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Developments and opportunities in fungal strain engineering for the production of novel enzymes and enzyme cocktails for plant biomass degradation

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Abstract

Fungal strain engineering is commonly used in many areas of biotechnology, including the production of plant biomass degrading enzymes. Its aim varies from the production of specific enzymes to overall increased enzyme production levels and modification of the composition of the enzyme set that is produced by the fungus. Strain engineering involves a diverse range of methodologies, including classical mutagenesis, genetic engineering and genome editing. In this review, the main approaches for strain engineering of filamentous fungi in the field of plant biomass degradation will be discussed, including recent and not yet implemented methods, such as CRISPR/Cas9 genome editing and adaptive evolution.

Keywords UV mutagenesis; chemical mutagenesis; adaptive evolution; genetic engineering; genome editing; epigenetics; omics

1. Introduction

Filamentous fungi are a diverse group of eukaryotic organisms, which have both positive and negative impacts on our society. Some of them are responsible for the contamination of food and fodder and infection of living organisms (Moretti et al., 2013). On the other hand, some filamentous fungi are potent producers of industrially relevant metabolites such as antibiotics, statins or immunosuppressive drugs as well as organic acids. Filamentous fungi have a central role in the production of various biotechnologically important enzymes, such as those degrading complex plant materials (de Vries, 2003; Lange et al., 2012). These fungal enzymes are used e.g. in the pulp and paper, food and feed, and textile and detergent industry. In addition, more recent application of fungal enzymes is in the biofuels and biochemicals industries (Mäkelä et al., 2014).

Due to the broad range of applications fungal enzymes are used for, the production level of the enzymes and the composition of the enzyme sets produced have been actively studied over the last decades, and have been the target of strain engineering in many academic and industrial studies (Archer, 2000; Archer et al., 1994; Juturu and Wu, 2012; MacCabe et al., 2002). Increasing enzyme production level in general is important for many applications, as it will directly affect the overall process costs. However, also ensuring that the right set of enzymes is produced, including all required activities and avoiding unwanted activities, is highly relevant. Often wild type strains do not produce the protein at levels required in industry, nor do they produce exactly the desired enzymes set. To solve this problem, strain engineering has generated industrial fungal strains that have superior performance compared to wild type strains. Genetic engineering approaches have been developed for industrially used fungal species and strains in order to increase their enzyme production (Ha et al., 2011; Tran et al., 2017). However, most of these methods are available for only a limited number of model or industrial fungi. In addition, the full capacity of fungi as enzyme factories depends on a detailed molecular level understanding of their physiology including regulatory mechanisms that govern enzyme production (de Vries, 2003). Although considerable progress has been made to improve the industrial potential of fungi, our knowledge remains limited and a number of questions have yet to be addressed. The availability of an increasing number of fungal genome sequences and omics data can lead to broader opportunities of genetic manipulations and research of potentially relevant industrial fungal species.

In this review, the approaches used for strain engineering of filamentous fungi in terms of production of enzymes and enzyme cocktails or plant biomass degradation will be discussed, using examples of the use of these strains to highlight their relevance and contribution to biotechnology. The availability of fungal (post-)genomics together with modern genome editing technology has provided new opportunities for strain engineering that will be especially emphasized. The examples of strain engineering in fungi for enzyme production are numerous and it is impossible to provide a full overview of this topic. This review will therefore focus on the main approaches used for strain engineering, each accompanied by some examples of the specific enzymes or regulators controlling enzyme production. It should be noted that due to legislation issues, the implementation of some of these technologies is delayed in certain applications. As this is highly dependent on the country in which the processes are performed, we will not discuss restrictions of the use of the engineered strains caused by legislation in this review.

2. Classical strain engineering approaches

2.1 UV and chemical mutagenesis to improve enzyme activity

Mutagenesis approaches have been used to obtain strains with improved plant biomass degrading enzyme production. While this approach has been used for a variety of enzymatic activities, especially improvement of cellulase production has received extensive attention. To achieve this, better production strains have been obtained by random mutagenesis methods that are simple and easy to perform. Classical mutagenesis has been the most widely accepted method of strain improvement, and has also been used to create most of the fungal strains employed for commercial cellulase production, although a few have been generated through genetic modification (Singh et al., 2017).

One type of classical mutagenesis is the use of physical mutagens, such as ultraviolet (UV) radiation, which has proved to be an efficient approach for strain improvement. The UVC rays cause mutations as they pass through DNA and excite the atoms of the DNA molecule. The loss of electrons causes a change in the covalent bonds between DNA nucleotides and induces two adjacent pyrimidines,

thymine and cytosine, to join and form a pyrimidine dimer (Fig. 1A), most commonly resulting in point mutations. If this DNA damage is not repaired immediately, DNA polymerase replicates the mutation, resulting it to be present in both strands of the DNA. In most cases, UV mutations are very harmful, but may sometimes lead to better adaptation of an organism to its environment or in improved biocatalytic performance (Irfan et al., 2011). For example, when a fungus is exposed to mutagens at a sub-lethal concentration, the level of cellulase activity has been shown to increase (Chand et al., 2005; Li, X.H. et al., 2010).

A second type of classical mutagenesis is the use of chemical agents, such as intercalating molecules (e.g. ethidium bromide - EtBr), that can insert themselves between DNA strands and stretch the DNA duplex in such a way that DNA polymerase will insert an extra nucleotide opposite an intercalated molecule. Intercalating agents therefore typically cause frameshift mutations (Ennis, 2001) (Fig. 1B). In addition, alkylating agents, such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and ethylmethane sulphonate (EMS), have been used as mutagens in strain engineering. These chemicals add alkyl groups to nucleotides at various positions, which may lead to transition mutations where one pyrimidine or purine base is substituted by the other. The consequences of nucleotide mutations in protein coding regions of a gene depend on the substitutions of the nucleobases as well as the location of the mutations. This may lead to alteration in the enzyme amino acid sequence that may either increase, decrease or abolish the activity of mutant enzymes (Ennis, 2001).

Use of UV radiation and chemicals, either separately or in combination, has been highly efficient in the generation of hypercellulolytic strains of filamentous fungi (Table 1). This is exemplified by the improvement of the cellulase titers of the *Trichoderma reesei* wild type strain QM6a (previously named *Trichoderma viride* QM6a) (Mandels and Reese, 1957), resulting in strains with superior qualities in terms of cellulase activity levels, protein secretion and catabolite derepression, which have been used at the industrial level (Bischof et al., 2016). First, the mutant QM9414 with four-fold increase in cellulase production and two-fold increase in extracellular protein level compared to the wild type QM6a strain was generated by UV irradiation of conidia (Dashtban et al., 2009; Montenecourt and Eveleigh, 1977b). After this, UV mutagenesis and screening for catabolite derepression resulted in the isolation of strain M7 (Montenecourt and Eveleigh, 1977a). A partially derepressed strain NG14 with increased production of extracellular protein and cellulase activity was obtained by chemical mutagenesis using N-nitroguanidine. Finally, the hypercellulolytic RUT-C30 strain was obtained after another round of UV mutagenesis together with screening for cellulase activity and catabolite derepression (Montenecourt and Eveleigh, 1979).

Other examples of increased cellulase production by classical mutagenesis were reported for another industrially used filamentous ascomycete, *Aspergillus*. A wild type *Aspergillus* strain was improved by two sequential treatments of Co^{60} γ-irradiation, UV irradiation and four sequential treatments with NTG (Vu et al., 2009). This resulted in a mutant strain that produced 2-, 3.2- and 1.8-fold higher activity of carboxymethyl cellulase (CMCase), filter paper cellulase (FPase) and β-glucosidase, respectively, compared to the wild type (Vu et al., 2009). Similarly, an *Aspergillus niger* mutant obtained by UV irradiation showed 3- and 2-fold increase in FPase and CMCase activity, respectively, compared to the parental strain (Irfan et al., 2011). Exposure of *Aspergillus oryzae* NRRL 3484 to sequential UV irradiation treatments followed by chemical treatments with NTG or EtBr, resulted in a mutant with a 4-fold higher FPase and CMCase activity than the wild type strain (El-Ghonemy et al., 2014).

The general suitability of classical mutagenesis is supported by its use for the improvement of cellulase production in several other fungi that are less commonly or not used in industry. A UV mutant strain of *Penicillium echinulatum* had a high filter paper activity (FPA) compared to the wild type strain

(Dillon et al., 1992), while successive mutagenic treatments with EMS followed by UV irradiation generated a *Penicillium janthinellum* mutant that showed 3-fold FPase and 2-fold CMCase activity levels compared to the parent strain (Adsul et al., 2007). UV treatment followed by chemical mutagenesis using NTG also generated a mutant of *Fusarium oxysporum* with 80% higher cellulolytic activity than its parent strain (Kuhad et al., 1994).

In addition to the improvement of cellulase production, classical mutagenesis has also been used to improve the production of other industrially relevant enzymes in fungi. For example, an *Aspergillus tubingensis* mutant generated by UV mutagenesis demonstrated improved xylanase activity, which was shown to be the result of a metabolic mutation (Nikolaev et al., 2013). In addition, UV irradiation has been successfully used to obtain *A. niger* mutants with increased pectinase production (Antier et al., 1993), whereas *A. niger* mutants showing improved lipase activity were generated with sequential exposure to UV radiation or nitrous acid (Mala et al., 2001). Of the biotechnologically interesting fungal oxidative enzymes, increased laccase production has been achieved with EtBr treatment in the basidiomycete fungi *Cyathus bulleri* (Dhawan et al., 2003) and *Pleurotus citrinopileatus* (Kushwaha et al., 2016).

These examples demonstrate the value of classical mutagenesis for improved enzyme production in filamentous fungi, and many of the mutants used at the industrial level have been obtained through this methodology. However, the use of classical mutagenesis also has disadvantages, in particular related to the non-targeted nature of this approach. The high dose of UV or chemical often used in industrial strain improvement strategies makes the generation of strains with single mutation highly unlikely. Improved production strains can acquire point mutations over several rounds of random mutagenesis leading e.g. to a reduced growth rate, sporulation defects or genomic instability. Some of these disadvantageous mutations will show up already in the screening of the progeny, and therefore strains containing them are likely discarded at this stage. However, other negative mutations may not show themselves until later stages of testing of the strains, such as reduced fermentation properties of the strain or reduced suitability for upscaling. Progeny with such negative characteristics would still be discarded, despite the amount of work already invested in them. In this context, directed genome manipulation can help to overcome the effect of deleterious point mutations (Kluge et al., 2018).

2.2 Genetically modified strains

After the discovery of DNA-mediated transformation procedures in the 1970s and 1980s, more targeted strategies were developed for the production of commercially valuable microbial strains. In addition, whole genome sequencing projects opened the possibility of genome mining, revealing a huge number of yet to be characterized genes encoding candidate plant biomass degrading enzymes (de Vries et al., 2017; Ohm et al., 2012; Rytioja et al., 2014). Together with the development of novel molecular tools, such as homologous recombination (HR) and RNA interference (RNAi), genetic engineering of fungal strains became a commonly used approach for the development of strains with improved characteristics. Both forward and reverse genetics have been used to improve fungal production of plant biomass degrading enzyme production. Two early examples of forward genetics are the identification of the starch- and xylan-related transcriptional activators, AmyR from *A. oryzae* (Petersen et al., 1999) and XlnR from *A. niger* (van Peij, N.N. et al., 1998), respectively. Examples of reverse genetics are also found for regulatory genes involved in the production of plant biomass degrading enzymes, such as *clr-1* and *clr-2* from *Neurospora crassa* (Coradetti et al., 2012), *rhaR* from *A. niger* (Gruben et al., 2014) and *gaaR* from *Botrytis cinerea* (Zhang, L. et al., 2016).

One of the genetic engineering strategies to manipulate the enzymatic spectrum of fungal strains is the introduction of additional gene copies. In *T. reesei*, the introduction of β-glucosidase genes from other fungi, such as *Penicillium decumbens* (Ma et al., 2011), *Aspergillus aculeatus* (Treebupachatsakul et al., 2015) and *Chaetomium atrobrunneum* (Colabardini et al., 2016), was able to compensate for the low native β-glucosidase activity in this species. In *Penicillium oxalicum*, overexpression of β-glucosidase encoding genes improved the activity of this enzyme in culture filtrates 65-fold (Yao et al., 2016), while in *Humicola insolens* overexpression of a major cellulase gene (*avi2*) resulted in an 8-fold higher Avi2 activity (Moriya et al., 2013). Also other genes contribute to cellulose degradation efficiency. It was shown in the dung fungus *Podospora anserina*, which produces enzymes that act synergistically with those of *T. reesei* (Couturier et al., 2011), that inactivation of cellobiose dehydrogenases reduced its ability to degrade cellulose (Tangthirasunun et al., 2017).

Another application of genetic engineering for strain improvement is the manipulation of promoters that drive the expression of enzyme encoding genes and therefore affect enzyme production. Replacing the binding sites for the major carbon catabolite repressor protein CRE1, in the *cbh1* promoter by binding sites for the transcription activators ACE2 (a cellulolytic activator) and the HAP2/HAP3/HAP5 complex (a general expression enhancer regulator) in *T. reesei*, enhanced transcription of a test gene (green fluorescent protein) under cellulase inducing conditions 7-fold (Zou et al., 2012). Similarly, introduction of additional copies of the enzyme encoding genes under control of string promoters can also improve enzyme production. Overexpression of cellobiohydrolases (*cbhI* and *cbhII*) in *T*. *reesei* under control of the *T. reesei cbh1* promoter achieved a 1.3- to 4-fold overexpression (Miettinen-Oinonen et al., 2005), while overexpression of its *bgl1* gene under control of the *egl3* or *xyn3* promoter, increased β-glucosidase activities by 4.0- to 7.5-fold (Barnett et al., 1991).

Combining the introduction of several genes can further enhance the effectivity of an enzyme mixture. Simultaneous expression of *bglI*, encoding a β-glucosidase from *A. niger* (AnBGL), and *eglIV*, encoding a lytic polysaccharide monooxygenase (LPMO) from *T. reesei* (TrLPMO), in *Penicillium verruculosum* under the control of the inducible *gla1* promoter resulted in more efficient hydrolysis of a lignocellulosic substrate than the control enzyme preparations (Bulakhov et al., 2017). Similarly, modification of the expression of a major regulator can also affect the enzyme mixture as a whole, such as the overexpression of *clrB* in *Penicillium oxalicum* using the *gpdA* promoter from *Aspergillus nidulans* that resulted in higher cellulase levels (Yao et al., 2015). Similar approaches have been performed for other regulators, such as the major (hemi-)cellulolytic regulator of *A. niger* (XlnR), resulting in increased levels of xylanases and cellulases (van Peij, N.N.M.E. et al., 1998). A combination of overexpression or activation of XlnR/Xyr1 and deletion of the major carbon catabolite repressor CreA/Cre1, resulted in even higher levels of (hemi-)cellulolytic enzymes in *A. niger* and *T. reesei* (Jiang et al., 2016). Similarly, overexpression of the amylolytic regulator AmyR, resulted in higher glucoamylase and α-amylase levels in *A. niger* (vanKuyk et al., 2012).

One of the drawbacks of genetic engineering in filamentous fungi has been the low frequency of targeted integration of the introduced gene. This is due to the fact that in these fungi DNA integration is mainly directed by non-homologous end joining (NHEJ), resulting in a very low frequency of site-specific recombination (Kück and Hoff, 2010). To improve this percentage, strains that are defective in NHEJ have been constructed in several fungal species. The NHEJ process is mediated by the DNA-dependent protein kinase catalytic subunit, the Ku70-Ku80 heterodimer, and the DNA ligase IV-Xrcc4 complex. A high percentage of homologous recombination is achieved when either the *ku70* or *ku80* gene is disrupted or deleted (Kück and Hoff, 2010). For example, deletion of the Ku70 homologue in *A. nidulans* (*nkuA*Δ) improved homologous integration from 13% to 90% (Nayak et al., 2006). Similar results have been reported for other fungi, such as *A. oryzae* (Takahashi et al., 2006), *Aspergillus fumigatus* (da Silva

Ferreira et al., 2006) and *Magnaporthe grisea* (Villalba et al., 2008). In *Penicillium decumbens*, deletion of *ku70* improved the targeting event to even 100% (Li, Z.H. et al., 2010). The availability of this methodology has also allowed the construction of gene knockout libraries of *Neurospora* and *Aspergillus*, leading to the identification of several new transcription factors involved in regulation of the production of cellulases and hemicellulases (Colot et al., 2006; Ogawa et al., 2012a; Tani et al., 2014). There is a potential risk in using this approach as Ku proteins are important to maintain telomere length in yeast and plants, and are necessary to ensure chromosome stability in mammals (Bailey et al., 1999; Boulton and Jackson, 1996). Phenotypic analysis of fungal strain defective in NHEJ demonstrated that these strains showed higher susceptibility to various toxins and irradiation (Nielsen et al., 2008). A recent study in *T. reesei* used transient silencing of NHEJ, to prevent those negative effects of *ku*-deletions (Chum et al., 2017). Such a transient system may be needed when applying removal of NHEJ in industrial production strains.

3. Novel strain engineering approaches

The availability of genome sequences and novel methodologies have strongly expanded the toolkit for fungal strain engineering, but also enable a higher level of control of the strain modifications as well as the analysis of the resulting progenies. In particular, highly precise genome editing technologies have broadened the range of modifications that can be done at a targeted locus. However, this would not be anywhere near as efficient without the current (post-)genomic methodologies that allow efficient design of genome editing approaches as well as detailed analysis of the resulting strains. These methodologies have also provided a better understanding of improved strains that are generated using non-targeted strain engineering methodologies (mutagenesis, evolutionary adaptation), providing additional leads for targeted strain engineering approaches.

3.1 CRISPR/Cas9 technology

3.1.1 Introduction to CRISPR/Cas9

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) system originates from bacterial and archaeal immune systems that contain *cas* genes and CRISPR array(s), which consist of short sequences that originated from foreign genetic material (also called spacers) interspaced with identical palindromic repeats (Jinek et al., 2012). The proteins encoded by the *cas* genes are responsible for acquisition of new foreign sequences into the CRISPR array(s) as well as for disruption of exogenous DNA through the activity of Cas proteins bearing endonuclease activity, such as the Cas9 protein (Richter et al., 2012).

In the CRISPR/Cas9 system, the Cas9 protein forms a complex with two RNA molecules: CRISPR RNA (crRNA) (Brouns et al., 2008), encoded by the random spacers found in the CRISPR array(s), and trans-activating CRISPR RNA (tracrRNA) (Deltcheva et al., 2011). It has been shown, that these two RNAs form a dual-tracrRNA:crRNA, which can also be designed as a single guide RNA (sgRNA) for genome editing purposes (Jinek et al., 2012). The Cas9-sgRNA complex is guided to a target DNA via homology of the crRNA to the protospacer region of the target sequence (Fig. 2). The Cas9 nuclease subsequently binds the target DNA through interaction with the protospacer adjacent motif (PAM) immediately downstream of the protospacer sequence. If the crRNA part of the sgRNA sequence successfully pairs with the target DNA, the Cas9 nuclease will perform a double strand break (DSB) three nucleotides upstream of the PAM motif (Garneau et al., 2010), which can be repaired either by the NHEJ or the homology-directed repair (HDR) pathway. The simple design and construction of a single guide RNA for

precise genome editing has rapidly expanded the toolbox of molecular methods, such as Zinc Finger Nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALENs) (Kim and Kim, 2014).

The SpCas9 from *Streptococcus pyogenes* is the most widely used Cas9 nuclease due to the abundant presence of its target PAM sequence (5'-NGG-3') (Jinek et al., 2012) throughout genomes of many types of organisms. This short, abundant PAM sequence provides many possibilities to edit the genes of interest, but it can also result in undesired mutations. The unintended mutations were mostly seen in organisms with large genomes, such as in human cell lines, where the CRISPR/Cas9 system has been reported to cause a wide variety of insertions, deletions and point mutations or even more complex genomic rearrangements (Cradick et al., 2013; Fu et al., 2013; Kosicki et al., 2018). However, there is no current evidence of complex CRISPR/Cas9-induced rearrangements in filamentous fungi. A comprehensive CRISPR/Cas9 off-target analysis would potentially decrease the probability of undesired mutations in these target organisms.

3.1.2 Implementation and improvement of CRISPR/Cas9 in filamentous fungi

CRISPR/Cas9 is a cost-efficient and simple platform to perform genetic manipulations with a single enzymatic activity guided by a pre-designed sgRNA molecule. Therefore, it has become a common genome editing method in a variety of organisms, such as yeast (DiCarlo et al., 2013) and human cell lines (Cong et al., 2013; Mali et al., 2013), and has been reported to work in numerous filamentous fungi, including some basidiomycete species (Table 2). In filamentous fungi, this system has been used to either target genes involved in metabolite or enzyme production (Deng et al., 2017a, b, 2018; Kuivanen et al., 2016; Liu, Q. et al., 2017; Liu, R. et al., 2017; Matsu-ura et al., 2015; Matsuda et al., 2018; Nielsen et al., 2017; Wang, Q. et al., 2018; Weber et al., 2017; Xu, G. et al., 2018) or to establish a CRISPR/Cas9 genome editing system in a new species (Arazoe et al., 2015; Fuller et al., 2015; Katayama et al., 2016; Liu et al., 2015; Matsu-ura et al., 2015; Nødvig et al., 2015; Pohl et al., 2016). In some cases, CRISPR/Cas9 was tested in closely or even distantly related fungal species to demonstrate the versatility of the system (Zheng et al., 2017).

In order to perform genetic manipulations with the CRISPR/Cas9 system, both the Cas9 nuclease and the sgRNA need to be present in the host organism. In fungi this can be achieved either through integration of *cas9* and sgRNA encoding constructs into the genome (Fuller et al., 2015), through expression from a replicative plasmid encoding both the *cas9* gene and sgRNA (Nødvig et al., 2015), transient expression of the system from a non-replicating plasmid (Matsu-ura et al., 2015), or by using *in vitro* assembled ribonucleoproteins (RNPs) (Pohl et al., 2016). In some cases the combination of *in vitro* and *in vivo* methods was applied with the utilization of *in vitro* synthesized sgRNA, while the *cas9* gene was either integrated into the genome of the host organism (Liu et al., 2015) or expressed from a replicative plasmid (Kuivanen et al., 2016). Additionally, genome editing in the mucoromycota species *Mucor circinelloides* was performed using *in vitro* synthesized sgRNA and purified Cas9 protein, but without *in vitro* RNP formation (Nagy et al., 2017).

The first report about application of the CRISPR/Cas9 system in a filamentous fungus was for the induction of mutagenesis in the industrially relevant enzyme producer *T. reesei* (Liu et al., 2015). The *cas9* gene of *S. pyogenes* was codon-optimized and fused with the SV40 nuclear localization signal (NLS), after which the resulting expression construct was randomly inserted into the genome of *T. reesei*. The *cas9* gene was expressed under the control of the constitutive promoter *Ppdc* or the inducible promoter *Pcbh1*, demonstrating that this system can work as an efficient and controllable genome editing tool in *T. reesei* (Liu et al., 2015). Shortly after the initial successful application of the CRISPR/Cas9 system in *T.*

reesei, Nødvig et al. published a self-replicating plasmid-based CRISPR/Cas9 transformation system, which was successfully applied in six *Aspergillus* species (Nødvig et al., 2015). For this, four plasmids were constructed, each containing a different fungal selection marker, either an auxotrophic marker (*AFUM_pyrG* and *AN_argB*) or an antibiotic selection marker (hyg^R and ble^R) (Nødvig et al., 2015). All these plasmids carried the *cas9* from *S. pyogenes*, which was codon optimized for *A. niger* and extended with the SV40 NLS (Nødvig et al., 2015), similar as in *T. reesei* (Liu et al., 2015). Importantly, a key component of this system established in Aspergilli is the *ama1* gene from *A. nidulans* (Gems et al., 1991), which enables plasmids to autonomously replicate in many fungal species and therefore prevents the need for integration of *cas9* gene into the fungal genome. Moreover, this self-replicating plasmid based method allows the possibility of marker-free genome editing, enabling multiple editing steps using the same selection marker (Nødvig et al., 2015). The sgRNA constructs were cloned into the CRISPR/Cas9 vectors in a single USER-cloning step (Nour-Eldin et al., 2010), resulting in *in vivo* expression of the guides under the control of the strong constitutive *PgpdA* promoter and the *TtrpC* terminator (Nødvig et al., 2015). Due to the lack of well-defined RNA polymerase III promoters in filamentous fungi, such as the U6 promoter (Miyagishi and Taira, 2002), the sgRNA was released from a larger polymerase II transcript by the action of two ribozymes (Nødvig et al., 2015). The plasmids of this study proved to be widely usable for genome editing in Aspergilli and even for the establishment of CRISPR/Cas9 transformation systems in phylogenetically distinct organisms (Alazi, Ebru et al., 2018; Kuivanen et al., 2017; Kuivanen and Richard, 2018; Kuivanen et al., 2016; Liu, N. et al., 2017; Matsuda et al., 2018; Nielsen et al., 2017; Wenderoth et al., 2017; Weyda et al., 2017).

The establishment of a CRISPR/Cas9 system in the industrially relevant ascomycete, *A. oryzae,* also provides possibilities for improved (heterologous) protein production (Katayama et al., 2016). While this system resulted in a low mutation rate (10-20%), it was demonstrated to work in two strains used for sake and soy sauce production, highlighting the opportunities for genome editing of industrially relevant strains (Katayama et al., 2016).

It was recently shown that using NHEJ deficient strains of *A. nidulans, A. niger* and *A. oryzae*, successful gene targeting was achieved with single-stranded 90-mer oligonucleotides as repair templates of Cas9-induced DNA double-strand breaks (Nødvig et al., 2018). This approach can be used to introduce precise modifications in the sequence of a gene of interest. Moreover, it was reported that using the *A. fumigatus* U3 promoter to mediate expression of sgRNAs, multiple gene alterations could be performed at the same time, which facilitates complex genetic engineering in filamentous fungi (Nødvig et al., 2018).

Basidiomycete fungi are less commonly used in biotechnology applications, partly due to the lack of genetic transformation systems for most species and relatively poor behavior in submerged fermentations of many species. More recently, the use of solid state fermentation of basidiomycete fungi for biological pre-treatment has gained increased attention (Pandey et al., 2018; Xu, X. et al., 2018; Zhou et al., 2015), which may soon result in increased attempts for strain engineering of these fungi. The availability of genome sequences has revealed the wealth of plant biomass degrading enzymes in basidiomycete fungi (Rytioja et al., 2014). This has raised interest in the development of these fungi for applications, requiring efficient strain engineering methodologies. So far, genome editing in basidiomycetes has only been described for few species, including *Coprinopsis cinerea* (Sugano et al., 2017), *Ganoderma lingzhi*, *Ganoderma lucidum* (Qin et al., 2017), *Ustilago maydis* (Schuster et al., 2018; Schuster et al., 2016) and *Ustilago trichophora* (Huck et al., 2018). It is worth to mention that the *U. maydis* CRISPR/Cas9 system (Schuster et al., 2016) involved the utilization of a plasmid carrying an autonomously replicating sequence (ARS) (Tsukuda et al., 1988) responsible for self-replication, which is a similar approach to the one developed for ascomycete fungi (Nødvig et al., 2015). In particular, wood-

degrading white rot basidiomycetes are essential for efficient degradation of lignin (Mäkelä et al., 2014) and the adaptation of a CRISPR/Cas9 transformation system in these organisms could facilitate the generation of hyperligninolytic strains for applications in which removal of lignin is needed.

3.1.3 Applications of CRISPR/Cas9 for lignocellulolytic enzyme production

Due to the broad range of mutations that can be introduced using CRISPR/Cas9, soon after its establishment in filamentous fungi it was used for the improvement of the production of lignocellulolytic enzymes (Fig. 2). Strain engineering to manipulate the regulatory system controlling lignocellulolytic enzyme production is an attractive way to not only understand the molecular mechanisms underlying production of these enzymes, but also to generate better enzyme production strains. Typically, these studies address species that are either industrial enzyme producers (e.g. *T. reesei*, *A. niger*, *Myceliophthora thermophila*) or well-established academic reference species (e.g. *N. crassa*). Some examples of this are given below.

The CRISPR/Cas9 system established in *T. reesei* (Liu et al., 2015) was recently used to study a negative regulator of xylanase activity, named SxlR (Liu, R. et al., 2017). The overexpression of this regulator resulted in reduced xylanase activity, but did not affect cellulase activity, while the deletion of *sxlR* gene resulted in a significant increase in expression of genes encoding GH11 endoxylanases. Similarly, in *A. niger* the auxotrophic *pyrG* marker containing plasmid (Nødvig et al., 2015) together with *in vitro* synthesized sgRNA were used to introduce a mutated version of the gene encoding the D-galacturonic acid regulator, GaaR, into the endogenous *gaaR* locus (Alazi, Ebru et al., 2018). The modified *gaaR* gene carried a single point mutation causing a W361R amino acid change and resulting in a constitutively active form of GaaR, leading to inducer-independent production of pectinolytic enzymes (Alazi, Ebru et al., 2018).

The CRISPR/Cas9 system was also successfully implemented in the ascomycete *M. thermophila* (Liu, Q. et al., 2017), an industrially relevant thermophilic species used for high-temperature fermentations and production of thermostable lignocellulolytic enzymes (Maheshwari et al., 2000). The target genes for deletions included a carbon catabolite repressor *cre-1*, an endoplasmic reticulum stress regulator *res-1*, a β-glucosidase *gh1-1* and an alkaline protease *alp-1* (Liu, Q. et al., 2017). The gene replacement frequency was 95%, which is much higher than the 20% frequency achieved in traditional transformations. In addition, this system was successfully applied for multiple simultaneous deletions, deleting up to four genes in one transformation event (Liu, Q. et al., 2017). All these deletions contributed to the improved (hemi)cellulase activity, which reached 13.3-fold increased activity compared to the wild type strain (Liu, Q. et al., 2017). This system was also used without modifications in *Myceliophthora heterothallica*, indicating the possibility of application in other related *Myceliophthora* species (Liu, Q. et al., 2017). Recently, the same CRISPR/Cas9 system (Liu, Q. et al., 2017) was used for the improved production of amylolytic and (hemi)cellulolytic enzymes through rational design of *M. thermophila* strains (Xu, G. et al., 2018). The deletion of a major amylase gene Mycth_72393, was shown to result in 23.6% lower amylase activity on starch, while the overexpression of the same gene resulted in 35% increased activity, highlighting the essential role of this enzyme in starch degradation. Overexpression of the key amylolytic enzyme regulator encoding gene *amyR* increased the amylase activity by 30%, while the deletion of this gene resulted in 23.7% reduced amylase activity in liquid culture supernatant (Xu, G. et al., 2018). The deletion of *amyR* also resulted in 3-fold increase in CMCase and xylanase activity.

The filamentous fungus *N. crassa* was also genetically engineered with the CRISPR/Cas9 system in 2015 (Matsu-ura et al., 2015). The *cas9* gene was expressed under the control of the *trpC* promoter and terminator region from *A. nidulans* after integration into the genome of *N. crassa*. The Small Nucleolar RNA 52 (SNR52) promoter from the yeast *Saccharomyces cerevisiae* (Harismendy et al., 2003) was successfully used to overexpress the single-guide RNA targeting the *clr-2*. The *clr-2* encodes a core transcription factor involved in the regulation of cellulase expression (Coradetti et al., 2012). By placing the *clr-2* gene under the control of a β-tubulin promoter, approximately two hundred-fold increase of *clr-2* mRNA expression was observed compared to the wild type strain, which consequently increased the expression of cellulase genes (Matsu-ura et al., 2015).

Overall, these results show that the CRISPR/Cas9 system has been efficiently applied in filamentous fungi to characterize transcription factors involved in the regulation of lignocellulose utilization and to increase the lignocellulolytic enzyme production. The establishment of this transformation system in a broad range of filamentous fungi can further increase the possibilities to generate more efficient enzyme producing strains suitable for industrial applications.

3.2 Adaptive evolution

In nature, fungi evolved to adapt optimally to their environment resulting in highly diverse physiologies for different species. Applying the possibility for adaptive evolution in strain engineering by repeated culturing on a selective medium is a relatively novel approach in fungal biotechnology. It was first demonstrated for the yeast *S. cerevisiae* by improvement of its ability to ferment D-xylose (Sonderegger and Sauer, 2003), and was then also applied to generate *S. cerevisiae* strains that efficiently co-fermented D-glucose, L-arabinose and D-xylose (Wisselink et al., 2009). This method was also applied for other features of this species, such as improved glycerol production and sulfite tolerance (Kutyna et al., 2012), improved growth on glycerol (Strucko et al., 2018) and acetic acid tolerance (Gurdo et al., 2018).

Less examples of the use of adaptive evolution have been reported for filamentous fungi, with the first being adaptive evolution of *Metarhizium anisopliae* towards strains showing robust growth at 37^{o}C (de Crecy et al., 2009). A similar approach was later used to obtain *A. nidulans* strains with increased growth rate due to adaptation to growth on solid media (Schoustra and Punzalan, 2012). More recently, adaptive evolution has been shown to enable higher production of plant biomass degrading enzymes in *Aspergillus* species.

A. niger grows poorly on pure cellulose, but successive growth of this species on agar plates with cellulose as the only carbon source resulted in significantly improved growth and sporulation (Patyshakuliyeva et al., 2016). Analysis of the best mutant demonstrated increased cellobiohydrolase and β-glucosidase activity, while transcriptome analysis revealed reduced expression of the ortholog of the cellulase repressor of *Podospora anserina* (Brun et al., 2009). The role of this gene in repressing cellulase production was confirmed by deleting it in *A. niger*, resulting in increased levels of cellobiohydrolase and β-glucosidase activity (Patyshakuliyeva et al., 2016).

Similarly, successive culturing of *A. oryzae* on agar plates with inulin resulted in significantly improved growth of the progeny (Culleton et al., 2016). Interestingly though, the best mutant did not only display increased inulinase activity, but also several other plant biomass degradation related activities. The molecular basis for this change is not yet clear, but may for instance be caused by an increased overall secretion capacity.

These examples indicate the high potential of adaptive evolution for strain engineering, especially when GMO approaches are not desired, such as in food-related applications.

3.3 Incorporation of omics technologies into fungal strain improvement

In the post-genomic era, the development of high-throughput analyses has proven them to be powerful tools to enhance our understanding on complex biological systems (Joyce and Palsson, 2006). The current omics approaches include genome sequencing, global transcriptomic profiling, proteomics and metabolomics, which allow a deeper examination of all components, interactions and functional states of the biological molecules in the cell. These new methodologies also provide a novel approach to strain engineering, not only in the analysis of the progenies of both forward and reverse genetics, but also in more strategic options to approach strain engineering.

Many of the omics methodologies were first implemented in the yeast *S. cerevisiae*. While *S. cerevisiae* has no significant ability to degrade plant biomass, we include some of the strain improvement studies of this species here, as they provide examples of the potential of omics methodologies that can be also applied to engineering of filamentous fungi. *S. cerevisiae* is a glucose fermenting species, which has been intensively used by the bioethanol industry. However, it is unable to utilize many compounds derived from the hydrolysis of lignocellulosic biomass, such as pentoses (D-xylose, L-arabinose) and the disaccharide cellobiose (Wang et al., 1980). To broaden its applicability, a large amount of research has been focused on engineering *S. cerevisiae* to convert xylose to ethanol (Ostergaard et al., 2000; Roca and Olsson, 2003; Tantirungkij et al., 1993). For example, two *S. cerevisiae* strains, TMB 3399 and 3400, were described that were both able to catabolize and ferment D-xylose to ethanol (Wahlbom et al., 2003b). These recombinant strains were constructed by chromosomal integration of the genes encoding D-xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK). *S. cerevisiae* TMB 3400 showed a 5-fold increase in growth rate and lower xylitol production than *S. cerevisiae* TMB 3399 when both were cultivated on D-xylose under oxygen limitation and anaerobic conditions. Subsequently, mRNA expression levels were compared in these strains showing a higher expression of a hexose transporter encoding gene *hxt*, a xylulokinase encoding gene *xks*, and genes *sol3, gnd1* and *tkl1* encoding enzymes involved in the pentose phosphate pathway (Wahlbom et al., 2003a). These early studies can be considered as first steps towards a more profound understanding of *S. cerevisiae* metabolic engineering and gene expression analyses, which strongly facilitate the strategies for strain improvement.

The lignocellulose pretreatment for the production of 2nd generation biofuels generates several inhibitory compounds that prevent fungi and their enzymes to efficiently hydrolyze the substrate. Comparative transcriptome analysis has been used to engineer *S. cerevisiae* strains with increased tolerance to inhibitors derived from lignocellulose (Thompson et al., 2016). More recently, a better fermentation performance was reported for a mutant strain of *S. cerevisiae* that is tolerant to acetic acid and furfural originating from lignocellulose by applying comparative proteomics and metabolomics analyses together with high-throughput phenotyping (Unrean et al., 2018). Changes were observed in the maintenance of energy and redox homeostasis as well as in the minimization of stress-induced cell damages.

As mentioned above, manipulation of regulators is an attractive approach of strain engineering to improve production of lignocellulolytic enzymes. The regulatory system driving this process is complex, including several transcription factors that respond to different inducers (Benocci et al., 2017). Omics analyses can provide a comprehensive understanding of signal compounds and key

transcriptional regulators, which may improve the engineering of industrial strains for higher productivity of target enzymes. A first requirement for this is the availability of genome sequences of relevant species, such as members of the genus *Aspergillus*. In 2005, the genome sequence of *A. oryzae* (Machida et al., 2005), *A. nidulans* (Galagan et al., 2005) and *A. fumigatus* (Nierman et al., 2005) were released. Some years later, the number of published and annotated fungal genomes increased exponentially (Andersen et al., 2011; de Vries et al., 2017; Futagami et al., 2011; Pel et al., 2007; Sato et al., 2011). As model organisms for basic research and major species for biotechnology, there is a considerable body of literature on Aspergilli and the recent advances in post genomic analyses has generated new knowledge for strain engineering (Battaglia et al., 2011; Borin et al., 2015; Coutinho et al., 2009; Khosravi et al., 2017; Kowalczyk et al., 2017). Genomics and post-genomics studies have accelerated progress in plant biomass degradation related research in these fungi, facilitating e.g. the discovery of novel regulators (Gruben et al., 2012; Gruben et al., 2014; Ogawa et al., 2012b, 2013) and enzymes (de Gouvea et al., 2018; de Souza et al., 2011; Mäkelä et al., 2017; Midorikawa et al., 2018; van den Brink et al., 2013). Some of these studies demonstrated the high variation in enzyme sets produced by *Aspergillus* species during growth on plant biomass, despite relatively similar genome content with respect to these enzymes (Benoit et al., 2015; de Vries et al., 2017). Currently, all species of the genus *Aspergillus* are being sequenced, with the first section recently published (Vesth et al., 2018), providing an unprecedented view into the diversity of a fungal genus. The differences in the plant biomass degrading approaches of Aspergilli revealed in these studies are perfect starting points for strategic strain improvement strategies for specific applications.

Omics techniques and data have already been used in several studies for strain improvement. Overexpression of gene encoding D-galacturonic acid responsive regulator GaaR in *A. niger* increased the transcription of genes encoding pectinases, D-galacturonic acid transporters and enzymes of the D-galacturonic acid pathway even under non-inducing conditions (Alazi, E. et al., 2018). Proteomic analysis of the *gaaR* overexpression strain showed high level of pectinases secretion when cultivated in fructose. The further deletion of the main carbon catabolite repressor gene *creA* also improved pectinase production. This modified *A. niger* strain with high pectinase production capacity showed high potential for industrial applications.

In another study, an *A. nidulans* hexokinase/glucokinase (*hxkA1*/*glkA4*) mutant was generated in order to prevent hexose consumption through glycolysis (Khosravi et al., 2018). A triple mutant was obtained through sexual crosses by combining these mutations with a deletion in *creA* (*creAΔ4 hxkA1 glkA4*). Transcriptomic and metabolomic analyses were performed to examine changes in gene expression profiles and identify metabolic profiles related to sugar catabolism. The results showed that the deletion of *creA* combined with blocking glycolysis resulted in an increased expression of two genes from pentose catabolic pathway (PCP) and five genes from the pentose phosphate pathway (PPP). In addition, several glycolytic genes were downregulated in both double and triple mutants when the mutant strains were grown on starch and cellulose. This strongly suggests that blocking glycolysis caused an initial negative feedback of D-glucose release and activated alternative metabolic conversion of this sugar and indicates that metabolic engineering of fungi for biotechnology applications will need to take into account additional pathways to obtain the desired result.

Re-annotation of the CAZy gene content of the *T. reesei* genome in combination with the gene expression analysis in the presence of different carbon sources has identified uncharacterized enzymes and new insights on enzymes needed for plant polysaccharide degradation (Häkkinen et al., 2012). Strain engineering of the industrial lineage of *T. reesei*, in order to include those enzymes in the commercial mixtures, is likely to increase the efficiency of the mixtures for plant biomass degradation. A gene co-expression network analysis was also performed (Borin et al., 2018) based on transcriptome

data of the *T. reesei* RUT-C30 (Dos Santos Castro et al., 2014) in order to identify new target genes involved in sugarcane bagasse degradation. The *xyr1* gene encoding major positive regulator of cellulases and hemicellulases was co-expressed with 50 upregulated cellulase, hemicellulase and oxidative enzyme encoding genes. When the *ace1* gene, encoding ACE1 repressor involved in regulation of cellulase gene expression, was replaced with the endoglucanase gene *egl1* in *T. reesei* RUT-C30, an increased expression of cellulolytic regulators was observed (Meng et al., 2018). Compared to the RUT-C30 strain, the mutant showed 90% and 132.7% increase in total cellulase and endoglucanase activities. Moreover, cellulases produced by the engineered strain were more efficient for hydrolyzing pretreated corn stover and Jerusalem artichoke stalk than those of RUT-C30.

Omics analysis has been widely used in other filamentous fungi as well. The genome of another industrially used fungus, *M. thermophila*, revealed a wide enzymatic repertoire including hydrolytic, oxidative and auxiliary enzymes (Berka et al., 2011), which offered a starting point for further investigation of molecular mechanisms and strain improvement (Kolbusz et al., 2014; Liu, Q. et al., 2017; Wang, J. et al., 2018). Similarly, the availability of a full genome of *N. crassa* has allowed the identification of two essential transcription factors, CLR-1 and CLR-2, which are required for the expression of cellulolytic genes (Coradetti et al., 2012), and the identification of the ortholog (XLR-1) (Sun et al., 2012) of the previously identified (hemi-)cellulolytic transcription factor XlnR from *A. niger* (van Peij, N.N. et al., 1998). Lately, chromatin immunoprecipitation (ChiPseq) and RNA sequencing were performed in order to identify biding regions for CLR-1, CLR-2 and XLR-1 (Craig et al., 2015). The results showed that CLR-1 bound to the regulatory regions of 293 genes in Avicel cellulose cultures, while CLR-2 bound to promoter sites of 164 genes in sucrose cultures when cellulase activity was not detectable. During growth on xylan, XLR-1 bound to the promoters of 84 genes, including genes encoding six hemicellulases, three acetylxylan esterases, one β-glucosidase and two β-xylosidases. The identification and functional analysis of these transcriptional regulators related to plant biomass degradation contributes to unraveling the molecular mechanisms underlying this process in filamentous fungi. Manipulation of transcriptional regulators is a highly promising approach for industrial strain engineering as it targets the system as a whole, rather than individual enzyme activities.

Advances in genome sequencing and innovative high-throughput technologies have also revealed new insights into the molecular basis of plant biomass degradation by basidiomycetes. While strain engineering of basidiomycetes is still in its infancy, a number of omics studies have opened up insights towards further development of these fungi for biotechnology. This includes the identification of the enzymatic sets employed by basidiomycete fungi for plant biomass degradation (Fernández-Fueyo et al., 2012; Hori et al., 2018; MacDonald and Master, 2012; Mahajan and Master, 2010; Martinez et al., 2009; Rytioja et al., 2017), as well as the identification of the small molecular mass inducers of the regulatory systems controlling this process (Casado López et al., 2018). Recently, a comparative analysis of basidiomycete transcriptome datasets was reported (Peng et al., 2017), which showed that a large set of conserved CAZymes encoding genes are expressed in plant biomass related substrates, suggesting that these enzymes are critical for degradation of any plant biomass, and should therefore always be present in commercial enzyme cocktails.

3.4. Epigenetics in fungi and its potential for strain engineering

Typically, three types of genetic phenomena are considered under the heading epigenetics: chromatin remodeling through histone modification, DNA methylation and RNA interference. All three phenomena have been demonstrated in fungi, although not always all three in the same fungal species (Smith et al., 2012). While no specific use of epigenetics for strain engineering have been reported, indication of its

potential have been obtained (Blenner, 2018). In *T. reesei*, the nucleosomes -1 and -2 downstream of the activating element of the *cbh2* promoter are loosened under inducing conditions, making the TATA box accessible (Zeilinger et al., 2003). Interestingly, deletion of the xylanase regulator encoding gene (*xyr1*) in this fungus significantly reduced the chromatin opening (Mello-de-Sousa et al., 2015). This is likely due to control of the expression of 15 putative chromatin remodeling genes by XYR1. Chromatin accessibility in *T. reesei* also seems to be affected by the global carbon catabolite repressor protein CRE1, as a deletion or a truncated version of this regulator resulted in a more open structure of the chromatin in the promoter regions of *cbh1* and *cbh2* (Mello-de-Sousa et al., 2014; Ries et al., 2014). The direct role of chromatin structure was confirmed by a deletion of a histone acetyltransferase in *T. reesei*, which not only resulted in decreased growth and morphological changes, but also in strong reduction of cellulolytic genes under inducing conditions (Xin et al., 2013). A similar result was obtained for a deletion strain of a histone lysine methyltransferase in *Pyricularia* (formerly *Magnaporthe*) *oryzae*, which resulted in significant reduction of the expression of a cellulase gene (Vu et al., 2013). An aspect related to that is the fact that certain regions of the genome provide higher expression levels than others (Blenner, 2018). Identification of these regions would provide better locations for genetic engineering, possibly ensuring higher enzyme production.

Indications for a role of DNA methylation in the expression of genes encoding plant biomass degrading enzymes have also been reported using an inhibitor of DNA methyltransferase, 5-aza-2'-deoxycitidine (Manfrão-Netto et al., 2018). In the presence of this inhibitor xylanase activity was increased during growth on wheat bran, while expression of cellobiohydrolase and xylanase encoding genes was increased during growth on glucose. It should however be noted that results from studies with inhibitors should be interpreted with caution due to side specificities they may have (Aghcheh and Kubicek, 2015).

While not studied in detail, the presence of antisense reads in transcriptomic studies of *T. reesei* (Ries et al., 2013) and *A. niger* (Delmas et al., 2012), suggests the possibility of RNA interference as a regulatory mechanism in these fungi. This is further strengthened by the observation that genes with mainly sense transcripts on wheat bran and antisense transcripts on glucose included several encoding plant biomass degrading enzymes (Delmas et al., 2012).

3.5 Selection and use of monokaryotic strains

Some fungi possess a sexual reproductive cycle, which provides an opportunity for strain selection and engineering through recombination during meiosis (Kothe, 1996). Dikaryotic fungal species produce monokaryotic offspring with diverse genetic combinations and therefore successive cycles of basidiome production and crosses can result in strain improvement without the need for mutagenesis (Wyatt and Broda, 1995). Several examples of this have been reported, such as for the white rot species, *Trametes versicolor*, where a monokaryon grew better than its parental dikaryon on glucose-soy agar and hardwood kraft pulp (Addleman and Archibald, 1993). Differences in laccase production were found for mono- and dikaryotic strains of *Pycnoporus cinnabarinus* (Herpoël et al., 2000), while this was also the case for laccases and other lignocellulolytic activities of *Pleurotus ostreatus* (Eichlerová et al., 2000; Linke et al., 2018). This offers interesting possibilities for strain improvement in these fungi, although the random nature of the genetic recombination may require the screening of an extensive set of progenies to obtain the best producing strain.

4. Selection of screening conditions

Irrespective of the approach chosen for strain engineering, the selection of the best resulting strain largely depends on the screening strategy chosen for the progenies. The ideal screening approach should provide a high probability of obtaining the desired alteration with a low chance of side-effects, such as changes that could later show to be disadvantageous. Typically, screening approaches can range from relatively simple (e.g. growth profiling) to more complicated and laborious (e.g. PCR-based analysis) methods. The design of the screen is likely the most important aspect of any strain engineering strategy, although technical limitations may be enforced by the engineering approach. In this section, some examples of screening approaches applied to filamentous fungal strains are presented.

If the desired improvement results in a growth phenotype, a direct selection of the progenies based on fungal growth on the screening media can be applied. This is for instance possible when the aim is to obtain strains with higher xylanase activity. Growth of progeny of the fungi on agar plates with partially soluble xylan as a carbon source, will result in clearing around the colony, with the largest clearing diameter indicating the highest xylanase activity (Ali et al., 2017).

However, in many cases growth on selective medium is combined with an indicator of enzyme activity. These can be highly specific substrates, such as 4-methylumbelliferyl glycosides, that can be used to screen for specific enzyme activities by detecting fluorescence of the released 4-methylumbelliferone (Ademark et al., 2001), but also staining of the screening/growth substrate is commonly used. After growth on agar plates supplemented with carboxymethylcellulose (CMC), the plates can be flooded with Gram's iodine, resulting in a bluish-black complex with cellulose, while a clear zone is visible where cellulose has been hydrolyzed, thus indicating cellulase activity (Kasana et al., 2008). Use of Congo-Red will also provide a similar clear zone on CMC as an indication of cellulase activity (Sarsaiya et al., 2018), while screening for increased starch hydrolysis can be done on starch agar plates using remazol brilliant blue (Vu et al., 2010), and for polygalacturonase activity on polygalacturonic acid agar plates using cetyltrimethylammonium bromide (Heerd et al., 2014).

When improving enzyme production in fungi, enzyme activity assay based screening methods are advantageous as they provide detailed information about the modification that occurred in the progenies. To screen strains with an improved ability to degrade plant biomass, the measurement of the amount of reducing sugars using dinitrosalicylic acid (DNS) (Miller, 1959) has become a commonly used method, and is highly suitable for a high-throughput (robotic) setup. The DNS method measures the overall release of sugars from the reducing-ends of oligo- and polysaccharides and is therefore not specific to a certain linkage of activity, but does exclude the detection of activity of oxidative enzymes, such as lytic polysaccharide monooxygenases (LPMOs). Some specificity can be obtained by not using crude plant biomass as a substrate, but pure polysaccharides (e.g. cellulose, xylan, polygalacturonic acid) (Batista-Garcia et al., 2017; da Silva Menezes et al., 2017; Ja'afaru, 2013; Zeni et al., 2011). However, this will still measure the combined activity of all (endo- and exo-acting) enzymes that depolymerize the used substrate. This method is therefore particularly useful for the selection of strains in which a number of enzyme activities or overall plant biomass degrading ability is improved. Variations on this method have also been described, some of which have a higher sensitivity than the original method. For example, the use of the formation of osazones from reducing sugars and *p*-hydroxybenzoic acid hydrazide was reported to be 5-fold more sensitive than a typical DNS assay and could therefore be efficiently used in 96-well plates and with low enzyme loading (Mellitzer et al., 2012). The use of micro-plate cultures and enzyme activity assays also allowed for the relatively simple analysis of both cellulase and xylanase activity (Cianchetta et al., 2012).

An example of a different method to screen for progenies is the use of the *cbh1* promoter in front of a gene encoding a fluorescent protein (DsRed) in *T. reesei*, which allowed for screening for

overproduction of cellulases by fluorescence (Gao et al., 2018). Recently, a novel screening method using micro-fluidics was reported, in which single spores of *A. niger* sorted by fluorescence-activated cell sorting (FACS) germinated and grew in 10 nl droplets and were suitable for fluorescence-based enzymatic screening, as demonstrated for strains with improved α-amylase activity (Beneyton et al., 2016). This method may facilitate high-throughput low volume screening that would improve current approaches.

5. Future perspective

Fungal strain engineering has a long history, but as indicated in the previous sections, the possible approaches to obtain strains with improved performance for the production of plant biomass degrading enzymes has broadened significantly in recent years. This is due to the recently established methods such as CRISPR/Cas9 genome editing and the potential of implementing epigenetics. These tools are still being further developed to higher efficiency and are accompanied by improved fungal genome sequences, exemplified by the gold-standard genome for *A. niger* (Aguilar-Pontes et al., 2018) and the recently initiated genome sequencing project of the Joint Genome Institute of the Department of Energy of the USA (https://jgi.doe.gov/csp-2019-finishing-genomes/). This diversity of possibilities for strain engineering will facilitate a more strategic choice in the best approach for a certain ultimate aim also keeping in mind legislation/public acceptance with respect to GMO methodology.

An important challenge is to make strain engineering applicable to a wider range of fungi, which requires the development of efficient genetic transformation systems for them. In addition, effective submerged or solid-state fermentation protocols are essential for application of fungi in industrial processes. With those hurdles removed, the potential of strain engineering will go far beyond the currently used fungal species and strains, and will likely significantly contribute to the establishment of a bio-based economy. Similarly, development of robotic screening methods for a wider range of enzyme activities would strongly stimulate selection of progenies, as this is now still quite laborious for several enzyme activities.

While combinations of different omics data (proteomics, transcriptomics, metabolomics) have already resulted in some deeper insights into the molecular mechanisms of plant biomass conversion of filamentous fungi (Daly et al., 2018; Edwards et al., 2017; Patyshakuliyeva et al., 2015; Rytioja et al., 2017; Samal et al., 2017), a strong development in this area can be expected in the coming years. This will not only be due to better correlations of such datasets, but also incorporation of other methodologies, such as ChIP-seq (Samal et al., 2017; Seiboth et al., 2012) and DAP-seq (Bartlett et al., 2017), which will provide many new leads for strain engineering.

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Table 1: Classical strain engineering approaches. This table includes some examples of the improvements obtained in the production of enzymes in filamentous fungi that were engineered by classical methods.

Species	Improvements	Method*	References
Aspergillus niger	2-fold increase in FPase and CMCase activity	UV mutagenesis	(Irfan et al., 2011)
Aspergillus oryzae	4-fold increase in FPase and CMCase activity	UV mutagenesis Chemical mutagenesis (NTG)	(El-Ghonemy et al., 2014)
Aspergillus tamarii	Increased pectinase activity from 59 U/ml to 65 U/ml	UV mutagenesis Chemical mutagenesis (Hydrogen peroxide)	(Akbar et al., 2015)
Aspergillus terreus	2- to 5-fold increase in CMCase, avicelase, FPase and β-glucosidase activity	UV mutagenesis Chemical mutagenesis (EMS)	(Kumar et al., 2015)
Chaetomium cellulolyticum	1.6-fold increase in CMCase activity	□ Irradiation	(Fawzi and Hamdy, 2011)
Fusarium oxysporum	80% higher cellulolytic activity	UV mutagenesis Chemical mutagenesis (NTG)	(Kuhad et al., 1994)
Gliocladium virens	Higher □-1,4-glucosidase, CMCase and FP cellulase activity	UV mutagenesis Chemical mutagenesis (EMS)	(Papavizas et al., 1990)
Humicola insolens	Increase of 115% in CMCase, 303% in FPase and 196% in β-glucosidase activity	UV mutagenesis Chemical mutagenesis (MNNG)	(Mariyam, 2011)
Penicillium echinulatum	High FPase activity	UV mutagenesis	(Dillon et al., 1992)
Penicillium janthinellum	3-fold increase in FPase and 2-fold increase in CMCase activity	UV mutagenesis Chemical mutagenesis (EMS)	(Adsul et al., 2007)

Penicillium oxalicum	2.7-fold increase in CMCase activity	UV mutagenesis Chemical mutagenesis (EtBr)	(Caniago et al., 2015)
Trichoderma reesei	2.7-fold increase in protein secretion, and 2.8-fold increase in FPase activity	UV mutagenesis Chemical mutagenesis (N-nitroguanidine)	(Peterson and Nevalainen, 2012)

*Only the methods used are mentioned, the methodology and the order in which the methods were used varies in each case and in many of them the strains were subjected to successive mutation steps. FPase = filter paper activity, CMCase = carboxymethyl cellulase activity, NTG = N-methyl-N'-nitro-N-nitrosoguanidine, EMS = ethylmethane sulphonate, MNNG = N-methyl-N-nitro-N-nitrosoguanidine, EtBr = ethidium bromide.

Table 2: CRISPR/Cas9 systems in filamentous fungi. This table includes the establishment of a CRISPR/Cas9 transformation system in different species, the utilization of CRISPR/Cas9 mediated genome editing for enzyme/metabolite production or investigation of metabolic pathways.

Species	Purpose/Relevance	Origin of Cas9	Origin of sgRNA	References
Alternaria alternata	Establishment of CRISPR/Cas9 system	AMA1 plasmid		(Wenderoth et al., 2017)
Aspergillus aculeatus	Establishment of CRISPR/Cas9 system	AMA1 plasmid		(Nødvig et al., 2015)
Aspergillus brasiliensis	Establishment of CRISPR/Cas9 system	AMA1 plasmid		(Nødvig et al., 2015)
Aspergillus carbonarius	Establishment of CRISPR/Cas9 system	AMA1 plasmid		(Nødvig et al., 2015)
	Comparison of gene targeting tools	AMA1 plasmid		(Weyda et al., 2017)
Aspergillus fumigatus	Establishment of CRISPR/Cas9 system	Integrative		(Fuller et al., 2015)
	Reconstitution of trypacidin production	Integrative		(Weber et al., 2017)
	Establishment of gene integration via microhomology	Integrative or AMA1 plasmid	Integrative, *in vitro* or AMA1 plasmid	(Zhang, C. et al., 2016)
	Application of RNP mediated transformation method	*In vitro* RNP[1]		(Al Abdallah et al., 2017)
	Off-target assessment	*In vitro* RNP[1]		(Al Abdallah et al., 2018)
	Investigation of azole resistance	*In vitro* RNP[1]		(Umeyama et al., 2018)
Aspergillus luchensis	Establishment of CRISPR/Cas9 system	AMA1 plasmid		(Nødvig et al., 2015)

Aspergillus nidulans	Establishment of CRISPR/Cas9 system	AMA1 plasmid		(Nødvig et al., 2015)
	Investigation of zaragozic acid A biosynthetic pathway	AMA1 plasmid		(Liu, N. et al., 2017)
	Identification of a novel promoter for sgRNA^2 expression; Efficient gene targeting with oligonucleotides; Multiplex genome editing	AMA1 plasmid		(Nødvig et al., 2018)
Aspergillus niger	Establishment of CRISPR/Cas9 system	AMA1 plasmid		(Nødvig et al., 2015)
	Efficient gene targeting with oligonucleotides	AMA1 plasmid		(Nødvig et al., 2018)
	Galactaric acid production	AMA1 plasmid	*In vitro*	(Kuivanen et al., 2016)
	Identification of enzymes involved in the D-glucuronic pathway	AMA1 plasmid	*In vitro*	(Kuivanen et al., 2017)
			AMA1 plasmid	(Kuivanen and Richard, 2018)
	Development of a gene integration system	AMA1 plasmid		(Sarkari et al., 2017)
	Identification of novel promoters for sgRNA^2 expression	Integrative		(Zheng et al., 2018a; Zheng et al., 2018b)
		AMA1 plasmid	AMA1 plasmid or integrative	(Song et al., 2018)
	Study of a constitutive GaaR regulator involved in pectinase production	AMA1 plasmid	AMA1 plasmid or *in vitro*	(Alazi, Ebru et al., 2018)

Aspergillus novofumigatus	Establishment of CRISPR/Cas9 system*;* *In vivo* and *in vitro* reconstitution of the biosynthetic pathway of novofumigatonin	AMA1 plasmid	AMA1 plasmid	(Matsuda et al., 2018)
Aspergillus oryzae	Establishment of CRISPR/Cas9 system	Integrative		(Katayama et al., 2016)
	Improvement of gene targeting efficiency in industrial strains; Activation of sclerotia formation	Integrative		(Nakamura et al., 2017)
	Demonstration of versatility of a system established in *Nodulisporium* sp.	Integrative	*In vitro*	(Zheng et al., 2017)
	Efficient gene targeting with oligonucleotides	AMA1 plasmid		(Nødvig et al., 2018)
Beauveria bassiana	Establishment of CRISPR/Cas9 system	Integrative	*In vitro*	(Chen et al., 2017)
Blastomyces dermatitidis	Establishment of CRISPR/Cas9 system; Study of zinc metabolism for fitness	Integrative		(Kujoth et al., 2018)
Cordyceps militaris	Establishment of CRISPR/Cas9 system	Integrative	*In vitro*	(Chen et al., 2018)
Fusarium graminearum	Establishment of CRISPR/Cas9 system	Integrative		(Gardiner and Kazan, 2018)
Fusarium oxysporum	Establishment of CRISPR/Cas9 system; Identification of the role of *bik1* gene in the synthesis of a red pigment, bikaverin	*In vitro* RNP[1]	*In vitro* RNP[1]	(Wang, Q. et al., 2018)
Myceliophthora heterothallica	Demonstration of versatility of a CRISPR/Cas9 system established in *Myceliophthora thermophila*	Integrative	Integrative	(Liu, Q. et al., 2017)
Myceliophthora thermophila	Establishment of CRISPR/Cas9 system; Engineering of a hyper-cellulase producing strain	Integrative		(Liu, Q. et al., 2017)
	Enhancement of amylolytic activity	Integrative		(Xu, G. et al.,

	through overexpression of a major amylolytic enzyme or the *amyR* transcription factor encoding gene; Increased lignocellulase activites through deletion of *amyR*			2018)
Neurospora crassa	Establishment of CRISPR/Cas9 system; Improved cellulase production through overexpression of *clr-2*	Transient expression from a non-replicative plasmid[3]		(Matsu-ura et al., 2015)
Nodulisporium sp. (No. 65-12-7-1)	Establishment of CRISPR/Cas9 system	Integrative	Integrative or *in vitro*	(Zheng et al., 2017)
Penicillium chrysogenum	Establishment of CRISPR/Cas9 system	AMA1 plasmid or *in vitro* RNP[1]	AMA1 plasmid, *in vitro* or *in vitro* RNP[1]	(Pohl et al., 2016)
Pyricularia oryzae (*Magnaporthe oryzae*)	Establishment of CRISPR/Cas9 system	Transient expression from a non-replicative plasmid[3]		(Arazoe et al., 2015)
	Application of RNP[1] mediated transformation method	*In vitro* RNP[1]		(Foster et al., 2018)
Sclerotinia sclerotiorum	Establishment of CRISPR/Cas9 system	Integrative		(Li et al., 2018)
Shiraia bambusicola	Establishment of CRISPR/Cas9 system	Integrative		(Deng et al., 2017b)
	Identification of genes involved in the hypocrellin biosynthetic pathway	Integrative		(Deng et al., 2017a, b, 2018)
Sporormiella minima	Demonstration of versatility of a system established in *Nodulisporium* sp.	Integrative	*In vitro*	(Zheng et al., 2017)
Talaromyces atroroseus	Establishment of CRISPR/Cas9 system; Identification of a novel gene responsible	AMA1 plasmid		(Nielsen et al., 2017)

	for production of polyketide-nonribosomal peptide hybrid products			
Trichoderma reesei	Establishment of CRISPR/Cas9 system	Integrative	*In vitro*	(Liu et al., 2015)
	Identification of a novel transcription factor; Enhancement of xylanase activity and higher hydrolytic activity on pretreated biomass	Integrative	*In vitro*	(Liu, R. et al., 2017)
Coprinopsis cinerea	Establishment of CRISPR/Cas9 system	Integrative	Integrative	(Sugano et al., 2017)
Ganoderma lingzhi	Demonstration of versatility of a system established in *G. lucidum*	Integrative	*In vitro*	(Qin et al., 2017)
Ganoderma lucidum	Establishment of CRISPR/Cas9 system	Integrative	*In vitro*	(Qin et al., 2017)
Ustilago maydis	Establishment of CRISPR/Cas9 system	ARS plasmid		(Schuster et al., 2016)
	Study of secreted proteins and development of CRISPR/Cas9 mediated multiplexing	ARS plasmid		(Schuster et al., 2018)
Ustilago trichophora	Establishment of CRISPR/Cas9 system	Transient expression from a non-replicative plasmid[3]		(Huck et al., 2018)

[1]RNP = ribonucleoprotein

[2]sgRNA = single guide RNA

[3]The plasmids were presumably not integrated into the host genome

Figure 1. Classical mutagenesis. Changes that can be produced with these mutagens are depicted. **A)** UV light leads to the formation of thymine dimers, which can introduce frameshift or point mutations. **B)** For chemical mutagenesis, the chemical agent (e.g. ethidium bromide) is intercalated and introduces a spacing between base pairs, causing deletion or insertion of base pairs.

Figure 2. Utilization of the CRISPR/Cas9 system in filamentous fungi for increased lignocellulolytic enzyme production. The artificial sgRNA is composed of a guide sequence (in green) and a scaffold sequence (in yellow), which are the corresponding parts of the naturally occurring crRNA and tracrRNA, respectively. The sgRNA forms a complex with the Cas9 nuclease (in blue), which will be directed to a targeted locus of a gene of interest. In case there is sequence homology between the guide sequence and the target sequence upstream of a PAM sequence (in red), a double strand break (DSB) will be performed. The DSB can be repaired in a targeted manner using repair templates, which can result in precise gene editing.

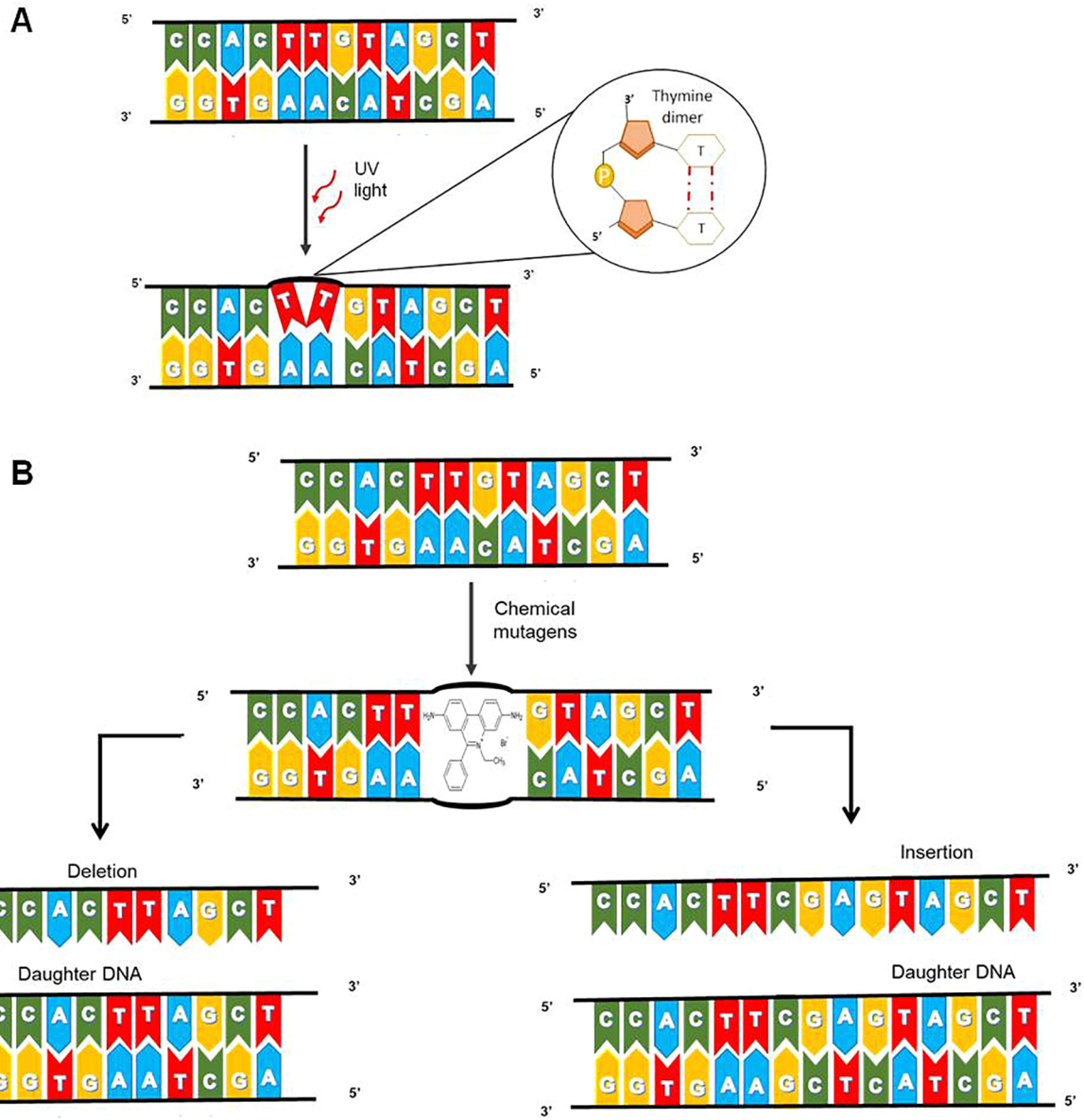
A
5'
3'
C C A C T T G T A G C T
G G T G A A C A T C G A
UV
light
Thymine
dimer
T
T
P
B
Chemical
mutagens
Deletion
C C A C T T A G C T
Daughter DNA
G G T G A A T C G A
Insertion
C C A C T T C G A G T A G C T
G G T G A A G C T C A T C G A

Figure 1

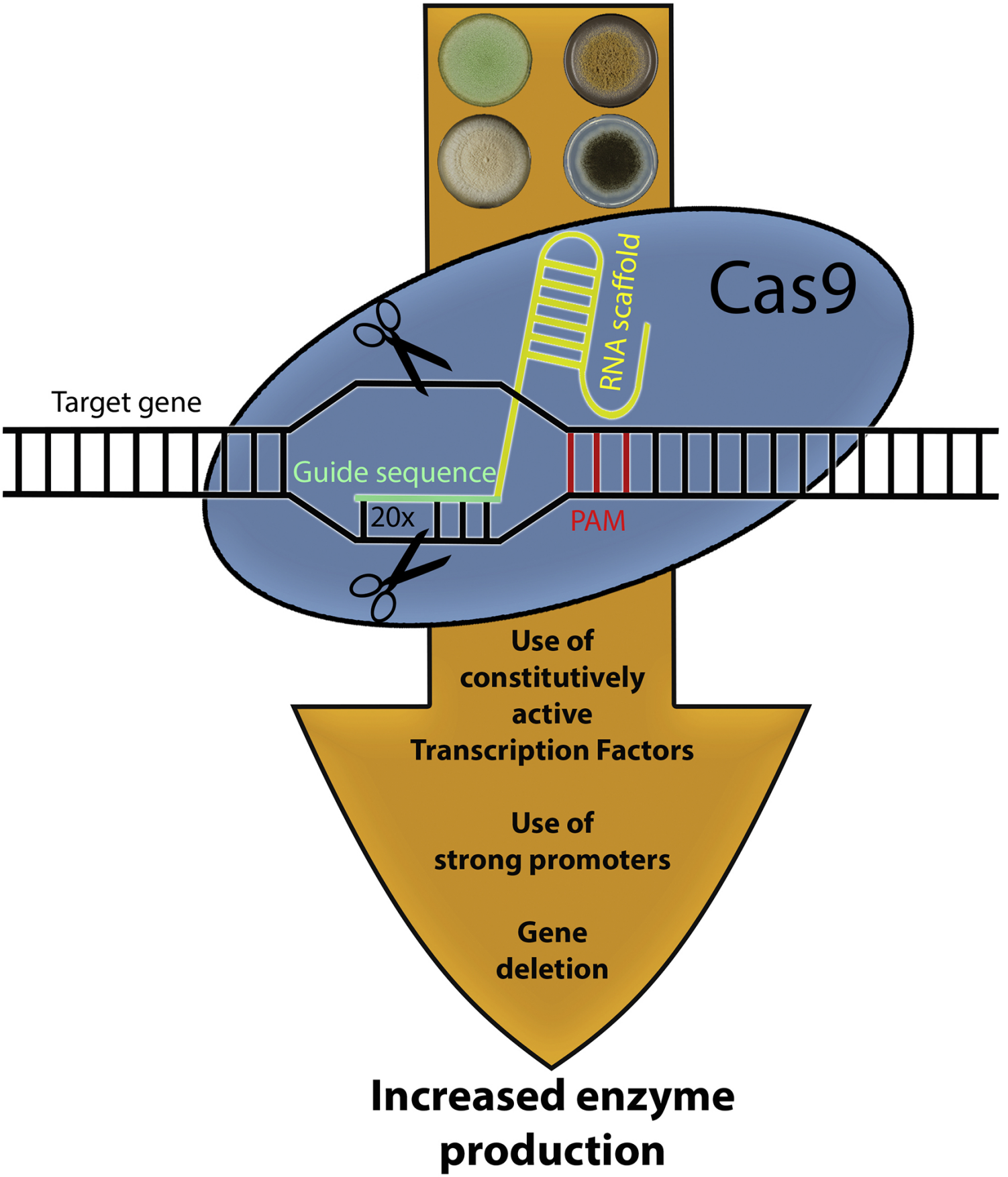
Cas9
RNA scaffold
Target gene
Guide sequence
20x
PAM
Use of
constitutively
active
Transcription Factors
Use of
strong promoters
Gene
deletion
Increased enzyme
production

Figure 2