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Publication Date

2022-10-01

DOI

10.1016/j.jclepro.2022.133488

Engineering of holocellulase in biomass-degrading fungi for sustainable biofuel production

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Abstract

Biofuels, such as bioethanol, are a clean and sustainable form of energy and have emerged as a viable alternative to fossil fuels. Plant biomass is an important raw material for the production of clean and renewable energy. The holocellulose contained in the composition of plants may be broken down into simple sugars, such as glucose, which are fermented by yeast to produce bioethanol. The conversion of glucose polymers into fermentable sugars is accomplished by enzymes known as holocellulases, which are produced by lignocellulolytic fungi. These enzymes act synergistically for the efficient degradation of cellulose polymers, and the fine and coordinated regulation of this process is performed by transcription factors (TFs). TFs are regulatory proteins that bind to the promoter region of their target genes (CAZymes, sugar transporters, signaling proteins, other TFs, etc.) to induce or repress their transcription. This review aims to understand the main regulatory mechanisms involved in plant biomass degradation by the most studied lignocellulolytic fungi *Trichoderma* sp., *Aspergillus* sp., *Penicillium* sp., and *Neurospora* crassa. In this context, the most studied TFs related to holocellulose degradation and genetic modification of TFs or promoters as a valuable tool to improve enzyme production for biotechnological purposes have been discussed. This review enables the expansion of knowledge on the regulation of the cellulolytic system of filamentous fungi and the application of this knowledge to the improvement of numerous bioproducts. Engineering TFs and promoters may yield more efficient strains that may be active in plant biomass hydrolysis. In this way, the technological

processes for obtaining ethanol from lignocellulose may become more commercially viable.

Keywords: Cellulosic ethanol, holocellulase, transcription factor, gene expression, transcriptional regulation, lignocellulolytic fungi, promoter engineering.

List of Abbreviations

AZFP - Artificial zinc finger protein

BGL – Beta-glucosidase

CAZymes – Carbohydrate active enzymes

CBH – Cellobiohydrolase

CCR – Carbon catabolite repression

CMCase - Carboxymethyl cellulase

DBD - DNA binding domain

DNA – Deoxyribonucleic acid

ED - Effector Domain

EG – Endoglucanase

FPase - Filter paperase

GH - Glycosyl hydrolase

HAP- Heme Activator Protein

LPMO – Lytic polysaccharide monooxygenase

pH - Potential of hydrogen

pNP - p-nitrophenyl

RNA – Ribonucleic acid

SCB - Sugarcane bagasse

71 TF - Transcription factor

72 ZF - Zinc finger

73

74 **1. Introduction**

75 In recent years, concerns about the finite nature of fossil fuel reserves and
76 environmental sustainability have led to an increased search for renewable and cleaner
77 energy sources. Second-generation fuels, such as bioethanol, have been highlighted as
78 environmentally friendly, sustainable, and cost-effective energy sources produced from
79 abundant raw materials in the environment, such as plant biomass, including corn bran
80 and sugarcane bagasse (SCB) (Robak and Balcerek, 2018). Biomass energy production
81 has a significant advantage because the carbon dioxide released during combustion does
82 not increase the CO_2 in the atmosphere as it is of biogenic origin (Tursi, 2019).

83 The structure of the plant biomass cell wall comprises holocellulose (cellulose
84 and hemicellulose) and lignin (Moreira et al., 2011). Cellulose is a complex
85 carbohydrate polymer composed of several hundred units of glucose and represents the
86 most abundant organic compound in nature. In contrast to cellulose, hemicellulose is a
87 heterogeneous branched polysaccharide formed by various pentoses and hexoses such
88 as mannans, arabinogalactans, xylans, and galactans. Lignin functions in the joining and
89 compacting the plant fibers, thus increasing their resistance (Tursi, 2019). The structure
90 of lignin comprises a complex amorphous aromatic polymer that is removed during the
91 production of bioethanol (Moreira et al., 2011). The proportion between
92 cellulose/hemicellulose/lignin that composes the vegetal biomass varies depending on
93 each plant. In wheat straw, for example, the concentration of cellulose ranges from 33–

40% (w/w); hemicelluloses range from 20–25% (w/w); and lignin ranges from 15–20% (w/w) (Srivastava et al., 2021).

One of the crucial steps in the production of bioethanol is ensuring the availability of sugars present in (hemi)cellulose for the subsequent fermentation by yeast. The release of simple sugars can occur chemically or enzymatically. Lignocellulolytic microorganisms play a crucial role in the latter. The biological pretreatment of plant biomass is environmentally safe, cost-effective, and may be carried out under environmental conditions that also have lower energy costs (Alvira et al., 2010). Among the microorganisms most commonly used in the production of holocellulolytic enzymes are filamentous fungi belonging to the genera *Trichoderma*, *Aspergillus*, *Neurospora*, and *Penicillium*.

Filamentous fungi transform plant residues, which are normally discarded by the agroindustry, into raw materials for the production of clean energy, as is the case with bioethanol. Bread residues, for example, may be bioconverted into ethanol efficiently, with the biomass hydrolysis process being carried out by the species *Neurospora intermedia* and *Aspergillus oryzae* (Kawa-Rygielska et al., 2022). Banana peels, wheat bran, and sawdust are also used as substrates for the cellulases produced by *Aspergillus terreus* and produce a high amount of ethanol with great purity (Sethi et al., 2017). Another suitable substrate for microorganism hydrolysis and ethanol production is the feedstock SCB. In a study by Huang *et al*., an engineered strain of *Trichoderma reesei* was used to produce ethanol directly from this feedstock (Huang et al., 2014). The cellulases and hemicellulases (referred together as holocellulases) produced by these fungi act synergistically to degrade vegetal polymers in a finely coordinated process (Gupta et al., 2016).

This review reports how the success of biomass hydrolysis is directly related to the microorganisms employed in the process and the use of genetic engineering to improve the ability of these microorganisms to degrade plant biomass. The regulatory mechanisms by which polysaccharides are degraded and transported into cells and the synthesis of regulatory proteins that control the transcription of specific genes involved in the production of holocellulases have further been discussed. This review also describes the most well-studied transcription factors (TFs) within lignocellulolytic fungi and how genetic engineering can be a key factor in optimizing the expression of these proteins and improve enzymatic production in the biotechnological industry. Several TFs involved in plant biomass degradation remain uncharacterized despite the advances discussed in this paper. It is therefore important to further investigate the regulatory mechanisms that control plant biomass degradation. All technologies involving the genetic engineering of TFs are important tools for optimizing the degradation of biomass by microorganisms. In the bioethanol production process, for example, such optimization is possible as the process makes the use of this clean and competitive energy source more commercially viable. This review assesses the latest findings regarding the regulation of the cellulolytic system and most recent technologies used in genetic engineering to obtain more efficient microorganisms that hydrolyze plant cell walls. Finally, future perspectives are presented to achieve a consolidated process for bioethanol production using lignocellulosic feedstock.

2. The holocellulolytic system in filamentous fungi

Technologies that use microorganisms for the production of renewable fuels have the advantage of reducing environmental waste and harmful greenhouse gas

emissions (Zabermawi et al., 2022). In filamentous fungi, the appropriate response to a given environmental stimulus is coordinated by different mechanisms. For example, stimuli can be caused by the release of sugars from the hydrolysis of cellulosic biomass, which involves four main steps: 1. The activation of specific intracellular signaling pathways responsible for the perception of signals in the environment; 2. Expression of TFs involved in the metabolism of different carbon sources; 3. Induction of the expression of enzymes capable of cleaving different substrates and releasing sugars for energy production; and 4. Expression of transporters in the cell membrane, which is responsible for sugar internalization and its incorporation in different metabolic pathways (Nogueira et al., 2020). These initial steps of the activation of the holocellulolytic system are regulated specifically by the available carbon source in the microenvironment of the microorganism. Thus, the recognition of the substrate by the fungus allows for the optimization of the production of degradative enzymes and is an essential process for the survival of microorganisms in nature (Bazafkan et al., 2014). The following discussion on this topic addresses the mechanisms involved in each step of the induction of the holocellulolytic system in the main filamentous fungi involved in the breakdown of lignocellulose.

Trichoderma sp. is a filamentous fungus involved in lignocellulosic biomass degradation (Peterson and Nevalainen, 2012) and is an important biocontrol agent against a number of plant pathogenic fungi (Sood et al., 2020). These organisms have developed specific mechanisms to use plant-derived polysaccharides as a major source of carbon and energy. Transcriptional regulation by specific TFs is a successful strategy employed by *Trichoderma reesei* to regulate the production of Carbohydrate-Active Enzymes (CAZymes) (Dos Santos Castro et al., 2016). Under specific conditions, TFs

bind to their sites at the target promoters and initiate the activation or repression of the holocellulase-encoding genes.

As shown in Figure 1, the promoter regions of all the CAZymes from *T. reesei* were selected and examined for potential specific binding motifs for the TFs XYR1, CRE1, ACE2, PacC, CLR1, and CLR2 using *in silico* analyses. XYR1 was observed as it is the main regulator of holocellulase production and a direct regulator of the genes involved in xylan/xyloglucan degradation. Almost no genes contained a binding site for CLR1 in the analyzed promoters, thus suggesting that CLR1 acts indirectly to regulate the enzymes involved in biomass degradation. For the other TFs, a varied profile was observed even when analyzing the same polysaccharide-related enzymes. Therefore, the finely tuned regulation performed by TFs is the key feature that allows *T. reesei* to adequately respond to environmental changes (Kunitake and Kobayashi, 2017).

In *T. reesei,* the induction of holocellulase expression is controlled at the transcriptional level and in a carbon source-dependent manner (Gupta et al., 2016). The holocellulolytic apparatus of *T. reesei* encompasses endoglucanases, exoglucanases, β-glucosidases, and other enzymes or proteins such as the lytic polysaccharide monooxygenases (LPMOs) (Song et al., 2018) and swollenin (Eibinger et al., 2016), which act synergistically to break down (hemi)cellulose compounds. Most of these enzymes are highly expressed in the presence of cellulose, sophorose, and SCB in comparison to glucose and glycerol (Dos Santos Castro et al., 2014).

Aspergillus sp. is another important industrial workhorse due to its ability to produce and secrete extracellular enzymes that are used in a wide range of biotechnological processes (De Gouvêa et al., 2018). In 2015, Udatha *et al*. performed a secretome analysis and found that one third of the glycosyl hydrolases (GHs) produced

by *A. oryzae* were up- or downregulated in the presence of oligosaccharide inducers. In addition, more than 120 CAZymes and genes related to diverse metabolic process were differentially expressed in response to these inducers. These findings suggest a sophisticated capacity for the adaptation of this species to survive with the available nutrients (Udatha et al., 2015). A comparative secretomic analysis of *A. niger* and *T. reesei* further revealed that the *A. niger* secretome was the most diverse and identified the 89 least-secreted enzymes in response to SCB (Borin et al., 2015). A secretome analysis of *Aspergillus* in response to less complex carbon sources showed important changes compared to those caused by SCB. A small number of hydrolytic enzymes was observed when the fungus was cultured in the presence of glucose (Adav et al., 2010).

Two other interesting examples of holocellulolytic fungi genera are *Neurospora* and *Penicillium. N. crassa* is an efficient plant biomass decomposer with a wide plant cell wall-degrading enzyme. Wu *et al*. showed that this fungus exhibited robust gene expression changes when cultured in the presence of monosaccharides, disaccharides, and complex biomass components (Wu et al., 2020). A quantitative proteomics analysis revealed that the *N. crassa* secretome is enriched with CBH1, GH6-1, GH5-1, and GH3-4 (the most abundant), hemicellulases, and LPMOs (Znameroski and Glass, 2013). Moreover, *P. oxalicum* (Song et al., 2016) and *P. funiculosum* (Ogunmolu et al., 2015) have been shown to be great holocellulolytic enzyme producers. Ribeiro *et al*. showed that *P. echinulatum* is a good producer of endoglucanase, cellobiohydrolase, and β-glucosidase (Ribeiro et al., 2012). Similarly, Schneider *et al*. showed that GHs are enriched in the *P. echinulatum* secretome (Schneider et al., 2016). A distinct holocellulolytic expression profile has been identified for *P. decumbens* and has a great

number and different types of holocellulolytic enzymes compared to other filamentous fungi (Liu et al., 2013).

Filamentous fungi can grow on a wide variety of substrates and adapt to diverse growth conditions. These characteristics make the fungi interesting targets for studies that assess the recycling of lignocellulosic residues, which were previously disposable, for the production of clean energy (Wikandari et al., 2022). Research on the cellulolytic system of the main filamentous fungi has been used to advance the technologies that contribute to the improvement of biomass hydrolysis and consequent sustainability and market competitiveness of bioethanol as a source of clean energy.

3. From sugar-sensing to its uptake: the crucial role of sugar transporters in regulating the holocellulolytic system

Proteins involved in sugar transport play an important role in the hydrolysis of plant biomass and are therefore important research targets aimed at improving the strains used in the production of bioenergy. The expression of an adequate set of transporters is necessary for sugar uptake and their incorporation into different metabolic pathways, which regulate (hemi)cellulase production (Figure 2). Different membrane proteins have been described as important players in sugar metabolism as they act in both sugar uptake and the activation of downstream signaling pathways that control (hemi)cellulase-encoding genes (Nogueira et al., 2020). To activate cellulolytic machinery, *T. reesei* senses the insoluble polysaccharides in the environment and produces cellobiose and its transglycosylated product, sophorose. These oligosaccharides act as inducers for the production of cellulases and hemicellulases (RG

De Paula et al., 2019). However, the mechanisms involved in sugar recognition and the intracellular inductor cascade remain unclear.

In *T. reesei*, several sugar transporters have been characterized due to their role in the transport of xylose, mannose, cellobiose, glucose, arabinose, xylitol, L-arabitol, or lactose (Huang et al., 2015; Nogueira et al., 2020) (Figure 2). In *N. crassa*, cellodextrin transporters have been shown to be important in the regulation of holocellulolytic genes. CDT-1 and CDT-2 are important for cellulose sensing and contribute to the secretion of cellulases (Znameroski et al., 2014). Similarly, CLP1 has been found to be essential for the activation of cellulase. Various sugar transporters of *N. crassa* may also be important in the secretion of cellulases that act as transceptors (Cai et al., 2015). For instance, two glucose transporters (HGT-1/2) are essential players in the regulation of sugar uptake and carbon catabolite repression (CCR) (Wang et al., 2017). Likewise, in *A. nidulans*, CltA and CltB are involved in cellobiose uptake and cellulose signaling (Dos Reis et al., 2016). In *P. decumbens*, the functional characterization of two cellodextrin transporters (*cdtC* and *cdtD*) revealed their crucial role in cellobiose consumption (Li et al., 2013). These findings clarify various aspects of the role of protein transporters in the control of (hemi)cellulolytic gene expression. The functional characterization of these novel transporters may therefore be necessary to reveal their potential in sensing and transducing the signals involved in biomass degradation.

As noted for the CAZymes, transporter-encoding genes can also be important targets of the TFs involved in the regulation of plant cell-wall degradation. These TFs include CRE1 and XYR1. *In silico* analyses of the promoter regions of the aforementioned transporters have uncovered a fine and orchestrated regulatory

mechanism that is essential for the correct functioning of holocellulolytic machinery (Figure 3). The carbon catabolite repressor, CRE1, has the greatest regulatory effect on sugar transporters, which has been evidenced by the higher density of potential binding motifs in the promoter regions of its target genes. The positive regulator, XYR1, also appears to be a direct regulator of most of the described transporters, especially for the proteins, CDT1, STR1, and CLTA. Similar to that found for the CAZymes, the TF CLR1 does not seem to be a direct regulator of the transporters involved in (hemi)cellulose degradation, and the remaining TFs have exhibited variable regulatory activities in the promoter regions of the analyzed transporters. Dos Santos Castros *et al*. further studied the transporters regulated by TFs and found that the expression of 77 genes encoding the transporters was modulated by XYR1 in a carbon source-dependent manner. These transporters included the ATP-binding cassette (ABC) transporter, sugar transporter, major facilitator superfamily (MFS) permeases, sugar permeases, and amino acid transporters (Dos Santos Castro et al., 2016). These studies indicate that sugar transporters are important potential targets of TFs relative to the holocellulolytic system. Thus, regulating the expression of these transporter-encoding genes is vital for the precise recognition and capture of nutrients present in the environment. Future work on efficient transport of nutrients should consider the development of mutant strains expressing multiple copies of specific transporters and the expression of transporters under the control of strong promoters. In addition, expression of disaccharide transporters such as cellobiose and xylose could be a good strategy to develop yeasts that can produce bioethanol in consolidated systems.

4. Transcription factors from lignocellulolytic fungi

The production of enzymes involved in the degradation of plant biomass requires orchestrated regulation dependent on the performance of several TFs. An overview of the regulation of plant cell wall degradation by activator and repressor proteins has been exemplified for the model fungus of cellulase production, *T. reesei* (Figure 4).

Biomass-degrading fungi are widely distributed in the environment and have evolved efficient means of recognizing and capturing available nutrients without wasting energy. TFs play a fundamental role in the regulation of these processes. They can activate or repress their target genes (including those encoding CAZymes, sugar transporters, signaling proteins, other TFs, etc.) in a direct or indirect manner. A phylogenetic analysis of the main TFs involved in biomass degradation in fungi is shown in Figure 5. Most of the proteins belong to the Cys_2His_2 zinc finger (ZF) class, including the main activator, Xyr1, and its homologues. The second most evident class comprises the Cys_2His_2 TFs, among which the carbon catabolite repressor CRE1 and its homologues are included. Homeodomain and GATA-type TFs appear in smaller numbers. Although these regulatory proteins have a highly conserved structure between species, some differences can be observed regarding their target genes or the mechanisms by which they are activated or repressed. In this section, the main TFs involved in the degradation of the holocellulose polymers by the most studied biomass-degrading fungi are described.

4.1. XYR1/XlnR/Xlr1

The protein XYR1 (xylanase regulator 1, ortholog of XlnR and Xlr1) is a TF that is considered to be the main activator of cellulase and hemicellulase expression in most

biomass-degrading fungi. Deletions of *xlnR* in *A. niger* impair the fungus during the colonization of wheat bran particles due to the reduced potential of *A. niger* to secrete arabinoxylan and cellulose-degrading enzymes in the mutant strain (Kowalczyk et al., 2017). Additionally, a global transcriptome analysis of a Δ*xyr1* mutant of *T. reesei* showed that most of the genes regulated by XYR1 and the induction of carbon sources, such as cellulose and sophorose, are CAZymes, other TFs, and sugar transporter families (Dos Santos Castro et al., 2016). Recently, Cao *et al.* showed that XYR1 interacts with the protein TrSNF12 and recruits the SWI-SNF chromatin-remodeling complex at cellulase gene promoters, thus allowing efficient cellulase gene transcription (Cao et al., 2019).

4.2. CRE1/CreA

The Cys_2His_2-type TF CRE1/CreA is the main regulator of CCR in fungi. The repressive role of CRE1 has further been evidenced in the hypercellulolytic *T. reesei* Rut-C30. In this strain, a truncated form of *cre1* was identified and allowed for the production of holocellulases under glucose conditions (Peterson and Nevalainen, 2012). In 2014, Antoniêto *et al.* showed that CRE1 regulates its target genes in a carbon source-dependent manner in *T. reesei*. CRE1 recognized the glucose content in the medium and modulated the access of cellulases to the plant cell wall or blocked the entry of cellulase inducers into the cell (Antoniêto et al., 2014). Interestingly, the main activator of cellulase and hemicellulase expression, *xyr1*, was repressed by CRE1 in both *T. reesei* (Antoniêto et al., 2014) and *A. nidulans* (Tamayo et al., 2008) under glucose-repressing conditions. The repressive role of CRE1 was also described in *N. crassa* (Sun and Glass, 2011) and *P. oxalicum* (Yao et al., 2015). In these fungi, the

CCR mechanisms mediated by CreA were also involved in the decreased gene expression of the main cellulase- and hemicellulase-encoding genes.

4.3. ACE1, ACE2, and ACE3

Aro *et al*. investigated the role of activator of cellulase expression 1 (ACE1) in cellulase and xylanase gene expression, observing that the deletion of *ace1* resulted in the increased expression of the major cellulolytic and xylanolytic genes during growth within carbon sources. ACE1 is therefore a repressor of cellulase and xylanase gene expression in *T. reesei* (Aro et al., 2003). The cellulase activator, ACE2, was first identified in *T. reesei* by Aro *et al*. in 2001. Genes encoding cellulases and xylanases were downregulated in the mutant strain Δ*ace2*, and cellulolytic activity was also reduced in the mutant strain during growth in cellulose (Aro et al., 2001). In 2014, Häkkinen *et al*. identified ACE3 and found that its overexpression improved both cellulase and xylanase production in *T. reesei*. In contrast, the deletion of this gene resulted in the abolishment of cellulase activity and decreased xylanase activity. The gene expression levels of cellulolytic and hemicellulolytic genes were also affected by ACE3, which confirmed its role in biomass degradation (Häkkinen et al., 2014).

4.4. HAP complex

The HAP (Heme Activator Protein) complex remodels the structure of chromatin, thus enhancing transcription (Tsukagoshi et al., 2001). In *A. nidulans* and *A. oryzae*, the HAP complex is called HAP B/C/E and comprises three subunits: HapB, HapC, and HapE. Several genes have been identified as targets for the HAP complex in these fungi, including genes that encode acetamidase and those related to penicillin

356 biosynthesis (Hortschansky et al., 2017). The HAP complex of *T. reesei* (called HAP
357 2/3/5) was assessed by Zeilinger *et al*. (Zeilinger et al., 2003, 2001), who found that the
358 three proteins HAP2, HAP3, and HAP5 were necessary to bind to the CCAAT box in
359 the promoter of *cbh2 in vitro*.
360
361 **4.5. CLR1, CLR2, and CLR3**
362 Along with Xlr1, the TFs CLR1 and CLR2 are considered the main regulators of
363 cell wall degradation in *N. crassa*. These two proteins were identified in 2012 by
364 Coradetti *et al*., who demonstrated that mutants with deletions of these genes exhibited
365 a severe growth defect during growth in Avicel (Coradetti et al., 2012). Recently, the
366 TF CLR3 was described as a repressor of CLR1 activity in *N. crassa*. CLR3 represses
367 the expression of *clr1* in the absence of cellulose. However, in the presence of cellulose,
368 the repressive function of CLR3 is relieved, and CLR1 is able to repress genes related to
369 plant cell wall degradation, including *clr2* (Huberman et al., 2017). Beier *et al*. further
370 demonstrated that CLR1 and CLR2 are involved in pectinase and xylanase gene
371 expression in *T. reesei* and that this regulation is light-dependent. Furthermore, these
372 TFs have less homology in comparison to other ascomycetes (Beier et al., 2020).
373
374 **4.6. LAE1/VEL1 (LaeA/VeA)**
375 The VELVET complex comprises three proteins, namely LaeA, VeA, and VelB,
376 which are involved in the development of and secondary metabolism in *A. nidulans* (G.
377 Wang et al., 2019). The orthologs of LaeA and VeA in *T. reesei*, known as LAE1 and
378 VEL1, are two other regulators of plant cell wall degradation. Seiboth *et al*. showed that
379 the regulation of cellulase gene expression by the methyltransferase, LAE1, is

dependent on the main positive regulator, XYR1. In addition, *xyr1* transcription is also dependent on LAE1. Mutants carrying *lae1* exhibit reduced cellulolytic and xylanolytic activities during growth in lactose and xylan carbon sources. Genes encoding CAZymes are also affected by the deletion of *lae1* in *T. reesei* (Seiboth et al., 2012) and *P. oxalicum* (Li et al., 2016). These data reinforce the crucial role of the VELVET complex as a regulator of plant cell wall degradation.

4.7. AraR

The TF AraR is a zinc binuclear transcriptional regulator, Zn_2Cys_6, identified in the *Aspergillus* species, which is involved in the regulation of L-arabinose catabolism and activation of genes encoding α-L-arabinofuranosidases. During cultivation of *A. niger* in SCB, AraR was shown to regulate several genes encoding biomass-degrading enzymes. Together with XlnR, they are responsible for regulating the genes related to the metabolism of simple and complex sugars, and this regulation is dependent on the complexity of the substrate and its derivatives (De Souza et al., 2013). The involvement of AraR in the production of α-L-arabinofuranosidases has also been described in *P. oxalicum*. Deletions of this TF cause reduced growth in the presence of L-arabinose and decreased α-L-arabinofuranosidase activity in wheat bran-containing media (Gao et al., 2019a).

4.8. PacC/Pac3/Pac1

The role of pH in the production of biomass-degrading enzymes was described for several fungal species, and the TF that regulates the pH signaling pathway is the protein PacC. An ortholog of PacC, known as Trpac1, was identified in *T. reesei*. Under

neutral pH, deletions of this gene resulted in increased cellulolytic activity and transcription levels of cellulase-encoding genes. The positive regulators of cellulase production, *xyr1* and *ace2*, were also regulated by Trpac1 (He et al., 2014). This indirect regulatory mechanism was also observed in *N. crassa*. Antoniêto *et al*. showed that the deletion of *pac3*, which is the ortholog of *pacC* in the *Aspergillus* species, resulted in decreased cellulolytic activity at alkaline pH. In addition, xilanase production was positively regulated by PAC3 under alkaline, neutral, and acidic pH. Furthermore, the TFs *xlr1*, *cre1*, *clr1*, and *clr2* were also regulated by PAC3 in a pH-responsive manner (Antoniêto et al., 2017).

4.9. Other TFs involved in biomass degradation

Many TFs have been identified and characterized in fungi, and many of these proteins are involved in the regulation of biomass-degrading genes. In *T. reesei*, the TFs, CTF1 (Q. Meng et al., 2020) and RCE1 (Cao et al., 2017), have been described as repressors of cellulase production. The latter binds in the same motif as Xyr1 in the *cbh1* promoter, thus acting as an antagonist of Xyr1. Xyr1 is also involved with the positive regulator, RXE1. This TF strongly binds to the *xyr1* promoter and regulates its transcriptional expression and the production of cellulase in *T. reesei* (L. Wang et al., 2019a). The expression of genes encoding β-glucosidases has been found to be positively regulated by the TF BglR, and mutants carrying the deletion of this regulator have exhibited the reduced capacity to hydrolyze cellobiose in *T. reesei* (Nitta et al., 2012). Another TF that has recently been described as a positive regulator of plant cell wall-degrading genes in *T. reesei* is AZF1. Deletions of *azf1* result in diminished transcriptional levels of genes encoding CAZymes, transporters, and other TFs during

428 growth in SCB (Antonieto et al., 2019). Similar to AZF1, the homeodomain CLP1 acts
429 as a positive regulator of cellulase genes in *T. reesei* (L. Wang et al., 2019b). The
430 calcium signaling pathway is also involved in (hemi)cellulase gene expression and
431 secretion in *T. reesei*, and the TF CRZ1 has further been identified as a key regulator of
432 the fungus (Martins-Santana et al., 2020). The TF ARE1 is also a positive regulator of
433 the main cellulase genes, including *cbh1*, *cbh2*, *egl1*, and *egl2*, in *T. reesei* (Qian et al.,
434 2019). XPP1 is described as a regulator of hemicellulose degradation, but no
435 involvement with cellulase production has been observed (Derntl et al., 2015). VIB1 is
436 another regulator involved in plant cell wall degradation in filamentous fungi. In *N.*
437 *crassa*, VIB1 regulates the transcription of CAZymes by affecting the expression of
438 *clr2* (Xiong et al., 2014). In *P. oxalicum*, deletions of the novel TF ATF1 have been
439 found to result in increased cellulase and xylanase production in media containing
440 wheat bran and rice straw (Zhao et al., 2019).
441
442 **5. Engineering gene expression to enhance cellulase production in filamentous**
443 **fungi**
444 Given the central role of TFs in the regulation of plant cell wall degradation,
445 these proteins are important targets in genetic engineering strategies for the construction
446 of strains to increase cellulase production and degrade lignocellulosic biomass (Renato
447 Graciano De Paula et al., 2019). The next section discusses the most recent and
448 significant studies involving TFs and promoter engineering aimed at improving the
449 production of holocellulase in filamentous fungi.
450
451 **5.1. TF engineering**

452 The overexpression of transcriptional activators and deleting or silencing
453 repressors are approaches that have been widely used to increase the production of
454 holocellulases. In addition, strategies involving the rational engineering of TFs have
455 emerged, including point and truncated mutations, the fusion of domains from different
456 TFs, and construction of artificial TFs (Figure 6).

457 In 2019, Liu *et al.* introduced the CRE1 truncated sequence from *T. reesei* RutC-
458 30 in the mutant strain SS-II and generated the strain SS-II-*cre1*$_{96}$, which exhibited
459 significantly higher cellulase activity than SS-II after 5 days of being cultured (Liu et
460 al., 2019). Another TF truncated in *T. reesei* RutC-30 is the positive regulator, ACE3. A
461 missense mutation was found to generate a stop codon and resulted in ACE3-723,
462 which is 11 amino acids shorter at the C-terminus. Similar to CRE1$_{96}$, ACE3-723 is
463 crucial for cellulase hyperproduction in RutC-30 (Chen et al., 2020). The *ace3* truncated
464 sequence was further inserted into the strain PC-3-7. This strain contains a mutation in
465 the gene, *bgl2*, that results in reduced hydrolysis activity and subsequent relief from
466 CCR. Corn straw saccharification by the mutant strain, PC-3-7-723 (carrying the
467 truncated *ace3*), produced more glucose than the controls, PC-3-7, and RutC-30. In fed-
468 batch fermentation using a mixture of glucose and β-disaccharides as a substrate, PC-3-
469 7-723 increased approximately 20–30% of cellulase activity compared to the parental
470 strain (Chen et al., 2020).

471 Mutagenesis further triggers mutations in the main cellulase expression
472 activator, XYR1, in *T. reesei.* The Iogen M8 strain was obtained after two rounds of
473 mutagenesis and exhibited elevated xylanase activity. This was caused by a point
474 mutation in XYR1 (A824V), which resulted in a high level of cellulase and
475 hemicellulase expression under inducer and non-inducer conditions (Derntl et al., 2013).

In *A. niger,* the mutation V756F in XlnR also resulted in elevated xylanase expression, even under repressing conditions (Hasper et al., 2004). An analysis of the effects of these point mutations in TFs allowed for the construction of strains to improve cellulase production (Figure 6). The substitution of the amino acid valine for phenylalanine at position 821 in XYR1 in *T. reesei* and the overexpression of this mutated TF increased the production of cellulases and xylanases when *T. reesei* was grown in lactose and glucose (Fonseca et al., 2020). In AraR, a close homolog of XlnR that regulates α-L-arabinofuranosidase genes, the mutation A731V led to the active expression of α-L-arabinofuranosidases (Gao et al., 2019a). Furthermore, when the mutations XYR1^{A824V} (from *T. reesei)* and XLR-1^{A828V} (from *N. crassa)* were introduced in *P. oxalicum*, the fungus was able to activate the expression of cellulolytic genes (Xia et al., 2019). Recently, Han *et al*. induced several mutations to modify phosphorylation sites in the C-terminus of CRE1 in *T. reesei* and found that the mutation, S388V, increased FPase and pNPCase activity when the fungus was cultured in glucose-containing media (Han et al., 2020b). Regulatory modifications, such as phosphorylation and dephosphorylation, are important for the effectiveness of TFs in cells. Therefore, mimicking them through point mutations may be an excellent method to relieve CCR and increase the production of (hemi)cellulases.

An analysis of the repressor CreA in the strain JUA10-1 of *P. oxalicum* further revealed a frameshift mutation at the C-terminus that is important for cellulase hyperproduction. The introduction of this mutation in *T. reesei* CRE1 alleviated CCR and increased the expression of *cel7a* in the presence of glucose (Han et al., 2020a). In addition, the truncation of Mig1, which is a homologue of CRE1/CreA and the main repressor of cellulolytic gene transcription in *Penicillium funiculosum* NCIM1288,

caused the loss of its ZF domain and catabolic carbon derepression (Figure 6G). The resulting strain, PfMig1^{88}, exhibited increased cellulase production than the control in inducing and non-inducing conditions (Randhawa et al., 2018). Comparative genomics analyses involving hypercellulolytic strains can therefore reveal the mutations that drive TF engineering with increased or constitutive activity in induced (Figure 6D) or repressed sources (Figure 6G) for the increased production of cellulases in filamentous fungi.

Another approach for rational TF engineering is domain fusion, which consists of the fusion of the N-terminus domain from a protein with the C-terminus domain from another protein by a linker, or the insertion of one domain into another at a specific position in a protein (Renato Graciano De Paula et al., 2019). The construction of these chimeric TFs is a potent strategy to modify the expression of target genes and yield strains with desired phenotypes (Figure 6). In 2009, Su *et al*. fused the DNA binding domain (DBD) of CRE1 to the effector domain (ED) of the activator, ACE2, and the latter was inserted into the DBD of another transcriptional repressor, ACE1, under the regulation of the CRE1 promoter itself. The engineered TF was expressed in the *T. reesei* strain, RutC-30, and resulted in elevated levels of cellulase and hemicellulase expression and the increased activity of these enzymes (Su et al., 2009).

Zhang *et al*. also used this approach to construct an artificial ZF protein (Azfp) library comprising four ZFs as DBDs (Zhang et al., 2020, 2016). They were fused to the Gal4 effector domain in *S. cerevisiae* and overexpressed in *T. reesei* using the robust promoter, P*pki*. In the obtained transformants, the activities of FPase and CMCase and biomass hydrolysis rate dramatically increased compared to those in the parental strains (Q. Meng et al., 2020). The utilization of the ED from XYR1 instead of the original

524 Gal4 from *S. cerevisiae* optimized the Azfp and resulted in efficient biomass conversion
525 (Q.-S. Meng et al., 2020). Likewise, promising results were obtained when the XYR1
526 ED was fused to the CRE1 DBD (Figure 6G). The resulting artificial regulator caused
527 constitutive cellulase and hemicellulase production when the strain was cultivated with
528 glucose as a carbon source, with a production that was 12.75-fold higher than that
529 yielded by the RutC30 strain (Zhang et al., 2017).

530 The production of cellulase using glucose as a carbon source was also yielded
531 when the DBD of CRE1 was fused with the strong activation domain VP16 of the
532 herpes simplex virus. The resulting strain presented the constitutive production of
533 cellulases that was 26.5-fold higher than that yielded by the parental strain (Zhang et al.,
534 2018b). The VP16 ED was also fused to the DBD of other *T. reesei* TFs, including
535 XYR1, ACE2, and ACE1. The artificial regulator TXYR1VP abolished cellulase
536 production but yielded increased xylanase activity per unit of biomass compared to
537 RutC-30, while the other chimeric TFs yielded the increased production of cellulase and
538 xylanase using inducing carbon sources (Zhang et al., 2018a, 2018b). The authors of
539 this study speculated that the absence of cellulase activity in the strain expressing
540 TXYR1VP occurred because of the formation of the heterodimer with the native XYR1
541 (Zhang et al., 2018a). To address this problem, an XYR1-deficient strain was
542 constructed for the insertion of artificial TFs. They, in turn, carried the DBD of XYR1
543 and ED of the Gal4-like TFs YPR1 and YPR2 (Yellow pigment regulator 1 and 2) from
544 *T. reesei*. The resulting strains exhibited xylanase and cellulase activity when grown in
545 different soluble carbon sources such as lactose, glucose, and glycerol. The strain TXY1
546 (XYR1::YPR1) presented high levels of β-glucosidase activity compared to the control
547 (Derntl et al., 2019).

548 Wang *et al*. further constructed new TFs by intercalating the DBDs of the
549 repressors, CRE1 and ACE1, with the EDs of the activators, XYR1, ACE2, ACE3, or
550 CLR2. The fused TFs presented higher levels of *cel7a* expression, especially those with
551 the XYR1 and ACE2 domains, which also exhibited the highest cellobiohydrolase
552 activity when using lactose as a carbon source (F. Wang et al., 2019). The construction
553 of chimeric TFs was applied to another cell factory, *P. oxalicum*. Gao *et al*. fused the
554 DBD of ClrB to the regulatory and activation domain of $XlnR^{A871V}$, which contains the
555 *T. reesei* homologous mutation, A824V. The overexpression of the new TF resulted in
556 the production of cellobiohydrolase without any carbon source and FPase activity that
557 was seven-fold higher than that of the parental strain when grown in media containing
558 cellulose and xylan (Gao et al., 2017).

559 The overexpression of native or artificial TFs is a great strategy to enhance
560 cellulase production. However, when a (hemi)cellulase repressor or non-inducing
561 soluble source is utilized, this production may not be significant. TFs contain a
562 regulatory domain in their middle region that plays a fundamental role in their activity,
563 subcellular localization, and interaction with DNA (Cziferszky et al., 2002). This
564 internal domain can be regulated by post-transcriptional modifications, which may be
565 involved in the inhibition of their activity in the presence of non-inducer sources
566 (Ribeiro et al., 2019). In *P. oxalicum*, this problem was solved by deleting the internal
567 regulatory region in the TF ClrB. The DBD at the N-terminus and activator domain at
568 the C-terminus were then directly fused. The internal deletion in ClrB moderately
569 enhanced cellulase production in cellulose-containing media and increased it by 10-fold
570 compared to the parental strain when cultivation was carried out using soluble sources
571 (Gao et al., 2019b).

572 Modulating the expression of genes involved in the degradation of
573 lignocellulosic biomass to obtain strains that produce larger amounts of cellulases and
574 hemicellulases is a promising approach. TF engineering permits the increase or
575 constitutive production of cellulases in engineered strains using different sources of
576 carbon. This strategy makes it possible to use carbon sources, including glucose
577 (holocellulase repressor) or glycerol (neutral). They may be significant in industrial
578 applications as they are easier to apply operationally, such as during mixing, feeding,
579 and filtering, than insoluble inducing sources (Randhawa et al., 2018).

580 Overexpression or deletion of TFs that up- or down-regulate holocellulase
581 expression seems to be a good strategy to obtain superproducing strains of
582 holocellulolytic enzymes. However, future work should consider the systems biology of
583 biomass-degrading fungi to make rational decisions. The network of gene expression
584 regulated by transcription factors in these fungi appears to be more complex than
585 thought.

586

587 **5.2. Promoter engineering**

588 Promoters are regulatory regions that are upstream of the transcription start
589 codon. TFs recognize specific sequences in these DNA regions and bind to these *cis*-
590 regulatory elements to activate or repress gene expression. Therefore, manipulating the
591 binding motifs of these regulators is a promoter engineering strategy in the construction
592 of new promoters with greater strength and different functions in modulating cellulase
593 gene expression (Jin et al., 2019). In filamentous fungi, there are several native
594 promoters that are used to drive gene expression. These include constitutive promoters,
595 such as housekeeping genes of the glycolytic pathway that have a constant rate of gene

596 expression, or tunable promoters with expression that is dependent on the presence or
597 absence of an inducer or repressor (Figure 7A and 7B) (Fitz et al., 2018). However,
598 there is not substantial research on promoter engineering in filamentous fungi.

599 A rational approach in promoter engineering is through the use of the *cis*-
600 regulatory elements present in target promoters. Their insertion or deletion is a way to
601 change the characteristics of the promoter and the activation or repression of its target
602 gene (Figure 7C) (Fitz et al., 2018). In *T. reesei*, the promoter of the *cbh1* gene (P*cbh1*)
603 is the most well-described and widely used for heterologous expression (Kiesenhofer et
604 al., 2018), with its expression induced by its degradation products and cellulose (Renato
605 Graciano De Paula et al., 2019). This promoter has three binding sites for CRE1, which
606 reduces *cbh1* transcription in the presence of glucose. Deletions of these CRE1 binding
607 sites in P*cbh1* allows for the detection of transcripts from reporter genes in glucose-
608 containing media (Ilmén et al., 1996). Zou *et al*. replaced these CRE1 sites with
609 activation sites, such as those from the positive regulators ACE2 or HAP2/3/5, thereby
610 generating the cbh1pM2 promoter, which exhibited increased gene expression and
611 reductions in catabolic carbon repression (Zou et al., 2012). P*cbh1* also possesses eight
612 binding sites for the repressor, ACE1. When using cbh1pM2 as the starting promoter,
613 Sun *et al*. replaced the sites for the binding motifs with the activators ACE2 or XYR1.
614 The new promoters presented a 3.6- and 5-fold increase, respectively, in the expression
615 of an *A. niger* mannanase, which was used as a reporter gene in this case (Sun et al.,
616 2020).

617 A comparative analysis of *cis*-regulatory elements for XYR1 in the promoters of
618 *cbh1* and *xyn1* revealed that the presence of *cis*-elements, which are inverted and
619 repeated sequences, plays a fundamental role in promoter inducibility. The insertion of

these sequences coupled to the rearrangement of XYR1 binding sites positively impacted the strength of the *cbh1* promoter, especially during induction by xylan and wheat bran (Kiesenhofer et al., 2018). The insertion and rearrangement of *cis*-elements for positive regulators (Figure 7D), such as the CCAAT binding motif, also increased the expression of heterologous genes in fungi other than *T. reesei* (Liu et al., 2008), such as *A. niger* (Liu et al., 2003) and *A. oryzae* (Minetoki et al., 1998). Synthetic promoters can also be built using synthetic biology with only minimal constituents that are essential for transcription and *cis*-regulatory elements that can optimize gene expression (Fitz et al., 2018) and independently regulate carbon sources (Figure 7E) (Renato Graciano De Paula et al., 2019).

6. Conclusions

Ethanol derived from plant biomass has exhibited benefits in the field of energy. It is considered a clean and sustainable form of energy in addition to being economically beneficial as it allows for less dependence on fossil fuels. Filamentous fungi possess a robust set of cellulases that act synergistically in the degradation of plant biomass. In this sense, TFs play a key role as they regulate the expression of cellulase genes. It is further laborious to apply strategies like those mentioned earlier for many promoters. Despite this, promoter engineering has proven to be a powerful tool for building hypercellulolytic strains with promoters with greater inducibility and independence from carbon sources. The transcriptional regulation of cellulolytic genes, including the TFs that are involved, has emerged as an important mechanism in the degradation of lignocellulose biomass by filamentous fungi. Deciphering these complex networks and regulatory mechanisms may allow for the development of new strains that yield the

increased or constitutive production of cellulases. Several approaches that have been developed to achieve this goal have been highlighted in this review. The rational engineering of the holocellulolytic system from industrially relevant microorganisms may improve the efficiency of saccharification and, consequently, ethanol production. It is essential to elucidate the molecular mechanisms underlying the regulation of this system to improve the industrial processes that are aimed at producing energy from clean and sustainable sources, such as bioethanol. The next step is to ensure that these biosources are suitable for industrial applications to overcome the bottlenecks present in the production of cellulases.

Acknowledgments

This work was supported by The State of São Paulo Research Foundation (FAPESP) (proc. 2019/11655-4 and 2018/03766-8). VKG would like to acknowledge the institutional funding sources supported by the SRUC, UK, for research and development. The work conducted by the US Department of Energy Joint Genome Institute is supported by the Office of Science of the US Department of Energy under contract no. DE-AC02-05CH11231.

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Conflicts of interest

The authors declare no conflicts of interest.

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Figure Legends

Figure 1. CAZyme-encoding genes are direct targets for transcription factors involved in biomass degradation. The binding motifs for the transcription factors

(TFs) XYR1, CRE1, ACE2, PacC, CLR1, and CLR2 were searched for in the promoter region (1.5 Kb) of genes encoding the CAZymes of *T. reesei*. The enzymes were grouped according to their related polysaccharides. TF binding motifs were counted by heatmaps created using the heatmap.2 function of gplots-R.

Figure 2. The key role of transporters during sugar uptake and the regulation of holocellulases. Holocellulose is mainly composed of cellulose and hemicellulose. Fungi belonging to genera *Trichoderma*, *Aspergillus*, *Neurospora*, and *Penicillium* have developed fine mechanisms to uptake the sugar released from the plant biomass and distinct transporter proteins responsible for sugar internalization. These sugars act by inducing the expression of specific TFs that are responsible for regulating the expression of holocellulolytic genes involved in holocellulose breakdown.

Figure 3. Transporter-encoding genes are direct targets for TFs involved in biomass degradation. The binding motifs for the TFs XYR1, CRE1, ACE2, PacC, CLR1, and CLR2 were searched for in the promoter region (1.5 Kb) of transporter encoding-genes. TF binding motifs were counted with heatmaps created using the heatmap.2 function of gplots-R.

Figure 4. Transcriptional regulation of the holocellulolytic system in the model fungus, *T. reesei*. In *T. reesei*, at least 13 positive regulators and six repressors of (hemi)cellulase production have been identified. The proteins, under inducing or repressing conditions, bind to their target genes (CAZymes, sugar transporters, signaling proteins, other TFs, etc.) to regulate their transcription.

Figure 5. Phylogenetic analysis of the main TFs related to (hemi)cellulase regulation in fungi. The analysis includes proteins from *Trichoderma*, *Aspergillus*, *Neurospora*, and *Penicillium*. The TFs cited in this review are highlighted in bold.

Protein sequences of selected TFs were aligned using MAFFT v7.407 (Katoh et al., 2018). Ambiguously aligned regions were removed using the -automated1 function of trimAl 1.4rev22 (Capella-Gutiérrez et al., 2009). The approximate maximum likelihood phylogenetic tree was inferred using FastTree v2.1.10 (Price et al., 2010) and visualized with Figtree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Figure 6. Rational engineering of TFs. The breakdown of cellulose by cellulases (A) releases cello-oligosaccharides that are transported to the cytosol and activate cell signaling (B). Positive TFs activate the transcription of cellulolytic genes (C). TFs with point or truncated mutations and chimeric TFs (with heterologous or artificial domains – Chimeric TF I) also activate the transcription of these genes (D), thereby increasing the production of cellulases (E). The glucose resulting from cellulose degradation activates the catabolic repression of carbon through cellular signaling (F). Mutant and chimeric TFs (with an ED from an activator and DBD from a repressor – Chimeric TF II) with constitutive activity can activate the transcription of cellulolytic genes (G). However, the repression of cellulase expression continues to occur and is mediated by negative TFs (H).

Figure 7. Toolset of promoters to drive gene expression. (A) Constitutive promoters. (B) Tunable promoters with expression that is activated/repressed in the presence of a stimulus. (C) Promoter engineering by the replacement of repressor binding sites (red squares) for activator binding sites (green squares). (D) Promoter engineering by the rearrangement of *cis*-regulatory elements (golden bars). (E) Synthetic promoter with minimal constituents. TF, transcription factor; TA, transcription activator; TR, transcription repressor.

Figure 1.

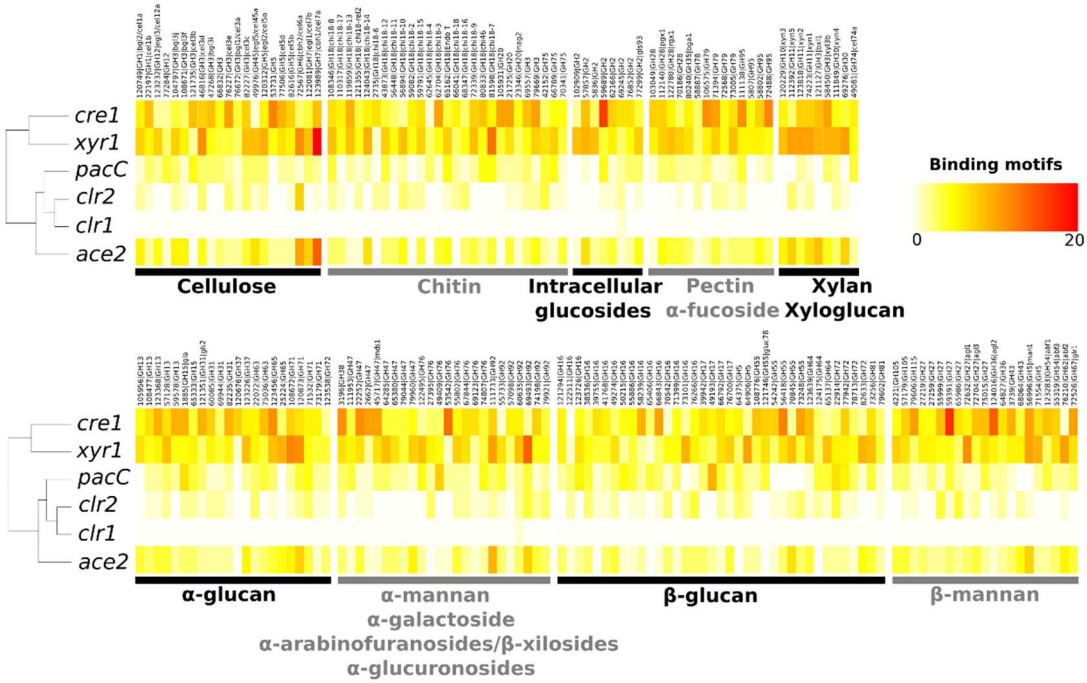

cre1
xyr1
pacC
clr2
clr1
ace2
Binding motifs
0
20
Cellulose
Chitin
Intracellular glucosides
Pectin
α-fucoside
Xylan
Xyloglucan
α-glucan
α-mannan
α-galactoside
α-arabinofuranosides/β-xilosides
α-glucuronosides
β-glucan
β-mannan

Figure 2.

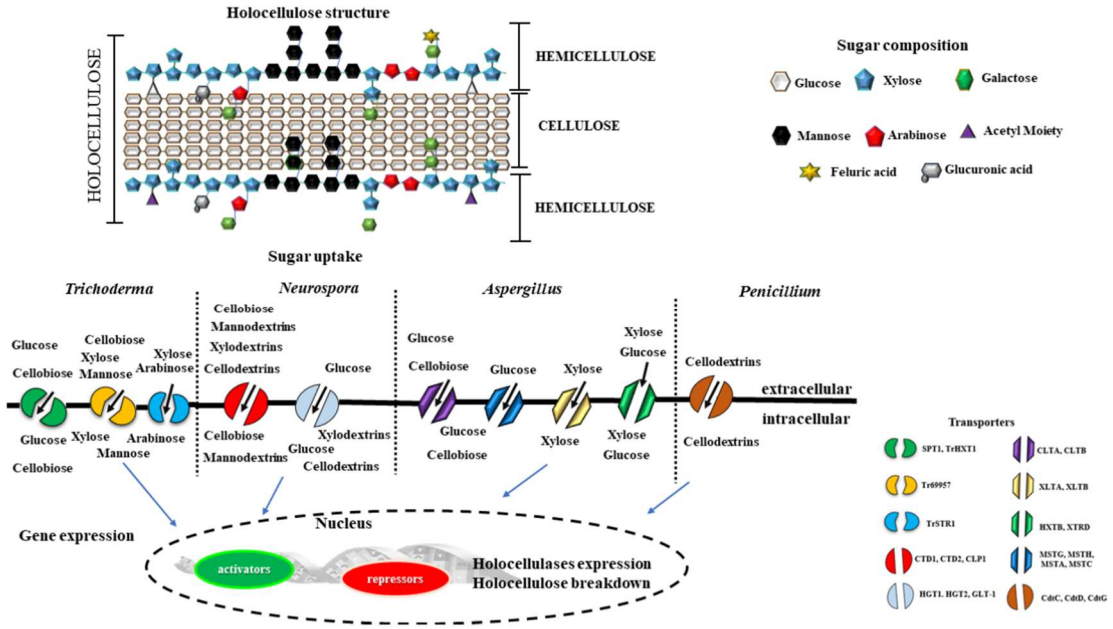

Holocellulose structure
HOLOCELLULOSE
HEMICELLULOSE
CELLULOSE
HEMICELLULOSE
Sugar composition
Glucose
Xylose
Galactose
Mannose
Arabinose
Acetyl Moiety
Feluric acid
Glucuronic acid
Sugar uptake
Trichoderma
Neurospora
Aspergillus
Penicillium
extracellular
intracellular
Gene expression
Nucleus
activators
repressors
Holocellulases expression
Holocellulose breakdown
Transporters

Figure 3.

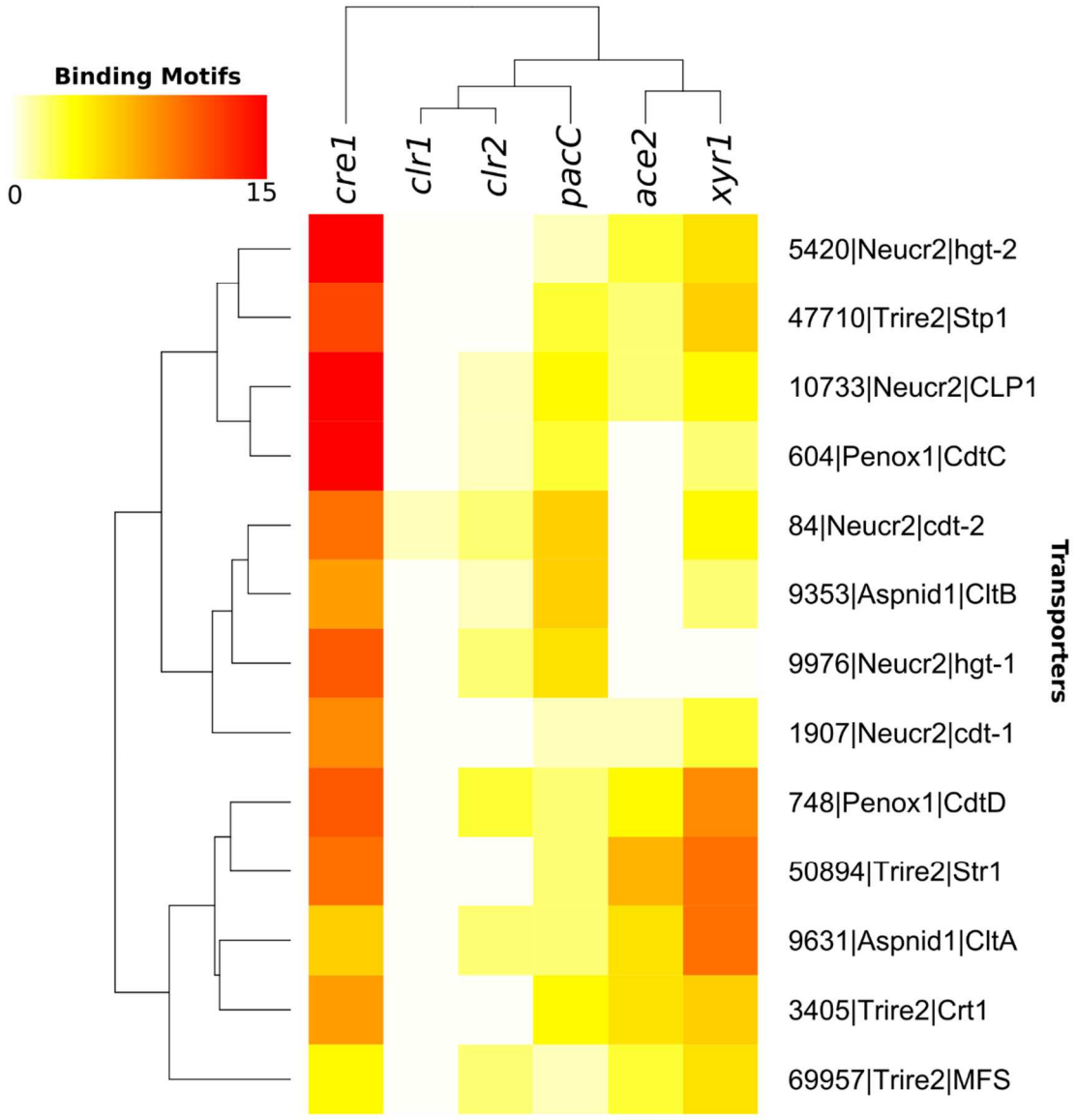

Binding Motifs
0
15
cre1
clr1
clr2
pacC
ace2
xyr1
5420|Neucr2|hgt-2
47710|Trire2|Stp1
10733|Neucr2|CLP1
604|Penox1|CdtC
84|Neucr2|cdt-2
9353|Aspnid1|CltB
9976|Neucr2|hgt-1
1907|Neucr2|cdt-1
748|Penox1|CdtD
50894|Trire2|Str1
9631|Aspnid1|CltA
3405|Trire2|Crt1
69957|Trire2|MFS
Transporters

Figure 4.

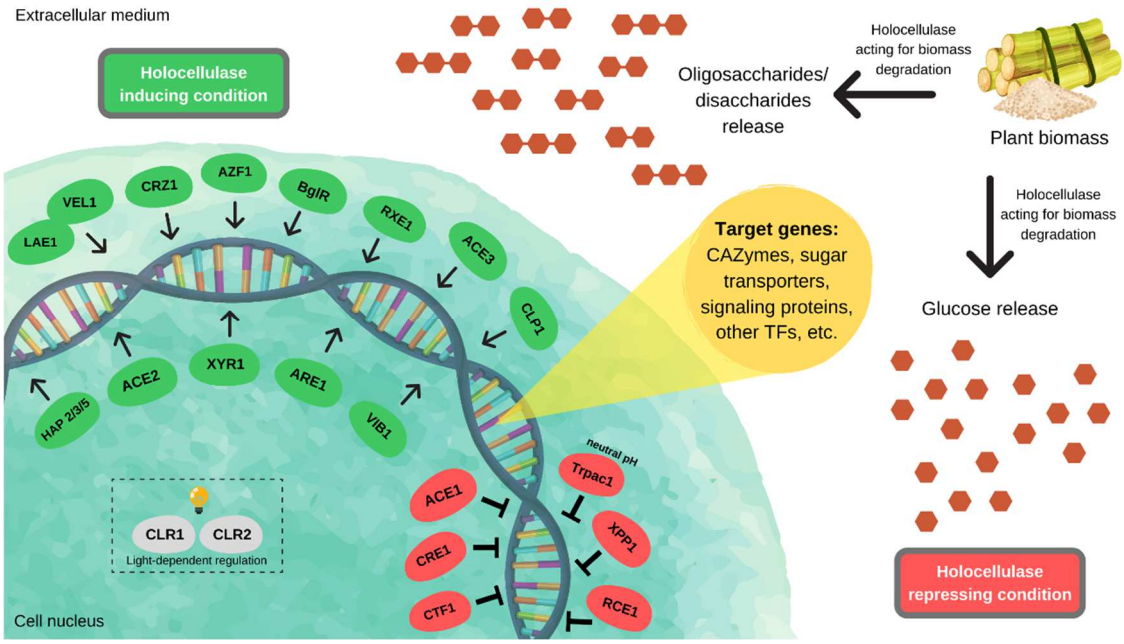

Extracellular medium
Holocellulase inducing condition
Oligosaccharides/ disaccharides release
Holocellulase acting for biomass degradation
Plant biomass
Holocellulase acting for biomass degradation
Glucose release
Target genes: CAZymes, sugar transporters, signaling proteins, other TFs, etc.
VEL1
LAE1
CRZ1
AZF1
BglR
RXE1
ACE3
CLP1
ACE2
XYR1
ARE1
VIB1
HAP 2/3/5
CLR1
CLR2
Light-dependent regulation
neutral pH
Trpac1
ACE1
CRE1
CTF1
XPP1
RCE1
Holocellulase repressing condition
Cell nucleus

Figure 5.

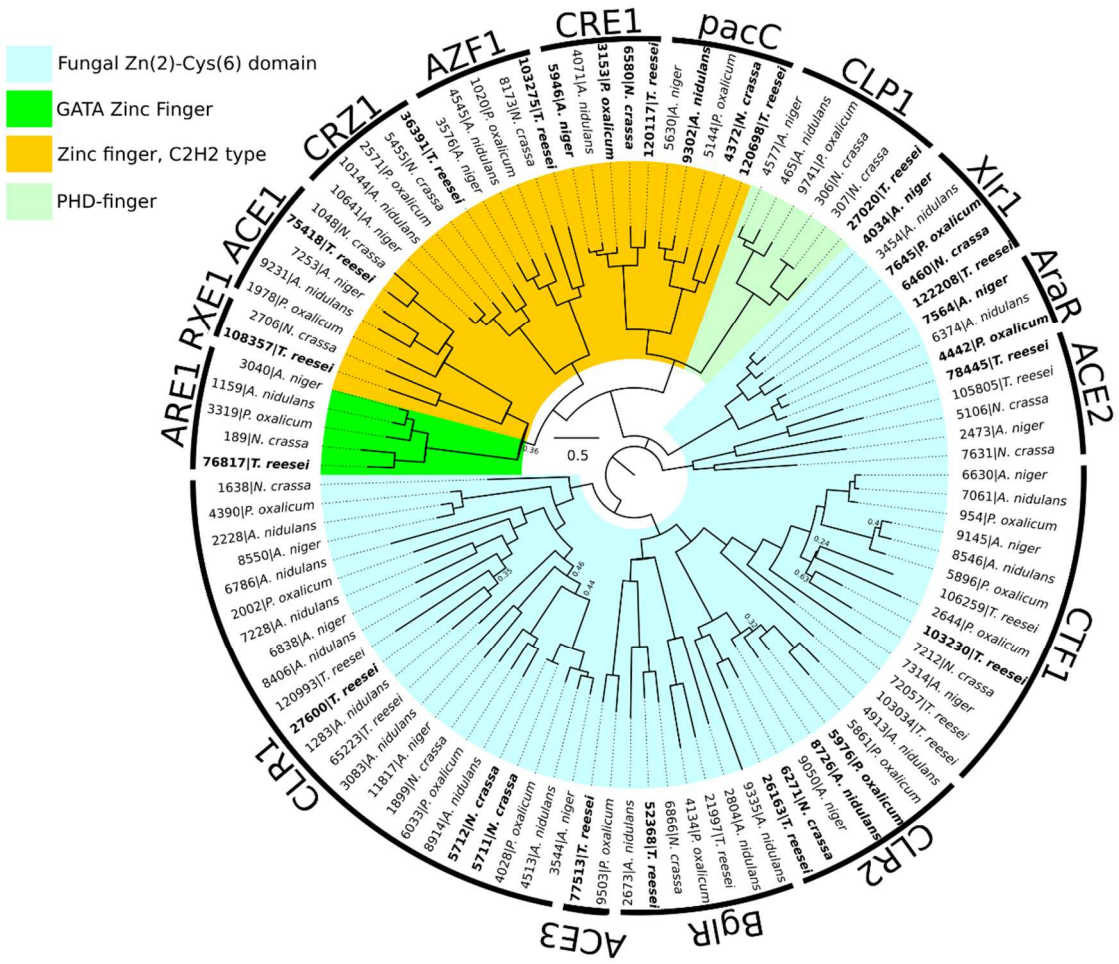
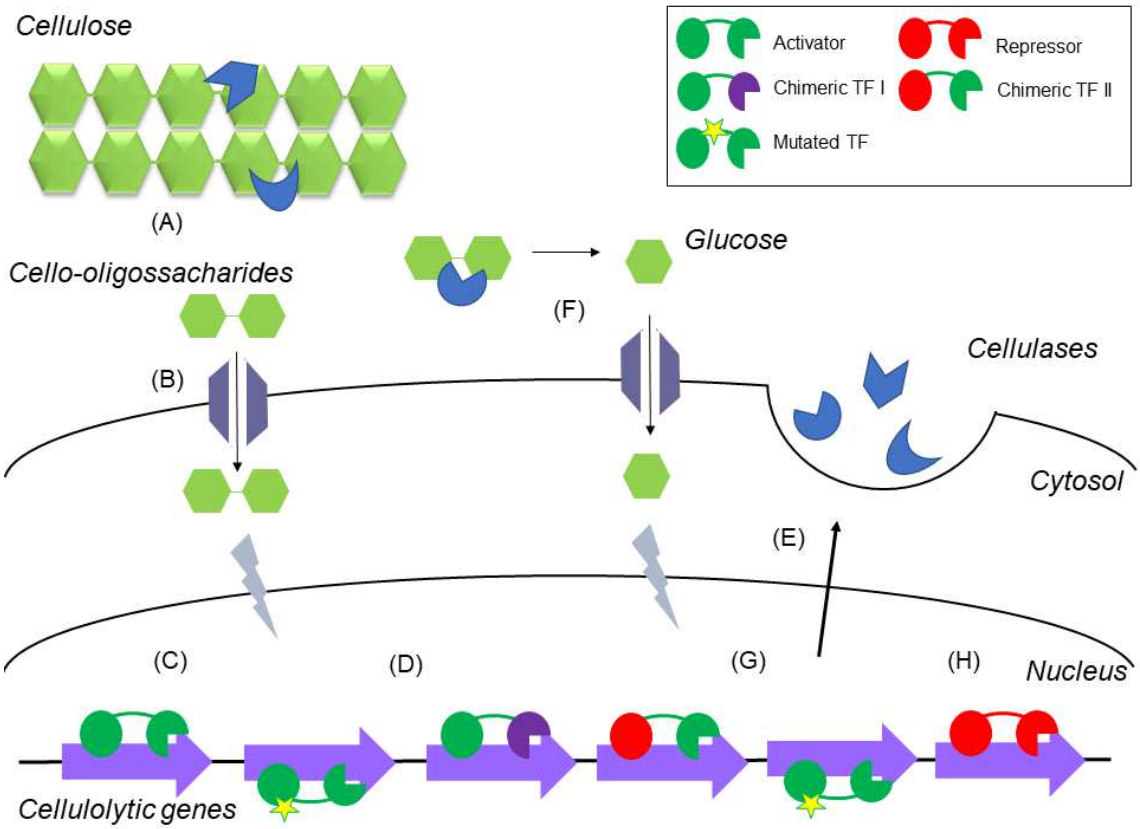
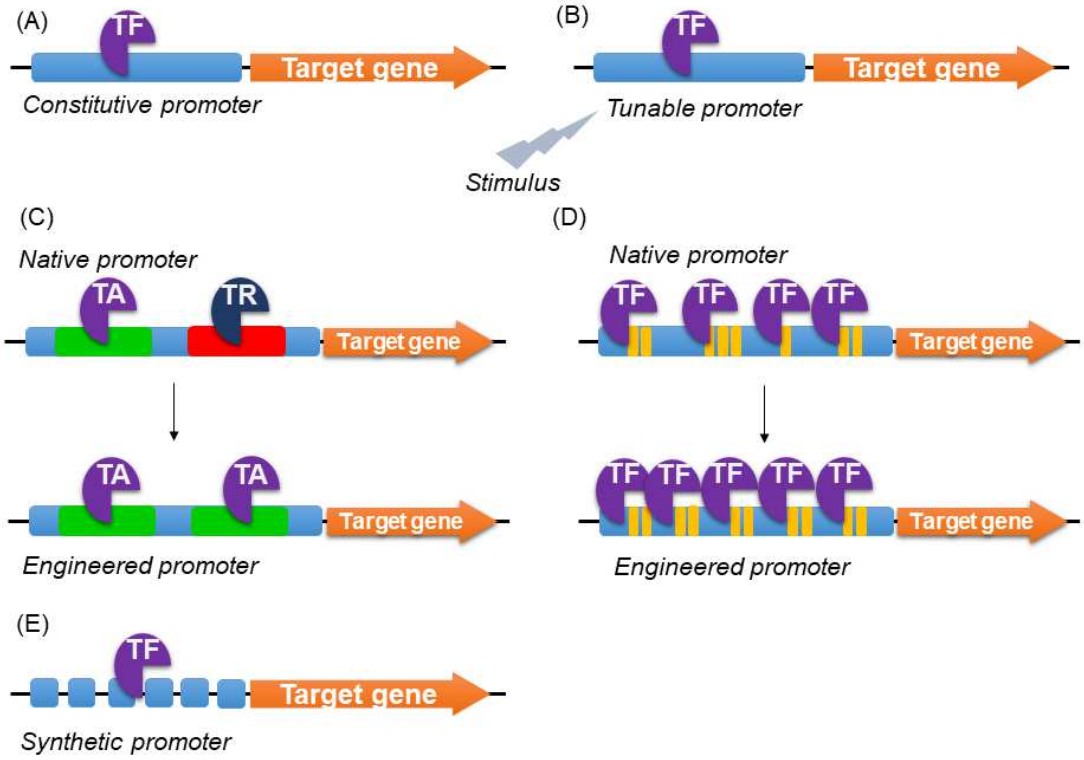

Fungal Zn(2)-Cys(6) domain
GATA Zinc Finger
Zinc finger, C2H2 type
PHD-finger
CRE1
pacC
CLP1
XlnR
AraR
ACE2
CTF1
CLR2
BglR
ACE3
CLR1
ARE1
RXE1
ACE1
CRZ1
AZF1
0.5

Figure 6.


Cellulose
Activator
Repressor
Chimeric TF I
Chimeric TF II
Mutated TF
(A)
Cello-oligossacharides
Glucose
(F)
(B)
Cellulases
Cytosol
(E)
(C)
(D)
(G)
(H)
Nucleus
Cellulolytic genes

1245 Figure 7.



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