Genetic engineering of *Trichoderma reesei* cellulases and their production

Irina S. Druzhinina* and Christian P. Kubicek†

Microbiology Group, Research Area Biochemical Technology, Institute of Chemical, Environmental and Biological Engineering, TU Wien, Vienna, Austria.

Summary

Lignocellulosic biomass, which mainly consists of cellulose, hemicellulose and lignin, is the most abundant renewable source for production of biofuel and biorefinery products. The industrial use of plant biomass involves mechanical milling or chipping, followed by chemical or physicochemical pretreatment steps to make the material more susceptible to enzymatic hydrolysis. Thereby the cost of enzyme production still presents the major bottleneck, mostly because some of the produced enzymes have low catalytic activity under industrial conditions and/or because the rate of hydrolysis of some enzymes in the secreted enzyme mixture is limiting. Almost all of the lignocellulytic enzyme cocktails needed for the hydrolysis step are produced by fermentation of the ascomycete *Trichoderma reesei* (Hypocreales). For this reason, the structure and mechanism of the enzymes involved, the regulation of their expression and the pathways of their formation and secretion have been investigated in *T. reesei* in considerable details. Several of the findings thereby obtained have been used to improve the formation of the *T. reesei* cellulases and their properties. In this article, we will review the achievements that have already been made and also show promising fields for further progress.

Introduction

Plant biomass is the most abundant renewable source for conversion to biofuel and biorefinery products. It consists of lignocellulose primary made of various polysaccharides and the aromatic polymer lignin, which provide mechanical strength to the plants and render them highly resistant to attack from pathogens (Houston *et al.*, 2016). The main polysaccharides in lignocellulose comprise the glucohompolyasaccharide cellulose (20–50%, w/w) and hemicelluloses (15–35%, w/w), which – depending on the plant–are primarily made up of a xylan, glucuronoxylan, xyloglucan, glucomannan and arabinoxylan backbones with heterogeneous side-chains (Kubicek, 2013; Álvarez *et al.*, 2016).

The use of the monosaccharides that constitute the plant biomass polymers implies their efficient hydrolysis, which – because of its recalcitrance and heterogeneity – is still a major technical challenge. To achieve this, the plant debris have first to be reduced in size by milling or chipping, followed by a mild chemical or physicochemical pretreatment step to make the biomass more susceptible to hydrolysis by the enzymes. Finally, enzymatic hydrolysis depolymerizes cellulose to D-glucose and the still present hemicelluloses to monosaccharides such as D-glucose, D-xylose and L-arabinose. These two steps are known to be the cost-intensive part of biomass conversion (Brethauer and Studer, 2015; Payne *et al.*, 2015; Gupta *et al.*, 2016a; Kubicek and Kubicek, 2016). The current situation and advances in the pretreatment technology have been subject to several excellent recent reviews and shall therefore not be covered here (Xu and Huang, 2014; Silveira *et al.*, 2015; Capolupo and Faraco, 2016; Rabemanolontsoa and Saka, 2016). As for the enzymatic hydrolysis step, some of the enzymes in the cellulase cocktail have too low catalytic activity, which impacts the amount needed for the saccharification step. In addition, the composition of the secreted enzyme mixture may not be optimal for certain applications because of the presence of some enzymes in limiting amounts (see below for details). Preparation and use of these enzymes are therefore a major cost factor (Chundawat *et al.*, 2011; Klein-Marcuschamer *et al.*, 2012; Gupta *et al.*, 2016a; Kubicek and Kubicek, 2016).
Enzymatic hydrolysis is nevertheless considered the best available procedure because it in contrast to chemical hydrolysis—it does not produce compounds that inhibit the further conversion of the hydrolysate to biofuels and platform chemicals by fermentation (Payne et al., 2015).

To remove the bottleneck at the enzymatic hydrolysis step, a significant amount of work has been performed to understand the performance of the respective enzymes (Beckham et al., 2014; Harris et al., 2014; Payne et al., 2015; Kubicek and Kubicek, 2016). As cellulose represents the most recalcitrant material of the plant biomass, and yields just α-glucose, the preferred monosaccharide for further use, the majority of work has been performed on cellulolytic enzymes. In the microbial world, there are two fundamentally different strategies for the hydrolysis of cellulose (Payne et al., 2015): the ‘bound enzyme paradigm’ (cellulosome) that is present in anaerobic microorganisms (Bae et al., 2013), and the ‘free enzyme paradigm’ that is used by aerobic microorganisms (Gupta et al., 2016a). The latter is responsible for the vast majority of plant cell wall degradation in nature (Kubicek, 2013). Among the organisms capable of doing this, the ascomycete Trichoderma reesei (Hypocreales) was the first microorganism selected for closer investigation because of its potential to produce an efficient cellulose-hydrolysing enzyme mixture (for review on the history of the detection and use of T. reesei see Druzhinina and Kubicek, 2016). Despite the fact that other fungi also produce powerful cellulolytic mixtures, which in some cases even exhibit properties superior to those produced by T. reesei (see e.g. Gusakov, 2011; Wang et al., 2012 for review), T. reesei is still almost exclusively used for cellulase production by industry because technologies for its use and handling are based on a seventy years of experience (Bischof et al., 2016).

For a long time, the canonical view of action of fungal cellulases was that endoglucanases (EGs; ‘nonprocessive cellulases’) act by cleaving cellulose chains in amorphous regions within the polymers chain, and cellobiohydrolases (CBHs; processive cellulases) hydrolyze cellulose chains at the end and release the disaccharide cellobiose (‘exo-end’ model). This disaccharide is then hydrolysed by β-glucosidases to glucose. According to their structure, these enzymes are grouped into various glycoside hydrolase (GH) families, which are archived in the CAZyme (carbohydrate active enzymes) database (Lombard et al., 2014). Cellulases produced by T. reesei belong to five GH families (Table 1): endo-β-1,4-D-glucanases are found in GH5, GH7, GH12 and GH45, and CBHs in GH6 and GH7. GH7 is the only family that contains both CBHs (CEL7A; previously named CBH1) and endo-β-1,4-D-glucanases (CEL7B, previously named EGL1).

GH5 cellulases are most abundant in fungi (Li and Walton, 2017), and also three members of this family are present in T. reesei. GH7 enzymes are common, and orthologues of CEL7A are the most prevalent cellulolytic enzymes in the secretomes of biomass-degrading fungi. Because of its processive mode of catalysis (like also CEL6A, which is however usually present in much smaller concentrations), it depolymerizes cellulose most rapidly and is also responsible for the majority of hydrolytic turnover. The GH6 family is currently the only known family that comprises cellulases that act from the

| Enzyme type | EC number | GH family | Abbreviated name | Full name | Previous name | Protein ID |
|-------------|-----------|-----------|-----------------|-----------|--------------|------------|
| Cellobiohydrolases | EC 3.2.1.91 | GH6 | CEL6A | Cellobiohydrolase II | CBHII | Trire2:72567 |
| EC 3.2.1.91 | GH7 | CEL7A | Cellobiohydrolase I | CBH1 | Trire2:123989 |
| Endo-β-1,4-D-glucanases | EC 3.2.1.4 | GH5 | CEL5A | Endoglucanase II | EGL2 | Trire2:120312 |
| EC 3.2.1.4 | GH5 | CEL5A | Endoglucanase I | EGL1 | Trire2:122081 |
| EC 3.2.1.4 | GH7 | CEL7B | Endoglucanase | EGL3 | Trire2:123232 |
| β-1,4-glucosidases | EC 3.2.1.21 | GH3 | CEL3A | β-glucosidase I | BGL1 | Trire2:45876 |
| EC 3.2.1.21 | GH3 | CEL3B | β-glucosidase | Trire2:121735 |
| EC 3.2.1.21 | GH3 | CEL3C | β-glucosidase | Trire2:8227 |
| EC 3.2.1.21 | GH3 | CEL3E | β-glucosidase | Trire2:76227 |
| EC 3.2.1.21 | GH3 | CEL3F | β-glucosidase | Trire2:104797 |
| EC 3.2.1.21 | GH3 | CEL3H | β-glucosidase | Trire2:108671 |
| EC 3.2.1.21 | GH3 | CEL3J | β-glucosidase | Trire2:66832 |

a. NN, no specific name given yet; the GH3 03B2-glucosidases CEL3D and CEL3G are not listed because they are intracellular enzymes (Guo et al., 2016).

b. Refers to the T. reesei genome database (http://genome.jgi.doe.gov/Trire2/Trire2.home.html).
nonreducing end of the cellulose chain. CEL7A and CEL6A therefore act in synergism and are considered as primary components in biomass degradation cocktails.

GH12 enzymes are typically characterized by a low molecular weight (25 kDa), and in contrast to most other cellulases – lack a cellulose-binding domain (CBM1) and glycosylation. They can therefore diffuse deeper into cellulosic material, which made them preferred candidates for the laundry industry. GH45 cellulases are also generally small but have a broader substrate specificity compared to GH5 and GH7 endo-β-1, 4-α-glucanases. Interestingly, GH45 enzymes are structurally and evolutionarily related to plant expansins.

Trichoderma reesei β-glucosidases are found in the GH1 and GH3. Those belonging to GH1 are exclusively intracellular enzymes, whereas seven of the nine GH3 β-glucosidases are secreted into the medium (Guo et al., 2016). CEL3A (previously named BGL1) accounts for most of the β-glucosidase secreted activity.

The ‘exo/endo’ model (for review see Kubicke, 2013) has been revised by Stahlberg et al. (1993) and Kurashin and Valjamäe (2011) who showed that CEL7A is also able to conduct ‘endo-initiation’ and is therefore not a true exocellulase. However, neither the EGs nor the CBHs from fungi can cause massive cellulose decomposition. They rather ‘peel one layer at a time’ (Payne et al., 2015). Elwyn T. Reese and co-workers had therefore in the fifties already proposed that a decrystallizing protein must exist, which does not hydrolyse the β-glycosidic linkage but possesses the ability to swell or disrupt cellulose (the so called ‘C1 factor’: Reese et al., 1950). The nature of this C1 protein was enigmatic for a long time. The recently detected lytic polysaccharide monooxygenases (LPMO; originally classified as endoglucanases GH61), attack the highly crystalline regions of cellulose and cleave cellulose by oxidation. They could therefore represent the earlier postulated C1 factor (Morgenstern et al., 2014; Johansen, 2016a,b). Another T. reesei protein, SWO1 which bears an expansin-like domain has also been considered to fulfill a function in preparing cellulase or hydrolytic attack. But recently published results with SWO1 that had been produced in a cellulase-negative T. reesei strain failed to show a synergy with cellulases in the degradation of pretreated lignocellulose (Eibinger et al., 2016). The authors therefore concluded that SWO1 is not a C1 factor of degradation of pure cellulose. A summary of our current understanding of enzymatic cellulose hydrolysis by ‘free cellulases’ is given in Fig. 1).

Not surprisingly, T. reesei has been the subject of intensive investigation towards the improvement of its cellulases and reducing the costs for their production and use. This involved attempts to increase their intrinsic activity, facilitating their production, and the reinforcement of existing cellulase preparations by auxiliary proteins and enzymes from other organisms (Wilson, 2009; Horn et al., 2012; Peterson and Nevalainen, 2012; Hu et al., 2015; Müller et al., 2015; Payne et al., 2015;
Kubicek and Kubicek, 2016). In this review, we will summarize the progress in these fields, thereby focusing exclusively on cellulose hydrolysis by *T. reesei*.

**Protein engineering of *T. reesei* cellulase**

Despite the progress that has been made in molecular understanding of the reaction mechanism of several cellulas and their binding to cellulose (for extensive review see Payne et al., 2015), these findings have (at least in the published literature) not yet led to strategies for improvement of cellulase activities of *T. reesei* by genetic engineering. Rather, the focus has been put on the stability of the enzymes at elevated temperatures, activity in broader pH ranges and the relief from inhibition by components present in the saccharification mixture.

**Engineering high-temperature tolerance of cellulas**

Thermostable cellulas are a major goal in the lignocellulose degradation technology. Performing lignocellulose saccharification at elevated temperatures would provide several benefits such as increased specific activity and stability, prevention of growth of contaminants and increased mass transfer rate due to lower fluid viscosity at high substrate concentrations (Viikari et al., 2007; Bluemer-Schuette et al., 2014). In addition, the biomass saccharification rate is inhibited by lignin that is still present, but this inhibition is less pronounced in thermostable enzymes (Rahikainen et al., 2013). *Trichoderma reesei* is mesophilic and its enzymes are consequently only moderately tolerant to temperatures above 50°C (Chokhawala et al., 2015). While the incorporation of respective cellulase genes from thermotolerant fungi such as *Acremonium thermophilum*, *Thermoascus aurantiacus*, *Chaetomium thermophilum*, *Myceliophthora thermophila*, or *Thielavia terrestris* is an option (Viikari et al., 2007; Voutilainen et al., 2008), there have also been several attempts to improve the thermostability of *T. reesei* cellulas by protein engineering: Sandgren et al. (2003) pioneered this area by comparing the amino acid sequence of CEL12A from *T. reesei* and its thermally much less stable orthologue from *T. citrinoviride*. The two proteins differed only in 14 amino acids, and exchanging each of them in *T. reesei* CEL12A by those from *T. citrinoviride* CEL12A identified an A35S substitution that most strongly decreases the thermal stability. Interestingly, a highly thermostable CEL12A from an unidentified *Streptomyces* sp. displayed an A35V substitution, and the introduction of this A35V mutation into *T. reesei* CEL12A resulted in a *T*<sub>m</sub> that was 7.7°C higher than that of the native enzyme. Mutations of neither A35, S35 nor V35 had an influence on the overall protein structure or hydration of the protein. However, the presence of a hydrophobic amino acid such as A or V at position 35 caused tighter van der Waals interactions with its three neighbouring amino acid side-chains and closed the entry to the β-sheet sandwich core. This behaviour, known as cavity-filling mutation, has been observed in thermal stabilizing mutations in other GHs too (Karshikoff et al., 2015).

Lantz et al. (2010) demonstrated that the two CBHs CEL6A and CEL7A of *T. reesei* are more thermostable than the other enzymes involved in cellulose breakdown (like endo-β-1,4-glucanase CEL5A and the β-glucosidase CEL3A). They are therefore likely rate limiting to the performance of the whole cellulase mixture at saccharification temperatures over 50°C. Consequently Day et al. (2007) compared the amino acid sequence and structure of 42 CEL7A family members from various fungi and identified 19 sites that could be involved in increased thermostability. Using site saturation mutagenesis, they indeed showed that mutations in 18 of these sites enhanced temperature stability, and the introduction of all 18 sites into CEL7A produced a variant, which exhibited an increase in *T*<sub>m</sub> of 14.8°C (*T*<sub>m</sub> 76.0°C; Lantz et al., 2010).

Arnold and colleagues from the California Institute of Technology have made substantial contributions to GH7 engineering primarily on the basis of computational prediction tools such as structure-guided recombination. They used non-contiguous recombination (NCR), a method that identifies pieces of structure that can be swapped among homologous proteins to create new chimeric proteins (Smith et al., 2013a). By this means they designed a library of chimeric enzymes, in which blocks of the structure of *T. reesei* CEL7A and the two thermostable CEL7A homologues from *Talaromyces emersonii* and *C. thermophilum*, respectively, were shuffled to create 531,438 possible variants (Smith et al., 2013b). Selecting a maximally informative subset of 35 chimeras for analysis, they found that these blocks contributed additively to the stability of a chimera. Two highly stabilizing blocks displayed the same two mutations (T360A and F362M), and they increased the thermal stability of CEL6A by 1 and 3°C, respectively (Smith et al., 2013b).

**Engineering cellulas for resistance towards ionic liquids**

As explained above, the recalcitrant nature of native lignocellulose makes a pretreatment by chemical, physical or biological means necessary. Classical pretreatment procedures, however, are performed in a separate step before hydrolysis, and so necessitate extensive washing (for review see Brethauer and Studer, 2015). Consequently, pretreatment and hydrolysis in a one-pot reaction would improve the process from an economic point
of view. Among the various pretreatment methods, the use of ionic liquids (ILs) would be most appropriate because it does not involve harsh conditions and does not lead to the formation of toxic by-products (Uju et al., 2017). However, the strongly nucleophilic ILs interact with the positively charged residues on the surface of the cellulases and inactivate them (Li et al., 2012). The enzymes from the last three thermotolerant species also are more stable at higher temperatures, thereby removing the limitation of saccharification by the low-temperature stability of T. reesei CEL1B during hydrolysis (Vikari et al., 2007). To this end, Xue et al. (2016) reported that a fusion of the N. fischeri NfBgl3A gene to the cbh1 structural gene resulted in β-glucosidase activities that were up to 175-fold higher than those of the parent strain.

Cellulbiose dehydrogenase (CBD, EC 1.1.99.18), a member of the enzyme arsenal auxiliary to glycoside hydrolases (family AA3; Levassuer et al., 2013), is an extracellular enzyme produced by various wood-degrading fungi (Henriksen et al., 2000). It oxidizes the reducing ends of cellulbiose and cellooligosaccharides to 1, 5 lactones, which are subsequently hydrolysed to the corresponding carboxylic acids. CBD orthologues have also been found in several Pezizomyotina genomes (Table S1). Although T. reesei contains several members of the AA3 family too (Druzhinina and Kubicek, 2016) – a CBD orthologue is absent. Ayers et al. (1978) first suggested that CBD (named ‘cellbiose oxidase’ then) from Phanerochaete chrysosporium could be involved in cellulose biodegradation. Bey et al. (2011) demonstrated that the addition of CBD from the basidiomycete Pycnoporus cinnabarinus synergistically stimulated the saccharification of wheat straw by a T. reesei commercial cellulase cocktail. This stimulation is due to relieving CBH1 from the competitive inhibition by cellbiose, which is oxidized by CBD to the non-inhibitory cellubiose lactone. Consequently, Wang and Lu (2016) overexpressed a CBD-encoding gene from P. chrysosporium under the cbh2 promoter in T. reesei, and obtained transformants with up to fourfold increased filter paper activity.

The identification of LPMOs as a group of enzymes that accelerate the breakdown of carbohydrate polymers like cellulose, chitin and starch by oxidative cleavage has been a breakthrough in lignocellulose conversion research (Johansen, 2016a,b). LPMOs belonging to the auxiliary enzyme family AA9 degrade cellulose nanofibris on the surface into shorter fragments. They therefore assist cellulases to attack otherwise highly resistant crystalline substrate areas, which results in a faster and more complete surface degradation (Eibinger et al., 2014). Supplementation of the commercial cellulase cocktail Cellic CTec1 (Novozymes) with AA9 enzymes improved the hydrolysis of lignocellulose (Sun et al.,
2015), which led to the new commercial cellulase preparations Cellic CTec2 and Cellic CTec3.

Because of the role of CBD in assisting the catalysis by AA9, it is possible that this stimulatory action of CBD (vide supra) is due to the stimulation of AA9. *Trichoderma reesei* has three AA9-encoding genes, of which one is strongly induced during growth under cellulase inducing conditions. The enzyme has been overproduced in *T. reesei* (Karlsson et al., 2001), but the resulting cellulose-hydrolysing activity of the secreted cellulolytic enzymes has not been identified (at that time, the enzyme was considered to be an endo-β-1,4-glucanase, CEL61A). The successful overexpression of AA9 – together with CBD – in *T. reesei* may directly lead to strains with improved cellulase performance.

Finally, *T. reesei* lacks invertases that hydrolyse sucrose (Berges et al., 1993), and therefore cannot grow on some cheap technical substrates such as sugarcane molasses. Ellilä et al. (2017) showed that this deficiency could be overcome by constitutively expressing an invertase gene from *Aspergillus niger* in *T. reesei*.

### Engineering cellulase gene expression

Various cellular levels have been identified which influence the expression of cellulases (for overview see Fig. 2), which will be described below.

#### Genetic engineering of transcriptional regulators

In *T. reesei*, the expression of all cellulases and of the majority of hemicellulases is induced in a coordinated manner by growth on carbon sources such as cellulolytic substrates, or some disaccharides such as lactose or sophorose, respectively. The expression of the enzymes can therefore be manipulated by engineering a small number of regulators that are responsible for the coordinated expression of cellulases. In *T. reesei*, unlike in *Neurospora crassa*, *Fusarium graminearum* or *Aspergillus* spp., the transcriptional activator XYR1 (Stricker et al., 2006) is the major transcriptional activator of both cellulase and xylanase gene expression. It belongs to the fungal specific class of transcription factors that contain a domain that binds Zn$^{2+}$ ions by six cysteine residues (Zn2Cys6). XYR1 binds to a 5'-GGCW4-3' DNA motif (Furukawa et al., 2009). Deletion of this gene eliminates cellulase induction by all known inducers, whereas *xyr1* overexpression enhances it (for review see Bischof et al., 2016; Gupta et al., 2016b; Druzhinina and Kubicek, 2016). Several strategies for manipulation of XYR1 (and of some other transcriptional regulators that will be described below) to enhance cellulase production or render its expression constitutive have therefore been presented (Table 2). Recently, da Silva Delabona et al. (2017) showed that a 26-fold constitutive overexpression
of xyr1 in another Trichoderma spp. (T. cf. harzianum) resulted in the production of an enzymatic complex that exhibited a 25% enhanced hydrolysis rate of sugarcane bagasse during the first 24 h of saccharification. Also, Ellilä et al. (2017) constitutively expressed a XYR1 (V821F) mutant (which leads to reduced glucose repression; Derntl et al., 2013) in T. reesei and obtained increased cellulase and xylanase production on sugarcane bagasse. Three other proteins or protein complexes (i.e. the Zn2Cys6 transcriptional activators ACE2 and ACE3, and the CCAAT-binding protein complex (HAP2/HAP3/HAP5) are also involved in the regulation of cellulase gene expression in T. reesei. So far, only the overexpression of ace3 has been shown to result in enhanced cellulase formation (Häkkinen et al., 2014). Other regulators may still be detected: introduction of multiple copies of the gene for the still uncharacterized Zn2Cys6 transcription factor Trire2:80291 into T. reesei was also shown to increase CBH1 formation twofold (Häkkinen et al., 2014). In addition, cellulase gene expression in N. crassa involves several other transcription factors, of which at least CLR2 and VIB1 (Coradetti et al., 2012; Xiong et al., 2014) have orthologues in T. reesei (Trire2:26163 and Trire2:54675, respectively; see genome annotation in Druzhinina et al., 2016). However, it is not known yet whether they also regulate cellulase gene expression in T. reesei, and – if so – whether their manipulation could be used to improve cellulase production.

Two interesting alternative perspectives for manipulation of cellulase gene expression have recently been presented: Zhang et al. (2017) constructed a hybrid cellulase regulator, which consisted of the DNA-binding domain of the glucose-repressor CRE1 (see below) fused to the XYR1 effector binding domains. Its overexpression in T. reesei led to an approximately 30-fold increase of constitutive cellulase and hemicellulase production on glucose. Zhang et al. (2016b) constructed an artificial zinc finger protein library and expressed it in T. reesei. One of the respective transformants showed a 55% rise in cellulase activity against filter paper and an 8.1-fold increased ß-glucosidase activity.

Cellulase gene expression has also been shown to be repressed by rapidly assimilated carbohydrates, which is due to the action of carbon catabolite repressor protein CRE1 (Nakari-Setälä et al., 2009). CRE1 interferes both with constitutive as well as induced cellulase and hemicellulase gene expression, but the degree of this repression varies for different cellulase and hemicellulase genes (see Druzhinina and Kubicek, 2016 for review). As an example, only the constitutive but not the inductive expression of the cellulobiohydrolase gene cel8A and of the xylanase gene xyn1 is repressed by CRE1 (Mach et al., 1996; Zeilinger et al., 2003). In contrast, CRE1 represses both constitutive and induced expression of the cellulobiohydrolase 1 gene cel7a (Nakari-Setälä et al., 2009). Elimination of the function of CRE1 had already been obtained by classical mutagenesis and resulted in a strain with increased cellulase formation in the presence of increased concentration of inducing carbon sources (cellulose or lactose; Eveleigh and Mon-tenecourt, 1979). This strain – known as T. reesei RUT C30 – bears a truncated version of the cre1 gene that expresses only the zinc finger but not the transactivating domains (CRE1-96; Ilmén et al., 1996). It is noteworthy that some strains used for the production of cellulosases and hemicellulases on the industrial scale are descendants of RUT C30. Recombinant strains of T. reesei with cre1 loss of function have been analysed too, but – in contrast to RUT C30 – they have a pleomorphic phenotype (Nakari-Setälä et al., 2009) and their use in cellulase production is therefore limited.

Another Zn2Cys6 transcription factor – ACE1 – acts as a partial repressor of cellulase and xylanase gene expression, and strains carrying a deleted allele displayed enhanced cellulase formation (Aro et al., 2003). The physiological conditions that trigger ACE1

| Table 2. Published examples of improvement of cellulase production in Trichoderma reesei by genetic engineering |
|-----------------|-----------------|-----------------|-----------------|
| Gene            | Manipulation method | Promoter        | References       |
| xyr1            | Constitutive overexpression | pki1            | Seiboth, Karimi et al. (2012) |
|                 | Constitutive overexpression | tcu1            | Lv et al. (2015)  |
|                 | ace1 downregulation | pdc1, downregulation by antisense with the same promoter  | Wang et al. (2014) |
| cbb1            | Promoter engineering | CRE1 binding sites exchanged against ACE2 and HAP2/3/5 binding sites | Zou et al. (2012) |
| cre1            | Deletion          |                | Nakari-Setälä et al. (2009) |
|                 | Truncation        |                | Nakari-Setälä et al. (2009); Mello-de-Sousa et al. (2014) |
| ace1            | Deletion          |                | Aro et al. (2003)  |
| ace3            | Introduction of multiple gene copies |                | Häkkinen et al. (2014) |
| lae1            | Constitutive overexpression | gpd1            | Seiboth-Karimi et al. (2012) |
| vel1            | Constitutive overexpression | tef1            | Karimi Aghcheh et al. (2014) |

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repression are not understood yet. ACE1 is an orthologue of Aspergillus nidulans StzA/Slt4 (Aro et al., 2003) that plays a role in Ca²⁺ homoeostasis (Spielvogel et al., 2008) and has further been implicated in nitrogen control (Chilton et al., 2008). It is however intriguing that the ACE1 orthologue of Colletotrichum gloeosporioides is mainly involved in appressorium formation (Dubey et al., 2016). An elucidation of the function of ACE1 in T. reesei will be essential before ace1 can be used for science-based strain engineering.

After this review had been submitted, Cao et al. (2017) reported on the identification of a further repressor of cellulase gene expression, RCE1. Disruption of its gene enhanced the induced expression of cellulase genes and led to a significant delay in termination of induction. RCE1 did not participate in CRE1-mediated catabolite repression, but antagonized the binding of XYR1 to the cellulase promoters.

Promoter engineering

Manipulation of the binding sites for transcriptional regulators in the cellulase genes is another strategy to modify their expression. Given the number of cellulases produced by T. reesei, this can be a time-consuming process. Nevertheless, replacing the CRE1 binding sites within the cbh1 promoter by the binding sites for the transcription activators ACE2 and the HAP2/HAP3/HAP5 complex enhanced transcription of a test gene (green fluorescent protein) under cellulase inducing conditions sevenfold (Zou et al., 2012). This approach may have its merit when the expression of only a few genes is targeted. On the other hand, engineering the xyrl promoter (XYR1 also needs to be induced for cellulase and hemicellulase formation; Lichius et al., 2014) – should provide a much more straightforward approach towards enhancement or modulation of cellulase production. Yet no such experiments have been published up to date.

Epigenetic engineering

Epigenetic engineering has recently been reviewed as a potentially new tool for industrial improvement of fungi (Aghcheh and Kubicek, 2015). The term has been coined by Waddington (1942) for heritable changes in gene expression that do not involve changes in the underlying DNA sequences. In the current understanding, two major levels of epigenetic regulation are important: DNA methylation; and chromatin remodelling by histone modification.

While evidence for a regulatory role of DNA-methylation in T. reesei is not available, some aspects of chromatin remodelling have been demonstrated.

Gupta et al. (2016b) have recently discussed the remodelling of chromatin by transcriptional activators and emphasized it as an emerging approach for improvement of cellulase formation. Under cellulase inducing conditions, the chromatin packing around the cellulase-encoding genes cbh1 and cbh2 opens, but this does not occur in a xyr1-deletion strain (Mello-de-Sousa et al., 2015, 2016). Consequently, Mello-de-Sousa et al. (2015) identified several genes encoding chromatin remodelling enzymes, including histone acetyltransferases, whose expression is significantly different in the Δxyr1 and its parental strain. Whether these genes could be used to improve cellulase production has not been tested yet. Another histone acetyltransferase (GCN5), however, has been shown to be necessary for cellulase gene expression, and the acetylations of K9 and K14 of histone H3 in the cbh1 promoter were strongly reduced in the T. reesei Δgcn5 strain (Xin et al., 2013).

The carbon catabolite repressor CRE1 has also been implicated in chromatin remodelling: strains expressing a non-functional CRE1 exhibit a loss of positioned nucleosomes within the cbh1 and cbh2 genes under repressing conditions only (Zeilinger et al., 2003; Ries et al., 2014). Interestingly, the truncated version of CRE1 (CRE1-96) of RUT C30 seems to trigger chromatin opening (Mello-de-Sousa et al., 2014), likely by regulating the expression of snf2/htf1 (Portnoy et al., 2011), a putative helicase that might participate in an ATP-dependent chromatin remodelling complex (Mello-de-Sousa et al., 2014). Identification and engineering of chromatin remodelling proteins are obviously a promising possibility for strain development.

In addition, there is another group of fungal genes that participate in chromatin modification and whose manipulation has been shown to modulate cellulase and hemicellulase production: the Velvet-LaeA/LAE1 complex (Seiboth, Karimi et al., 2012; Karimi Aghcheh et al., 2014; Liu et al., 2016). LaeA, originally identified in A. nidulans as a regulator of secondary metabolite gene expression, has characteristics of an S-adenosyl-L-methionine arginine protein methyltransferase (Sarikaya-Bayram et al., 2015). However, LaeA appears not to methylate histones. Rather it seems to function by interaction with the transcription factors of the Velvet protein complex. Constitutive overexpression of both lae1 as well as vefl in T. reesei has been shown to enhance cellulase expression and secretion (Seiboth, Karimi et al., 2012; Karimi Aghcheh et al., 2014).

Metabolic engineering

A significant body of work has been dedicated to the characterization of the metabolic steps that are involved in the signalling of cellulase gene expression. The current state of knowledge in this area has recently been reviewed (Druzhinina and Kubicek, 2016; Gupta et al.,
2016b). Despite a fair amount of progress, an essential improvement of cellulase production by the manipulation of individual genes involved in this process has not yet occurred. This is in part due to the fact that manipulation of genes involved in central metabolism and particularly in signal transduction often produce pleiotropic effects that are difficult to use for strain improvement. Whole-genome dedicated strategies, such as the use of whole-cell metabolic models, likely offer the best available strategy for the identification of the limiting steps for cellulase production.

To this end, Castillo et al. (2016) applied the CoReCo (comparative metabolic reconstruction framework) pipeline (Pitkänen et al., 2014) for the construction of a high-quality metabolic model of T. reesei (BIOMODELS database). The model contains a biomass equation, reaction boundaries and uptake/export reactions, which make it ready for the simulation of protein production processes. It was applied by Pakula et al. (2016) for analysis of the flux balances of its metabolism, using a transcriptomic analysis of protein production by T. reesei in chemostat cultivations. The study showed that high protein (cellulase) production might be limited at the level of biosynthesis of sulfur-containing amino acids, which identifies a clear target for metabolic engineering that was so far not detected by other means.

The engineering toolbox of T. reesei

A suitable molecular tool box that enables easy exchange and manipulation of genes is a prerequisite for engineering T. reesei or individual components of its cel lulolytic cocktail, as reviewed above. This topic has been subject of several exhaustive recent reviews (Steiger, 2013; Bischof and Seiboth, 2014; Bischof et al., 2016; Gupta et al., 2016b), and we shall therefore discuss it only very briefly. The low efficiency of gene targeting has for a long time been a major bottleneck in this regard, but was finally – like in several other fungi – solved by inactivating components of the non-homologous end joining pathway of DNA repair such as tku70 or tmus53 (Guangtao et al., 2009; Steiger et al., 2011; Schuster et al., 2012). More recently, Ouedraogo et al. (2015, 2016) demonstrated that an I-SceI mediated double strand break in a T. reesei tku70 gene improved transformation efficiencies and increased homologous integration up to 90–100%. Also the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system (D’Ago stino and D’Aniello, 2017) has been adapted for use with T. reesei (Liu et al., 2015). It depends only on the Cas9 (CRISPR-associated) nuclease which uses a single chimeric guide RNA for targeting, and so introduces specific DNA double strand breaks that stimulate gene targeting.

Further, a number of new approaches were offered that allowed the insertion of expression cassettes at defined genomic regions. Demtl et al. (2015) constructed T. reesei strains bearing truncated versions of several genes resulting in auxotrophic phenotypes, and tested the rescue of prototrophy by transformation with a complementary fragment of the same gene. Among them, they used the ade2 locus (encoding a phosphoribosylaminomimidazole carboxylase necessary for purine biosynthesis) because integration into this locus destroys ade2, the Δade2 disruptants accumulate polymerized 5-aminoimidazole ribonucleotide (Ugolini and Bruschi, 1996) and can therefore easily be identified by the red colour. Demtl et al. (2015) also successfully demonstrated the applicability of the asl1 (encoding argininosuccinate lyase of arginine biosynthesis), the hah1 (encoding homoaconitase dehydratase involved in lysine biosynthesis) and the pyr4 genes (encoding the oroticid-5-phosphate decarboxylase of the uridine biosynthesis pathway) for this approach to obtain site-directed integration in T. reesei.

Even if the transformation frequency is high, identification of the transformants that express the target protein at desired levels can still be a tedious task because the expression levels between transformants can be very variable. Subramanian et al. (2017) have therefore adapted the 2A peptide system from the foot-and-mouth disease virus to T. reesei. This tool allows multiple independent genes to be transcribed as a single mRNA. Upon translation, the 2A peptide sequence causes a ‘ribosomal skip’ between its two C-terminal amino acids and generates two independent gene products. A target protein can therefore be cotranslated with a readily testable marker protein, and colonies with desired expression levels can so be identified. The authors tested this by coexpression of CEL7A and an enhanced green fluorescent protein as a marker and obtained similar levels of expression of both proteins. This system therefore offers an efficient strategy to test the expression of heterologous proteins in T. reesei, and provides a novel platform for multi-protein-expression in approximately equimolar ratios using a single polycistronic gene expression cassette.

A further essential tool needed for strain engineering is promoters that allow high constitutive expression without dependence on special physiological conditions. An important issue is thereby that the transcriptions factors that bind to the respective regulatory elements in these promoters are present in sufficient quantities in the cells because their use would otherwise be limiting the expression by titration effects. Table 3 summarizes such promoters and their use in T. reesei.

Also, there is a demand for promoters that could be temporarily turned either on or off. Traditionally, the T. reesei cbh1 and xyn1 promoters (of the genes encoding the cellobiohydrolase CEL7A and the endo-β-1,
Table 3. *Trichoderma reesei* promoters used

| Promoter | Condition |
|----------|-----------|
| Constitutive expression | Glycolytic genes (*pki1, gpd1, pgd1, eno1, pdc1*) |
| cre1:xyr1 hybrid | Copper deficiency |
| cDNA1 | Methionine deficiency |
| tef1 | Copper deficiency |
| TauD-dioxigenase | Methionine deficiency |
| Inducible expression | tcu1 copper transporter |
| Li et al. (2012); Seiboth, Karimi et al. (2012); Wang et al. (2014); Linger et al. (2015) | |
| Zhang et al. (2017) | |
| Uzbas et al. (2012) | |
| Seiboth, Karimi et al. (2012) | |
| Lv et al. (2015) | |
| Bischof et al. (2015) | |

a. Promoters of cellulase and xylanase genes are not listed.

4-xylanase XYN1, respectively) have been used for this purpose, but the respective inducers or repressors also act on other cellulases and xylanases and are therefore not sufficiently specific (for review see Bischof and Seiboth, 2014). To solve this problem, Bischof et al. (2015) introduced the promoter of a gene encoding Trire2:123979 (a putative dioxigenase of unknown function) which in *T. reesei* can be strongly repressed by addition of 0.1 mM l-methionine. An alternative strategy was presented by Lv et al. (2015), using the promoter of the gene encoding the copper transporter TCU1 (Trire2:52315), which can be repressed by Cu²⁺ ions of > 0.2 μM. This system was recently used to manipulate the expression of the histone acetyltransferase GCN5 (Trire2:64680) in *T. reesei* (Zheng et al., 2016).

Towards the development of an inducible expression system, Zhang et al. (2016a) constructed a modular synthetic regulator by fusing the gene fragments encoding the DNA-binding domains of yeast Gal4, the light responsive domain of *N. crassa* Vivid, and the *Herpex simplex* VP16 transactivation domains. The fusion gene was put under the control of a synthetic promoter carrying five copies of the Gal4 consensus binding site. It was successfully expressed in *T. reesei* in the presence of light. However, the operation of this system at an industrial scale with high-density suspensions of cellulosic substrates and fungal mycelium has not been tested yet.

Conclusions

The potential of lignocellulosic biomass as renewable feedstock for substituting a significant fraction of today’s fossil fuel consumption has been an attractive theory for many years. In recent years, the first industrial-scale cellulosic ethanol plants have started to operate. Yet competition with technologies based on fossil resources is still hampered by the high costs for the pretreatment and enzymatic saccharification steps due to the natural recalcitrance of lignocellulosic biomass. In this article, we have focused on saccharification, and discussed how the production and properties of the cellulases from the major industrial producer *T. reesei* could be changed towards a better process economy. In this regard, it is interesting to note that – despite the amount of work dedicated to the understanding of the catalytic mechanisms of some of the major cellulases – comparatively little work has been published that shows whether the introduction of activity increasing mutations indeed lead to strains that produce better enzyme preparations. Thereby it should also be borne in mind that cellulases function at a solid–liquid interface of a physically and chemically heterogeneous substrate, i.e. native or pretreated plant cell walls, which clearly impact the activity of the enzyme, and makes the enzyme dependent on other enzyme partners. Finally, it is still an open question whether (and if so why) the multiplicity of some cellulases (e.g. the endo-β-1,4-glucanases) and the LPMOs are essential for the overall hydrolysis of commercially used lignocellulose substrates. Answers to these questions are needed for genetic engineering of *T. reesei* strains to produce more active cellulase mixtures.

Engineering of cellulase production is a related, yet different issue: industrial strains producing extremely high cellulase concentrations (over 120 g l⁻¹ total protein) have been reported (Gupta et al., 2016a), and it is worth of discussion whether a further increase (or shortening of the fermentation time needed to achieve this) is feasible and required. Smaller companies or newcomers may, however, want to construct their own producer strain, and can now select from a variety of conditions for this purpose. Also, the carbon source for production of the enzymes needs not to be the same that is used for saccharification, and the producer strain, therefore, may have to be tailored to form cellulases on media not containing lignocellulose or containing a mixture of lignocellulose and other carbon sources. Unfortunately, most of the alterations achieved by metabolic engineering, promoter or transcription factor engineering and epigenetic engineering have been tested only under laboratory conditions, frequently using minimal media, and by only measuring the relative abundance of cellulase mRNA. The presence of a heterogenous polymer and the chemical changes that occur in the medium during its...
hydrolysis will likely have a strong impact on the function of these engineered strains. It is therefore difficult to predict whether the introduced mutation would indeed be beneficial for the industrial process. We strongly recommend that such engineered strains should always also be tested on proper lignocellulose substrates. In addition, an in vitro saccharification experiment with the enzymes produced in these cultivations should also be included.

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Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. 100 best hits in a blastp analysis* using the H. insolens cellobiose dehydrogenase as a query.