CHAPTER 4

Transcriptional Regulation and Responses in Filamentous Fungi Exposed to Lignocellulose

Paul Daly, Jolanda M van Munster, Roxane Raulo and David B Archer*

School of Life Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, UK

Abstract: Biofuels derived from lignocellulose are attractive alternative fuels but their production suffers from a costly and inefficient saccharification step that uses fungal enzymes. One route to improve this efficiency is to understand better the transcriptional regulation and responses of filamentous fungi to lignocellulose. Sensing and initial contact of the fungus with lignocellulose is an important aspect. Differences and similarities in the responses of fungi to different lignocellulosic substrates can partly be explained with existing understanding of several key regulators and their mode of action, as will be demonstrated for *Trichoderma reesei*, *Neurospora crassa* and *Aspergillus* spp. The regulation of genes encoding Carbohydrate Active enZymes (CAZymes) is influenced by the presence of carbohydrate monomers and short oligosaccharides, as well as the external stimuli of pH and light. We explore several important aspects of the response to lignocellulose that are not related to genes encoding CAZymes, namely the regulation of transporters, accessory proteins and stress responses. The regulation of gene expression is examined from the perspective of mixed cultures and models are presented for the nature of the transcriptional basis for any beneficial effects of such mixed cultures. Various applications in biofuel technology are based on manipulating transcriptional regulation and learning from fungal responses to lignocelluloses. Here we critically access the application of fungal transcriptional responses to industrial saccharification reactions. As part of this chapter, selected regulatory mechanisms are also explored in more detail.

Keywords: Accessory proteins, *Aspergillus*, biofuel, CAZyme, gene regulation, inducer, light, lignocellulose, mixed culture, model, *Neurospora*, nucleosome, pH, saccharification, signalling, stresses, transcription factor, transporter, *Trichoderma*, XlnR/XYR1/XLR-1.

INTRODUCTION

There is great impetus to develop second generation biofuels, which involves the production of liquid fuels from various types of non-food lignocellulosic

*Corresponding author David B Archer: School of Life Sciences, University of Nottingham, University Park, Nottingham, NG7 2RD, UK; Tel: 0044 (0)115 951 3313; Fax: 0044 (0)115 951 3251; E-mail: david.archer@nottingham.ac.uk

Roberto Nascimento Silva (Ed)

biomasses, often from agricultural or waste residues [1-3]. Second generation biofuels are made by breaking down the polysaccharides in lignocellulose to simple sugars, in a process called saccharification, using enzymes produced industrially by filamentous fungi. The sugars are subsequently fermented to produce a biofuel such as ethanol or butanol. Filamentous fungi are suitable suppliers of carbohydrate-active enzymes (CAZymes) [4] because the saccharification of lignocellulose is a natural function for many fungal species and the commercial production of enzymes from several fungi has already been achieved. Furthermore, molecular tools to exploit many relevant fungal species have been developed [5, 6]. However, the saccharification step is inefficient and expensive with the cost of enzymes a major factor in the expense [2, 7]. Analysis of the costs associated with the production of enzymes highlighted a breadth of contributory factors [7]. In relation to the subject of this chapter, reductions in the costs of enzymes could be achieved by improving their functionalities (efficiency of saccharification) on the target lignocellulose materials and by producing them at higher yield on cheaper substrates. Improved enzyme functionalities (activities) and cheaper production could come from understanding better the fungal response to lignocellulose. This response occurs through the regulation of gene expression that leads to the production and secretion of the derived enzymes. Therefore, this chapter assesses current knowledge on gene regulation of fungi exposed to lignocellulose.

The main industrial fungus used to supply cellulases is *Trichoderma reesei*, which is also used as a research model. Other ascomycete fungi studied as models as well as being exploited for commercial enzyme production include *Aspergillus niger*, *Penicillium* spp., *Talaromyces versatilis*, amongst others [8]. *Neurospora crassa* is used as a research model but is not commercially exploited for enzyme production [9]. The basidiomycetes are less well explored and the molecular tools are less well developed, although this gap is closing and the enzymatic capabilities of basidiomycetes (*e.g.* in the deconstruction of lignin) can be expected to play a major role in the near future [10]. This chapter will focus on the ascomycetes. There are already several extensive reviews related to the regulatory responses of fungi to small molecules as well as, but less so, to lignocellulose [9, 11-18]. We will therefore refer to the reviewed information where applicable and expand the discussion to focus on exposure of fungi to lignocellulose.

The genome sequences of fungal species of interest for this chapter, *T. reesei* [19], *N. crassa* [20] and *A. niger* [21, 22], provide a catalogue of the genes encoding

CAZymes as well as other relevant functionalities such as transcriptional regulators and signalling proteins. Genome sequences are available from (near to) wild-type strains of those species and a comparison is available for *T. reesei* of a wild-type and the carbon catabolite de-repressed mutant strain RUT-C30 [23]. Commercial enzyme producers use mutagenised and genetically-modified strains whereas most laboratory-based research is conducted with un-improved strains, although *T. reesei* RUT-C30 has been explored extensively both commercially and in the lab [24].

A large number of factors affect 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, corn stover, 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 [9, 13, 25]. 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 [26] and light [27]. We aim in this chapter to make comparisons of published data on transcriptional responses of fungi to lignocellulose and to take into account the many variables that obscure inter-study comparisons.

Finally, fungal species do not saccharify lignocellulose in isolation in nature and they are component parts of a complex microbial community. Therefore, saccharolytic functions may not be, under all conditions, optimised within a single species. We will therefore also discuss the options for combining the capabilities of different ascomycete species, based on their transcriptional responses to lignocellulose.

SECTION I – 'FIRST CONTACT' BETWEEN FUNGI AND LIGNOCELLULOSE

Lignocellulose is a complex material that is composed mainly of cellulose, hemicellulose, pectin and lignin [2]. Fungi face a complex task when they are

exposed to lignocellulose and need to degrade this substrate to grow. The fungi need to detect the presence of the lignocellulose and respond to its composition by secreting a set of appropriate hydrolytic enzymes. Subsequently, fungi need to take up the resulting small sugars released from the lignocellulose to sustain their growth. All of this requires a considerable investment of energy and resources and, unsurprisingly, it is a carefully regulated process. When more easily-metabolised sugars are available in sufficient amounts, carbon catabolite repression (CCR) [28, 29] represses a large number of genes [30], including genes encoding lignocellulose-degrading CAZymes under conditions where lignocellulose and other inducers are present [31, 32]. How the detection and signalling of the presence or absence of such easily metabolised carbon sources can prepare the fungus for the degradation of lignocellulose is discussed below. Subsequently, models are presented that describe how a fungus may release inducers of CAZymes during its 'first contact' with lignocellulose, and we focus on the identity of these inducers. Finally, the 'first contact' of a spore with a carbon source, which triggers germination, is explored.

Signalling Cascades Related to Nutrient Sensing and Expression of CAZy Genes

Recently the literature on signalling cascades related to nutrient sensing and lignocellulolytic enzyme production was extensively reviewed by Brown *et al.* [15] and in this section some of the key aspects will be highlighted. Although filamentous fungi are the focus of this chapter, at present there is more complete information available on signalling cascades for nutrient sensing in the yeast *Saccharomyces cerevisiae*. Fungi detect the presence of glucose, an easily metabolised carbon source, predominantly *via* the cAMP-dependent protein kinase A (PKA) pathway *via* two main mechanisms; G-protein coupled receptor (GPCR) signalling and the phosphorylation of imported glucose [15].

With regard to the GPCR signalling mechanism; in *S. cerevisiae*, the GPCR Gpr1p senses glucose and signals downstream to activate adenylate cyclase Cyr1p which produces a burst of cAMP. This cAMP activates PKA which subsequently translocates to the nucleus and regulates transcription factors, as reviewed by Zaman *et al.* [33]. The PKA pathway is also involved in detection in filamentous fungi [34] but it is unclear whether it is similarly activated by the two mechanisms as orthologues of the GPCR glucose sensor have yet to be identified in filamentous fungi [15]. With regard to the other mechanism involving glucose phosphorylation, in *A. nidulans* and *T. reesei* glucose is taken up into the hyphae

and phosphorylated by the sugar kinases GlkA/GLK1 and HxkA/HXK1. Deletion of both these genes results in de-repression of genes normally under the control of CCR in *T. reesei* [35] and *A. nidulans* [36]. In *S. cerevisiae*, as reviewed by Zaman *et al.* [33] and Brown *et al.* [15], phosphorylated glucose induces RAS signalling which results in cAMP production and PKA activation. Phosphorylated glucose furthermore causes inactivation of the kinase Snf1p which is a sensor of cellular energetic state in *S. cerevisiae* and known to be required for growth on alternative carbon sources. The inactivation of Snf1p leads to a reduction of phosphorylation of the CCR regulator Mig1p and promotes the localisation of non-phosphorylated Mig1p in the nucleus where it represses target genes. In filamentous fungi, CCR is mediated by the CreA/CRE1 protein [28], which is a functional homologue of Mig1p. CreA/CRE1 is localized in the cytoplasm under non-repressing conditions and is shuttled to the nucleus under repressing conditions [37, 38]. Its localization is regulated by its phosphorylation state [37], similar to Mig1p. As noted by Brown *et al.* [15], the phosphorylation state of CreA/CRE1 has a different effect in *T. reesei* compared to other filamentous fungi where in *T. reesei* phosphorylation of CRE1 leads to repression (instead of de-repression) of genes [39].

A number of other protein kinases with a role in nutrition-state signalling have been identified in filamentous fungi. Brown *et al.* [37] studied two non-essential protein kinases SnfA (the homologue of yeast Snf1p) and SchA in *A. nidulans*. They demonstrated that the deletion of *snfA* or *schA* decreased the production of hydrolytic enzymes by decreasing the ability of *A. nidulans* to unlock the CreA repression mechanism under de-repressing conditions (either starvation or Avicel cellulose). Detecting the absence of easily metabolised sugars, and subsequent derepression of CCR is required but not sufficient to induce the production of CAZymes for lignocellulose degradation. The fungus also needs to detect and respond to the presence of lignocellulose.

How Fungi Detect Lignocellulose

Many details of how fungi detect lignocellulose are unknown. As lignocellulose is a large, insoluble structure, it cannot enter the cell. Consequently, partial degradation products of the lignocellulose, such as carbohydrate monomers or small oligosaccharides are considered to be released and imported into the cell, where they act as inducers of the subsequent degradative response. Two, not mutually exclusive, models can explain the generation of the small carbohydrate molecules from the lignocellulose (Fig. **1**).

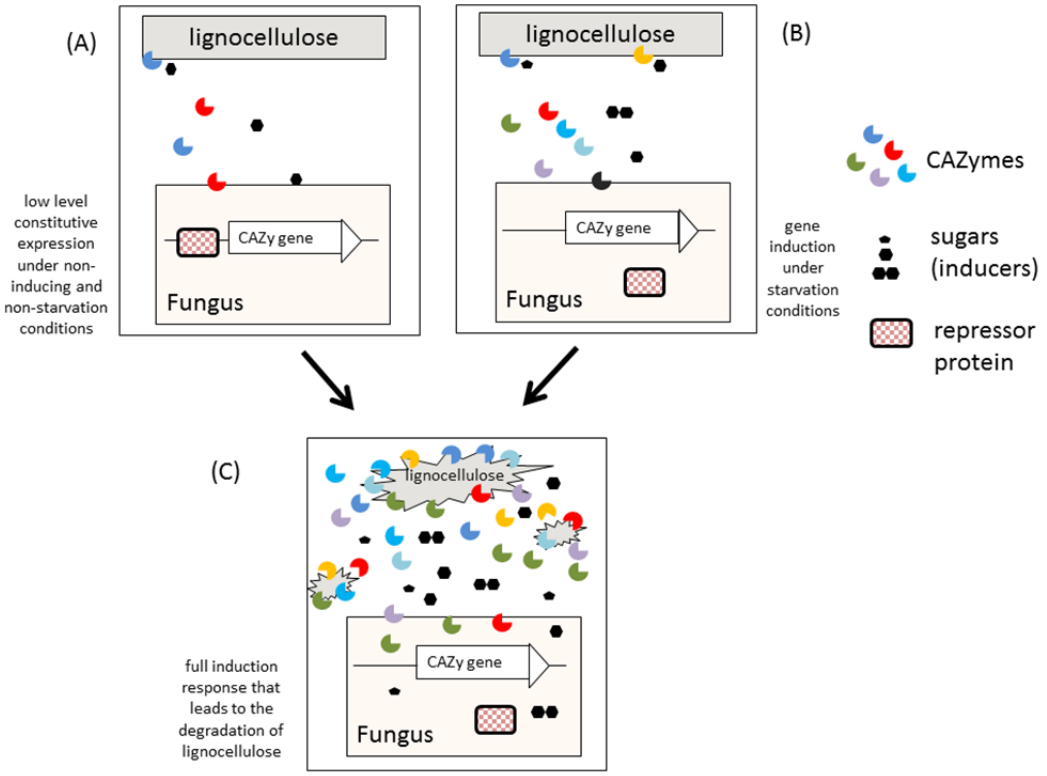


Figure 1: Two, not mutually exclusive, models can explain the generation of the small carbohydrate inducing molecules from the lignocellulose. (A) Low level constitutive expression of CAZyme-encoding genes and (B) starvation induced expression of CAZyme-encoding genes can result in the release of inducers from lignocellulose leading to (C) the full induction response.

In the first model, an important role is reserved for a set of enzymes whose genes are constitutively expressed at a low level under non-inducing and non-starvation conditions. These enzymes, such as *A. niger* endopolygalacturonase PgaA, *T. reesei* cellobiohydrolase CBH1 and endoglucanase EGL1, are considered to partially degrade lignocellulose and release inducers [29, 40, 41]. In the second model, soluble, low molecular weight carbohydrates are considered to be produced by enzymes encoded by genes that are responsive to CCR. These enzymes are thought to 'scout' the environment for available carbon sources. Alleviation of CCR, either by inactivation of the CreA/CRE1 regulator or by lack of a carbon source in the medium, results in the increase of transcription of genes encoding CAZymes that are active on plant-derived carbohydrates in a number of fungi [42-48]. A large proportion of these genes was expressed both during carbon starvation conditions and during exposure to lignocellulose for example in

N. crassa exposed to Avicel [45] or *A. niger* exposed to wheat straw [42]. This transcriptional response to carbon starvation results in secretion of enzymes that are active on plant-derived carbohydrates [42]. These enzymes release small carbohydrate monomers and oligosaccharides from plant-derived carbohydrates, some of which are known inducers of genes encoding lignocellulose-degrading CAZymes [42]. These models can work together by using enzymes provided by both low constitutive gene expression as well as genes with increased transcription through carbon catabolite derepression, to generate inducers for the full degradative response.

As an extension of the models discussed above, Benz *et al.* [49] proposed a 'tasting' model for a group of *N. crassa* genes that were induced during exposure to the polysaccharides xylan, Avicel and pectin. These genes encoded esterases, endo- and exo-acting hydrolases including those that release monosaccharides from oligosaccharides. The 'tasting' model proposed that this group of enzymes could release inducers from a wide range of polysaccharides thus allowing the fungus to then fine-tune its response to the so called 'flavours' of the environment based on the released inducers. Most of the genes in this group were lowly induced in carbon starvation conditions, and thus may overlap with the scouting response under these conditions [49].

Inducers and Induction Mechanisms

Inducers released from the lignocellulose are considered to be of key importance in the induction of CAZyme-encoding genes. There can be different small molecule inducers of the same or similar CAZyme-encoding genes in different fungal species. A recent review by Amore *et al.* [11] summarises many of these inducers and the genes that they induce in filamentous fungi. Other reviews also summarises some of the relevant inducers and induction mechanisms [13, 29]. In *A. niger*, xylose (a sugar that forms the backbone of xylan and is found in other hemicelluloses and pectins) induces cellulase as well as hemicellulase-encoding genes [50]. The effect of this molecule is concentration dependent where higher xylose concentrations can be repressive in a CreA-dependent manner, rather than inductive [51]. In *T. reesei*, the disaccharide sophorose which is made when cellobiose (a disaccharide of glucose) is transglycosylated by a β-glucosidase, functions as an inducer of cellulase encoding genes [52]. Lactose (a disaccharide of glucose and galactose which is not a component of lignocellulose) can also induce cellulases as well as other CAZymes in *T. reesei* [53]. Xylanases in *T. reesei* can be induced by xylose and arabinose (a sugar that is a minor component of hemicelluloses and pectins) but independently of each other and by using

different metabolites [25]. Interestingly, although industrially used strains are often subjected to rounds of mutagenesis, a recent study claimed that the induction mechanism in the industrial *T. reesei* strain RUT-C30 is still largely intact [54]. In *N. crassa*, cellobiose is the inducer primarily of cellulases [55]. Xylose in *N. crassa* induced fewer hemicellulase-encoding genes than a xylan polymer indicating that additional small molecules from hemicellulose are required for the full induction response or that the size or structure of the polymer is important [56]. Section II of this chapter will describe the response of fungi to xylan and other complex polymers (containing many different small molecule inducers). Finally, gentiobiose (a disaccharide of glucose joined *via* a β-1,6 linkage) is an inducer of cellulases in the ascomycete *Penicillium purpurogenum* [57]. As was the case for the sensing of glucose, the sensing of inducers involves intracellular signalling cascades involving phosphorylation. In *A. oryzae*, xylose (the inducer of cellulase and hemicellulase genes in *A. oryzae*) triggered reversible phosphorylation of a major CAZyme-encoding gene regulator XlnR [58]. In *N. crassa*, there were changes in the phosphorylation levels of the major regulator CLR-1 in the presence of Avicel [59]. In *T. reesei*, Wang *et al.* [60] suggested that the kinase TMK3 may be involved in cellulase and hemicellulase production by phosphorylating and activating transcription factors responsible for CAZyme induction. Deletion of *tmk3* down-regulated the transcript level of *cbh1*, *cbh2*, *egl1*, *egl2* and *bgl1* encoding, respectively, two cellobiohydrolases, two endoglucanases and one β-glucosidase [60].

Exploration of a Relevant Regulatory Mechanism – Regulation of Spore Germination by Sugars

The majority of the literature reviewed in this chapter is with mycelial fungi but, in addition, there is contact between lignocellulose and fungal spores. Ascomycetes such as *A. niger* and *T. reesei* can produce and disperse large numbers of asexual conidiospores. These stress-resistant structures remain dormant during adverse environmental conditions and thus ensure survival of the fungus under these circumstances. When times change for the better, the conidiospore needs to germinate in response, thus allowing another round of mycelial growth. Due to their ubiquitous nature and saprophytic lifestyle, conidiospores are likely to germinate on, or near, plant material. The germination of fungal condiospores starts with the breaking of dormancy, after which the conidiospore swells, takes up water and activates carbon storages. Subsequently, cell polarity is established and a germ tube is formed [61-63]. The breaking of dormancy is regulated by a ‘germination trigger’ or inducer, usually a sugar molecule, that results in mobilisation of internal energy stores such as trehalose

and the swelling of the conidiospore and activation of metabolism. The sugar providing the germination trigger does not have to be identical to the carbohydrate that subsequently supports outgrowth of the mycelium because triggering germination and supporting mycelial growth are separate events. D-glucose, D-xylose and D-mannose trigger germination of *A. niger* as well as support growth of the germ tube [64]. The concentration of D-glucose needed to trigger germination is much lower (≥10 nM) than the concentration needed to support growth of the germ tube (≥10 μM). Other sugars, such as 2-deoxy-D-glucose trigger germination but do not support growth. The sugars D-galactose and L-arabinose are unable to trigger germination but they are taken up by the conidiospore and support outgrowth of a germ tube in the presence of a germination trigger [64]. The identity and concentration of sugars and other small molecules such as amino acids [65] that are encountered in the environment thus provide important signals to the conidiospore, regulating whether conidiospore germination can be initiated on lignocellulose and mycelial growth supported.

SECTION II – TRANSCRIPTIONAL REGULATION OF GENES ENCODING CAZYMES

Global transcriptional analyses of the responses of fungi to lignocellulosic substrates are often used to define sets of genes, often termed 'regulons', that are induced or modulated in expression on particular substrates. Genes that encode CAZymes 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 CAZyme-encoding genes. As well as the polymers, environmental factors such as light and pH can play crucial roles in determining their expression. The role of nucleosome positioning in the regulation of a CAZyme-encoding gene will be explored in more detail.

Studies of the CAZy Gene Responses to Polysaccharides and Lignocelluloses

For the purposes of Section II, more robust conclusions can be drawn by comparing the responses within a single fungal species to different substrates rather than also describe the responses of different fungi to the same substrate. Inter-species differences in gene content and regulation hamper the drawing of robust conclusions.

Responses of T. Reesei to Polysaccharides and Lignocellulosic Substrates

An extensive study with the *T. reesei* RUT-C30 strain, which is a CCR de-repressed strain and a hypersecreter of cellulases, compared the transcriptional

responses to untreated substrates (bagasse, Avicel and two xylans) and pre-treated substrates (stream exploded bagasse, wheat straw and spruce) [66]. In an analysis of the gene expression patterns, a 'common core' of induced genes was defined as those induced by both Avicel and xylan and induced on a least 70% of the substrates [66]. This 'common core' included genes encoding a large range of activities such as endoglucanases, cellobiohydrolases, endoxylanases and several other activities that hydrolyse components of polysaccharide backbones and side chains and the linkages between the cell wall components [66]. In this common core were genes encoding chitinases, which are active towards the fungal cell wall rather than the plant cell wall, that probably have a cell wall remodelling or starvation related role. As well as genes encoding for hydrolases, a gene encoding for lytic polysaccharide monoxygenase activity (AA9) [67] was also in this 'common core' of induced genes. Outside of this 'common core', there were differences in the levels of the induction of CAZyme-encoding genes but no clear examples of genes that were induced on only one substrate but not another with some of the differences due to time rather than substrate [66]. There are two examples from the study of Hakkinen *et al.* [66] of differences in the transcriptional response of CAZyme-encoding genes that are in part dependent on the pre-treatment of the substrate. Firstly, the CAZyme-encoding gene expression patterns from the untreated compared to the pre-treated bagasse clustered separately. On the pre-treated bagasse, there was a cluster of CAZyme-encoding genes which had increased abundance but have not yet been characterised in *T. reesei* [66]. Secondly, Hakkinen *et al.* [66] also analysed steam exploded spruce which was enriched in cellulose and had very few monomeric sugars. The CAZyme-encoding gene expression patterns concurred with this enrichment of cellulose in the pre-treated spruce whereby the patterns on the pre-treated spruce clustered with the patterns on the Avicel (mainly cellulose) substrate for two time points [66]. It is worthwhile noting that the expression patterns of the CAZyme-encoding genes in this study are likely to be mainly due to the presence of inducers from the lignocelluloses rather than the accumulation of released sugars to repressive levels because RUT-C30 is a CCR depressed strain.

Responses of N. Crassa to Polysaccharides and Lignocellulosic Substrates

Benz *et al.* [49] compared the transcriptional responses of *N. crassa* to cellulose, xylan and pectin. That study defined the 'pectin regulon' and compared this to the 'Avicel regulon' from Coradetti *et al.* [45] and the 'xylan regulon' - the xylan study was a replication of the microarray experiment from Sun *et al.* [56] using RNAseq. All three substrates significantly induced a common set of 29 genes and three quarters of these genes encoded CAZymes. These genes encoded esterases, exo- and endoglucanases,

β-xylosidases and β-galactosidases but genes encoding β-glucosidases were absent. Clustering analyses identified clusters containing genes predominantly induced on a particular substrate. When 21 of the genes that clustered in the predominantly pectin-related expression clusters were deleted separately, four of the mutants showed reduced growth on pectin [49]. These deletion mutants demonstrated the utility of the expression clustering in predicting gene function. Benz *et al.* [49] excluded the genes that were induced by carbon starvation from their analysis of substrate induced genes. This is an important exclusion because the similarities in the CAZy responses may not necessarily be because the fungus is responding transcriptionally in a specific manner to different polysaccharides or lignocelluloses but instead the response could be one related to an initial starvation [42].

Responses of A. Niger to Polysaccharides and Lignocellulosic Substrates

For *A. niger*, there are studies that examine the transcriptional response to the lignocellulosic substrates wheat straw (at two time points) [31, 42], willow [68] and pre-treated sugar cane bagasse [69]. The responses to two polysaccharides which are components of lignocellulose (oat spelt xylan and arabinan) were studied by Andersen *et al.* [70] and compared to cultures where monomers (xylose and arabinose) were the carbon source. There were many similarities in the CAZy transcriptional responses of *A. niger* to wheat straw (at the later time point), willow and pretreated bagasse [31, 68, 69]. Most of the genes encoding the CAZymes required to break down cellulose and the hemicellulose backbones and side chains were induced. This probably reflects the ability of xylose (present in each of these lignocelluloses) to induce both genes encoding cellulases and hemicellulases as described in Section I. Although the responses of *A. niger* to wheat straw compared to wood from a willow tree were broadly similar, there were some notable differences in expression levels [68]. Genes that had higher expression on wheat straw compared to willow included a GH62 arabinofuranosidase and two feruloyl esterases and these increases in expression could be related to compositional differences in the substrates [68]. A temporal trend was also observed in the transcriptional responses of *A. niger* to untreated wheat straw where, generally, genes encoding enzymes with activity towards hemicelluloses were induced earlier than genes encoding enzymes with activity towards pectins [42]. Andersen *et al.* [70] described differences in the response of *A. niger* to different polysaccharide substrates including CAZyme-encoding genes that were only induced on arabinan [70]. Comparisons between the study with the defined polysaccharides [70] to the lignocellulose studies [31, 42, 68, 69] is complicated because the concentrations of xylose in the xylan cultures at the time of sampling have the potential to have a repressing effect on some CAZyme-encoding genes as well as an inducing effect on others. A previous study in *A.*

niger showed that concentrations of xylose higher than 1 mM can have a repressive effect on CAZyme-encoding genes [51].

Responses of Myceliophthora Thermophila to Lignocellulosic Substrates

Kolbusz *et al.* [71] investigated the responses of *M. thermophila* to three agricultural straws from dicots (alfalfa, flax and canola) and three straws from monocots (barley, triticale and oat). Using a principal component analysis (PCA), Kolbusz *et al.* [71] found that the expression patterns from the monocots clustered separately from the dicots. They then analysed the expression dataset as if the straws from different monocot or dicot species were replicates of a group. In total, 95 genes encoding CAZymes were induced on the lignocelluloses compared to the glucose control with 59 CAZyme-encoding genes induced on both monocot and dicot straws, 22 CAZyme-encoding genes induced only on the monocot straws and 14 CAZyme-encoding genes induced only on the dicot straws [71]. Correspondence was found between the genes that were induced and the differences between the composition of the monocot and dicot straws [71]. The dicot straws induced more genes encoding pectinolytic activity than the monocot straws, which corresponds well with the increased proportion of pectins in dicots [71]. The monocot but not dicot straws induced a carbohydrate esterase 1 (CE1) CAZy family feruloyl esterase which corresponds well with the higher proportion of feruloyl linkages in monocot cell walls as reviewed by Vogel [72]. Knowledge of the molecular basis of the regulation of these genes is required to understand how the straws induce different responses but regulation at this level is largely unexplored in *M. thermophila*. Kolbusz *et al.* [71] observed a temporal trend, when they examined a time course study of proteins secreted by *M. thermophila* cultured with lignocellulose to complement their transcriptomic study. The enzymes with activity towards cellulose and hemicelluloses tended to be secreted before enzymes with activity towards pectins. Kolbusz *et al.* [71] also noted that in these types of studies, there are CAZyme-encoding genes that are not induced by any lignocellulose substrate or other polysaccharides which they referred to as 'cryptic' CAZyme-encoding genes because what induces these genes has not been elucidated yet. Kolbusz *et al.* [71] suggest that some of these 'cryptic' CAZyme-encoding genes may be expressed at specific temperatures, times or pH emphasising the need for more extensive transcriptionally profiling studies.

Repressors and Activators of CAZyme-Encoding Gene Transcription

The responses of fungi to lignocellulose, described in detail above, are mediated by transcriptional repressors and activators. Fewer repressors than activators relevant to lignocellulose degradation have been identified. There are three main

fungal species where repressors and activators relevant to lignocellulose have been studied: *Aspergillus* spp., mainly *A. niger*, the industrially used *T. reesei* and the model *N. crassa*.

Transcriptional Repressors

Section I introduced a key repressor in fungi relevant to transcriptional regulation related to lignocellulose CreA/CRE1 [28, 30]. CreA/CRE1 functions in CCR where, in the presence of sufficient glucose and in some cases other sugars, it suppresses the transcription of genes encoding enzymes involved in the metabolism of more complex polysaccharides [28]. The binding sites for CreA/CRE1 are two neighbouring palindromic consensus sequences in the promoter of genes (5'-SYGGRG-3' in *A. nidulans* [73] and *T. reesei* [74]), and binding of CreA/CRE1 thus hinders their transcription. CreA/CRE1 is a master regulator insofar as CreA/CRE1 regulates other transcription factors such as activators of genes encoding CAZymes [51, 75]. A CreA/CRE1 orthologue was found in the genomes of most of the 108 ascomycete and basidiomycete species analysed by Todd *et al.* [76]. Another well-known repressor is the counter intuitively named, ACE1 (activator of cellulase expression 1) from *T. reesei* [77]. Deletion of *ace1* resulted in increased expression of the genes encoding the main cellulases and some xylanases in *T. reesei* [77]. Todd *et al.* [76] identified *ace1* orthologues in two thirds of the ascomycete genomes but did not identify orthologues in any of the 31 basidiomycete genomes they analysed.

Transcriptional Activators Overview

Filamentous fungi have a large range of transcriptional activators that function in lignocellulose degradation. Some of these are functionally conserved across large phylogenetic groups in the fungal kingdom, while others are more lineage specific. Many of the activators have only been identified in ascomycetes and not basidiomycetes [76]. Seven activators relevant to lignocellulose degradation in ascomycetes, XlnR, AmyR, InuR, AraR, GalR, GalX and RhaR, had no orthologues in any of the 31 basidiomycetes analysed by Todd *et al.* [76]. Many of the well-known activators involved in plant biomass degradation are members of the Zn_2Cys_6 family of transcription factors and this family is particularly expanded in ascomycetes compared to basidiomycetes [76]. Todd *et al.* [76] concluded that the expansion of various activator families is likely to have occurred after the divergence of ascomycetes and basidiomycetes. A number of important regulators are discussed in more detail below.

XlnR/XYR1 Activators in Aspergillus spp. and T. Reesei

One of the major transcription factors, considered a master regulator, involved in regulation of CAZyme-encoding genes in response to lignocellulose is a binuclear zinc finger protein named XlnR (xylanase regulator) primarily in *Aspergillus* spp. and XYR1 (xylanase regulator 1) primarily in *T. reesei* and related species. XlnR/XYR1 is the key activator in *Aspergillus* spp. and *T. reesei* of genes encoding cellulase and hemicellulase enzymes [18, 78-80]. One of the early studies showed that XlnR as well as regulating genes encoding xylanolytic enzymes (when xylose was the inducer) regulated the two genes encoding endoglucanases (*eglA* and *eglB*) that were analysed [78]. There are significant differences in how XlnR in *A. niger* and XYR1 in *T. reesei* regulate [18]. In *T. reesei*, XYR1 interacts with the co-regulators ACE1 and ACE2 (activator of cellulase expression 2) [81] whereas *A. niger* lacks an *ace2* orthologue [18, 76]. 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* [82]. The relationship between inducer, transcription factor and the regulated protein encoding genes is complex if not enigmatic. Section I described how some of the inducers of CAZymes are different in *T. reesei* compared to *A. niger*, namely the effects of lactose and sophorose, but these different inducers can still signal through the XYR1 transcription factor in *T. reesei*. Measurements of the shuttling of XYR1 in *T. reesei* provide insights into the functioning of this activator [38]. The XYR1 protein was synthesised in the cytoplasm as part of the induction process and when induction ceased the XYR1 protein in the nucleus was rapidly degraded [38]. Finally, a recent analysis in five ascomycetes emphasised some of the functional diversity of XlnR/XYR1; there were substantial differences in the CAZyme-encoding genes regulated by the XlnR/XYR1 orthologues in response to induction by xylan [83].

CLR-1 and CLR-2 activators in N. 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 Δ*xlr-1* mutant, growth on cellulose and cellulolytic activity is only slightly affected [56]. Instead in *N. crassa*, two other Zn_2Cys_6 family transcription factors CLR-1 and CLR-2 were found to be the predominant regulators of the expression of cellulase-encoding genes [45]. Orthologues of *clr-1* and *clr-2* have been identified in many fungal species [45] but some of the orthologues have been demonstrated to be functionally different. *A. nidulans* ClrB has more limited functions than CLR-2 in *N. crassa* [84]. Expression of *clr-2* driven by a non-endogenous promoter) in *N. crassa*

led to inducer independent increases in CAZyme-encoding gene expression, whereas similar expression of *clrB* in *A. nidulans* did not have such an effect [84]. Also, *A. nidulans clrB* cannot complement a Δ*clr-2 N. crassa* strain [84]. In *A. oryzae,* the orthologue of CLR-2 called ManR was characterised as a regulator of genes encoding mannan degrading enzymes [85]. Coradetti *et al.* [84] suggested that the differences in cellulase gene regulation in filamentous fungi may reflect an ancient divergence in the regulatory mechanisms between the Sordariomycetes (includes *N. crassa* and *T. reesei*) and the Eurotiomycetes (includes *Aspergillus* spp.). The characterisation of the functions of the orthologues of *clr-1* and *clr-2* in *T. reesei* could substantiate whether the differences are related to an ancient evolutionary divergence or otherwise.

Other Relevant Transcription Factors

AraR (arabinolytic regulator), a Zn_2Cys_6 transcription factor, is a relatively recent duplication of XlnR as it is only found in certain fungal lineages [86]. AraR is considered to work in co-operation with XlnR in the regulation of the pentose catabolic pathway [87]. The roles of XlnR and AraR were analysed in *araR* and *xlnR* deletion strains of *A. niger* [88]. Whilst the expression of a range of CAZyme-encoding genes was partly dependent on AraR, there were few clear examples of CAZyme-encoding genes where the expression was solely dependent on AraR [88]. BglR (beta-glucosidase regulator) positively regulates the expression of genes encoding β-glucosidases in *T. reesei* [89]. This transcriptional activation could eventually lead to the repression of CAZyme-encoding genes: the β-glucosidases will hydrolyse cellobiose to glucose and, unless the glucose is metabolised by the fungus, repression of CAZyme-encoding genes *via* CRE1 will occur. ClbR (cellobiose response regulator) is another Zn_2Cys_6 transcription factor, which was characterised in *A. aculeatus* [90]. ClbR regulated CAZyme-encoding genes in response to cellulose and cellobiose (but not xylose) in an XlnR-dependent and independent manner [90]. Orthologues of *clbR* were only found in other species of the *Eurotiales* order [90]. Recently, ACE3 (activator of cellulase expression 3), which contains the Pfam Fungal specific transcription factor domain, was identified in *T. reesei* [91]. The ACE3 transcription factor was considered to be a master regulator as the data from deletion and over-expression of *ace3* indicates that the expression of *xyr1* was altered [91]. RhaR (L-rhamnose-responsive regulator) is another recently identified activator of pectinase-encoding genes but further pectin-related regulators remain to be identified [92]. Many gaps remain in understanding of transcriptional repression and activation, such as unidentified repressors and activators, cross-talk between different regulators and unravelling the functional differences in orthologous transcription factors in fungi.

Environmental Regulation of CAZyme-Encoding Genes

Light Regulation of CAZyme-Encoding Gene Expression

In addition to starvation and small molecule inducers, expression of CAZyme-encoding genes can be influenced by environmental factors such as light and pH. Light affects the transcription of CAZyme-encoding genes and secretion of CAZymes in both *N. crassa* [93] and *T. reesei* [94]. Homologous proteins in both species mediate this light responsiveness, while the regulatory mechanism differs slightly between these fungi. In *N. crassa*, the blue light photoreceptors white collar 1 (WC-1) and WC-2 are transcription factors which 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 [95]. The VVD (VIVID) photoreceptor interacts with the WCC and modulates the response to light [96]. In *N. crassa,* the WCC binds both the *clr-1* promoter and the *cre-1* promoter, regulating their expression through light [93, 97]. In *T. reesei*, the homologues of WC-1, WC-2 and VIVID, which are BLR1, BLR2 and ENV (ENVOY) affect CAZyme-encoding gene expression. In darkness, deletion of some of these genes increased the cellulase activity in the culture filtrate [98]. Gyalai-Korpos *et al.* [98] considered that the effect on cellulase activity of *blr1* and *blr2* deletion was due to differences in protein secretion and that of *env1* deletion due to adjustments in response to the environment. Tisch and Schmoll [27] 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 [27]. Also, BLR1 and BLR2 in *T. reesei* are not considered to act as a complex under darkness [98]. 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 [99].

pH Regulation of CAZyme-Encoding gene Expression

There are many examples demonstrating a connection between ambient pH and differences in CAZyme production with one of the first demonstrated by Bailey *et al.* [100] in *T. reesei*. The authors showed that a higher pH was favourable to xylanase production whereas a lower pH was favourable to cellulase production.

In a recent study, Li *et al.* [101] showed that the optimum pH levels for production of endoglucanases, exoglucanases and β-glucanases by *T. reesei* were different. Adav *et al.* [102] also showed with *T. reesei* that the secreted CAZy profiles changed with different pH levels. The differences in CAZyme 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 CAZyme-encoding genes.

A. nidulans is one of the main model systems where pH regulation has been studied [103]. The ambient pH is considered to signal through a plasma membrane complex [104, 105] 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 *palI* [106-108]. 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 [109] 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 [110]. 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 [106, 107]. So in effect in *A. nidulans* during alkaline conditions, PacC activates alkaline-expressed genes and represses acidic-expressed genes [111].

The active PacC binds the core target sequence 5'-GCCARG-3' in the promoters of its target genes [112], however to our 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 found at alkaline pH [111, 113]. A study using the thermophilic ascomycete *Humicola grisea* investigated the pH-dependent transcriptional regulation of CAZyme-encoding genes with sugarcane bagasse as the substrate [113]. Here the transcript level of *cbh1.1*, *cbh2.2*, *egl1*, *egl2*, *bgl4* and *xyn1* increased at alkaline pH (pH8) and Mello-de-Sousa *et al.* [113] identified one or multiple PacC binding sites in the promoters of these genes. Other CAZyme-encoding genes regulated by PacC include the *A. nidulans* xylanase genes (*xlnA* and *xlnB*) [114] and alpha-L-arabinofuranosidase gene (*abfB*) [115]. In a recent global transcriptomic study in *T. reesei*, Hakkinen [26] investigated the influence of extracellular pH and of

PAC1 (the *T. reesei* orthologue of *pacC*) during growth on Avicel. Among the large number of pH-responsive genes were 60 CAZyme-encoding genes (from glycosyl hydrolase (GH), CE or polysaccharide lyase (PL) classes). The authors showed that of the genes encoding proteins with the same enzymatic activity, some were up-regulated at low pH and others at high pH levels**.** It is possible that the genes up-regulated at the low pH encode for enzymes that have higher activity at the low pH. However, Hakkinen [26] 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 CAZyme-encoding genes.

Exploration of a Relevant Regulatory Mechanism – Nucleosome Positioning

Regulation of the responses of filamentous fungi to lignocellulose is not only mediated simply through binding of transcription factors in promoter regions of target genes; the organisation of DNA also plays an role. There are changes in nucleosome positioning in the promoter and coding regions of the *cbh1* gene in *T. reesei* during CCR repressing compared to cellulase-encoding gene inducing conditions [116]. Nucleosomes, which are made of histones and associated DNA, are the basic organisational unit of chromatin, which is packaged or compacted DNA. The positioning and presence of nucleosomes is not random; it has a role in regulating gene expression [117, 118]. The gene *cbh1* encodes one of the major cellobiohydrolases in *T. reesei* [19]. Ries *et al.* [116] investigated the positions of nucleosomes in the promoter and coding sequences of *cbh1* primarily using MNase enzyme digestion (micrococcal nuclease digestion) of DNA. The MNase digestion technique cleaves linkages between nucleosomes allowing the position of the nucleosomes to be mapped. Under repressing conditions in *T. reesei* QM6a, the nucleosomes are at particular positions in the promoter region and in the coding region of *cbh1* (Fig. **2**). In the presence of the inducer sophorose, the nucleosomes are no longer present in the coding region of *cbh1* and are also re-positioned in the promoter region of *cbh1* [116]. The re-positioning of the nucleosomes correlates to an extent with induction of expression in terms of the activator binding sites that were exposed and the repressor binding sites that were blocked (Fig. **2**). One of the nucleosomes is re-positioned at sites that the repressor ACE1 binds and the other nucleosome is re-positioned at sites that the repressor CRE1 binds (Fig. **2**).

Ries *et al.* [116] suggested a regulatory basis for the positioning by showing that the repressor CRE1 regulated the positioning within the coding sequence of *cbh1*.

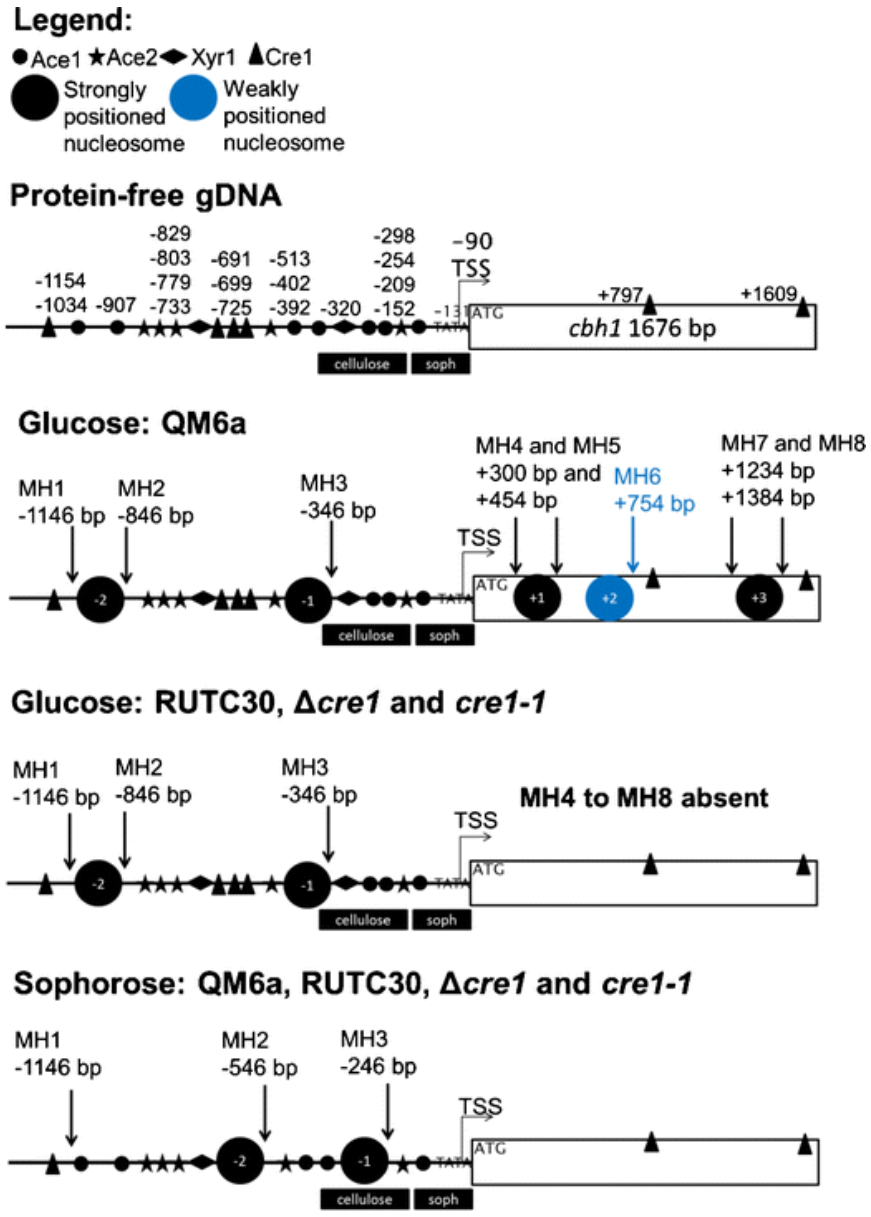


Figure 2: The following is the caption text from Ries *et al.* [116]: Predicted nucleosome positioning (black and blue spheres) according to the strong MNase cutting sites (black arrows) and weak MNase cutting sites (blue arrow) in the cbh1 promoter in the presence of glucose and sophorose in the wild-type strain and the cre1 mutant strains. As a reference, the promoter region of cbh1 is also presented when protein-free and with all known transcription factor binding motifs. The locations of DNA sequences thought to mediate induction by sophorose (soph.) and cellulose are indicated with two black boxes. The TSS (transcription start site) and TATA box are also shown. Reproduced with permission. © Springer-Verlag Berlin Heidelberg 2014.

Deletion or truncation of *cre1* resulted in a loss of the positioned nucleosomes in the coding sequence under repressive conditions (Fig. **2**). Thus, under repressive conditions a type of double lock regulatory mechanism regulates gene expression. One lock is the classic role for CRE1 in binding to the promoter and coding sequences preventing binding of activators and RNA polymerases. The second lock is where CRE1 has a role in positioning the nucleosomes in the *cbh1* coding sequence to prevent transcription. Ries *et al.* [116] suggest this could occur either *via* the CRE1 protein binding to the coding sequence and recruiting chromatin remodelling complex proteins or through CRE1 regulation of other genes that affects the nucleosome positioning such as chromatin remodelling factor genes. Two other studies are worth noting in the context of this regulatory mechanism. The role of chromatin (which nucleosomes are a component of) is described in a study that screened for novel regulators of cellulase production [91]. Another gene relevant to this regulatory mechanism, *lae1,* encodes a methyltransferase involved in chromatin modifications and is a regulator of genes encoding CAZymes [119]. Future studies are likely to confirm an important role for chromatin in the response of fungi to lignocellulose.

SECTION III – TRANSCRIPTIONAL REGULATION OF GENES ENCODING TRANSPORTERS, ACCESSORY PROTEINS AND THOSE INVOLVED IN STRESS RESPONSE

Apart from the CAZyme-encoding genes, other major groups of genes that are regulated in response to lignocellulose include those encoding transporters and non-hydrolytic accessory proteins. Transporters can have an effect on lignocellulose degradation by influencing inducer uptake whereas the accessory proteins can improve the efficiency of the lignocellulose saccharification. Also, expression of genes involved in stress responses such as nutrient limitation stresses and endoplasmic reticulum (ER) stress is affected in response to lignocellulose. In addition, this section will explore the emerging regulatory mechanism of antisense RNA in more detail.

Regulation of Transporter Genes

Introduction to Transporters

Transporters are essential to the utilisation of lignocellulose by fungi. They transport parts of the lignocellulose broken down in the extracellular environment into the fungus. Most of the relevant transporters are sugar transporters belonging to the major facilitator superfamily (MFS) which includes hexose and pentose sugar and short oligosaccharide transporters [120]. Other relevant transporters

include those involved in iron homeostasis which were induced on lignocellulose in *T. reesei* [121]. The transporter classification database (www.tcdb.org/) provides an extensive classification system for transporters [122]. Regulation of transporters occurs at the transcriptional level but is also likely to be influenced substantially at the post-transcriptional level such as through the activation of transporters already deposited in the membrane. Filamentous fungi, unlike unicellular fungi such as *S. cerevisiae,* possess xylose transporters as well as transporters of other hemicellulosic sugars. The identification of these genes is not only important in understanding the fungal responses to lignocellulose, but also provides a resource for engineering species such as *S. cerevisiae* for improved performance in ethanolic fermentations [123].

Transporter Regulation Insights from Global Transcriptional Analyses

During growth of *N. crassa* on Avicel, transporters were transcriptionally induced and the expression of the majority of these transporters was fully or partly dependent on two of the key transcription factors that are responsible for regulating cellulase gene expression, *clr-1* and *clr-2* [45]. Coradetti *et al.* [45] described an 'Avicel' regulon as genes with higher expression in *N. crassa* cultured with Avicel compared to either 'no carbon' or sucrose conditions. This 'Avicel regulon' contained 13 genes encoding transporters, including the characterised transporters *cellodextrin transporter-1* (*cdt-1)* and *cdt-2* [45, 124]. The regulation of these 13 genes was investigated with the Δ*clr-1* and Δ*clr-2* transcription factor mutants and 10 out of 13 of the genes were either dependent on one or both of *clr-1* and *clr-2* or had their expression modulated by these transcription factors [45]. In a further study in *N. crassa*, Benz *et al.* [49] described the 'pectin regulon' and compared this to the 'Avicel regulon' [45] and the 'xylan regulon'. Interestingly, there were no genes encoding transporters in the 29 genes that overlapped between the three regulons, but there were transporter encoding genes in each of the three two-way comparisons of the Avicel, xylan and pectin regulons [49]. These comparisons suggest that *N. crassa* lacks a gene encoding a sugar transporter that is induced by diverse polysaccharide substrates. However, transporter-encoding genes that were either constitutively expressed or were only transiently induced could have played a role. These transiently induced genes may not have been detected at the time points sampled in these *N. crassa* studies. In *A. niger* Δ*xlnR* and Δ*araR* deletion strains cultured with xylose and arabinose, the expression of six genes encoding transporters was modulated suggesting that their expression was dependent on either AraR or XlnR regulators [88].

Transporter Regulation Insights from Characterised Transporters

Transporters are difficult to characterise functionally because of their redundancy. As a result, it is challenging to draw firm conclusions from global transcriptional analyses as this includes uncharacterised transporters with ambiguous or possibly erroneous annotations. Focusing on the regulation of reliably characterised transporters can give further insights. Two recent studies describe the regulation of characterised transporters of xylan breakdown products in *N. crassa* and *A. nidulans*. These studies demonstrate that the regulators of the genes encoding the transporters can be the same as the regulators of the genes encoding the CAZymes that break down the polysaccharides.

In *N. crassa*, the transporters CDT-1 and CDT-2 were induced on Avicel, and when expressed heterologously in yeast, were shown to function in the uptake of cellodextrins [124, 125]. More recently, the work of Cai *et al.* [126] showed that CDT-1 is primarily involved in the transport of cellulose components, due to defects in growth of *Δcdt-1* mutants on cellulose but not on xylan. The same study implicated CDT-2 in the transport of hemicellulose components due to defects in growth of *Δcdt-2* mutants on xylan. The *N. crassa* XLR-1 transcription factor – a regulator of hemicellulase encoding genes – was the primary regulator of *cdt-2*, which encodes the transporter of breakdown components of hemicellulose. The CLR-1 transcription factor – a regulator of cellulase genes – was the primary regulator of *cdt-1*, which encodes the transporter of breakdown products of cellulose (although CLR-1 also has a role in regulating *cdt-2*) [126]. In *A. nidulans*, a gene encoding a high affinity xylose transporter, *xtrD,* was induced by xylose in a XlnR-dependent manner [127]. When fungi are exposed to lignocellulose, high affinity transporters are induced because the concentrations of free sugars are generally low [120]. The regulation of genes encoding sugar transporters characterised as either of high or low sugar affinity is partly dependent on CreA/CRE1. In *A. niger*, the gene encoding the high affinity sugar transporter MstA is regulated by CreA-mediated CCR ensuring that it is expressed under conditions when concentrations of monomers are low [128]. The *xtrD* gene from *A. nidulans* described above is also regulated in a CreA-dependent manner [127].

Transcription Factor Binding Motifs of Transporter Genes

A survey of transcription factor binding sites of genes encoding transporters can give insight into their regulation. CreA/CRE1 binding sites are present in the promoters of genes encoding high affinity sugar transporters such as *mstA* from *A. niger* [128]. XlnR binding sites are present in the promoters of genes encoding

sugar transporters in aspergilli. Andersen *et al.* [129] compared the transcriptional responses of three *Aspergillus* spp. (*A. niger*, *A. oryzae* and *A. nidulans*) cultured with either xylose or glucose. Of the 23 genes that were differentially expressed as well as being orthologous across the three *Aspergillus* spp., six of the genes encoded sugar transporters with five of the genes up-regulated on xylose [129]. The XlnR binding motif, defined by the authors as 5′-GGNTAAA-3′, was present in all five of the promoters of the up-regulated transporter genes from *A. niger* and *A. oryzae* and in two of the promoters from *A. nidulans* [129]. To our knowledge, a survey of transcription factor binding sites of genes encoding transporters relevant to lignocellulose is not available for filamentous fungi but such a study would be of use, ahead of functional characterisation.

Regulation Insights from Clustering Analyses of Global Transcriptional Datasets

Co-expression clustering analyses of global transcriptomic datasets is a powerful tool to gain new insights into gene function and regulation [130]. Novel transporters have been identified based on co-expression with other genes of known function, such as *lat-1* in *N. crassa* which encodes an arabinose transporter [49]. In the analyses of transcriptomic datasets of *T. reesei* exposed to various lignocelluloses, the expression profile of genes encoding transporters were found in transcriptional clusters with genes encoding CAZymes and transcription factors [91]. Interestingly, some of the transporter-encoding genes were not only co-regulated with genes encoding transcription factors and CAZymes but also located in the same chromosomal regions [91]. There are limitations of this co-expression clustering analysis insofar as transporters are concerned; genes encoding constitutively expressed or transiently induced transporters will not cluster with induced CAZyme-encoding genes. The transient nature of the induction of some transporters highlights the need for more extensive time-point transciptomic profiling studies.

Transporters Functioning as Receptors – 'Transceptors'

A 'transceptor' is a transporter that also functions as a receptor for signal transduction [131]. This 'transceptor' concept is significant as it shifts the model of regulation of transporters to include the regulation of the sensing as well as the transport of small molecules. In *N. crassa*, a recent study showed how the cellodextrin transporters CDT-1 and CDT-2 could function as 'transceptors' [132]. When CDT-1 and CDT-2 were mutated to remove their ability to transport cellobiose, the cellulase gene induction was not correspondingly reduced indicating a secondary sensing and signalling role for the transporters [132].

Znameroski *et al.* [132] described other studies in the literature regarding 'transceptors' relevant to the response of *T. reesei* to lignocellulose. Whilst there is indirect evidence supporting 'transceptors' in the examples from *N. crassa* and *T. reesei*, direct evidence as far as we are aware for the molecular basis of the signalling role of these 'transceptors' related to lignocellulose is lacking. This injects a note of caution into whether the cell's signalling apparatus is actually regulated when 'transceptors' are regulated.

Accessory Protein Encoding Genes

Swollenins

Swollenins are fungal proteins that have similarity to plant expansins and have an ability to disrupt the crystalline structure of cellulose to make amorphous cellulose [133]. The first swollenin protein was characterised in *T. reesei* [133]. SWO1 has a modular structure that includes a carbohydrate binding module (CBM) and was shown to disrupt the crystalline structure of cellulose fibers [133]. Swollenins were also shown to aid in the amorphogenesis step during the enzymatic hydrolysis of pre-treated biomass [134]. The swollenin gene *swo1* was highly induced in *T. reesei* when cultured with wheat straw [135]. Saloheimo *et al.* [133] showed *swo1* in *T. reesei* was regulated in a similar manner to cellulases with *swo1* induced by cellulose, lactose, sophorose and cellobiose.

Hydrophobic Surface Interacting Proteins (HSIPs)

We define hydrophobic surface interacting proteins (HSIPs) as proteins that have the ability to interact with a hydrophobic surface. HSIPs include proteins such as hydrophobins and other proteins with similar functional properties. Genes that encoded HSIPs, were induced in *A. niger* in response to wheat straw [31], willow [68] and sugar cane bagasse [69] and in *T. reesei* in response to wheat straw [135]. The induction of HSIPs suggests a possible role for HSIPs in improving the efficiency of saccharification of lignocellulose. Hydrophobins are a diverse family of small, amphipathic, secreted proteins that are unique to filamentous fungi [136]. The hydrophobins have proven functions in fungal development, surface interaction, pathogenicity and evasion of host responses [136, 137]. The functionality that may be relevant to the process of saccharification of lignocellulose (a polymer with hydrophobic properties) is the ability of a HSIP to improve the degradation of another hydrophobic polymer, polybutylene succinate-coadipate (PBSA) [138, 139]. Maeda *et al.* [139] identified the polyesterase CutL1, that degrades PBSA, in supernatant from *A. oryzae* cultured with PBSA. Subsequently, to search for other factors involved in the degradation of PBSA,

Takahashi *et al.* [138] analysed the transcriptomic response of *A. oryzae* when cultured with PBSA and found that one of the induced genes encoded a hydrophobin RolA. By examining the localisation of these proteins in culture with *A. oryzae* grown on PBSA, it was determined that the secreted RolA was capable of adsorbing to the surface of both hyphal cell walls and the PBSA film and was likely to result in the formation of amphipathic hydrophobin monolayers [138]. *In vitro*, RolA was found to adsorb to the surface of PBSA and specifically recruit soluble CutLI, increasing the amount of CutLI-mediated PBSA hydrolysis compared to soluble CutLI only [138]. This suggested to Takahashi *et al.* [138] that RolA exhibits two distinct functions relating to the degradation of PBSA: (1) improving physical substrate breakdown by improving hyphal-substrate interactions by increase hyphal hydrophobicity and (2) improving enzymatic substrate degradation by recruiting degradative enzymes to the substrate's surface. With regard to HSIPs in *A. niger*, the genome of *A. niger* contains eight genes encoding hydrophobins [140] among which *hyp1* (the orthologue of *A. oryzae rolA*) and *hfbD* are induced on wheat straw, together with a gene encoding a hydrophobic surface-binding protein *hsbA* [31]. Although direct evidence is not available to support this, an hypothesis with regard to saccharification in the *A. niger* cultures with lignocellulose is that HSIPs could perform a similar function with lignocellulose and improve the efficiency of its saccharification as has been shown previously for *A. oryzae* RolA with PBSA.

Stress Responses as Part of the Response to Lignocellulose

Nutrient Limitation Stresses

The lack of nutrients such as carbon or iron 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 is sequential [42, 66]. This may lead to the easily degraded carbohydrates being hydrolysed and 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 [47, 48, 141]. 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 [42]. Also in *T. reesei* grown on wheat straw, autophagy was induced and cell wall remodelling enzymes were up-regulated [121], indicating nutrient limitation. With regard to iron limitation, growth in the

presence of lignocellulose can lead to stress due to lack of available iron. Wheat straw binds iron thereby decreasing its availability to the fungus. Furthermore, iron consumption by *T. reesei* is ~ 3-fold higher on wheat straw compared to growth on lactose [121]. In response to the iron limitation, growth of *T. reesei* on wheat straw was accompanied by increased expression of genes encoding proteins involved in iron transport, siderophore transporters and siderophore biosynthesis as well as ferric reductases [121].

Endoplasmic Reticulum (ER) Stress

Fungi can be subject to ER stress when exposed to lignocellulose. The secretion of proteins from eukaryotic cells requires that the proteins enter the ER, where a process of assisted folding, formation of disulfide bonds and glycosylation occurs before the proteins are translocated to the cell exterior by vesicular trafficking. Fungi that secrete proteins are subject to ER stress when the load on the system is high and particularly when non-native proteins are expressed. The associated homeostatic responses to ER stress are collectively called the unfolded protein response (UPR) and the UPR has been well-described in filamentous fungi and particularly so in *A. niger* [142, 143] and *T. reesei* [144]. The UPR is 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* [31] or *T. reesei* [135] were transferred from glucose-grown conditions to wheat straw, where the secretion of CAZy enzymes is induced, there was no clear impact on the transcript levels of selected UPR marker genes such as *hacA/1, bipA/1 or pdiA/1,* although the UPR involves transcript level changes to several hundreds of genes, *e.g.* in *A. niger* [145]. The apparent lack of induction of the UPR was presumably because the CAZymes 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 comprehensive study with *N. crassa* showed induction of the UPR (including enhanced transcript level from the *hac-1* gene and enhanced splicing of the *hac-1* mRNA) when exposed to Avicel [49]. 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* [42]. The carbon starvation response involves the induction of ER-autophagy genes and CAZyme-encoding genes (many of which are predicted to be involved in cell autolysis) [47, 146]. Wheat straw also up-regulated the transcript levels of ER-autophagy genes in *T. reesei* [121]. While there is a link between the ER stress and ER-autophagy [147, 148],

an induction of UPR marker genes was not reported during ER-autophagy. High protein production (which can occur when fungi are exposed to lignocellulose) is not clearly linked with the UPR according to a study of the rates of growth and specific protein production in *T. reesei* [149]. 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* [150, 151]. To summarise, it is 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.

Exploration of a Relevant Regulatory Mechanism – 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 [152]. One type of regulatory RNA is natural antisense transcripts (NATs), where RNA complementary in sequence to the sense RNA is transcribed [153]. Amongst other possible mechanisms, the antisense RNA is considered to have a regulatory role by binding to the sense RNA to promote degradation of the sense RNA. 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 [31, 135]. 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 [31]. In the conditions of the study of Delmas *et al.* [31], ~ 2% of the total RNAseq reads were antisense transcripts. The amount of the antisense RNA has been measured but whether there is a functional role in terms of regulation remains to be proven in these examples, *i.e.* if the antisense transcripts are no longer transcribed, does this change the abundance of the sense transcripts, protein levels and the phenotype of the fungus? 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 [154]. Xue *et al.* [154] showed that transcriptional interference by antisense RNA is required for circadian clock function in *N. crassa* [154]. 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 as described in Section II. The transcription factors WC-1 and WC-2 also regulate the gene *frequency* (*frq*) which functions in maintaining rhythmicity and also has antisense expression [154]. Xue *et al.* [154] demonstrated that the antisense transcript of *frq,* named

qrf, supresses the expression of *frq*. The antisense gene *qrf* has a promoter that can bind the same transcription factors that the sense gene *frq* promoter can bind. When the promoter of the antisense gene *qrf* was mutated, there were more transcripts of the sense gene and more protein present [154]. Xue *et al.* [154] provided evidence supporting a mechanism by which the antisense transcripts regulate sense expression through premature termination of transcription of the *frq* transcript. Their measurements of the polymerase positions indicated that the RNA polymerase on the sense transcript stalls because of the transcription of the antisense *qrf* [154].

SECTION IV – MIXED CULTURES; COMBINING GENES AND THEIR REGULATORY SYSTEMS

To understand fully both transcriptional regulation of fungi and their responses to lignocellulose, the natural environment where fungi exist as multi-species communities should be considered. The benefits of combining different parts of fungal responses can be seen most simply in examples where an individual gene from one fungus is combined with another fungus such as the expression of a laccase from a basidiomycete in *T. reesei* [155]. Notwithstanding the successes, one of the limitations of combining individual genes is that some or the entire regulatory context of the combined gene is lost (although in some cases this is beneficial such as where the native promoter is not optimal). A more complex combination is that of mixed cultures where entire fungal species are combined. Understanding transcriptional regulation and responses of fungi in mixed cultures may provide insights that are relevant to fungal biotechnology for biofuels.

Introduction to Mixed Cultures and Associated Terminology

Fungal mixed cultures are mixtures of two or more individual fungal species or strains. Part of the rationale for using mixed cultures to degrade lignocellulose is that different fungal species can be found in the same lignocellulose-containing ecological niche such as a hollow tree stump [156] or leaves [157]. In nature, competition rather than co-operation may well dominate amongst fungi. For example, Boddy [158] states that competition is the most common type of interaction occurring between wood decaying higher fungi. There are some reasons why competition might not necessarily prevail in the degradation of lignocellulose in nature or in mixed cultures in the laboratory. Firstly, in nature there could be a selective advantage for co-existing and co-operating fungi when their enzymatic activities are complementary and can degrade different parts of the lignocellulose. Secondly, in the laboratory the competition that occurs in

nature could be moderated to a co-existence by optimisation of culture conditions. For example, Kolasa *et al.* [159] used plate-based assays to determine compatibility between different fungal species and showed that compatibility partially depended on the carbon source. The literature on fungal mixed cultures with lignocellulose and where secreted enzymes from single cultures are combined can be summarised in the context of synergistic, additive and subtractive effects on saccharification. Synergistic effects can be broadly defined as where the whole is greater than the sum of the individual parts [160]. In a mixed culture, a synergistic effect is where the fungi co-operate and the effect on saccharification is greater than the sum of the effects in the individual monocultures. An effect is additive in a mixed culture when it is equivalent to the sum of the effects in the individual monocultures, which could involve a co-existence of the two fungal species in the mixed culture. A subtractive effect in the mixed culture results in less than the sum of the effects in the individual monocultures, which involves some form of antagonism or competition between the fungal species. The effects are dynamic; what appears to be an additive effect in the mixed culture may for example be a combination of synergistic and subtractive effects with the subtractive effects masking the synergism.

Mixed Cultures at the Enzymatic Level

Assessing data from the literature on whether there is a synergistic effect of mixed cultures on saccharification is complicated for several reasons; there is often a lack 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 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. So perhaps the more ambiguous term of ‘potential beneficial effect’ for saccharification can be used when summarising some of the literature related to mixed cultures relevant to lignocellulose degradation.

There is substantial literature on the effects at the enzymatic level of mixed cultures with *T. reesei*, which is one of the most studied fungal species in mixed cultures [159, 161-164]. Two of these studies combined ascomycetes and basidiomycetes [161, 163] with one of the studies performing saccharification assays using enzyme cocktails from the mono and mixed cultures [163]. In the study of Ma and Ruan [163], 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*. Here the de-lignifying enzymes

of the basidiomycete resulted in the saccharification of a greater proportion of the polysaccharides by *T. reesei* CAZymes. Several studies show beneficial effects of mixed cultures for enzymatic activities but do not culture the fungi with lignocellulose or do not perform saccharification assays with lignocellulose. One of the earlier studies showed there were beneficial effects when *T. reesei* was cultured with *Aspergillus* spp. where *T. reesei*, which is deficient in secreting β-glucosidases (or transcribing genes that encode β-glucosidases), was complemented by the secreted activities of *A. niger* [164]. In the study of Hu *et al.* [161] beneficial effects on enzymatic activities relevant to lignocellulose degradation were observed in some of the mixed culture combinations compared to the monocultures. Ahamed and Vermette [162] reported a beneficial effect with higher volumetric filter paper activity in a mixed culture of *A. nige*r and *T. reesei,* but not a higher filter paper activity per amount of fungal biomass compared to an *A. niger* monoculture.

Although not involving mixed cultures *per se*, saccharification assays where supernatants from single cultures are combined are useful to demonstrate potential benefits of combining fungi. Antagonistic effects in the mixed cultures may mask some of the synergistic effects in the following examples. In one study, saccharification assays using a combination of equal volumes of supernatants from *A. niger* and *T. reesei* lignocellulosic monocultures released synergistically more sugars than the sum of the amounts released from saccharification assays using the enzymes from monocultures in separate assays [165]. In another similar study using volumetric blends of supernatants of *T. reesei* and *Aspergillus awamori* monocultures, synergistic improvement of the saccharification of sugar cane bagasse was measured [166]. In both of these studies, the reduction in cellobiose inhibition of cellulases in the *T. reesei* cocktail by the β-glucosidase activity from the *Aspergillus* spp. cocktails probably played a role in the synergy. Fortes Gottschalk *et al.* [166] also noted the useful role of ferulic acid esterases secreted by aspergilli.

Mixed Cultures at the Transcriptional Level

Transcriptomic studies could elucidate how gene expression changes in a mixed culture compared to single cultures. However, there is no literature on the transcriptional responses of mixed cultures relevant to lignocellulose degradation where the fungi co-operate. Arfi *et al.* [167] studied, using a standard complex laboratory medium, mixed cultures with basidiomycetes that competed. Here RNAseq analysis was performed on the out-competing fungus from the mixed culture, *Pycnoporus coccineus*, showing that genes involved in detoxification of

secondary metabolites had higher transcript abundance compared to the single cultures of *P. coccineus* [167].

Models for Transcriptional Regulation and Responses in Mixed Cultures

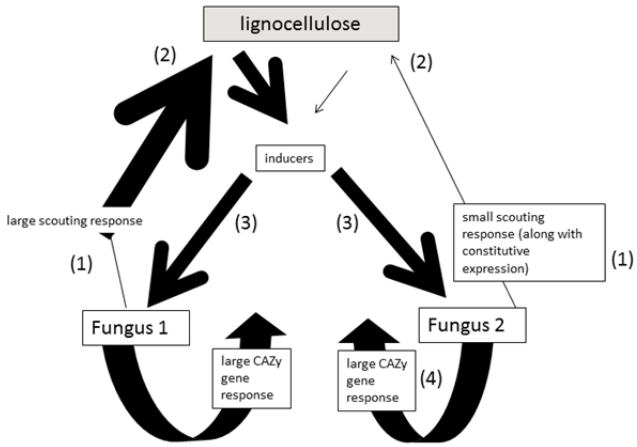
How transcriptional regulation and responses of fungi to lignocellulose can have beneficial effects on saccharification in mixed cultures, can be explained by models which draw upon the information in the previous sections of this chapter from single culture studies. In the absence of transcriptional data from relevant mixed cultures, these models are a best guess as to what could be the transcriptional bases for the observed beneficial effects. Fig. **3** illustrates two of these models.

Firstly, in the absence of simple carbon sources, fungi could have different abilities to scout the environment and these scouting enzymes could release complementary inducers. The enzymes secreted as part of a scouting response from one of the fungi could release an inducer from the lignocellulose that activates genes in the other fungus. These genes may have otherwise not been activated at all or not until later on in the culture (Fig. **3**). *A. niger* up-regulated various CAZyme-encoding genes including those encoding cellulases in response to carbon starvation [42]. Other fungi may lack a 'scouting' response or have one that is composed of different enzyme activities, and/or have a different cohort of CAZyme-encoding genes constitutively expressed at a low level.

Secondly, beneficial effects of mixed cultures may be explained by differences in sensitivity of fungal regulatory mechanisms to sugars. As a result, sugars may have an inductive effect at particular concentrations in some species, but a repressive effect in others (Fig. **3**). For example in *A. niger* and *T. reesei*, concentrations of xylose (a major inducer or cellulases and hemicellulases) higher than 1mM can have a repressive effect on CAZyme-encoding genes [51, 168] but these concentrations of xylose may not be repressive in other fungi.

Thirdly, fungi in mixed cultures encounter toxic substances, such as phenolic compounds from lignocellulose. Fungi with high expression or activity of tyrosinases, the enzymes that de-toxify phenolic compounds [169], could detoxify the mixed culture efficiently thus preventing inhibition of the other fungus. Thus, de-toxifying mechanisms originating from one fungus could have beneficial effects on growth of both fungi, and thus on saccharification in mixed cultures. A gene annotated as encoding a tyrosinase is induced (albeit the expression is relatively low) in *T. reesei* on wheat straw [135] and transcripts for tyrosinases were found in soil samples from a hardwood forest [170].

(A)



(B)

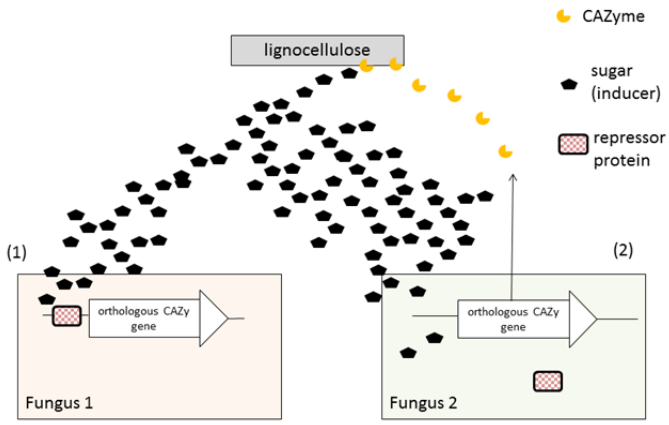


Figure 3: Illustrations of two models for how transcriptional regulation and responses of fungi to lignocellulose could have beneficial effects on saccharification in mixed cultures. (A) beneficial effects on saccharification in a mixed culture due to the scouting response of one fungus inducing CAZyme-encoding genes in the other fungus: (1) the scouting responses, (2) release of inducers from lignocellulose, (3) inducers induce large scale CAZyme-encoding gene expression and (4) induction of the large scale response earlier in the fungus that had the smaller scouting response. Note: The thicknesses of the arrows indicate the magnitude of the action. (B) Beneficial effects, on saccharification, of different levels of sensitivity to repression of CAZyme-encoding genes. (1) The concentration of an inducer reaches a level that becomes repressive in one of the fungi in a mixed culture whereas (2) the concentration is still inductive in the other fungus. Although in 'Fungus 1', the gene here is now repressed, the mixed culture has the benefit of other genes in 'Fungus 1' that may not be present in the genome of 'Fungus 2' or still being induced in 'Fungus 1' by other mechanisms contributing to the saccharification of the lignocellulose.

Technologies to Study Mixed Cultures

At the individual gene level there are methods to quantify or estimate relative amounts of each fungus based on quantifications of RNA using quantitative PCR [167]. At the global level, Dual-RNAseq or simultaneous RNAseq is a technique that allows the quantification of transcripts from multiple organisms simultaneously and the technique is primarily applied to host pathogen interactions [171]. Dual-RNAseq has potential to be applied to mixed species cultures also. A key requirement for the success of Dual-RNAseq is that there are sufficient differences in sequence of the 50-200bp RNAseq reads to facilitate discrimination of the genes from different species in the analysis.

SECTION V – APPLICATIONS IN BIOFUEL TECHNOLOGY

The transcriptional regulation of genes encoding CAZymes in fungi has been exploited before its details were understood. This occurred by classical strain improvement using mutagenesis and screening. More recently, applications have emerged that build on the prior understanding of the regulatory mechanisms described in this chapter. Both these approaches are discussed below in the context of transcriptional regulation. Furthermore, considerations are outlined on whether what is transcriptionally induced in fungi on lignocellulose is a guide to optimise industrial saccharification reactions.

Applications in Biofuel Technology Related to Transcriptional Regulation

Applications without Prior Understanding of the Regulatory Mechanisms

T. reesei is a widely used fungus for saccharolytic enzyme production and a recent review charts the progress in strain improvement in *T. reesei* for cellulase production [24]. Various changes are required to a wild-type fungus to improve its functionality in industrial fermentation conditions for cellulase production. The *T. reesei* wild-type strain QM6a was subjected to random mutagenesis to improve strain performance [24]. One of the strains to emerge from this mutagenesis was RUT-C30, which secreted cellulases at high yields under induction conditions despite the presence of glucose, indicating the strain was carbon catabolite derepressed. Subsequently, the *T. reesei* RUT-C30 strain was shown to have a truncated CRE1, thus preventing CCR [172]. The RUT-C30 strain has other mutated and deleted genes including nine other transcription factors [23]. This number is higher than would be expected by chance given the mutation frequency in the RUT-C30 strain and Le Crom *et al.* [23] speculate that these transcription

factor genes are unlikely to be unrelated to the selection process and cellulase production.

Another biotechnology application uses a quirk of the transcriptional regulation machinery of *T. reesei*. Lactose induces cellulases and other CAZymes in *T. reesei* [53]. The disaccharide lactose, consisting of glucose and galactose connected *via* a β-1,4 linkage, is a cheap by-product from cheese manufacturing or whey processing [53]. Lactose is not part of the plant cell wall [173] and so is a surprising inducer of CAZymes. In recent years, many detailed studies on the composition of plant cell walls have provided information of the linkages present in polysaccharides and these are summarised in the review of Scheller and Ulvskov [173]. Ivanova *et al.* [53] speculate that the basis for the induction *via* lactose could be how the molecule mimics the sensing or metabolism of β-galactosides, which are present in the plant cell walls. These β-galactosides may serve as a signal to the fungus for the presence of lignocellulose in the environment. It is possible that there is a plethora of other inducers of cellulases that are derived from non-cellulose parts of lignocellulose waiting to be discovered.

Applications with Prior Understanding of the Regulatory Mechanisms

Understanding of gene regulation *via* XlnR and PacC led to the deployment of a transcription factor engineering approach in *A. nidulans* that enhanced CAZyme production [174]. Constitutively over-expressing XlnR led to earlier and increased protein production [174]. Under alkaline conditions, PacC (as described in Section III), activates alkaline-expressed genes and supresses acidic-expressed genes [103]. In the deregulated XlnR background, the activation of PacC was impeded by preventing the cleavage of the inactive form of PacC. This resulted in increased activity of a heterologously expressed protein whose expression was driven by the promoter of an acidic-expressed CAZyme-encoding gene [174]. This study in *A. nidulans* highlights the potential benefit of employing a transcription factor engineering approach in an industrially relevant strain, and applies knowledge on the transcriptional regulation of fungi exposed to lignocellulose.

Interpreting Transcriptional Data to Guide Optimisation of Saccharification

One concept related to models for an optimised saccharification reaction is that of the minimal enzyme concept [175]. The minimal enzyme concept is defined by its authors as concerning the identification of the minimal number, minimal levels and the optimal combination of the best performing mono-active enzymes to

saccharify lignocellulose [175]. There are arguments both in support of and opposing using what is transcriptionally induced and when for an optimised saccharification reaction.

The high level of transcriptional induction of the *cbh1* and *cbh2* genes from *T. reesei* provides some support for the use of the transcriptional data to guide the protein requirements for an optimised model for saccharification. In *T. reesei* QM6a, the cellobiohydrolase genes *cbh1* and *cbh2* are amongst the most highly expressed genes on wheat straw [135]. Those genes are so highly expressed in *T. reesei* that the signal in microarray analyses can be saturated [66]. The enzymes CBH1 and CBH2 are found in some of the highest abundances amongst the *T. reesei* secreted proteins [176]. Rosgaard *et al.* [177] surveyed previous studies and described how CBH1 can comprise up to 60% and CBH2 up to 20% by weight of the total proteins secreted by the *T. reesei* RUT-C30 strain. Based on levels of transcription and secretion, these cellobiohydrolases may be required in substantial amounts for an optimised saccharification reaction. Indeed, in an analysis determining the optimal amounts of four *T. reesei* proteins (CBH1, CBH2, EGL1 and EGL2) for the saccharification of pre-treated barley straw substrates, CBH1 was the protein required in the largest amount [175].

Access to lignocellulose limits the efficiency of saccharification [178] and improving this access is an important part of an optimised saccharification reaction. Genes encoding proteins that improve access to the lignocellulose are induced in fungi on lignocellulosic 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 [179]. Swollenins (described in Section III) are induced in *T. reesei* in the presence of wheat straw [135] and have a demonstrated role in improving the efficiency of saccharification [134]. CBMs have carbohydrate binding activity and can increase interaction of an attached enzyme with its substrate [179]. Some CBMs can have disruptive or loosening activity on the polysaccharides [179]. Many studies show that genes encoding enzymes with attached CBMs are induced on lignocellulosic substrates [31, 66, 135]. Whilst beneficial in nature, these CBMs may not be as relevant in an industrial setting for an optimised saccharification reaction [180]. Varnai *et al.* [180] 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. Another recent study strongly implicates

CBMs in non-productive binding to lignin as a reason for irreversible cellulase loss during the saccharification of pre-treated biomass [181].

Transcriptomic studies show that on some substrates genes are induced that encode enzyme activities than are not necessarily required. For example, genes encoding enzymes with endo-acting activities are induced in *A. niger* grown on xylose [88]. In optimised saccharification reactions for the depolymerisation of some polymers, endo-acting activities are redundant. Meyer *et al.* [175] showed that for a vinasse substrate (an industrial waste residue), which contains arabinoxylan with a degree of polymerisation (DP) of less than ~36, endo-1,4-β-xylanase was not required. β-xylosidase and two arabinofuranosidases were sufficient to depolymerise the arabinoxylan. It's likely if the fungi whose regulatory systems related to lignocellulosic substrates have been described in detail in this chapter (*T. reesei*, *A. niger* and *N. crassa*) when cultured with a substrate like this vinasse, would transcribe genes encoding endoxylanases along with the genes encoding the β-xylosidase and arabinofuranosidases. Fungi do not seem to possess mechanisms that can sense the DP of a carbon source. The understanding of how fungal mechanosensing or contact sensing functions is limited [182] but there is no evidence to show these sensing abilities are relevant to the induction of CAZymes that degrade lignocellulose.

There are well documented discrepancies between transcriptional and proteomic responses as reviewed by Zhang *et al.* [183]. These discrepancies reject the use of what is transcriptionally induced to guide inputs for optimised saccharification reactions unless there is supporting proteomic evidence. However, one has to interpret with caution any lack of supporting proteomics evidence for two reasons. Firstly, the conditions in the laboratory that try to replicate what happens in nature often use wild-type strains, which secrete substantial amounts of proteases. These can degrade other secreted proteins to levels undetectable by proteomics. Fungal strains used for enzyme production, such as RUT-C30 (also commonly used in transcriptomic studies) are often protease deficient compared to wild-type strains. Secondly, the protein products from genes that are transcriptionally up-regulated may not be translated sufficiently for proteomic detection due to a limitation of the carbon required to synthesise these proteins. Culture conditions on lignocellulose have similarities with carbon-starved conditions [42, 121].

CONCLUDING REMARKS

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

experimental approaches now begin with genome-wide transcriptomic studies and thus 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. Even within the best-annotated genome sequences there are many uncharacterised genes, annotated as encoding proteins of 'unknown function', that are induced in the presence of lignocellulose. There are also CAZyme-encoding genes that remain un-induced under most conditions; the so-called 'cryptic' CAZyme-encoding genes. More extensive integration of transcriptomic and proteomic studies will also be beneficial in unravelling the complexities of fungal responses to lignocellulose. This integration 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 key. Lignocellulose structures and their accessibility to enzymes are affected by pre-treatments, type of the lignocellulose feedstock and change over time in the saccharification reaction. 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. Finally, one cannot forget that production of biofuels from an industrial perspective is ultimately 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 in the functionality of the cocktail (by better understanding the response of fungi to lignocelluloses) so less enzymes are required for the saccharification.

CONFLICT OF INTEREST

The authors confirm that this chapter contents have no conflict of interest.

ACKNOWLEDGEMENTS

We gratefully acknowledge support from the Biotechnology and Biological Sciences Research Council (BBSRC) (Grant refs. BB/G01616X/1 and

BB/K01434X/1). We also acknowledge fruitful discussions regarding the subject area of this chapter with Matt Kokolski, Steve Pullan and Stephane Delmas.

REFERENCES

[1] Daly P, Maluk M, Zwirek M, Halpin C. Lignin Biosynthesis and Lignin Manipulation. In: Harding SE, Ed. Stability of Complex Carbohydrate Structures: Biofuels, Foods, Vaccines and Shipwrecks: The Royal Society of Chemistry; 2013. p. 153-9.

[2] Gomez LD, Steele-King CG, McQueen-Mason SJ. Sustainable liquid biofuels from biomass: the writing's on the walls. New Phytol. 2008; 178(3): 473-85.

[3] US-DOE. Breaking the biological barriers to cellulosic ethanol: a joint research agenda. http://genomicsgtl.energy.gov/biofuels/2005workshop/b2blowres63006.pdf. 2006.

[4] Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 2014; 42(D1): D490-D5.

[5] Meyer V. Genetic engineering of filamentous fungi — Progress, obstacles and future trends. Biotechnol Adv. 2008; 26(2): 177-85.

[6] Meyer V, Wu B, Ram AFJ. Aspergillus as a multi-purpose cell factory: current status and perspectives. Biotechnol Lett. 2011; 33(3): 469-76.

[7] Klein-Marcuschamer D, Oleskowicz-Popiel P, Simmons BA, Blanch HW. The challenge of enzyme cost in the production of lignocellulosic biofuels. Biotechnol Bioeng. 2012; 109(4): 1083-7.

[8] Gusakov AV. Alternatives to Trichoderma reesei in biofuel production. Trends Biotechnol. 2011; 29(9): 419-25.

[9] Znameroski E, Glass N. Using a model filamentous fungus to unravel mechanisms of lignocellulose deconstruction. Biotechnol Biofuels. 2013; 6(1): 6.

[10] Rytioja J, Hildén K, Yuzon J, Hatakka A, de Vries RP, Mäkelä MR. Plant-polysaccharide-degrading enzymes from basidiomycetes. Microbiol Mol Biol Rev. 2014; 78(4): 614-49.

[11] Amore A, Giacobbe S, Faraco V. Regulation of cellulase and hemicellulase gene expression in fungi. Curr Genomics. 2013; 14(4): 230-49.

[12] Glass NL, Schmoll M, Cate JHD, Coradetti S. Plant cell wall deconstruction by ascomycete fungi. Annu Rev Microbiol. 2013; 67(1): 477-98.

[13] Kubicek CP. Regulation of Formation of Plant Biomass-Degrading Enzymes in Fungi. Fungi and Lignocellulosic Biomass: Wiley-Blackwell; 2012. p. 129-47.

[14] Aro N, Pakula T, Penttilä M. Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiol Rev. 2005; 29(4): 719-39.

[15] Brown NA, Ries LNA, Goldman GH. How nutritional status signalling coordinates metabolism and lignocellulolytic enzyme secretion. Fungal Genet Biol. 2014; 72: 48–63.

[16] Culleton H, McKie V, de Vries RP. Physiological and molecular aspects of degradation of plant polysaccharides by fungi: What have we learned from Aspergillus? Biotechnol J. 2013; 8(8): 884-94.

[17] Kowalczyk JE, Benoit I, de Vries RP. Chapter Two - Regulation of Plant Biomass Utilization in Aspergillus. In: Sariaslani S, Gadd GM, Eds. Adv Appl Microbiol. Volume 88: Academic Press; 2014. p. 31-56.

[18] Stricker AR, Mach RL, de Graaff LH. Regulation of transcription of cellulases- and hemicellulases-encoding genes in Aspergillus niger and Hypocrea jecorina (Trichoderma reesei). Appl Microbiol Biotechnol. 2008; 78(2): 211-20.

[19] Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, *et al.* Genome sequencing and analysis of the biomass-degrading fungus Trichoderma reesei (syn. Hypocrea jecorina). Nat Biotech. 2008; 26(5): 553-60.

[20] Galagan JE, Calvo SE, Borkovich KA, Selker EU, Read ND, Jaffe D, *et al.* The genome sequence of the filamentous fungus Neurospora crassa. Nature. 2003; 422(6934): 859-68.

[21] Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, *et al.* Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. Nat Biotech. 2007; 25(2): 221-31.

[22] Andersen MR, Salazar MP, Schaap PJ, van de Vondervoort PJI, Culley D, Thykaer J, *et al.* Comparative genomics of citric-acid-producing Aspergillus niger ATCC 1015 *versus* enzyme-producing CBS 513.88. Genome Res. 2011; 21(6): 885-97.

[23] Le Crom S, Schackwitz W, Pennacchio L, Magnuson JK, Culley DE, Collett JR, *et al.* Tracking the roots of cellulase hyperproduction by the fungus Trichoderma reesei using massively parallel DNA sequencing. Proc Natl Acad Sci U S A. 2009; 106(38): 16151-6.

[24] Peterson R, Nevalainen H. Trichoderma reesei RUT-C30 - thirty years of strain improvement. Microbiology. 2012; 158(1): 58-68.

[25] Herold S, Bischof R, Metz B, Seiboth B, Kubicek CP. Xylanase gene transcription in Trichoderma reesei is triggered by different inducers representing different hemicellulosic pentose polymers. Eukaryot Cell. 2013; 12(3): 390-8.

[26] Hakkinen M. Transcriptional analysis of Trichoderma reesei under conditions inducing cellulase and hemicellulase production, and identification of factors influencing protein production: University of Helsinki, Finland; 2014.

[27] Tisch D, Schmoll M. Targets of light signalling in Trichoderma reesei. BMC Genomics. 2013; 14: 657.

[28] Ruijter GJG, Visser J. Carbon repression in aspergilli. FEMS Microbiol Lett. 1997; 151(2): 103-14.

[29] Suto M, Tomita F. Induction and catabolite repression mechanisms of cellulase in fungi. J Biosci Bioeng. 2001; 92(4): 305-11.

[30] Portnoy T, Margeot A, Linke R, Atanasova L, Fekete E, Sandor E, *et al.* The CRE1 carbon catabolite repressor of the fungus Trichoderma reesei: a master regulator of carbon assimilation. BMC Genomics. 2011; 12: 269.

[31] Delmas S, Pullan ST, Gaddipati S, Kokolski M, Malla S, Blythe MJ, *et al.* Uncovering the genome-wide transcriptional responses of the filamentous fungus Aspergillus niger to lignocellulose using RNA sequencing. PLoS Genet. 2012; 8(8): e1002875.

[32] Sun J, Glass NL. Identification of the CRE-1 cellulolytic regulon in Neurospora crassa. PLoS One. 2011; 6(9): e25654.

[33] Zaman S, Lippman SI, Zhao X, Broach JR. How Saccharomyces responds to nutrients. Annu Rev Genet. 2008; 42: 27-81.

[34] Dong W, Yinbo Q, Peiji G. Regulation of cellulase synthesis in mycelial fungi: Participation of ATP and cyclic AMP. Biotechnol Lett. 1995; 17(6): 593-8.

[35] Kubicek CP, Mikus M, Schuster A, Schmoll M, Seiboth B. Metabolic engineering strategies for the improvement of cellulase production by Hypocrea jecorina. Biotechnol Biofuels. 2009; 2: 19.

[36] Flipphi M, van de Vondervoort PJ, Ruijter GJ, Visser J, Arst HN, Jr., Felenbok B. Onset of carbon catabolite repression in Aspergillus nidulans. Parallel involvement of hexokinase and glucokinase in sugar signaling. J Biol Chem. 2003; 278(14): 11849-57.

[37] Brown NA, de Gouvea PF, Krohn NG, Savoldi M, Goldman GH. Functional characterisation of the non-essential protein kinases and phosphatases regulating Aspergillus nidulans hydrolytic enzyme production. Biotechnol Biofuels. 2013; 6(1): 91.

[38] Lichius A, Seidl-Seiboth V, Seiboth B, Kubicek CP. Nucleo-cytoplasmic shuttling dynamics of the transcriptional regulators XYR1 and CRE1 under conditions of cellulase and xylanase gene expression in Trichoderma reesei. Mol Microbiol. 2014; 94(5): 1162–78.

[39] Cziferszky A, Mach R, Kubicek C. Phosphorylation positively regulates DNA binding of the carbon catabolite repressor Cre1 of Hypocrea jecorina (Trichoderma reesei). J Biol Chem. 2002; 277: 14688 - 99.

[40] Carle-Urioste JC, Escobar-Vera J, El-Gogary S, Henrique-Silva F, Torigoi E, Crivellaro O, *et al.* Cellulase induction in Trichoderma reesei by cellulose requires its own basal expression. J Biol Chem. 1997; 272(15): 10169-74.

[41] Parenicova L, Benen JA, Kester HC, Visser J. pgaA and pgaB encode two constitutively expressed endopolygalacturonases of Aspergillus niger. Biochem J. 2000; 345 (3): 637-44.

[42] van Munster JM, Daly P, Delmas S, Pullan ST, Blythe MJ, Malla S, *et al.* The role of carbon starvation in the induction of enzymes that degrade plant-derived carbohydrates in Aspergillus niger. Fungal Genet Biol. 2014; 72: 34-47.

[43] Foreman PK, Brown D, Dankmeyer L, Dean R, Diener S, Dunn-Coleman NS, *et al.* Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus Trichoderma reesei. J Biol Chem. 2003; 278(34): 31988-97.

[44] Margolles-Clark E, Ilmén M, Penttilä M. Expression patterns of ten hemicellulase genes of the filamentous fungus Trichoderma reesei on various carbon sources. J Biotechnol. 1997; 57(1-3): 167-79.

[45] Coradetti ST, Craig JP, Xiong Y, Shock T, Tian C, Glass NL. Conserved and essential transcription factors for cellulase gene expression in ascomycete fungi. Proc Natl Acad Sci U S A. 2012; 109(19): 7397-402.

[46] Martens-Uzunova ES, Schaap PJ. Assessment of the pectin degrading enzyme network of Aspergillus niger by functional genomics. Fungal Genet Biol. 2009; 46 Suppl 1: S170-S9.

[47] Nitsche B, Jorgensen T, Akeroyd M, Meyer V, Ram A. The carbon starvation response of Aspergillus niger during submerged cultivation: Insights from the transcriptome and secretome. BMC Genomics. 2012; 13(1): 380.

[48] Szilagyi M, Miskei M, Karanyi Z, Lenkey B, Pocsi I, Emri T. Transcriptome changes initiated by carbon starvation in Aspergillus nidulans. Microbiology. 2013; 159(Pt 1): 176-90.

[49] Benz JP, Chau BH, Zheng D, Bauer S, Glass NL, Somerville CR. A comparative systems analysis of polysaccharide-elicited responses in Neurospora crassa reveals carbon source-specific cellular adaptations. Mol Microbiol. 2014; 91(2): 275-99.

[50] Mach-Aigner AR, Omony J, Jovanovic B, van Boxtel AJB, de Graaff LH. d-Xylose concentration-dependent hydrolase expression profiles and the function of CreA and XlnR in Aspergillus niger. Appl Environ Microbiol. 2012; 78(9): 3145-55.

[51] de Vries RP, Visser J, de Graaff LH. CreA modulates the XlnR-induced expression on xylose of Aspergillus niger genes involved in xylan degradation. Res Microbiol. 1999; 150(4): 281-5.

[52] Sternberg D, Mandels GR. Induction of cellulolytic enzymes in Trichoderma reesei by sophorose. J Bacteriol. 1979; 139(3): 761-9.

[53] Ivanova C, Bååth JA, Seiboth B, Kubicek CP. Systems analysis of lactose metabolism in Trichoderma reesei identifies a lactose permease that is essential for cellulase induction. PLoS One. 2013; 8(5): e62631.

[54] Poggi-Parodi D, Bidard F, Pirayre A, Portnoy T, Blugeon C, Seiboth B, *et al.* Kinetic transcriptome analysis reveals an essentially intact induction system in a cellulase hyper-producer Trichoderma reesei strain. Biotechnol Biofuels. 2014; 7(1): 173.

[55] Znameroski EA, Coradetti ST, Roche CM, Tsai JC, Iavarone AT, Cate JHD, *et al.* Induction of lignocellulose-degrading enzymes in Neurospora crassa by cellodextrins. Proc Natl Acad Sci U S A. 2012.

[56] Sun J, Tian C, Diamond S, Glass NL. Deciphering transcriptional regulatory mechanisms associated with hemicellulose degradation in Neurospora crassa. Eukaryot Cell. 2012; 11(4): 482-93.

[57] Kurasawa T, Yachi M, Suto M, Kamagata Y, Takao S, Tomita F. Induction of cellulase by gentiobiose and its sulfur-containing analog in Penicillium purpurogenum. Appl Environ Microbiol. 1992; 58(1): 106-10.

[58] Noguchi Y, Tanaka H, Kanamaru K, Kato M, Kobayashi T. Xylose triggers reversible phosphorylation of XlnR, the fungal transcriptional activator of xylanolytic and cellulolytic genes in Aspergillus oryzae. Biosci Biotechnol Biochem. 2011; 75(5): 953-9.

[59] Xiong Y, Coradetti ST, Li X, Gritsenko MA, Clauss T, Petyuk V, *et al.* The proteome and phosphoproteome of Neurospora crassa in response to cellulose, sucrose and carbon starvation. Fungal Genet Biol. 2014; 72(0): 21-33.

[60] Wang M, Zhao Q, Yang J, Jiang B, Wang F, Liu K, *et al.* A mitogen-activated protein kinase Tmk3 participates in high osmolarity resistance, cell wall integrity maintenance and cellulase production regulation in Trichoderma reesei. PLoS One. 2013; 8(8): e72189.

[61] D'Enfert C. Fungal spore germination: Insights from the molecular genetics of Aspergillus nidulans and Neurospora crassa. Fungal Genet Biol. 1997; 21(2): 163-72.

[62] Novodvorska M, Hayer K, Pullan ST, Wilson R, Blythe MJ, Stam H, *et al.* Trancriptional landscape of Aspergillus niger at breaking of conidial dormancy revealed by RNA-sequencing. BMC Genomics. 2013; 14: 246.

[63] van Leeuwen MR, Krijgsheld P, Bleichrodt R, Menke H, Stam H, Stark J, *et al.* Germination of conidia of Aspergillus niger is accompanied by major changes in RNA profiles. Stud Mycol. 2013; 74(1): 59-70.
[64] Hayer K, Stratford M, Archer DB. Structural features of sugars that trigger or support conidial germination in the filamentous fungus Aspergillus niger. Appl Environ Microbiol. 2013; 79(22): 6924-31.
[65] Hayer K, Stratford M, Archer DB. Germination of Aspergillus niger conidia is triggered by nitrogen compounds related to L-amino acids. Appl Environ Microbiol. 2014; 80(19): 6046-53.
[66] Hakkinen M, Arvas M, Oja M, Aro N, Penttilä M, Saloheimo M, *et al.* Re-annotation of the CAZy genes of Trichoderma reesei and transcription in the presence of lignocellulosic substrates. Microb Cell Fact. 2012; 11(1): 134.
[67] Levasseur A, Drula E, Lombard V, Coutinho P, Henrissat B. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. Biotechnol Biofuels. 2013; 6(1): 41.
[68] Pullan ST, Daly P, Delmas S, Ibbett R, Kokolski M, Neiteler A, *et al.* RNA-sequencing reveals the complexities of the transcriptional response to lignocellulosic biofuel substrates in Aspergillus niger. Fungal Biol Biotech. 2014; 1(3).
[69] de Souza WR, de Gouvea PF, Savoldi M, Malavazi I, de Souza Bernardes LA, Goldman MH, *et al.* Transcriptome analysis of Aspergillus niger grown on sugarcane bagasse. Biotechnol Biofuels. 2011; 4: 40.
[70] Andersen M, Giese M, P R, Nielsen J. Mapping the polysaccharide degradation potential of Aspergillus niger. BMC Genomics. 2012; 13(1): 313.
[71] Kolbusz MA, Di Falco M, Ishmael N, Marqueteau S, Moisan M-C, Baptista CdS, *et al.* Transcriptome and exoproteome analysis of utilization of plant-derived biomass by Myceliophthora thermophila. Fungal Genet Biol. 2014; 72: 10-20.
[72] Vogel J. Unique aspects of the grass cell wall. Curr Opin Plant Biol. 2008; 11(3): 301-7.
[73] Cubero B, Scazzocchio C. Two different, adjacent and divergent zinc finger binding sites are necessary for CREA-mediated carbon catabolite repression in the proline gene cluster of Aspergillus nidulans. EMBO J. 1994; 13(2): 407-15.
[74] Takashima S, Iikura H, Nakamura A, Masaki H, Uozumi T. Analysis of Cre1 binding sites in the Trichoderma reesei cbh1 upstream region. FEMS Microbiol Lett. 1996; 145(3): 361-6.
[75] Tamayo EN, Villanueva A, Hasper AA, de Graaff LH, Ramón D, Orejas M. CreA mediates repression of the regulatory gene xlnR which controls the production of xylanolytic enzymes in Aspergillus nidulans. Fungal Genet Biol. 2008; 45(6): 984-93.
[76] Todd R, Zhou M, Ohm R, Leeggangers H, Visser L, de Vries R. Prevalence of transcription factors in ascomycete and basidiomycete fungi. BMC Genomics. 2014; 15(1): 214.
[77] Aro N, Ilmén M, Saloheimo A, Penttilä M. ACEI of Trichoderma reesei is a repressor of cellulase and xylanase expression. Appl Environ Microbiol. 2003; 69(1): 56-65.
[78] van Peij NNME, Gielkens MMC, de Vries RP, Visser J, de Graaff LH. The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in Aspergillus niger. Appl Environ Microbiol. 1998; 64(10): 3615-9.
[79] Stricker AR, Grosstessner-Hain K, Würleitner E, Mach RL. Xyr1 (Xylanase Regulator 1) regulates both the hydrolytic enzyme system and d-Xylose metabolism in Hypocrea jecorina. Eukaryot Cell. 2006; 5(12): 2128-37.
[80] van Peij NNME, Visser J, De Graaff LH. Isolation and analysis of xlnR, encoding a transcriptional activator co-ordinating xylanolytic expression in Aspergillus niger. Mol Microbiol. 1998; 27(1): 131-42.
[81] Aro N, Saloheimo A, Ilmén M, Penttilä M. ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of Trichoderma reesei. J Biol Chem. 2001; 276(26): 24309-14.
[82] Chilton IJ, Delaney CE, Barham-Morris J, Fincham DA, Hooley P, Whitehead MP. The Aspergillus nidulans stress response transcription factor StzA is ascomycete-specific and shows species-specific polymorphisms in the C-terminal region. Mycol Res. 2008; 112(12): 1435-46.
[83] Klaubauf S, Narang HM, Post H, Zhou M, Brunner K, Mach-Aigner AR, *et al.* Similar is not the same: Differences in the function of the (hemi-)cellulolytic regulator XlnR (Xlr1/Xyr1) in filamentous fungi. Fungal Genet Biol. 2014; 72: 73-81.

[84] Coradetti ST, Xiong Y, Glass NL. Analysis of a conserved cellulase transcriptional regulator reveals inducer-independent production of cellulolytic enzymes in Neurospora crassa. Microbiologyopen. 2013; 2(4): 595–609.

[85] Ogawa M, Kobayashi T, Koyama Y. ManR, a transcriptional regulator of the β-Mannan utilization system, controls the cellulose utilization system in Aspergillus oryzae. Biosci Biotechnol Biochem. 2013; 77(2): 426-9.

[86] Battaglia E, Visser L, Nijssen A, van Veluw GJ, Wösten HA, de Vries RP. Analysis of regulation of pentose utilisation in Aspergillus niger reveals evolutionary adaptations in Eurotiales. Stud Mycol. 2011; 69(1): 31-8.

[87] Battaglia E, Hansen S, Leendertse A, Madrid S, Mulder H, Nikolaev I, *et al.* Regulation of pentose utilisation by AraR, but not XlnR, differs in Aspergillus nidulans and Aspergillus niger. Appl Microbiol Biotechnol. 2011; 91(2): 387-97.

[88] de Souza WR, Maitan-Alfenas GP, de Gouvêa PF, Brown NA, Savoldi M, Battaglia E, *et al.* 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. Fungal Genet Biol. 2013; 60(0): 29-45.

[89] Nitta M, Furukawa T, Shida Y, Mori K, Kuhara S, Morikawa Y, *et al.* A new Zn(II)2Cys6-type transcription factor BglR regulates β-glucosidase expression in Trichoderma reesei. Fungal Genet Biol. 2012; 49(5): 388-97.

[90] Kunitake E, Tani S, Sumitani J-i, Kawaguchi T. 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. 2012: 1-12.

[91] Hakkinen M, Valkonen M, Westerholm-Parvinen A, Aro N, Arvas M, Vitikainen M, *et al.* Screening of candidate regulators for cellulase and hemicellulase production in Trichoderma reesei and identification of a factor essential for cellulase production. Biotechnol Biofuels. 2014; 7(1): 14.

[92] Gruben BS, Zhou M, Wiebenga A, Ballering J, Overkamp KM, Punt PJ, *et al.* Aspergillus niger RhaR, a regulator involved in L-rhamnose release and catabolism. Appl Microbiol Biotechnol. 2014; 98(12): 5531-40.

[93] Schmoll M, Tian C, Sun J, Tisch D, Glass NL. Unravelling the molecular basis for light modulated cellulase gene expression - the role of photoreceptors in Neurospora crassa. BMC Genomics. 2012; 13(1): 127.

[94] Tisch D, Kubicek CP, Schmoll M. The phosducin-like protein PhLP1 impacts regulation of glycoside hydrolases and light response in Trichoderma reesei. BMC Genomics. 2011; 12: 613.

[95] He Q, Liu Y. Molecular mechanism of light responses in Neurospora: from light-induced transcription to photoadaptation. Genes Dev. 2005; 19(23): 2888-99.

[96] Chen CH, Ringelberg CS, Gross RH, Dunlap JC, Loros JJ. Genome-wide analysis of light-inducible responses reveals hierarchical light signalling in Neurospora. EMBO J. 2009; 28(8): 1029-42.

[97] Smith KM, Sancar G, Dekhang R, Sullivan CM, Li S, Tag AG, *et al.* Transcription factors in light and circadian clock signaling networks revealed by genomewide mapping of direct targets for neurospora white collar complex. Eukaryot Cell. 2010; 9(10): 1549-56.

[98] Gyalai-Korpos M, Nagy G, Mareczky Z, Schuster A, Reczey K, Schmoll M. Relevance of the light signaling machinery for cellulase expression in Trichoderma reesei (Hypocrea jecorina). BMC Res Notes. 2010; 3: 330.

[99] Tisch D, Kubicek CP, Schmoll M. 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. 2011; 48(6): 631-40.

[100] Bailey MJ, Buchert J, Viikari L. Effect of pH on production of xylanase by Trichoderma reesei on xylan-based and cellulose-based media. Appl Microbiol Biotechnol. 1993; 40(2-3): 224-9.

[101] Li C, Yang Z, Zhang RH, Zhang D, Chen S, Ma L. Effect of pH on cellulase production and morphology of Trichoderma reesei and the application in cellulosic material hydrolysis. J Biotechnol. 2013; 168(4): 470-7.

[102] Adav SS, Ravindran A, Chao LT, Tan L, Singh S, Sze SK. Proteomic analysis of pH and strains dependent protein secretion of Trichoderma reesei. J Proteome Res. 2011; 10(10): 4579-96.

[103] Peñalva MA, Arst HN. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. Microbiol Mol Biol Rev. 2002; 66(3): 426-46.

[104] Herranz S, Rodriguez JM, Bussink HJ, Sanchez-Ferrero JC, Arst HN, Jr., Peñalva MA, *et al.* Arrestin-related proteins mediate pH signaling in fungi. Proc Natl Acad Sci U S A. 2005; 102(34): 12141-6.

[105] Hervas-Aguilar A, Galindo A, Peñalva MA. Receptor-independent Ambient pH signaling by ubiquitin attachment to fungal arrestin-like PalF. J Biol Chem. 2010; 285(23): 18095-102.

[106] Arst HN, Jr., Bignell E, Tilburn J. Two new genes involved in signalling ambient pH in Aspergillus nidulans. Mol Gen Genet. 1994; 245(6): 787-90.

[107] Caddick MX, Brownlee AG, Arst HN, Jr. Regulation of gene expression by pH of the growth medium in Aspergillus nidulans. Mol Gen Genet. 1986; 203(2): 346-53.

[108] Calcagno-Pizarelli AM, Negrete-Urtasun S, Denison SH, Rudnicka JD, Bussink HJ, Munera-Huertas T, *et al.* Establishment of the ambient pH signaling complex in Aspergillus nidulans: PalI assists plasma membrane localization of PalH. Eukaryot Cell. 2007; 6(12): 2365-75.

[109] Diez E, Alvaro J, Espeso EA, Rainbow L, Suarez T, Tilburn J, *et al.* Activation of the Aspergillus PacC zinc finger transcription factor requires two proteolytic steps. EMBO J. 2002; 21(6): 1350-9.

[110] Hervas-Aguilar A, Rodriguez JM, Tilburn J, Arst HN, Jr., Peñalva MA. Evidence for the direct involvement of the proteasome in the proteolytic processing of the Aspergillus nidulans zinc finger transcription factor PacC. J Biol Chem. 2007; 282(48): 34735-47.

[111] Tilburn J, Sarkar S, Widdick DA, Espeso EA, Orejas M, Mungroo J, *et al.* The Aspergillus PacC zinc finger transcription factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO J. 1995; 14(4): 779-90.

[112] Espeso EA, Tilburn J, Sanchez-Pulido L, Brown CV, Valencia A, Arst HN, *et al.* Specific DNA recognition by the Aspergillus nidulans three zinc finger transcription factor PacC. J Mol Biol. 1997; 274(4): 466-80.

[113] Mello-de-Sousa TM, Silva-Pereira I, Pocas-Fonseca MJ. Carbon source and pH-dependent transcriptional regulation of cellulase genes of Humicola grisea var. thermoidea grown on sugarcane bagasse. Enzyme Microb Technol. 2011; 48(1): 19-26.

[114] MacCabe AP, Orejas M, Perez-Gonzalez JA, Ramón D. Opposite patterns of expression of two Aspergillus nidulans xylanase genes with respect to ambient pH. J Bacteriol. 1998; 180(5): 1331-3.

[115] Gielkens M, Gonzalez-Candelas L, Sanchez-Torres P, van de Vondervoort P, de Graaff L, Visser J, *et al.* The abfB gene encoding the major alpha-L-arabinofuranosidase of Aspergillus nidulans: nucleotide sequence, regulation and construction of a disrupted strain. Microbiology. 1999; 145 (Pt 3): 735-41.

[116] Ries L, Belshaw NJ, Ilmén M, Penttilä ME, Alapuranen M, Archer DB. The role of CRE1 in nucleosome positioning within the cbh1 promoter and coding regions of Trichoderma reesei. Appl Microbiol Biotechnol. 2014; 98(2): 749-62.

[117] Arya G, Maitra A, Grigoryev SA. A structural perspective on the where, how, why, and what of nucleosome positioning. J Biomol Struct Dyn. 2010; 27(6): 803-20.

[118] Radman-Livaja M, Rando OJ. Nucleosome positioning: How is it established, and why does it matter? Dev Biol. 2010; 339(2): 258-66.

[119] Karimi-Aghcheh R, Bok JW, Phatale PA, Smith KM, Baker SE, Lichius A, *et al.* Functional analyses of Trichoderma reesei LAE1 reveal conserved and contrasting roles of this regulator. G3 (Bethesda). 2013; 3(2): 369-78.

[120] Kubicek CP. Catabolic Pathways of Soluble Degradation Products from Plant Biomass. Fungi and Lignocellulosic Biomass: Wiley-Blackwell; 2012. p. 119-28.

[121] Bischof R, Fourtis L, Limbeck A, Gamauf C, Seiboth B, Kubicek C. Comparative analysis of the Trichoderma reesei transcriptome during growth on the cellulase inducing substrates wheat straw and lactose. Biotechnol Biofuels. 2013; 6(1): 127.

[122] Saier MH, Reddy VS, Tamang DG, Västermark Å. The transporter classification database. Nucleic Acids Res. 2014; 42(D1): D251-D8.

[123] Runquist D, Hahn-Hagerdal B, Radstrom P. Comparison of heterologous xylose transporters in recombinant Saccharomyces cerevisiae. Biotechnol Biofuels. 2010; 3(1): 5.

[124] Galazka JM, Tian C, Beeson WT, Martinez B, Glass NL, Cate JHD. Cellodextrin transport in yeast for improved biofuel production. Science. 2010; 330(6000): 84-6.

[125] Tian C, Beeson WT, Iavarone AT, Sun J, Marletta MA, Cate JHD, *et al.* Systems analysis of plant cell wall degradation by the model filamentous fungus Neurospora crassa. Proc Natl Acad Sci U S A. 2009; 106(52): 22157-62.

[126] Cai P, Gu R, Wang B, Li J, Wan L, Tian C, *et al.* Evidence of a critical role for cellodextrin transporte 2 (CDT-2) in both cellulose and hemicellulose degradation and utilization in *Neurospora crassa*. PLoS One. 2014; 9(2): e89330.

[127] Colabardini A, Ries L, Brown N, dos Reis T, Savoldi M, Goldman M, *et al.* Functional characterization of a xylose transporter in Aspergillus nidulans. Biotechnol Biofuels. 2014; 7(1): 46.

[128] van Kuyk PA, Diderich JA, MacCabe AP, Hererro O, Ruijter GJ, Visser J. Aspergillus niger mstA encodes a high-affinity sugar/H+ symporter which is regulated in response to extracellular pH. Biochem J. 2004; 379(Pt 2): 375-83.

[129] Andersen MR, Vongsangnak W, Panagiotou G, Salazar MP, Lehmann L, Nielsen J. A trispecies Aspergillus microarray: Comparative transcriptomics of three Aspergillus species. Proc Natl Acad Sci U S A. 2008; 105(11): 4387-92.

[130] D'Haeseleer P. How does gene expression clustering work? Nat Biotech. 2005; 23(12): 1499-501.

[131] Kriel J, Haesendonckx S, Rubio-Texeira M, Van Zeebroeck G, Thevelein JM. From transporter to transceptor: Signaling from transporters provokes re-evaluation of complex trafficking and regulatory controls. Bioessays. 2011; 33(11): 870-9.

[132] Znameroski EA, Li X, Tsai JC, Galazka JM, Glass NL, Cate JHD. Evidence for transceptor function of cellodextrin transporters in Neurospora crassa. J Biol Chem. 2014; 289(5): 2610-9.

[133] Saloheimo M, Paloheimo M, Hakola S, Pere J, Swanson B, Nyyssönen E, *et al.* Swollenin, a Trichoderma reesei protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. Eur J Biochem. 2002; 269(17): 4202-11.

[134] Gourlay K, Hu J, Arantes V, Andberg M, Saloheimo M, Penttilä M, *et al.* Swollenin aids in the amorphogenesis step during the enzymatic hydrolysis of pretreated biomass. Bioresour Technol. 2013; 142(0): 498-503.

[135] Ries L, Pullan S, Delmas S, Malla S, Blythe M, Archer D. Genome-wide transcriptional response of Trichoderma reesei to lignocellulose using RNA sequencing and comparison with Aspergillus niger. BMC Genomics. 2013; 14(1): 541.

[136] Wösten HA. Hydrophobins: multipurpose proteins. Annu Rev Microbiol. 2001; 55: 625-46.

[137] Kim S, Ahn IP, Rho HS, Lee YH. MHP1, a Magnaporthe grisea hydrophobin gene, is required for fungal development and plant colonization. Mol Microbiol. 2005; 57(5): 1224-37.

[138] Takahashi T, Maeda H, Yoneda S, Ohtaki S, Yamagata Y, Hasegawa F, *et al.* The fungal hydrophobin RolA recruits polyesterase and laterally moves on hydrophobic surfaces. Mol Microbiol. 2005; 57(6): 1780-96.

[139] Maeda H, Yamagata Y, Abe K, Hasegawa F, Machida M, Ishioka R, *et al.* Purification and characterization of a biodegradable plastic-degrading enzyme from Aspergillus oryzae. Appl Microbiol Biotechnol. 2005; 67(6): 778-88.

[140] Jensen BG, Andersen MR, Pedersen MH, Frisvad JC, Sondergaard I. Hydrophobins from Aspergillus species cannot be clearly divided into two classes. BMC Res Notes. 2010; 3: 344.

[141] Jorgensen TR, Nitsche BM, Lamers GE, Arentshorst M, van den Hondel CA, Ram AF. Transcriptomic insights into the physiology of Aspergillus niger approaching a specific growth rate of zero. Appl Environ Microbiol. 2010; 76(16): 5344-55.

[142] Geysens S, Whyteside G, Archer DB. Genomics of protein folding in the endoplasmic reticulum, secretion stress and glycosylation in the aspergilli. Fungal Genet Biol. 2009; 46(1, Supplement): S121-S40.

[143] Conesa A, Punt PJ, van Luijk N, van den Hondel CAMJJ. The secretion pathway in filamentous fungi: A biotechnological view. Fungal Genet Biol. 2001; 33(3): 155-71.

[144] Saloheimo M, Pakula TM. The cargo and the transport system: secreted proteins and protein secretion in Trichoderma reesei (Hypocrea jecorina). Microbiology. 2012; 158(1): 46-57.

[145] Guillemette T, van Peij N, Goosen T, Lanthaler K, Robson G, van den Hondel C, *et al.* Genomic analysis of the secretion stress response in the enzyme-producing cell factory Aspergillus niger. BMC Genomics. 2007; 8(1): 158.

[146] Nitsche B, Burggraaf-van Welzen A-M, Lamers G, Meyer V, Ram AJ. Autophagy promotes survival in aging submerged cultures of the filamentous fungus Aspergillus niger. Appl Microbiol Biotechnol. 2013; 97(18): 8205-18.
[147] Bernales S, Papa FR, Walter P. Intracellular signaling by the unfolded protein response. Annu Rev Cell Dev Biol. 2006; 22: 487-508.
[148] Whyteside G, Alcocer MJC, Kumita JR, Dobson CM, Lazarou M, Pleass RJ, *et al.* Native-state stability determines the extent of degradation relative to secretion of protein variants from Pichia pastoris. PLoS One. 2011; 6(7): e22692.
[149] Arvas M, Pakula T, Smit B, Rautio J, Koivistoinen H, Jouhten P, *et al.* Correlation of gene expression and protein production rate - a system wide study. BMC Genomics. 2011; 12(1): 616.
[150] Pakula TM, Salonen K, Uusitalo J, Penttilä M. The effect of specific growth rate on protein synthesis and secretion in the filamentous fungus Trichoderma reesei. Microbiology. 2005; 151(1): 135-43.
[151] Kwon MJ, Jorgensen T, Nitsche B, Arentshorst M, Park J, Ram A, *et al.* The transcriptomic fingerprint of glucoamylase over-expression in Aspergillus niger. BMC Genomics. 2012; 13(1): 701.
[152] Morris KV, Mattick JS. The rise of regulatory RNA. Nat Rev Genet. 2014; 15(6): 423-37.
[153] Lapidot M, Pilpel Y. Genome-wide natural antisense transcription: coupling its regulation to its different regulatory mechanisms. EMBO Rep. 2006; 7(12): 1216-22.
[154] Xue Z, Ye Q, Anson SR, Yang J, Xiao G, Kowbel D, *et al.* Transcriptional interference by antisense RNA is required for circadian clock function. Nature. 2014; 514: 650–3.
[155] Zhang J, Qu Y, Xiao P, Wang X, Wang T, He F. Improved biomass saccharification by Trichoderma reesei through heterologous expression of lacA gene from Trametes sp. AH28-2. J Biosci Bioeng. 2012; 113(6): 697-703.
[156] Tian B-Y, Huang Q-G, Xu Y, Wang C-X, Lv R-R, Huang J-Z. Microbial community structure and diversity in a native forest wood-decomposed hollow-stump ecosystem. World J Microbiol Biotechnol. 2010; 26(2): 233-40.
[157] Das M, Royer TV, Leff LG. Diversity of fungi, bacteria, and actinomycetes on leaves decomposing in a stream. Appl Environ Microbiol. 2007; 73(3): 756-67.
[158] Boddy L. Interspecific combative interactions between wood-decaying basidiomycetes. FEMS Microbiol Ecol. 2000; 31(3): 185-94.
[159] Kolasa M, Ahring BK, Lübeck PS, Lübeck M. 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. Bioresour Technol. 2014; 169: 143-8.
[160] Slinker BK. The statistics of synergism. J Mol Cell Cardiol. 1998; 30(4): 723-31.
[161] Hu HL, van den Brink J, Gruben BS, Wösten HAB, Gu JD, de Vries RP. Improved enzyme production by co-cultivation of Aspergillus niger and Aspergillus oryzae and with other fungi. Int Biodeterior Biodegradation. 2011; 65(1): 248-52.
[162] Ahamed A, Vermette P. 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. 2008; 42(1): 41-6.
[163] Ma K, Ruan Z. Production of a lignocellulolytic enzyme system for simultaneous bio-delignification and saccharification of corn stover employing co-culture of fungi. Bioresour Technol. 2015; 175: 586-93.
[164] Duff SJB, Cooper DG, Fuller OM. Effect of media composition and growth conditions on production of cellulase and β-glucosidase by a mixed fungal fermentation. Enzyme Microb Technol. 1987; 9(1): 47-52.
[165] van den Brink J, Maitan-Alfenas GP, Zou G, Wang C, Zhou Z, Guimarães VM, *et al.* Aspergillus niger and Trichoderma reesei enzymes have a synergistic effect on saccharification of wheat straw and sugarcane bagasse. Biotechnol J. 2014; 9(10): 1329–38.
[166] Fortes Gottschalk LM, Oliveira RA, da Silva Bon EP. Cellulases, xylanases, beta-glucosidase and ferulic acid esterase produced by Trichoderma and Aspergillus act synergistically in the hydrolysis of sugarcane bagasse. Biochem Eng J. 2010; 51(1-2): 72-8.
[167] Arfi Y, Levasseur A, Record E. Differential gene expression in Pycnoporus coccineus during interspecific mycelial interactions with different competitors. Appl Environ Microbiol. 2013; 79(21): 6626-36.

[168] Mach-Aigner AR, Pucher ME, Mach RL. d-Xylose as a repressor or inducer of xylanase expression in Hypocrea jecorina (Trichoderma reesei). Appl Environ Microbiol. 2010; 76(6): 1770-6.
[169] Halaouli S, Asther M, Sigoillot JC, Hamdi M, Lomascolo A. Fungal tyrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. J Appl Microbiol. 2006; 100(2): 219-32.
[170] Kellner H, Vandenbol M. Fungi unearthed: Transcripts encoding lignocellulolytic and chitinolytic enzymes in forest soil. PLoS One. 2010; 5(6): e10971.
[171] Westermann AJ, Gorski SA, Vogel J. Dual RNA-seq of pathogen and host. Nat Rev Micro. 2012; 10(9): 618-30.
[172] Ilmén M, Thrane C, Penttilä M. The glucose repressor gene cre1 of Trichoderma: Isolation and expression of a full-length and a truncated mutant form. Mol Gen Genet. 1996; 251(4): 451-60.
[173] Scheller H, Ulvskov P. Hemicelluloses. Annu Rev Plant Biol. 2010; 61: 263 - 89.
[174] Tamayo-Ramos J, Orejas M. Enhanced glycosyl hydrolase production in Aspergillus nidulans using transcription factor engineering approaches. Biotechnol Biofuels. 2014; 7(1): 103.
[175] Meyer AS, Rosgaard L, Sorensen HR. The minimal enzyme cocktail concept for biomass processing. J Cereal Sci. 2009; 50(3): 337-44.
[176] Adav SS, Chao LT, Sze SK. Quantitative secretomic analysis of Trichoderma reesei strains reveals enzymatic composition for lignocellulosic biomass degradation. Mol Cell Proteomics. 2012; 11(7): M111 012419.
[177] Rosgaard L, Pedersen S, Langston J, Akerhielm D, Cherry JR, Meyer AS. Evaluation of minimal Trichoderma reesei cellulase mixtures on differently pretreated barley straw substrates. Biotechnol Prog. 2007; 23(6): 1270-6.
[178] Mansfield SD, Mooney C, Saddler JN. Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnol Prog. 1999; 15(5): 804-16.
[179] Arantes V, Saddler J. Access to cellulose limits the efficiency of enzymatic hydrolysis: the role of amorphogenesis. Biotechnol Biofuels. 2010; 3(1): 4.
[180] Varnai A, Siika-aho M, Viikari L. Carbohydrate-binding modules (CBMs) revisited: reduced amount of water counterbalances the need for CBMs. Biotechnol Biofuels. 2013; 6(1): 30.
[181] Gao D, Haarmeyer C, Balan V, Whitehead T, Dale B, Chundawat S. Lignin triggers irreversible cellulase loss during pretreated lignocellulosic biomass saccharification. Biotechnol Biofuels. 2014; 7(1): 175.
[182] Kumamoto CA. Molecular mechanisms of mechanosensing and their roles in fungal contact sensing. Nat Rev Micro. 2008; 6(9): 667-73.
[183] Zhang W, Li F, Nie L. Integrating multiple 'omics' analysis for microbial biology: application and methodologies. Microbiology. 2010; 156(2): 287-301.