrolysis is highly dependent on their structure, morphology and composition. To optimize the kinetics and the yield of sugars from this key step, a pre-treatment of the biomass is necessary for the enzymes to access cellulose and hemicellulose. Enzymatic hydrolysis is a key step in the biofuel production process but it is also the most costly step (Klein-Marcuschamer *et al.* 2012). This step represents 30% of the ethanol production cost, and therefore studies are carried out to try to improve this step (Agrawal *et al.* 2013).

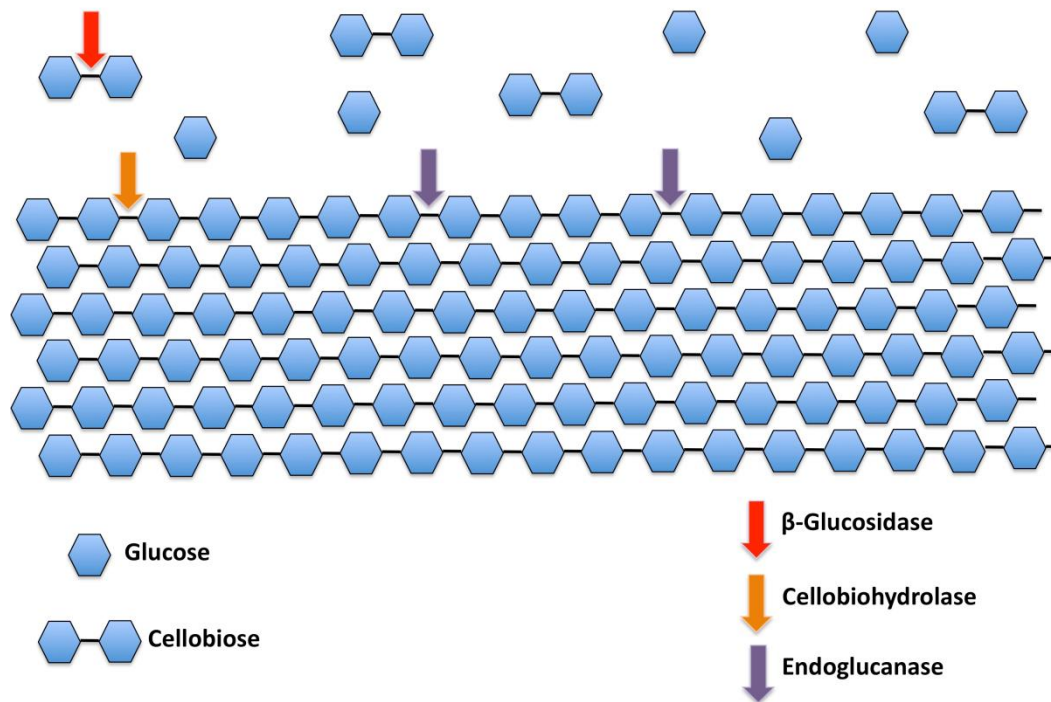


**Figure 1.5:** Schematic view of cellulose degradation. Endoglucanases hydrolyse cellulose bonds internally, while cellobiohydrolases cleave cellobiose units from the ends of the polysaccharide chains. The released cellobiose units (disaccharide) are finally hydrolysed by  $\beta$ -glucosidases, releasing glucose, the main carbon source readily metabolisable by fungi (Souza 2013).

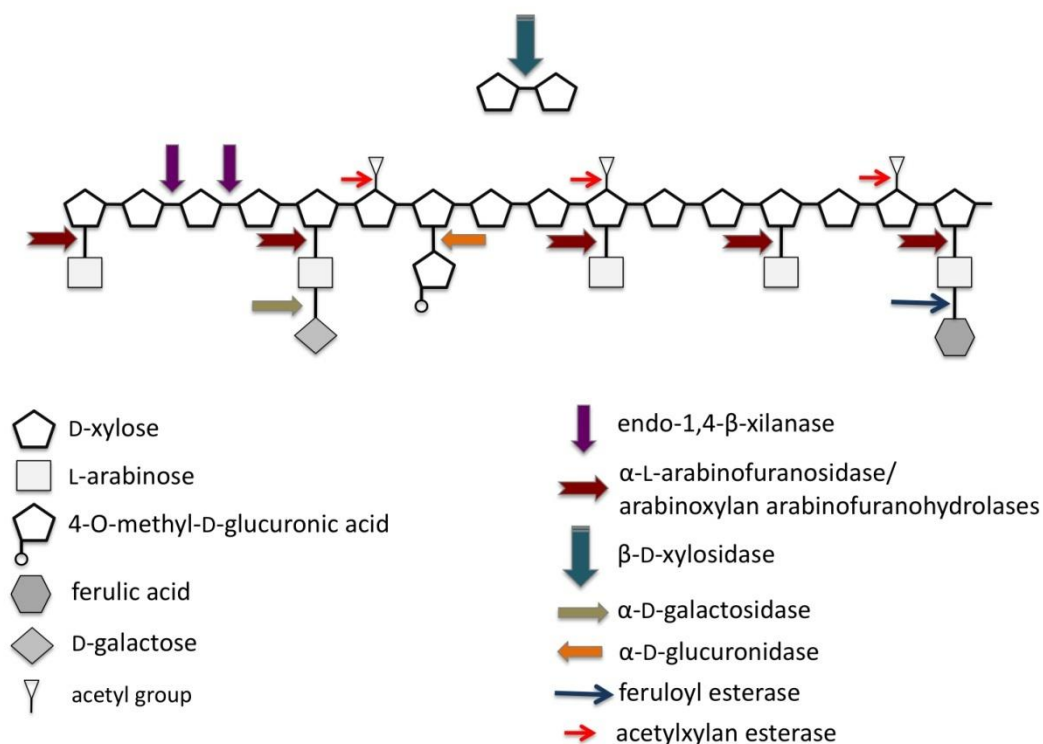


**Figure 1.6:** Schematic view on a hemicellulolytic system, degradation of arabinoxylan is depicted. The arrows represent each enzyme active for a determined substrate (Souza 2013).

#### 1.2.2.4 Carbohydrate active enzymes (CAZy)

CAZy enzymes catalyse the breakdown, formation or modification of carbohydrates. These enzymes are organised into different families based on structurally related catalytic functions or Carbohydrate Binding Motifs (CBMs) present in the amino acid sequence. Various enzyme classes described in the CAZy database (<http://www.cazy.org/>) have been mentioned in the previous sections as they are abundantly present in many microorganisms. The database describes the glycoside hydrolase (GHs), glycosyl transferase (GT), polysaccharide lyase (PL) and carbohydrate esterase (CE) enzyme classes. In addition the CAZy database also describes CBMs that are involved with adhesion to carbohydrate. Using a sequence-based classification, with algorithmic methods to assign sequences to various families, the GHs have been assigned to more than 100 families (Bairoch 1999; Henrissat 1999; Henrissat *et al.* 1995a; Henrissat *et al.* 1995b). These methods can provide help in the classification of proteins for which no biochemical evidence has been obtained (Withers 2011).

The genome of *A. niger* encodes a large set of plant cell wall degrading enzymes: 251 Glycoside Hydrolases (GHs) (55 families), 25 Carbohydrate Esterases (CEs), 8 Polysaccharide Lyases (PLs) and 29 Hemicellulases (Cantarel *et al.* 2009).

### 1.2.2.4.1 Glycoside hydrolases (GHs)

Glycoside hydrolases are classified under the Enzyme Commission Number EC 3.2.1 as enzymes that catalyse the hydrolysis of O-, N- and S- linked glycosides to release smaller sugars (Withers 2011). They are extremely common enzymes with roles in nature including degradation of biomass such as cellulose and hemicellulose. Together with glycosyltransferases, glycosidases form the major catalytic machinery for the synthesis and breakage of glycosidic bonds.

In Eukaryotes, glycoside hydrolases are found within the endoplasmic reticulum and Golgi apparatus where they are involved in processing of N-linked glycoproteins, and in the lysosome as enzymes involved in the degradation of carbohydrate structures. Two reaction mechanisms are most commonly found for the retaining and inverting enzymes, at first outlined by Koshland (1953) and then by Sinnott *et al.* (1990). Inverting enzymes utilize two enzyme residues, typically carboxylate residues that act as acid and base respectively whereas retaining glycosidases operate through a two-step mechanism, with each step resulting in inversion. Again, two residues are involved with one acting as a nucleophile and the other as an acid/base. In the first step the nucleophile attacks the anomeric centre, resulting in the formation of a glycosyl enzyme intermediate, with acidic assistance provided by the acidic carboxylate. In the second step the now deprotonated acidic carboxylate acts as a base and assists a nucleophilic water to hydrolyse the glycosyl enzyme intermediate, giving the hydrolyzed product. Several interesting variations on these mechanisms have been found. One fundamentally different mechanism has also been described that is catalyzed by an NADH cofactor (Rajan *et al.* 2004; Yip *et al.* 2004).

Glycoside hydrolases can also be classified as exo- or endo-acting, depending on whether they act at the (usually non-reducing) end or in the middle, respectively, of an oligo/polysaccharide chain. Glycoside hydrolases may also be classified by sequence or structure based methods (CAZy website). Recent progress in glycosidase sequence analysis and 3D structure comparison has allowed the proposal of an extended hierarchical classification of the glycoside hydrolases (Naumoff 2011).

### 1.2.2.4.2. Cellulases

Cellulases are cellulose degradative enzymes. Three different types of cellulases can be distinguished. Firstly, the endoglucanases cleave internal bonds in the amorphous region of the cellulose to create new chain ends. Then, the cellobiohydrolases act to cleave two to four units from the ends of the chains produced by endoglucanases, resulting in polysaccharides, such as cellobiose. Finally, the beta-glucosidases hydrolyse the cellobiose into glucose (Dashtban *et al.* 2009; Liu *et al.* 2011; Mathew *et al.* 2008).

#### 1.2.2.4.2.1 Hemicellulases

Hemicellulases are enzymes degrading hemicelluloses which include xyloglucans, xylans, galactomannans and pectins. Xylan backbones are degraded by endoxylanases which hydrolyse xylan into oligosaccharides and  $\beta$ -xylosidase degrades the oligopolymers of xylose (up to 4 residues) into xylose. The endoxylanases have been reported to have both specific and non-specific cleavage sites, the specific ones dependent on the substituents on the xylose residues neighbouring the cleaving site, while others cleave at random sites.

The other hemicelluloses, galactoglucomannans, are degraded by  $\beta$ -endomannases into mannoooligosaccharides, and further hydrolysed to mannose residues by  $\beta$ -mannosidases. The smooth region (i.e. unbranched) of pectin is degraded by three groups of enzymes: pectin lysases, pectate lysases and polygalacturonases, while several enzymes are responsible for degrading the hairy regions (i.e. branched). Moreover, *Aspergillus* species produce enzymes specific for the many residues in the side chains of hemicellulose and pectin, and which cleave the side chains either at the branching point next to the backbone, or between residues in the side chain. These side chain degrading enzymes have been shown to have synergistic interactions between them and the backbone of degrading enzymes (de Vries *et al.* 2001).

#### 1.2.2.4.1.2 Ligninases

Ligninases are enzymes degrading lignin. They can also be found under the name Lignin-modifying Enzymes (LMEs) as they are not hydrolytic but oxidative enzymes. Ligninases can be classified into two families: peroxidases, such as lignin peroxidases, and phenol-oxidases of Laccase type (Dashtban *et al.* 2009). These enzymes catalyse several types of reactions such as cleaving the  $\beta$ -O-4 ether bonds

in lignin which is essential for the depolymerisation of lignin. White-rot basidiomycetes are known to be the most efficient producers of ligninases in fungi.

### 1.2.2.4.3 Carbohydrate esterases (CEs)

Carbohydrate Esterases are enzymes catalyzing the de-O- or de-N-acylation of substituted saccharides releasing sugar halves. Acetylxytan esterases are common components of hemicellulolytic and cellulolytic enzyme systems of microorganisms proliferating on plant biomass residues. Their function is to deesterify partially acetylated hardwood 4-O-methyl-D-glucuronoxylan and xylans of annual plants (Biely *et al.* 1986; Biely *et al.* 1985). These esterases are part of hemicellulolytic enzyme systems and found in 8 of 15 carbohydrate esterase (CE) families. Most of the CEs are serine-type esterases, also attacking low-molecular-mass substrates such as 4-nitrophenyl (4-NP) or 4-methylumbelliferyl acetate (Biely *et al.* 1999; Tenkanen *et al.* 2003). An exception in this regard is the acetylxytan esterases of CE family 4, which also contain chitin deacetylases. These enzymes do not operate on the two above-mentioned aryl acetates and also do not possess the Ser-His-Asp catalytic triad. They are aspartate deacetylases, activated with bivalent metal cations coordinated with two histidines (Puchart *et al.* 2006; Taylor *et al.* 2006).

### 1.2.2.4.4 Polysaccharide lyases (PLs)

Polysaccharide Lyases (EC 4.2.2.-) are a ubiquitous group of enzymes in nature (Sutherland, 1995). These enzymes cleave uronic acid-containing polysaccharide using a  $\beta$ -elimination mechanism instead of a hydrolytic mechanisms (intervention of a water molecule) to generate an unsaturated hexenuronic acid residue and a new reducing end. These enzymes are often complementary to the action of GHs for the breakdown of C6-carboxylated polysaccharides (Lombard *et al.* 2010).

### 1.2.2.4.5 Copper-dependent monooxygenases (Family AA9 – formerly GH61)

The enzymes in this family were originally classified as a glycoside hydrolases (GH61) based on very weak endo-1,4-beta-D-glucanase activity in one family member. They are now reclassified into Auxiliary Activity Family 9 (AA9) in CAZy (Levasseur *et al.* 2013). The first AA9 protein was identified in the fungus *Agaricus bisporus* where the gene encoding the protein was found to be induced on growth on cellulose and a CBM domain was found in its protein sequence (Raguz *et al.* 1992). Since, AA9 family members have been described in other fungi such as *T. reesei*, *Aspergillus* species and *Neurospora crassa* with their co-induction with classical

cellulases upon growth on cellulose suggested that the AA9 was involved in lignocellulose degradation (Raguz *et al.* 1992; Wymelenberg *et al.* 2002). Enzymes of the family AA9 are copper-dependent oxidases which require the presence of small molecule redox-active co-factors such as gallate and ascorbate for enhanced activity (Quinlan *et al.* 2011). They also strongly bind a copper ion which is essential for enzymatic function (Phillips *et al.* 2011; Quinlan *et al.* 2011; Westereng *et al.* 2011). AA9 family members initiate cellulose degradation by generating oxidised and non-oxidised cellodextrin products which are easily acted upon by cellulases and thus differ significantly in their mode of action from conventional GHs which use acid/base catalysis to break glycosidic bonds. The *A. niger* genome encodes 7 AA9 proteins of which three have been characterized in more detail. The genes An15g04570 and An14g02670 encoding two of these proteins have been shown to contain a CBM1 domain. The EglD (gene An15g04900) protein has been described to have a CBM1 domain as well as having an endoglucanase activity (Pel *et al.* 2007).

### 1.1.2.5 Non enzymatic degradation

Some higher fungi are able to degrade lignocellulose by the production of free hydroxyl radicals through the Fenton reaction. This reaction allows the release of hydroxyl radicals from hydrogen peroxide. The free radicals produced are highly reactive and attack polysaccharides and lignin in a non-specific manner. This provides cleavages which make it easier for cellulolytic enzymes to access lignocellulose (Dashtban *et al.* 2009).

Three main pathways have been described for the production of free radicals: (1) cellobiose dehydrogenase (CDH); (2) low molecular weight peptide/quinone redox cycling; (3) glycopeptide-catalyzed Fenton reaction.

- (1) CDHs are able to modify and degrade the three main components of lignocellulose, cellulose, hemicellulose and lignin, by producing free hydroxyl radicals in a Fenton type reaction (Baldrian *et al.* 2008).
- (2) Some fungi such as *Gloerophyllum trabeum* produce a low molecular weight peptide which can degrade cellulose into short fibres by an oxidative reaction (Yang *et al.* 2004). Some of these low molecular weight compounds are quinones that are converted to hydroquinones by some fungal enzymes. Free radicals will be produced from these hydroquinones through Fenton reaction (Wang *et al.* 2008a).

- (3) Other glycopeptides have been found in other fungi such as *Phanerochaete chrysosporium* that are able to catalyse redox reaction and thus produce free hydroxyl radicals (Tanaka *et al.* 2007).

### 1.3 Regulation of genes encoding glycoside hydrolases in *A. niger*

A large number of factors affect the expression of CAZyme-encoding genes during exposure to lignocellulose. These include the fungal species (and derived strains), the source of lignocellulose (such as sugar cane bagasse, wheat straw or willow, amongst others), lignocellulose pre-treatments (e.g. maceration/grinding, heat, acid/alkaline hydrolysis, ionic liquids and others) and the small regulatory molecules that are released from the substrate. There is increasing knowledge of the small molecules that serve as regulators of genes that encode CAZymes, as well as the transcription factors that mediate that regulation (Herold *et al.* 2013; Kubicek 2012; Znameroski *et al.* 2013). It can be overly simplistic to refer to repressing (e.g. glucose) and inducing (e.g. xylose) monomers for three reasons. Firstly, the monomer concentrations may also be relevant. Secondly, in natural environments and during the saccharification of lignocellulose by fungi, a mixture of these sugars is present. Thirdly, disaccharides as well as monomers can serve as regulatory molecules, such as the disaccharide sophorose in *T. reesei*. In their responses to lignocellulose, fungi respond to the interface at the surface and are exposed to a succession of changing conditions over time as the lignocellulosic material is degraded. It is apparent too that transcriptional regulation of CAZyme-encoding genes is affected by pH (Hakkinen 2014) and light (Tisch *et al.* 2013).

### 1.4 Aims of the study

This project aims to explore the regulatory networks involved in the breakdown of lignocellulosic materials by fungi. This will provide new areas of potential improvement for industrial processes for production of second generation biofuels.

Specific Aims are:

- Identify homologues in *A. niger* of the regulatory genes *clr-1*, *clr-2* and *clbR* previously described in other fungal species.
- Functionally characterize those regulatory genes in *A. niger* and explore their interactions in regulatory networks.

- Identify new regulators using RNA-sequencing technology and phosphoproteomics tools
- Investigate the role of hydrophobic surface interacting proteins (HSIPs) in the breakdown of lignocellulose and the expression of genes encoding GH enzyme.

## Chapter 2. Materials and methods

## 2.1 Materials

### 2.1.1 Biological materials

#### 2.1.1.1 Fungal strains

The *A. niger* strain AB4.1 ( $\Delta$ pyrG) was used in this work (van Hartingsveldt *et al.* 1987). The derived mutants of the AB4.1 strain used in this work are the  $\Delta$ xlnR,  $\Delta$ clrA,  $\Delta$ creA,  $\Delta$ hyp1,  $\Delta$ hfbD,  $\Delta$ hsbA and  $\Delta$ hyp1 $\Delta$ hfbD strains. The  $\Delta$ clrB and  $\Delta$ farA strains were kindly provided by A. Ram (Leiden University, The Netherlands). They were made in *A. niger* MA234.1 derived from MA169.4 (Carvalho *et al.* 2010) and contain a *hygB* marker (pGpdA-*hygB*-tTrpC). The AB4.1,  $\Delta$ xlnR,  $\Delta$ clrA,  $\Delta$ creA,  $\Delta$ hyp1,  $\Delta$ hfbD,  $\Delta$ hsbA and  $\Delta$ hyp1 $\Delta$ hfbD strains were grown on Potato Dextrose Agar (PDA, Oxoid) supplemented with 10mM uridine (final concentration) for routine cultures. The  $\Delta$ clrB and  $\Delta$ farA strains were grown on PDA. Agar slopes were inoculated with *A. niger* conidia and incubated at 28°C for 4 days with loose caps, after which time the caps were tightened and the slopes with visible conidiated growth of *A. niger* were stored at room temperature.

#### 2.1.1.2 Bacterial strain

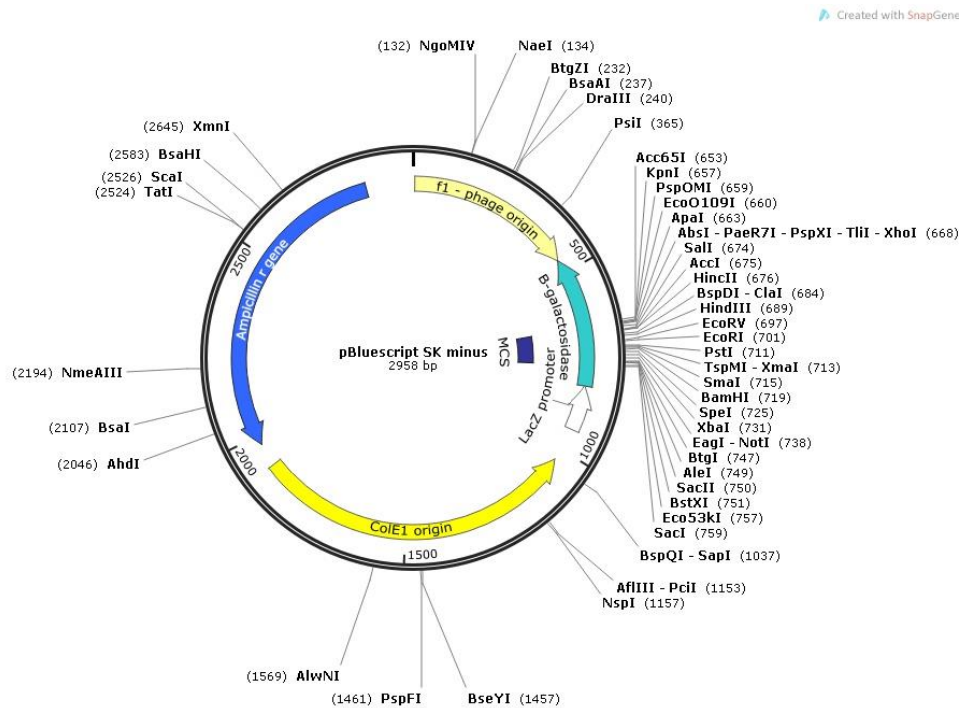
*Escherichia coli* XL-1 Blue strain (Invitrogen) was used in this study as it allows blue-white colour screening and preparation of high-quality plasmid DNA.

### 2.1.2 Nucleic acid materials

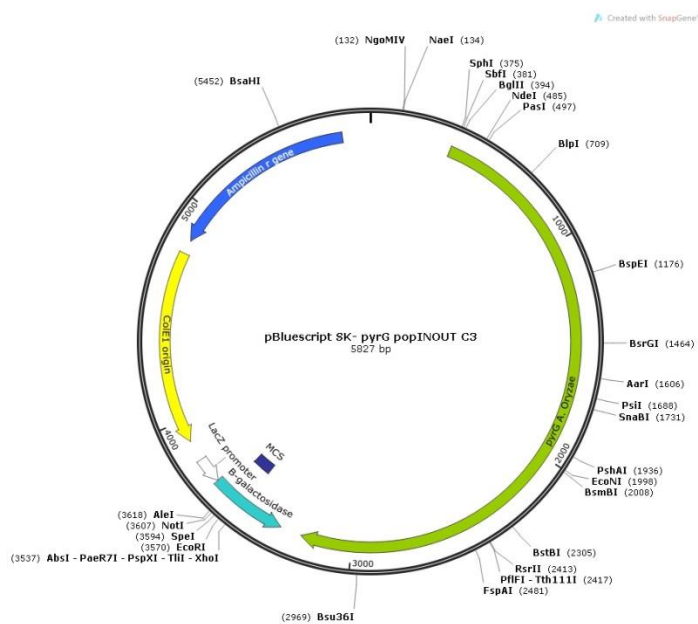
#### 2.1.2.1 Vectors

##### 2.1.2.1.1 PopIN/PopOut plasmids

The plasmids pBluescript SK – PyrG PopIN/PopOut C3 or C7 (pC3 and pC7) were used in this work (Delmas *et al.* 2014). They were derived from the pBluescript SK minus plasmid which contains several useful sequences for use in cloning methods. The sequences include a polylinker sequence (MCS), antibiotic resistance sequence to ampicillin and an *E. coli* and f1 helper phage origin of replication. The polylinker sequence is located within a LacZ-controlled gene designed to provide a blue coloration when expressed in bacteria allowing a blue-white screening. The pC3 and pC7 plasmids were modified to include a *pyrG* gene to permit the selection of *A. niger* transformants in the auxotrophic strain AB4.1 (**Figure 2.1**).



**A**



**B**

**Figure 2.1:** Schematics of the pBluescript SK plasmid with the Multiple Cloning Site (MCS) (A) and of the PyrG PopIN/OUT pC3 plasmid (B) containing the *pyrG* gene from *A. oryzae*.

### 2.1.2.2 Plant material

The wheat straw (Cordiale variety) used in this work was obtained from the Bioenergy and Brewing Science department (Sutton Bonington, Leicestershire, U.K.) and milled using a Laboratory Mill (Laboratory Mill 3600, Perten, Sweden) and passed through a sieve with a mesh size of 700  $\mu$ m for size reduction prior to ball

milling. 5 g of pre-milled wheat straw was ball-milled in 80 mL stainless steel grinding bowls with 10mm diameter steel balls in a Planetary Mill (Pulverisette 5 classic line, Fritsch, Germany), at 400 rpm for a grinding time of 20 min, resulting in an average particle size of 75  $\mu\text{m}$ .

## 2.2 Methods

### 2.2.1 Culture of *A.niger*

For cultures of the AB4.1 strain and of the mutants derived strains of *A. niger*, spores were washed with 0.01% (v/v) Tween-80 solution and  $10^6$  spores/ml (final concentration), in solution, were inoculated into a 250ml sterile conical, Erlenmeyer, flask containing 100ml *Aspergillus* Minimum Medium (AMM) (6g sodium nitrate ( $\text{NaNO}_3$ ); 20ml *Aspergillus* salt solution [all  $\text{l}^{-1}$ : KCl, 0.52 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.52 g;  $\text{KH}_2\text{PO}_4$ , 1.52 g;  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ , 0.008 mg;  $\text{Cu-SO}_4 \cdot 5\text{H}_2\text{O}$ , 0.16 mg;  $\text{FePO}_4 \cdot \text{H}_2\text{O}$ , 0.16 mg;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 0.16 mg;  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.16 mg;  $\text{ZnSO}_4$ , 1.6 mg];  $\text{dH}_2\text{O}$  up to 1L, pH6.5) 1% (w/v) glucose solution and incubated for 48h at 28°C on a shaker (New Brunswick Scientific) set to 150rpm. Cultures were set up containing single carbon source substrates, added at 1% (w/v) to AMM. Substrates used were glucose and ball-milled wheat straw. Each medium was autoclaved at 117°C instead of 121°C so as not to result in the sugars caramelising. For studies involving time-points, the *A. niger* strain used was grown in AMM containing 1% (w/v) glucose for 48h before being transferred to AMM supplemented with 1% (w/v) of the desired carbon source and incubated further for 24h or 48h. Supernatants were collected at each time point by separating the mycelia and the insoluble straw by filtration through Miracloth (Calbiochem) for enzymatic analysis.

### 2.2.2 Extraction of nucleic acids

#### 2.2.2.1 gDNA extraction

*A. niger* was grown in 250ml conical flasks containing 100ml *Aspergillus* Complete Media (ACM, 20ml *Aspergillus* salt solution, 10ml *Aspergillus* vitamin solution (400mg p-aminobenzoic acid, 2mg d-biotin, 1.4g choline chloride, 100mg nicotinic acid, 250mg pyridoxine hydrochloride, 100mg riboflavin and 50mg thiamine hydrochloride acid per litre), 0.075g adenine, 1g casmino acids, 10g glucose, 2g peptone and 1g yeast extract per litre, adjusted to pH 6.5 with NaOH). The flasks were inoculated with  $10^6$  spores/ml (final concentration) and incubated at 28°C shaking at 150rpm for 24h. Mycelia were collected through Miracloth (Calbiochem), washed with ACM and flash frozen in liquid nitrogen prior to DNA extraction. The mycelia were re-

suspended in 600µl Fungal DNA Extraction Buffer (50mM Tris-HCL (pH7.5), 10mM EDTA, 50mMNaCl, 1% (w/v) SDS) and, after 1min of vortex, the suspension was incubated at 65°C for 30min. The samples were centrifuged at 16060g for 5min in a Biofuge pico centrifuge (Heraeus). 500µl of supernatant was then mixed with 400µl phenol:chloroform:isoamyl alcohol (25:24:1v/v/v, Sigma) and transferred in 2ml phase-lock tubes (5Prime) and centrifuged again at 16060g for 10min. 500µl of the aqueous phase, upper layer, was then dispensed with an equal volume of isopropanol (propan-2-ol, Fisher Scientific) to precipitate the genomic DNA(gDNA) for 20min at 4°C before being centrifuged at 16060g for 10min. The pellets were washed with 70% (v/v) ethanol and centrifuged at 16060g before drying in a laminar air flow cabinet and the gDNA was then dissolved in 100µl TE buffer (10mM Tris (pH8), 1mM EDTA). The gDNA samples obtained were stored at -20°C. The concentrations of gDNA were measured using a Nanoview Plus (Labtech) at 260nm.

### *2.2.2.2. Plasmid DNA extraction*

The different plasmids studied were isolated from the cultures using Zyppy™ Plasmid Miniprep Kit (Zymo Research) according to the manufacturer's instructions.

### *2.2.2.3 RNA extraction*

At each given time, mycelia were collected through Miracloth (Calbiochem), washed with AMM containing no carbon source and flash frozen in liquid nitrogen prior to RNA extraction. For long term storage mycelia were kept at -80°C. Frozen *A. niger* mycelia were ground to a powder using a mortar and pestle. The powder obtained for each sample was added to tubes containing 1mL TriZol (Invitrogen) and the suspensions were incubated at room temperature for 5 minutes before the addition of 200 µL of chloroform. The mixtures were vortexed for 15 seconds and incubated at room temperature for 5 minutes. The two layers were separated by centrifugation at 16060g for 10 minutes. The upper phase was then transferred to tubes containing 750 µL of isopropanol and incubated for 10 minutes at room temperature. The tubes were centrifuged (16060g for 10 min) before the supernatant was removed and discarded. The pellets were washed with 75% v/v ethanol, air-dried for 2-5minutes and then re-dissolved in 100 µl DEPC-treated H<sub>2</sub>O for 20 min at 4°C. All solutions, unless otherwise stated, were prepared in DEPC-treated H<sub>2</sub>O. The RNA was purified again after extraction using a NucleoSpin RNA II kit (Macherey-Nagel) including the additional on-column DNase digestion according to the manufacturer's protocol to eliminate genomic DNA contamination.

### 2.2.3 DNA analysis

#### 2.2.3.1 Hydrolysis with restriction enzymes

DNA restriction analysis required the utilisation of different endonucleases used between 1 to 2 Units per µg of DNA according to the manufacturer's instructions.

#### 2.2.3.2 Electrophoresis

DNA fragments from PCR or from restriction were separated according to their sizes on 1% (w/v) agarose gels in 1x TAE (stock 50x: 242 g Tris base/L, 57.1 ml glacial acetic acid/L and 100 ml of 500 mM EDTA/L). Gels were stained using ethidium bromide at a final concentration of 0.1 mg/ml. All the pictures were taken from BioRad Chemidoc XRS+ using the Quantity One 4.6.6 program (BioRad).

The buffer used for electrophoresis of RNA samples was 1x TBE buffer (stock 10x: 107.8g/L Tris base, 55 g/L boric acid and 5.8 g/L EDTA (20mM final concentration)).

The expected sizes of PCR products were calculated *in silico* using the target gDNA sequence with the corresponding primers used for the PCR. The estimation of sizes of amplified fragments was done using molecular weight standards for agarose gel electrophoresis. The digested standard DNA includes fragments ranging from 0.5-10.0 kilobases (kb). The exact size of the obtained product was confirmed by sequencing the PCR product.

#### 2.2.3.4 PCR amplification

##### 2.2.3.4.1 RT-PCR

DNA was amplified in a 50µl reaction volume containing 100 nM of each primer, 10mM of dNTP mix, 5x High Fidelity Buffer, 0.5U Phusion® polymerase (New England Biolabs), approximately 50ng of template DNA (gDNA or cDNA), and filtered sterile distilled water (filtered SDW). The PCR amplifications were carried out in Techne thermocyclers after an initial denaturation at 98°C for 5 minutes, followed by 30 to 35 cycles and a final extension at 72°C for 5 minutes. Each cycle was composed of a denaturation step at 98°C for 20 seconds, annealing at 60°C (depending on the primers) for 20 seconds, and extension at 72°C for 30 seconds.

##### 2.2.3.4.2 qRT-PCR

qRT-PCR amplifications were carried out using the Applied Biosystems 7500 Fast Real-Time PCR system. The PCR reaction mixture (10 µl) contained 1 µl of cDNA, 0.4 µl of specific primer sets (175 nM final concentration), and FAST SYBR-Green

Master Mix (Applied Biosystems). PCRs were carried out for 40 cycles; denaturation at 95°C for 15 seconds, annealing at 67°C for 30 seconds, and extension at 60°C for 60 seconds. All measurements were independently conducted 3 times. The specificity of primer sets used for qRT-PCR amplification was evaluated by melting curve analysis. The Standard Curve Method was used for quantification against a known concentration of genomic DNA (Li *et al.* 2009). The results were expressed in percentage of the *actA* (actin) gene or relative concentrations were expressed in arbitrary units. All primers used, and sequences are listed in Table 2.1 (Appendix 2.1).

### 2.2.4 RNA analysis

#### 2.2.4.1 cDNA amplification by RT-PCR

cDNA was synthesised from the isolated RNA by reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Oligo(dT) was used as a primer on 0.5 µg of RNA per reverse transcription reaction. The cDNA was checked by PCR using the Phusion polymerase (New England Biolabs) with 0.5 µl of cDNA in a 20 µl PCR reaction. The PCR program used had an initial denaturation step at 98 °C for 2 min. After the initial denaturation the program continued with 30 cycles of denaturation at 98 °C for 20 sec, hybridisation at 60 °C for 20 sec, and elongation at 72 °C for 20 sec. The final extension was at 72 °C for 5 min.

### 2.2.5 Cloning of amplified fragments

#### 2.2.5.1 Ligation

The fragments to ligate together were mixed, at a molar DNA ratio of 3:1, in a 20µl reaction using 1µl T4 DNA Ligase and 2µl 10x T4 DNA Ligase Buffer (New England Biolabs) and left to ligate at room temperature for 18 hours.

#### 2.2.5.2 Bacterial transformation by electroporation

5µL of the ligation mixes were used to transform competent XL-1 Blue *E. coli* cells by electroporation, using a Micro Pulser TM (BioRad). 1mL of SOC medium (10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride, 10mM magnesium sulphate, 20mM glucose, 2% w/v tryptone, 0.5% w/v yeast extract) was immediately added to the transformation mixes and mixed by pipetting up and down a few times. The SOC/transformation mixes were then transferred to a shaking incubator (250rpm) at 37°C for 1 hour.

### 2.2.5.3 Selection and analysis of recombinant clones

To select for transformants the SOC/transformation mixes were spread onto Petri dishes containing LB medium plus agar (25mL) and Ampicillin (1µg/mL). The plates were incubated at 37°C for 24 hours. Single white colonies were picked and streaked on new plates containing the same amount of LB plus agar supplemented with Ampicillin (1µg/mL) and incubated for 24 hours. Colonies from the plates where positive clones had been identified were inoculated in 30mL tubes containing 10mL LB and 1µg/mL Ampicillin and incubated at 37°C on a shaker (250rpm) for 16 hours. Restriction digests, using appropriate restriction enzymes according to the plasmid to digest, were used initially to identify positive clones.

### 2.2.6 Transformation of *A. niger*

#### 2.2.6.1 Preparation of protoplasts

For preparation of *A. niger* AB4.1 protoplasts, 400ml of protoplast media (AMM containing no carbon source, 0.1% (w/v) Bacto™ Casamino Acids, 0.1% (w/v) glucose, 10mM uridine) was inoculated with  $10^8$  spores (re-suspended in 0.1% (v/v) Tween-20) and the culture was incubated at 28°C, on a shaker at 150rpm for 18h. The cultures were harvested through Miracloth (Calbiochem), washed with cold Mycelium Wash buffer (0.6M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) and weighed after being blotted dry. The cell pellet was re-suspended in cold MM buffer (1.2M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 20mM 2-(N-Morpholino) ethane sulfonic acid sodium salt ( $\text{C}_6\text{H}_{12}\text{NNaO}_4\text{S}$ , MES Sodium Salt)). Lysing Enzyme (Sigma Aldrich) (100mg/ml in MM; sterilised through a 0.2µm filter), Chitinase (Sigma Aldrich) (4mg/ml in MM; sterilised through a 0.2µm filter) and Bovine Serum Albumin (Sigma Aldrich) (100mg/ml in MM, filter sterilised) were added to the cell culture, to protect the protoplasts from protease activity, and incubated at 30°C on an Innova 2300 platform shaker (New Brunswick Scientific) set to 80rpm for 30min, and 50rpm thereafter checking microscopically after 2h and then at 30min intervals. By the second 30min interval, protoplasting was visible throughout the sample under the microscope. The protoplasts were purified through 2 columns of sterile polyallomer wool (one loosely packed set atop the other column, tightly packed) and washed with cold NM buffer (1M NaCl, 20mM MES) before being centrifuged at 900g in a PK121R multispeed centrifuge (ALC) swing-out rotor for 10min. The pellet was re-suspended in 1ml cold STC (1.2M sorbitol, 10mM Tris base, 50mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ; adjusted to pH7.5, autoclaved at 117°C), topped up to 50ml with STC and mixed. The protoplasts were then centrifuged again, only at 550g

in a swing-out rotor, and the protoplast pellet formed was re-suspended in 1ml cold STC. A protoplast count was then done on a  $10^2$  dilution in a haemocytometer.

#### 2.2.6.2 Transformation

For each transformation 3µg of selection vector was added to 100µl of protoplast suspension (containing  $2 \times 10^7$  protoplasts) for a 25min incubation at room temperature (minus-DNA control with STC instead was also done alongside). A total of 1250µl of polyethylene glycol 4000 grade (PEG 4000) was then added and left to incubate a further 20min. STC was then added up to 10ml to dilute the PEG and the samples pelleted by centrifugation at 550g. The pellets were re-suspended in STC and 100µl were spread onto sorbitol-containing minimal agar plates (made up to 1 litre containing 20ml *Aspergillus* salt solution; 6g NaNO<sub>3</sub>, 1% (w/v) glucose, 1.2M sorbitol, pH 6.5, 15g agar and autoclaved at 117°C before aseptically added 20ml 50x MgSO<sub>4</sub> (6.5g MgSO<sub>4</sub>·7H<sub>2</sub>O in 250ml SDW, autoclaved) prior to pouring plates, 25ml per plate). The plates were incubated at 30°C for 6-8 days to allow cells to rebuild their cell wall and grow to sporulation.

#### 2.2.6.3 Selection of transformants

The AB4.1 strain used for the transformation lacks the *pyrG* gene and is therefore auxotrophic for uridine. The lack of uridine in the sorbitol-containing plates is a first selection for integration of the plasmid into the AB4.1 genome as transformants should express the *pyrG* gene after integrating the plasmid and are then able to grow on media lacking uridine. The colonies that grew were picked out and streaked for two successive rounds on glucose-containing minimal media agar slopes with no uridine to allow selection and purification of the colonies containing an integrated plasmid. In the next stage the selective pressure for maintaining the integrated *pyrG* gene was released by growing the cells on PDA slopes containing uridine. This step, also done in two successive rounds, allows the excision of the plasmid from the AB4.1 genome, as illustrated in **Figure 2.2**. Finally, colonies that have excised the plasmid from the genome were selected by transferring the cells onto a plate containing 5-fluorouracil-6-carboxylic acid monohydrate (5'-FOA). Growth can only occur from colonies that have excised the plasmid containing the *pyrG* gene, leaving no active *pyrG* gene (the AB4.1 strain is *pyrG*-deficient) present in the genome. Colonies still expressing the *pyrG* gene will be able, on plates containing uridine, to convert the 5'-FOA into fluorodeoxyuridine, which is toxic to the cell. Colonies that do grow throughout the 5'-FOA screening will be either wild-type revertant strains, or

mutant strains. This is because the second crossover event can occur using either of the flanking DNA regions, upstream or downstream, as illustrated in **Figure 2.2**.

Genomic DNA extractions were carried out with the colonies of *A. niger* grown on 5-FOA plates, and PCR screening was done on the samples to identify any successful deletion mutants as well as DNA sequencing.

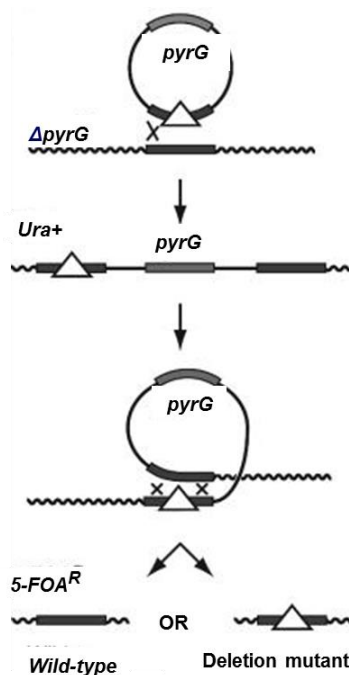


**Figure 2.2:** Schematic diagram of the PopIN/OUT transformation procedure.

This method was used for the deletion of the *xlnR*, *clrA*, *An11g06730*, *hyp1*, *hsbA*, and *hfbD* genes as well as for the double deletion of *hyp1* and *hfbD*. The primers used for the deletion of the mentioned genes are listed in Table 2.1 (Appendix 2.1).

## 2.2.7 Growth related assays

### 2.2.7.1 pH measurement

pH was measured, using a pH 210 Microprocessor pH meter (Hanna instruments), in the cultures as a wide range of organic acids is produced by *A. niger* during fermentation which results in the acidification of the medium. Thus the measurement of pH, which lowers due to acidification of the culture medium, can serve as an indirect indicator of growth for *A. niger* (Archer *et al.* 1990, Pedersen *et al.* 2000, Rosling *et al.* 2004).

### 2.2.7.1.1 pH-controlled experiment

A 2L fermenter (FerMac 320, Electrolab) was used for growing the cultures of *A. niger* strains where the pH was maintained at 5 using NaOH (200rpm, air 1L/min, 28°C). Glucose cultures were set up as described previously in 1L sterile Erlenmeyer flasks containing 400ml AMM, 1% (w/v) glucose solution and incubated for 48h at 28°C on a shaker (New Brunswick Scientific) set to 150rpm. The cultures were transferred to AMM supplemented with 1% (w/v) wheat straw to which was added 0.1M uridine. Samples were taken at 6h, 24h and 48h. Supernatants were collected at each time points for enzymatic analysis.

### 2.7.1.2 Glucosamine assay

The contents of flasks were filtered and freeze-dried in 50ml centrifuge tubes, including an insoluble substrate only control. Samples (0.1g dry weight for the glucose cultures and the full content of the flask for the wheat straw cultures) were hydrolyzed by 2 ml of concentrated sulphuric acid (98%) at room temperature for 24 hours. Similarly, N-acetylglucosamine (GlcNAc) standards were placed into 50 ml centrifuge tubes and 2ml of concentrated sulphuric acid (98%) was added at room temperature for 24 hours. The mixtures were transferred into 100ml bottles with 35ml of dH<sub>2</sub>O already inside and then autoclaved at 121°C for 15 minutes. After cooling, the solution was neutralized with NaOH to pH 7 and further diluted to 100 ml with dH<sub>2</sub>O. 1 ml of the above sample solutions was transferred into a 2ml tube. 1 ml acetyl acetone reagent (4% (v/v) acetyl acetone in 1.25 N Na<sub>2</sub>CO<sub>3</sub>) was added and then incubated at 100°C for 20 minutes. The samples were transferred into a 15 ml centrifuge tube. After cooling to room temperature, 6 ml of absolute ethanol was added and then mixed gently. 1 ml of Ehrlich reagent (1.6 g of N-N dimethyl-p-aminobenzaldehyde in 60 ml solution containing 50:50 (v/v) of absolute ethanol: concentrated HCl) was added and mixed gently. The mix was incubated at 65°C for 10 minutes and the absorbance value was determined at 530 nm.

## 2.2.8 Cell wall analysis

### 2.2.8.1 Cell wall stress

To test for a cell wall stress, spores of the strains of interest were washed with 0.01% (v/v) Tween-80 solution and 10<sup>6</sup> spores/ml, in solution, were inoculated into a 96-well plate, each well containing 150µl of AMM 1% (w/v) glucose solution supplemented with Sodium Dodecyl Sulfate (SDS, final concentration 70µg/ml, Sigma Aldrich), CalcoFluor White (CFW, final concentration 100µg/ml, Sigma Aldrich) or Congo Red

(CR, final concentration 250µg/ml, Sigma Aldrich) and incubated at 28°C on a shaker (New Brunswick Scientific) set to 150rpm. The OD<sub>600</sub> was read at 0h, 24h, 48h and 72h after inoculation.

The strains sensitivity to cell wall stressors was also performed by inoculating 10<sup>6</sup> spores/ml (final concentration) on AMM 1% (w/v) glucose agar plates containing SDS (final concentration 70µg/ml), CFW (final concentration 50µg/ml) and CR (final concentration 250µg/ml). The plates were incubated for 4 days at 28°C in the dark.

### 2.2.8.2 Sugar composition analysis using DIONEX

For the preparation of cell wall samples, mycelia were collected through Miracloth (Calbiochem), washed with AMM containing no carbon source and flash frozen in liquid nitrogen prior to cell wall preparation. Frozen *A. niger* mycelia were ground to a powder using a mortar and pestle. The resulting powder was washed with 5ml of protein extraction buffer (2% w/v SDS, 40mM β-mercaptoethanol, 50mM Tris-HCl, 5mM EDTA, pH 7.4) and incubated at room temperature for 10 minutes before being centrifuged in a PK121Rmultispeed centrifuge (ALC) swing-out rotor for 10min at 4000g. The pellets were then resuspended in 1ml H<sub>2</sub>O before being freeze-dried overnight using a ThermoSavant Modulyo® Freeze-dryer coupled to an Edwards RV8 vacuum pump. 10mg of dry cell wall mass was then resuspended in 225µl 72% (w/w) sulphuric acid and incubated for 3h at room temperature with gentle shaking by hand throughout the incubation to allow complete contact of the acid solution with the polysaccharides. The samples were then further diluted to 1M sulphuric acid by addition of 2.85ml ultra-pure H<sub>2</sub>O containing 1mg/ml fucose (internal standard) and homogenised. The suspensions were incubated 4h at 100°C using a boiling water bath. After incubation, the tubes were cooled to room temperature and 1ml of the hydrolysate was transferred to a new centrifuge tube. The hydrolysate was neutralised by dropwise addition of a saturated barium hydroxide solution (40g/L Ba(OH)<sub>2</sub> in ultra-pure H<sub>2</sub>O) until the pH reached neutrality and incubated overnight at 4°C. After incubation the pH was checked, the volume adjusted to 10ml with ultra-pure H<sub>2</sub>O and the samples were centrifuged at 4000g for 10min in a PK121Rmultispeed centrifuge (ALC) swing-out rotor. 0.1ml of the supernatant was diluted 10 times and 1ml of the sample was taken and placed into a numbered HPLC vial for sugar analysis using the HPLC (DIONEX ICS 3000) Reagent-Free™ Ion Chromatograph equipped with Dionex ICS-3000 system, electrochemical detection using ED 40 and computer controller. A CarboPac™ PA 20 column (3 x 150 mm)

was used for the separation with a mobile phase of 10 mM NaOH and a flow rate of 0.5 mL/min. The injection volume was 10 µl and the column temperature was 30°C.

### 2.2.8.3 Microscopy

#### 2.2.8.3.1 Cell wall permeability assay using Phloxine B

1ml of 16 hours-old germinating conidia was incubated with Phloxine B (Sigma Aldrich) solution (5µM final concentration) and observations were performed under a fluorescent microscope with blue and green filter combinations. This dye stains cytosolic material due to a loss of the integrity of the cell making it more permeable to Phloxine B (Rasooly 2007). The images were taken using a GXML3201 LED microscope (Suffolk, United Kingdom) equipped with a GT Vision camera (Suffolk, United Kingdom). A 100× objective lens and a final magnification of ×1000 were used. Microscope images were viewed and recorded using GXCapture version 7.3 software (Suffolk, United Kingdom). The fluorescence intensity was determined using the ImageJ software on 50 images taken for each condition from 3 biological repeats.

#### 2.2.8.3.2 Transmission Electron Microscopy

Samples were grown in AMM 1% (w/v) glucose solution and incubated for 48h at 28°C on a shaker (New Brunswick Scientific) set to 150rpm. They were then fixed with EM fixative buffers (3% glutaraldehyde in 0.1 M sodium cacodylate buffer). After two rinses in 0.1 M cacodylate buffer (pH 7.2), samples were placed in 1% osmium tetroxide in the same buffer for 1hr, then rinsed in distilled water for 5 min. They were subsequently dehydrated in graded ethanol series, culminating in two changes in propylene oxide. The samples were then infiltrated, polymerized with resin and sectioned. They were stained with ethanolic uranyl acetate followed by lead acetate and examined using a Tecnai bio twin digital transmission electron microscope run at 100 Kv.

### 2.2.9 Mitochondrial assay and ROS accumulation

#### 2.2.9.1 Inhibition of the mitochondrial complexes

To test for the functionality of the mitochondrial complexes, spores of the strains of interest were washed with 0.01% (v/v) Tween-80 solution and  $10^6$  spores/ml, in solution, were inoculated into a 96-well plate, each well containing 150µl of AMM 1% (w/v) glucose solution supplemented with Antimycin A (AntiA, Sigma Aldrich) (final concentration 60µg/ml) and/or Salicylhydroxamic acid (SHAM, Sigma Aldrich) (final concentration 5mM) and incubated at 28°C on a shaker (New Brunswick Scientific)

set to 150rpm. AntiA is an inhibitor of the complex III of the respiratory chain (Ahmad *et al.* 1950; Rieske *et al.* 1962) whereas SHAM is an inhibitor of the AOX (Murphy *et al.* 1999). The OD<sub>600</sub> was read at 0h, 24h, 48h and 54h after inoculation. The no drug control was AMM supplemented with the solvents of AntiA and SHAM, Ethanol and Dimethyl Sulfoxide (DMSO, Sigma Aldrich).

### 2.2.9.2 Intracellular ROS accumulation

1ml of 16 hours-old germinating conidia suspension was incubated with 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Sigma Aldrich) solution (5 µM final concentration) and observations were performed under a fluorescent microscope with blue and green filter combinations. H2DCF-DA passively diffuses into cells and is retained in the intracellular level after cleavage by intracellular esterases (Kristiansen *et al.* 2009; Xie *et al.* 1999). Upon oxidation by ROS, the non-fluorescent H2DCF-DA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) (Kristiansen *et al.* 2009). The images were taken using a GXML3201 LED microscope (Suffolk, United Kingdom) equipped with a GT Vision camera (Suffolk, United Kingdom). A 100× objective lens and a final magnification of ×1000 were used. Microscope images were viewed and recorded using GXCapture version 7.3 software (Suffolk, United Kingdom). The fluorescence intensity was determined using the ImageJ software on 50 images taken for each condition from 3 biological repeats.

### 2.2.10 Protein analysis

#### 2.2.10.1 Enzymatic assays

##### 2.2.10.1.1 Congo Red Assay

The Congo Red assay was used to visualise the production of extracellular enzymes by *A. niger* on different substrates. Radial diffusion of enzyme into substrate incorporated in an agar gel produces zones of hydrolysis which can be visualized by staining non degraded substrate with Congo red dye (Carder 1986). 10 µl of the supernatants collected were spotted on CMC or xylan plates (0.5% w/v CMC or xylan, 1% Agar) and incubated for 2h at 37°C. The plates were then washed with 5ml of SSC solution (1M NaCl, 0.1M sodium citrate). 3ml of Congo Red solution (5mg/ml in water) was then added to stain the polysaccharides but no single sugars. Plates were left on a shaker for 15min before the liquid was poured off and replaced with SSC solution. Plates were left on a shaker for 15min to allow de-staining to occur. Pictures were taken using a HP ScanjetG4010 scanner.

**2.2.10.1.2 3,5-dinitrosalicylic acid (DNS) assay for reducing sugars**

The reaction was started by mixing 500  $\mu$ L of supernatant from fungal cultures with 750  $\mu$ L of 2% wheat straw made in 50mM Citrate buffer (pH 4.8) in 2ml tubes. The mixture was incubated overnight at 50°C under agitation at 250rpm. The reaction was stopped by heating the samples at 100°C. The samples were centrifuged for 10 minutes at 16060g and the supernatants were transferred to a new tube. The amount of reducing sugars liberated in the enzyme reaction was assayed by mixing 80  $\mu$ L of the enzyme reaction with 120  $\mu$ L DNS solution in a 96-well PCR microplate, heating to the boiling point for 5 min and cooling on ice for 5 minutes. 150  $\mu$ L of each samples was transferred to a new 96-well microplate before measuring the absorbance at 540 nm using an ELISA micro-plate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Scientific). Ranges of glucose standards run at the same time were used to calculate the saccharification activity of the samples. All experiments were done in biological and technical triplicates.

**2.2.10.2 Protein extraction and quantification**

Pellets of *A. niger* mycelia were ground to a powder using a mortar and pestle under liquid nitrogen. The powder obtained for each sample was lysed by mixing with in 150  $\mu$ L of solution containing 4% (w/v) SDS, 100mM Tris/HCl pH 7.6, 0.1M DTT (lysis solution) and incubated at 95°C for 3 min. The DNA was then sheared by sonication to reduce the viscosity of the sample. Before starting sample processing the lysates were clarified by centrifugation at 16,060 g for 5 min.

100  $\mu$ L of the lysates obtained from the protein extraction was used for protein quantification assay. This was performed using the RC/DC (reducing agent compatible (RC) and detergent compatible (DC)) assay (BioRad) according to the manufacturer's instructions.

**2.2.10.3 SDS PAGE gels and staining**

Proteins in culture supernatant from relevant times were separated on 4–20% Tris-glycine Novex SDS-PAGE gels (Invitrogen). 15 ml Tris-glycine-SDS loading buffer was added to 12 ml supernatant plus 3 ml 1 M DTT. The sample mixture was heated to 100°C for 5 minutes before being loaded onto the gel with running conditions of 100 V for 3 hours. The gel was subsequently stained with silver according to the method of Yan *et al.* (2000).

### 2.2.10.4 Western blot analysis

An equal concentration of protein samples (2mg/ml) were mixed with 5µl of NuPAGE LDS Sample Buffer (4X) (Life Technologies), 2µl of NuPAGE Reducing Agent (10X) (Life Technologies) and sterile dH<sub>2</sub>O up to 20µl and heated at 70°C for 10 minutes. The samples were then loaded onto a 10% Bis-Tris Novex SDS-PAGE gel (Life Technologies) with running condition of 200V for about 35 minutes in 1X MES Running Buffer (dilution of 20X MES Running Buffer in dH<sub>2</sub>O, Life Technologies) in a XCell SureLock Mini-Cell System (Life Technologies). As samples were reduced, 500µl of NuPAGE antioxidant (Life Technologies) were added in the upper buffer chamber. 10µl of Color Prestained Protein Standard, Broad Range (11-245kDa) (NEB) was loaded onto the gel for size control.

The gel was then placed over 2 sponges and 3 layers of Whatman paper 3MM (Sigma-Aldrich) and covered with a nitrocellulose membrane (GE Healthcare Life Sciences), 3 layers of Whatman paper 3MM and 3 sponges that had been previously soaked in the transfer buffer (10ml 20X NuPAGE Transfer Buffer (Life Technologies), 20ml methanol, 200µl NuPAGE antioxidant (Life Technologies), dH<sub>2</sub>O up to 200ml). The system was placed in an XCell SureLock Mini-Cell System and the upper buffer chamber was filled with the remaining transfer buffer and the tank was filled with 600ml dH<sub>2</sub>O. The running conditions were set at 30V constant for 1h.

After transfer, the membrane was washed with 1X Phosphate Buffered Saline (PBS), 0.1% v/v Tween 20, 3% milk (PBSTM) and further incubated with PBSTM for 1h at room temperature under agitation (MidiMix, Denley). The membrane was then incubated with the primary antibody (ABI), diluted in PBSTM to 1/2500 for the antiTyr ABI (NEB), overnight at 4°C under agitation (MidiMix, Denley). After incubation with the ABI, the membrane was washed 3 times (1x15min and 2x5min) in 1X PBS, 0.1% Tween 20 (PBST). The polyHRP secondary antibody (ThermoFisher) was then prepared to a dilution of 1/5000 in PBSTM and incubated with the membrane for 1h at room temperature under agitation (MidiMix, Denley). The membrane was then washed 3 times (1x15min and 2x5min) in PBST.

The membrane was finally incubated for 5min in the dark with the Pierce ECL Western Blotting Substrate (Thermo Scientific) before being revealed using the BioRad Chemidoc XRS+ using the Quantity One 4.6.6 program (BioRad).

### 2.2.10.5 *Phosphostaining using ProQ and SYPRO reagents*

**ProQ Diamond staining.** Gels were stained using ProQ Diamond (Molecular Probes, Invitrogen) to detect the phosphoproteome. Incubation was performed overnight under gentle agitation. To avoid background, gels were extensively destained in 50 mM sodium acetate, pH 4.0, containing 15% 1,2-propanediol for 6h. After several washes with ultrapure water, gels were scanned using a BioRad Chemidoc XRS+ with the Quantity One 4.6.6 program (BioRad). All steps were performed in dark, and gels were immediately scanned after the end of the protocol. All staining and destaining steps were optimized according to the detection of Peppermint Stick markers (Molecular Probes).

**Sypro ruby staining.** Total levels of proteins were revealed using Sypro Ruby (Molecular Probes, Invitrogen) staining. Gels were incubated in staining solution overnight at room temperature under gentle agitation. Gels were successively washed once with ultrapure water, twice in destaining solution [10% methanol (v/v), 7% acetic acid (v/v)], and finally twice with ultrapure water, each bath with a duration of 10 min. All steps were performed in dark, and gels were immediately scanned after the end of the protocol.

### 2.2.10.6 *Phosphoproteomics*

Proteomics was carried out by Dr Andrew R Bottrill at the University of Leicester Proteomics Facility (PNAFL, University of Leicester). Trypsin digestion was carried out using the Filter Aided Sample Preparation (FASP) protocol.

Phosphopeptide enrichment was performed by immobilised metal affinity chromatography. Peptides were bound to PHOS-Select iron affinity beads (Sigma, UK) in 250mM acetic acid / 30% acetonitrile and the pH carefully adjusted to 2.5. Washes were carried out with 250mM acetic acid / 30% acetonitrile and with water, and peptides eluted using 400mM ammonium hydroxide. Samples were lyophilized in a speedvac and resuspended in 0.1% (w/v) formic acid / 0.05% (w/v) trifluoroacetic acid / 2% (v/v) acetonitrile. LC-MS/MS was carried out using an RSLCnano HPLC system (Dionex, UK) and an LTQ-Orbitrap-Velos mass spectrometer (Thermo Scientific). Samples were loaded at high flow rate onto a reverse-phase trap column (0.3mm i.d. x 1mm), containing 5µm C18 300 Å Acclaim PepMap media (Dionex) maintained at a temperature of 37 °C. The loading buffer was 0.1% (w/v) formic acid / 0.05% (w/v) trifluoroacetic acid / 2% (v/v) acetonitrile.

Peptides were eluted from the trap column at a flow rate of 0.3 $\mu$ l/min and through a reverse-phase capillary column (75 $\mu$ m i.d. x 250mm) containing Symmetry C18 100 Å media (Waters, UK) that was manufactured in-house using a high pressure packing device (Proxeon Biosystems, Denmark). The output from the column was sprayed directly into the nanospray ion source of the LTQ-Orbitrap-Velos mass spectrometer.

LC-MS/MS was carried out using an LTQ-Orbitrap-Velos mass spectrometer (Thermo Scientific). The LTQ-Orbitrap-Velos mass spectrometer was set to acquire a 1 microscan FTMS scan event at 60000 resolution over the m/z range 350-1250 Da in positive ion mode. Accurate calibration of the FTMS scan was achieved using a background ion lock mass for polydimethylcyclsiloxane (445.120025Da). Subsequently up to 20 data dependent CID MS/MS were triggered from the FTMS scan. The isolation width was 2.0 Da, normalized collision energy 35.0. Dynamic exclusion was enabled.

The .raw data file obtained from each LC-MS/MS acquisition was processed using Proteome Discoverer (version 1.4, Thermo Scientific), searching each file in turn using Mascot3 (version 2.2.04, Matrix Science Ltd.) against the UniProtKB-Swissprot4 database (2010). The peptide tolerance was set to 10 ppm and the MS/MS tolerance was set to 0.4 Da. Fixed modifications were set as carbamidomethyl (C) with variable modifications of carbamidomethyl (N-term), oxidation (M) and phosphorylation (S/T/Y).

The output from Proteome Discoverer was further processed using Scaffold Q+S5 (version 4.0.5, Proteome Software) (Searle, 2010). Upon import, the data was searched using X!Tandem6 (The Global Proteome Machine Organization). PeptideProphet7 and ProteinProphet8 (Institute for Systems Biology) probability thresholds of 95% were calculated from the decoy searches (Figure 5.1 in Appendix) and Scaffold was used to calculate an improved 95% peptide and protein probability threshold based on the data from the two different search algorithms, Mascot and X!Tandem.

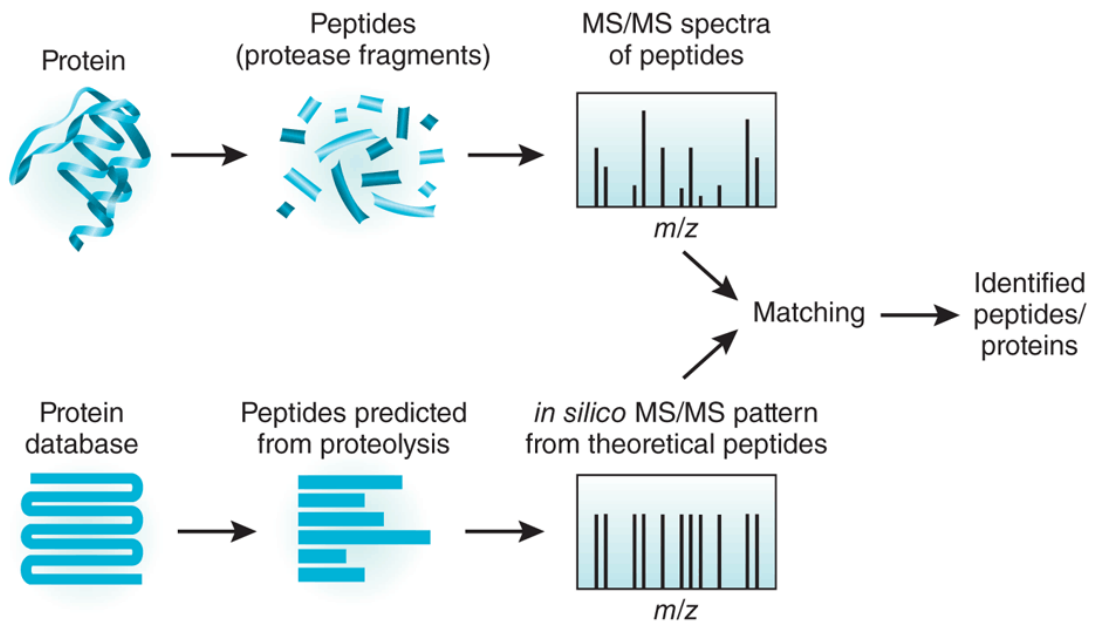


**Figure 2.3:** General approach used by peptide-centric MS technologies for the identification of protein complex mixtures. After proteolysis of a protein, or complex mixture of proteins, the spectra associated with protease fragments are matched with spectra generated in silico using information obtained from protein databases (Duncan *et al.* 2010).

### 2.2.11 *In silico* analysis tools and statistics

#### 2.2.11.1 Design of primers

Primers used for the deletion process were designed to bind to sequences upstream and downstream from the genes of interest and at each end of the gene region. Sequence files for each of the three genes were obtained from CADRE (<http://www.cadre-genomes.org.uk/index.html>), and uploaded into Vector NTI 10.3.0 for primer design. The primers were designed with restriction sites and checked on Vector NTI to verify the restriction sites were inserted correctly in the primers. The designed primers (although excluding their restriction site recognition sequences) were tested on Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to obtain the predicted product size. For complementation of the  $\Delta xlnR$  strain, a complementation cassette was obtained by amplifying the upstream region of the *xlnR* gene, the *xlnR* gene and the downstream region of the gene. The primers used were the ones used to amplify the up and down flanking regions for the deletion process. Primers used in this work are presented in Table 2.1 (Appendix 2.1).

### 2.2.11.2 BLAST analysis

Search for homology between sequences was done using the algorithm BLAST (Altschul *et al.* 1990) on the public database NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). BLASTn and BLASTp allow the search for homologies between DNA sequences and protein sequences respectively.

### 2.2.11.3 Sequence alignments

Sequences alignments were done using the default parameters of the MULTALIN programme available online (<http://multalin.toulouse.inra.fr/multalin/>).

### 2.2.11.4 Protein motif scanning

The scanning of protein sequences for the presence of known motifs was performed using the ScanProsite tool by ExPASy (<http://prosite.expasy.org/scanprosite/>), InterProScan version 4.8 by MBL-EBI (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) and SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). The ScanProsite tool was used against the PROSITE motif collection, excluding motifs with a high probability of occurrence from the scan. The InterProScan was performed against the InterPro collection of protein signature database, using the default protein motif scanning parameters. SignalP 4.1 was used against the organism group Eukaryotes, with default settings.

### 2.2.11.5 TFs binding site prediction

The prediction of PacC binding sites was done using the TF prediction tool available at <http://www.yeasttract.com/tfsbindingsites.php> and the MatInspector prediction tool from Genomatix Suite (<http://www.genomatix.de/>). The YEASTRACT (Yeast Search for Transcriptional Regulators And Consensus Tracking) information system is a tool for the analysis and prediction of transcription regulatory associations in *Saccharomyces cerevisiae*. Last updated in June 2013, this database contains over 200 000 regulatory associations between TFs and target genes, including 326 DNA binding sites for 113 TFs. The MatInspector tool comes with a large library of weight matrices representing transcription factor binding sites which can be searched in DNA sequences.

**Chapter 3. Measurement of fungal growth on  
lignocellulosic substrates**

### 3.1 Introduction

To access nutrients, fungi have developed the capacity to penetrate or access complex substrates by either or both enzymatic processes and physical pressure. The result is that hyphae can access nutrients unavailable to other microorganisms in a range of different environments (Carlile *et al.* 1994). Environments can be highly variable and fungi have evolved to occupy a wide diversity of habitats. The heterogeneity of environments is caused by variation in deposition of organic nutrients, availability of each nutrient and water and the response of the thallus at each point to environmental factors. Growth in heterogeneous conditions results in the mycelium having different parts in different stages of response (Carlile *et al.* 1994). Some parts of the mycelium may respond to available nutrients with exponential growth, others will be static, and others declining. However, a range of environmental factors influences the response of fungi beyond the overriding nutritional requirements (**Figure 3.1**).

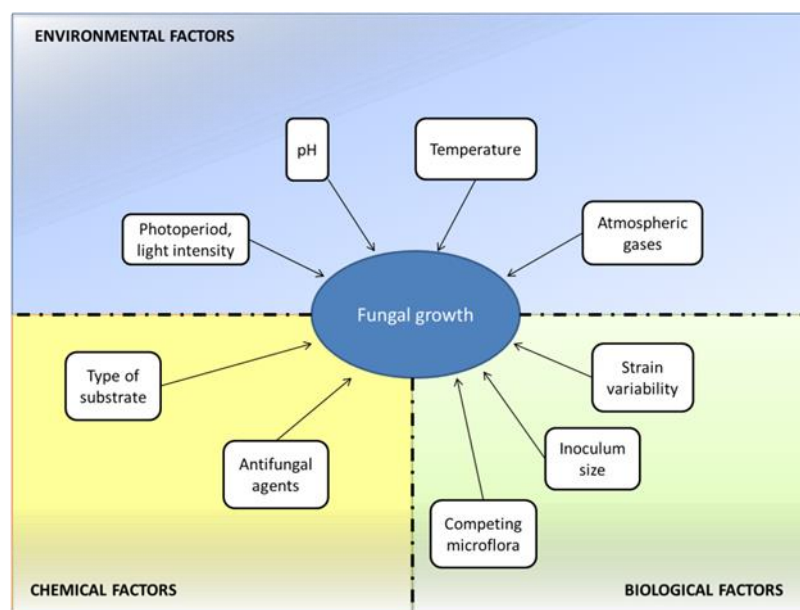


**Figure 3.1:** Principal factors influencing fungal growth (modified from European Commission (1994)).

Measuring growth of fungi can be a difficult task. Growth may be defined as an increase in mass of the whole part of a living organism by the synthesis of macromolecules. Organisms can grow by increasing the mass of their constituent cells followed by cell division and an increase in cell number. In this chapter, a range of assays have been tested when *A. niger* is grown in a simple substrate, glucose, and in a complex substrate, ball-milled wheat straw. The AB4.1 strain of *A. niger* as

well as the  $\Delta xlnR$  strain were used in this work. Hasper *et al.* (2000) showed that the deletion of  $xlnR$  in both *A. niger* and *A. oryzae* resulted in a strong decrease of the transcript levels of genes encoding several hemicellulose-degrading enzymes. Moreover, the authors showed that the growth of the *A. niger*  $\Delta xlnR$  strain was strongly reduced on hemicellulosic substrates (Hasper *et al.* 2000).

#### 3.1.1 Indicators of fungal growth

##### 3.1.1.1 Acidification of the medium - pH

Many species of fungi change the pH as they grow with some species increasing and others decreasing the pH of their medium as the use of inorganic nitrogen sources (ammonium and nitrates) give different pH responses. Conventional culture media used for physiological studies of fungi (e.g. Potato Dextrose Agar) have low buffering capacities and the effects of pH on fungal growth on such media are difficult to assess (Child *et al.* 1973). The pH of the medium is important as it influences mineral availability, enzyme activity and membrane function. As for enzyme activity, enzymes govern all metabolic processes and the functioning of enzymes is influenced by the pH of the environment or media. The ambient pH has been shown to strongly influence abiotic factors such as carbon availability (Andersson *et al.* 2000; Kemmitt *et al.* 2006), nutrient availability (Kemmitt *et al.* 2006; Kemmitt *et al.* 2005; Pietri *et al.* 2008) and the solubility of metals (Firestone *et al.* 1983; Flis *et al.* 1993). In addition, pH may control biotic factors, such as the biomass composition of fungi and bacterial populations (Fierer *et al.* 2006) in agricultural soils, for example (Arao 1999; Bardgett *et al.* 2001). Generally speaking, fungi can tolerate a wide range of pH values, as the majority of fungi have been found to grow well at a pH range from 4.2 to 9.3 (Rousk *et al.* 2010).

A wide range of acids (e.g. citric acid, gluconic acid) are produced by *A. niger* during fermentation by the utilisation of the carbohydrate sucrose. *A. niger* can convert as much as 95% of available carbon to organic acids (Karaffa *et al.* 2003). Secretion of acids helps in degrading the plant cell walls on which the fungus grows, but it also slows the growth of competing organisms (Pedersen *et al.* 2000). Thus pH plays a very significant role in fungal growth and acidification of the medium can be a good indicator of growth for *A. niger* (Archer *et al.* 1990, Pedersen *et al.* 2000, Rosling *et al.* 2004) (**Figure 3.2**).

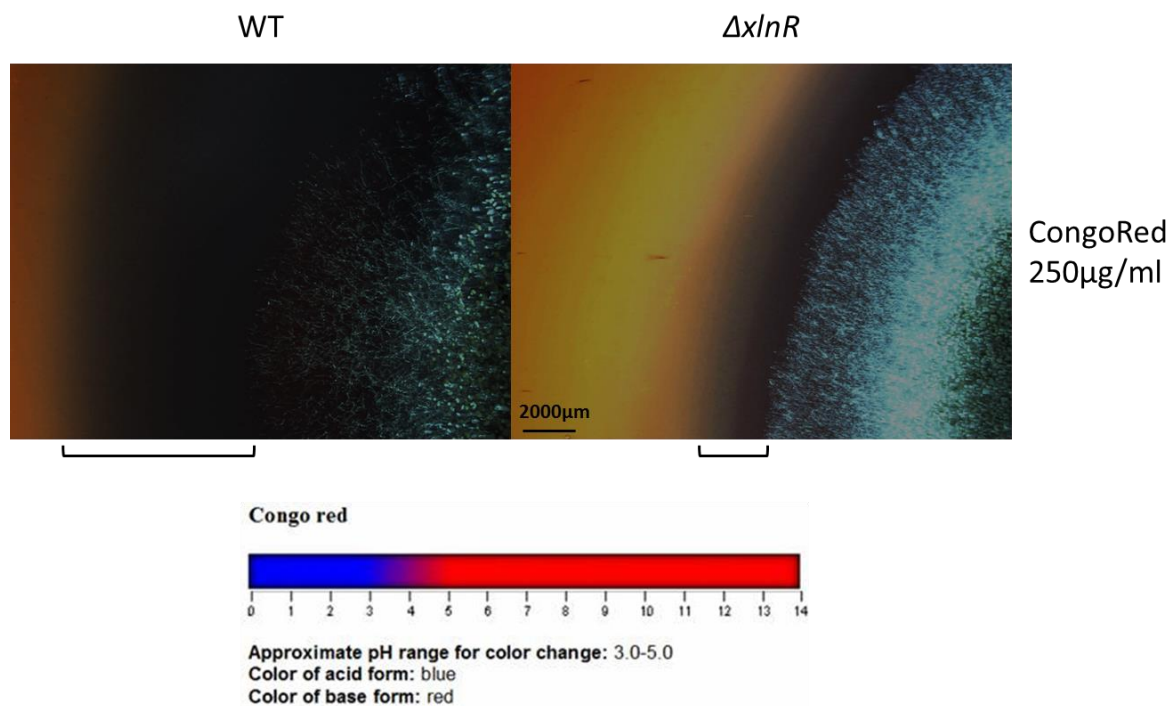


**Figure 3.2:** Production of acids by the *A. niger* WT and  $\Delta xlnR$  strains on AMM + Congo Red plates. Congo red can be used as a pH indicator and the medium turns from red to blue when it becomes acid. The production of acids from *A. niger* colonies resulted in the production of a blue halo. — indicates the size of the halo produced. Congo Red image for pH indicator from <http://individual.utoronto.ca/jenna/B42/BA.htm>.

#### 3.1.1.2 Dry weight

Dry weight measurement is probably the most widely used and most generally applicable method for assaying growth of fungi. Growth of filamentous fungi in liquid culture is usually measured as an increase in dry weight, using either stationary or shaken cultures in Erlenmeyer flasks. Dry weight measurement requires separation of biomass from the growth medium which is relatively straightforward in liquid cultures containing a soluble carbon source such as glucose. However, when filamentous fungi are grown on lignocellulosic substrates, the fungus is mixed with the substrate and dry weight measurement is not useful to determine the fungal biomass. In this case, there are few methods that can provide representative and reproducible values for fungal growth.

### 3.1.2 Measurement of cellular components composition

The biomass can also be estimated from measurements of a specific component, as long as the cell component constitutes a constant proportion of the biomass during growth. Chemical estimation of its concentration can be used to estimate the growth of the fungus. Whipps *et al.* (1982) suggested potentially suitable compounds such as total nitrogen, chitin, proteins or ergosterol contents to provide indirect ways of measuring fungal biomass. Cell wall components such as chitin, glucan and mannan have been the most widely used. The disadvantages of these methods include variation in cell composition which is influenced by specific growth rate of some fungi or the medium composition.

#### 3.1.2.1 Glucosamine assay

Glucosamine is a useful compound for the estimation of fungal biomass, taking advantage of the presence of chitin, poly-N-acetylglucosamine, in the cell walls of many fungi. Interference with this method may occur when using complex agricultural substrates containing glucosamine in glucoproteins. It has been used in solid state fermentation (SSF) systems to estimate fungal biomass (Aidoo *et al.* 1981) as the amount present is known to be characteristic of the species and will remain constant throughout the fermentation (Pusztai 1964). Mycelial glucosamine contents ranged from 67 to 126 mg per g dry mycelium (Andersen 2008).

#### 3.1.2.2 Ergosterol

Ergosterol is the predominant sterol found in the cell membranes of fungi. Ergosterol estimation was therefore compared with the estimation of glucosamine for the determination of growth of *Agaricus bisporus* (Matcham *et al.* 1985). In solid cultures, directly proportional relationships for glucosamine and ergosterol during the linear extension of the mycelium were obtained. The determination of ergosterol was claimed to be more convenient than glucosamine as it can readily be measured by gas chromatography, high performance liquid chromatography (HPLC) or ultra violet spectrometry (Seitz *et al.* 1979). The determination of ergosterol is also valuable in correlating fungal activity to synthesis of fungal secondary metabolites such as aflatoxins, alternariol and zearalenone (Seitz *et al.* 1979; Zill *et al.* 1988).

#### 3.1.2.3 Total protein concentration

The most readily measured biomass component is protein. Protein is a biomass component which has commonly been used as an indicator of growth. Protein

measurements are simple if a predominantly starchy substrate is used, but cannot be used if the substrate contains significant amounts of protein, since the hydrolysis of the substrate proteins will counter-balance the production of biomass proteins. Generally, dry wheat straw is composed of around 3% (by weight) of proteins (Sarkar *et al.* 2012). The protein content in the biomass of microscopic fungi and yeasts depends on the species and the type of medium. For *A. niger*, proteins represent ca. 26% (w/w) of dry biomass (Andersen 2008).

#### 3.1.2.4 Enzymatic assay

The production of extracellular enzymes can also be used as an index of the amount of growth. Filamentous fungi are good secretors of metabolites and hydrolytic enzymes, which has granted them an important role in biotechnology (Archer *et al.* 2008). Many of the hydrolytic enzymes secreted by fungi have evolved to degrade the complex structure of plant cell walls (McCann *et al.* 2008). The most prominent enzymes that are secreted in the process of plant cell wall decomposition are hemicellulases, cellulases, pectinases and proteases. These enzymes degrade complex organic matter such as hemicellulose or cellulose to simple sugars that are used as a source of carbon, energy and nutrients (Burns *et al.* 2013). Therefore, measuring extracellular enzymatic activity as the ability of *A. niger* to degrade wheat straw to support its growth can serve as an assay of growth. However, methods using extracellular activities as a measure of growth have been found to vary in their results. For example, extracellular laccase (polyphenoloxidase) activities have been used to estimate growth of *A. bisporus* on composted wheat straw (Wood 1979). In a subsequent study, Wood (1980) found that laccase production by *A. bisporus* mycelium in liquid culture varied by a factor of 4 between simple and complex culture media raising doubts over the use of the laccase method as a means of accurately determining unknown quantities of *A. bisporus* mycelium in solid substrates.

#### 3.1.2.5 Molecular methods

They are based on nucleic acid sequences that are specific to the target fungi. The most useful method is called nucleic acid hybridisation and it involves the selection, cloning and chemical labelling of sequences specific to the target organism. These are then used as probes to detect RNA or DNA of the fungus in the substrate considered. DNA content has been used to estimate the biomass of *A. oryzae* on rice (Bajracharya *et al.* 1980). The method was calibrated using the DNA contents of fungal mycelia obtained in submerged culture. DNA contents were higher during early growth and then decreased, levelling off as the stationary phase was

approached. The method was corrected for the DNA content of unfermented rice, which remained unchanged since *A. oryzae* did not produce extracellular DNases (Smith *et al.* 1969).

Recently, Simeng *et al.* (2015) developed a qRT-PCR-based method to quantify fungal genomic DNA to assess the fungal biomass during SSF. In fact, due to its high specificity and sensitivity, qRT-PCR allows quantification of specific DNA targets, at potentially any phylogenetic level, from single genotypes to large taxonomic groups or even all fungi. Recently, several studies have shown the reliability of this method for monitoring and quantifying ochratoxin A producing fungal strains in several food matrices (Rodriguez *et al.* 2011) or for quantifying fungus biomass in soil (Feinstein *et al.* 2009; Snajdr *et al.* 2011). Simeng *et al.* (2015) used this method to compare the ability of three basidiomycetes to grow on wheat straw and their suitability for wheat straw pre-treatment. They concluded that the qRT-PCR-based method provided a reliable alternative to conventional methods to estimate mycelium quantity during SSF. However, Song *et al.* (2014) and Wallander *et al.* (2013) identified two main limitations to this method: poor reproducibility in nucleic acid extraction and variation of the conversion factors used to convert genomic DNA quantities to mycelium dry weight between strains.

#### 3.1.3 Aims

The aim of this chapter is to find a fast, reproducible, reliable and practical technique for determining fungal biomass as a routine assay to phenotype *A. niger* deletion strains when grown on wheat straw.

## 3.2 Material and Methods

See Chapter 2:

- 2.2.7.1 for pH measurement
- 2.2.7.2 for glucosamine assay
- 2.2.10.2 for protein extraction and concentration measurement
- 2.2.10.1.2 for DNS assay

### 3.3 Results

#### 3.3.1 Indicators of fungal growth

##### 3.3.1.1 Acidification of the medium - pH

The pH was measured in cultures grown for 48h in glucose (starting pH of the glucose medium pH6.5) before the mycelium was transferred to wheat straw medium (starting pH of the wheat straw medium was 6.5). After 24h and 48h in wheat straw medium, the pH was measured in the cultures. For the glucose condition, after 48h of culture, the pH had dropped from 6.5 to 2.87 for the *A. niger* WT strain and from 6.5 to 3.64 for the  $\Delta xlnR$  strain. In wheat straw, after transfer to the medium, the pH had dropped from 6.5 to 3.37 after 24h and to 2.84 after 48h for the WT strain when the pH had increased from 6.5 to 6.55 after 24h and to 6.73 after 48h for the  $\Delta xlnR$  strain (**Figure 3.3**). A two-way ANOVA test was performed within a same condition (e.g. glucose 48h) to show differences between the two strains. The results from the test showed that for the three conditions the differences between the WT strain and the  $\Delta xlnR$  strain were significant.

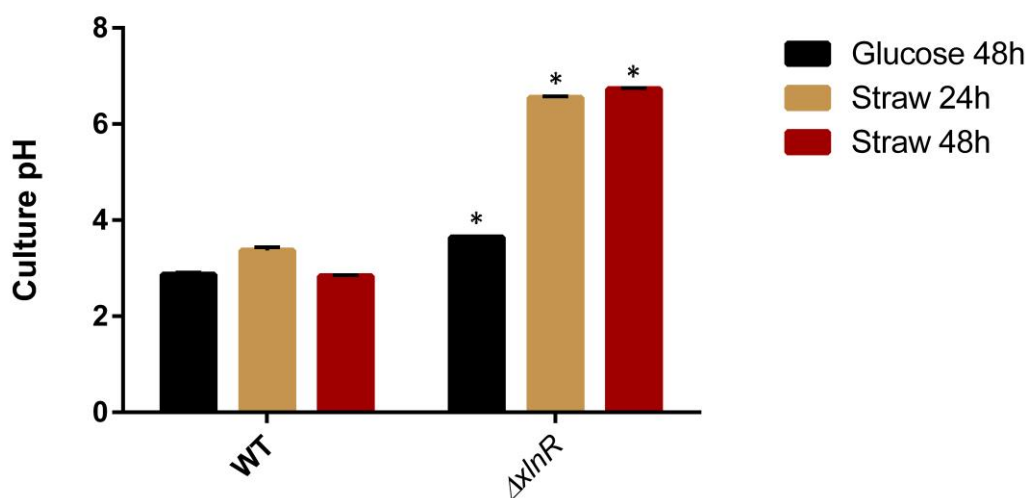


**Figure 3.3:** Culture pH measurements in the glucose 48h cultures as well as at 24h and 48h after transfer of the mycelium to wheat straw 1% (w/v) cultures. A two-way ANOVA test was performed. \* indicates a significant difference within one condition (e.g. glucose 48h) when the WT and the  $\Delta xlnR$  strains were compared ( $p < 0.05$ ).

## 3.3.2 Measurement of cellular components

## 3.3.2.1 Glucosamine assay

The amounts of glucosamine in fungal cultures were measured at each time point and converted back to a dry weight biomass. After 48h in glucose (1% w/v) the dry weight (DW) for the WT strain was 0.103g and 0.087g in the  $\Delta xlnR$  strain. The DW (estimated from glucosamine assay and standard curves, glucosamine vs dry weight, see Appendix 3.2) after 24h and 48h in wheat straw (1% w/v) were 0.21g and 0.34g respectively for the WT strain and 0.15g and 0.197g for the  $\Delta xlnR$  strain (**Figure 3.4**). For the WT strain the biomass increased by 2 times between the glucose condition and the 24h wheat straw time point and by 3.3 times after 48h. The increase in biomass was similar for the  $\Delta xlnR$  strain with a 1.7 times increase at 24h and 2.26 at 48h in wheat straw. However the WT biomass increased 1.4 times more than the  $\Delta xlnR$  strain for the 24h time point and 1.7 times more for the 48h sample. A two-way ANOVA test was performed within a same condition (e.g. glucose 48h) to show differences between the two strains. The results from the test showed that for the three conditions the differences between the WT strain and the  $\Delta xlnR$  strain were significant.

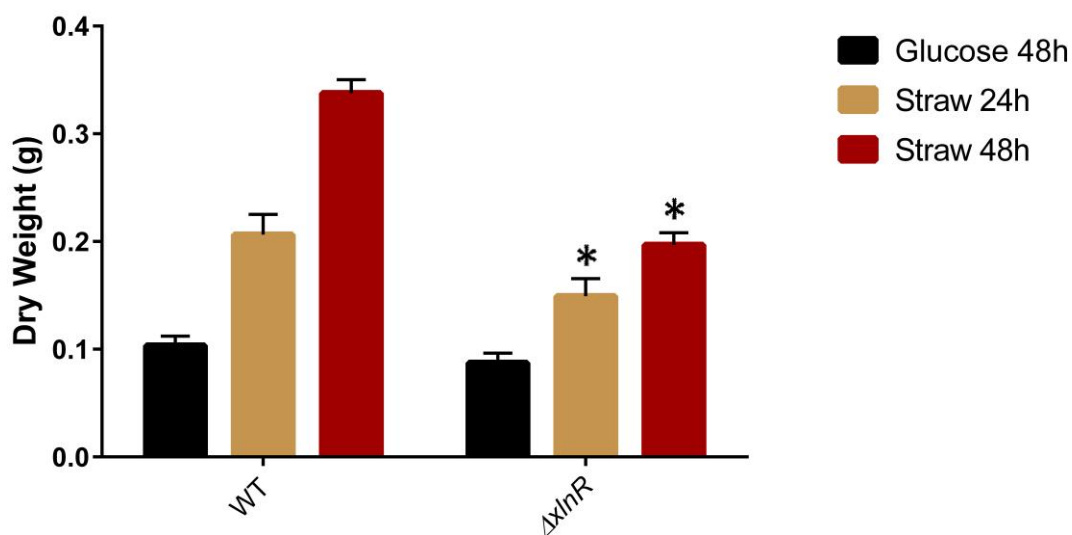


**Figure 3.4:** Glucosamine content measurements converted to dry weights in the glucose 48h cultures as well as at 24h and 48h after transfer of the mycelium to wheat straw 1% (w/v) cultures. The 48h glucose mycelia were considered as a 0h time point samples in wheat straw. A two-way ANOVA test was performed. \*

indicates a significant difference within one condition (e.g. glucose 48h) when the WT and the  $\Delta xlnR$  strains were compared ( $p < 0.05$ ).

#### 3.3.2.2 Total protein concentration

The concentrations of total proteins in fungal cultures were measured at each time point and converted back to a dry weight biomass. After 48h in glucose (1% w/v) the dry weight (DW) for the WT strain was 0.041g and 0.044g in the  $\Delta xlnR$  strain. The DW (estimated from protein assay and standard curves, total protein concentrations vs dry weight) after 24h and 48h in wheat straw (1% w/v) were 0.09g and 0.06g respectively for the WT strain and 0.12g and 0.05g for the  $\Delta xlnR$  strain (**Figure 3.5**). For the WT strain the biomass increased by 3 times between the glucose condition and the 48h wheat straw time point. The increase in biomass was lower for the  $\Delta xlnR$  strain with a 1.1 times increase between the glucose condition and the 48h wheat straw one. A two-way ANOVA test was performed within a same condition (e.g. glucose 48h) to show differences between the two strains. The results from the test showed that for the the 24h and 48h wheat straw time points the differences between the WT strain and the  $\Delta xlnR$  strain were significant.

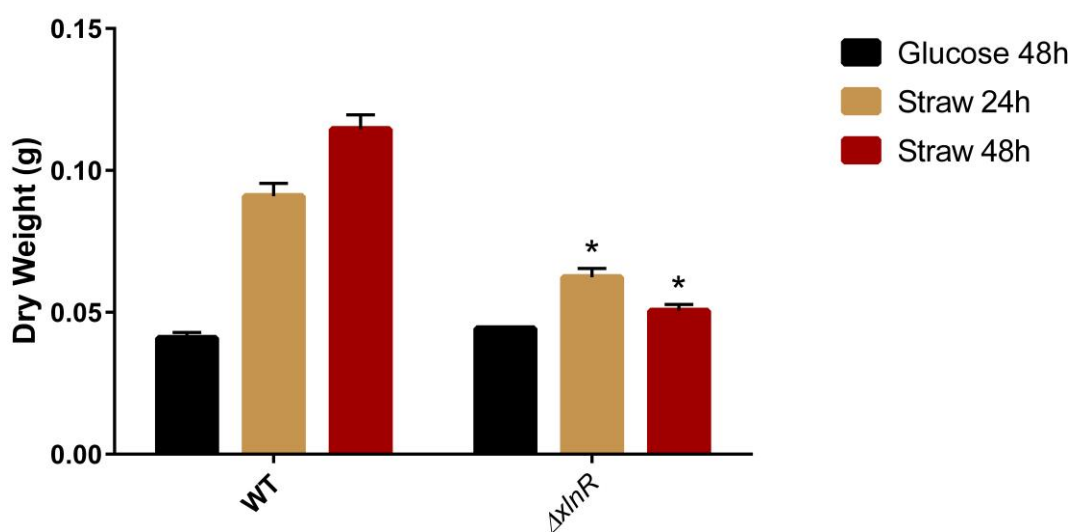


**Figure 3.5:** Total protein concentration measurements converted to dry weights in the glucose 48h cultures as well as at 24h and 48h after transfer of the mycelium to wheat straw 1% (w/v) cultures. A two-way ANOVA test was performed. \* indicates a significant difference within one condition (e.g. glucose 48h) when the WT and the  $\Delta xlnR$  strains were compared ( $p < 0.05$ ).

## 3.3.2.3 Enzymatic activity

The measurement of the enzyme activity from the supernatants of the WT and  $\Delta xlnR$  wheat straw cultures was done using the DNS assay measuring the reducing sugar ends released from the incubation of the supernatants (500 $\mu$ l) with wheat straw. The results showed that fewer reducing sugar ends were released from the incubation of the supernatants from the  $xlnR$  deletion strains when compared to the WT supernatant. For the WT strain, 1.06 and 1.27 glucose equivalents were released from the 24h and 48h supernatants respectively. For the *A. niger*  $xlnR$  deletion strain, 0.51 and 0.73 reducing sugar ends were released from the incubation with wheat straw (Figure 3.6).

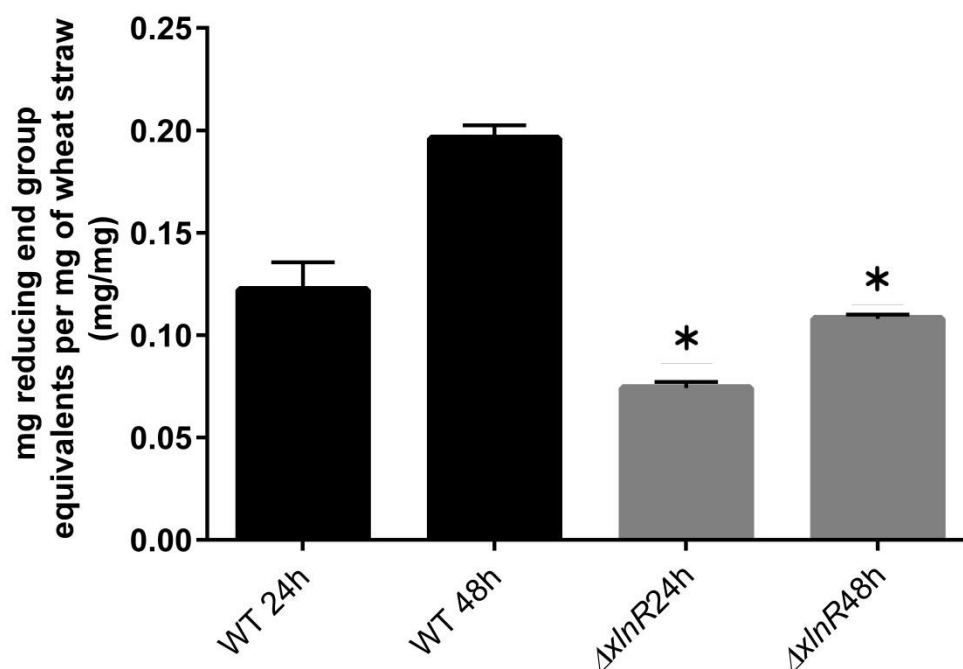


**Figure 3.6:** Measurement of reducing sugar ends released after incubation of equal volumes of the supernatants (500 $\mu$ l) of the WT and  $\Delta xlnR$  wheat straw cultures with wheat straw done by DNS assay. A two-way ANOVA was performed for each condition in comparison to the WT strain. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. \* represent the condition for which the two-way ANOVA test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

## 3.3.2.4 Relative growth in glucose cultures

The proportions of glucosamine and total proteins were calculated for the glucose-grown mycelial samples in relation to the dry weight measurements. The proportion of glucosamine in the glucose cultures was 4.78% (w/w) for the WT strain and 2.39% (w/w) for the  $\Delta xlnR$  strain (**Figure 3.7**). For the total protein concentration, the proportion in the WT strain was 31.9% (w/w) and 34.8% (w/w) for the  $\Delta xlnR$  strain (**Figure 3.8**). A t-test was performed to show the levels of confidence in any difference between the two strains. For the glucosamine and the protein proportions the differences observed between the WT and the  $\Delta xlnR$  strains were significant.

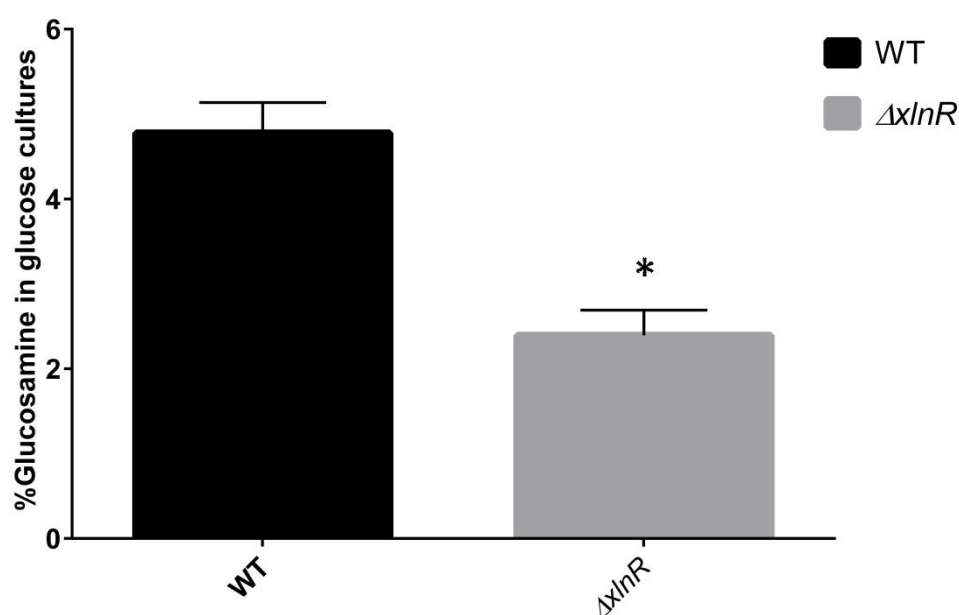


**Figure 3.7:** Proportion of glucosamine in the glucose 48h cultures compared to the expected amount of glucosamine in pure culture of *A. niger* (5%) (Andersen *et al.* 2008). A t-test was performed for statistics analysis. \* indicates a significant difference between the WT and the  $\Delta xlnR$  strains ( $p < 0.05$ ).

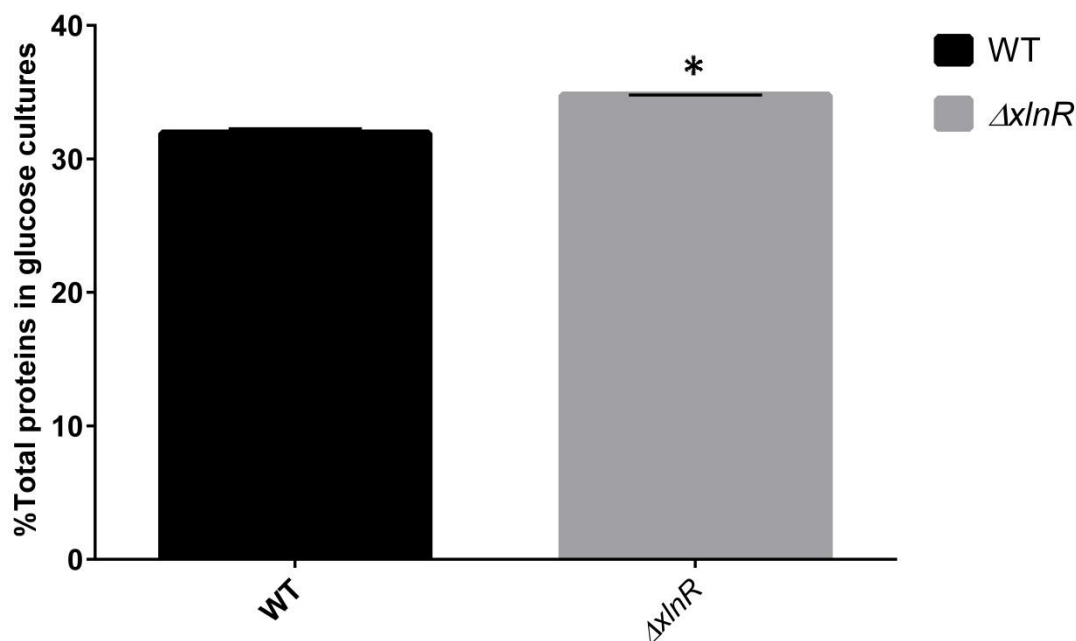


**Figure 3.8:** Proportion of total proteins in the glucose 48h cultures compared to the expected amount of intracellular proteins in pure culture of *A. niger* (26%) (Andersen *et al.* 2008). A t-test was performed for statistics analysis. \* indicates a significant difference between the WT and the  $\Delta xlnR$  strains ( $p < 0.05$ ).

### 3.4 Discussion

Various biomass determination methods are available for the quantification of fungal biomass. Each method has its advantages and drawbacks. A perfect method does not exist yet for measuring growth in complex substrates but some methods are more suitable than others. Most of the tested methods are based on the quantification of cell-specific components, which are known to change in response to environmental changes (Scotti *et al.* 2001). Steudler *et al.* (2015) studied the effect of 3 main cultivation variables on the estimation of fungal biomass: temperature, mycelium age and the substrate. In their study they used the Basidiomycete fungus *Trametes hirsuta* to investigate which biomass estimation assay would be the most suitable in solid-state fermentation (SSF). They found that biomass estimation based on glucosamine content was influenced by mycelium age confirming the results of Matcham *et al.* (1985). However, in the case of Ascomycetes, such as *A. niger*, it was shown that the age of the mycelium did not influence the glucosamine estimation making it a suitable method for biomass estimation (Desgranges *et al.* 1991; Scotti *et al.* 2001). In addition to the age of the mycelium, other studies showed that biomass

values, obtained by all applied methods, were influenced by the medium and the substrate (Desgranges *et al.* 1991; Klamer *et al.* 2004; Matcham *et al.* 1985; Miller *et al.* 1998; Ooijkaas *et al.* 1998; Scotti *et al.* 2001). Substrates directly influence the growth of the fungus or more precisely, the formation of fungal biomass, through their nutrient contents, degradability and impurities (Steudler *et al.* 2015). Thus, total biomass, cell component levels and metabolic activities would all be affected by the substrate. For instance, straw is less decomposable than corn silage or orange peel, thus more extracellular enzymes, different enzymes or a longer incubation time are required to obtain nutrients from it (Steudler *et al.* 2015). Also, other parameters can affect the biomass estimation such as (1) interference from components in the substrate other than the target compounds or (2) interference resulting from the presence of the target compounds in the substrate. In this perspective, ergosterol content measurement seems to be the most suitable method, as ergosterol does not occur in significant amount in plants (Matcham *et al.* 1985), no interference is expected in the analysis of ergosterol content. Also, it is the predominant sterol in fungal cell membranes (Klamer *et al.* 2004) so the samples are likely to contain sufficient amounts for robust analysis. The ergosterol method is regarded as a major approach for the estimation of living fungal biomass but that depends on the assumption that ergosterol is rapidly degraded upon death of fungal hyphae. Nevertheless, Mille-Lindblom *et al.* (2003) showed that ergosterol was persisting independently of the presence of living fungi and further confirmed this result by showing that ergosterol was very stable and slowly degraded even after 2 months in environmental samples without living fungi (Mille-Lindblom *et al.* 2004). Therefore, the possibility that a significant fraction of the measured ergosterol does not represent living fungal biomass should be considered. In the perspective of using biomass estimation as an indicator of fungal growth, ergosterol may not be the best method to phenotype strains based on their growth rates, as dead hyphae could be present in the culture of a strain that would not be able to grow well on complex substrates such as the  $\Delta xlnR$  strain. Glucosamine is also a fungal specific compound that is not found in plants and it should provide a suitable assay for the analysis of fungal growth. However, hexosamines are known to interfere with glucosamine content analysis (Matcham *et al.* 1985) and can be present in plant cell walls as in the cell wall of rice coleoptile that was found to contain several hundred micrograms hexosamine per gram of dry weight (Hoson *et al.* 1983). As for wheat straw, the level of hexosamines is not yet known but it is likely that some hexosamines would be present in wheat straw and would interfere with the glucosamine assay. However, all the samples were grown all under the same condition and this interference should be

the same in all the samples and be reflected in the results of all samples allowing for phenotyping analysis. As for ergosterol, it is possible that glucosamine remains in dead hyphae but to my knowledge no measurement of the stability of glucosamine in time has been reported. When dry weights were calculated back from the measurement of glucosamine contents, the values obtained were similar to the dry weight measured from 1.5g wet weight glucose culture suggesting that the interference from wheat straw for the glucosamine content analysis is null or very low. Here the glucosamine assay was tested to find out if it could be a suitable phenotypic routine assay. The results obtained for the WT and the  $\Delta xlnR$  strains showed differences in growth for the 2 strains suggesting that measuring glucosamine content is a sensitive enough approach for growth phenotyping. However, the results showed that the  $\Delta xlnR$  strain was growing according to the assay which was not expected to this extent as this strain is known to grow poorly on complex substrates such as wheat straw (Hasper *et al.* 2002). A sugar composition analysis of the WT and  $\Delta xlnR$  strains was carried out (see Chapter 4) and the results showed that the cell wall of the  $\Delta xlnR$  strain contained around 3% more glucosamine, by weight, than the WT cell wall. This suggests that the growth rate observed for the  $xlnR$  deletion strain should be lowered as more glucosamine is present in the cell wall and therefore increases the values measured for glucosamine content. Nonetheless, as the  $\Delta xlnR$  strain is not degrading straw as efficiently as the WT strain, more wheat straw is present at the time of measurement and the interference due to the substrate could also be greater in the  $\Delta xlnR$  strain sample, increasing the measurement of the glucosamine content. The advantage of using the glucosamine assay is that it can be easily used routinely in the laboratory as it does not require any specific equipment such as HPLC which is required in the case of the ergosterol content measurement.

The measurements of total protein concentration as well as enzymatic activities were also used to assess the growth of *A. niger* in wheat straw. The measurement of extracellular enzymatic activity seemed to be a suitable assay in the case of the  $\Delta xlnR$  strain. However, the  $xlnR$  gene encodes the main regulator XlnR that is known to be necessary for the expression of most CAZy-encoding genes for an efficient degradation of lignocellulosic substrates (Hasper *et al.* 2000). Therefore, the supernatants of the cultures obtained from the deletion strain do not include a lot of secreted enzymes and so the assay allows for good phenotyping results. In the perspective of using this assay for the phenotyping of other deletion strains, it may not be as sensitive as in this case if the deletion strains to phenotype are not mutants of genes that would be involved in the regulation of secreted enzymes. Measuring

the total concentration of proteins was also a good indicator of growth as the results showed differences between the 2 strains tested, yet there are some limiting factors to the use of this method. The measure of the total protein content is not a fungal specific method as the substrate can potentially contain proteins as well. In the case of wheat straw, it was shown that dry wheat straw is composed of around 3% (by weight) of proteins (Sarkar *et al.* 2012) which could lead to overestimation of the protein content or could prevent the detection of small phenotypic differences. In addition, protein assays are usually colorimetric assays that have been shown to be sensitive to interferences (Peterson 1979) and their accuracy greatly depends on the method used to extract proteins (Guerlava *et al.* 1998). Also, it is not known how wheat straw could interfere with the colorimetric reaction.

Other more straightforward methods were used to measure fungal growth such as measuring the pH of the culture as the result of the acidification of the medium or measuring the dry weight of the flasks for each time point. *A. niger* produces a range of acids as it grows during fermentation and thus measuring pH is a good indicator of growth for this species (Archer *et al.* 1990; Pedersen *et al.* 2000) unless the medium is heavily buffered. It is a reliable and reproducible way of measuring growth for *A. niger* and is appropriate to use in both simple substrates such as glucose or in complex substrates such as wheat straw. However, as expected, dry weight measurement is not a suitable method for measuring growth in wheat straw as the mycelium is mixed with the substrate. In order to use dry weight as an indicator of growth in complex substrates, a way of separating the mycelium of the substrate would be needed.

The proportions of total proteins and glucosamine were calculated relative to the dry weight of the samples (from 1.5g wet weight samples). The total protein proportion was shown to be around 26% for *A. niger* (Andersen 2008) and the results obtained here showed that this proportion was correct in both the WT strain (around 30%) and the  $\Delta xlnR$  strain (around 29%). However, the proportion of glucosamine measured was around the correct value for the WT strain (around 5%) but was much lower for the  $\Delta xlnR$  strain. This is in contradiction with the analysis of the cell wall sugars composition. It could be explained by the fact that the dry weight of the  $\Delta xlnR$  strain samples (from 1.5g wet weight samples) were lower than the WT one, therefore it could decrease the proportion of glucosamine measured relative to dry weight. In the case of the sugar analyses, it was done from the preparation of cell wall samples from the content of a full culture which should make the measurement more reliable and accurate.

In most cases, it seems to be advantageous to use a combination of different methods. This has two advantages. Firstly, it can increase the reliability of the results. Secondly, it can provide additional information about the state of the culture and allow for better measurements.

### 3.5 Future work

Among the different methods tested, pH seems to be the most appropriate to phenotype strains from *A. niger* based on the particularity of this species to acidify the medium on which it grows. The advantage of using pH measurement is that it is suitable for both simple and complex substrates and that no interference would occur from the complex substrate in the measurement. As for the fungal specific methods to estimate fungal biomass, even if none of the described methods is perfect, the glucosamine assay was the easiest to use in the laboratory as a routine assay for relatively quick phenotyping. It is a quite straightforward and cheap method that does not require extra equipment such as the use of HPLC in the case of the measurement of ergosterol. In any case, the best approach is to combine different methods and having a third assay to phenotype the strains would make the results more reliable. However, how wheat straw interferes with the different assays described in this chapter is not known and more investigation should be done to find out how this interference is impacting the results of the different methods.

**Chapter 4. Transcriptional regulation of genes  
encoding Glycoside Hydrolases (GHs) in *A. niger***

## 4.1 Introduction

### 4.1.1 Transcriptional regulation and responses – CAZy genes and associated regulatory mechanisms

Mechanisms of transcriptional regulation of genes encoding cellulases and hemicellulases in filamentous fungi have been studied, in *Aspergillus* spp. (Noguchi *et al.* 2009; van Peij *et al.* 1998b) and *Trichoderma* spp. (Stricker *et al.* 2008) amongst other species. Producing these extracellular enzymes is energy-consuming for the fungus, so the enzymes are produced only under conditions in which the fungus needs to use plant polymers as an energy and carbon source. Moreover, production of many of these enzymes is coordinately regulated, and they are induced in the presence of the substrate polymers. Molecules such as gentiobiose in *Penicillium* spp. (Kurasawa *et al.* 1992), xylose in *A. niger* (Mach-Aigner *et al.* 2012) and sophorose in *T. reesei* (Sternberg *et al.* 1979) have been shown to be inducers of genes encoding cellulases and hemicellulases. These molecules, gentiobiose and sophorose, are products of the hydrolysis and/or transglycosylation of xylan and/or cellulose (Hrmova *et al.* 1991; Mandels *et al.* 1962).

Transcription factors are proteins involved in the process of converting, or transcribing, DNA into RNA. Transcription factors include a wide number of proteins, excluding RNA polymerase that initiate and regulate the transcription of genes (Roeder 1996). One distinct feature of transcription factors is that they have DNA-binding domains that give them the ability to bind to specific sequences of DNA called enhancer or promoter sequences. Some transcription factors bind to a DNA promoter sequence near the transcription start site and help form the transcription initiation complex. Other transcription factors bind to regulatory sequences, such as enhancer sequences, and can either stimulate or repress transcription of the related gene. These regulatory sequences can be thousands of base pairs upstream or downstream from the gene being transcribed. Regulation of transcription is the most common form of gene control. The action of transcription factors allows for unique expression of each gene in different cell types and during development (Latchman 1997).

The zinc finger family of transcription factors is one of the most important families of transcription factors, and many have regulatory functions in development or metabolism (Caddick 2008). The Zn binuclear cluster motif, Zn<sub>2</sub>Cys<sub>6</sub>, is considered

as fungal specific (Todd *et al.* 1997). Zinc finger TFs have a conserved DNA-binding motif in their N-terminus (CX<sub>2</sub>CX<sub>6</sub>CX<sub>5-16</sub>CX<sub>2</sub>CX<sub>6-8</sub>). This conserved motif is followed by a less conserved domain which was identified as a fungal specific TF domain (Yuan *et al.* 2008).

Sequencing of the *A. niger* CBS 513.88 genome (Pel *et al.* 2007) has permitted the identification of 286 ORFs predicted to have the Zn<sub>2</sub>Cys<sub>6</sub> motif in their sequence. Nevertheless, the majority of these TFs have not been studied in detail and only a few of them have been described (**Table 4.1**).

**Table 4.1:** Known regulators of genes encoding CAZy enzymes and involved in pH regulation in filamentous fungi.

| Factor | Function                                                                                                         | Binding domain                   | Recognition motif     | References                     |
|--------|------------------------------------------------------------------------------------------------------------------|----------------------------------|-----------------------|--------------------------------|
| CreA   | Carbon Catabolite Repression                                                                                     | C2H2 Zinc finger                 | SYGGRG                | (Drysdale <i>et al.</i> 1993)  |
| XlnR   | Regulator of xylanolytic genes encoding enzymes required for xylan degradation and endoglucanase gene expression | Zn <sub>2</sub> Cys <sub>6</sub> | GGCTAAA               | (van Peij <i>et al.</i> 1998b) |
| AraR   | Regulator of genes encoding enzymes required for arabinose degradation genes encoding enzymes                    | Zn <sub>2</sub> Cys <sub>6</sub> | Not determined        | (de Vries <i>et al.</i> 1999b) |
| AmyR   | Regulator of genes encoding enzymes required for starch degradation                                              | Zn <sub>2</sub> Cys <sub>6</sub> | CGGN <sub>8</sub> CGG | (Yuan <i>et al.</i> 2008)      |
| InuR   | Regulator of genes encoding enzymes required for inulin degradation                                              | Zn <sub>2</sub> Cys <sub>6</sub> | CGGN <sub>8</sub> CGG | (Yuan <i>et al.</i> 2008)      |
| PacC   | Regulator of genes activated in response to external pH                                                          | C2H2 Zinc finger                 | GCCARG                | (MacCabe <i>et al.</i> 1996)   |
| CLR-1  | Regulator of genes encoding enzymes required for cellulose degradation A                                         | Zn <sub>2</sub> Cys <sub>6</sub> | Not determined        | (Coradetti <i>et al.</i> 2012) |
| CLR-2  | Regulator of genes encoding enzymes required for cellulose degradation B                                         | Zn <sub>2</sub> Cys <sub>6</sub> | Not determined        | (Coradetti <i>et al.</i> 2012) |
| ClbR   | Regulator of genes encoding enzymes required for cellobiose degradation                                          | Not determined                   | Not determined        | (Kunitake <i>et al.</i> 2012)  |

#### 4.1.2 Transcriptional responses of *A. niger* to lignocellulosic substrates

The responses of *A. niger* to different lignocellulosic substrates have been studied in detail in the recent years. Transcriptional analyses have been carried out for various

substrates such as wheat straw (at two time points) (Delmas *et al.* 2012; van Munster *et al.* 2014), willow (Pullan *et al.* 2014), and pre-treated sugar cane bagasse (de Souza *et al.* 2011). The analyses showed many similarities in the CAZy transcriptional responses of *A. niger* to wheat straw (at the later time point), willow and pre-treated bagasse (de Souza *et al.* 2011; Delmas *et al.* 2012; Pullan *et al.* 2014). Most of the genes encoding the CAZy enzymes required to breakdown cellulose and the hemicellulose backbones and side chains were induced. When the responses of *A. niger* to wheat straw were compared to the responses to willow in more detail, a similar pattern was observed, though there were some notable differences in expression levels (Pullan *et al.* 2014). Genes that had higher expression on wheat straw compared to willow included a GH62 arabinofuranosidase and two feruloyl esterases. These increases in expression could be related to compositional differences in the substrates (Pullan *et al.* 2014). A sequential response was also suggested in the transcriptional responses of *A. niger* to wheat straw where most of the genes encoding enzymes degrading hemicelluloses were induced earlier than genes encoding enzymes with activity towards pectins (van Munster *et al.* 2014). Andersen *et al.* (2012) described differences in the response of *A. niger* to different polysaccharide substrates but comparisons between the study with the different polysaccharides (Andersen *et al.* 2012) to the lignocellulose studies (de Souza *et al.* 2011; Delmas *et al.* 2012; Pullan *et al.* 2014; van Munster *et al.* 2014) is complicated because the concentrations of xylose in the xylan cultures at the time of sampling have the potential to also have a repressing as well as an inducing effect. A previous study in *A. niger* showed that concentrations of xylose higher than 1mM can have a repressive effect on CAZy genes (de Vries *et al.* 1999a).

#### 4.1.3 Activators and repressors of CAZy gene transcription

##### 4.1.3.1 Overview of transcriptional activators and repressors

There are some common and unique activators in fungi relevant to lignocellulose degradation. Todd *et al.* (2014) showed that most of these activators are found in Ascomycetes and not in Basidiomycetes. In a set of 64 characterised activators in Ascomycetes, the authors found that among the 7 relevant to the breakdown of lignocellulose (XlnR, AmyR, InuR, AraR, GalR, GalX and RhaR), none of them had any orthologues in the 31 Basidiomycetes species that they considered (Todd *et al.*

2014). Moreover, Todd *et al.* (2014) showed that most of these regulators belong to the  $\text{Zn}_2\text{Cys}_6$  TF family which is prevalent in Ascomycetes compared to Basidiomycetes.

A key repressor of transcriptional regulation in fungi related to lignocellulose is the repressor CreA/CRE1 (Portnoy *et al.* 2011; Ruijter *et al.* 1997). This regulator functions in Carbon Catabolite Repression (CCR), and, in the presence of sufficient glucose, triggers the transcriptional suppression of genes encoding enzymes responsible for the degradation of complex polysaccharides (Ruijter *et al.* 1997). Contrary to Ascomycetes activators, CreA/CRE1 orthologues were found in both Ascomycetes and Basidiomycetes fungi (Todd *et al.* 2014). However, for the other well-known repressor ACE1 (Activator of Cellulase Expression 1) of *T. reesei* (Aro *et al.* 2003), its presence was confirmed in Ascomycetes but no orthologues were found in Basidiomycetes species analysed by Todd *et al.* (2014).

#### 4.1.3.2 *XlnR/XYR1*

The xylanolytic activator XlnR is the most studied of the transcriptional activators involved in the regulation of glycoside hydrolase (GH) encoding genes. It encodes a polypeptide of 875 amino acids with a  $\text{Zn}_2\text{Cys}_6$  binuclear cluster at the N-terminal region. Based on electrophoretic mobility shift assays, DNase I footprinting and comparison of various xylanolytic promoters, the binding consensus motif was proposed to be GGCTAAA (van Peij *et al.* 1998b). It should be noted that while most  $\text{Zn}_2\text{Cys}_6$  motifs bind as a dimer to an inverted or direct repeat of CGG, XlnR binds to a GCC triplets. It remains to be clarified whether XlnR functions as a dimer. Functional analysis of the *A. niger* XlnR has revealed that the putative C-terminal coiled-coil region is involved in the nuclear import of XlnR, and the C-terminus downstream of the coiled-coil region has an inhibitory function in XlnR-controlled transcription (Hasper *et al.* 2004).

XlnR regulates the expression of genes encoding cellulases, hemicellulases and also accessory enzymes. It is the main regulator of xylan degradation in *Aspergillus* spp. It is also the orthologue of the *xyr1* gene of *T. reesei* (van Peij *et al.* 1998b). van Peij *et al.* (1998b) showed that XlnR was involved in the regulation of three major genes associated with xylan degradation: *xlnB*, *xlnC* and *xlnD*, encoding endoxylanases B, C and  $\beta$ -xylosidases, respectively. Accessory enzymes regulated by XlnR are involved mainly in hemicellulose degradation such as the acetylxylan esterase A or

the feruloyl esterase A. XlnR is also associated with the expression of some cellulase-encoding genes such as endoglucanases *eglA* and *eglB*. Hasper *et al.* (2002) showed that the endoglucanase (GH74)-encoding gene *eglC* was transcriptionally regulated by XlnR in *A. niger*.

van Peij *et al.* (1998b) and Hasper *et al.* (2002) carried out studies to demonstrate the importance of XlnR on the activation of the expression of GH-encoding genes in *A. niger*. They used a wild-type strain, an *xlnR* deletion strain and a strain containing multiple copies of *xlnR*. The studies showed that, in a strain lacking a functional *xlnR*, the genes encoding XlnB, XlnC and XlnD (van Peij *et al.* 1998b) and EglC (Hasper *et al.* 2002) were not expressed. In contrast, the transcription levels of these genes were increased in the multiple-copy strains. This highlights the role of XlnR in the regulation of their expression. Also, in *A. niger*, the constitutive activation of XlnR resulted in constant xylanase production even under repressing conditions (Hasper *et al.* 2004).

Brunner *et al.* (2007) and Endo *et al.* (2008) first showed in their studies that several genes encoding cellulolytic enzymes were controlled by an XlnR-independent pathway. They showed this for *Fusarium graminearum* and *A. nidulans* respectively. Recently, Tani *et al.* (2012) provided evidence of two distinct pathways in *A. aculeatus*: an XlnR-dependent and an XlnR-independent pathway. In *A. aculeatus*, Tani *et al.* (2012) showed that the XlnR-dependent pathway regulates the expression of genes encoding cellulases whereas the XlnR-independent pathway controls the expression of genes encoding hemicellulases. This study suggests that the different signals from cellulose degradation to both the XlnR-dependent and -independent signalling pathways are at the beginning of the signalling pathway in *A. aculeatus*.

#### **4.1.3.3 CreA/CRE1**

In addition to induction by mono- and oligo-saccharides, genes encoding hydrolytic enzymes involved in plant cell wall deconstruction in filamentous fungi can be repressed during growth in the presence of easily metabolized carbon sources, such as glucose. CreA is a global carbon regulatory protein of CCR in *Aspergillus* species. CCR is an important mechanism to repress the production of plant cell wall degrading enzymes during growth on preferred carbon sources (de Vries *et al.* 2001; Kubicek *et al.* 2009).

CreA is a Cys<sub>2</sub>His<sub>2</sub>- type TF (Dowzer *et al.* 1991; Ilmén *et al.* 1996). CreA has been shown to repress the expression of genes encoding cellulases, arabinases, xylosidases, feruoyl esterases and also pectinases (de Vries *et al.* 2001). Glucose is not the only easily metabolized sugar that leads to CreA-mediated repression. High concentrations of xylose have been demonstrated to activate CreA repression which leads to decreasing expression levels of xylanolytic and cellulolytic genes in *A. niger* (de Vries *et al.* 1999a) and *A. terreus* (Ali 1992).

In *A. nidulans*, mutations in the genes encoding a hexokinase and a glucokinase led to the CCR mediated by CreA. This suggests a potential role of these two protein kinases in the sugar signalling regulation (Flippin *et al.* 2003).

#### 4.1.3.4 Roles of XlnR and CreA in *A. niger*

Previous work carried out in the laboratory showed that the regulation of the expression of responsive genes in *A. niger* was sequential. Firstly, the lack of easily available carbon source leads to the alleviation of CreA repression and to the induction of a subset of starvation- induced genes. A subset of CAZy genes was found to be expressed upon starvation, when there is no XlnR induction or CreA repression. Those derived CAZy enzymes, resulting of the activation of the CAZy genes, are hypothesized to have a scouting role, to degrade carbon sources in the vicinity to release inducing sugars. These sugars can in turn induce the expression of XlnR-dependent CAZy genes (**Figure 4.1**) (Delmas *et al.* 2012). However, transcriptional regulation of cellulase and hemicellulase genes is incompletely understood in filamentous fungi, such as *A. niger*, capable of degrading lignocellulose. Other possible regulators have been identified such as ClbR in *A. aculeatus* or CLR-1 and CLR-2 in *Neurospora crassa*.

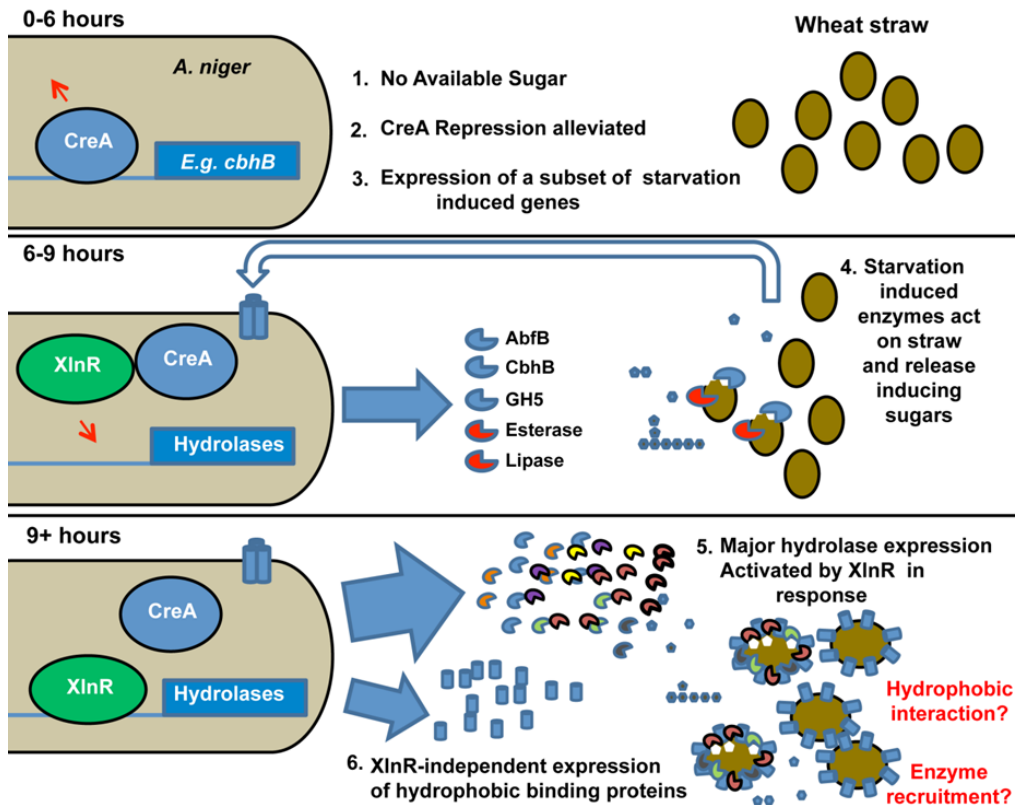


**Figure 4.1:** Induction model based on the sequential expression of responsive genes. The sequence of events is illustrated and key events are numbered. The upper panel represents the transcriptional events in *A. niger* upon exposure (0–6 h) to straw represented by filled ovals. The lack of easily-available carbon source leads to the alleviation of CreA repression (represented by the arrow above CreA) and induction of a subset of starvation-induced genes represented by *cbhB*. At 6–9 h exposure to straw (middle panel), the expressed hydrolases and other enzymes act upon the wheat straw, releasing small quantities of inducing sugars such as xylose (filled pentamer) as well as glucose (filled hexamer). Transporters for the sugars are induced (indicated by the trans-membrane cylinders and un-filled large arrow). By 9 hours (lower panel) the presence of xylose has caused activation of XlnR and, thereby, large-scale expression of hydrolases genes. Also induced by 9 hours, in an XlnR-independent manner, is the hydrophobic binding protein HsbA. The hydrophobin HfbD is induced by 12 hours. A physical association of hydrophobic binding proteins with straw and degradative enzymes is hypothesised and represented (see Chapter 5). Note that the functionality of XlnR and CreA is indicated by attachment to recognition sequences in target promoters and is meant only to indicate the functional control of those promoters by the transcription factors.

Modifications to those transcription factors (e.g. phosphorylation of XlnR in *A. oryzae* (Noguchi *et al.* 2011) may occur without necessarily implying that their location changes.

#### 4.1.3.5 CLR-1 and CLR-2 in *Neurospora crassa*

In *N. crassa*, the *xlnR/xyr1* orthologue *xlr-1* does not have the same function in regulating expression of cellulase encoding genes as in *A. niger* or *T. reesei*. In the  $\Delta xlr-1$  mutant, growth on cellulose, and cellulolytic activity, are only slightly affected (Sun *et al.* 2012). Instead in *N. crassa*, two other Zn<sub>2</sub>Cys<sub>6</sub> family transcription factors CLR-1 and CLR-2 were found to be the predominant regulators of the expression of cellulase-encoding genes (Coradetti *et al.* 2012). This study showed that genes encoding these TFs are conserved among the genomes of most filamentous fungi capable of degrading cellulose. This suggests that homologues of CLR-1 and CLR-2 play an important role in plant cell-wall degradation. Coradetti *et al.* (2012) demonstrated that these two TFs are important regulators of genes encoding both cellulases and hemicellulases in the presence of cellulose (Avicel, 97% cellulose and 3% xylan) as carbon source, but they are not required for growth or hemicellulase production in the presence of xylan. In this work, Coradetti *et al.* (2012) showed that CLR-1 is the crucial element in a cellobiose sensing mechanism when *N. crassa* is grown on Avicel. CLR-1 promotes expression of several genes necessary for cellobiose utilization, as well as the expression of *clr2*. CLR-2, maybe in a complex with CLR-1, directly induces cellulase and hemicellulase gene expression when *N. crassa* is grown on Avicel. *clr1* and *clr2*, as for *xlnR*, are regulated by CCR, and their expression is induced only when the CCR is relieved. Moreover, Coradetti *et al.* (2012) showed that *clr2* is under transcriptional regulation by CLR-1 and that the relative abundance of CLR-1 under de-repressed conditions is insufficient for the induction of the expression of *clr2* (**Figure 4.2**) (Coradetti *et al.* 2012). In *A. oryzae*, the orthologue of CLR-2 called ManR was characterised as a regulator of genes encoding mannan-degrading enzymes (Ogawa *et al.* 2013).

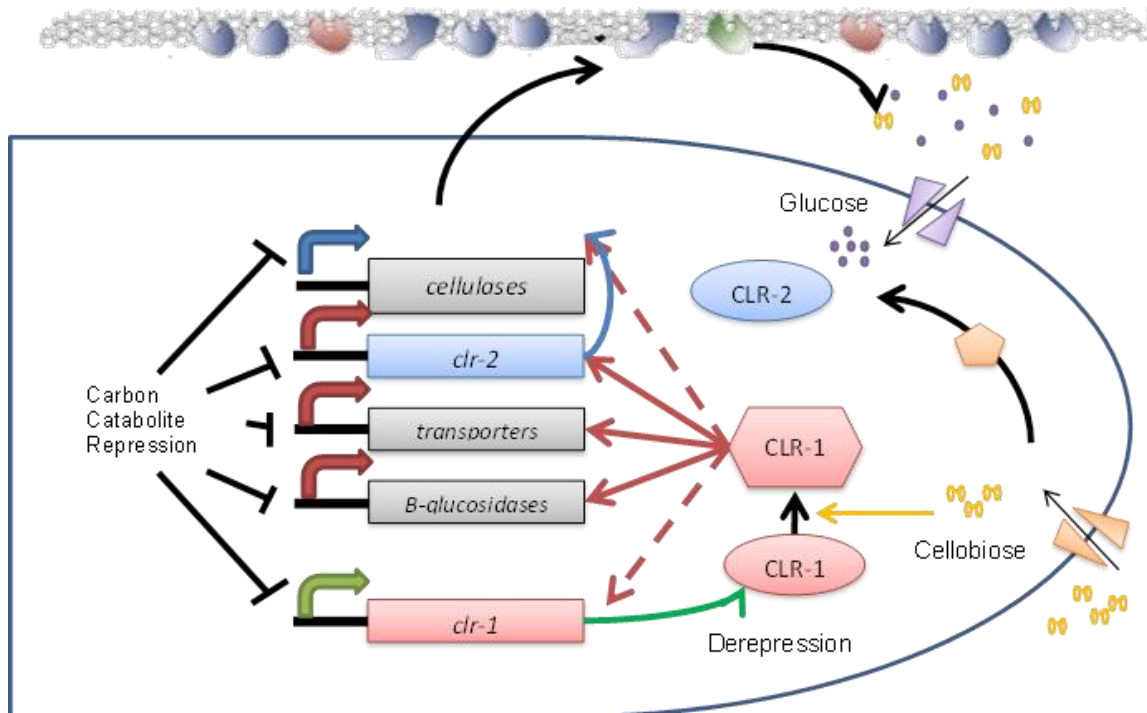


**Figure 4.2:** Model of cellulase induction mediated by CLR-1 and CLR-2 in *N. crassa*. When de-repressed, genes encoding cellulases,  $\beta$ -glucosidases, cellobiose transporters, and CLR-1 are expressed at low levels. CLR-1 is activated in the presence of cellobiose, promoting expression of cellobiose transporters and  $\beta$ -glucosidase genes. Activated CLR-1 is necessary for increased expression levels of *clr-2* and may also regulate its own expression. CLR-2 (possibly in concert with CLR-1) drives expression of the Avicel regulon, releasing more cellobiose in a positive feedback loop. Adapted from Coradetti *et al.* (2012).

#### 4.1.3.6 Other relevant transcription factors

Among the other TFs found in Ascomycetes to be relevant to lignocellulose degradation, AraR (Arabinolytic Regulator) seems to be of importance. AraR is a  $\text{Zn}_2\text{Cys}_6$  TF resulting from a recent duplication event of *xlnR* and is only found in certain fungal species (Battaglia *et al.* 2011b). Battaglia *et al.* (2011a) showed that AraR was working in co-operation with *XlnR* in the regulation of the pentose catabolic pathway. In *A. niger*, the deletions of *araR* and *xlnR* showed that the expression of CAZy genes was partly dependent on AraR. However, there was no clear evidence that some genes would be under the sole regulation of AraR (de Souza *et al.* 2011; de Souza *et al.* 2013).

Another Zn<sub>2</sub>Cys<sub>6</sub> TF identified is ClbR (Cellobiose Response Regulator) which was characterised in *A. aculeatus* (Kunitake *et al.* 2012). In this study, Kunitake *et al.* (2012) showed that ClbR regulated CAZy genes in response to cellulose and cellobiose (but not xylose) in an XlnR-dependent and independent manner. Moreover, the authors showed that the deletion of *clbR* leads to a decrease of the cellobiose and cellulose responsive induction of the *cbhl*, *cmc1*, *cmc2*, *xyn1b* and *xyn1a* genes encoding respectively a cellobiohydrolase, two carboxymethylcellulases and two xylanases.

Two other TFs, ACE1 and ACE2, were identified in *T. reesei* and were shown to be potentially involved in the regulation of hydrolase formation (Aro *et al.* 2001; Saloheimo *et al.* 2000). In their study, Rauscher *et al.* (2006) showed that ACE1 acted as a repressor antagonist of XYR1 function by competing for one of its binding sites in the *xyn1* promoter. In contrast, the deletion of *ace2* led to a decrease of the expression levels of the main cellulose encoding genes but did not affect induction by sophorose (Aro *et al.* 2001). ACE2 was also shown to contact the xylanase-activating element XAE (essential for *xyn2* expression) in the *xyn2* promoter (Wurleitner *et al.* 2003) but is not involved in *xyn1* transcription (Aro *et al.* 2001; Rauscher *et al.* 2006). Finally, a new regulator, ACE3 (Activator of Cellulase Expression 3) which contains a PFam Fungal specific TF domain, was identified in *T. reesei* (Hakkinen 2014). The results of the study showed that when *ace3* was deleted or over-expressed, the expression of *xyr1* (*xlnR* homologue) was altered, suggesting that ACE3 could be a major regulator in *T. reesei* (Hakkinen 2014).

#### 4.1.4 Environmental regulation of CAZy genes

Factors other than starvation and small molecule inducers can trigger the expression of CAZy genes in fungi and include environmental factors such as light or pH. Fungi use light as a source of information (and not as a source of energy like plants) as the ability to sense light is essential to recognize and anticipate conditions unfavourable for vegetative growth, such as loss of water or deprivation of nutrient availability on the soil surface. About one hundred fungal species have been shown to react to light (Marsh 1959) with perception mechanisms for blue, near UV, green and red light (Herrera-Estrella *et al.* 2007; Purschwitz *et al.* 2006). Light has been demonstrated to affect the transcription of CAZy genes and secretion of CAZy enzymes in both *N. crassa* (Schmoll *et al.* 2012) and *T. reesei* (Tisch *et al.* 2011b). The signalling

pathways responsible for this light responsiveness are mediated by homologous proteins in both species although the regulatory mechanism behind this response differs slightly between these two fungi. In *N. crassa*, the blue light photoreceptors white collar 1 (WC-1) and WC-2 which are transcription factors, can form a complex (white collar complex (WCC)) that regulates transcription of light-responsive genes through binding to light responsive elements (LREs) in their promoters (He *et al.* 2005). The VVD (VIVID) photoreceptor interacts with the WCC and modulates the response to light (Chen *et al.* 2009). In *T. reesei*, CAZy gene expression in response to light is regulated by the homologues of WC-1, WC-2 and VIVID which are BLR1, BLR2 and ENV (ENVOY). In darkness, deletion of some of these genes increased the amount of cellulase activity in the culture filtrate (Gyalai-Korpos *et al.* 2010). Gyalai-Korpos *et al.* (2010) considered that the effect on cellulase activity of *blr1* and *blr2* deletion was due to differences in protein secretion and that of *env1* deletion was due to adjustments in response to the environment. Tisch *et al.* (2013) described a key difference in the functioning of the homologous proteins in *T. reesei* and *N. crassa*. Transcriptional analysis of deletion mutants under light and dark conditions showed that photoreceptor regulation of carbon metabolism is mediated by BLR1 and BLR2 stimulation of *env1* transcription in *T. reesei*, whereas in *N. crassa* VIVID had a negative effect on the WCC (Tisch *et al.* 2013). Also, BLR1 and BLR2 in *T. reesei* are not considered to act as a complex under darkness (Gyalai-Korpos *et al.* 2010). The *T. reesei* ENVOY protein has an additional function; it affects the expression of G-protein alpha subunits GNA1 and GNA3. Changes in signalling of these proteins in the heterotrimeric G-protein pathway affect cAMP and consequently the cAMP-PKA pathway that influences cellulase expression. ENV1 can also adjust cAMP levels, probably via a phosphodiesterase, a protein that degrades cAMP (Tisch *et al.* 2011a).

#### 4.1.4.1 pH regulation of CAZy gene expression

There are many examples demonstrating a connection between ambient pH and differences in CAZy enzyme production with one of the first examples demonstrated by Bailey *et al.* (1993) in *T. reesei*. The authors showed that a higher pH (pH 6.0 – 7.0) was favourable for xylanase production whereas a lower pH (pH 4.0) was favourable for cellulase production. In a recent study, Li *et al.* (2013) showed that the optimum pH levels for production of endoglucanases, exoglucanases and  $\beta$ -glucanases by *T. reesei* were different. Adav *et al.* (2011) also showed with *T. reesei*

that the secreted CAZy profiles changed with different pH levels ranging from pH 3.0 to pH 9.0. The differences in CAZy enzyme production in the above examples can partly be explained by the homeostatic system by which fungi respond to pH fluctuations in their environment and PacC, the transcription factor effector of this system, which can regulate some of the CAZy genes.

*A. nidulans* is the main model systems where pH regulation has been studied. In *A. nidulans*, the ambient pH is considered to signal through a plasma membrane complex (Herranz *et al.* 2005; Hervas-Aguilar *et al.* 2010) where this signal is passed on through a signalling cascade made of six Pal proteins encoded by the genes *palA*, *palB*, *palC*, *palF*, *palH* and *pall* (Arst *et al.* 1994; Caddick *et al.* 1986; Calcagno-Pizarelli *et al.* 2007). The target of the signalling cascade is the zinc finger transcription factor PacC. Three different forms of PacC have been described: in acidic conditions the full-length form of PacC predominates (Diez *et al.* 2002) whereas in alkaline pH conditions, the activation of the pathway leads to two subsequent cleavage steps resulting in two shorter forms of PacC. The product of the PalB-mediated cleavage step, PacC53 is then cleaved to create the active form of PacC (Hervas-Aguilar *et al.* 2007). The deletion of *pacC* or *pal* genes leads to a phenotype mimicking that displayed in acidic conditions whereas a constitutively active PacC results in alkaline-expressed genes and repression of acid-expressed genes regardless of pH (Arst *et al.* 1994; Caddick *et al.* 1986). So in effect in *A. nidulans* during alkaline conditions, PacC activates alkaline-expressed genes and represses acidic-expressed genes (Tilburn *et al.* 1995).

The active PacC binds the core target sequence 5'-GCCARG-3' in the promoters of its target genes (Espeso *et al.* 1997), however to my knowledge a comprehensive search for this motif has not been reported in species such as *T. reesei*, *N. crassa* or *Aspergillus* spp. The promoter of *pacC* contains PacC binding sites confirming that PacC autoregulates with higher abundance of *pacC* transcripts shown at alkaline pH (pH 8.0) (Mello-de-Sousa *et al.* 2011; Tilburn *et al.* 1995). A study with the thermophilic ascomycete *Humicola grisea* investigated the pH-dependent transcriptional regulation of CAZy enzyme encoding genes with sugarcane bagasse as the substrate (Mello-de-Sousa *et al.* 2011). The transcript levels of *cbh1.1*, *cbh2.2*, *egl1*, *egl2*, *bgl4* and *xyn1* increased at alkaline pH (pH 8.0) and Mello-de-Sousa *et al.* (2011) identified one or multiple PacC binding sites in the promoters of these genes. Other CAZy genes regulated by PacC include the *A. nidulans* xylanase genes (*xlnA*

and *xlnB*) (Maccabe *et al.* 1998) and alpha-L-arabinofuranosidase gene (*abfB*) (Gielkens *et al.* 1999). In a recent global transcriptomic study in *T. reesei*, Hakkinen (2014) investigated the effects of extracellular pH and of *PAC1* (the *T. reesei* orthologue of *pacC*). A large number of genes responded to changes of pH and 60 CAZy genes (from GH, CE or polysaccharide lyase (PL) classes) were among the pH-responsive genes. The authors showed that of the genes encoding proteins with the same enzymatic activity, some were up-regulated at low pH (pH 4.0) and others at high pH levels (pH 6.0-7.0). It is possible that the genes upregulated at the low pH encode for enzymes that have higher activity at the low pH. However, Hakkinen (2014) found that the pH-responsive genes were not only under *PAC1* regulation. There were likely to be other regulatory mechanisms functioning at the same time, affecting the pH-dependent expression of CAZy genes.

#### 4.1.5 Other regulatory mechanisms

##### 4.1.5.1 Stress responses

##### **Nutrient limitation stresses**

Situations of nutrient starvation, such as carbon limitation, can lead to a stress response in fungi exposed to lignocellulose. When *A. niger* and *T. reesei* are exposed to lignocellulose, expression of genes encoding plant polysaccharide-degrading enzymes was shown to be sequential (Delmas *et al.* 2012; van Leeuwen *et al.* 2013; van Munster *et al.* 2014). This may lead to the easily degraded carbohydrates being hydrolysed and the products imported first, leaving the fungus with the recalcitrant part of the substrate. This recalcitrance of lignocellulose can result in carbon limitation or starvation, and thus nutrient stress. In response to carbon starvation, recycling of fungal cell material and asexual sporulation are induced (Kim *et al.* 2005; Nitsche *et al.* 2012; Szilagyi *et al.* 2013). Genes involved in asexual sporulation and autolytic cell wall recycling were induced in *A. niger* after exposure to wheat straw for 24 hours, suggesting the fungus indeed experiences carbon limitation or starvation when growing on wheat straw (van Munster *et al.* 2014).

##### **Endoplasmic reticulum stress**

The endoplasmic reticulum (ER), which represents 50% of the total cellular membranes, contributes to several vital functions for homeostasis and cell survival. It

is within the ER that secreted proteins, as well as proteins involved in cell wall composition and intracellular organelles, are synthesised, modified and folded into their native conformation. Indeed, the ER lumen provides an oxidative environment allowing the formation of disulphide bonds. The ER lumen is also particularly rich in chaperone proteins and in specialised enzymes for the folding of proteins that are  $\text{Ca}^{2+}$  dependent for their catalytic activity. Finally, it is within the ER that proteins undergo post-translational modifications (e.g. N-glycosylation, oligomerisation). The ER is therefore an important cellular component and only the correctly folded proteins will pass the quality control system and be transported to the Golgi apparatus for their secretion or transport to other components of the cell. The proteins identified as incorrectly folded are degraded through a process called ER-associated-degradation (ERAD) (Ellgaard *et al.* 2003; Sitia *et al.* 2003). To do so, proteins are dislocated to the cytoplasm where they are degraded through the proteasome pathway. Any event leading to a modification of the ER function, such as the accumulation of unfolded proteins or the modification of the calcium homeostasis of the cell triggers a physiological response called the Unfolded Protein Response (UPR) or ER stress (Kaufman 2002; Schroder *et al.* 2005). The UPR aims to: (1) slow down the protein synthesis to avoid the arrival of other proteins in the ER lumen; (2) synthesise new chaperone proteins or foldases to stimulate folding processes; (3) lead unfolded proteins to degradation; (4) trigger cell apoptosis when all of the previous processes have failed. The UPR pathway was first described in the yeast *S. cerevisiae*. The UPR pathway implies the activation of an ER transmembrane protein Ire1 (Inositol-requiring enzyme-1) (Rutkowski *et al.* 2004). Ire1 is the only ER stress sensor present in all Eukaryotes and therefore reflects the most ancient and conserved branch of the UPR (Mori 2009). The activation of this protein requires its dissociation from the chaperone protein BipA that keeps it in an inactive form within the ER membrane. The increased arrival of proteins into the ER lumen triggers the dissociation of BipA which can then associate to proteins that are being folded allowing for the UPR pathway activation. Recent evidence demonstrated that the yeast Ire1p also directly binds to unfolded proteins that can trigger its activation suggesting that Ire1 can sense ER stress by directly monitoring the concentration of unfolded proteins (Credle *et al.* 2005; Zhou *et al.* 2006; Gardner and Walter 2011). Therefore, the higher-order oligomerisation of Ire1, which is required for downstream activation, is influenced by two factors: binding to unfolded proteins, which shifts Ire1 toward an active, oligomeric state, and binding to BiPA, which stabilizes Ire1 in the

inactive, monomeric state. Ire1 has serine/threonine kinase and endoribonuclease activities in its cytoplasmic domain (Rutkowski *et al.* 2004) and even though its oligomerisation triggers its auto-transphosphorylation, Ire1 does not signal downstream via phosphorylation. Instead, a conformational change occurring upon nucleotide binding acts as a molecular switch to activate Ire1's RNase domain (Aragon *et al.* 2009; Korennykh *et al.* 2009; Papa *et al.* 2003). Ire1 plays an important role in the processes of apoptosis and survival when an ER stress is observed in physiological or physiopathological conditions. Once activated Ire1 triggers the splicing of a non-conventional intron present in the mRNA of the bZIP TF Hac1 releasing a translational block for the production of Hac1. Hac1 binds ER Stress Element (ERSE) sequences present in the promotor regions of many, but not all, of the UPR pathway target genes, to up-regulate genes encoding ER quality control components (Bouchecareilh *et al.* 2009; Sidrauski *et al.* 1996; Travers *et al.* 2000; Yoshida *et al.* 2001).

The UPR is therefore an important factor in the optimised secretion of proteins at very high yields in industrial production. The significance of the UPR is under-explored in relation to the saccharification of lignocellulose by fungi. For example, under conditions where *A. niger* (Delmas *et al.* 2012; Suto *et al.* 2001) or *T. reesei* (Kriel *et al.* 2011) were transferred from glucose-grown conditions to wheat straw there was no clear impact on the transcript levels of selected UPR marker genes such as *hac1*, *bipA* or *pdi1*, although the UPR involves transcript level changes to several hundreds of genes, e.g. in *A. niger* (Guillemette *et al.* 2007). The apparent lack of induction of the UPR was presumably because the GH enzymes secreted were native proteins that the fungi naturally secrete well and that the enzyme levels secreted did not cause undue load on the system at the time-points studied. In contrast, a study in *N. crassa* showed induction of the UPR pathway (increase in the transcript level of the *hac-1* gene and increase splicing of the *hac-1* mRNA) when exposed to Avicel (Szilagyi *et al.* 2013). Transcriptomic data supports the occurrence of ER-autophagy, which is linked to ER stress, when fungi are exposed to lignocellulose. The transition from nutrient-rich growth conditions to lignocellulose involves a carbon starvation response in *A. niger* (Parenicova *et al.* 2000). The carbon starvation response involves the induction of ER-autophagy genes and GH-encoding genes (Geysens *et al.* 2009; Martens-Uzunova *et al.* 2009) and it was also shown that wheat straw up-regulated the transcript levels of ER-autophagy genes in

*T. reesei* (Ries *et al.* 2014). Other studies have shown that increased flux of native protein through the secretory system can lead to induction of the UPR in both *T. reesei* and *A. niger* (Bernales *et al.* 2006; Nitsche *et al.* 2013). It is therefore likely that there is at least a transient ER stress when fungi are transferred from conditions where simple sugars are available to one where lignocellulose is the sole source of carbon, but detection of the UPR probably depends on sampling time and possibly the composition of the lignocellulose.

#### **4.1.5.2 Antisense RNA**

A less well studied mechanism of transcriptional regulation is that involving regulatory RNAs but it is emerging as a major focus of research on regulation (Morris *et al.* 2014). One type of regulatory RNA is natural antisense transcripts (NATs), where RNA complementary in sequence to the sense RNA is transcribed (Lapidot *et al.* 2006). There are several examples of genes in *A. niger* and *T. reesei* for which antisense transcripts exist and where the amount of antisense transcripts changes in the presence of lignocellulose (Delmas *et al.* 2012; Ries *et al.* 2013). One example described in *A. niger* is a putative acetate permease transporter, for which the majority of the gene transcripts were antisense in a glucose rich medium, while in a wheat straw medium the majority of the transcripts were in the sense orientation (Delmas *et al.* 2012). In the conditions of the study of Delmas *et al.* (2012), ~ 2% of the total RNAseq reads were antisense transcripts. Recent research provides support for a functional role of antisense transcription and for a mechanism for how antisense RNAs regulate gene expression involving the stalling of RNA polymerases (Xue *et al.* 2014). The authors showed that transcriptional interference by antisense RNA is required for circadian clock function in *N. crassa* (Xue *et al.* 2014). The circadian clock has relevance to transcriptional responses to lignocelluloses as light has a role in regulating transcription related to cellulases via WC-1 and WC-2. The transcription factors WC-1 and WC-2 also regulate the gene frequency (*frq*) which functions in maintaining rhythmicity and also has antisense expression (Xue *et al.* 2014).

#### **4.1.6 Aims**

The aims of this chapter were to (1) characterise the orthologues of the newly discovered TFs CLR-1 and CLR-2 of *N. crassa* in *A. niger*, (2) Discover new TFs by

further analysing the RNA-Sequencing dataset and (3) further phenotype deletion strains of already known regulators.

## 4.2 Material and Methods

See Chapter 2:

- 2.2.11 for the bioinformatics analysis
- 2.2.5 and 2.2.6 for the gene deletion procedure
- 2.2.7.1 for pH measurement
- 2.2.7.2 for glucosamine assay
- 2.2.10.3 for SDS PAGE gel on the culture supernatants
- 2.2.10.1.2 for DNS assay
- 2.2.3.4.2 for qRT-PCR
- 2.2.8 for cell wall analysis
- 2.2.9 for the mitochondrial assay and the ROS measurement
- 2.2.7.1.1 for the pH controlled experiment

## 4.3 Results

### 4.3.1 Identification and analysis of the orthologues of *clr-1* and *clr-2* in *A. niger*

Based on the work of Coradetti *et al.* (2012), the orthologues of the *clr-1* and *clr-2* genes in *A. niger* were predicted in the genome using the algorithm BLAST from NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The orthologue of *clr-1* was identified as the gene An05g00020 (*clrA*). This gene is located on chromosome VII in the genome of *A. niger*. It is 1956 bp long and encodes a 621aa protein. The orthologue of *clr-2* is the gene An12g01870 (*clrB*). This gene is located on the chromosome III in the genome of *A. niger*. It is 2127 bp long and encodes a 708 aa protein (<http://www.cadre-genomes.org.uk/index.html>).

The software ProSite (<http://prosite.expasy.org/>) was used to determine the protein domains present in the protein sequences of ClrA. Prosite is a database of protein families and domains (Sigrist *et al.* 2002). It consists of biologically significant sites, patterns and profiles that help to reliably identify to which known protein family (if any) a new sequence belongs. The results show that the ClrA protein contains a Zinc finger type DNA-binding domain as for the CLR-1 protein of *N. crassa*. A Zinc finger

DNA-binding domain was also identified in the ClrB sequence of *A. niger* using the tool search for conserved domain of the NCBI website (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (Appendix 4.1-4.5).

#### 4.3.2 Characterising these genes in *A. niger*

##### 4.3.2.1 RNA-sequencing data

An overview of the protocol used for each set of experiments performed by Delmas *et al.* (2012) using glucose and wheat straw, together with the use of willow and no carbon source (Pullan *et al.* 2014; van Munster *et al.* 2014) is shown in **Figure 4.3**.

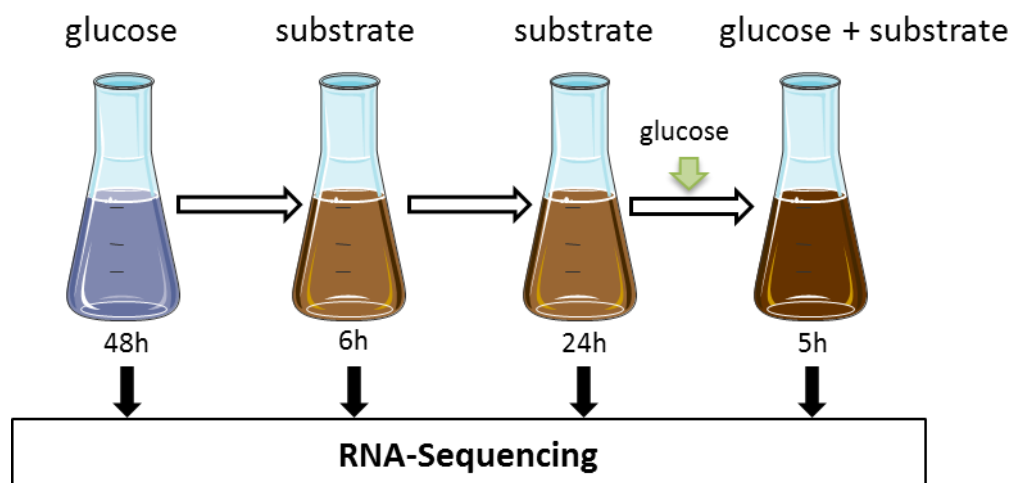


**Figure 4.3:** *A. niger* was grown on a sole carbon source of glucose for 48hrs, followed by either wheat straw, willow or no carbon source (substrate) for 24hrs, and then the substrate supplemented with glucose for 5hrs, and RNA extracted from mycelia at each of the three stages for RNA-seq, in biological replicate. Transcription data was then mapped to the *A. niger* ATCC1015 and CBS 513.88 genomes to produce a transcriptome and expressed as RPKM (Reads Per Kilobase of exon model per Million mapped reads). Data were collected and analysed for wheat straw substrate by Delmas *et al.* (2012), the carbon free dataset by van Munster *et al.* (2014) and the data from the willow substrate by Pullan *et al.* (2014).

When looking at the RPKM values for the different transcription factors of interest, *xlnR* is the most transcriptionally induced on wheat straw when compared to *clrA* or *clrB*. There was a 13 times fold change in the transcript level of the *xlnR* gene when the strain was switched from glucose (repressing condition) to wheat straw for 6h. In the same conditions the fold change for the *clrA* gene was 1.25 times and 6.2 times

for the *clrB* gene (**Table 4.2**). RPKM values for all genes were calculated for each of the separate biological replicates, as well as for the combined mapping of all three. The results shown were from the combined mapping scores. Three statistical significance tests were applied to changes in gene expression. The increase in RPKM values for the *xlnR* and the *clrB* genes were significant but not the value for the *clrA* gene.

**Table 4.2:** RPKM values for the *xlnR*, *clrA* and *clrB* genes of *A. niger*

| <i>RPKM values</i> | <i>xlnR</i> | <i>clrA</i> | <i>clrB</i> |
|--------------------|-------------|-------------|-------------|
| Glucose 48h        | 8.55        | 17.27       | 71.73       |
| Straw 6h           | 110.54      | 21.67       | 446         |
| <b>Fold change</b> | <b>13</b>   | <b>1.25</b> | <b>6.2</b>  |

#### 4.3.2.2 Deletion of the *clr* genes in *A. niger*

##### 4.3.2.2.1 Construction of the ligated flanking regions of the *clrA* gene from *A. niger*

The primers used to build the deletion cassette were designed to have restriction enzyme tags, to allow for precise ligations of the up and down flanking regions of the *clrA* gene, and between the product of the ligation of the up and the down fragments (named *clrA* updown fragment) with the pC3 plasmid (**Figure 4.4**).

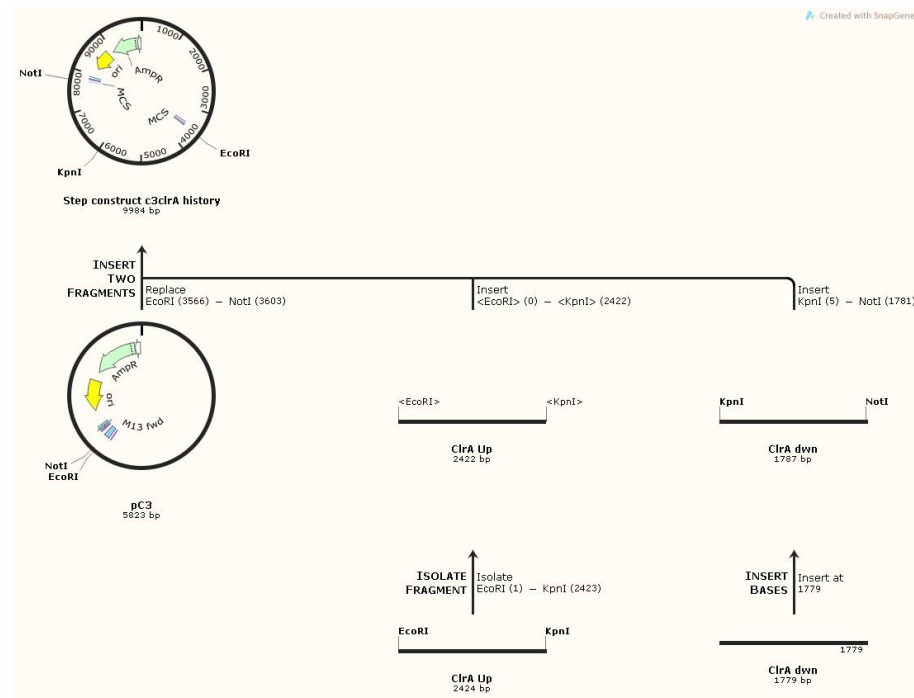


**Figure 4.4:** Schematic of the deletion cassette for the *clrA* gene and its ligation in the pC3 plasmid. Firstly the up and down fragments were amplified by PCR before being digested with the following restriction enzymes: *EcoRI* and *KpnI* for the up fragment and *KpnI* and *NotI* for the down fragment. The up and down inserts were then ligated together. The pC3 plasmid was digested with *EcoRI* and *NotI* and the updown fragment was inserted by ligation.

PCRs were carried out on samples of genomic DNA from *A. niger* strain N402 using primers for either the upstream DNA fragment of the gene of interest, or the downstream DNA fragment. The sizes of the expected PCR products were estimated *in silico* based on the sequence of the gene of interest and the set of primers used for the PCR reaction. **Figure 4.5** below shows the results from these initial PCRs.

After purification, the upstream and the downstream DNA fragments were digested using the restriction enzyme *KpnI*. After digestion, the DNA was then purified and the upstream and downstream DNA regions of the *clrA* gene were ligated using the T4 DNA ligase enzyme. The products of the ligation were then separated by agarose gel electrophoresis and the ligated *clrA* updown DNA fragments were extracted. The ligation product obtained was then amplified by PCR and double digested with the restriction enzymes *EcoRI* and *NotI* to allow the ligation in the pC3 plasmid which had been digested using the same enzymes.

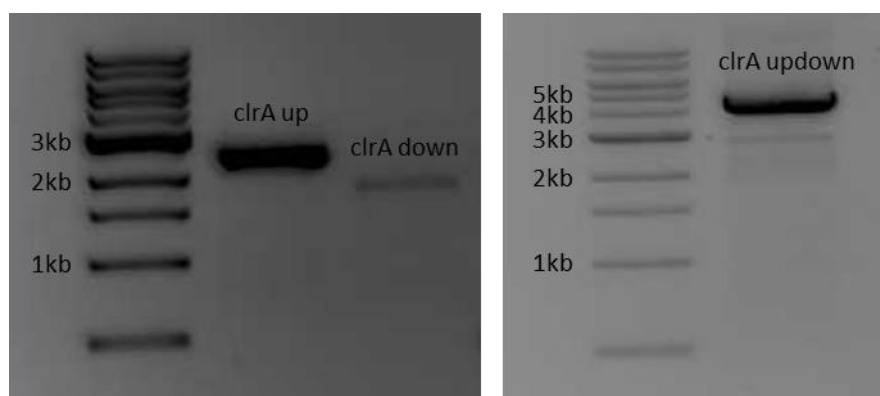


**Figure 4.5:** RT-PCR of the upstream and downstream flanking DNA regions of the *clrA* gene. The expected sizes for the PCR products of the flanking regions were 2412bp for the upstream fragment, and 1772bp for the downstream fragment. The *clrA* updown ligation product's size estimated *in silico* is 4184bp and a product of around 4kb was obtained as shown in the figure. The exact size of the obtained product was calculated from the sequencing result of the PCR product.

#### 4.3.2.2.2 Cloning

The double-digested fragment obtained was then ligated in the Multiple Cloning Site (MCS) of the pC3 plasmid. The predicted map of the pC3 plasmid with the correct *clrA* updown fragment insertion is shown in **Figure 4.6**.

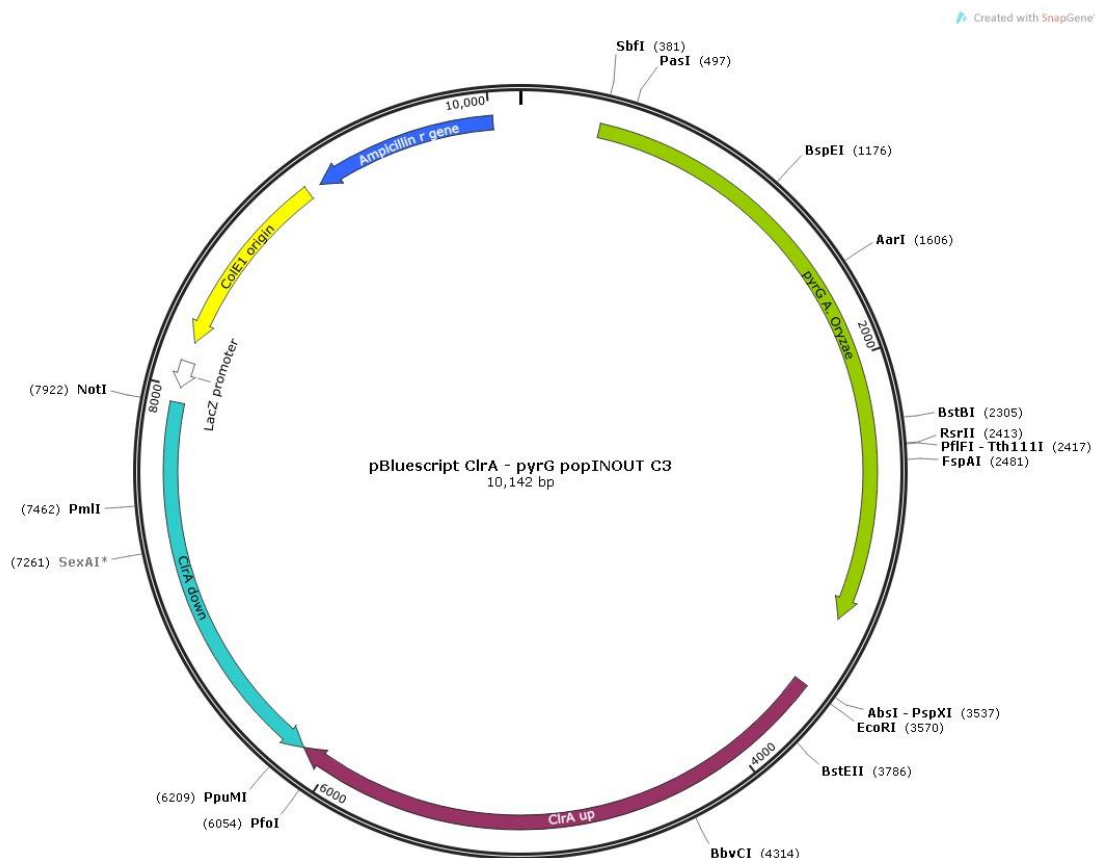


**Figure 4.6:** Predicted map of the pC3 plasmid with the *clrA* deletion cassette correctly inserted.

The ligation mix was transformed in *E. coli* XL1-Blue for the blue-white screening. White colony transformants obtained from these transformations were purified and the plasmids were isolated from transformants using the Miniprep procedure. In **Figure 4.7**, the results show that the predicted plasmid for the *clrA* deletion cassette was obtained.

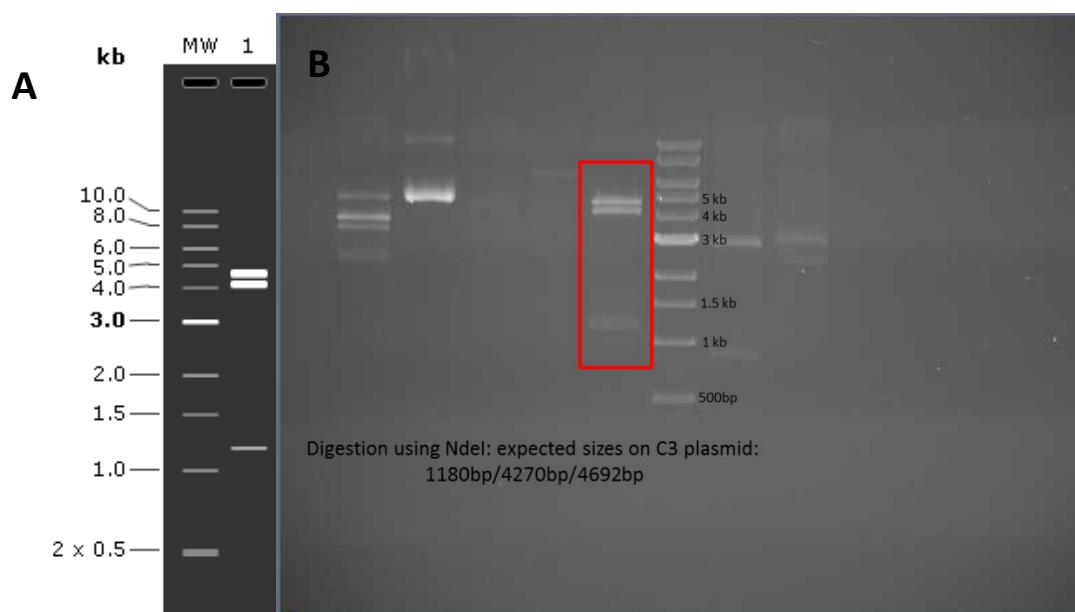


**Figure 4.7:** (A) *In silico* prediction of the pC3\_clrA plasmid digested with the restriction enzyme *NdeI* (B) Positive screening of the pC3 plasmid containing the *clrA* deletion cassette after digestion with the *NdeI* restriction enzyme.

#### 4.3.2.2.3 Transformation of *A. niger* and screening for transformants

Genomic DNA extractions were carried out with the colonies of *A. niger* grown on 5-FOA plates, and PCR screening was done on the samples to identify any successful deletion mutants of  $\Delta clrA$  using the *clrA* external primers (Appendix **Table 2.1**). From 15 colonies tested 3 colonies were positive for the gene deletion. The electrophoresis profiles of the DNA fragments obtained after PCR are shown below in **Figure 4.8**.

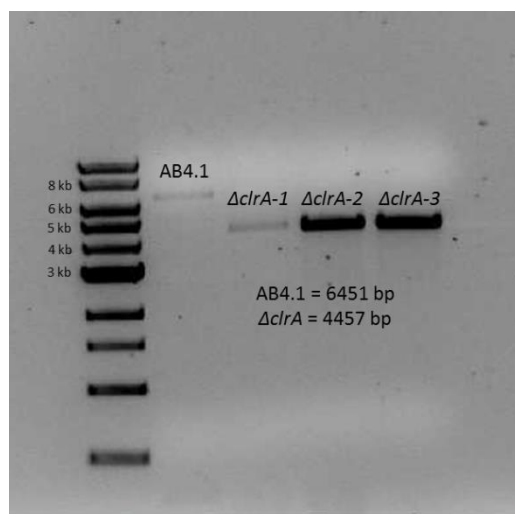


**Figure 4.8:** PCR screening using the external *clrA* gene primers. The estimated size (based on *in silico* analysis) for the PCR product with the AB4.1 strain was 6451bp, and 4457bp for the  $\Delta clrA$  mutant strain. Here a band between 6kb and 7kb was obtained for the WT strain another one between 4kb and 5kb for the  $\Delta clrA$  mutant strains tested confirming the deletion of the *clrA* gene.

#### 4.3.3 Strain phenotypes

##### 4.3.3.1 Culture pH

The pH was measured in cultures grown for 48h in glucose (starting pH of the glucose medium pH6.5) before the mycelium was transferred to wheat straw medium (starting pH of the wheat straw medium pH6.5). After 24h and 48h in wheat straw, the pH was measured in the cultures. For the glucose condition, after 48h of culture, the pH had dropped from 6.5 to 2.87 for the WT strain, from 6.5 to 2.73 for the  $\Delta clrA$  strain, from 6.5 to 3.08 for the  $\Delta clrB$  strain and from 6.5 to 3.64 for the  $\Delta xlnR$  strain. In wheat straw, after transfer to the medium, the pH had dropped from 6.5 to 3.37 after 24h and to 2.84 after 48h for the WT strain, from 6.5 to 3.48 after 24h and to 2.89 after 48h for the  $\Delta clrA$  strain and from 6.5 to 4.63 after 24h and to 3.47 after 48h for the  $\Delta clrB$  strain. In contrast, the pH had increased from 6.5 to 6.55 after 24h and to 6.73 after 48h for the  $\Delta xlnR$  strain (**Figure 4.9**). A two-way ANOVA test was performed within the same condition (e.g. glucose 48h) to show differences between the two strains. The results from the test showed that for the three conditions the differences between the WT strain and the  $\Delta xlnR$  and the  $\Delta clrB$  strains were significant.

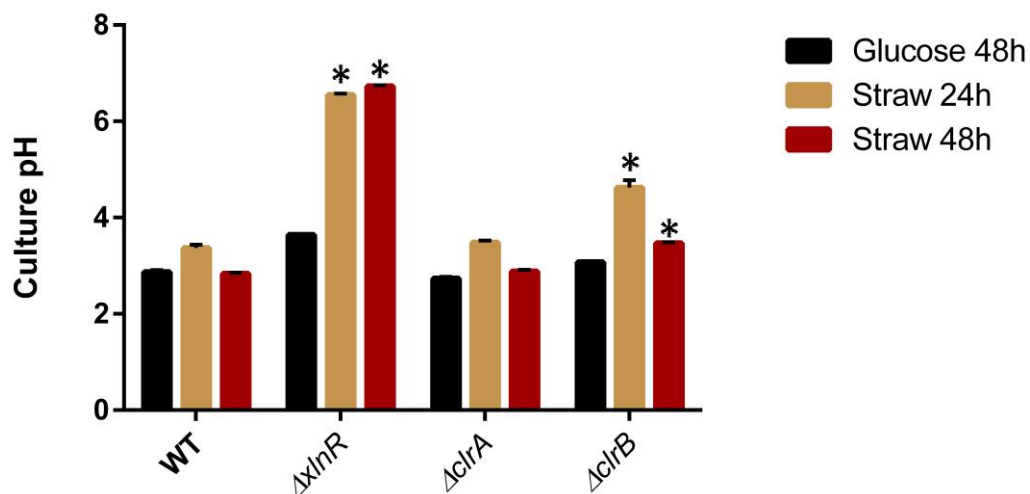


**Figure 4.9:** pH measurements from glucose and wheat straw cultures. For both substrates the starting pH of the medium was set up at pH6.5. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. A two-way ANOVA was performed for each condition in comparison to the WT strain. \* represents the condition for which the two-way ANOVA test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

#### 4.3.3.2 Glucosamine content

The content of the cultures in glucosamine was measured at each time point and the equivalent dry weight was calculated back using a standard curve of known glucosamine content of known dry weight samples. After 48h in glucose the dry weight of the WT strain (from 1.5g wet weight) was 0.103g, 0.093g for the  $\Delta clrA$  strain, 0.1g for the  $\Delta clrB$  strain and 0.087g in the  $\Delta xlnR$  strain. The dry weights, calculated back from glucosamine contents of each flask, after 24h and 48h in wheat straw were 0.207g and 0.338g respectively for the WT strain, 0.213g and 0.353g for the  $\Delta clrA$  strain, 0.159g and 0.229g for the  $\Delta clrB$  strain and 0.149g and 0.197g for the  $\Delta xlnR$  strain (**Figure 4.10**). A two-way ANOVA test was performed within a same condition (e.g. glucose 48h) to show differences between the two strains. The results from the test showed that for the glucose condition the differences between the WT strain and all the deletion strains were significant. On wheat straw only the differences between the WT strain and the  $\Delta xlnR$  and the  $\Delta clrB$  strains were significant.

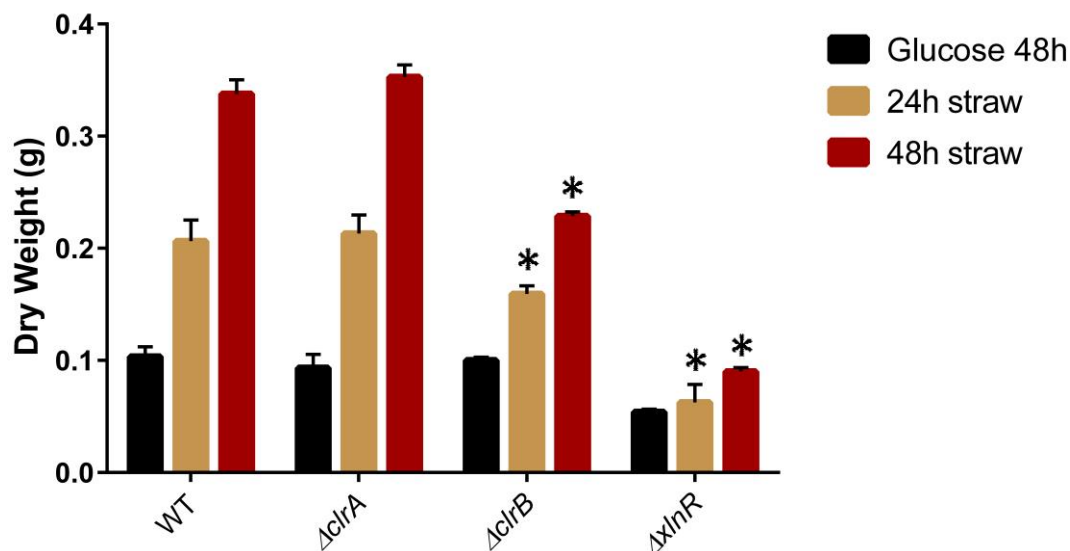


**Figure 4.10:** Increase in the biomass content of the  $\Delta xlnR$ ,  $\Delta clrB$  and  $\Delta clrA$  cultures in glucose and wheat straw. The wheat straw cultures were inoculated with 1.5g wet weight mycelium from the corresponding glucose culture. The dry weight conversion of wet weight is around 15 times less than the wet weight. The glucosamine content was measured from 1.5g (wet weight) for the glucose culture and from the content of the full culture for the wheat straw cultures and the dry weight was calculated back using a standard curve of the glucosamine content against the dry weight. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. \* represents the condition for which the T-test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

#### 4.3.3.3 Supernatant protein profiles

The supernatants from cultures grown in wheat straw for 24h and 48h were analysed on SDS-PAGE gels for differences in the protein secretion profiles for the different deletion strains (**Figure 4.11**). The secretion profile observed for the  $\Delta clrA$  deletion strain is very similar to the WT one with the main bands being the same at the two time points. However, the supernatants of the  $\Delta clrB$  culture seem to contain fewer secreted proteins as some bands are missing when compared to the WT supernatants, especially at the 48h time point. As for the  $\Delta xlnR$  deletion strain, the secretion profile observed is very different to the WT one or the other deletion strains with only faint bands appearing on the gel and only few of them seem to be common

to the WT strain showing that the deletion of *xlnR* prevents the secretion of degrading enzymes. Interestingly, some bands appear stronger after 48h of exposure to wheat straw for the WT and the  $\Delta clrA$  deletion strain suggesting that the expression of enzymes is still up-regulated at this time point.

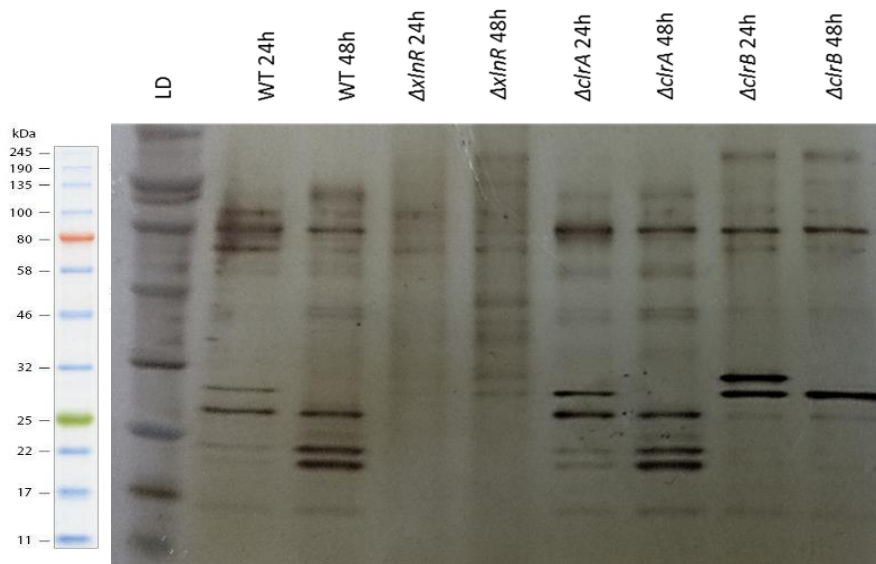


**Figure 4.11:** Silver-stained protein gel on supernatants from cultures transferred to wheat straw for 24h and 48h. LD stands for Ladder.

#### 4.3.3.4 3,5-dinitrosalicylic acid (DNS) Assay

The measurement of the enzyme activity from the supernatants of the  $\Delta xlnR$ ,  $\Delta clrB$  and  $\Delta clrA$  wheat straw cultures was done using the DNS assay measuring the reducing sugar ends released from the incubation of the supernatants with wheat straw. The results showed that fewer reducing sugar ends were released from the incubation of the supernatants from the three deletion strains when compared to the WT supernatant. For the WT strain, 0.12 and 0.2 mg of reducing sugars per mg of wheat straw were released from the 24h and 48h supernatants respectively. For the deletion strains, 0.11 and 0.15 mg of reducing sugars per mg of wheat straw were released for the  $\Delta clrA$  strain, 0.1 and 0.12 mg of reducing sugars per mg of wheat straw for the  $\Delta clrB$  strain and 0.07 and 0.11 mg of reducing sugars per mg of wheat straw for the  $\Delta xlnR$  strain. Statistical analysis showed that the result was significantly different to the WT strain for the *xlnR* deletion strain after 24h and the results were significantly different to the WT strain for the 3 deletion strains after 48h (**Figure 4.12**).

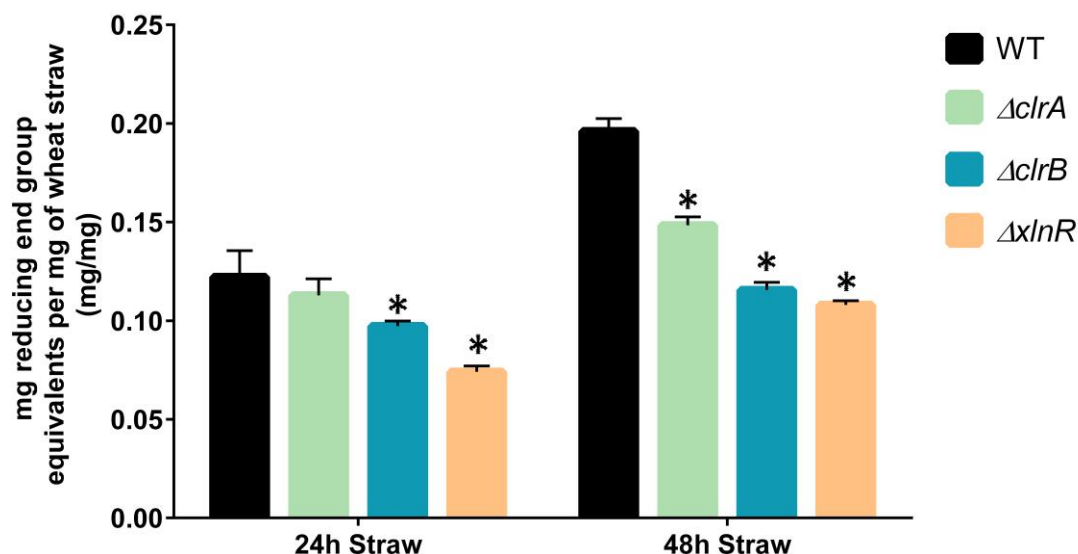


**Figure 4.12:** Measurement of sugars reducing ends released after incubation of equal volumes of the supernatants of the  $\Delta xlnR$ ,  $\Delta clrB$  and  $\Delta clrA$  wheat straw cultures with straw done by the DNS assay. A two-way ANOVA was performed for each condition in comparison to the WT strain. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. \* represents the conditions for which the two-way ANOVA test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

#### 4.3.4 Expression of TF-encoding genes and GH-encoding genes

##### 4.3.4.1 Expression of the *clrA* and *clrB* genes in the WT and $\Delta xlnR$ strain

The transcript levels of the *clrA* and *clrB* genes were measured in the WT and the  $\Delta xlnR$  strains using qRT-PCR. The results for the WT strain confirmed the RNA-sequencing data with the *clrB* gene being more highly induced than the *clrA* gene. However, the transcript levels of the two transcripts were lowered in the  $\Delta xlnR$  strain with around a one-fold change induction for both genes when compared to the glucose condition (**Figure 4.13**).

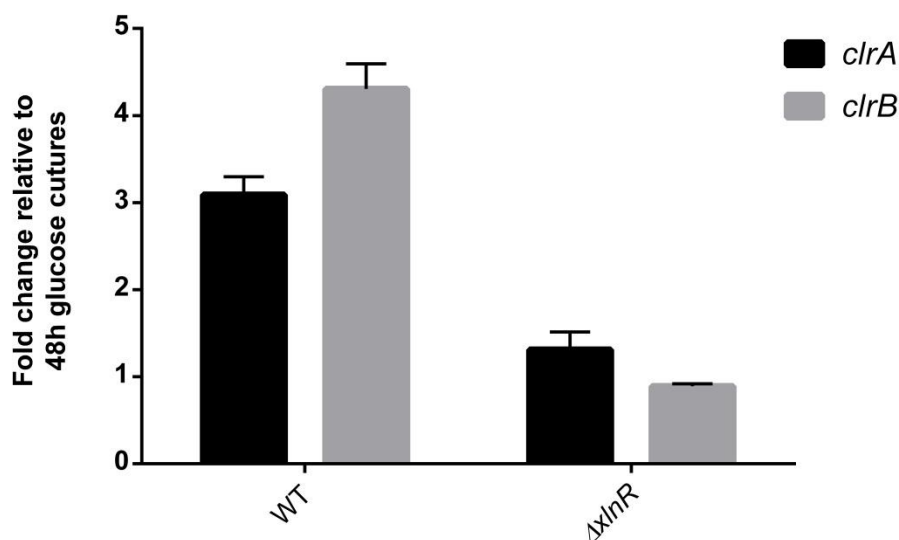


**Figure 4.13:** Expression of the *clrA* and *clrB* genes in *A. niger* grown in wheat straw media. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. The expression values represent the expression fold change relative to the 48h glucose culture.

#### 4.3.4.2 Expression of GH-encoding genes in shake flask cultures

The transcript levels of the genes *cbhA*, *bglB*, *xynA* and *eglC* were measured by qRT-PCR in the  $\Delta xlnR$ ,  $\Delta clrB$  and  $\Delta clrA$  strains after 24h of incubation in wheat straw in shake flask cultures. The fold changes relative to the 48h glucose cultures were measured and compared to the WT strain. For the *cbhA* gene, the fold change relative to the glucose culture was 4273 for the WT strain, 49 for the  $\Delta clrA$  strain, 2.9 for the  $\Delta clrB$  strain and 0.6 for the  $\Delta xlnR$  strain. For the *eglC* gene, the fold change relative to the glucose culture was 330 for the WT strain, 115 for the  $\Delta clrA$  strain, 0.056 for the  $\Delta clrB$  strain and 0.035 for the  $\Delta xlnR$  strain (**Figure 4.14**). For the *bglB* gene, the fold change relative to the glucose culture was 101 for the WT strain, 0.73 for the  $\Delta clrA$  strain, 0.21 for the  $\Delta clrB$  strain and 0.20 for the  $\Delta xlnR$  strain. For the *xynA* gene, the difference was not significant between the WT and the  $\Delta clrA$  strain. However, the fold change relative to the glucose culture decreased to 802 for the  $\Delta clrB$  strain and to 12.2 for the  $\Delta xlnR$  strain when the WT strain had a fold change of 1660 compared to glucose cultures.

Chapter 4. Transcriptional regulation of genes encoding Glycoside Hydrolases (GHs) in  
*A. niger*

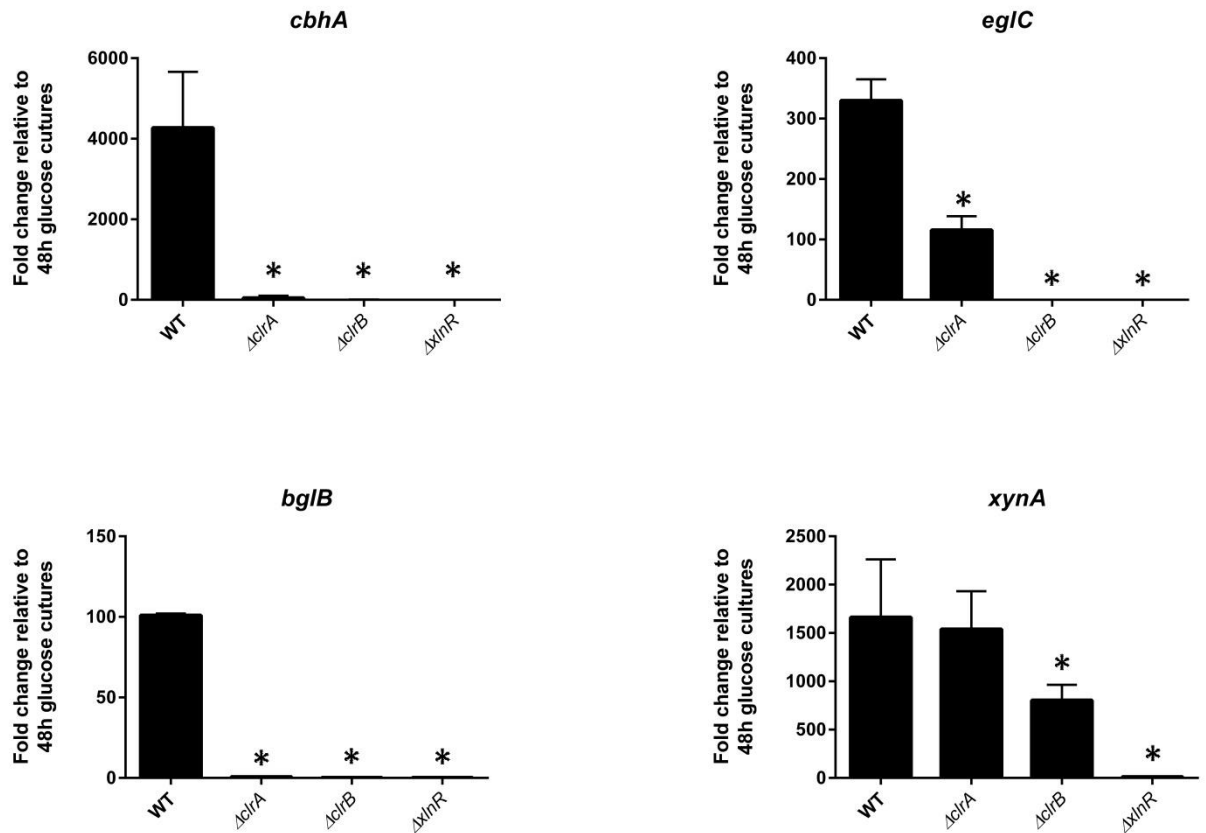


**Figure 4.14:** Transcript levels from the *cbhA*, *egIC*, *bglB* and *xynA* genes in *A. niger* grown in wheat straw media. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. The transcript levels values represent the fold change relative to the 48h glucose culture. \* represents the condition for which the T-test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

#### 4.3.4.3 qRT-PCR in pH-controlled environment

To investigate if the decrease of the transcript levels of the *cbhA*, *egIC*, *bglB* and *xynA* genes observed in the qRT-PCR experiment (Figure 4.14) could be due to the non-acidification of the culture (Figure 4.9), the  $\Delta xlnR$  and the  $\Delta clrB$  strains were grown in a fermenter with the pH maintained at pH5. For the *cbhA* gene, the fold change relative to the glucose culture was 0.60 in the non-controlled experiment and 0.50 for the pH-controlled experiment for the  $\Delta xlnR$  strain and 2.91 under non-controlled condition and 1.25 in the pH-controlled experiment for the  $\Delta clrB$  strain. For

the *eglC* gene, the fold change relative to the glucose culture was 0.035 in the non-controlled experiment and 0.48 for the pH-controlled experiment for the  $\Delta xlnR$  strain and 0.056 under non-controlled condition and 1.6 in the pH-controlled experiment for the  $\Delta clrB$  strain. For the *bglB* gene, the fold change relative to the glucose culture was 0.20 in the non-controlled experiment and 1.6 for the pH-controlled experiment for the  $\Delta xlnR$  strain and 0.21 under non-controlled condition and 0.48 in the pH-controlled experiment for the  $\Delta clrB$  strain. For the *xynA* gene, the fold change relative to the glucose culture was 12.2 in the non-controlled experiment and 45.6 for the pH-controlled experiment for the  $\Delta xlnR$  strain and 802.2 under non-controlled condition and 781.2 in the pH-controlled experiment for the  $\Delta clrB$  strain (**Figure 4.15**). The fold change measured for the WT strain in the fermenter condition was similar to the one measured in the non-controlled condition.

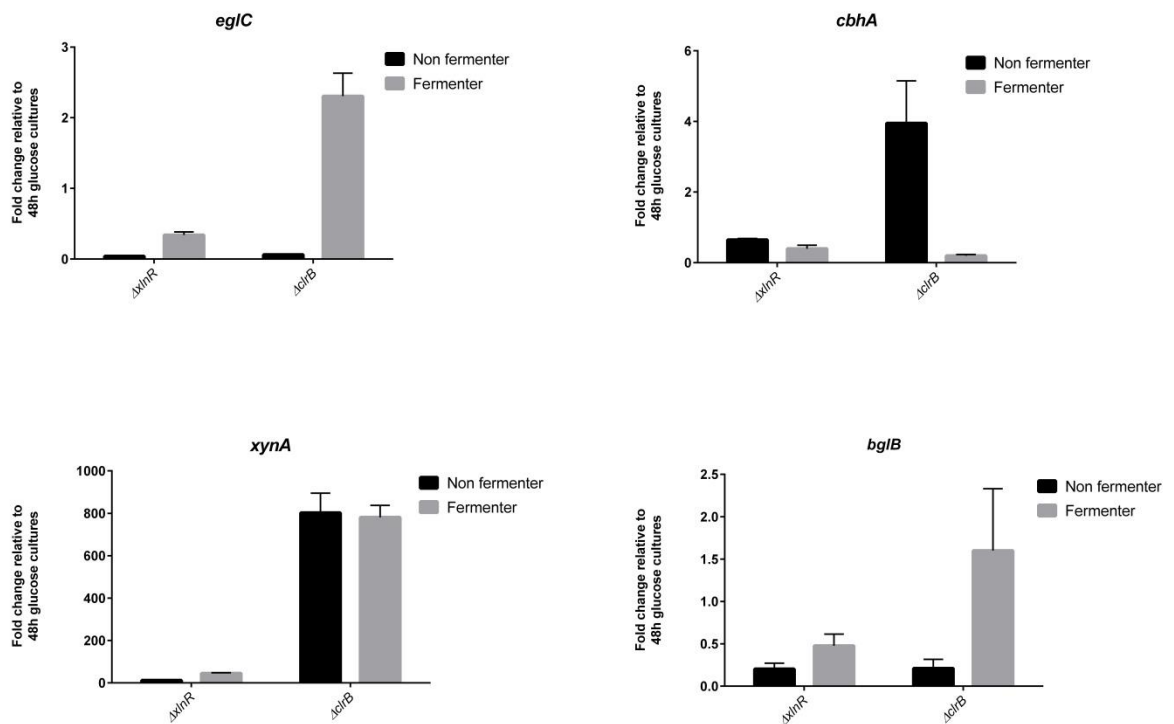


**Figure 4.15:** Comparison of the transcript levels of the *cbhA*, *xynA*, *bglB* and *eglC* genes in *A. niger* grown in wheat straw media under pH-controlled condition (pH5.0 in fermenter) and no control of pH (flask). The results shown represent triplicates of two independent biological samples and the error bars indicate the standard error of the mean. For both experiments, the transcript level values represent the fold change relative to the 48h glucose culture. \* represents the condition for which the T-test

result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

#### 4.3.4.4 Prediction of PacC binding sites

The prediction of PacC binding sites was done using the TF prediction tool available at <http://www.yeasttract.com/tfsbindingsites.php>.

**Table 4.3:** Prediction of PacC binding sites in the promoter sequences of GH-encoding genes and TF-encoding genes. The positions are from the ATG translational start codon of the gene.

| Gene        | PacC motif | Binding Sequence | Position             | Strand      |
|-------------|------------|------------------|----------------------|-------------|
| <i>cbhA</i> | Yes        | TGCCAAG          | -1471                | Reverse (R) |
| <i>cbhB</i> | No         |                  |                      |             |
| <i>eglC</i> | Yes        | TGCCAAG          | -301                 | Forward (F) |
| <i>bglB</i> | No         |                  |                      |             |
| <i>xynA</i> | No         |                  |                      |             |
| <i>xlnR</i> | No         |                  |                      |             |
| <i>creA</i> | Yes        | TGCCAAG          | -177                 | F           |
| <i>clrA</i> | No         |                  |                      |             |
| <i>clrB</i> | No         |                  |                      |             |
| <i>pacC</i> | Yes        | TGCCAAG          | -1291 / -1186 /<br>0 | F / F / R   |

#### 4.3.5 Role of XlnR in the fungal cell integrity

##### 4.3.5.1 Cell permeability

The cell permeability of the *xlnR* deletion strain was assessed using the dye Phloxine B. This dye stains cytosolic material due to a loss of the integrity of the cell making it more permeable to Phloxine B (Rasooly 2007). The fluorescence intensity was measured for 50 images for each strain in three biological repeats. The average intensity measured for the *clrB* deletion strain was around the WT one whereas the fluorescence intensity of the *xlnR* deletion strain was increased by 2.2 times in the

deletion strain when compared to the WT intensity. An increase of the fluorescence means that more dye has entered the cell. (**Figure 4.17**).

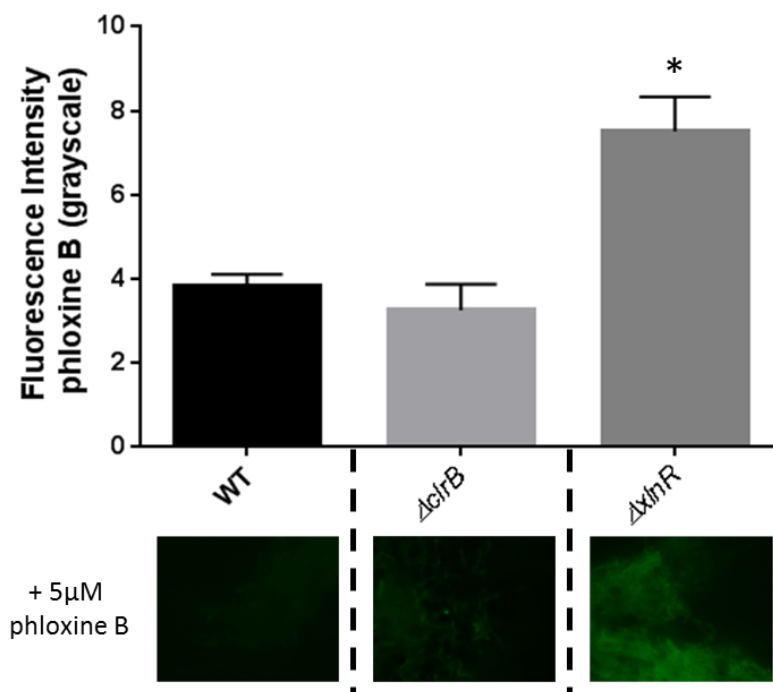


**Figure 4.17:** Cell permeability measurement using the dye phloxine B. Phloxine B stains the cytosolic material due to a more permeable cell in the  $\Delta xlnR$  and  $\Delta clrB$  strains. The fluorescence intensity was measured using the ImageJ software. The results shown represent 50 measurements of three independent biological samples and the error bars indicate the standard error of the mean. \* represents the condition for which the t-test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

#### 4.3.5.2 Cell wall composition and imaging

An analysis of the sugars composing the cell wall of the WT,  $\Delta xlnR$  and the  $xlnR$  complemented strains was done using HPLC. The results showed differences in the content of glucosamine and galactose with the  $\Delta xlnR$  strain containing around 3% more glucosamine and around 4% more galactose than the WT strain (**Figure 4.18**). There was no significant differences for the content of glucose, mannose and galactosamine between the two strains. Concerning the  $xlnR$  complemented strain,

the galactose content of the strain was similar to the *xlnR* deletion strain whereas the amount of glucose was increased when compared to the WT and  $\Delta xlnR$  strains.

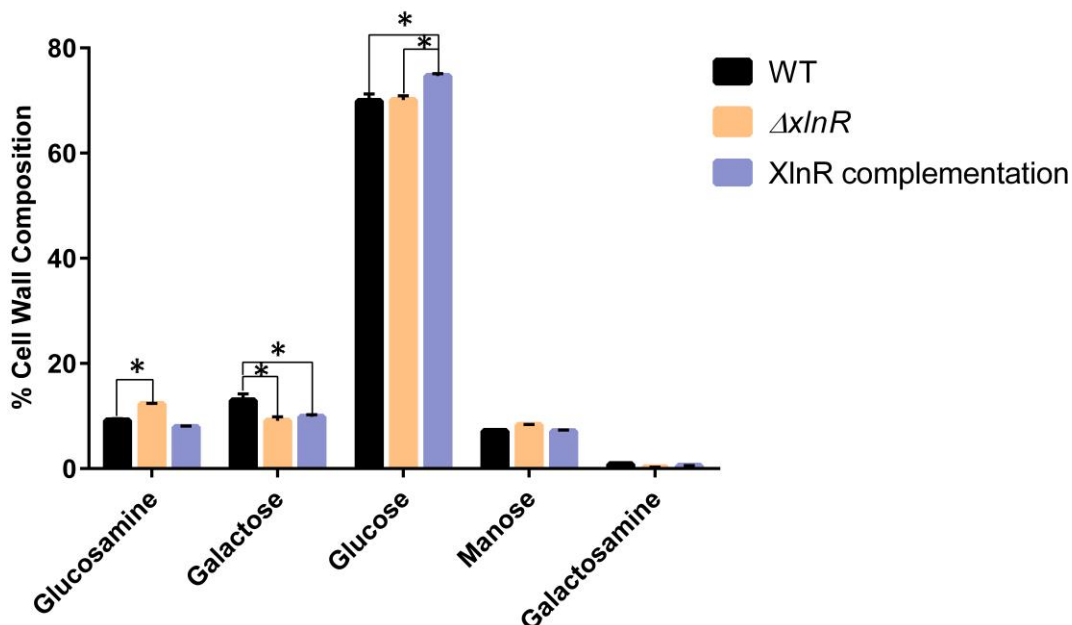


**Figure 4.18:** Sugar analysis of the cell wall of the WT,  $\Delta xlnR$  and the *xlnR* complemented strains using HPLC. A two-way ANOVA was performed for each condition in comparison to the WT strain. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. \* represents the condition for which the two-way ANOVA test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

Transmission Electron Microscopy (TEM) was used to look at the cell wall thickness of the WT and  $\Delta xlnR$  strains. On the images, the cell wall of the  $\Delta xlnR$  strain appears whiter than the WT strain one but no difference in thickness was observed from the images (**Figure 4.19**). The thickness of the cell wall of the strains was measured and showed a significant difference between the WT and the  $\Delta xlnR$  strains with the  $\Delta xlnR$  strain cell wall being around 50nm thinner than the WT strain one (**Figure 4.19**). However, although the phenotype of the *xlnR* complemented strain was similar to the WT one in terms of colour, the cell wall of the complemented strain was also thinner even though the difference between the  $\Delta xlnR$  strain and the complemented strain was significant.

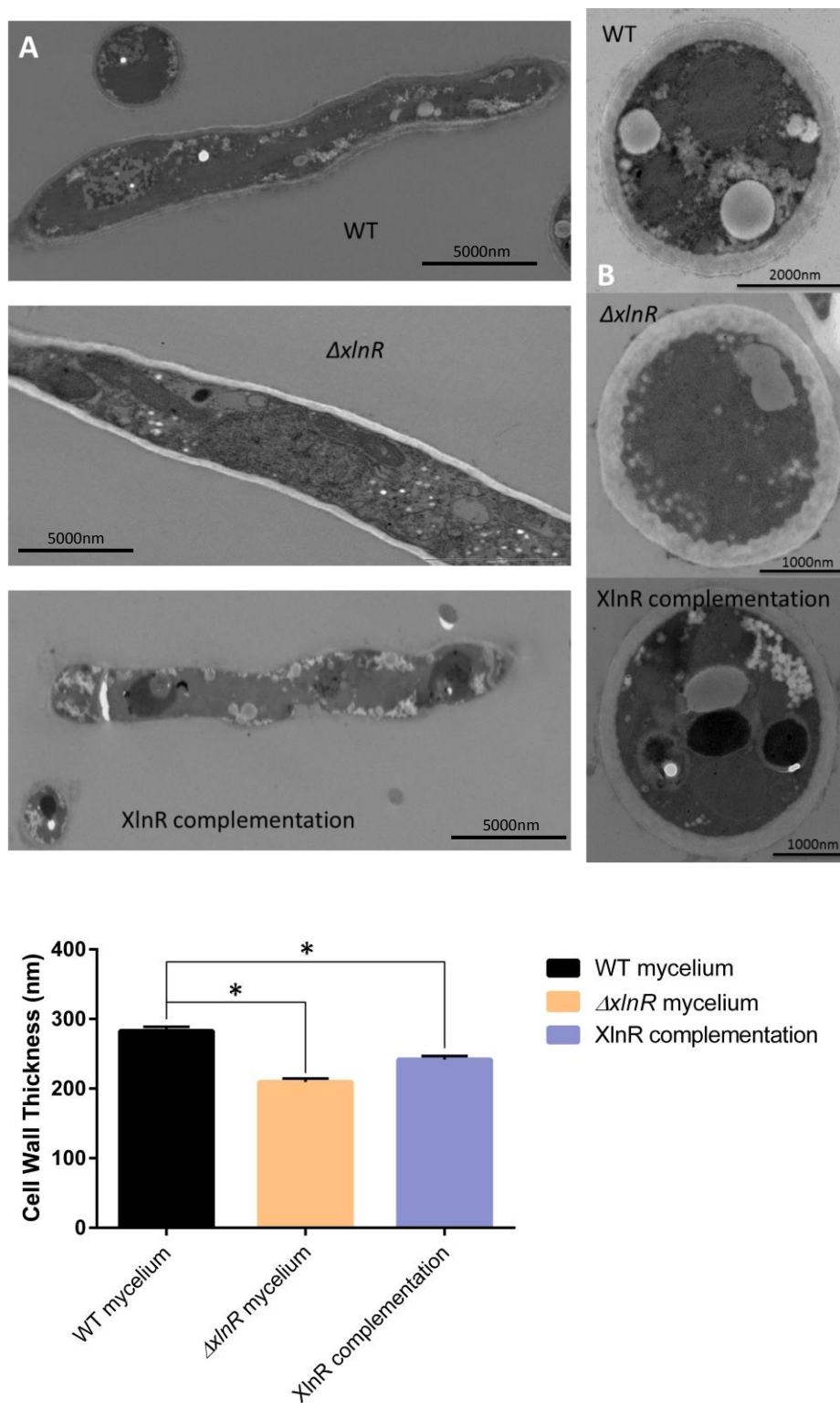


**Figure 4.19:** Microscopy and cell wall thickness.

#### *4.3.5.3 Induction of the Cell Wall Integrity Pathway (CWI)*

To investigate if the differences in the cell wall of the different strains could be due to a deficiency of the CWI pathway, a range of different cell wall stressors were tested in liquid cultures and on plates. If the CWI pathway is affected in the deletion strains, no response should be activated and the strains should be sensitive to the stressors such as Sodium Dodecyl Sulfate (SDS), CalcoFluor White (CFW) and Congo Red (CR). In both assays, liquid cultures (**Figure 4.20A**) and on plates (**Figure 4.20B**), no significant differences in the growth of the strains were observed in the presence of stressors. This suggests that the CWI pathway is functional in the deletion strains and that a compensatory response is activated in response to stressors.

## Chapter 4. Transcriptional regulation of genes encoding Glycoside Hydrolases (GHs) in *A. niger*

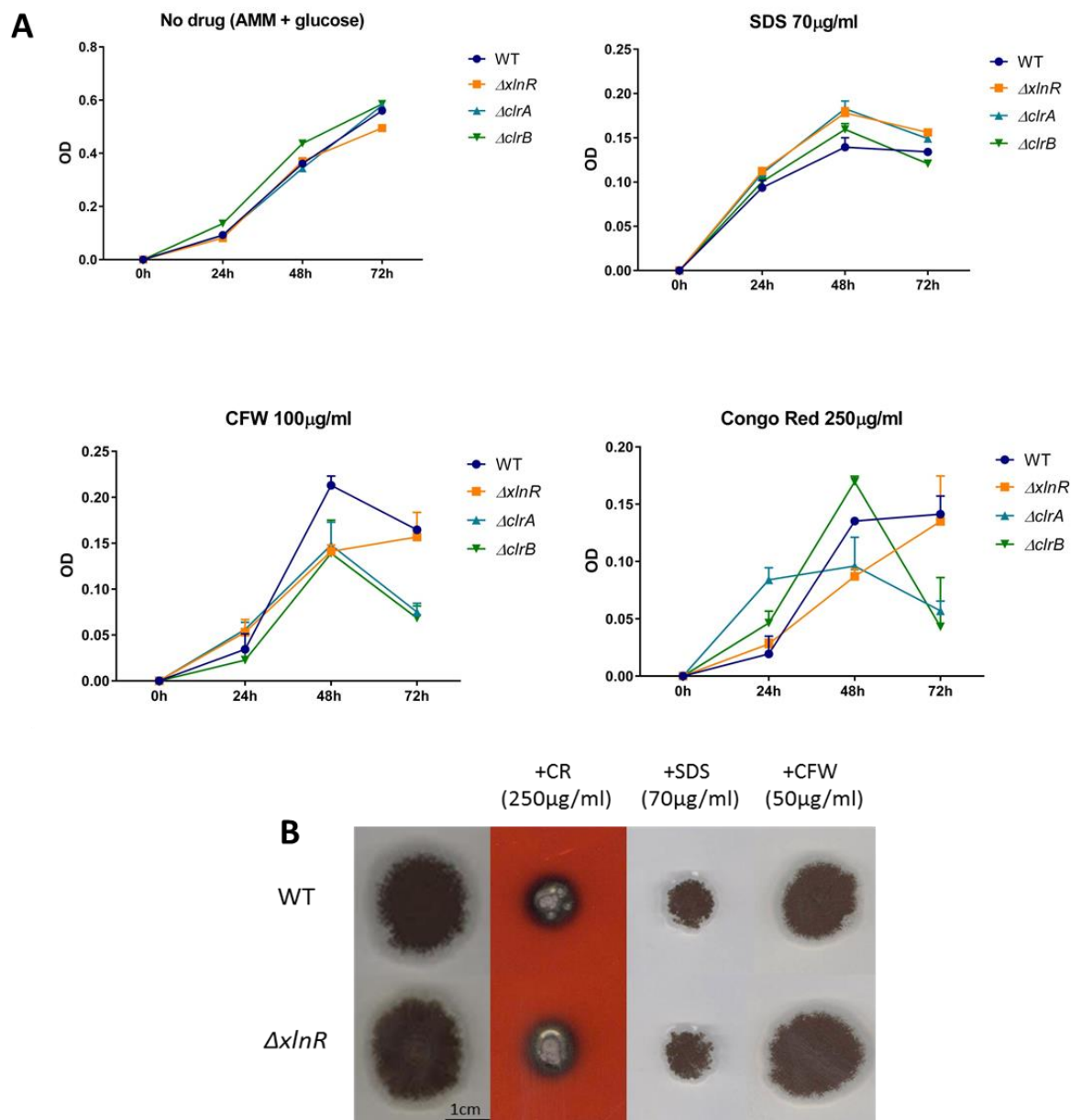


**Figure 4.20:** Test for a cell wall stress by using cell wall stressors such as Sodium Dodecyl Sulfate (SDS, 70μg/ml), CalcoFluor White (CFW, 50-70μg/ml) and Congo Red (CR, 250μg/ml) in a 96-well plate assay (**A**) or in Petri dishes (**B**).

### 4.3.5.4 Activation of the Alternative Oxidase (AOX) pathway and accumulation of Reactive Oxygen Species (ROS) in the *A. niger* $\Delta xlnR$ strain

Few studies have associated oxidative stress with the induction of AOX at the transcript and protein level (Morgan *et al.* 2008; Welchen *et al.* 2012). As a result,

AOX is now often used as a general marker of mitochondrial dysfunction and/or cellular oxidative stress. Further, numerous abiotic and biotic stress conditions are known to elevate AOX amount, supporting the idea that such stresses impact mitochondrial function and that AOX might represent an important acclimation response. Therefore, to confirm that an oxidative stress and accumulation of ROS in the  $\Delta xlnR$  strain cells may be happening, the transcript level of the *aox1* gene was measured. In the WT strain, in the glucose condition, the relative expression level of the *aox1* gene was 5.2 and it was 287 in the  $\Delta clrB$  strain and 310  $\Delta xlnR$  strain. This corresponds to a fold change of 55.2 between the WT strain and the  $\Delta clrB$  strain and of 59.6 between the WT strain and the  $\Delta xlnR$  strain (**Figure 4.21**).

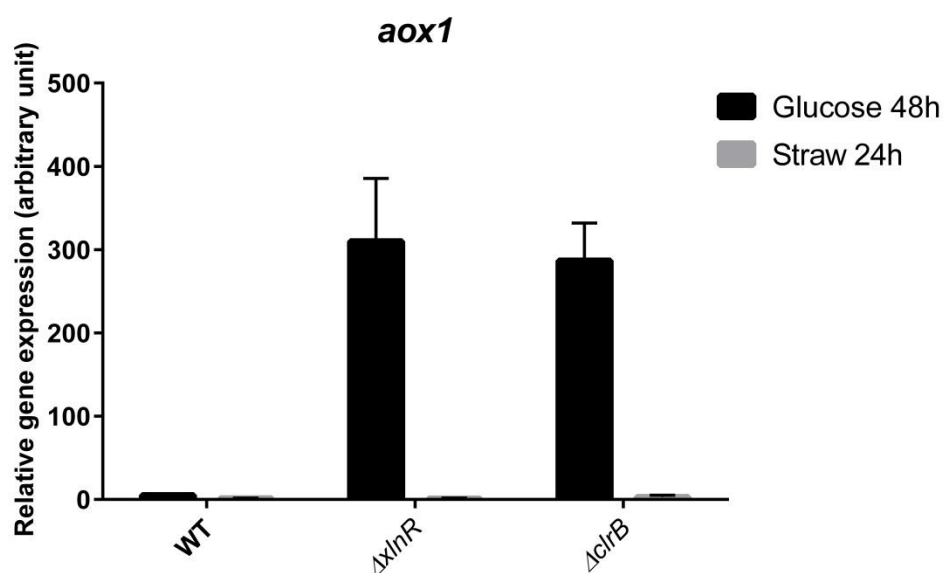


**Figure 4.21:** Expression of the alternative oxidase gene (*aox1*) measured by qRT-PCR in the WT and the  $\Delta xlnR$  and  $\Delta clrB$  strains. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean.

The accumulation of ROS was assessed using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA). H<sub>2</sub>DCF-DA passively diffuses into cells and is retained in the intracellular level after cleavage by intracellular esterases. Upon oxidation by ROS, the non-fluorescent H<sub>2</sub>DCF-DA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF). The fluorescence intensity was measured for 50 images for each strain in three biological repeats. The average intensity measured for the WT strain was 0.87, 1.74 for the  $\Delta clrB$  strain and 3.2 for the  $\Delta xlnR$

strain suggesting that the  $\Delta xlnR$  strain may be exposed to an oxidative stress with an accumulation of intracellular ROS in the fungal cells (**Figure 4.22**).

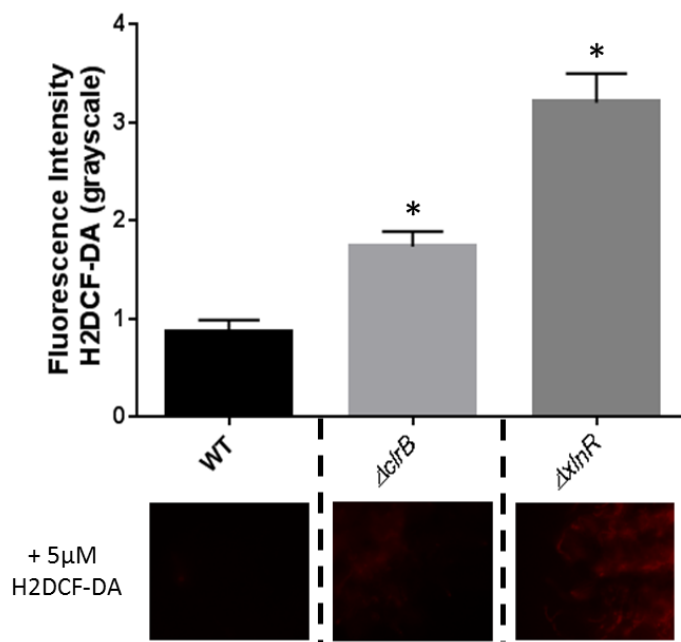


**Figure 4.22:** Reactive Oxygen Species (ROS) accumulation using the dye H<sub>2</sub>DCF-DA. H<sub>2</sub>DCF-DA emits a green fluorescence when it is oxidised by ROS in the cell. The fluorescence intensity was measured using the ImageJ software. The results shown represent 50 measurements of three independent biological samples and the error bars indicate the standard error of the mean. \* represents the condition for which the T-test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

#### 4.3.5.5 Inhibition of the mitochondrial complexes by Antimycin A (*AntiA*) and salicylhydroxamic acid (*SHAM*)

To verify that the activation of the AOX pathway and the accumulation of intracellular ROS were due to a decrease in the cell membrane permeability rather than to a mitochondrial dysfunction, the integrity of the mitochondrial complexes was assayed using inhibitors. In the case of a mitochondrial dysfunction, the inhibition of complexes, especially the complex III located after the alternative oxidase in the respiratory chain (**Figure 4.23B**), should result in no growth of the strains. Antimycin A (*antiA*) was used to inhibit the complex III of the respiratory chain and salicylhydroxamic acid (*SHAM*) was used to inhibit the AOX. The results showed that

when the AOX is inhibited, the different strains tested were growing well though a bit less than the control with no inhibitors. However when the complex III was inhibited, the growth rates of the strains were much lower showing that the complex III was functional and that there was no mitochondrial dysfunction in the deletion strains (Figure 4.23A).

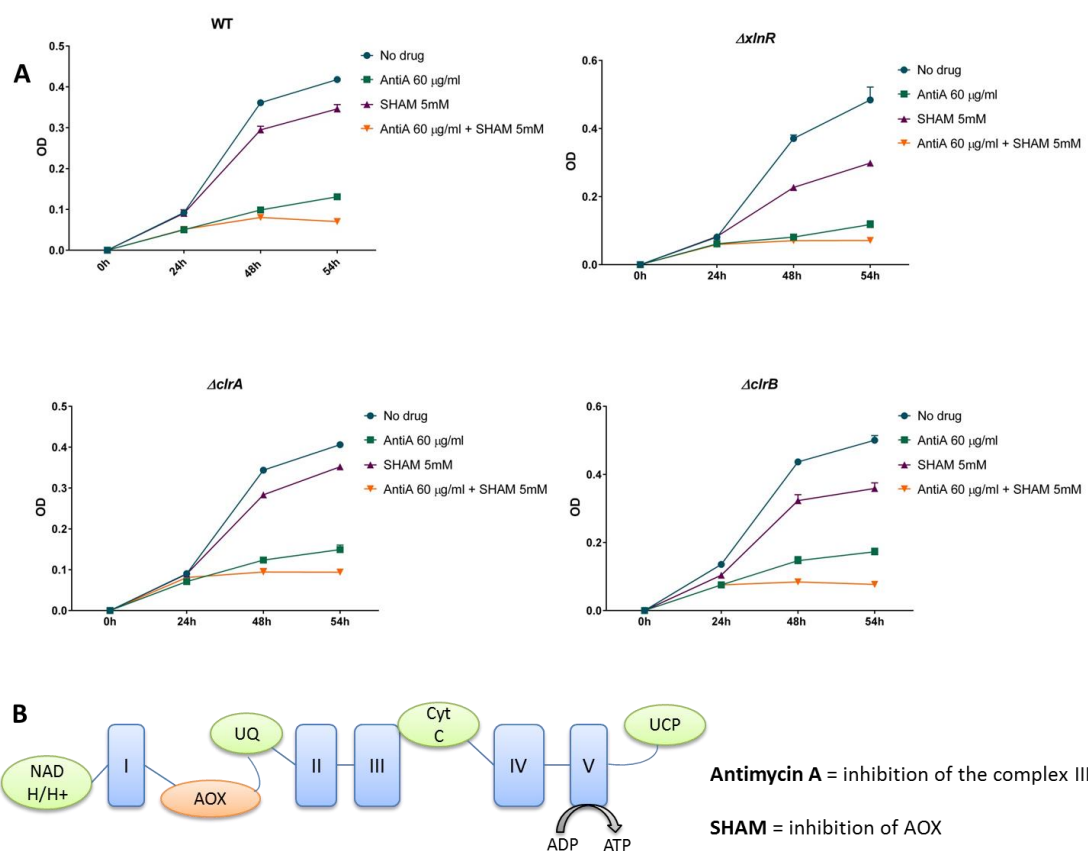


**Figure 4.23:** (A) Inhibition of the mitochondrial complexes using Antimycin A (AntiA) which targets the complex III of the respiratory chain and salicylhydroxamic acid (SHAM) which targets the Alternative Oxidase (AOX) pathway. (B) Mitochondrial respiratory chain.

## 4.4 Discussion

Genes that encode CAZy enzymes active towards polysaccharides are one of the major groups of genes induced in response to lignocellulose. Transcription factors that function as repressors and activators regulate the expression of these CAZy-encoding genes. XlnR/XYR1 is the key activator in *Aspergillus* spp. and *T. reesei* of genes encoding cellulase and hemicellulase enzymes (Stricker *et al.* 2006; Stricker *et al.* 2008; van Peij *et al.* 1998a; van Peij *et al.* 1998b). In *N. crassa*, in contrast, the *xlnR/xyr1* orthologue *xlr-1* does not have the same function in regulating expression of cellulase-encoding genes as in *A. niger* or *T. reesei*. Instead, two other transcription factors CLR-1 and CLR-2 were found to be the predominant regulators of the expression of cellulase-encoding genes in *N. crassa* (Coradetti *et al.* 2012). This suggests that homologues of CLR-1 and CLR-2 play an important role in plant cell-wall degradation by fungi. Coradetti *et al.* (2012) demonstrated that these two TFs are important regulators of genes encoding both cellulases and hemicellulases in the presence of cellulose (Avicel) as carbon source, but they are not required for growth or hemicellulase production in the presence of xylan. In *A. oryzae*, the orthologue of CLR-2 called ManR was characterised as a regulator of genes encoding mannan degrading enzymes (Ogawa *et al.* 2013). The aims of this chapter were to (1) characterise the orthologues of the newly discovered TFs CLR-1 and CLR-2 of *N. crassa* in *A. niger*, (2) discover new TFs by further analysing the RNA-Sequencing dataset and (3) further phenotype deletion strains of already known regulators.

In *A. niger*, the roles of ClrB in the *Aspergilli* are distinct from the roles in *N. crassa*. When comparing the roles of the *clr-2/clr-B* orthologues in both *N. crassa* and *A. nidulans*, Coradetti *et al.* (2012) showed that the induction pattern of major cellulase-encoding genes in the  $\Delta clr-B$  deletion strain was several thousand-fold less than WT but, in the  $\Delta clr-A$  deletion strain, the transcript level of the studied genes was two- to fourfold less. In the results presented here, the same induction pattern was observed for both the *eglC* and the *xynA* genes. The *clrA* deletion strain showed a 3-fold decrease in its transcript level for the *eglC* gene and a similar transcript level for the *xynA* gene when compared to the WT. This suggests that, as in *A. nidulans* for which the induction of cellulase genes required *clr-B* but not *clr-A* (Coradetti *et al.* 2012), the *A. niger* *clrA* orthologue may not be involved in the induction of GH-encoding genes. However, for all the genes tested, the transcript level of the  $\Delta clrB$  deletion

strain was significantly decreased when compared to the WT strain showing similarities with the regulatory patterns observed in *A. nidulans* (Coradetti *et al.* 2012).

The CAZy enzymes active upon wheat straw are enzymes that are secreted by the fungus to degrade wheat straw and release compounds that would support its growth, and also release inducer molecules. The lack of induction of the enzymes essential for growth on the substrate would result in an impaired growth of the fungus as well as less enzyme activity from the supernatants of the cultures grown on wheat straw. Therefore, the growth of the *A. niger clrA*, *clrB* and *xlnR* deletion strains was assayed by measuring the pH and the glucosamine content of the cultures. In our data, both the *A. niger xlnR* and the *clrB* deletion strains did not lower the pH of the cultures in wheat straw as low as in the WT or the  $\Delta clrA$  strains. As presented in the Chapter 3, glucosamine is a useful compound for the estimation of fungal biomass, taking advantage of the presence of chitin, poly-N-acetylglucosamine, in the cell walls of many fungi and was found to range from 67 to 126 mg glucosamine per g dry mycelium (Andersen 2008). The measurement of the glucosamine content of the cultures as a measure of growth showed that the *clrB* and *xlnR* deletion strains had a lower glucosamine content supporting the results from the pH measurements suggesting that these strains grew less well in those conditions. The *xlnR* deletion strain is known to grow poorly on wheat straw as it was shown that the growth of the  $\Delta xlnR$  strain was impaired on plates containing xylan and was not producing xylanolytic enzymes anymore (van Peij *et al.* 1998a; van Peij *et al.* 1998b). The increase in the glucosamine content observed for the 48h culture for the *A. niger*  $\Delta xlnR$  strain could be due to interference of the wheat straw with the assay (e.g. from hexosamines (Hoson *et al.* 1983), see Chapter 3 for details) and as the substrate is not being degraded as efficiently as for the other strains, the interference of wheat straw with the assay could be greater than for the other strains. Also, the sugar analysis of the cell wall of the  $\Delta xlnR$  strain showed that it was composed of around 3% more glucosamine and of around 4% less galatose than the WT strain which could increase the glucosamine value measured with the assay. A Congo red assay was carried out on a cellulosic substrate, CarboxyMethylCellulose (CMC), and xylan to evaluate if there was enzymatic activity in the supernatants of the cultures on these substrates (Appendix 4.6). The results showed that the  $\Delta xlnR$  strain did not produce any xylanolytic enzymes as no halo could be observed on the plates.

However, some cellulolytic activity remained even if it was less than for the WT strain. This suggests that some cellulases are still being produced in the  $\Delta xlnR$  strain and that could explain why the strain shows some growth on wheat straw based on the glucosamine content.

As the functioning of enzymes can also be influenced by the pH of the environment or the media, the *A. niger*  $\Delta xlnR$  and  $\Delta clrB$  strains were grown in a fermenter controlled at pH5.0 in order to confirm that the growth results, as well as the expression data, were the outcome of the lack of growth by the non-expression of the degrading enzymes rather than a pH effect on the enzyme activities. There are many examples demonstrating a connection between ambient pH and differences in CAZy enzyme production with one of the first examples demonstrated by Bailey *et al.* (1993) in *T. reesei*. In a recent study, Li *et al.* (2013) showed that the optimum pH levels for production of endoglucanases, exoglucanases and  $\beta$ -glucanases by *T. reesei* were different. Adav *et al.* (2011) also showed with *T. reesei* that the secreted CAZy profiles changed with different pH levels ranging from pH 3.0 to pH 9.0. The differences in CAZy enzyme production in the above examples can partly be explained by the homeostatic system by which fungi respond to pH fluctuations in their environment and PacC, the transcription factor effector of this system, which can regulate some of the CAZy genes. The active PacC binds the core target sequence 5'-GCCARG-3' in the promoters of its target genes (Espeso *et al.* 1997). The prediction of PacC binding sites in the four genes studied showed that both *cbhA* and *eglC* sequences contained a PacC binding site while *xynA* and *bglB* do not seem to be regulated by pH conditions as no PacC binding sites were found in their sequences. The data showed that the transcript levels from the *cbhA*, *eglC* and *xynA* genes were the same in the flask cultures (where the pH values dropped to 2.84 for the WT strain after 48h and remained at 6.5 for the  $\Delta xlnR$  strain) as in the fermenter condition (pH5.0). This was noticeable, for example, with the  $\Delta xlnR$  strain where the pH was lower by 1.5 units in the fermenter when compared to the pH in the flasks. Therefore, the phenotypes observed in the qRT-PCR experiment (Figure 4.14) were due to the deletion of the genes encoding XlnR and ClrB showing the importance of both TFs in the degradation of wheat straw in *A. niger*. To confirm these transcriptomic results at a functional level, the enzymatic activity from the supernatants of cultures grown in wheat straw was assayed. The measurement of glucose molecule released from the incubation of wheat straw cultures with wheat

straw showed less saccharification activity on wheat straw in the supernatants of the cultures for the *A. niger*  $\Delta xlnR$ ,  $\Delta clrA$  and  $\Delta clrB$  strains compared to the wild-type strain with the  $\Delta xlnR$  and  $\Delta clrB$  strains being the least able to release reducing sugar ends. This result was confirmed by SDS-PAGE run with the culture supernatants. This showed that the profiles of the *clrA* deletion strain, from both the 24h and 48h samples, were very similar to the WT one while the one from the *clrB* deletion strain showed fewer bands and the one from the *xlnR* deletion strain showed a completely different pattern with fainter bands and very few bands were similar to the WT strain especially at 48h where there were more bands present. Based on the genes up-regulated in the transcriptomic dataset (Delmas *et al.* 2012), assumptions of the proteins present for each strain can be made. At 24h and 48h, a band of an approximate size of 36kDa is present for both the WT and the  $\Delta clrA$  strain, very faint for the  $\Delta clrB$  strain and absent for the  $\Delta xlnR$  strain. This protein could correspond to EglC that has an estimated size of 36.5kDa although two close bands are present at 48h at around 28 and 30kDa for both the WT and the  $\Delta clrA$  strain but absent for the other strains. The proteins of these estimated sizes could be a GH61 of 29.4kDa and a feruloyl esterase of 28.6kDa. Also, a protein of around 80kDa that could be a  $\beta$ -glucosidase precursor, is present for all the strains but fainter for the  $\Delta xlnR$  strain. The presence of stronger bands at the 48h time point for both the WT and the  $\Delta clrA$  strain suggests that enzymes are still produced at this later time point. This means that these enzymes are of potential interest for the degradation of wheat straw for biofuels production as they could be added to the enzyme mixture to increase the efficiency of the degradation after 48h. Similarly, enzymes such as GH61, the feruloyl esterase or EglC, that seem to be the most highly induced, could be added to the enzyme mixture for a better degradation at 24h of exposure to wheat straw. Interestingly a protein of around 170kDa seems to appear on the profiles of both the  $\Delta xlnR$  (at 48h) and  $\Delta clrB$  (at both time points) strains but is not identified. To identify the proteins cited, a 2-dimensional electrophoresis should be done, in order to separate proteins based on their sizes and their isoelectric points. The corresponding bands can then be excised from the gel and identified using protein mass spectrometry.

There are significant differences in how XlnR in *A. niger* and XYR1 in *T. reesei* regulate transcription of their target genes (Stricker *et al.* 2008). In *T. reesei*, XYR1 interacts with the co-regulators ACE1 and ACE2 (activator of cellulase expression 2)

(Aro *et al.* 2001) whereas *A. niger* lacks an *ace2* orthologue (Stricker *et al.* 2008; Todd *et al.* 2014). Furthermore, the *ace1* orthologue does not have the same function in *Aspergillus* spp. as shown by deletion of the *ace1* orthologue *stzA* in *A. nidulans* (Chilton *et al.* 2008). Chilton *et al.* (2008) showed that if in *T. reesei* ACE1 was involved in cellulose utilisation, its orthologue in *A. nidulans*, StzA, was controlling the abiotic stress response in this species. The RNA-sequencing data showed that the *clrB* gene was more highly induced on wheat straw than the *clrA* gene. This was confirmed by measuring the expression levels of the two genes by qRT-PCR for the WT strain. However, in the *xlnR* deletion strain, the transcript levels of both *clrA* and *clrB* were decreased to around one-fold change compared to the glucose time point. This suggests that XlnR may regulate the two genes. In *N. crassa*, CLR-1 and CLR-2 interact for the induction of specific genes. As in *A. niger*, *clrA* is not highly induced, an interaction between ClrA and ClrB might not be happening. Instead, another possibility would be that XlnR and ClrB interact together for the induction of a subset of genes. Several methods allow for the identification of protein-protein interactions. *In vitro* methods include co-immunoprecipitation (co-IP) and pull-down assay. The co-IP method is used to identify physiologically relevant protein-protein interaction by using target protein-specific antibody to indirectly capture proteins that are bound to a specific target protein. These protein complexes can then be analysed to identify new binding partners, binding affinities, the kinetics of binding and the function of target proteins. The pull-down assay is used to selectively extract a protein-DNA complex from a sample. Typically the pull-down assay uses a DNA probe labelled with a high affinity tag, such as biotin, which allows the probe to be recovered or immobilised. The proteins are then eluted from the DNA and detected by western blot or identified by mass spectrometry. Pull down assays are a form of affinity purification and are similar to immunoprecipitation, except that a "bait" protein is used instead of an antibody. If XlnR and ClrB interact together, these methods would allow for the identification of their complex and of the targeted DNA sequence in the case of the pull-down assay. *In vivo* assays can also be used to detect protein-protein interactions. One of these methods is the Fluorescence Resonance Energy Transfer (FRET). FRET is a phenomenon that occurs when 2 fluorophores are in sufficient proximity ( $<100\text{\AA}$ ) and an appropriate relative orientation such that an excited fluorophore (donor) can transfer its energy to a second, longer-wavelength fluorophore (acceptor) in a non-radiative manner. Thus, excitation of the donor can produce light emission from the acceptor, with attendant loss of emission from the

donor. Because FRET is a non-destructive spectroscopic method for measuring molecular interactions, it can be done in living cells as represented by the ratio between donor and acceptor emission intensities (Pollok *et al.* 1999) (Figure 4.24).

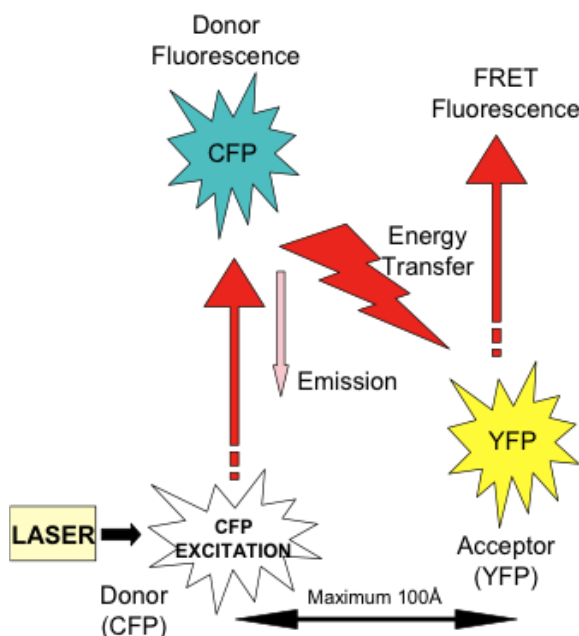


**Figure 4.24:** The principle of FRET. Energy transfer from excitation of the donor molecule (CFP) to the acceptor molecule (YFP) will occur only if the molecules are less than 100Å apart. This causes quenching of donor fluorescence and excitation of the acceptor at its emission wavelength.  
(<http://www.bristol.ac.uk/biochemistry/halestrap/FRET.html>).

Genes encoding the homologue proteins of the sensors of the High Osmolarity Glycerol (HOG) pathway, *SSK1* and *SSK2*, were found to be up-regulated in the  $\Delta xlnR$  strain in Sugarcane Exploded Bagasse (SEB) by de Souza *et al.* (2011). Hog1p is a crucial participant in osmotic stress response in *S. cerevisiae* but does not appear to play a significant role in the oxidative stress (Ferrigno *et al.* 1998). However, Kawasaki *et al.* (2002) showed that the HOG pathway was required for normal oxidative resistance in *A. nidulans* with the deletion of the *HOG1* homologue, *sakA*, resulting in a high sensitivity to oxidative stress. Recently, Mesa-Arango *et al.* (2014) demonstrated that when the cell membrane of *A. fumigatus* is changed (e.g. by the action of fungicides), the membrane was more permeable and resulted in the accumulation of ROS. Few studies have associated oxidative stress with the induction of AOX at the transcript and protein level (Morgan *et al.* 2008; Welchen *et*

*al.* 2012). In our results, the gene *aox1* is highly induced in both the *clrB* and *xlnR* deletion strains. The use of the dye H2DCF-DA, as a marker of oxidative stress, showed the accumulation of ROS with an increased in the dye fluorescence for the two strains. These results suggest that the  $\Delta clrB$  and the  $\Delta xlnR$  strains undergo an oxidative stress when grown on glucose. These results are supported by the transcriptomic data from de Souza *et al.* (2011) where several genes encoding proteins with oxidoreductase activity were found to be up-regulated in the  $\Delta xlnR$  strain. In addition to being sensitive to oxidative stress, the  $\Delta xlnR$  strain displayed cell wall defects with increased sensitivity to phloxine B.

Several signalling pathways act to ensure the cell wall integrity (CWI), for example the Protein Kinase C and HOG pathways (Eisman *et al.* 2006; Munro *et al.* 2007; Paravicini *et al.* 1996) and the  $Ca^{2+}$ -calcineurin pathway (Munro *et al.* 2007; Singh *et al.* 2009; Wiederhold *et al.* 2005). Dagley *et al.* (2011) showed that defective cell walls were linked to dysfunctional mitochondria and phospholipid imbalance in *Candida albicans* and mutants tested were hypersensitive to cell wall targeting drugs. However, when the *xlnR* and *clrB* deletion strains were tested for mitochondrial dysfunction, by inhibiting the different complexes of the respiratory chain, or for cell wall stress, no significant differences were observed between the deletion strains and the WT strain (Figure 4.23). Nonetheless, a phosphoproteomics analysis showed that several proteins involved in lipid metabolism were activated in the glucose condition for the  $\Delta xlnR$  strain (see Chapter 5) suggesting that a deregulation of the lipid metabolism may be occurring in the deletion strain. On the contrary, protein kinases of the  $Ca^{2+}$ -calcineurin pathway, known to be involved in the CWI (Munro *et al.* 2007; Singh *et al.* 2009; Wiederhold *et al.* 2005), were found to be activated in the WT strain under the same condition. This is supported by the study of Chen *et al.* (2010) which showed that mutants affected in the phospholipid biosynthesis pathway were not sensitive to cell wall stressors in *C. albicans* even though displaying defects in CWI. Additionally, the deletion of the gene *PGS1*, involved in the synthesis of the mitochondrial phospholipid cardiolipin, has been shown to have cell wall defect (Zhong *et al.* 2005; Zhong *et al.* 2007) in *S. cerevisiae*. How lipid metabolism affects CWI is unknown, but effects of phosphatidylethanolamine on glycosylphosphatidylinositol (GPI) anchor synthesis and compromised signalling through the PKC or calcineurin pathways in the phospholipid biosynthesis mutants have been proposed mechanisms by Chen *et al.* (2010). Phospholipid imbalance

affects the activity of  $\beta$ -glucan synthase. 1,3- $\beta$ -glucan synthase catalyses the formation of a  $\beta$ -1,3-glucan polymer that is a major component of the yeast cell wall (Mio *et al.* 1997).  $\beta$ -glucan synthase is an integral membrane protein and earlier biochemical work indicates a critical role for the phospholipid environment in ensuring proper  $\beta$ -glucan synthase activity (Saugy *et al.* 1988; Sloan *et al.* 1987; Wasserman *et al.* 1986). Therefore, one hypothesis is that XlnR, in addition to be essential for CAZy enzyme-encoding genes expression, may also be involved in the cell wall synthesis by maintaining the lipid homeostasis necessary for cell wall synthase proteins activity. The deletion of *xlnR* leads to the incapacity of the strain to grow on straw but also could lead to a lipid imbalance that would cause cell wall defects and make the strain to undergo oxidative strain with exposure to ROS.

Lipid imbalance could be due to fewer lipids being present or to a change in the lipid composition of the membranes. Phospholipid Fatty Acids (PFLAs) (and sterols) are the integral hydrophobic components of membranes and can be considered as the insulating material between proteins and other charged molecules. The content in PFLAs can be measured by fractionating the lipids on a solid-phase extraction column and then the phospholipid phase can be dried prior to esterification to form the fatty acid methyl esters (FAMES) that can be identified and quantified using gas chromatography coupled to mass spectrometry. This method would allow for identification of the fatty acids present in both the WT and the *xlnR* deletion strain to see whether or not there are differences in the lipid composition of the two strains. If one of the targets of XlnR is synthesis of the mitochondrial lipids, the quantification of the cardiolipin could be assayed using HPLC (Ruggiero *et al.* 1984).

Altogether, the results presented in this chapter confirm the importance of XlnR as the main regulator of GH-encoding genes. However, ClrB seems to also have an important role in this regulation with the  $\Delta$ *clrB* strain being strongly compromised in its ability to grow on wheat straw. On the contrary to the  $\Delta$ *xlnR* strain, the  $\Delta$ *clrB* strain did not display any change in the cell wall and was, overall, less sensitive to oxidative stress, which may be a reason why the strain is able to grow better on wheat straw than the *xlnR* deletion strain. The data also suggest that XlnR may have other role than being the regulator of GH-encoding genes. A role in cell wall synthesis and/or lipid homeostasis is hypothesised and further work would be needed to test this hypothesis.

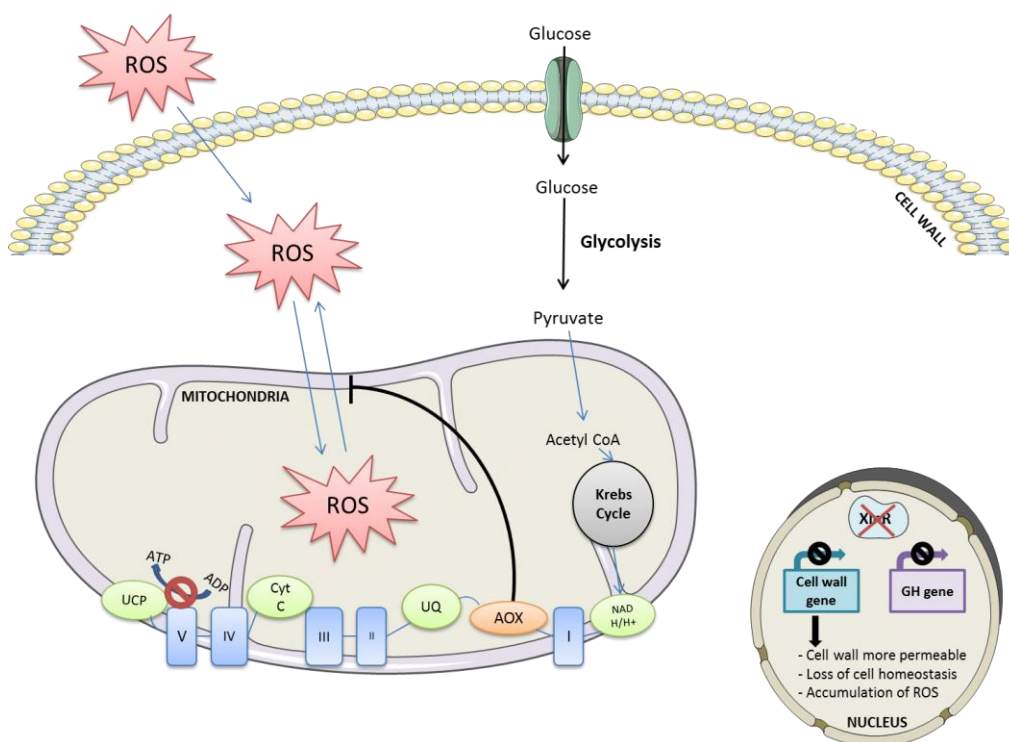


**Figure 4.25:** Proposed model of the effect of the deletion of *xlnR* in *A. niger*. XlnR, in addition to be essential for CAZy enzyme-encoding genes expression, may also be involved in the cell wall synthesis by maintaining the lipid homeostasis necessary for cell wall synthase proteins activity. The deletion of *xlnR* leads to the incapacity of the strain to grow on straw but also could lead to a lipid imbalance that would cause cell wall defects and make the strain to undergo oxidative strain with exposure to ROS. In response to the increased level of ROS, the Alternative Oxidase (AOX) is activated to quench the ROS.

## 4.5 Future work

The results presented in this chapter show that the transcriptional response of *A. niger* to wheat straw is not solely controlled by the TF XlnR. ClrB, and to some extent ClrA, seem to play a role in this response. To further phenotype the corresponding deletion strains, the  $\Delta clrA$  and  $\Delta clrB$  strains could be tested on other substrates such as willow or sugarcane bagasse to investigate whether or not their role is specific to wheat straw. Also the complementation strains of the *clrA* and *clrB* deletions should

be done to confirm their phenotypes. However, other TFs must be involved and further screening of transcriptomic and proteomic datasets would help identifying and characterising these new TFs. Those studies will explore both the molecular basis of the fungal responses (to include sensing, signalling and transcriptional regulation) but will also provide pointers to the optimised saccharification of target lignocellulosic materials. A better understanding of the regulatory networks involved in the transcriptional response to lignocellulosic substrates will help to reduce costs of inducing enzymes and improving the functionality of the enzymatic cocktail used in the biofuel production process.

**Chapter 5: Role of phosphorylation in the breakdown  
of lignocellulose by *A. niger***

## 5.1 Introduction

### 5.1.1 Phosphorylation of proteins, a modification involved in signalling

Phosphorylation is a post-translational modification of proteins that occurs in a wide range of cellular processes (e.g. differentiation, cell division, proliferation, and apoptosis) and particularly in signalling mechanisms. Phosphorylated proteins have been known for over a century (Levene 1906), followed by the discovery of a phosphoserine in the yolk sac (Lipmann 1932) and finally by the first description of a protein phosphorylation event (Burnett *et al.* 1954). The phosphorylation brings about important functional modifications of the target protein, such as amplification or inhibition of its enzymatic activity, change in its cellular localisation and structural changes that allow the association with other proteins. The phosphorylation reaction is the esterification of the serine, tyrosine or threonine side chain, by adding one or several phosphate groups (**Figure 5.1**).

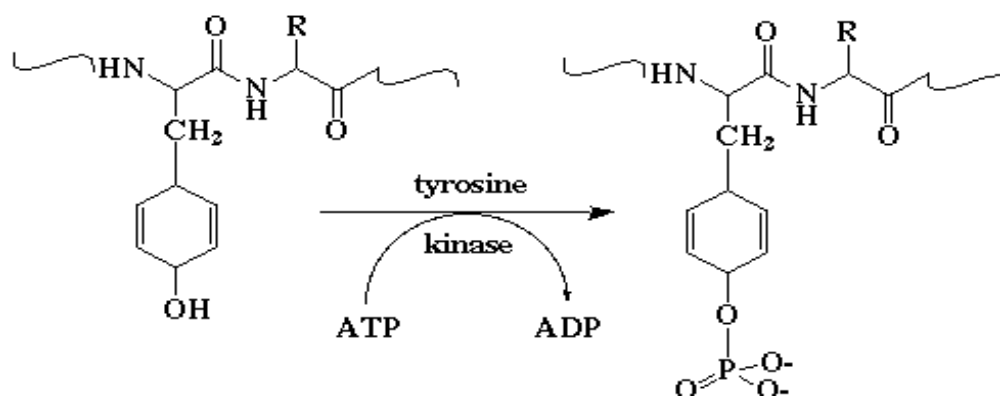


**Figure 5.1:** phospho-esterification of tyrosine (<http://biochimej.univ-angers.fr/>). Phosphorylation is the esterification reaction on the side chain of serine, threonine or tyrosine by adding one or several phosphate groups.

Phosphorylation is mediated by protein kinases. The added phosphate groups are then removed (de-phosphorylation) by phosphatase enzymes. The processes of phosphorylation and de-phosphorylation provide a mechanism by which metabolic or signalling pathways are regulated.

### 5.1.2 Protein kinases families

The 27 eukaryotic families of protein kinases represent one of the most important protein super-families. They account for around 2% of the genes in eukaryotic genomes. These proteins are involved in the regulation of most of the metabolic pathways and seem to phosphorylate around 30% of the proteome. Up to date, 2000 kinases have been identified and over 100 tri-dimensional structures have been reported in the Protein Data Bank (PDB) (Sussman *et al.* 1998).

#### 5.1.2.1 Tyrosine kinases

Over 90 tyrosine kinases have been identified in the human genome and they are part of a multigenic family (Robinson *et al.* 2000). Tyrosine kinases are divided into 2 groups:

- Cytoplasmic tyrosine kinases (32 of that type – EC 2.7.10.2).
- Transmembrane receptor-linked tyrosine kinases (58 of that type – EC 2.7.10.1 – EC 2.7.1.112) e.g. insulin receptor.

These proteins have an extracellular domain that can link to a specific ligand and an intracellular catalytic domain (CD) that links to and phosphorylates the targeted proteins. The binding of the ligand to the extracellular domain leads to structural modifications of tyrosine kinase that trigger their activation.

#### 5.1.2.2 Serine/Threonine kinases

Examples of Serine/Threonine kinases are:

- Protein kinases regulated by cyclic nucleotides (cAMP, cGMP): protein kinase A (PKA – Calmodulin dependent kinase EC 2.7.1.123) and protein kinase G
- Protein kinase C (PKC - Calmodulin dependent kinase EC 2.7.1.123) activated by diacylglycerol
- Mitogen-activated protein kinases (MAPK - EC 2.7.11.24): Extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase, MAP Kinase p38
- Protein kinases [calcium – calmodulin]-dependent (CaM kinases – EC 2.7.1.123) in animals
- Calcium-dependent protein kinases (CDPK - EC 2.7.11.1) in plants and protozoans
- Glycogen phosphorylase kinase (EC 2.7.11.1)

### 5.1.2.3 Histidine kinases (EC 2.7.13.3)

These proteins do not have the same structure as the other protein kinases and are mainly found in prokaryotes and plants (Nongpiur *et al.* 2012). They work in relay together with an aspartate residue that receives the phosphate group transferred from ATP to the histidine.

### 5.1.3 Mitogen Activated Protein Kinases (MAPKs - EC 2.7.11.24)

Mitogen-activated protein kinases are also known as MAP kinases that are serine/threonine-specific protein kinases belonging to the CMGC (CDK/MAPK/GSK3/CLK) kinase group. The closest relatives of MAPKs are the cyclin-dependent kinases (CDKs) (Manning *et al.* 2002). MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock and proinflammatory cytokines. They regulate cell proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis, among many others (Pearson *et al.* 2001). MAP kinases are found in eukaryotes only, but they are fairly diverse and encountered in all animals, fungi and plants, and even in an array of unicellular eukaryotes.

#### 5.1.3.1 Activation

MAPKs are kinases that catalyse the phosphorylation of MAPs. MAPK signalling pathways involve a series of kinases that are activated in a cascade: a MAPK is activated by a MAPKK, itself activated by a MAPKKK. The MAPKKK is activated by another protein kinase activated, also by phosphorylation, in response to different signals (Madden *et al.* 1997). These cascades of activation are composed of different modules. MAPKs (MAPKKK, MAPKK, and MAPK) have a conserved Thr-X-Tyr motif, composed of a threonine, a variable amino acid depending on the MAPK type, and a tyrosine in their activation loop. This motif needs to be phosphorylated on both the threonine and the tyrosine residues in order to lock the kinase domain in a catalytically competent conformation. In humans, the variable amino acid is essential as it allows each MAPK to be specifically recognised by its activating kinase which induces an adapted response to the external signal (Robbins *et al.* 1993). The tandem activation loop phosphorylation is performed by members of the MAPKK or Ste7 family. MAPKK kinases in turn, are also activated by phosphorylation, by a number of different upstream serine-threonine kinases, the MAPKKK. Because

MAPKK kinases display very little activity on substrates other than their cognate MAPK, classical MAPK pathways form multi-tiered, but relatively linear pathways. These pathways can effectively convey stimuli from the cell membrane (where many MAPKKs are activated) to the nucleus (where only MAPKs may enter) or to many other subcellular targets. The specificity of interaction between the kinase and the protein to phosphorylate is due to the recognition of the tertiary structure of proteins. Three-dimensional structural analysis showed that phosphorylation can affect protein activity by inducing allosteric conformational changes (Sprang *et al.* 1998). It is also quite clear that the activity of the protein kinases is linked to their level of phosphorylation. Thus, it has been shown that for mammalian MAPK, 2 distinct phosphorylation sites are present, at tyrosine and threonine residues, and only the double phosphorylation of the protein leads to a full and efficient activation (Anderson *et al.* 1990). In a similar way, MAPKK require a double phosphorylation on both serine residues or on one serine residue and one threonine residue to be activated and be able to phosphorylate the MAPK downstream of the cascade (Huang *et al.* 1994). The MAPKKK activation seems to be more complex due to the diversity of signals and effectors (e.g. G protein coupled receptors, phosphorelays, membrane sensors, protein kinase) leading to the activation of the cascade. Moreover, some MAPKKK can associate in oligomers resulting in their auto-phosphorylation (Tobiume *et al.* 2002).

Each activated MAPK phosphorylates various substrates (TFs, other protein kinases, phospholipases and different proteins associated with cytoskeleton) that will be necessary for the cell to react efficiently to a change in its environment (Payne *et al.* 1991).

### 5.1.3.2 Signalling cascade

The first elements of the signalling pathway are the receptors coupled to the cell membrane (Gray *et al.* 1997; Verna *et al.* 1997). In fact, the activation of the protein kinase is passed on *via* these receptors associated with the membrane (**Figure 5.2**).

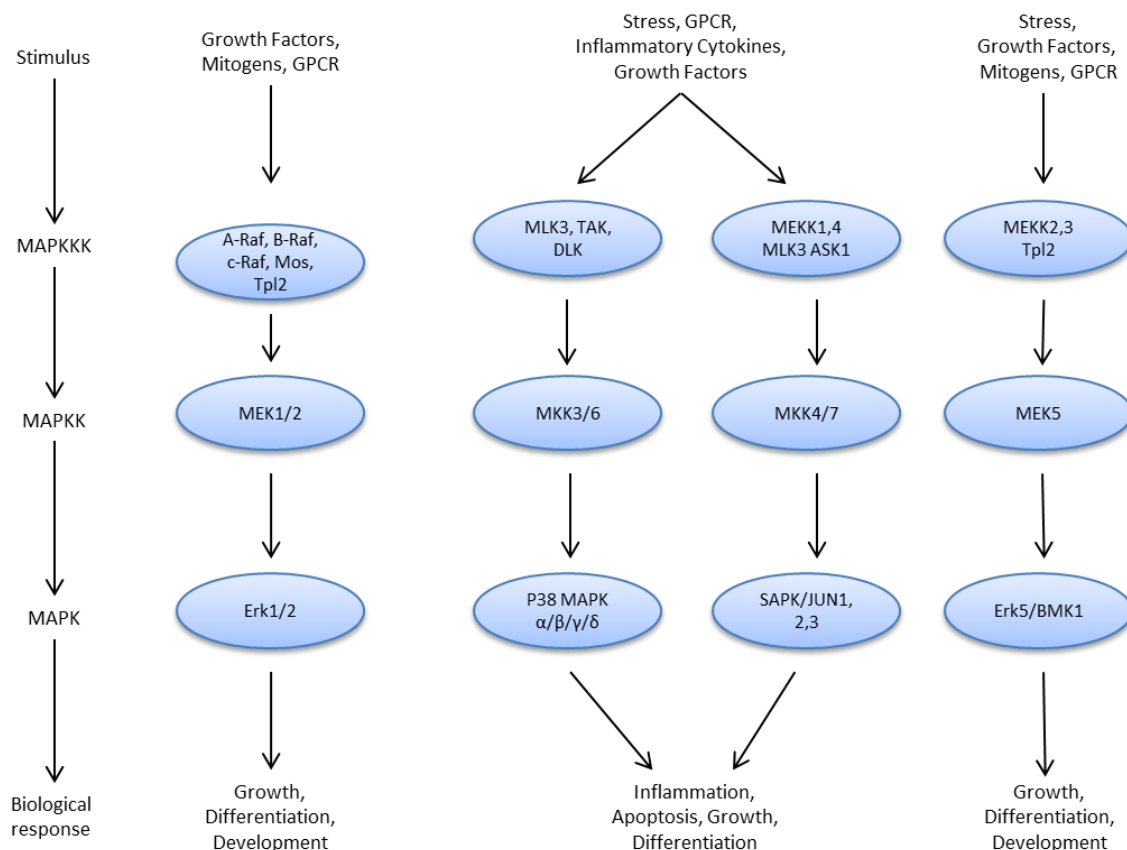


**Figure 5.2:** MAPK signalling cascades (adapted from [www.cellsignal.com](http://www.cellsignal.com)). Signalling via the conventional MAPKs follows a classical three-tiered kinase cascade: MAPKKK→ MAPKK→MAPK. A broad range of extracellular stimuli including mitogens, cytokines, growth factors, and cellular stressors stimulate the small GTPases of the Ras/Rho family, leading to activation of a MAPKK kinase (MAPKKK). MAPKKK phosphorylates and activates a downstream MAPK kinase (MAPKK), which in turn phosphorylates and activates a MAPK. Activation of MAPKs leads to the phosphorylation and activation of specific MAPK-activated protein kinases, such as members of the ERK, SAPK, or p38 families. These MAPK-activated protein kinases function to amplify the signal and mediate the broad range of biological processes regulated by the different MAPKs.

#### 5.1.3.3 MAPKs in animals

All Eukaryotic cells have multiple MAPK pathways regulating gene expression, mitosis, metabolism, survival, apoptosis and differentiation. Five MAPK modules have been described up to now: extracellular signal-Regulated Kinases (ERKs) 1 and 2 (ERK1/2), c-Jun amino-terminal Kinases (JNKs) 1, 2 and 3, p38 isoforms α, β, γ and δ, ERKs 3 and 4, and ERK5. These five pathways share proteins involved in the

different signalling cascades but lead to different specific biological responses (Denhardt 1996; Woodgett *et al.* 1996).

### 1- Extracellular signal-Regulated Kinases (ERK1, ERK2):

The ERK1/2 pathway of mammals is probably the best-characterized MAPK system. This pathway is preferentially activated in response to growth factors and to phorbol esters (tumour inducer). It also regulates cells proliferation and differentiation.

### 2- C-Jun N-terminal Kinases (JNKs) (MAPK8, MAPK9, MAPK10):

These kinases are also known as Stress-Activated Protein Kinases (SAPKs).

### 3- P38 isoforms (MAPK11, MAPK12 (=ERK6), MAPK13, MAPK14):

JNKs and p38 signalling pathways are responsive to stress stimuli such as cytokines, UV exposure or osmotic and heat shocks. These pathways are involved in cell differentiation and apoptosis.

### 4- ERK5 (MAPK7):

This MAPK is activated in response to growth factors and to stress. It participates in cell proliferation.

### 5- ERK3 (MAPK6) and ERK4 (MAPK7):

ERK3 and ERK4 are distinguished from other MAP kinases in that they lack the conserved TXY motif in their activation loop, possessing instead an SEG motif (Boulton 1991; Cheng *et al.* 1996). Phosphorylation at Ser189 in the SEG motif has been reported (Cheng *et al.* 1996; Coulombe *et al.* 2003). With limited information about its upstream kinases and downstream substrates, the significance of this phosphorylation remains to be elucidated (Coulombe *et al.* 2004; Coulombe *et al.* 2003; Julien *et al.* 2003). Erk3 is an inherently unstable protein, rapidly degraded through amino-terminal ubiquitination and proteasome degradation while ERK4 is more stable (Bind *et al.* 2004; Coulombe *et al.* 2003; Kant *et al.* 2006). Accumulating evidence suggests that Erk3 is involved in cell differentiation (Julien *et al.* 2003).

#### 5.1.3.4 MAPKs in fungi

Survival of fungi in the environment involves coping with different kind of stresses. This implies a complex network of cellular mechanisms to adapt and deal with changes occurring in the environment. Signal transduction pathways involving MAPK cascades probably constitute the major mechanism by which fungi sense such changes and develop adaptive responses. Studies carried out on the model organism *Saccharomyces cerevisiae* showed that five MAPK pathways were implicated in different processes: mating, adjustment to nutrient limiting conditions,

survival under hyperosmotic conditions, repair of cell wall injuries and ascospore cell wall assembly (Chen *et al.* 2007). Two pathways have been particularly well characterized in *S. cerevisiae*: the High Osmolarity Glycerol pathway (HOG) and the Cell Wall Integrity pathway (CWI) (**Figure 5.3**).

### 5.1.3.4.1 HOG pathway

The HOG pathway is involved in cellular adaptation to hyperosmotic environments. Eukaryotic cells respond to osmotic stress by increasing their internal osmolarity. *S. cerevisiae* responds to these conditions of stress by activating the synthesis of glycerol and by decreasing its cellular permeability. This triggers an important accumulation of glycerol in the yeast's cytoplasm. The HOG pathway is mainly composed of three MAPKKKs encoded by the genes *SSK2/22* or *STE11*, and MAPKK and MAPK encoded respectively by the genes *PBS2* and *HOG1* (Hohmann 2002). Two crucial elements are involved in the fast and transitory activation of the HOG pathway in response to hyperosmolarity and these elements provide specific responses to different stress conditions (O'Rourke *et al.* 2002). The first one is a system that is comparable to the two-component bacterial system. It is composed of a membrane sensor and a cytosolic regulator. In this system, the transmembrane sensor is the protein Sln1p (group VI histidine kinase). In the absence of stress, Sln1p autophosphorylates and transfers the phosphate group to the phosphorelay protein Ypd1p which can then phosphorylate Ssk1p. This leads to the inactivation of the HOG pathway. In conditions of stress, Ssk1p is dephosphorylated which allows the activation of the MAP kinase cascade. The triggering signal is passed on to the MAPKKK Ssk2/22 and then to the MAPKK Pbs2p (Singh 2000). Another sensor involved in the activation of the HOG pathway in response to osmolarity is the protein Sho1p which has a SH3 domain allowing protein interactions. Through this SH3 domain Sho1p directly interacts with Pbs2p via Ste11p (MAPK involved in the response to pheromone and filamentous growth) which leads to the activation of Hog1p by phosphorylation. It seems that the interaction between Ste11p and Sho1p is necessary for Pbs2p interaction. It has been suggested that Pbs2p may act as a scaffold protein by maintaining Sho1p, Ste11p and Hog1p in an assembly. Such an association enables the cells to avoid any crosslink activation between shared components of different pathways (O'Rourke *et al.* 2002). In addition to its activation in response to hyperosmolarity, the Sho1p part of the HOG pathway is also activated in response to heat shock (Winkler *et al.* 2002). The MAPKK Pbs2p, then, phosphorylates the MAPK Hog1p. The phosphorylated form of Hog1p can be either

cytoplasmic or transferred to the nucleus assisted by Gsp1p, a protein involved in transferring to the nucleus proteins with a nuclear localisation signal (Oki *et al.* 1998). In that case Hog1p has a role of transcription factor (TF). The targets of the phosphorylated Hog1p are yet to be clearly identified. However, the main targets of the TF Hog1p seem to be genes involved in cell wall synthesis such as *EXG1* or other TFs such as Msn2/7p, Hot1p, Sko1p, Mcm1p and Smp1p (Reiser *et al.* 1999; Rep *et al.* 1999; Xu 2000). Three phosphatases Ptc1p, Ptp2p and Ptp3p are involved in the negative regulation of the HOG pathway (Hohmann 2002).

In *A. fumigatus*, the Sho1 homologue was shown to be involved in osmotic stress (Ma *et al.* 2008). The *sho1* gene also plays a role in radial growth, germination and polarity establishment in the same species (Ma *et al.* 2008). In *A. nidulans*, Pbs2 was also shown to be involved in stress responses (Furukawa *et al.* 2005). In *A. fumigatus*, the homologue of yeast Hog1p, SakA, is involved in the response to osmotic stress. However, in *A. nidulans*, SakA is not required for surviving in hyperosmotic conditions which indicates that different mechanisms may be involved in these two species (Kawasaki *et al.* 2002). Finally, Tmk3, a homologue of yeast Hog1p in *T. reesei*, has been shown to act in the CWI pathway similarly to Slit2-type MAPK showing again differences in the regulation in filamentous fungi (Wang *et al.* 2013).

### 5.1.3.4.2 CWI pathway

The CWI pathway has been well described in the yeast *S. cerevisiae*. The activation of the CWI pathway by a stress that compromises the cell wall integrity leads to, via a MAPK cascade (Bck1p, Mkk1p/Mkk2p), the phosphorylation of the MAPK Slit2p (also named Mpk1p). This triggers the expression of genes involved in the synthesis and the restructuring of cell wall components and to the establishment of a mechanism called “compensatory mechanism” (Baetz *et al.* 2001). This response is characterised by an increase in the cell wall contents of  $\beta$ -glucans and chitin, changes in the cell wall polymers association and by an increase in the quantity of proteins necessary for cell wall synthesis. This response can be triggered by chemical reagents interfering with the cell wall (Calcofluor White, Congo Red, glucanases) or by oxidative stress, osmotic or heat shocks (Bermejo *et al.* 2008). The CWI pathway activation is controlled by five cellular membrane proteins (Wsc1p, Wsc2p, Wsc3p, Mid2p and Mtl1p). Under stress conditions, Wsc1p and Mid2p interact with Rom2p, a guanine exchange factor that activates the small GTPase Rho1p. Rho1p, once activated, interacts with Pkc1p (Protein kinase 1) leading to its

activation (Bermejo *et al.* 2008). The active form of Pkc1p starts the signalling cascade by phosphorylating Bck1p (MAPKKK). Bck1p, then, phosphorylates and activates two MAPKK, Mkk1p and Mkk2p. In turn, these MAPKK activate their target, Slt2p. Phosphorylated Slt2p acts upon two TFs, Rlm1p (Watanabe *et al.* 1995) and SBF (Baetz *et al.* 2001). SBF is a heterodimer made of two proteins, Swi4p and Swi6p, involved in the regulation of genes entailed in the G1/S transition in the cell cycle. Swi4p and Swi6p as well as Rlm1p TFs act upon the expression of genes involved in cell wall biosynthesis and restructuring (Jung *et al.* 1999).

The CWI pathway has been shown to be the main pathway activated in response to a cell wall stress in *Alternaria brassicicola* (Sellam *et al.* 2007). In the same way, in *S. cerevisiae*, studies showed that the CWI pathway was activated by cell wall damage when caused by zymolyase (Bermejo *et al.* 2008) but also by a hyperosmotic shock in a HOG1-dependent manner (Garcia *et al.* 2009).

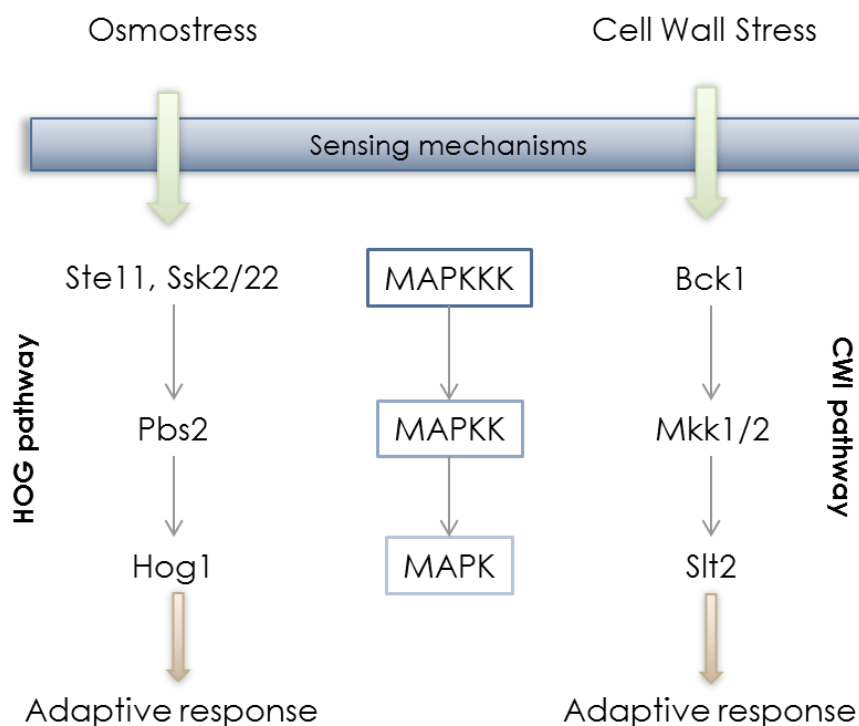


**Figure 5.3:** HOG and CWI pathways in *S. cerevisiae*.

#### 5.1.3.5 MAPKs in plants

The genome of *Arabidopsis thaliana* is predicted to encode around 1100 protein kinases, corresponding to 4% of the 25,500 predicted genes. This proportion is double that observed in human, *S. cerevisiae* or *Caenorhabditis elegans*. In *A.*

*thaliana*, the protein kinases that are transmembrane receptors phosphorylate the serine and threonine residues (they phosphorylate mainly the tyrosine residues in animals and fungi).

Despite the high number of MAPK genes, MAPK pathways of higher plants have been studied less than animal or fungal ones. Although their signalling appears very complex, the MAPK3, MAPK4 and MAPK6 kinases of *A. thaliana* are key mediators of responses to osmotic shock, oxidative stress, to cold and are involved in anti-pathogen responses (Rodriguez *et al.* 2010; Sinha *et al.* 2011). In addition, they are also involved in morphogenesis, since MPK4 mutants display severe dwarfism (Kosetsu *et al.* 2010).

### 5.1.4 Transcription factor activation

TF may be activated (or deactivated) through their signal sensing domain by a number of mechanisms including:

- **Ligand binding:** nuclear receptors have the ability to directly bind to DNA and regulate the expression of adjacent genes. Hence, these receptors are classified as transcription factors (TFs) (Evans 1988; Olefsky 2001). The regulation of gene expression by nuclear receptors generally only happens when a ligand is present. More specifically, ligand binding to a nuclear receptor results in a conformational change in the receptor which, in turn, activates the receptor resulting in up-regulation or down-regulation of gene expression.

- **Phosphorylation:** Phosphorylation of a transcription factor, often at multiple sites, is a common mechanism for responding to signalling events (Karin *et al.* 1995). This modification can lead to changes in transcription factor concentration or activity in the nucleus (Calkhoven *et al.* 1996). Three main levels of regulation modulate TF activity through phosphorylation. Firstly, TFs can be kept in a latent state in the cytoplasm and are then translocated into the nucleus upon activation. Secondly, the nuclear binding activity of nuclear TFs can be modulated either positively or negatively by phosphorylation. Finally, phosphorylation can affect the interaction of transcription factor transactivation domains with the transcriptional machinery (Hunter *et al.* 1992) (Figure 5.4).

– **Interaction with other TFs or co-regulatory proteins:** TFs may interact with DNA indirectly through protein-protein interactions with other transcription factors or coregulatory proteins (Metivier *et al.* 2003).

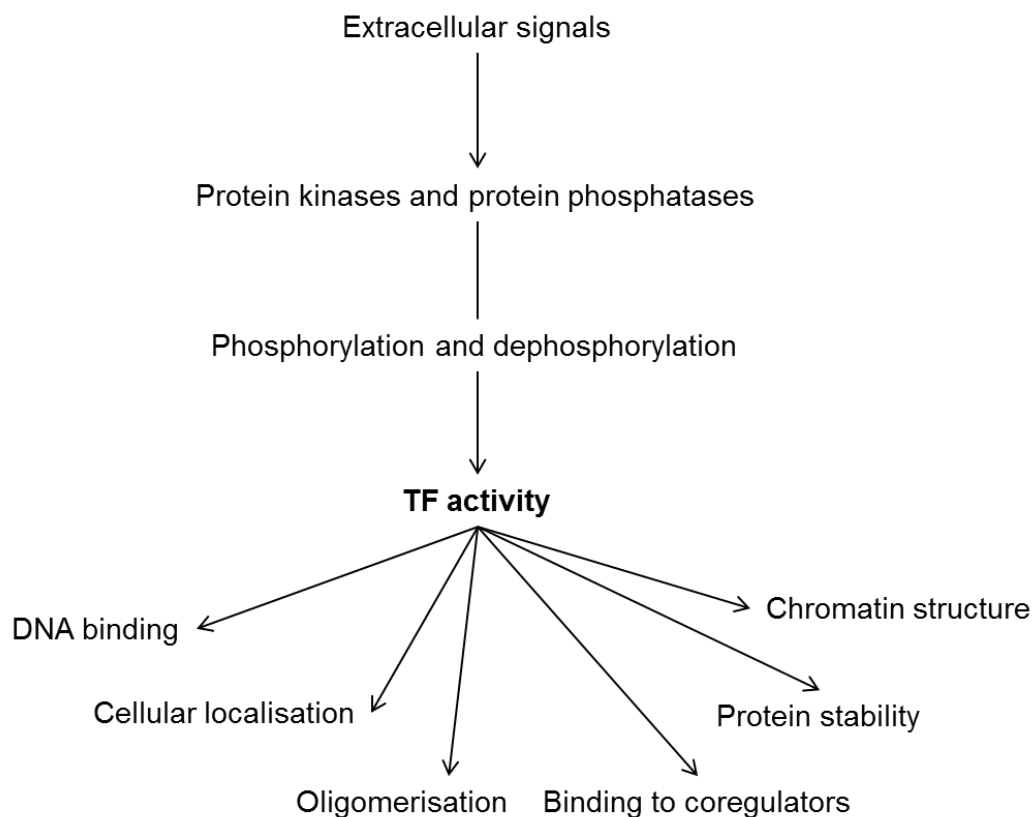


**Figure 5.4:** Protein phosphorylation and protein dephosphorylation regulate the activities of TFs through multiple mechanisms. In response to extracellular signals, the phosphorylation state of TFs and co-regulators is modulated by the activities of protein kinases and protein phosphatases. These modifications either positively or negatively affect TF activity by regulating distinct aspects of TF function (Whitmarsh *et al.* 2000).

#### 5.1.5 MAPK signalling cascades and expression of GHs

In fungi, lignocellulolytic enzyme production is tightly controlled at the transcriptional level by the action of transcriptional activators and repressors. Wang *et al.* (2013) showed that the deletion of *tmk3*, homologue of *hog1* in *T. reesei*, down-regulated the transcript level of genes encoding GH enzymes. The authors observed a decrease in the transcript levels of *cbh1*, *cbh2*, *egl1*, *egl2* and *bgl1* encoding, respectively, two cellobiohydrolases, two endoglucanases and one  $\beta$ -glucosidase when *tmk3* was deleted. They, therefore, suggested that Tmk3 may be involved in

cellulase and hemicellulase production and/or induction. Tmk3 may also regulate cellulase production by phosphorylating and activating TFs responsible for GH induction in *T.reesei*.

Brown *et al.* (2013) studied SnfA, the homologue of Snf1, a sensor of cellular energetic state in *S. cerevisiae*, in *A. nidulans*. Snf1 is known to be required for growth on alternative carbon sources. The authors studied the effect of *snfA* deletion upon expression of GHs. They demonstrated that the deletion of *snfA* decreased the production of hydrolytic enzyme production by decreasing the ability of *A. nidulans* to utilise cellulose and transcribe genes encoding hydrolytic enzymes. Snf1 has also been shown to regulate the expression of 400 genes in response to carbon exhaustion (Young *et al.* 2003) (**Figure 5.5**).

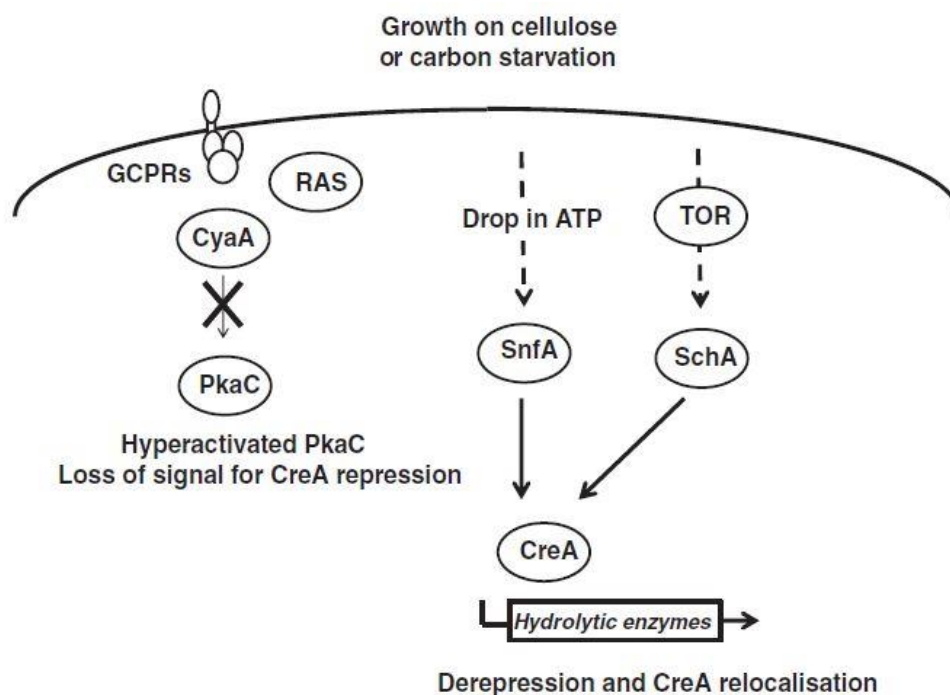


**Figure 5.5:** A schematic for the mechanisms by which non-essential protein kinases regulate CreA repression and hydrolytic enzyme production in *A. nidulans*. Upon growth on cellulose as a sole carbon source, a drop in phosphorylated glucose and RAS-mediated PKA activation, results in the loss of the positive signal for CreA nuclear localisation and repression. Subsequently, the drop in intracellular ATP and an alteration in TOR signalling, results in SchA and SnfA controlling CreA relocalisation and hydrolytic enzyme production. Bold arrows represent experimentally proven, while dotted arrow represent hypothetical, connections (Brown *et al.* 2013).

### **5.1.6 Aims**

A phosphoproteomics-based analysis has been carried out on both the WT and the *xlnR* deletion strains of *A. niger*. The aims of the analysis were to:

- Identify key regulators of signalling pathways activated in response to exposure to wheat straw in *A. niger*.
- Improve our knowledge of the mechanisms involved in the degradation of wheat straw in *A. niger*.

## **5.2 Material and methods**

See Chapter 2:

- 2.2.10.6 for the phosphoproteomics analysis
- 2.2.10.4 for western blot analysis
- 2.2.3.4.2 for qRT-PCR
- 2.2.5 and 2.2.6 for the gene deletion procedure
- 2.2.7.1 for pH measurement
- 2.2.7.2 for glucosamine assay

## **5.3 Results**

As highlighted in the Chapter 3, the wheat straw substrate seems to interact with the colorimetric measurement of the protein concentrations using the RC/DC assay. The protein extractions carried out for the phosphoproteomics study were quantified for concentrations using this assay. Therefore, the results presented in this chapter may be due to a difference in the protein concentration estimation with some samples (e.g.  $\Delta xlnR$  glucose condition) having more proteins detected during the phosphoproteomics analysis than others.

A phosphoproteomic study was carried out on both the WT and the  $\Delta xlnR$  deletion strains of *A. niger* in order to identify key regulators of the signalling pathways involved in the breakdown of a lignocellulosic substrate, wheat straw. The analysis consisted in comparing the phosphoproteome profile of the strains when grown in a simple, easily available carbon source, glucose, with the phosphoproteome profile of the same strains when exposed to a complex substrate, wheat straw, for 6h, 12h

and 24h to identify the proteins that could be involved in signalling pathways. The data analysis was done on the phosphopeptides that were found in both biological repeat for each sample.

### 5.3.1 Phosphoproteomics datasets

Noticeable differences in the WT strain and the  $\Delta xlnR$  strain were detected (**Figure 5.6**). In the WT strain, there were 15 phosphopeptides in the glucose condition, and a constantly increasing number of phosphopeptides in the wheat straw conditions between 6h (7 phosphopeptides), 12h (18 phosphopeptides) and 24h (35 phosphopeptides) of exposure to the substrate. In the  $\Delta xlnR$  strain, 147 phosphopeptides were found in the glucose condition, 15 phosphopeptides in the 6h wheat straw condition, 22 after 12h of exposure to wheat straw and 13 phosphopeptides in the 24h of exposure to wheat straw sample. In addition, none of the phosphopeptides found in the glucose condition for the two strains was found in either of the 6h, 12h or 24h of exposure to straw samples (data not shown).

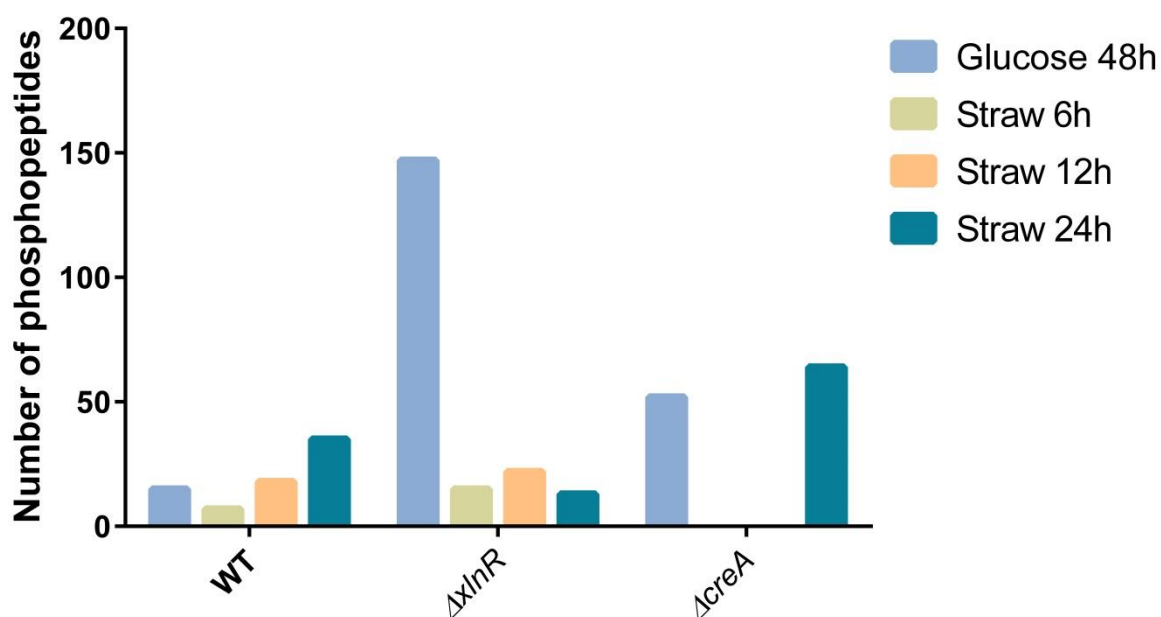


**Figure 5.6:** Diagram showing the number of phosphopeptides found in the WT, the  $\Delta xlnR$  and the  $\Delta creA$  strains for each condition: 48h glucose, 6h wheat straw, 12h wheat straw and 24h wheat straw. The phosphopeptides were found in both biological repeat samples.

The comparison was then made between the same condition for the two strains to identify if the phosphopeptides activated in response to a specific condition were the same in the WT strain and the  $\Delta xlnR$  strain (**Figure 5.7**). The data show that these phosphopeptides were different between the two strains. In the glucose condition only 5 phosphopeptides were shared between the WT and the  $\Delta xlnR$  strain. In wheat straw, there were no shared phosphopeptides at 6h of exposure to the substrate, 1 common phosphopeptide in the 12h sample and 3 in the 24h sample.

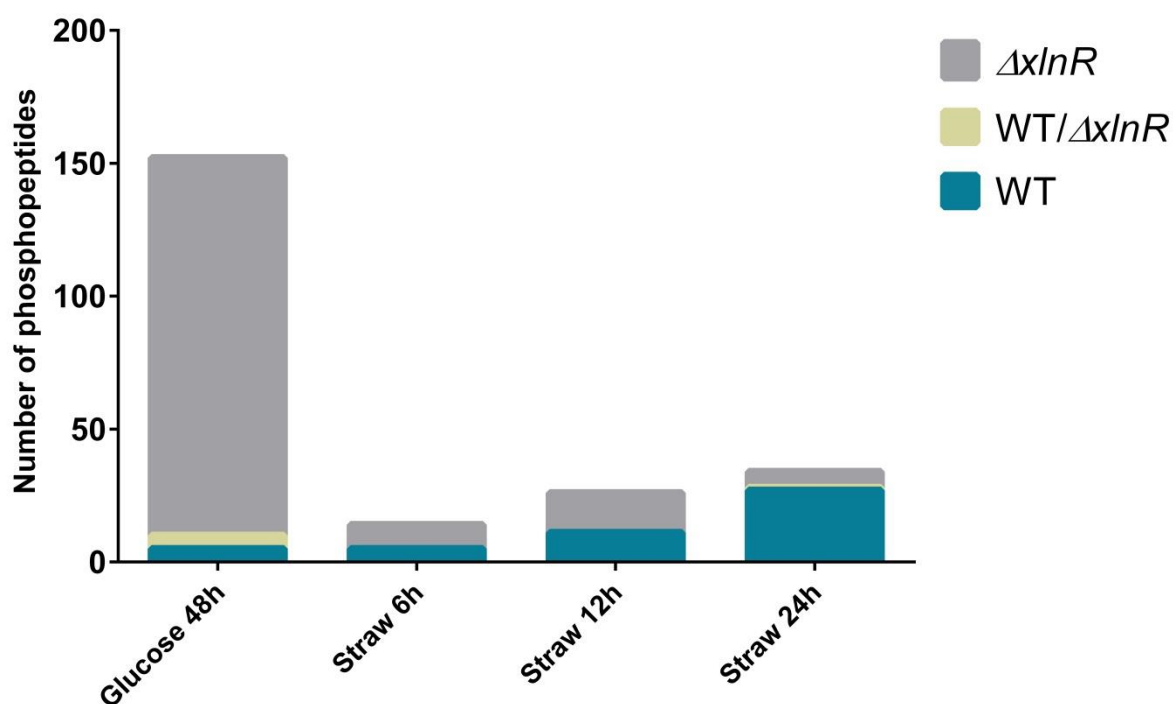
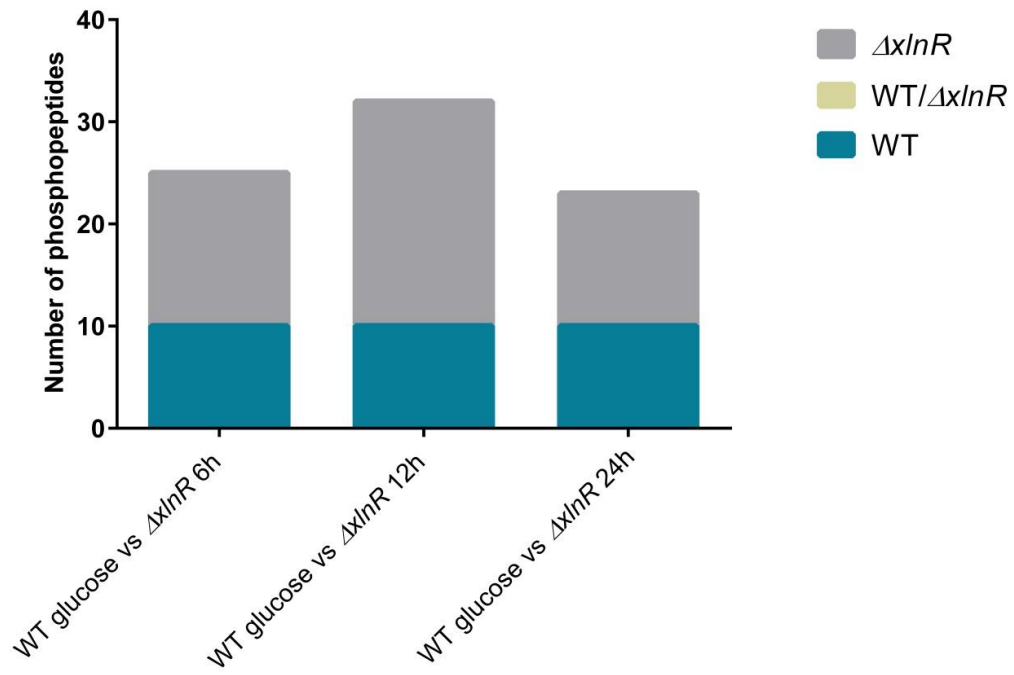


**Figure 5.7:** A comparison of the number of phosphopeptides found in the WT and the  $\Delta xlnR$  strains of *A. niger* in the different conditions: 48h of exposure to glucose, 6h, 12h and 24h of exposure to wheat straw respectively. The figure in the blue bar corresponds to the number of phosphopeptides found in the WT strain, the one in the grey bar to the number of phosphopeptides found in the  $\Delta xlnR$  deletion strain, and the one in the yellow bar corresponds to phosphopeptides found in both the WT and  $\Delta xlnR$  strains.

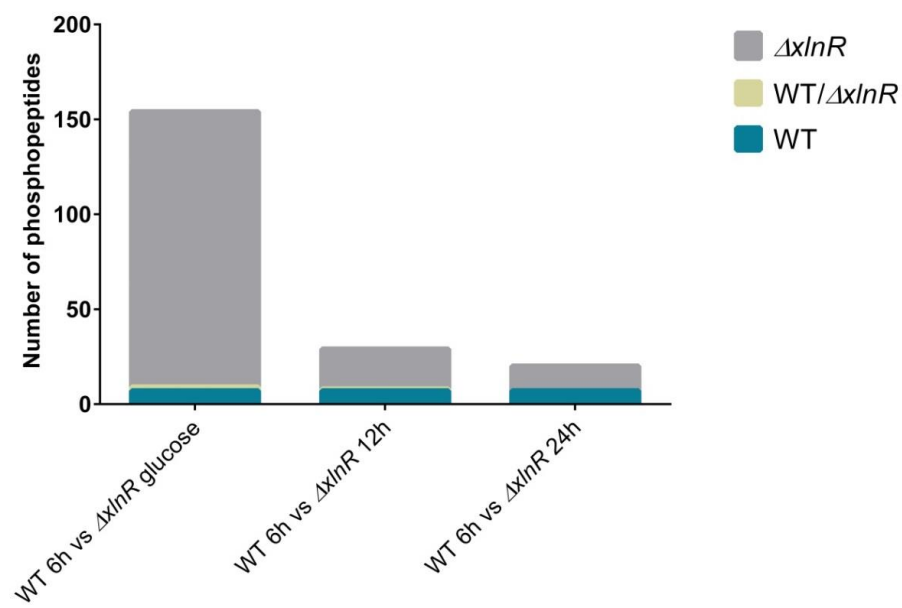
A shift in the response timing sequence can sometimes occur due to the deletion of an important regulator such as XlnR. To investigate whether or not the deletion of *xlnR* provoked such a change, the comparison of phosphopeptides found in the WT glucose condition was compared to the one found in the different  $\Delta xlnR$  conditions. The same comparison was made for all the conditions (**Figure 5.8**). Overall, there

was no increase in the number of phosphopeptides shared between 2 different conditions. The maximum number of phosphopeptides found between 2 conditions was 9 when the WT 24h condition is compared to the  $\Delta xlnR$  glucose condition and 15 when the WT 12h condition was compared with the  $\Delta xlnR$  glucose condition.

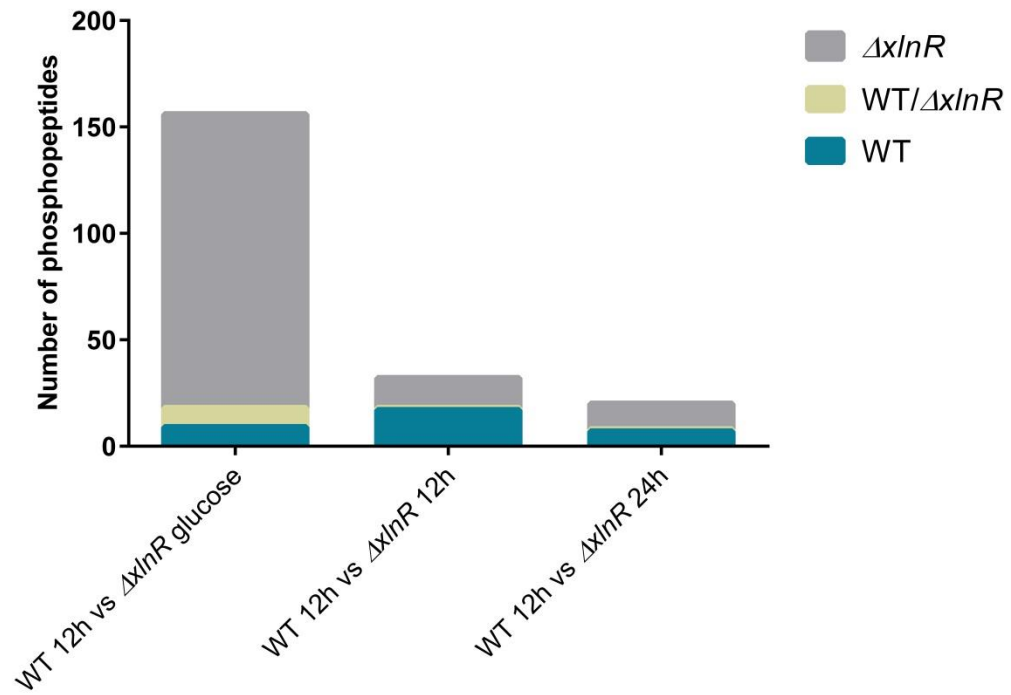
**A**



**B**



C



D

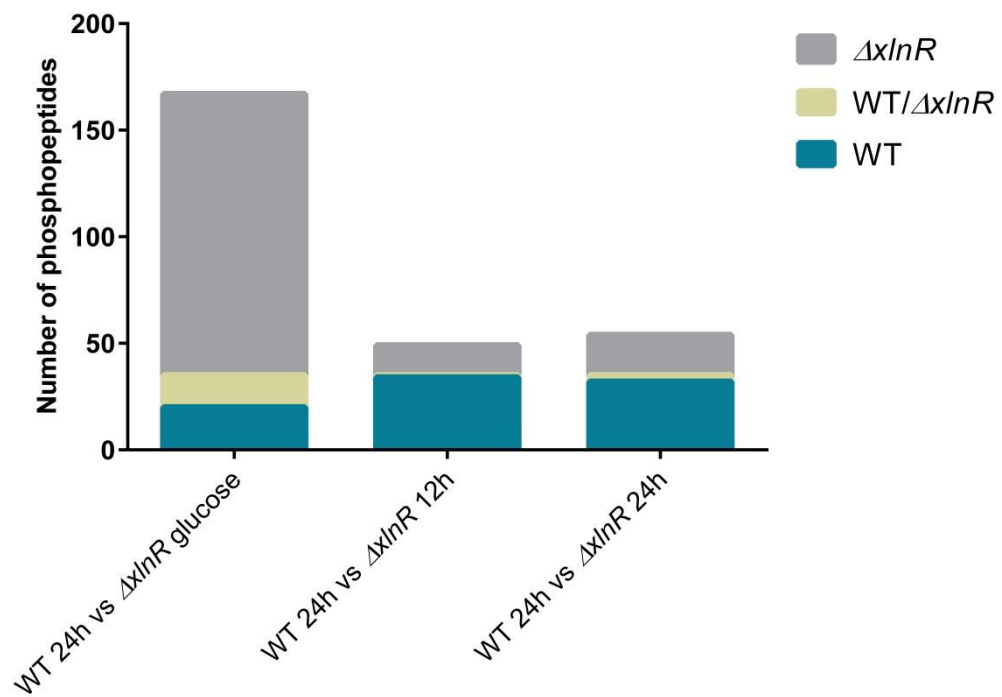


**Figure 5.8:** Comparison of the number of phosphopeptides found between the different conditions. (A) Diagram comparing the number of phosphopeptides found in the WT glucose and the  $\Delta xlnR$  strains after 6h, 12h and 24h of exposure to wheat straw respectively. (B) Diagram comparing the number of phosphopeptides found in the WT after 6h of exposure to wheat straw and the  $\Delta xlnR$  strains after 48h in glucose and 12h and 24h of exposure to wheat straw respectively. (C) Diagram comparing the number of phosphopeptides found in the WT after 12h of exposure to wheat straw and the  $\Delta xlnR$  strains after 48h in glucose and 6h and 24h of exposure to wheat straw respectively. (D) Diagram comparing the number of phosphopeptides found in the WT after 24h of exposure to wheat straw and the  $\Delta xlnR$  strains after 48h in glucose and 6h and 24h of exposure to wheat straw respectively.

### *Categories*

Phosphoproteins were classified using the KEGG categories. In the WT glucose 48h condition there were phosphoproteins in the following categories: protein kinase, protein involved in endoplasmic reticulum processing, protein involved in steroid biosynthesis, transporter, transcription factors and other proteins. After 6h of exposure to wheat straw, the categories found were one involved in meiosis in yeast, in the methane metabolism, transcription factor, transporter and other proteins. After 12h, there were also proteins involved in cell wall biogenesis, germination, linked to the Golgi apparatus as well as transcription factor, transporter and other proteins. Finally, after 24h of exposure to wheat straw, proteins involved in cell wall biogenesis, spliceosome, linked to the citrate cycle as well as transcription factor, transporter, ribosome, protein kinase and other proteins were found. At that time point the main categories were the transcription factors with 5 proteins, the protein kinases with 4 and other proteins with 11 phosphoproteins found (**Figures 5.9 and 5.10**).

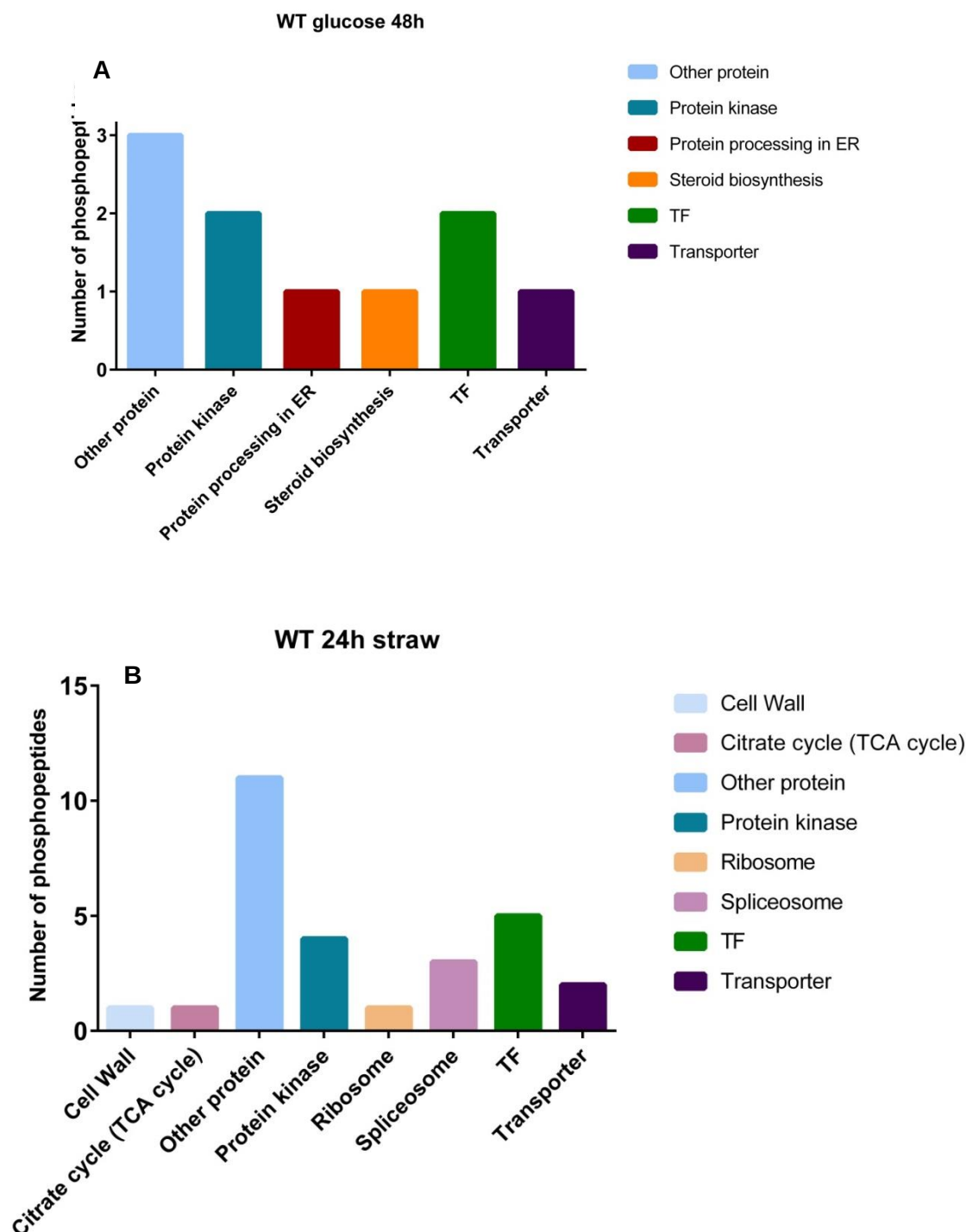


**Figure 5.9:** KEGG categories of *A. niger* phosphopeptides found after 48h of exposure to glucose (A) and after 24h of exposure to wheat straw (B) for the WT strain. Scaffold (version Scaffold\_4.0.7, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. The software calculated an improved 95% peptide and protein probability threshold based on the data from the two different search algorithms, Mascot and X!Tandem.

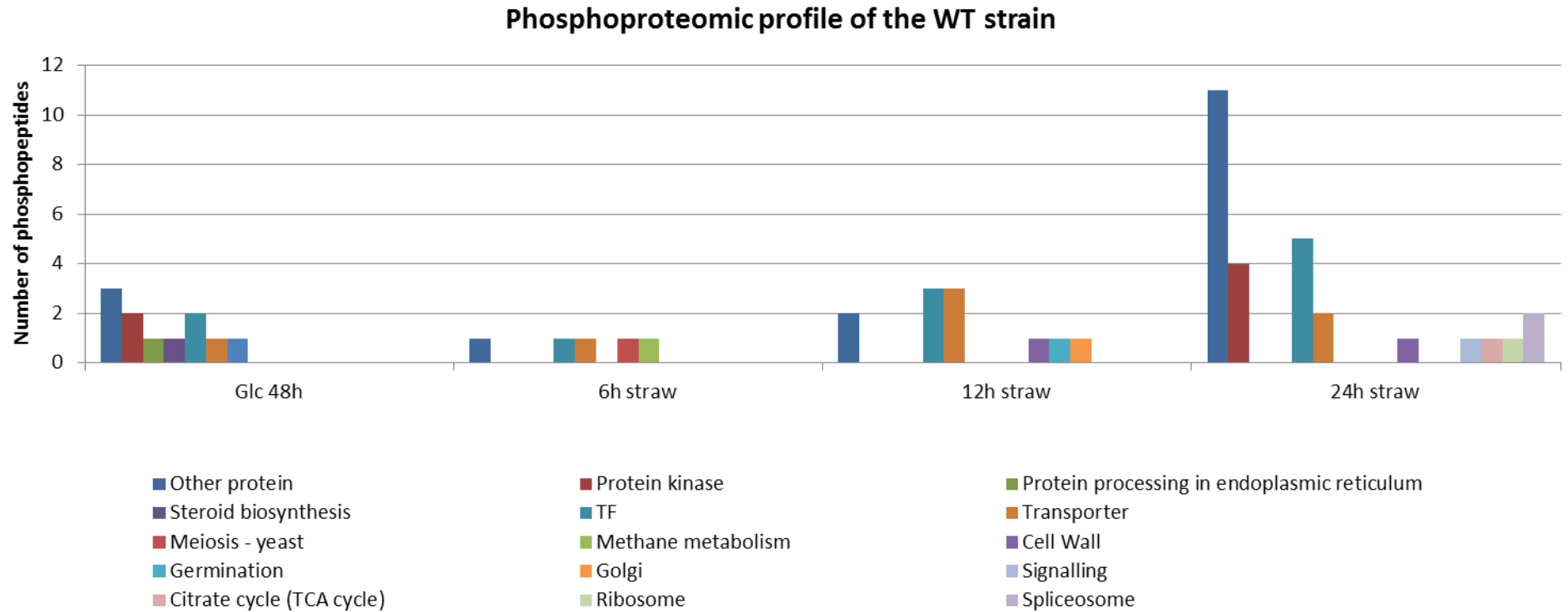


**Figure 5.10:** KEGG categories of *A. niger* phosphopeptides found after 48h of exposure to glucose and after 6h, 12h and 24h of exposure to wheat straw respectively for the WT strain. Scaffold (version Scaffold\_4.0.7, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. The software calculated an improved 95% peptide and protein probability threshold based on the data from the two different search algorithms, Mascot and X!Tandem.

## Chapter 5. Role of phosphorylation in the breakdown of lignocellulose by *A. niger*

Concerning the  $\Delta xlnR$  deletion strain, there were 25 categories when the strain was exposed for 48h to glucose. Among these 25 categories the most represented one was the transcription factor one with 15 phosphoproteins, the transporter one with 11 proteins, the protein kinase with 10 and finally the category grouping the other proteins with 69 phosphoproteins found. After 6h of exposure to wheat straw, different categories than the one found for the WT strain were found and included proteins involved in amino sugar and nucleotide sugar metabolism or SNARE interaction in vesicular transport. There were also transcription factors, transporters and other proteins. After 12h of exposure to wheat straw, the proteins found were homologues of proteins involved in meiosis in yeast, but also transcription factors, transporters, protein kinases and other proteins. At 24h, only 2 categories were found: proteins involved in the spliceosome and a category called 'other proteins' (**Figures 5.11 and 5.12**).

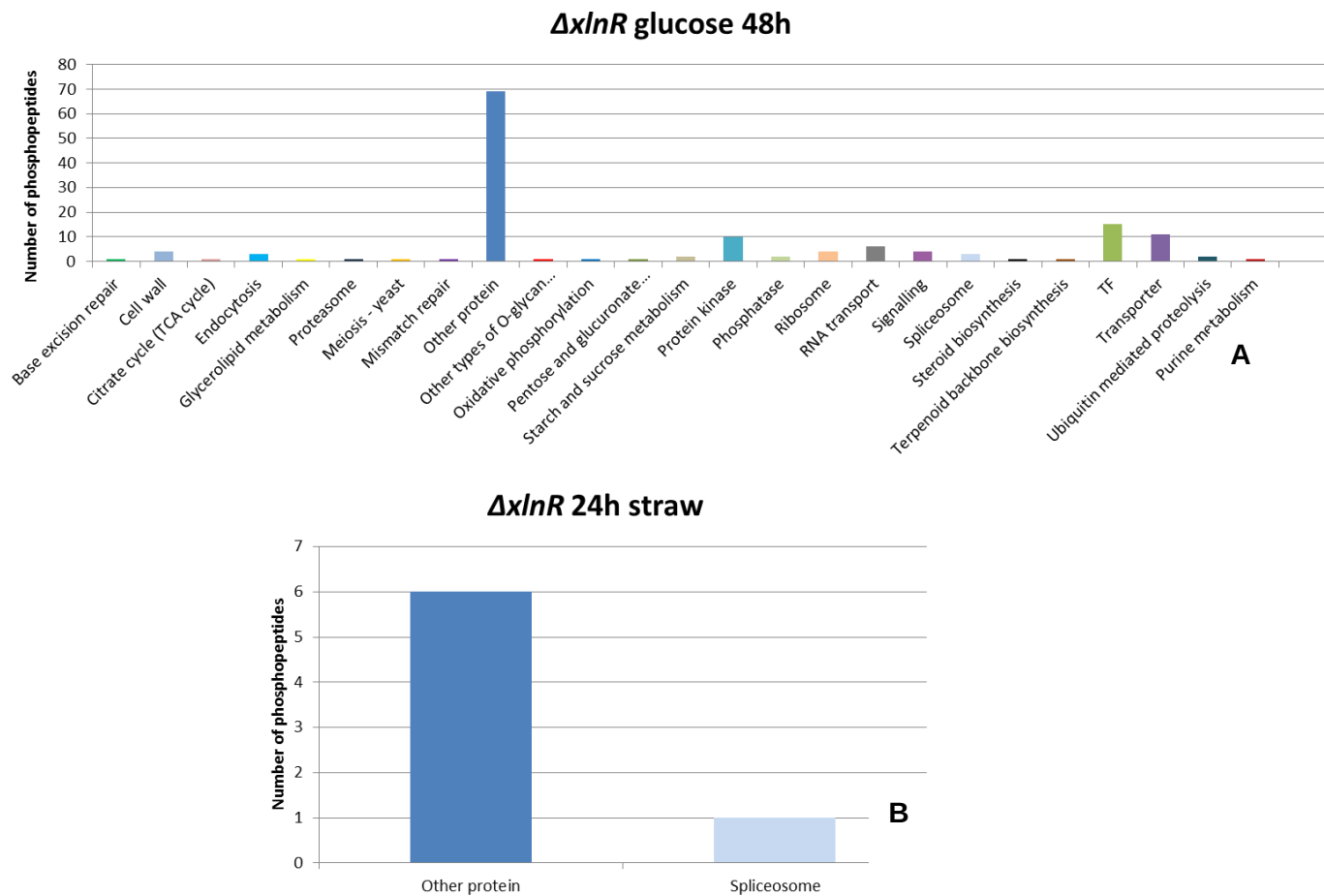


**Figure 5.11:** KEGG categories of *A. niger* phosphopeptides found after 48h of exposure to glucose (A) and after 24h of exposure to wheat straw (B) for the *ΔxlnR* deletion strain. Scaffold (version Scaffold\_4.0.7, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. The software calculated an improved 95% peptide and protein probability threshold based on the data from the two different search algorithms, Mascot and X!Tandem.

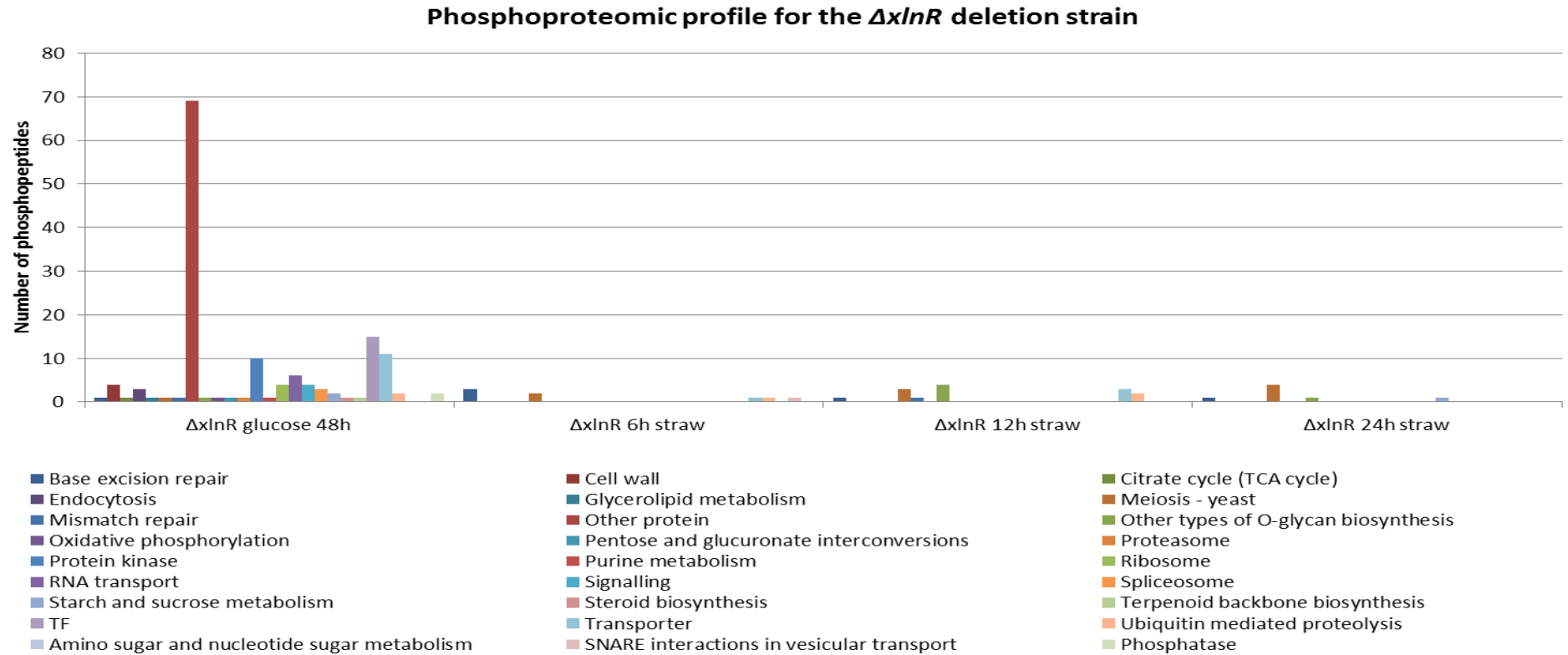
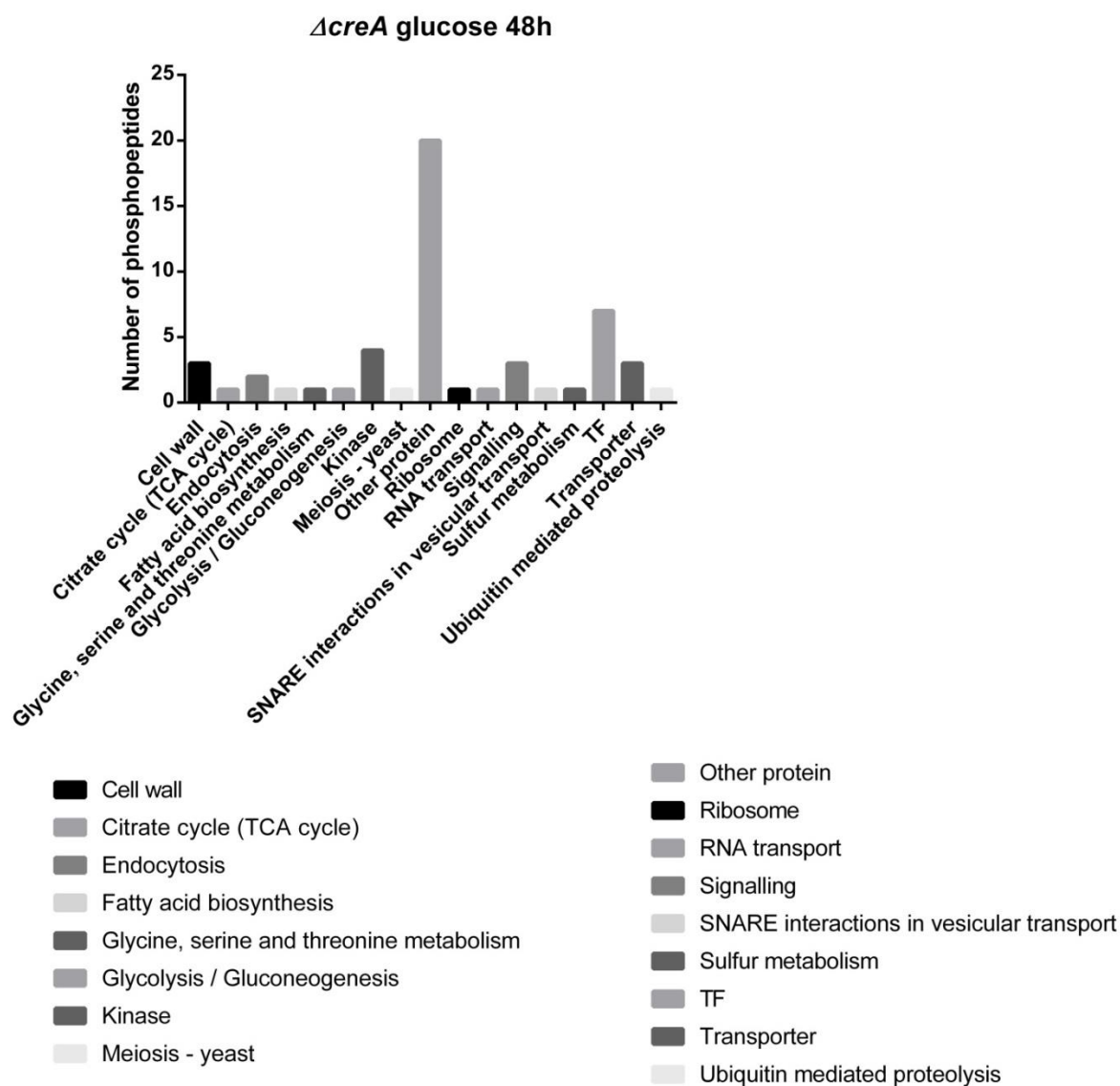


**Figure 5.12:** KEGG categories of *A. niger* phosphopeptides found after 48h of exposure to glucose and after 6h, 12h and 24h of exposure to wheat straw respectively for the  $\Delta xlnR$  deletion strain. Scaffold (version Scaffold\_4.0.7, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. The software calculated an improved 95% peptide and protein probability threshold based on the data from the two different search algorithms, Mascot and X!Tandem.



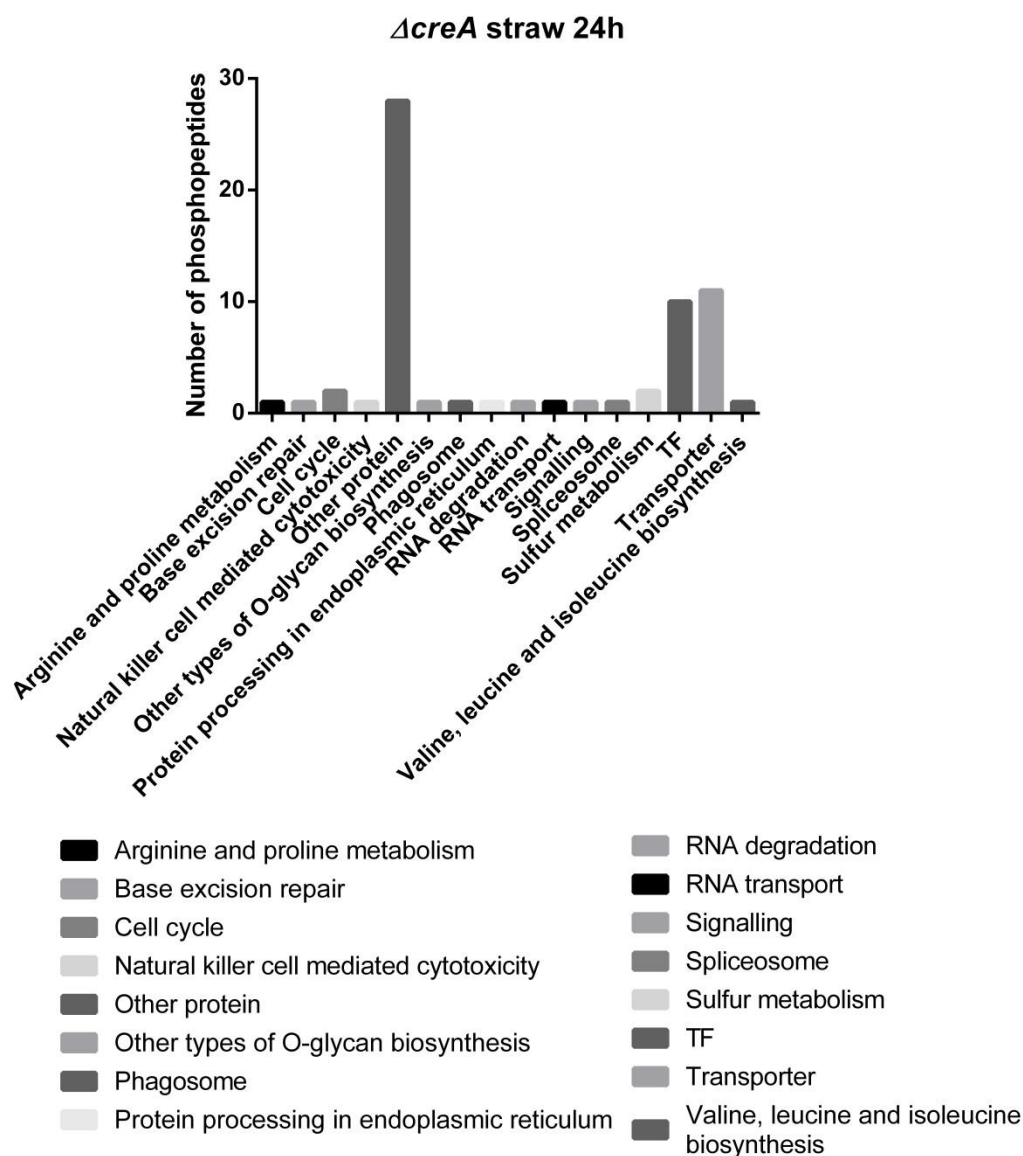


**Figure 5.13:** KEGG categories of *A. niger* phosphopeptides found after 48h of exposure to glucose (A) and after 24h of exposure to wheat straw (B) for the  $\Delta creA$  deletion strain. Scaffold (version Scaffold\_4.0.7, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. The software calculated an improved 95% peptide and protein probability threshold based on the data from the two different search algorithms, Mascot and X!Tandem.

### 5.3.2 Confirmation of the phosphoproteomics results

#### 5.3.2.1 Differences in the phosphoproteomes of the different strains

##### 5.3.2.1.1 Western blot analysis

To show differences in the phosphoproteomes of the WT and the  $\Delta xlnR$  strains, western blot and phosphostaining analyses were performed. Western blot analysis was carried out using anti-Phospho-Tyrosine Mouse (P-Tyr-100) antibody (Ref #9417 New England Biolabs) but did not show any bands on gels (data not shown). Phosphostaining, using the ProQ Diamond method, showed bands but did not show differences between samples (data not shown). For the western blot analysis, a range of protein amounts from 7 $\mu$ g to 100 $\mu$ g from the glucose culture were used and resulted each time in no detection of Tyrosine-phosphorylated (Tyr-P) proteins using the anti-Phospho-Tyrosine Mouse (P-Tyr-100) antibody. Different dilutions of the primary and secondary antibody were also assayed to try increasing the sensitivity of the detection but did not give any results either. This may be due to a lack of sensitivity of the antibody towards fungal phosphoproteins rather than a technical problem as the proteins in the positive control (EGF Receptor Control Cell Extracts) were detected. Also, the ladder was used as a negative control in the experiment as no Tyr-P proteins are present in it (NEB technical service communication).

##### 5.3.2.1.2 Induction of two phosphatase-encoding genes in the $\Delta xlnR$ strain at 48h in glucose

Two phosphatase-encoding genes were found to be phosphorylated in the  $xlnR$  deletion strain in the glucose condition. These genes have not been characterised in filamentous fungi but are annotated as phosphatase-encoding genes in the UniProt database for *A. niger*. The results showed an increase of the transcript levels of the 2 phosphatase encoding genes in the  $\Delta xlnR$  glucose condition when compared to the WT strain. The transcript levels of the two genes were 3 times higher in the  $xlnR$  deletion strain than in the WT strain in the glucose condition. In the wheat straw time point, the phosphatase 1 gene (An18g03660) had a transcript level increased when compared to the WT strain. However, the phosphatase 2 gene (An11g07970) had a transcript level lower than for the WT strain for the same condition (Figure 5.14).

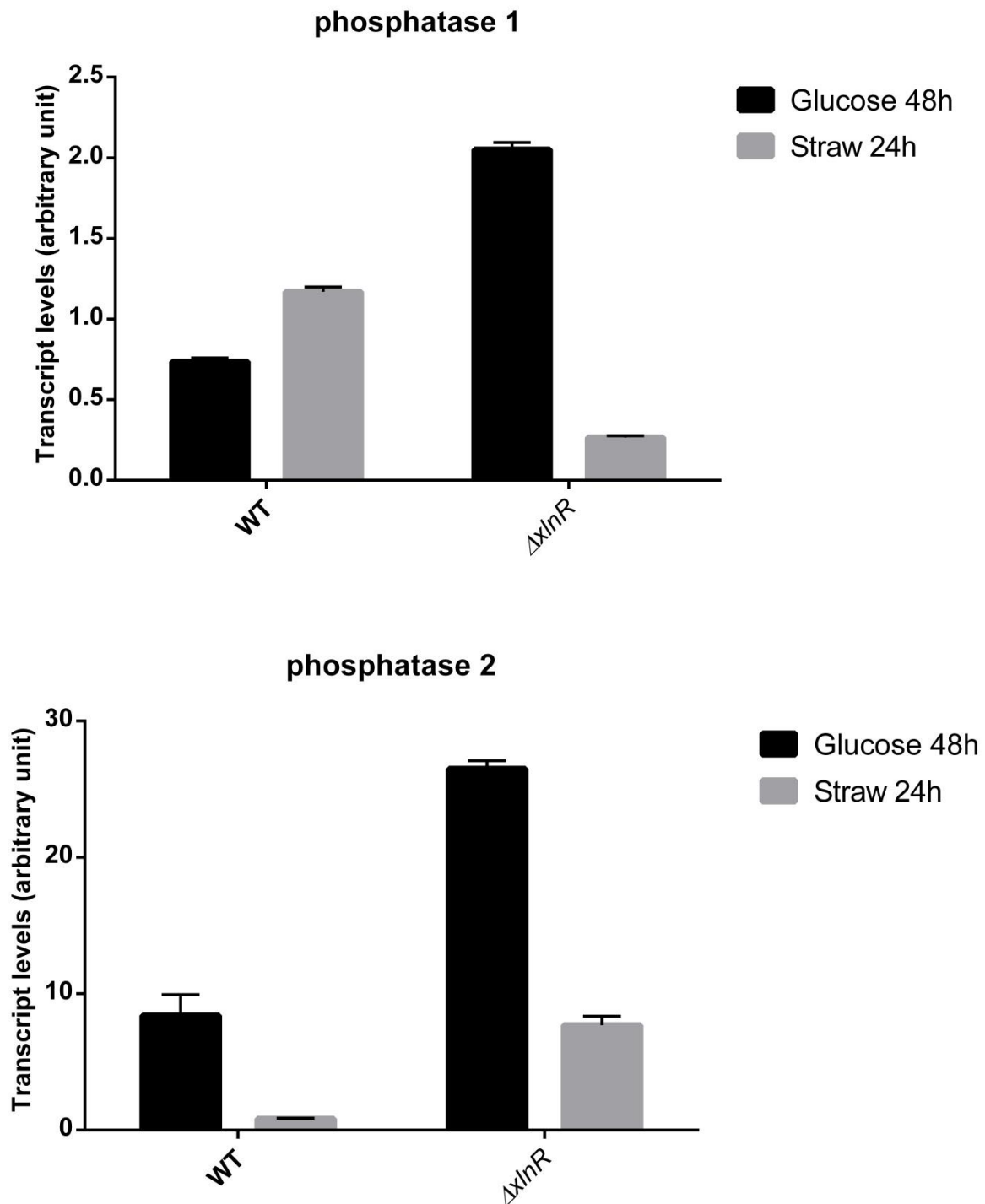


**Figure 5.14:** Expression of two phosphatase encoding genes in *A. niger* grown in glucose and wheat straw media for the WT and the  $\Delta xlnR$  strains. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. The expression values represent the expression fold change relative to the 48h glucose culture. \* represents the condition for which the T-test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

5.3.2.1.3 Induction of *slt2* and *hog1* in the  $\Delta xlnR$  strain at 48h in glucose

The *slt2* gene encodes the last MAPK of the CWI pathway whose main function is the maintenance of the cell wall integrity. When looking at the transcript level of the gene in both the WT and the  $\Delta xlnR$  strains in glucose and in wheat straw, a 5 times increase in the transcript level was observed in the glucose condition for the  $\Delta xlnR$  strain when compared to the WT strain (**Figure 5.15**). This indicates an activation of the CWI pathway in the *xlnR* deletion strain when exposed to glucose for 48h.

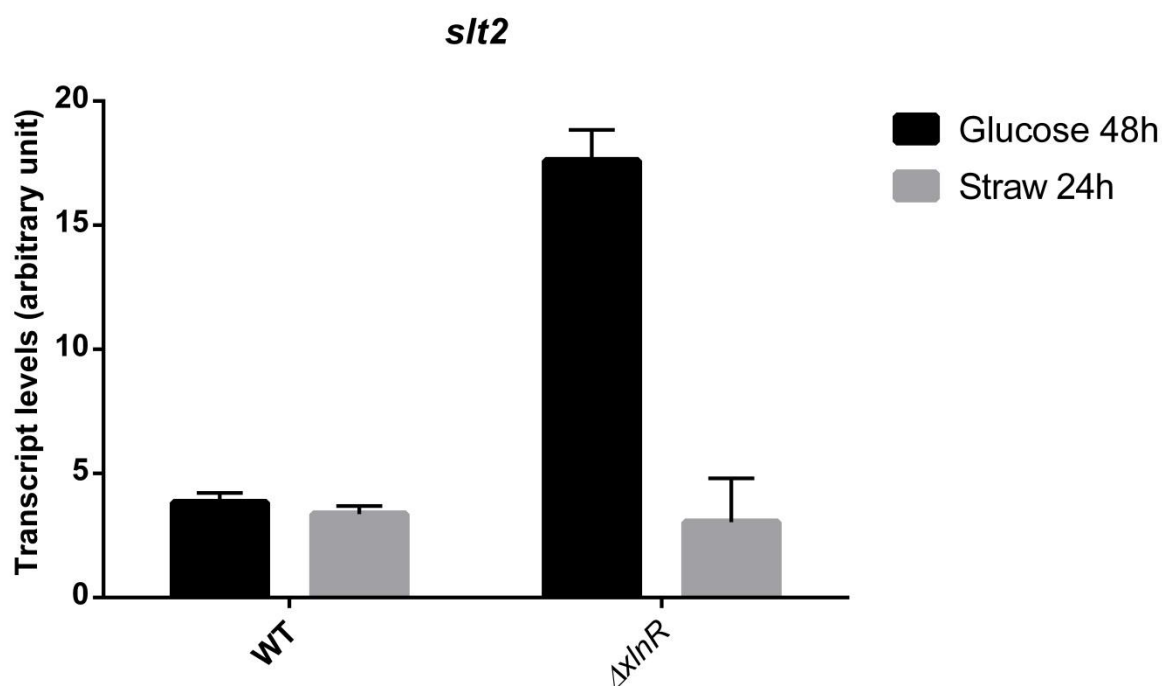


**Figure 5.15:** Expression of the *slt2* gene in *A. niger* grown in glucose and wheat straw media for the WT and the  $\Delta xlnR$  strains. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. The expression values represent the expression fold change relative to the 48h glucose culture. \* represents the condition for which the T-test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

The *hog1* gene encodes the last MAPK of the HOG pathway whose main function is the response to hyper or hypo-osmotic conditions. When looking at the transcript level of the gene in both the WT and the  $\Delta xlnR$  strains in glucose and in wheat straw, a 6 times increase was observed in the glucose condition for the  $\Delta xlnR$  strain when compared to the WT strain

(Figure 5.16). This indicates an activation of the HOG pathway in the *xlnR* deletion strain when exposed to glucose for 48h.

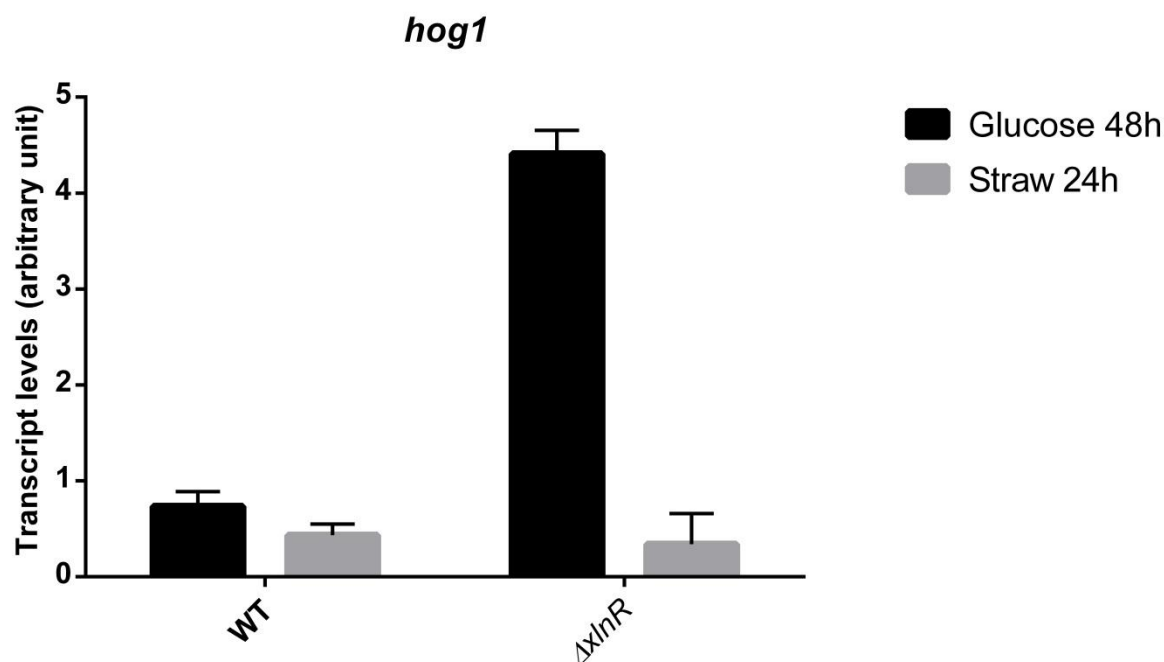


**Figure 5.16:** Expression of the *hog1* gene in *A. niger* grown in glucose and wheat straw media for the WT and the  $\Delta xlnR$  strains. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. The expression values represent the expression fold change relative to the 48h glucose culture. \* represents the condition for which the T-test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

### 5.3.3 Deletion of the An11g06750 gene in *A. niger*

The gene An11g06750 encodes a Zn<sub>2</sub>Cys<sub>6</sub> TF. In the phosphoproteomic dataset, the protein was found in the WT condition but not in the  $\Delta xlnR$  samples. This suggests that the TF coded by this gene could be under the regulation of XlnR. This gene has not been previously characterised in *A. niger* or other filamentous fungi. It is the homologue of the *CTI6* gene of *S. cerevisiae* which encodes a component of the Rpd3L histone deacetylase complex.

#### 5.3.3.1 Growth phenotype of the $\Delta$ An11g06750 strain

The pH was measured in cultures grown for 48h in glucose before the mycelium was transferred to wheat straw medium. After 24h and 48h in wheat straw, the pH was measured

in the cultures. For the glucose condition, after 48h of culture, the pH had dropped from 6.5 to 2.87 for the WT strain and from 6.5 to 3 for the  $\Delta An11g06750$  strain. In wheat straw, after transfer to the medium, the pH had dropped from 6.5 to 3.37 after 24h and to 2.84 after 48h for the WT strain and from 6.5 to 3.2 after 24h and to 3.4 after 48h for the  $\Delta An11g06750$  strain (**Figure 5.17**). A two-way ANOVA test was performed within the same condition (e.g. glucose 48h) to show differences between the two strains. The results from the test showed that only the wheat straw 48h time point had significant difference between the WT and the  $\Delta An11g06750$  strains.

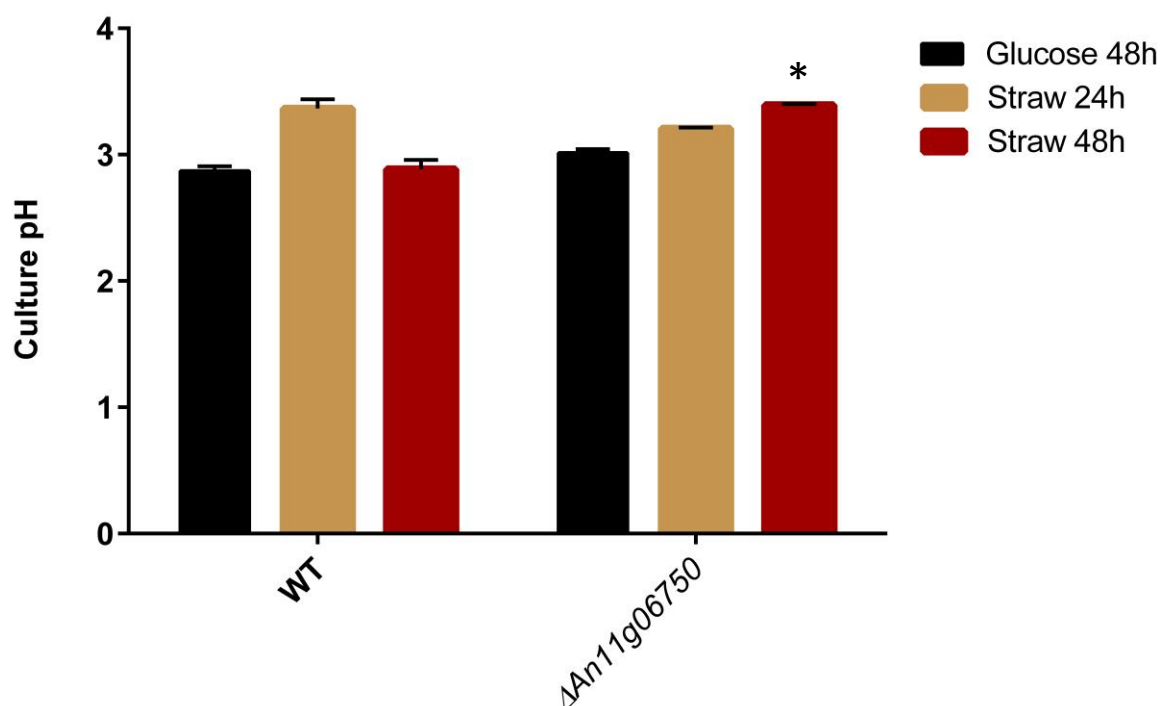


**Figure 5.17:** pH measurements from glucose and wheat straw cultures. For both substrates the starting pH of the medium was set up at pH6.5. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. A two-way ANOVA was performed for each condition in comparison to the WT strain. A significant difference was observed between the WT and the  $\Delta An11g06750$  strain for the wheat straw 48h time point.

The content of the cultures in glucosamine was measured at each time point and the equivalent dry weight was calculated back using a standard curve of known glucosamine content of known dry weight samples. After 48h in glucose the dry weight of the WT strain (from 1.5g wet weight) was 0.105g and 0.1g in the  $\Delta An11g06750$  strain. The dry weights after 24h and 48h in wheat straw were 0.16g and 0.19g respectively for the WT strain and

0.14g and 0.175g for the  $\Delta An11g06750$  strain (**Figure 5.18**). A two-way ANOVA test was performed within a same condition (e.g. glucose 48h) to show differences between the two strains. The results from the test showed that the differences between the WT strain and the  $An11g06750$  deletion strain were not significant.

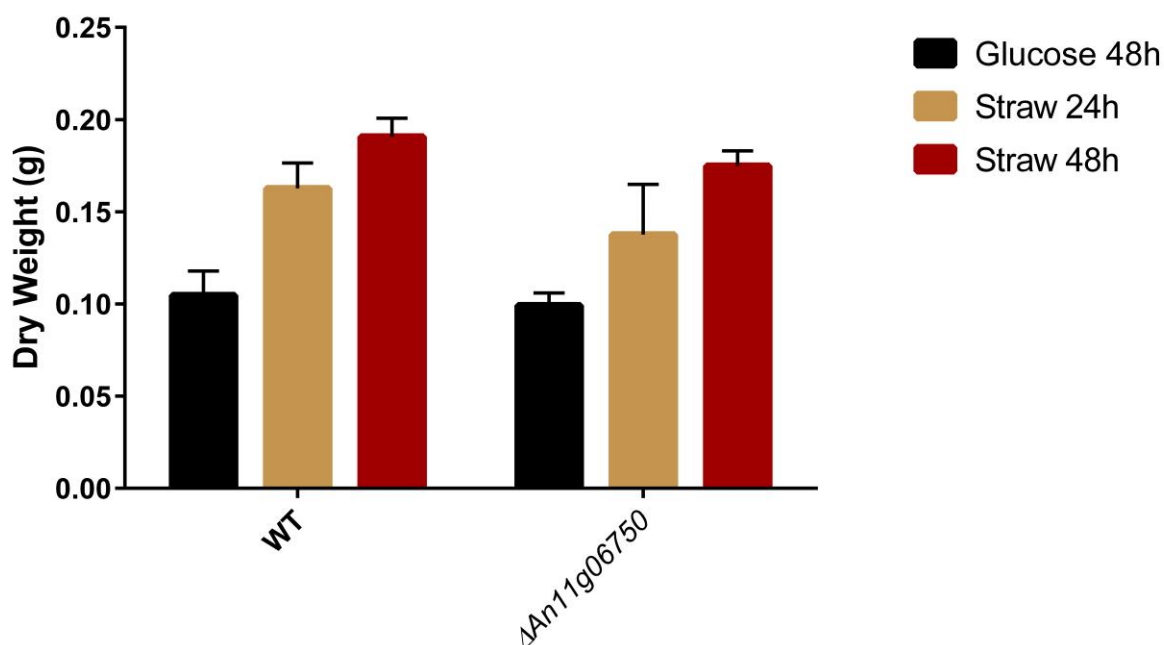


**Figure 5.18:** Increase in the biomass content of the WT and the  $\Delta An11g06750$  cultures in glucose and wheat straw. The wheat straw cultures were inoculated with 1.5g wet weight mycelium from the corresponding glucose culture (ca. 0.1g dry weight). The glucosamine content was measured from 1.5g (wet weight) for the glucose culture and from the content of the full culture for the wheat straw cultures and the dry weight was calculated back using a standard curve of the glucosamine content against the dry weight. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. No significant differences were observed between the WT and the  $\Delta An11g06750$  strain.

#### 5.3.3.2 Expression of GH-encoding genes in the $\Delta An11g06750$ strain

The transcript levels of the *cbhA*, *eglC* and *bglB* genes were measured in the WT and the  $\Delta An11g06750$  strains. The fold change of *cbhA* transcript levels, between the glucose culture and the wheat straw culture was 88, 7 for the *eglC* gene and 5 for the *bglB* gene in the  $\Delta An11g06750$  strain (**Figure 5.19**). This is 49 times (*cbhA*), 47 times (*eglC*) and 20 times (*bglB*) less than for the WT strain when compared to the glucose cultures.

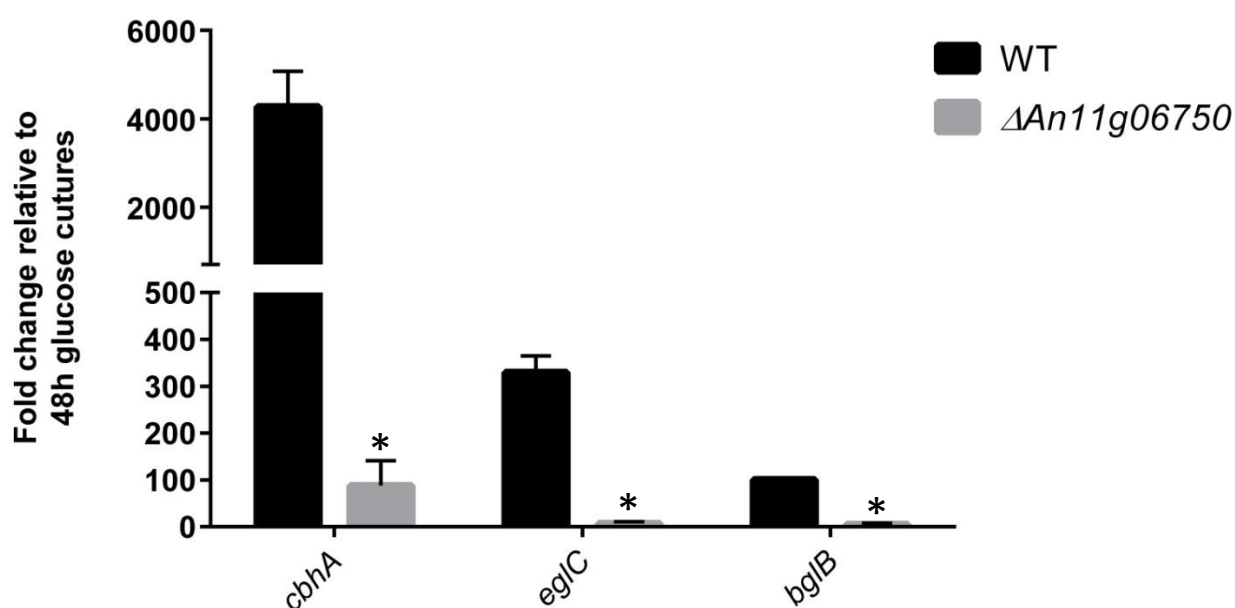


**Figure 5.19:** Expression of the *cbhA*, *egIC* and *bglB* genes in *A. niger* grown in wheat straw media for the WT and the  $\Delta An11g06750$  strains. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. The expression values represent the expression fold change relative to the 48h glucose culture. \* represents the condition for which the T-test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

#### 5.3.3.3 Expression of the *xlnR* gene in the $\Delta An11g06750$ strain

When looking at the transcript level of the *xlnR* gene in the  $\Delta An11g06750$  strain in glucose and in wheat straw, a decrease in the transcript level was observed in the glucose and wheat straw conditions for the  $\Delta An11g06750$  strain when compared to the WT strain (**Figure 5.20**).

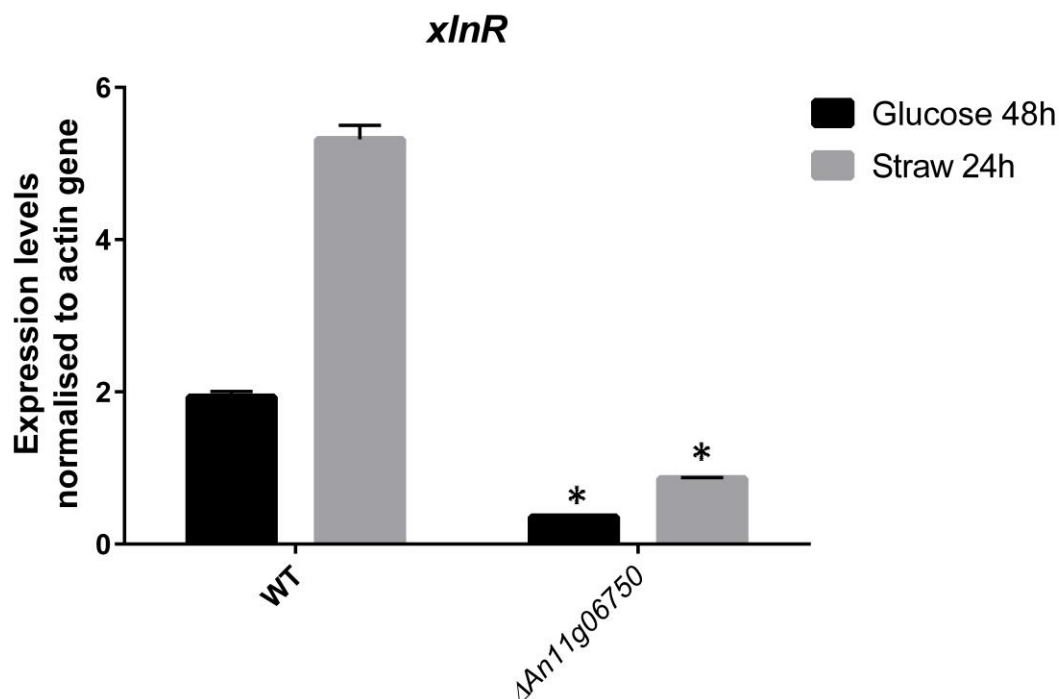


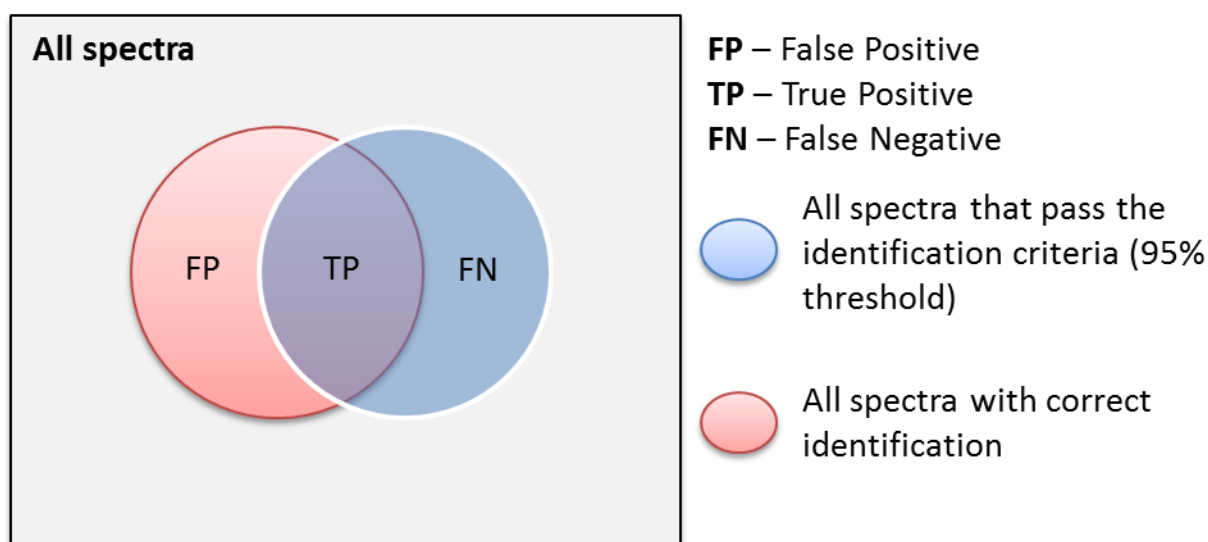
**Figure 5.20:** Expression of the *xlnR* gene in *A. niger* grown in glucose and wheat straw media for the WT and the  $\Delta An11g06750$  strains. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. The expression values represent the expression fold change relative to the 48h glucose culture. \* represents the condition for which the T-test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

## 5.4 Discussion

Phosphorylation is a key reversible modification that regulates protein function, subcellular localisation, complex formation, degradation of proteins and therefore cell signalling networks. With all of these modification results, it is assumed that up to 30% of all proteins can be phosphorylated, some multiple times. Phosphoproteomics provides clues on what protein or pathway might be activated because a change in phosphorylation status almost always reflects a change in protein activity. Therefore, exposing *A. niger* to glucose, in a non-starving condition for 48h and then transferring it to wheat straw, can lead to changes in the phosphoproteomic profile of cells in the two conditions.

Mass spectrometry is the most widely used method for the study of phosphoproteomes (Mann *et al.* 2001). After digestion of the proteins, the peptides are marked and enriched before being analysed by LC MS/MS. The spectra obtained are then compared to

theoretical spectra generated *in silico* from theoretical peptides. Only a fraction of the MS/MS spectra obtained leads to the identification of the corresponding peptides. This step depends on the spectrometer performance, on the quality of the MS/MS spectra obtained and on the presence of the peptide in the database used for the identification. These parameters could explain why only a low number of phosphopeptides were found in this work. Also, the target-decoy strategy was used for the identification of the phosphopeptides. It relies on the creation of a “target-decoy” database which is firstly achieved by obtaining all the sequences of the target proteins of the studied samples (Uniprot *A. niger*). Then, a decoy database is generated that preserves the amino acid composition of the target database. The target-decoy database is then obtained by combining the target and the decoy databases together (Figure 5.1 Appendix). This strategy allows for the estimation of the probability for an identification to be correct which depends on the accuracy of the identification and on the sensitivity of the analysis (Elias *et al.* 2010). Therefore, depending on the parameters used for the analysis, the number of phosphopeptides can vary significantly. In the case of this study, a choice for an optimised accuracy of the identification could have led to a number of true positive identifications lower than in the case of an optimisation of the sensitivity of the identification (**Figure 5.21** and **5.2** Appendix).



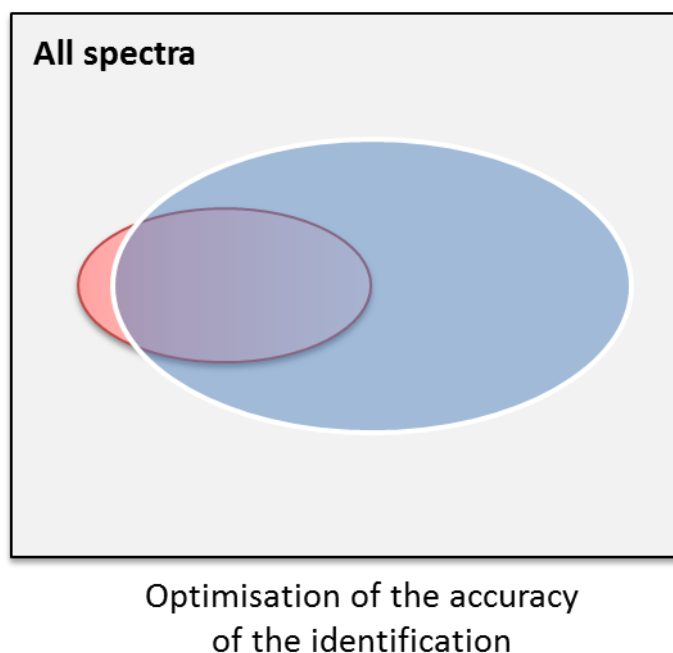


**Figure 5.21:** Selection criteria applied to score distributions (left) determine the form of the Venn diagrams. Application of a single score threshold, which excludes most incorrect identifications can yield an acceptable precision rate, but yields sub-optimal sensitivity (adapted from Elias *et al.* (2010)).

When the phosphoproteins in *A. niger* in the glucose condition were compared with those found in the 3 time points of exposure to straw it was observed that there were no proteins commonly found in the two conditions for both the WT and the  $\Delta xlnR$  strain. This suggests a specific response for each of the substrates with a pool of phosphoproteins being activated specifically in response to glucose and another pool activated in response to wheat straw. The phosphoproteomic profiles of the WT and  $\Delta xlnR$  strains of *A. niger* showed very few phosphoproteins common to the 2 strains for the same condition. In the WT strain, after 48h of exposure to glucose there was a phosphorylated monosaccharide transporter and two protein kinases. The first one was a phosphotransferase that is an enzyme that catalyses the reaction of phosphorylation. The phosphotransferase is part of the phosphotransferase system (PTS) that transports sugars (glucose/mannose/mannitol) into the cell. It acts by phosphorylating the substrate *via* the phosphotransferase during transport. In the case of glucose, the product obtained after phosphorylation is Glucose-6-Phosphate (G6P), the product of the first reaction of glycolysis which degrades glucose to pyruvate (Deutscher *et al.* 2006; Roseman 1969). The second protein kinase found was a  $\text{Ca}_2^+$ /CaM dependent kinase. These protein kinases are activated by a release of  $\text{Ca}_2^+$ . The formation of G6P (via the phosphotransferase) generates Adenosine TriPhosphate (ATP) whose accumulation

leads to the closure of ATP-dependent channels and to a depolarization of the cell. This cellular depolarization triggers the opening of the  $\text{Ca}_2^+$  channels and the increase of intracellular  $\text{Ca}_2^+$  activating the  $\text{Ca}_2^+/\text{CaM}$  dependent kinase (Hook *et al.* 2001). Only 10 phosphoproteins were found in the glucose condition but it shows consistency for a specific response to glucose.

After 24h of exposure to wheat straw, among the phosphoproteins found there were 4 protein kinases essential for growth of *A. niger*. One of them is a RIM15-like protein that positively regulates the entry into stationary phase of cells under starvation condition such as when exposed to wheat straw (van Munster *et al.* 2014). In *S. cerevisiae*, Rim15p phosphorylates Igo1p and Igo2p that are part of TORC1, a complex of proteins that regulates cell growth and metabolism in response to environmental cues. The second protein kinase was a phototropin-like Ser/Thr protein kinase. This protein is the homologue of *nrc-2* in *Neurospora crassa*. *Nrc-2* in *N. crassa* has a role in growth and development by controlling the entry into the asexual developmental program (Kothe *et al.* 1998). The presence of these proteins as well as maltose and fucose permeases suggests an adaptation of *A. niger* to the new substrate to support its growth. Moreover, Delmas *et al.* (2012), showed that the major enzymes for degradation of wheat straw were induced after 24h of exposure to straw which is consistent with the transport of sugars like maltose and fucose.

XlnR is the most studied of the transcriptional activators involved in the regulation of glycosyl hydrolase genes. XlnR regulates the expression of cellulases, hemicellulases and also accessory enzymes (Hasper *et al.* 2000; van Peij *et al.* 1998b). It is the main regulator of xylan degradation in *Aspergillus spp*, therefore studying the phosphoproteomic profile of the  $\Delta\text{xlnR}$  strain when exposed to wheat straw can provide important information on this regulation. When the *A. niger*  $\Delta\text{xlnR}$  strain was exposed to glucose for 48h, 147 phosphoproteins were detected in contrast to only 10 phosphoproteins detected for the WT strain in the same condition. Among these phosphoproteins there were protein kinases such as an aspartokinase or a glycogen synthase kinase. Aspartokinase is an enzyme that catalyzes the phosphorylation of the amino acid aspartate. This reaction is the first step in the biosynthesis of three essential amino acids: methionine, lysine, and threonine, known as the "aspartate family" (Viola 2001). It has been shown in a WT strain of *Klebsiella aerogenes* that the presence of glucose stimulates the loss of aspartokinases I and II (Fulks *et al.* 1985). The presence of glucose may have the same effect in *A. niger* as no aspartokinases were found in the glucose 48h condition for the WT strain. Another protein kinase found was a glycogen synthase kinase (GSK). Phosphorylation of a protein by GSK-

3 usually inhibits the activity of its downstream target (Woodgett 1994; Woodgett 2001). GSK-3 is active in a number of central intracellular signaling pathways, including cellular proliferation, glucose regulation, and apoptosis. Orena *et al.* (2000) showed that when the human GSK3 is inhibited by LiCl the transport of glucose is stimulated. This suggests that in the WT strain XlnR may, under the non-starvation condition inhibit the GSK protein to allow glucose transport. The homologue of the *gsk* gene in *A. niger* is the gene An15g00170. If the protein is found to be phosphorylated in the dataset, the gene itself was not found to be induced in the RNA-Seq dataset. The RPKM values corresponding to the An15g00170 gene were the same in the glucose 48h time point and after 24h of exposure to wheat straw. Moreover 2 phosphatases were found in this condition. Phosphatases are enzymes that remove a phosphate group from its substrate by hydrolysing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group (dephosphorylation) (Hunter 1995). The phosphorylation of a protein can make it active or inactive. Therefore, a phosphatase dephosphorylating a protein can make it active in the  $\Delta xlnR$  strain when the same protein was inactive in the WT strain suggesting that XlnR may have a role in maintaining these proteins in an inactive state.

After 24h of exposure to wheat straw the number of phosphoproteins found dropped to 7 in the  $\Delta xlnR$  strain. None of them are involved in the growth of *A. niger* or in the regulation as no protein kinase or transcription factors were found. This is consistent with other studies carried out on the XlnR protein. Van Peij *et al.* (1998b) showed that XlnR was involved in the regulation of three major genes associated with xylan degradation: *xlnB*, *xlnC* and *xlnD*, encoding endoxylanases B, C and  $\beta$ -xylosidases, respectively. Accessory enzymes regulated by XlnR are involved mainly in hemicellulose degradation such as the acetylxylan esterase A or the feruloyl esterase A (de Vries *et al.* 1999a; de Vries *et al.* 1999b). XlnR is also associated with the expression of some cellulase genes such as engluconases *eglA* and *eglB*. Hasper *et al.* (2002) showed that the endoglucanase (GH74)-encoding gene *eglC* was transcriptionally regulated by XlnR in *A. niger*. Van Peij (1998b) and Hasper (2002) carried out studies to demonstrate the importance of XlnR in the activation of the expression of GH-encoding genes in *A. niger*. They used a wild-type strain, an *xlnR* deletion strain and a strain containing multiple copies of *xlnR*. The studies showed that, in a strain lacking a functional *xlnR*, the genes encoding XlnB, XlnC and XlnD (van Peij *et al.* 1998b) and EglC (Hasper *et al.* 2002) were not expressed. In contrast, the transcription levels of these genes were increased in the *xlnR* multiple-copy strains. This highlights the role of XlnR in the regulation of their expression and in the ability of *A. niger* to grow in presence of a complex substrate such as wheat straw. In the  $\Delta xlnR$  strain, these enzymes are not produced anymore and, as a result, *A. niger* cannot grow on wheat straw explaining why after 24h

proteins involved in its development were not found. These results are consistent with the results presented in Chapter 3 showing that the  $\Delta xlnR$  strain was not able to grow on wheat straw with its glucosamine content being much lower than for the WT strain. Also, the measurement of the pH in the  $\Delta xlnR$  cultures showed that there was no drop of pH in the wheat straw cultures suggesting that the enzymes necessary for wheat straw degradation are not produced. This was confirmed in Chapter 4 with transcript level measurements of the *eglC*, *bglB*, *cbhA* and *xynA* genes in the *xlnR* deletion strain. The results showed that all the genes tested had their transcript levels substantially decreased in the  $\Delta xlnR$  strain compared to the WT strain.

The inductions of the *slt2* and *hog1* genes also confirm the results obtained in Chapter 4. The results presented in the previous chapter showed that the *xlnR* deletion strain was more permeable possibly due to a change in its cell wall composition. By having increased cell permeability the deletion strain is likely to be more sensitive to osmotic stress. In *A. fumigatus*, the homologue of yeast Hog1p, SakA, was shown involved in the response to osmotic stress (Kawasaki *et al.* 2002). Also, Tmk3, the homologue of yeast Hog1p in *T. reesei*, has been shown to act in the CWI pathway similarly to SlT2-type MAPK showing again differences in the regulation in filamentous fungi (Wang *et al.* 2013). It is not clear in *A. niger* if the homologue of Hog1p or SlT2p would be responsible for the response to osmotic stress. The deletion of these two genes could help deciphering their respective roles.

The results described in this chapter have not been confirmed using alternative methods which is necessary to do to confirm the changes in the phosphoproteome that have been observed. One of the methods used for the confirmation of phosphoproteomic data is the use of two-dimensional (2-D) gel electrophoresis. In the first dimension, proteins can be separated based on differences in isoelectric point (pI). In the second dimension, they can be separated according to molecular weights. Following separation, 2-D electrophoresis gels are stained for protein visualization and analysis. The proteins of interest can then be identified using mass spectrometry. Another method is to detect phosphoproteins according to their phosphorylation sites using antibodies. Different antibodies exist that target the tyrosine, threonine or serine phosphorylation sites but most of them have been designed to be specific for human proteins and were shown to lack of specificity for *A. niger* proteins in this study. Therefore in order to be able to use western blot as a confirmation for the data presented here, specific antibodies for *A. niger* proteins should be produced. An antibody is a large, Y-shape protein produced by plasma cells that is used by the immune system to identify and neutralize pathogens such as bacteria and viruses. The antibody recognizes a

unique molecule of the pathogen, called an antigen, via the variable region (Janeway 2001; Litman *et al.* 1993). Monoclonal antibodies are monospecific antibodies that are made by identical immune cells that are all clones of a unique parent cell, in contrast to polyclonal antibodies which are made from several different immune cells. Monoclonal antibodies have monovalent affinity, in that they bind to the same epitope where as polyclonal antibodies are a collection of immunoglobulin molecules that react against a specific antigen, each potentially identifying a different epitope. The production of antibodies for *A. niger* proteins for the different sites of phosphorylation would allow for the identification of differences in the phosphoproteomes of the WT strain and the *xlnR* and *creA* deletion strains.

Nevertheless, the data obtained in this work showed that the deletion of *xlnR* led to an increase in the number of proteins phosphorylated in the glucose condition. This is in line with the results presented in the Chapter 4 that showed that the  $\Delta xlnR$  strain had increased cell permeability and a different cell wall composition. These results are confirmed with the induction of the *slt2* and *hog1* gene that are involved in the response to osmotic stress. Therefore, a suggested model is that XlnR, in addition to regulate GH-encoding genes expression, may act upon an Upstream Activating System (UAS) to activate protein kinase or TFs that would be involved in different cellular processes (**Figure 5.23**).

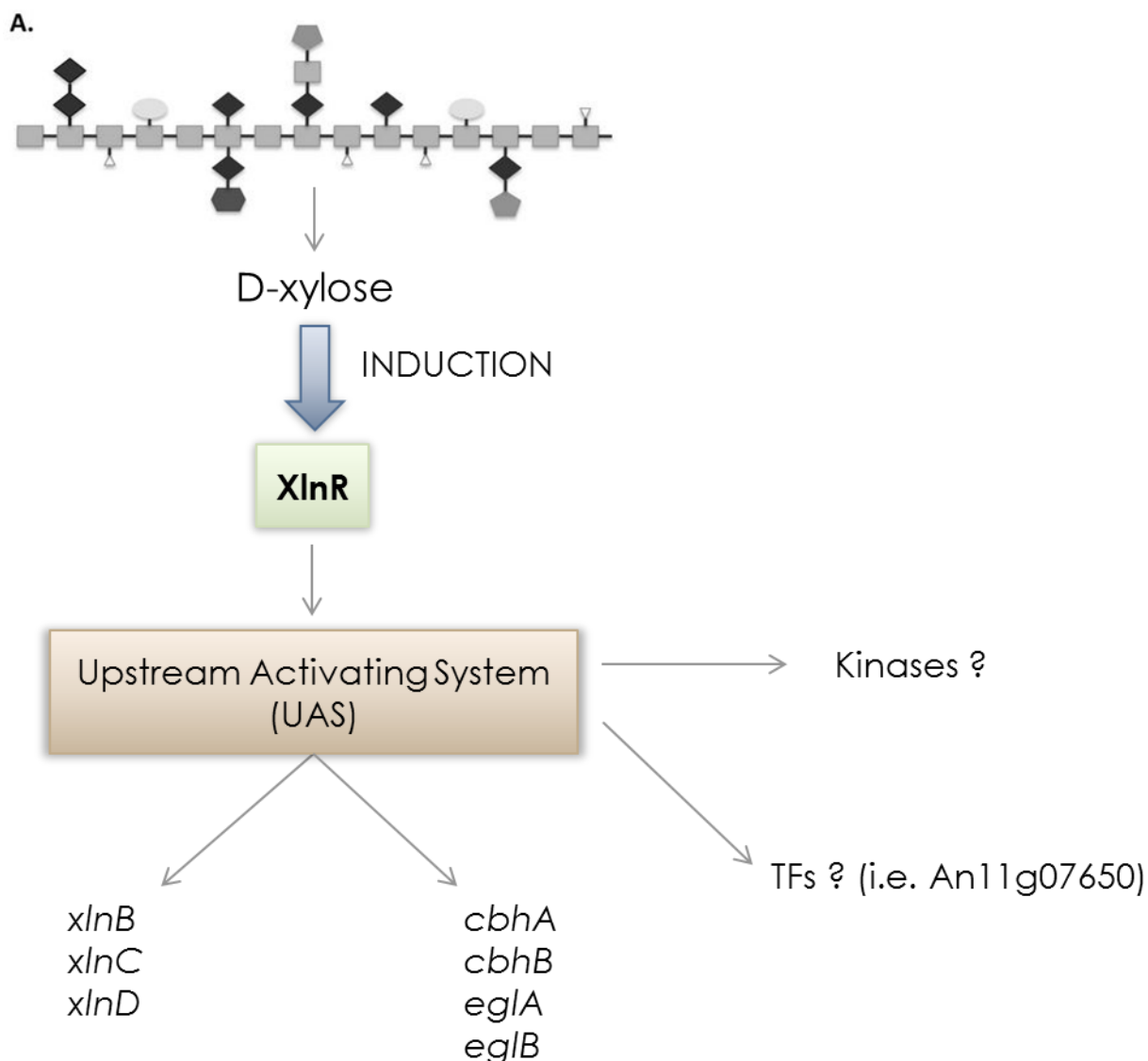


**Figure 5.23:** The release of xylose leads to the induction of XlnR that triggers the activation of an upstream activating system (UAS). According to the dataset presented here, this UAS could be composed of kinases or TFs. This system then leads to the expression of genes encoding GH enzymes such as *cbhA* or *eglB* for example.

## 5.5 Future work

The preliminary data of this study showed interesting results and may provide new information on the importance of XlnR in the regulation of GH-encoding genes expression but also in controlling the environment to which *A. niger* is exposed depending on nutrient availability. Further analysis of the dataset will have to be done as well as some qRT-PCR to examine the expression of genes encoding some of the phosphoproteins found in both the WT and the  $\Delta xlnR$  strain especially on the 2 phosphatases found in the glucose

condition for the  $\Delta xlnR$  strain. The characterisation of the TF encoded by the gene An11g06750 should be done in depth as this gene has not been characterised previously. This could lead to the description of a new TF of potential interest for biofuels production. Confirmation at the proteomic level of the dataset will also have to be done. This could be done by using the 2-D electrophoresis coupled to mass spectrometry for the identification of the main different proteins in the different dataset.

**Chapter 6: Possible roles of hydrophobic surface  
interacting proteins in the breakdown of  
lignocellulose by *A. niger***

## 6.1 Introduction

In this study, the term 'hydrophobic surface interacting protein' (HSIP) is used to denote secreted, amphipathic proteins that are recruited (adsorbed) to and interact with hydrophobic surfaces and hydrophobic:hydrophilic interfaces. The fungal proteins of this nature that are described in this work belong to two distinct protein families; hydrophobins (major class of HSIPs) and the HsbA family.

### 6.1.1 The hydrophobins: general features and biological roles

Hydrophobins are a large family of small proteins (around 100 amino acids), produced by filamentous fungi at different developmental stages, self-assembling at hydrophobic/hydrophilic interfaces into amphipathic biofilms. Hydrophobins can be secreted into the surroundings or retained in the fungal structures, such as fruiting bodies or mycelium. Their biological functions seem to be diverse, but always related to interactions with interfaces or surfaces. Described as the most surface active proteins ever known, hydrophobins play a role as a coating/protective agent, in adhesion, surface modification, or other type of functions that require surfactant-like properties (Linder 2009). For instance, they allow fungi to escape an aqueous environment, lowering the surface tension (**Figure 6.1**), and mediate attachment of fungi to hydrophobic surfaces (Wessels 1997).

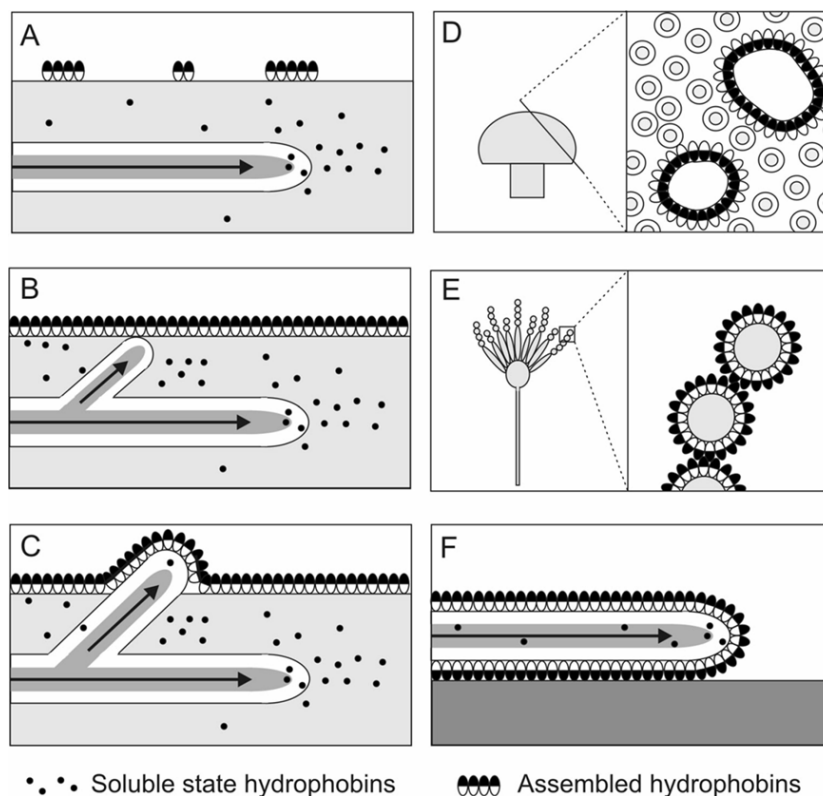
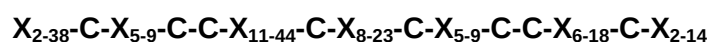


**Figure 6.1:** Model of the biological role of hydrophobins during growth and development of filamentous fungi. Soluble-state hydrophobin is secreted at the tip of the growing, submerged hypha and diffuses into the aqueous environment (A). The water surface tension is lowered due to the assembly of hydrophobins into an amphipathic membrane at the medium-air interface (B), allowing the hypha to breach the medium-air interface and grow into the air. The hydrophobin secreted by emerging aerial hyphae cannot diffuse into the environment and assembles at the cell wall-air interface (C). Aerial cavities in fruiting bodies are lined with an assembled hydrophobin layer preventing water to fill these cavities (D). Hydrophobins also play a role in the dispersal of spores by coating them with an amphipathic hydrophobin layer (E). Via assembly at the interface between the cell wall and a hydrophobic surface, hydrophobins mediate attachment to the surface, for example, during infection of host tissue (F) (Wösten *et al.* 2015).

Despite the low sequence similarity between hydrophobins, they have a characteristic pattern of eight conserved cysteine (Cys) residues forming four non-sequential disulphide bonds that stabilize the structure.



The distribution of the Cys residues, the hydropathy pattern, the morphology and the stability of assembled biofilm allow the division of hydrophobins into two classes: class I and class II.

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Class I hydrophobin genes have been isolated from Ascomycetes and Basidiomycetes, while the genes encoding class II hydrophobins have not been identified in Basidiomycetes. Class I hydrophobins generate very insoluble assemblies, which can be solubilised only in strong acids such as trifluoroacetic acid or formic acid, whilst assemblies of class II hydrophobins can be solubilized in ethanol or sodium dodecyl sulphate aqueous solutions (Wösten *et al.* 2000).

### 6.1.1.1 Structure of hydrophobins

The structural analysis of proteins and the resulting better understanding of structure-function relationships would help understanding their potential biological roles. In the conserved pattern of eight Cys residues (**Figure 6.2**), the second and third, and the sixth and seventh Cys residues are invariably adjacent. The inter-Cys regions of class II hydrophobins are well conserved. In contrast, in class I hydrophobins the region between the third and the fourth Cys (loop Cys3-Cys4) varies from 4 to 44 residues, whilst the region between the fourth and the fifth Cys (loop Cys4-Cys5) varies from 8 to 23 residues (Sunde *et al.* 2008).

#### Class I hydrophobins

```

SC4  CNSG-PVQ--CCNETTT--VANAQ-KQGLLG---LLGVVV---GPITGLVGLNCSP---ISVVG---LTGNSCTA-QTVCCDHVTQNG-----LVN--VGC
PRI2  CNNG-SLQ--CCNSSMTQDRGNLQIAQGVVLGGLLGLGGLLDLVDLNLALIGVQCSP---ISIVG-----NANTCTQ-QTVCCSNNNFNG-----LIA--LGC
SC3   CTTG-SLS--CCNQVQS---ASSPVTALG---LLGIV---LDLNVLVGISCSPP---LTVIG---VGGSGCSA-QTVCCENTQFNG-----LIN--IGC
ABH1  CDVG-EIH--CCDTQQT---PDHTSAAASG---LLGVP---INLGAFLGFDCTP---ISVLG---VGGNNCAA-QPVCCGTGNQFTA-----LINA-LDC
EAS   CSID-DYKPYCCQSMMSG-----PAGSPGL-----LNLIP---VDLSASLG--C-----VVG---VIGSQCGA-SVKCCCKDDVTNTGNSFLIINA-ANC
HCF1  CAVGSQIS--CCTTNSS-----GSD-----ILGNV-----LGGSCLLDN--VSLISSLN-----SNCPAGNTFCPPS-NQDG-----TLNINVSC
MPG1  CGAEKVVS--CCNSKELK--NSKSGAE-----IPIDV-----LSGECKNIPINILTINQLI--PINNFCSD-TVSCCSGEQIG-----LVN--IQC
RODA  CGDQAQLS--CCNKATYAG-DVTDIDEGILAGTLKNLIGGGS---GTEGLGLFNQCSKLDLQIPVIGIPIQALVNQKCKQ-NIACCQNSPSPDASG--SLIGLGLFC

```

#### Class II hydrophobins

```

HFBII CPTG-LFSNPCCCATQVLGLGLDCKVPSQNVYDGTDFRNVCAKTGA-QPLCCVAP-VAGQALLC
HFBII CPTG-LFSNPCCCATNVLDLIGVDCKTPTIAVDTGAIQAHCAASKGS-KPLCCVAP-VAGQALLC
SRH1  CPNG-LYSNPCCGANVLGVAALDCHTPRVDVLTGPIFQAVCAEAGGKQPLCCVVP-VAGQDLLC
CU     CTGL-LQSEACCCNTDILGVANLDCGPPSPVPTSPSQFQASCVAADGGRSARCCCTLS-LLGLALVC
CRP    CSST-LYSEACCCATDVLGVADLDCETVPETPTSPSSSFESICATSG-RDAKCCCTIP-LLGQALLC
MGP    CSG--LYGSAQCCATDILGLANLDCGQPSDAPVDADNFSEICAAIG-QRARCCLVP-ILDQGILC
HCF6   CPAN---RVPCQCCQLSVLGVADVTCASPSSGLTSSVSAFEADCAANDG-TTACCLIP-VLGLGLFC
HYD4   CPDGLIGTQCCLDLVGLVSGECSSPSKTFNSAKEFQEICAAAG-QKARCCFLSEVFTLGAPC

```

**Figure 6.2:** Amino acid sequence comparison of class I and II hydrophobins. Only amino acids between the first and last Cys residues are shown due to high sequence variations at the termini. The conserved Cys residues are highlighted in yellow with the conserved disulphide bonding pattern indicated with brackets. The relatively low degree of sequence conservation is apparent. The abbreviations used are: SC3, *Schizophyllum commune* (accession P16933); SC4, *S. commune* (accession P16934); EAS, *Neurospora crassa* (accession Q04571); MPG1, *Magnaporthe grisea* (accession P52751); HCF1, *Cladosporium fulvum* (accession Q00367); ABH1, *Agaricus bisporus* (accession P49072); PRI2, *Agrocybe aegerita* (accession Q9Y8F0); RODA, *Aspergillus fumigatus* (accession P41746); HFBII, *Trichoderma reesei* (accession P52754); HFBII, *T. reesei* (accession P79073); CU, *Trichoderma reesei* (accession P52754); HFBII, *T. reesei* (accession P79073); CU, *Trichoderma reesei* (accession P52754).

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*Ophiostoma ulmi* (accession Q06153); CRP, *Cryphonectria parasitica* (accession P52753); HCF6, *C. fulvum* (accession Q9C2X0); MGP, *M. grisea* (accession O94196); HYD4, *Gibberella moniliformis* (accession Q6YF29) and SRH1, *Trichoderma harzianum* (accession P79072) (Sunde *et al.* 2008).

In general, the primary structure of class I hydrophobins comprises 100-125 amino acids and there can be glycosylation. The most detailed structural work on a class I hydrophobin has been carried out for the EAS hydrophobin from *Neurospora crassa*, studied by Nuclear Magnetic Resonance (NMR) (Kwan *et al.* 2006). EAS forms a four stranded  $\beta$ -barrel structure, an additional two stranded  $\beta$ -sheet, interrupted by some disordered regions (**Figure 6.3A**). The structure (about 3nm of diameter) displays a complete segregation of charged and hydrophobic residues on its surface, forming a hydrophobic patch and making the protein amphipathic.

Class II hydrophobins have shorter sequences comprising 50-100 amino acids, and are more easily handled, because of their reduced tendencies to aggregate. The crystal structures of two class II hydrophobins, Hfbl and HfbII of *Trichoderma reesei* have been determined, and several other studies on these two proteins have been carried out (Hakanpää *et al.* 2006). The class II hydrophobin fold is compact and globular (about 2nm of diameter) and consists of a small antiparallel  $\beta$ -barrel formed by two hairpins connected by a stretch of an  $\alpha$ -helix, without disordered loop. The helix occupies the same region of space as the additional  $\beta$ -sheet in the EAS protein of *N. crassa* EAS protein (Zampieri *et al.* 2010). The overall topology is determined by the cross-linking of the secondary structure via disulphide bonds giving stability thus forming the peculiar amphiphilic structure of the proteins (**Figure 6.3B**).

Therefore, by a structural superimposition of the proteins EAS and HfbII, it has been shown that class I and class II hydrophobins share the same fold despite a very low sequence similarity (**Figure 6.3C**). Large differences are however found in the loops of the  $\beta$ -hairpins of the central  $\beta$ -barrel, indicating a structural reason for different molecular assembling between the two classes. Moreover, hydrophobins can be post-translationally modified. For instance, the protein SC3 from *Schizophyllum commune* contains 16-22 O-linked mannose units. These sugar molecules influence the properties of the assembled protein (de Vocht *et al.* 1998; Scholtmeijer *et al.* 2002).

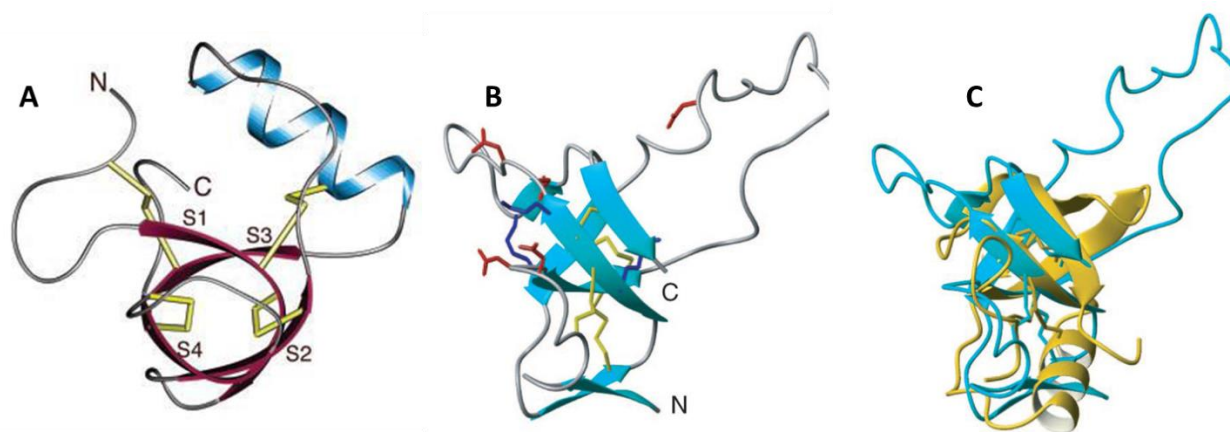


**Figure 6.3:** (A) The X-ray crystal structure of the *T. reesei* Class II hydrophobin HfbII determined by crystallography, containing an  $\alpha$ -helix (blue), and the four  $\beta$ -strands of the  $\beta$ -barrel (S1-4) that is stabilised by four disulphide bridges (yellow) (Hakanpää *et al.* 2004). (B) The NMR structure of the *N. crassa* Class I hydrophobin EAS, containing three  $\beta$ -strands in addition to the four conserved  $\beta$ -strands of the hydrophobin  $\beta$ -barrel, which is stabilised by four disulphide bridges (yellow) (Kwan *et al.* 2006). (C) An overlay of the NMR structure of *N. crassa* EAS (blue) with the X-ray crystal structure of *T. reesei* HfbII (yellow), highlighting both the structural variation between these hydrophobins and the conservation of the  $\beta$ -barrel (Kwan *et al.* 2006).

#### 6.1.1.2 Hydrophobin self-assembly

Hydrophobins can self-assemble in solutions, forming oligomers, or at hydrophobic/hydrophilic interfaces (water/air, water/oil, or water/solid surfaces) into amphiphilic biofilms.

Experimentally characterized hydrophobins have been observed to exhibit a wide range of tertiary structures (Kallio *et al.* 2007), with their monomers capable of forming (hetero and/or homo) dimers, multimers and/or rodlets. Rodlets are amyloidogenic hydrophobin multimers in which a single  $\beta$ -sheet from each hydrophobin subunit come together to form an amyloidogenic cross- $\beta$  core (Macindoe *et al.* 2012). In the case of the hydrophobin EAS, this  $\beta$ -sheet is distinct from those of the  $\beta$ -barrel and not present in monomeric EAS, instead located on a poorly conserved but highly amyloidogenic domain ( $X_n$ ) between Cys<sub>7</sub> and Cys<sub>8</sub>.

Hydrophobin monomers or multimers can then further assemble into polymeric hydrophobin monolayers, one surface of which is hydrophilic and the other hydrophobic (due to the exposed hydrophobic domain of hydrophobin  $\beta$ -barrels), that are then able to bind to both hydrophobic and hydrophilic surfaces respectively via hydrophobic and hydrophilic

interactions. Research into the *T. reesei* hydrophobin HfbII suggests that its polymerisation into monolayers occurs as part of a wider multimerisation system (**Figure 6.4**), in which HfbII monomers are able to self-assemble into numerous, distinct multimers (Hakanpää *et al.* 2006; Kallio *et al.* 2007).

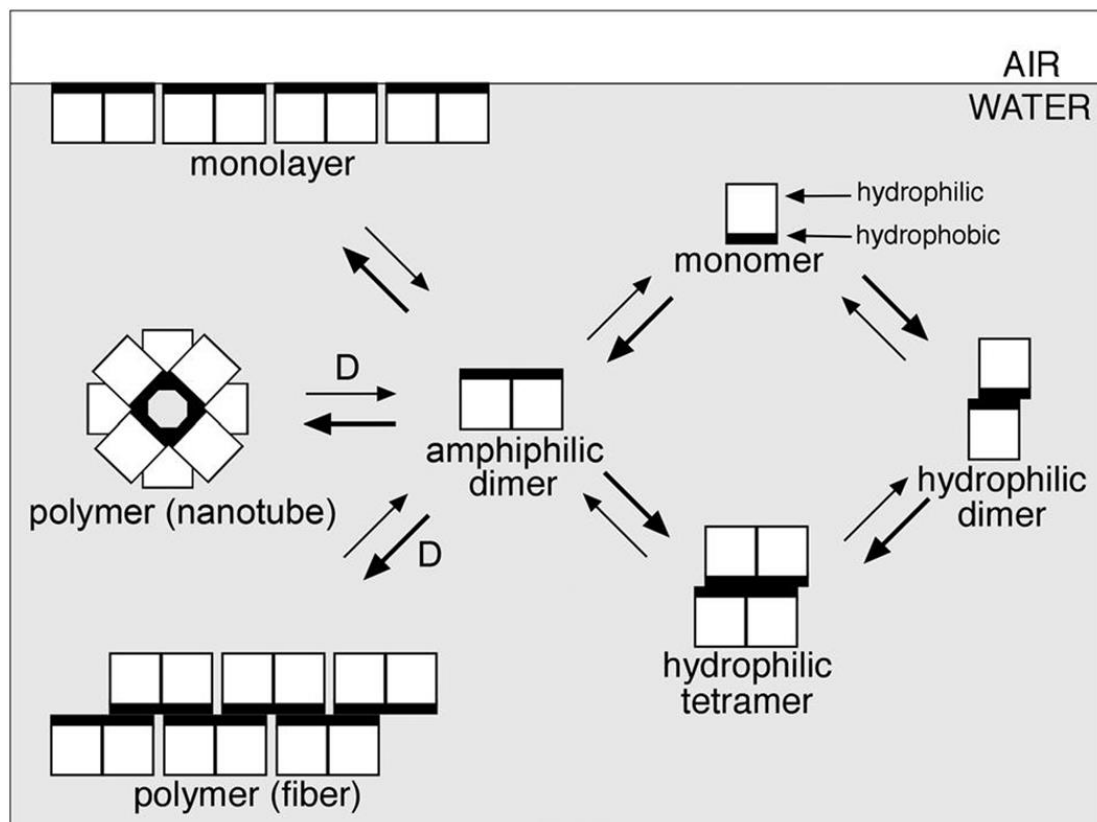


**Figure 6.4:** The functional mechanism for the multimer formation of the Class II hydrophobin HfbII, proposed by Kallio *et al.* (2007), with the example of air:water interface monolayer formation. *D* represents the requirement of detergent for structure formation. Note that this mechanism may not apply to all Class II hydrophobins.

As not all of these forms are capable of polymerisation, this serves to modulate hydrophobin monolayer formation by sequestering (amphipathic) unpolymerised hydrophobins into (hydrophilic) dimers and multimers, in the absence of a suitable hydrophilic:hydrophobic interface. It has also been suggested that, as rodlet-forming hydrophobins have been observed to initially assemble into a functional rodlet-less monolayer before their multimerisation into rodlets (de Vocht *et al.* 2002), rodlet formation may be another strategy of preserving hydrophobins (Kallio *et al.* 2007); sequestering them at hydrophilic:hydrophobic interfaces instead of, or in addition to soluble (hydrophilic) dimers and multimers.

**6.1.1.3 Functional diversity of hydrophobins**

Despite differences in their physical properties, both class I and II hydrophobins have been observed to have a broad range of functions, many of which relate to fungal development. Although not universal (Mosbach *et al.* 2011), it has been demonstrated that hydrophobins are responsible for conidial hydrophobicity in numerous filamentous fungi (including aspergilli) by coating the surface of hydrophilic conidia with amphipathic monolayers (Paris *et al.* 2003; Wösten *et al.* 2000). This hydrophobic conidial surface decreases their affinity for hydrophilic surfaces, making them more difficult to suspend in water and thereby promoting their aerial dispersal. Post-germination, hydrophobins have also been found to assemble into monolayers at air:water interfaces, allowing hyphae to breach this interface by reducing the surface tension of water (Wessels *et al.* 1991; Wösten *et al.* 2000). The resultant hydrophobin-coated aerial hyphae are then prevented from re-submersion by their hydrophobicity, which may then further develop into reproductive structures, such as conidiophores, for the continuation of the life cycle (Adams *et al.* 1998). Hydrophobins have also been observed to play an analogous role in the utilisation of solid hydrophobic substrates, whereby the coating of hyphae in a monolayer makes them hydrophobic and so better to adhere to the substrate (Wösten 2001). Once bound, hyphae are then able to physically and enzymatically degrade the substrate more effectively, improving the potential for substrate utilisation by the fungi.

Hydrophobins have also been found to perform several functions in pathogenic fungi, relating to host invasion, response evasion and toxicity (Whiteford *et al.* 2002; Wösten 2001). Hydrophobins have been shown to mediate the attachment of pathogenic, hydrophobin-coated conidia to hydrophobic host surfaces (Boucias *et al.* 1988). It has also been found that the hyphal secretion hydrophobins onto host surfaces mediates the attachment of pathogenic fungi to their hosts (Talbot *et al.* 1996). Additionally, this hydrophobin coat also prevents conidia and hyphae from being recognised by their host immune system and thereby prevents the activation of a host response against them (Aimanianda *et al.* 2010). Pathogenic fungi often need to cross a host's protective barrier for successful invasion, mediated by invasive structures such as appressoria or stachels. The hydrophobin MPG1 has been shown to be necessary for the successful invasion of rice by *Magnaporthe grisea* (Talbot *et al.* 1996), whereby a hydrophobin monolayer strongly attaches the fungus to its host and allows the pressure of hyphal growth within the appressorium to overcome the host's protective barrier. Once invasion is successful, the host barrier often needs to be crossed again for the sporulation of the fungus to occur, a process in which hydrophobins such as Cryparin of the plant pathogen *Cryphonectria*

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*parasitica* has been demonstrated to play a role (Kazmierczak *et al.* 2005). In addition to their involvement in the life cycle of pathogenic fungi, the *Ophiostoma ulmi* hydrophobin cerato-ulmin (CU) has been shown to exhibit toxicity to its host, linking it to pathogenicity of the fungus (Richards 1993). It should also be noted that hydrophobins have been demonstrated to play similar roles to pathogenicity in the symbiosis of filamentous fungi (Whiteford *et al.* 2002).

Perhaps the hydrophobin function that is the most relevant for the process of lignocellulose degradation is their ability to improve the degradation of hydrophobic polymers, first described by Takahashi *et al.* (2005) in the *Aspergillus oryzae* hydrophobin RolA. It had already been demonstrated that *A. oryzae* was able to grow on micro-particles of the hydrophobic polymer polybutylene succinate-coadipate (PBSA) as a sole carbon source (Maeda *et al.* 2005), and by analysing transcription under these conditions using cDNA microarrays, various genes were discovered to be highly transcribed as a result of growth on PBSA. One of these genes encoded CutLI, a secreted cutinase that was previously shown to hydrolyse PBSA (Maeda *et al.* 2005), whilst another gene encoded class I hydrophobin RolA. By examining the localisation of these proteins in-culture with *A. oryzae* grown on PBSA, it was determined that secreted RolA is capable of adsorbing to the surface of both hyphal cell walls and PBSA film, likely to result in the formation of amphipathic hydrophobin monolayers. Recently it was also shown that RolA and CutLI interacted through an ionic interaction between amino acid residues of the 2 proteins (Takahashi *et al.* 2015). The adsorption of RolA to the hyphal cell wall is likely to produce hydrophobic hyphae that are able to bind to a hydrophobic substrate, improving the efficiency of physical substrate breakdown by hyphal-substrate interactions. *In vitro*, RolA was found to adsorb to the surface of PBSA and specifically recruit soluble CutLI, greatly increasing the rate of CutLI mediated PBSA hydrolysis compared to soluble CutLI. This suggested to Takahashi *et al.* (2005) that RolA exhibits two distinct functions relating to the degradation of PBSA (1) improving physical substrate breakdown by improving hyphal-substrate interactions and (2) improving enzymatic substrate degradation by recruiting degradative enzymes to the substrate's surface.

Delmas *et al.* (2012) used RNA sequencing to map the transcriptional changes that take place in the fungus when its mycelia are transferred from a simple sole carbon source of glucose, onto the lignocellulosic biomass wheat straw. This research highlighted the potential involvement of numerous genes, many of which were predicted to encode products from a wide range of protein families. Most of the strongly-induced transcripts in response to wheat straw were predicted to be derived from genes encoding Carbohydrate Active

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Enzymes (CAZy) based on their appearance in the CAZy database (Cantarel *et al.* 2009), including glycoside hydrolases, carbohydrate esterases and polysaccharide lyases; enzymes associated with the degradation of lignocellulose. However, it was also demonstrated that various other non-CAZy genes were also induced by these conditions such as hydrophobins and surface interacting proteins. The genome of *Aspergillus niger* encodes 8 hydrophobins among which Hyp1 (homologue of RolA) and HfbD as well as a hydrophobic surface-binding protein HsbA (**Table I**) (Jensen *et al.* 2010). Given the possible similarity between PBSA and lignocellulose degradations, as well as the numerous other roles that hydrophobins play in plant pathogenicity by filamentous fungi and the utilisation of plant biomass as part of their life cycle, a suggested role for hydrophobic surface binding proteins and hydrophobins that these proteins could have a role in recruiting degradative enzymes to the straw surface.

**Table I:** Hydrophobins in *Aspergillus niger* (from Jensen *et al.* (2010))

| Species                    | Gene       | Size (Da) | n(AA) | n(Cys) | Eight cystein pattern           | Theoretical class |
|----------------------------|------------|-----------|-------|--------|---------------------------------|-------------------|
| <i>A. niger</i> CBS 513.88 | An03g02360 | 12486     | 122   | 8      | CN{6}CCN{32}CN{25}CN{5}CCN{4}C  | I                 |
|                            | An03g02400 | 13063     | 131   | 8      | CN{6}CCN{31}CN{23}CN{5}CCN{6}C  | Intermediate      |
|                            | An04g08500 | 14397     | 146   | 8      | CN{7}CCN{39}CN{20}CN{5}CCN{17}C | I                 |
|                            | An15g03800 | 13225     | 130   | 8      | CN{5}CCN{32}CN{6}CN{5}CCN{13}C  | Intermediate      |
|                            | An01g10940 | 10693     | 100   | 8      | CN{14}CCN{17}CN{11}CN{7}CCN{8}C | Intermediate      |
|                            | An07g03340 | 16207     | 162   | 8      | CN{7}CCN{39}CN{21}CN{5}CCN{17}C | I                 |
|                            | An09g05530 | 20465     | 202   | 9      | CN{8}CCN{33}CN{11}CN{5}CCN{16}C | Intermediate      |
|                            | An08g09880 | 9169      | 91    | 9      | CN{7}CCN{16}CN{6}CN{5}CCN{10}C  | Intermediate      |

### 6.1.2 HsbA family: Novel amphipathic proteins

Members of the HsbA family are small, secreted, amphipathic proteins that are capable of binding to hydrophobic surfaces. They were first described in *A. oryzae* by Ohtaki *et al.* (2006). They demonstrated that *A. oryzae* HsbA mediated PBSA degradation by recruiting the cutinase CutLI to the polymer's surface. Despite this apparent functional similarity to the *A. oryzae* hydrophobin RolA, HsbA family proteins are distinct from hydrophobins in both their structure and mechanism of action. Discovered to be an extracellular protein when *A. oryzae* is grown in the presence of micro-particles, it was inferred that HsbA is processed

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and secreted via the Golgi apparatus due to the presence of a KexB cleavage site in the protein's primary sequence. This cleavage site relates to a subtilisin-like processing enzyme encoded by the *kexB* gene in *A. oryzae* (Mizutani *et al.* 2004). The orthologue of the *kexB* gene has been experimentally characterized in *A. niger* (Jalving *et al.* 2000) and is associated with the *A. niger* KEX processing pathway (Spencer *et al.* 1998).

The protein sequence inferred by examining the *A. oryzae* *hsbA* gene did not contain the disulphide bond-forming cysteine residues that are conserved between hydrophobins, confirming that HsbA belonged to a distinct protein family and leading to the characterisation of the HsbA domain (Ohtaki *et al.* 2006). As *A. oryzae* HsbA is the only member of this family that has been functionally characterized, the role and properties of its homologues in other species are still relatively uncertain. HsbA was found to be an amphipathic extracellular protein that, unlike hydrophobins, has yet to be described to either self-assemble into hydrophobic monolayers at hydrophobic:hydrophilic interfaces nor attach to hyphal cell walls. Instead, HsbA attaches to hydrophobic surfaces, demonstrated by adsorption to the surface of PBSA, via antichaotropic salt-dependent hydrophobic interactions. Although HsbA is capable of adsorption to hydrophobic surfaces without them, this was greatly improved by the presence of antichaotropic salts such as NaCl or CaCl<sub>2</sub>, which was suggested by Ohtaki *et al.* (2006) to induce a conformational change in HsbA that increased the protein's affinity for hydrophobic. Although soluble HsbA was found to be unable to bind to CutLI, HsbA that had been adsorbed to the surface of PBSA was capable of binding CutLI and localising it to PBSA. This greatly increased the rate of PBSA degradation and thereby facilitated the polymer's degradation. This adsorption-dependent CutLI was found to be specific, as the substitution of the cutinase by another protein (BSA) did not result in the recruitment of the protein to the PBSA surface. By considering PBSA as a model for hydrophobic substrate, this suggests that the function of HsbA in *A. oryzae*, and potentially other HsbA family proteins, is to specifically recruit degradative enzymes to hydrophobic substrates to increase their efficiency at degrading it. Furthermore, given the analogous relationship between PBSA and enzymatic lignocellulose degradations, there may be similarities between the roles of HSIPs in the degradation of PBSA and lignocellulose.

### 6.1.3 Possible roles of HSIPs in the degradation of lignocellulose

The degradation of PBSA may be compared to the breakdown of natural hydrophobic biomass, such as lignocellulose (Ohtaki *et al.* 2006). The discovery of surface-active proteins could potentially be applied to the saccharification of wheat straw for biofuel production. Hyp1, found in *A. niger*, is homologous to RolA. Hyp1, HfbD and HsbA may possibly play a

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role in the degradation of wheat straw by *A. niger* (Delmas *et al.* 2012). A model has been proposed whereby, under starvation conditions, a subset of hydrolases are targeted to the plant cell wall and perform a “scouting” role. The hydrolases test for available complex polysaccharides and liberate sugars and oligosaccharides, which then cause larger-scale expression of themselves, other hydrolases and HSIPs. In this way, when faced with starvation conditions, the organism can explore its environment and sense the presence of a degradable substrate from the release of inducing sugars. The results obtained by Delmas *et al.* (2012), as well as previous research on the response of *A. niger* to pectin and starch, support the suggested model. This could point to a conserved response pattern to insoluble substrates (Delmas *et al.* 2012). It has been proposed that HSIPs have three possible functions in the use of lignocellulosic biomass by *A. niger*. The suggested mechanisms are that HSIPs may help recruiting enzymes for the enzymatic saccharification, physical degradation and/or the induction of the fungal response to the lignocellulosic biomass.

HSIPs may mediate lignocellulose saccharification by recruiting enzymes. Furthermore, the physical degradation of lignocellulosic biomass could be improved by facilitating fungal-substrate interactions. HSIPs form amphipathic monolayers which facilitates the adhesion of hyphae to host surfaces, required for host penetration. HSIPs may thus allow adhesion and physical degradation of the host's surface. Thirdly, the response of *A. niger* to the presence of lignocellulose could be affected by HSIPs. In the proposed model, the inducing sugars released by the enzymes are detected by the fungus. The result is the induction of various genes involved in biomass utilisation, including HSIPs. HSIPs could improve the induction of this response by recruiting enzymes, leading to an increased rate of inducing sugars release. In addition, the fungal adhesion to the substrate, mediated by HSIPs, may stimulate receptors present on the hyphal surface which recognise the substrate. The response of *A. niger* to lignocellulosic biomass would thereby also be induced. Not only transcriptional but also physiological changes which improve physical degradation could occur (**Figure 6.5**) (Fetherston 2013).

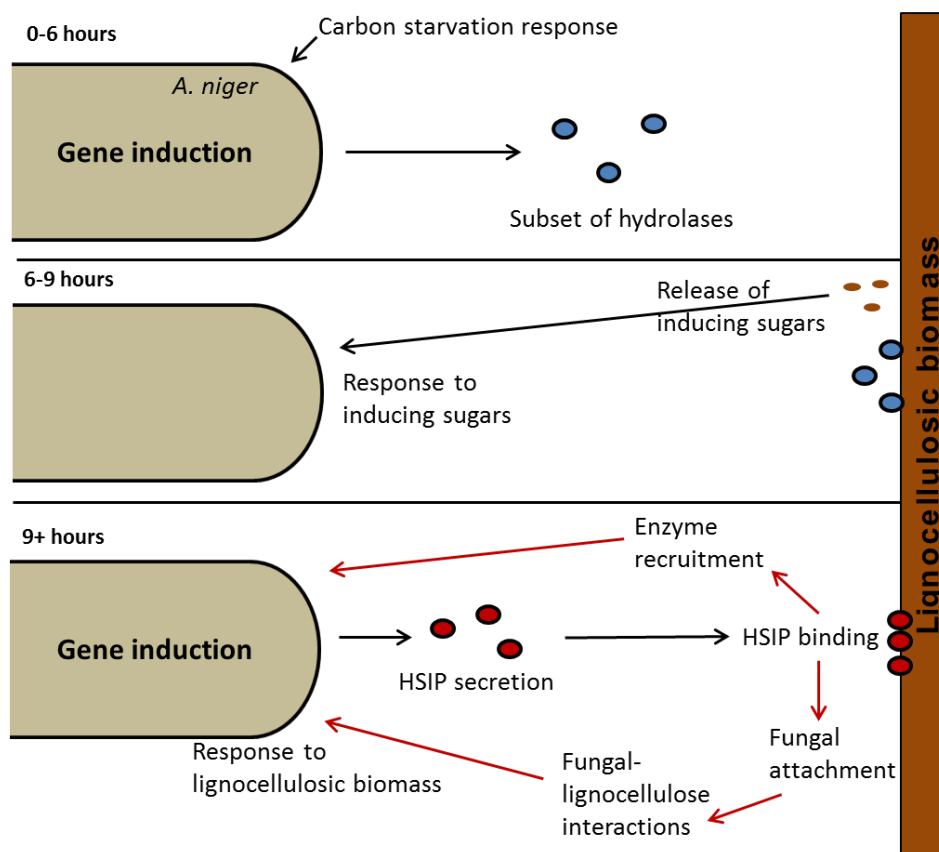


**Figure 6.5:** Proposed mechanisms of HSIP function in the utilisation of lignocellulosic biomass by *A. niger* (adapted from Delmas *et al.* (2012) and Fetherston (2013). In the upper panel (0-6h) carbon starvation response induces the expression of a subset of hydrolases. At 6-9h (middle panel) the expressed hydrolases degrade the lignocellulosic biomass and release inducing sugars, which elicit a response in the fungus. After 9h (lower panel) large scale expression of hydrolases and HSIPs occurs. HSIPs bind to the biomass, recruit enzymes and mediate fungal attachment. The induction of lignocellulose specific responses is then triggered, which leads to the improved utilisation of lignocellulosic biomass.

The function of HSIPs was further investigated in the current study. Deletion strains, in which the genes encoding the HSIPs Hyp1, HfbD and HsbA had been deleted, as well as a double deletion strain for the genes *hyp1* and *hfbD*, were phenotyped as these proteins were previously found to be expressed by *A. niger* in response to wheat straw (Delmas *et al.* 2012).

#### 6.1.4. Aims

The aim of this chapter is to investigate the roles of the hydrophobic surface interacting proteins Hyp1, HfbD and HsbA in the saccharification of wheat straw by *A. niger*. The following hypothesis was tested:

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hydrophobic surface interacting proteins (HSIPs) could facilitate the utilisation of lignocellulosic substrate by *A. niger* by improving the interaction between the fungus and the substrate and/or increasing the rate of enzymatic hydrolysis.

The specific aims of the study were to:

- determine whether there was a difference in growth rate between WT *A. niger* and the  $\Delta hyp1$ ,  $\Delta hfbD$ ,  $\Delta hyp1hfbD$  and  $\Delta hsbA$  deletion strains
- determine the roles of HSIPs in the saccharification of lignocellulose by *A. niger*
- study the gene expression upon exposure to wheat straw of the  $\Delta hyp1$ ,  $\Delta hfbD$  and  $\Delta hyp1hfbD$  deletion strains compared to the WT strain.

## 6.2 Material and Methods

See Chapter 2:

- 2.2.11 for bioinformatics analysis
- 2.2.7.1 for pH measurement
- 2.2.7.2 for glucosamine assay
- 2.2.10.1.2 for DNS assay
- 2.2.10.3 for SDS-PAGE gel on the culture supernatants
- 2.2.3.4.2 for qRT-PCR
- 2.2.3.4.1 for RT-PCR
- 2.2.7.1.1 for pH controlled experiment

## 6.3 Results

### 6.3.1 Surface Interacting Protein RNA-seq Data

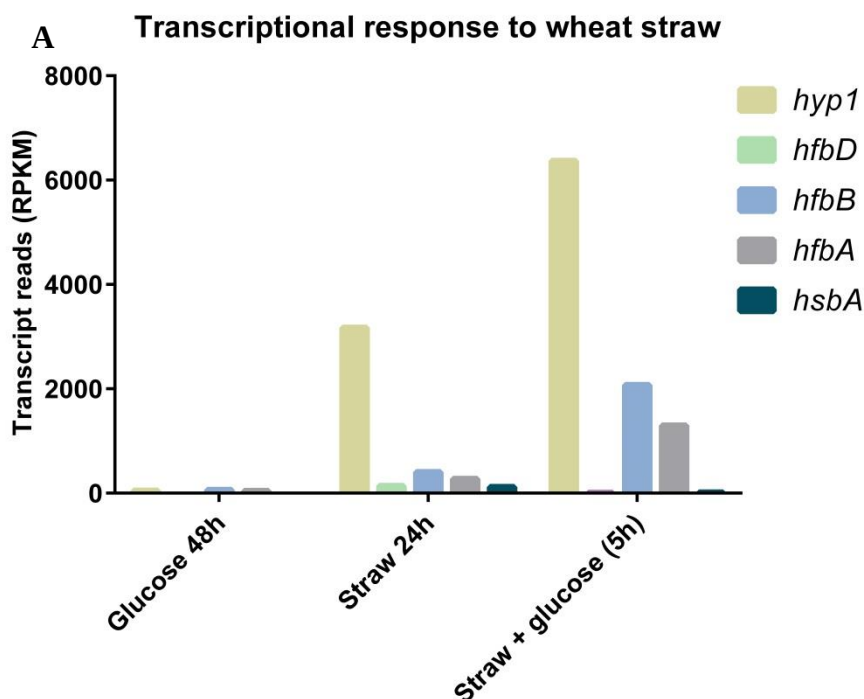
A RNA-Seq experiment was performed by Delmas *et al.* (2012). Transcript data for a total of 9 HSIPs were examined by Delmas *et al.* (2012), including the eight predicted hydrophobins in *A. niger* CBS 513.88 (Jensen *et al.* 2010) and the predicted HsbA family protein. The CBS 513.88 strain was chosen because it has the higher number of predicted hydrophobins of the three *A. niger* genomes that have been sequenced, with only seven predicted in the ATCC 1015 strain.

Although each of the putative surface interacting protein-encoding genes showed a relatively low number of transcript reads (<5RPKM (Reads Per Kilobase of exon model per Million

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mapped reads)) when *A. niger* was grown on minimal glucose conditions, five HSIP-encoding genes were found to have substantially raised levels of transcript reads (defined in here as a >5x in RPKM, of >10RPKM) in response to transfer of *A. niger* onto various other carbon conditions; *hfbA* (An03g02400), *hfbB* (An03g02360), *hfbD* (An08g09880), *hsbA* (An09g00840) and *hyp1* (An07g03340).

When comparing the transcriptional responses on the different substrates, different profiles of induction for the HSIP-encoding genes can be observed. On wheat straw, the most transcriptionally induced HSIP-encoding gene was *hyp1* with a fold change of 69 when compared to the glucose culture. The addition of glucose to the wheat straw culture after 24h did not result in a repression of the expression of *hyp1* as the transcript level kept increasing up to a 139 fold change compared to the glucose culture (**Figure 6.6A**). However on both willow and no carbon, the most induced gene was *hsbA* with an increase of the transcript level of the gene of 828 when compared to the glucose time point and 427 in the no carbon condition when also compared to the glucose time point. In the case of willow the transcript level of the *hsbA* gene was repressed by the addition of glucose. For the no carbon condition, after 24h the fold change of the gene was 79 when compared to glucose (**Figure 6.6B and C**).



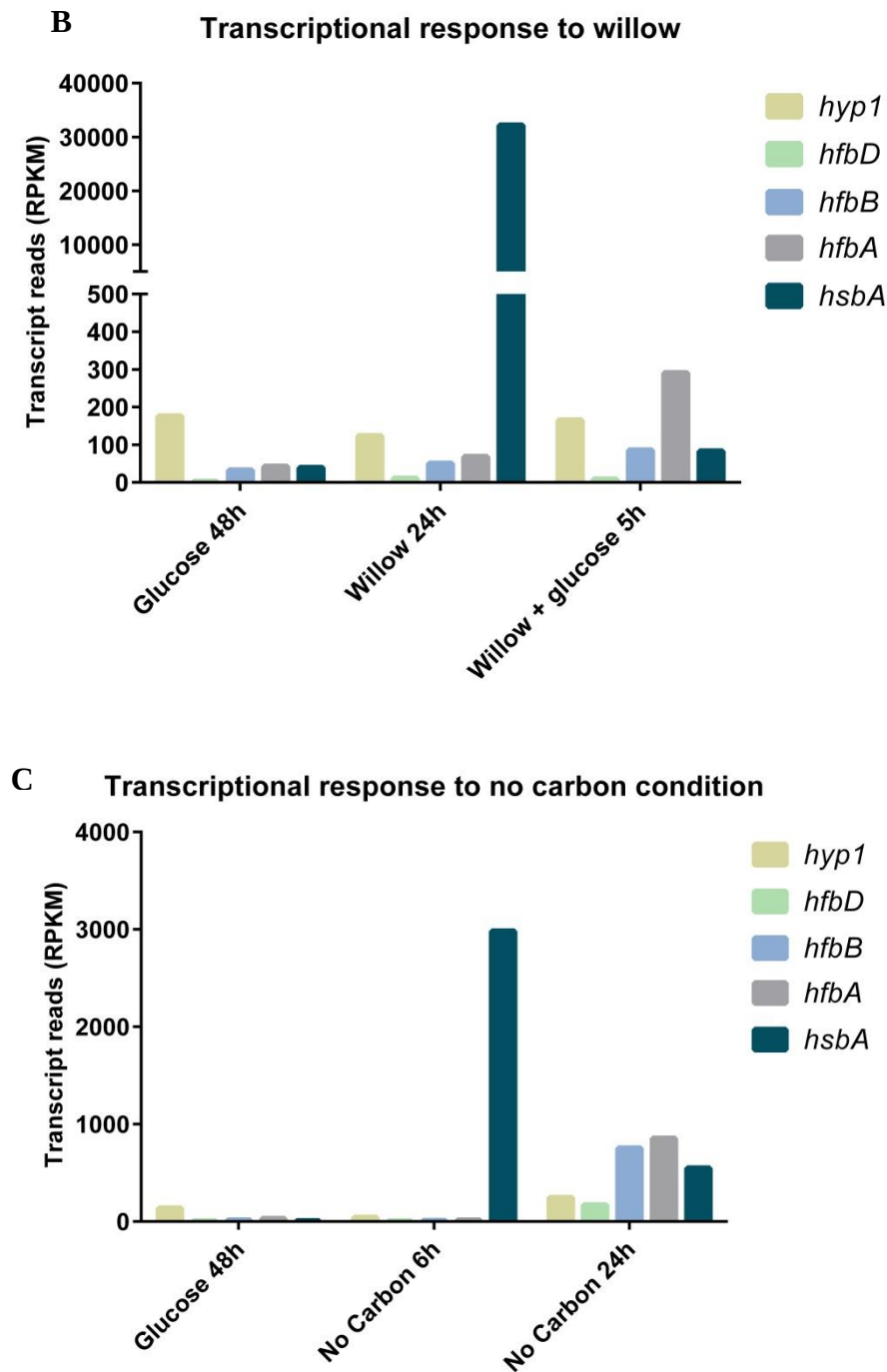
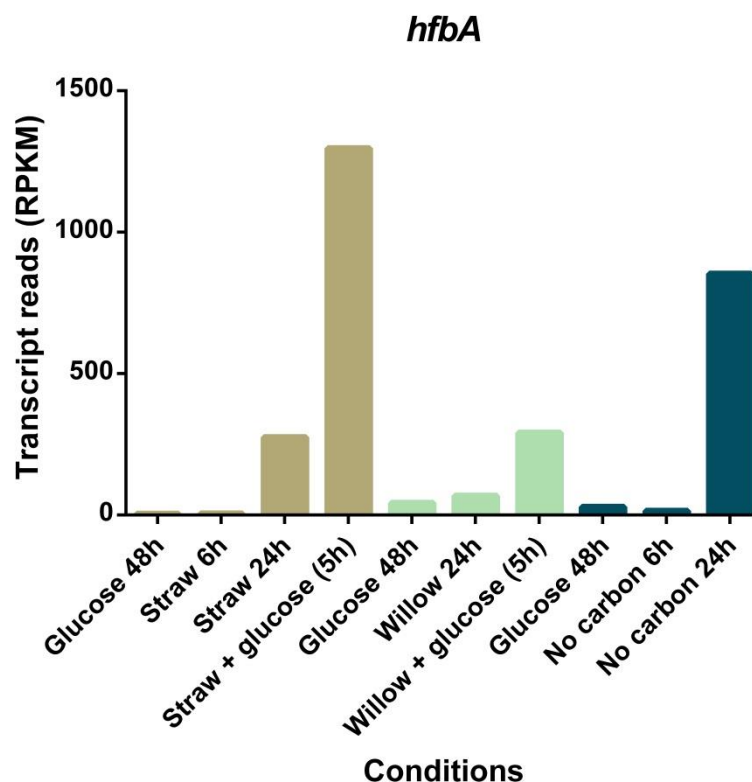
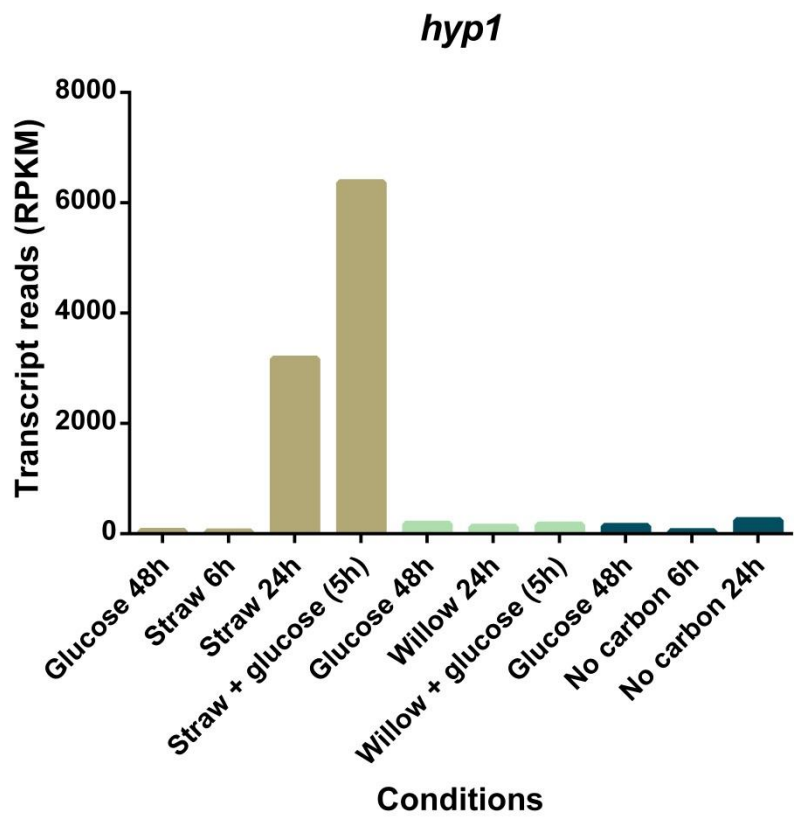
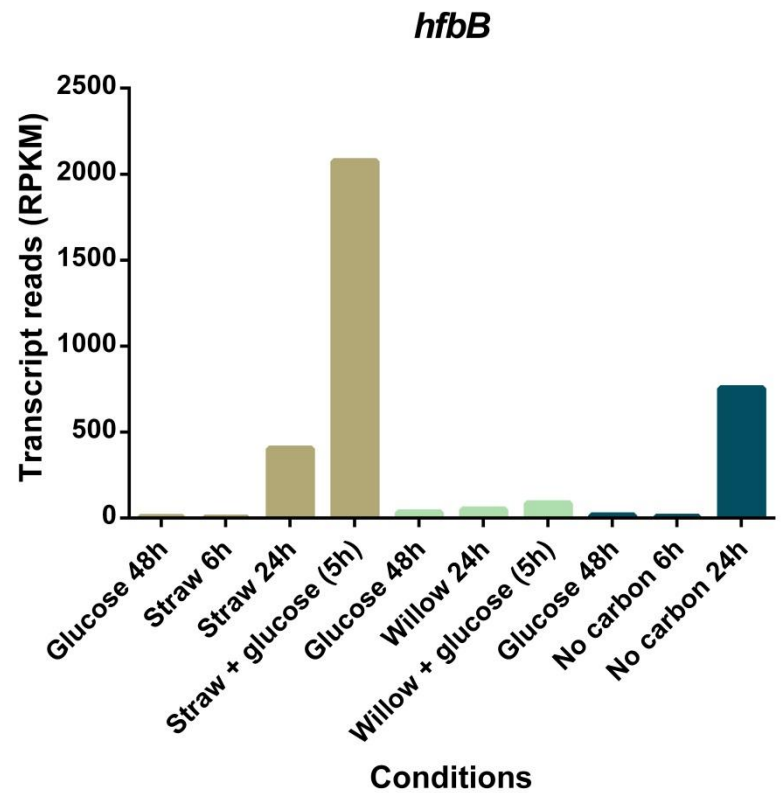


**Figure 6.6:** Transcript reads (RPKM) of the HSIP-encoding genes *hyp1*, *hfbD*, *hfbB*, *hfbA* and *hsbA* in glucose (48h) and after transfer to wheat straw (24h) (A), willow (24h and 24h+5h glucose) (B) and to a no carbon medium (6h and 24h) (C). Four statistical tests were performed on the three biological repeats with an adjusted p-value of  $\leq 0.05$  for the significance threshold. Only the genes that passed the four tests were considered for the RNA-Seq analysis.

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When looking at the profiles of each gene on the different substrates, it can be noticed that both the *hfbA* and the *hfbB* genes have their highest transcript levels at the latter time point which is consistent with their roles in sporulation and development. However, the induction of *hyp1* on wheat straw and *hsbA* on both willow and at 6h in the no carbon medium seems to be substrate specific as *hyp1* is not found to be highly induced in the other substrates and neither is *hsbA* (Figure 6.7).





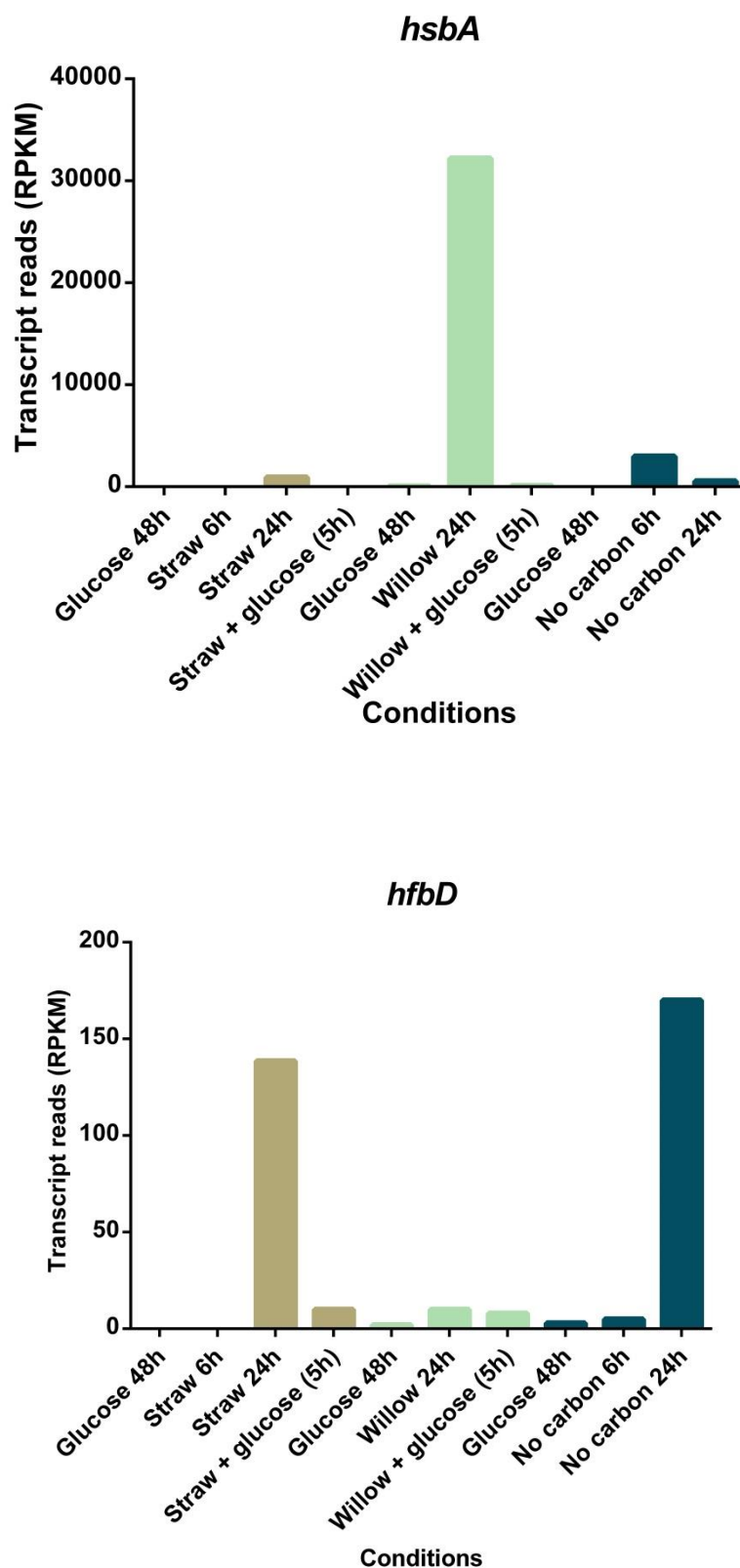


**Figure 6.7:** Transcript reads (RPKM) of the HSIP-encoding genes *hfbA*, *hfbB*, *hyp1*, *hsbA* and *hsfbD* on different lignocellulosic substrates, wheat straw and willow, and in a no carbon medium.

### 6.3.2 Bioinformatics analysis of *A. niger* HSIPs

Bioinformatics techniques can be used to predict possible protein functions, for example by scanning the protein for known motifs and searching for homologous proteins that have already been functionally characterised (Luscombe *et al.* 2001). A commonly used method of discovering homologous genes and proteins is to perform a BLAST (Basic Local Alignment Search Tool) search on a nucleotide or amino acid sequence, against a database of known sequences to find those that exhibit the closest similarity (Altschul *et al.* 1990). Once homologues or motifs have been discovered, they can be searched for in the existing literature and tentatively used to infer the function of a predicted protein. However, the accuracy of predicting a function in this way often depends on the specific homologues, families and motifs that are associated with the protein. As the hydrophobin family is poorly conserved and has a diverse range of functions in various fungal species (Wösten *et al.* 2000), it is necessary to confirm their similarity with alternative techniques such as multiple sequence alignments.

#### 6.3.2.1 Confirming Annotation of *A. niger* Surface Interacting Proteins

##### 6.3.2.1.1 Protein Motif Scanning

To confirm the previously-assigned identities of the putative hydrophobin encoding genes *hfbD* and *hyp1*, and putative HsbA family protein encoded by *hsbA*, the predicted proteins were analysed with protein motif scanning to identify domains specific to their respective protein families. Motif scanning of the predicted hydrophobins HfbD and Hyp1 using MBL-EBI InterProScan (Quevillon *et al.* 2005) returned a match for an uncharacterised eukaryotic signal peptide (SignalP-NN(euk)) of 18 amino acids in length at the N-terminus of both proteins, and Hyp1 also returned a match for two overlapping motifs from the hydrophobin family (InterPro entry IPR001338) (**Figure 6.8**). Although the total overlap of the two motifs implies that both motifs identify the same hydrophobin domain, its identification by both distinct motifs supports the previous assignment of *hyp1* as encoding a hydrophobin in previous research (Pel *et al.* 2007). Scanning of HsbA using MBL-EBI InterProScan (Zdobnov *et al.* 2001) returned a match for a single motif associated with the hydrophobic surface binding protein A (*hsbA*) family supporting HsbA's previous characterisation as a member of the HsbA protein family. Although the presence of a signal-peptide domain was detected for Hyp1 and HfbD, suggesting their secretion by *A. niger*, this was not the case for HsbA. To determine if a signal-peptide domain is present in HsbA, the signal peptide domain scanning tool SignalP 4.1 (Petersen *et al.* 2011) was also used to analyse these HSIPs (Appendix 6.1). Based on the discrimination scores of HfbD (0.904), HsbA (0.903) and Hyp1 (0.774), this suggests that a signal peptide is present in each HSIP and that they are being

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targeted to the ER which could lead to their secretion. This is consistent with their previous characterisation as HSIPs (Delmas *et al.* 2012).

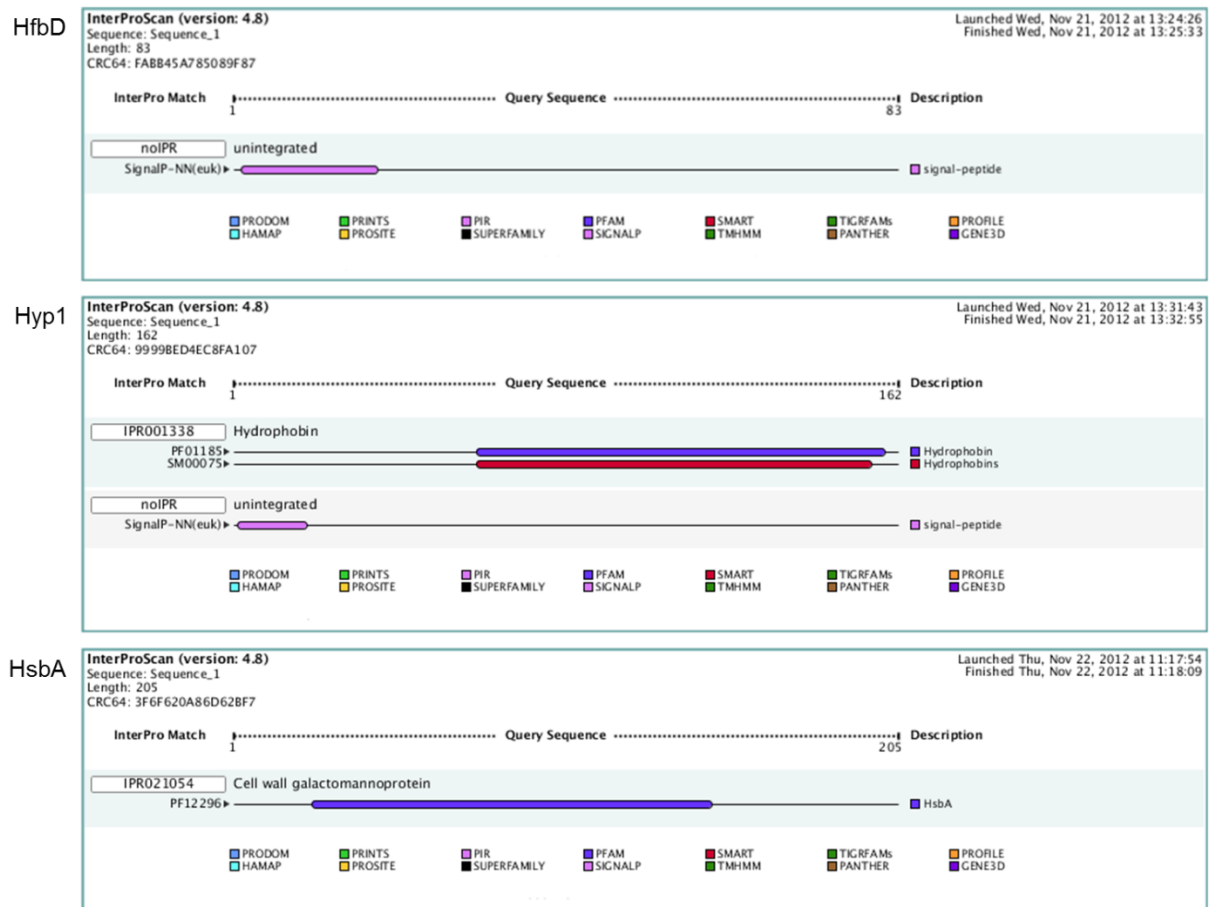


**Figure 6.8:** Visual representation of the results of protein motif scanning the *A. niger* surface interacting proteins HfbD (An08g0980), HsbA (An09g00840) and Hyp1 (An07g03440) using InterProScan (Zdobnov *et al.* 2001). Left to right; N-terminus to C-terminus. Motif colour represents source database.

### 6.3.2.1.2 Reciprocal BLAST Searches of *A. niger* and *A. oryzae* HSIPs

A reciprocal BLAST search (Rivera *et al.* 1998) was done by Richard Fetherston (Fetherston 2013) to characterize the *A. niger* HSIPs in order to determine if they are not only homologues but also orthologues of the *A. oryzae* HSIPs. Homology between the *A. niger* HSIPs HsbA and Hyp1, and the *A. oryzae* HSIPs HsbA (Ao\_HsbA) and RoIA (Ao\_RoIA) was discovered by Delmas *et al.* (2012).

BLAST searches were performed for the genes and predicted protein products of *hyp1* and *hsbA* against the *A. oryzae* RIB40 genome (**Table 2**). Although a nucleotide BLAST found no significant similarity (E-value <10) between *hsbA* and the *A. oryzae* genome, *Ao\_rolA*

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(CBS Gene ID AO090020000588) was determined to be the closest homologue of *hyp1* in *A. oryzae* (e-value 3.0e-49). Comparisons at the protein level for HsbA and Hyp1 against the *A. oryzae* protein database found that their closest homologues are the predicted protein products of *Ao\_hsbA* (e-value 2.0e-25) and *Ao\_rolA* (e-value 9.0e-31) respectively.

Then, BLAST searches were performed for the genes and predicted protein products of *Ao\_hsbA* and *Ao\_rolA* against the *A. niger* CBS 513.88 genome (Table 2). Nucleotide BLAST searches found no significantly similar (e-value <10) genes sequences to *Ao\_hsbA* in the *A. niger* genome, but did find that the closest homologue of *Ao\_rolA* in *A. niger* is *hyp1* (e-value 3.0e-49). A protein BLAST search of the predicted proteins of *Ao\_hsbA* and *Ao\_rolA* demonstrated that their closest homologues in *A. niger* are the products of *hsbA* (e-value 8.0e-21) and *hyp1* (e-value 1.0e-38) respectively. The results of these reciprocal BLAST searches confirm that HsbA and Hyp1 are respectively the closest homologues of *Ao\_HsbA* and *Ao\_RolA* in *A. oryzae*, and therefore their likely orthologues, are provided in Table 2.

**Table 2:** Genomes used for BLAST search were *A. niger* CBS 513.99 and *A. oryzae* RIB40. Score is expressed in 'bits'.

| BLAST Input sequence |                |        | Search Database  | Top BLAST output |       |         |                |
|----------------------|----------------|--------|------------------|------------------|-------|---------|----------------|
| CBS gene ID          | Name           | Search |                  | CBS gene ID      | Score | e-value | Name           |
| An07g03340           | <i>hyp1</i>    | BLASTn | <i>A. oryzae</i> | AO090020000588   | 194   | 3.0e-49 | <i>Ao_rolA</i> |
|                      | Hyp1           | BLASTp | <i>A. oryzae</i> | AO090020000588   | 128   | 9.0e-31 | <i>Ao_rolA</i> |
| An09g00840           | <i>hsbA</i>    | BLASTn | <i>A. oryzae</i> | -                | -     | >10     | -              |
|                      | HsbA           | BLASTp | <i>A. oryzae</i> | AO090023000804   | 111   | 2.0e-25 | <i>Ao_hsbA</i> |
| AO090020000588       | <i>Ao_rolA</i> | BLASTn | <i>A. niger</i>  | An07g03340       | 194   | 3.0e-49 | <i>hyp1</i>    |
|                      | <i>Ao_RolA</i> | BLASTp | <i>A. niger</i>  | An07g03340       | 154   | 1.0e-38 | <i>hyp1</i>    |
| AO090023000804       | <i>Ao_hsbA</i> | BLASTn | <i>A. niger</i>  | -                | -     | >10     | -              |
|                      | <i>Ao_HsbA</i> | BLASTp | <i>A. niger</i>  | An09g00840       | 95    | 8.0e-21 | <i>hsbA</i>    |

### 6.3.3 Phenotyping of the deletion strains

#### 6.3.3.1 pH

The growth of the  $\Delta hyp1$ ,  $\Delta hfbD$ ,  $\Delta hsbA$  and  $\Delta hyp1\Delta hfbD$  strains was assayed by measuring the pH of the cultures as the strains were growing in wheat straw and by measuring the glucosamine content of the cultures. The pH was measured in cultures grown for 48h in glucose before the mycelia were transferred to wheat straw medium. After 24h and

48h in wheat straw, the pH was measured in the cultures. For the glucose condition, after 48h of culture, the pH had dropped from 6.5 to 2.9 for the *A. niger* WT strain, and respectively from 6.5 to 2.7 for the  $\Delta hyp1$  strain, 3 for the  $\Delta hfbD$  strain, 2.6 for the  $\Delta hsbA$  strain and 3.6 for the  $\Delta hyp1\Delta hfbD$  strain. In wheat straw, after transfer to the medium, the pH had dropped from 6.5 to 3.4 after 24h and to 2.8 after 48h for the WT strain. For the cultures of the  $\Delta hyp1$ ,  $\Delta hfbD$ ,  $\Delta hsbA$  and  $\Delta hyp1\Delta hfbD$  strains, the pH decreased to 3.7, 4, 3.5 and 3.9 after 24h and to 3.1, 4, 3 and 3.3 after 48h respectively (**Figure 6.9**). A two-way ANOVA test was performed within the same condition (e.g. glucose 48h) to show differences between the strains.

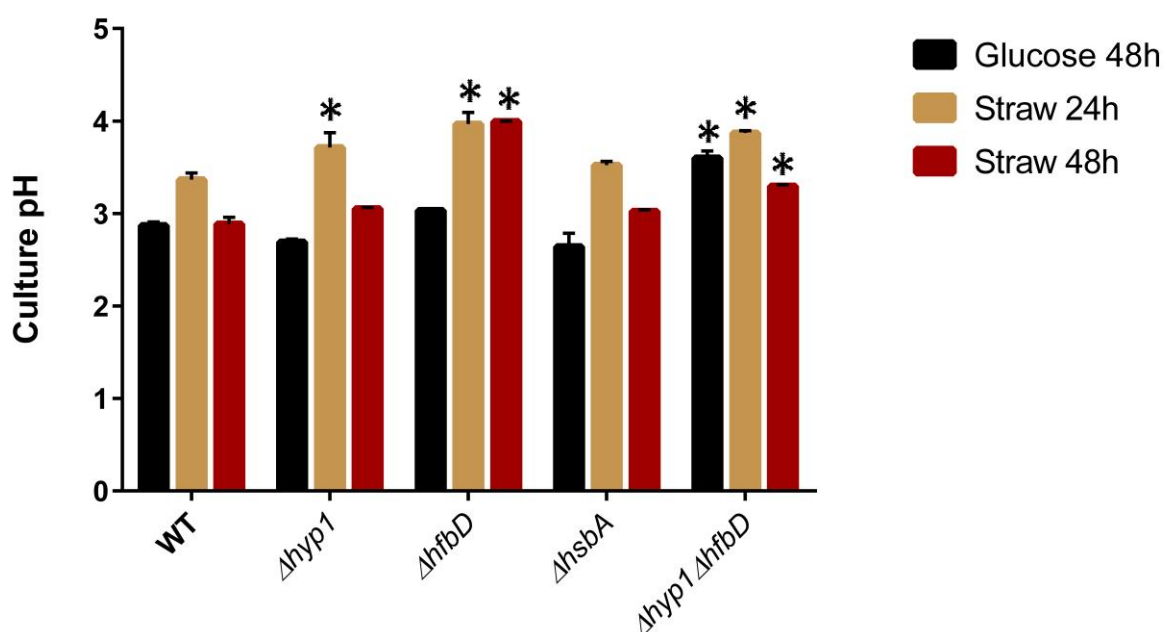


**Figure 6.9:** pH measurements from glucose and wheat straw cultures. For both substrates the starting pH of the medium was set up at pH6.5. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. A two-way ANOVA was performed for each condition in comparison to the WT strain. \* represent the condition for which the two-way ANOVA test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

#### 6.3.3.2. Glucosamine assay

Glucosamine is a useful compound for the estimation of fungal biomass, taking advantage of the presence of chitin, poly-N-acetylglucosamine, in the cell walls of many fungi (Aidoo *et al.* 1981; Pusztai 1964). The measurement of the glucosamine content for the different strains was done from cultures inoculated with 1.5g wet weight of mycelium from the corresponding

48h glucose culture and incubated for 24h and 48h in wheat straw. The dry weight corresponding to the glucosamine content was calculated back using a standard curve of know dry weights of mycelium against their glucosamine contents. After 48h in glucose the dry weight, corresponding to 1.5g of wet weight mycelium, for the WT strain was 0.103g, 0.1g for the  $\Delta hyp1$  strain, 0.112g for the  $\Delta hfbD$  strain, 0.103g for the  $\Delta hsbA$  strain and 0.105g for the  $\Delta hyp1\Delta hfbD$  strain. The dry weights after 24h and 48h in wheat straw were 0.207g and 0.338g respectively for the WT strain, 0.09g and 0.113g for the  $\Delta hyp1$  strain, 0.188g and 0.182g for the  $\Delta hfbD$  strain, 0.127g and 0.112g for the  $\Delta hsbA$  strain and 0.119g and 0.152g for the  $\Delta hyp1\Delta hfbD$  strain (**Figure 6.10**).

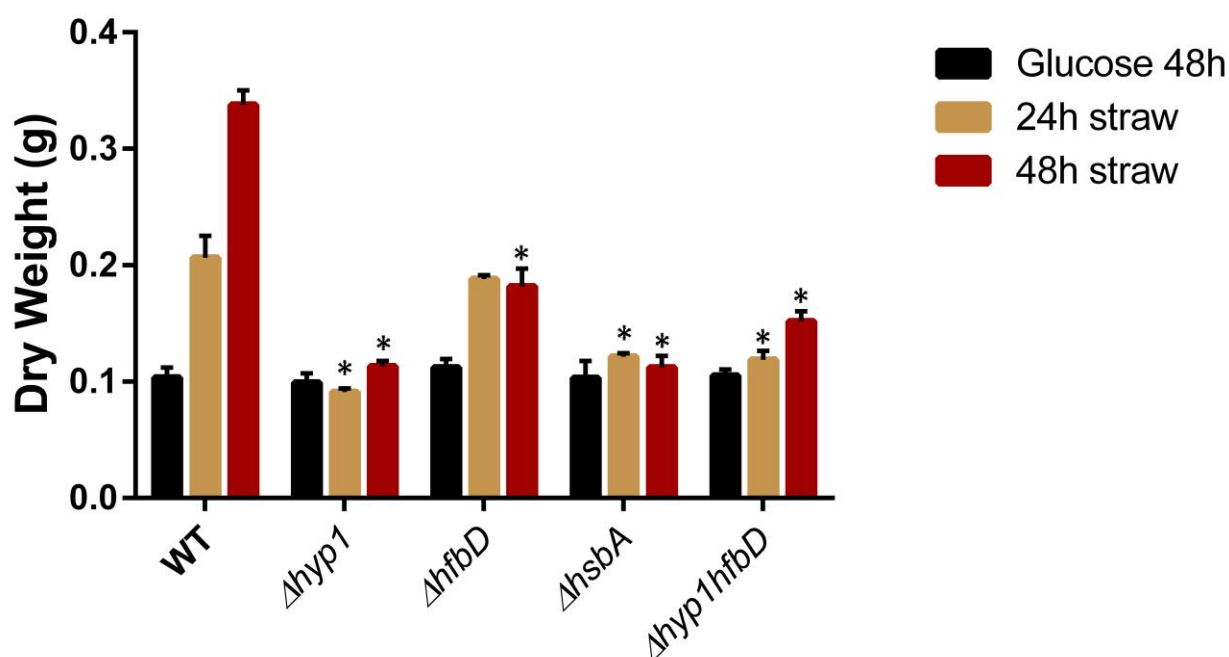


**Figure 6.10:** Increase in the biomass content of the  $\Delta hyp1$ ,  $\Delta hfbD$ ,  $\Delta hsbA$  and  $\Delta hyp1\Delta hfbD$  cultures in glucose and wheat straw. The wheat straw cultures were inoculated with 1.5g (wet weight) of mycelium from the corresponding glucose culture. The glucosamine content was measured from 1.5g (wet weight) for the glucose culture and from the content of the full culture for the wheat straw cultures and the dry weight was calculated back using a standard curve of the glucosamine content against the dry weight of different standard samples. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. \* represent the condition for which the T-test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

### 6.3.3.3 Enzymatic assay and banding pattern of the supernatant proteins

The measurement of the enzyme activity from the supernatants of the *A. niger*  $\Delta hyp1$ ,  $\Delta hfbD$ ,  $\Delta hsbA$  and  $\Delta hyp1\Delta hfbD$  wheat straw cultures was done using the DNS assay measuring the reducing sugar ends released from the incubation of the supernatants with wheat straw. The results showed that fewer reducing sugar ends were released from the incubation of the supernatants from the three deletion strains  $\Delta hyp1$ ,  $\Delta hfbD$  and  $\Delta hyp1\Delta hfbD$  when compared to the WT supernatant. For the WT strain, 0.12 and 0.2 mg of reducing sugars per mg of wheat straw were released from the 24h and 48h supernatants respectively. For the deletion strains, 0.09 and 0.09 mg of reducing sugars per mg of wheat straw were released for the  $\Delta hyp1$  strain, 0.09 and 0.19 mg of reducing sugars per mg of wheat straw for the  $\Delta hfbD$  strain, 0.22 and 0.36 mg of reducing sugars per mg of wheat straw for the  $\Delta hsbA$  strain and 0.2 and 0.18 mg of reducing sugars per mg of wheat straw for the  $\Delta hyp1\Delta hfbD$  strain (**Figure 6.11**). A two-way ANOVA test was performed within the same condition (e.g. glucose 48h) to show differences between the strains.

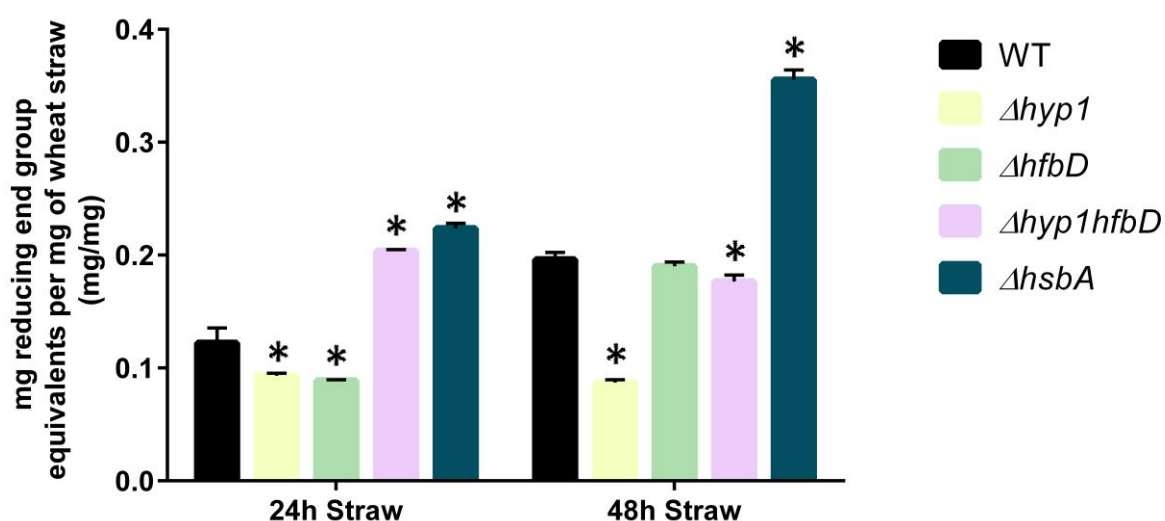


**Figure 6.11:** Measurement of reducing sugar ends released after incubation of equal volumes of the supernatants of the  $\Delta hyp1$ ,  $\Delta hfbD$ ,  $\Delta hsbA$  and  $\Delta hyp1\Delta hfbD$  wheat straw cultures with straw done by DNS assay. A two-way ANOVA was performed for each condition in comparison to the WT strain. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean. \* represent the condition for which the two-way ANOVA test result had a p-value < 0.05 which means that the difference observed is significant when compared to the WT.

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The same samples were analysed on a SDS-PAGE gel which showed that the supernatants from the *hsbA* deletion strain had extra bands in its secretion profile (**Figure 6.12**). This was consistent with the measurement of reducing sugar ends that showed that more sugar reducing ends were released from the incubation of the  $\Delta$ *hsbA* supernatants with wheat straw.

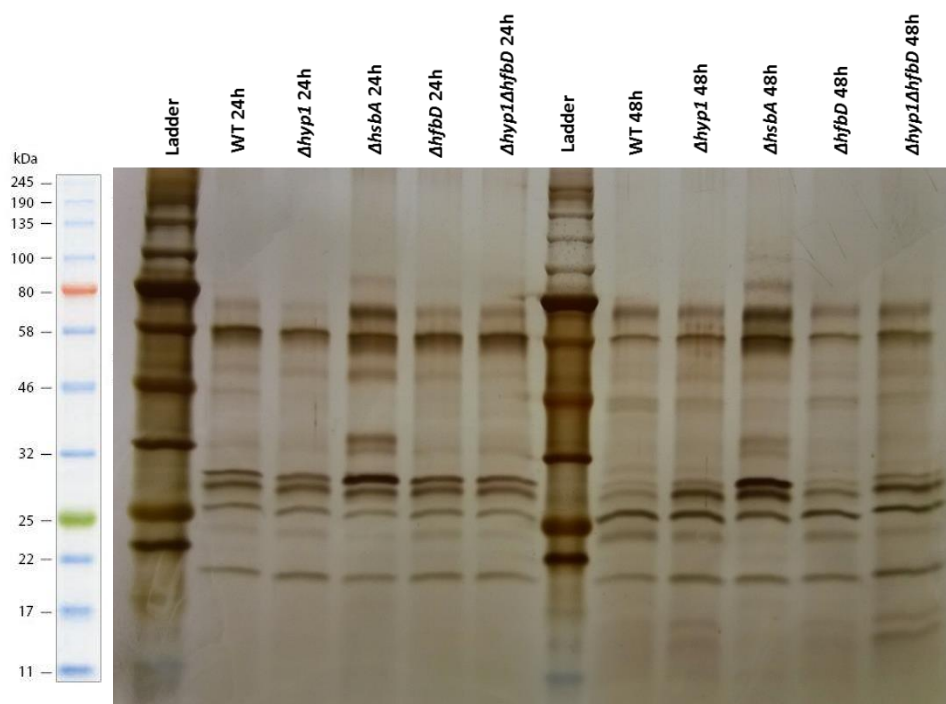


**Figure 6.12:** Silver stained protein gel on supernatants from cultures transferred to wheat straw for 24h and 48h.

### 6.3.4 GH-encoding gene expression in the HSIPs strains

The transcript levels of the genes *cbhA*, *eglC* and *bglB* were measured by qRT-PCR in the  $\Delta$ *hyp1*,  $\Delta$ *hfbD*,  $\Delta$ *hsbA* and  $\Delta$ *hyp1* $\Delta$ *hfbD* strains after 24h of incubation in wheat straw. The fold changes relative to the 48h glucose cultures were measured and compared to the WT strain. For the *cbhA* gene, the fold change relative to the glucose culture was 4273 for the WT strain, 4.5 for the  $\Delta$ *hyp1* strain, 1.1 for the  $\Delta$ *hsbA* strain, 458.6 for the  $\Delta$ *hfbD* strain and 366.7 for the  $\Delta$ *hyp1* $\Delta$ *hfbD* strain. For the *eglC* gene, the fold change relative to the glucose culture was 330 for the WT strain, 63.8 for the  $\Delta$ *hyp1* strain, 20.5 for the  $\Delta$ *hsbA* strain, 16.8 for the  $\Delta$ *hfbD* strain and 10 for the  $\Delta$ *hyp1* $\Delta$ *hfbD* strain. For the *bglB* gene, the fold change relative to the glucose culture was 101 for the WT strain, 0.3 for the  $\Delta$ *hyp1* strain, 21.9 for the  $\Delta$ *hsbA* strain, 1.6 for the  $\Delta$ *hfbD* strain and 9.2 for the  $\Delta$ *hyp1* $\Delta$ *hfbD* strain (**Figure 6.13**). To summarize, transcripts of the genes *cbhA*, *eglC* and *bglB* were detected at very low

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levels in the WT strain grown on glucose, while an > 100-fold increase in transcription was measured during grown on wheat straw. For all the mutant strains an at least 5-fold less increase in transcription was detected on wheat straw, as compared to the expression in the WT strain in a glucose culture. In the glucose samples, the transcript levels of the different genes were low but not zero so the fold changes measured are representative of an induction of the genes.

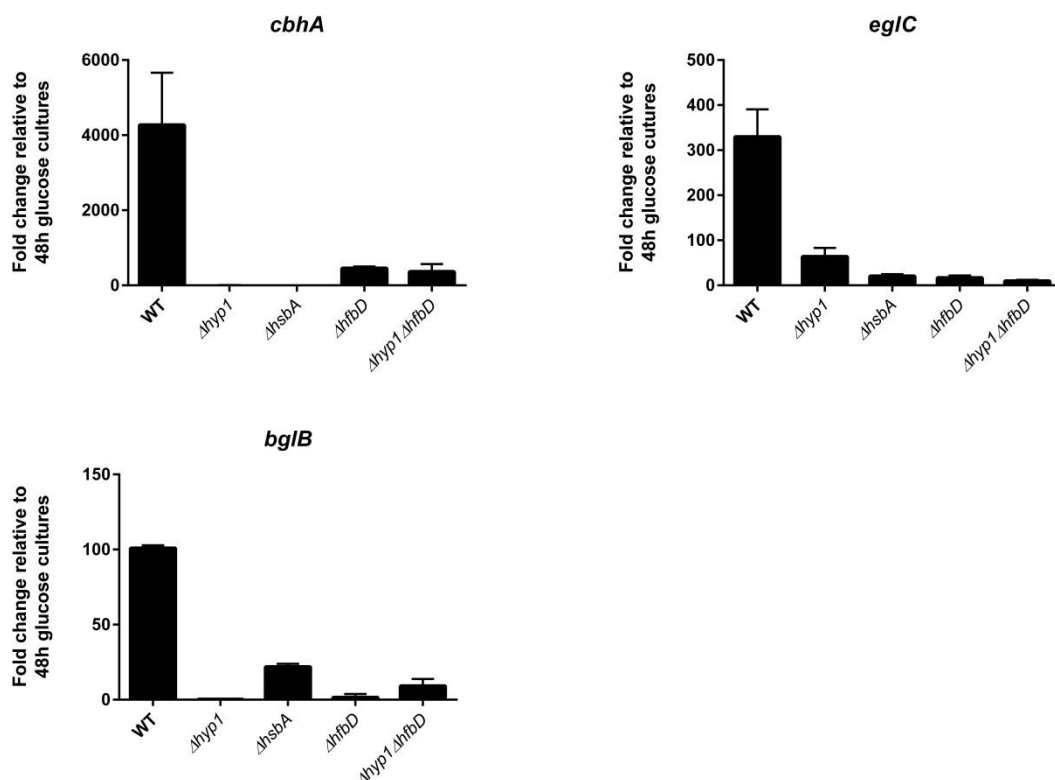


**Figure 6.13:** Expression of the *cbhA*, *egIC* and *bglB* genes in *A. niger* grown in wheat straw media for 24h. The transcript levels of the different genes in the 48h glucose condition were low but not equal to zero so the fold change calculated is representative of the genes induction. The results shown represent triplicates of three independent biological samples and the error bars indicate the standard error of the mean.

### 6.3.4.1 Influence of pH on GH-encoding gene expression

To investigate whether differences in biomass accumulation and gene expression between the WT and the deletion of *hyp1* and *hfbD* was caused by differences in acidification during growth, the WT and the  $\Delta hyp1 \Delta hfbD$  strains were exposed to wheat straw in a fermenter where the pH that was maintained at 5 (**Figure 6.14**).

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The results showed an increase over time in the transcript levels of the *cbhA*, *cbhB*, *bglB* and *eglC* genes for both the WT and the  $\Delta hyp1\Delta hfbD$  strains. However, the transcript levels of all the tested genes were substantially lower in the double deletion strain after 12h of exposure to wheat straw. The transcript levels of the genes were 80 times lower for *cbhB*, 8 times for *bglB*, 263 times for *cbhA* and 232 times lower for *cbhA* when compared to the WT strain. The *cbhA* and *cbhB* genes were induced around the 6h time point and the *eglC* and *bglB* around the 9h time point.

To investigate whether these transcriptional changes are translated into differences in secreted CAZy enzymes, the culture filtrates were analysed on SDS-PAGE (**Figure 6.15**). No differences could be identified between the profiles of the proteins in the culture filtrates of the WT and the  $\Delta hyp1\Delta hfbD$  strains at 6, 24 or 48 h. This suggests that either there are no differences in secreted proteins during cultivation, for example due to limitations in translational capacity, or that any differences in accumulation of secreted proteins may only be visible at a time-point between 6h and 24 h of cultivation, or that SDS-PAGE analysis is too crude to show differences (which might be better revealed by enzymic assay).

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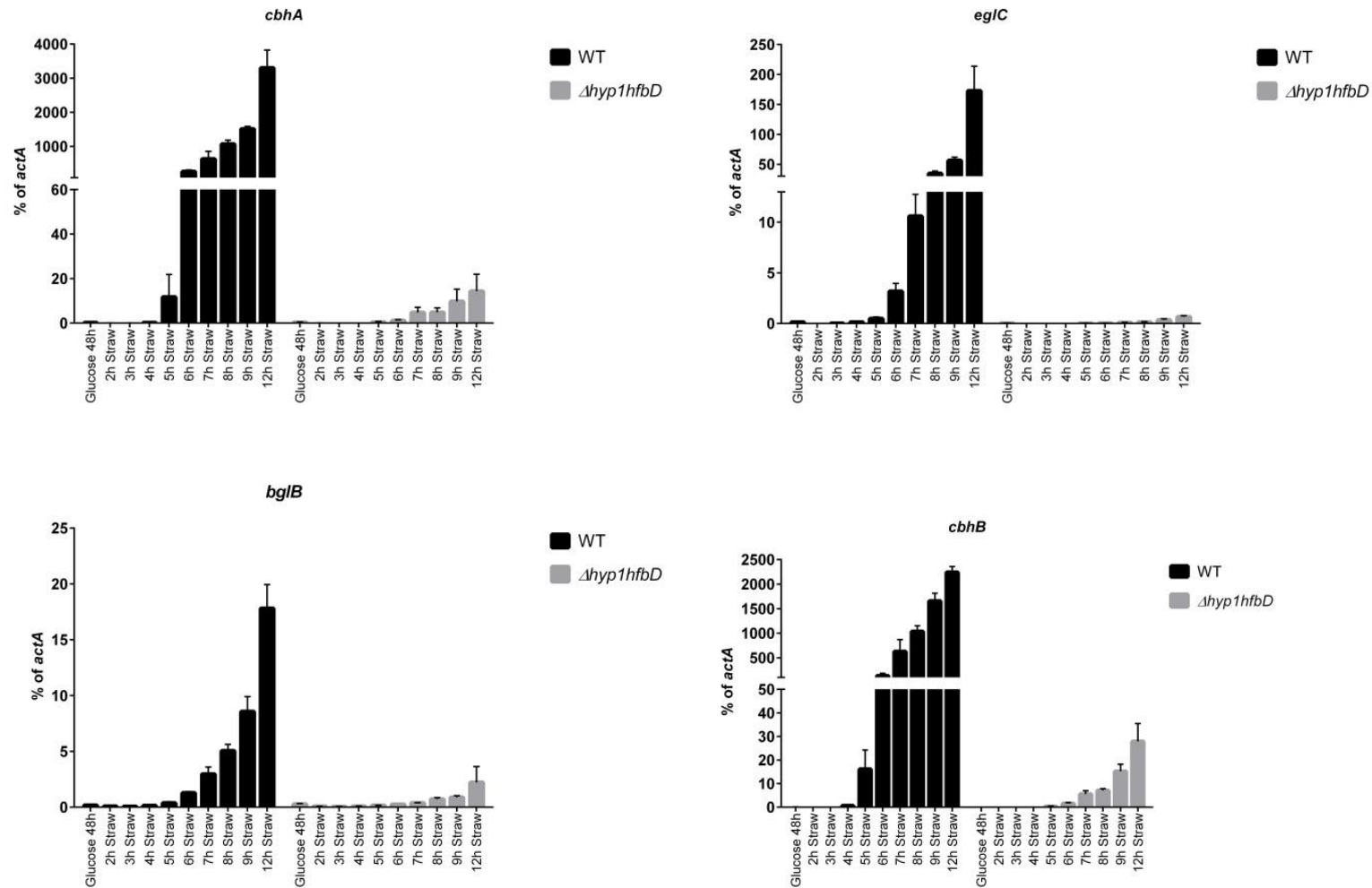


**Figure 6.14:** Expression of the genes *cbhA*, *egIC*, *bglB* and *cbhB* during growth of *A. niger* strains AB4.1 (black bars) and  $\Delta hyp1\Delta hfbD$  (grey bars) on wheat straw at pH 5. The results shown are given as the mean of 3 biological replicates, normalised for the expression of reference gene *actA*, with error bars representing the standard deviation.

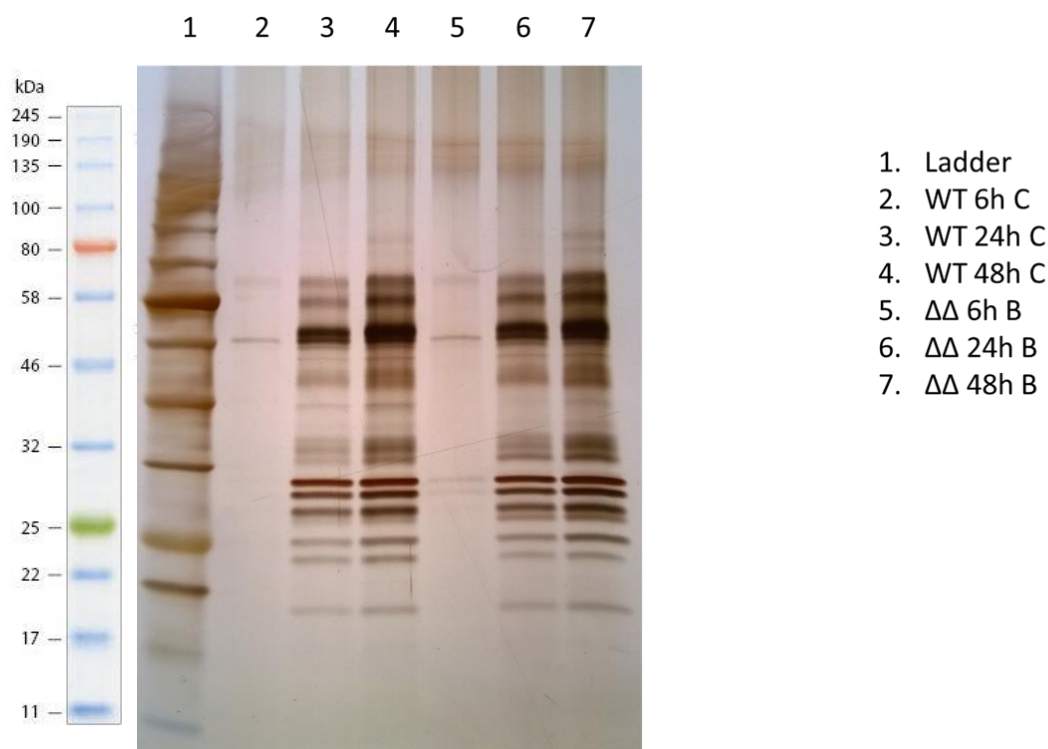


**Figure 6.15:** SDS-PAGE analysis of the culture filtrates of *A. niger* AB4.1 and  $\Delta hyp1\Delta hfbD$  grown at pH5 (done by Matthew Kokolski).

### 6.3.5 Regulation of HSIP-encoding genes

The expression of the *hyp1*, *hfbD* and *hsbA* genes was analysed in the WT and in the  $\Delta xlnR$ ,  $\Delta clrA$ ,  $\Delta clrB$  and  $\Delta farA$  strains by RT-PCR. The samples tested were from both glucose and wheat straw cultures. For the *hfbD* and the *hsbA* genes, the transcripts of the genes were detected in all the TFs deletion strains similar to the WT level. However, for the *hyp1* gene the transcript level of the gene was clearly decreased in the wheat straw sample for both the  $\Delta clrB$  and the  $\Delta farA$  strains. This was then measured using qRT-PCR. The *hyp1* gene was induced by a 65 fold change when the WT strain was transferred from glucose to wheat straw. For the  $\Delta clrB$  and the  $\Delta farA$  strains, the *hyp1* was induced by 1.6 and 1.2 times respectively when compared to the glucose culture. In the  $\Delta xlnR$  and the  $\Delta clrA$  strains, the transcript level of the *hyp1* gene was around half of the WT level (**Figure 6.16**).

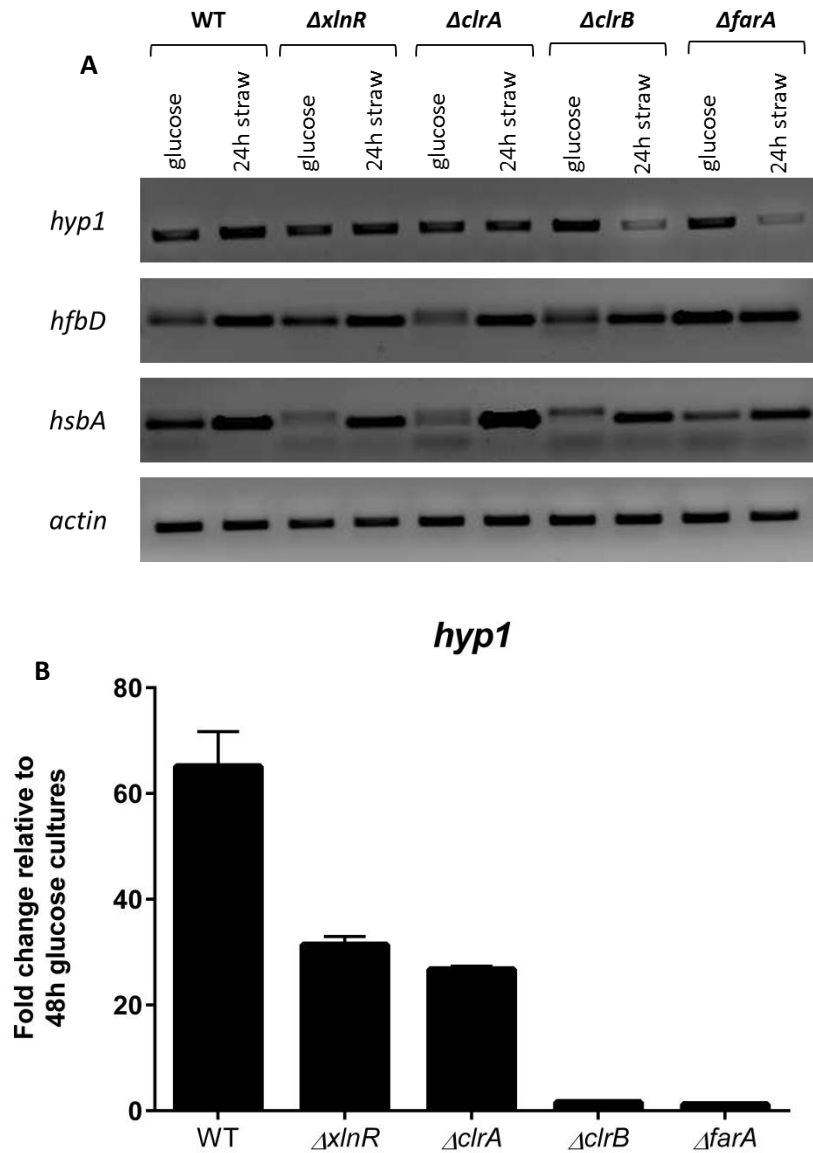


**Figure 6.16:** (A) RT-PCR showing the expression of the *hyp1*, *hfbD* and *hsbA* genes in the WT and in the  $\Delta xlnR$ ,  $\Delta clrA$ ,  $\Delta clrB$  and  $\Delta farA$  strains. (B) Expression of the *hyp1* gene measured by qRT-PCR in the WT and in the  $\Delta xlnR$ ,  $\Delta clrA$ ,  $\Delta clrB$  and  $\Delta farA$  strains. The results shown represent the mean of three independent biological samples and the error bars indicate the standard error of the mean.

## 6.4 Discussion

Genes that encode HSIPs, were found to be induced in *A. niger* in response to wheat straw (Delmas *et al.* 2012), willow (Pullan *et al.* 2014), in sugar cane bagasse (de Souza *et al.* 2011) and in a no carbon condition (van Munster *et al.* 2014) and in *T. reesei* in response to wheat straw (Ries *et al.* 2013). The potential induction of HSIPs under those conditions suggests a possible role for HSIPs in improving the efficiency of saccharification of lignocellulose. In this study the roles of the hydrophobins Hyp1 and HfbD, as well as the hydrophobic surface binding protein HsbA, from *A. niger* were investigated in the degradation of a lignocellulosic substrate, wheat straw.

The increases in transcript levels from the putative HSIP-encoding genes *hfbA*, *hfbB*, *hfbD* and *hyp1* 24h after *A. niger* was transferred from a sole carbon source of glucose, representing a repressing control state, onto wheat straw (lignocellulosic biomass) suggested their possible involvement in either the starvation response or utilisation of lignocellulose. As the addition of glucose to wheat straw cultures caused a decrease in *hfbD* transcript level this suggests that the gene's regulation is likely to be glucose-dependent. In contrast, the further increase in *hfbA*, *hfbB* and *hyp1* transcript levels that occurred in the 5h after the addition of glucose to wheat straw cultures implies that these genes are not repressed by glucose and supports the concept of a specific response to lignocellulose or other complex substrates. However, the lack of increase in hydrophobin transcript levels when *A. niger* was transferred onto willow biomass (another lignocellulosic biomass) instead of wheat straw made their general involvement in lignocellulose utilisation less likely, as increase in transcript levels would then be expected on any lignocellulosic carbon source.

The potential role of *hfbA*, *hfbB* and *hfbD* in the carbon starvation response is further suggested by their substantial increase in transcript levels in the absence of a carbon source, likely to be glucose dependent for *hfbD* but glucose independent for *hfbA* and *hfbB*. Conversely, *hyp1* is less likely to be involved in the starvation response as it did not exhibit an increase in transcript levels in *A. niger* when exposed to either willow or no carbon media, making it more likely to be induced by the presence of an inducer relating specifically to wheat straw than lignocellulose in general.

*HsbA* transcript levels were found to increase following the transfer of *A. niger* from glucose onto wheat straw, willow and no carbon media, and then returned to original transcript levels with the introduction of glucose. This repression of transcript levels by glucose was found to be CreA-independent by Delmas *et al.* (2012), suggesting that another signalling pathway may be involved. This possibility of a distinct regulatory system is supported by the greater

## Chapter 6. Possible roles of hydrophobic surface interacting proteins in the breakdown of lignocellulose by *A. niger*

RPKM of *hsbA* at 6h of growth on no carbon media than at 24h, which differs from that of both glucose-dependent *hfbD* and glucose-independent *hfbA* and *hfbB*. The particularly large (>100x) increase in transcript levels of *hsbA* when *A. niger* is introduced to willow biomass from glucose media, suggests that the mechanism that regulates this change is particularly sensitive to willow biomass, potentially by an inducer that is specific to, or more abundant in, willow biomass than wheat straw. Given the variation observed in the transcriptional responses of *A. niger* with respect to *hsbA*, it is also possible that the gene is regulated by more than a single pathway relating to carbon starvation and/or lignocellulosic biomass utilisation.

Pullan *et al.* (2014) showed that the CAZy proportion of the transcriptome is around one third greater in response to wheat straw compared to willow after 24h of exposure to the substrates. These differences in gene expression could possibly be due to differences in the inducing signals that are generated from the substrates, probably through the generation of small inducing molecules from the respective lignocellulosic substrates. Wheat straw is a gramineous monocot whereas willow is a woody dicot. The two species are separated by around 400 million years of evolution and in that time have evolved different plant cell wall properties. Therefore, differences between hemicelluloses of monocots and dicots can be observed (Scheller *et al.* 2010) with an increased amount of decoration of glucuronoxyran in monocots with arabinose to form glucuronoarabinoxylan. The increased levels of arabinoxylans within monocots may explain the particularly increased expression level seen for the GH62 family on wheat straw as compared to willow. Arabinofuranosidases cleave arabinofuranose side chains from the xylan backbone of arabinoxylans (Gielkens *et al.* 1997). Apart from differences in the decoration of hemicelluloses, differences in cell wall components were also observed. One example is the presence of ferulic acid linkages that connect lignin and hemicelluloses in monocots such as wheat, but are largely absent from dicots such as willow (Buanafina 2009; Vogel 2008). Delmas *et al.* (2012) confirmed the importance of ferulic acid in wheat straw with the gene *faeA*, encoding a protein with ferulic acid esterase activity, being one of the most induced on wheat straw. On the contrary, the expression of *faeA* was 14 fold lower on willow than on wheat straw. These transcriptional data were consistent with the enzymatic activities measured toward arabinoxylan being greater on wheat straw than on willow. Also, the supernatants of the wheat straw cultures were shown to have the higher protein concentration. This is consistent with the study from Delmas *et al.* (2012) that demonstrated that there was more free glucose present initially and released from the substrate over time in the willow media compared to wheat straw. This led to a delayed transcriptional response and to less enzymes being secreted in the supernatants of the willow cultures. These transcriptional observations support the

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hypothesis that the response of *A. niger* to lignocellulose is fine-tuned by, and linked to, the substrate composition and could explain the specific induction of some HSIP-encoding genes towards wheat straw (e.g. Hyp1) or willow (e.g. HsbA).

The deletion strains were phenotyped for their ability to grow on wheat straw, by measuring the pH of the cultures as well as their glucosamine content. The examination of liquid culture ambient pH of the  $\Delta hyp1$ ,  $\Delta hfbD$  and  $\Delta hyp1\Delta hfbD$  strains displayed some differences in the capacity of *A. niger* WT and HSIP deletion strains to acidify cultures in lignocellulosic wheat straw after 24h, but not cultures in simpler substrates glucose (this study) and xylan (Fetherston 2013). Biomass accumulation has been linked to ambient pH in *A. niger* (Archer *et al.* 1990), so the variation in culture acidification between the *A. niger* strains examined in this study may be due to the effects of HSIP gene deletion on the growth rate of the fungus. Growth rate was firstly measured on plates but didn't give significant results (Fetherston 2013). However the assessment of growth by measuring the glucosamine content of the deletion strains after 24h of exposure to wheat straw showed some differences when compared to the WT strain. The results showed that all the deletion strains were less able to grow on wheat straw compared to the WT strain at both 24h and 48h which is consistent with the acidification results except for the  $\Delta hsbA$  strain. The  $\Delta hsbA$  deletion strain did not show any significant differences when compared with the WT strain for acidification of the culture. Similarly, when culture supernatants of the different deletion strains were assayed for their capacity to release reducing sugar ends from wheat straw, the  $\Delta hsbA$  strain was found to be more efficient than the WT. This result is consistent with the SDS-PAGE result showing that the *hsbA* deletion strain had more proteins present in its supernatant. In contrast, the other deletion strains were less able to release reducing sugar ends from the incubation of their supernatants with wheat straw confirming the growth phenotyping results. This could suggest that Hyp1 and HfbD, both classified as hydrophobin proteins, would be involved in the attachment to the substrate, whereas HsbA, a hydrophobic surface interacting protein, could have another role. In pathogenic filamentous fungi, the capacity of hydrophobins to form amphipathic monolayers has been observed to mediate the adhesion of hyphae and invasive structures to the host surfaces, which is used to great effect in penetrating the barriers of their hosts (Talbot *et al.* 1993). In regards to an insoluble lignocellulosic substrate such as wheat straw, hydrophobin monolayers may allow *A. niger* to adhere to the substrate's surface and physically degrade it as the fungus grows, increasing the surface area for enzymatic saccharification. Ohtaki *et al.* (2006) showed that HsbA, alongside RolA (Hyp1 homologue), was specifically recruiting cutinases to the surface of the plastic PBSA and facilitate its degradation in *A. oryzae*. However, HsbA was only been found to be secreted and not adsorbed to the surface of PBSA in contrast to RolA that was able to

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adsorb at the plastic surface (Takahashi *et al.* 2005). This suggests that HsbA may have a role in recruiting enzymes to the substrate, together with hydrophobins, but without attaching to the surface of the substrate. Also, in *A. nidulans*, the homologue of the Hyp1 protein was tagged with a red fluorescent protein and detected by western blot analysis in a protein sample extracted from the mycelium but was not detected in the supernatant of the culture (Neil Brown, personal communication) confirming the hypothesis that Hyp1, and other hydrophobins, may mediate the attachment to the substrate.

Such a role would imply that HsbA is not involved in sensing the environment by interacting with the substrate and would only be interacting with the secreted enzymes. In this case, the CAZy enzymes necessary to degrade wheat straw would be expressed in the deletion strain. However, when the transcript levels of the genes *cbhA*, *eglC* and *bglB* were measured by qRT-PCR in the HSIP deletion strains, the results showed that in all the mutants the transcript levels of the 3 genes were substantially decreased when compared to the WT strain. This means that another regulatory mechanism may be involved or that HsbA may be helping to recruit the other hydrophobins to the surface of the substrate to promote the induction of the degradative enzymes. This could be tested by identifying the proteins that HsbA is interacting with by using a method for protein interaction screening such as the Bimolecular Fluorescence Complementation (BiFC) method. BiFC is a technology used to validate protein interactions. It is based on the association of fluorescent protein fragments that are attached to components of the same macromolecular complex. Proteins that are postulated to interact are fused to unfolded complementary fragments of a fluorescent reporter protein and expressed in live cells. Interaction of these proteins will bring the fluorescent fragments within proximity, allowing the reporter protein to reform in its native three-dimensional structure and emit its fluorescent signal (Kerppola 2006a). This fluorescent signal can be detected and located within the cell using an inverted fluorescence microscope that allows imaging of fluorescence in cells (**Figure 6.17**). In addition, the intensity of the fluorescence emitted is proportional to the strength of the interaction, with stronger levels of fluorescence indicating close or direct interactions and lower fluorescence levels suggesting interaction within a complex (Morell *et al.* 2008). Therefore, through the visualisation and analysis of the intensity and distribution of fluorescence in these cells, one can identify both the location and interaction partners of proteins of interest. Other methods such as the pull-down assay or the FRET technique, as described in the Chapter 3, could also be used.

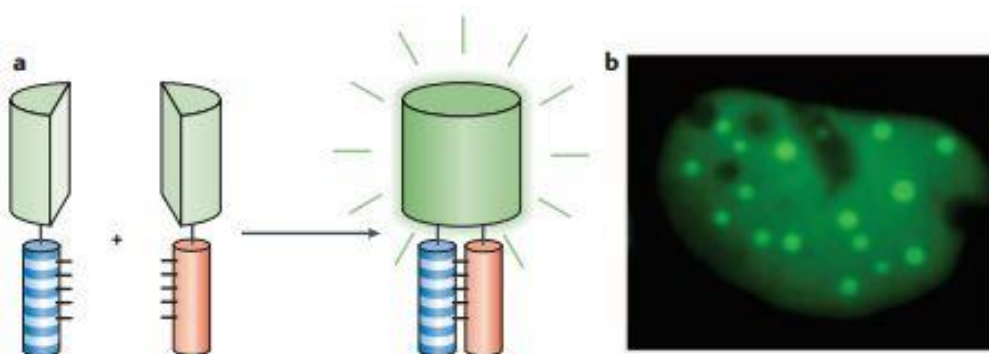


**Figure 6.17:** Protein complex formation using BiFC. Interaction between protein A and protein B occurs first, followed by the re-formation and fluorescence of fluorescent reporter protein. From (Kerppola 2006b).

The pH effect on gene expression was also tested using a fermenter, as for the TF deletion strains (Chapter 4). A time course over 12h (time point every hour until 9h and one at 12h) was done for both the WT and the  $\Delta hyp1\Delta hfbD$  strains at pH5. The results confirmed the previous data from Delmas *et al.* (2012) showing the early induction of *cbhB* after 6h in wheat straw and a bit later for *bglB* and *eglC* in the WT strain. For the  $\Delta hyp1\Delta hfbD$  strain there is an increase of all the transcript levels of the genes tested over time but much lower than for the WT strain suggesting that there is no pH effect on the expression of the gene in the deletion strain. This is supported by the fact that the homologue of the *cbhB* gene was shown to be not pH-dependent in *T. reesei* (Hakkinen *et al.* 2015) and that the other genes tested have similar transcript levels to the one observed for the *cbhB* gene.

However, it may be noticed that these results were not always in line with what was obtained by other members of the group using the same deletion strains. Variations were observed in terms of growth phenotypes (e.g. pH of the culture) or GH-encoding genes expression. Spores are rendered hydrophobic because they have thick walls, comprising a melanin and hydrophobic/rodlet layer (Dague *et al.* 2008). Hydrophobins in the rodlet layer coat the surfaces of spores conferring hydrophobicity and water resistance, helping to facilitate the dispersal of spores in the air (Bayry *et al.* 2012). They help keep dormant spores dry and help reduce the surface tension of water and may play a role in enabling the fungus to grow more easily into the air. The single and double deletions of the HSIPs described in this work may have reduced the hydrophobicity of the conidia and may be affecting the ability of the spores to germinate and some variation in the hydrophobicity of the different deletion strains may be occurring when assayed for growth or GH-encoding genes expression that would lead to different results. Therefore, the hydrophobicity of the different strains should be

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assayed. One way of testing for hydrophobicity is to use a technique known as the Microbial Adhesion to Hydrocarbons (MATH) test (Rosenberg 2006). This method is based on determination of microbial hydrophobicity by differential partitioning at an aqueous-hydrocarbon interface and the results yield the hydrocarbon interaction affinity of the microbes. Such an assay would help to know if some of the deletions led to a significant loss of hydrophobicity.

From the results presented in this chapter, the HSIP-encoding genes do not seem to be regulated by XlnR, which is the main regulator of GH-encoding genes for the degradation of wheat straw. However, the transcript level of the *hyp1* gene was strongly decreased in the  $\Delta clrB$  and  $\Delta farA$  strains. Hynes *et al.* (2006) showed that FarA (and FarB) are TFs that serve as activators of genes involved in fatty acid utilisation in *A. nidulans*. Recently, Martins *et al.* (2014) showed that FarA was also regulating major suberin (plant secondary cell walls specialised tissues) degrading enzymes in *A. nidulans*. In *A. oryzae*, FarA was showed to regulate *hsbA*, which is involved in the recruitment of the cutinase CutL1 to the fatty acid surface (Garrido *et al.* 2012). The authors also demonstrated that in the  $\Delta farA$  strain, no HsbA protein and no CutL1 protein were produced. They also detected a lower transcript level of *rolA* (*hyp1* homologue) but no *cutL1* transcript. In the data presented here, a lower transcript level of *hyp1* is also detected in the  $\Delta farA$  strain but the *hsbA* gene is still being induced in the deletion strain to the WT level. This suggests that the regulation of HSIP-encoding genes is different between *A. oryzae* and *A. niger*. This could also be substrate related, as shown with the transcriptomic data, displaying the induction of different HSIP-encoding genes depending on the substrate type (Delmas *et al.* 2012; Pullan *et al.* 2014; van Munster *et al.* 2014). In this case, FarA could be involved in the induction of *hyp1* when *A. niger* is grown on wheat straw but may induce other HSIPs on another substrate, such as *hsbA* on PBSA in *A. oryzae* (Garrido *et al.* 2012). As the transcript level of *hyp1* is decreased in both the  $\Delta clrB$  and the  $\Delta farA$  strains, an interaction between the two TFs may be necessary for the induction of HSIP-encoding genes. A double deletion strain for *clrB* and *farA* could be obtained and tested for expression of *hyp1*, and other HSIP-encoding genes, to see if the gene is still expressed in the double deletion strain.

In the model proposed by Delmas *et al.* (2012), the carbon starvation of *A. niger* that occurs when it is first exposed to a sole carbon source of lignocellulosic biomass triggers the production and secretion of degradative enzymes that play a scouting role in the detection of complex substrates. The degradation of these substrates by scouting enzymes results in the release of inducing sugars that detected by the fungus, triggering the induction of various

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genes (including CAZy genes, lipases, metabolic enzymes and HSIPs) involved in the substrate's utilisation. In this mechanism, HSIPs would improve the induction of this lignocellulose-specific response by recruiting scouting enzymes to the substrate's surface and increasing the rate of inducing sugars release (Delmas *et al.* 2012). The results obtained in this chapter are consistent with this model, as the deletion of the different HSIP-encoding genes led to no induction of the CAZy-encoding genes necessary for wheat straw degradation (**Figure 6.18**). Equally, the HSIP-mediated adhesion of the fungus to lignocellulosic biomass may promote the substrate's recognition by receptors on the hyphal surface that are sensitive to the presence of lignocellulose, leading to the induction of *A. niger* response to lignocellulosic biomass. It is important to note that as well as triggering transcriptional changes, this response may lead to physiological changes that improve the capacity of the fungus for lignocellulosic biomass degradation. A localisation of the HSIPs in *A. niger* would be useful, as done by Neil Brown in *A. nidulans*, to confirm the physical interaction of HSIPs with the substrate. Perhaps the most relevant example of this is the activation of the *M. grisea* membrane protein Pth11 in response to hydrophobic surfaces and cell wall components, resulting in the induction of appresoria formation that facilitates host invasion (DeZwaan *et al.* 1999). Interestingly, the *A. niger* gene that encodes the homologue of Pth11 has been demonstrated by (Delmas *et al.* 2012) to be upregulated (RPKM) when *A. niger* is exposed to wheat straw.

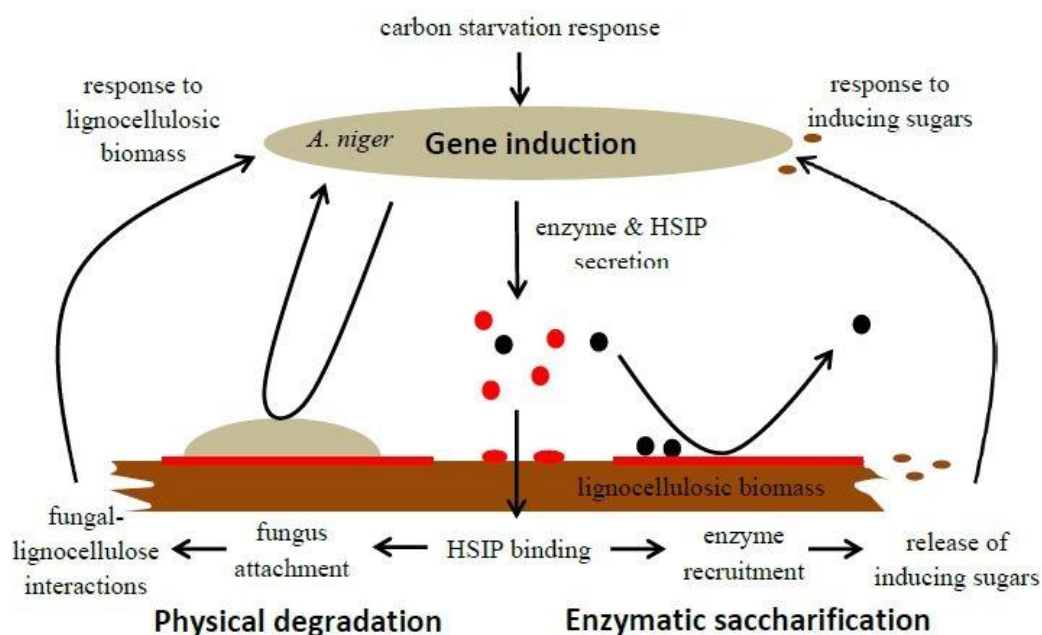


**Figure 6.18:** Proposed mechanisms of HSIP activity in the utilisation of lignocellulosic biomass by *A. niger*. Carbon starvation induces the fungal expression and secretion of HSIPs (red), which bind to the surface of lignocellulosic biomass, and degradative enzymes (black) that play a scouting role. Bound HSIPs then mediate the enzymatic saccharification and physical degradation of the substrate, respectively leading to the release of inducing sugars and the activation of lignocellulosic biomass receptors on the fungal surface. This then triggers the induction of lignocellulose specific responses, leading to the improved utilisation of lignocellulosic biomass.

Although it should be stressed that the mechanism proposed by Delmas *et al.* (2012) is speculative, it is an extension of the enzyme recruitment observed by HSIPs in *A. oryzae* (Ohtaki *et al.* 2006; Takahashi *et al.* 2005). The pH measurement data suggested that the HSIP-deletion strains were less growing on wheat straw when compared to the WT strain. However, it cannot be excluded that these HSIPs may mediate the induction of other genes involved in the wheat straw response of *A. niger*. If this includes the induction of genes involved in the modulation of ambient pH by the fungus, such as those relating to the production and secretion of organic acids (Jorgensen *et al.* 2009), then HSIP deletion would be expected to influence the culture acidification by the fungus. Furthermore, the variety in HSIP functions of experimentally characterised hydrophobins, the distinct transcriptional changes that they undergo in response growth of various carbon conditions and the differential effects of their deletion with respect to culture acidification suggest that the regulation and functions of HfbD, HsbA and Hyp1 are likely to be diverse, with each showing

either distinct mechanisms of action or, if HSIPs are involved in gene induction, significant differences in the genes they are involved in regulating.

## **6.5 Future work**

The results presented in this chapter give some support to the proposed hypothesis that HSIPs may play a role in the utilisation of lignocellulosic biomass by *A. niger*, but more research will need to be carried out on these proteins and their respective deletion mutants to provide a direct link between HSIP activity and lignocellulose utilisation. As Takahashi *et al.* (2005) have had success in categorising the *A. oryzae* hydrophobin RolA when purified, this suggests that it may be useful to develop methods for the production and purification of *A. niger* HSIPs for an accurate assessment of their effect on saccharification activity, potential for substrate/enzyme/fungal interactions and the properties of their amphipathic monolayer formation (hydrophobins), as well as their capacity for rescuing aberrant phenotypes in *A. niger* HSIP deletion mutants. In this case, having the complemented strains for the different deletion strains would be another way to confirm the phenotypes observed. To date, the complemented strains for  $\Delta hyp1$  and  $\Delta hfbD$  have been obtained by Jolanda van Munster and are going to be tested for the different phenotypes. As the ability of HSIPs to bind to the surface of lignocellulose is a key feature of the mechanisms that have been proposed for their roles in lignocellulose utilisation, it will be very important to determine the propensity of HSIPs for binding to this complex substrate. If indeed HSIPs are involved in the saccharification activity of the fungus with respect to lignocellulose degradation, then there may be prospects for the use of these small, secreted proteins in the production of second generation biofuels. HSIPs may be useful in the process of converting lignocellulosic biomass into fermentable sugars in two ways, by improving the fungal pre-treatment and/or the enzymatic degradation of lignocellulosic biomass to simple sugars for fermentation, potentially improving the efficiency of bioethanol production and so their viability for use as transport fuels. Considering the impact of the deletion of the genes encoding HSIPs on the transcription of GH-encoding genes, adding HSIPs to the saccharification process could greatly increase the efficiency of the degradation of wheat and could help to reduce the cost associated with the enzymatic hydrolysis.

## **Chapter 7. General discussion**

A key issue for the biofuels production is the cost-effective conversion of carbohydrates contained in lignocellulosic biomass into fermentable sugars which will provide a viable route to biofuels and bioproducts. Many different raw materials, conversion methods, and process configurations have been studied for the generation of sugars from lignocellulosic biomass. The lignocellulosic biomass conversion normally includes a pre-treatment step to increase the digestibility of the substrates and an enzymatic hydrolysis process. Therefore, different enzymes are involved in the degradation of the substrates and appropriate combinations of different enzymatic activities are required for complete hydrolysis.

Understanding how fungi respond to lignocellulosic substrates can provide new perspectives in the application of fungal enzymes in biofuel technology. Many early studies were done without prior knowledge of the mechanisms of transcriptional regulation. One example is the engineering of strains improved for saccharolytic enzyme production and a recent review charts the progress in strain improvement in *T. reesei* for cellulase production (Peterson *et al.* 2012). Various changes are required to a WT strain to improve its functionality in industrial fermentation conditions for cellulase production. The *T. reesei* WT strain QM6a was subjected to random mutagenesis to improve the strain performance (Peterson *et al.* 2012). One of the strains to emerge from this mutagenesis was the RUT-C30 strain which was found to secrete high yields of cellulases in the presence of glucose (along with an inducer of cellulases) indicating the strain was carbon catabolite derepressed. Subsequently, the *T. reesei* RUT-C30 strain was shown to have a truncated CRE1 transcription factor making CRE1 mediated CCR no longer possible in this strain (Ilmén *et al.* 1996). The RUT-C30 strain has other mutated and deleted genes including changes affecting nine other transcription factors (Le Crom *et al.* 2009). The number of transcription factor-encoding genes that were mutated was higher than would be expected by chance given the mutation frequency in the RUT-C30 strain. Le Crom *et al.* (2009) speculate that these transcription factor genes are unlikely to be unrelated to the selection process and cellulase production.

More recently, applications with a prior understanding were made which points to the potential of exploiting this understanding in future applications. Understanding of gene regulation via XlnR and PacC led to the deployment of a transcription factor engineering approach in *A. nidulans* that enhanced CAZy enzyme production (Tamayo-Ramos *et al.* 2014). Here the XlnR transcription factor was de-regulated by constitutively over-expressing XlnR which led to earlier and increased protein production (Tamayo-Ramos *et al.* 2014). The functioning of the PacC transcription factor was also modulated. PacC, under alkaline conditions, activates alkaline expressed genes and suppresses acidic expressed genes (Arst *et al.* 2003). The activation of PacC was impeded by preventing the cleavage of the inactive form of PacC, leading to increased activity of a heterologously expressed protein whose

expression was driven by the promoter of an acidic expressed CAZy gene in the deregulated XlnR background (Tamayo-Ramos *et al.* 2014). The results presented in this thesis showed that other TFs may be involved in the response to lignocellulose, and more particularly in the response to wheat straw, such as ClrA and ClrB. These results as well as the study from Tamayo-Ramos *et al.* (2014) in *A. nidulans* highlight the potential benefit of employing this transcription factor engineering approach in an industrially relevant strain whereby the knowledge gained from studies on the transcriptional regulation of fungal genes can be applied. Also, the identification of highly induced enzymes at both 24h and 48h (Chapter 4) such as EglC, GH61 or a feruloyl esterase provide insight on which enzymes to add to the mixture to increase the efficiency of the wheat straw degradation. HSIPs could also potentially be added to the mixture to facilitate the interaction with the substrates as the deletion of the genes encoding HSIPs had an important impact on the level of transcripts of these genes.

Apart from strain engineering, another possibility for the improvement of biofuels production is the optimisation of the saccharification reaction. One concept related to models for an optimised saccharification reaction is that of the minimal enzyme concept (Meyer *et al.* 2009). The minimal enzyme concept is defined by its authors as the identification of the minimal number, minimal levels and the optimal combination of the best performing mono-active enzymes to saccharify lignocellulose (Meyer *et al.* 2009). Timing of induction of genes encoding CAZymes and accessory activities can provide a guide for when to introduce enzymes into a saccharification reaction to have the maximum benefit. For example, one study describes a successive secretion of oxidative and hydrolytic enzymes when a basidiomycete is cultured with lignocellulosic substrates (Navarro *et al.* 2014) with the oxidative enzymes making breaks in the polysaccharide chains that improve the efficiency of the hydrolytic enzymes. The high level of transcriptional induction of the *cbh1* and *cbh2* genes from *T. reesei* provided some support for the use of the transcriptional responses to lignocelluloses to guide the protein requirements for an optimised model for saccharification. In *T. reesei* WT strain QM6a, the cellobiohydrolase genes *cbh1* and *cbh2* were amongst the most highly expressed genes on wheat straw (Ries *et al.* 2013) and the corresponding enzymes, CBH1 and CBH2, were among the most abundant proteins in the culture supernatants (Adav *et al.* 2012). Based on levels of transcription and secretion, it would be expected that these cellobiohydrolases would be required in substantial amounts for an optimised saccharification reaction. Indeed, Meyer *et al.* (2009) showed that among four *T. reesei* proteins (CBH1, CBH2, EGL1 and EGL2), CBH1 was the protein required in the largest amount for the saccharification of three pre-treated barley straw substrates.

Access to the lignocellulose limits the efficiency of saccharification (Mansfield *et al.* 1999) and optimising this access is a part of an optimised saccharification reaction. Genes that improve access to the lignocellulose and other accessory proteins are induced in fungi on lignocellulose substrates. One review describes various non-hydrolytic proteins such as swollenins and carbohydrate binding modules (CBMs) that can loosen the structure of cellulose and thereby improve access (Arantes *et al.* 2010). Swollenin is a non-hydrolytic protein which contains a CBM module and is thought to enhance cellulase activity by disrupting cellulose microfibrils. Swollenins are induced in *T. reesei* in the presence of wheat straw (Ries *et al.* 2013) and have a demonstrated role in improving the efficiency of saccharification (Gourlay *et al.* 2013). A CBM is defined as a 'contiguous amino acid sequence within a carbohydrate-active enzyme with a discreet fold having carbohydrate-binding activity' and as well as binding activity, some CBMs can have disruptive or loosening activity on the polysaccharides (Arantes *et al.* 2010). There is evidence that genes encoding enzymes with a CBM are induced before genes encoding enzymes without a CBM (Delmas *et al.* 2012). Although there is evidence to suggest that these CBMs, whilst beneficial in nature and for some saccharification reactions, they may not be relevant in an industrial setting for an optimised saccharification reaction (Varnai *et al.* 2013). The authors showed that reducing the amount of water in saccharification reactions, through high solids loadings as used in industry, counterbalanced the need for CBMs. The frequency of enzyme interaction with the substrate is higher under these conditions, and the benefit of a CBM is outweighed by non-productive binding of CBMs to lignin.

Transcriptomics and proteomics data may indicate a strong correlation between mRNA expression and protein abundance but discrepancies between transcriptomic and proteomic responses have been found in several studies (Zhang *et al.* 2010). It has been suggested that the discrepancy comes from several factors, including protein regulation by post-translational modification, post-transcriptional regulation of protein synthesis, differences in the half-lives of mRNA and proteins, possible functional requirement for protein binding, and significant levels of experimental error (Park *et al.* 2005). Phosphorylated proteins were not always encoded by genes that were highly induced in the transcriptomic dataset for the WT strain (no RNAseq from  $\Delta xlnR$ ). However, this discrepancy also highlights the importance of applying transcriptomic analysis in combination with proteomic analysis, since the biological systems may not be faithfully represented by either of the single individual approaches (Park *et al.* 2005). These discrepancies reject the use of what is transcriptionally induced to guide inputs for optimised saccharification reactions unless there is supporting proteomic evidence.

Transcriptomic studies show that small molecules, often monosaccharides or disaccharides, induce genes encoding endo-acting activities even though there is not a requirement for these activities such as for example where a xylose medium induces genes encoding endo-acting activities in *A. niger* (de Souza *et al.* 2013). Fungi do not seem to possess mechanisms that can sense the degree of polymerisation of a carbon source. Therefore, the understanding with regard to how fungal mechanosensing and contact sensing functions is limited (Kumamoto 2008) and there is no evidence to show these senses are relevant to the induction of CAZy enzymes that degrade lignocellulose. It is generally accepted that filamentous fungi sense and respond to extracellular signals such as pheromones, lipids, nucleotides, peptides, nitrogen, glucose and other nutrients by G-protein signalling (Kulkarni *et al.* 2005). G-proteins are transmembrane proteins, consisting of a G-protein coupled receptor (GPCR) and an associated heterotrimeric G-protein ( $\alpha$ ,  $\beta$  and  $\gamma$ ) and are divided into 9 different classes. The GPCRs contain 7 transmembrane helices connected by intracellular and extracellular loops, with an extracellular amino terminus and the carboxy terminus extending into the cytoplasm (Li *et al.* 2007). The extracellular amino terminus interacts in most cases with the ligand and the carboxyl terminus with the G-proteins. G-protein mediated signalling is transmitted via one or more of the following pathways (Yu, 2006): 1) activated protein kinase A (PKA) through elevated cAMP levels, 2) mitogen-activated protein MAPK pathways and 3) IP3 (inositol triphosphate)-Ca<sup>2+</sup>-DAG (diacylglycerol)-dependent protein kinase C (PKC). Equally, the HSIP-mediated adhesion of the fungus to lignocellulosic biomass may promote recognition of the substrate by receptors on the hyphal surface that are sensitive to the presence of lignocellulose, leading to the induction of the fungal response to lignocellulosic biomass. The results presented in this work support this idea with the deletion of genes encoding HSIPs leading to decreased in transcript level of GH-encoding genes.

Different fungal and non-fungal species co-exist in the environment in a competitive or co-operative relationship. Therefore, in order to fully understand both transcriptional regulation of fungi and their responses to lignocellulose, the natural environment where these fungi exist as communities should be considered. Combining species can be most simply seen in examples where an individual gene from one fungus is expressed in another fungus such as the expression of a laccase from a basidiomycete in *T. reesei* (Zhang *et al.* 2012). However, a more complex combination is that of mixed cultures where two or more fungal species or strains are combined. Understanding transcriptional regulation and responses of fungi in mixed cultures may provide insights that are relevant to fungal biotechnology for biofuels. In nature, competition rather than co-operation may well dominate amongst fungi. However, there are some reasons why competition might not necessarily prevail in the degradation of

lignocellulose in nature or in mixed cultures in the laboratory. In nature there could be a selective advantage in fungi for co-existing and co-operating fungi when, as their enzymatic activities are complementary, different fungi can degrade different parts of the lignocellulose. In a fungal biotechnology approach, the competition that occurs in nature could be moderated to a co-existence by optimisation of culture conditions. Kolasa *et al.* (2014) showed that whether there was compatibility partly depended on the carbon source used. Evidence of synergistic effects on saccharification has been detected when the enzymes from single cultures of *A. niger* and *T. reesei* are combined in saccharification assays. The results showed that when enzyme mixtures were combined, more sugars were released than the sum of the amounts released from saccharification assays using the enzymes from monocultures in separate assays (van den Brink *et al.* 2014). There is substantial literature on the effects at the enzymatic level of mixed cultures with *T. reesei*, as *T. reesei* is one of the most studied fungal species in mixed cultures (Ahamed *et al.* 2008; Duff *et al.* 1987; Hu *et al.* 2011; Kolasa *et al.* 2014; Ma *et al.* 2015). Two of these studies tested a combination of enzymes from ascomycetes and basidiomycetes (Hu *et al.* 2011; Ma *et al.* 2015) with one of the studies performing saccharification assays using enzyme cocktails from the mono and mixed cultures (Ma *et al.* 2015). In this study, Ma *et al.* (2015), showed that there was a clear synergistic effect on saccharification using culture supernatant from a mixed culture of an ascomycete *T. reesei* and a basidiomycete *Coprinus comatus* as the action of lignin-degrading enzymes of the basidiomycete resulted in the saccharification of a greater proportion of the polysaccharides by *T. reesei* CAZy enzymes. Assessing whether there is a synergistic effect of mixed cultures on saccharification from many studies in the literature is complicated for several reasons; there is an absence of data on actual saccharification, protein amount in the cultures, the relative amounts of each fungal species in the mixed culture and an appropriate monoculture control. Furthermore, the likelihood of the dynamic nature of the mixed culture, where there are likely to be antagonistic effects as well as co-operative effects occurring simultaneously, could mask a synergy.

Many recent advances have been made in the area of transcriptional regulation and responses to lignocellulose in fungi but much remains to be understood. Many approaches now begin with genome-wide transcriptomic studies and they depend on accurate and well-annotated genome sequences. Fortunately, genomes from an increasing number of species are being sequenced even if accurate annotation lags behind. There are many genes annotated as encoding proteins of 'unknown function' that are induced in the presence of lignocellulose that have yet to be investigated. There are also CAZy genes that remain un-induced under most conditions. More extensive integration of transcriptomic and proteomic studies will also be beneficial in unravelling the complexities of fungal responses to

lignocellulose. Those studies will explore both the molecular basis of the fungal responses (to include sensing, signalling and transcriptional regulation) but will also provide pointers to the optimised saccharification of target lignocellulosic materials. For the latter, an integration of the fungal responses with a detailed analysis of the structure and composition of the lignocellulosic substrates will be important. Lignocellulose structures and their accessibility to enzymes are affected by pre-treatments and will also vary not only with source of lignocellulose but also with time during the course of enzymatic deconstruction. The majority of current research is focused on major model systems such as *T. reesei*, *N. crassa* and *Aspergillus* spp. but these species may not be representative of the array of transcriptional regulatory systems and responses found in nature. Furthermore, the main species studied at this level are primarily ascomycetes but a tractable basidiomycete model system relevant to lignocellulose degradation and with amenable molecular tools should emerge from the systems currently under study. The ultimate aim of biofuel production is about making a profit through keeping costs competitive with alternative fuels. Further research in this field should lead to reductions in the costs of the enzymes by (1) reducing the costs of inducing the enzymes (by better understanding how induction works) and (2) improving the functionality of the cocktail (by better understanding of the response of fungi to lignocelluloses) so less enzymes are required for the saccharification.

# Appendices

## Chapter 2

### Primers used in this study

|                    | Sequence (5' - 3')                       | Function                                 |
|--------------------|------------------------------------------|------------------------------------------|
| <b><i>xlnR</i></b> |                                          |                                          |
| <i>xlnR</i> up F   | ATGCATGCGGCCGCTCCCAACTTTATTCACTCCC       | <i>xlnR</i> deletion upstream external   |
| <i>xlnR</i> up R   | ATGCATAAGCTTGGAATTCGCAAGGAAGTGG          | <i>xlnR</i> deletion upstream internal   |
| <i>xlnR</i> down F | ATGCATAAGCTTTTTTGCAGTAACACGGCTG          | <i>xlnR</i> deletion downstream internal |
| <i>xlnR</i> down R | ATGCATACTAGTACTCACGGGATCCACGAAG          | <i>xlnR</i> deletion downstream external |
| <i>xlnR</i> gene F | CACCATCTGAGCTCGCAGCC                     | Screen <i>xlnR</i> deletion, internal    |
| <i>xlnR</i> gene R | GCAGTTTCTGGCATACACG                      | Screen <i>xlnR</i> deletion, internal    |
|                    |                                          |                                          |
| <b><i>clrA</i></b> |                                          |                                          |
| <i>clrA</i> up F   | tattatGAATTCACGCGGCTTTCAACATATC          | <i>clrA</i> deletion upstream external   |
| <i>clrA</i> up R   | tattatGGTACCCACCGAGGGCTGAAAGCATC         | <i>clrA</i> deletion upstream internal   |
| <i>clrA</i> down F | tattatGGTACCAAGAGGCACCTTGACTTGCAG        | <i>clrA</i> deletion downstream internal |
| <i>clrA</i> down R | tatatGCGGCCGCGCTCATTGTCCCGAGAAGAA        | <i>clrA</i> deletion downstream external |
| <i>clrA</i> gene F | CCCTCTACAACCCTCACAA                      | Screen <i>clrA</i> deletion, internal    |
| <i>clrA</i> gene R | TGAGGCCTGGACGCTACTAT                     | Screen <i>clrA</i> deletion, internal    |
|                    |                                          |                                          |
| <b>An11g06750</b>  |                                          |                                          |
| An11g06750 up F    | GCGAGCTCATTCCGAAGTAA                     | An11g06750 deletion upstream external    |
| An11g06750 up R    | GTCAATTCCAGCAGCGGCTTGATGGAAGTATATAAGCTGA | An11g06750 deletion upstream internal    |
| An11g06750 down F  | acacggcacaattatccatcgTGAGACAATAAAAAAGAGA | An11g06750 deletion downstream internal  |
| An11g06750 down R  | TCGCTGGTAAACATTGGTG                      | An11g06750 deletion downstream external  |
| An11g06750 gene F  | GCGAATCTAAGTCGGTCGAA                     | Screen An11g06750 deletion, internal     |
| An11g06750 gene R  | GGAGCAGCAGTAGAGGATGG                     | Screen An11g06750 deletion, internal     |
|                    |                                          |                                          |
| <b>qRT-PCR</b>     |                                          |                                          |
| qPCR <i>bglB</i> F | AGGTGGAGATTGTGGTGGAG                     | qRT-PCR <i>bglB</i>                      |
| qPCR <i>bglB</i> R | CCCAATACCTCGTCCTCTCA                     | qRT-PCR <i>bglB</i>                      |
| qPCR <i>clrA</i> F | GCCTCTCATTGAGAACTGG                      | qRT-PCR <i>clrA</i>                      |
| qPCR <i>clrA</i> R | CGTCAATGGGTTGACACAAG                     | qRT-PCR <i>clrA</i>                      |
| qPCR <i>clrB</i> F | GGACGGCAATTGAAAGATGT                     | qRT-PCR <i>clrB</i>                      |
| qPCR <i>clrB</i> R | AGCTGTGCTCTTGGATCAT                      | qRT-PCR <i>clrB</i>                      |
| qPCR <i>eglC</i> F | CGATGGTGTTACCGGTCTCT                     | qRT-PCR <i>eglC</i>                      |
| qPCR <i>eglC</i> R | GCCGAAGTCGAACTCTTCAC                     | qRT-PCR <i>eglC</i>                      |
| qPCR <i>xynA</i> F | TTATGCCTGGGATGTTGTCA                     | qRT-PCR <i>xynA</i>                      |
| qPCR <i>xynA</i> R | TGCCGGTCAATTTAGGGTAG                     | qRT-PCR <i>xynA</i>                      |
| qPCR <i>cbhA</i> F | CCAGCAAGCCGGAACGCTCA                     | qRT-PCR <i>cbhA</i>                      |
| qPCR <i>cbhA</i> R | AACGCGCCGTTTAGCCACACA                    | qRT-PCR <i>cbhA</i>                      |
| qPCR <i>cbhB</i> F | GTCGTACCCAGTTCATCA                       | qRT-PCR <i>cbhB</i>                      |
| qPCR <i>cbhB</i> R | GCCGTTGACACTGGAGTA                       | qRT-PCR <i>cbhB</i>                      |
| qPCR <i>actA</i> F | CCTCTCATATCCGCTGTG                       | qRT-PCR <i>actA</i>                      |
| qPCR <i>actA</i> R | GTGCTAACATGACGGGTAATTC                   | qRT-PCR <i>actA</i>                      |

**Table 2.1:** Primers used for the deletion of *xlnR*, *clrA* and An11g06750 and the primers used for the qRT-PCR experiment.

## Chapter 3

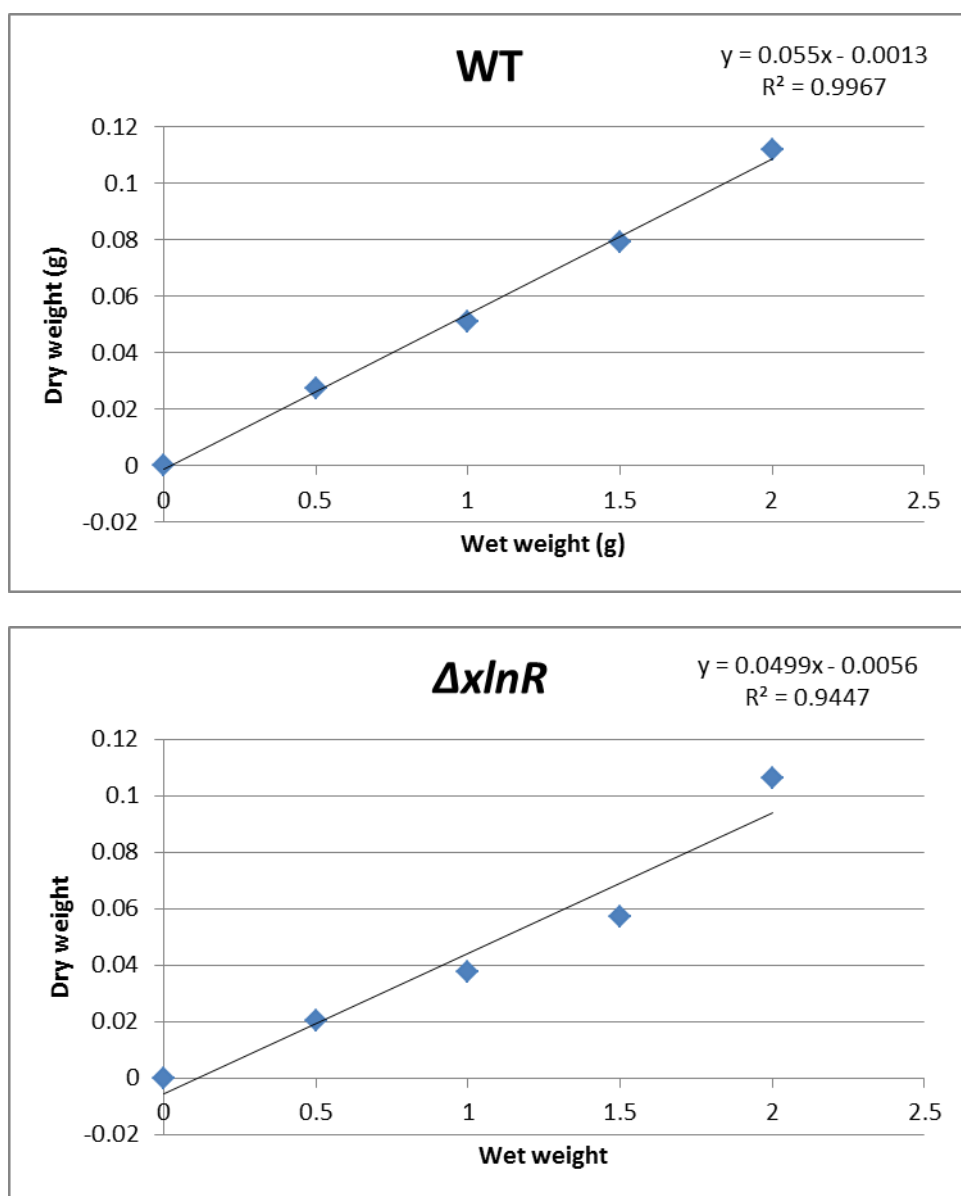
Dry weight against wet weight standard curves

**Figure 3.1:** Standard curves of dry weights against wet weights for the WT and  $\Delta xlnR$  strains in glucose.

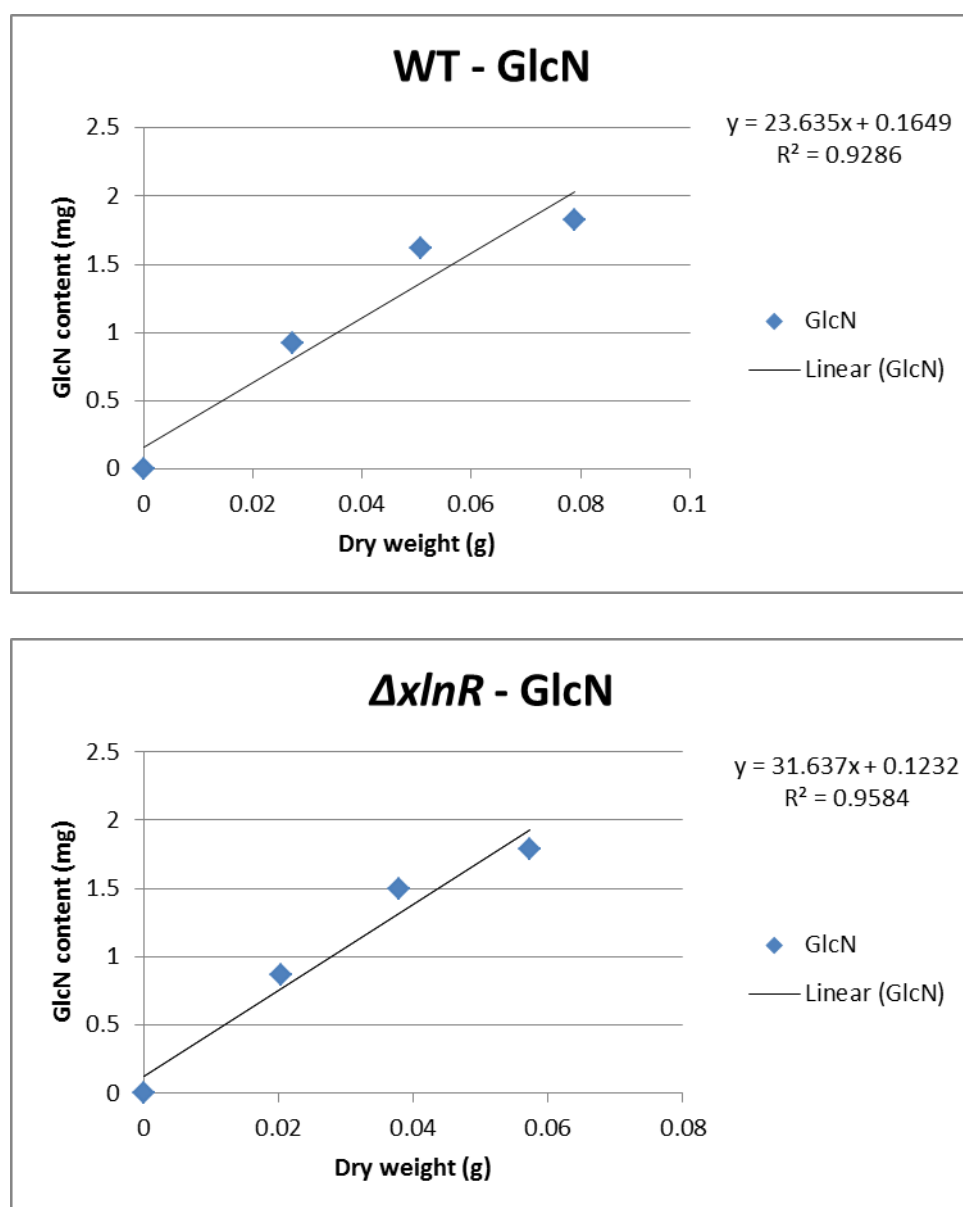
Dry weight against glucosamine content standard curves

**Figure 3.2:** Standard curves of dry weights against glucosamine content for the WT and  $\Delta xlnR$  strains in glucose.

### Dry weight against total protein concentration standard curves

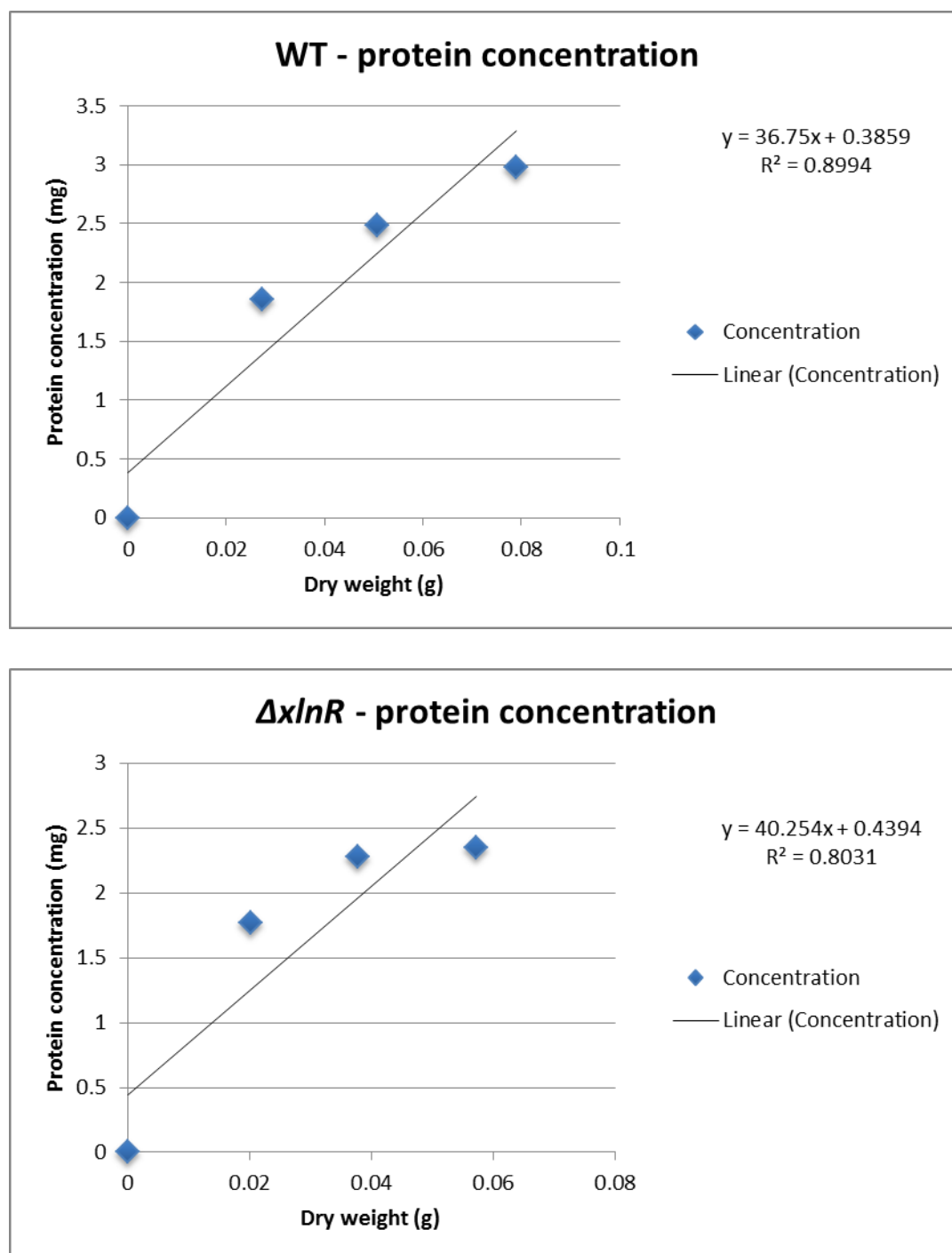


**Figure 3.3:** Standard curves of dry weights against the total protein concentration for the WT and  $\Delta xlnR$  strains in glucose.

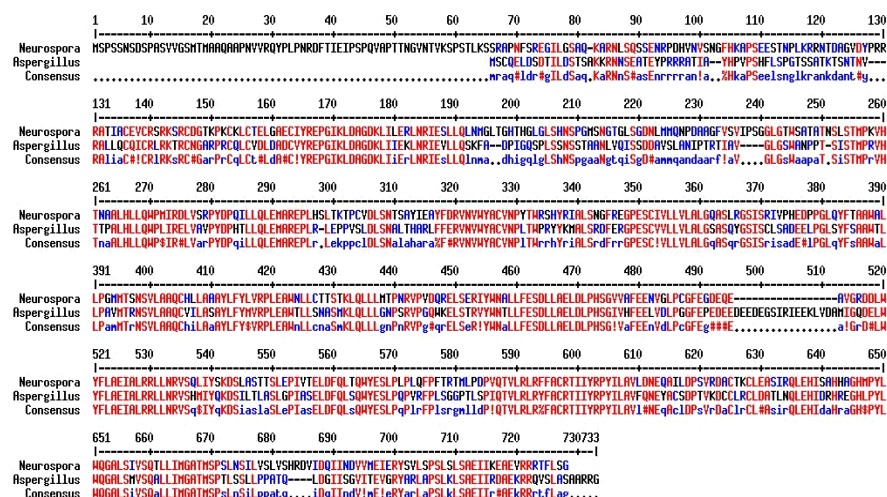
## Chapter 4

### Characterisation of the *A. niger* *clrA* gene

**Table 4.1:** BLASTn result for the *clrA* gene

| Max score | Tot score | Query cover | E-value | Max id | Accession  |
|-----------|-----------|-------------|---------|--------|------------|
| 693       | 693       | 83%         | 0.00    | 56%    | CAK44822.1 |

The Total score is the sum of scores of all aligned sequences. The query cover corresponds to the percentage of query sequence that is covered. The e-value gives a measure of the similarity of sequences. The lower the e-value is, the higher the congruity of the query sequence and the retrieved sequence. An e-value of 0 means that there is an exact match for the sequence.



|                     |         |
|---------------------|---------|
| Identical positions | 360     |
| Identity            | 49.113% |
| Similar positions   | 155     |

**Figure 4.2:** Alignment result between the CLR-1 *N. crassa* sequence and the ClrA *A. niger* sequence. The red colour with capital letters means that there is a high consensus between the two sequences. The blue colour with lower case letters corresponds to a low consensus between the two sequences whereas the black colour means that no characteristics were reported. Similar position is a count of the number of positions over the length of the alignment where  $\geq 51\%$  of the residues or bases at that position are similar.

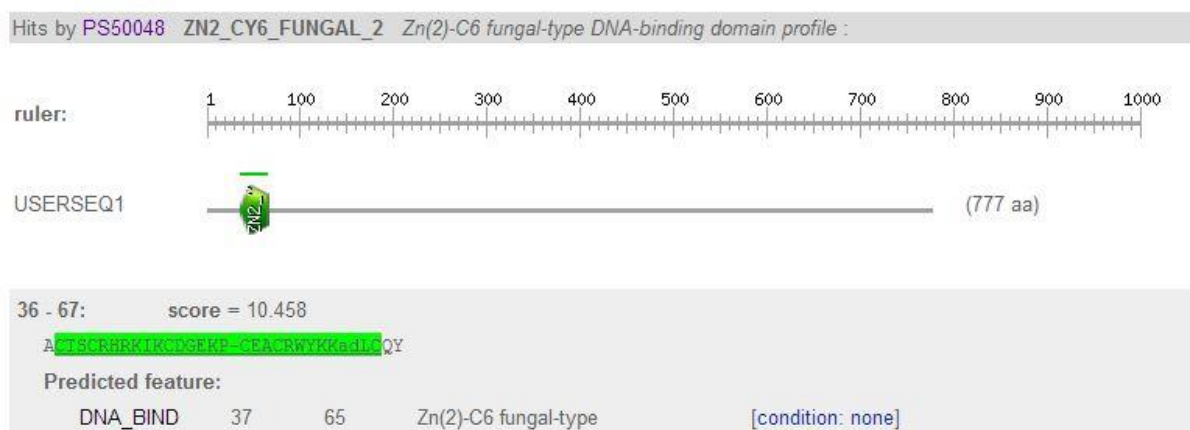


**Figure 4.3:** Identification of a Zinc finger DNA-binding domain in the ClrA sequence of *A. niger*. The ruler represents the length of the sequences. The green motif illustrates the Zinc finger domain. In the box below, the program identified that for this sequence the motif is located between the 36 and the 67 aa and highlighted, in green, the DNA-binding motif which goes from the 37 aa to the 65 aa. Prosite was run against all the motifs of the database except the ones with a high probability of occurrence. By default, hits from Prosite profiles with a score greater than the profile defined cut-off score are shown.

#### Characterisation of the *A. niger* *clrB* gene

**Table 4.2:** BLASTn result for the *clrB* gene

| Max score | Tot score | Query cover | E-value   | Max id | Accession  |
|-----------|-----------|-------------|-----------|--------|------------|
| 452       | 452       | 73%         | 8.00E-147 | 41%    | CAK48469.1 |



**Figure 4.4:** Alignment result between the CLR-2 *N. crassa* sequence and the ClrB *A. niger* sequence. The red colour with capital letters means that there is a high consensus between the two sequences. The blue colour with lower case letters corresponds to a low consensus between the two sequences whereas the black colour means that no characteristics were reported.

Search for conserved domain on the NCBI website (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>).

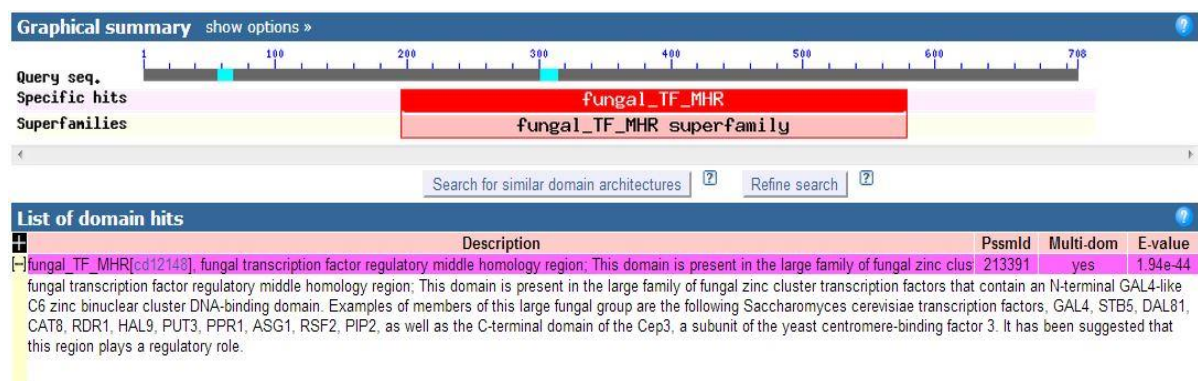
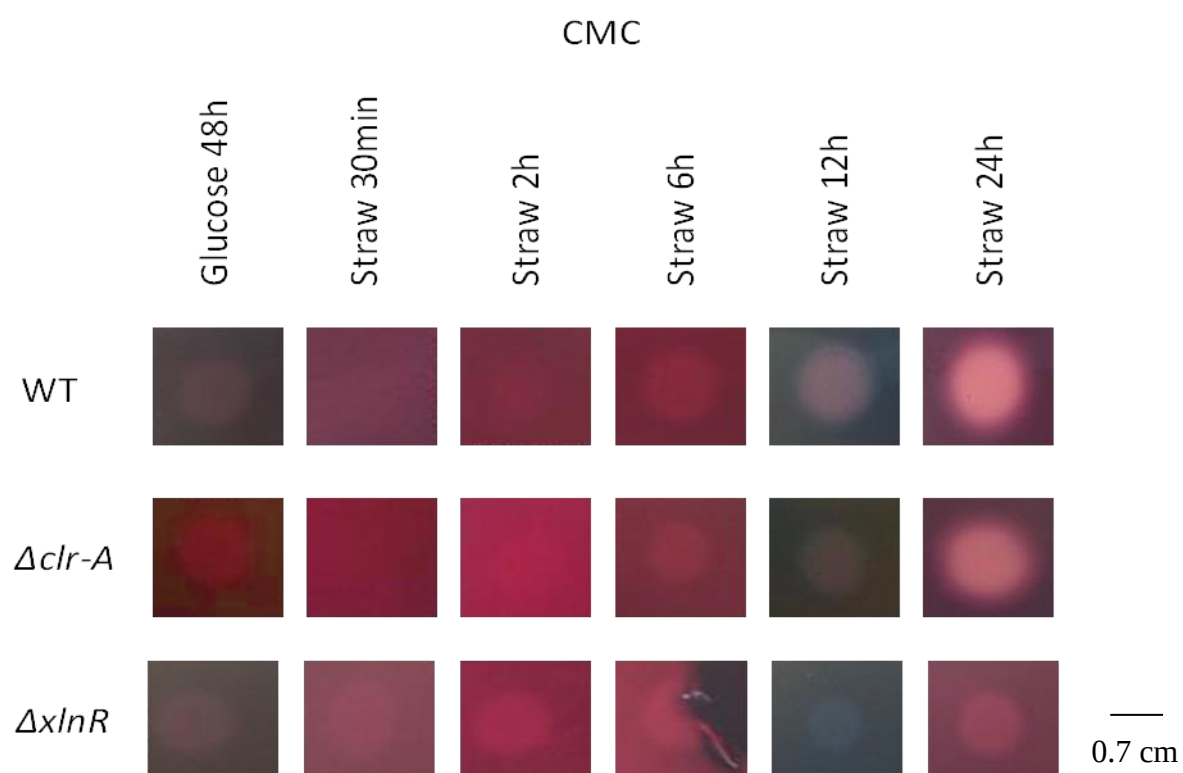


**Figure 4.5:** Identification of a Zinc finger binuclear cluster, with an e-value using the tool search for conserved domain of the NCBI website (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). This software gives a description of the type of motif identified, here one of a Zinc finger binuclear cluster, with an e-value

associated. The lowest the e-value is, the highest the congruity of the query sequence and the retrieved sequence.

Enzymatic assay using Congo Red stained plates with CarboxyMethylCellulose (CMC) and xylan substrates



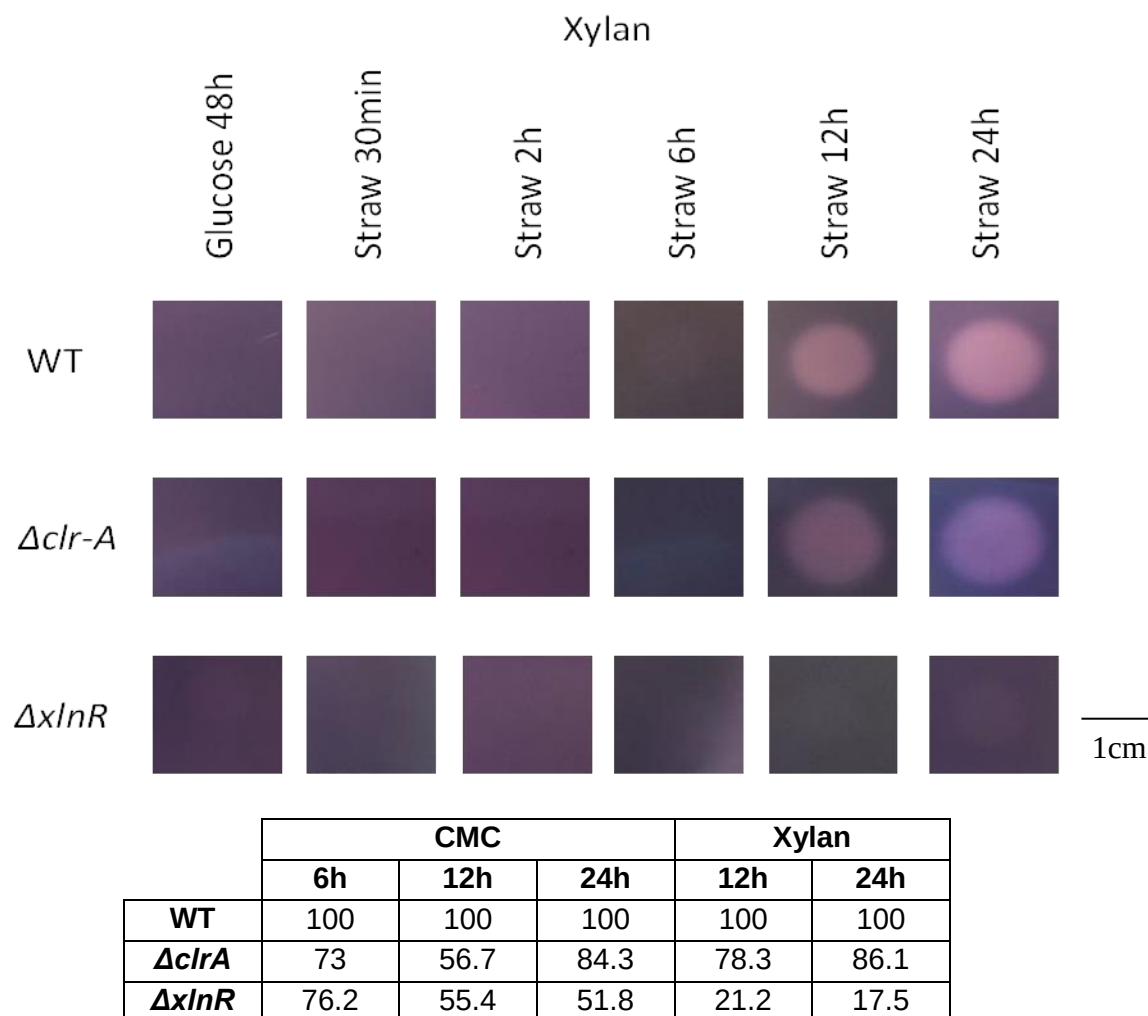


**Figure 4.6:** Congo Red assay carried out on two substrates: CMC and xylan. The samples tested were cultures supernatants (normalised for a protein loading of 100μg in a volume of 10μl) obtained from the time courses carried out on the WT, *ΔxlnR* and *ΔclrA* strains. Table of the ratio of intensities of each clearance when compared to the WT (100%).

## Chapter 5

### Creation of target-decoy databases for decoy searches

#### **Original sequence**



#### **Reversed sequence**

Each sequence of the target database is reversed



#### **Shuffled sequence**

Each sequence of the target database is transformed by mixing the amino acids of the sequence but keeping the composition of the original sequence



#### **Randomised sequence**

Each sequence of the target database is transformed into a new random sequence based on the amino acid composition of the database



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**Figure 5.1:** The different methods used for the creation of target-decoy databases for the identification of peptides after LC MS/MS.

# Optimisation of the target-decoy identifications

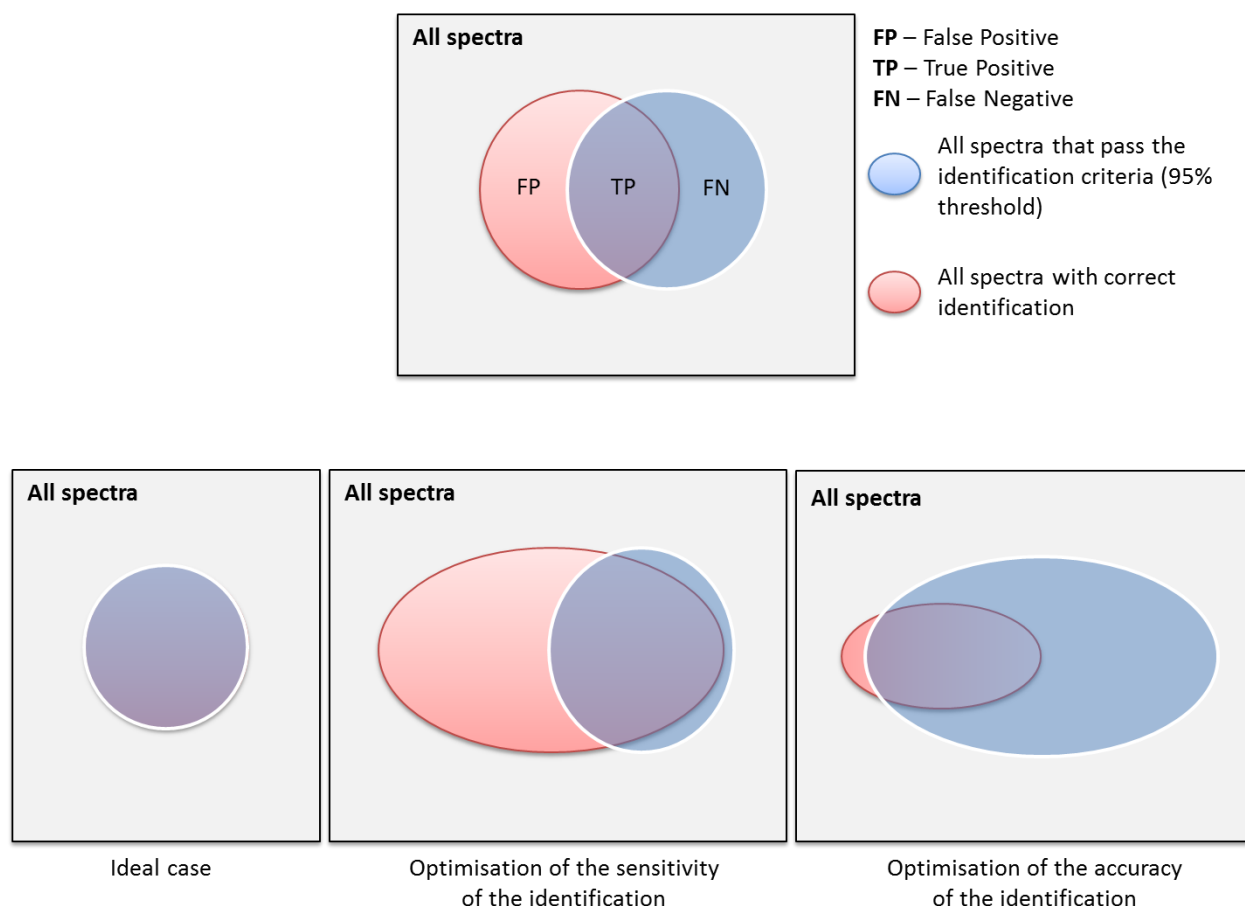


**Figure 5.2:** Selection criteria applied to score distributions (top) determine the form of the Venn diagrams. In the ideal scenario, all identifications are true positive hits (left). In the case of an optimisation of the sensitivity of the identification, the number of false positive increased as the number of false negative hits decreases (middle). In the case of the optimisation of the accuracy of the identification, the number of peptides correctly identified decreases but the accuracy of the identification is improved (right) (adapted from Elias *et al.* (2010)).

## Chapter 6

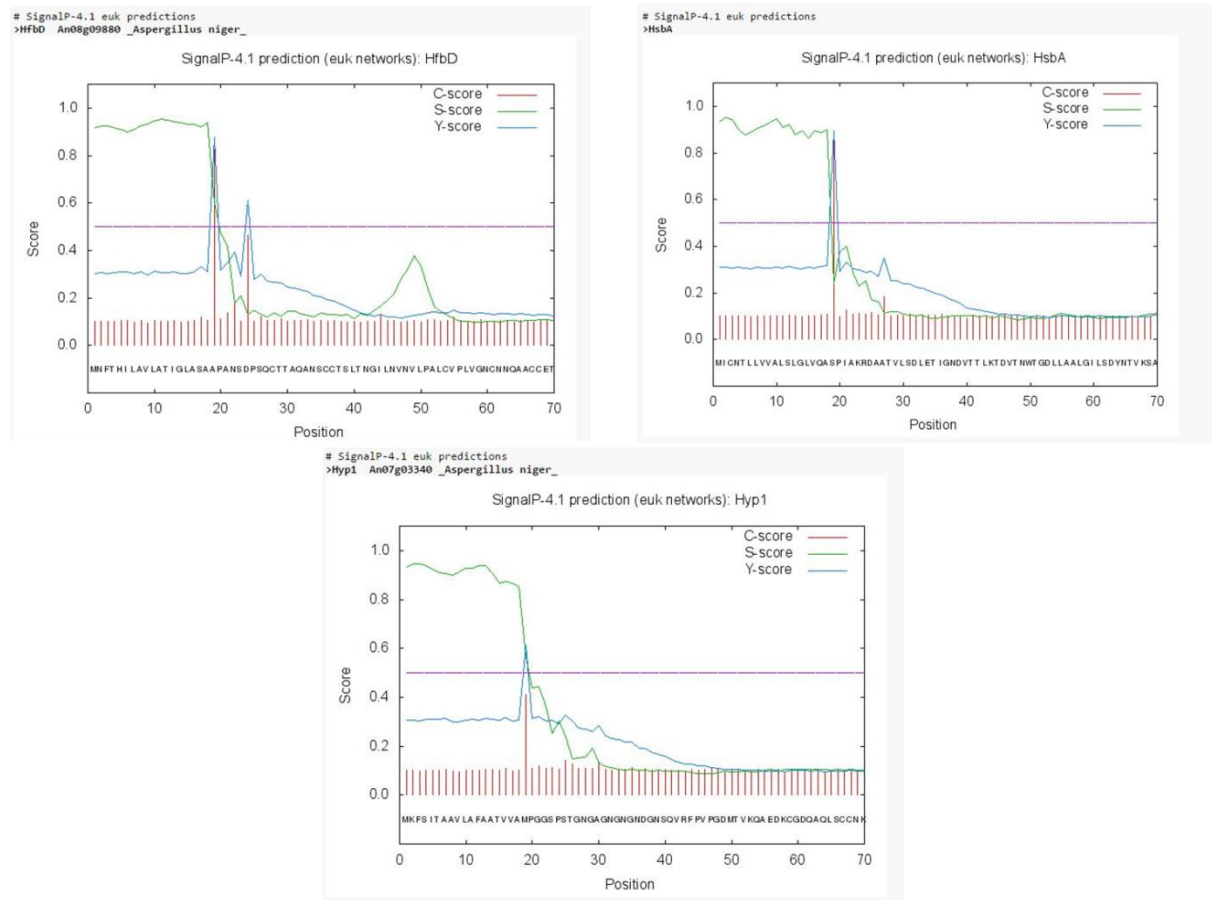


**Figure 6.1:** Visual representation of the results of protein motif scanning for the *A. niger* surface interacting proteins HfbD (An08g0980), HsbA (An09g00840) and Hyp1 (An07g03440) using SignalP (Petersen *et al.*, 2011). C-score (raw cleavage site score), S-Score (signal peptide score) and Y-Score (combined cleavage site score) are displayed for each residue (position from N-terminus) against the default signal peptide discrimination cut-off (0.450). As the discrimination scores of HfbD (0.904), HsbA (0.903) and Hyp1 (0.774) are greater than the discrimination cut-off, then this suggests that they each contain N-terminal signal peptides.

## References

- Abarca ML, Bragulat MR, Castella G, Cabanes FJ (1994) Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. Appl Environ Microbiol 60:2650-2652.
- Adams TH, Wieser JK, Yu JH (1998) Asexual sporulation in *Aspergillus nidulans*. Microbiol Mol Biol Rev 62:35-54.
- Adav SS, Chao LT, Sze SK (2012) Quantitative secretomic analysis of *Trichoderma reesei* strains reveals enzymatic composition for lignocellulosic biomass degradation. Mol Cell Proteomics 11:M111 012419. doi:10.1074/mcp.M111.012419
- Adav SS, Ravindran A, Chao LT, Tan L, Singh S, Sze SK (2011) Proteomic analysis of pH and strains dependent protein secretion of *Trichoderma reesei*. J Prot Res 10:4579-4596. doi:10.1021/pr200416t
- Agrawal, Satlewal, Verma (2013) Production of an extracellular cellobiase in solid state fermentation. J Microbiol Biotech Food Sci 2:2339-2350.
- Ahamed A, Vermette P (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. Biochem Eng J 42:41-46. doi:10.1016/j.bej.2008.05.007
- Ahmad K, Schneider HG, Strong FM (1950) Studies on the biological action of Antimycin A. Arch Biochem 28:281-294.
- Aidoo KE, Hendry R, Wood BJB (1981) Estimation of fungal growth in a solid-state fermentation system. Eur J Appl Microbiol Biotechnol 12:6-9. doi:10.1007/Bf00508111
- Aimanianda V, Latge JP (2010) Fungal hydrophobins form a sheath preventing immune recognition of airborne conidia. Virulence 1:185-187. doi:10.4161/viru.1.3.11317
- Ali S (1992) Regulation of cellulase biosynthesis in *Aspergillus terreus*. World J Microbiol Biotech 8:73-75.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic Local Alignment Search Tool. J Mol Biol 215:403-410. doi:10.1006/jmbi.1990.9999
- Andersen M, Giese M, P R, Nielsen J (2012) Mapping the polysaccharide degradation potential of *Aspergillus niger*. BMC Genomics 13:313. doi:10.1186/1471-2164-13-313
- Andersen MR (2008) Systems biology studies of *Aspergilli* - From sequence to science. Technical University of Denmark
- Andersen MR, Salazar MP, Schaap PJ, van de Vondervoort PJ, Culley D, Thykaer J, Frisvad JC, Nielsen KF, Albang R, Albermann K, Berka RM, Braus GH, Braus-Stromeyer SA, Corrochano LM, Dai Z, van Dijck PW, Hofmann G, Lasure LL, Magnuson JK, Menke H, Meijer M, Meijer SL, Nielsen JB, Nielsen ML, van Ooyen AJ, Pel HJ, Poulsen L, Samson RA, Stam H, Tsang A, van den Brink JM, Atkins A,

- Aerts A, Shapiro H, Pangilinan J, Salamov A, Lou Y, Lindquist E, Lucas S, Grimwood J, Grigoriev IV, Kubicek CP, Martinez D, van Peij NN, Roubos JA, Nielsen J, Baker SE (2011) Comparative genomics of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88. *Genome Res* 21:885-897. doi:10.1101/gr.112169.110
- Andersen MR, Vongsangnak W, Panagiotou G, Salazar MP, Lehmann L, Nielsen J (2008) A trispecies *Aspergillus* microarray: Comparative transcriptomics of three *Aspergillus* species. *Proc Nat Acad Sci* 105:4387-4392. doi:10.1073/pnas.0709964105
- Anderson NG, Maller JL, Tonks NK, Sturgill TW (1990) Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* 343:651-653. doi:10.1038/343651a0
- Andersson BE, Welinder L, Olsson PA, Olsson S, Henrysson T (2000) Growth of inoculated white-rot fungi and their interactions with the bacterial community in soil contaminated with polycyclic aromatic hydrocarbons, as measured by phospholipid fatty acids. *Bioresource Technol* 73:29-36. doi:10.1016/S0960-8524(99)00134-0
- Aragon T, van Anken E, Pincus D, Serafimova IM, Korennykh AV, Rubio CA, Walter P (2009) Messenger RNA targeting to endoplasmic reticulum stress signalling sites. *Nature* 457:736-740. doi:10.1038/nature07641
- Arantes V, Saddler J (2010) Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. *Biotechnol Biofuels* 3:4. doi: 10.1186/1754-6834-3-4
- Arao T (1999) *In situ* detection of changes in soil bacterial and fungal activities by measuring C-13 incorporation into soil phospholipid fatty acids from C-13 acetate. *Soil Biol Biochem* 31:1015-1020. doi:10.1016/S0038-0717(99)00015-2
- Archer DB, Connerton IF, MacKenzie DA (2008) Filamentous fungi for production of food additives and processing aids. *Food Biotechnol* 111:99-147. doi:10.1007/10\_2007\_094
- Archer DB, Roberts IN, Mackenzie DA (1990) Heterologous protein secretion from *Aspergillus niger* in phosphate-buffered batch culture. *Appl Microbiol Biotechnol* 34:313-315.
- Aro N, Ilmén M, Saloheimo A, Penttilä M (2003) ACEI of *Trichoderma reesei* is a repressor of cellulase and xylanase expression. *Appl Environ Microbiol* 69:56-65. doi:10.1128/aem.69.1.56-65.2003
- Aro N, Pakula T, Penttilä M (2005) Transcriptional regulation of plant cell wall degradation by filamentous fungi. *FEMS Microbiol Rev* 29:719-739. doi:10.1016/j.femsre.2004.11.006

- Aro N, Saloheimo A, Ilmén M, Penttilä M (2001) ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. J Biol Chem 276:24309-24314. doi:10.1074/jbc.M003624200
- Arst HN, Jr., Bignell E, Tilburn J (1994) Two new genes involved in signalling ambient pH in *Aspergillus nidulans*. Mol Gen Genet 245:787-790. doi: 10.1007/BF00297286
- Arst HN, Peñalva MA (2003) pH regulation in *Aspergillus* and parallels with higher eukaryotic regulatory systems. Trends Genet 19:224-231. doi:10.1016/S0168-9525(03)00052-0
- Baetz K, Moffat J, Haynes J, Chang M, Andrews B (2001) Transcriptional coregulation by the cell integrity mitogen-activated protein kinase Slt2 and the cell cycle regulator Swi4. Mol Cell Biol 21:6515-6528. doi: 10.1128/MCB.21.19.6515-6528.2001
- Bai ZH, Zhang HX, Qi HY, Peng XW, Li BJ (2004) Pectinase production by *Aspergillus niger* using wastewater in solid state fermentation for eliciting plant disease resistance. Bioresour Technol 95:49-52. doi:10.1016/j.biortech.2003.06.006
- Bailey MJ, Buchert J, Viikari L (1993) Effect of pH on production of xylanase by *Trichoderma reesei* on xylan-based and cellulose-based media. Appl Microbiol Biotechnol 40:224-229. doi: 10.1007/BF00170371
- Bairoch A (1999) The enzyme data bank in 1999. Nucleic Acids Res 27:310-311. doi: 10.1093/nar/27.1.310
- Bajracharya R, Mudgett RE (1980) Effects of controlled gas environments in solid-substrate fermentations of rice. Biotechnol Bioeng 22:2219-2235. doi:10.1002/bit.260221103
- Baker SE (2006) *Aspergillus niger* genomics: past, present and into the future. Med Mycol 44 Suppl 1:S17-21. doi:10.1080/13693780600921037
- Baldrian P, Valaskova V (2008) Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev 32:501-521. doi:10.1111/j.1574-6976.2008.00106.x
- Bardgett RD, Anderson JM, Behan-Pelletier V, Brussaard L, Coleman DC, Ettema C, Moldenke A, Schimel JP, Wall DH (2001) The influence of soil biodiversity on hydrological pathways and the transfer of materials between terrestrial and aquatic ecosystems. Ecosystems 4:421-429. doi:10.1007/s10021-001-0020-5
- Battaglia E, Hansen S, Leendertse A, Madrid S, Mulder H, Nikolaev I, Vries R (2011a) Regulation of pentose utilisation by AraR, but not XlnR, differs in *Aspergillus nidulans* and *Aspergillus niger*. Appl Microbiol Biotechnol 91:387-397. doi:10.1007/s00253-011-3242-2
- Battaglia E, Visser L, Nijssen A, van Veluw GJ, Wösten HA, de Vries RP (2011b) Analysis of regulation of pentose utilisation in *Aspergillus niger* reveals evolutionary adaptations in Eurotiales. Stud Mycol 69:31-38. doi:10.3114/sim.2011.69.03
- Bayry J, Aïmanianda V, Guijarro JI, Sunde M, Latge JP (2012) Hydrophobins-unique fungal proteins. PLoS Pathog 8:e1002700. doi:10.1371/journal.ppat.1002700

- Bennett JW (2010) An Overview of the Genus *Aspergillus*. *Aspergillus: Mol Biol Genom* -17.
- Bermejo C, Rodriguez E, Garcia R, Rodriguez-Pena JM, Rodriguez de la Concepcion ML, Rivas C, Arias P, Nombela C, Posas F, Arroyo J (2008) The sequential activation of the yeast HOG and SLT2 pathways is required for cell survival to cell wall stress. *Mol Biol Cell* 19:1113-1124. doi:10.1091/mbc.E07-08-0742
- Bernales S, Papa FR, Walter P (2006) Intracellular signaling by the unfolded protein response. *Annu Rev Cell Dev Biol* 22:487-508. doi:10.1146/annurev.cellbio.21.122303.120200
- Biely P, Cote GL, Kremnicky L, Greene RV (1999) Differences in catalytic properties of acetylxyloxy esterases and non-hemicellulolytic acetyl esterases. *Recent Adv Carbohydrate Bioeng* 73-81.
- Biely P, Mackenzie CR, Puls J, Schneider H (1986) Cooperativity of esterases and xyloxyases in the enzymatic degradation of acetyl xyloxy. *Bio Technol* 4:731-733. doi:10.1038/Nbt0886-731
- Biely P, Puls J, Schneider H (1985) Acetyl xyloxy esterases in fungal cellulolytic systems. *FEBS Lett* 186:80-84. doi:10.1016/0014-5793(85)81343-0
- Bind E, Kleyner Y, Skowronska-Krawczyk D, Bien E, Dynlacht BD, Sanchez I (2004) A novel mechanism for mitogen-activated protein kinase localization. *Mol Biol Cell* 15:4457-4466. doi:10.1091/mbc.E04-03-0234
- BIOMASS Energy Centre (2013) Crops are grown specifically for use as fuel. <http://www.biomassenergycentre.org.uk/>
- Blumer-Schuette SE, Lewis DL, Kelly RM (2010) Phylogenetic, microbiological, and glycoside hydrolase diversities within the extremely thermophilic, plant biomass-degrading genus *Caldicellulosiruptor*. *Appl Environ Microbiol* 76:8084-8092. doi:10.1128/Aem.01400-10
- Bokinsky G, Peralta-Yahya PP, George A, Holmes BM, Steen EJ, Dietrich J, Lee TS, Tullman-Ercek D, Voigt CA, Simmons BA, Keasling JD (2011) Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered *Escherichia coli*. *Proc Natl Acad Sci USA* 108:19949-19954. doi:10.1073/pnas.1106958108
- Bouchecareilh M, Chevet E (2009) Endoplasmic reticulum stress: light my FIRE. *M S-Med Sci* 25:281-287. doi:10.1051/medsci/2009253281
- Boucias DG, Pendland JC, Latge JP (1988) Nonspecific factors involved in attachment of entomopathogenic deuteromycetes to host insect cuticle. *Appl Environ Microbiol* 54:1795-1805.
- Boulton NS, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD (1991) ERKs: a family of protein-

- serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 17:663-675. doi: 10.1016/0092-8674(91)90098-J
- Brown NA, de Gouvea PF, Krohn NG, Savoldi M, Goldman GH (2013) Functional characterisation of the non-essential protein kinases and phosphatases regulating *Aspergillus nidulans* hydrolytic enzyme production. *Biotechnol Biofuels* 6:91. doi:10.1186/1754-6834-6-91
- Brunner K, Lichtenauer AM, Kratochwill K, Delic M, Mach RL (2007) Xyr1 regulates xylanase but not cellulase formation in the head blight fungus *Fusarium graminearum*. *Curr Genet* 52:213-220. doi:10.1007/s00294-007-0154-x
- Buanafina dO (2009) Feruloylation in grasses: current and future perspectives. *Mol Plant* 2:861-872. doi:10.1093/mp/ssp067
- Burnett G, Kennedy EP (1954) The enzymatic phosphorylation of proteins. *J Biol Chem* 211:969-980.
- Burns RG, DeForest JL, Marxsen J, Sinsabaugh RL, Stromberger ME, Wallenstein MD, Weintraub MN, Zoppini A (2013) Soil enzymes in a changing environment: Current knowledge and future directions. *Soil Biol Biochem* 58:216-234. doi:10.1016/j.soilbio.2012.11.009
- Caddick MX (2008) Gene regulation in the *Aspergilli*: Genomics, medical aspects, biotechnology, and research methods. edited by G H Goldman and S A Osmani. CRC Press.
- Caddick MX, Brownlee AG, Arst HN, Jr. (1986) Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. *Mol Gen Genet* 203:346-353.
- Calcagno-Pizarelli AM, Negrete-Urtasun S, Denison SH, Rudnicka JD, Bussink HJ, Munera-Huertas T, Stanton L, Hervas-Aguilar A, Espeso EA, Tilburn J, Arst HN, Jr., Penalva MA (2007) Establishment of the ambient pH signaling complex in *Aspergillus nidulans*: PalH assists plasma membrane localization of PalH. *Eukaryot Cell* 6:2365-2375. doi:10.1128/EC.00275-07
- Calkhoven CF, Ab G (1996) Multiple steps in the regulation of transcription-factor level and activity. *Biochem J* 317 329-342. doi: 10.1042/bj3170329
- Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res* 37:D233-238. doi:10.1093/nar/gkn663
- Carder JH (1986) Detection and quantitation of cellulase by Congo red staining of substrates in a cup-plate diffusion assay. *Anal Biochem* 153:75-79.
- Carlile MW, Watkinson SC, Gooday GW (1994) *The fungi* (second edition). Academic Press
- Carvalho ND, Arentshorst M, Jin Kwon M, Meyer V, Ram AF (2010) Expanding the ku70 toolbox for filamentous fungi: establishment of complementation vectors and recipient

- strains for advanced gene analyses. *Appl Microbiol Biotechnol* 87:1463-1473. doi:10.1007/s00253-010-2588-1
- Chen CH, Ringelberg CS, Gross RH, Dunlap JC, Loros JJ (2009) Genome-wide analysis of light-inducible responses reveals hierarchical light signalling in *Neurospora*. *EMBO J* 28:1029-1042. doi:10.1038/emboj.2009.54
- Chen RE, Thorner J (2007) Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* 1773:1311-1340. doi:10.1016/j.bbamcr.2007.05.003
- Chen YL, Montedonico AE, Kauffman S, Dunlap JR, Menn FM, Reynolds TB (2010) Phosphatidylserine synthase and phosphatidylserine decarboxylase are essential for cell wall integrity and virulence in *Candida albicans*. *Mol Microbiol* 75:1112-1132. doi:10.1111/j.1365-2958.2009.07018.x
- Cheng M, Zhen E, Robinson MJ, Ebert D, Goldsmith E, Cobb MH (1996) Characterization of a protein kinase that phosphorylates serine 189 of the mitogen-activated protein kinase homolog ERK3. *J Biol Chem* 271:12057-12062. Doi: 10.1074/jbc.271.20.12057
- Child JJ, Knapp C, Eveleigh DE (1973) Improved pH control of fungal culture media. *Mycologia* 65:1078-1086.
- Chilton IJ, Delaney CE, Barham-Morris J, Fincham DA, Hooley P, Whitehead MP (2008) The *Aspergillus nidulans* stress response transcription factor StzA is ascomycete-specific and shows species-specific polymorphisms in the C-terminal region. *Mycol Res* 112:1435-1446. doi:10.1016/j.mycres.2008.06.028
- Coradetti ST, Craig JP, Xiong Y, Shock T, Tian C, Glass NL (2012) Conserved and essential transcription factors for cellulase gene expression in ascomycete fungi. *Proc Natl Acad Sci USA* 109:7397-7402. doi:10.1073/pnas.1200785109
- Council for Agricultural Science and Technology (2003) Mycotoxins: Risks in Plants, Animal and Human Systems. In: 139 TFR (ed). Iowa: CAST
- Coulombe P, Rodier G, Bonneil E, Thibault P, Meloche S (2004) N-Terminal ubiquitination of extracellular signal-regulated kinase 3 and p21 directs their degradation by the proteasome. *Mol Cell Biol* 24:6140-6150. doi:10.1128/MCB.24.14.6140-6150.2004
- Coulombe P, Rodier G, Pelletier S, Pellerin J, Meloche S (2003) Rapid turnover of extracellular signal-regulated kinase 3 by the ubiquitin-proteasome pathway defines a novel paradigm of mitogen-activated protein kinase regulation during cellular differentiation. *Mol Cell Biol* 23:4542-4558. doi: 10.1128/MCB.23.13.4542-4558.2003
- Credle JJ, Finer-Moore JS, Papa FR, Stroud RM, Walter P (2005) On the mechanism of sensing unfolded protein in the endoplasmic reticulum. *Proc Natl Acad Sci USA* 52:18773-18784. doi:10.1073/pnas.0509487102

- Dagley MJ, Gentle IE, Beilharz TH, Pettolino FA, Djordjevic JT, Lo TL, Uwamahoro N, Rupasinghe T, Tull DL, McConville M, Beaurepaire C, Nantel A, Lithgow T, Mitchell AP, Traven A (2011) Cell wall integrity is linked to mitochondria and phospholipid homeostasis in *Candida albicans* through the activity of the post-transcriptional regulator Ccr4-Pop2. *Mol Microbiol* 79:968-989. doi:10.1111/j.1365-2958.2010.07503.x
- Dague E, Delcorte A, Latge JP, Dufrene YF (2008) Combined use of atomic force microscopy, X-ray photoelectron spectroscopy, and secondary ion mass spectrometry for cell surface analysis. *Langmuir* 24:2955-2959. doi:10.1021/la703741y
- Dashtban M, Schraft H, Qin W (2009) Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. *Int J Biol Sci* 5:578-595. doi: 10.7150/ijbs.5.578
- de Souza WR, de Gouvea PF, Savoldi M, Malavazi I, de Souza Bernardes LA, Goldman MH, de Vries RP, de Castro Oliveira JV, Goldman GH (2011) Transcriptome analysis of *Aspergillus niger* grown on sugarcane bagasse. *Biotechnol Biofuels* 4:40. doi:10.1186/1754-6834-4-40 [pii]10.1186/1754-6834-4-40 [doi]
- de Souza WR, Maitan-Alfenas GP, de Gouvêa PF, Brown NA, Savoldi M, Battaglia E, Goldman MHS, de Vries RP, Goldman GH (2013) The influence of *Aspergillus niger* transcription factors AraR and XlnR in the gene expression during growth in d-xylose, l-arabinose and steam-exploded sugarcane bagasse. *Fung Genet Biol* 60:29-45. doi:10.1016/j.fgb.2013.07.007
- de Vocht ML, Reviakine I, Ulrich WP, Bergsma-Schutter W, Wösten HA, Vogel H, Brisson A, Wessels JG, Robillard GT (2002) Self-assembly of the hydrophobin SC3 proceeds via two structural intermediates. *Protein Sci* 11:1199-1205. doi:10.1110/ps.4540102
- de Vocht ML, Scholtmeijer K, van der Vegte EW, de Vries OMH, Sonveaux N, Wösten HAB, Ruyschaert JM, Hadziioannou G, Wessels JGH, Robillard GT (1998) Structural characterization of the hydrophobin SC3, as a monomer and after self-assembly at hydrophobic/hydrophilic interfaces. *Biophysic J* 74:2059-2068. doi: 10.1016/S0006-3495(98)77912-3
- de Vries RP, Visser J (1999a) Regulation of the feruloyl esterase (*faeA*) gene from *Aspergillus niger*. *Appl Environ Microbiol* 65:5500-5503.
- de Vries RP, Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol Mol Biol Rev* 65:497-522. doi:10.1128/MMBR.65.4.497-522.2001
- de Vries RP, Visser J, de Graaff LH (1999b) CreA modulates the XlnR-induced expression on xylose of *Aspergillus niger* genes involved in xylan degradation. *Res Microbiol* 150:281-285. doi: 10.1016/S0923-2508(99)80053-9

- Delmas S, Llanos A, Parrou JL, Kokolski M, Pullan ST, Shunburne L, Archer DB (2014) Development of an unmarked gene deletion system for the filamentous fungi *Aspergillus niger* and *Talaromyces versatilis*. *Appl Environ Microbiol* 80:3484-3487. doi:10.1128/AEM.00625-14
- Delmas S, Pullan ST, Gaddipati S, Kokolski M, Malla S, Blythe MJ, Ibbett R, Campbell M, Liddell S, Aboobaker A, Tucker GA, Archer DB (2012) Uncovering the genome-wide transcriptional responses of the filamentous fungus *Aspergillus niger* to lignocellulose using RNA sequencing. *PLoS Genet* 8:e1002875. doi:10.1371/journal.pgen.1002875
- Denhardt DT (1996) Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. *Biochem J* 318 729-747. doi: 10.1042/bj3180729
- Desgranges C, Vergoignan C, Georges M, Durand A (1991) Biomass estimation in solid-state fermentation .1. Manual biochemical methods. *Appl Microbiol Biotechnol* 35:200-205.
- Deutscher J, Francke C, Postma PW (2006) How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol Mol Biol Rev* 70:939-1031. doi:10.1128/MMBR.00024-06
- DeZwaan TM, Carroll AM, Valent B, Sweigard JA (1999) *Magnaporthe grisea* Pth11p is a novel plasma membrane protein that mediates appressorium differentiation in response to inductive substrate cues. *Plant Cell* 11:2013-2030. doi:10.1105/tpc.11.10.2013
- Diez E, Alvaro J, Espeso EA, Rainbow L, Suarez T, Tilburn J, Arst HN, Jr., Penalva MA (2002) Activation of the *Aspergillus* PacC zinc finger transcription factor requires two proteolytic steps. *EMBO J* 21:1350-1359. doi:10.1093/emboj/21.6.1350
- Dodd D, Cann IK (2009) Enzymatic deconstruction of xylan for biofuel production. *Glob Change Biol Bioenergy* 1:2-17. doi:10.1111/j.1757-1707.2009.01004.x
- Dowzer CE, Kelly JM (1991) Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. *Mol Cell Biol* 11:5701-5709.
- Drysdale MR, Kolze SE, Kelly JM (1993) The *Aspergillus niger* carbon catabolite repressor encoding gene, *creA*. *Gene* 130:241-245.
- Duff SJB, Cooper DG, Fuller OM (1987) Effect of media composition and growth conditions on production of cellulase and  $\beta$ -glucosidase by a mixed fungal fermentation. *Enzyme Microb Technol* 9:47-52. doi:10.1016/0141-0229(87)90048-2
- Duncan MW, Aebersold R, Caprioli RM (2010) The pros and cons of peptide-centric proteomics. *Nat Biotechnol* 28:659-664. doi:10.1038/nbt0710-659
- Eisman B, Alonso-Monge R, Roman E, Arana D, Nombela C, Pla J (2006) The Cek1 and Hog1 mitogen-activated protein kinases play complementary roles in cell wall

- biogenesis and chlamydospore formation in the fungal pathogen *Candida albicans*. Eukaryot Cell 5:347-358. doi:10.1128/EC.5.2.347-358.2006
- Elias JE, Gygi SP (2010) Target-decoy search strategy for mass spectrometry-based proteomics. Methods Mol Biol 604:55-71. doi:10.1007/978-1-60761-444-9\_5
- Ellgaard L, Helenius A (2003) Quality control in the endoplasmic reticulum. Nat Rev Mol Cell Biol 4:181-191. doi:10.1038/nrm1052
- Endo Y, Yokoyama M, Morimoto M, Shirai K, Chikamatsu G, Kato N, Tsukagoshi N, Kato M, Kobayashi T (2008) Novel promoter sequence required for inductive expression of the *Aspergillus nidulans* endoglucanase gene *eglA*. Biosci Biotechnol Biochem 72:312-320.
- Espeso EA, Tilburn J, Sanchez-Pulido L, Brown CV, Valencia A, Arst HN, Penalva MA (1997) Specific DNA recognition by the *Aspergillus nidulans* three zinc finger transcription factor PacC. J Mol Biol 274:466-480. doi:10.1006/jmbi.1997.1428
- European Commission Report (1994) Mycotoxins in human nutrition and health. Agroindustrial research division of the European Commission Directorate-General XII for scientific research and development 36.
- Evans RM (1988) The steroid and thyroid hormone receptor superfamily. Science 240:889-895.
- Feinstein LM, Sul WJ, Blackwood CB (2009) Assessment of bias associated with incomplete extraction of microbial DNA from soil. Appl Environ Microbiol 75:5428-5433. doi:10.1128/AEM.00120-09
- Ferrigno P, Posas F, Koepp D, Saito H, Silver PA (1998) Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. EMBO J 17:5606-5614. doi:10.1093/emboj/17.19.5606
- Fetherston R (2013) Investigating the role of hydrophobic surface interacting proteins in the saccharification of lignocellulose by *Aspergillus niger*. University of Nottingham
- Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. Proc Natl Acad Sci USA 103:626-631. doi:10.1073/pnas.0507535103
- Firestone MK, Killham K, Mccoll JG (1983) Fungal toxicity of mobilized soil aluminum and manganese. Appl Environ Microbiol 46:758-761.
- Flipphi M, van de Vondervoort PJ, Ruijter GJ, Visser J, Arst HN, Jr., Felenbok B (2003) Onset of carbon catabolite repression in *Aspergillus nidulans*. Parallel involvement of hexokinase and glucokinase in sugar signaling. J Biol Chem 278:11849-11857. doi:10.1074/jbc.M209443200
- Flis SE, Glenn AR, Dilworth MJ (1993) The interaction between aluminum and root nodule bacteria. Soil Biol Biochem 25:403-417. doi:10.1016/0038-0717(93)90066-K

- Fulks RM, Stadtman ER (1985) Regulation of glutamine synthetase, aspartokinase, and total protein turnover in *Klebsiella aerogenes*. *Biochim Biophys Acta* 843:214-229.
- Furukawa K, Hoshi Y, Maeda T, Nakajima T, Abe K (2005) *Aspergillus nidulans* HOG pathway is activated only by two-component signalling pathway in response to osmotic stress. *Mol Microbiol* 56:1246-1261. doi:10.1111/j.1365-2958.2005.04605.x
- Gao D, Chundawat SP, Krishnan C, Balan V, Dale BE (2010) Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover. *Bioresour Technol* 101:2770-2781. doi:10.1016/j.biortech.2009.10.056
- Garcia R, Rodriguez-Pena JM, Bermejo C, Nombela C, Arroyo J (2009) The high osmotic response and cell wall integrity pathways cooperate to regulate transcriptional responses to zymolyase-induced cell wall stress in *Saccharomyces cerevisiae*. *J Biol Chem* 284:10901-10911. doi:10.1074/jbc.M808693200
- Garrido SM, Kitamoto N, Watanabe A, Shintani T, Gomi K (2012) Functional analysis of FarA transcription factor in the regulation of the genes encoding lipolytic enzymes and hydrophobic surface binding protein for the degradation of biodegradable plastics in *Aspergillus oryzae*. *J Biosci Bioeng* 113:549-555. doi:10.1016/j.jbiosc.2011.12.014
- Geysens S, Whyteside G, Archer DB (2009) Genomics of protein folding in the endoplasmic reticulum, secretion stress and glycosylation in the *Aspergilli*. *Fung Genet Biol* 46:S121-S140. doi:10.1016/j.fgb.2008.07.016
- Gielkens M, Gonzalez-Candelas L, Sanchez-Torres P, van de Vondervoort P, de Graaff L, Visser J, Ramon D (1999) The *abfB* gene encoding the major alpha-L-arabinofuranosidase of *Aspergillus nidulans*: nucleotide sequence, regulation and construction of a disrupted strain. *Microbiol* 145 735-741.
- Gielkens MM, Visser J, de Graaff LH (1997) Arabinoxylan degradation by fungi: characterization of the arabinoxylan-arabinofuranohydrolase encoding genes from *Aspergillus niger* and *Aspergillus tubingensis*. *Curr Genet* 31:22-29.
- Gilkes NR, Henrissat B, Kilburn DG, Miller RC, Jr., Warren RA (1991) Domains in microbial beta-1, 4-glycanases: sequence conservation, function, and enzyme families. *Microbiol Rev* 55:303-315.
- Gourlay K, Hu J, Arantes V, Andberg M, Saloheimo M, Penttilä M, Saddler J (2013) Swollenin aids in the amorphogenesis step during the enzymatic hydrolysis of pretreated biomass. *Biores Technol* 142:498-503. doi:10.1016/j.biortech.2013.05.053
- Graboski MS, McCormick RL (1998) Combustion of fat and vegetable oil derived fuels in diesel engines. *Prog Energ Combust Sci* 24:125-164. doi:10.1016/S0360-1285(97)00034-8

- Gray JV, Ogas JP, Kamada Y, Stone M, Levin DE, Herskowitz I (1997) A role for the Pkc1 MAP kinase pathway of *Saccharomyces cerevisiae* in bud emergence and identification of a putative upstream regulator. *EMBO J* 16:4924-4937. doi:10.1093/emboj/16.16.4924
- Guerlava P, Izac V, Tholozan JL (1998) Comparison of different methods of cell lysis and protein measurements in *Clostridium perfringens*: application to the cell volume determination. *Curr Microbiol* 36:131-135.
- Guillemette T, van Peij N, Goosen T, Lanthaler K, Robson G, van den Hondel C, Stam H, Archer D (2007) Genomic analysis of the secretion stress response in the enzyme-producing cell factory *Aspergillus niger*. *BMC Genomics* 8:158. doi: 10.1186/1471-2164-8-158
- Gyalai-Korpos M, Nagy G, Mareczky Z, Schuster A, Reczey K, Schmoll M (2010) Relevance of the light signaling machinery for cellulase expression in *Trichoderma reesei* (*Hypocrea jecorina*). *BMC Res Notes* 3:330. doi:10.1186/1756-0500-3-330
- Hakanpää J, Linder M, Popov A, Schmidt A, Rouvinen J (2006) Hydrophobin HFBII in detail: ultrahigh-resolution structure at 0.75 Å. *Acta Crystallogr D Biol Crystallogr* 62:356-367. doi:10.1107/S0907444906000862
- Hakanpää J, Parkkinen TAA, Hakulinen N, Linder M, Rouvinen J (2004) Crystallization and preliminary X-ray characterization of *Trichoderma reesei* hydrophobin HFBII. *Acta Crystallogr D Biol Crystallogr* 60:163-165. doi:10.1107/S0907444903024430
- Hakkinen M (2014) Transcriptional analysis of *Trichoderma reesei* under conditions inducing cellulase and hemicellulase production, and identification of factors influencing protein production. University of Helsinki, Finland
- Hakkinen M, Sivasiddharthan D, Aro N, Saloheimo M, Pakula TM (2015) The effects of extracellular pH and of the transcriptional regulator PACI on the transcriptome of *Trichoderma reesei*. *Microb Cell Fact* 14:63. doi:10.1186/s12934-015-0247-z
- Hasan HAH (1995) *Alternaria* mycotoxins in black rot lesion of tomato fruit - Conditions and regulation of their production. *Mycopathol* 130:171-177. doi:10.1007/Bf01103101
- Hasper AA, Dekkers E, van Mil M, van de Vondervoort PJ, de Graaff LH (2002) EglC, a new endoglucanase from *Aspergillus niger* with major activity towards xyloglucan. *Appl Environ Microbiol* 68:1556-1560. doi: 10.1128/AEM.68.4.1556-1560.2002
- Hasper AA, Visser J, de Graaff LH (2000) The *Aspergillus niger* transcriptional activator XlnR, which is involved in the degradation of the polysaccharides xylan and cellulose, also regulates D-xylose reductase gene expression. *Mol Microbiol* 36:193-200. doi: 10.1046/j.1365-2958.2000.01985.x

- Hasper AA, Trindade LM, van der Veen D, van Ooyen AJ, de Graaff LH (2004) Functional analysis of the transcriptional activator XlnR from *Aspergillus niger*. Microbiol 150:1367-1375. doi: 10.1099/mic.0.26557-0
- Hattori T, Kino K, Kirimura K (2009) Regulation of alternative oxidase at the transcription stage in *Aspergillus niger* under the conditions of citric acid production. Curr Microbiol 58:321-325. doi:10.1007/s00284-009-9369-z
- Hayer K (2013) Germination of *Aspergillus niger* conidia. University of Nottingham
- He Q, Liu Y (2005) Molecular mechanism of light responses in *Neurospora*: from light-induced transcription to photoadaptation. Genes Dev 19:2888-2899. doi:10.1101/gad.1369605
- Henrissat B (1999) Classification of chitinases modules. EXS 87:137-156. doi: 10.1007/978-3-0348-8757-1\_10
- Henrissat B, Callebaut I, Fabrega S, Lehn P, Mornon JP, Davies G (1995a) Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proc Natl Acad Sci U S A 92:7090-7094. doi: 10.1073/pnas.92.15.7090
- Henrissat B, Romeu A (1995b) Families, superfamilies and subfamilies of glycosyl hydrolases. Biochem J 311 350-351. doi: 10.1042/bj3110350
- Hernández MRR, Nelson Pérez Guerra, Renato Pérez Rosés (2006) Amylase production by *Aspergillus niger* in submerged cultivation on two wastes from food industries. J Food Eng 73:93-100. doi: 10.1016/j.jfoodeng.2005.01.009
- Herold S, Bischof R, Metz B, Seiboth B, Kubicek CP (2013) Xylanase gene transcription in *Trichoderma reesei* is triggered by different inducers representing different hemicellulosic pentose polymers. Eukaryot Cell 12:390-398. doi:10.1128/ec.00182-12
- Herranz S, Rodriguez JM, Bussink HJ, Sanchez-Ferrero JC, Arst HN, Jr., Penalva MA, Vincent O (2005) Arrestin-related proteins mediate pH signaling in fungi. Proc Natl Acad Sci U S A 102:12141-12146. doi:10.1073/pnas.0504776102
- Herrera-Estrella A, Horwitz BA (2007) Looking through the eyes of fungi: molecular genetics of photoreception. Mol Microbiol 64:5-15. doi:10.1111/j.1365-2958.2007.05632.x
- Hervas-Aguilar A, Galindo A, Penalva MA (2010) Receptor-independent ambient pH signaling by ubiquitin attachment to fungal arrestin-like PalF. J Biol Chem 285:18095-18102. doi:10.1074/jbc.M110.114371
- Hervas-Aguilar A, Rodriguez JM, Tilburn J, Arst HN, Jr., Penalva MA (2007) Evidence for the direct involvement of the proteasome in the proteolytic processing of the *Aspergillus nidulans* zinc finger transcription factor PacC. J Biol Chem 282:34735-34747. doi:10.1074/jbc.M706723200

- Herve C, Rogowski A, Blake AW, Marcus SE, Gilbert HJ, Knox JP (2010) Carbohydrate-binding modules promote the enzymatic deconstruction of intact plant cell walls by targeting and proximity effects. *Proc Natl Acad Sci U S A* 107:15293-15298. doi:10.1073/pnas.1005732107
- Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol Mol Biol Rev* 66:300-372. doi: 10.1128/MMBR.66.2.300-372.2002
- Hook SS, Means AR (2001) Ca(2+)/CaM-dependent kinases: from activation to function. *Annu Rev Pharmacol Toxicol* 41:471-505. doi:10.1146/annurev.pharmtox.41.1.471
- Hoson T, Wada S (1983) Possible role of hexosamine-containing cell wall component in growth regulation of rice coleoptiles. *Plant Cell Physiol* 24:1421-1430.
- Hrmova M, Petrakova E, Biely P (1991) Induction of cellulose- and xylan-degrading enzyme systems in *Aspergillus terreus* by homo- and heterodisaccharides composed of glucose and xylose. *J Gen Microbiol* 137:541-547.
- Hu HL, van den Brink J, Gruben BS, Wösten HAB, Gu JD, de Vries RP (2011) Improved enzyme production by co-cultivation of *Aspergillus niger* and *Aspergillus oryzae* and with other fungi. *Internatl Biodeter Biodegrad* 65:248-252. doi:10.1016/j.ibiod.2010.11.008
- Huang W, Erikson RL (1994) Constitutive activation of Mek1 by mutation of serine phosphorylation sites. *Proc Natl Acad Sci U S A* 91:8960-8963. doi: 10.1073/pnas.91.19.8960
- Hunter T (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 80:225-236. doi: 10.1016/0092-8674(95)90405-0
- Hunter T, Karin M (1992) The regulation of transcription by phosphorylation. *Cell* 70:375-387. doi: 10.1016/0092-8674(92)90162-6
- Hyeon JE, Jeon WJ, Whang SY, Han SO (2011) Production of minicellulosomes for the enhanced hydrolysis of cellulosic substrates by recombinant *Corynebacterium glutamicum*. *Enzyme Microb Technol* 48:371-377. doi:10.1016/j.enzmictec.2010.12.014
- Hynes MJ, Murray SL, Duncan A, Khew GS, Davis MA (2006) Regulatory genes controlling fatty acid catabolism and peroxisomal functions in the filamentous fungus *Aspergillus nidulans*. *Eukaryot Cell* 5:794-805. doi:10.1128/EC.5.5.794-805.2006
- Ilmén M, Thrane C, Penttilä M (1996) The glucose repressor gene cre1 of *Trichoderma*: Isolation and expression of a full-length and a truncated mutant form. *Mol Gen Genet* 251:451-460. doi:10.1007/BF02172374
- Jalving R, van de Vondervoort PJ, Visser J, Schaap PJ (2000) Characterization of the kexin-like maturase of *Aspergillus niger*. *Appl Environ Microbiol* 66:363-368. doi: 10.1128/AEM.66.1.363-368.2000

- Janeway CA (2001) How the immune system protects the host from infection. *Microbes Infect* 3:1167-1171. doi: 10.1016/S1286-4579(01)01477-0
- Jensen BG, Andersen MR, Pedersen MH, Frisvad JC, Sondergaard I (2010) Hydrophobins from *Aspergillus* species cannot be clearly divided into two classes. *BMC Res Notes* 3:344. doi:10.1186/1756-0500-3-344
- Jordan DB, Bowman MJ, Braker JD, Dien BS, Hector RE, Lee CC, Mertens JA, Wagschal K (2012) Plant cell walls to ethanol. *Biochem J* 442:241-252. doi:10.1042/BJ20111922
- Jorgensen TR, Goosen T, Hondel CA, Ram AF, Iversen JJ (2009) Transcriptomic comparison of *Aspergillus niger* growing on two different sugars reveals coordinated regulation of the secretory pathway. *BMC Genomics* 10:44. doi:10.1186/1471-2164-10-44
- Juhasz T, Szengyel Z, Reczey K, Siika-Aho M, Viikari L (2005) Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. *Process Biochemistry* 40:3519-3525. doi:10.1016/j.procbio.2005.03.057
- Julien C, Coulombe P, Meloche S (2003) Nuclear export of ERK3 by a CRM1-dependent mechanism regulates its inhibitory action on cell cycle progression. *J Biol Chem* 278:42615-42624. doi:10.1074/jbc.M302724200
- Jung US, Levin DE (1999) Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. *Mol Microbiol* 34:1049-1057. doi: 10.1046/j.1365-2958.1999.01667.x
- Kallio JM, Linder MB, Rouvinen J (2007) Crystal structures of hydrophobin HFBII in the presence of detergent implicate the formation of fibrils and monolayer films. *J Biol Chem* 282:28733-28739. doi:10.1074/jbc.M704238200
- Kant S, Schumacher S, Singh MK, Kispert A, Kotlyarov A, Gaestel M (2006) Characterization of the atypical MAPK ERK4 and its activation of the MAPK-activated protein kinase MK5. *J Biol Chem* 281:35511-35519. doi:10.1074/jbc.M606693200
- Karaffa L, Kubicek CP (2003) *Aspergillus niger* citric acid accumulation: do we understand this well working black box? *Appl Microbiol Biotechnol* 61:189-196. doi:10.1007/s00253-002-1201-7
- Karin M, Hunter T (1995) Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr Biol* 5:747-757.
- Karp A, Shield I (2008) Bioenergy from plants and the sustainable yield challenge. *New Phytol* 179:15-32. doi:10.1111/j.1469-8137.2008.02432.x
- Kaufman RJ (2002) Orchestrating the unfolded protein response in health and disease. *J Clin Invest* 110:1389-1398. doi:10.1172/Jc1200216886

- Kawasaki L, Sanchez O, Shiozaki K, Aguirre J (2002) SakA MAP kinase is involved in stress signal transduction, sexual development and spore viability in *Aspergillus nidulans*. *Mol Microbiol* 45:1153-1163.
- Kazmierczak P, Kim DH, Turina M, Van Alfen NK (2005) A Hydrophobin of the chestnut blight fungus, *Cryphonectria parasitica*, is required for stromal pustule eruption. *Eukaryot Cell* 4:931-936. doi:10.1128/EC.4.5.931-936.2005
- Kemmitt SJ, Wright D, Goulding KWT, Jones DL (2006) pH regulation of carbon and nitrogen dynamics in two agricultural soils. *Soil Biol Biochem* 38:898-911. doi:10.1016/j.soilbio.2005.08.006
- Kemmitt SJ, Wright D, Jones DL (2005) Soil acidification used as a management strategy to reduce nitrate losses from agricultural land. *Soil Biol Biochem* 37:867-875. doi:10.1016/j.soilbio.2004.10.001
- Kerppola TK (2006a) Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. *Nat Protoc* 1:1278-1286. doi:10.1038/nprot.2006.201
- Kerppola TK (2006b) Visualization of molecular interactions by fluorescence complementation. *Nat Rev Mol Cell Biol* 7:449-456. doi:10.1038/nrm1929
- Kim S, Ahn IP, Rho HS, Lee YH (2005) MHP1, a *Magnaporthe grisea* hydrophobin gene, is required for fungal development and plant colonization. *Mol Microbiol* 57:1224-1237. doi:10.1111/j.1365-2958.2005.04750.x
- Kim S, Dale BE (2004) Global potential bioethanol production from wasted crops and crop residues. *Biomass Bioenergy* 26:361-375. doi:10.1016/j.biombioe.2003.08.002
- Klamer M, Baath E (2004) Estimation of conversion factors for fungal biomass determination in compost using ergosterol and PLFA 18 : 2 omega 6,9. *Soil Biol Biochem* 36:57-65. doi:10.1016/j.soilbio.2003.08.019
- Klein-Marcuschamer D, Oleskiewicz-Popiel P, Simmons BA, Blanch HW (2012) The challenge of enzyme cost in the production of lignocellulosic biofuels. *Biotechnol Bioeng* 109:1083-1087. doi:10.1002/bit.24370
- Klich MA (2009) Health effects of *Aspergillus* in food and air. *Toxicol Ind Health* 25:657-667. doi:10.1177/0748233709348271
- Knob A, Terrasan CRF, Carmona EC (2010) Beta-xylosidases from filamentous fungi: an overview. *World J Microbiol Biotechnol* 26:389-407. doi:10.1007/s11274-009-0190-4
- Kolasa M, Ahring BK, Lübeck PS, Lübeck M (2014) Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. *Biores Technol* 169:143-148. doi:10.1016/j.biortech.2014.06.082

- Korennykh AV, Egea PF, Korostelev AA, Finer-Moore J, Zhang C, Shokat KM, Stroud RM, Walter P (2009) The unfolded protein response signals through high-order assembly of Ire1. *Nature* 457:687-693. doi:10.1038/nature07661
- Kosetsu K, Matsunaga S, Nakagami H, Colcombet J, Sasabe M, Soyano T, Takahashi Y, Hirt H, Machida Y (2010) The MAP kinase MPK4 is required for cytokinesis in *Arabidopsis thaliana*. *Plant Cell* 22:3778-3790. doi:10.1105/tpc.110.077164
- Koshland (1953) Stereochemistry and the mechanism of enzymatic reactions. *Biol Rev Camb Philos Soc* 28:416–436.
- Kothe GO, Free SJ (1998) The isolation and characterization of *nrc-1* and *nrc-2*, two genes encoding protein kinases that control growth and development in *Neurospora crassa*. *Genet* 149:117-130.
- Kriel J, Haesendonckx S, Rubio-Teixeira M, Van Zeebroeck G, Thevelein JM (2011) From transporter to transceptor: Signaling from transporters provokes re-evaluation of complex trafficking and regulatory controls. *Bio Essays* 33:870-879. doi:10.1002/bies.201100100
- Kristiansen KA, Jensen PE, Moller IM, Schulz A (2009) Monitoring reactive oxygen species formation and localisation in living cells by use of the fluorescent probe CM-H(2)DCFDA and confocal laser microscopy. *Physiol Plant* 136:369-383. doi:10.1111/j.1399-3054.2009.01243.x
- Kubicek CP (2012) Regulation of formation of plant biomass-degrading enzymes in fungi *Fungi and Lignocellulosic Biomass*. Wiley-Blackwell, pp 129-147.
- Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B (2009) Metabolic engineering strategies for the improvement of cellulase production by *Hypocrea jecorina*. *Biotechnol Biofuels* 2:19. doi:10.1186/1754-6834-2-19
- Kulkarni RD, Thon MR, Pan H, Dean RA (2005) Novel G-protein-coupled receptor-like proteins in the plant pathogenic fungus *Magnaporthe grisea*. *Genome Biol* 6:R24. doi:10.1186/gb-2005-6-3-r24
- Kumamoto CA (2008) Molecular mechanisms of mechanosensing and their roles in fungal contact sensing. *Nat Rev Micro* 6:667-673. doi: 10.1038/nrmicro1960
- Kunitake E, Tani S, Sumitani J-i, Kawaguchi T (2012) A novel transcriptional regulator, ClbR, controls the cellobiose- and cellulose-responsive induction of cellulase and xylanase genes regulated by two distinct signaling pathways in *Aspergillus aculeatus*. *Appl Microbiol Biotechnol* 1-12. doi:10.1007/s00253-012-4305-8
- Kurasawa T, Yachi M, Suto M, Kamagata Y, Takao S, Tomita F (1992) Induction of cellulase by gentiobiose and its sulfur-containing analog in *Penicillium purpurogenum*. *Appl Environ Microbiol* 58:106-110.

- Kwan AH, Winefield RD, Sunde M, Matthews JM, Haverkamp RG, Templeton MD, Mackay JP (2006) Structural basis for rodlet assembly in fungal hydrophobins. *Proc Natl Acad Sci U S A* 103:3621-3626. doi:10.1073/pnas.0505704103
- Lapidot M, Pilpel Y (2006) Genome-wide natural antisense transcription: coupling its regulation to its different regulatory mechanisms. *EMBO Rep* 7:1216-1222. doi:10.1038/sj.embor.7400857
- Latchman DS (1997) Transcription factors: an overview. *Int J Biochem Cell Biol* 29: 1305-1312. doi: 10.1016/S1357-2725(97)00085-X
- Latge JP (1999) *Aspergillus fumigatus* and aspergillosis. *Clinical Microbiol Rev* 12:310-+. doi: 10.1128/9781555815523
- Le Crom S, Schackwitz W, Pennacchio L, Magnuson JK, Culley DE, Collett JR, Martin J, Druzhinina IS, Mathis H, Monot F, Seiboth B, Cherry B, Rey M, Berka R, Kubicek CP, Baker SE, Margeot A (2009) Tracking the roots of cellulase hyperproduction by the fungus *Trichoderma reesei* using massively parallel DNA sequencing. *Proc Natl Acad Sci USA* 106:16151-16156. doi:10.1073/pnas.0905848106
- Levasseur A, Drula E, Lombard V, Coutinho P, Henrissat B (2013) Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnol Biofuels* 6:41. doi: 10.1186/1754-6834-6-41
- Levene A (1906) The cleavage products of vitellin. *J Biol Chem* 2:127 – 133.
- Li C, Yang Z, Zhang RH, Zhang D, Chen S, Ma L (2013) Effect of pH on cellulase production and morphology of *Trichoderma reesei* and the application in cellulosic material hydrolysis. *J Biotechnol* 168:470-477. doi:10.1016/j.jbiotec.2013.10.003
- Li L, Wright SJ, Krystofova S, Park G, Borkovich KA (2007) Heterotrimeric G protein signaling in filamentous fungi. *Annu Rev Microbiol* 61:423-452. doi:10.1146/annurev.micro.61.080706.093432
- Li W, Abad JA, French-Monar RD, Rascoe J, Wen A, Gudmestad NC, Secor GA, Lee IM, Duan Y, Levy L (2009) Multiplex real-time PCR for detection, identification and quantification of '*Candidatus Liberibacter solanacearum*' in potato plants with zebra chip. *J Microbiol Methods* 78:59-65. doi:10.1016/j.mimet.2009.04.009
- Li Y, Tschaplinski TJ, Engle NL, Hamilton CY, Rodriguez M, Jr., Liao JC, Schadt CW, Guss AM, Yang Y, Graham DE (2012) Combined inactivation of the *Clostridium cellulolyticum* lactate and malate dehydrogenase genes substantially increases ethanol yield from cellulose and switchgrass fermentations. *Biotechnol Biofuels* 5:2. doi:10.1186/1754-6834-5-2
- Linder MB (2009) Hydrophobins: proteins that self assemble at interfaces. *Curr Opin Colloid Interf Sci* 14:356-363. doi:10.1016/j.cocis.2009.04.001

- Lipmann FaL, PA (1932) Serinephosphoric acid obtained on hydrolysis of vitellinic acid. J Biol Chem 98:109-114.
- Litman GW, Rast JP, Shamblott MJ, Haire RN, Hulst M, Roess W, Litman RT, Hinds-Frey KR, Zilch A, Amemiya CT (1993) Phylogenetic diversification of immunoglobulin genes and the antibody repertoire. Mol Biol Evol 10:60-72.
- Liu YS, Baker JO, Zeng Y, Himmel ME, Haas T, Ding SY (2011) Cellobiohydrolase hydrolyzes crystalline cellulose on hydrophobic faces. J Biol Chem 286:11195-11201. doi:10.1074/jbc.M110.216556
- Lombard V, Bernard T, Rancurel C, Brumer H, Coutinho PM, Henrissat B (2010) A hierarchical classification of polysaccharide lyases for glycogenomics. Biochem J 432:437-444. doi:10.1042/BJ20101185
- Luscombe NM, Greenbaum D, Gerstein M (2001) What is bioinformatics? A proposed definition and overview of the field. Meth Inform Med 40:346-358.
- Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: an update. Curr Opin Biotechnol 16:577-583. doi:10.1016/j.copbio.2005.08.009
- Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506-577. doi: 10.1128/MMBR.66.3.506-577.2002
- Ma K, Ruan Z (2015) Production of a lignocellulolytic enzyme system for simultaneous bio-delignification and saccharification of corn stover employing co-culture of fungi. Biores Technol 175:586-593. doi:10.1016/j.biortech.2014.10.161
- Ma Y, Qiao J, Liu W, Wan Z, Wang X, Calderone R, Li R (2008) The Sho1 sensor regulates growth, morphology, and oxidant adaptation in *Aspergillus fumigatus* but is not essential for development of invasive pulmonary aspergillosis. Infect Immun 76:1695-1701. doi:10.1128/IAI.01507-07
- Maccabe AP, Orejas M, Perez-Gonzalez JA, Ramon D (1998) Opposite patterns of expression of two *Aspergillus nidulans* xylanase genes with respect to ambient pH. J Bacteriol 180:1331-1333.
- Maccabe AP, Van den Hombergh JP, Tilburn J, Arst HN, Jr., Visser J (1996) Identification, cloning and analysis of the *Aspergillus niger* gene *pacC*, a wide domain regulatory gene responsive to ambient pH. Mol Gen Genet 250:367-374.
- Mach-Aigner AR, Omony J, Jovanovic B, van Boxtel AJB, de Graaff LH (2012) D-Xylose concentration-dependent hydrolase expression profiles and the function of CreA and XlnR in *Aspergillus niger*. Appl Environ Microbiol 78:3145-3155. doi:10.1128/aem.07772-11

- Macindoe I, Kwan AH, Ren Q, Morris VK, Yang WR, Mackay JP, Sunde M (2012) Self-assembly of functional, amphipathic amyloid monolayers by the fungal hydrophobin EAS. *Proc Natl Acad Sci USA* 109:E804-E811. doi:10.1073/pnas.1114052109
- Madden K, Sheu YJ, Baetz K, Andrews B, Snyder M (1997) SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. *Science* 275:1781-1784.
- Maeda H, Yamagata Y, Abe K, Hasegawa F, Machida M, Ishioka R, Gomi K, Nakajima T (2005) Purification and characterization of a biodegradable plastic-degrading enzyme from *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 67:778-788. doi:10.1007/s00253-004-1853-6
- Mandels M, Parrish FW, Reese ET (1962) Sophorose as an inducer of cellulase in *Trichoderma viride*. *J Bacteriol* 83:400-408.
- Mann M, Pandey A (2001) Use of mass spectrometry-derived data to annotate nucleotide and protein sequence databases. *Trends Biochem Sci* 26:54-61. doi: 10.1016/S0968-0004(00)01726-6
- Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. *Science* 298:1912-1934. doi:10.1126/science.1075762
- Mansfield SD, Mooney C, Saddler JN (1999) Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnol Prog* 15:804-816. doi:10.1021/bp9900864
- Marsh T, Bassler (1959) A guide to the literature on certain effects of light on fungi: reproduction, morphology, pigmentation, and phototropic phenomena. *Plant Disease Reprtr Suppl* 261:251-312.
- Martens-Uzunova ES, Schaap PJ (2009) Assessment of the pectin degrading enzyme network of *Aspergillus niger* by functional genomics. *Fungal Genet Biol* 46 Suppl 1:S170-S179. doi: 10.1016/j.fgb.2008.07.021
- Martins I, Garcia H, Varela A, Nunez O, Planchon S, Galceran MT, Renaut J, Rebelo LP, Silva Pereira C (2014) Investigating *Aspergillus nidulans* secretome during colonisation of cork cell walls. *J Proteomics* 98:175-188. doi:10.1016/j.jprot.2013.11.023
- Matcham SE, Jordan BR, Wood DA (1985) Estimation of fungal biomass in a solid substrate by 3 independent methods. *Appl Microbiol Biotechnol* 21:108-112.
- Mathew GM, Sukumaran RK, Singhania RR, Pandey A (2008) Progress in research on fungal cellulases for lignocellulose degradation. *J Sci Ind Res* 67:898-907.
- McCann MC, Carpita NC (2008) Designing the deconstruction of plant cell walls. *Curr Opin Plant Biol* 11:314-320. doi:10.1016/j.pbi.2008.04.001
- Mello-de-Sousa TM, Silva-Pereira I, Pocas-Fonseca MJ (2011) Carbon source and pH-dependent transcriptional regulation of cellulase genes of *Humicola grisea* var.

- thermoidea* grown on sugarcane bagasse. *Enzyme Microb Technol* 48:19-26. doi:10.1016/j.enzmictec.2010.08.007
- Mesa-Arango AC, Trevijano-Contador N, Roman E, Sanchez-Fresneda R, Casas C, Herrero E, Arguelles JC, Pla J, Cuenca-Estrella M, Zaragoza O (2014) The production of reactive oxygen species is a universal action mechanism of amphotericin B against pathogenic yeasts and contributes to the fungicidal effect of this drug. *Antimicrob Agents Chemother* 58:6627-6638. doi:10.1128/Aac.03570-14
- Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F (2003) Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* 115:751-763.
- Meyer AS, Rosgaard L, Sorensen HR (2009) The minimal enzyme cocktail concept for biomass processing. *J Cereal Sci* 50:337-344. doi: 10.1016/j.jcs.2009.01.010
- Mille-Lindblom C, Tranvik LJ (2003) Antagonism between bacteria and fungi on decomposing aquatic plant litter. *Microb Ecol* 45:173-182. doi:10.1007/s00248-002-2030-z
- Mille-Lindblom C, von Wachenfeldt E, Tranvik LJ (2004) Ergosterol as a measure of living fungal biomass: persistence in environmental samples after fungal death. *J Microbiol Methods* 59:253-262. doi:10.1016/j.mimet.2004.07.010
- Miller M, Palojarvi A, Rangger A, Reeslev M, Kjoller A (1998) The use of fluorogenic substrates to measure fungal presence and activity in soil. *Appl Environ Microbiol* 64:613-617.
- Mio T, Adachi-Shimizu M, Tachibana Y, Tabuchi H, Inoue SB, Yabe T, Yamada-Okabe T, Arisawa M, Watanabe T, Yamada-Okabe H (1997) Cloning of the *Candida albicans* homolog of *Saccharomyces cerevisiae* GSC1/FKS1 and its involvement in beta-1,3-glucan synthesis. *J Bacteriol* 179:4096-4105.
- Mizutani O, Nojima A, Yamamoto M, Furukawa K, Fujioka T, Yamagata Y, Abe K, Nakajima T (2004) Disordered cell integrity signaling caused by disruption of the *kexB* gene in *Aspergillus oryzae*. *Eukaryot Cell* 3:1036-1048. doi:10.1128/EC.3.4.1036-1048.2004
- Morell M, Espargaro A, Aviles FX, Ventura S (2008) Study and selection of in vivo protein interactions by coupling bimolecular fluorescence complementation and flow cytometry. *Nat Protoc* 3:22-33. doi:10.1038/nprot.2007.496
- Morgan MJ, Lehmann M, Schwarzlander M, Baxter CJ, Sienkiewicz-Porzucek A, Williams TC, Schauer N, Fernie AR, Fricker MD, Ratcliffe RG, Sweetlove LJ, Finkemeier I (2008) Decrease in manganese superoxide dismutase leads to reduced root growth and affects tricarboxylic acid cycle flux and mitochondrial redox homeostasis. *Plant Physiol* 147:101-114. doi:10.1104/pp.107.113613

- Morris KV, Mattick JS (2014) The rise of regulatory RNA. *Nat Rev Genet* 15:423-437. doi:10.1038/nrg3722
- Mosbach A, Leroch M, Mendgen KW, Hahn M (2011) Lack of evidence for a role of hydrophobins in conferring surface hydrophobicity to conidia and hyphae of *Botrytis cinerea*. *BMC Microbiol* 11:10. doi:10.1186/1471-2180-11-10
- Moss RB (2002) Allergic bronchopulmonary aspergillosis. *Clinical Rev Allergy Immunol* 23:87-104. doi:10.1385/Criai:23:1:087
- Munro CA, Selvaggini S, de Bruijn I, Walker L, Lenardon MD, Gerssen B, Milne S, Brown AJ, Gow NA (2007) The PKC, HOG and Ca<sup>2+</sup> signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*. *Mol Microbiol* 63:1399-1413. doi:10.1111/j.1365-2958.2007.05588.x
- Murphy AD, Lang-Unnasch N (1999) Alternative oxidase inhibitors potentiate the activity of atovaquone against *Plasmodium falciparum*. *Antimicrob Agents Chemother* 43:651-654.
- Naumoff DG (2011) Hierarchical classification of glycoside hydrolases. *Biochem* 76:622-635. doi:10.1134/S0006297911060022
- Navarro D, Rosso M-N, Haon M, Olive C, Bonnin E, Lesage-Meessen L, Chevret D, Coutinho P, Henrissat B, Berrin J-G (2014) Fast solubilization of recalcitrant cellulosic biomass by the basidiomycete fungus *Laetisaria arvalis* involves successive secretion of oxidative and hydrolytic enzymes. *Biotechnol Biofuels* 7:143.
- Nitsche B, Burggraaf-van Welzen A-M, Lamers G, Meyer V, Ram AJ (2013) Autophagy promotes survival in aging submerged cultures of the filamentous fungus *Aspergillus niger*. *Appl Microbiol Biotechnol* 97:8205-8218. doi:10.1007/s00253-013-4971-1
- Nitsche B, Jorgensen T, Akeroyd M, Meyer V, Ram A (2012) The carbon starvation response of *Aspergillus niger* during submerged cultivation: Insights from the transcriptome and secretome. *BMC Genomics* 13:380. doi: 10.1186/1471-2164-13-380
- Noguchi Y, Sano M, Kanamaru K, Ko T, Takeuchi M, Kato M, Kobayashi T (2009) Genes regulated by AoXlnR, the xylanolytic and cellulolytic transcriptional regulator, in *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 85:141-154. doi:10.1007/s00253-009-2236-9
- Noguchi Y, Tanaka H, Kanamaru K, Kato M, Kobayashi T (2011) Xylose triggers reversible phosphorylation of XlnR, the fungal transcriptional activator of xylanolytic and cellulolytic genes in *Aspergillus oryzae*. *Biosci Biotechnol Biochem* 75:953-959. doi:JST.JSTAGE/bbb/100923 [pii]

- Nongpiur R, Soni P, Karan R, Singla-Pareek SL, Pareek A (2012) Histidine kinases in plants: cross talk between hormone and stress responses. *Plant Signal Behav* 7:1230-1237. doi:10.4161/psb.21516
- O'Donohue (2008) La production de carburants a partir de biomasse lignocellulosique par voie biologique: etat de l'art et perspectives. OCL 15.
- O'Rourke SM, Herskowitz I, O'Shea EK (2002) Yeast go the whole HOG for the hyperosmotic response. *Trends Genet* 18:405-412. doi: 10.1016/S0168-9525(02)02723-3
- Ogawa M, Kobayashi T, Koyama Y (2013) ManR, a transcriptional regulator of the  $\beta$ -mannan utilization system, controls the cellulose utilization system in *Aspergillus oryzae*. *Biosci Biotechnol Biochem* 77:426-429. doi:10.1271/bbb.120795
- Ohtaki S, Maeda H, Takahashi T, Yamagata Y, Hasegawa F, Gomi K, Nakajima T, Abe K (2006) Novel hydrophobic surface binding protein, HsbA, produced by *Aspergillus oryzae*. *Appl Environ Microbiol* 72:2407-2413. doi:10.1128/AEM.72.4.2407-2413.2006
- Oki M, Noguchi E, Hayashi N, Nishimoto T (1998) Nuclear protein import, but not mRNA export, is defective in all *Saccharomyces cerevisiae* mutants that produce temperature-sensitive forms of the Ran GTPase homologue Gsp1p. *Mol Gen Genet* 257:624-634.
- Olefsky JM (2001) Nuclear receptor minireview series. *J Biol Chem* 276:36863-36864. doi:10.1074/jbc.R100047200
- Ooijskaas LP, Tramper J, Buitelaar RM (1998) Biomass estimation of *Coniothyrium minitans* in solid-state fermentation. *Enzyme Microb Technol* 22:480-486. doi:10.1016/S0141-0229(97)00246-9
- Orena SJ, Torchia AJ, Garofalo RS (2000) Inhibition of glycogen-synthase kinase 3 stimulates glycogen synthase and glucose transport by distinct mechanisms in 3T3-L1 adipocytes. *J Biol Chem* 275:15765-15772. doi:10.1074/jbc.M910002199
- Papa FR, Zhang C, Shokat K, Walter P (2003) Bypassing a kinase activity with an ATP-competitive drug. *Science* 302:1533-1537. doi:10.1126/science.1090031
- Paravicini G, Mendoza A, Antonsson B, Cooper M, Losberger C, Payton MA (1996) The *Candida albicans* PKC1 gene encodes a protein kinase C homolog necessary for cellular integrity but not dimorphism. *Yeast* 12:741-756. doi:10.1002/(SICI)1097-0061(19960630)12:8<741::AID-YEA967>3.0.CO;2-G
- Parenicova L, Benen JA, Kester HC, Visser J (2000) *pgaA* and *pgaB* encode two constitutively expressed endopolygalacturonases of *Aspergillus niger*. *Biochem J* 345:637-644.

- Paris S, Debeaupuis JP, Crameri R, Carey M, Charles F, Prevost MC, Schmitt C, Philippe B, Latge JP (2003) Conidial hydrophobins of *Aspergillus fumigatus*. Appl Environ Microbiol 69:1581-1588. doi: 10.1128/AEM.69.3.1581-1588.2003
- Park C, Marqusee S (2005) Pulse proteolysis: a simple method for quantitative determination of protein stability and ligand binding. Nat Methods 2:207-212. doi:10.1038/nmeth740
- Payne DM, Rossomando AJ, Martino P, Erickson AK, Her JH, Shabanowitz J, Hunt DF, Weber MJ, Sturgill TW (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J 10:885-892.
- Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22:153-183. doi:10.1210/edrv.22.2.0428
- Pedersen H, Christensen B, Hjort C, Nielsen J (2000) Construction and characterization of an oxalic acid nonproducing strain of *Aspergillus niger*. Metabolic Engineering 2:34-41. doi:10.1006/mben.1999.0136
- Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijk PW, van Dijk A, Dijkhuizen L, Driessen AJ, d'Enfert C, Geysens S, Goosen C, Groot GS, de Groot PW, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CA, van der Heijden RT, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJ, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NN, Ram AF, Rinas U, Roubos JA, Sagt CM, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJ, Wedler H, Wösten HA, Zeng AP, van Ooyen AJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 25:221-231. doi:10.1038/nbt1282
- Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8:785-786. doi:10.1038/nmeth.1701
- Peterson GL (1979) Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. Anal Biochem 100:201-220.
- Peterson R, Nevalainen H (2012) *Trichoderma reesei* RUT-C30 - thirty years of strain improvement. Microbiol 158:58-68. doi:10.1099/mic.0.054031-0

- Phillips CM, Beeson WT, Cate JH, Marletta MA (2011) Cellobiose dehydrogenase and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by *Neurospora crassa*. ACS Chem Biol 6:1399-1406. doi:10.1021/cb200351y
- Pietri JCA, Brookes PC (2008) Relationships between soil pH and microbial properties in a UK arable soil. Soil Biol Biochem 40:1856-1861. doi:10.1016/j.soilbio.2008.03.020
- Pollok BA, Heim R (1999) Using GFP in FRET-based applications. Trends Cell Biol 9:57-60.
- Poole DM, Morag E, Lamed R, Bayer EA, Hazlewood GP, Gilbert HJ (1992) Identification of the cellulose-binding domain of the cellosome subunit S1 from *Clostridium thermocellum* YS. FEMS Microbiol Lett 78:181-186.
- Portnoy T, Margeot A, Linke R, Atanasova L, Fekete E, Sandor E, Hartl L, Karaffa L, Druzhinina IS, Seiboth B, Le Crom S, Kubicek CP (2011) The CRE1 carbon catabolite repressor of the fungus *Trichoderma reesei*: a master regulator of carbon assimilation. BMC Genomics 12:269. doi:10.1186/1471-2164-12-269
- Puchart V, Gariépy MC, Shareck F, Dupont C (2006) Identification of catalytically important amino acid residues of *Streptomyces lividans* acetylxyloxyesterase A from carbohydrate esterase family 4. Biochim Biophys Acta Prot Proteom 1764:263-274. doi:10.1016/j.bbapap.2005.11.023
- Pugnet R, Blanchet R, Salençon J, Carpentier A (2010) Le changement climatique Institut de France. vol Académie des Sciences,
- Pullan ST, Daly P, Delmas S, Ibbett R, Kokolski M, Neiteler A, van Munster JM, Wilson R, Blythe MJ, Gaddipati S, Tucker GA, Archer DB (2014) RNA-sequencing reveals the complexities of the transcriptional response to lignocellulosic biofuel substrates in *Aspergillus niger*. Fung Biol Biotechnol 1. doi: 10.1186/s40694-014-0003-x
- Punekar NS, Vaidyanathan CS, Rao NA (1984) Mechanisms of citric-acid fermentation by *Aspergillus niger*. J Sci Ind Res 43:398-404.
- Punt PJ, Drint-Kuijvenhoven A, Lokman BC, Spencer JA, Jeenes D, Archer DA, van den Hondel CA (2003) The role of the *Aspergillus niger* furin-type protease gene in processing of fungal proproteins and fusion proteins. Evidence for alternative processing of recombinant (fusion-) proteins. J Biotechnol 106:23-32.
- Purschwitz J, Müller S, Kastner C, Fischer R (2006) Seeing the rainbow: light sensing in fungi. Curr Opin Microbiol 9:566-571. doi:10.1016/j.mib.2006.10.011
- Pusztai A (1964) Hexosamines in the seeds of higher plants (Spermatophytes). Nature 201:1328-1329.
- Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R, Lopez R (2005) InterProScan: protein domains identifier. Nucleic Acids Res 33:W116-120. doi:10.1093/nar/gki442

- Quinlan RJ, Sweeney MD, Lo Leggio L, Otten H, Poulsen JC, Johansen KS, Krogh KB, Jorgensen CI, Tovborg M, Anthonsen A, Tryfona T, Walter CP, Dupree P, Xu F, Davies GJ, Walton PH (2011) Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. *Proc Natl Acad Sci USA* 108:15079-15084. doi:10.1073/pnas.1105776108
- Raguz S, Yague E, Wood DA, Thurston CF (1992) Isolation and characterization of a cellulose-growth-specific gene from *Agaricus bisporus*. *Gene* 119:183-190.
- Rajan SS, Yang X, Collart F, Yip VL, Withers SG, Varrot A, Thompson J, Davies GJ, Anderson WF (2004) Novel catalytic mechanism of glycoside hydrolysis based on the structure of an NAD<sup>+</sup>/Mn<sup>2+</sup> -dependent phospho- $\alpha$ -glucosidase from *Bacillus subtilis*. *Structure* 12:1619-1629. doi:10.1016/j.str.2004.06.020
- Rasooly R (2007) Phloxine B, a versatile bacterial stain. *FEMS Immunol Med Microbiol* 49:261-265. doi:10.1111/j.1574-695X.2007.00188.x
- Rauscher R, Wurleitner E, Wacenovský C, Aro N, Stricker AR, Zeilinger S, Kubicek CP, Penttilä M, Mach RL (2006) Transcriptional regulation of *xyn1*, encoding xylanase I, in *Hypocrea jecorina*. *Eukaryot Cell* 5:447-456. doi:10.1128/Ec.5.3.447-456.2006
- Read ND, Jeffree CE (1991) Low-temperature scanning electron microscopy in biology. *J Microsc* 161:59-72.
- Reiser V, Ammerer G, Ruis H (1999) Nucleocytoplasmic traffic of MAP kinases. *Gene Expr* 7:247-254.
- Rep M, Reiser V, Gartner U, Thevelein JM, Hohmann S, Ammerer G, Ruis H (1999) Osmotic stress-induced gene expression in *Saccharomyces cerevisiae* requires Msn1p and the novel nuclear factor Hot1p. *Mol Cell Biol* 19:5474-5485.
- Richards WC (1993) Cerato-ulmin - a unique wilt toxin of instrumental significance in the development of Dutch elm disease. *Dutch Elm Disease Res* 89-151.
- Ries L, Belshaw NJ, Ilmén M, Penttilä ME, Alapuranen M, Archer DB (2014) The role of CRE1 in nucleosome positioning within the *cbh1* promoter and coding regions of *Trichoderma reesei*. *Appl Microbiol Biotechnol* 98:749-762. doi:10.1007/s00253-013-5354-3
- Ries L, Pullan S, Delmas S, Malla S, Blythe M, Archer D (2013) Genome-wide transcriptional response of *Trichoderma reesei* to lignocellulose using RNA sequencing and comparison with *Aspergillus niger*. *BMC Genomics* 14:541. doi: 10.1186/1471-2164-14-541
- Rieske JS, Zaugg WS (1962) The inhibition by antimycin A of the cleavage of one of the complexes of the respiratory chain. *Biochem Biophys Res Commun* 8:421-426.
- Rivera MC, Jain R, Moore JE, Lake JA (1998) Genomic evidence for two functionally distinct gene classes. *Proc Natl Acad Sci USA* 95:6239-6244. doi: 10.1073/pnas.95.11.6239

- Robbins DJ, Zhen E, Owaki H, Vanderbilt CA, Ebert D, Geppert TD, Cobb MH (1993) Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. *J Biol Chem* 268:5097-5106.
- Robinson DR, Wu YM, Lin SF (2000) The protein tyrosine kinase family of the human genome. *Oncogene* 19:5548-5557. doi:10.1038/sj.onc.1203957
- Rodriguez A, Rodriguez M, Luque MI, Justesen AF, Cordoba JJ (2011) Quantification of ochratoxin A-producing molds in food products by SYBR Green and TaqMan real-time PCR methods. *Int J Food Microbiol* 149:226-235. doi:10.1016/j.ijfoodmicro.2011.06.019
- Rodriguez MC, Petersen M, Mundy J (2010) Mitogen-activated protein kinase signaling in plants. *Annu Rev Plant Biol* 61:621-649. doi:10.1146/annurev-arplant-042809-112252
- Roeder RG (1996) The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem Sci* 21:327-335. doi: 10.1016/0968-0004(96)10050-5
- Roseman S (1969) The transport of carbohydrates by a bacterial phosphotransferase system. *J Gen Physiol* 54:138-184.
- Rosenberg M (2006) Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. *FEMS Microbiol Lett* 262:129-134. doi:10.1111/j.1574-6968.2006.00291.x
- Rosling A, Lindahl BD, Taylor AFS, Finlay RD (2004) Mycelial growth and substrate acidification of ectomycorrhizal fungi in response to different minerals. *FEMS Microbiol Ecol* 47:31-37. doi:10.1016/S0168-6496(03)00222-8
- Rousk J, Baath E, Brookes PC, Lauber CL, Lozupone C, Caporaso JG, Knight R, Fierer N (2010) Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J* 4:1340-1351. doi:10.1038/ismej.2010.58
- Ruggiero FM, Landriscina C, Gnoni GV, Quagliariello E (1984) Lipid composition of liver mitochondria and microsomes in hyperthyroid rats. *Lipids* 19:171-178. doi:10.1007/Bf02534794
- Ruijter GJG, Visser J (1997) Carbon repression in *Aspergilli*. *FEMS Microbiol Lett* 151:103-114. doi:10.1111/j.1574-6968.1997.tb12557.x
- Rutkowski DT, Kaufman RJ (2004) A trip to the ER: coping with stress. *Trends Cell Biol* 14:20-28. doi:10.1016/j.tcb.2003.11.001
- Saloheimo A, Aro N, Ilmén M, Penttilä M (2000) Isolation of the *ace1* gene encoding a Cys(2)-His(2) transcription factor involved in regulation of activity of the cellulase promoter *cbh1* of *Trichoderma reesei*. *J Biol Chem* 275:5817-5825.
- Sarkar N, Ghosh SK, Bannerjee S, Aikat K (2012) Bioethanol production from agricultural wastes: an overview. *Renewable Energy* 37:19-27. doi:10.1016/j.renene.2011.06.045

- Saugy M, Farkas V, Maclachlan G (1988) Phosphatases and phosphodiesterases interfere with 1,3-beta-D-glucan synthase activity in pea epicotyl membrane preparations. *Eur J Biochem* 177:135-138.
- Scheller H, Ulvskov P (2010) Hemicelluloses. *Annu Rev Plant Biol* 61:263 - 289. doi: 10.1146/annurev-arplant-042809-112315
- Schmoll M, Tian C, Sun J, Tisch D, Glass NL (2012) Unravelling the molecular basis for light modulated cellulase gene expression - the role of photoreceptors in *Neurospora crassa*. *BMC Genomics* 13:127. doi: 10.1186/1471-2164-13-127
- Scholtmeijer K, Janssen MJ, Gerssen B, de Vocht ML, van Leeuwen BM, van Kooten TG, Wösten HAB, Wessels JGH (2002) Surface modifications created by using engineered hydrophobins. *Appl Environ Microbiol* 68:1367-1373. doi:10.1128/Aem.68.3.1367-1373.2002
- Schroder M, Kaufman RJ (2005) ER stress and the unfolded protein response. *Mutat Res Fund Mol Mechan Mutagen* 569:29-63. doi:10.1016/j.mrfmmm.2004.06.056
- Scotti CT, Vergoignan C, Feron G, Durand A (2001) Glucosamine measurement as indirect method for biomass estimation of *Cunninghamella elegans* grown in solid state cultivation conditions. *Biochem Eng J* 7:1-5. doi:10.1016/S1369-703x(00)00090-5
- Seiboth B, Metz B (2011) Fungal arabinan and L-arabinose metabolism. *Appl Microbiol Biotechnol* 89:1665-1673. doi:10.1007/s00253-010-3071-8
- Seitz LM, Sauer DB, Burroughs R, Mohr HE, Hubbard JD (1979) Ergosterol as a measure of fungal growth. *Phytopathol* 69:1202-1203. doi:10.1094/Phyto-69-1202
- Sellam A, Dongo A, Guillemette T, Hudhomme P, Simoneau P (2007) Transcriptional responses to exposure to the brassicaceous defence metabolites camalexin and allyl-isothiocyanate in the necrotrophic fungus *Alternaria brassicicola*. *Mol Plant Pathol* 8:195-208. doi:10.1111/j.1364-3703.2007.00387.x
- Sidrauski C, Cox JS, Walter P (1996) tRNA ligase is required for regulated mRNA splicing in the unfolded protein response. *Cell* 87:405-413.
- Sigrist CJ, Cerutti L, Hulo N, Gattiker A, Falquet L, Pagni M, Bairoch A, Bucher P (2002) PROSITE: a documented database using patterns and profiles as motif descriptors. *Brief Bioinform* 3:265-274. doi: 10.1093/bib/3.3.265
- Simeng Z, Sacha G, Isabelle HG, Marie-Noelle R (2015) A PCR-based method to quantify fungal growth during pretreatment of lignocellulosic biomass. *J Microbiol Methods* 115:67-70. doi:10.1016/j.mimet.2015.05.024
- Singh KK (2000) The *Saccharomyces cerevisiae* Sln1p-Ssk1p two-component system mediates response to oxidative stress and in an oxidant-specific fashion. *Free Radic Biol Med* 29:1043-1050. doi: 10.1016/S0891-5849(00)00432-9

- Singh SD, Robbins N, Zaas AK, Schell WA, Perfect JR, Cowen LE (2009) Hsp90 governs echinocandin resistance in the pathogenic yeast *Candida albicans* via calcineurin. PLoS Pathog 5:e1000532. doi:10.1371/journal.ppat.1000532
- Sinha AK, Jaggi M, Raghuram B, Tuteja N (2011) Mitogen-activated protein kinase signaling in plants under abiotic stress. Plant Signal Behav 6:196-203. doi:10.4161/psb.6.2.14701
- Sinha P, Sharma RP, Roy MK (1994) Management of storage rot in onion through gamma-irradiation and chemicals. J Food Sci Technol Mys 31:341-343.
- Sinnott (1990) Catalytic mechanism of enzymic glycosyl transfer. Chem Rev 90:1171–1202.
- Sitia R, Braakman I (2003) Quality control in the endoplasmic reticulum protein factory. Nature 426:891-894. doi:10.1038/nature02262
- Sloan ME, Rodis P, Wasserman BP (1987) CHAPS solubilization and functional reconstitution of beta-glucan gynthase from red beet root (*Beta vulgaris* L.) storage tissue. Plant Physiol 85:516-522.
- Smith PB, Hancock GA, Rhoden DL (1969) Improved medium for detecting deoxyribonuclease-producing bacteria. Appl Microbiol 18:991-993.
- Snajdr J, Dobiasova P, Vetrovsky T, Valaskova V, Alawi A, Boddy L, Baldrian P (2011) Saprotrophic basidiomycete mycelia and their interspecific interactions affect the spatial distribution of extracellular enzymes in soil. FEMS Microbiol Ecol 78:80-90. doi:10.1111/j.1574-6941.2011.01123.x
- Song ZW, Vail A, Sadowsky MJ, Schilling JS (2014) Quantitative PCR for measuring biomass of decomposer fungi in planta. Fung Ecol 7:39-46. doi:10.1016/j.funeco.2013.12.004
- Souza WR (2013) Microbial degradation of lignocellulosic biomass, sustainable degradation of lignocellulosic biomass - techniques, applications and commercialization. Dr. Anuj Chandel (Ed.), InTech, doi: 10.5772/54325.
- Spencer JA, Jeenes DJ, MacKenzie DA, Haynie DT, Archer DB (1998) Determinants of the fidelity of processing glucoamylase-lysozyme fusions by *Aspergillus niger*. Eur J Biochem 258:107-112.
- Sprang SR, Coleman DE (1998) Invasion of the nucleotide snatchers: structural insights into the mechanism of G protein GEFs. Cell 95:155-158.
- Stephanopoulos G (2007) Challenges in engineering microbes for biofuels production. Science 315:801-804. doi:10.1126/science.1139612
- Sternberg D, Mandels GR (1979) Induction of cellulolytic enzymes in *Trichoderma reesei* by sophorose. J Bacteriol 139:761-769.

- Steudler S, Bley T (2015) Biomass estimation during macro-scale solid-state fermentation of basidiomycetes using established and novel approaches. *Bioprocess Biosyst Eng* 38:1313-1323. doi:10.1007/s00449-015-1372-0
- Stricker AR, Grosstessner-Hain K, Würleitner E, Mach RL (2006) Xyr1 (Xylanase Regulator 1) regulates both the hydrolytic enzyme system and D-xylose metabolism in *Hypocrea jecorina*. *Eukaryot Cell* 5:2128-2137. doi:10.1128/ec.00211-06
- Stricker AR, Mach RL, de Graaff LH (2008) Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*). *Appl Microbiol Biotechnol* 78:211-220. doi:10.1007/s00253-007-1322-0
- Sun J, Tian C, Diamond S, Glass NL (2012) Deciphering transcriptional regulatory mechanisms associated with hemicellulose degradation in *Neurospora crassa*. *Eukaryot Cell* 11:482-493. doi:10.1128/EC.05327-11
- Sunde M, Kwan AH, Templeton MD, Beever RE, Mackay JP (2008) Structural analysis of hydrophobins. *Micron* 39:773-784. doi:10.1016/j.micron.2007.08.003
- Sussman JL, Lin D, Jiang J, Manning NO, Prilusky J, Ritter O, Abola EE (1998) Protein Data Bank (PDB): database of three-dimensional structural information of biological macromolecules. *Acta Crystallogr D Biol Crystallogr* 54:1078-1084.
- Suto M, Tomita F (2001) Induction and catabolite repression mechanisms of cellulase in fungi. *J Biosci Bioeng* 92:305-311. doi: 10.1016/S1389-1723(01)80231-0
- Szilagyi M, Miskei M, Karanyi Z, Lenkey B, Pocsi I, Emri T (2013) Transcriptome changes initiated by carbon starvation in *Aspergillus nidulans*. *Microbiol* 159:176-190. doi:10.1099/mic.0.062935-0
- Takahashi T, Maeda H, Yoneda S, Ohtaki S, Yamagata Y, Hasegawa F, Gomi K, Nakajima T, Abe K (2005) The fungal hydrophobin RolA recruits polyesterase and laterally moves on hydrophobic surfaces. *Mol Microbiol* 57:1780-1796. doi:10.1111/j.1365-2958.2005.04803.x
- Takahashi T, Tanaka T, Tsushima Y, Muragaki K, Uehara K, Takeuchi S, Maeda H, Yamagata Y, Nakayama M, Yoshimi A, Abe K (2015) Ionic interaction of positive amino acid residues of fungal hydrophobin RolA with acidic amino acid residues of cutinase CutL1. *Mol Microbiol* 96:14-27. doi:10.1111/mmi.12915
- Talbot NJ, Ebbole DJ, Hamer JE (1993) Identification and characterization of MPG1, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 5:1575-1590. doi:10.1105/tpc.5.11.1575
- Talbot NJ, Kershaw MJ, Wakley GE, De Vries O, Wessels J, Hamer JE (1996) MPG1 encodes a fungal hydrophobin involved in surface interactions during infection-

- related development of *Magnaporthe grisea*. Plant Cell 8:985-999. doi:10.1105/tpc.8.6.985
- Tamayo-Ramos J, Orejas M (2014) Enhanced glycosyl hydrolase production in *Aspergillus nidulans* using transcription factor engineering approaches. Biotechnol Biofuels 7:103. doi: 10.1186/1754-6834-7-103
- Tanaka H, Yoshida G, Baba Y, Matsumura K, Wasada H, Murata J, Agawa M, Itakura S, Enoki A (2007) Characterization of a hydroxyl-radical-producing glycoprotein and its presumptive genes from the white-rot basidiomycete *Phanerochaete chrysosporium*. J Biotechnol 128:500-511. doi:10.1016/j.jbiotec.2006.12.010
- Tani S, Kanamasa S, Sumitani J, Arai M, Kawaguchi T (2012) XlnR-independent signaling pathway regulates both cellulase and xylanase genes in response to cellobiose in *Aspergillus aculeatus*. Curr Genet 58:93-104. doi:10.1007/s00294-012-0367-5
- Taylor EJ, Gloster TM, Turkenburg JP, Vincent F, Brzozowski AM, Dupont C, Shareck F, Centeno MSJ, Prates JAM, Puchart V, Ferreira LMA, Fontes CMGA, Biely P, Davies GJ (2006) Structure and activity of two metal ion-dependent acetylxyln esterases involved in plant cell wall degradation reveals a close similarity to peptidoglycan deacetylases. J Biol Chem 281:10968-10975. doi:10.1074/jbc.M513066200
- Tenkanen M, Eyzaguirre J, Isoniemi R, Faulds CB, Biely P (2003) Comparison of catalytic properties of acetyl xylan esterases from three carbohydrate esterase families. Appl Microbiol Biotechnol 61:211-229. doi:10.1007/s00253-003-0855-0
- Tilburn J, Sarkar S, Widdick DA, Espeso EA, Orejas M, Mungroo J, Penalva MA, Arst HN, Jr. (1995) The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J 14:779-790.
- Tisch D, Kubicek CP, Schmoll M (2011a) New insights into the mechanism of light modulated signaling by heterotrimeric G-proteins: ENVOY acts on *gna1* and *gna3* and adjusts cAMP levels in *Trichoderma reesei* (*Hypocrea jecorina*). Fungal Genet Biol 48:631-640. doi:10.1016/j.fgb.2010.12.009
- Tisch D, Kubicek CP, Schmoll M (2011b) The phosducin-like protein PhLP1 impacts regulation of glycoside hydrolases and light response in *Trichoderma reesei*. BMC Genomics 12:613. doi:10.1186/1471-2164-12-613
- Tisch D, Schmoll M (2013) Targets of light signalling in *Trichoderma reesei*. BMC Genomics 14:657. doi:10.1186/1471-2164-14-657
- Tobiume K, Saitoh M, Ichijo H (2002) Activation of apoptosis signal-regulating kinase 1 by the stress-induced activating phosphorylation of pre-formed oligomer. J Cell Physiol 191:95-104. doi:10.1002/jcp.10080

- Todd R, Zhou M, Ohm R, Leeggangers H, Visser L, de Vries R (2014) Prevalence of transcription factors in ascomycete and basidiomycete fungi. *BMC Genomics* 15:214. doi: 10.1186/1471-2164-15-214
- Todd RB, Andrianopoulos A (1997) Evolution of a fungal regulatory gene family: the Zn(II)<sub>2</sub>Cys<sub>6</sub> binuclear cluster DNA binding motif. *Fungal Genet Biol* 21:388-405. doi:10.1006/fgbi.1997.0993
- Tomme P, Driver DP, Amandoron EA, Miller RC, Jr., Antony R, Warren J, Kilburn DG (1995) Comparison of a fungal (family I) and bacterial (family II) cellulose-binding domain. *J Bacteriol* 177:4356-4363.
- Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 101:249-258. doi: 10.1016/S0092-8674(00)80835-1
- van den Brink J, Maitan-Alfenas GP, Zou G, Wang C, Zhou Z, Guimarães VM, de Vries RP (2014) *Aspergillus niger* and *Trichoderma reesei* enzymes have a synergistic effect on saccharification of wheat straw and sugarcane bagasse. *Biotechnol J* n/a-n/a. doi:10.1002/biot.201400317
- van Hartingsveldt W, Mattern IE, van Zeijl CM, Pouwels PH, van den Hondel CA (1987) Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. *Mol Gen Genet* 206:71-75.
- van Leeuwen MR, Krijgsheld P, Bleichrodt R, Menke H, Stam H, Stark J, Wösten HA, Dijksterhuis J (2013) Germination of conidia of *Aspergillus niger* is accompanied by major changes in RNA profiles. *Stud Mycol* 74:59-70. doi:10.3114/sim0009
- van Munster JM, Daly P, Delmas S, Pullan ST, Blythe MJ, Malla S, Kokolski M, Noltorp EC, Wennberg K, Fetherston R, Beniston R, Yu X, Dupree P, Archer DB (2014) The role of carbon starvation in the induction of enzymes that degrade plant-derived carbohydrates in *Aspergillus niger*. *Fungal Genet Biol*. doi:10.1016/j.fgb.2014.04.006
- van Peij NNME, Gielkens MMC, de Vries RP, Visser J, de Graaff LH (1998a) The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. *Appl Environ Microbiol* 64:3615-3619.
- van Peij NNME, Visser J, De Graaff LH (1998b) Isolation and analysis of *xlnR*, encoding a transcriptional activator co-ordinating xylanolytic expression in *Aspergillus niger*. *Mol Microbiol* 27:131-142. doi:10.1046/j.1365-2958.1998.00666.x
- Varnai A, Siika-aho M, Viikari L (2013) Carbohydrate-binding modules (CBMs) revisited: reduced amount of water counterbalances the need for CBMs. *Biotechnol Biofuels* 6:30. doi: 10.1186/1754-6834-6-30

- Verna J, Lodder A, Lee K, Vagts A, Ballester R (1997) A family of genes required for maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* 94:13804-13809. doi: 10.1073/pnas.94.25.13804
- Viola RE (2001) The central enzymes of the aspartate family of amino acid biosynthesis. *Acc Chem Res* 34:339-349. doi: 10.1021/ar000057q
- Vogel J (2008) Unique aspects of the grass cell wall. *Curr Opin Plant Biol* 11:301-307. doi: 10.1016/j.pbi.2008.03.002
- Volk A, White, Neuhauser, Gray, Demeter, Lindsey, Jarnefeld, Aneshansley, Pellerin and Edick (2000) Developing a willow biomass crop enterprise for bioenergy and bioproducts in the United States. *Proc Bioenerg* 2000.
- Vonberg RP, Gastmeier P (2006) Nosocomial aspergillosis in outbreak settings. *J Hosp Infect* 63:246-254. doi:10.1016/j.jhin.2006.02.014
- Wallander H, Ekblad A, Godbold DL, Johnson D, Bahr A, Baldrian P, Bjork RG, Kieliszewska-Rokicka B, Kjoller R, Kraigher H, Plassard C, Rudawska M (2013) Evaluation of methods to estimate production, biomass and turnover of ectomycorrhizal mycelium in forests soils - A review. *Soil Biol Biochem* 57:1034-1047. doi:10.1016/j.soilbio.2012.08.027
- Wang L, Yan W, Chen J, Huang F, Gao P (2008a) Function of the iron-binding chelator produced by *Coriolus versicolor* in lignin biodegradation. *Sci China C Life Sci* 51:214-221. doi:10.1007/s11427-008-0033-9
- Wang L, Zhang Y, Gao P (2008b) A novel function for the cellulose binding module of cellobiohydrolase I. *Sci China C Life Sci* 51:620-629. doi:10.1007/s11427-008-0088-3
- Wang M, Zhao Q, Yang J, Jiang B, Wang F, Liu K, Fang X (2013) A mitogen-activated protein kinase Tmk3 participates in high osmolarity resistance, cell wall integrity maintenance and cellulase production regulation in *Trichoderma reesei*. *PLoS One* 8:e72189. doi:10.1371/journal.pone.0072189
- Wasserman BP, McCarthy KJ (1986) Regulation of plasma membrane Beta-glucan synthase from red beet root by phospholipids : reactivation of Triton X-100 extracted glucan synthase by phospholipids. *Plant Physiol* 82:396-400.
- Watanabe Y, Irie K, Matsumoto K (1995) Yeast RLM1 encodes a serum response factor-like protein that may function downstream of the Mpk1 (Slt2) mitogen-activated protein kinase pathway. *Mol Cell Biol* 15:5740-5749. doi: 10.1128/MCB.15.10.5740
- Welchen E, Hildebrandt TM, Lewejohann D, Gonzalez DH, Braun HP (2012) Lack of cytochrome C in *Arabidopsis* decreases stability of Complex IV and modifies redox metabolism without affecting Complexes I and III. *Biochim Biophys Acta* 1817:990-1001. doi:10.1016/j.bbabi.2012.04.008

- Wessels J, De Vries O, Asgeirsdottir SA, Schuren F (1991) Hydrophobin genes involved in formation of aerial hyphae and fruit bodies in *Schizophyllum*. *Plant Cell* 3:793-799. doi:10.1105/tpc.3.8.793
- Wessels JGH (1997) Hydrophobins: Proteins that change the nature of the fungal surface. *Adv Microb Physiol* 38:1-45.
- Westereng B, Ishida T, Vaaje-Kolstad G, Wu M, Eijsink VG, Igarashi K, Samejima M, Stahlberg J, Horn SJ, Sandgren M (2011) The putative endoglucanase PcGH61D from *Phanerochaete chrysosporium* is a metal-dependent oxidative enzyme that cleaves cellulose. *PLoS One* 6:e27807. doi:10.1371/journal.pone.0027807
- Whipps JM, Haselwandter K, McGee EEM, Lewis DH (1982) Use of biochemical markers to determine growth, development and biomass of fungi in infected-tissues, with particular reference to antagonistic and mutualistic biotrophs. *Trans Br Mycol Soc* 79:385-400.
- Whiteford JR, Spanu PD (2002) Hydrophobins and the interactions between fungi and plants. *Mol Plant Pathol* 3:391-400. doi:10.1046/j.1364-3703.2002.00129.x
- Whitmarsh AJ, Davis RJ (2000) Regulation of transcription factor function by phosphorylation. *Cell Mol Life Sci* 57:1172-1183. doi:10.1007/PL00000757
- Wiederhold NP, Kontoyiannis DP, Prince RA, Lewis RE (2005) Attenuation of the activity of caspofungin at high concentrations against *Candida albicans*: possible role of cell wall integrity and calcineurin pathways. *Antimicrob Agents Chemother* 49:5146-5148. doi:10.1128/AAC.49.12.5146-5148.2005
- Wilson DB (2011) Microbial diversity of cellulose hydrolysis. *Curr Opin Microbiol* 14:259-263. doi:10.1016/j.mib.2011.04.004
- Winkler A, Arkind C, Mattison CP, Burkholder A, Knoche K, Ota I (2002) Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress. *Eukaryot Cell* 1:163-173. doi: 10.1128/EC.1.2.163-173.2002
- Withers SG (2011) Glycoside Hydrolase Family 1. CAZypedia available at <http://www.cazypedia.org/>.
- Wood DA (1979) Method for estimating biomass of *Agaricus bisporus* in a solid substrate, composted wheat straw. *Biotechnol Lett* 1:255-260. doi:10.1007/Bf01386998
- Wood DA (1980) Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J Gen Microbiol* 117:327-338.
- Woodgett JR (1994) Regulation and functions of the glycogen synthase kinase-3 subfamily. *Semin Cancer Biol* 5:269-275.
- Woodgett JR (2001) Judging a protein by more than its name: GSK-3. *Sci STKE* 2001:re12. doi:10.1126/stke.2001.100.re12

- Woodgett JR, Kyriakis JM, Avruch J, Zon LI, Zanke B, Templeton DJ (1996) Reconstitution of novel signalling cascades responding to cellular stresses. *Philos Trans R Soc Lond B Biol Sci* 351:135-141; discussion 142. doi:10.1098/rstb.1996.0009
- Wösten HA (2001) Hydrophobins: multipurpose proteins. *Annu Rev Microbiol* 55:625-646. doi:10.1146/annurev.micro.55.1.625
- Wösten HA, Scholtmeijer K (2015) Applications of hydrophobins: current state and perspectives. *Appl Microbiol Biotechnol* 99:1587-1597. doi:10.1007/s00253-014-6319-x
- Wösten HAB, de Vocht ML (2000) Hydrophobins, the fungal coat unravelled. *Biochimica Et Biophysica Acta Rev Biomembranes* 1469:79-86. doi:10.1016/S0304-4157(00)00002-2
- Wurleitner E, Pera L, Wacenovsky C, Cziferszky A, Zeilinger S, Kubicek CP, Mach RL (2003) Transcriptional regulation of *xyn2* in *Hypocrea jecorina*. *Eukaryot Cell* 2:150-158. doi: 10.1128/EC.2.1.150-158.2003
- Wymelenberg AV, Denman S, Dietrich D, Bassett J, Yu X, Atalla R, Predki P, Rudsander U, Teeri TT, Cullen D (2002) Transcript analysis of genes encoding a family 61 endoglucanase and a putative membrane-anchored family 9 glycosyl hydrolase from *Phanerochaete chrysosporium*. *Appl Environ Microbiol* 68:5765-5768. doi: 10.1128/AEM.68.11.5765-5768.2002
- Xie Z, Kometiani P, Liu J, Li J, Shapiro JI, Askari A (1999) Intracellular reactive oxygen species mediate the linkage of Na<sup>+</sup>/K<sup>+</sup>-ATPase to hypertrophy and its marker genes in cardiac myocytes. *J Biol Chem* 274:19323-19328.
- Xu JR (2000) Map kinases in fungal pathogens. *Fungal Genet Biol* 31:137-152. doi:10.1006/fgbi.2000.1237
- Xue Z, Ye Q, Anson SR, Yang J, Xiao G, Kowbel D, Glass NL, Crosthwaite SK, Liu Y (2014) Transcriptional interference by antisense RNA is required for circadian clock function. *Nature advance online publication*. doi:10.1038/nature13671
- Yan JX, Wait R, Berkelman T, Harry RA, Westbrook JA, Wheeler CH, Dunn MJ (2000) A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis* 21:3666-3672. doi:10.1002/1522-2683(200011)21:17<3666::AID-ELPS3666>3.0.CO;2-6
- Yang W, Liu J, Wang W, Zhang Y, Gao P (2004) Function of a low molecular peptide generated by cellulolytic fungi for the degradation of native cellulose. *Biotechnol Lett* 26:1799-1802. doi:10.1007/s10529-004-4612-y
- Yip VL, Varrot A, Davies GJ, Rajan SS, Yang X, Thompson J, Anderson WF, Withers SG (2004) An unusual mechanism of glycoside hydrolysis involving redox and

- elimination steps by a family 4 beta-glycosidase from *Thermotoga maritima*. J Am Chem Soc 126:8354-8355. doi:10.1021/ja047632w
- Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M, Mori K (2001) Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6-alpha and 6-beta that activates the mammalian unfolded protein response. Mol Cell Biol 21:1239-1248. doi:10.1128/MCB.21.4.1239-1248.2001
- Young ET, Dombek KM, Tachibana C, Iderer T (2003) Multiple pathways are co-regulated by the protein kinase Snf1 and the transcription factors Adr1 and Cat8. J Biol Chem 278:26146-26158. doi:10.1074/jbc.M301981200
- Yuan XL, Roubos JA, van den Hondel CA, Ram AF (2008) Identification of InuR, a new Zn(II)<sub>2</sub>Cys<sub>6</sub> transcriptional activator involved in the regulation of inulinolytic genes in *Aspergillus niger*. Mol Genet Genomics 279:11-26. doi:10.1007/s00438-007-0290-5
- Zampieri F, Wösten HAB, Scholtmeijer K (2010) Creating surface properties using a palette of hydrophobins. Materials 3:4607-4625. doi:10.3390/Ma3094607
- Zdobnov EM, Apweiler R (2001) InterProScan-an integration platform for the signature-recognition methods in InterPro. Bioinformatics 17:847-848. doi:10.1093/bioinformatics/17.9.847
- Zhang J, Qu Y, Xiao P, Wang X, Wang T, He F (2012) Improved biomass saccharification by *Trichoderma reesei* through heterologous expression of *lacA* gene from *Trametes* sp. AH28-2. J Biosci Bioeng 113:697-703. doi:10.1016/j.jbiosc.2012.01.016
- Zhang W, Li F, Nie L (2010) Integrating multiple 'omics' analysis for microbial biology: application and methodologies. Microbiol 156:287-301. doi:10.1099/mic.0.034793-0
- Zhong Q, Gvozdenovic-Jeremic J, Webster P, Zhou J, Greenberg ML (2005) Loss of function of KRE5 suppresses temperature sensitivity of mutants lacking mitochondrial anionic lipids. Mol Biol Cell 16:665-675. doi:10.1091/mbc.E04-09-0808
- Zhong R, Ye ZH (2007) Regulation of cell wall biosynthesis. Curr Opin Plant Biol 10:564-572. doi:10.1016/j.pbi.2007.09.001
- Zhou J, Liu CY, Back SH, Clark RL, Peisach D, Xu Z, Kaufman RJ (2006) The crystal structure of human Ire1 luminal domain reveals a conserved dimerization interface required for activation of the unfolded protein response. Proc Natl Acad Sci USA 103:14343-14348. doi:10.1073/pnas.0606480103
- Zhu JY, Pan XJ, Zalesny RS (2010) Pretreatment of woody biomass for biofuel production: energy efficiency, technologies, and recalcitrance. Appl Microbiol Biotechnol 87:847-857. doi:10.1007/s00253-010-2654-8

- Zill G, Engelhardt G, Wallnofer PR (1988) Determination of ergosterol as a measure of fungal growth using Si-60 HPLC. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung* 187:246-249. doi:10.1007/Bf01043348
- Znameroski E, Glass N (2013) Using a model filamentous fungus to unravel mechanisms of lignocellulose deconstruction. *Biotechnol Biofuels* 6:6. doi: 10.1186/1754-6834-6-6