Fungal Biology

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Gene Expression Systems in Fungi: Advancements and Applications

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Preface

Strain improvement with fungal gene expression systems has a tradition of decades regarding modern fermentation industry and even millennia when we think of brewing processes for beer, wine or enzymatic processes in baking. Genetic engineering techniques developed in the last century have revolutionized fermentation industry and enabled production of enzyme mixtures and secondary metabolites with strongly increased efficiency, first by random mutagenesis, but soon also with knowledge-based targeted genome modification. Thereby, economic and optimized production of various compounds originating from diverse organisms became possible in filamentous fungi, from novel, thermostable, or cold active enzymes to potent antibiotics and antibodies to efficient anticancer drugs. At the same time, these possibilities opened up new challenges to be solved, like precise regulation of gene expression during the growth phase for optimized product yield and expression of heterologous proteins with unusual characteristics in a production organism.

This volume aims to give a broad overview on gene expression systems in biotechnological workhorses for production of enzymes and metabolites from the phyla of ascomycetes and basidiomycetes and the subphylum mucoromycotina. Additionally, we include model organisms that have provided valuable insights into the physiology of fungi, also with respect to industrial applications and, for example, the corn smut fungus *Ustilago maydis* as an alternative expression system. For the diversity in potential fungal hosts for production of a given compound, strain collections and for fungi particularly the Fungal Genetics Stock Center represent an invaluable resource for research and application, which is discussed in a dedicated chapter.

The "Tools" section summarizes current and versatile methods for strain manipulation, such as high throughput construction of genetically modified fungi and strategies for activation of silent secondary metabolite gene clusters and targets for improvement such as the mechanism of carbon catabolite repression. However, also novel strategies and tools that became available in recent years or have raised increased attention are highlighted. The use of inteins in protein synthesis is one example of such intriguing avenues for protein expression, and signal transduction pathways of fungi still remain to be exploited further for optimization of production processes.

In the last few years also, sexual development was achieved with industrial fungi, which were believed to develop only clonally for decades. *Trichoderma reesei* was the first one, for which this tool became available and more and more others are following thanks to considerable effort of the fungal scientific community. In this volume we included two chapters on the perspectives and challenges of application of sexual development for strain improvement.

Last but not least, the section on "Challenges" aims to provide insight into the perspectives and needs in industry and discusses safety issues with recombinant production organisms.

With this volume we aim to provide a profound resource for scientists in research and industry alike as well as for students starting their work on application-oriented research with fungi. We intend to inspire researchers to combine classical and novel strategies to meet the challenges with fungal biotechnology in economical and environmentally safe production of the diverse products that make our life more convenient like enzymes or to even save it with novel antibiotics and drugs.

Tulln, Austria

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Part I
Organisms

Gene Expression Systems in Industrial Ascomycetes: Advancements and Applications

Jonas Ramoni, Verena Seidl-Seiboth, Robert H. Bischof, and Bernhard Seiboth

Abbreviations

CAZyme	Carbohydrate-active enzyme
CBM	Carbohydrate binding module
CPP	Cerato-platanin protein
DOX	Doxycycline
ER	Endoplasmatic reticulum
GH	Glycoside hydrolase
PB	Protein bodies
PMO	Polysaccharide monooxygenase
TRE	Tetracycline responsive elemen
tUPR	Unfolded protien response

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1 Introduction

One of the characteristics of the lifestyle of many filamentous fungi is that they secrete a wide array of extracellular enzymes and proteins to interact with their environment and thereby utilizing different polymers as carbon and energy source in their habitats. This intrinsic property makes them excellent hosts for the industrial production of such depolymerizing enzymes. But although the total number of fungal species is higher than one million in nature, only a few species are regularly used for industrial protein production while the genomic resources of the other fungi are mainly exploited as source for new proteins and enzymes which are expressed heterologously in other hosts. The product levels of most proteins in natural occurring strains are usually too low for commercial application and therefore strain improvement programs by mutagenesis and selection together with optimization of the protein production conditions in industrial scale fermentations were necessary. These efforts raised the levels of exported proteins already in the 1980s of the last century to levels of about 30 g/L for selected enzymes such as cellulases or glucoamylases (Durand et al. 1988; Finkenstein et al. 1989). Today it is generally accepted that e.g. industrial strains of *Trichoderma reesei* produce at least 100 g/L, but the strains and exact conditions leading to this improvement were not reported (Cherry and Fidantsef 2003). Filamentous fungi, such as different *Aspergillus spp., T. reesei* and recently *Myceliophthora thermophila* also known under the former species name *Chrysosporium lucknowense* (Visser et al. 2011) were established as expression platforms for the production of industrial enzymes. Their status is a consequence of their capability of producing and secreting large amounts of enzymes, their amenability to large-scale fermentation as well as their long history of safe use in industrial enzyme production. Consequently the production of several enzymes has obtained the generally recognized as safe (GRAS) status by the U.S. Food and Drug Administration FDA.

2 Debottlenecking Fungal Protein Production

Gene based studies including simple transformation, gene cloning or their functional analysis were and are in general more laborious and tedious than with some of the major competing microbial cell factories such as *Escherichia coli*, *Saccharomyces cerevisiae* or *Pichia pastoris*. In addition while at least the first two are accepted as model organisms with a large community, their counterparts as model organisms in the filamentous fungal world are *Aspergillus nidulans* and *Neurospora crassa* which have only limited application as biotechnological organism. Therefore it is often necessary to transfer methods or tools to the fungal cell factories *A. niger* or *T. reesei*. Although this situation is unlikely to change in the near future, the advent of the different omics technologies including mainly genomics, transcriptomics and with increasing frequency also proteomics, hold the

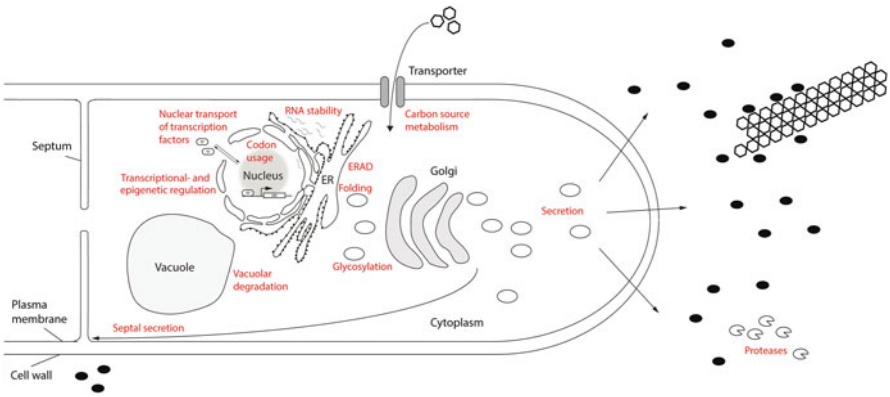

Fig. 1 Extracellular protein production and secretion in filamentous fungi. A number of potential bottlenecks in fungal protein expression are depicted starting with the transcription of the gene, its translation, passage through the secretory pathway and its export

potential to tackle bottlenecks in protein production with a greater pace. A growing number of wild-type genomes, genomes of improved producer strains and other mutants are available and allow together with transcript data to use systems biological approaches to study protein production (Meyer et al. 2015; Schmoll et al. 2014).

The biggest competitive advantage of filamentous fungi over other microbial cell factories is their enormous potential to produce high quantities of extracellular enzymes. Most of the industrial protein production with fungi is therefore targeted to the outside of the cell and a common strategy to improve translation and post-translational processes is to genetically fuse the target protein with an endogenous protein carrier. Strain improvement programs to use the highly efficient protein synthesis, folding and export machinery of these industrial fungi has so far mainly succeeded for endogenous proteins but the level of heterologously expressed proteins especially non-fungal proteins remains generally one to two orders of magnitude lower. Methods and tools to improve the expression of heterologous proteins in these fungi are therefore highly desirable. Several bottlenecks in these production process have been identified to date and a few limiting factors have been overcome by genetically modifying the expression host (Fig. 1). To debottleneck protein production basic genetic strategies including (1) the introduction of multiple copies of the gene of interest (2) the use of strong constitutive or inducing promoters (3) efficient secretion signals (4) construction of protease deficient strains and (5) the optimization of codon usage were developed and tested within the last years with variable success. A number of reviews have summarized this in detail (Punt et al. 2000; van den Hombergh et al. 1997; Archer and Peberdy 1997; Gouka et al. 1997). These mentioned strategies usually are suitable to raise protein yields but still heterologous protein yields remain low indicating that additional barriers are present and that our knowledge of the restricting factors is still limited.

Apart from the protein yield itself, it is equally important that the protein of interest is produced in its active form. To produce extracellular proteins eukaryotes have

developed a highly specialized secretion pathway (Fig. 1) which accomplishes many functions to convert the protein into its active form. These include folding, proteolytic processing to the mature protein and the addition and processing of *N*- and *O*-glycans and many other posttranslational modifications. Following the intracellular protein translation most extracellular proteins are imported in the endoplasmic reticulum (ER) where proteolytic processing, folding and the addition of glycans are accomplished before they are further transported through the Golgi apparatus and exported. These proteins are then secreted through the hyphal tip. In recent years it became also evident that some proteins follow different routes and are transported to other parts of the hyphae including septa. The fact that these proteins are transported in different ways is likely to contribute to the success or failure in recombinant protein production (Nevalainen and Peterson 2014). Another field which deserves more attention in the future is the role of the molecular chaperones and foldases that assist in the non-covalent folding of proteins. Increased target protein synthesis may lead to e.g. overloading of the folding capacity of the ER and can result in a downregulation of the expression of the recombinantly produced protein and of other proteins by triggering the unfolded protein response (UPR) or repression under secretion stress (RESS) (Pakula et al. 2003). Manipulation of their expression levels including the molecular chaperone BiP or protein disulfide isomerases can improve the yield of heterologous proteins by increasing the folding capacity. Accumulation of unfolded protein in the ER often results in the triggering of the UPR and induces the expression of molecular chaperones, vesicular traffic components, and ER-associated degradation (ERAD) proteins, to resolve the ER stress by increasing the folding, transport, and degradation of proteins. UPR is controlled by the transcription factor Hac1/A and its constitutive expression is one way to improve the production of heterologous proteins (Valkonen et al. 2003; Nakajima et al. 2006). When exported to the medium proteins are exposed to the harsh environment and especially proteases in the supernatant of fermentations can lead to high protein losses. Therefore it is often necessary to reduce the protease activity to limit the degradation of heterologous proteins (van den Hombergh et al. 1997; Yoon et al. 2011). Heterologous protein production can also be improved by preventing vacuolar protein sorting and autophagy (Yoon et al. 2010, 2013).

Especially for the production of human proteins which are applied as therapeutics a correct glycosylation is often essential as this affects immunogenicity, the functionality of the protein or its half-life in the patient. A drawback in the effort to produce human proteins in fungi is that the glycosylation pattern differs considerably. The structure of glycans added by fungi is of the high mannose type whereby fungi have small high mannose type *N* and *O*-glycans whereas humans have a complex type glycosylation including *N*-acetylneuraminic acid. In contrast to *S. cerevisiae* the adverse hyperglycosylation is not a typical feature of filamentous fungi although it has been detected in some cases. In addition fungi secrete a number of glycosidases which further modify the glycans and thereby leading to a considerable heterogeneity of the glycan structure (Maras et al. 1999). Although it is possible to introduce the human glycosylation pathway in certain organisms including *P. pastoris*, it appears that the race for humanizing the glycosylation pattern in fila-

mentous fungi seems to be lost for the moment as many therapeutic enzymes are produced with improved yields in mammalian cell lines (Nevalainen and Peterson 2014).

3 Fungal Enzymes, Proteins and New Players

Enzymes and proteins produced by fungi find their application in virtually all kind of industries. Within the last years there is also an increasing demand for enzymes and proteins employed in the saccharification of cellulosic plant biomass to simple sugars which are further transformed to biofuels or value added biochemicals (Choi et al. 2015; Wyman 2007). As enzymes constitute an important cost factor in the transformation of cellulosic biomass, research efforts to find novel or design improved enzymes or production hosts is supported by large government and industrial funding. The importance of fungi as enzyme producers is underlined by an analysis of the producing organism from the 2014 list of technical enzymes published by the Association of Manufacturers and Formulators of Enzyme Preparations (http://www.amfep.org/). Of 246 enzyme formulations 138 (56.1 %) are produced by fungi. Of these, 94.2 % are produced by Ascomycota with Mucoromycotina (5.1 %) and Basidiomycota (0.7 %) accounting for the rest of the enzymes. Among the Ascomycota, Eurotiomycetes account for 59.4 % and Sordariomycetes for 26.9 % of all fungal enzyme preparations. Thermophilic species (*T. emersonii*, *H. insolens* and *K. lactis*) account for 8.7 % of all enzymes. Table 1 gives an overview of important fungi and their main products.

Beside these enzymes and other proteins (see later) a new class of products summarized here as surface-active proteins has received increasing attention. Proteins used as foaming agents in foods contribute to the formation and stability of the foam structure (Cooper and Kennedy 2010; Foegeding et al. 2006; Wilde et al. 2004) but these are mainly denaturated proteins that do not have surfactant properties in their native state, e.g. the β-lactoglobulin (egg white). The use of proteins that are natural surfactants for biotechnological applications is still not frequently encountered yet. The probably most prominent example for natural surfactants for which a wide range of applications has already been envisaged and tested is a protein family that is exclusively found in the fungal kingdom, namely the hydrophobins. Hydrophobins are small, extracellular proteins with low molecular masses in the range of 7–12 kDa. They self-assemble into protein layers and large protein aggregates at hydrophobic/hydrophilic interfaces (Wösten 2001; Linder et al. 2005; Sunde et al. 2008). This is due to hydrophobic and hydrophilic patches on the protein surface that trigger self-assembly under appropriate environmental conditions. Despite this conserved feature that can be considered as the main characteristic of hydrophobins, they exhibit considerable variability in their amino-acid sequences. However, they contain eight cysteine residues that can be found at extremely conserved positions and thereby contribute to the structural organization of the mature proteins (Wösten and Wessels 1997; Linder et al. 2005). Hydrophobins are grouped into two classes

Table 1 Commercial enzyme formulations produced by yeast and fungi as of 2014

Production organism	Enzyme formulations
Aspergillus melleus	Aminoacylase, AMP deaminase, protease
Aspergillus niger[a]	Aminopeptidase, α-amylase, α-L-arabinofuranosidase, asparaginase, carboxypeptidase (serine-type), catalase, cellulase, α-galactosidase, ß-glucanase glucoamylase, α-glucosidase, ß-glucosidase, glucose oxidase, glucosyltransferase, hemicellulase, inulase, lipase triacylglycerol, mannanase, pectin lyase, pectin methylesterase, pentosanase, polygalacturonase, peroxidase, phospholipase B, phytase, protease, tannase, xylanase
Aspergillus oryzae	α-Amylase, ß-glucanase, ß-galactosidase, pectin methylesterase, aminopeptidase, asparaginase, glucose oxidase, laccase, lipase triacylglycerol, phospholipase A2, phytase, protease, xylanase
Candida spp.	Lipase triacylglycerol
Cryphonectria parasitica	Protease
Disporotrichum dimorphosporum	Xylanase
Fusarium venenatum	Cellobiose dehydrogenase, protease
Humicola insolens	ß-Glucanase, pentosanase, xylanase
Kluyveromyces lactis	Lactase or ß-galactosidase, protease
Ogataea angusta	Hexose oxidase, lipase triacylglycerol
Penicillium spp.	Cellulase, glucose oxidase, ß-glucosidase, lipase monoacylglycerol, lipase triacylglycerol, phosphodiesterase, rhamnosidase
Pichia pastoris	Phospholipase C
Rhizomucor miehei	Lipase triacylglycerol, protease
Rhizopus spp.	Aminopeptidase, lipase triacylglycerol, protease
Saccharomyces cerevisiae	α-Acetolactate decarboxylase, invertase, α-galactosidase
Schizosaccharomyces pombe	Phytase
Talaromyces emersonii	Cellulase, ß-glucanase, xylanase
Trichoderma harzianum	ß-Glucosidase, ß-glucanase
Trichoderma reesei	α-Amylase, cellulase, catalase, ß-glucanase, glucoamylase, α- and ß-glucosidase, hemicellulase, laccase, ß-mannanase, pectin lyase, pectin methylesterase, pentosanase, phospholipase A2 and B, phytase, polygalacturonase, protease, xylanase

Source: http://www.amfep.org/

[a]The *Aspergillus niger* group covers strains known under the names *A. aculeatus*, *A.awamori*, *A. ficuum*, *A. foetidus*, *A. japonicus*, *A. phoenicis*, *A. saitoi*, *A. tubingensis* and *A. usamii*

(class I and II) according to their solubility in solvents, hydropathy profiles, and spacing between their conserved cysteines (Linder et al. 2005). In general, protein aggregates of class I hydrophobins are more stable and can only be dissolved in strong acids, while class II aggregates can be dissolved using aqueous dilutions of organic solvents. Initially class I hydrophobins were only reported in Basidiomycota, with SC3 from *Schizosaccharomyces pombe* as the most prominent example, which is among those hydrophobins that have already been studied in great detail.

Among other hydrophobins that have already been subject to detailed investigations are the class II hydrophobins HFBI and HFBII from *T. reesei* (e.g. Linder et al. 2005; Askolin et al. 2006; Lienemann et al. 2015; Magarkar et al. 2014) and the class I hydrophobin EAS from *N. crassa* (Macindoe et al. 2012; Kwan et al. 2008). While typical class I hydrophobins are considered to be rather rare in ascomycetes, in the past few years a phylogenetically separate clade of class I hydrophobins was described in Ascomycota, i.e. *Trichoderma* species (Seidl-Seiboth et al. 2011) and *Aspergillus* species (Jensen et al. 2010).

Hydrophobins are amphiphilic proteins and are able to invert the polarity of other surfaces very effectively. Therefore, they are handled as candidates for large scale applications, spanning from non-wetting coatings to biocompatible surfactants. Biotechnologically, the numerous applications of hydrophobins include their use as biosurfactants, as foaming agents and for protein immobilization in the food industries and in biosensors or to improve the degradation of polyester polymers (Khalesi et al. 2012; Espino-Rammer et al. 2013). Besides functions related to foam stabilization, immobilization of flavor compounds or the encapsulation of trace ingredients in beverages are further applications. The study of hydrophobins might also lead to a better understanding of the gushing phenomenon in beverages like beers, wines and ciders, which causes great economic losses in those fields and also needs to be considered for the positive applications listed above. The use of hydrophobins in pharmaceutical formulations and in medicine is another interesting application as they cause an increased stabilization of drugs. The controlled molecular modification of surfaces is a key in the preparation of function-oriented micro-patterned advanced materials. Researchers suggest that hydrophobin coating might be an intermediate to attach cells, proteins, or other type of molecules to hydrophobic surfaces in biosensors.

Hydrophobins are interesting for enhancing the biocompatibility of medical implants in order to avoid the rejection of the implants (Hektor and Scholtmeijer 2005). Low-friction surfaces are required in various biomedical applications (Misra et al. 2006). To reduce the friction of materials, hydrophobins can be used safely. It has been shown that by covering the surface of conidia, hydrophobins shield antigens in the cell wall, thereby protecting the fungal structure against the immune system (Aimanianda et al. 2009). These observations suggest that the use of hydrophobins in medical applications will possibly not elicit immunogenic reactions.

Another family with surface-activity altering properties are members of the cerato-platanin protein family (Gaderer et al. 2014). At first glance, cerato-platanin proteins (CPPs) and hydrophobins exhibit similar features: CPPs are also small, secreted proteins with cysteine residues at strongly conserved positions that are exclusively found in fungi. However, it has been documented in the past few years that the amino-acid sequences, the cysteine-pattern as well as the three-dimensional structure of CPPs are completely unrelated to hydrophobins. Structurally they are rather related to carbohydrate-binding proteins such as barwin proteins and expansins (de Oliveira et al. 2011). For CPPs binding to chitin and chito-oligosaccharides has already been shown (de Oliveira et al. 2011; de O. Barsottini et al. 2013) and it was established that CPPs are very important for fungal-plant interactions and

stimulate the induction of defense responses in plants (Gaderer et al. 2014; Pazzagli et al. 2014).

In view of the carbohydrate-binding properties of CPPs it is even somewhat surprising that they have also additional features and are also surface-active proteins. They are able to self-assemble at hydrophobic/hydrophilic interfaces and form protein layers e.g. on the surface of aqueous solutions, thereby altering the polarity of solutions and surfaces (Frischmann et al. 2013; Bonazza et al. 2015; Gaderer et al. 2014). Interestingly, the surface activity-altering properties of CPPs are the opposite of what can be observed for hydrophobins. EPL1, a member of the CPP family from the fungus *Trichoderma atroviride*, was reported to rather enhance the polarity/apolarity properties of the respective surfaces, e.g. they enhance the polarity of aqueous solutions (Frischmann et al. 2013; Bonazza et al. 2015) and form highly ordered monolayers at a hydrophobic surface/liquid-interface (Bonazza et al. 2015). When class I hydrophobins were mixed with EPL1 in solution a hybrid layer is formed. This mixed protein layer is on one hand not inverting but enhancing the hydrophobicity of HOPG (highly oriented pyrolytic graphite), typical for EPL1, and on the other hand, it is stable and water insoluble, which is reminiscent of hydrophobin layers (Bonazza et al. 2015). This unprecedented mixture of properties provides a promising starting point for future investigations of their detailed structure and organization as well as for potential biotechnological applications of CPPs.

The surface-activity altering effects of CPPs are potentially interesting for biotechnological applications such as enhancing the wettability-properties of solutions, which can be of interest for applications where a uniform moistening of a moderately hydrophobic surface is of interest, e.g. spraying of plant protection products or in cleaning agents. A possible use as additives for the induction of plant resistance and defense mechanisms in fertilizers would also be an interesting possibility. The strong foaming of solution of CPP, as has been reported for EPL1 (Frischmann et al. 2013), could be applied for the stabilization of foams and emulsions and similar to the hydrophobins, CPP might contribute to the unwanted foaming during the fermentation of filamentous fungi.

4 Controlling Gene Expression

Promoters initiate transcription and are therefore rightly regarded as a central element in gene expression and protein production. One of the simplest approaches to increase the production of a certain protein is therefore to swap its promoter. For this purpose a number of strong promoters were isolated within the last decades. However, increased target protein synthesis may lead to e.g. overloading of the folding capacity of the ER which in turn results in a downregulation of the expression of the recombinantly produced protein. It is therefore advantageous to test not only the strongest promoters available but also other promoters with intermediate expression strengths to increase expression of a certain protein. Based on their regulation promoters are either regarded as constitutive or tunable. Whereas constitutive

expression is, mostly because of the ease of use, the technique of choice, this strict "expression on" mechanism can lead to ambiguous phenotypes. Attention has to be paid when the produced protein is toxic or at least harmful for the cell or when the production of the protein leads to a considerably higher metabolic burden for the cell. In these cases cells which have either lost the expression cassette or turned off the production of the desired protein have a clear growth advantage and will overgrow the producers during the fermentation process with the consequence of an overall lower enzyme yield. Therefore tunable promoters provide a useful alternative to allow a transient modulation of gene expression which can be achieved by either inducible or repressible promoters. The stimuli that trigger the change in gene transcription can be diverse in nature and include pH level (Denison 2000; Häkkinen et al. 2015; Penalva et al. 2008), temperature (Matsushita et al. 2009), light (Tisch and Schmoll 2010) or the presence or absence of a favored nutrient (Marzluf 1997; Kubicek et al. 2009).

In many filamentous fungi, inducible promoters are derived from genes which encode the most abundant extracellular proteins. For *T. reesei* it is the promoter of the major cellulase CBH1/CEL7A (Rahman et al. 2009; Harkki et al. 1989; Paloheimo et al. 2003). This cellobiohydrolase makes up about 60 % of total secreted proteins upon cellulase inducing conditions. The *cbh1* promoter is induced by cellulose but also by disaccharides such as sophorose and lactose or monosaccharides such as L-sorbose (Kubicek et al. 2009). Lactose, a cheap waste product from the milk processing industries, is in contrast to cellulose soluble and has the advantage over sophorose to be much cheaper. A number of cellulase hyperproducing *T. reesei* strains derived from QM6a by random mutagenesis are available in the public domain which have a considerable higher *cbh1* expression compared to the wild-type strain QM6a including RUT-C30 (Portnoy et al. 2011; Peterson and Nevalainen 2012). Presence of easily metabolizable sugars such as D-glucose lead to carbon catabolite repression of the *cbh1* promoter in a CRE1 mediated manner (Ilmén et al. 1996a, b). This repression is lower in *cre1* deleted strains such as RUT-C30 where a considerable level of *cbh1* expression is found even under repressing conditions (Ilmén et al. 1996a) but expression does not reach the induced transcript level. The equivalent to the *cbh1* promoter in *A. niger*is the *glaA* promoter which controls the starch hydrolyzing enzyme glucoamylase (GlaA). Expression is induced by starch or its hydrolytic products, including glucose (Ganzlin and Rinas 2008; Ward et al. 1990; Dunn-Coleman et al. 1991; Fleissner and Dersch 2010).

Another useful alternative for conditional gene expression is the *A. nidulans alcA-alcR* system. Induction of the alcohol dehydrogenase gene *alcA* is mediated by the $Zn(II)_2Cys_6$ transcriptional activator AlcR (Felenbok 1991; Fleissner and Dersch 2010). This expression system, consisting of the *alcA* promoter and the *alcR* regulator, can be transferred to other fungal species including *A. niger* (Nikolaev et al. 2002), *A. fumigatus* (Romero et al. 2003) or even to plants (Roslan et al. 2001). For *A. niger* high basal levels of *alcA* transcription were observed, accompanied by only a low ratio of *alcA* inducibility, a fact which has to be considered for certain applications. An advantage of the system is that the titration effect caused by too many AlcR targets (such as multi-copy integrations) can be easily surpassed by overexpression of the activator (Gwynne et al. 1989).

For *T. reesei* the promoter of the copper transporter gene *tcu1* was recently described (Lv et al. 2015). Its activity is controlled by copper availability and tightly repressible by copper concentrations ≥500 nM. The applicability of this system was demonstrated by the tunable expression of two cellulases on D-glucose. However, this system might suffer from a limited applicability when cheap and complex carbon sources such as wheat straw are used in biorefinery applications and when copper containing enzymes such as the polysaccharide monooxygenases (PMOs) are part of the enzyme cocktail to be produced.

In a recent study a transcriptomic approach was used to identify L-methionine repressible genes whose promoters can be used under industrially relevant cellulase inducing conditions, i.e. when *T. reesei* is grown on wheat straw. It was shown that the promoter region of a putative TauD like monooxygenase was tightly repressible by the addition of 40 μM of L-methionine while the frequently used *met3* repressible promoter system was not working under these conditions. Addition of L-methionine did also not affect the expression level of the cellulases or xylanases and this novel repressible promoter system was also active on the cellulase repressing carbon source D-glucose and the cellulase neutral carbon source glycerol (Bischof et al. 2015).

For a uniform expression over the whole production period, constitutive promoters that are independent of the addition of inducers or repressors are usually applied. Some of these promoters including a number of glycolytic genes are described as constitutive but were mainly isolated on D-glucose medium and were usually not tested for their expression level and strength on a wider range of carbon sources and might not fulfill the criteria of a constitutive promoter by definition. For *T. reesei* a number of such promoters from genes active on glucose were identified including *tef1* (transcription elongation factor) and *hfb1* (hydrophobin1; (Nakari-Setälä et al. 1993)), *cDNA1* (unknown protein (Nakari-Setälä and Penttilä 1995)), or *hex1* (major protein of the Woronin bodies (Curach et al. 2004)). Li et al. (2012) quantified the expression of a number of genes involved in glucose metabolism and showed that among these *pdc1* (pyruvate decarboxylase) and *eno1* (enolase) provided highest expression of the *T. reesei* endoxylanase XYN2 on D-glucose containing medium.

The combination of strong carbon catabolite repressing conditions found during growth on D-glucose together with promoters active on D-glucose is a useful approach to get rid of a number of extracellular proteins which would otherwise disturb the enzymatic assays for the produced protein or its further downstream processing. This approach was used to produce e.g. cellulases but can be extended to other extracellular enzymes usually repressed by glucose (Nakari-Setälä and Penttilä 1995; Kitamoto et al. 1999). Uzbas et al. (2012) refined this strategy by overexpression of individual cellulase in a cellulase free *T. reesei* host caused by the deletion of the major transcriptional activator for cellulases and xylanases XYR1. In this way native and improved cellulases can be easily tested without the need of a further purification step. Several constitutive promoters in *Aspergillus* ssp. research are regularly used in literature, among them the P*adhA* and the P*pkiA* for *A. niger* (Roth and Dersch 2010), the P*gpdA* for *A. nidulans* (Punt et al. 1990), and the

P*gdhA* for *A. awamori* (Moralejo et al. 1999). However, for protein production as well as optimization of complex networks towards metabolic engineering, a well sorted genetic toolbox containing strong promoters but also those of intermediate activity is necessary as described for *A. niger* (Blumhoff et al. 2013). The authors selected their promoters based on publicly available transcriptome data and characterized the expression levels of six novel constitutive promoters which cover a range of two to three orders of magnitude.

A disadvantage of many of the above described promoters and expression systems is that they are often integrated in the host's metabolism and react to changes in the host's physiology. Within the last years other systems were developed with the objective to function independent from the host's metabolism. For different *Aspergilli* a system was tested which makes use of the activation of the human estrogen receptor by different estrogenic substances to initiate inducible gene transcription. This hERα-ERE system was successfully applied to drive β-galactosidase expression in an inducer-concentration dependent manner (Pachlinger et al. 2005). Another example is the Tet-On and Tet-Off system, a widely applied expression system from *E. coli* to animal cell cultures, tested now also for different *Aspergilli* spp. (Vogt et al. 2005; Meyer et al. 2011). This system is applicable in an inducer dose dependent manner with expression rates comparable to the strong *gpdA* promoter in *A. niger*. The tetracycline operator system is based on the *E. coli* tetracycline-resistance operon, which senses tetracycline or its analogue doxycycline (Dox) and results in resistance response. When no tetracycline is present, the repressor protein (tetR) binds the operator sequence (*tetO*) and thereby inhibits transcription. In contrast, when the cell faces tetracycline, tetR is detached from *tetO* which results in expression of the gene. The tunable expression system described for Aspergilli (Fig. 2) contains a constitutively, synthetic transcriptional hybrid transactivator (tTA in Tet-Off) or reverse hybrid transactivator (rtTA^{S}-M2 in Tet-On). They both consist of tetR fused to the VP16 of the herpes simplex virus and the expression system was optimized towards high level gene expression by placing seven *tetO* sequences (*tetO7*) in front of a minimal promoter (Pmin). The Tet-On system is sometimes preferred over Tet-Off for its faster responsiveness.

Promoters that are regulated in an inducer concentration dependent tunable manner are not only beneficial for recombinant protein production but will be also needed in functional genomics. Especially with the advancement of synthetic and systems biology standardized, modular and orthogonal tools yielding predictable results will be needed. Using such promoters, protein production can be split into two separated phases: a cell growth phase followed by, upon addition of an inducer substance, a protein production phase. The ideal inducer in this context would be a low cost molecule which has no impact on cell physiology but a concentration dependent stimulation of the expression of the gene regulated under the tunable promoter.

Promoter strength is often a critical value and depends amongst other factors on the location of the expression cassette in the chromosome. Therefore it is often necessary to target the expression cassettes to specific loci in the genome to quantify their strength. Jorgensen et al. (2014) have therefore presented an elegant approach

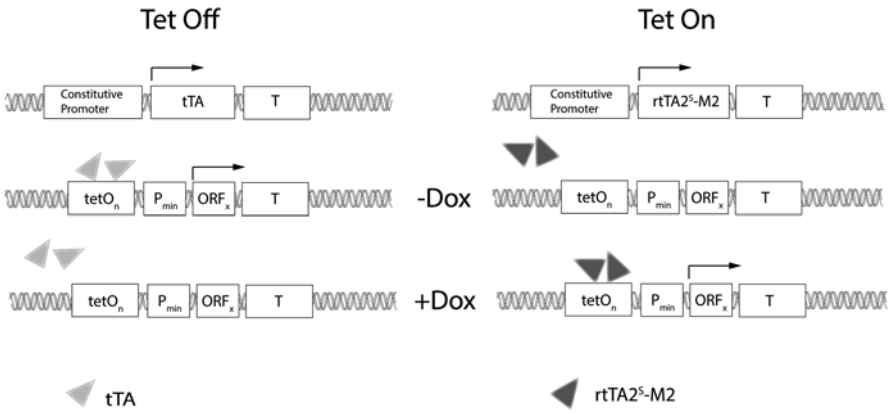

Fig. 2 The tunable Tet-Off and Tet-On expression systems. The Tet-Off system uses the tetracycline transactivator tTA, a fusion of the tetracycline repressor TetR with the activation domain of the Herpes Simplex Virus VP16. The tTA protein is able to bind to specific *TetO* operator sequences. Usually several repeats of such *TetO* sequences are placed upstream of a minimal promoter making up the tetracycline response element *(TRE)*. Repression of the tTA/*TRE*-controlled genes is initiated by the addition of doxycyclin which binds tTA and renders it incapable of binding to the target sequence. The Tet-On system is based on the same components as the Tet-off system with the difference that a reverse hybrid transactivator (rtTA^S-M2) is used which binds the *tetO7* sequence in the presence of Dox and binding is impaired in its absence. Thus the addition of Dox initiates the transcription of the genetic product

by selecting the *ade2* locus as integration site. *Ade2* mutants develop red pigment that facilitates easy and rapid detection of correctly targeted transformants. This system includes a *tku70* (Guangtao et al. 2009) deleted host strain which enables increased gene targeting efficiency and positive transformants, containing the desired gene inserted into the defined expression site, are obtained with an efficiency close to 100 %.

5 Targeted Protein Production

One of the advantages of filamentous fungi compared to other cell factories is the high capacity secretion system for extracellular enzymes such as cellulases, xylanases or amylases. This high secretion capacity is exploited in the gene fusion approach to improve the yields of many non-fungal proteins. Thereby the heterologous gene is linked as a translational fusion to a gene encoding a highly abundant secreted protein. There are several fungal proteins or domains available that can be attached. Especially the modular structure present in many glycoside hydrolases (GHs) is suitable for producing fusion proteins. Their structure is usually defined by the sequential order of the following motifs: a signal sequence followed by the catalytic domain of the GH, which is connected via a linker to the carbohydrate binding module (CBM). This C-terminal binding module is usually replaced by a

heterologous protein although fusions to the full-length proteins have also been successful. Fusion of the target protein to such a secretion carrier has been proposed to have a positive effect on production levels by stabilizing mRNA, improving import in and trafficking through the secretory pathway, and proximity shielding against proteolysis (Eini and Sirkka 1995). A further advantage of the fusion might be that the heterologous protein is also less susceptible to stick to the cell wall.

For the first translational fusions the *T. reesei* CBH1/CEL7A or the *A. niger* GlaA were used as carrier and fused to the bovine chymosine driven by the strong inducible *cbh1* and *glaA* promoters in *T. reesei* and *A. awamori*, respectively (Harkki et al. 1989; Ward et al. 1990). Additional proteins used later as carriers are e.g. the *A. awamori* α-amylases AmyA and AmyB. Translational fusions have resulted in improvement of the expression levels of a number of proteins including bovine prochymosin, porcine pancreatic phospholipase A2, human interleukin-6, hen egg-white lysozyme and human lactoferrin. The increase of the expression levels varies (up to 1000-fold) and up to 1–2 g/L are reported when production strains were in addition subjected to several rounds of mutagenesis (Gouka et al. 1997). To release the carrier from the protein of interest an artificial protease cleavage site is often introduced at the linker part. This approach ensures that the recombinant protein is on the one hand efficiently passed through the cell machinery with the aid of the fungal GH part and on the other hand is later detached of this GH part by e.g. Kex protease. This protease recognizes and cleaves dibasic and acid/basic residues on proteins targeted for secretion (Jalving et al. 2000). In the case of chymosin separation from the recombinant fusion partner is achieved by autoproteolysis.

As the extracellular export of the proteins exposes them to a harsh environment which often leads to protein losses by degradation, new strategies to produce proteins intracellularly but within the secretory pathway to guarantee correct folding and glycosylation were tested. The focus in these strategies is to direct the protein to specific compartments or protein bodies (PBs). One of these variations of the above discussed gene fusion technique is based on the already mentioned fungal hydrophobins, which are fused to the ER retention signal XDEL to allow localization of the protein in the ER. Fusion of the hydrophobin 1 coding region, *hfb1*, to the coding region of GFP driven by the strong *cbh1* promoter led to the intracellular accumulation of HFBI-GFP fusion proteins as micelle shaped protein-body like structures in the ER. A further advantage of this system is that the agglomerated PBs can easily be purified by altering the hydrophobicity of their fusion partners using a surfactant-based aqueous two-phase system which is cheap and applicable for large scale fermentation volumes (Linder et al. 2004; Collén et al. 2002; Mustalahti et al. 2013; Joensuu et al. 2010). This hydrophobin based technique is not limited to fungi but was also efficiently tested for plants to improve protein production (Joensuu et al. 2010).

In addition to these promising findings, a recent study showed, that the titer of recombinantly expressed human proteins in fungi can be increased when the fused carrier protein is targeted for intracellular accumulation in PBs (Smith et al. 2014). This strategy is derived from plants and uses plant seed storage proteins as carriers. In plants these proteins are concentrated in PBs in the lumen of the ER waiting for

their mobilization during germination. The PBs contain mainly zeins which account for more than half of the total seed protein mass. Zein proteins are imported in the ER and localize to the periphery of the PBs surrounding aggregates of other zein proteins (Corchero et al. 2013; Nyyssonen et al. 1993). Expression of γ-zein in plant leaves results in the formation of membrane-bound structures strongly resembling cereal PBs. Fusion of proteins to synthetic zera peptides contribute to PB biogenesis in plants and in fungi. The PBs like structures have tight conformation, surrounded by the ER membrane, thereby protecting the heterologous proteins from proteolysis to some extent during cell disruption and PB harvesting (Joensuu et al. 2010; Torrent et al. 2009; Smith et al. 2014).

While often replaced in translational fusions, the CBM domain itself represents a convenient fusion-partner in protein purifications. Due to their strong capability to bind to cellulose or other natural polymers like xylan or chitin, such domains can be used for simple, effective and scalable affinity purification of proteins of interest using e.g. cellulose columns (Sugimoto et al. 2012). Furthermore, several studies showed impressive improvements of enzymatic activities when this domain was fused to enzymes which do not have a CBM (Thongekkaew et al. 2013; Mahadevan et al. 2008; Ravalason et al. 2009; Reyes-Ortiz et al. 2013; Tang et al. 2013). The linker domain that connects the GH part with the CBM part might be a further player in the creation of designer enzymes with enhanced activity. They are characterized by a low conservation and typically consist of glycine, proline, serine and threonine residues, the latter two are often O-glycosylated, thereby impairing proteolytic degradation and extending the linker peptides (Hu et al. 2001; Harrison et al. 1998; Stals et al. 2004). Several studies described the physiological function of linkers to be a flexible connector of structure domains but also to facilitate interaction between them (Sammond et al. 2012; Gustavsson et al. 2001; Lin et al. 2007; Sonan et al. 2007; Yuzawa et al. 2012; Robinson and Sauer 1998). In addition, recent studies indicate that the linker domain is not only optimized in length, but also in sequence towards the type of cellulase (Sammond et al. 2012; Payne et al. 2013). These findings could be a useful tool for the creation of recombinant CAZymes with enhanced or modified carbohydrate recognition. However, the perfect connector between GH and CBM only makes sense if used in the right context. Enzymes lacking a CBM domain might be, due to higher recovery recycling rates, even more suitable than ones containing it (Pakarinen et al. 2014) and it might be sufficient if the CBM domain is supplemented as an unconnected molecule to enzyme mixtures (Mello and Polikarpov 2014).

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Production of Industrial Enzymes in *Trichoderma reesei*

Marja Paloheimo, Thomas Haarmann, Susanna Mäkinen, and Jari Vehmaanperä

1 Introduction: From the Solomon Islands to Industrial Bioreactors

The mesophilic filamentous fungus *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) has become a major cell factory in the enzyme industry, and the benchmark organism for production of cellulases, especially for biomass conversion. The strain was originally isolated during the second World War from US Army tent canvas in the Solomon Islands and initially identified as *Trichoderma viride*. The isolate was designated QM6a since the strain was part of a collection at the US Army QuarterMaster Research and Development Centre at Natick, Massachusetts (Mandels and Reese 1957). Later QM6a was shown to be distinct from the already known *T. viride* and was renamed *T. reesei* in honour of the Natick laboratory researcher Elwyn T. Reese.

For a long time, *T. reesei* was used only as a model organism for cellulose degradation studies. The worldwide demand for alternative fuel sources in the mid1970s meant that the potential of *T. reesei* to produce cellulases to hydrolyse cellulose-rich biomass into fermentable sugars was of great interest, as it is again today. In general, many filamentous fungi have the potential to produce high amounts of extracellular proteins. Yet, the production level of any protein of interest in naturally occurring strains is usually too low for commercial exploitation, rendering substantial strain improvement programs essential (Punt et al. 2002). Therefore, using ran-

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dom mutagenesis, academic and industrial research programs have over the decades produced several strain pedigrees of *T. reesei* whose enzyme productivity was several times higher than that of the "original" *T. reesei* strain QM6a (Bailey and Nevalainen 1981; Durand et al. 1988).

In the mid 1980s genetic engineering tools became available for *T. reesei* (Penttilä et al. 1987), and since the 1990s *T. reesei* has increasingly been applied as a host of homologous and heterologous enzymes for feed, textile and other industries (Eveleigh and Montenecourt 1979; Tolan and Foody 1999; Paloheimo et al. 2011; Viikari et al. 2012; Puranen et al. 2014) and it has now become one of the major production platforms for industrial enzyme manufacturing. During the last decade or so the interest in using cellulases for production of second generation bioethanol has been revived, with substantial public investments in *T. reesei* bioinformatics and enzyme development, particularly by the U.S. Department of Energy (DoE). Also, the use of *T. reesei* as one of the fungal model organisms for protein secretion as well as the discovery of other *T. reesei* strains in nature including the sexual form *H. jecorina* and the establishment of mating in the species have kept *T. reesei* in the focus of both academic and industrial research teams.

2 Industrial Enzymes

The global industrial enzyme market is estimated to be worth above €3 billion (Novozymes 2013). It is conventionally divided into feed, technical and food segments according to the main application area. Figure 1a displays further refinement of the segments into different industries (Adrio and Demain 2014; Sarrouh et al. 2012; Jari Vehmaanperä, Roal Oy, personal communication).

The great majority of industrial enzymes belong to microbial secreted hydrolytic enzymes and are produced with highly developed microbial hosts. Most industrial enzymes are produced by genetically modified micro-organisms (GMMs) and their production is approved for contained use (Nielsen et al. 2007). The main cell factories used by the established enzyme companies are selected proprietary strains of *Aspergillus* (*A. oryzae* or *A. niger*), *T. reesei* (some *T. reesei* strains have previously been incorrectly taxonomically assigned *T. viride* or *T. longibrachiatum;* (Kuhls et al. 1996; 1999)) or *Bacillus* (*B. subtilis, B. amyloliquefaciens* or *B. licheniformis*), *Streptomyces vialoceoruber* and *Humicola insolens* (Tolan and Foody 1999; Outtrup and Jorgensen 2002; Østergaard and Olsen 2011; AMFEP 2014). Recent entries to this list are, e.g., the fungal platform C1, which has been promoted by the Dyadic corporation (now the strain has been re-identified as *Myceliophthora thermophila* (Gusakov et al. 2007; Visser et al. 2011)), and the methylotrophic yeast *Pichia pastoris* (http://www.rctech.com; http://www.lifetechnologies.com). With the fast growth of the feed segment, fungal cell factories now probably produce about 40–50 % of the value of industrial enzymes.

Table 1 indicates some of the most important classes for industrial enzymes and their applications, most of which are also produced in *T. reesei*. In total, the latest AMFEP database (AMFEP 2014) reports about 70 different industrial enzyme

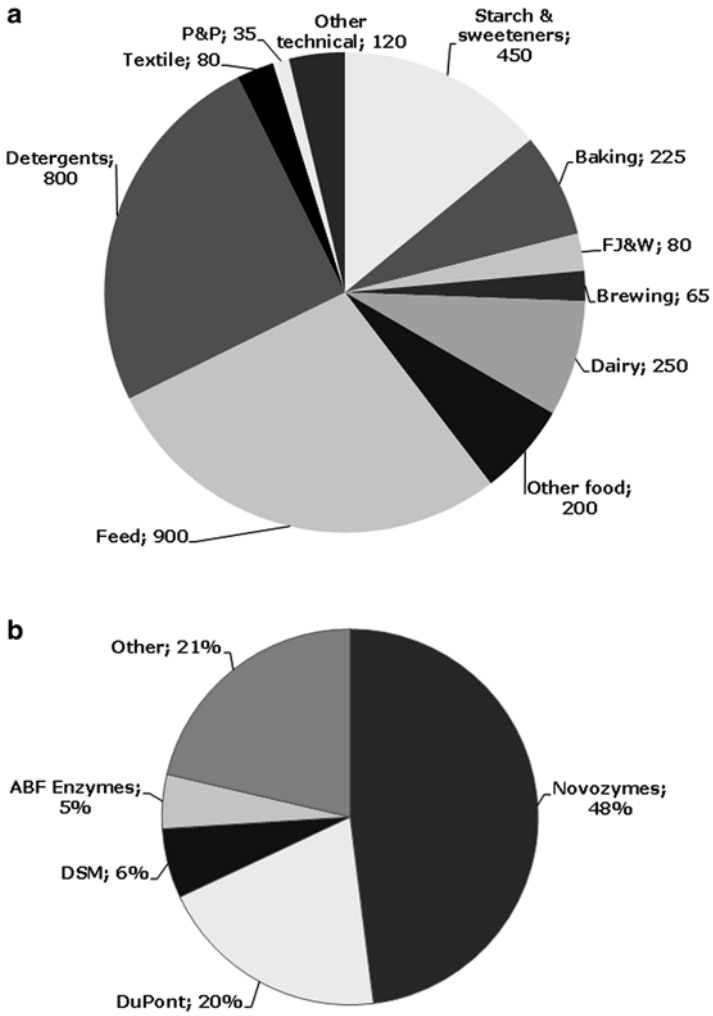

Fig. 1 (**a**) Estimation of industrial enzymes sale (€3.0 billion) per application segment. Starch & sweeteners, baking, FJ&W (fruit juice & wine), brewing and dairy segments belong to the food enzymes, and detergents, textile and P&P (paper & pulp) segments are classified as technical enzymes. (**b**) The main enzyme companies and their approximate shares of sales

classes based on the IUB Enzyme Nomenclature (Bairoch 2000), including 25 glycosyl hydrolases (EC 3.2.1.x).

The biggest manufacturer and supplier of industrial enzymes by far is Novozymes (Denmark) with almost a 50 % share of the markets, followed by DuPont (former Danisco/Genencor) (USA), DSM (the Netherlands) and ABF Enzymes, the group of AB Enzymes (Germany), Roal (Finland) and AB Vista (UK) (Fig 1b). All these are established players in the market with a long track record. Companies such as Gist-brocades, Miles, Solvay, Rhodia, Valley Research, Röhm Enzyme, Alko/Primalco

Table 1 Examples of main industrial enzyme classes and their applications. Enzyme classes which are also commercially produced by *T. reesei* are indicated

Enzyme class	in Tr[a]	Application industry	Benefit
Amylase (A)	Yes	– Starch processing	– Fermentable sugars from starch
Glucoamylase (GA)	Yes	– First generation biofuel	– Manufacture of High Fructose Corn Syrup (HFCS)
(Glucose isomerase)	–	– Baking (A)	– Fermentable sugars from starch
			– Increased shelf life
			– Increased bread volume
Protease	Yes	– Detergent	– Protein soil removal
Rennet, chymosin		– Dairy	– Milk coagulation for cheese manufacturing
Cellulase	Yes	– Textile	– Stonewashing, biofinishing
Beta-glucanase	Yes	– Brewing	– Increasing rate of filtration, viscosity reduction
		– Second generation biofuel	– Fermentable sugars from lignocellulose
		– Feed	– Nutrient release, prebiotic effect
		– Detergent	– Anti-greying, fibre care
		– Pulp and Paper (P&P)	– Energy saving in pulp refining
Xylanase	Yes	– Baking	– Increased bread volume, better dough management
		– Feed	– Nutrient release, prebiotic effect
		– Pulp and Paper (P&P)	– Improvement of Kraft pulp bleachability
Pectinase	Yes	– Fruit juice and wine (FJ&W)	– Yield improvement from pulp (maceration)
			– Clarification of juice
Phytase	Yes	– Feed	– Phosphorus release from phytin
Lipase (L)	–	– Detergent	– Lipid stain removal (L)
Phospholipase, lysophospholipase (PL, LPL)	Yes	– Baking	– Dough stabilisation (L)
		– Food	– Transesterification (L)
		– Pulp and Paper (P&P)	– Oil degumming (PL, LPL)
			– Wheat-based glucose syrup production (PL, LPL)
			– Pitch control (L)

[a]Produced in *Trichoderma* as indicated in the AMFEP list 2014 (AMFEP 2014)

The list is not intended to be exhaustive

Biotec, BioPract and Diversa/Verenium have been acquired by the larger companies over the last 20 or so years. Typically, from time to time new entrants with a promising novel technology in their toolbox have sought growth in the field of industrial enzymes, but failed to gain ground, apparently because they have lacked other crucial elements to run the business, such as production platforms, manufacturing facilities, experience in regulatory affairs, application expertise and access to markets.

Enzymes from exotic sources, such as Archaea may have interesting characteristics, but if you cannot produce them, you cannot sell them.

As the majority of microbial industrial enzymes are secreted into the growth medium by the host, the enzyme preparations in their simplest form are concentrated spent media from which the cell biomass has been removed. They can be sold as monocomponents with only one major activity, or as mixture of multiple activities, the recipes of which are kept as trade secrets. The great majority of microbial industrial enzymes are produced in large (40–400 m^3 or 10,000–100,000 gal) bioreactors by submerged cultivation. In general, in enzyme manufacturing larger cultivation volumes have the benefit of scale, and fungi and yeasts also perform well in the biggest bioreactors.

Enzymes are by definition biocatalysts and the typical dosage in industrial applications is in the range of 100 ppm or often significantly less. Enzymes are supplied in different package sizes, ranging from 25 kg canisters to big bags or even tank trucks. The prices range from few Euros to several hundreds of Euros per kilogram of the product depending on the enzyme concentration, the differentiation provided, the source of the enzyme and the value it brings to the customer.

2.1 Case: Second Generation Biofuel Enzymes

Using sucrose derived from sugar cane or sugar beet, or converting starch to simple sugars with the help of amylase and glucoamylase, and then fermenting the sugars to ethanol with yeast is known as first generation biofuel technology. However, the process itself is old and has been used in making potable and technical alcohol for decades. Second generation biofuel technology comprises conversion of the cellulose, xylan and other sugar polymers in lignocellulolytic feedstocks to fermentable sugars with the help of enzymes, or by other means. Enzymatic hydrolysis of biomass for making bioethanol, or other high-value products in biorefineries, is an emerging new enzyme market, but has not grown to a significant sales segment as yet. However, the potential is huge, because of the large volumes of biomasses needed for the generation of the transport fuels and the high quantity of enzymes required (Viikari et al. 2012; Kim and Kim 2014). Due to the recalcitrant nature of lignocellulose, pretreatment of the feedstock is necessary and yet 40–100 equally high dosages of enzyme protein with longer hydrolysis times (several days) are required as compared to starch hydrolysis (Merino and Cherry 2007). An enzyme preparation with multiple activities—e.g., cellobiohydrolase, endoglucanase, β-glucosidase and xylanase—is needed for complete conversion (Viikari et al. 2007). The cellulase composition produced by *T. reesei* is the industry standard against which all the improved cocktails are compared, and *T. reesei* is the benchmark organism for the production of the required enzyme protein (Viikari et al. 2007, 2012).

The manufactured bioethanol must maintain a competitive price versus the conventional fuels, and with the current dosage the enzymes account for a significant part of the ethanol manufacturing costs. A demo manufacturing site producing

20 million gallons of ethanol annually would already require one dedicated large size bioreactor for the enzyme production. To ensure low-cost, reliable and flexible delivery the enzyme production facility would preferably be situated on-site or near-site. To further lower the costs it has been suggested to eliminate the cell separation, concentration and formulation costs, and use whole broth—that is, the spent medium and the biomass as such—in the hydrolysis (Merino and Cherry 2007). It has also been suggested to use the fermentable sugars generated or some fractions thereof, such as xylose, for the enzyme production. However, if this lowers the enzyme titres in the broth, as is likely to be the case, more production capacity is needed, which may remove any benefits; furthermore, the sugars should be available at high concentrations for the fed-batch operations, and such concentration adds costs. As a novel approach to produce all required enzymes with one host ('multiactivity strain') we and others have constructed *T. reesei* strains expressing several cloned enzyme genes simultaneously at the desired ratios using selected promoters (Terhi Puranen, Roal Oy, personal communication; Merino and Cherry 2007).

If the second generation biofuel enzyme market really takes off, it could become one of the largest markets in the world and could be a game-changer in terms of lowering enzyme production costs and could also have an influence on other conventional enzyme businesses. It would also make *T. reesei* the leading production platform. The business model for production of biomass-hydrolysing enzymes for second generation bioethanol is very different from standard industrial enzyme manufacturing, due to the attempts to match the high enzyme dosage with the low value of the end product (ethanol), high investment needs, need for close partnership with the ethanol manufacturer and uncertainty regarding the long-term price development of ethanol, and it has as yet remained a small business (Merino and Cherry 2007). The improvements in enzyme performance and manufacturing costs during recent years have been incremental despite some headlines and have not lowered the enzyme cost to a completely different level. However, the research into biomass enzymes has resulted in one novel discovery, the family of AA9 (formerly GH61) enzymes, which are copper-dependent lytic polysaccharide monooxygenases (LPMOs) and cleave cellulose chains with oxidation of various carbons (C-1, C-4 and C-6) (Merino and Cherry 2007; Hemsworth et al. 2013).

3 *T. reesei* as a Cell Factory for Industrial Enzymes

3.1 Manufacturing

T. reesei has established its position as one of the two main fungal cell factories for production of industrial enzymes over the last 40 years (Tolan and Foody 1999; Merino and Cherry 2007). The reasons are partly historical, but it is mainly because *T. reesei* fulfills the prerequisites required from an industrial production host very well:

Capacity

- *T. reesei* naturally produces high levels of cellulases (Montenecourt et al. 1980)
- mutant strain lineages have been developed which are capable of secreting 40–100 g of total enzyme protein per litre of spent medium (Pourquie and Warzywoda 1993; Cherry and Fidantsef 2003)
- with the strong *cbh1/cel7A* promoter, the majority of the secretion capacity can be directed towards the target enzyme production
- the strains can be tailored to be deleted for genes encoding undesired side activities

Safety

- *T. reesei* is a saprophytic fungus and non-pathogenic to humans, and therefore safe to use (Nevalainen et al. 1994; Blumenthal 2004)
- all current industrial strains can be traced back to one single isolate, QM6a (Mandels and Reese 1957; Durand et al. 1988)
- the species is only found in nature within a narrow belt of ±20° around the equator (Kubicek et al. 2008) and therefore, in most countries, the production strain is not expected to propagate in nature if accidentally released from manufacturing (Providenti et al. 2004)
- genomic data is available from several strains (Kubicek 2013), allowing identification of potential pathways for secondary metabolites

Robustness

- *T. reesei* enzyme production is supported in cheap and easily available raw materials and with simple sugars as the carbon source
- the manufacturing can be upscaled to reactor volumes larger than 100 m^3 without compromising productivity
- the strains tend to have manageable viscosity, produce no acids and the clones can be stably maintained as uninucleated conidia, which also allows clonal screening approaches for rejuvenation

Track Record

- *T. reesei* has been used for industrial enzyme production since the early 1970s (Bailey and Nevalainen 1981; Tolan and Foody 1999; Hjortkjaer et al. 1986; Pourquie et al. 1988)
- the species has served in academia as a model for lignocellulose degradation and fungal protein secretion (Saloheimo and Pakula 2012)

When the absolute levels of the secreted protein are low, higher relative improvements are more easily achieved in strain improvement programs (described in more detail in chapter 4.1) and in medium optimisation. At commercially relevant production levels the combination of the strain capacity with a carefully tailored production process becomes the key factor. With *T. reesei* as a production host the aim is generally to either maximise the cellulase complex production (e.g. for biomass hydrolysis) or to optimise the heterologous gene expression under the main cellulase

gene promoter *cbh1/cel7A* (for industrial monocomponent semifinals). Commonly reported carbon sources, which induce *T. reesei* cellulase and *cbh1* expression are cellulose, lactose and sophorose (Pourquie et al. 1988; dos Santos et al. 2014). Cellulose is a natural substrate and can be applied in high concentrations as it adds little to the osmotic pressure, but it is not soluble, which makes feeding problematic and it is also relatively expensive; it probably serves best as an additional inducing substrate in the batch phase. Lactose is a soluble sugar and relatively cheap, and in spite of not being a building block of lignocellulose, promotes cellulase expression. At high enzyme production levels, the yield of enzyme protein per gram of sugar is relevant as this determines how much sugar needs to be available for the strain[1] and the low solubility of lactose (25 %) may set a ceiling for use of lactose (Pourquie et al. 1988).

Glucose is an ideal carbon source as it is cheap and has high solubility, but has a catabolic repression effect on cellulase production. Sophorose serves as a potent inducer but the list price is high (Viikari et al. 2012). Use of a glucose-sophorose mixture which has been produced on-site using β-glucosidase (Vaheri et al. 1979) together with a strain which is genetically glucose-derepressed has been claimed to result in high cellulase yields and low costs (Mitchinson 2004; England et al. 2010).

3.2 Safety Aspects

Enzyme manufacturing has to be safe for the operators and the products for the end-users, meaning that they have to be free from mycotoxins, antibiotics or other activities potentially harmful to humans, animals or the environment. The above secondary metabolites may provide protection against predators (e.g. other fungi) and give an advantage to the fungus in its survival in its ecological niches (Fox and Howlett 2008).

T. reesei belongs to the group of biosafety level 1 microbes, has a long history of safe use, and several enzyme products originating from *T. reesei* strains have obtained a GRAS (Generally Regarded As Safe) status in the FDA's (U.S. Food and Drug Administration) evaluation (http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices). There are two groups of mycotoxins within the *Trichoderma* genus of relevance to humans, namely gliotoxins (belonging to the epipolythiodioxopiperazine class of peptides (Patron et al. 2007)) and trichothecenes/Trichodermin (belonging to sesquiterpenes (Godtfredsen and Vangedal 1965)). The *T. reesei* genome contains a GliP cluster counterpart but it is clearly smaller in size compared to that in species producing gliotoxin/gliovirin and the genes in this cluster are not expressed (Patron et al. 2007; Mukherjee et al. 2012). In one publication (Watts et al. 1988) it was suggested that a mutant derived from *T. reesei* QM9414 produces Trichodermin. However, according to a recent review

[1] If 4 g of sugar are consumed for each 1 g of secreted enzyme, 400 g sugar/L would be required to achieve 100 g of secreted enzyme /L (Pourquie et al. 1988; Cherry and Fidantsef 2003).

publication only a few *Trichoderma* species can produce Trichodermin (Hermosa et al. 2014) and it has also recently been described that the gene responsible for the first committed step in trichothecene biosynthesis (trichodiene synthase, *tri5*) is absent from the *T. reesei* genome: a BLASTP analysis with the *tri5* gene product from *T. brevicompactum* (a strain producing trichothecenes) results in no hits on the *T. reesei* genome sequence (Christian Kubicek, personal communication). This indicates that *T. reesei* is unable to initiate trichothecene biosynthesis. Taken together it is concluded that *T. reesei* does not produce mycotoxins (Nevalainen et al. 1994; Blumenthal 2004).

With respect to secondary metabolites, *T. reesei* is capable of producing peptaibols (e.g. several paracelsin analogues) which are linear or cyclic peptide antibiotics synthesised by non-ribosomal peptide synthetases, NRPSs (Degenkolb et al. 2012). *T. reesei*'s genome contains ten NRPS-encoding genes altogether (Martinez et al. 2008), of which two encode peptaibol synthetases with sequence homology to synthetases of other *Trichoderma* species (Degenkolb et al. 2012; Kubicek et al. 2011). Efficient biosynthesis of peptaibols is described as occurring predominantly in solid cultivations (Kubicek et al. 2007; Komon-Zelazowska et al. 2007). Moreover, peptaibols have mostly been isolated from very old and already strongly sporulating cultures of *Trichoderma* (Kubicek et al. 2007), not representing the typical industrial fermentations in which the cultivation is optimised for the fungus both in terms of length and conditions (controlled feed of nutrients and oxygen, suitable pH and temperature).

In addition to the abovementioned NRPSs, the genome of *T. reesei* contains ten genes encoding polyketides and two genes encoding NRPS-PKS hybrid enzymes (Martinez et al. 2008; Kubicek et al. 2011). However, the role of these compounds in relation to safety has not been discussed in the literature so far.

In connection to production strain safety it has to be stressed that there are no reports on changing a non-toxic production strain into a toxic strain (Blumenthal 2004).

4 Improvement of *T. reesei* Strains for Industrial Enzyme Production: Techniques and Tools in Use

Each filamentous fungal species has its special characteristics regarding its use as a host and as an enzyme producer. However, the filamentous fungi as a group also share properties regarding expression and secretion. Thus, general approaches used to improve enzyme production in some of the fungal species can often be exploited in another species. For example, mutagenesis, use of strong promoters, selection of multicopy strains, choice of integration site, optimisation of the codon usage and use of carriers are techniques routinely used in developing *T. reesei* strains. Usually, homologous enzymes or enzymes from taxonomically closely related fungal species are relatively easily produced in high yields in *T. reesei*, as is true for other industrial filamentous fungal hosts. Yields of enzymes from taxonomically less related fungi, bacteria and mammals are usually much lower, one of

the reasons being their higher sensitivity to host proteases. In such cases additional modifications to the protein in question or to the host are necessary to achieve industrially feasible yields.

4.1 Mutagenesis

Strain improvement by mutagenesis has remained one of the elementary tools for industrial *T. reesei* host and GMM strain development. One of the advantages is that no detailed knowledge of the underlying mechanisms of the desired feature is needed. Basically, the mutagenesis approach consists of subjecting the fungus to sub-lethal doses (e.g. 1–5 % survival level) of mutagens and subsequent screening of a large number (typically at least >10,000 clones) of survivors for the improved characteristic (Bailey and Nevalainen 1981; Alikhanian 1962; Rowlands 1984). As the specific DNA damage leading to the mutation occurs only in one genome and often in only one of the DNA strands, uninucleated haploid conidia are the preferred starting material, and careful clonal purification after the mutagenesis is required to allow segregation of the mutations and to avoid mosaicism (Rowlands 1984). Because of the large number of clones, the screening needs to be run on a small scale to try to mimic real culture conditions. If the expected improvements are quantitative and incremental, the standard deviation of the screen needs to be tight in order to avoid excluding the improved mutants due to the background noise. Sophisticated screening methods taking advantage of various high throughput methods have been designed for *T. reesei* (Toyama et al. 2002; Zhang et al. 2006; Throndset et al. 2010). However, the small scale screening assay may always result in a selection of strains which are superior in the screen, but fail to perform under relevant commercial conditions: "what you screen is what you get".

A major constraint of the random mutagenesis approach is that the mutations cannot be directed on distinct target genes. Thus, sublethal mutations may accumulate in long mutant lineages, causing strain degeneration. Mating (chapter 5.2) may now provide a tool to rejuvenate these lineages in *T. reesei*.

Strains which have been developed only by means of mutagenesis and screening are called CMOs (CMO = Classically Modified Organism) or non-GMOs (GMO = Genetically Modified Organism) and may find more acceptance in certain markets due to customer perceptions. However, the term 'classical' here refers to techniques developed for microbes only about 70 years ago when developing the high penicillin-producing strains, such as the Wisconsin series (Alikhanian 1962).

For *T. reesei* the screening for cellulase hyperproducers derived by classical mutagenesis has led to essentially two different lineages of publicly available mutated strains, known as the Rutgers lineage (Eveleigh and Montenecourt 1979) and the Natick series (Reese 1975). One of the best-known cellulase producer strains from the Rutgers lineage that is publicly available is Rut-C30 (recently reviewed in Peterson and Nevalainen (2012)). This strain has been obtained through

three classical mutagenesis steps, starting with QM6a. RL-P37 is another mutant strain derived from the same parent, RutNG-14, and used by NREL and Genencor (now DuPont Industrial Biosciences) (Tholudur et al. 1999; Foreman et al. 2003) in particular in studies using *T. reesei* for second generation biofuel manufacturing.

The most widely distributed and best-known isolate from the Natick series is QM9414, which has been obtained through two rounds of classical mutagenesis and screening, starting with QM6a. This strain has been the parental strain for several major commercial strain improvement campaigns: the VTT/ALKO series (Bailey and Nevalainen 1981; Mäntylä et al. 1992, 1998), the CAYLA series (Durand et al. 1988), the Cetus series (Shoemaker et al. 1983), and the Kyowa series (Nevalainen et al. 1994; Kawamori et al. 1985); the publication by Durand and coworkers (Durand et al. 1988) displays a detailed pedigree of these mutant families. The most studied strains of these lineages are:

• VTT/ALKO[2]:	VTT-D-79125 and ALKO2221 (low protease mutant)
• CAYLA[3]:	CL 847
• Cetus[4]:	L27
• Kyowa[5]:	PC-3-7 (Kawamori et al. 1985; Mordcawa et al. 1985; Fujii et al. 2010)

Electrophoretic karyotyping studies have shown that *T. reesei* mutants show rearrangements between the chromosomes (Mäntylä et al. 1992; Carter et al. 1992). Comparison between the genome sequences to reveal exact changes between the mutant cell lines and data analysis for making biological interpretations from the differences in public and proprietary genomes are now possible. Bioinformatics and genome-wide approaches currently provide tools for identifying the often complex genetic traits which are the basis for the improved features in the best mutants. The current status and published outcome of sequencing projects is described in more detail in chapter "Homologous and Heterologous Expression of Basidiomycete Genes Related to Plant Biomass Degradation."

In recent years the further development of *T. reesei* with respect to enhancement of cellulase production or its use as a production host for heterologous proteins has mainly been done at industrial research laboratories. Thus, most of the results have stayed as proprietary information and have not been communicated to the public. The Dyadic corporation has recently reported a low viscosity morphological *T. reesei* mutant X-252 claimed to have benefits in high throughput screening and in large scale cultivations (Emalfarb et al. 2010; *T reesei* is designated as *T. longibrachiatum* in the patent).

[2] VTT = Technical Research Centre of Finland, Espoo, Finland. ALKO = State Alcohol Monopoly, Helsinki, Finland. In 1995, the enzyme business was moved to Primalco Biotec at the Altia Group, Helsinki, Finland.

[3] CAYLA = Société CAYLA, Toulouse, France

[4] CETUS = Cetus Corporation, Berkeley, CA, USA

[5] Kyowa = Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan

4.2 Gene Deletions

Gene deletions are routinely used to improve industrial production strains by increasing the relative yield of the enzyme in question (to obtain "monocomponent products"), to delete unwanted side activities that would be detrimental in the targeted application or harmful to the enzyme product itself (e.g., host proteases), or to remove pathways for undesired secondary metabolites. The deletions are normally done by replacing the target gene with a selectable marker with the aid of the gene's 5′- and 3′-flanking regions and homologous recombination (Mäntylä et al. 1998). Proprietary industrial *T. reesei* host strains have been tailored to carry deletions, e.g., in all major cellulase and xylanase genes, which facilitates monitoring the main activity in quality control and allows easy detection of the target enzyme. In our experience, the multiple deletion strains still grow and produce enzymes to similar levels under standard conditions as their parents.

The homologous integration frequency varies depending on the strain, locus, insertion site and flanking regions (Guangtao et al. 2009; Schuster et al. 2012). *T. reesei* strains with deletions in genes responsible for the pathway for (ectopic) integration of exogenous DNA into chromosomal DNA (nonhomologous end joining, NHEJ), *tku70* (Guangtao et al. 2009; Joergensen et al. 2014), and *tmus53* (Steiger et al. 2011) have been shown to have elevated targeting frequencies.

With the current technology, *T. reesei* strains with multiple deletions need to be constructed in a successive manner requiring either the use of several marker genes or a bidirectional selection system with recyclable markers. One example of the latter is *pyr4* coding for an orotidine 5′-monophosphate decarboxylase (Seidl and Seiboth 2010). Strains positive for *pyr4* are prototrophic for uracil/uridine and at the same time sensitive to the 5-fluoro-orotic acid (5-FOA) that is metabolised to 5-fluorodeoxyuridine monophosphate, a compound acting as an inhibitor of the thymidylate synthase essential for DNA synthesis. Therefore it is possible to screen either for the gain (growth on plates without uracil/uridine supplementation) or the loss (resistance to 5-FOA) of the *pyr4* marker. A recently published paper describes a new bidirectional marker, *pyr2*, encoding an orotate phosphoribosyl transferase that also allows selection of transformants using uridine and 5-FOA plates (Joergensen et al. 2014). For improved marker recycling, a Cre/loxP excision system adapted from bacteriophage P1 has been applied for *T. reesei* (Steiger et al. 2011). Dominant marker genes are preferred over auxotrophic markers as no starting auxotrophic strains are thus needed. The dominant markers in use or reported to be useful in *Trichoderma* are as follows: *amdS* (*Aspergillus nidulans* acetamidase, enables strain to grow on acetamide as a sole nitrogen source; Penttilä et al. 1987), *hph* (hygromycin B phosphotransferase giving resistance to hygromycin, originating from *Klebsiella* and isolated from *E. coli*; Mach et al. 1994), *ble* (phleomycin-bleomycin binding protein for resistance to phleomycin and bleomycin, isolated from *Streptoalloteichus hindustanus*; Durand et al. 1988), *suc1* (*Aspergillus niger* β-D-fructofuranosidase or invertase, enables growth on sucrose as the only carbon

source; Berges et al. 1993), *npt2* (neomycin phosphotransferase isolated from *Escherichia coli* conferring resistance to Geneticin; Gruber et al. 2012) and *ptrA* (gene product confers resistance to pyrithiamine, isolated from *Aspergillus oryzae*; Kubodera et al. 2002). The use of genes for antibiotic resistance (ARMs or Antibiotic Resistance Markers) is generally avoided in construction of industrial production strains for regulatory reasons.

4.3 Low Protease Hosts and Production Strains

In some cases, high production of in particular heterologous (but also homologous) recombinant proteins is hampered by degradation of the protein of interest by host protease(s), affecting both the yield of the enzyme and the stability of the enzyme product (Braaksma and Punt 2008). The amounts of native proteases in industrial *T. reesei* hosts are usually relatively low, due to selection of low protease mutants as production hosts/strains, use of strains from which the selected protease genes have been genetically deleted or disrupted and optimisation of the raw materials and cultivation conditions in such a way that represses or at least does not enhance protease production (Mäntylä et al. 1998; Wiebe 1999). However, some enzymes are exceptionally sensitive even to low amounts of proteases and may need further modifications to remain stable in products. For example, with cellulases carrying a CBM, the junction point between the core and the linker may be particularly susceptible to protease cleavage and to develop a product with acceptable shelf life requires careful engineering of the joining sequence in addition to using a low protease host (Vehmaanperä et al. 2006).

Mutants with lowered protease production can be screened by using selection plates with protease substrates such as haemoglobin or casein and picking up the colonies with the smallest halo. One of the recently published substrates for screening low protease strains is the proprietary suicide chemical known as "SUI" (Braaksma and Punt 2008). The mutants with low protease production are more resistant to SUI than the parent strains and can thus be easily screened on SUI plates. The chemical identity of this substrate is not published.

Low protease strains are of great importance for cost-effective industrial enzyme business. However, to the best of our knowledge, no similar global protease regulator gene such as the *Aspergillus* sp. *prtT* (Punt et al. 2008) has yet been published from *Trichoderma*. As proteases have different specificities and each enzyme product differs in its sensitivity to proteases, a case-by-case analysis to identify the most harmful protease activity is usually necessary. Exploitation of the proteomic and transcriptomic data (array and RNA-seq) enables identification of host proteases which are the most detrimental to the target enzyme(s). Thus, it is expected that these techniques will offer additional possibilities for strain development by making further tailoring of the production strains more straightforward.

4.4 Factors Affecting Transcription/Expression

The expression of target genes under the regulation of the *cbh1* promoter can be positively affected by modifying/amplifying the regulator binding site(s) in the promoter or by up- or down-regulating the expression of regulators binding to the promoter (see chapter 4.6). In addition, overexpression of a global regulator of several secondary metabolite gene cluster genes in *T. reesei*, the *lae1* encoding the putative protein methytransferase Lae1, has been described to significantly increase transcription of cellulase genes in QM9414 (Seiboth et al. 2012). However, according to a recent publication, this effect is possibly an indirect effect of a change in the growth rate observed in the Lae1 overproduction strains compared to the host and a Δ*lae1* strain and needs further confirmation (Fekete et al. 2014).

The properties of the gene to be expressed may have an effect on transcription efficiency and mRNA stability as well as translation efficiency. The codon usage of the heterologous gene should be adjusted to that of the host to confirm high enzyme yield. The native, efficiently expressed *T. reesei* genes show a strong bias in codon usage towards C at the wobble position (Te'o et al. 2000; Bergquist et al. 2002). The codon usage of a heterologous gene to mimic that of the host has been shown to be relevant in expression of, e.g., efficient transcription of the AT-rich *xynB* gene from *Dictyoglomus thermophilum*. Xylanase B was detected only after change of 20 codons to resemble those generally used by *T. reesei* (Te'o et al. 2000). Several reasons for low yields of heterologous proteins from filamentous fungi, due to the mRNA sequence/structure have been suggested including premature termination of transcription, incorrect processing, instability of the mRNA, occurrence of a strong secondary structure and underrepresentation of isoacceptor tRNAs for efficient translation (e.g., Gouka et al. 1996, 1997a).

In recent years, the components of mRNA and their contribution to the formation of stabilising secondary structures and eventually their effect on protein production have been studied extensively in yeast (e.g., Curran et al. 2013; Trotta 2013; Zur and Tuller 2012). Also, there is some published data on the regulatory effects of the 5′- and 3′-UTRs and their efficiency on translation and enzyme yields (Tamayo-Ramos et al. 2013; Koda et al. 2004, 2006; Platt et al. 1996). The cassettes used in *T. reesei* expression usually contain either the 3′-UTR from the host (e.g., the *cbh1* terminator region) or in case of a fungal gene donor, that of the native gene. However, research targeted to the influence of the UTR region(s) on mRNA stability, translation efficiency and enzyme yield would be valuable.

Most genes in filamentous fungi and other eukaryotes contain introns. Genes of *T. reesei* contain, on average, two introns (Martinez et al. 2008). There are indications in some older publications of the production of enzyme being better when a genomic gene is used in the expression cassette instead of a cDNA (Joutsjoki et al. 1993). However, literature and unpublished results are also available showing identical production levels with the genomic gene and cDNA constructions (Marja Paloheimo, Roal Oy, unpublished results). Introns have been found to significantly affect gene expression in plants, but the phenomenon termed Intron-Mediated

Enhancement (IME) has not been conclusively proven to exist in a fungal species. IME results in mRNA accumulation independently of the transcription initiation rate, especially when an intron is positioned near the 5′ end of the transcript or at the 5′-UTR (Rose 2008; Akua and Shaul 2013; Parra et al. 2011). Introns in the *Malbranchea cinnamomea* protease coding region have been shown to positively affect its production in *T. reesei*. The effect of the removal of the three endogenous introns of the protease gene was cumulative and the most drastic effect was achieved with the deletion of the intron nearest to the 5′ end of the gene (Paloheimo 2013). These results seem consistent with IME similar to plants, although they are very much preliminary.

As discussed above there is growing evidence suggesting that all the elements (promoter, gene and introns, terminator) need to be carefully optimised and positioned in the expression cassette to ensure the highest possible production yields. The compatibility of the elements with each other may affect protein yield more than the strength of the promoter. Knowledge of the factors affecting the mRNA stability and translation efficiency is still scarce and it can thus be expected that there is still room to improve enzyme yields by better design of these elements. As filamentous fungi are used extensively for production of proteins they would serve as excellent objects to study the effects of UTRs and introns on protein production.

4.5 Approaches to Improving Enzyme Production by Modifying the Secretion Pathway

Starting from the end of the 1990s, intensive research has been ongoing regarding fungal secretion machinery and its control mechanisms (for reviews, see, e.g., Saloheimo and Pakula 2012; Conesa et al. 2001; Shoji et al. 2008). The quality control in the endoplasmic reticulum (ER) system for correct folding, letting only properly folded proteins proceed and efficient removal of non-folded proteins—is presumed to be one of the most relevant bottlenecks for the production of (heterologous) proteins and has thus been a self-evident target for modifications to achieve yield improvements.

The proteins destined for the secretion pathway (ER, Golgi complex, vesicles) enter it *via* the ER and the signal sequence plays an essential role in targeting. In some cases the pro sequences are essential for proper folding of the enzyme (Chen and Inouye 2008) and thus may also affect the secretion efficiency of different classes of enzymes. To our knowledge, however, systematic studies on the signal and/or pro sequence(s) and their engineering to improve protein production in filamentous fungi have not been published. The signal sequences used for production in *T. reesei* usually either derive from the protein of interest or a signal sequence from a host protein (e.g. CBHI) is used. In some studies the native heterologous signal sequence has been reported to lead to better yield in *T. reesei* (Joutsjoki et al. 1993)

but, according to our studies, replacing the native signal sequence with that of CBHI most often does not make any difference (Roal Oy, unpublished results). However, the heterologous fungal protease from *Fusarium* represents an exception: a drastic decrease in protease production was detected when the CBHI signal sequence instead of the native signal sequence was used (Susanna Mäkinen, Roal Oy, unpublished result). The reasons for the effect have not been studied in detail but possibly the native (but not the CBHI) signal sequence is compatible with the protease pro sequence, required for correct folding of the mature protease.

The folding in the ER is aided by ER resident chaperones, e.g. the heat shock proteins of the Hsp70 family (BiP/Kar2p), calnexin and foldases, the members of the protein disulphide isomerase (PDI) family and peptidyl-prolyl *cis-trans*-isomerases (PPIase) (reviewed, e.g. in Conesa et al. 2001; van Anken and Braakman 2005). The core *N*-glycan is also attached in the ER. Several genes encoding chaperones, chaperone binding proteins and foldases have been overexpressed to improve yields of heterologous proteins in filamentous fungi in particular (reviewed in Conesa et al. 2001; Moralejo et al. 2001; Valkonen et al. 2003). Unfortunately the results from the experiments have remained inconsistent and sometimes even contradictory and no generally usable tools for enzyme yield improvements have been obtained.

The ER also contains the mechanisms to maintain and control the folding capacity and to efficiently remove and target misfolded or persistently unfolded proteins to the ER-associated protein degradation (ERAD) pathway (reviewed e.g. in Goldberg 2003; Meusser et al. 2005). The ER quality control is regulated by highly specific signaling pathways known together as the Unfolded Protein Response (UPR) (reviewed in e.g. Rutkowski and Kaufman 2004). The UPR is induced when the ER homeostasis is perturbed due to accumulation of unfolded/misfolded proteins (resulting, e.g., from overproduction of heterologous enzymes). UPR induction results in at least three effects: the protein folding and transport is improved, the unfolded proteins are effectively degraded and fewer secretory cargo proteins are allowed to enter the ER. These effects are due to elevated levels of transcription from the genes encoding proteins involved in protein folding, inhibition of protein synthesis and other pathways relevant to releasing ER from the stress. UPR in *T. reesei* is mediated by the transcription factor Hac1. An active form of Hac1 is formed upon UPR induction by a dual mechanism: an unconventional intron from the mRNA is spliced and an upstream ORF from the 5′-untranslated end is removed (Saloheimo et al. 2003). Attempts have been made to improve yields of enzymes by overexpressing genes encoding UPR sensors and by constitutively inducing the UPR pathway (Valkonen et al. 2003, 2004). The outcome has been similar to that in studies of chaperone/foldase overexpression and no breakthrough regarding industrial enzyme production has yet been published.

In addition to UPR, filamentous fungi have been proposed to have at least two feedback mechanisms which lead to reduced amounts of new proteins being targeted to ER during stress conditions. “RESS” (REpression under Secretion Stress) acts by downregulating genes encoding secreted proteins (e.g. the *T. reesei* major

cellulase genes) leading to a decreased protein load of the secretory pathway (Pakula et al. 2003; Al-Sheikh et al. 2004). "Differential translation" has been suggested to act by reducing on the one hand translation of several secreted proteins and proteins functioning in ribosomal biogenesis and assembly, and on the other hand enhancing translation of proteins functioning as part of the secretory system (Guillemette et al. 2007). Vesicle transport including correct sorting of the vesicles to defined compartments seems to play another important role in (heterologous) protein production. One example is the sorting receptor Vps10 that was shown in yeast to be responsible for the recognition and delivery of several vacuolar proteins and is also involved in targeting recombinant and aberrant proteins for vacuolar degradation (Holkeri and Makarow 1998). The knock-out of *vps10* in *Aspergillus oryzae* led to enhanced production and secretion of heterologous proteins most likely because the aberrant proteins were no longer targeted to the vacuole for degradation (Yoon et al. 2010).

The majority of proteins in eukaryotes are glycoproteins (Apweiler et al. 1999). *T. reesei* glycoproteins have diverse structures, depending on the strain, culture media and conditions (Stals et al. 2004a, b; Goto 2007). Glycans have a role in secretion as they promote folding of glycoproteins by increasing the hydrophilicity and are important in ER quality control by acting as recognition signals ("tags") for chaperones in the calnexin cycle (reviewed in Helenius and Aebi 2004; Molinari 2007). In older literature it has been suggested that *O*-glycosylation but not *N*-glycosylation would be required for secretion in *T. reesei* (Kubicek et al. 1987) and that yields of secreted enzymes could be increased by increasing the amount of sugar precursors in cells (Kruszewska et al. 1999). These effects have not been studied further in newer literature. However, glycans may also in some cases have a positive effect on the yields of some enzymes due to their positive effect on enzyme stability (Wang et al. 1996; Neustroev et al. 1993).

Filamentous fungi are believed to secrete the majority of proteins from the growing tips (Wösten et al. 1991; Mueller et al. 2002) but there are also published examples of exocytosis taking place at fungal septa or other regions of the hyphae (Hayakawa et al. 2011; Read 2011). In *T. reesei* the existence of at least one alternative secretion pathway has been suggested in which the proteins would exit (also) from sub-apical regions (Nykänen et al. 1997; Valkonen et al. 2007). The alternative pathway(s) may be regulated and be specific for certain types of transport vesicles and their cargo but there are not yet publications available on the exploitation of such pathways for enzyme production.

The results from the studies on modifications targeted at improving efficiency of fungal secretion pathways show that suitable modification(s) need(s) to be tested on a case-by-case basis for each enzyme or protein, suggesting that different proteins have their own special requirements and limiting step(s) for secretion. Possibly a coordinated increase in expression of several genes encoding suitable folding catalysts would overcome rate-limiting steps and result in significant improvements. In addition it has to be kept in mind that the effects obtained depend greatly on the host: some of the modifications resulting in improvements in public strains are not directly transferrable to industrial mutants.

4.6 CBH1/CEL7A, *Improved and Alternative Promoters*

Generally a strong inducible host promoter is used for high-yield enzyme production. In *T. reesei* more than half of the secreted proteins consist of cellobiohydrolase I (CBHI/Cel7A) (McFarland et al. 2007). For this reason, the *cbh1*/*cel7A* promoter is most often used to drive protein production in the strain and is considered a benchmark for *T. reesei*. It has been used successfully for years, achieving grams per litre yields even for heterologous bacterial proteins (Peterson and Nevalainen 2012; Paloheimo et al. 2007); it is safe to assume that in industry the production levels are a magnitude higher as several bacterial enzymes produced in *T. reesei* have been registered as commercial products (AMFEP 2014; Bento et al. 2012; Maurer et al. 2013).

A precise analysis of the different binding sites present in the *cbh1* promoter sequence has uncovered several potential glucose repressor Cre1 binding sites. Deletion or point mutation of these Cre1 binding sites completely abolished glucose repression (Ilmen et al. 1996a). In line with this it has been shown that deletion of *cre1* or exchange with the truncated variant gene found in Rut-C30 leads to derepressed cellulase and hemicellulase expression in cultures with glucose as the carbon source (Nakari-Setälä et al. 2009). Ace1, another cellulase regulator with a binding site in the *cbh1* promoter sequence was discovered to act as a repressor since the deletion of *ace1* results in an increase in the expression of all main cellulase and xylanase genes in cultures under cellulose-inducing conditions (Aro et al. 2003). Binding sites for the transcriptional activator Ace2 are also present and shown to be functional (Aro et al. 2001). Deletion of *ace2* in a hypercellulolytic mutant of *T. reesei* led to lowered induction kinetics of mRNAs encoding the major cellulases and endoglucanases, and to an overall reduction in cellulase activity under inducing conditions by 30–70 %. The other regulators known to bind to the *cbh1* promoter are the positive regulator Xyr1 for cellulase and xylanase expression (Rauscher et al. 2006) and the CCAAT binding complex Hap 2/3/5. The CCAAT binding motif is a common element found in the promoter and enhancer regions of a large number of eukaryotic genes and is, for example, also present in the *cbh2* promoter (Zeilinger et al. 2001). Elevated enzyme yields have been obtained in *T. reesei* QM9414 and Rut-C30 by constitutively expressing the *xyr1* gene (Portnoy et al. 2011), and by combining this with the downregulation of *ace1* (Wang et al. 2013).

The knowledge of these different binding sites present in the *cbh1* promoter sequence has been exploited to construct improved variants of this promoter (Liu et al. 2008). The rationale was to delete regions with binding sites for repressing transcription factors (Cre1, Ace1) and at the same time to insert several copies of sequences that are known to bind activating transcription factors such as Ace2. These modifications significantly increased the heterologous expression of a reporter gene. Later on the modified promoter was successfully used to express human erythropoietin in *T. reesei* (Ivanova et al. 2013) and to construct cellulase hyper-expressing *T. reesei* strains (Zou et al. 2012). A similar strategy has been proven to be successful for the optimisation of different promoters in *Aspergillus niger*, where the insertion of multiple copies of an activator transcription factor

binding site improved the expression of both heterologous and endogenous genes (Minetoki et al. 1998; Liu et al. 2003).

Usually, introducing multiple copies of a *cbh1* or other promoter-driven expression cassette to a host strain increases the protein production over a single copy transformant. In case of the *cbh1* promoter the effect has been shown to be saturated after three to four copies. One proposed theory for this saturation is the depletion of transcription factors required by the promoter (Karhunen et al. 1993; Margolles-Clark et al. 1996). Introduction of a heterologous gene into several different loci using multiple different promoters has been suggested to increase productivity (Miyauchi et al. 2014). However, random integration of the cassettes may directly affect the expression of the target gene and in some cases indirectly production by disrupting the endogenous gene(s) at the integration site. Without monitored integration, it is difficult to conclude whether multiple promoters can truly relieve the titration of transcription factors when solely the *cbh1* promoter is used for expressing the target gene.

One drawback of using the *cbh1* promoter is that efficient protein production from the promoter requires an inducer, the most common being cellulose, lactose or sophorose. In industrial scale protein production the need for an additional inducer can add to the cost of production. Therefore there has been interest in screening for alternative promoters that could be used when the strain is cultivated on glucose without additional inducers. Recently, Li et al. (2012) screened for constitutive promoters by assaying genes that are highly expressed in the presence of glucose. They found two promising gene promoters from the glycolysis pathway, *pdc* (pyruvate decarboxylase) and *eno* (enolase), and successfully expressed the *T. reesei xyn2* gene encoding xylanase II under these promoters. In addition to avoiding the use of an inducer in production, the recombinant strains produced very little background proteins, which is beneficial in some applications. This is a promising result as the constitutive promoters studied earlier, such as *tef1* (translation elongation factor 1 alpha, (Nakari-Setälä and Penttilä 1995; Uzbas et al. 2012)) and *cDNA1* (hypothetical protein Trire2: 110879, (Nakari-Setälä and Penttilä 1995; Uzbas et al. 2012)), were at best over tenfold weaker in comparison. Also the *pki1* (pyruvate kinase) gene promoter (Kurzatkowski et al. 1996) is weaker in comparison and inferior especially at higher glucose concentrations, even though it is from the same glycolysis pathway as *pdc* and *eno*. At this moment there is no information available on the strength of these new promoters compared to the *cbh1* promoter in real enzyme production conditions. It is known, however, that the protein levels expressed from the *cDNA1* promoter in glucose cultivations are clearly lower when compared to those obtained from the *cbh1* promoter in cellulase-inducing conditions (Penttilä 1998). In addition to the above promoters, the *pgk1* (encoding 3-phosphoglycerate kinase) and *hex1* (encoding Hex1 protein in Woronin bodies, a dominant protein in the cell envelope in *T. reesei*) promoters have been characterised (Vanhanen et al. 1991; Curach et al. 2004). However, the *pgk1* promoter seems to be unsuited for high-level protein production (Penttilä 1998) and publications studying the potential of the *hex1* promoter for enzyme production are not yet available.

Another reason why one would choose a promoter other than *cbh1* is that the promoter strength can act as a double-edged sword when expressing heterologous proteins, e.g. from taxonomically remote organisms such as bacteria. In one published example the *Aspergillus nidulans* hydrophobin I (DewA) was produced in *T. reesei* when expressed from the *hfb2* (hydrophobin II) promoter, but not from the *cbh1* promoter even though *dewA* was found to be transcribed in both cases and no secretion stress could be confirmed (Schmoll et al. 2010).

4.7 Use of Carriers to Improve Yields of Heterologous Proteins

Gene fusions have been successfully used as tools to improve heterologous protein production in filamentous fungi (reviewed e.g. in Gouka et al. 1997b). The carriers have been suggested to aid secretion by stabilising the mRNA, facilitating the translocation in and/or into the secretory pathway, by aiding folding and giving protection from degradation. The carrier is generally attached to the amino terminal end of the protein of interest and it most often consists of a module (or modules) of a host protein that is naturally produced in high amounts and has the ability to fold independently. The polypeptides used as carriers in *T. reesei* are the core/linker modules of cellobiohydrolase I (CBHI, Cel7A) (Penttilä 1998) and mannanase I (MANI, Man5A) (Paloheimo et al. 2003) and the cellulose (carbohydrate) binding modules (CBMs) of cellobiohydrolase II (CBHII, Cel6A) and endoglucanase II (EGII, Cel5A) (Paloheimo et al. 2003; Miyauchi et al. 2013). The CBM carriers derive from proteins which are encoded by a "tail-first" orientation (CBM-linker prior to the core part). The original idea behind using CBMs as carriers came from reasoning that using small-sized modules without catalytic activity saves energy for the fungus and doesn't interact with the application. Sequences coding for shorter polypeptides, pro-sequences and *N*-terminal amino acids of the full-length fungal proteins have also been tested as carriers. Using these "non-modular carriers", however, improvements in yields have not been obtained or the improvements have not been as high as with a core or CBM carrier (Penttilä 1998; Paloheimo et al. 2003).

Most often a linker and an engineered cleavage site of Kex2 endopeptidase (e.g. Lys-Arg) is added between the fusion partners to attain efficient cleavage. The hinge (linker) naturally separating the catalytic and substrate-binding domains in the native *T. reesei* proteins has been shown to have a positive effect on production, possibly due to better separation of the two folding sequences and/or more efficient access of the proteases to recognise the cleavage site. The most suitable cleavage site, however, may depend on the structures of both the fusion partners and the fusion protein and not only the sequence of the linker as has been suggested e.g. in Spencer et al. (1998) and Paloheimo et al. (2003). Another interesting option for cleavage is to use the FMDV 2A sequence in *T. reesei* as described in Nguyen et al. (2008).

The carrier approach has been shown to improve yield (with few exceptions) in *T. reesei* of bacterial (Paloheimo et al. 2003, 2007; Löbel et al. 2008) and mammalian

proteins (reviewed in Penttilä (1998)). However, clear improvements in yields of fungal-derived enzymes, xylanase from *Humicola grisea* and laccases from *Melanocarpus albomyces* and *Thielavia arenaria* have not been obtained (de Faria et al. 2002; Kiiskinen et al. 2004; M. Paloheimo, Roal Oy, unpublished results). Thus, the success of the fusion approach seems also to be dependent on the cargo protein.

5 Latest Developments

5.1 Bioinformatics

The wild type *T. reesei* isolate QM6a genome sequence was published in 2008 (Martinez et al. 2008) and knowledge of the strain has grown rapidly over the last few years. The initial automated gene prediction, functional annotation, and manual curation added up to 9129 genes, from which the genomic inventory has since been refined by the addition of 23 completely new genes (Arvas et al. 2010) and an updated CAZyome (Häkkinen et al. 2012). Both the latter studies utilised the widely used and studied mutant Rut-C30. High protein production capabilities of *T. reesei* have fueled research in the past, so it is self-evident that genomes of high-yield mutants are and will continue to be of great interest. The genomes of the first mutant strains from the Rutgers series, NG14 and its descendant Rut-C30, were published soon after the finalisation of the genome of the wild type isolate in 2009 (Le Crom et al. 2009). Le Crom et al. (2009) found in total 223 single nucleotide variants (SNVs) between QM6a and Rut-C30, leading to 44 non-synonymous mutations. Three of these mutations had been described earlier: a truncation of the gene *cre1* responsible for catabolite repression (Ilmen et al. 1996b), a frameshift mutation in the gene *gls2* coding for the glucosidase II alpha subunit involved in protein glycosylation (Geysens et al. 2005), and a 85 kb-deletion that eliminated 29 genes including transporters and transcription factors (Seidl et al. 2008). At about the same time, the Rut-C30 genome was also investigated using array Comparative Genomic Hybridization (aCGH). Although the Rut-C30 genome sequence from 2009 was of high quality at the time, aCGH analysis managed to reveal 16 additional mutations (Vitikainen et al. 2010). During their assessment of the aCGH results, Vitikainen et al. (2010) reproduced the massive 85 kb-deletion detected in Rut-C30 in the wild type strain QM6a and deleted the gene encoding a transcription factor ID72076 which is disrupted by a frameshift mutation in Rut-C30. Neither of the modifications increased the protein production of QM6a. Thus, the full picture of the genetic and physiological alterations behind the improved cellulase production capacity of Rut-C30 is still not yet solved.

Establishment of quick and cost-efficient next-generation sequencing (NGS) technology is rapidly leading to massive expansion of genomic information from *T. reesei* strains. In addition to QM6a and Rut-C30 there were 11 *T. reesei* mutant genomes reported to be complete and/or drafted on the Genomes Online Database (http://www.genomesonline.org) and the Joint Genome Institute web-page

(http://genome.jgi.doe.gov/genome-projects/) by April of 2015 (PC1-4, RL-P37, X-31, MCG77, 25-4, QMY-1, PC-3-7, CBS999.79, QM9136, QM9978, QM9414). None of the 11 genomes have a full web-portal for their use and most were unpublished at the time. Reports from the sequencing of the mutant genomes have just recently started to emerge, like from QM9136 (Lichius et al. 2015) and from a Japanese *T. reesei* mutant PC-3-7 derived from QM9414 (Porciuncula et al. 2013). Technical developments in the field have also improved the quality of the data. For example, re-sequencing of the Rut-C30 genome with higher genome coverage resulted in the identification of 34 previously unreported mutations in protein coding regions (Koike et al. 2013). In the three mutagenesis steps from QM6a to Rut-C30 altogether over 130 proteins might be affected by mutations in their respective gene sequences, from which a total of 90 proteins have a mutation in their amino acid sequences (Kubicek 2013). Many of these proteins are potentially relevant to protein production, such as transcription factors and proteins related to RNA processing and transporters, but very few of these mutations have been investigated thoroughly.

Besides the abovementioned work, in-depth reports on the effects of mutations found from the genome projects are strikingly scarce. Even though the vast amount of data possesses challenges in extracting the relevant information at the moment, it can be presumed that more publications from the field will emerge after a lag phase. It is likely that much of the data will stay proprietary or unpublished until the IP on the most relevant results has been secured.

Next-generation sequencing has revolutionised not only genome research but transcriptome analysis too. Advantages of deep RNA sequencing (RNA-seq) over microarrays, such as low background signal, excellent sensitivity for low and high transcripts, and the ability to screen for novel transcripts, splice variants, and natural antisense transcripts (NATs) have made the method increasingly popular (Wang et al. 2009). RNA-seq has already been applied to *T. reesei* by several groups, who have investigated the induction of genes under complex lignocellulosic substrates and/or simple inducing sugars to refine which CAZy genes are related to which substrate (dos Santos et al. 2014; Chen et al. 2014; Zhang et al. 2013; Ries et al. 2013). However, the focus is also more and more directed towards the regulatory networks behind these responses. Regulatory genes have been screened from both microarray (Häkkinen et al. 2014) and RNA-seq based datasets (dos Santos et al. 2014). Even though the above searches resulted in listing of a few identical transcription factors, the majority of the genes obtained in the studies were different. In general, RNA-seq can be regarded as especially useful in the regulatory network studies due to the ability of the technique to detect low-abundance transcripts. Use of RNA-seq also gave the first proof of NATs existing in *T. reesei*, obtained from a study using straw-induced mycelia as starting material for RNA isolation (Ries et al. 2013). NATs were reported for genes involved in variety of cellular functions. The proportion of antisense reads from all reads corresponded to that of *Aspergillus niger* where NATs had already been observed earlier. Even though the inventory or level of antisense transcripts is not unusual in *T. reesei* this information opens up a new area of research to focus on in the future.

In addition to gene-level methods, exploration of proteomes is currently also used as a tool to better understand differences between strains, strain characteristics, and the physiology of production strains in different types of cultivations and as a basis to design further developments in *T. reesei* production strains (Herpoël-Gimbert et al. 2008). Up to 15–22 % of the total secreted proteins by *T. reesei* have been reported to consist of proteases and peptidases (Adav et al. 2012; Marx et al. 2013). As the pool of proteolytic activities varies greatly depending on the strain and culture conditions and due to the fact that the target proteins have differing sensitivities to different proteases, choice of the most detrimental specific protease activities to be inactivated in production strains can be done only on a case-by-case basis according to results from a proteomics approach (Kari Juntunen, Roal Oy, unpublished results).

5.2 Mating

Sexual development in fungi occurs between compatible mating partners and with the need for specific conditions such as nutrient availability, temperature, humidity, pH, and light (Debuchy et al. 2010). Seidl et al. (2009) were the first to describe the ability of *T. reesei* to perform sexual reproduction. Taxonomically, *T. reesei* (and its teleomorph *H. jecorina*) belong to the group of Ascomycetes (class of Sordariomycetes) and within this group to those fungi which are heterothallic with a bipolar mating type. Heterothallism means that successful sexual reproduction occurs between two compatible partners and self-fertilisation is not possible. The two mating type loci MAT1-1 and MAT1-2 of *T. reesei* are two different sequences ("idiomorphs") which occupy the same genomic region (Metzenberg and Glass 1990). During the process of sexual development *T. reesei* takes over the male or female role independently from its mating type, but one of the mating partners has to act as a male (fertilisation of female reproductive structures) and the other has to act as a female (production of reproductive structures to be fertilised).

Since all strains of *T. reesei* which are nowadays used in industry can be traced back to strain QM6a they all carry—as does the strain QM6a—the MAT1-2 locus. Crossing of different industrial strains with each other to further improve them by introducing favorable traits or to rid strains of mutations of genes or undesired genes such as genes conferring e.g. resistance to antibiotics or coding undesired products whose presence may interfere with regulatory requirements is therefore not possible at present.

A possibility to overcome the inability to cross different *T. reesei* mutant strains would be to exchange the mating type locus for the opposite one at the same genomic locus. In case of *T. reesei* QM6a this implies to exchange the MAT1-2 locus for the MAT1-1 locus.

Seidl et al. (2009) introduced the complementary mating type locus (MAT1-1) ectopically into *T. reesei* strain QM6a thereby generating a strain carrying both mating type loci (MAT1-1 and MAT1-2). This strain was fertile in crossings with wild type

strains of the *T. reesei* teleomorph *H. jecorina* carrying either the MAT1-1 or the MAT1-2 locus. However, in crossings with strain QM6a and its derivatives (all MAT1-2) this strain was found to be sterile. From these results it is concluded that *T. reesei* QM6a is able to act as a male partner but that it cannot produce fruiting bodies and is therefore female-sterile. Probably its maintenance in the labs for over 60 years without selective pressure acting to maintain mating competence has resulted in mutations in one or more of the genes necessary for sexual recombination.

The phenomenon of female sterility is not uncommon in nature since asexual reproduction is less resource consuming than sexual reproduction. Therefore, it could be speculated that the female sterile part of a population could have a certain evolutionary advantage over their sexual counterparts, at least in environments with little nutrients or harsh conditions (Taylor et al. 1999).

If sexual reproduction between industrial strains becomes possible it would have several advantages. By crossing strains from separated strain lineages which have their own unique history of classical mutagenesis the favorable traits of the lineages could be combined in the offspring. Mating would be an alternative for extension and acceleration of classical mutagenesis and screening approaches in this case.

Another option would be the "rejuvenation" of strains which have acquired mutations that lead e.g. to growth defects. Crossing with *T. reesei* wild-type strains from nature with a MAT1-1 mating type is already possible, but the disadvantage of this combination is that the wild-type strains are very much different to the industrial strains which can all be traced back to QM6a. This leads to an unpredictable outcome of these crossings and many problems in established industrial processes, and may also require extensive documentation for the regulatory bodies.

Probably the biggest advantage of successful crossings between industrial strains would be the possibility to combine strains expressing different enzyme activities to construct multi-activity strains. If this has to be done by normal transformation with different expression plasmids it would take much more time. Moreover, since, for example, the availability of selection markers is limited, successful mating would circumvent this problem too (Kubicek et al. 2014).

6 Concluding Remarks

T. reesei is a high-producing and safe industrial host for production of different types of enzymes and proteins. It fulfills the key elements required for cost-effective production of industrial enzymes: high-capacity proprietary mutant lines have been developed, genetic technology is in use and optimised processes are available. These represent the core technology of industrial biotechnological companies and cannot be fully protected by patents or other enforceable means. As a result, that information is often kept as trade secrets and neither the mutant strains nor the processes are published. This leads to the fact that the strains and the technology used in academia differ significantly from those used for commercial exploitation and the innovations with model systems are sometimes obsolete for the established

industrial players. Interestingly, it seems that the fungus needs to have a general makeup fit for high productivity of secreted enzymes as several fungi which have been used extensively as model organisms in academia such as *Aspergillus nidulans* and *Neurospora crassa,* or *Penicillium chrysogenum* for production of antibiotics, have not established themselves as industrial enzyme production hosts.

The current advanced sequencing technologies and bioinformatics tools enable in-depth comparison and absorption of new information from public and proprietary mutant lines. This information is and can be used as basis for choosing novel targets for strain modifications that are relevant for enzyme production to fully exploit the strains' capacity. Also, it gives outstanding possibilities to novel types of developments, e.g. metabolic engineering of *T. reesei* for improved production of enzymes and also other types of products.

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The Renaissance of *Neurospora crassa*: How a Classical Model System is Used for Applied Research

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1 Introduction

Fungi have been exploited for biotechnological applications for centuries and are still considered one of the most versatile groups of organisms for this purpose. Already early on, societies began to utilize fungi (albeit unknowingly) by capitalizing on their metabolic properties and the metabolites they produced. One of the oldest known examples of this sort is the use of yeasts in baking and the production of alcoholic beverages (dating back to about 5000 BC). Nowadays fungi still play an essential role in drug production, as bio-control agents, in food processing, and enzyme biotechnology.

Modern industries continuously strive to improve the economics of their production processes. A major focus is to increase the efficiency of known processes and thus to minimize the production costs while maximizing the amount of the desired product. Traditional ways to optimize microbial production strains were based on random mutagenesis followed by selection of improved organisms. Today, genetic tools and molecular biology techniques can be used to selectively alter cellular metabolism to achieve over-production of certain metabolites or to discover and synthesize new components that could not be previously produced. Thus, new ways of using yeasts and other fungi as cell factories evolved. But besides those techniques,

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which can be exploited to increase titers and yields, it is important to understand the organism itself. The better the insight into an organism, the more accessible it becomes for industrial uses. The community around the filamentous ascomycete species *Neurospora crassa* (*N. crassa*) offers an enormous resource of knowledge, since the fungus has been used in labs around the world for almost 100 years. Studies combining the power of *N. crassa* as a well-known model system for eukaryotic cell biology and genetics with modern "omics" techniques provide fundamental data for a better understanding of the biology of filamentous fungi. Moreover, many findings have already enabled progress of industrial bioprocesses or can inspire solutions for biotechnological applications using filamentous fungi.

1.1 Neurospora Research from Past to Present

Early studies with *N. crassa* and its exceptional biological traits were fundamental and paved the way in a number of important areas in eukaryotic biology. The initial description of *Neurospora* goes back more than 170 years to 1843 when bakeries in Paris reported contamination of uncountable orange spores (Payen 1843), and the causative fungus was called the red bread mold. In the mid-1920s, the mycologist Bernard O. Dodge and his colleague Cornelius L. Shear assigned this fungus to the genus *Neurospora* (Shear and Dodge 1927). The two scientists described sexual reproductive structures (perithecia) of different *Neurospora* species and they gave a clear account of the existence of mating types. Furthermore, Dodge started the first crossing experiments along with the first tetrad analysis, worked out the basic genetics of the fungus and therefore introduced *N. crassa* to the fields of genetic and cytological studies (Davis and Perkins 2002). In the following age of biochemistry, genetics and molecular biology, *N. crassa* advanced to become an excellent model organism to study numerous aspects of biology. In 1941, Beadle and Tatum obtained the first biochemical mutants and proposed what later became known as the "one-gene-one-enzyme" hypothesis (Beadle and Tatum 1941). It was the first time that fungal mutants of a certain kind could be selectively isolated and subjected to complementation analysis. This work on *N. crassa*, and particularly the approaches to study those mutants, aided the development of biochemical microbiology and genetic recombination also in bacteria (Horowitz 1985; Tatum and Lederberg 1947), and in 1958 Beadle, Tatum and Lederberg won the Nobel Prize for their pioneering research in microbial genetics. Thereafter *N. crassa* quickly became the subject of intensive investigations, contributing to various aspects of basic cellular as well as genetic mechanisms, such as studies on meiosis, crossing over, and the first proof of gene conversion (Mitchell 1955). In the following decades, *Neurospora* research provided essential findings in mitochondrial genetics (Griffiths et al. 1995), photobiology and circadian rhythms (Dunlap and Loros 2006; Linden et al. 1997), polar growth (Riquelme et al. 2011), cell fusion (Fleissner et al. 2008), genome defense systems like RIP (Selker 1990), DNA methylation (Freitag and Selker 2005) as well

as cell development and differentiation (Davis 2000). The major basis for the success of this model system is the simplicity of its nutritional requirements and the ease of its manipulation in the laboratory that has been developed by the *N. crassa* community over the last decades.

Another important feature of the saprophyte *N. crassa* is its well-investigated life cycle that includes both asexual and sexual cycles. *Neurospora* vegetative hyphae are syncytia built of incompletely septated cells, allowing the movement of cytoplasm and organelles between compartments. The hyphae are multinucleate, tip-growing structures that undergo regular branching in order to develop an extensive mycelium (Gow 1995; Turner et al. 1999). In response to various environmental cues, the vegetative hyphae form specialized aerial structures (the conidiophores) which then produce long chains of asexual macroconidia, multinucleate orange spores that are important for the dispersal of the fungus (Springer and Yanofsky 1989). *Neurospora* may also undergo another asexual cycle known as microconidiation. The microconida differentiate from vegetative hyphae or microconidiophores and are uninucleate (Rossier et al. 1977). Like many other filamentous fungi, *N. crassa* is a heterothallic fungus with two opposite mating types, encoded by loci called *mat A* and *mat a* (Metzenberg and Glass 1990). Upon nitrogen limiting conditions, *Neurospora* enters its sexual phase and induces the formation of fruiting bodies (protoperithecia). A specialized hypha (trychogyne) emerges from the protoperithecium and can only be fertilized by a cell of the opposite mating type (Nelson 1996). Cell fusion and fertilization results in the development of mature fruiting bodies (perithecia) within which the sexual ascospores are formed (Raju 1992). Both sexual and asexual developments are highly regulated and influenced by environmental conditions. Through most of its life cycle, *N. crassa* is haploid and at least 28 morphologically different cell types are described in the literature (Bistis et al. 2003).

The life cycle provides several favorable properties for studying genetics and sexual biology: First, the haploid vegetative tissue can be forced to undergo heterokaryon formation, which is useful for complementation assays. Second, the two distinct mating types enable genetic crosses, and finally, the large asci are excellent for studying meiotic processes. In addition to those traits, an efficient transformation system was developed for *N. crassa* already in the 1970s (Mishra and Tatum 1973; Case et al. 1979), and the process soon became a well-established protocol to effectively transfer genetic material and subsequently analyze gene functions (Davis 2000). By now a variety of selectable markers and promotors are available and are routinely used to screen and control expression of transgenes.

A major milestone in the history of *Neurospora* was the founding of the Fungal Genetic Stock Center (FGSC) in 1960 to ensure that strains from earlier studies remained available to following generations of researchers (McCluskey 2003). David Perkins started systematic sampling of natural isolates of *Neurospora* species from all over the world (Perkins and Turner 1988; Perkins et al. 1976). These efforts contributed to a large strain collection that is provided by the FGSC and enables the community to study distribution, ecology, systematics and genetics of natural

populations of *Neurospora* and to gain valuable information on evolutionary biology. The collection furthermore provides genetically distinct variants for investigations regarding questions such as the stability of genetic polymorphism or useful allelic protein differences (Perkins et al. 1976; Turner et al. 2001). Surely, the availability of those fungal stocks contributed to the growth of the large cooperative and interactive *Neurospora* community. The strong communal support was an essential driver for the *N. crassa* genome sequencing project, which led to the *Neurospora* genome being the first to be sequenced of all filamentous fungi (Galagan et al. 2003). Its >40 Mb haploid genome is comprised of approximately 10,000 protein-coding genes and comparative analyses revealed that *N. crassa* possesses numerous genes without homologues in *Saccharomyces cerevisiae (S. cerevisiae),* indicating that *Neurospora* could be more suitable as a model for higher eukaryotes in various aspects of cell biology (Borkovich et al. 2004). Further, whole genome sequencing of *N. crassa* wild isolate collections (present at the FGSC) also enabled experimental analyses of naturally occurring genetic variations to identify population and ecotype specific traits by genome-wide association studies (GWAS) (McCluskey 2012; Palma-Guerrero et al. 2013). An additional benefit is that *N. crassa* seems to have very little redundancy within its genome, unlike most other eukaryotes (Borkovich et al. 2004; Galagan and Selker 2004). This way, deleterious mutations of single genes often show strong phenotypic effects, thus enabling researchers to quickly correlate specific genes to their particular function. The availability of the complete genome sequence of *N. crassa* was essential for another community-driven project in which all annotated open reading frames were systematically disrupted through targeted gene replacements (Dunlap et al. 2007). Thus, a nearly full genome deletion strain set for *N. crassa* exists, carrying single gene deletions that enable the functional characterization of individual genes (Collopy et al. 2010). All of the strains are easily accessible through the FGSC, where nearly 21,000 *Neurospora* strains are currently maintained (McCluskey et al. 2010; Roche et al. 2014a).

Due to the undeniable contributions in the past and the available tools for genetics, biochemistry and molecular biology, *N. crassa* still has a great potential for further discoveries. Regarding industrial issues, *Neurospora* is undoubtedly of particular importance, since many other filamentous ascomycetes, such as *Trichoderma reesei* (*T. reesei*) or *Aspergillus niger* (*A. niger*), are prominent fungal workhorses in many biotechnological applications (Cullen 2007; Schuster and Schmoll 2010). Additionally, in particular *Neurospora* and *T. reesei* are phylogenetically related, as both belong to the class of Sordariomycetes (Fig. 1) (Fitzpatrick et al. 2006). Unravelling regulatory networks or signaling cascades controlling gene expression in a model organism like *N. crassa* provides knowledge that can potentially be applied to currently used industrial fungi and development of new species with desired industrial traits.

One major focus in fungal biotechnological research is the elucidation of plant biomass degradation. Filamentous fungi have the capacity to produce large amounts of enzymes for lignocellulose deconstruction that release fermentable sugars from plant biomass. In nature, *N. crassa* colonizes burned vegetation and trees and is

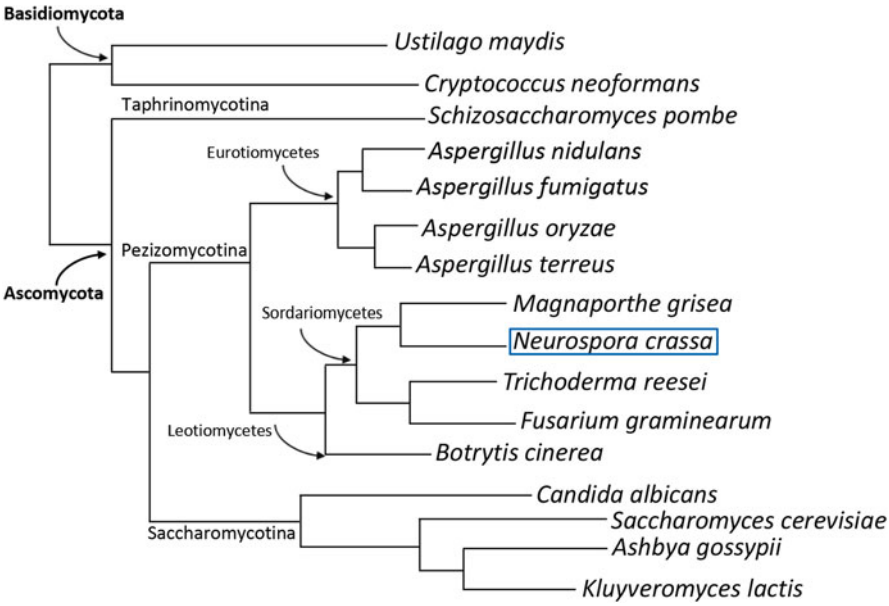

Fig. 1 Phylogenetic tree showing the relation of *N. crassa* to a selection of industrially and scientifically important fungal species based on whole genome data sets (Modified and redrawn based on Fitzpatrick et al. 2006)

found on residues of sugar cane processing or on carbohydrate-rich foods (Davis and Perkins 2002). Recent studies indicate additionally that *Neurospora* also possesses an endophytic phase associated with Scots pine and is even able to switch to a pathogenic lifestyle (Kuo et al. 2014). Crucial for a life on plant biomass, *Neurospora* has the ability to secrete all main enzymes involved in lignocellulose biomass degradation (Dogaris et al. 2013). With these versatile features and the availability of numerous molecular, genetic, and biochemical tools, *N. crassa* can help to expedite analyses of fungal plant biomass deconstruction. In this chapter, we try to highlight not only the capacity of *N. crassa* as a biomass degrader and how recent development in genomic, proteomic and transcriptomic analyses promote the understanding of the underlying processes, but also to introduce the biotechnological potential of *N. crassa* itself.

1.2 Plant Biomass Composition and Decomposition

Lignocellulosic biomass is the most abundant biopolymer on earth and it is assumed that it comprises about 50 % of the world biomass with available reserves of approximately 10–50 billion tons per year (Demain 2009; Sánchez and Cardona 2008). It

consists primarily of plant cell walls and is of high economic value for food, fiber, and the paper industries. More recently, it is gaining increased attention as feedstock for the production of second-generation, carbon-neutral biofuels (Pauly and Keegstra 2008; Somerville et al. 2010; Youngs and Somerville 2012). Plant cell walls are built up of four major polymeric components: the polyphenolic substance lignin and three major polysaccharides: cellulose, hemicellulose, and pectin. Along with numerous enzymes, proteoglycans and structural proteins, these building blocks form a complex network that give the plant cell wall its rigidity and fulfils multiple essential functions for the plant (Somerville et al. 2004; Popper et al. 2011). Most fungal species utilize plant biomass as their carbon source. They digest the plant cell wall contents extracellularly by secreting a large amount and diverse set of enzymes that release small degradation products, which are then absorbed (Conesa et al. 2001).

The most abundant component in plant cell walls is cellulose, a highly ordered linear polysaccharide composed of long unbranched chains of β-1,4-linked D-glucose units. These chains are bundled via hydrogen bonds to form microfibrils and give the cell wall its tensile strength (Frey-Wyssling 1954). Traditionally, the biodegradation of cellulose was thought to be catalyzed by three enzyme classes: endoglucanases, exoglucanases (or cellobiohydrolases), and β-1,4-glucosidases. Only recently, another enzyme class—that had long been considered enigmatic—was added to this group, comprising the lytic polysaccharide monooxygenases (LPMOs) (Beeson et al. 2012; Phillips et al. 2011; Harris et al. 2010; van den Brink and de Vries 2011; Agger et al. 2014). The LPMOs have received much attention in the last years due to their huge potential for cellulose degradation enhancement and have spurred many research projects aimed at finding and characterizing new family members in different microorganisms (Phillips et al. 2011; Vaaje-Kolstad et al. 2010; Quinlan et al. 2011; Westereng et al. 2011; Bey et al. 2013; Morgenstern et al. 2014). Research using *Neurospora* has been tremendously helpful to elucidate the catalytic mechanism of the lignocellulolytic LPMOs (Phillips et al. 2011; Agger et al. 2014; Isaksen et al. 2014; Eibinger et al. 2014; Li et al. 2012; Kittl et al. 2012). The four mentioned enzyme groups work synergistically towards cellulose deconstruction and are classified based on their amino acid sequence, structure and their mode of action (Table 1) (Carbohydrate Active Enzyme database; http://www.cazy.org/; Lombard et al. 2014). The lignocellulolytic LPMOs seem to be conserved in all cell wall degrading fungi and were re-grouped into CAZy family AA9 from their former classification as GH61s based on their oxidative cleavage mechanism (Levasseur et al. 2013).

The more complex polysaccharide classes in plant cell walls are hemicelluloses and pectins. Hemicelluloses include xylans, xyloglucans, glucomannans, galactomannans, arabinoxylans and glucuronoxylans and form extensive cross-links between cellulose fibers to provide extra stability to the plant cell (Somerville et al. 2004). Pectins are the most structurally and functionally complex polysaccharides in plant cell walls and together with hemicelluloses build up the matrix in which the cellulose microfibrils of primary cell walls are embedded (Mohnen 2008; Harholt

Table 1 Enzyme families involved in degradation of the three major plant cell wall polysaccharides (cellulose, hemicellulose and pectin) in fungi (Adapted from Coutinho et al. (2009))

Substrate	Code	Enzyme activity	CAZy family
Cellulose	EGL	β-1,4-Endoglucanase	GH5, GH7, GH12, GH45
	CBH	Cellobiohydrolase/exoglucanase	GH6, GH7
	BGL	β-1,4-Glucosidase[a]	GH1, GH3
	LPMO	Lytic polysaccharide monooxygenase[a]	AA9, formerly GH61
Xylan	XLN	β-1,4-Endoxylanase	GH10, GH11
	BXL	β-1,4-Xylosidase[a]	GH3, GH43
	ABF	α-Arabinofuranosidase[a]	GH43, GH51, GH54
	AXH	Arabinoxylan arabinofuranohydrolase	GH62
	AGU	α-Glucuronidase	GH67, GH115
	AGL	α-1,4-Galactosidase[a]	GH27, GH36
	LAC	β-1,4-Galactosidase[a]	GH2, GH35
	AXE	Acetyl xylan esterase	CE1, CE3
	FAE	Feruloyl esterase[a]	CE1
Xyloglucan	XEG	Xyloglucan-active β-1,4-endoglucanase	GH12, GH74
	ABF	α-Arabinofuranosidase[a]	GH43, GH51, GH54
	AXL	α-Xylosidase	GH31
	AFC	α-Fucosidase	GH29, GH95
	AGL	α-1,4-Galactosidase[a]	GH27, GH36
	LAC	β-1,4-Galactosidase[a]	GH2, GH35
	XGAE	Xyloglucan acetylesterase	
	LPMO	Lytic polysaccharide monooxygenase[a]	AA9, formerly GH61
Galactomannan	MAN	β-1,4-Endomannanase	GH5, GH26
	MND	β-1,4-Mannosidase	GH2
	LAC	β-1,4-Galactosidase[a]	GH2, GH35
	AGL	α-1,4-Galactosidase[a]	GH27, GH36
	BGL	β-1,4-Glucosidase[a]	GH1, GH3
	CMAE	Galactomannan acetyl esterase	

(continued)

Table 1 (continued)

Substrate	Code	Enzyme activity	CAZy family
Pectin	ABF	α-Arabinofuranosidase[a]	GH43, GH51, GH54
	ABN	Endoarabinanase	GH43
	ABX	Exoarabinanase	GH93
	BXL	β-1,4-Xylosidase[a]	GH3, GH43
	FAE	Feruloyl esterase[a]	CE1
	GAL	β-1,4-Endogalactanase	GH53
	GLN	β-1,6-Endogalactanase	GH5
	LAC	β-1,4-Galactosidase[a]	GH2, GH35
	PAE	Pectin acetyl esterase	CE12, CE13
	PEL	Pectin lyase	PL1
	RGL	Rhamnogalacturonan lyase	PL4, PL11
	PGA	Endopolygalacturonase	GH28
	PGX	Exopolygalacturonase	GH28
	PLY	Pectate lyase	PL1, PL3, PL9
	PME	Pectin methyl esterase	CE8
	RGAE	Rhamnogalacturonan acetyl esterase	CE12
	RGX	Exorhamnogalacturonase	GH28
	RHA	α-rhamnosidase	GH78
	RHG	Rhamnogalacturonan hydrolase	GH28
	UGH	D-4,5-Unsaturated β-glucuronyl hydrolase	GH88
	URH	Unsaturated rhamnogalacturonan hydrolase	GH105
	XFG	β-1,4-Exogalactanase	–
	XSG	β-1,6-Exogalactanase	–
	XTG	β-1,3-Exogalactanase	GH43

[a]Enzymes that are involved in the degradation of different types of substrates

et al. 2010; Bonnin et al. 2014). Four major structural classes are commonly differentiated: homogalacturonan (HG), rhamnogalacturonan I (RG-I), xylogalacturonan (XG) and rhamnogalacturonan II (RG-II). As a result of the extremely complex structure of hemicelluloses and pectic polysaccharides, fungi need to secrete a large set of enzymes with a variety of activities (Table 1) to efficiently degrade these plant cell wall components (for detailed reviews see: van den Brink and de Vries 2011; Bonnin et al. 2014; Benoit et al. 2012; Glass et al. 2013; Kubicek 2012).

The biological degradation of plant biomass by the action of microbial degraders such as saprobic filamentous fungi is a pivotal process and of fundamental importance for global carbon turnover. Due to the recalcitrance of the cell wall polysaccharides described above, only a small fraction of microorganisms are capable of efficiently degrading lignocellulosic biomass. Basidiomycete and ascomycete fungi play a dominant role in this respect and for this reason have become the main source of commercially used hydrolytic enzymes (Kubicek et al. 2009). However, besides increasing yield, industry is constantly thriving to optimize the composition of their enzyme cocktails to better tailor these for the various feedstocks at hand, which can differ widely depending on the regional circumstances surrounding a biofuel plant. For both of these reasons a better understanding of the molecular processes and signaling cascades that fungi use to regulate their enzymatic secretome is of great interest. The knowledge in this regard is still incomplete, but significant advances have been made in recent years, with important contributions from research on *N. crassa*.

2 Systems Analysis: Combining the Genetic Power of *Neurospora* with Modern Sequencing Technologies

In the last decades, traditional qualitative studies of individual gene function were succeeded by quantitative analyses of complex cellular processes at the systems level. This shift was achieved by advances in high-throughput technologies and allows today's scientists to monitor the abundances of various biological molecules and measure their variation between biological states (Nie et al. 2007). By using these system analyzing "omics" methods—namely transcriptomics, proteomics, and metabolomics—in *Neurospora*, it has been possible to analyze the fundamental processes occurring during plant cell wall degradation. *N. crassa* particularly lends itself to systems analyses, due to its fully sequenced and almost completely annotated genome (Davis and Perkins 2002; Davis 2000; Borkovich et al. 2004; Dunlap et al. 2007).

A transcriptome constitutes the total amount of mRNA in a cell at any given moment and the corresponding protein complement is thus called proteome (Hegde et al. 2003). It is worth noting that the correlation between mRNA and protein levels is often poor (for further information, see: Anderson and Seilhamer 1997; Gygi et al. 1999). Much of this discrepancy can be attributed to regulatory processes in

the cell, e.g. mRNA degradation, alternative RNA splicing, processing and modification of proteins and varying protein turnover rates (Hegde et al. 2003; Gygi et al. 1999). A combined transcriptome and proteome profiling is helpful, because solitary approaches will never fully unravel complex processes in cell biology and biochemistry. Additionally, good correlations can confirm the discovery of an induced response to a treatment, while the lack of a strong correlation can help to detect flaws in the experimental processes or may show a biological uncoupling between the corresponding levels of the respective mRNA and protein species (for further information, see: Nie et al. 2007; Hegde et al. 2003; Cox et al. 2005). Despite the aforementioned issues, "omics" approaches are becoming increasingly popular in the research field of fungal plant cell wall degradation. Through the generation of large data sets in different filamentous fungi, like *T. reesei* or *A. niger*, and comparison of the obtained results with data from *N. crassa,* it is possible to detect similarities or differences in gene expression and protein secretion.

Plant cell walls are mainly composed of cellulose microfibrils embedded in a matrix of pectin, hemicellulose, lignin, and structural proteins (see above, Part 1.2). To elucidate how *Neurospora* recognizes and reacts to different cell wall components systems analysis has been employed, unveiling the complex molecular events leading to their degradation. Early studies by Tian *et al*. were directed towards an elucidation of the cellulolytic machinery of *N. crassa* (Tian et al. 2009). Gene expression levels were recorded when *Neurospora* was grown on *Miscanthus* × *giganteus* (*Miscanthus*) or microcrystalline cellulose (Avicel). While the overall upregulated gene pools were similar in size (231 vs. 187 upregulated genes, respectively) about half (114 genes) were found to be common on both substrates. This result is not unexpected, since *Miscanthus* is a complex substrate comprised of cellulose, hemicellulose and pectin, while Avicel consists of purified crystalline cellulose (with little hemicellulose impurities). Therefore, the difference in gene expression could be accounted to the additional activation of hemicellulases and pectinases on *Miscanthus*. These findings were further supported by a shotgun proteomics approach (Tian et al. 2009).

Though cellulose is the most abundant component of plant cell walls, hemicelluloses and pectin are vital parts of the cell wall structure, too. If filamentous fungi like *N. crassa* want to penetrate the protective shell around the plant cell, they have to dissolve all parts of the cell wall. Therefore, *Neurospora* should have the ability to recognize and discern between the different cell wall components as well as temporally and spatially regulate the corresponding enzyme production. Through the application of transcriptome analysis, Sun *et al*. were able to elucidate the gene expression of *N. crassa* when grown on hemicellulose (Sun et al. 2012; Benz et al. 2014a). In those studies, beechwood xylan and its major hydrolytic degradation product xylose were used as hemicellulosic substrates. Transcriptome analyses showed that 353 genes were significantly induced by beechwood xylan. A portion of the upregulated genes were xylose metabolism pathway genes, consistent with the fact that the main product of xylan degradation is D-xylose or xylose derivatives (Sun et al. 2012; Chiang and Knight 1960; Hasper et al. 2000). More importantly

however, exposure of *N. crassa* to xylan did not significantly induce predicted cellulase genes. This result is in contrast to that in *A. niger and T. reesei,* where D-xylose was shown to be an inducer for hemicellulase and cellulase genes (Gielkens et al. 1999; Mach-Aigner et al. 2010). In *N. crassa*, only 30 genes showed significantly increased expression levels under 2 % xylose conditions, half of which were also induced by xylan (Sun et al. 2012; Gielkens et al. 1999; Marui et al. 2002). Therefore, xylose does not seem to act as a potent inducer of hemicellulose degradation at least at the concentrations applied. Nonetheless, the observations of Sun and colleagues demonstrated that *N. crassa* reacts substantially different to hemicellulose than to cellulose (Tian et al. 2009; Sun et al. 2012).

Recent studies by Li *et al*. employed next-generation sequencing technology to examine the transcriptome of *N. crassa* grown on L-arabinose and compared it to the respective response to D-xylose (Li et al. 2014a). While many filamentous fungi can utilize both pentoses as a sole carbon source, their catabolic pathways significantly overlap, and D-xylose is preferred over L-arabinose when present in combination (Li et al. 2014a; Seiboth and Metz 2011). Whereas D-xylose usage starts already at low D-glucose levels, the uptake of L-arabinose is prevented until D-xylose and/or D-glucose are completely depleted (Li et al. 2014a). Therefore, L-arabinose seems to be the least favored monosaccharide. This observation was supported by transcriptomic data, which showed that gene expression profiles on L-arabinose were dramatically different from those on D-xylose, partly due to a parallel induction of the carbon starvation response. However, using these data, Li and colleagues identified three novel sugar transporters as well as a transcription factor associated with the regulation of hemicellulases (see below). These factors are possible targets for improving hemicellulose degradation in filamentous fungi and for pentose utilization engineering in sugar fermenting yeasts, since for example the three major released monosaccharides D-glucose, D-xylose and L-arabinose cannot be completely utilized by *S. cerevisiae* (Znameroski and Glass 2013).

While cellulose and hemicellulose degradation is relatively well understood and used on an industrial scale, pectin is another source of inexpensive and efficiently produced monosaccharides (Olsson and Hahn-Hägerdal 1996; Sun and Cheng 2002; Mabee et al. 2011; Benz et al. 2014b). Although not prominent in mature (secondary) plant cell walls, pectin is abundant in the primary cell walls of soft and growing tissues and the main polysaccharide of the middle lamella, which acts as the "glue" between neighboring plant cells. Pectin is probably the most structural and functional complex hetero-polysaccharide in the plant cell wall and thus fungi need a sophisticated biochemical apparatus for its degradation (Mohnen 2008; Harholt et al. 2010; Willats et al. 2001; Vincken et al. 2003; Caffall and Mohnen 2009). Due to its abundance in agricultural wastes, pectin has high potential as a biofuel feedstock (Doran et al. 2000; Canteri-Schemin et al. 2005; Rivas et al. 2008). Benz *et al*. analyzed the pectin degradation "toolbox" and described the *N. crassa* secretome and gene expression profile when exposed to pectin (Benz et al. 2014a). By taking the complexity of pectin modifications into account, it was not

unexpected to see that growth on pectin led to the most complex secreted protein complement of all three major plant cell wall polysaccharides (a total of 80 proteins were identified) (Benz et al. 2014a). Additionally, by comparing pectin, xylan and cellulose secretomes, significant differences were observed, with 55 proteins being uniquely identified on pectin (Tian et al. 2009; Sun et al. 2012; Benz et al. 2014a). Transcriptional profiling revealed that all predicted pectinase genes, along with several genes encoding enzymes with the potential to degrade pectin side-chains, were strongly upregulated. However, while the pectinolytic machinery was largely independent of the cellulose 'regulon' and also different to the hemicellulosic counterpart, secretome and transcriptome data showed a significant overlap between the hemicellulosic response and the cellulolytic or pectinolytic ones, respectively. Taken together, Benz and colleagues demonstrated that *Neurospora* possesses (and utilizes) an almost complete set of specifically regulated genes and secreted proteins for the degradation of pectin (Benz et al. 2014a). This result was somewhat surprising, since the natural substrates of *N. crassa* are grasses and trees, which are poor in pectin. Moreover, galacturonic acid (D-GalA), as the major backbone sugar of pectin, is not a favorite carbon source of *N. crassa* as can be seen in comparative growth assays (Fungal Growth Database: http://www.fung-growth.org). However, pectin degradation might still be beneficial, since the weakening of middle lamellae will help to macerate plant tissue structures, and the fact that *N. crassa* continues to sustain a gene complement for D-GalA metabolism could indicate that a selective pressure exists to deal with the potentially toxic metabolites (Huisjes et al. 2012a).

In summary, systems analyses revealed that through the discrete regulation of the polysaccharide regulons, *Neurospora* is capable to degrade all major parts of the plant cell wall. While the cellulolytic and pectinolytic regulons are more specific, the hemicellulases appear to be activated more constitutively, probably reflecting the fact that hemicelluloses are distributed more evenly throughout the plant cell wall layers. Moreover, it was shown that many hydrolases were expressed differently over time, which leads to the conclusion that the complete hydrolysis of plant biomass most probably requires a cascade-like action of different enzymes (Borkovich et al. 2004; Benz et al. 2014a).

The studies presented in this chapter provide a solid foundation for the rational engineering of filamentous fungi to improve their ability for the deconstruction of plant cell walls. *N. crassa* contributes to unravel the function of plant cell wall degrading enzyme systems and therefore possibly expedites the creation of an optimal enzyme composition for the industrial liquefaction of lignocellulosic biomass (Tian et al. 2009). Furthermore, through the investigation of induction mechanisms, the production of lignocellulolytic enzymes from other cellulolytic filamentous fungi can potentially be simplified (Znameroski et al. 2012) (see below). However, our knowledge of cell wall degrading bioprocesses is still incomplete, and additional work determining the underlying transcriptional networks under various conditions, will be necessary to achieve further advances in the generation of biofuels.

3 The Transcriptional Network of Polysaccharide Degradation in *Neurospora*

Complex organic matter like the plant cell wall is difficult to deconstruct. Fungi commit an enormous amount of resources to degrade plant biomass, and in order to do this efficiently, they have developed systems to identify and differentiate the different polysaccharides of the cell wall, which involves the up- or downregulation of a large number of genes. For that reason, if an easier to metabolize substrate like glucose is available, this will be utilized preferentially and all genes involved in polysaccharide degradation are actively repressed (Aro et al. 2005; Gancedo 1998; Ronne 1995; Ruijter and Visser 1997). The process regulating this choice is accordingly called carbon catabolite repression (CCR), and it strongly affects the production of hydrolytic enzymes in all filamentous fungi (Aro et al. 2005; Ruijter and Visser 1997; Ebbole 1998; Flipphi and Felenbok 2004; Strauss et al. 1995; Sun and Glass 2011; Xiong et al. 2014a).

In case of starvation, genes encoding sugar transporters and hydrolytic enzymes, but also factors involved in their production and secretion will be de-repressed, leading to relaxed expression at a low level (de-repressed phase) (Tian et al. 2009; Kelly 2004; Nakari-Setälä et al. 2009; Coradetti et al. 2012; Delmas et al. 2012). The small amount of enzymes secreted into the environment enable the fungus to 'scout' for available carbon sources in the surrounding area. Degradation of suitable polysaccharides will release inducer molecules (mono- or oligosaccharides) that are needed for the induction of an appropriate set of genes encoding for substrate specific hydrolytic enzymes. By perceiving these inducing molecules, fungi can discern between different cell wall polysaccharides and elicit a specifically tailored enzymatic response for the efficient utilization of the cell wall components at hand (inductive phase) (Znameroski et al. 2012; Mandels and Reese 1960; Vaheri et al. 1979; Chikamatsu et al. 1999).

Even though CCR has been investigated for several years now, our knowledge of this important regulatory mechanism is still insufficient. To further improve industrial output of lignocellulosic enzymes and achieve a full degradation of cell wall polysaccharides, mechanisms like CCR and other involved transcriptional regulators have to be examined thoroughly. Thus, recent research has focused on investigating the degradative response of filamentous fungi towards plant cell wall components (Benz et al. 2014a; Kubicek 2013; Ries et al. 2013; de Souza et al. 2011) and regulatory mechanisms behind those responses (Xiong et al. 2014a; de Souza et al. 2013; Coradetti et al. 2013).

It is known that cellobiose, the major soluble end product of cellulases, induces cellulase gene expression and activity in *T. reesei* and *Aspergillus* species (Mandels and Reese 1960; Vaheri et al. 1979; Chikamatsu et al. 1999). However, for a long time, the nature of the cellulolytic inducer was enigmatic in *Neurospora*. At least in the *N. crassa* WT, incubation with cellobiose did not lead to induction of cellulases (Znameroski et al. 2012). By crossing deletion strains for three key genes identified

via transcriptomics, two predicted extracellular and one intracellular β-glucosidases were disabled in *N. crassa* (Δ*gh1-1*, Δ*gh3-3*, and Δ*gh3-4*; forming the Δ*3βG* strain). These β-glucosidases hydrolyze cellobiose into two glucose molecules and could therefore delay the activation of the cellulose degradation due to activation of CCR. This was confirmed by experiments with the Δ*3βG* strain, which showed similar relative expression levels of cellulase genes when shifted to cellobiose as did a WT culture shifted to Avicel. According to these results, cellodextrins like cellobiose (or a related metabolite) are indeed the inducer for cellulase gene expression in *N. crassa* (Znameroski et al. 2012).

Large groups of genes, as is the case in CCR, are mainly regulated by transcription factors, which act as switches enhancing or decreasing gene expression in a developmental or environmental context (Aro et al. 2005). *N. crassa* encodes about 200 predicted transcription factors (Galagan et al. 2003; Borkovich et al. 2004; Shiu et al. 2005). Of these, 178 contain a clear DNA-binding motif, which can be allocated into six families: the basic helix-loop-helix (bHLH), bZIP, C2H2 zinc finger, GATA factor, Myb and Zn binuclear cluster families. The other 22 transcription factors may contain a non-typical DNA-binding motif (Tian et al. 2011). The past has shown that fungi have developed similar, but usually non-identical, ways to regulate their plant cell wall degradation machinery (Glass et al. 2013; Amore et al. 2013), probably as an adaptation to different ecological niches. Nevertheless, by comparing the regulated genes of orthologous transcription factors in different ascomycete species, it is possible to elucidate the differences and similarities in regulatory networks, which will facilitate the research on industrially used fungi.

CCR is mediated by Mig1/CreA/CRE1, a conserved zinc-finger transcription factor that functions primarily as a repressor of lignocellulolytic genes (Ebbole 1998; Sun and Glass 2011; Dowzer and Kelly 1991; Ilmén et al. 1996; Nehlin and Ronne 1990; Ruijter et al. 1997). Whereas Mig1 functions to repress transcription of 90 genes associated with utilization of alternative carbon sources in *S. cerevisiae* under conditions of glucose sufficiency, CreA/CRE1 directly regulates genes involved in xylose, xylan, arabinose, proline and ethanol utilization in *Aspergilli* and *T. reesei* (Kubicek et al. 2009; Ruijter et al. 1997; de Vries and Visser 2001; Santangelo 2006). CRE1/CreA and its homolog in *N. crassa*, CRE-1, additionally regulate cellulose utilization (Znameroski et al. 2012; Sun and Glass 2011; de Vries and Visser 2001; Mach et al. 1996). In the case of *Neurospora*, Sun and Glass showed that a deletion of *cre-1* caused sustained expression of cellulase genes, resulting in higher cellulolytic enzyme activity during growth on Avicel. CRE-1 binds directly to motifs in promoter regions of its regulated genes and therefore probably competes for binding with positive regulatory factors (Sun and Glass 2011). For example, CRE-1 binds to the promoter region of *cbh-1* in *N. crassa* and may compete for binding with cellulolytic regulators required for induction. CRE-1 was also found to regulate the expression level of some, but not all, hemicellulase genes in *N. crassa* under Avicel conditions (Sun and Glass 2011) and is furthermore involved in regulating glycogen metabolism under carbon repressing and non-repressing conditions (Cupertino et al. 2015). Altogether, these data provide strong

evidence that CRE-1 functions as a global transcription factor in *Neurospora* and affects large-scale gene expression directly as well as indirectly (Sun and Glass 2011). Moreover, *cbh-1* and *xylanase A* have also been identified as CRE-1 targets in industrial fungi like *T. reesei* and *A. nidulans* (Mach et al. 1996; Takashima et al. 1996; Orejas et al. 1999). Therefore, CRE-1 and its homologs seem to play a general role in the regulation of the production of many plant cell wall degrading enzymes.

While CRE-1 is the main known repressor in the cellulolytic response of *Neurospora*, gene induction upon perception of inducers is conveyed by specialized activators. Through a screen of the *N. crassa* transcription factor deletion collection, Coradetti *et al.* identified two formerly uncharacterized zinc binuclear cluster transcription factors that were essential for growth and enzymatic activity on cellulose, but were dispensable for growth on xylan (Coradetti et al. 2012). Based on these features, the transcription factors were named cellulose degradation regulator 1 and 2 (*clr-1* and *clr-2*, respectively). Through transcriptomics, Coradetti and colleagues identified a 212-gene set that specifically responded to crystalline cellulose, the so-called Avicel regulon. Of these 212 genes 135 were regulated by CLR-1/CLR-2, including cellulase and hemicellulase genes, genes encoding cellodextrin transporters and genes likely to be involved in degrading more complex sources of lignocellulose. Additional experiments demonstrated that the Δ*clr-1* mutant exhibited an impaired growth on cellobiose, the major hydrolytic product of cellulose degradation and key factor for cellulose sensing in *N. crassa* (Znameroski et al. 2012; Coradetti et al. 2012). Intriguingly, expression of *clr-2* was abolished in the Δ*clr-1* mutant, while the Δ*clr-2* mutant showed no growth deficiency on cellobiose and the expression levels of *clr-1* were unaffected in this mutant. Transcriptional profiling revealed that *clr-1* showed a stronger expression in the de-repressed phase, whereas *clr-2* was significantly higher expressed in the inductive phase. Taken together, the following model view of cellulase induction emerged: CLR-1 is somehow activated in the presence of cellodextrins (cellulose degradation products) and subsequently induces its target genes. These include several genes necessary for efficient import and utilization of cellobiose as well as *clr-2*. CLR-2 then acts as the main inducer of cellulase and hemicellulase gene expression. Homologs for CLR-1/CLR-2 can be found in the genomes of most filamentous ascomycete species. For example, the *A. nidulans* Δ*clrB* (*clr-2* homolog) deletion strain was deficient for cellulase activity as well. However, the Δ*clrA* (*clr-1* homolog) strain was unaffected in cellulase activity, but exhibited a growth deficiency on cellobiose (Coradetti et al. 2013). These data exemplify the diversity found in the signaling pathways that regulate plant cell wall degradation in fungi (Coradetti et al. 2012, 2013).

Two further transcription factors, vegetative incompatibility blocked 1 (VIB-1) and colonial 26 (COL-26), are important for plant cell wall deconstruction, glucose sensing/metabolism, and regulation of CCR in *N. crassa*, respectively (Xiong et al. 2014a; Colot et al. 2006). VIB-1 is required for extracellular protease secretion in response to both carbon and nitrogen starvation and acts as a mediator of nonself recognition as well as cell death in *Neurospora* (Xiang and Glass 2002; Dementhon

et al. 2006). Additionally, a Δ*vib-1* mutant was observed to exhibit severe growth defects on cellulose as well as a lack of cellulolytic enzyme activity (Xiong et al. 2014a). Since the p53-like transcription factor VIB-1 is conserved among filamentous ascomycete fungi, the ortholog TrVIB-1 of *T. reesei* (49 % amino acid identity with *N. crassa* VIB-1) was constitutively expressed in the *Neurospora* Δ*vib*-1 background and was indeed able to fully restore the growth and cellulolytic enzyme phenotype to WT levels (Xiong et al. 2014a). Transcriptional profiling of the Δ*vib-1* strain revealed that the expression of the Avicel regulon was significantly reduced in this mutant. One of the downregulated genes was *clr-2*, suggesting that *vib-1* functions upstream of this central cellulose regulator. However, a Δ*3βG*/Δ*vib-1* quadruple mutant was still able to induce cellulase gene expression upon exposure to cellobiose, and thus VIB-1 does not seem to be involved in inducer signal processing or perception that lead to activation of CLR-1/CLR-2. Instead, these results indicate that VIB-1 functions upstream of regulators that mediate inducer-dependent signal transduction (Xiong et al. 2014a).

It was observed that the Δ*vib-1* mutant exhibited an increased expression of genes functioning in metabolism, energy and mediation of CCR under Avicel conditions. However, a strain carrying a *cre-1* and *vib-1* deletion could not restore WT growth on Avicel, although CRE1-mediated CCR was eliminated. In addition to *cre-1* and *vib-1,* the transcription factor *col-26*, a homolog of the CCR mediating *bglR* in *T. reesei*, had to be deleted for a rescue of the growth defect of Δ*vib-1* on Avicel. Experiments by Xiong *et al.* suggest that COL-26 might play a role in glucose sensing and/or metabolism. Considering that CRE-1 mediated CCR is active in Δ*col-26* strains and that both *col-26* and *cre-1* had to be deleted to rescue the growth defect of Δ*vib-1* on cellulose, the regulatory function of VIB-1 on CRE-1-mediated CCR and COL-26-mediated glucose sensing/metabolism might be sequentially exerted. In the proposed model by Xiong and colleagues, VIB-1 and COL-26 act at the induction stage of the cellulolytic response, negatively regulating CRE-1 mediated CCR. This leads to a relief of CCR and the efficient induction of cellulolytic genes in response to cellulose. Later on, when glucose is released from cellulose, glucose sensing/signaling via COL-26 may begin to repress the cellulolytic response, while VIB-1 counteracts this inhibition. In conclusion, while COL-26 may influence the strength of cellulase expression according to current glucose levels, VIB-1 could be a global repressor of CCR by suppressing CRE-1 and COL-26 activity. Therefore, in the latter stages of cellulase utilization, VIB-1 and COL-26 seem to act antagonistically, thereby fine-tuning the extent of cellulose degradation by *Neurospora*. Intriguingly, the hyper-secreting *T. reesei* strains RUT-C30 and PC-3-7 exhibit mutations in *cre1*/*cre-1* and *bglR*/*col-26*, even though synergistic effects between those two transcription factors and the influence of *Trvib1*/*vib-1* were not explored (Xiong et al. 2014a; Le Crom et al. 2009; Nitta et al. 2012; Porciuncula et al. 2013). An identification and characterization of the possible regulatory interactions in these hyper-producers of lignocellulose degrading enzymes could potentially further increase enzyme yields.

While most of the aforementioned transcription factors regulate the gene expression of *Neurospora* on cellulose, most hemicellulases are part of a different regu-

lon. A transcription factor called xylan degradation regulator 1 (*xlr-1*), was found to be essential for hemicellulose degradation in *N. crassa* (Sun et al. 2012). This transcription factor is part of a conserved fungal transcription factor family that contains a Zn_2Cys_6 binuclear cluster domain. In *A. niger*, loss-of-function mutations in *xlnR*, an *xlr-1* homolog, results in strains showing significantly reduced xylanolytic activities; XlnR regulated approximately 10 genes encoding xylan and cellulose degrading enzymes (Hasper et al. 2000; Gielkens et al. 1999; van Peij et al. 1998). Another *xlr-1* homolog, *xyr-1*, acts as the central regulator for plant cell wall degradation in *T. reesei*, regulating cellulase, hemicellulase, as well as xylose metabolism (Stricker et al. 2006). Moreover, XYR1 was found to be responsible for the induction of cellulases in *T. reesei* by exposure to lactose (Stricker et al. 2007). In contrast, the *Neurospora* homolog *xlr-1* is only essential for xylan and xylose utilization. When exposed to xylan or xylose, Δ*xlr-1* mutants did not secrete protein nor displayed any endoxylanase activity (Sun et al. 2012). The secretome of the Δ*xlr-1* strain on cellulose on the other hand was not significantly altered as compared to the WT secretome and only a minor growth defect and slightly lower endoglucanase activity was observed. However, transcriptional profiling on Avicel showed that besides seven hemicellulase genes, seven predicted cellulase genes displayed a reduced (or delayed) induction in the Δ*xlr*-1 strain, indicating that XLR-1 in *N. crassa* retains a modulating role regarding the cellulolytic response. It was furthermore observed that the gene set induced by exposure to xylan (353 genes) did not completely overlap with the gene set dependent on functional XLR-1 (245 genes), suggesting that additional transcription factors are involved in hemicellulose decomposition. It is interesting to note in this respect that a *N. crassa* Δ*cre-1* strain grown on Avicel exhibited increased expression levels also of several hemicellulase genes, indicating that genes involved in hemicellulose degradation are under control of CCR (Sun and Glass 2011). Still, the expression of *xlr-1* was not influenced in a Δ*cre-1* strain, suggesting that this control is apparently not mediated via direct transcriptional repression of *xlr-1* by CRE-1 (Sun et al. 2012).

Recently, another novel C2H2 family transcription factor was found to regulate the expression of hemicellulase genes on L-arabinose and xylan, and therefore was called hemicellulase regulator 1 (HCR-1) (Li et al. 2014a). This transcription factor was identified via transcriptional profiling of *Neurospora* during growth on L-arabinose (see above). A deletion strain of *hcr-1* showed a significant upregulation of seven hemicellulase genes, suggesting that this transcription factor acts as a hemicellulase gene repressor. Additional experiments with Δ*hcr-1* on xylan and Avicel revealed that the protein secretion and hemicellulase activity were increased comparable to Δ*cre-1* levels, whereas the cellulase activity was not affected in the Δ*hcr-1* mutant. These results suggest that HCR-1 is a hemicellulase-specific regulator, and phylogenetic analysis revealed that HCR-1 homologs are present in a variety of cellulose degrading fungi, such as *T. reesei* and *A. niger* (Li et al. 2014a).

In 2015, a study investigating the transcriptome of *N. crassa* grown on five major crop straws revealed that RCA-1 (NCU01312) is involved in the lignocellulase

production of *Neurospora* (Wang et al. 2015). It is a homolog of the sporulation regulator *flbD* in *A. nidulans*, although no effect on sporulation could be observed in a *Neurospora* Δ*rca-1* deletion strain (Arratia-Quijada et al. 2012; Shen et al. 1998). Instead, Wang *et al*. could show that the Δ*rca-1* strain exhibited significantly increased transcription levels of major cellulases and xylanases when grown on corn straw. Intriguingly, when glucose, sucrose or Avicel were used as carbon source, Δ*rca-1* displayed reduced growth rates. Moreover, the transcription factor was de-repressed in the presence of high extracellular glucose levels. These results suggest that RCA-1 integrates signals derived from complex plant biomass rather than reacts to cellulose alone and might additionally play a role in glucose metabolism/signaling (Wang et al. 2015).

Mechanistically, RCA-1 seems to regulate the expression of plant polysaccharide degrading enzymes in an indirect manner, by acting upstream on *clr-2* during growth on complex plant biomass. This is based on qRT-PCR results demonstrating that the major lignocellulase regulator *clr-2* was twofold upregulated in Δ*rca-1*, while the expression of *rca-1* itself was not affected in Δ*clr-1/2*, Δ*xlr-1* or Δ*cre-1* mutants (Sun et al. 2012; Sun and Glass 2011; Coradetti et al. 2012; Wang et al. 2015). Whether RCA-1 exerts its control of *clr-2* directly or indirectly via regulation/interaction with CLR-1 is currently unknown.

A lot has been learned about the regulation of cellulases and hemicellulases already. The regulators of genes encoding for pectin degrading enzymes on the other hand are mostly unknown in fungi. In *A. niger,* the $Zn(II)_2Cys_6$ transcription factor RhaR (An13g00910) was reported to be involved in the release of L-rhamnose from the pectin substructure rhamnogalacturonan I, as well as the catabolism of this monosaccharide (Gruben et al. 2014). Homologs of this gene can be found in *Neurospora* and *A. nidulans*, and their role in these organisms seems similar (Pardo and Orejas 2014). However, due to the complex composition of pectin it is not unlikely that several additional, and as yet unidentified, transcription factors exist that interact to mediate the degradation of the remaining pectin structures. Novel approaches will have to be taken to uncover these enigmatic regulators.

The industrial fungal strains used today were generally created by random mutagenesis. Together with process engineering, those strains were refined to yield a high rate of hydrolytic enzymes (Himmel et al. 2007). However, further progress by undirected evolution might be difficult due to the accumulation of undesired secondary effects in the background. The identification of regulatory networks governed by CRE-1, VIB-1, COL-26, XLR-1, HCR-1, as well as CLR-1/CLR-2 as described above (Fig. 2) opens up new paths for the modification of enzyme production (Glass et al. 2013; Sun et al. 2012; Znameroski and Glass 2013; Sun and Glass 2011; Coradetti et al. 2012; Amore et al. 2013). In combination with the knowledge about substrate specificities and inducer molecules, new and better production strains could be tailored and thereby industrial processes during degradation of lignocellulosic material further improved (Glass et al. 2013; Znameroski and Glass 2013; Znameroski et al. 2012).

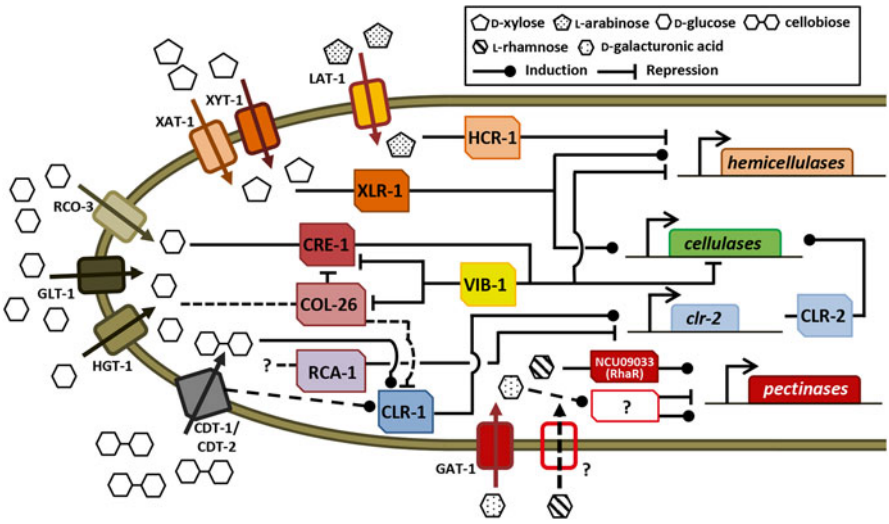

Fig. 2 Schematic diagram summarizing the sugar transport and signaling processes involved in the polysaccharide perception of *N. crassa*. Model view of the regulation of (hemi-) cellulase genes by transcription factors, as described in the literature. The known transporters for the four main monosaccharides, D-glucose, D-xylose, L-arabinose, and D-galacturonic acid, as well as cellobiose have been incorporated in this model. The transporter for L-rhamnose is unknown (*question mark*). Each signaling pathway starts next to a potential inducing molecule. CDT-1/CDT-2 are described as possible transceptors and could therefore regulate cellulase gene expression by influencing CLR-1/CLR-2 mediated gene induction (*dashed line* from CDT-1/CDT-2 to CLR-1). COL-26 is involved in glucose sensing/metabolism and reduces the expression of cellulases, possibly by influencing CLR-1/CLR-2 (*dashed line* from D-glucose over COL-26 to CLR-1). RCA-1 was reported to be responsible for complex biomass induction, but the inducer is unknown (indicated by a *question mark*). This model is by no means complete, as several transcription factors and sugar transporters have not yet been characterized. For example, apart from the L-rhamnose utilization regulator RhaR, currently no pectinase transcription factors are described in the literature (*dashed line* from D-galacturonic acid to the unknown pectinase regulator depicted by a *question mark*)

4 Sugar Transport Systems in *Neurospora*

As discussed above, mono- and oligosaccharides can play a dual role as metabolites and signaling molecules. Since sugars cannot freely permeate cell walls and biological membranes, active uptake or facilitation via transport proteins located to the cell membrane is required. For that purpose, fungi have evolved elaborate and versatile transporters (Romano 1986). Transport systems therefore play an important role in all aspects of lignocellulose perception, degradation and utilization, explaining the interest they attract in many fields of biotechnologically driven research.

Genome sequence analysis revealed that *N. crassa* has about 354 genes encoding for different types of transport proteins of which 141 are predicted to belong to the major facilitator superfamily (MFS) type transporters (complete listing: http://www.membranetransport.org). MFS type proteins are secondary active proteins and

play a pivotal role in a multitude of metabolic processes as well as in energy homeostasis in pro- and eukaryotes (Yan 2013). Their specificities cover a wide spectrum of different substrates, such as ions, lipids, amino acids, peptides and the like. Moreover, many of the known fungal sugar transport proteins for mono- and oligosaccharides belong to the ubiquitous MFS transporter family (Benz et al. 2014a, b; Andre 1995; Dos Reis et al. 2013; Nelissen et al. 1997; Vankuyk et al. 2004; Zhang et al. 2013; Galazka et al. 2010). Approximately 60 of the *Neurospora* MFS-type transporters are members of the family 1, the only MFS type family that is assigned to sugar transport (Pao et al. 1998). Phylogenetic analysis of fungal sugar transporters suggests that the *N. crassa* transporters are remarkably divergent compared to the transport proteins of the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* (*S. pombe*) (Galagan et al. 2003). Only a few, if any, are homologs of characterized sugar transporters in *S. cerevisiae*. This could indicate that the sugar transport capacity of *Neurospora* is more functionally diverse and thus able to utilize a greater variety of different sugars, which would coincide with the expanded range of polysaccharide hydrolases encoded in the *Neurospora* genome (Borkovich et al. 2004).

Despite the high number of transport protein-encoding genes in *N. crassa,* still only very few have been functionally characterized (Table 2). The first approach to study sugar transport in *N. crassa* revealed two distinct glucose transport systems: a constitutive low affinity transport system and a high-affinity glucose-repressible system, even though the individual transport proteins contributing to each system were not known (Scarborough 1970; Schneider and Wiley 1971). Sequence analysis suggested RCO-3 (NCU02582) to be a glucose transporter and as such the protein

Table 2 List of hitherto identified and characterized sugar transport proteins involved in mono- or oligosaccharide metabolism in *N. crassa*

Locus	Protein	Properties	Key references
NCU02582	RCO-3	D-glucose, glucose sensor	Madi et al. (1997)
NCU10021	HGT-1	D-glucose, high-affinity	Xie et al. (2004)
NCU00821	An25	D-xylose	Du et al. (2010)
NCU04963	An29-2	D-xylose, D-glucose	
NCU00801	CDT-1	Cellodextrins, proton-symport, possible transceptor function	Galazka et al. (2010), Kim et al. (2014), Znameroski et al. (2014)
NCU08114	CDT-2	Cellodextrins and xylodextrins, facilitator, possible transceptor function	Galazka et al. (2010), Kim et al. (2014), Cai et al. (2014), Li et al. (2015a), Znameroski et al. (2014)
NCU00988	GAT-1	Galacturonic acid, high-affinity glucuronic acid	Benz et al. (2014b)
NCU02188	LAT-1	L-arabinose	Benz et al. (2014a), Li et al. (2015b)
NCU01633	GLT-1	D-glucose	Li et al. (2014a)
NCU01132	XAT-1	D-xylose and L-arabinose	
NCU05627	XYT-1	D-xylose	
NCU05853	CBT-1/ CLP-1	Cellobionic acid	Xiong et al. (2014b), Cai et al. (2015)

was characterized. It was found to act as a glucose sensor as well, transducing information about extracellular glucose levels, and is therefore involved in regulating gene expression of both high- and low affinity glucose transport systems (Madi et al. 1997). One of the few characterized sugar transporters in *N. crassa* is HGT-1 (NCU10021), a high-affinity glucose transporter identified by microarray analysis and sugar transport assays (Xie et al. 2004).

In the last few years, many attempts have been made to study specific transport proteins in *N. crassa* involved in the uptake of plant biomass derived mono- and oligosaccharides. Partly, this increased interest is based on the fact that yeast (*S. cerevisiae*), one of the most employed organisms for large-scale industrial fermentation, is derived from fruit-inhabiting wild isolates, and thus is able to naturally take up and utilize only a few hexose sugars like glucose, fructose, mannose and galactose. However, to achieve high-yield, high-rate fermentation from plant biomass hydrolysates (consisting of ~70 % cellodextrins and D-glucose, and ~30 % pentoses, such as D-xylose) (Carroll and Somerville 2009) research has focused on the construction of metabolically engineered yeast strains with improved capability for effective sugar utilization (van Maris et al. 2006; Subtil and Boles 2012). D-xylose, for instance, can only enter wild-type yeast cells through non-specific hexose transporters with much lower affinity for xylose than for glucose (Kötter and Ciriacy 1993). In 2010, a novel D-xylose-specific transporter (An25, NCU00821) was discovered in *N. crassa*, and heterologously expressed and characterized in *S. cerevisiae* (Du et al. 2010). While this study discovered one of the first naturally occurring D-xylose-specific transporters in cellulolytic fungi (besides other less specific proteins like An29-9), the attempt to increase the utilization rate of D-xylose in recombinant yeasts via directed transport of the substrate failed, most likely due to the absence of utilization pathways (Du et al. 2010). Numerous additional studies have since then been published aiming to improve D-xylose fermentation or to relieve D-glucose inhibition (see Subtil and Boles 2012; Kim et al. 2013; Latimer et al. 2014; Farwick et al. 2014; Li et al. 2013 and references therein). *Neurospora* research opened up an alternative solution, when two MFS-type cellodextrin transporters, CDT-1 and CDT-2 (cellodextrin transporter 1 and 2; NCU00801 and NCU08114), were identified in *N. crassa* through microarray analysis (Tian et al. 2009; Galazka et al. 2010). These were further characterized to be a proton-symporter (CDT-1) and a facilitator (CDT-2), respectively (Kim et al. 2014). Cellodextrins are glucose oligomers, which cannot be naturally metabolized by *S. cerevisiae*. By introducing CDT-1 or CDT-2 along with the β-glucosidase GH1-1 of *N. crassa* into *S. cerevisiae*, a cellodextrin-assimilating yeast strain was constructed that was able to efficiently ferment cellobiose (Galazka et al. 2010). With this approach, cellobiose is actively transported into yeast cells, and by the action of the heterologously expressed intracellular β-glucosidase, glucose is released. Uptake of cellodextrins instead of D-glucose thus bypassed problems caused by glucose repression and led to engineered *S. cerevisiae* strains capable of simultaneous cellobiose and xylose fermentation (Ha et al. 2011a; Li et al. 2010). Ha *et al*. were subsequently able to show that this successful concept for the CDT-1 and GH1-1-containing strain was extendable to the co-fermentation

of cellobiose with galactose (Ha et al. 2011b). This opened the possibility of engineering cellobiose utilization by microorganisms and the implementation of simultaneous saccharification and fermentation (SSF) (Galazka et al. 2010; Fox et al. 2012; Jin et al. 2013).

Several current studies have been focusing on the directed evolution and better characterization of the cellobiose transporters CDT-1 and CDT-2 from *N. crassa* to increase their potential in industrial processes (Ha et al. 2013; Lian et al. 2014; Ryan et al. 2014). Notably, CDT-2 was found to be not entirely specific for cellodextrins, but to also transport xylodextrins (Cai et al. 2014; Li et al. 2015a). While this might explain it's relatively lower cellodextrin transport rates compared to CDT-1, the full substrate range for CDT-1 remains to be systematically elucidated as well and might include further structurally related plant cell wall oligosaccharides. Intriguingly, both transporters seem to have a dual function and also be involved in signal transduction for differential regulation. Recent findings suggest that they act as so-called "transceptors" and function in downstream activation of cellulolytic gene expression (Znameroski et al. 2014). The concept of signal transduction via transceptors was already shown to be required during nutrient sensing in *S. cerevisiae* (Kriel et al. 2011; Thevelein and Voordeckers 2009). Likewise, some transporters of *T. reesei* seem to be acting as transceptors to induce cellulase gene expression (Zhang et al. 2013; Ivanova et al. 2013). Due to these results, the model of lignocellulosic polysaccharide degradation and regulation in *N. crassa* needs to be extended (Fig. 2). Traditionally, mono- or oligomeric residues derived from plant cell wall polysaccharides are believed to be transported into the hyphae and sensed by intracellular components, resulting in the activation of variable transcription factors (e.g. CLR-1, CLR-2), which then induce expression of specific target gene pools. As suggested now, transceptors like CDT-1 or CDT-2 might be capable of activating cascades themselves in addition to the transport of their substrates (cellodextrins) (Znameroski et al. 2014). Since signaling and transport could be separated by creating certain transporter mutants, the inducer binding sites might be non-identical with the translocation channel, but further research is necessary to clarify the exact mechanism. Intriguingly, two recent reports suggest a similar dual role also for the closest homolog of CDT-1 and CDT-2 in *Neurospora*, NCU05853. The function of this transporter had been enigmatic for the last years, since it is co-induced with the cellulolytic regulon, but did not show any detectable activity towards the known cellulose degradation products. This situation is reminiscent of that of the LPMOs (formerly GH61s; see Part 1.2), which eventually were shown to cleave cellulose in an endo-like fashion, however not hydrolytically but in a copper-dependent redox mechanism in concert with the cellobiose dehydrogenase (CDH-1) producing a mixture of oxidized oligosaccharides such as aldonic acids (Beeson et al. 2012; Phillips et al. 2011). As is clear now, the roles of NCU05853 and the LPMOs/CDH-1 are indeed linked, since NCU05853 was shown to transport aldonic acids, and was consequently named CBT-1 (cellobionic acid transporter) (Xiong et al. 2014b). A parallel study showed that a deletion of NCU05853/CBT-1 (here termed CLP-1 for cellodextrin transporter-like protein 1), in the Δ3βG/Δ*cdt-2* quadruple knockout background led to a dramatic upregulation

of the cellodextrin transporter *cdt-1* gene and hyper-secretion of cellulases (Cai et al. 2015). It was thus suggested that CBT-1/CLP-1 is involved in cellulase induction and (in WT) acts as a suppressor of *cdt-1* expression. The mechanism of this suppression is not clear at the moment, but one hypothesis is that in the absence of CBT-1/CLP-1, cellobionolactone/cellobionic acid produced for example by CDH-1 accumulate. It is possible that CDT-1, as a putative transceptor (Znameroski et al. 2014; see above), can bind but not transport cellobionic acid, and that this (unproductive) binding can lead to cellulase induction. Even though it is intriguing to speculate, more research is necessary to provide evidence and elucidate the details. Moreover, it will be interesting to test whether the use of CBT-1/CLP-1 can help to develop yeast strains capable of catabolizing the oxidized degradation products of LPMOs, which are increasingly used in industrial enzyme cocktails due to their synergistic cellulase activity.

Strategies to use specific transporters of *N. crassa* for metabolic engineering of yeast are not restricted to produce advanced biofuels from renewable resources, but can be used for value-added chemical production as well. The identification and characterization of the first eukaryotic galacturonic acid (D-GalA) transporter in *Neurospora* provided a step towards a more complete utilization of plant biomass for biofuel and commodity chemical production (Benz et al. 2014b). D-GalA is the main backbone component of pectin with a content of up to 70 % (Mohnen 2008). Agricultural waste streams including citrus peels, sugar beet pulp and apple pomace are pectin-rich feedstocks (Doran et al. 2000; Rivas et al. 2008; Kennedy et al. 1999), but are mainly used as animal feed or fertilizers and thus are of low economic value. Therefore, finding alternative applications to raise this value is appealing (Canteri-Schemin et al. 2005; Edwards and Doran-Peterson 2012; Angel Siles Lopez et al. 2010; Pourbafrani et al. 2010). Metabolic engineering of *S. cerevisiae* to utilize D-GalA is in fact a promising route for converting an abundant but underutilized sugar during industrial fermentation processes. Although background uptake of D-GalA through an unknown, channel-type transport system in *S. cerevisiae* was demonstrated to occur under certain conditions (Souffriau et al. 2012) it was suggested to be the limiting step in D-GalA utilization engineering in yeast (van Maris et al. 2006; Huisjes et al. 2012b). The galacturonic acid transporter GAT-1 (NCU00988) from *N. crassa* was the first eukaryotic D-GalA transporter to be identified. It was demonstrated to be a high-affinity permease, and therefore showed best transport kinetics at low substrate concentrations (µM range). However, when engineered into *S. cerevisiae*, GAT-1 also increased bioproduct conversion at high D-GalA concentrations more likely to be encountered in real hydrolysates (Benz et al. 2014b). By co-expressing either a D-galacturonate reductase (GAAA) or uronate dehydrogenase (UDH) in this yeast strain, a transporter-dependent conversion of D-GalA into either L-galactonic acid (L-GalOA) or *meso*-galactaric acid (GalAA) was achieved. GalAA is an isomer of glucaric acid, a component that can be converted into high-value materials such as hydroxylated nylons (Werpy and Petersen 2004; Kiely et al. 2000). L-GalOA has similar properties to D-gluconic acid, and correspondingly holds potential in a multitude of industries such as pharmaceutics, textile, food and cosmetics (Werpy and Petersen

2004; Ramachandran et al. 2006). Although homologs of GAT-1 from other fungi isolated from pectin-rich environments could be better suited for industrial purposes, exhibiting a low-affinity—high-capacity kinetics, the identification of the first eukaryotic family member in *N. crassa* was an important step towards a more complete and effective utilization of lignocellulosic feedstocks through bioengineering processes.

The same comparative systems analysis that led to the identification of GAT-1 was also successful in the identification of an L-arabinose transporter (LAT-1, NCU02188) in *N. crassa* (Benz et al. 2014a). Moreover, in another recently published study three further sugar transporters were identified and characterized (Li et al. 2014a). This approach, being the first transcriptomic analysis of filamentous fungi grown on L-arabinose, provided crucial information on the response of *N. crassa* regarding this monosaccharide. The thereby identified transporters, namely GLT-1 (D-glucose transporter 1, NCU01633), XAT-1 (D-xylose and L-arabinose transporter, NCU01132), and XYT-1 (D-xylose transporter 1, NCU05627) were heterologously expressed in yeast and their substrate affinity and transport capacity compared. XAT-1 and XYT-1 showed higher substrate affinity than the previously described xylose transporter An25 (Li et al. 2014a; Du et al. 2010). These novel pentose transport proteins are promising candidates to improve pentose fermentation in yeast and thus to expedite the complete utilization of lignocellulosic plant matter.

Overall, the large number and the suggested functional diversity of transport proteins encoded by the *Neurospora* genome (Borkovich et al. 2004) may provide important tools for metabolic engineering either for the efficient and cost-competitive production of lignocellulosic biofuels or other products deriving from biomass hydrolysis, like commodity chemicals.

5 Contributions of *Neurospora* to Bioprocess Engineering

The industrial use of mycelial fungi in the manufacture of pharmaceuticals, chemicals or enzymes is a multibillion dollar industry and thus of great economic importance (Loros and Dunlap 2006; Nielsen 2013; McPherson 2012; Yoder and Lehmbeck 2004). *N. crassa* has been used as a model system for genetics and cell biology for almost 100 years, but not as a production organism in industrial applications. However, the toolbox that has been established for *Neurospora* (genetics, biochemical and molecular studies, systems approaches etc.) includes a plethora of resources, which can be used to investigate the industrial application potential of filamentous fungi. In the last years, *Neurospora* has triggered the interest in particular of researchers working to improve the enzymatic conversion of biofuel feedstocks, partly since isolates of *N. crassa* have often been recovered from sugar cane (Turner et al. 2001; Pandit and Maheshwari 1996), a plant closely related to *Miscanthus*, which is an attractive feedstock for industrial biofuel production (Somerville et al. 2010). Fungi are the main source of cellulolytic enzymes to

depolymerize plant biomass into fermentable sugars (Cherry and Fidantsef 2003; Adrio and Demain 2014). The costs associated with the conversion of the insoluble lignocellulosic feedstocks into fermentable sugars still represents a significant barrier to the production of cost-competitive biofuels. For economic reasons it is therefore crucial to synthesize cellulolytic enzymes with highest efficiency in the fungal production strains.

The induction of cellulase secretion in many fungi generally involves the incubation on feedstock substrates, which poses problems due to their insoluble nature, associated costs, and the fact that the secreted enzymes might bind strongly to the lignocellulosic particles, potentially leading to low recovery rates. Some fungi are inducible with soluble, cheap and abundant industrial residues like lactose (for example T. reesei, see Ivanova et al. 2013; Bischof et al. 2013). However, this advantageous feature is not generalizable, and does not lead to induction in many fungi. Research in *N. crassa* has shown that by deletion of the major β-glucosidase genes an induction of the complete repertoire of cellulases could be achieved upon exposure to cellobiose, recapitulating the wild type response to insoluble cellulosic substrates (Znameroski et al. 2012) (see above, Part 3). Taking advantage of soluble carbon sources such as cellodextrins to induce cellulase gene expression could reduce costs and simplify industrial enzyme production. This knowledge achieved by *Neurospora* research could thus be used to transfer it into other currently used industrial fungi.

One of the major improvements regarding lignocellulolytic enzyme cocktails in the last years was the elucidation of the role of the lytic polysaccharide monooxygenases (LPMOs) (see above, Part 1.2). Their functional characterization extended the traditional view of cellulose degradation. As is now known, LPMOs are key polysaccharide-degrading enzymes acting in an oxidative mechanism, thereby creating synergistic effects in combination with the "traditional" cellulases (Harris et al. 2014). The unique reactivity on cellulose and hemicellulose and the diversity of LPMOs in fungi (as well as in bacteria, comprising family AA10) makes them powerful tools for enzymatic biomass conversion technologies. Unsurprisingly, heterologously expressed LPMOs have already been incorporated into commercial cellulase cocktails such as produced by Novozymes (called Cellic CTec2 and CTec3) and were shown to increase the cellulose conversion yield (Harris et al. 2014; Dimarogona et al. 2012). The variety of LPMOs encoded by the genome of *Neurospora* (Tian et al. 2009; Martinez et al. 2008), their suggested functional diversity, and the numerous studies contributing to the elucidation of their structures and precise mechanisms (Beeson et al. 2012; Phillips et al. 2011; Agger et al. 2014; Quinlan et al. 2011; Bey et al. 2013; Isaksen et al. 2014; Li et al. 2012; Langston et al. 2011; Sygmund et al. 2012; Vu et al. 2014) have already provided substantial advances for the application in enzyme cocktails required for saccharification of lignocellulosic biomass. However, a deeper mechanistic understanding of those enzyme families will be necessary to further enhance their utility in industrial processes.

The efficient production of lignocellulases does not only depend on their transcriptional induction, but also on the coordination of a number of downstream

processes such as translation, folding, glycosylation and export. Filamentous fungi are capable of very high secretion rates, but these come at a price and elicit endoplasmatic reticulum (ER) stress. In order for these processes not to become bottlenecks in biotechnological production settings, a better understanding of the cross talk between ER stress and lignocellulase synthesis is paramount. A recent study by Fan *et al*. coupled the unique genetic screening tools available for *Neurospora* to high-throughput mRNA sequencing and addressed this issue in a systematic fashion (Fan et al. 2015). This way, an ER stress regulon under lignocellulase producing conditions could be described, and numerous novel factors with a likely impact on cellulase secretion were uncovered. Among these are 100 formerly hypothetical genes and three novel transcriptional regulators mediating cellulase secretion and ER stress response (RES-1, RES-2, and RRG-2).

Research using *Neurospora* has already extended beyond the production and characterization of enzymes for plant cell wall saccharification to include the conversion to energy-rich downstream metabolites. To this end, recent studies demonstrated *N. crassa* could serve to study lipid production from lignocellulosic feedstocks (Roche et al. 2013, 2014b). Microbially produced triacylglycerol (TAG) synthesized from low-cost fermentation substrates such as lignocellulose is a potential resource for the production of biodiesel (Yousuf 2012). Unfortunately, cost-effective lignocellulosic substrates cannot be directly utilized by many oleaginous production platforms without pretreatment and enzymatic hydrolysis of the appropriate biomass (Ruan et al. 2012, 2013; Economou et al. 2011; Gong et al. 2013). With a multi-gene approach to engineer *N. crassa,* Roche *et al*. succeeded in developing a *Neurospora* strain with enhanced cellulose degrading capacities and increased lipid accumulation (Roche et al. 2013, 2014b). This approach could be utilized for direct industrial conversion of lignocellulose to TAG from low-cost cellulosic feedstocks without supplementation of exogenous cellulases. Another recently published study exemplified a novel and efficient conversion strategy using an engineered strain of *N. crassa* for the production of cellobionic acid, a stereoisomer of the high value-added chemical lactobionic acid (Hildebrand et al. 2015a, b).

The direct biotechnological production of ethanol by *N. crassa* as an alternative production route to bioethanol without using yeast fermentation was reviewed not long ago (Dogaris et al. 2013) with the conclusion that so far no *Neurospora* strain is able to compete with the presently used industrial fungal workhorses. However, recently a new isolate of *N. crassa* from oil palm empty fruit bunch (EFB) was shown to be a promising strain for commercially efficient saccharification of EFB as a cheap waste product of the palm oil industry (Li et al. 2014b). But even if *Neurospora* should not be able to compete with industrially used fungi in the near future, the aforementioned mechanistic insights gained from *N. crassa* research, such as on the direct conversion of lignocellulosic feedstocks as well as regarding the regulation and production of lignocellulosic enzymes may have a significant impact on industrial strain design and production economics.

6 Conclusion and Outlook

The renaissance of *N. crassa* mentioned in the title has shifted research on this fascinating microorganism beyond understanding fundamental science questions to include applied research goals, in particular regarding the industrial bioprocessing of lignocellulosic biomass. Even though most of these advances have been made only recently, using the genetic tools *N. crassa* can offer as a developed model system has proven to be promising for industrially relevant science and will hopefully allow much more progress in the near future. However, as becomes more and more obvious from the data, fungal biomass degradation is a complex process, and our understanding of the respective regulatory networks is still limited. To address these issues, the Joint Genome Institute (JGI) has funded "The Fungal Nutritional ENCODE Project" in 2013 (http://jgi.doe.gov/the-fungal-nutritional-encode-project/). The transcriptional response of *N. crassa* to a variety of carbon sources (mono-, oligo-, and polysaccharides up to complex biomass) as well as to variations in light and the availability of sulphur, phosphate and nitrogen will be recorded. Analysis of the huge data set will again require a community effort. The goal is to provide high-resolution models of gene regulation at a genome-wide scale. Through the development of a nutritional regulatory map, new factors and regulons linked to carbon utilization will be identified, and these targets will aid towards the rational engineering of improved industrial fungal strains for the production of second generation biofuels.

With these studies, *Neurospora* continues to develop in its role as a model microbe. However, fungi are extremely divergent, and each fungus evolved for a specific niche with varying requirements. Therefore, in a next step, it will be essential to embrace these differences, and by comparative analyses and continued genome sequencing of new species (for example in the "1000 Fungal Genomes Project"; http://genome.jgi-psf.org/programs/fungi/1000fungalgenomes.jsf) bridge this apparent gap. Only then will we be able to grasp how filamentous fungi perceive their carbon-biased environment and adapt to changes during plant biomass deconstruction.

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Improvement of Industrially Relevant Biological Activities in Mucoromycotina Fungi

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1 Industrial Use of Mucoromycotina

Mucoromycotina is an *incerte sedis* subphylum emerged from the former Zygomycota and includes the classical core zygomycetes groups, Mucorales, Mortierellales and Endogonales (Hibbett et al. 2007). Several Mucoromycotina species, such as *Mucor circinelloides*, *Mucor hiemalis*, *Lichtheimia corymbifera*, *Phycomyces blakesleeanus* and *Rhizopus oryzae*, have been used as model organisms in genetic and molecular biological studies to examine, among others, light induction and regulation mechanisms, carotene biosynthesis, sexual differentiation, fungal morphogenesis or pathogenicity (Papp et al. 2010). Recently, there is an increasing interest in the exploitation of the biotechnological potential of Mucoromycotina fungi. They are used primarily as producers of hydrolytic enzymes (e.g. lipases, proteases, endoglucanases, glucoamylases and polygalacturonases), organic acids (e.g. lactic, fumaric and malic acids), alcohol, carotenoids and polyunsaturated fatty acids. Most significant species are *R. oryzae* used to produce organic acids and ethanol (John et al. 2007; Roa Engel et al. 2008; Meussen et al. 2012) as well as lipase and other enzymes (Resina et al. 2004; Li et al. 2011), *Rhizomucor miehei* and *Rhizomucor pusillus* producing rennin-like aspartyl protease and lipase (Nouani et al. 2009; Yegin et al. 2011), *Mortierella alpina* used for the production of arachidonic acid and other polyunsaturated fatty acids (Kavadia et al. 2001; Bellou et al. 2012) and *Blakeslea trispora*, which is an industrial source

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of β-carotene and lycopene (Dufossé 2006). Some species belonging to the genera *Actinomucor*, *Amylomyces*, *Mucor* and *Rhizopus* are applied in cheese ripening (Hermet et al. 2012) and in oriental soybean-based food fermentations (e.g. production of tempeh, oncom and sufu) (Nout and Aidoo 2002; Babu et al. 2009).

2 Genetic Transformation and Gene Expression Systems in Mucoromycotina

Both basic research and biotechnological developments require routinely applicable tools for functional analysis, expression and manipulation of genes. These techniques include procedures for genetic transformation and selection of the transformants, well-characterized molecular markers and expression signals, etc. Genetic transformation systems available for Mucoromycotina fungi were previously reviewed in detail by Ibrahim and Skory (2007) and Papp et al. (2008, 2010).

To transform Mucoromycotina fungi, the most frequently used method is the polyethylene glycol (PEG)-mediated transformation of protoplasts (Ibrahim and Skory 2007; Papp et al. 2010). Protoplast formation occurs from young hyphae or germ tubes. Electroporation was successfully used to transform *Absidia glauca* (Schilde et al. 2001), *M. circinelloides* (Gutiérrez et al. 2011) and *Rhizopus delemar* (Xu et al. 2014) and it may be a good alternative of the PEG-mediated method. *Agrobacterium tumefaciens*-mediated transformation (ATMT) has also been adapted to several Mucoral fungi, such as *Backusella lamprospora*, *M. circinelloides*, *Rm. miehei* and *R. oryzae* (Monfort et al. 2003; Michielse et al. 2004; Nyilasi et al. 2005, 2008; Ibrahim and Skory 2007; Papp et al. 2012). This method often led to unstable transformants, except for *Mortierella*, where it proved to be the most efficient technique resulting increased transformation frequency and stable transformants (Ando et al. 2009b). For *Umbelopsis*, the *Agrobacterium rhizogenes*-mediated transformation (ARMT) was developed (Wei et al. 2010). Successful electroporation or ATMT of most Mucoral fungi also requires protoplast formation. Biolistic particle bombardment directly of sporangiospores is well established for *A. glauca* (Bartsch et al. 2002), *M. circinelloides* (González-Hernández et al. 1997), *Mo. alpina* (Takeno et al. 2005a, b; Ando et al. 2009a) and *R. oryzae* (Ibrahim and Skory 2007).

For successful expression of the introduced exogenous gene, it should be placed under the control of appropriate regulatory sequences (i.e. promoters and terminators) of the host. Although several promoters have been identified in Mucoromycotina fungi, only a few of them have been analysed in detail (Larsen et al. 2004). One of the first zygomycetous promoters used for heterologous expression was that of the *Rhizopus niveus* phosphoglycerate kinase 2 (*pgk2*), which was applied to express the *Escherichia coli* β-glucuronidase gene (*uidA*) in *R. niveus* (Takaya et al. 1995). Promoter and terminator sequences of the *M. circinelloides* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd1*) are maybe the best-characterized and most frequently used regulator sequences in this fungal group. These regions proved to be

useful to drive the expression of different fungal and bacterial genes in *M. circinelloides, Mucor rouxii* and *Rm. pusillus* (Wolff and Arnau 2002; Larsen et al. 2004; Appel et al. 2004). *Gpd1* is regulated by glucose; its transcription is stronger at higher glucose concentrations (Wolff and Arnau 2002). By the removal of the sequences responsible for glucose regulation, Larsen et al. (2004) constructed a 361-bp derivative of the *gpd1* promoter, which was able to constitutively drive the exogenous genes with high expression level regardless of the carbon source used. In *R. oryzae*, the most frequently used promoters are those of glucoamylase A (*amyA*), pyruvate decarboxylase (*pdcA*) and phosphoglycerate kinase (*pgk1*) (Gao and Skeen 2002; Mertens et al. 2006). Mertens et al. (2006) found that the potential of these promoters to drive the green fluorescent protein gene (*gfp*) showed the following order: *pdcA* > *amyA* > *pgk1*. Recently, various promoters of *Mo. alpina* was tested using the β-glucuronidase (*GUS*) reporter gene assay and constitutive, time-dependent and inducible promoters were found (Okuda et al. 2014a, b). These promoters drove the *GUS* expression more efficiently than the histone H4 promoter, which is traditionally used to express genes in *Mortierella*. Moreover, *GAL10* promoter, which can be induced by galactose even in the presence of other sugars, increased GUS expression to 50-fold by adding galactose in the culture medium (Okuda et al. 2014b).

3 Production of Hydrolytic Enzymes

In the group Mucoromycotina, there are several excellent exoenzyme producers, especially among *Mucor*, *Rhizomucor* and *Rhizopus* species. As most of these fungi are saprotrophic, they can produce biomass degrading hydrolases in high amounts under solid conditions using a wide range of cheap plant-waste substrates (Kotogán et al. 2014). An up-to-date review by Ferreira et al. (2013) summarized Mucoromycotina sources of amylase, pectinase, cellulase, protease, phytase and hydroxylase and pointed out the application of lipases as biocatalyzers in organic reactions. Although several recent studies have reported wild-type Mucoromycotina hydrolases including *Mucor* and *Thermomucor* lipases (Mohamed et al. 2011; Ülker and Karaoğlu 2012; Ferrarezi et al. 2014), *Rhizomucor* β-glucosidase (Krisch et al. 2012) and *Rhizopus* aspartic protease (Hsiao et al. 2014), new biocatalysts are being developed by adapting of these native enzymes to a desired application and reaction condition. According to the 2015 data of Association of Manufacturers and Formulators of Enzyme Products (AMFEP; http://www.amfep.org/content/list-enzymes), about 5 % of the commercial enzymes are derived from Mucoral fungi; among them, only three *Rm. miehei* hydrolases, an amyloglucosidase, an aspartic protease and a lipase, are produced in other microbial hosts (i.e. in *Aspergillus niger* and *Aspergillus oryzae*). Recombinant triacylglycerol lipase derived from *Rm. miehei* (RML) and expressed in *A. oryzae* is currently used in the food industry (Adrio and Demain 2010). RML production in *A. oryzae* was first reported in an early study of Huge-Jensen et al. (1989) where the RML gene was driven by

A. oryzae α-amylase promoter and *A. niger* glucoamylase terminator sequences. Application of modified RML as a technical enzyme is also well known (Rodrigues and Fernandez-Lafuente 2010) and novel studies pointed out that this enzyme has outstanding ability to convert various oil residues to biodiesel (Huang et al. 2012, 2014; Calero et al. 2014). Aspartic protease of *Rm. miehei* and its recombinant form produced in *Aspergillus* are also important commercial enzymes used in cheese manufacturing for milk clotting. One of the most important non-recombinant commercial enzymes in Mucoromycotina is the lipase of *R. oryzae*. Other non-recombinant commercial enzymes include aminopeptidase of *R. oryzae*, amyloglucosidases of *R. delemar* and *R. oryzae*, lipases of *Mucor javanicus*, *Rm. miehei*, *R. delemar*, *R. niveus* and *R. oryzae* and protease of *R. niveus*.

Apart from the abovementioned applications, there are only a few examples for expression of hydrolase genes of Mucoral fungi in other filamentous fungi. Takó et al. (2010) expressed the *Rm. miehei* β-glucosidase gene (*bgl*) placed under the control of the *M. circinelloides gpd1* promoter and terminator in *M. circinelloides*. Although transformants showed significantly higher β-glucosidase activity in both liquid and solid-state cultures than the untransformed *Mucor* strain, *M. circinelloides* secreted the heterologous enzyme with somewhat lower efficiency than the donor *Rm. miehei* strain.

Heterologous expressions of Mucoromycotina hydrolases and studies to modify their characteristics have been carried out mainly in the methylotrophic yeast *Pichia pastoris*. From this point of view, lipase of *R. oryzae* (ROL) is the best studied enzyme. ROL was firstly expressed in *P. pastoris* by Minning et al. (1998). The constructed pPICZαA-ROL vector, in which the mature ROL (mROL) sequence was fused with the α-factor prepro signal sequence of *Saccharomyces cerevisiae*, has been used in several further studies. In this study, methanol feeding was linked to the dissolved oxygen content of the medium and a lipase activity of 500,000 U/L could be achieved. Recombinant ROL (rROL) had similar specific activity to that of the native lipase. Later, the same vector was transformed into the wild type *P. pastoris* X-33 (Mut^+) strain (Invitrogen) and different methanol feeding strategies during enzyme production were tested (Minning et al. 2001). By an optimized method involving simultaneous feeding of glycerol and methanol followed by a single methanol feed, a productivity of 12,888 U/L/h was achieved. This ROL-expressing strain has been used in various studies to optimize the conditions for culturing and enzyme production (Minning et al. 2001; Ramón et al. 2007; Surribas et al. 2007). Using pPICZαA-ROL, the lipase gene is controlled by the methanol induced *AOX1* (alcohol oxidase 1) promoter. Resina et al. (2004) constructed the vector pPICZFLDα-ROL, in which the *ROL* gene is driven by the promoter of the *P. pastoris FLD1* (formaldehyde dehydrogenase) gene. This promoter can be induced by methylamine as a sole nitrogen source providing a basis for further design of methanol-free expression strategies. Cos et al. (2005a) compared the *AOX1* and *FLD1* promoters using the abovementioned *P. pastoris* X-33-derived strains containing either pPICZαA-ROL or pPICZFLDα-ROL in batch and fed-batch cultures and found that the two systems had very similar productivity under the tested conditions. To improve folding and secretion of ROL, the plasmid pPICZFLDα-ROL was

transformed in *P. pastoris* strains, which expressed the induced form of the *S. cerevisiae* unfolded protein response transcriptional factor Hac1 and/or hold deletion of the *GAS1* gene encoding β-1,3-glucanosyltransglycosylase playing important role in the assembly of the yeast cell wall (Resina et al. 2009). Overexpression of ROL in the strains expressing HAC1 and containing *Δgas1* led to a three and fourfold increase in the total process specific productivity, respectively, while this increment was sevenfold in the ROL-expressing HAC1/*Δgas1* strain.

Using the *P. pastoris* strains GS115 (Mut^+) and KM71 (Mut^s) (Invitrogen), strains containing the *ROL* gene in single and multiple copies were constructed (Serrano et al. 2001; Cos et al. 2005b). Although increased *ROL* gene dosage had a deleterious effect on Mut^s strains in bioreactor cultures (Cos et al. 2005b), methanol and sorbitol co-feeding helped to maintain the cell growth and enzyme production (Ramón et al. 2007).

The *P. pastoris* KM71-derived strain containing a single copy of pPICZαA-ROL (Cos et al. 2005b) was used to study the effect of the methanol set-point and sorbitol feeding rate during fed-batch fermentation on the lipase production (Arnau et al. 2010). This strain was also used to compare the biochemical properties of rROL and the native ROL (nROL) (Guillén et al. 2011). In the latter study, rROL showed partly different characteristics from those of nROL due to the effect of the pre-prosequence of ROL and the post-translational modifications of *Pichia*. Specific activity of rROL was more than 40-fold higher than that of nROL and it had lower molecular weight.

The prosequence is a key factor for the folding and secretion of lipases and its engineering may improve secretion and enzyme properties. When ProROL (ROL prosequence and the mROL) was expressed in *P. pastoris*, the resulting crude recombinant lipase could be directly used for the transesterification of plant oils at high-water content and seemed to be promising for biodiesel production (Li et al. 2011). Yu et al. (2013) constructed a chimeric lipase consisting of the prosequence of *Rhizopus chinensis* lipase (RCL) and mROL and expressed it in *P. pastoris*. The produced chimera lipase reached its maximum activity at 4050 U/mL and it was successfully used for biodiesel production from tung oil in an immobilized form. RCL has also been used for heterologous expression studies (Yu et al. 2009). Although recombinant RCL is a very promising enzyme, the low thermostability restricts its potential for industrial use. Thus possibilities to improve the thermostability of RCL by directed evolution, N-glycosylation or engineering disulfide bonds in the lid hinge region of the protein are extensively studied (Yu et al. 2012a, b; Yang et al. 2015).

4 Production of Organic Acids and Ethanol

Today, lactic acid bacteria are primarily used for industrial lactic acid production (Liaud et al. 2015). Contrary to *Lactobacillus* spp., which require cost-increasing supplements (i.e. yeast extract) in the medium and produces typically mixed

isomers of lactic acid, filamentous fungi are able to produce lactic acid on low-cost carbon sources, such as plant biomass or waste paper, and produces only L-(+)-lactic acid (Park et al. 2004; Zhang et al. 2007b; Yadav et al. 2011; Liaud et al. 2015). Synthesis of lactic acid from pyruvate is catalysed by a NAD-dependent lactic acid dehydrogenase (LDH). In *R. oryzae*, two LDH genes, *ldhA* and *ldhB*, were identified (Skory 2000). *R. oryzae* strains having both *ldhA* and *ldhB* genes accumulate predominantly lactic acid (LA), while those isolates, which lack functional *ldhA* accumulate predominantly fumaric and malic acids (FMA) (Skory and Ibrahim 2007). In a molecular phylogenetic study, lactic acid producing strains and FMA producers clearly belonged to two distinct clades and the name *R. delemar* was proposed for the FMA producing strains (Abe et al. 2007). Lactic acid production of *R. oryzae* could be increased by the introduction of the endogenous *ldhA* gene into the fungus in multiple copies using autonomously replicating plasmids (Skory 2004). Lactic acid production competes with ethanol and fumaric acid (FA) formation; thus, transformants with increased lactic acid accumulation showed decreased ethanol and FA production.

Certain *Aspergillus* species are used to produce organic acids (primarily citric- and itaconic acids) at industrial scales. Expression by multicopy integration of the *R. oryzae ldhA* gene in the *Aspergillus brasiliensis* BRFM103 genome resulted in a large amount (13.9 g/L) of lactic acid production and a 27 % L-lactic acid conversion yield (Liaud et al. 2015). *R. oryzae ldhA* was also expressed in *S. cerevisiae* under the control of the yeast *adh1* (alcohol dehydrogenase 1) promoter (Skory 2003). The recombinant yeast strain produced 38 g/L lactic acid with a yield of 0.44 g lactic acid/g glucose. Interestingly, if *R. oryzae ldhA* was expressed in *S. cerevisiae* mutants with reduced pyruvate decarboxylase activity and disrupted alcohol dehydrogenase activity, besides the reduced ethanol production, lactic acid production was also decreased (Skory 2003). The same gene was integrated into the genome of an engineered *S. cerevisiae* strain capable of fermenting xylose and overexpressed under the control of *pgk1* promoter. When cultured on xylose, the recombinant strain produced lactic acid as the main product with a high yield (0.69 g/g xylose) and with a very low amount of ethanol (<0.01 g/g xylose) indicating that lactic acid can be fermented from xylose by *S. cerevisiae* (Turner et al. 2015). The L-lactate dehydrogenase of three species, *R. oryzae*, *Lactobacillus helveticus* and *Bacillus megaterium* was expressed in the yeast *Candida sonorensis* and lactate production of the resulting transformants were compared (Ilmén et al. 2013). Strains harbouring the *R. oryzae ldhA* gene produced significantly less lactate than the strains harbouring the *L. helveticus* or *B. megaterium ldh* genes. *R. oryzae ldhA* gene was also expressed in acid-resistant *Candida magnolia*. The mutant yeast was able to grow at pH 2.5, while the optimum for lactic acid production was pH 3.5 (Zhang et al. 2011).

Ethanol and FA are also synthesized from glucose via pyruvate and their production competes with that of lactic acid. Three enzymes, pyruvate carboxylase (RoPYC), malate dehydrogenase (RoMDH) and fumarase (RoFUM) are involved in the FA biosynthesis. Expression of the *R. oryzae RoPYC* gene encoding a pyruvate carboxylase under the control of its own promoter in uracil auxotrophic

R. oryzae resulted in low yield of FA (less than 0.05 g/g glucose) and ethanol (0.13–0.36 g/g glucose) accumulation (Zhang et al. 2012). FA accumulation could be increased (from 0.62 to 0.78 g/g glucose) by expressing the *E. coli pepc* gene (encoding a phosphoenolpyruvate carboxylase described only in bacteria and plants) in *R. oryzae* under the regulation of endogenous *pgk1* promoter and *pdcA* terminator to increase the carbon flux towards oxaloacetic acid (Zhang et al. 2012).

Transformation of *R. oryzae* by plasmids containing the endogenous fumarase encoding gene (*fumR*) under the control of its own promoter significantly increased the fumarase activity in the transformants (Zhang and Yang 2012). However, instead of FA production, malic acid (MA) accumulation increased in the transformants by 2–2.5 times compared to the wild-type strain, because the overexpressed fumarase also catalysed the hydration of FA to MA (Zhang and Yang 2012). In contrast, when the *R. oryzae fumR* was expressed in *E. coli* BL21, and the enzyme was purified and characterized, FUMR has been found to have a preference to convert L-MA to FA and an overexpression of the gene may result increased FA accumulation (Song et al. 2011). Moreover, the presence of both cytosolic and mitochondrial isozymes of the *R. oryzae* fumarase has been presumed (Goldberg et al. 2006; Song et al. 2011). The FUMR characterized by Song et al. (2011) can be the cytosolic enzyme, with a 15-amino acid deletion compared to the other enzyme described by Zhang and Yang (2012), which may cause its different activity and distribution (Song et al. 2011).

The *RoPYC*, *RoMDH* and *RoFUM1* genes of the *R. oryzae* FM19 strain were expressed in the xylose-utilizing *Scheffersomyces (Pichia) stipitis* to overexpress the reductive pathway (Wei et al. 2015). Under oxygen-limited condition and using xylose as the sole carbon source, the transformants produced 14.5 times higher amount of FA than the untransformed strain. The enzyme activity could be further improved by codon optimization. FA accumulation of the host, *S. stipitis* was also enhanced by blocking the FA conversion in the tricarboxylic acid cycle by double knock-out of the endogenous fumarase genes (*Psfum1* and *Psfum2*) and improving the FA transportation by overexpressing of the heterologous *YMAE1* (encoding a codon optimized *Schizosaccharomyces pombe* transporter) under the control of the strong constitutive *TEF1* (translation elongation factor 1) promoter. This mutant transformed with the codon optimized *R. oryzae* genes accumulated 37.92 times higher amount of FA (4.67 g/L) than the original strain (Wei et al. 2015).

Expression of the *RoMDH* and its co-expression with *RoFUM1* in *S. cerevisiae* led to an FA accumulation of 0.54 and 0.38 g/L, respectively (Xu et al. 2012a). At the same time, overexpression of the endogenous *PYC2* gene together with the expression of *RoMDH* increased the FA accumulation with 489 % reaching 3.18 g/L (Xu et al. 2012a). In another study, expression of the *RoPYC* in *fum1* knock out mutant *S. cerevisiae* increased FA production from 0.6 to 1.1 g/L (Xu et al. 2012b). Transformation with the endogenous *SFC1* gene encoding a succinate—fumarate transporter led to an FA accumulation of 1.6–1.7 g/L (Xu et al. 2012b). Co-expression of the *RoPYC*, *RoMDH* and *RoFUM1* genes in the *fum1* mutant *S. cerevisiae* increased the FA accumulation from 0.25 to 2.45 g/L; by optimizing the C/N ratio this strain produced 5.64 g/L FA (Xu et al. 2013). Interestingly, expression of only

the *RoMDH* and *RoFUM1* genes in the same recipient strain led to a significant decrease in FA accumulation (Xu et al. 2013).

5 Gene Expression Studies for Production of Carotenoids

Members of the order Mucorales are known to be β-carotene producing fungi, especially *B. trispora*, *P. blakesleeanus* and *M. circinelloides*. Although *B. trispora* is an industrial source of β-carotene and *P. blakesleeanus* is a traditional model organism of the carotenoid biosynthesis, no successful genetic transformation has been carried out in them (Obraztsova et al. 2004; Sanz et al. 2011; Garre et al. 2015). In contrast, *M. circinelloides* is more amenable to molecular techniques than the other two species; efficient transformation systems are available for this fungus and it is able to maintain and express exogenous genes (Papp et al. 2006, 2013; Garre et al. 2015). The biosynthetic process is well-resolved in all three organisms and structural carotenogenic genes have been identified in them (Fig. 1a; Velayos et al. 2000a,b; Rodríguez-Sáiz et al. 2004; Almeida and Cerdá-Olmedo 2008). The function of these biosynthetic genes was examined and proven by their heterologous expression in *M. circinelloides* (Rodríguez-Sáiz et al. 2004; Sanz et al. 2011).

Similar to some other fungi, structural genes of the carotenoid specific biosynthetic pathway are induced by blue light in *Mucor*, thus, this fungus requires light for carotenoid production. A negative regulator of the carotenoid biosynthesis is the protein encoded by the *crgA* gene (Navarro et al. 2001). Deletion of *crgA* led to overexpression of carotenogenic genes under both light and dark conditions (Silva et al. 2008). In *M. circinelloides* strain MU202 lacking lycopene cyclase activity and accumulating lycopene, deletion of *crgA* resulted in enhanced lycopene accumulation (Nicolás-Molina et al. 2008). This modified strain was able to produce 54 g/L lycopene in a complex enriched liquid medium.

Similar to other Mucoral fungi, *M. circinelloides* produces β-carotene as the main carotenoid. However, it is able to produce the hydroxylated derivatives of β-carotene, such as zeaxanthin and β-cryptoxanthin, in small amounts (Papp et al. 2006). The amount of these hydroxy-carotenoids can be increased and production of other oxygenated β-carotene derivatives (i.e. xanthophylls) can be achieved by expressing exogenous genes. The cytochrome P-450 hydroxylase gene (*crtS*) is responsible for the formation of astaxanthin in the basidiomycetous yeast, *Xanthophyllomyces dendrorhous.* Expression of this gene under the control of the *Blakeslea carRA* (phytoene synthase-lycopene cyclase) promoter increased the β-cryptoxanthin and zeaxanthin content of *M. circinelloides* (Álvarez et al. 2006). When *crtS* was driven by the *Mucor gpd1* promoter, *M. circinelloides* transformants were able to produce the keto-derivatives of β-carotene (i.e. echinenone, canthaxanthin and a small amount astaxanthin) besides an increased level of β-cryptoxanthin and zeaxanthin (Csernetics et al. 2015). *Paracoccus* sp. N81106 is a marine astaxanthin producing bacterium. Its β-carotene ketolase (*crtW*) and hydroxylase genes *(crtZ*) were placed under the control of the *Mucor gpd1* promoter and terminator

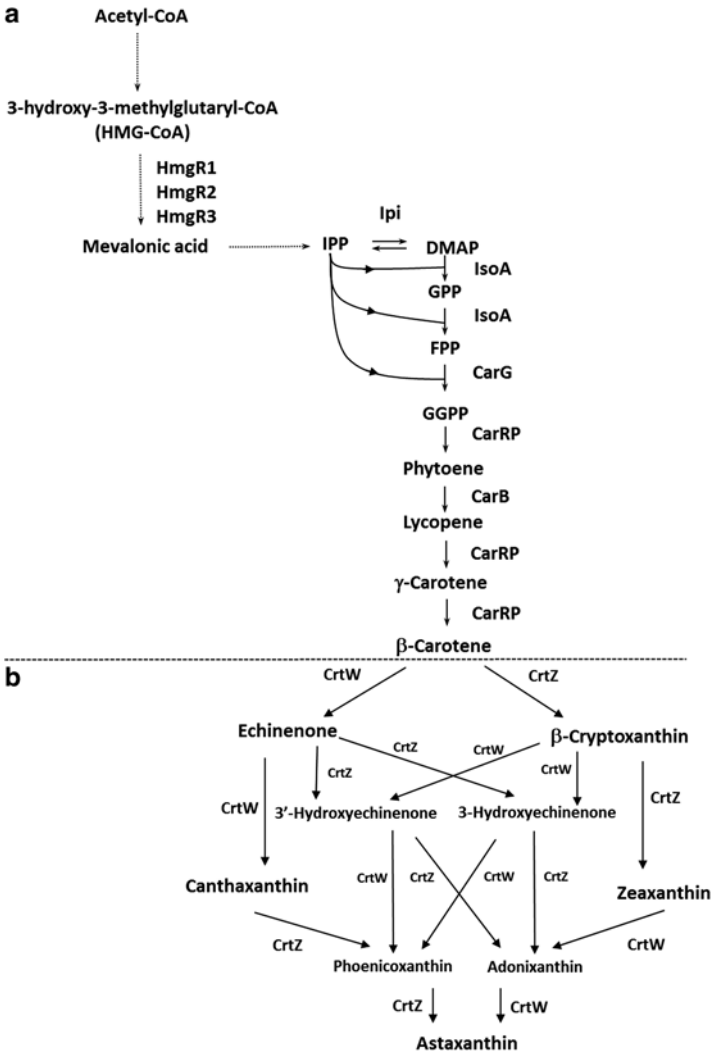

Fig. 1 Carotenoid biosynthesis pathway in *M. circinelloides* (**a**) and possible steps of the β-carotene—astaxanthin conversion in *M. circinelloides* transformants harbouring the *Paracoccus* sp. N81106 *crtW* and *crtZ* genes (**b**). HMG-CoA—3-hydroxy-3-methylglutaryl-CoA; IPP—isopentenyl pyrophosphate; DMAP—dimethylallyl pyrophosphate; GPP—geranyl pyrophosphate; FPP—farnesyl pyrophosphate; GGPP—geranylgeranyl pyrophosphate. *M. circinelloides* enzymes: HmgR1-3—HMG-CoA reductases, Ipi—IPP isomerase; IsoA—FPP synthase; CarG—GGPP synthase; CarRP—phytoene synthase/lycopene cyclase; CarB—phytoene desaturase. *Paracoccus* sp. N81106 enzymes: CrtZ—β-carotene hydroxylase and CrtW—β-carotene ketolase

and they were transformed into *M. circinelloides* on autonomously replicating plasmids (Papp et al. 2006; Csernetics et al. 2011). Transformants produced β-cryptoxanthin, zeaxanthin, canthaxanthin and astaxanthin in low amounts (Fig. 1b). The *crtW* gene was later integrated into the *M. circinelloides* genome by gene replacement and restriction enzyme-mediated integration and transformants producing canthaxanthin as the main carotenoid could be selected. These strains produced more than 200 μg/g (dry mass) of canthaxanthin (Papp et al. 2013).

Carotenoids are terpenoid compounds and their precursors are synthesized via the so-called mevalonic acid (MVA) pathway in fungi (Fig. 1a). Therefore, expression of the enzymes of this pathway may affect the carotenoid level as well. Overexpression of the endogenous genes of isopentenyl pyrophosphate isomerase (*ipi*), farnesyl pyrophosphate synthase (*isoA*) and geranylgeranyl pyrophosphate synthase (*carG*) by increasing their copy numbers using autonomously replicating vectors and changing their promoters to that of the *Mucor gpd1* significantly enhanced the carotenoid production of *M. circinelloides* (Csernetics et al. 2011). The highest carotenoid content, more than 2 mg/g (dry weight), was achieved in those strains, which expressed the *carG* gene driven by the *gpd1* promoter. Similarly, enhanced carotenoid production was observed when the isopentenyl pyrophosphate isomerase (*IPI*) and the geranylgeranyl pyrophosphate synthase (*GGPS*) genes of *B. trispora* were expressed in engineered, carotenoid producing *E. coli* (Sun et al. 2012). A rate-limiting step of the MVA pathway is the conversion of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonic acid catalysed by the HMG-CoA reductase (HmgR) (Fig. 1a). The *M. circinelloides* genome contains three HmgR genes (*hmgR1*, *hmgR2* and *hmgR3*) (Nagy et al. 2014). Increasing the copy number of *hmgR2* and *hmgR3* significantly enhanced the carotenoid and ergosterol production in *M. circinelloides* (Nagy et al. 2014).

6 Production of Polyunsaturated Fatty Acids (PUFAs)

Oleaginous microorganisms, as alternatives to agricultural and animal oil sources, have been intensively studied. Among Mucoromycotina fungi, *Mortierella* species are well-known producers of long-chain PUFAs, especially arachidonic acid (AA). *Mo. alpina* is an industrial PUFA producer synthesizing mainly AA besides other ω-3 and ω-6 PUFAs, which can be used by the food- and pharmaceutical industries (Dyal and Narine 2005). Members of the genera *Mucor*, *Rhizopus* and *Umbelopsis* can also be considered as oleaginous fungi but they are capable to produce only 18 carbon atomic PUFAs, such as γ-linolenic acid (GLA), α-linolenic acid (ALA), linoleic acid (LA) and oleic acid (OA).

PUFA biosynthesis is well revealed in *Mo. alpina*. Mono- and polyunsaturated fatty acids are formed from saturated fatty acids (i.e. stearic acid or palmitic acid) by different desaturases and elongases, which form double bonds or elongate the carbon chains by two-carbon-atomic units (Fig. 2). *Mo. alpina* genes encoding the different fatty acid desaturases and elongases were identified in the nineties.

Fig. 2 PUFA biosynthetic pathways in *Mo. alpina*. The major pathway (ω-6) is signed with encircled fatty acids. Certain ω-3 fatty acids (ALA, EPA) can be also formed at low temperature, they were signed with dashed lines. The heterologously expressed PavELO elongase and its products signed with grey shades

Function of *Mo. alpina* Δ5-, Δ12-, Δ6-, Δ9- and ω3-fatty acid desaturase genes were confirmed by their heterologous expression in *S. cerevisiae*, which resulted in accumulation of AA, LA, GLA, OA and ω3-PUFAs, respectively (Knutzon et al. 1998; Huang et al. 1999; MacKenzie et al. 2002). A *Mo. alpina* cDNA expression library was also constructed in a yeast expression vector, by which the so-called GLELO elongase was identified and its activity was measured by the conversion of GLA to dihomo-γ-linolenic acid (DGLA) (Parker-Barnes et al. 2000). By its expression in *S. cerevisiae*, the *MAELO* gene from *Mo. alpina* was confirmed to encode a fatty acid elongase involved in the elongation of very long-chain saturated fatty acids (VLCSFAs) (Sakuradani et al. 2008). Function of *Mo. alpina* ω3-desaturase was also studied in *S. cerevisiae*, and it was found to be a trifunctional Δ12/Δ15/ω3-desaturase having Δ12- and Δ15-desaturation activities for 16-carbon fatty acid,

in addition to its major function, which is ω3-desaturation (Kikukawa et al. 2013). Function of PUFA biosynthetic genes were also studied by expressing them in *Aspergillus oryzae* using the maltose inducible α-amylase or glucoamylase promoters (Sakuradani et al. 1999a, b, c; Sakuradani and Shimizu 2003; Abe et al. 2005, 2006; Takeno et al. 2005a). Expression of the genes caused significant changes in the lipid composition of the *Aspergillus* transformants and revealed the desaturase or elongase activities of the corresponding enzymes. Heterologous expression of *Mo. alpina* Δ6-desaturase gene in *Pichia pastoris* was also performed resulting in GLA accumulation in the yeast cells (Li et al. 2004b; Liu et al. 2011).

PUFA biosynthetic genes were also identified in other Mucoromycotina species. For example, the Δ12-desaturase gene of *Umbelopsis isabellina* has been cloned and its function was proven by expressing the gene in *S. cerevisiae* and *E. coli*, the produced enzyme converted OA to LA (Li et al. 2006). To elucidate the function and substrate specificity of Δ9-desaturases from *Rhizopus arrhizus* and *Cunninghamella echinulata* and Δ6-desaturases from *M. rouxii*, *R. arrhizus*, *Rhizopus nigricans*, *R. oryzae*, *Rhizopus stolonifer* and *Thamnidium elegans*, the identified genes were also successfully expressed in *S. cerevisiae* and *P. pastoris* (Wei et al. 2009; Wan et al. 2013; Laoteng et al. 2000; Wang et al. 2006, 2007; Zhang et al. 2004, 2005; Lu et al. 2007, 2009; Zhu and Zhang 2013).

Biosynthetic genes isolated from Mucoromycotina fungi have been used for metabolic engineering of yeasts to improve their PUFA production. By co-expressing Δ12- and Δ6-desaturase genes of *M. rouxii* in *S. cerevisiae*, LA and GLA accumulation could be achieved (Ruenwai et al. 2011). However, it was revealed that PUFAs can interfere with important cellular pathways in yeast, such as the oxidative stress response or proteasomal activity, suggesting that membrane composition has a fundamental role in these processes. Eicosapentaenoic acid (EPA) production was also carried out in *S. cerevisiae* by expressing *Mo. alpina* Δ5-desaturase (Tavares et al. 2011). Genetic engineering of *Yarrowia lipolytica* was performed using *Mo. alpina* genes; transformation by the Δ12- and Δ6-desaturase genes on a dual cassette vector led to a significant increase in the GLA level (Chuang et al. 2010). In the same yeast, co-expression of *Mo. alpina* Δ12-desaturase gene and the codon-optimized linoleic acid isomerase gene (*opai*) from *Propionibacterium acnes* enhanced the production of trans-10, cis-12 conjugated LA up to 10 % of total fatty acids (Zhang et al. 2013). The thermotolerant yeast *Hansenula polymorpha* is also a possible host for producing different PUFAs; GLA production has been successfully achieved by expressing Δ6-desaturase gene from *M. rouxii* (Laoteng et al. 2005). Moreover, using of a mutated version of the same gene further enhanced the GLA production (Khongto et al. 2010). The *ACC* gene encoding acetyl-CoA carboxylase from *M. rouxii* was also expressed in *H. polymorpha* resulting in a 40 % increase in the total fatty acid content (Ruenwai et al. 2009). GLA production in the oleaginous yeast *Lipomyces kononenkoae* was also achieved by overexpressing the Δ6-desaturase gene of *C. echinulata*, however, the proportion of GLA remained very low (1.2 %) in the total fatty acids of the transformed yeast (Wang et al. 2011).

Mortierella and *Umbelopsis* PUFA biosynthetic genes were also expressed in different oil producing plants. Canola oil is made from the seeds of *Brassica napus*.

This oil is low in saturated fatty acids and contains both ω-6 and ω-3 fatty acids in a ratio of 2:1, mostly OA (61 %), LA and ALA. Expression of the *Mo. alpina* Δ5-desaturase gene resulted in transgenic canola seeds producing taxoleic acid (C18:2Δ5,9) and pinolenic acid (C18:3Δ5,9,12), the Δ5-desaturation products of OA and LA, respectively (Knutzon et al. 1998). Soybean oil is extracted from *Glycine max* seeds and contains mostly LA (51 %), OA and ALA. Expression of *U. isabellina* Δ6-desaturase gene in transgenic soybean resulted in the presence of GLA (27 %) in the fatty acid profile (Li et al. 2004a). Seed-specific expression of the genes encoding Δ6- and Δ5-desaturases and GLELO elongase from *Mo. alpina* and the down-regulation of the endogenous Δ15-desaturase resulted in GLA, 8,11-eicosadienoic acid (EDA), DGLA and AA production (Chen et al. 2006). In soybean, amount of the seed-stored oil and protein is inversely correlated as an increase in the amount of stored oil causing decreasing protein level and vice versa. Lardizabal et al. (2008) solved this problem by expressing a codon-optimized diacylglycerol acyltransferase 2A gene from *Umbelopsis ramanniana* in soybean obtaining transgenic soy crop with increased oil (1.5 %) in the mature seeds with no impact on protein content or yield. Besides soybean, GLA producing transgenic plants could be created by expressing *U. isabellina* and *Mo. alpina* Δ6-desaturase genes in tobacco (Li et al. 2003), adzuki bean and *Lotus japonicus* (Chen et al. 2005). Since Δ6-desaturase is able to use both LA and ALA as substrates, octadecatetraenoic acid (OTA) production could also be achieved in some plants (Li et al. 2003). Recently, high level of GLA production (50 % of total fatty acids) was reached by expressing *Mo. alpina* Δ6- and Δ12-desaturase genes in transgenic safflower seeds (Nykiforuk et al. 2012). These findings revealed that metabolic engineering of the PUFA biosynthetic pathway in order to produce specific PUFAs in oilseed crops can be a promising tool.

PUFA production also can be improved in *Mortierella*, *Umbelopsis* and *Mucor* strains by overexpressing of homologous or heterologous PUFA biosynthetic genes. Overexpression of the homologous *GLELO* elongase gene in *Mo. alpina* increased the AA production in the transformants (Takeno et al. 2005a), while overexpression of the endogenous ω3-desaturase in *Mo. alpina* increased the EPA content up to 35 % of total lipids and decreased the AA production (Ando et al. 2009b). In a recent study, the endogenous Δ12-desaturase and *malce1* elongase genes are reported to be overexpressed in *Mo. alpina* 1S-4 and its mutant strain resulting in elevated AA production (Sakuradani et al. 2013).

NADP(+)-dependent malic enzyme (ME) catalyses the oxidative decarboxylation of L-malate to pyruvate and provides NADPH for intracellular metabolism, such as fatty acid synthesis (Hao et al. 2014a). In *M. circinelloides*, the endogenous ME coding *malE* gene and the corresponding gene from *Mo. alpina* was overexpressed by using the *Mucor gpd1* promoter. Expression of both *malE* genes enhanced fatty acid biosynthesis and formation of unsaturated fatty acids, including GLA (Zhang et al. 2007a). Similarly, heterologous expression of the *M. circinelloides malE* gene increased the lipid accumulation in the oleaginous yeast *Rhodotorula glutinis* (Li et al. 2013). Thus, the previous hypothesis that the rate-limiting step of fatty acid biosynthesis is the supply of NADPH generated by ME, seemed to be

considerably strengthened by these results. At the same time, overexpression of the endogenous *malA*, which were previously associated with lipid accumulation, did not increase the lipid content of *M. circinelloides* (Rodríguez-Frómeta et al. 2013). It should be mentioned that *M. circinelloides* has five ME genes encoding six isoforms, which may have different role and/or importance in the fatty acid biosynthesis. The gene *malA* encodes isoform III, from which the isoform IV is formed by post-translational modifications. Recently, overexpression of the endogenous cytosolic and mitochondrial ME genes, *malE1* and *malE2*, respectively, led to different results in *Mo. alpina* (Hao et al. 2014a, b). Enhanced expression of *malE1* increased the total fatty acid content by 30 %, while overexpression of *malE2* increased the AA production by 60 % and did not affect the total fatty acid content. Based on these studies, Hao et al. (2014a) suggested *malE2* and the encoded mitochondrial ME as effective tools to improve industrial AA production in *Mo. alpina*.

Gene silencing may also be a possible tool to modify PUFA production in *Mo. alpina*. Transformants, in which the Δ12-desaturase gene was silenced by RNAi technique, accumulated primarily ω-9 fatty acids instead of ω-6 PUFAs (Takeno et al. 2005b) and RNAi silencing of the Δ12-desaturase gene in a mutant, which was defective in MALCE1 elongase activity, led to the accumulation of ω-7 PUFAs (Fig. 2) (Sakuradani et al. 2013). Silencing the *MAELO* gene by RNAi resulted in the production of 20-, 22-, and 24-carbon saturated fatty acids in low and stearic acid in high amount in the transformants (Sakuradani et al. 2008).

Heterologous expression of genes from other oleaginous organisms in *Mo. alpina* has also been achieved recently. Sakuradani et al. (2013) expressed the *PavΔ5* and *OstΔ6* desaturase genes from microalgae *Pavlova salina* and *Ostreococcus lucimarinus*, respectively, resulting in higher rates of DGLA and AA in the total fatty acids. Furthermore, by the co-expression of the endogenous ω3-desaturase and *PavElO* elongase genes from *Pavlova*, production of C22 PUFAs, such as ω-6 docosatetraenoic acid (22:4n–6) and ω-3 docosapentaenoic acid (22:5n–3) was achieved in *Mo. alpina* (Fig. 2). In another study, expression of the *Saprolegnia diclina* Δ17-desaturase gene (*sdd17m*) in a ω3-desaturation-defective mutant *Mo. alpina* caused EPA accumulation (20 % of total lipids) in the transformants at low and ordinary temperature (Okuda et al. 2015).

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Homologous and Heterologous Expression of Basidiomycete Genes Related to Plant Biomass Degradation

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1 Introduction

The necessity to move towards a bio-based economy creates many challenges for industry and academia. Industries dedicated to supplying the population with food, feed, detergents, textile and other products collaborate with researchers to develop efficient biotechnological tools. This includes the development of optimal enzyme sets to convert plant biomass into valuable products. Recently there has been a strong increase in the demand of these enzymes due to their growing biotechnological significance (Harris et al. 2014).

Fungi play an important role in plant biomass degradation; therefore improving the production of fungal enzymes is of great interest for biotechnology. Fungal enzymes have been mainly studied in saprobic ascomycetes, and much less extensively in basidiomycetes. This is largely caused by the better manageability of ascomycetes in industrial fermentations and the availability of transformation systems for many ascomycetes, while these aspects are still a major challenge in basidiomycetes. Despite this, basidiomycetes form a promising source of novel enzymes with different biochemical properties than ascomycete enzymes.

Plant biomass consists mainly of polysaccharides, proteins and lignin (Pauly and Keegstra 2010). Ascomycete enzyme systems focus largely on the polysaccharide

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fraction but they rarely modify the aromatic lignin polymer, due to the absence of lignin-modifying peroxidases in their genomes (Floudas et al. 2012). They do produce laccases, which have been implicated to potentially modify lignin (Xie et al. 2014). In contrast, basidiomycetes include the most efficient lignin decomposers, but they also degrade polysaccharides. The lignin-modifying enzymes of basidiomycetes are of fundamental importance for efficient bioconversion of plant residues that are rich in lignin, such as wheat straw. They are therefore highly relevant for various biotechnological applications, for example pulp and paper, and textile industries, bioremediation, and the production of biofuels and biochemicals (Jolivalt et al. 2005; Raghukumar et al. 2008). With respect to plant biomass degradation, basidiomycetes are classified either as white-rot, brown-rot or litter-decomposing fungi. White- and brown-rot refer to the appearance of wood after fungal decomposition. Brown-rot fungi efficiently degrade wood polysaccharides but are only slightly capable of modifying lignin, resulting in a brownish residue of partly decayed wood. In contrast, white-rot fungi are able to decompose all wood polymers, including lignin, resulting in a white residue, consisting mainly of cellulose (Lee et al. 2014; Ruiz-Dueñas et al. 2013; Rytioja et al. 2014).

1.1 Plant Polysaccharide Degrading Enzymes

Cellulose is the most abundant polysaccharide in plant biomass. This polymer gives rigidity to the plant cell wall and is formed by chains of β-1,4-linked D-glucose molecules that create linear crystalline structures (microfibrils) and less crystalline, amorphous regions (Kolpak and Blackwell 1976).

Several types of hemicelluloses are present in plant biomass: xyloglucan, xylan, mannan, glucomannan, and β-1,3/1,4-glucans. They are named after the monosaccharides that form their backbone (de Vries and Visser 2001). In addition, they contain various branches consisting of monomers and short oligomers.

Pectin is a heteropolysaccharide mainly composed of three principal structures (Caffal and Mohnen 2009). The simplest one is homogalacturonan, a galacturonic acid based linear polysaccharide. Xylogalacturonan is modified form of homogalacturonan in that it contains xylose branches. Rhamnogalacturonan I has a backbone of alternating galacturonic acid and rhamnose residues, with arabinan and (arabino-)galactan side chains attached to the rhamnose residues.

Each plant polysaccharide degrading fungus has a specific set of carbohydrate-active enzymes involved in the degradation of the backbone and branching structures (Culleton et al. 2013; van den Brink and de Vries 2011). The different enzymes involved in this process with their CAZy family (Lombard et al. 2014), EC number and substrate are listed in Table 1. While some enzymes act only on a single polysaccharide (e.g. cellobiohydrolase on cellulose), others are involved in the degradation of several polysaccharides (e.g. β-galactosidase for xylan, pectin, xyloglucan and galactomannan). An overview of the available knowledge on plant polysaccharide degrading enzymes from basidiomycetes was recently presented (Rytioja et al. 2014).

Table 1 Fungal CAZymes involved in plant biomass degradation. Based on (Coutinho et al. 2009; Lombard et al. 2014)

CAZy families	Enzyme code	Enzyme	EC number	Substrate
Glycoside hydrolases				
GH1,3	BGL	β-1,4-D-Glucosidase	3.2.1.21	Cellulose, xyloglucan, galactoglucomannan
GH2,35	LAC	β-1,4-D-Galactosidase	3.2.1.23	Xylan, xyloglucan, galactomannan, pectin
GH2	MND	β-1,4-D-Mannosidase	3.2.1.25	Galactomannan
GH3,39,43	BXL	β-1,4-D-Xylosidase	3.2.1.37	Xylan, pectin
GH5,7,12,61,131	EGL	β-1,4-D-Endoglucanase	3.2.1.4	Cellulose
GH5,26	MAN	β-1,4-D-Endomannanase	3.2.1.78	Galactomannan
GH5	GLN	β-1,6-Endogalactanase	3.2.1.164	Pectin
GH6,7	CBH	Cellobiohydrolase	3.2.1.91	Cellulose
GH10,11	XLN	β-1,4-D-Endoxylanase	3.2.1.8	Xylan
GH12,74	XEG	Xyloglucan-active β-1,4-D-endoglucanase	3.2.1.151	Xyloglucan
GH13	AMY	α-Amylase	3.2.1.1	Starch
GH15	GLA	Glucoamylase	3.2.1.3	Starch
GH27,36	AGL	α-1,4-D-Galactosidase	3.2.1.22	Xylan, xyloglucan, galactomannan
GH28	PGA	Endopolygalacturonase	3.2.1.15	Pectin
GH28	PGX	Exopolygalacturonase	3.2.1.67	Pectin
GH28	RGX	Rhamnogalacturonan galaturonohydrolase/ exorhamnogalacturonase	3.2.1.40	Pectin
GH28	RHG	Rhamnogalacturonan hydrolase/endorhamnogalacturonase	3.2.1.–	Pectin
GH29,95	AFC	α-L-Fucosidase	3.2.1.51	Xyloglucan
GH30	XBH	Xylobiohydrolase	3.2.1.–	Xylan

(continued)

Table 1 (continued)

CAZy families	Enzyme code	Enzyme	EC number	Substrate
GH31	AGD	α-1,4-D-Glucosidase	3.2.1.20	Starch
GH31	AXL	α-D-Xylosidase	3.2.1.–	Xyloglucan
GH32	INU	Endo-inulinase	3.2.1.7	Inulin
GH32	INX	Exo-inulinase	3.2.1.80	Inulin
GH32	SUC	Invertase/fructofuranosidase	3.2.1.26	Inulin
GH43,51,54	ABF	α-L-Arabinofuranosidase	3.2.1.55	Xylan, xyloglucan, pectin
GH43	ABN	Endoarabinanase	3.2.1.99	Pectin
GH43	XTG	β-1,3-Exogalactanase	3.2.1.145	Pectin
GH53	GAL	β-1,4-Endogalactanase	3.2.1.89	Pectin
GH62	AXH	Arabinoxylan arabinofuranohydrolase	3.2.1.55	Xylan
GH67,115	AGU	α-Glucuronidase	3.2.1.139	Xylan
GH78	RHA	α-Rhamnosidase/rhamnogalacturonan rhamnohydrolase	3.2.1.40	Pectin
GH88	UGH	D-4,5-Unsaturated glucuronyl hydrolase	3.2.1.–	Pectin
GH93	ABX	Exoarabinanase	3.2.1.–	Pectin
GH105	URH	Unsaturated rhamnogalacturonan hydrolase	3.2.1.–	Pectin
GH131	AMG	Amylo-α-1,6-glucosidase	3.2.1.33	Starch
–	XFG	β-1,4-Exogalactanase	–	Pectin
–	XSG	β-1,6-Exogalactanase	–	Pectin
Carbohydrate esterases				
CE1,2,3,4,5,6	AXE	Acetyl xylan esterase	3.1.1.72	Xylan
CE1	FAE	Feruloyl esterase	3.1.1.73	Xylan, pectin
CE8	PME	Pectin methyl esterase	3.1.1.11	Pectin
CE12	RGAE	Rhamnogalacturonan acetyl esterase	3.1.1.–	Pectin
CE12	PAE	Pectin acetyl esterase	3.1.1.–	Pectin
CE15	GE	Glucuronoyl esterase	3.1.1.–	Xylan

–	GMAE	Galactomannan acetyl esterase	–	Galactomannan
–	XGAE	Xyloglucan acetylesterase	–	Xyloglucan
Polysaccharide lyases				
PL1	PEL	Pectin lyase	4.2.2.10	Pectin
PL1,3,9	PLY	Pectate lyase	4.2.2.2	Pectin
PL4,11	RGL	Rhamnogalacturonan lyase	4.2.2.–	Pectin
Auxiliary activities				
AA1_1	LCC	Laccase	1.10.3.2	Lignin
AA2	MnP	Manganese peroxidase	1.11.1.13	Lignin
AA2	VP	Versatile peroxidase	1.11.1.16	Lignin
AA2	LiP	Lignin peroxidase	1.11.1.14	Lignin
AA3,8	CDH	Cellobiose dehydrogenase	1.1.99.18	Cellobiose
AA9,10,13	LPMO	Lytic polysaccharide monooxygenase		Cellulose , xylan, xyloglucan, starch

1.2 Lignin-Modifying Enzymes

Lignin is a complex heterogenous aromatic polymer that is among the most abundant natural materials on earth (Martinez et al. 2004). Lignin is present in all layers of woody cell walls and is composed of three monomers: coniferyl alcohol, sinapyl alcohol and *p*-coumaryl alcohol. Plant laccases and peroxidases polymerize these monomers to guaiacyl-, syringyl- and hydroxyphenyl-type of lignin subunits (Higuchi 2006). The most common linkage between the subunits is a β-aryl-ether linkage (Adler 1977), but other linkages also occur (Brunow et al. 1998). The structure and composition of lignin varies significantly depending on the plant species (Martínez et al. 2008; Ralph et al. 2004). Lignin is an undesirable component e.g. in the conversion of wood into pulp and paper and remova1 of lignin is a major step in the paper making process (Campbell and Sederoff 1996). White-rot basidiomycetes are studied due to their efficient and complete degradation of woody plant cell walls (Ruiz-Dueñas et al. 2008). In contrast to other fungi and bacteria they are capable of mineralising lignin to carbon dioxide and water by producing extracellular, oxidative lignin-modifying enzymes. These include class II heme-containing peroxidases (AA2 in CAZy database), i.e. manganese peroxidases (MnPs), lignin peroxidases (LiPs) and versatile peroxidases (VPs), which catalyse H_2O_2-dependent unspecific reactions (Table 1) (Mäkelä et al. 2014). Various phenolic compounds are oxidised to organic radicals via the action of MnPs, while LiPs are able to oxidise non-phenolic lignin substructures. VP is a hybrid type of peroxidase combining the activities of both MnP and LiP (Kamitsuji et al. 2005; Ruiz-Dueñas et al. 2001).

Fungal laccases (AA1_1 in CAZy database) belong to the widely distributed family of multicopper oxidases and participate in many processes from fungal morphogenesis to melanin synthesis (Giardina et al. 2010; Kües and Rühl 2011). Laccases catalyse the oxidation of phenolic compounds and aromatic amines coupled to the reduction of molecular oxygen to water (Giardina et al. 2010). In the presence of small molecular weight mediator molecules laccases can also oxidise non-phenolic compounds. Although the role of laccases in lignin degradation is still debatable, they are usually regarded as lignin-modifying enzymes (Lundell et al. 2010). Laccases can be used in various biotechnological processes. For example the white-rot fungus *Trametes pubescens* is considered an excellent producer of industrial laccases (Galhaup et al. 2002). Applications of laccases include bleaching of textiles (Vinod 2001) and wood pulp (Widsten and Kandelbauer 2008), clearing of fruit juice, beer and wine (Minussi et al. 2002), biosensors (Kulys and Vidziunaite 2003), hair dyes, degradation of plastics and decontamination of soils (Kunamneni et al. 2008).

Despite the huge biotechnological potential of fungal lignin-modifying enzymes, the production of both laccases and peroxidases to levels required by industry using homologous and heterologous expression remains a challenge and has been the topic of numerous research projects.

2 Homologous and Heterologous Expression of Basidiomycete Genes in Basidiomycetes

Overexpression of genes in basidiomycetes is less commonly performed than in ascomycetes. This is largely due to the availability of transformation systems for many ascomycetes, while only a relatively small number of basidiomycetes can be transformed. A well-developed transformation system based on auxotrophic mutants was generated for the white-rot fungus *Phanerochaete chrysosporium* that is the best studied basidiomycete with respect to plant biomass degradation (Mayfield et al. 1994). Transformation systems are also being developed for other basidiomycete species and significant progress has been made (e.g. Burns et al. 2005, 2006; Eastwood et al. 2008; Salame et al. 2012; Sharma and Kuhad 2010; Shi et al. 2012; Wang et al. 2008), but their efficiency is still significantly less than those of ascomycetes.

2.1 Homologous Expression

Homologous transformation of plant cell wall degradation related genes is limited to a small number of basidiomycete species and this involves mainly genes related to lignin degradation. The advantage of homologous overexpression is that the genes do not need to be modified to accommodate optimal codon usage or gene structure as is sometimes the case for heterologous overexpression (Kajita et al. 2004). Homologous expression in basidiomycetes has been reported for several genes (Irie et al. 2001; Li et al. 2000; Ma et al. 2003; Mayfield et al. 1994; Sollewijn Gelpke et al. 1999), and some examples are discussed in more detail below and listed in Table 2.

Laccases have been overproduced in some basidiomycete species. The genome of the white-rot fungus *Trametes* (*Coriolus*) *versicolor* contains several laccases but the highest secretion level was observed for laccase III (CVL3, Kajita et al. 2004). Improved production of this enzyme was achieved by transformation using a chimeric gene for laccase III under glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter of *T. versicolor* and hygromycin B phosphotransferase (*hph*) gene that encodes resistance to phleomycin and hygromycin B as a selection marker. In addition, a further improvement was made by the addition of copper (II) sulphate to the growth medium (Kajita et al. 2004).

Overexpression of laccase genes in the white-rot species *Polyporus brumalis* resulted in a three to fourfold increase in laccase activity (Ryu et al. 2008b). In this case, transformants were generated using the restriction enzyme mediated integration (REMI) method (Leem et al. 1999) and using hygromycin resistance selection. The *lcc1* gene of the coprophilic fungus *Coprinopsis cinerea* has been homologously expressed using different homologous and heterologous promoters. The highest activity of one of the *C. cinerea* transformants was reached under control of *gpd* II promoter from the litter-decomposing fungus *Agaricus bisporus* and with addition of copper to the medium (Kilaru et al. 2006).

Table 2 Homologous overproduction of basidiomycete enzymes involved in plant biomass degradation

	Enzyme	Enzyme	Protein/gene accession number	Species	Production level	References
Lignin modifying enzymes	Laccase (AA1_1)	Cvl3	BAA22153	*Coriolus* (*Trametes*) *versicolor*	0.95 μmol/min/mL oxidized guiacol	Kajita et al. (2004)
		Pblac1	EF362634	*Polyporus brumalis*	nr	Ryu et al. (2008b)
		Pblac2	EF362635		nr	
		Lcc1	AAR01242	*Coprinopsis cinerea*	3 μmol/min/mL ABTS[a] radical	Kilaru et al. (2006)
		Lac1	AAT28344	*Pycnoporus cinnabarinus*	4660 nkat/mL, 1.2 g/L	Alves et al. (2004)
	Lignin peroxidase (AA2)	LiPH8	ACY82388	*Phanerochaete chrysosporium*	2 mg/L	Sollewijn Gelpke et al. (1999)
		YK-LiP1	AB455006	*Phanerochaete sordida*	0.09 nkat/mL	Sugiura et al. (2009)
		YK-LiP2	AB455007	*P. sordida*	nr	Sugiura et al. (2009)
	Versatile peroxidase (AA2)	MnP4	AAK69187	*Pleurotus ostreatus*	0.12 μmol/min/mL oxidized phenol red	Salame et al. (2012)
	Manganese peroxidase (AA2)	MnP3	AAB39652	*P. ostreatus*	1.560 U/mL[b]	Irie et al. (2001)
		MnP4	AB585997	*P. sordida*	nr	Sugiura et al. (2012)

nr production level (mg/L) not reported

[a]2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

[b]One unit was defined as the amount of enzyme that results in an increase of one absorbance unit per minute at 465 nm

The production of a laccase of the white-rot fungus *Pycnoporus cinnabarinus* was significantly increased by overexpression under control of its own promoter, and *Schizophyllum commune GPD* and hydrophobin (*SC3*) promoters (Alves et al. 2004). The maximum levels (4660 nkat mL^{-1}) are the highest reported so far, highlighting the potential of this species for enzyme production.

As mentioned above, *P. chrysosporium* has been the most extensively studied white-rot basidiomycete and a transformation system for this fungus was already developed many years ago (Mayfield et al. 1994). The *P. chrysosporium* genome does not have laccase encoding genes, but this species produces a number of lignin-modifying heme peroxidases (Martinez et al. 2004).

Homologous production of lignin peroxidase (LiPH8) of *P. chrysosporium* has been successful (Sollewijn Gelpke et al. 1999). LiPs are major extracellular components of the lignin-degrading system of *P. chrysosporium*, but there are still open questions about their mechanisms of action, like the presumptive role of veratryl alcohol as a mediator in LiP-catalysed reactions.

Another species of the genus *Phanerochaete*, *Phanerochaete sordida*, exhibits greater ligninolytic selectivity during growth on beech wood than either *P. chrysosporium* or *T. versicolor*, and produces two LiPs (YK-LiP1 and YK-LiP2) under manganese-deficient conditions. Cloning and homologous expression of the YK-LiP1 and YK-LiP2 encoding genes in *P. sordida* YK-624 were carried out to obtain higher levels of these enzymes allowing characterization of the recombinant protein (Sugiura et al. 2009).

A manganese peroxidase (MnP3) from the white-rot fungus *Pleutorus ostreatus* was also homologously overproduced using a drug-resistant selection marker. This marker was based on a nucleotide substitution in *P. ostreatus sdi1*, encoding an iron sulfur protein that confers dominant resistance to the fungicide carboxin (Honda et al. 2000). The recombinant *mnp3* was expressed under control of the *P. ostreatus sdi1* promoter and terminator (Irie et al. 2001).

Due to the lower transformation efficiency of (filamentous) basidiomycetes compared to ascomycetes, relatively few studies have been reported on gene disruption of plant biomass degradation related genes in basidiomycetes. However, a homologous gene-replacement system was developed for *P. ostreatus* (Salame et al. 2012). This highlighted the limitations of homologous recombination in basidiomycetes due to the low frequency (2.3 %) of site-specific recombination of DNA integration and the low transformation efficiency using protoplast-mediated transformation.

2.2 Heterologous Expression in Other Basidiomycetes

Because of the limited number of easily transformable basidiomycete species only few basidiomycete genes encoding plant biomass degrading enzymes have been heterologously expressed in other basidiomycetes (Table 3). One example is the expression of a MnP encoding gene from the white-rot fungus *Dichomitus squalens* in *P. chrysosporium*. Most (82 %) of the transformants exhibited MnP activity and further analysis demonstrated that the recombinant protein was fully functional (Li et al. 2001)

Table 3 Heterologous production of basidiomycete enzymes involved in plant biomass degradation in filamentous fungi

	Enzyme	Enzyme	Protein accession number	Host	Source of the native protein	Highest production level	References
Lignin modifying enzymes	Laccase (AA1_1)	Lac1	AF170093	*Aspergillus niger*	*Pycnoporus cinnabarinus*	0.7 1 µmol/min/mL ABTS radical, 85 mg/L	Record et al. (2002)
						7.1 µmol/min/mL ABTS radical, 51 mg/L	Sigoillot et al. (2004)
		Pel3	AY686700		*Pleurotus eryngii*	2.4 U/mg[a] protein	Rodríguez et al. (2008)
		Lcc2	Q12718		*Trametes versicolor*	2.7 µmol/min/mL ABTS radical	Bohlin et al. (2006)
		Lcc4	U44431			0.6 µmol/min/mL ABTS radical	Téllez-Jurado et al. (2006)
		POXA1b	CAA06291		*Pleurotus ostreatus*	583.3 nkat/mL, 13 mg/L	Macellaro et al. (2014)
		1H6C	–			1000 nkat/mL, 20 mg/L	
		Lcc1	AAD30964	*Aspergillus oryzae*	*Coprinopsis cinerea*	3 µmol/min/mL ABTS radical, 135 mg/L	Yaver and Golightly (1996)
		Lcc1, Lcc4	P56193, Q02081		*Rhizoctonia solani*	nr	Wahleithner et al. (1996)
		LacA	AB015758		*Schizophyllum commune*	774 U/mL[b]	Hatamoto et al. (1999)
		Lac1	AF170093		*P. cinnabarinus*	7.8 µmol/min/mL ABTS radical, 51 mg/L	Sigoillot et al. (2004)
		Lcc1	AF170093		*Pycnoporus coccineus*	2.8 U/mL	

		LacA	AY839936.1	*Trichoderma reesei*	*Trametes* sp.	3.62 µmol/min/mL ABTS radical	Zhang et al. (2012)
		Lcc	AAR21094		*P. ostreatus*	237.134 U/mL[a]	Dong et al. (2012)
		Lac1	CAE00180		*Melanocarpus albomyces*	193 nkat/mL, 230 mg/L	Kiiskinen et al. (2004)
		Lcc	CAA36379		*Phlebia radiata*	7.7 nkat/mL, 19.5 mg/L	Saloheimo and Niku-Paavola (1991)
		Lac	ACC43989	*Penicillium canescens*	*Trametes hirsuta*	3 U/mL[c]	Abianova et al. (2010)
	Versatile peroxidase (AA2)	VPl	AF007222	*Aspergillus nidulans*	*P. eryngii*	466 U/L[d]	Eibes et al. (2009)
	Manganese peroxidase (AA2)	MnP1	Q02567	*A. niger*	*Phanerochaete chrysosporium*	3 U/L[a]	Cortes-Espinosa et al. (2011)
				A. oryzae	*P. chrysosporium*	0.33 µmol/min/mL	Stewart et al. (1996)
				Amylomyces rouxii	*P. chrysosporium*	130 U/L[b]	Montiel-González et al. (2009)
		MnP2	AAF31330	*P. chrysosporium*	*Dichomitus squalens*	0.65 µmol/min/mL	Li et al. (2001)
	Lignin peroxidase (AA2)	LiP	M27884	*A. rouxii*	*P. chrysosporium*	72.14 U/L	Montiel-González et al. (2009)
			CAA32616	*Phlebia radiata*	*T. reesei*	nd	Saloheimo et al. (1989)
	Aryl-alcohol oxidase (AA3_2)	AAO	AAC72747	*A. nidulans*	*P. eryngii*	400–500 mU/mL[a]	Varela et al. (2001)

(continued)

Table 3 (continued)

	Enzyme	Enzyme	Protein accession number	Host	Source of the native protein	Highest production level	References
(Hemi-) cellulose active enzymes	Cellobiohydrolase I (GH7)	CBH1	AB103461	*A. oryzae*	*Athelia* (*Corticium*) *rolfsii*	0.122 U/mL[a]	Yasokawa et al. (2003)
	β-Glucosidase (GH3)	Uebgl3A	AB618734	*A. oryzae*	*Ustilago esculenta*	Nr	Nakajima et al. (2012)
	Cellobiose dehydrogenase (AA3)	CDH	XP_001835032	*A. niger*	*Coprinopsis cinerea*	127 nkat/mL	Turbe-Doan et al. (2013)
	Pyranose dehydrogenase	PDH1	AY753307	*A. niger*	*Agaricus meleagris*	nr	Pisanelli et al. (2010)
				A. nidulans		5.5 U/ mL[a]	
	Glucuronoyl esterase (carbohydrate esterase)	GE1	130517 (JGI[f])	*Aspergillus vadensis*	*P. chrysosporium*	0.036 U/mL[e]	Duranová et al. (2009)
				P. cinnabarinus		0.417 U/mL[e]	
				S. commune		0.453 U/mL[e]	
		GE2	6482 (JGI[f])	*S. commune*	*P. chrysosporium*	0.530 U/mL[e]	

nr production level not reported, *nd* not detected

[a]Unit definition unknown

[b]One unit of enzyme activity is defined as a change in A_{690} of 0.001 per minute

[c]One unit of enzyme activity equals with an increase in the optical density of the reaction mixture over 1 min calculated per mg of protein

[d]One unit of activity is defined as the amount of enzyme oxidizing 1 µmol of Mn^{2+} per minute

[e]One unit of activity is defined as the amount of enzyme de-esterifying 1 µmol of 4-nitrophenyl-2-*O*-(methyl-4-*O*-methyl-α-D-glucopyanosyluronate)-β-D-xylopyranoside per minute

[f]Available through the fungal genome portal of the Joint Genome Institute of the Department of Energy of the USA (http://genome.jgi.doe.gov/programs/fungi/index.jsf)

Glucuronoyl esterases are suggested to break the linkage between lignin and xylan through glucuronic acid. Two genes (*ge1* and *ge2*) from *P. chrysosporium* encoding these enzymes were successfully expressed in *P. cinnabarinus* under control of the *S. commune GPD* promoter and in *S. commune* under control of the *S. commune SC3* promoter to produce the recombinant protein (Duranová et al. 2009). The highest glucoronoyl esterase activity was found in the transformants of *ge2* in *S. commune*.

3 Heterologous Expression of Basidiomycete Genes in Ascomycetes

3.1 Expression in Yeasts

Pichia pastoris and *Saccharomyces cerevisiae* are the most commonly used yeast hosts for heterologous expression of plant biomass modifying enzyme encoding genes from basidiomycetes. This is because of the various production strains and vectors that are available for these species. In few cases, *Kluyveromyces lactis*, *Pichia methanolica* and *Yarrowia lipolytica* have been employed as hosts for the production of basidiomycete plant biomass modifying enzymes. *K. lactis*, *S. cerevisiae* and *Y. lipolytica* are generally regarded as safe (GRAS) organisms, which is beneficial when producing food or medical related proteins. The methylotrophic yeasts *P. pastoris* and *P. methanolica* both have two alcohol oxidase (AOX, AUG) encoding genes and can utilize methanol as a sole carbon source (Cregg et al. 1989; Raymond et al. 1998). The strong and regulated *AOX* (or *AUG*) promoter regions of these yeasts are widely employed for the heterologous expression of recombinant proteins.

In contrast to the other eukaryotic heterologous protein production systems, the yeast systems are fast and inexpensive ways to achieve recombinant proteins. One of the main advantages is also the availability of a wide variety of well-developed tools for genetic manipulation of yeasts. In addition, unicellular yeasts are easy to cultivate in bioreactors, unlike filamentous fungi. Yeasts can be cultivated on a comparatively simple and inexpensive medium at a relatively fast growth rate (Daly and Hearn 2005). As eukaryotic organisms, yeasts are more suitable for the production of eukaryotic proteins than bacteria, since yeasts are able to perform several post-translational modifications, which are often crucial for the biochemical properties of the target proteins. These include e.g. phosphorylation, glycosylation, formation of disulfide bonds and proteolytic processing of the signal sequence (Cregg et al. 2000; Gasser et al. 2008; Huang et al. 2012).

Although yeasts are able to perform these modifications, the patterns of O- and N-linked sugar units added to the synthesized proteins by yeasts differ from those of other eukaryotes (Berends et al. 2009; Cereghino and Cregg 2000; Gasser et al. 2008; Gellissen et al. 2005; Hamilton and Gerngross 2007; Herscovics and Orlean 1993). This can be a drawback when using yeasts as production hosts since the different glycosylation patterns may alter the folding of the recombinant protein and

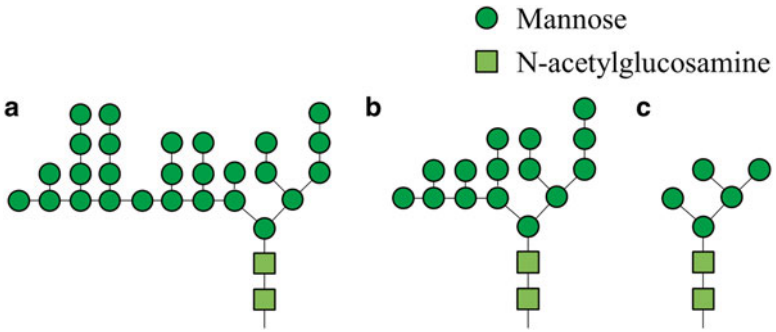

Fig. 1 Simplified structure of N-linked glycosylation pattern of (**a**) *Saccharomyces cerevisiae*, (**b**) *Pichia pastoris*, and (**c**) basidiomycete fungi (Berends et al. 2009; Hamilton and Gerngross 2007; Herscovics and Orlean 1993)

by that change its biochemical and functional properties (Berends et al. 2009; Bohlin et al. 2006; Hamilton and Gerngross 2007; Herscovics and Orlean 1993). The O-linked glycosylation of the proteins produced by yeasts consist only of mannose subunits. In contrast, higher eukaryotes, including basidiomycete fungi, add also other O-linked sugar units, such as galactose, N-acetylgalactosamine and N-acetylglucosamine, to the proteins (Lengeler et al. 2008). Yeasts may also attach excess N-linked mannose subunits to the recombinant proteins (Fig. 1) (Berends et al. 2009; Hamilton and Gerngross 2007; Herscovics and Orlean 1993). Especially, *S. cerevisiae* is known to hyperglycosylate secreted proteins (Mattanovich et al. 2009).

Yeast can produce intracellular recombinant proteins or secrete proteins out of the cells (Macauley-Patrick et al. 2005). However, secretion of the recombinant proteins is usually preferred, since targeting of the recombinant protein into the yeast eukaryotic secretory pathway is necessary especially when the recombinant protein requires post-translational modifications. Yeasts also secrete relatively low levels of native proteins, thus simplifying the recovery and purification of the target protein from the extracellular culture medium (Cregg et al. 2000; Huang et al. 2011a).

3.1.1 Yeast Expression Systems Used for the Heterologous Production of Plant Biomass Modifying Enzymes from Basidiomycetes

Recombinant Protein Production in Saccharomyces Cerevisiae

Multiple *S. cerevisiae* strains (such as CEN.PK2-1C, CEN.PK113-17A, W303-1A, INVSc1, Y294, BJ5465 and BJ3505) have been used for the heterologous expression of basidiomycete plant cell wall modifying enzymes (Table 4). From these strains, BJ5465 and BJ3505 are protease deficient, thus making the recombinant proteins less susceptible to degradation by extracellular proteases. All the previously mentioned *S. cerevisiae* strains are auxotrophic mutants which are unable to synthetize uracil or uridine, indispensable compounds for ribonucleic acids, or one of the essential amino acids tryptophan or leucine. These features have been exploited as selection markers in the *S. cerevisiae* expression vectors (Table 5). Most of the

Table 4 Yeast strains used for the heterologous expression of plant biomass modifying enzymes from basidiomycetes

Host	Strain	Genotype	Phenotype	References
Saccharomyces cerevisiae	CEN.PK2-1C	MATa; ura3-52; trp1-289; leu2-3112; his3Δ 1; MAL2-8^{c}; SUC2	Unable to synthetize uracil, tryptophan, leucine and histidine	Bleve et al. (2008, 2013)
	CEN. PK113-17A	MATa; ura3; leu2	Unable to synthetize uracil and leucine	Cassland and Jönsson (1999)
	CEN. PK113-13D	MATa; ura3-52; MAL2-8c; SUC2	Unable to synthetize uracil	Wilde et al. (2012)
	W303-1A	MATa; leu2-3112; trp1-1; can1-100; ura3-1; ade2-1; his3-11,15	Unable to synthetize leucine, tryptophan, uracil, adenine and histidine; sensitive to canavanine	Klonowska et al. (2005), Necochea (2005), Pezzella et al. (2009)
	INVSc1	MATa; his3D1; leu2; trp1-289; ura3-52	Unable to synthetize histidine, leucine, tryptophan and uracil	Cassland and Jönsson (1999), Tang et al. (2001)
	BJ5465	MATa; ura3-52; trp1; leu2-delta1; his3-delta200; pep4::HIS3; prb1-delta1.6R; can1; GAL	Unable to synthetize uracil, tryptophan, leucine and histidine; sensitive to canavanine; protease-deficient	Ryu et al. (2008a)
	BJ3505	pep4::HIS3; prb-δ1.6R HIS3; lys2-208: trp1-δ101; ura3-52; gal2; can1	Unable to synthetize tryptophan and uracil; sensitive to canavanine; protease-deficient; able to utilize alpha-aminoadipate as a nitrogen source	Toda et al. (2005)
Pichia pastoris	X-33	Wild type		Bey et al. (2011), Ding et al. (2002), Garg et al. (2012), Herter et al. (2011), Huy et al. (2013c), Joo et al. (2008), Nishibori et al. (2013), Salinas et al. (2011), Toda et al. (2008), Wang and Wen (2009)

(continued)

Table 4 (continued)

Host	Strain	Genotype	Phenotype	References
	GS115	his4	Unable to synthetise histidine	Brown et al. (2002), Colao et al. (2006, 2009), Ding et al. (2002), Hildén et al. (2013), Hong et al. (2006), Huang et al. (2011b), Huy et al. (2011, 2013a, b, c), Jiang et al. (2008), Jönsson et al. (1997), Kim et al. (2012), Li et al. (2007), Lin et al. (2013), O'Callaghan et al. (2002), Saito et al. (2013), Sun et al. (2012), You et al. (2013)
	KM71H	aox1::ARG; arg4	Reduced ability to metabolise methanol	Bao et al. (2013), Igarashi et al. (2008)
	KM71	his4; aox1::ARG4; arg4	Unable to synthetise histidine; reduced ability to metabolise methanol	Kawai et al. (2003), Kotake et al. (2009), Tang et al. (2001), Yoshida et al. (2001)
	SMD1168H	pep4	Protease-deficient	Gu et al. (2003)
	SMD1168	his4; pep4	Unable to synthetise histidine; protease-deficient	Bohlin et al. (2006), Gelo-Pujic et al. (1999), Jönsson et al. (1997)
Pichia methanolica	PMAD11	ade2-11	Unable to synthetize adenine	Guo et al. (2006, 2008)
	PMAD16	ade2-11; pep4; prb1	Unable to synthetize adenine; protease-deficient	Guo et al. (2005, 2006)
Kluyveromyces lactis	CMK5	MATa; thr; lys; pgi1; adh3; adh1::URA3; adh2::URA3	Unable to synthetize threonine and lysine	Camattari et al. (2007), Faraco et al. (2008), Festa et al. (2008), Pezzella et al. (2009), Piscitelli et al. (2005), Ranieri et al. (2009)
Yarrowia lipolytica	Polg	MATa; leu2-270; ura3-302c; xpr2-322; axp-2	Unable to synthetise leucine and uracil; protease-deficient	Jolivalt et al. (2005), Madzak et al. (2000, 2005, 2006)

Table 5 Yeast expression vectors used for the heterologous expression of basidiomycete plant biomass modifying enzymes

Host	Vector	Promoter		Selection marker	Signal peptide	References
S. cerevisiae	pYES2	GAL1	Inducible	Uracil synthesis	Native	Bleve et al. (2008, 2013), Tang et al. (2001), Wilde et al. (2012)
	pSAL4	CUP1	Inducible	Uracil synthesis	Native	Autore et al. (2009), Festa et al. (2008), Pezzella et al. (2009)
	pFL60	PGK	Constitutive	Uracil synthesis	Native	Cassland and Jönsson (1999)
	pAJ401	PGK	Constitutive	Uracil synthesis	Native	Cassland and Jönsson (1999)
	pDP51	GAL10	Inducible	Uracil synthesis	Native	Cusano et al. (2009), Klonowska et al. (2005)
	pAC1	ADH1	Constitutive	uracil synthesis	Native	Klonowska et al. (2005)
	pYEX-S1	PGK	Constitutive	Uracil synthesis	Native	Ryu et al. (2008a)
	YEp-FLAG-1	ADH2	Inducible	Tryptophan synthesis	*S. cerevisiae* mating type α-leader sequence	Toda et al. (2005)
	YEp352	PGK	Constitutive	Uracil synthesis	Native	Hill et al. (1986)
	Yep351	GAL10	Inducible	Leucine synthesis	Native	Hill et al. (1986)
	pJRoC30-α	GAL1	Inducible	Uracil synthesis	*S. cerevisiae* mating type α-leader sequence	Garcia-Ruiz et al. (2012)
	pBM258	GAL1	Inducible	Uracil synthesis	Native	Cusano et al. (2009)
P. pastoris	pPIC9	AOX1	Inducible	Histidine synthesis	*S. cerevisiae* mating type α-leader sequence	Bao et al. (2013), Brown et al. (2002), Colao et al. (2006, 2009), Hong et al. (2006), Huang et al. (2011b), Jönsson et al. (1997), Joo et al. (2008), Kawai et al. (2003), Lin et al. (2013), Sun et al. (2012), Yoshida et al. (2001), You et al. (2013)
	pPIC3.5	AOX1	Inducible	Histidine synthesis	Native	Huang et al. (2011b), Saito et al. (2013)
	pPICZ	AOX1	Inducible	Zeocin resistance	Native	Ding et al. (2002), Hildén et al. (2013), Huy et al. (2013b), Lu et al. (2009), Nishibori et al. (2013)

(continued)

Table 5 (continued)

Host	Vector	Promoter		Selection marker	Signal peptide	References
	pPICZα	AOX1	Inducible	Zeocin resistance	*S. cerevisiae* mating type α-leader sequence	Bey et al. (2011), Ding et al. (2002), Garg et al. (2012), Hildén et al. (2013), Huy et al. (2013b), Igarashi et al. (2008), Kim et al. (2012), Kotake et al. (2009), Salinas et al. (2011), Tang et al. (2001), Toda et al. (2008), Wang and Wen (2009)
	pHIL-D2	AOX1	Inducible	Histidine synthesis	Native	Brown et al. (2002), Colao et al. (2006, 2009), Gelo-Pujic et al. (1999), Jönsson et al. (1997), O'Callaghan et al. (2002)
	pGAPZ	GAP	Constitutive	Zeocin resistance	Native	Bohlin et al. (2006), Gu et al. (2003)
	pGAPZα	GAP	Constitutive	Zeocin resistance	*S. cerevisiae* mating type α-leader sequence	Gu et al. (2003), Herter et al. (2011), Jiang et al. (2008)
P. methanolica	pMETαA	AUG1	Inducible	Adenine synthesis	*S. cerevisiae* mating type α-leader sequence	Guo et al. (2005, 2006, 2008), Raymond et al. (1998)
K. lactis	pYG132	KlADH4	Inducible	Geneticin resistance	Native	Faraco et al. (2008), Pezzella et al. (2009), Piscitelli et al. (2005), Saliola et al. (1999)
	pLC12	KlADH4	Inducible	Uracil synthesis	Native	Camattari et al. (2007)
Y. lipolytica	pINA1267	pLEU2	Inducible	Leucine synthesis	XPR2 extracellular protease prepro sequence	Madzak et al. (2000, 2005)
	pINA1296	pLEU2	Inducible	Leucine synthesis	XPR2 extracellular protease prepro sequence	Jolivalt et al. (2005), Madzak et al. (2000, 2005, 2006)

vectors applied for the expression of the basidiomycete lignocellulose modifying genes contain the *ura3* gene enabling the uracil auxotrophic strain to grow on a medium that lacks uracil or uridine.

The *S. cerevisiae* expression vectors employ both constitutive and inducible promoter regions of the yeast. The constitutive promoters applied are alcohol dehydrogenase I (*ADH1*) and phosphoglycerate kinase (*PGK*) (Derynck et al. 1983; Hitzeman et al. 1981; Romanos et al. 1992). These promotes are active in the presence of glucose and the genes cloned downstream of them are then expressed. The inducible promoters include galactokinase (*GAL1*), UDP galactose-4-epimerase (*GAL10*), alcohol dehydrogenase II (*ADH2*) and copper-metallothionein (*CUP1*) (Butt et al. 1984; Johnston and Davis 1984; Mascorro-Gallardo et al. 1996; Romanos et al. 1992; West et al. 1984). The *GAL1* and *GAL10* promoters are activated by galactose and repressed by glucose. The expression of *ADH2* promoter is activated by a non-fermentable carbon source such as ethanol or glycerol, whereas the addition of Cu^{2+} ions activates the expression of *CUP1* promoter region.

Recombinant Protein Production in Pichia Pastoris

All *P. pastoris* strains are derivatives of the wild type strain Y-11430 from Northern Regional Research Laboratories (NRRL, Peoria, IL, USA). Both wild type and auxotrophic strains of *P. pastoris* have been applied for the production of lignocellulose converting basidiomycete enzymes (Table 4). X-33 is a wild type strain, whereas GS115, KM71 and SMD1168 have a mutated histidinol dehydrogenase (*his4*) gene and are unable to grow without histidine supplementation. In addition, SMD1168 and SMAD1168H are protease deficient strains that lack protease A activity, thus often improving the yield of recombinant proteins. The strains KM71 and KM71H contain a mutated *AOX1* promoter. The remaining *AOX2* promoter is weaker and therefore the cells exhibit a slower growth rate with decreased consumption of toxic methanol (Chiruvolu et al. 1997; Cregg et al. 1989). The *his4* gene of *P. pastoris* and a gene encoding resistance against the antibiotic zeocin have been employed as selection markers in the expression vectors (Table 5). In these vectors, alcohol oxidase (*AOX*) and glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter regions of *P. pastoris* are used. The *AOX1* promoter is induced when methanol is used as the sole carbon source (Cregg et al. 1989) while the constitutive *GAP* promoter is active in the presence of glucose (Waterham et al. 1997). *P. pastoris* vectors pPIC3.5, pHIL-D2, pPICZ and pGAPZ use the native signal sequence of the heterologously produced protein for the secretion, whereas in the vectors pPIC9, pPICZα and pGAPZα the signal sequence of *S. cerevisiae* mating type α-factor is included (Cregg and Higgins 1995).

Compared to other yeasts, *P. pastoris* is capable of growing at higher cell density (OD_{600} 500) therefore often resulting in elevated yield of recombinant protein (Cereghino and Cregg 2000; Jahic et al. 2002). In fact, the amount of the target protein can be up to 30 % of all the extracellular proteins secreted by *P. pastoris* (Cregg and Higgins 1995; Gellissen 2000).

Other Recombinant Protein Production Systems

K. lactis, *P. methanolica* and *Y. lipolytica* are yeast species more rarely applied for the production of plant biomass modifying proteins from basidiomycetes. *K. lactis* CMK5 strain is a uracil auxotroph and possess only one functional alcohol dehydrogenase (KlADH) encoding gene (Table 4) (Saliola et al. 1999). *K. lactis* expression vectors include the inducible *KlADH4* promoter region (Table 5), which is activated in the presence of ethanol (Saliola et al. 1991). In *K. lactis* expression vectors, *ura3* and a gene encoding antibiotic resistance against geneticin are used as selection markers (Van Ooyen et al. 2006).

The *P. methanolica* PMAD11 and PMAD16 strains are adenine auxotrophic mutants. In addition, PMAD16 strain is protease deficient. In the *P. methanolica* expression vector, the *ade2* gene from *S. cerevisiae* has been used to complement adenine auxotrophy in the host strain (Table 5) (Raymond et al. 1998). Similarly with *P. pastoris*, the methanol inducible promoter region of alcohol oxidase (*AUG*) has been applied for the production of recombinant proteins in *P. methanolica* (Raymond et al. 1998). The pMETα vector utilizes the signal sequence of *S. cerevisiae* mating type α-factor for the secretion of the recombinant protein.

The protease deficient and leucine auxotrophic strain of *Y. lipolytica*, Polg, has been used for the production of recombinant plant cell wall degrading enzymes from basidiomycetes (Table 4) (Madzak et al. 2004). The expression vectors pINA1267 and pINA1296 both contain the inducible leucine promoter region (*pLEU2*) of *Y. lipolytica* which is activated by the presence of leucine precursors (Table 5) (Madzak et al. 2000, 2004). These vectors complement *leu2* deficiency providing a wild type phenotype. Recombinant proteins are secreted by employing the signal sequence of extracellular protease (XPR2) of *Y. lipolytica* (Madzak et al. 2000).

3.1.2 Examples of Recombinant Proteins Involved in Plant Biomass Degradation Produced in Yeasts

Biochemically active plant biomass modifying enzymes including hydrolases, oxidases and lyases from various basidiomycete species has been successfully produced in *S. cerevisiae* and *P. pastoris* (Table 6). Most of these enzymes originate from wood-decaying white-rot fungal species. Recently, the *P. pastoris* expression system has gained an increasing interest when compared to *S. cerevisiae* and other yeast systems.

Laccases (AA1_1) are the most commonly produced basidiomycete lignin modifying enzymes in yeasts (Table 6). From lignin modifying oxidoreductases, the highest activity levels, up to 7200 U/L, have been achieved with laccase from the saprobic fungus *Cyathus bulleri* produced in *P. pastoris* (Garg et al. 2012). The highest protein yield, up to 340 mg/L, has been reported when laccase of the white-rot fungus *Trametes trogii* has been produced in *P. pastoris* (Colao et al. 2009). Laccase production in *P. pastoris* has been significantly optimized. Supplementation

Table 6 Basidiomycete enzymes involved in plant biomass degradation produced in yeasts as recombinant enzymes

	Enzyme	Gene accession number(s)	Expression host	Source of the native protein	Production level	References
Lignin modifying enzymes	Laccase (AA1_1)	M60560	*S. cerevisiae*	*Colorius (Trametes) hirsutus*	nr	Kojima et al. (1990)
		AM773999 AM774000		*Pleurotus eryngii*	30–88 U/L[a]	Bleve et al. (2008, 2013)
		AJ005018 Z34848 Z34847 AJ344434		*Pleurotus ostreatus*	75 U/L[a]	Autore et al. (2009), Festa et al. (2008), Pezzella et al. (2009), Piscitelli et al. (2005)
		AB072703		*Pycnoporus coccineus* (syn. *Pycnoporus sanguineus*, *Trametes sanguinea)*	nr	Hoshida et al. (2001, 2005)
		Y18012 X84683 AAC49828 AY210894		*Trametes versicolor*	35 mU/L[a]	Cassland and Jönsson (1999), Necochea (2005), Uldschmid et al. (2003)
		AKY160 EMY162 EMY164		*Trametes* sp. C30	1120 U/L[a], 2 mg/L	Cusano et al. (2009), Klonowska et al. (2005)
		XM_001829459	*P. pastoris*	*Coprinopsis cinerea*	nr	Lin et al. (2013)
		EF439894 EF439893 HG764548 HG764549		*Coprinus comatus*	1465 U/L[a]	Bao et al. (2013), Gu et al. (2014)
		EU195884		*Cyathus bulleri*	600–7200 U/L[a]	Garg et al. (2012)
		AB253791		*Flammulina velutipes*	nr	Saito et al. (2013)
		AY167042		*Fome lignosus*	nr	Hu et al. (2007), Liu et al. (2003)
		AF185275 FJ473385		*Ganoderma lucidum*	680 U/L[a]	Joo et al. (2008), Sun et al. (2012), You et al. (2013)

(continued)

Table 6 (continued)

	Enzyme	Gene accession number(s)	Expression host	Source of the native protein	Production level	References
		JQ027726 JQ027727		*Physisporinus rivulosus*	nr	Hildén et al. (2013)
		AF297228		*Pleurotus sajor-caju*	nr	Soden et al. (2002)
		FJ473384		*Polyporus grammocephalus*	320 U/L[a]	Huang et al. (2011b)
		AF170093		*Pycnoporus cinnabarinus*	nr	Herter et al. (2011), Otterbein et al. (2000)
		AB072703		*Pycnoporus sanguineus (Trametes sanguinea)*	1733–4706 U/L[a]	Lu et al. (2009), Hoshida et al. (2001)
		AJ294820 AM292415		*Trametes trogii*	17–340 mg/L	Colao et al. (2006, 2009)
		X84683 Y18012 U44431 U44851 AY693776 Y18012 AB539566 AY049725		*T. versicolor*	14–1500 U/L[a]	Bohlin et al. (2006), Brown et al. (2002), Gelo-Pujic et al. (1999), Herter et al. (2011), Jönsson et al. (1997), Nishibori et al. (2013), O'Callaghan et al. (2002)
		AF388910 AY846842		*Trametes* sp. AH28-2	5440 U/L[a]	Hong et al. (2006), Li et al. (2007)
		AY839942		*Trametes* sp. 420	nr	Hong et al. (2007), Xu et al. (2007), Zhou et al. (2007)
		AJ005018 Z34848 Z34847 AJ344434	*K. lactis*	*P. ostreatus*	12 U/L[a]	Faraco et al. (2008), Festa et al. (2008), Pezzella et al. (2009), Piscitelli et al. (2005)
		AJ294820		*T. trogii*	nr	Camattari et al. (2007), Ranieri et al. (2009)
		AF170093	*Y. lipolytica*	*P. cinnabarinus*	20 mg/L	Madzak et al. (2005)
		AF414109		*T. versicolor*	2.5 mg/L	Jolivalt et al. (2005), Madzak et al. (2006)
		na	*P. methanolica*	*T. versicolor*	nr	Guo et al. (2005, 2006, 2008)

	Lignin peroxidase (AA2)	na	*S. cerevisiae*	*Phanerochaete chrysosporium*	nr	Ryu et al. (2008a)
		X15599	*P. pastoris*	*P. chrysosporium*	nr	Wang and Wen (2009)
	Versatile peroxidase (AA2)	na	*S. cerevisiae*	*P. eryngii*	21 mg/L	Garcia-Ruiz et al. (2012)
	Manganese peroxidase (AA2)	J04980	*P. pastoris*	*P. chrysosporium*	nr	Gu et al. (2003), Jiang et al. (2008)
Oxalate degrading enzymes	Oxalate decarboxylase (EC 4.1.1.2)	na	*P. pastoris*	*Dichomitus squalens*	nr	Sietiö et al. (2015)
	Oxalate oxidase (EC 1.2.3.4)	AJ746412	*P. pastoris*	*Ceriporiopsis subvermispora*	4 mg/L	Moomaw et al. (2013), Moussatche et al. (2011)
Cellulose degrading enzymes	Cellobiohydrolase I (GH7)	na	*S. cerevisiae*	*P. chrysosporium*	nr	Van Rensburg et al. (1998)
	Cellobiohydrolase II (GH6)	AB370872	*P. pastoris*	*Irpex lacteus*	nr	Toda et al. (2008)
	Endoglucanase (GH5, GH45)	AB194135	*S. cerevisiae*	*I. lacteus*	2 mg/L	Toda et al. (2005)
		AB378504	*P. pastoris*	*P. chrysosporium*	nr	Igarashi et al. (2008)
		HM052796		*Gloeophyllum trabeum*	nr	Kim et al. (2012)
		na		*T. versicolor*	0.5 mg/L	Salinas et al. (2011)
		AF329732		*Volvariella volvacea*	65–100 mg/L	Ding et al. (2002)
	β-Glucosidase (GH1)	na	*S. cerevisiae*	*P. chrysosporium*	nr	Wilde et al. (2012)
		AB081121	*P. pastoris*	*P. chrysosporium*	nr	Kawai et al. (2003)
	Cellobiose dehydrogenase (AA3, AA8)	HQ825322	*P. pastoris*	*P. cinnabarinus*	52–350 mg/L	Bey et al. (2011)
		X88897		*P. chrysosporium*	79 mg/L	Yoshida et al. (2001)
		AY187939		*T. versicolor*	nr	Stapleton et al. (2004)

(continued)

Table 6 (continued)

	Enzyme	Gene accession number(s)	Expression host	Source of the native protein	Production level	References
Hemicellulose degrading enzymes	β-Mannanase (GH5)	AJ271862	*S. cerevisiae*	*Agaricus bisporus*	nr	Tang et al. (2001)
		AJ271862	*P. pastoris*	*A. bisporus*	nr	Tang et al. (2001)
		na		*Armillariella tabescens*	440 mg/L	Wang et al. (2009)
	Arabinanase (GH43)	JQ838072	*P. pastoris*	*P. chrysosporium*	8 mg/L	Huy et al. (2013b)
	Xylosidase/ arabinofuranosidase (GH43)	JX625152		*P. chrysosporium*	14 mg/L	Huy et al. (2013a)
	Acetyl xylan esterase (CE1)	JQ031636		*P. chrysosporium*	26 mg/L	Huy et al. (2013c)
	Endo-1,4-β-xylanase (GH10)	AQ993045		*P. chrysosporium*	nr	Huy et al. (2011)
	β-Galactanase (GH43)	AB461394		*I. lacteus*	nr	Kotake et al. (2009)

na gene accession number not available
nr production level (mg/L) not reported
[a]one unit (U) is defined as the amount of enzyme required to degrade 1 μmol of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) in 1 min

of copper ions and lowered growth temperature (15–25 °C) has resulted in increased laccase activity (Cassland and Jönsson 1999; Hildén et al. 2013; Liu et al. 2003; O'Callaghan et al. 2002). While copper ions are indispensable for laccase activity by forming the enzyme catalytic site (Baldrian 2006), lower temperature may prevent denaturation and aggregation of the native proteins which decreases the need for protein folding mechanisms resulting in higher and more efficient secretion capacity (Dragosits et al. 2009). In addition, improved production of two laccase isoenzymes of *Coprinus comatus* has been obtained by using an N-terminal fusion of 10 amino acids from a *Volvariella volvacea* xylanase (Gu et al. 2014).

Different class II heme peroxidases (AA2), lignin peroxidases (LiPs), manganese peroxidases (MnPs) and versatile peroxidases (VPs) have been produced in *S. cerevisiae* and *P. pastoris* (Garcia-Ruiz et al. 2012; Gu et al. 2003; Jiang et al. 2008; Ryu et al. 2008a; Wang and Wen 2009). The highest production level, 21 mg/L, has been described for the white-rot fungus *Pleurotus eryngii* VP produced in *S. cerevisiae* (Garcia-Ruiz et al. 2012). Recombinant production of class II heme peroxidases has been challenging in yeasts (Conesa et al. 2002). However, they have been produced successfully in *Escherichia coli* and *Aspergillus* species (Conesa et al. 2002; Fernández-Fueyo et al. 2012, see 3.2.1). The main obstacle in producing recombinant class II heme peroxidases is the availability and incorporation of iron-containing heme as the prosthetic group (Franken et al. 2011; Gu et al. 2003). While the production of heme peroxidases increased by supplementation of hemoglobin or hemin to the medium (Conesa et al. 2000; Gu et al. 2003), the mechanism of heme uptake is poorly understood. Better knowledge of the heme biosynthetic pathway and its regulation is needed to overcome the limiting effect of heme.

The production of active oxalate metabolizing enzymes, oxalate decarboxylase (ODC) and oxalate oxidase (OXO), from the white-rot fungi *Dichomitus squalens* (Sietiö et al. 2015) and *Ceriporiopsis subvermispora* (Moomaw et al. 2013; Moussatche et al. 2011), respectively, has been successful in *P. pastoris* (Table 6). In wood-decaying fungi these enzymes have been associated with the regulation of intra- and extracellular concentrations of oxalic acid, which is one of the key components in biological decomposition of wood. The production level of *C. subvermispora* OXO was 4 mg/L (Moussatche et al. 2011).

In addition to the lignin modifying oxidoreductases, plant cell wall polysaccharide degrading enzymes of basidiomycetes have been produced in *S. cerevisiae* and *P. pastoris*. The production of cellulose hydrolysing enzymes such as cellobiohydrolase I (GH7), cellobiohydrolase II (GH6), endoglucanases (GH5, GH45) and β-glucosidase (GH1) have been described (Table 6). From those, the highest yield (100 mg/L) was obtained for endoglucanase from the straw-degrading basidiomycete *V. volvacea* produced in *P. pastoris* (Ding et al. 2002). Oxidative cellobiose dehydrogenases (CDH; AA3 and AA8) have been produced only in *P. pastoris* (Bey et al. 2011; Stapleton et al. 2004; Yoshida et al. 2001), and the highest yield (350 mg/L) was achieved for CDH from the white-rot fungus *P. cinnabarinus* (Bey et al. 2011).

Some hemicellulose degrading enzymes, such as β-mannanase (GH5), endo-β-1,4-xylanase (GH10), β-galactanase (GH43), arabinanase (GH43), an enzyme possessing

both xylosidase and arabinofuranosidase activities (GH43), and acetyl xylan esterase (CE1) have also been produced in yeasts (Table 6). The best production level, up to 440 mg/L, in *P. pastoris* has been reported for a β-mannanase from the plant pathogen *Armillariella tabescens* (Wang et al. 2009).

3.2 Expression in Filamentous Ascomycetes

The limitations of overexpression of genes in basidiomycetes has stimulated attempts to heterologously express basidiomycete genes in filamentous ascomycetes, in particular those species used in industry, due to their high secretion capacity and good fermentation properties. However, production levels of basidiomycete enzymes in these ascomycetes are often very low. In this section we will provide examples of successful expression and the production levels that were reached.

Many yeasts and filamentous fungi have been used as hosts to develop different heterologous protein production systems for basidiomycete enzymes (Hoshida et al. 2005). Particular emphasis has been on lignin-modifying enzymes, as they are largely absent in ascomycete fungi. However, most lignin-modifying enzymes are generally difficult to overproduce heterologously in an active form (Hoshida et al. 2005). Heterologous production of active lignin-modifying peroxidases has been reported in several species (Eibes et al. 2009; Montiel-González et al. 2009; Stewart et al. 1996), but in no cases the levels have been satisfactory enough taking into account the amount of enzyme required for its biotechnological application. One of the reasons of this setback is factors related to the hemoprotein nature of the peroxidases.

3.2.1 Heterologous Expression in *Aspergillus* and *Penicillium*

Aspergillus niger is a well-known fungal host that produces high levels of recombinant enzymes for many industrial applications. It is therefore not surprising that it has also been used for the expression of basidiomycete genes.

The enzyme that has received the most attention for heterologous production in ascomycetes is laccase, due to its potential in a variety of industrial applications (Kunamneni et al. 2008). While overall expression of basidiomycete laccases in *Aspergillus* and *Penicillium* is regularly successful, the production levels vary strongly (Table 3). Expression of a laccase from *P. cinnabarinus* in *P. pastoris* resulted in a protein that differed in some biochemical properties (e.g. molecular mass, isoelectric point, optimal temperature and pH) from the native enzyme (Otterbein et al. 2000). However, expression of this laccase in *A. niger* demonstrated highly similar properties to the native enzyme, suggesting that *A. niger* is a more suitable production host for this enzyme (Record et al. 2002). When comparing the production of the *P. cinnabarinus* laccase in *A. niger* and *Aspergillus oryzae*, higher protein yields were obtained for *A. oryzae* (Sigoillot et al. 2004). While the

biochemical properties were similar between the native and the recombinant enzymes, the recombinant enzymes had a higher K_m value than the native enzyme. Also, in the presence of a redox mediator, 1-hydroxybenzotriazole, both the native enzyme and the recombinant enzyme produced in *A. niger* were able to delignify Kraft pulp almost to 75 %, while no delignification was observed with the *A. oryzae* produced enzyme (Sigoillot et al. 2004). Other basidiomycete laccases were also expressed in *A. niger*, such as one from *P. eryngii* (*pel*3), although the activity level obtained was low (Rodríguez et al. 2008). The best result was obtained when the native signal peptide was replaced by the signal peptide from *A. niger* glucoamylase and a protease deficient *A. niger* strain was used, suggesting that the native signal sequence was not well recognized and/or processed by *A. niger*. Expression in a low-protease producing *A. niger* strain also resulted in high production levels for a laccase from *P. ostreatus* and a variant thereof that was obtained through directed evolution (Macellaro et al. 2014). Using a low protease strain was also beneficial for the production of a laccase from *S. commune*. Several ascomycete strains were tested in this study, but the best production was achieved with *Aspergillus sojae* 1860, which has low extracellular protease levels (Hatamoto et al. 1999). These results indicate that ascomycete proteases can significantly reduce the production of basidiomycete enzymes.

The fermentation approach also affects the production of laccases, as was demonstrated for the production of a *T. versicolor* laccase in *A. niger*. Solid state fermentation resulted in a much higher yield of laccase than submerged fermentation (Téllez-Jurado et al. 2006). In a different study, expression of two laccase genes from *T. versicolor* in *A. niger* under control of the *gpdA* promoter demonstrated much higher yield compared to expression of these genes in *P. pastoris* (Bohlin et al. 2006), confirming the high potential of *A. niger* for industrial enzyme production, while *P. pastoris* is more suitable for academic studies.

A *Trametes villosa* laccase (*lcc1*) was successfully expressed in another industrial *Aspergillus* species, *A. oryzae* and the properties of the recombinant enzyme were the same as those of the native enzyme (Yaver and Golightly 1996). This demonstrates that while there are differences in the glycosylation patterns of ascomycetes and basidiomycetes (Berends et al. 2009), these do not necessarily affect the biochemical properties of the heterologously produced enzymes. *A. oryzae* was also used for the heterologous production of a laccase from the white-rot fungus *Pycnoporus coccineus* using the maltose inducible promoter of α-amylase encoding *amyB* gene (Taka promoter) (Hoshida et al. 2005). The activity of this laccase was highly copper-dependent, even if the expression of the gene under control of the *amyB* promoter was not. When three laccase genes from the plant pathogenic basidiomycete *Rhizoctonia solani* were expressed in *A. oryzae* under control of the Taka amylase promoter, good production levels were only obtained for two of the enzymes (Wahleithner et al. 1996). For the third gene an initial activity level could be detected, but this disappeared in time, suggesting that this enzyme was not stable in *A. oryzae* cultures.

Penicillium is a sister genus of *Aspergillus* and species of this genus are more commonly used for the production of secondary metabolites than of enzymes.

However, heterologous production of a laccase from *Trametes hirsuta* in *Penicillium canescens* resulted in a high level of secreted protein (Abianova et al. 2010), indicating that species of this genus could be good alternatives for *Aspergillus* species with respect to enzyme production.

Heterologous expression of genes encoding lignin-modifying peroxidases from *P. chrysosporium* has been performed in several Aspergilli (Cortés-Espinosa et al. 2011). Stewart et al. (1996) were able to successfully produce active MnP and LiP from *P. chrysosporium* in *A. oryzae* under control of the Taka amylase promoter. To achieve this hemin was added to the culture medium. The same *mnp* containing expression vector was also introduced in *A. niger* where active protein was similarly detected when the culture medium contained hemoglobin (Cortés-Espinosa et al. 2011). However, the best *A. niger* transformant had less activity than the native *P. chrysosporium* strain.

The importance of temperature during bioreactor cultivation for the improvement of the recombinant proteins was demonstrated in a recent study using a versatile peroxidase from *Pleurotus eryngii*. The production of this enzyme in *A. nidulans* and *A. niger* was performed under different temperatures and the highest activity was obtained at 19 °C, which is significantly below the optimum growth temperature for *A. niger* (Eibes et al. 2009). Also in these cultures, hemin was added to the media.

Production levels of the flavoenzyme aryl-alcohol oxidase (AAO), also involved in lignin degradation, in *P. eryngii* are quite low and unsuitable for industrial applications. Heterologous production of the *P. eryngii* AAO encoding gene in *A. nidulans* was performed using the strong inducible alcohol dehydrogenase promoter (*alcA*). The recombinant protein had the same molecular mass and isoelectric point as the native protein, suggesting no significant modifications. In addition, the production of the recombinant enzyme was 10–50 times higher than in the original fungus, indicating that this strategy is highly promising for obtaining industrially relevant levels of lignin-related enzymes (Varela et al. 2001).

Cellobiose dehydrogenases (CDHs) are produced by many plant biomass degrading and phytopathogenic fungi, but the function of these enzymes are not fully deciphered. A putative *cdh* gene from *Coprinopsis cinerea* as well as one from the ascomycete *Podospora anserina* were successfully expressed in *A. niger* to enable comparison of their enzymatic properties (Turbe-Doan et al. 2013). Interestingly a much higher activity was observed for the transformant carrying the *C. cinerea cdh* and taking into account the specific activity of the enzymes, a fivefold higher enzyme production was observed for the basidiomycete protein (Turbe-Doan et al. 2013).

In many studies, the main purpose of heterologous expression of the gene is to allow the detailed characterization of the enzyme, since the production level in the original species is too low or the purification is too complicated to obtain sufficient amounts of pure enzyme. This was the case for the CDH described above, but for instance also for the heterologous expression of a cellobiohydrolase gene (*cbh1*) from the plant pathogen *Athelia rolfsii* (synonym *Corticium rolfsii*) in *A. oryzae*

under control of the phosphoglycerate kinase promoter *pgkA* (Yasokawa et al. 2003). The same motivation resulted in the expression of the UeBgl3A gene encoding a β-glucosidase from the plant pathogen *Ustilago esculenta*, in *A. oryzae* under control of the *amyB* promoter (Nakajima et al. 2012). The production levels are highly variable though (Table 3), and reflect the problems associated with the heterologous production of basidiomycete proteins in ascomycetes. While some are produced at high levels, others result in only minor amounts of protein that is barely sufficient for extensive characterization.

The major pyranose dehydrogenase (PDH) from the litter-decomposing fungus *Agaricus meleagris* was heterologously expressed in *A. nidulans* and *A. niger* under control of the glucoamylase promoter of the respective species (Pisanelli et al. 2010). Two versions were introduced in *A. nidulans*, one with its native signal sequence and one with the signal sequence of the *A. nidulans* glucoamylase, with the native signal sequence resulting in the highest extracellular activity. This is the opposite results as that obtained for the *P. eryngii* laccase (*pel*3, see above), where the glucoamylase signal peptide improved the production of the enzyme (Rodríguez et al. 2008). Introduction of this modified version of PDH in *A. niger* resulted in yields that were 10 times lower than those obtained for the best *A. nidulans* transformant (Pisanelli et al. 2010). As expression of *P. eryngii pel*3 was also performed in *A. niger*, this could imply that *A. nidulans* is more capable to processing basidiomycete signal peptides.

Expression of a *P. cinnabarinus* glucuronoyl esterase encoding gene was attempted in *A. niger* and *Aspergillus vadensis* under control of the *A. niger* glucoamylase promoter (Duranová et al. 2009). Activity was only detected in transformants of *A. vadensis*, although 10–20-fold lower than in transformants of *S. commune* and *P. cinnabarinus* (see 2.2). The better result for *A. vadensis* compared to *A. niger* could be due to the very low extracellular protease production in *A. vadensis* and the absence of acidification of the medium (de Vries et al. 2004), which may be the main reasons why no active protein was detected in *A. niger* transformants.

3.2.2 Heterologous Expression in *Trichoderma reesei*

Trichoderma reesei is with *Aspergillus* the most commonly used fungus for industrial production of heterologous and homologous proteins, in particular cellulases. Some successful examples of heterologous production of basidiomycete enzymes in *T. reesei* have been reported (Table 3). One example is the expression of a laccase gene (*lacA*) from *Trametes* sp. The gene was placed under control of the strong and constitutive *gpdA* promoter from *A. nidulans* and its native signal peptide was replaced by the signal peptide of *T. reesei* cellobiohydrolase I (CBHI). The production levels obtained for this construct were higher than those of most other studies (Zhang et al. 2012).

Dong et al. (2012) obtained a 28.6-fold higher production of a *P. ostreatus* laccase by producing it heterologously in *T. reesei*. The gene was codon optimized for *T. reesei* and expressed under control of the *cbh1* promoter. When this promoter and species was used for the heterologous production of a *Phlebia radiata* laccase, three additional isoforms were detected, suggesting variable phosphorylation or glycosylation of the recombinant enzyme (Saloheimo and Niku-Paavola 1991). Fusion of heterologous proteins of interest to homologous proteins of the expression host can improve the production of the target protein. To test this, a native form of a laccase from an ascomycete fungus *Melanocarpus albomyces* and one fused to a hydrophobin were expressed in *T. reesei* under control of the *cbh1* promoter (Macellaro et al. 2014). In this case, the fusion protein was accumulating intracellularly, likely due to the difficulties in secretion, and higher production of the native form was observed.

3.2.3 Promoters Used for Heterologous Expression of Basidiomycete Genes in Ascomycetes

The promoters used for expression of heterologous genes in filamentous ascomycetes can be divided in constitutive and inducible promoters, but all originate from genes that are highly expressed in the intended hosts. The constitutive promoters have the advantage that they result in high expression under most culture conditions, allowing more flexibility in the enzyme production. In contrast, inducible promoters require specific inducing conditions, but often result in higher enzyme production than the constitutive promoters. In addition, inducible promoters can be used for production of proteins that have a negative effect on the production hosts. In such a case, the production strain can initially be grown under non-inducing conditions and when sufficient growth has occurred the inducer can be added to result in a short period of high protein production.

Both types of promoters have been used for heterologous expression of basidiomycete genes encoding plant biomass degrading enzymes (Table 7). The most commonly used inducible promoters are the glucoamylase promoter from *A. niger* (Conesa et al. 2000) and the Taka amylase promoter from *A. oryzae* (Stewart et al. 1996), which both respond to the presence of maltose or starch. The most commonly used constitutive promoter is the glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) promoter from *A. nidulans* (Punt and van den Hondel 1992), although this promoter has also been used from other species. Recently, a new set of constitutive promoters from *A. niger* and *A. vadensis* were tested for enzyme production, resulting in several with a higher production levels than *gpdA* (Culleton et al. 2014), but these have not yet been tested for the expression of basidiomycete genes.

Table 7 Promoters used for heterologous expression of basidiomycetes genes in filamentous basidiomycetes and ascomycetes

Promoter	Corresponding gene	Species of origin	Reference	Host species	Expressed gene	Donor species	Reference
alcA	Alcohol dehydrogenase	*A. nidulans*	Fernández-Cañón and Peñalva (1995)	*A. nidulans*	Versatile peroxidase	*Pleurotus eryngii*	Eibes et al. (2009)
					Aryl-alcohol oxidase	*P. eryngii*	Varela et al. (2001)
				A. niger[a]	Versatile peroxidase	*P. eryngii*	Eibes et al. (2009)
amyB	α-Amylase	*A. oryzae*	Tada et al. (1991)	*A. oryzae*	β-Glucosidase	*Ustilago esculenta*	Nakajima et al. (2012)
					Laccase	*Pycnoporus cinnabarinus*	Hoshida et al. (2005)
amy	α-Amylase	*A. nidulans*	Téllez-Jurado et al. (2006)	*A. niger*	Laccase	*Trametes versicolor*	Téllez-Jurado et al. (2006)
bgaS	β-Galactosidase	*Penicillium canescens*	Nikolaev et al. (1992)	*P. canescens*	Laccase	*Trametes hirsuta*	Abianova et al. (2010)
cbh1	Cellobiohydrolase	*T. reesei*	Saloheimo et al. (1989)	*T. reesei*	Laccase	*Phlebia radiata*	Saloheimo and Niku-Paavola (1991)
						Pleurotus ostreatus	Dong et al. (2012)
						Melanocarpus albomyces	Kiiskinen et al. (2004)
glaA	Glucoamylase	*A. nidulans*	Pisanelli et al. (2010)	*A. nidulans*	Pyranose dehydrogenase	*Agaricus meleagris*	Pisanelli et al. (2010)
glaA	Glucoamylase	*A. niger*	Pisanelli et al. (2010)	*A. niger*	Pyranose dehydrogenase	*A. meleagris*	Pisanelli et al. (2010)
glaA	Glucoamylase		Conesa et al. (2000)	*A. vadensis*	Glucuronoyl esterase	*P. cinnabarinus*	Duranová et al. (2009)
				A. niger	Cellobiose dehydrogenase	*Coprinopsis cinerea*	Turbe-Doan et al. (2013)
gpdA	Glyceraldehyde-3-phosphate dehydrogenase	*A. nidulans*	Punt and van den Hondel (1992)	*T. reesei*	Laccase	*Trametes* sp.	Zhang et al. (2012)
				A. niger	Laccase	*P. cinnabarinus*	Record et al. (2002)
							Sigoillot et al. (2004)
						P. eryngii	Rodríguez et al. (2008)
						T. versicolor	Bohlin et al. (2006)
						P. ostreatus	Macellaro et al. (2014)

(continued)

Table 7 (continued)

Promoter	Corresponding gene	Species of origin	Reference	Host species	Expressed gene	Donor species	Reference
GPD	Glyceraldehyde-3-phosphate dehydrogenase	*S. commune*	Alves et al. (2004)	*P. cinnabarinus*	Glucuronoyl esterase	*P. cinnabarinus*	Duranová et al. (2009)
GPD	Glyceraldehyde-3-phosphate dehydrogenase	*P. chrysosporium*	Li et al. (2001)	*P. chrysopsorium*	Manganese peroxidase	*Dichomitus squalens*	Li et al. (2001)
pgkA	Phosphoglucokinase	*A. oryzae*	Ogawa et al. (1994)	*A. oryzae*	Cellobiohydrolase	*Athelia* (*Corticium*) *rolfsii*	Yasokawa et al. (2003)
SC3	Hydrophobin	*S. commune*	Alves et al. (2004)	*S. commune*	Glucuronoyl esterase	*P. cinnabarinus*	Duranová et al. (2009)
Taka amylase	α-Amylase	*A. oryzae*	Christensen et al. (1988)	*A. oryzae*	Laccase	*Rhizoctonia solani*	Wahleithner et al. (1996)
					Laccase	*Trametes villosa*	Yaver and Golightly (1996)
					Lignin peroxidase	*P. chrysopsorium*	Stewart et al. (1996)
					Manganese peroxidase	*P. chrysopsorium*	Stewart et al. (1996)
					Lignin peroxidase	*P. chrysopsorium*	Montiel-González et al. (2009)
				A. rouxii	Manganese peroxidase	*P. chrysopsorium*	Montiel-González et al. (2009)
				A. niger	Manganese peroxidase	*P. chrysopsorium*	Cortés-Espinosa et al. (2011)
tanA	Tannase	*A. oryzae*	Hatamoto et al. (1996)	*A. oryzae*	Laccase	*S. commune*	Hatamoto et al. (1999)

[a]In this *A. niger* strain the *alcR* gene from *A. nidulans* has been introduced

4 Heterologous Expression in Zygomycetes

Only one example of heterologous expression of basidiomycete genes in zygomycetes has been described (Montiel-González et al. 2009) (Table 3). *Amylomyces rouxii* was used as a cell factory for heterologous expression of a LiP and a MnP encoding genes from *P. chrysosporium* under control of the Taka amylase promoter of *A. oryzae*. *A. rouxii* is an efficient degrader of organopollutants such as pentachlorophenol (PCP) through its ability to produce phenoloxidases. As no heme peroxidases were found in cultures of this fungus, the introduction of heterologous peroxidases was believed to further improve its ability to degrade PCP, which was confirmed in this study. Interestingly, no exogenous heme or hemoglobin was added to the media to ensure active forms of the heme-containing lignin-modifying peroxidases, as has been used in filamentous ascomycetes.

5 Conclusions and Outlook

Homologous and heterologous overexpression of basidiomycete genes is a challenging research field. The low number of transformable basidiomycete species strongly limits the options for homologous overexpression, as well as heterologous expression in basidiomycete hosts. Development of better transformation systems for a larger number of basidiomycetes will be required to develop them as an enzyme production platform.

In contrast, ascomycete expression systems are well established and cover both yeasts and filamentous fungi as described in this chapter. However, the levels of basidiomycete enzymes produced in these systems are often much lower than those of ascomycete enzymes. No systematic studies into the reasons behind this have been performed, but it is likely that differences in basidiomycete and ascomycete gene models (basidiomycete genes often have more introns with less conserved start and stop sequences) or sensitivity to ascomycete proteases contribute to this. A better understanding of the factors affecting the production of basidiomycete enzymes in ascomycete hosts is required to reach the levels that are suitable for industrial applications.

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Prospects for Bioprocess Development Based on Recent Genome Advances in Lignocellulose Degrading Basidiomycetes

Chiaki Hori and Daniel Cullen

1 Introduction

Efficient and complete degradation of woody plant cell walls requires the concerted action of hydrolytic and oxidative systems possessed by a relatively small group of filamentous basidiomycetous fungi. Among these wood decay species, *Phanerochaete chrysosporium* was the first to be sequenced (Martinez et al. 2004). In the intervening 10 years, over 100 related saprophytes have been sequenced. These genomes have revealed impressive sequence diversity, and recent functional analyses are providing a deeper understanding of their roles in the deconstruction of plant cell walls and the transformation of xenobiotics.

Wood cell walls are primarily composed of cellulose, hemicellulose and lignin. Many microbes are capable of hydrolyzing the linkages in cellulose and hemicellulose, even though crystalline regions within cellulose can be rather challenging substrates (reviewed in Baldrian and Lopez-Mondejar 2014; van den Brink and de Vries 2011). In contrast, few microbes possess the oxidative enzymes required to efficiently degrade the recalcitrant lignin, a complex, amorphous, and insoluble phenylpropanoid polymer (Higuchi 1990; Ralph et al. 2004). These unusual wood decay fungi secrete extracellular peroxidases with impressive oxidative potential. Potential applications have focused primarily on lignocellulosic bioconversions to

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high-value, low molecular weight products and on organopollutant transformations. Voluminous literature covers the physiology and genetics of wood decay (for reviews see Cullen 2013; Cullen and Kersten 2004; Eriksson et al. 1990; Hatakka and Hammel 2010; Kersten and Cullen 2013; Kirk and Farrell 1987). This chapter does not provide a comprehensive treatment of the field, but rather highlights recent genome progress relevant to bioprocess development.

2 Diversity and Microbiology of Wood Decay Fungi

Two distinct forms of wood decay were generally recognized. White rot fungi degrade cellulose, hemicellulose, and lignin, although the patterns vary depending on the wood species and fungal strain (Blanchette 1991; Daniel 1994; Eriksson et al. 1990; Schwarze 2007). White rot fungi such as *P. chrysosporium*, simultaneously degrade all cell wall polymers, while *Ceriporiopsis subvermispora* selectively degrades lignin ahead of cellulose and hemicellulose (Akhtar et al. 1992; Behrendt and Blanchette 1997; Srebotnik and Messner 1994). A few white rot fungi, including *Phlebiopsis gigantea*, are able to rapidly colonize freshly cut conifers by metabolizing and tolerating resins, triglycerides and fatty acids. In contrast to white rot, brown rot fungi modify, but do not remove, lignin. Instead, a polymeric residue is left (Niemenmaa et al. 2007; Yelle et al. 2008, 2011) and the cellulose is rapidly depolymerized. Seemingly consistent with decay patterns, initial genome analyses revealed multiple genes encoding extracellular peroxidases in white rot fungi, but none in brown rot. In addition to wood decay fungi, certain litter decomposers also degrade lignin (reviewed in Eriksson et al. 1990; Hatakka 2001), and these saprophytes may play an essential role in the transformation and degradation of humic substances (Kluczek-Turpeinen et al. 2005; Snajdr et al. 2010; Steffen et al. 2002). The white rot fungus *Trametes* sp. degrades humic substances in a process likely involving Fenton-based mechanisms (Grinhut et al. 2011a, b).

Importantly, brown rot depolymerization of cellulose (Gilbertson 1981; Kirk et al. 1991; Kleman-Leyer et al. 1992; Worrall et al. 1997) proceeds rapidly in advance of extensive colonization and substrate weight loss. This observation and the limited porosity of cell walls strongly argue for the involvement of diffusible, small molecular weight oxidants (Blanchette et al. 1997; Cowling 1961; Flournoy et al. 1993; Srebotnik et al. 1988; Srebotnik and Messner 1991). Highly reactive hydroxyl radical, generated via the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + \cdot OH$) has been implicated in this process (Akhtar et al. 1992; Cohen et al. 2002, 2004; Xu and Goodell 2001). The precise mechanisms supporting sustainable hydroxyl radical production remain unclear although plausible redox systems have been proposed (reviewed in Arantes et al. 2012; Goodell 2003), and considerable evidence supports hydroquinone redox cycling (Arantes et al. 2011; Paszczynski et al. 1999; Suzuki et al. 2006) (Fig. 1a).

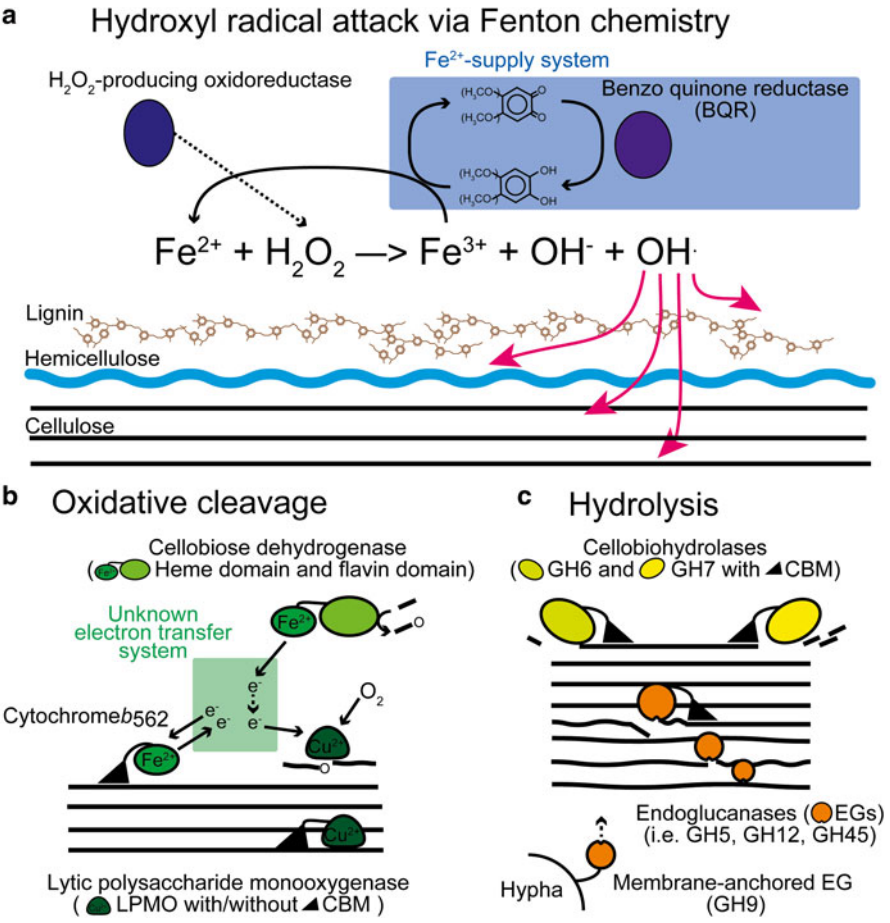

Fig. 1 Simple diagram of cellulose attack by wood decay fungi with hydroxyl radical (**a**), oxidative enzymes (**b**) and glycoside hydrolases (**c**)

3 Enzymes and Oxidative Processes Involved in Lignocellulose Conversions

3.1 Lignin Degradation

Owing to their high oxidation potential, the Class II peroxidases lignin peroxidase (LiP), versatile peroxidase (VP) and manganese peroxidases (MnP) modify lignin and related aromatic molecules. LiP and VP have redox potentials of approximately 1.5 V, and are able to directly oxidize non-phenolic lignin model compounds by a single electron (Kersten et al. 1985; Kirk et al. 1986; Miki et al. 1986). LiP and VP feature a surface tryptophan mediating long-range electron transfer

(LRET) of larger sterically-hindered non-phenolic substrates (Choinowski et al. 1999; Doyle et al. 1998). Depending on the structure of substrate, complex patterns of intermediates are formed (Miki et al. 1986; Tien and Kirk 1983). Peroxidases and ligninolysis have been reviewed (Hammel and Cullen 2008; Higuchi 1990; Martinez et al. 2014).

In contrast to LiPs and VPs, MnPs lack a conserved surface Trp and cannot directly oxidize non-phenolic aromatics. However, in the presence of H_2O_2 and a suitable Mn^{3+} chelator, conserved Mn-binding sites in the vicinity of a heme propionate allow MnP to catalyze the oxidation of Mn^{2+} to Mn^{3+} (Sundaramoorthy et al. 1994; Wariishi et al. 1992). These Mn-binding sites and the abovementioned surface Trp are conserved in VPs which thereby have hybrid characteristics of LiPs and MnPs (Ruiz-Dueñas et al. 2009). Diffusible Mn^{3+} chelates will oxidize phenolics directly or generate other oxidizing species, and oxalate is a likely physiological Mn^{3+} chelator for the MnP and VP reactions (Kuan and Tien 1993). Superoxide and perhydroxyl radicals might, in the presence of lipids, initiate radical chain reactions producing ligninolytic radicals (Kapich et al. 1999).

All lignin degrading fungi were thought to possess some combination of genes encoding class II peroxidases, but recent analysis of *Botryobasidium botryosum* and *Jaapia argillacea* has shown that these wood decay fungi slowly degrade lignin in the absence of high oxidation potential peroxidases (Riley et al. 2014) (Fig. 2). Similarly, no class II peroxidases were detected in the genome of *Schizophyllum commune* (Ohm et al. 2010), a white rot fungus exhibiting weak to non-existent lignin degradation (Boyle et al. 1992; Schmidt and Liese 1980). These observations undermine the strict dichotomous classification of white rot and brown rot wood decay, although it should be noted that efficient lignin degrading fungi typically have multiple class II peroxidase genes while brown rot fungi and phylogenetically related ectomycorrhizae have none (Floudas et al. 2012; Martin et al. 2008; Martinez et al. 2009). Among the highly ligninolytic white rot fungi, the number of genes encoding class II peroxidases varies sharply. For example, LiP, MnP, and VP gene numbers are 10, 5, and 0, respectively in *P. chrysosporium*, but 0, 9, and 3 in *Dichomitus squalens* (summarized in Cullen 2013). The role of this genetic multiplicity is uncertain, but differential regulation in response to substrate composition was observed prior to the 'genomics era' (Holzbaur and Tien 1988; Stewart and Cullen 1999) and, of particular relevance here, transcripts corresponding to specific *P. chrysosporium* LiP and MnP genes were identified in colonized soil containing polycyclic aromatic hydrocarbons (PAHs) (Bogan et al. 1996a, b). *Pleurotus ostreatus* lacks LiP-encoding genes, but transcript levels of MnP and VP are influenced by media composition (Knop et al. 2014) and the VP4 dominates under Mn deficient conditions.

The degradation of PAHs and other organopollutants has been attributed to the striking oxidation potential and low substrate specificity of the class II peroxidases (reviewed in Cullen 2002; Hadar and Cullen 2013; Hammel 1995a, b; Higson 1991; Pointing 2001). PAHs such as benzopyrene, pyrene and anthracene have ionization potentials below 7.6 eV and serve as substrates for LiP (Hammel 1995a; Hammel et al. 1986). LiPs will also transform chlorinated phenols (Hammel and Tardone 1988;

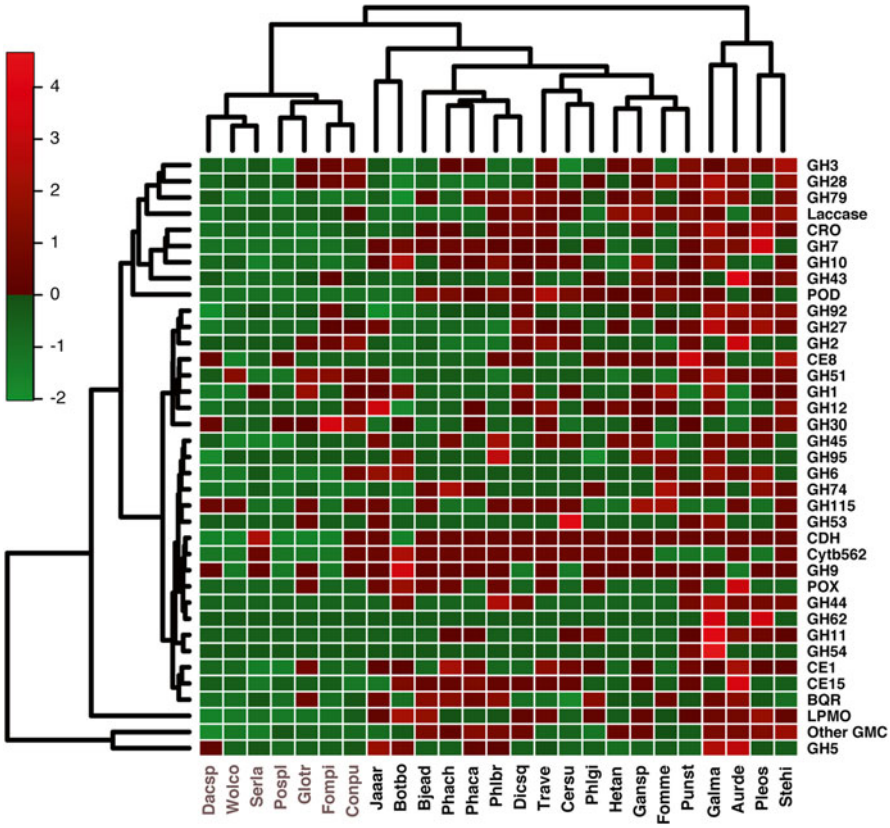

Fig. 2 The numbers of lignocellulose-degrading enzymes predicted in 25 wood decay fungal genomes. Heatmap color scale shown on *upper left*. Cluster dendrogram was performed by R using gene numbers and clearly separates seven brown-rot fungi and sixteen white-rot fungi. Unusual white rot fungi, *Jaapia argillacea* (Jaaar) and *Botryobasidium botryosum* (Botbo), which lack POD genes, cannot be easily assigned to white- or brown-rot categories (Riley et al. 2014). Seven species of brown-rot fungi include *Dacryopinax* spp. (Dacsp), *Wolfiporia cocos* (Wolco), *Serpula lacrimans* (Serla), *Postia placenta* (Pospl), *Gloeophyllum trabeum* (Glotr), *Fomitopsis pinicola* (Fompi) and *Coniophora puteana* (Conpu). The 16 species of white-rot fungi are: Bjead, *Bjerkandera adusta*; Phach, *Phanerochaete chrysosporium*; Phaca, *Phanerochaete carnosa*; Phlbr, *Phlebia brevispora*; Dicsq, *Dichomitus squalens*; Trave, *Trametes versicolor*; Cersu, *Ceriporiopsis subvermispora*; Phlgi, *Phlebiopsis gigantea*; Hetan, *Heterobasidion annosum*; Gansp, *Ganoderma* spp.; Fomme, *Fomitiporia mediterranea*; Punst, *Punctularia strigosozonata*; Galma, *Galerina marginata*; Aurde, *Auricularia delicate*; Pleos, *Pleurotus ostreatus*; Stehi, *Stereum hirsutum.* This aggregated data set was derived from (Floudas et al. 2012; Hori et al. 2013, 2014c; Riley et al. 2014)

(Mileski et al. 1988; Reddy and Gold 2000; Valli and Gold 1991), tetrahydrofurans (Vazquez-Duhalt et al. 1994), dioxins (Hammel et al. 1986; Valli et al. 1992b), methoxybenzenes (Kersten et al. 1985), and chloro- and nitro-methoxybenzenes (Teunissen et al. 1998; Valli et al. 1992a, b; Valli and Gold 1991). MnP can decolorize azo dyes (Heinfling et al. 1998) and oxidize pentachlorophenol and 2,4,6-trinitrotoluene

(TNT)(Reddy and Gold 2000; Scheibner and Hofrichter 1998; Van Aken et al. 1999). VPs will also transform azo dyes (Salame et al. 2010, 2012b) as well as carbamazepine (Golan-Rozen et al. 2011).

The PAHs phenanthrene and flourene are not MnP or LiP substrates (Bogan et al. 1996a, b, c; George and Neufield 1989; Hammel et al. 1992; Vazquez-Duhalt et al. 1994), but peroxidation of unsaturated lipids can generate transient lipoxyradical intermediates that oxidize non-phenolic lignin model compounds, flourene (Bogan et al. 1996a, b, c) and phenanthrene (Moen and Hammel 1994). The efficient production of class II peroxidases in *E. coli* (Doyle and Smith 1996; Nie et al. 1998) has substantially advanced understanding of the catalytic properties of individual isozymes (Martinez et al. 2014), and directed evolution using a *S. cerevisiae* expression system has been used to alter temperature-, peroxide-, and pH-tolerance of *P. eryngii* VP (Garcia-Ruiz et al. 2012).

Although not class II peroxidases, recent studies also suggest that heme thiolate peroxidases (HTPs) and the dye decolorization peroxidases (DyPs) (Hofrichter et al. 2010; Lundell et al. 2010) may serve as useful biocatalysts for organopollutant degradation. HTPs include chloroperoxidases and peroxygenases and these catalyze various reactions (Gutierrez et al. 2011; Ullrich and Hofrichter 2005). The number of genes predicted to encode these enzymes varies substantially among fungi, and they are particularly abundant in the genomes of the saprotrophs *Agaricus bisporus* and *Coprinopsis cinereus*. In the case of the button mushroom *A. bisporus*, 16 of the 24 putative HTP genes were upregulated in compost (Morin et al. 2012). Potential applications for fungal peroxidases were recently reviewed (Martinez et al. 2014).

Laccases have also been implicated in lignin degradation through the oxidation of phenolic units in lignin, but the major non-phenolic substructures can only be cleaved in the presence of auxiliary substrates such as ABTS (2,2′-azino-bis-3-ethylthiazoline-6-sulfonate) (Bourbonnais et al. 1997, 1998; Collins et al. 1999) (reviewed in Giardina et al. 2010). Similarly, organophosphorous insecticides are degraded by *P. ostreatus* laccase (Amitai et al. 1998), and high ionization potential PAHs are oxidized by *Coriolopsis gallica* and *T. versicolor* laccases (Johannes et al. 1996; Pickard et al. 1999) in the presence of synthetic mediators. Laccase activity and stability were improved using *S. cerevisiae*-based directed evolution (Mate et al. 2010).The genomes of most white-rot fungi feature multiple laccase genes but some, notably *P. chrysosporium* and *Phlebiopsis gigantea*, contain none (Fig. 2).

Considerable research has examined PCP degradation by ligninolytic fungi and their purified enzymes. Peroxidases and laccases can initiate oxidation via para-quinone formation and release of chlorine. Remediation studies have demonstrated detoxification by humification into organic matter, and LiP, MnP and laccase polymerization of labeled PCP led to soil-bound products (Ruttimann-Johnson and Lamar 1996). Field remediation of PCP-contaminated soils has been demonstrated (Ford et al. 2007a, b; Lamar and Dietrich 1990; Lamar et al. 1990a, b, 1994; Lestan and Lamar 1996).

Numerous investigations have focused on the degradation of azo dyes, the largest class of synthetic dyes (Singh and Arora 2011), and wood decay fungi have shown considerable promise (Kaushik and Malik 2009; Stolz 2001; Wesenberg et al. 2003).

Both class II peroxidases and laccases will oxidize certain azo dyes and site specific mutagenesis verified the importance of Trp164 in *Pleurotus eryngii* VP (Camarero et al. 2005; Ruiz-Duenas et al. 2008). Random mutagenesis has improved degradative performance of a *P. ostreatus* laccase (Miele et al. 2010).

3.2 Peroxide Generation

Several enzymes have been proposed as the source of extracellular H_2O_2, a key requirement for class II peroxidases and for Fenton chemistry. Considerable evidence supports glyoxal oxidase (GLOX), a radical-copper oxidase (Whittaker et al. 1996) first reported in *P. chrysosporium* cultures (Kersten and Kirk 1987). Considerable evidence supports a close physiological connection between GLOX and LiP (Kersten 1990) and the enzyme is found in most, but not all, white rot fungi (Cullen 2013). Brown rot fungi do not feature GLOX-encoding genes although, as in the case of the white rot fungi *C. subvermispora, H. annosum* and *F. mediterranea*, structurally related copper radical oxidases (Vanden Wymelenberg et al. 2006) may compensate (Cullen 2013).

Other potential sources of extracellular H_2O_2 include glucose-methanol-choline oxidases (GMCs) such as aryl alcohol oxidase (AAO), methanol oxidase and various sugar oxidases (reviewed in Hernandez-Ortega et al. 2012). Methanol oxidase, in particular, is highly expressed by white rot and by the brown rot fungus, *Gloeophyllum trabeum*. In this case, the enzyme is proposed to generate the H_2O_2 required as a Fenton reactant (Daniel et al. 2007) ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + \cdot OH$). The biological role of cellobiose dehydrogenase (CDH) remains uncertain (Henriksson et al. 2000; Zamocky et al. 2006), but this multi-domain enzyme (Hallberg et al. 2000) warrants mention because of its ability to enhance cellulose oxidation by lytic polysaccharide monooxygenases (LPMOs) (Harris et al. 2010; Langston et al. 2011). LPMOs were originally classified as 'hydrolases' but subsequently shown to be copper-dependent monooxygenases (Quinlan et al. 2011; Westereng et al. 2011) (Fig. 1b).

3.3 Other Oxidative Enzymes

Beyond secreted peroxidases and laccases, wood decay fungi possess a plethora of intracellular systems for transforming an impressive array of organopollutants. The genomes generally contain approximately 150 or more cytochrome P450 encoding genes (Cullen 2013), but their precise role(s) are uncertain. Nevertheless, advances have been made (Doddapaneni et al. 2013; Syed and Yadav 2012).

White rot P450s have been implicated in the degradation of 4-methyldibenzothiophene (Ichinose et al. 1999), endosulfan (Kullman and Matsumura 1996) and various PAHs (Bezalel et al. 1996a, b; Masapahy et al. 1999; Syed et al. 2010,

2011). In some instances, the combined activities of ligninolytic peroxidases and P450s has been demonstrated (Bezalel et al. 1997). Similarly, the transformation of carbamazepine (CBZ), a widely occurring pharmaceutical pollutant, involves *Pleurotus ostreatus* MnP and P450(s) (Cruz-Morato et al. 2012; Golan-Rozen et al. 2011; Marco-Urrea et al. 2009). In *P. chrysosporium* cultures, 2,4,6-trichlorophenol (Reddy et al. 1998) and PCP (Hammel and Tardone 1988; Mileski et al. 1988; Reddy and Gold 1999, 2000) are degraded by peroxidase-driven dechlorination and then intracellular reductive dechlorination and hydroxylation. The catalytic activity of a *P. chrysosporium* P450 has been improved via mutagenesis (Syed et al. 2013). Activity of membrane bound P450s against PAHs has been assessed in *Pichia* by coexpression of a reductase partner (Syed et al. 2010).

In addition to the intensively studied degradative activities of wood decay fungi, certain enzymes hold considerable promise as biosynthetic catalysts. For example, tandemly arranged genes encoding pyranose 2 oxidase and pyranosome dehydratase have been observed in several genomes including *P. gigantea* (Hori et al. 2014b) and *P. chrysosporium* (Martinez et al. 2004). Their precise function remains uncertain, but they can produce the antibiotic cortalcerone (de Koker et al. 2004; Giffhorn 2000). These enzymatic reactions have been coupled to bifunctional catalysts containing Bronsted and Lewis acid sites to produce furylglycolic acid, a compound suitable for copolymerization with lactic acid (Schwartz et al. 2013).

4 Hydrolytic Systems Involved in Cell Wall Degradation

Fungal hydrolases and related carbohydrate active enzymes (CAZys) represent a major portion of the commercial enzyme market. Based on well established fermentation technologies, the ascomycetes *Trichoderma* and *Aspergillus* produce copious quantities of cellulases including exocellobiohydrolase I (CBHI), exocellobiohydrolase II (CBHII), β-1,4-endoglucanase (EG) and β-glucosidase (β-Glu) (Baldrian and Valaskova 2008; Kirk and Cullen 1998). The genetic characterization of the *T. reesei* cellulases was established long before the initial genome analysis (Martinez et al. 2008) and the hydrolases were known to be encoded by relatively few genes within glycoside hydrolase families GH6 (CBHII), GH7 (CBHI, EG), GH12 (EG), GH5 (EG) and β-Glu (GH1, GH3) (Fig. 1c). This stands in sharp contrast to the genetic diversity encountered in wood decay fungi such as *P. chrysosporium* where six CBH1 isozymes are produced. Possibly, this structural diversity reflects functional differences (Munoz et al. 2001). It is now clear that such genetic multiplicity is the norm in white rot fungi (Floudas et al. 2012; Hori et al. 2014b), and this extends to hydrolases, esterases and lyases involved in hemicellulose degradation (Kirk and Cullen 1998; van den Brink and de Vries 2011).

Likely owing to their reliance on oxidative depolymerization of cellulose, brown rot fungi have few, if any, exocellobiohydrolases and endoglucanses. *Serpula lacrymans* appears exceptional in that three GH5s with cellulose binding domains (putative EGs) have been identified in the genome (Eastwood et al. 2011). Several GH5 EG-like genes have been identified in other brown rot species but, in the

absence of binding domains and any exocellobiohydrolases (CBH1 or CBH2), it is unclear how such enzymes could efficiently hydrolyze crystalline cellulose.

The repertoire of genes and their regulation vary substantially among wood decay species and the substrate colonized. For example, *P. chrysosporium* transcript and secretome patterns differ markedly when cultured in media containing complex woody substrates, especially those genes encoding a GH92 α-1,2-mannosidase, α-GH27 α-galactosidase, a GH5 1,4 β-mannan endohydrolase, and two carbohydrate esterases (CE15) (Vanden Wymelenberg et al. 2011). Although closely related to *P. chrysosporium*, the complement of *Phanerochaete carnosa* genes (Suzuki et al. 2012) and their regulation (Macdonald and Master 2012; MacDonald et al. 2012) differ substantially. These patterns are, at least in part, related to a *P. carnosa's* preference for softwood; a characteristic even more pronounced by *P. gigantea*. Development of conversion processes may also be aided by elucidating transcript patterns on conventional forestry feedstocks (Vanden Wymelenberg et al. 2011) as well as gene expression responses to specific syringyl-rich transgenic derivatives of hybrid poplar (Gaskell et al. 2014). These recent studies demonstrate that lignin composition substantially altered gene expression suggesting that commercial enzyme mixtures could be improved by tailoring enzyme components to specific feedstocks. Such studies facilitate development of enzymatic systems for biomass conversions and may help guide *Populus* breeding programs.

In addition to polysaccharide degradation certain wood-inhabiting fungi efficiently remove resinous extractives from freshly harvested and/or wounded pine. Often problematic in paper manufacturing as pitch deposits, these extractives include resin acids, long chain fatty acids and triglycerides. Among white rot fungi, *Phlebiopsis gigantea* is particularly well adapted to colonizing conifer wood and recent genome analyses (Hori et al. 2014b) have identified highly expressed lipases and active β-oxidation pathways likely involved in the metabolism of the triglycerides (Fig. 3). Such lipolytic systems, particularly the lipases, may be useful for commercial bioprocesses.

5 Emerging Experimental Tools and Future Prospects

5.1 Genetic Transformation Systems

Strain improvement and fundamental research on lignocellulose degrading fungi have been hampered by the availability of tenable genetic transformation systems. Auxotroph complementation and drug resistance have been used for the model white rot fungus *P. chrysosporium*, but the efficiency is quite low and multiple, ectopic integration events are typically obtained. Encouraging advances along these lines include gene silencing via RNA interference (Matityahu et al. 2008) and the uptake of DNA by shock waves (Magana-Ortiz et al. 2013). The latter approach has been employed to generate *P. chrysosporium* transformants producing increased levels of LiP, MnP and a VP (Coconi-Linares et al. 2014). In *P. ostreatus*, enhanced ligninolytic activity was obtained by over expression of the gene encoding VP,

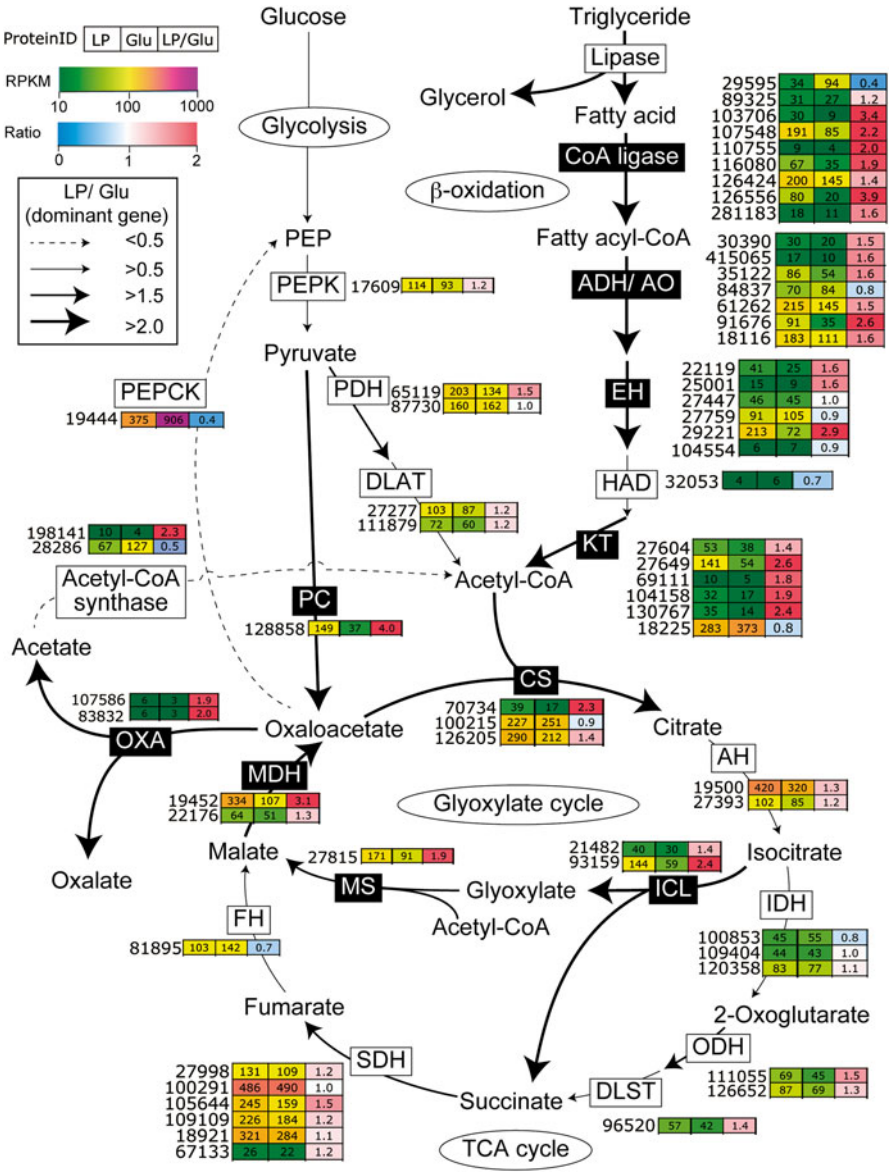

Fig. 3 Adapted from Hori et al. (Hori et al. 2014c), pathways illustrating lipid oxidation and related metabolism when *P. gigantea* is cultivated on medium containing loblolly pine (LP) relative to glucose (Glu) as sole carbon source. Enzymes encoded by upregulated genes are highlighted in *black* and associated with *thickened arrows*. Abbreviations: *ADH/AO* Acyl-CoA dehydrogenase/oxidase, *AH* aconitate hydratase, *CoA ligase* long fatty acid-CoA ligase, *DLAT* dihydrolipoyllysine-residue acetyltransferase, *DLST* dihydrolipoyllysine-residue succinyltransferase, *EH* enoyl-CoA hydratase; *FH* fumarate hydratase, *KT* ketothiolase (acetyl-CoA C-acyltransferase), *HAD* 3-Hydroxyacyl-CoA dehydrogenase, *ICL* isocitrate lyase, *IDH* isocitrate dehydrogenase, *MDH* malate dehydrogenase, *MS* malate synthase, *ODH* oxoglutarate dehydrogenase, *OXA* oxaloacetase, *PC* pyruvate carboxylase, *PDH* pyruvate dehydrogenase, *PEP* phosphoenolpyruvate, *PEPCK* phosphoenolpyruvate carboxykinase, *PEPK* phosphoenolpyruvate kinase, *SDH* succinate dehydrogenase

mnp4 (Salame et al. 2012a), and peroxidase regulation is influenced by calmodulin as shown by treatment with CaM inhibitors, RNAi suppression and overexpression (Suetomi et al. 2014).

The inability to target genes has been a longstanding experimental limitation among filamentous basidiomycetes. However, impaired nonhomologous end joining has been demonstrated in Ku knockout strains of non ligninolytic strains of *S. commune* (de Jong et al. 2010) and *C. cinereus* (Nakazawa et al. 2011) and, more recently, in the efficient lignin degrader, *P. ostreatus* (Salame et al. 2012b). The latter Δ*ku80* strain allows 100% homologous recombination among the stable transformants which have been useful for defining the catalytic roles of VP and MnP genes (Knop et al. 2014; Salame et al. 2013, 2014).

Such powerful experimental tools offer unprecedented opportunities to establish the function of a wide range of genes and thereby directly contribute to our understanding of lignocellulose conversions. Importantly, this analysis could help resolve the challenging problem of 'hypothetical genes', a common feature of fungal genomes. For perspective, of 11,891 predicted *P. gigantea* genes, 4744 encode 'hypothetical' or 'uncharacterized' proteins. RNA-seq and secretome analyses have shown that many of these genes are regulated and/or encode secreted proteins when cultivated on woody substrates (Hori et al. 2014b). Clearly, these proteins have significant roles, and targeted disruptions/replacements could provide meaningful functional insight along these lines. The regulation or expression levels of promising genes could be altered to improve, for example, bioremediation performance. Additionally, the 'hypothetical' protein of interest might be amenable to production in heterologous systems, in which case detailed biochemical investigations and bioprocess evaluation would be possible.

5.2 Metagenomes and Metatranscriptomes

The availability of an increasing number of fungal genomes and concomitant expansion of databases (Cantarel et al. 2009; Levasseur et al. 2013) facilitate metagenome, and metatranscriptome investigations of complex microbial communities (Baldrian and Lopez-Mondejar 2014). For examples, fungal transcripts associated with PAH degradation were identified in a soil microcosm amended with the phenanthrene (de Menezes et al. 2012), and eukaryote transcripts in spruce and beech forest soil included diverse fungal CAZys along with metazoan homologs (Damon et al. 2012). Focusing on cellulose degradation in coniferous forests, Baldrian and co-workers showed that fungal decomposition dominated in litter while bacteria were mainly represented in the soil (Baldrian et al. 2012; Stursova et al. 2012). Of relevance to biomass utilization, a diverse array of exocellobiohydrolase (CBH1) genes had been identified (Baldrian et al. 2012).

Recent database expansions, enhanced mass spectrometry performance and improved bioinformatic methods have made possible high throughput identification of extracellular proteins in pure cultures of *P. chrysosporium* (Hori et al. 2011; Manavalan et al. 2011; Ravalason et al. 2008; Sato et al. 2007; Vanden Wymelenberg

et al. 2005, 2009, 2010) and other white rot fungi (Floudas et al. 2012; Hori et al. 2014a, b; Martinez et al. 2009). Metaproteomic analysis of more complex samples will increasingly be used for defining the structure and functional activities within microbial communities (Hettich et al. 2012; Mueller and Pan 2013). For example, Schneider and co-workers (Schneider et al. 2012), have determined that, relative to bacteria, fungi were the main enzyme producers in forest litter samples, and they observed shifts in the relative abundance of ascomycetes to basidiomycetes over time. In these studies, mass spectrometry-identified peptide patterns were associated with activities of hemicellulases and cellulases. Collectively, these studies reveal a rich diversity of microbes and physiological activities in complex substrates.

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The Corn Smut Fungus *Ustilago maydis* as an Alternative Expression System for Biopharmaceuticals

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1 Introduction

1.1 Overview of the Current Expression Systems

Currently more than 200 marketed biopharmaceutical products exist. With the global sales of recombinant proteins crossing the 100 billion dollar mark, this emphasizes the significant impact of protein production on the overall pharmaceutical market (Berlec and Štrukelj 2013). A repertoire of excellent platforms is available to produce recombinant proteins for applications in therapeutics, research, and industry. Living cells are harnessed as factories to synthesize proteins based on molecular engineering. The bacterium *Escherichia coli* and mammalian systems are currently the main workhorses of biopharmaceutical production (Andersen and Krummen 2002; Walsh 2010), although new and emerging systems like fungi (Punt et al. 2002; Mattanovich et al. 2012), plants (Ma et al. 2003; Twyman et al. 2013), transgenic animals (Houdebine 2009) and cell-free synthesis (He et al. 2011) also account for some of these products. The selection of the expression system depends on the type of protein to be expressed, its requirements for functional activity and the desired yield (Berlec and Štrukelj 2013; Demain and Vaishnav 2009). Moreover,

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it is worthwhile to mention that the downstream processing can amount up to 80 % of the total manufacturing costs (Roque et al. 2004). Thus, the systems in which a protein is secreted into the medium are favorable to use because of the simplicity in downstream processing and thus, reduced overall expenses (Moir and Mao 1990).

Among the prokaryotic expression systems, *E. coli* is most widely employed due to its excellent properties such as the huge variety of genetic tools available for cloning and expression, quick growth in inexpensive media, and high product yields (Swartz 2001; Waegeman and Soetaert 2011; Huang et al. 2012). Hence, 31 % of the biopharmaceuticals approved till now are produced in this system (Berlec and Štrukelj 2013). However, it is apt for the production of small and structurally simple proteins but mostly not suitable for expressing huge proteins with complex tertiary structures, or for proteins requiring disulfide bridges or other post-translational modifications (Evan et al. 1985). The proteins produced either accumulate intracellularly or are secreted into the periplasmic space. Some examples of protein secretion in the medium have also been reported (Parente et al. 1998; Mergulhão et al. 2005; Zhang et al. 2006; Ni and Chen 2009; Schwarz et al. 2012). The tendency for protein aggregation in the form of inclusion bodies, however, necessitates employing cumbersome protein purification and refolding methods to obtain active protein (Lilie et al. 1998; Sørensen and Mortensen 2005).

Mammalian systems are preferred expression hosts due to their ability to carry out correct post-translational modifications required for many eukaryotic proteins. These include important human therapeutic proteins like e.g. erythropoietin, interferons or monoclonal antibodies (Wurm 2004; Zhu 2012). Accordingly, 43 % of the approved biopharmaceuticals are produced in this system. Mainly rodent cells such as Chinese hamster ovary (CHO), baby hamster kidney (BHK) and mouse myeloma (NS0) cells are employed. Less frequently, cells of human descent like human embryonic kidney (HEK-293) and human-retina-derived cells (PER-C6) are also applied (Berlec and Štrukelj 2013). However, high cultivation expenses, lengthy generation of stable expression clones and the risk of viral contamination still remain bottlenecks, which can account for very high costs of protein drugs produced in these systems (Berlec and Štrukelj 2013; Wurm 2004; Spadiut et al. 2014).

Fungal expression systems not only offer the advantage of high density culture capacity in inexpensive media as for bacterial systems, but also possess the ability to perform post-translational modifications as for mammalian systems (Mattanovich et al. 2012). Furthermore, these systems provide a simpler and cheaper downstream processing because proteins are secreted into the medium (Gerngross 2004). The most commonly used fungi are the yeasts *Saccharomyces cerevisiae* (15 % of approved biopharmaceuticals) and *Pichia pastoris* (1 % of approved biopharmaceuticals) while several other yeasts such as *Hansenula polymorpha, Kluyveromyces lactis, Schizosaccharomyces pombe, Yarrowia lipolytica* and *Arxula adeninivorans* are reported only in rare cases (Berlec and Štrukelj 2013; Çelik and Çalık 2012). Besides the yeasts, some species of filamentous fungi originating for example from the genera *Trichoderma*, *Aspergillus* or *Penicillium* have been exploited for their efficient secretion capacity and hence, their excellent ability of extracellular enzyme synthesis (Conesa et al. 2001; Ward 2012). Despite this promising potential, there

are some downsides to fungal expression platforms that often turn out difficult to eliminate. Especially in the case of filamentous fungi, proteolytic degradation by host proteases has been one of the major problems limiting the yields of heterologous proteins (Ward 2012). Furthermore, although post-translational modifications are performed in these systems, inappropriate glycosylation patterns like hypermannosylation can result in an interference with protein activity or impart harmful immunogenicity in humans (Gerngross 2004). Humanization of the glycosylation machinery has been successfully performed in yeast cells to produce various glycoproteins like recombinant erythropoietin in *P. pastoris* (Hamilton et al. 2006; Hamilton and Gerngross 2007) or IgGs (Li et al. 2006). Strategies to engineer the glycosylation machinery also exist for other fungi (De Pourcq et al. 2010).

Many alternate platforms are being explored but their commercialization has been slow till now (Demain and Vaishnav 2009; Corchero et al. 2013). In general, every system has its drawbacks and none of them is suitable for the production of all heterologous proteins. Hence, there is a strong demand for exploring alternative, novel cell factories to fill the existing gaps and to expand the repertoire of expression systems (Corchero et al. 2013; Feldbrügge et al. 2013). In the future, this will broaden the selection and bring down the costs of clinically relevant biopharmaceuticals, making them more affordable.

1.2 Exploiting Novel Routes of Secretion for Biotechnological Applications

Downstream processing can be simplified if heterologous proteins are secreted into the culture medium. In a eukaryotic cell, proteins are usually exported by the conventional secretory pathway which starts from recognition and hence translocation of signal peptide-containing proteins across the membrane of the endoplasmic reticulum (ER). After folding and quality control, secretory proteins are packaged into transport vesicles, followed by delivery of the cargo to the Golgi apparatus. After further modification of the cargo, post-Golgi transport carriers fuse with the plasma membrane eventually resulting in the release of the secretory proteins into the extracellular milieu (Nickel 2005).

Additionally, in some eukaryotes, protein secretion via non-conventional pathways is observed. Unlike the conventional pathway, this route does not require a canonical N-terminal secretion signal (Nickel 2005; Cleves 1997). Non-classical secretion of proteins has been described to occur either via vesicular or non-vesicular transport (Rabouille et al. 2012). Representatives of both types have already been shown for higher eukaryotes like mammalian cells. Examples are the vesicle-independent secretion of fibroblast growth factor 2 (FGF2) by direct translocation across the plasma membrane in a range of mammalian cells (Nickel 2011) or the export of Interleukin 1β via secretory lysosomes from monocytes during inflammation (Nickel and Rabouille 2009; Malhotra 2013). Different other vesicular transport routes have been discovered in lower eukaryotes. One well-described example

is the secretion of the acyl-CoA binding protein in *S. cerevisiae* (Acb1) and *Dictyostelium* (AcbA) via autophagosome-like vesicles (Rabouille et al. 2012). Exploiting these alternate secretion pathways in biotechnology by either presenting heterologous fusion proteins on the cell surface or secreting them into the extracellular medium would be a novel step towards broadening the repertoire of expression platforms (Nickel 2010).

This book chapter focusses on the exploitation of unconventional secretion in the fungus *Ustilago maydis* and compiles some aspects like the basic research that has initially inspired the idea, the advantages of the system, and the steps of its establishment and optimization.

2 The Corn Smut Fungus *Ustilago maydis*

2.1 U. maydis: A Model Microorganism on the Rise

U. maydis is a dimorphic basidiomycete that causes smut disease on corn. This fungus is well known through extensive research conducted for several years in various fields of molecular biology such as DNA recombination and repair (Holliday 2004; Kojic et al. 2013; Yu et al. 2013), plant-pathogen interaction (Bölker 2001; Brefort et al. 2009; Djamei and Kahmann 2012), post-transcriptional regulation (Vollmeister and Feldbrügge 2010) or molecular transport (Göhre et al. 2012; Steinberg 2014; Vollmeister et al. 2012a). Its genome is sequenced and manually annotated with very high quality (Kämper et al. 2006). As a consequence, this dimorphic fungus develops more and more towards a popular fungal model system (Dean et al. 2012; Steinberg and Perez-Martin 2008; Vollmeister et al. 2012b).

U. maydis cells can grow in a yeast-like form dividing by budding (Fig. 1a) while for pathogenic development cells switch to filamentous growth. This filamentous form infects corn plants turning the cobs into tumors filled with fungal spores (Vollmeister et al. 2012b) (Fig. 1b). Interestingly, these infected corn cobs have been served as a delicacy for centuries in Central America suggesting that this fungus is harmless for human consumption (Fig. 1c). Hence, *U. maydis* could be safely used as an expression host for production of protein biopharmaceuticals (Feldbrügge et al. 2013).

With respect to its use in biotechnology, *U. maydis* is known as a promising host for the production of secondary metabolites like glycolipids or itaconic acid (Hewald et al. 2006; Teichmann et al. 2007; Klement et al. 2012). Furthermore, the fungus harbors hydrolytic enzymes that could be of use (Feldbrügge et al. 2013). For example, a novel lipase (Uml2) was recently described that was discovered by genome mining and displays homology to the industrially important lipase CalB from *Candida antarctica*. Uml2 not only exhibits esterase activity comparable to CalB but it also harnesses an additional ability to hydrolyze phospholipids making it a potential alternate biocatalyst for biotechnological purposes (Buerth et al. 2014).

U. maydis is a robust organism and well suited for fermentation studies. Therefore, several groups are employing it to produce bio-based platform chemicals. Aerobic

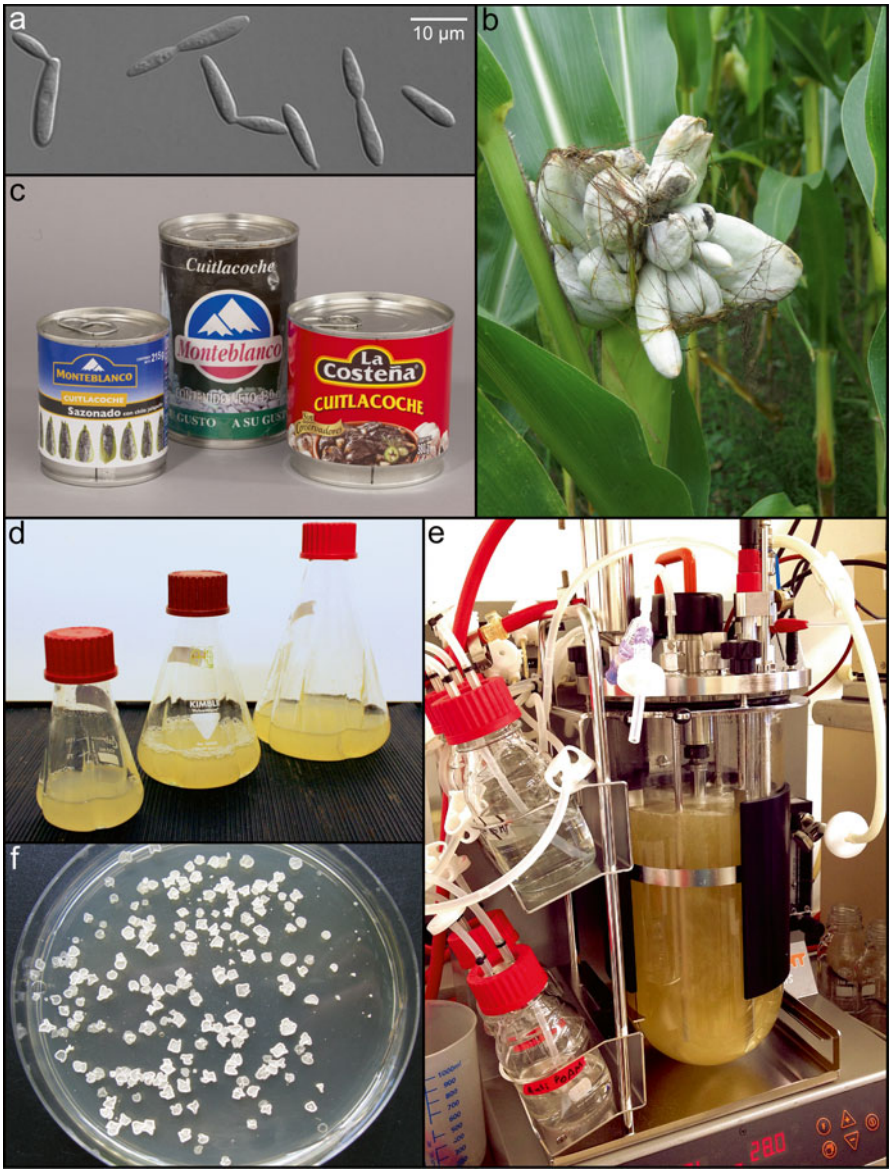

Fig. 1 The corn smut fungus *U. maydis*. (**a**) Microscopic image of yeast-like cells growing in liquid medium. (**b**) Infected maize plant showing tumor development on corn cobs (picture taken near Regensburg, Germany). (**c**) Corn smut galls conserved in tins and sold as the delicacy "cuitlacoche" (picture taken by Steffen Köhler, HHU Düsseldorf). (**d**) Yeast-like cells in shake flask cultures. (**e**) Three liter bioreactor cultivation of yeast-like cells. (**f**) Transformants on a regeneration agar plate five days after transformation of protoplasts

fermentation of cellulosic biomass has been successfully employed for production of itaconic acid, a platform chemical for the synthesis of potential biofuels (Klement et al. 2012; Maassen et al. 2014). A metabolic model for optimized itaconic acid production has been developed which can eventually be helpful in reactor studies and downstream processing of broth for protein expression purposes (Voll et al. 2012).

2.2 *Microtubule-Dependent Transport and Unconventional Secretion*

Besides the omnipresent eukaryotic secretion apparatus, a second, alternative secretion process was uncovered a few years ago in *U. maydis* (Koepke et al. 2011; Stock et al. 2012). In particular, unconventional secretion of a bacterial-type endochitinase (Cts1) was found to be dependent on long distance transport of mRNAs along microtubules in fungal filaments. This cytoskeletal trafficking of mRNAs is mediated by the key RNA binding protein Rrm4 (Becht et al. 2005, 2006). Deletion of *rrm4* led to impaired unipolar growth and reduced virulence, indicating the significance of this microtubule-dependent mRNA transport for polar growth and thus, successful infection of corn plants (Vollmeister and Feldbrügge 2010; Becht et al. 2005, 2006; Zarnack and Feldbrügge 2007).

Meanwhile, the molecular process of mRNA shuttling is well described. mRNA binding by Rrm4 is accomplished by three N-terminal RNA recognition motifs (RRMs) present in the Rrm4 protein while a C-terminal MademoiseLLE domain (MLLE) is required for the formation of shuttling mRNA particles (Becht et al. 2006). mRNA trafficking along microtubules mediated by Rrm4 requires the molecular motor-based movement of endosomes using Dyn1/2 for minus-end directed transport and Kin3 for plus-end delivery (Steinberg 2007; Schuster et al. 2011a). Intriguingly, co-localization studies demonstrated that Rrm4 and bound mRNAs hitchhike on endosomes that were known to shuttle along microtubules (Baumann et al. 2012; Schuster et al. 2011b). Recently, live-cell imaging studies on polarized fungal filaments further showed co-localization of septin mRNA and encoded septin protein as well as the presence of ribosomes on shuttling endosomes supporting the hypothesis of local translation on moving endosomes for delivery of proteins to distinct subcellular sites (Baumann et al. 2014; Higuchi et al. 2014).

To further characterize the role of Rrm4 during pathogenic development, differential proteomic studies were performed on parental and *rrm4* deletion filaments. Several proteins were differentially abundant in *rrm4Δ* filaments such as three mitochondrial proteins, a ribosomal protein and the potential bacterial-type endochitinase Cts1. In particular, Cts1 was shown in increased protein amounts in *rrm4Δ* filaments while losing its usual extracellular activity. Although its localization at the growth pole was not affected, secretion of Cts1 was drastically reduced in the absence of Rrm4. These results illustrate the importance of a functional microtubule-dependent mRNP transport for efficient secretion of Cts1 which is probably achieved by mRNA transport of the respective interacting export factors (Koepke et al. 2011).

Interestingly, according to bioinformatics predictions Cts1 does not harbor a conventional N-terminal secretion signal. It thus constitutes an interesting candidate for a novel carrier that can be tested for its use in applied research.

3 *U. maydis* as an Alternative Expression System

3.1 Exploiting Unconventional Secretion for Protein Export

Since Cts1 lacks a predictable N-terminal secretion signal needed for conventional secretion, the possibility of secretion by an alternate pathway was investigated. This assumption was proved using β-glucuronidase (Gus) from *E. coli* as a reporter enzyme (Stock et al. 2012). This bacterial enzyme by chance contains a eukaryotic N-glycosylation site at Asn_{345} which renders the enzyme inactive upon glycosylation. Therefore, after secretion via the conventional pathway involving passage through ER and Golgi apparatus, the enzyme is no longer active (Iturriaga et al. 1989). If in contrast it takes a non-conventional route bypassing the ER, it would circumvent glycosylation and the active enzyme would be secreted into the medium.

Based on this principle, Gus activity in strains expressing fusion proteins of Cts1 and Gus was assayed. The enzyme was indeed active in the supernatant when fused to the N-terminus of Cts1, suggesting that the fusion protein was secreted by a non-conventional route (Stock et al. 2012). Importantly, the observation that about 100 amino acids of the N-terminus are dispensable for Cts1 secretion further strengthened this hypothesis since the signal sequence for classical secretion is usually present at the immediate N-terminus (Stock et al. 2012).

The discovery of non-conventional Cts1 secretion prompted the idea to test the feasibility of *U. maydis* as a protein expression system. As an alternative to the commonly employed signal recognition particle (SRP)-dependent export of glycosylated proteins, unglycosylated heterologous proteins with therapeutic or industrial use can be secreted as Cts1 fusion proteins in this system (Stock et al. 2012). Besides the original finding in fungal filaments, unconventional secretion was later also demonstrated in yeast-like cells, permitting usage of both types of cells for protein expression (Feldbrügge et al. 2013; Stock et al. 2012).

3.2 Genetic Engineering of U. maydis

The prime prerequisite for a ubiquitous expression host is an easy handling along with low demands with respect to growth conditions. *U. maydis* meets these criteria quite well: in its yeast form, the fungus grows by budding in standard complete as well as minimal medium (Holliday 1974) with a growth rate of about two hours in shake flask cultures (Fig. 1d). Furthermore, cells are robust and well suited for

bioreactors which can be grown to high densities in a short time, without loss of strain fitness (Maassen et al. 2014) (Fig. 1e). Albeit in nature filamentous growth is restricted to the plant stage, engineered strains can also grow in the hyphal form in axenic culture. The coordinated induction of filamentous growth in these strains can be achieved by a simple medium switch (Brachmann et al. 2001).

As an important basis for strain generation, molecular cloning techniques are well established in *U. maydis* (Stock et al. 2012; Brachmann et al. 2001, 2004; Kämper 2004; Kojic and Holloman 2000; Terfrüchte et al. 2014). Deletion and insertion mutants can be generated easily. A versatile set of genetic tools is available that includes not only integrative plasmids allowing targeted insertion at defined genomic loci but also self-replicating plasmids as well as constitutive and inducible promoters (Stock et al. 2012; Brachmann et al. 2001; Terfrüchte et al. 2014; Spellig et al. 1996). During the past years, different genetic engineering strategies were established and a comprehensive vector library with replacement modules exists to enable various gene expression studies. The plasmid collection encompasses five different resistance-cassettes. Specific modules containing these cassettes were designed for different purposes like to simply carry out gene deletion, or express genes under the control of different heterologous promoters, or to study expression or localization using reporter genes like Gfp or mCherry. Also, combining different modules is possible (Baumann et al. 2012; Brachmann et al. 2004; Kojic and Holloman 2000).

Initially, gene deletion constructs were generated using a SfiI-based cloning strategy utilizing the feature of this enzyme that any five nucleotides can be selected between its recognition sequences (5´-GGCCNNNN↓NGGCC-3´). Since homologous recombination is utilized for carrying out genetic manipulation in this fungus, homologous flanking regions upstream and downstream of the gene to be deleted are amplified by PCR. Subsequently, the flanks are directionally ligated to a cassette of choice by using distinct core sequences (Brachmann et al. 2004; Kämper 2004). The construct is then introduced into a vector for amplification. The linear construct used for transformation can be regenerated using appropriate restriction sites located close to the insertion sites. Note that by this method, short non-homologous sequences remain at each end of the two flanks.

Fig. 2 (continued) restriction sites (i.e., SspI) can be used to remove the linear transformation construct from the resulting deletion plasmid generating perfect homologous ends (Terfrüchte et al. 2014) (*HygR* hygromycin resistance cassette, *GentR* gentamycin resistance, *AmpR* ampicillin resistance, *UF* upstream flank, *DF* downstream flank). (**b**) Scheme showing deletion of the *goi* by homologous recombination. Protoplasts are transformed with a linear fragment of the deletion plasmid and positive clones are selected on regeneration agar containing the appropriate antibiotic. For marker recycling the deletion mutant (Δ) is transformed with a free replicating plasmid expressing FLP under control of an inducible promoter (P_{crg1}). This leads to excision of the DNA sequence inserted between the FRT sites rendering the strain sensitive again (ΔR). Later, the FLP expression plasmid is removed by repetitive growth in medium free of antibiotics. wt, wild type genomic locus; Δ, mutant locus in which the *goi* was replaced by HygR; ΔR, mutant locus after resistance cassette recycling. UARS, *U. maydis* autonomously replicating sequence; *FLP* flippase, *NatR* nourseothricin resistance cassette

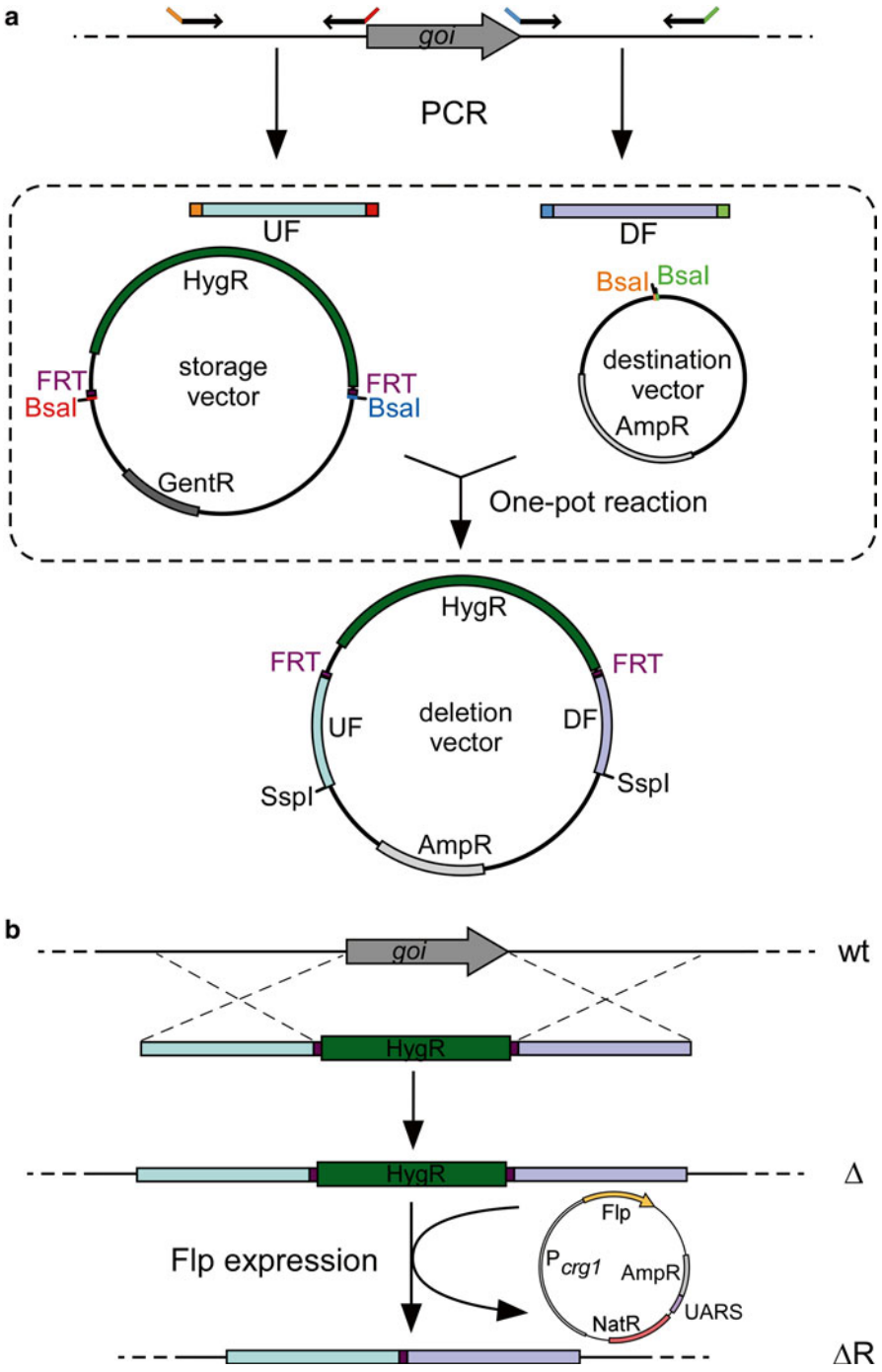

Fig. 2 Genetic engineering of expression strains. (**a**) Gene deletion using a BsaI-mediated Golden Gate cloning strategy. Deletion constructs for a gene-of-interest (*goi*) are generated by amplifying flanking sequences, concomitantly inserting appropriate BsaI sites (colored primer appendages). Subsequently, flanks are combined with an appropriate resistance cassette in a one-pot reaction. At the same time, the deletion construct is inserted into a vector backbone for amplification. Specific

Recently, a more elegant and high-throughput method based on Golden Gate cloning to generate deletion constructs was described (Terfrüchte et al. 2014) (Fig. 2a). The principle of this technique is based on the special property of type II restriction enzymes like BsaI. This enzyme cleaves few base pairs downstream of its recognition sequence allowing seamless joining of DNA fragments without introducing foreign nucleotides (Engler et al. 2008). Again, flanking regions are amplified by PCR introducing distinct BsaI sites at the ends. The flanks are then ligated to a resistance cassette of choice and inserted into a vector backbone. To achieve this, a specific set of two vectors namely storage and destination vectors is needed. The storage vector contains a resistance cassette, mediating for example hygromycin resistance for selection in the fungal host, surrounded by distinct BsaI sites. The destination vector contains compatible BsaI sites for insertion of the deletion construct. To assemble the final deletion vector, a one-pot reaction is carried out in which the amplified flanks along with storage and destination vectors are added in a reaction mix containing the enzymes BsaI and DNA ligase (Fig. 2a). This enables restriction by BsaI and ligation of the resulting appropriate fragments in a single step, while the respective recognition sites are lost. As an additional improvement the linear deletion construct to be used for transformation is designed to contain blunt end restriction sites so that after excision of the linear construct for transformation perfect homologous ends are generated. This increases the rate of homologous recombination (Terfrüchte et al. 2014) (Fig. 2a).

Due to the limited number of resistance markers available for *U. maydis*, an efficient resistance-marker recycling system has been invented a few years back that enables multiple gene deletions (Khrunyk et al. 2010). This marker recycling method is based on a property of the yeast recombinase flippase (FLP) to recognize and catalyze recombination between specific 34 bp sequences called FRT sites resulting in cleaving off the intervening DNA sequence and leaving one recombination site behind (Hamers-Casterman et al. 1993). This FLP/FRT system has recently been adapted for Golden Gate cloning (Terfrüchte et al. 2014). Storage vectors are in this case designed in a way that the resistance cassettes are surrounded by specific FRT sequences which are then introduced in the deletion construct (Fig. 2b). The resulting gene deletion strain is transformed with a free replicating FLP expression plasmid. Induction of FLP expression leads to removal of the resistance cassette between the FRT sites leaving the strain marker-free. Hence, the same selectable marker can now be used for subsequent rounds of gene manipulation (Fig. 2b). Subsequently, the marker-free strain is grown in complete medium without antibiotics to eliminate the FLP expression plasmid and making the strain available for the next gene deletion round (Khrunyk et al. 2010). In the future, both steps of gene deletion and FLP induction will be combined to generate strains faster.

In summary, genetically modified *U. maydis* strains can be generated quickly in around two to three weeks (Fig. 1f) with verification using simple PCR reactions and Southern blot analysis. This results in genetically stable transgenic strains, without the need to keep selective conditions. Its fast growth rate coupled with excellent molecular handling thus makes this fungus a good starting point to develop it as a valuable protein production factory.

3.3 Applying Unconventional Secretion for the Production of Single Chain Antibodies

To investigate whether unconventional secretion of Cts1 can be exploited for the export of heterologous proteins, antibody fragments were selected as a first target. Single-chain variable fragments (scFvs) are a popular format of antibody fragments in which the V_H and V_L domains are joined with a flexible polypeptide linker preventing dissociation (Bird et al. 1988). Such antibody fragments (25–30 kDa) are smaller than intact immunoglobulin molecules (150 kDa) and retain the specific, monovalent, antigen-binding affinity of the parent IgG, while showing improved pharmacokinetics for tissue penetration (Wörn and Plückthun 2001; Nelson and Reichert 2009). They show a reduced plasma half-life resulting in less whole body exposure to the drug, faster body clearance, and improving target to non-target ratios (Carter 2006). Due to their excellent pharmacokinetic properties, scFvs have varied applications such as in diagnostics, i.e. medical imaging, therapeutics like cancer, infections, inflammatory diseases, and also in immuno-detection, purification, and bioseparation applications (de Marco 2011; Holliger and Hudson 2005).

To study scFv expression and export via unconventional secretion in *U. maydis*, different scFv-Cts1 expression cassettes were developed using established techniques mentioned above (Stock et al. 2012; Sarkari et al. 2014). These constructs contain specific sequences to be used for integration of the expression vector at a defined locus by homologous recombination, leading to mitotically stable strains. The ip^s locus used for this purpose codes for the iron-sulphur subunit of a succinyl dehydrogenase. An amino acid exchange ($His_{253}Leu$) encoded by the ip^r allele in the expression cassette confers carboxin resistance to the enzyme (Broomfield and Hargreaves 1992; Keon et al. 1991). After transformation of yeast-like cells, linearized integrative plasmids harboring the ip^r allele are integrated by homologous recombination at this defined locus either in a single or in multiple copies. Importantly, multiple insertion of the plasmids leads to higher protein expression which can be advantageous from the industrial point of view (Stock et al. 2012). In the future, the system can be further improved by targeting expression constructs to other candidate loci making it even more flexible for genetic manipulation.

All integrative expression vectors were designed in a way that they harbor sequences encoding different tags which could be used for the purpose of protein purification and analysis. In the first generation expression plasmids (pRabX1), tags were placed in the middle of the fusion gene to minimize the risk of their loss due to proteolytic degradation (Stock et al. 2012). However, to simplify purification, the second generation plasmids (pRabX2) provide His or Strep tags at the N-terminus (Sarkari et al. 2014). These have been shown to promote efficient purification yielding active secreted protein. In the future, an internal TEV protease cleavage site will furthermore allow the excision and removal of Cts1 from the fusion protein to obtain pure heterologous protein. All vectors are moreover designed in a way that they provide unique restriction sites for the easy exchange of promoter regions, the target gene or the sequences encoding tags. Hence, in summary, a versatile vector system now allows the quick generation of different expression constructs.

3.4 Optimization for Efficient Protein Production

Initially, protein yields of scFv-Cts1 were found to be very low in *U. maydis* (Stock et al. 2012). To be used at an industrial level in the future, protein production needs to be improved so as to approach the yields of existing expression systems. Strain optimization to enhance protein production is a crucial step in developing expression systems (Archer et al. 1994). Before fine-tuning production at bioprocess level (Wang et al. 2005), it is imperative to generate an optimized strain with improved amounts of secreted protein by manipulation at the transcriptional, post-transcriptional, and post-translational levels (Archer et al. 1994; Kück and Hoff 2010).

Usually, selection of the right promoter for obtaining optimum expression constitutes the first step in system optimization. Depending on the type of protein to be produced, either constitutive or inducible promoters can be used (Van den Hondel et al. 1991). Constitutive promoters offer an advantage in terms of maintaining protein expression during the entire growth phase. During establishment of the expression system based on Cts1-mediated secretion of Gus, such type of constitutive synthetic promoter called P_{otef} was used (Stock et al. 2012). This promoter is made up of two direct repeats of a synthetic fragment consisting of seven tetracycline-responsive elements placed upstream of the transcription elongation factor 2 promoter P_{tef} (Spellig et al. 1996). Despite the successful secretion of Gus-Cts1 protein, low yields were obtained compared to other systems (Stock et al. 2012). As low expression is a main reason for low amounts of secreted protein, in the next step different promoters have been analyzed to check for higher protein expression levels. Again using Gus as a reporter, an alternative synthetic promoter called P_{oma} was identified as the yet most promising candidate. It is assembled from a minimal promoter of the pheromone *mfa1* gene and eight repetitive 40 bp upstream activating elements (UAS) taken from the *prf1* transcription factor promoter (Flor-Parra et al. 2006; Hartmann et al. 1999). This promoter showed an about 30-times higher Gus activity in the culture broth of strains expressing Gus-Cts1 compared to P_{otef} expression strain. Moreover, scFv production could be enhanced strongly when this promoter was applied (Sarkari et al. 2014). This demonstrates that optimizing transcriptional levels enhances Cts1-mediated protein secretion in *U. maydis*.

One additional important factor to be considered in the optimization of fungal expression systems is protein degradation. Typically, fungal systems encounter the problem of low protein yields due to degradation by host proteases (van den Hombergh et al. 1997a). Thus, deletion of key proteases is a promising step to reduce proteolytic degradation thereby improving the yield of secreted heterologous proteins (Idiris et al. 2010). Bioinformatic analysis has shown that *U. maydis* has 31 secreted protease genes (Mueller et al. 2008) (PEDANT, http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t237631_Ust_maydi_v2, accessed at 06/12/2014). This is a low number in comparison to other fungal expression systems which often contain more than 100 protease genes (Yoon and Kimura 2009). Deletion of several key proteases in various fungal hosts such as *S. cerevisiae*, *S. pombe*, *A. niger, A. oryzae* has led to successful improvements

in heterologous protein yields (Yoon and Kimura 2009; van den Hombergh et al. 1997b; Idiris and Tohda 2006; Yoon 2011; Newstead et al. 2007).

To study the impact of proteases in *U. maydis*, homologous counterparts of these proteases were eliminated. As mentioned above, Golden Gate cloning combined with FRT-mediated resistance cassette recycling can be employed to carry out multiple gene deletions in a short time (Terfrüchte et al. 2014; Khrunyk et al. 2010). Hence, sequential elimination of five protease genes was carried out in scFv production strains. The resulting strains showed a strongly reduced proteolytic activity without impaired strain fitness. Furthermore, increased amounts of active secreted scFvs were observed in this background (Sarkari et al. 2014). Using secretome analysis, more key proteases could be identified and deleted, thereby completely diminishing the proteolytic activity of expression strains.

4 Conclusions and Future Prospects

Establishing novel expression systems to expand the repertoire always constitutes an advantage in overcoming existing bottlenecks, and in the end provides better quality biopharmaceuticals at affordable prices (Corchero et al. 2013). The expression platform presented here enables protein export using a novel secretory mechanism and thus helps avoiding unspecific glycosylation which may be critical for certain therapeutic or industrial proteins (Stock et al. 2012). Furthermore, this simple eukaryotic fungal system harnesses the power of protein secretion not only by an unconventional mechanism but also by the conventional pathway which could be exploited to produce glycosylated proteins in the near future. Here, the glycosylation machinery could likely be humanized with fewer efforts as compared to other fungal species (Fernández-Álvarez et al. 2010). Till now, the expression system is at its nascent stage and has been used to show secretion of active proteins like antibody fragments (i.e., scFvs), enzymes like Gus, and several others in the pipeline (Stock et al. 2012; Sarkari et al. 2014). Optimization of the system led to improved secreted protein yields from about 1 to 40 μg/L at shake flask level with working densities of culture not higher than OD_{600} of 1.0 (Sarkari et al. 2014). Hence, expression studies will be scaled up from shake flask to the bioreactor level to achieve higher cell densities and subsequently, much better yields of secreted proteins reaching levels in mg/L scale.

Although the role of endosome-coupled mRNA shuttling is known to be essential in establishing unipolar growth mediated through local translation of transported mRNAs (Baumann et al. 2014), its connection to unconventional secretion of Cts1 is poorly understood. To improve protein secretion, one needs to understand the biology of the host even more. Hence, once the secretion pathway is deciphered, it will open more room for optimization of the system for example by manipulation of the trafficking factors to improve secretion rates. In summary, *U. maydis* holds a great biotechnological potential as an interesting platform for protein expression.

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Gene Expression in Filamentous Fungi: Advantages and Disadvantages Compared to Other Systems

Nada Kraševec and Mojca Benčina

1 Introduction: Genomics, a New Push in Fungal Research

Filamentous fungi have played an important role in the history of food-making processes. In Asia, fungi, especially *Aspergillus oryzae*, *A. sojae*, and *A. awamori*, have been used to enhance the taste of rice, soybeans, and other plant-based foods for thousands of years (Machida et al. 2008). Awareness of the commercial importance of filamentous fungi started with two events. At the very beginning of the twentieth century, after the discovery of penicillin and the identification of producer *Penicillium rubens*, filamentous fungi entered into a new era of use as organisms that could produce high value compounds for human health. This coincides with the first reported commercial production of citric acid in 1919 by Pfizer in Brooklyn, NY and later by Citrique Belge (now DSM) in Europe. Following those reports, filamentous fungi have been exploited extensively for production of homologous proteins. After the first report of DNA-mediated genetic transformation of *Neurospora crassa* in 1973 (Mishra and Tatum 1973), fungal biotechnology became a dominant technology. In addition to fungal metabolites and natural products exploited by the pharmaceutical industry, fungi and the proteins that are produced by fungi are used in bioconversions and bioprocess chemicals (e.g., bioplastics, biodiesel) and as food and feedstock in agriculture. Many industrially-important products that are

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produced by *Aspergillus niger*, *A. aculeatus*, *A. sojae*, *A. oryzae*, *Trichoderma reesei*, *T. harzianum*, *Fusarium venenatum*, *Rhizopus oryzae*, *Penicillium camembertii*, and *Humicola insolens* have obtained a generally regarding as safe (GRAS) status (http://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices). Consequently, these fungi are principal organisms used in food fermentation and cellulose degradation.

After many decades of completing the toolbox for the genomic manipulations of fungi, one can now observe rapid development in the area of fungal research. This is a consequence of at least two parallel events that took place: re-emerging interest for clean, renewable energy and growing interest in sustainable carbon resources for building-block chemicals using fungi and their enzymes to break-down plant biomass to fermentable sugars, which play an important part in these processes (Grigoriev et al. 2011). The second push definitely comes from the application of low-cost, high-throughput DNA sequencing technologies (Berka 2013).

2 Hosts for Producing Recombinant Proteins

The steps used to produce recombinant proteins include cloning a target DNA, amplification of proteins in an expression host, and adapting the host to meet the criteria for obtaining the most effective level of protein production. Protein quality, functionality, production speed, and yield are factors to consider when choosing the right host. Yeast and filamentous fungi are generally employed for the production of proteins that are used in the food and feedstock industry and in the chemical industry; they are also used for bioconversions. Non-glycosylated small proteins are mainly produced by *Escherichia coli* and *Bacillus subtillis*. Glycosylated human proteins are mainly, but not exclusively, made in mammalian cells. In the past, many studies have been published that have examined several aspects related to improvements in recombinant protein production and the prospects of using filamentous fungi as protein producers. Recently published research studies that summarize the production of recombinant proteins by filamentous fungi are listed in Table 1.

2.1 Filamentous Fungi

For protein production on an industrial scale, filamentous fungi, yeast, and bacteria are primarily employed. Filamentous fungi show an enormous nutritional flexibility as well as metabolic and secretion capacity. Among the hosts that are used for protein production, the highest levels of proteins can be found in the homologous proteins that are secreted into cultivation medium and in the heterologous protein of fungal origin that are produced by filamentous fungi under optimized fermentation conditions (Visser et al. 2011). Therefore, it is not surprising that fungi are employed in many commercial protein production processes. According to the Association of Manufacturers and Formulators of Enzyme products (AMFEP), over 60 % of the

Table 1 Research studies summarizing advances in recombinant proteins production using filamentous fungi

Title	Reference
Heterologous protein expression in filamentous fungi	Nevalainen et al. (2005)
A comparative evaluation of the advantages and limitations of frequently used expression systems	Yin et al. (2007)
Production of recombinant proteins by microbes and higher organisms	Demain and Vaishnav (2009)
Recombinant protein production systems for *Aspergillus*	Fleissner and Dersch (2010)
Biotechnology of *Trichoderma*	Schuster and Schmoll (2010)
New tools for genetic manipulation of fungi	Kück and Hoff (2010)
Genetic manipulation of fungal strains for the improvement of heterologous genes expression	Koushki et al. (2011)
Fungal protein production: Design and production of chimeric proteins	Punt et al. (2011)
Aspergillus as a multi-purpose cell factory: Current status and perspectives	Meyer et al. (2011b)
Production of recombinant proteins by filamentous fungi	Ward (2012)
Heterologous gene expression in filamentous fungi	Su et al. (2012)
Engineering fungal secondary metabolism	Scharf and Brakhage (2013)
Recent advances in the heterologous expression of microbial natural product biosynthetic pathway	Ongley et al. (2013)
Integrated approaches for assessment of cellular performance in industry relevant filamentous fungi	Workman et al. (2013)
Alternative hosts for protein production in structural biology	Fernández and Vega (2013)
Physical methods for genetic transformation of fungi and yeast	Rivera et al. (2014)
Making recombinant proteins in filamentous fungi—are we expecting too much?	Nevalainen and Peterson (2014)

industrial enzymes made with filamentous fungi are produced in *Aspergillus*. The following commercial enzymes are produced: aminopeptidase, amylase, arabinofuranosidase, asparaginase, catalase, cellulase, galactosidase, glucanase, glucoamylase, glucose isomerase and glucose oxidase, glucosidase, chymosine, hemicellulase, inulase, laccase, galactosidase, lipase, pectate lyase and pectin lyase, and esterase, pectinesterase, phytase, protease, xylanase (AMFEP, www.amfep.org; Enzyme Technical Association (ETA) http://enzymetechnicalassoc.org/fcclist/.pdf), and human lactoferrin, calf chymosin, plant thaumatin, and plant neoculin.

The main advantages of filamentous fungi as a protein producer include the high yields and efficient secretion capacity of its bioactive proteins with post-translational modifications, the affordable growth media, and the relatively simple genetic manipulation required in comparison to mammalian, plant, and insect cell lines. On the other hand, the yields of recombinant proteins of non-fungal origin are remarkably low. A considerable number of non-fungal proteins are lost in the secretory pathway due to incorrect post-translational processing. If the recombinant proteins of mammalian origin successfully pass folding, disulfide bond formation, and proteolytic processing, high-mannose type glycosylation without terminal sialylation occurs. Therefore, glycosylation is definitely one of the challenges that

must be overcome before filamentous fungi (Gerngross 2004) can become effective producers of pharmaceutical proteins for human consumption. Various improved fungal host strains have been developed for recombinant protein production, such as protease deficient strains (e.g., *A. niger* prtT deletion strain (Braaksma and Punt 2008; Punt et al. 2008), strains reducing unfolded protein response (UPR), repression under secretion stress (RESS), and endoplasmic reticulum-associated protein degradation (ERAD) (Nevalainen et al. 2005), and strains with altered protein secretion mechanisms (Sagt 2012). Regulation of gene expression using strong tunable promoters and production of non-fungal proteins, such as chimeric proteins, in combination with highly-expressed well-secreted fungal proteins are the steps that are required to improve yields of heterologous proteins, especially for therapeutics (Gasser and Mattanovich 2007; Gouka et al. 1997). Attempts for expressing pharmaceutical proteins have been made in *Aspergillus niger* for human interleukin 6 (Punt et al. 2008), immunoglobulin G1-kappa (Ward et al. 2004), non-glycosylated proteins like TNFα (Kraševec and Komel 2008), G-CSF (Kraševec et al. 2014), and others (Frenzel et al. 2013).

2.2 Yeast

Unlike other types of fungi, yeasts are single cell eukaryotic microorganisms that have a short generation time and are easy to genetically manipulate. They do not produce bacterial endotoxins and some of their products (invertase and urea amidolyase produced by *Saccharomyces cerevisiae*, lipase from *Candida rugosa*, phospholipase from *Pichia pastoris*, and lactase from *Kluyveromyces marxianus*) are generally regarded as safe (GRAS). *S. cerevisiae* and a methylotrophic yeast *P. pastoris*, are the two yeast species that are most frequently used for both recombinant protein production and therapeutic proteins (Table 2) (Celik and Calık 2012; Demain and Vaishnav 2009; Gasser et al. 2013; Mattanovich et al. 2012). For example *P. pastoris* expresses 8 g/L of a functional scFV antibody fragment under optimized growth conditions with co-expression of a BiP chaperone (Damasceno et al. 2007). Yeasts have the capacity to perform post-translational modifications, like glycosylation and the formation of disulfide bonds; their products are also proteolytically processed in a way that is similar to mammalian cells. Both *S. cerevisiae* and *P. pastoris* are genetically well-characterized with a well-established genetic toolbox. Targeted gene insertion into the genome and generation of stable production strains are promoted by high frequency of homologous recombination. Protein production in these two species of yeast is also cost effective, due to their high density growth in chemically-defined media. Nevertheless, the production of full-size immunoglobulins remains a challenge. Insufficient secretion of larger proteins and proteolysis of the secreted proteins are the main disadvantages of using a yeast system for expression of human glycoproteins. Inappropriate hyper-glycosylation of glycoproteins by *S. cerevisiae* could be partially overcome by (humanized) methylotrophic

P. pastoris, which also offers the advantage that it can be grown in media containing methanol that inhibits the growth of other types of microorganisms.

2.3 Bacteria

Bacteria are used for the production of many commercially important proteins (Table 2) and many biopharmaceuticals (PEGylated urate oxidase-Krystexxa; botulinum toxin-A (Xeomin®; meningococcal conjugate vaccine (Menveo); clostridial collagenase (Xiaflex); TNF-α inhibitor-Crtmzia; Thrombopoietin (Nplate®) [FDA approval 2008 and later]; insulins, IFNs, growth hormones)

Table 2 Filamentous fungi hosts for production of homologous and heterologous proteins at industrial scale (Association of Manufacturers and Formulators of Enzyme products (AMFEP), www.amfep.org; Enzyme Technical Association (ETA), http://enzymetechnicalassoc.org/fcclist/.pdf)

Filamentous fungi	Yeast	Bacteria
Aspergillus niger, *A. oryzae*, *A. aculeatus*, *A. sojae*, *A. mellus*, *A. awamori*, *A. ficuum*, *A. foetidus*, *A. japonicas*, *A. phoenicis*, *A. saitoi*, *A. tubingensis*, *A. usamii*	*Saccharomyces cerevisiae*	*Bacillus subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. lentus*, *B. stearothermophilus*, *B. circulans*, *B. halodurans*, *B. clausii*, *B. acidopullulyticus*, *B. brevis*, *B. alcalophilus,*
Trichoderma reesei, *T. harzianum*, *T. longibrachiatum*, *T. viride*	*Pichia pastoris*, *P. angusta* alias *Hansenula polymorpha*, *P. stipitis*	*Streptomyces vialoceoruber*, *S. lividans*, *S. werraensis*, *S. murinus*, *S. olivochromogenes*, *S. rubiginosus*, *S. chromofuscus*, *S. cinnamoneus*
Fusarium venenatum, *Penicillium camemberti*, *P. lilacinum*, *P. chrysogenum*, *P. multicolor*, *P. funiculosum*, *P roqueforti*, *P. funiculosum*, *P. citrinum*, *Humicola insolens*, *Rizopus oryzae*, *R. niveus*, *Trametes hirsute*, *T. versicolor*, *Rizomucor miehei*, *Mucor michei*, *M. pusillus*, *M. javanicus*, *Disporotrichum dimorphosporum*, *Endothia parasitica*, *Leptographium procerum*, *Cheatomium erraticum*, *Chrysosporium lucknowense*, *Cryphonectria* sp.	*Kluyveromyces lactis*, *K. marxianus*, *Schizosaccharomyces pombe*, *Candida lipolytica*, *C. rugosa*, *Debaryomyces hansenii*, *Lipomyces spp.*, *Yarrowia lipolytica*, *Schwanniomyces occidentalis*	*Escherichia coli*, *Klebsiella planticola*, *Microbacterium imperial*, *Sphingobacterium multivorum*, *Lactobacillus fermentum*, *L. casei* , *L. paracasei*, *Streptoverticillium mobaraense*, *Leuconostoc mesenteroides*, *Chryseobacterium proteolyticum*, *Geobacillus caldoproteolyticus*, *Mycrococcus luteus*, *M. lysodeikticus*

GRAS status for the produced products was obtained for the organisms that are underlined in this table

(Demain and Vaishnav 2009; Frenzel et al. 2013; Kern et al. 2007; Liu et al. 2013). Bacteria have many advantages over fungi, including rapid growth, high yields, cost effective production, and well-developed and extensive genetic tools. Knowledge of bacterial genetic elements, promoters, translational initiation sites, transcriptional factors, transcription, translation, and protein folding makes them valuable host organisms for the expression of small non-glycosylated proteins. Moreover, the bacterial genome can be easily modified. However, imperfect expression of proteins with disulfide bonds, the presence of endotoxin in the protein isolates, and acetate formation causing cell toxicity are the main disadvantages of using bacteria for protein production. Proteins produced in the form of inclusion bodies are usually inactive, insoluble, and require refolding. A reduction in the formation of inclusion bodies can be accomplished by: (1) reducing the rate of protein synthesis by employing different promoters and using a lower cultivation temperature, (2) optimizing the growth media, (3) coexpression of chaperones and foldases, (4) protein expression in the form of chimeric proteins, and (5) using other host bacteria. Higher rates of recombinant proteins are usually produced in the cytoplasm rather than in the periplasm, except for proteins with disulfide bonds that are directed to the periplasmic space with signal peptides at the N-terminus. Some examples of high level protein production in *E. coli* include: alkaline phosphatase at 5.2 g/L (periplasm) (Choi et al. 2000), human granulocyte colony-stimulatory factor at 3.2 g/L (periplasm) (Jeong and Lee 2001), and insulin-like growth factor 2.5 g/L (periplasm) (Joly et al. 1998).

Gram-positive bacteria from genus *Bacillus* are another promising type of bacteria that can be used for the production of heterologous proteins (Liu et al. 2013) (Table 2). The advantages of *B. subtilis* are: (1) it is a non-pathogenic form of bacteria that produces endotoxin-free products, (2) the products often obtain GRAS status (e.g., amylomaltase, asparaginase, xylanases, maltotetraohydrolase, glycosyltransferase, acyltransferase, pullulanase, pectate lyase, and alpha-amylase produced in *Bacillus* spp.), and (3) recovery of the proteins is cost effective. *B. subtilis* offers a genetically well-characterized system with a highly efficient transformation method, and genome reduction. To diminish the unwanted protease activity that is characteristic of *B. subtilis*, a strain has been developed that is deficient in eight extracellular proteases (Murashima et al. 2002). Two database collections provide information on the transcriptional regulation of *Bacillus* spp. (http://dbtbs.hgc.jp/ (Sierro et al. 2008) and its gene sequence, expression, regulation, and metabolic pathway with regulation mechanisms (http://subtiwiki.uni-goettingen.de/ (Lammers et al. 2010).

2.4 Cell Cultures

Mammalian Cells: The majority of biopharmaceuticals are produced in mammalian cell cultures. An approach that is similar to the approach used to optimize microbial production has also been used to improve production yields in mammalian cells. Developments of media and feeding strategies for cells in a bioreactor, along with

the genetic engineering of host cells, have contributed to higher product titers. Recent advances in global expression profiling at both the transcriptome and proteome levels have provided a better understanding of the physiology of mammalian cells and have further led to the identification of new targets for host cell line improvements. Metabolic engineering (Lim et al. 2010) has focused on enhancing energy metabolism and reducing waste products, such as lactate and ammonia, which was achieved through lower concentrations of glucose and glutamine in the growth media and by the expression manipulation of several enzymes: lactate dehydrogenase, pyruvate kinase, glutamine synthase, and glucose transporters. To accumulate sufficient cells in a short time and to obtain a prolonged production phase with little growth, different approaches, including gene-inhibiting apoptosis, have been used to modify the cell cycle control genes. To overcome the potential productivity limitations imposed by secretory bottlenecks, chaperones or foldases, targets associated with endoplasmic reticulum stress response and exocytic vesicle trafficking, have been manipulated. Glycosylation is a highly variable post-translational modification that also results in glycoprotein heterogeneity in mammalian cells. A uniform glycosylation pattern of therapeutic glycoproteins is preferred; therefore, cell engineering strategies have been used to enhance glycosylation and sialylation in proteins.

The best cell lines that can be used as expression hosts to produce human recombinant proteins that meet quality and productivity demands are listed in Table 3. In mammalian cells, the production of antibodies could reach up to 5 g/L. However, there are several drawbacks using mammalian expression hosts; the process is expensive, it results in poor yields for the secreted proteins, and the resulting product might potentially be contaminated by viruses.

Plant Cells: Foreign non-plant proteins, including therapeutic proteins and industrial enzymes, can be expressed in dedifferentiated plant cells, such as the suspended tobacco, rice, and carrot cells, and in differentiated plant tissues and organ cultures, such as moss and hairy root cultures (see Huang and McDonald's (2012) study emphasizing the benefits of plant cell protein production) (Table 3). Plant cell suspension cultures are derived from stably transformed plant tissues by *Agrobacterium*-mediated transformation. In comparison to animal cell cultures, the production of recombinant proteins in plants and plant cells is much safer and less expensive. Doubling cell time varies from 12 h for tobacco BY-2 cells to up to 3 days for rice cells.

Insect Cells: Insect cells are a well-established instrument for recombinant protein expression. The baculovirus expression vector (BEV) system relies on the infection of lepidopteran cells derived from the fall armyworm, *Spodioptera frugiperda* (Sf9/Sf21, Super SF9), or from the fruit fly, *Drosophila melanogaster* (DS2). Cell lines from the cabbage looper, *Trichoplusia ni* (High Five, BTI-Tnao38, BTI-TN-5B1-4) can also be used for infection with a recombinant baculovirus derived from the nuclear polyhedrosis virus, *Autographa californica*, which contains circular double-stranded DNA (Demain and Vaishnav 2009; Frenzel et al. 2013). The BEV systems become more accessible with improved plaque selection of the recombinant virus.

Table 3 Cell lines for the production of recombinant proteins, with examples (Liu et al. 2013)

Human cells
HEK293 cells: embryonic kidney cells immortalized by adenovirus 5 (Ad5) (activated protein C and viral vectors); Per-C6 cells: embryonic retinoblasts immortalized by Ad5 E1A and E1B (adenoviral vectors, influence vaccine, IgM, and IgG 27 g/L); CAP cells: amniocytes immortalized by Ad5, E1/pIX functions; HKB-11:fused HEK293 and 2B8 cells (interleukin 2, IL-4, ICAM-1, and rFVIII); HT-1080: fibrosarcoma with epithelial-like phenotype (epoetin delta, iduronate-2-sulfatase, α-galactosidase A, and velaglucerase alpha)
Other mammalian cells
Chinese hamster ovary (CHO) cells (human tPA 34 mg/L); baby hamster kidney (BHK) cells; NS0-mouse myeloma cells (monoclonal antibodies 3 g/L); Sp2/0-mouse myeloma cells
Plant cells
Tobacco (anti-rabies Mab, Newcastle virus vaccine), carrots (glucocerebrosidase), rice cell cultures, aquatic plants (Specht and Mayfield 2014) host proteins
Insect cells
Fall armyworm *Spodioptera frugiperda* (Sf9/Sf21, Super SF9), fruit fly *Drosophila melanogaster* (DS2), cabbage looper *Trichoplusia ni* (High Five, BTI-Tnao38, BTI-TN-5B1-4)

Removing the nonessential genes that are involved in the viral life cycle from the baculovirus genome and inserting the enhancer sequences within the strong polyhedron promoter increases the stability of the recombinant viruses and enhances the yields of the target proteins. This insect cell expression system offers many advantages, including eukaryotic post-translational modifications with phosphorylation, N- and O-glycosylation, correct signal peptide cleavage and proteolytic processing, acylation, myristoylation, amidation, proper protein folding with disulfide bond formation, and no limits on protein size with the simultaneous expression of multiple genes. However, a specific pattern of glycosylation and improper protein folding are two of the disadvantages of using an insect cell system.

2.5 Transgenic Animals and Plants

Animals: In the future, genetically engineered animals may provide an important source of the protein based therapeutics. Transgenic animals are being used for the production of recombinant proteins found in milk, egg whites, blood, urine, plasma, and silk worm cocoons (Table 4) (Demain and Vaishnav 2009; Frenzel et al. 2013). In 2006, anti-thrombin III derived from the milk of genetically engineered goats (ATryn) was approved by the EU commission for use in treating patients. The production titer is 14 g of anti-thrombin III per L of goat milk. In most cases, the recombinant protein produced by the transgenic animal is as active as the native protein. Recombinant protein production in milk is also more cost effective than it is in a cell culture. However, poorly controlled expression of the introduced genes,

Table 4 Advantages and disadvantages of cell lines for the production of heterologous proteins on an industrial scale

Advantages	Disadvantages
Mammalian cells	
Proper folding	High costs
Post-translational modifications with addition of fatty acid chains, phosphorylation of tyrosines, threonine and serine hydroxyl groups	Complex scale-up
Correct glycosylation pattern	Risk of human pathogen infection
Plant cells	
Controlled growth	Reactor scale-up
Consistency of product yield	Low concentrations and instability of secreted proteins
Quality and homogeneity	High costs compare to field production
Insect cells	
Complex post-translational modifications: phosphorylation, O-glycosylation, N-glycosylation, proteolytic processing, acylation	Specific pattern of post-translational processing with incorrect glycosylation
Folding mammalian proteins	Differences in proteins expressed in mammalian and insect cells
S-S bond formation	
Solubility of proteins	

the costs of breeding and maintaining transgenic livestock using Good Agricultural Practice (GAP) guidelines, the lengthy period needed to access production yields, and the risk of human pathogen infection are some of the negative aspects of producing proteins using transgenic animals. Plant hosts have been used for the expression of human therapeutics, nutraceuticals, antibodies, industrial enzymes, vaccine antigens, and biopolymers (Table 4) (Frenzel et al. 2013; Wilken and Nikolov 2012).

Plants: Plants are efficient protein producers; they are capable of post-translational modifications. The United States Department of Agriculture has approved the field release of transgenic seeds expressing human lysozyme, lactoferrin, and serum albumin in rice (http://www.isb.vt.edu/search-release-data.aspx). The ability to generate biomass using low cost media, low production and storage costs, easy scalability, low risk of human pathogen infection, and the production of endotoxin-free products are some of the main advantages of using plants to produce proteins. However, low protein yields and the novelty of technologies are the main challenges of using plant hosts for protein production. Transgenic plant production time, regulatory issues (e.g., open field production), and public concerns are the main disadvantages of using transgenic plants for the production of human therapeutics.

Table 5 shows transgenic animals and plants for the production of human proteins on an industrial scale.

Table 5 Transgenic animals and plants for the production of human proteins on an industrial scale

Animals	
Sheep-milk (α-1-antytripsin 35 g/L, fibrinogen 5 g/L, and activated protein C 0.4 g/L); goat-milk (tPA 35 g/L; anti-trombin III 14 g/L); rabbit–milk (α-glucosidase 8 g/L); mice-milk (growth hormone 4 g/L, and anti-trombin III 2 g/L); pig (hemoglobin 40 g/L)	
Advantages	**Disadvantages**
High production of recombinant proteins	Required lactation, which is periodic
Time required for the start of production	
Upkeep costs for the transgenic animals	
Seed crops and fruits	
Corn (avidin, trypsin, aprotinin, collagen 120 mg/kg germ fraction, IgG), rapeseed (chymosin), rice seed (human lysozyme, lactoferin, and transferring 10 g/kg), soya bean (glucuronisidase), canola, maize, sunflower, and barley http://orfgenetics.com, *Carica papaya, Actinida chinensis, Cucurbita pepo, Ananas bracteatus, Ananas comosus*; proteins from the host organisms	
Advantages	**Disadvantages**
Accumulation within storage organelles	Fear of potential cross-contamination
Stability of seed-expressed proteins	Mixing transgenic and non-transgenic grain
Separated from proteases	Aversion of producing biopharmaceuticals in feed and food crops
Minimization of purification	
Requirements for GRAS crops (rice, corn)	
Leaf crops	
Tobacco leaves (aprotinin 750 mg/kg, glucuronisidase, IgG 1.5 g/kg), alfalfa, lettuce, and *Ficus glabrata*; proteins from the host organisms	
Advantages	**Disadvantages**
High biomass yield	High water content
	Storage stability of harvested biomass

3 Protein Data Bank Analysis of Fungal Hosts for Protein Production in Structural Biology

The high-yields and low-cost of protein production are guidelines for industrial protein production. Consequently, the host selection is highly dependent upon the costs of cultivation and product isolation. Therefore, the number of hosts is limited to a few organisms and an emphasis is placed on organisms whose products are generally regarded as safe (see chapter "High-Throughput Construction of Genetically Modified Fungi"). In structural biology, on the other hand, protein quality and purity are the primary requirements for proteins. For that reason, numerous expression hosts are used and each has specific advantages and disadvantages for protein production. One has to decide which properties of the heterologous expressed protein are important for the outcome and then select the host

accordingly. If the glycosylation of a protein is important, then a structural system that is similar to the original species should be used. For example, a cell free system is the system of choice for the production of membrane proteins. Detergents and lipids in the reaction mixture form a bicelle complex comprised of detergent-protein-lipid, which anchors and stabilizes the membrane proteins (Murray and Baliga 2013; Zheng et al. 2014). A comparison of the expression systems/hosts for protein production is presented in Table 6 (adopted from (Fernández and Vega 2013) and modified).

We evaluated the structural biology of the organisms used for recombinant protein production that were obtained from the Protein Data Bank (PDB) (as of February 18, 2014). Out of a total of 89,025 PDB entries, only the entries with a non-empty "Expression Organism (Gene Source)" field were considered. *E. coli*, baculovirus-infected insect cells, and mammalian cells represent the three main expression host groups, and together they account for 86 % of the expression hosts used in this study (Fig. 1a). These three host groups are followed by: yeasts (1.9 %)—methylotrophic and non-methylotrophic—and cell-free systems (0.4 %). The other alternative hosts are combined and categorized as "other" (11.8 %). Only a few filamentous fungi (0.2 %) have been used as expression hosts for structural biology. Those filamentous fungi belong to Ascomycota, genus *Aspergillus* (*A. oryzae*, *A. niger*, *A. nidulans*, *A. awamori* and *A. kawachi*), followed by genus *Trichoderma* (species *T. reesei* and *T. longibrachiatum*), and basidiomycota, genus *Penicillium* (*P. canescenses* and *P. funiculosum*) and *Phanerochaete chrysosporium* (Fig. 1b). The origin of two-thirds of the proteins expressed in the filamentous fungi is fungal, followed by bacterial. Only a few of the PDB entries have mammalian origins or originate from yeasts, grasses, shrimp, and birds (Table 7). The fungi-produced proteins belong to three classes: oxidoreductases, hydrolases, and lyases according to enzyme classifications (Table 8).

4 Emerging Opportunities of Systems and Synthetic Biology to Improve Protein Production

Systems biology and synthetic biology are complementary disciplines. Background knowledge of high-throughput bioinformatics technologies (Andersen and Nielsen 2009)—which includes genomics, transcriptomics, proteomics, interactomics, fluxomic states, metabolomic models, meta-analysis of promoters and expression regulatory sequences, software for protein structure visualization, and multi-domain protein engineering—provides the basis for reengineering the hosts using synthetic parts and pathways to improve production. Synthetic biology uses mathematical models to engineer novel biological synthetic networks that are able to perform tasks and change the behavior of biological systems (Cameron et al. 2014). Recent developments in synthetic biology have extended the toolset used in classical genetic engineering. Expression hosts have been tempered by implementing transcription, translation, and post-translational modifications and by introducing

Table 6 Comparison of expression systems/hosts for the production of proteins for structural biology

Expression host				
Bacteria	Unicellular fungi (yeast)	Filamentous fungi	Insect cells	Mammalian cells
Doubling time (h)				
0.3–0.5	1.2–3.0	3.0–4.0 h	16–72	14–36
Cultivation time				
Hours to days	Days to 1 week	Days to 1 week	Days to 1 week	Weeks
Cost of medium				
Low	Low	Low	High	High
Secretion capability				
To periplasm	To medium	To medium	To medium	To medium
Vectors				
Episomal	Episomal, integrative	Episomal, integrative	Bacmid	Episomal, integrative
Gene regulatory sequences (activation)				
Inducible	Inducible, constitutive	Inducible, constitutive	Viral life cycle	Constitutive
Proteolytic processing				
No	Some	Some	Yes	Yes
N-linked glycosylation				
None	High mannose	Mammalian-type core	Complex	Complex
	No sialic acid	No sialic acid	No sialic acid	
	Non-human sugars added	Non-human sugars added	Non-human sugars added	
O-linked glycosylation, phosphorylation, acetylation, acylation				
None	Yes	Yes	Yes	Yes
Advantages				
Scale-up, low cost, easy operation	Scale-up, eukaryotic protein processing, simple media	Scale-up, eukaryotic protein processing, simple media, good secretion	Better yields than mammalian systems, near mammalian protein processing	Optimal post-translational processing for human proteins
Disadvantages				
Protein solubility, suitable for small proteins	Not optimal post-translational processing for mammalian proteins	Not optimal post-translational processing for mammalian proteins	Demanding and expensive cultivation, low-high yields	Demanding and expensive cultivation, low-moderate yields

synthetic regulatory networks. Moreover, synthetic biology provides tools that can be used to create new multi-component systems that mirror natural biology and to construct organisms with novel features.

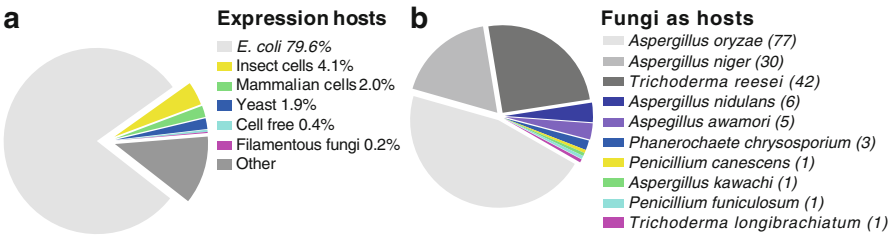

Fig. 1 Analysis of hosts for protein production in structural biology. (**a**) Expression hosts for protein synthesis; "other" refers to the alternative hosts. (**b**) Filamentous fungi as the expression host; the number of entries for the protein structures in the PDB are depicted (Data were collected from Protein Data Bank (PDB) on February, 18, 2014)

Table 7 Origin of the proteins expressed in filamentous fungi in structural biology

Fungi (66.6 %)
Aspergillus aculeatus, A. amstelodami, A. japonicas, A. kawachii, A. nidulans, A. niger, A. oryzae, A. tubingensis, Bjerkandera adusta, Chaetomium thermophilum, Coprinopsis cinerea, Fusarium graminearum, Humicola grisea, H. insolens, Limnoria quadripunctata, Myceliophthora thermophila, Penicillium canescens, P. citrinum, Phanerochaete chrysosporium, Pseudoplectania nigrella, Rasamsonia emersonii, Thermopolyspora flexuosa, Thielavia arenaria, T. terrestris, Trichoderma citrinoviride, and *T. reesei*
Bacteria (17.9 %)
Citrobacter braakii, Hafnia alvei, Microdochium nivale, Streptomyces coelicolor, Thermoascus aurantiacus, and *Yersinia kristensenii*
Higher eukaryotes (12.9 %)
Melanocarpus albomyces, Triticum aestivum, Gallus gallus, Bos taurus, Camelus dromedaries, and *Homo sapiens*
Yeast (2.6 %)
Saccharomyces cerevisiae

Table 8 Proteins expressed in filamentous fungi for structural biology, grouped according to enzyme classification

Oxidoreductases 1.x.x.x
Amine oxidase, catechol oxidase, cellobiose dehydrogenase, dye decolorizing peroxidase, fungal peroxidase (ligninase), galactose oxidase, glucose oxidase, laccase, manganese peroxidase, and quercetin 2, 3-dioxygenase
Hydrolases 3.x.x.x
Alpha-amylase, cellobiohydrolase i and ii, cellulase, endocellulase, endopolygalacturonase i, endo-xylogalacturonan hydrolase a, feruloyl esterase, beta-1,4-galactanase, glucoamylase, beta-glycoside i, lactoferrin, alpha-1,2-mannosidase, beta-mannanase, 4-phytase, rhamnogalacturonan acetylesterase, rhamnogalacturonase a, xylanase, and beta-1,4-xylanase
Lyases 4.x.x.x
Alpha-carbonic anhydrase, isocitrate lyase, and rhamnogalacturonan lyase

Filamentous fungi have served as industrial cell factories for a century and they are attractive cells for re-engineering for industrial use. We witnessed immense improvements in production yields, reducing the costs of fermentations as a consequence of implementing a strain improvement program (e.g., citric acid production by *A. niger*, hydrolyses synthesis by *T. reesei*, and penicillin production by *Penicillium*). We can predict that research being conducted in the fields of synthetic biology and systems biology will result in the development of innovations in strain improvements.

4.1 Systems Biology Resources for Filamentous Fungi

The systems biology resources for filamentous fungi have improved in the past ten years. Several platforms/databases offer information about fungal genomes (Table 9). The data could be used for gene-mining and gene-replacement searches and for

Table 9 Fungal "omics" resources

Database	Link
NCBI, National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
MycoCosm, Joint Genome Institute (JGI) ~350 fungal genomes	http://genome.jgi-psf.org/
AspGD, *Aspergillus* genomes database	http://www.aspgd.org/
DOGAN project, *A. oryzae* genome	http://www.bio.nite.go.jp/dogan/project/view/AO
EMBL-EBI, Ensembl Fungi, Ensembl Genomes, European Bioinformatics Institute; support for analysis and visualization of fungal genomic data	http://fungi.ensembl.org/index.html
FGI, Fungal Genome Initiative, Broad Institute ~50 fungal genomes	http://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/fungal-genome-initiative
CADRE, Central *Aspergillus* REsource & AsperCyc; metabolic pathways for the *Aspergillus* genus	http://www.cadre-genomes.org.uk/index.html
CoReCo, Comparative metabolic reconstruction	http://esaskar.github.io/CoReCo/

global genome analysis and comparison. In addition to NCBI, the MycoCosm at JGI is the largest fungal database that stores about 350 fungal genomes with the goal to scale up to 1000 fungal genomes (Grigoriev et al. 2014). The Ensembl Fungi database, developed by EMBL-EBI and accessed via the Ensembl Genomes browser, serves as an interface for the analysis and visualization of genomic data. The specific AspGD database and the Japanese database, DOGAN, provide information about the *A. niger, A. nidulans, A. fumigatus,* and *A. oryzae* genomes in comparison to 15 other *Aspergilli* species (Arnaud et al. 2012; Cerqueira et al. 2014; Machida et al. 2005). The FGI at the Broad Institute enables sequence data analysis for the fungi (50 fungi have been sequenced) that are important to medicine, agriculture, and industry. The CADRE database of the AsperCyc genome is a resource that provides predicted metabolic pathways for *Aspergilli* using data about the *Aspergillus* genomes (Mabey Gilsenan et al. 2012). A novel computational approach for comparative metabolic reconstruction (CoReCo) provides genome-scaled metabolic network models for 49 important fungal species (Pitkänen et al. 2014).

4.2 Molecular Toolbox for Synthetic Biology

The transition to synthetic biology from recombinant DNA technology came with cheap DNA sequencing. While DNA synthesis provides powerful ways to harness biology for our use, it is very expensive so the construction of large gene libraries and synthetic genomes is still out of reach for most. To facilitate and simplify building new complex DNA devices, the repositories of DNA elements—the Registry of Biological parts (http://parts.igem.org/Main_Page)—offer characterized biological modules, including promoters, ribosomal binding sites, coding sequences, and terminators. DNA building blocks can be assembled into larger constructs of predictable biological functions according to standard molecular cloning. Furthermore, restriction-site-independent technology—the DNA assembly method by Gibson et al. (Gibson et al. 2009)—for assembling DNA is replacing restriction enzyme-based cloning and is speeding up synthesis of complex DNA elements. Unfortunately, the verification of DNA assembly is still a requirement that is time-consuming, logistically challenging, and expensive.

Tools that simplify the manipulation of a host genome have been developed. Deletions of the ku70 gene and the ku80 gene have been shown to significantly increase the frequency of homologous integration for *Aspergillus* spp. (Zhang et al. 2011) and yeast *Pichia pastoris* (Näätsaari et al. 2012). Gene replacement with marker recycling using self-excising knock out cassettes in a *ku70/ku80* deficient strain enables subsequent rounds of strain engineering with only one selective marker (Hartmann et al. 2010; Mizutani et al. 2012). Recently, new targeted integration technologies using Zinc-fingers, TALEN, or Cas/CRISPR (Westra et al. 2014) may facilitate specific site integration (Gaj et al. 2013).

4.3 Synthetic Regulatory Tools

One way to improve protein expression is to place a protein of interest downstream from transcription regulatory sequences that would enable the expression of the protein under given fermentation conditions. At the transcriptional, translational, and post-translational levels the control is based on genetically encoded information, like promoter strength, gene copy number, mRNA turnover rate, and enzyme concentration.

Transcriptional Control: Transcriptional control is a critical step in gene expression and it has been widely used in synthetic biology to optimize metabolic pathways and genetic circuits. Through promoter engineering, promoter libraries (Carninci et al. 2006; Hartner et al. 2008; Siegl et al. 2013) can offer synthetic promoters with different strengths and regulations that are the cumulative effect of short nucleotide sequences, which mediate binding of transcription machinery, and upstream activator sequences, which fine-tune gene expression (Blazeck and Alper 2013). For example, Juven-Gershon et al. (2006) fused the AOX1 promoter to activating transcription factor binding sites for *Pichia*. A minimal promoter was fused to binding sequences for transcription activator-like effector (TALE) in combination with synthetic transcription activators or repressors; fusions of TALE DNA-binding domain and, for example VP16 (Sadowski et al. 1988) or KRAB (Witzgall et al. 1994), respectively, were found to efficiently regulate mammalian gene expression (Gaber et al. 2014). TALEs have already been used in yeast (Aouida et al. 2014); however, whether or not these types of expression regulators work in fungi must still be tested.

Serial analysis of gene expression (SAGE) has been used to identify the promoters of highly expressed genes by determining the amount of proteins that are produced under a defined condition (Harbers and Carninci 2005). Various "omics" technologies have been used to identify the numerous natural, endogenous promoters that drive gene expression under given cultivation conditions in filamentous fungi (Lorito et al. 2010; de Oliveira and de Graaff 2011). Sibthorp et al. (2013) performed whole transcriptome sequencing of *A. nidulans*. Global analysis of 5′-transcript ends provided an extensive map of the promoters across the fungal genome. Todd et al. (2014) analyzed 37 classes of transcription factors across hundreds of fungal genomes and demonstrated a significant difference in the regulatory repertoire of ascomycete fungi and basidiomycete fungi.

Post-transcriptional Regulation: In post-transcriptional regulation, the structural elements around the translational initiation region of mRNA play an important role in determining the amount of protein that is produced from a particular mRNA sequence (Pfleger et al. 2006). Software for designing synthetic ribosome-binding sites and 5'-untranslated regions can predict the expression levels of protein (RBS calculator, https://salislab.net/software/ (Salis et al. 2009); the RBS designer, http://ssbio.cau.ac.kr/web/?page_id=195 (Na and Lee 2010); and the UTR designer for prokaryotic translation efficiency, http://sbi.postech.ac.kr/utr_designer (Seo et al. 2013)). In addition, to decuple cell growth and protein production, orthogonal ribosomes that

function independently from the native ribosome in the cells can be used to translate specific mRNA molecules (Callura et al. 2012; Rackham and Chin 2005).

Protein Sequence Characteristics: Re-optimizing codons of the target genes based on codon usage of the host is an approach that is widely used to improve protein expression. However, synonymous codon replacement in regions of inherently slow mRNA translation, ranging from rare to abundant, can affect the three-dimensional structure of protein (Zhang and Ignatova 2011). To resolve this problem, Angov et al. (2008) developed an algorithm to identify regions of slowly translated mRNA. Recently, a machine learning data modeling tool has been developed that can be used to predict the production of secreted protein. Van den Berg et al. (2012) performed a detailed analysis of the protein features that might influence protein production and secretion. That study used 600 homologous genes and 2000 heterologous fungal genes that were over-expressed in *A. niger*. The authors developed software Hipsec (http://bioinformatics.tudelft.nl/hipsec), which predicts if gene expression of an extracellular protein will lead the successful high-level production in filamentous fungus *A. niger*.

4.4 Regulatory Controls

Regulation of gene expression can be driven by environmental changes as well as by extracellular and intracellular signals. Environmental signals, like pH, temperature, and light, can modulate the expression of many genes (e.g., PacC-pH of *Trichoderma* sp., temperature, light; (Hughes et al. 2012; Kennedy et al. 2010; Müller et al. 2014; Trushina et al. 2013). In synthetic biology, the environmental sensors can be exploited in order to regulate metabolic pathways.

Extracellular Signals: The simplest control of gene expression is achieved with promoters, which respond to the external signals (e.g., antibiotics and nutrients). Gene expression control could be upgraded to several levels using orthogonal promoters, like arabinose and IPTG-inducible promoters in bacteria (Lee et al. 2007), tetracycline in fungi (Meyer et al. 2011a), and pristinamycin- and erythromycin-inducible promoters for eukaryotic systems (Gaber et al. 2014). Ideally, induction of protein expression would occur without the need for expensive inducers. Natural promoters (e.g., glucose-CreA (Dowzer and Kelly 1989), nitrogen-AreA (Caddick et al. 1986), and xylose-XlnR (Gonzalez et al. 1997; Mach-Aigner et al. 2012; van Peij et al. 1998) are limited since they do not always maximize the transcription levels under the given growth conditions. However, with the help of synthetic biology, bioinformatics facilitates the generation of promoters that would modulate gene expression on external stimuli.

Intracellular Signals: Intracellular stimuli are probably the most difficult signals to use for the regulation of gene expression; however, using them would make it possible to efficiently balance metabolic fluxes with limited cellular resources. For

example, intracellular stimuli can be used in bacteria to redirect acetate synthesis towards lycopene synthesis (Farmer and Liao 2000). Response to intracellular pH (Young et al. 2010) and redox potential (McLaughlin et al. 2010) can also be used when applying sensors coupled to transcription factors.

More complex regulation of metabolic fluxes can be driven through transcriptional logics and genetic circuits. These dynamic regulations have been tested in bacteria and in mammalian cells. In mammalian cells, orthogonal DNA binding domains of TALENs and Zinc fingers, in combination with a suitable promoter design, have been used to build logic devices (Gaber et al. 2014) and genetic circuits (Brophy and Voigt 2014). These technologies can also be valuable for regulating changes in cellular response in fungi.

4.5 Engineering Post-translational Modifications

Production of human therapeutic proteins has had a profound impact on the pharmaceutical industry. While a few of these proteins are produced in bacteria, yeast, and fungi, most are produced in mammalian cells. The majority of therapeutic proteins are post-translationally modified and glycosylation is the most common and complex post-translational modification. Filamentous fungi and yeast can perform typical eukaryotic glycosylation; unfortunately, the final glycosylation pattern in them differs from the final glycosylation pattern in humans. Humanization of glycosylation in *P. pastoris* requires the elimination of hyperglycosylation by deleting appropriate yeast genes; it also requires the introduction of additional galactosidases and glycosyltransferases and the biosynthetic pathways and transporters for sugars that are not present in yeast (Vogl et al. 2013). Moreover, optimal spatial positioning along the secretory pathway in the endoplasmic reticulum and Golgi is necessary (De Pourcq et al. 2010). Nett et al. (2011) published a comprehensive analysis of fusion protein libraries ranging between 66 N-terminal targeting sequences of fungal type II membrane proteins to 33 catalytic domains of heterologous glycosylation enzymes from fungi, worms, fruit flies, mice, rats, and humans. Attempts have been made to humanize the filamentous fungi glycosylation pattern with minor success (Kainz et al. 2008; Maras et al. 1999).

4.6 Spatial Organization of Enzymes to Channel Metabolic Flux

The synthesis of new compounds that are not usually made by biological systems and the creation of novel pathways that can synthesize natural products and molecules is an important intersection between metabolic engineering and synthetic biology. Through the use of fused and scaffolded enzymes synthetic biology significantly extends the reach of metabolic engineering to direct flux, and it also enhances yields

(Conrado et al. 2012; Delebecque et al. 2011; Dueber et al. 2009; Lee et al. 2012, 2013; Moon et al. 2010; Steen et al. 2010; Zhang et al. 2006) Compartmentalization and channeling improves the synthesis rates and limits the side reactions. Examples of substrate channeling have been achieved with DNA-guided assembly in bacteria (Conrado et al. 2012; Lee et al. 2013), RNA scaffolding (Delebecque et al. 2011), or protein scaffolding (Dueber et al. 2009; Moon et al. 2010; Steen et al. 2010).

5 Conclusions: A Look Towards the Future

Over the last century it has become obvious that no universal host is suitable for the expression of every protein. Some disadvantages of certain systems cannot be avoided. Therefore, the choice of a preferred expression system depends upon the required quality and quantity of the protein of interest. Furthermore, the cost and convenience of an optimal system should be considered. If a high yield of protein is more relevant than the glycosylation pattern, then expression systems like bacteria, yeast, or fungi should be used. For industrial proteins, especially those that are fungal in origin, filamentous fungi are the expression system of choice due to their highly efficient secretion machinery. If the post-translational modifications are important, then an expression host that is most similar to the origin species should be selected.

The integration of systems biology and "omics"-based studies with synthetic biology will drive development in our ability to customize not only fungal cell factories but also other hosts. Furthermore, we believe that the whole genome-scale engineering of expression systems and synthetic biology (Esvelt and Wang 2013) will impact the host's capacity to synthesize new products.

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Yeast Expression Systems for Industrial Biotechnology

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1 Introduction

Employing budding yeasts and fission yeasts as important expression systems has advantages for industrial biotechnology. Due to their excellent abilities and well-characterized for molecular biology and cell physiology, nowadays yeasts are not only recognized as laboratories' expression hosts, but also regarded as workhorses for industrial production of biofuels, chemicals, pharmaceuticals, enzymes and food ingredients.

Considering yeasts as expression hosts, currently there are various systems for genetic manipulation through either autonomously replicating plasmids or genomically integrated DNA. Comparing the yeast expression system with bacterial expression systems, such as *Escherichia coli*, yeast expression allows post-translational modification, such as N-linked glycosylation, phosphorylation, acetylation and acylation. In contrast with other eukaryotic expression systems, such as insect and mammalian cells, yeast expression offers several advantages, for example yeasts have a rapid cell growth and require simple cultivation techniques for maintenance, in particular for recombinant cells (Buckholz and Gleeson 1991; Gellissen et al. 2005). Like yeast, fungal expression systems have been exploited according to their excellent potential for commercial production of recombinant proteins (van Hartingsveldt et al. 1987). However, employing fungal expression systems for the production of heterologous proteins is often limited due to the poorly characterized secretory pathway in filamentous fungi (Sims et al. 2005).

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In the area of industrial biotechnology, the yeast expression system is used for different kinds of industrial product formation. Towards systems biology, it is mostly directed to improve production of chemicals, including organic acids, fatty acids, pigments, pharmaceuticals and therapeutic molecules, vitamins and other food ingredients, and production of enzymes and biofuels. Traditionally, the yeast properties used for industrial fermentation were improved through random mutagenesis, strain selection and genetic engineering. Based on classical approaches, however, not all engineering target possibilities can be exploited due to deficiency of knowledge of metabolic and regulatory processes in the cells. In addition, it is time-consuming and side-effects may appear including unwanted by-products from strain construction, strain selection or product formation, respectively. Certainly, systems biology can be a promising tool for designing and development of yeast expression systems that can be used for improved production for both yield and productivity.

In this chapter, we summarize different kinds of yeasts as important workhorses for heterologous expression with respect to genetics, molecular tools and methods towards systems biology. Figure 1 illustrates the whole concept and perspectives of establishing yeast expression systems. Besides, we also show different examples of case studies using yeast expression as a model system for fundamental molecular biology and advancements in systems biology through integrative analysis.

2 Yeasts as Workhorses for Heterologous Expression

There are large numbers of yeasts that have been recognized as workhorses in industrial biotechnology, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia stipitis*, *Schizosaccharomyces pombe*, *Schwanniomyces occidentalis*, *Yarrowia lipolytica*, and *Arxula adeninivorans* as the heterologous expression hosts for the production of recombinant products. In principle, there are several features that make an ideal yeast expression system. For example, yeast can express heterologous genes either through episomal plasmids or by chromosomal integration of an expression cassette, and it is therefore a versatile host for genetic modifications. Concerning the life cycle, yeast has both haploid and diploid forms. Moreover, sexual crossing or clonal division (budding or fission) are possible, which are supporting genetic manipulation and screening. Considering yeast physiological properties, it is easy to cultivate with a short generation time and inexpensive culture media. Beyond the post-genomics era, there are lots of multi-level omics data for yeast through high-throughput technologies available. In the following, we provide some examples for benefits using yeast expression systems in industrial biotechnology.

An initial yeast expression system developed for heterologous gene expression is often found in *S. cerevisiae* (Liu et al. 2012; Mattanovich et al. 2012). A set of expression vectors consists of different leader sequences, promoter and selectable markers for recombinant amylase and insulin precursor to be expressed in *S. cerevisiae*. This set of expression vectors allows an evaluation of the crucial factors

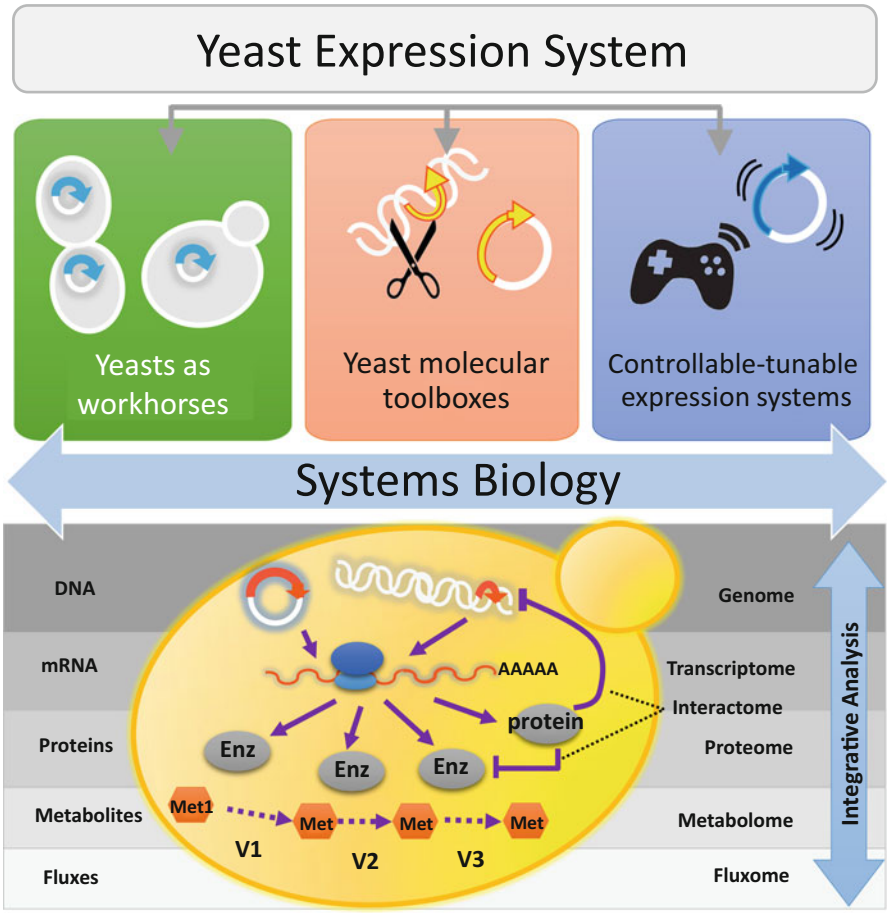

Fig. 1 Illustration shows development of yeast expression systems which requires yeasts as workhorses, molecular toolboxes, controllable-tunable expression systems and systems biology throughout integrative analysis

for production and secretion of recombinant proteins in *S. cerevisiae* which is a combination of effects of transcription and translation levels, protein uniqueness and leader sequences (Liu et al. 2012). However, post-translational modifications (PTM) are often an issue for heterologous protein expression in *S. cerevisiae.* Hyperglycosylation occurs frequently and results in alteration of protein binding properties which potentially leads to allergenic recombinant protein, particularly for biopharmaceutical proteins. Therefore, the methylotrophic yeast *P. pastoris* has arisen as a choice for heterologous protein expression due to an expression of human-like N-glycosylation patterns (Li et al. 2007; Vogl et al. 2013). The elimination of hyperglycosylation was conducted by deleting the appropriate associated genes and introducing additional glycosidases and glycosyltransferases, including the missing biosynthetic pathway for achieving humanized glycosylation patterns in yeast. The introduction of 14 heterologous genes for glycoengineering in a

quadruple knock-out of *P. pastoris* was able to remove all four endogenous β-mannosyltransferases, therefore removed undesired β-linked mannose residues from the complex terminally sialylated human glycoproteins (Hamilton et al. 2006). Later on, deletion of the remaining members of the β-mannosyltransferase (BMT) family culminating in the quadruple knock-out enabled removal of β-Man-associated glycans and their related antigenicity in the recombinant human erythropoietin (Hopkins et al. 2011). These comprehensive studies have emphasized the strategies in developing *P. pastoris* as a host for tailor-made recombinant protein production.

Extending the choice of hosts from the model systems, such as *S. cerevisiae* and *P. pastoris* (Li et al. 2007), many efforts have been taken to develop unconventional yeasts, for example *H. polymorpha*, *Y. lipolytica*, *S. pombe*, *A. adeninivorans* as the recombinant hosts to overcome the limitation of using traditional *S. cerevisiae* (Gellissen et al. 2005; Fennessy et al. 2014). An attractive example for developing an expression system using an oleaginous yeast, *Yarrowia lipolytica*, is presented by Juretzek et al. (2000). In this study, various promoters of the genes *G3P, ICL1, POT1, POX1, POX2* and *POX5* were investigated for their regulations and activities and literally be selected as the suitable promoters for high expression of heterologous genes in *Y. lipolytica*.

Despite the outstanding features of these recombinant hosts described earlier, there is clearly no single host system that is optimal for the production of all possible recombinant products. Therefore, the availability of a vector system that could be targeted to the various host candidates and would greatly facilitate a comparative assessment and establish the expression system for the target of interest related to heterologous products.

3 Advancements in Genetics, Molecular Tools and Methods for Yeast Expression Systems

During the last decades, the exploitation of yeast genetics and recombinant DNA technology has expedited the development of yeast expression system for heterologous production. In the following, we describe the current development of yeast molecular toolboxes which are often used for construction of yeast expression systems. In addition, we also describe assessing controllable and tunable yeast expression systems.

3.1 The Development of Yeast Molecular Toolboxes

Currently, genetics and molecular tools are important for developing the yeast expression systems. A basic genetic manipulation in yeast is mostly carried out using expression vectors which can be divided into two categories: (1) autonomously replicating plasmids containing the origin of replication and the propagation

of plasmid, which is independent from the host's genome, (2) integrating plasmids which require the incorporation into the host genome via homologous recombination. Relating to recombinant yeast as a host, the core elements in expression vectors must contain the suitable expression signals (species-species expression) which include a strong promoter and a termination codon, a selectable marker and an origin of replication. Focusing on yeast expression systems, novel fluorescent proteins, tagging proteins, selectable markers and promoters are identified and characterized (Janke et al. 2004). For some specific cases, several dual gene expression cassette vectors, either with dual antibiotic selection markers or dual promoters are developed (Partow et al. 2010; Vickers et al. 2013). It is worth to note that the risk of using dominant antibiotic selection markers is an occurrence of spontaneous resistance over time. The choice of a selectable marker has been changed from antibiotic dominant markers to auxotrophic markers. Nonetheless, there are some weaknesses in engineering auxotrophic host strains and preparing plasmids which carry the corresponding auxotrophy-complementing genes specifically in yeast species with less annotated genome databases (Pronk 2002).

Regarding development of yeast molecular toolboxes, the highly efficient homologous recombination in *S. cerevisiae* has facilitated the development of site-specific integrations, gene-deletion/replacements and marker recycling strategies. In order to apply the well-established toolboxes developed in *S. cerevisiae* to *S. pombe* or *H. polymorpha* which is less efficient in homologous recombination, the removal of *pku70*$^+$ and *pku80*$^+$ in *S. pombe* and the mutation of *yku80* in *H. polymorpha* which encoded DNA end binding protein required for non-homologous DNA end joining and DNA repair pathway were conducted to enhance the efficiency for targeted integration in *S. pombe* and *H. polymorpha*, respectively (Fennessy et al. 2014; Saraya et al. 2012). For further details on the current molecular toolboxes for *H. polymorpha* and *S. pombe* can be seen in different studies (Gellissen et al. 2005; Fennessy et al. 2014; Saraya et al. 2012). Due to extensive molecular characterization in *S. cerevisiae*, yeast molecular toolboxes for cross species complementation were performed. A *S. pombe leu1* mutant was crossed with the *S. cerevisiae LEU2*$^+$ gene and is hence an example for applying the molecular toolboxes transferring existing budding yeast technology to the fission yeast (Beach and Nurse 1981).

3.2 Assessing the Controllable and Tunable Yeast Expression System

The development of basic genetics and molecular methods, such as transformation, gene deletion/knockouts, episomal/integrative plasmids have enabled the establishment of a controllable and tunable yeast expression system. Despite the fact that inducible expression systems (i.e. *GAL1*/*GAL10* and *MET25* in *S. cerevisiae*, *AOX1* in *P. pastoris* or *MOX* in *H. polymorpha*) have been widely used for controlling gene expression in metabolic pathway engineering, their rigid control over the whole system results in an inefficiency to adjust the host metabolic function upon

encounter of alternative environments (Solow et al. 2005; Weinhandl et al. 2014; Yocum et al. 1984). Consequently, the fine-tuning of the promoter strength and regulation in yeast expression systems becomes a fascinating field for optimizing methodologies. Substitution of natural promoters with synthetic promoters results in enhanced expression and improved folding of recombinant proteins by modifying transcription, translation, post-translation modifications and designing synthetic regulatory networks (Vogl et al. 2013; Blount et al. 2012; Juven-Gershon et al. 2006). Creating the synthetic hybrid promoters by tandem fusing of an upstream activation sequence (UAS) to a separate core promoter region has been reported as the tunable expression system in *Y. lipolytica* (Blazeck et al. 2011). A similar strategy was applied to regulate the constitutive expression of endogenous promoters by tandem fusion of upstream activating sequences (UASs) from *CLB2*, *CIT1*, *GAL1/GAL10* and *TEF1* to the core promoters of *S. cerevisiae* (Blazeck et al. 2012). Furthermore, the diminishing of nucleosome affinity enables redesign of native promoters as well as *de novo* design of synthetic promoters and hence increased yeast promoter activity and expression levels (Curran et al. 2014). Recently, a strategy to modulate eukaryotic transcription at natural and synthetic promoters using programmable and tunable synthetic transcription factors based on a bacterial CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas system has been reported by Farzadfard et al. (2013). This CRISPR/Cas system has been successfully established as a powerful tool for genome engineering of *S. cerevisiae* (Bao et al. 2014; DiCarlo et al. 2013). Despite the outstanding strategies described here, there is clearly no single method which is ideal for expressing of all possible target genes. Therefore, systems biology through integrative analysis for advancing yeast expression system is prerequisite.

4 Moving Towards Systems Biology for Advancing Yeast Expression Systems

Systems biology aims at a comprehensive understanding the genotype and phenotype relationships of biological system. Thereby, quantitative description through analysis of relationships among different levels (i.e. genes, mRNA, enzymes, proteins, metabolites, and fluxes) enables creation of complex networks. With the development in high-throughput omics technologies, there is now again an increasing focus on investigation of the cellular behavior and consequences. Today, systems biology is often used as a powerful tool for integrative analysis of mathematical models with experimental data to gain new insight and a basic understanding of cells and to advance development of biological processes. In yeast expression systems, it is mostly directed towards enhancing production of chemicals and protein drugs through the use of systems biology. In the following, we describe two broad applications through using systems biology for (1) genome-scale modeling of yeast metabolism and (2) integrative analysis of global regulation of yeast metabolism with overall aim at developing and/or improving yeast expression system.

4.1 Genome-Scale Modeling of Yeast Metabolism

The genomes of a large number of important yeasts have been sequenced and nowadays the number of available genomes is continuously growing. The rapid release of yeast genomes subsequently opens an opportunity for development of a yeast model at a genome-scale. Through the actual challenge in systems biology, a genome-scale model of yeast therefore is developed for aiming at precise prediction of the outcome of genetic or environmental changes and can then be used as scaffolds to understand phenotypic behaviors in yeast cells. The developed model serves as an important resource for gaining further insight into our understanding of yeast physiology. Up to date, there are different genome-scale models of yeasts that have been developed for various kinds of scopes. As presented in Table 1, we provide a list of genome-scale models of yeasts with different scopes and references.

Considering developing/enhancing yeast expression systems, we highlight the yeast *S. cerevisiae* as it generally serves as a unique experimental system for functional studies of heterologous genes allowing for generation of over-expression phenotypes. The first genome-scale metabolic model of *S. cerevisiae* *i*FF708 devel-

Table 1 Genome-scale models of yeasts and their different scopes

Model name	Scopes	References
S. cerevisiae		
*i*FF708[a]	First yeast genome-scale model	Forster et al. (2003)
*i*ND750	Expanding 8 compartments	Duarte et al. (2004)
*i*LL672	Model reduction	Kuepfer et al. (2005)
*i*MH805/775	Transcriptional regulation	Herrgard et al. (2006)
*i*IN800[a]	Expanding lipid metabolism	Nookaew et al. (2008)
*i*MM904	Metabolome analysis	Mo et al. (2009)
Yeast 1.0	Consensus network	Herrgard et al. (2008)
Yeast 4	Improved consensus network	Dobson et al. (2010)
Yeast 5	Improved consensus network	Heavner et al. (2012)
*i*TO977	Comprehensive model	Osterlund et al. (2013)
Yeast 7	Improved fatty acid metabolism	Aung et al. (2013)
Y. lipolytica		
*i*NL895	First oleaginous yeast (lipid accumulating)	Loira et al. (2012)
P. pastoris		
PpaMBEL1254	Heterologous protein production	Sohn et al. (2010)
*i*PP668	Heterologous protein production	Chung et al. (2010)
*i*LC915[a]	Heterologous protein production	Caspeta et al. (2012)
P. stipitis		
*i*SS884	Substrate utilization	Caspeta et al. (2012)

[a]Model has been used for developing/enhancing yeast expression system

oped by Förster et al. was used for heterologous expression studies (Forster et al. 2003). As a case study can be presented by Wattanachaisaereekul in 2007 (Wattanachaisaereekul 2007), the genome-scale metabolic model of *S. cerevisiae* suggested by Förster et al. (2003) was used as stoichiometric model of yeast metabolism to evaluate the potential for production of 6-MSA as polyketides (Forster et al. 2003). The reactions towards the polyketides naturally produced by e.g. *Penicillium patulum* were added into the *S. cerevisiae i*FF708 model and both the flux towards 6-MSA production and the specific growth rate were optimized using linear programming. After modeling was performed, the productivity of 6-MSA could be substantially increased due to the increase of NADPH and acetyl-CoA which are used as precursors for 6-MSA biosynthesis, especially by deletion of *GDH1* and *SER3* resulting in 6-MSA specific productivity. The *in silico* gene deletion analysis through genome-scale modeling could therefore eventually be a platform for developing and/or enhancing yeast expression system for the improved production of polyketides.

Concerning other yeasts, the genome-scale metabolic model of *P. pastoris i*LC915 presented by Caspeta et al. was also emphasized due to its use in recombinant protein production (Caspeta et al. 2012). The protein was the human monoclonal antibody 3H6 Fab fragment (FAB). FAB production through genome scale modeling of *P. pastoris* was investigated using different carbon sources and nitrogen sources as well as the effect of oxygen uptake rates on the physiological state of yeast cells. The results showed that *i*LC915 can be used not only to gain understanding on its metabolic capabilities, but also that it is a versatile tool for designing an attractive expression system for the heterologous protein production.

4.2 Integrative Analysis of Global Regulation of Yeast Metabolism

Systems biology integrates high-throughput omics data (e.g. genomics, transcriptomics, proteomics, metabolomics, fluxomics) and uses genome-scale models as scaffolds for integrative analysis and interpretation of yeast metabolism. Focusing on developing/enhancing yeast expression systems, integrative analysis of global regulation of yeast metabolism is also concerned. A good case example is presented by Ruenwai and others in 2011 (Ruenwai et al. 2011), their studies constructed the poly unsaturated fatty acids (PUFA)-producing yeast strains by expression of Δ^{12}-desaturase gene of *M. rouxii* and co-expression of the fungal Δ^{12}- and Δ^{6}-desaturase genes under control of the constitutive *M. rouxii* Δ^{9}-desaturase promoter. Through transcriptome analysis and flux distribution, interestingly, the global responses of the yeast cell to endogenous PUFAs were detected consistency in relation to the lipid biosynthesis and fatty acid biosynthetic pathway. Thereafter the reporter metabolites and subnetworks algorithm was used to identify highly regulated metabolites and significantly correlated metabolic subnetworks. This analysis was

based on the reconstructed genome-scale metabolic network of *S. cerevisiae* iIN800. More interestingly, significant reporter metabolites and key genes encoding enzymes were identified that are involved in the pentose phosphate pathway, amino acid metabolism, energy metabolism and lipid metabolism. Through integrative analysis, the detected pathway targets and key candidate genes encoding enzymes could be a guideline for enhancing yeast expression system to endogenous PUFAs.

5 Summary and Perspectives on the Yeast Expression Platform

As previously described, it is clear that there are currently various direct applications of yeast expression systems for industrial biotechnology, but as indicated there are promising perspectives for preferring yeast expression systems in the production of biofuels, chemicals, pharmaceuticals, enzymes and food ingredients more than bacterial and other eukaryotic expression systems due to yeast's features in terms of simple genetic manipulation and screening as well as fast growth in inexpensive culture media. Towards development of yeast molecular toolboxes and controllable-tunable expression systems, the yeast expression platform can be efficiently constructed with a yeast strain under the desirable characteristics regarded as an attractive workhorse for heterologous expression. Due to the complexity of the yeast expression platform, design and development therefore further leads to a requirement of systems biology. Throughout integrative analysis, a range of efficient yeast expression platforms have been designed and developed for desirable recombinant products formation. With these systems, we hope to see the use of yeast producing strains in screening, modification and expression systems that have not only high genetic stability (either episomal plasmid or genome integrated system), but also show reproducibly high productivity with economically inexpensive and environmentally friendly.

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Part II
Tools

High-Throughput Construction of Genetically Modified Fungi

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1 Introduction

Once the concept of the central dogma (DNA-RNA-protein) was established in biology, study of gene functions immediately followed, in order to understand the genetic basis of complicated biological mechanisms. Due to their complex life cycles and diverse metabolic capabilities, fungi have long been considered as good model systems for functional studies of genes in eukaryotes. This was demonstrated well by the Nobel prize study by Beadle and Tatum, in which the one-gene one-polypeptide theory was supported by work with *Neurospora* (Beadle and Tatum 1941). In addition, functional genomic studies of fungi are required for improving human life, as many fungi are economically important for industry, medicine and agriculture (EspinelIngroff 1996; Yarden et al. 2003; Ghorai et al. 2009).

Gene function studies in fungi have been often carried out using mutants and methodologies for generating mutants are advancing continuously. Before genome sequences were available, gene mutagenesis was mostly focused on random point and insertional mutations. Since the first genome sequence for a fungus, *Saccharomyces cerevisiae*, was completed in 1996, an increasing number of fungal genome sequences have been released every year. According to an analysis by Aguilar-Pontes et al., sequences of about 820 fungal genomes are currently available (Aguilar-Pontes et al. 2014). Functional studies in the post genomic era are often performed in high throughput manner, producing a new area of study, "-omics"

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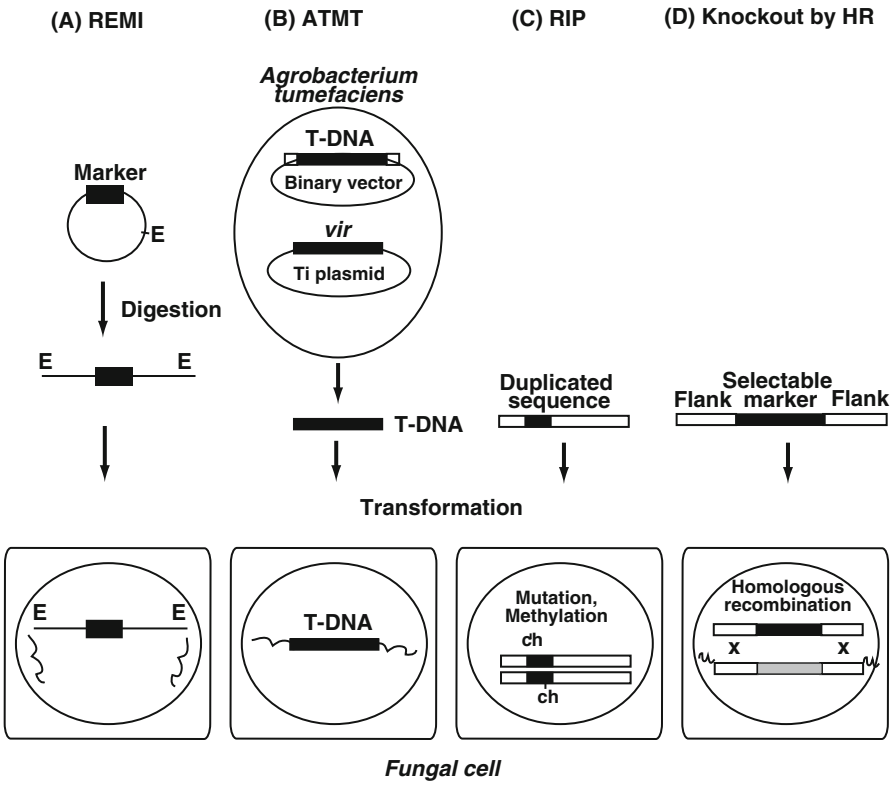

Fig. 1 Schematic view of methodologies of fungal gene mutation. (**a**) Restriction enzyme mediated-integration (REMI), (**b**) *Agrobacterium tumefaciens* mediated transformation (ATMT), (**c**) Repeat-induced point mutation (RIP), (**d**) Targeted gene replacement by homologous recombination

(de Wouw and Howlett 2011). Targeted mutagenesis and identification of mutated genes in random mutant libraries is more easily accomplished using sequence information. In this chapter, we summarize the development in methodologies for gene mutagenesis in fungi (Fig. 1), a rapidly growing area of research in the post-genomic era. Unfortunately, we can not refer to all related publications in this chapter due to space limitations.

2 Gene Mutations in Fungi During the Pre-genomic Era

2.1 Generation of Mutants Using Radiation

Since radiation, such as UV, X-rays, γ-rays, and ion beams, is known to cause DNA damage, generation of mutants using radiation has been routinely practiced in fungi. Solar ultraviolet light (UV) contains UVA (315–400 nm), UVB (280–315 nm), and UVC (below 280 nm), and more than 90 % of UV is UVA. UVA and UVB induce

the formation of pyrimidine dimers in DNA and this results in C to T transition mutations (Brash et al. 1991; Sage et al. 2012).

While UV (greater than 200 nm) is non-ionizing radiation, X-rays, γ-rays, and ion beams are ionizing radiation that emits higher energy (Genuis 2008). Compared to UV, ionizing radiation transfers higher energy and leads to significant damage to DNA, including double- or single-strand breaks, resulting in substantial gene deletions or rearrangements. Ion beams can induce double strand DNA breaks because of high energy transfer to the target, whereas X-rays and γ-rays transfer relatively low energy and mostly produce DNA single strand breaks and oxidative damage (Hagen 1994; Goodhead 1995).

Mutagenesis using non-ionizing and ionizing radiation has been frequently used in construction of random mutant libraries that are often very useful resources for functional study of fungal genomes (Ma et al. 2013). Radiation is also applied to useful fungi in industry and biocontrol, in order to produce fungicide-resistant and functionally improved strains (Ma et al. 2011; Shinohara et al. 2013; Kramer et al. 2014; Fitriana et al. 2014).

2.2 *Restriction Enzyme Mediated-Integration*

REMI (Restriction Enzyme Mediated Integration) is an advanced insertional mutagenesis technique using restriction enzymes *in vivo* (Schiestl and Petes 1991; Kuspa and Loomis 1992). In REMI mutagenesis, plasmid DNA that has been linearized with a restriction endonuclease is transformed into the target organism in the presence of the same or a different restriction enzyme. After transformation, the plasmid DNA is integrated into the compatible sites in the genome generated by the action of the restriction enzyme. To identify the mutated sites, inserted plasmid is recovered with flanking genome sequences by digesting genomic DNA isolated from transformants using another restriction enzyme. Requirements for successful REMI mutation are single copy integration, increase in transformation rate, and complete plasmid rescue. Studies shows that transformation frequency increases when restriction enzymes are used (Schiestl and Petes 1991).

REMI mutation in fungi was first developed in *S. cerevisiae* and then successfully established in other non-pathogenic and pathogenic fungi (Kahmann and Basse 1999). This mutagenesis technique has been frequently used in phytopathogenic fungi, such as *Cochliobolus heterostrophus*, *Magnaporthe grisea*, *Ustilago maydis*, *Pyrenophora teres*, *Cladosporium fulvum*, *Colletotrichum lindemuthianum*, and contributed greatly to isolation of pathogenicity genes (Kahmann and Basse 1999).

2.3 Agrobacterium tumefaciens *Mediated T-DNA Insertion Mutations*

Originally developed as an efficient DNA delivery method in plant cell systems (Stafford 2000), T- DNA insertion mediated by *Agrobacterium tumefaciens* has been implemented as a mutagenesis tool in fungi (Michielse et al. 2005).

A. tumefaciens causes crown gall tumors in plants by transferring a part of the DNA (T-DNA) located on tumor inducing plasmid (Ti plasmid) into the plant nucleus. Transferred T-DNA is integrated into the plant genome and the products of genes expressed from the T-DNA are involved in inducing tumors.

The principle of T-DNA delivery by *A. tumefaciens* and integration into the host genome has been actively applied to fungal gene disruption (Michielse et al. 2005). Compared to conventional random mutagenesis methods, it has several advantages, including use of intact cells as starting materials and a high rate of single-copy integration. The procedure starts with construction of DNA delivery vectors. The required components in a vector are the *vir* genes on the Ti plasmid that promote DNA transfer and the T-DNA that will be transferred. In current practice, a binary vector system is used, in which the *vir* genes and T-DNA are placed in two separate plasmids (Hoekema et al. 1983). By the action of *vir* genes, single-stranded T-DNA is copied and passed through the bacterial cell membrane and wall via a Type IV secretion mechanism (Regensburg-Tuink and Hooykaas 1993; Christie 1997). Once inside the fungal cell, T-DNA is then transferred to the nucleus using a nuclear localization signal and integrated into the fungal genome, presumably by the action of host proteins (Bundock et al. 1995; Gelvin 2000).

Agrobacterium tumefaciens-mediated transformation (ATMT) has been successfully used in targeted and insertional mutagenesis schemes and continues to be an efficient tool even after sequences of fungal genomes are available. Due to high frequencies of transformation and homologous recombination, ATMT using vectors harboring gene knockout cassette DNA has a high efficiency for targeted gene mutation in many fungi (Michielse et al. 2005). Vectors with selectable markers in the T-DNA region are used to generate random insertional mutant libraries in yeasts and fungi (Michielse et al. 2005). For efficient insertional mutagenesis, ATMT should satisfy two requirements, a high rate of random insertion and single or low-copy integration.

2.4 *Repeat-Induced Point Mutation (RIP) in* Neurospora crassa

Repeat induced point mutation (RIP) was first discovered in *N. crassa* (Selker 1990). Evidence suggests that it evolved as a defense mechanism to relieve the detrimental effects of genome duplication caused by transposable elements during the sexual cycle (Selker 2002). However, gene silencing using RIP as a mutagenesis tool has emerged as a more studied topic. RIP starts with a premeiotic scanning mechanism that allows identification of sequence duplications in the genome. Subsequently, C:G pairs are mutated to T:As in both copies of duplicated sequences and these mutations preferentially occur in CpA dinucleotides (Cambareri et al. 1989; Grayburn and Selker 1989). In order to produce RIP mutants, cloned DNA corresponding to the sequence to be mutated is transformed into the fungal cell,

along with a selectable marker (Glass and Lee 1992; Arganoza et al. 1994). Transformants are crossed to wild type, during which time the duplicated copies in the transformant nuclei are subjected to point mutations. DNA from cross progeny is analyzed using Southern analysis with methylation-sensitive restriction enzymes and the duplicated sequence as a probe (Glass and Lee 1992; Arganoza et al. 1994). Progeny with a Southern hybridization band pattern that differs significantly from wild type are further analyzed by sequencing the genome region in the duplication. From sequence analysis, the investigator can select those mutants that either have multiple mutations and/or premature stop codon(s) (effectively null mutations) or a small number of point mutations that may have more subtle effects on gene function. The latter is because in contrast to other mutational tools, RIP can generate partially inactivated mutants. Analysis of such leaky mutants has contributed to identifying genetic basis for fungal development and metabolism (Barbato et al. 1996).

Since discovered, RIP has been actively used in generating mutants for many genes in *N. crassa*, including *am*, *nmr*, *nit-3*, *mtA-1*, and *het-C* (Glass and Lee 1992; Arganoza et al. 1994; Fincham 1990; Jarai and Marzluf 1991; Okamoto et al. 1993). Interestingly, RIP is not found in other fungi, but a similar gene inactivation mechanism called methylation induced premeiotically (MIP) has been observed in *M. grisea*, *A. fumigatus*, *P. anserina*, and *Fusarium* species (Nakayashiki et al. 1999; Neuveglise et al. 1996; Graia et al. 2001; Hamann et al. 2000; Hua-Van et al. 1998). In MIP, duplicated DNA sequences are inactivated, but inactivation is accomplished by DNA methylation, with no point mutations.

3 Advances in Methodology for Fungal Gene Deletion (Post-genomic Era)

3.1 Construction of Vectors (or Cassettes) for Gene Deletion

The ability to create gene replacement mutants in fungi, in which the entire open reading frame (ORF) is replaced with a selectable marker, was pioneered in the yeast *S. cerevisiae* (Rothstein 1983). Early vectors for creating such "gene knockout" mutants were constructed through restriction enzyme digestion of cloned fragments of genomic DNA and then ligating with a selectable marker cassette and insertion into a *E. coli* cloning vector (Rothstein 1983). In yeast, it is now known that flanking regions can be quite short to achieve homologous recombination (Raymond et al. 2002), although it was standard to use ~0.5-1 kb flanks in earlier applications of the method (Kataoka et al. 1984). The advent of genome sequences made it possible to isolate the flanking regions surrounding the ORF through amplification of genomic DNA in polymerase chain reactions, with primers corresponding to the ends of the flanks.

3.2 Yeast Recombinational Cloning

The high rate of homologous recombination in *S. cerevisiae* has been exploited to use yeast as "factories" for assembly of inserts into plasmid vectors without restriction digest/ligation approaches. This method has been called "yeast recombinational cloning" or YRC and was developed by C. Raymond and colleagues (2002). In this approach, a yeast-*E. coli* high-copy shuttle vector such as pRS426 (Sikorski and Hieter 1989) is used for replication and amplification of the inserted DNA in yeast.

To implement YRC for construction of gene knockout cassettes, two gene-specific PCR products corresponding to the 5′ and 3′ flanks of the gene(s) of interest and an antibiotic resistance cassette must be amplified. There should be approximately 25–30 bp of overlapping complementary sequence on the ends of the fragments to be assembled (Raymond et al. 2002). For each knockout cassette, four gene-specific primers are needed: 5′ flank forward (5For), 5′ flank reverse (5Rev), 3′ flank forward (3For), and 3′ flank reverse (3Rev). Each primer also contains sequence complementary to the yeast-*E. coli* shuttle vector or the antibiotic resistance cassette at the 5' end. The 5For and 3Rev primers contain sequence corresponding to the different ends of the vector, while the 5Rev and 3For primers include sequence complementary to the 5' and 3' ends of the antibiotic resistance cassette. For the *N. crassa* knockout project, plasmid pCSN44 was used as the source of the antibiotic resistance cassette. This vector contains the dominant drug-resistance marker gene hygromycin B phosphotransferase (*hph*) from *E. coli*, that is expressed in fungi using the promoter and terminator of the *trpC* gene from *A. nidulans* (Staben et al. 1989; Gritz and Davies 1983).

The pRS426 vector is digested with two different restriction enzymes in the polylinker region, in order to generate two ends without homologous sequence. The vector is then co-transformed with the three PCR products into yeast, with selection on medium lacking uracil. Typically, the entire population of yeast transformants is collected and DNA extracted (Collopy et al. 2010), followed by PCR amplification of the knockout cassette, using primers complementary to the vector that flank the inserted PCR fragments.

3.3 Fusion PCR

Fusion PCR is a rapid method to create gene knockout cassettes by linking long stretches of genomic sequence flanking the gene of interest to a selectable marker (Wach 1996). In this approach, PCR is used to amplify flanking regions with primers that include homology to the end of selectable marker. These flanking region fragments are then used as long primers to amplify the selectable marker from a DNA template. High fidelity polymerases are typically used during this approach, in order to minimize the incidence of errors in the final knockout cassettes.

This approach has been used successfully to create knockout constructs in several filamentous fungi, including *Cochliobolus heterostrophus* and *Gibberella zeae* (Catlett et al. 2003) and *A. nidulans* (Szewczyk et al. 2006).

3.4 Selectable markers

Effective selectable markers are critical for identification of fungal knock-out mutants. The common markers used in fungi have been the subject of a recent review (Jiang et al. 2013). Genes that confer resistance against antibiotics are widely used, including hygromycin B (*hph*; Blochlinger and Diggelmann 1984), phosphinothricin/blasticidin (*bar*: Pall 1993), nourseothricin (*nat*; Kück and Hoff 2006), phleomycin (*ble*: Gatignol et al. 1987) and neomycin (*neo*: Sureka et al. 2014). Other classes of marker genes include visual proteins, such as Gus, LacZ and eGFP; nutritional markers, including *pyrG* (orotidine-5′-monophosphate decarboxylase), *hxk1* (hexokinase), *niaD* (nitrate reductase), *argB* (ornithine carbamoyltransferase), *CBS1* (cystathionine β-synthase), *HPD4* (phydroxyphenylpyruvate dioxygenase), *ptrA* (pyrithiamine resistance) and *amdS* (acetamidase), and conditional lethal genes, such as HSVtk (herpes simplex virus thymidine kinase) (rev. in Jiang et al. 2013).

4 Increasing the Frequency of Homologous Recombination in Fungi

4.1 Use of Strains Deficient in Recombination

Wild-type *N. crassa* strains, like other filamentous fungi, have a very low rate of homologous recombination (Paietta and Marzluf 1985). Fortunately, this barrier has been overcome through deletion of genes homologous to *ku70* and *ku80* (Critchlow and Jackson 1998; Walker et al. 2001). *ku70* and *ku80* are required for non-homologous end joining DNA repair, a process that leads to insertion of incoming DNA at double-strand breaks in the genome of eukaryotic organisms, including yeast (Ooi et al. 2001).

In *N. crassa*, a seminal study showed that mutation of *mus-51* or *mus-52*, genes homologous to *ku70* and *ku80*, greatly reduces ectopic integration and improves the incidence of homologous recombination by more than 90 % with a low incidence of ectopic integration (Ninomiya et al. 2004). The degree of homologous recombination depended on the length of homologous DNA flanking the insert, with 500–1000 bp leading to nearly 100 % homologous recombination (Ninomiya et al. 2004). These observations were used to create gene knockouts in *N. crassa* (Colot et al. 2006) and *A. nidulans* (De Souza et al. 2013) (see below).

4.2 Split Marker Recombination

In filamentous fungi, split marker recombination was first used to create gene knockouts in the corn pathogens *Cochliobolus heterostrophus* and *Gibberella zeae* (Catlett et al. 2003). In this procedure, two different fragments are transformed into the fungal cell, each containing overlapping regions of a selectable marker gene. One of the fragments also contains homologous sequence 5′ to the region of insertion, while the second fragment contains homologous sequence 3′ to the insertion. Resistance is only achieved by recombination between the two selectable marker regions of the fragments and between the 5′ and 3′ regions with the fungal genome. The split marker approach works using either fusion PCR or plasmid-based strategies to make the knockout constructs (Catlett et al. 2003). The method resulted in rates of homologous recombination of 50–100 % for six candidate genes from *C. heterostrophus* and *G. zeae* (Catlett et al. 2003).

Selectable markers employed for the split marker approach include antibiotic resistance genes, such as hygromycin resistance, in *Aspergillus fumigatus* (Gravelat et al. 2012) and *Fusarium oxysporum* (Liang et al. 2014). In the *F. oxysporum* study, a second marker, neomycin resistance, was inserted 5′to the 5′ flanking region on one fragment. The neomycin resistance cassette was incorporated in the construct to allow later identification of those transformants with ectopic copies of the fragment. Transformants were selected on hygromycin-containing medium and then tested for resistance to neomycin. Only transformants that were resistant to hygromycin, but sensitive to neomycin, were carried forward. Using this strategy, homologous recombination rates of 77–90 % were achieved for three RNA-dependent RNA polymerase genes (Liang et al. 2014).

5 Examples of High-Throughput Gene Deletion Projects in Filamentous Fungi

The genome of *N. crassa* has nearly 10,000 predicted genes (Galagan et al. 2003). A high-throughput knockout project was initiated to create gene replacement mutations for all of the genes (Collopy et al. 2010; Colot et al. 2006; Dunlap et al. 2007; Park et al. 2011). Yeast recombinational cloning (Raymond et al. 2002) was implemented to make all constructs for the project, using primer sequences generated from a primer prediction software program. Recipient strains were generated with gene replacements of *mus-51* and *mus-52*, using *bar* as the selectable marker (Ninomiya et al. 2004). The final knockout cassettes were designed with 1 kb flanking DNA on either side of the hph selectable marker. These cassettes were amplified from yeast vectors using PCR and then used to electroporate the Δ*mus-51*::*bar*$^{+}$ or Δ*mus-52*::*bar*$^{+}$ strain. The recipient strain was chosen to avoid linkage between the *mus* mutation and the gene being knocked out, so that the frequency of homokaryotic knockout mutants without the *mus* mutation would be higher after crossing the

primary transformants to wild type. After the sexual cross, progeny were screened for resistance to phosphinothricin (*bar*$^{+}$ gene) and mating type (*mat A* or *mat a*). The goal was to obtain hygromycin-resistant, phosphinothricin-sensitive progeny in both mating types.

In the first stage of the *N. crassa* project, transformants were screened using Southern analysis to validate that the appropriate gene was deleted and that there were no ectopic insertions (Colot et al. 2006). However, after several thousand genes, it became clear that the number of strains with ectopic copies in addition to the correct integration event was vanishingly small. Therefore, the project switched to a PCR-based method for confirming knockout mutants. By taking advantage of a high-throughput gene deletion method (Colot et al. 2006; Park et al. 2011), all genes have been tried at least once (Ouyang and Borkovich, unpublished observations), and approximately 85 % of genes have been knocked out successfully. The knockouts are available for the whole community from FGSC.

The general method used to create the *N. crassa* knockouts has also been utilized to produce knockout cassettes for 9851 genes representing 93.3 % of the protein-coding genes in *A. nidulans* (De Souza et al. 2013). Major differences were that the *pyrG* marker was used in place of *hph* and flanks as short as 0.6 kb were used. The cassettes for 128 genes were used to create mutants in all predicted protein kinase genes, with the resulting strains analyzed for a variety of phenotypes (De Souza et al. 2013).

The filamentous fungal pathogen *Magnaporthe oryzae* (*Magnaporthe grisea*) is the most severe fungal disease of rice throughout the world (Valent 1990). In this organism, a library of 21,070 ATMT-mediated insertional mutants has been assembled and analyzed for a variety of phenotypes, including pathogenicity (Jeon et al. 2007). Other species in which insertional libraries have been created that contain at least 1000 mutants include *Fusarium oxysporum*, *Aspergillus fumigatus*, *Monacrosporium sphaeroides, Ustilago maydis*, *Colletotrichum sansevieriae* and *Beauveria bassiana* (rev. in Jiang et al. 2013).

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Awakening of Fungal Secondary Metabolite Gene Clusters

Juliane Fischer, Volker Schroeckh, and Axel A. Brakhage

1 Introduction

Multispecies microbial communities play important roles in nature, health or industry (Fig. 1). A hallmark is the formation of secondary metabolites (SMs), compounds that often have interesting chemical or pharmaceutical properties, ranging from antibiotics and immunosuppressants to potential anti-cancer drugs, but are also of importance due to their toxicological characteristics, e.g. in agriculture and industrial food production (Brakhage 2013). During the last decade SMs became a focal point of interest from another aspect: the need for new antibiotics to fill the gap that arose by a dramatically increase of antibiotic resistance (Rahman et al. 2010; Nathan and Cars 2014; Laxminarayan et al. 2013; Bell et al. 2014). As a consequence, methods to identify novel SMs, to elucidate both their structure as well as their natural function and thereby the regulation of production became a matter of intense research (Netzker et al. 2015). An expanding area is research on filamentous fungi including *Aspergillus* species. Genome sequences for several Aspergilli have been determined (Knuf and Nielsen 2012), thus providing a wealth of information about potential SM production. Surprisingly, numerous SM gene clusters were identified in the various *Aspergillus* sp., displaying a discrepancy between the

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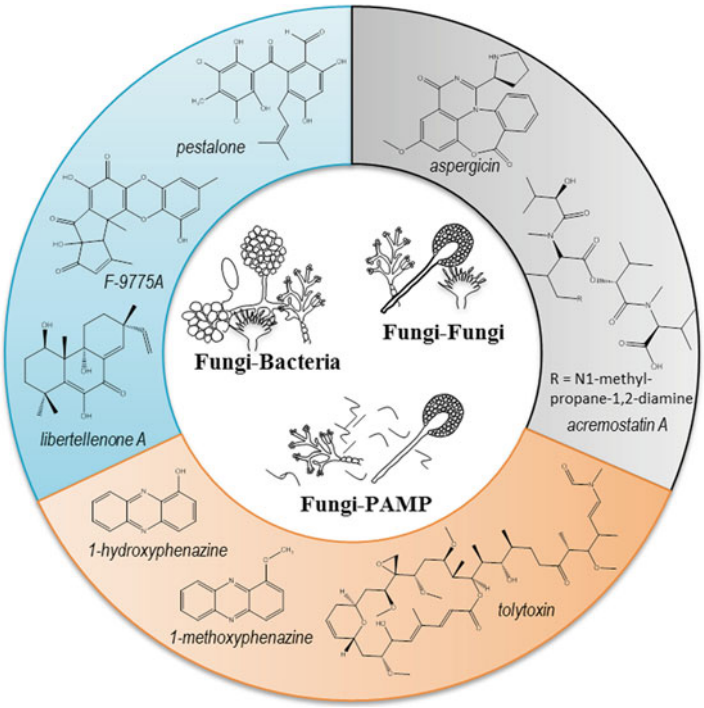

Fig. 1 Interspecies, fungal-bacterial interactions or co-incubation with PAMP can trigger the activation of silent SM gene clusters and thereby can lead to the activation of so-far unknown natural products

number of SM gene clusters and the so far known SMs of a single species (Craney et al. 2013; Bergmann et al. 2007), leading to the concept of silent SM gene clusters. These clusters impressively illustrate that SMs are only produced under specific physiological conditions, and that their biosynthesis is subject to diverse regulatory controls that await further elucidation. The variety of genetic mechanisms that co-regulate transcription of SM gene clusters range from specific regulation by DNA binding transcription factors to global regulation via chromatin modulation. In this review, we describe the various mechanisms by that fungal secondary metabolism is regulated and discuss how the identified regulatory networks can contribute to natural product research.

2 Secondary Metabolite Gene Clusters

SMs are structurally heterogeneous, low-molecular-mass compounds. They are mostly derived from either polyketides or non-ribosomal peptides and the genes for their biosynthesis are, with a few exceptions, located in clusters (Smith et al. 1990).

SM gene clusters can span several 10,000 bases (Brakhage 2013) and can comprise more than 20 genes, e.g., the aflatoxin gene cluster of *A. flavus* is composed of approximately 25 genes (Brown et al. 1996). Characteristic for most of the clusters is the involvement of at least one central biosynthesis gene encoding an extremely large multidomain, multimodular enzyme belonging to the polyketide synthases (PKSs) or the non-ribosomal peptide synthetases (NRPSs) (Hertweck 2009a; Crawford and Townsend 2010) surrounded by genes for tailoring enzymes such as oxidases, transporters and regulatory proteins (see below) (Hertweck 2009a). Typically, the PKS and NRPS enzymes use simple building blocks such as malonyl-CoA (PKS) and amino acids (NRPS) to synthesize the core structure (a polyketide or a peptide backbone) of a given SM that is further modified by tailoring enzymes—oxygenases, glycosyltransferases, methylases etc.—into the final natural product. The highly conserved features of the SM biosynthesis machinery allows for genomics-guided natural product discovery (Hertweck 2009b; Brakhage and Schroeckh 2011). In combination with the growing number of available fungal genome sequences (the *Aspergillus* Genome Database, AspGD, provides a central repository for *Aspergillus* sp.) and the development of bioinformatic algorithms such as SMURF (Khaldi et al. 2010), antiSMASH (Medema et al. 2011) or FungiFun (Priebe et al. 2011) enabling the identification of SM gene clusters in a fungal genome. A surprising result of these analyses was the discovery that the number of SM gene clusters exceeds those of known SMs in a single fungus by far. For example, the model fungus *A. nidulans* is potentially able to produce 32 polyketides, 14 non-ribosomal peptides and two indole alkaloids (Brakhage et al. 2008). When determined by SMURF, antiSMASH, by manual annotation and experimental characterization the number of SM gene clusters in a microorganism can increase to 71 (Inglis et al. 2013; Andersen et al. 2013). Because only few products of SMs are known this implies that a significant number of the gene clusters are silent and a multitude of SMs encoded by these clusters are still unknown. Even for the well-studied model fungus *A. nidulans* the percentage where both the SM and the corresponding cluster are known is at about 50 %. The residual clusters, referred to as cryptic or orphan (Hertweck 2009b) and their encoded natural products form an enormous untapped reservoir of the SM discovery pipeline. To lift this hidden treasure it is important to study how SM formation is regulated in nature, e.g. by global and specific regulators or via interplay with other organisms and to exploit the identified triggers to discover the compounds produced by these clusters.

3 Global Regulators

In their environments, fungi are constantly challenged by different stressors such as nutrient deprivation, other (micro)organisms that share the same habitat, changes of pH and temperature. The responses in fungi are mediated *via* transcription factors that link environmental cues and SM formation. About half of the current gene clusters do not contain obvious regulatory genes. Such gene clusters are controlled by global regulatory proteins, which in addition to a particular gene cluster also

regulate other genes not belonging to secondary metabolism (Brakhage 2013; Sanchez et al. 2012). Several of such regulators were identified when regulation and optimization of beta-lactam antibiotic formation was investigated. Fungal beta-lactam biosynthesis gene clusters lack a regulatory gene (Then Bergh and Brakhage 1998). For example, the transcriptional regulator PacC is the key factor of pH regulation (Tilburn et al. 1995). At alkaline pH PacC activates the transcription of the penicillin biosynthesis genes *acvA* and *ipnA* (Then Bergh and Brakhage 1998; Espeso and Penalva 1996). Similarly, the CCAAT-binding complex (CBC), consisting of the core subunits HapB, HapC and HapE integrates physiological signals such as the redox status and, by addition of another subunit HapX, iron deprivation and iron excess (Hortschansky et al. 2007; Thön et al. 2010; Gsaller et al. 2014). CBC also binds to CCAAT boxes in the promoters of *ipnA* and *aatA* and exerts a positive regulatory effect on penicillin biosynthesis in *A. nidulans.* A third example is CpcR1, the cephalosporin C regulator 1 (Schmitt et al. 2004) that controls arthrospore formation in *Acremonium chrysogenum* (Hoff et al. 2005) and, together with another transcription factor, Fkh1, regulates the cephalosporin biosynthesis in the same fungus.

Studies on gene regulation in response to nitrogen revealed that nitrogen availability not only affects physiology and morphology but also triggers the formation of a broad range of secondary metabolites (reviewed in: Tudzynski 2014). In all fungal species studied, the major GATA transcription factor AreA is the central player of the nitrogen regulatory network (Wiemann and Tudzynski 2013; Caddick and Arst 1998). Studies in *A. nidulans* showed the ability of AreA to mediate chromatin remodeling by increasing histone H3 acetylation (Berger et al. 2008). The finding that AreA is essential for both the expression of gibberellin biosynthesis genes in *Fusarium fujikuroi* (Mihlan et al. 2003) and for other SMs in different *Fusarium* species (Giese et al. 2013) made AreA an accepted regulator of SMs. A second GATA transcription factor, AreB, that was proposed to compete with AreA for binding to GATA elements, acts both as positive and negative regulator. Recent work on *F. fujikuroi* demonstrated that AreB regulates common target genes together with AreA, but also AreB-specific target genes. Whereas both transcription factors are essential for the expression of the gibberellin gene cluster (Michielse et al. 2014), AreB is sufficient to positively affect apicidin F and fusaric acid biosynthesis in *Fusarium* sp. (Niehaus et al. 2014).

The nuclear methyltransferase-domain protein LaeA was found to affect a number of SM gene clusters (Bok and Keller 2004). The true molecular function of LaeA remains to be elucidated. By generating *laeA* null mutants (Δ*laeA*), *e.g.* in *A. nidulans*, *A. fumigatus* and *A. flavus*, it has been shown that LaeA plays two roles in filamentous fungi. One is directly connected to SMs, *i.e.*, LaeA controls approximately 50 % of the SM gene clusters in fungi and loss of LaeA results in decreased SM formation (Perrin et al. 2007; Butchko et al. 2012; Kale et al. 2008). The second role of LaeA is its involvement in fungal morphology and development (Bok et al. 2005; Kamerewerd et al. 2011). For example, a Δ*laeA* mutant of *A. nidulans* hardly produces any Hülle cells as well as the mutant produces smaller cleistothecia compared to the wild-type strain (Sarikaya Bayram et al. 2010; Shaaban et al. 2010).

Together with the two velvet family proteins VelB and VeA LaeA forms the heterotrimeric VelB–VeA–LaeA complex. As investigated in *A. nidulans*, the complex coordinates light signal dependent fungal development with secondary metabolism (Bayram et al. 2008). This is performed through controlling the velvet family regulatory proteins for both light-dependent development and fungal cell-type specificity (Sarikaya Bayram et al. 2010). The fact that the velvet complex proteins are highly conserved among fungal pathogens suggests that besides the functions in development, sporulation and secondary metabolism the LaeA containing velvet complex also has important functions in pathogenicity. This is supported by the finding that until now all examined Δ*laeA* mutants of pathogenic fungi displayed reduced virulence (Bok et al. 2005; Amaike and Keller 2009; Wu et al. 2012; Lopez-Berges et al. 2013). As LaeA contains a methyltransferase domain it was hypothesized to impact SM gene cluster expression *via* epigenetic modification of the chromatin structure (Perrin et al. 2007). This hypothesis was confirmed by the discoveries that an *A. nidulans* Δ*laeA* mutant displayed more heterochromatin protein A (HepA, see below) occupancy with increased repressive histone 3 lysine 9 trimethylation (H3K9me3) in genes of the sterigmatocystin cluster (Reyes-Dominguez et al. 2010). Furthermore, numerous genes of *Trichoderma reesei* showed LAE1-regulated changes in the histone mark H3K4me3 (Karimi-Aghcheh et al. 2013). However, a substrate directly methylated by LaeA has not yet been identified with the exception of an automethylation reaction that produced S-methylmethionine in *A. nidulans* (Patananan et al. 2013). The finding that methyltransferases other than LaeA can associate with the velvet complex (e.g. LlmF, VipC–VapB) (Sarikaya-Bayram et al. 2015) indicates that a highly dynamic cellular network controls SM formation in response to a given environmental signal. This presumption is strengthened by the COP9 signalosome (CSN), another conserved protein complex acting at the interface of fungal development and secondary metabolism. Primarily, CSN controls protein degradation by the proteasome (Braus et al. 2010) and, thus, the protein turnover in the cell. As a result, numerous pleiotropic effects occur, influencing the cell cycle and differentiation, oxidative stress response, DNA repair, the circadian clock or light signaling, but also secondary metabolism.

4 Cluster-Specific Regulators

In addition to global regulators many SM gene clusters are regulated by cluster-specific regulators, whose genes are located in the cluster they regulate (Brakhage 2013). In some cases these cluster-specific regulators were found to regulate more than one cluster (Bergmann et al. 2010). Cluster-specific transcription factors usually are sequence-specific DNA-binding proteins of the Zn_2Cys_6 type (Chang and Ehrlich 2013). A well-known cluster-specific activator is AflR, one of about 25 genes of the aflatoxin and sterigmatocystin (*stc*) clusters in various *Aspergillus* spp. (Brown et al. 1996). The gene *aflR* encodes a typical Zn_2Cys_6 protein required for transcriptional activation of most, if not all, structural genes in the *afl* cluster in *A.*

flavus and *st* cluster in *A. nidulans* (Flaherty and Payne 1997; Yin and Keller 2011). Both clusters contain a second regulatory protein, *aflS*, adjacent to *aflR* (Brown et al. 1996) whose precise role is not yet clear. When a transcriptional regulator gene is part of a cluster, its expression is usually required for transcriptional activation of all other genes of the concomitant cluster. This holds true for GliZ of the gliotoxin biosynthesis gene cluster of *A. fumigatus* that activates all *gli* genes, except of *gliT*, encoding gliotoxin oxidase, which confers resistance to gliotoxin (Schrettl et al. 2010). Another example is the regulator ApdR, that regulates all genes of the aspyridone biosynthesis cluster in *A. nidulans* (Bergmann et al. 2007).

As already mentioned, even SM cluster-specific transcription factors were found to activate more than their 'own' cluster genes thus leading to the concept of cross talk between SM clusters. For example, the 'silent' *afo* cluster which encodes enzymes for the asperfuranone biosynthesis of *A. nidulans* is induced by targeted expression of ScpR, a secondary metabolism cross pathway regulator of the *inp* cluster of the same fungus (Bergmann et al. 2010). Similarly, the above described AflR is involved in both sterigmatocystin and asperthecin biosynthesis in *A. nidulans* (Yin et al. 2012). Altogether, as in most cases the signals triggering the activation of the pathway-specific regulators remain to be identified or even the pathway regulators itself are unknown, a crosstalk between fungal SM clusters may exist more often than anticipated, adding not only another level of complexity but also forming a further basis for the discovery of new compounds, *e.g.* by linking of pathways *via* transcription factors.

5 Genetic Engineering

In recent years, genetic engineering of natural product gene clusters has been further developed. There are different ways to interfere with the regulatory circuit of SM biosynthesis. By genome mining, a plethora of gene clusters have been identified which putatively encode for the biosynthesis of so far unknown natural products. However, most of these genes remain silent under standard laboratory conditions. A method to elucidate such silent gene clusters employs the exchange of the endogenous promoters of cluster genes by strong inducible promoters (Brakhage 2013). This was successfully applied for the *acvA* gene encoding the tripeptide synthetase whose activity is rate-limiting for the penicillin biosynthesis. The *acvA* promoter was exchanged against the strong inducible alcohol dehydrogenase promoter *alcA*. Thereby, the penicillin production of transformant strains was drastically increased (Kennedy and Turner 1996). Similarly, ectopic expression of the brevianamide F synthetase gene in *A. fumigatus* under the same *alcA* promoter, caused the accumulation of the corresponding biosynthesis product brevianamide F (Maiya et al. 2006). However, overexpression of a single cluster-specific gene does not always succeed in increased yield of a compound because this is then often limited by the expression of other genes in the same cluster. Additionally, homologous recombination often is a rate-limiting step in the genetic manipulation as it occurs

in very low frequencies in some fungi (Bergmann et al. 2007). A superior method is the overexpression of pathway-specific transcription factor genes as this often allows the concerted activation of all genes of a given cluster. For fungi, this method was first published by Bergmann et al. in 2007, leading to the discovery of novel PKS-NRPS hybrid metabolites, the aspyridones. By genome mining the authors identified the silent *apd* gene cluster containing a hybrid PKS-NRPS gene, surrounded by a putative oxidoreductase gene as well as genes encoding an exporter and activator. Overexpression of the identified regulatory gene under control of the *alcA* promoter led to the formation of aspyridones, which showed moderate cytotoxic effects (Bergmann et al. 2007). Similarly, overexpression of *afoA*, another transcriptional regulator of *A. fumigatus*, led to the discovery of the unknown compound asperfuranone. By genome mining the genomic position of the asperfuranone gene cluster was revealed. *AfoA* was set under the control of the inducible *alcA* promoter which in turn, by activation of the promoter, led to the biosynthesis of the so far unknown compound (Chiang et al. 2009). Interestingly, Bergmann and colleagues (2010) found that the asperfuranone cluster on chromosome VII was also induced by the transcription factor ScpR (secondary metabolism cross pathway regulator) located in the *inp* cluster on chromosome II. Overproduction of ScpR induced the biosynthesis of asperfuranone (Bergmann et al. 2010). In a different study the *gpdA* promoter was used for overexpression of a putative SM gene cluster transcription factor in *A. nidulans*. By genome mining the authors found a novel cluster harboring a $Zn(II)_2Cys_6$–type transcription factor designated PbcR. Overexpression of the respective gene led to the onset of seven genes from the predicted SM gene cluster as well as the biosynthesis of the diterpene compound, ent-pimara-8(14),15-diene (Bromann et al. 2012). Even though SM genes are usually clustered in the genome, the available genome data suggest that about 50 % of gene clusters do not contain a pathway specific-transcription factor (Unkles et al. 2014). Furthermore, some transcription factors require post-translational modification for full function, such as AflR, the transcription factor of the sterigmatocystin biosynthesis gene cluster in *A. nidulans* (Shimizu et al. 2003). Therefore, another interesting attempt, especially for biotechnological applications, is the expression of SM gene clusters in a heterologous host. For example, Maiya et al. (2006) showed successful expression of the brevianamide F synthetase gene by the inducible *alcA* promoter. The same construct was also inserted into the *A. nidulans* genome, which lacks the brevianamide F synthetase gene. While testing the recipient strain and the conditional mutant under inducing conditions the compound was only detected in the strain harboring the respective synthetase (Maiya et al. 2006). In another proof-of-concept study *A. niger* was used as the recipient strain to express the enniatin NRPS synthetase (ESYN) gene. In *Fusarium oxysporum* ESYN is responsible for the biosynthesis of cyclic depsipeptides belonging to the enniatin family. The compounds exhibit antimicrobial, antiviral and anticancer activities. Heterologous production of the corresponding NRPS was controlled by the Tet-ON system allowing the induction and repression of the cluster gene at any time during the fermentation process. Identity and purity of the peptide product were proven by tandem MS, NMR spectroscopy and X-ray crystallography. By applying this

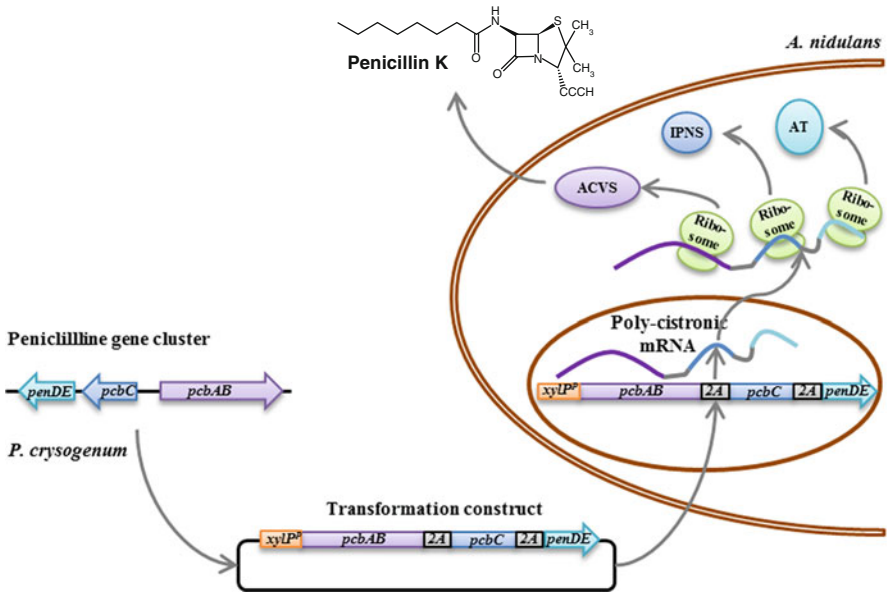

Fig. 2 The depicted construct was used for the heterologous expression of the penicillin clusters genes in *A. nidulans.* The vector consists of the penicillin cluster genes from *P. crysogenum* separated by 2A peptides and the inducible xylose promoter (Unkles et al. 2014). This vector was transferred into the recipient strain *A. nidulans* and expressed as a single poly-cistronic mRNA the complete penicillin biosynthesis gene cluster. Ribosomal translation is interrupted by 2A peptides aiding the co-translation of the three penicillin biosynthesis enzymes in *A. nidulans* and thereby leading to the production of penicillin

method, production of enniatins in *A. niger* drastically increased compared to the initial yields (Richter et al. 2014).

However, a greater challenge is the expression of a complete biosynthesis pathway in a heterologous host. The difficulty lies in the enormous size of both NRPS and PKS genes as well as the whole cluster itself making conventional cloning strategies cumbersome. Also, the stepwise introduction of each gene into a heterologous host would require another selectable marker for each recombination step plus an inducible promoter in front of each gene. Therefore, to generate a single polycistronic DNA containing all pathway-specific genes under control of a single inducible promoter is a straight-forward approach for the heterologous biosynthesis of natural products. A proof-of-concept study reported the expression of the penicillin biosynthesis gene cluster from *Penicillium chrysogenum* in an *A. nidulans* strain lacking the corresponding gene cluster (Fig. 2). A large polycistronic mRNA was generated and expressed under control of the xylose-inducible promoter. Subsequent cleavage of the pathway-specific enzymes was achieved by insertion of viral 2A peptide sequences between the enzyme-encoding sequences. Viral 2A peptide sequences induce co-translational cleavage of eukaryotic polycistronic mRNAs (Fig. 2). The production of the large polycistronic mRNA and of penicillin by the generated mutants was verified (Unkles et al. 2014).

Taken together, several strategies for activation of SM gene clusters identified by genome mining are available. Overexpression of transcription factor genes is a straight-forward method which often leads to activation/overexpression of a complete gene cluster. It also helps to increase the amount of products, which is often necessary for structure elucidation. However, only half of the putative gene clusters encode a regulatory protein and overexpression of single enzyme-encoding genes is cumbersome. Consequently, the expression of a whole SM gene cluster as a polycistronic mRNA in a heterologous host, driven by a single promoter, can be a feasible method to detect the products of such clusters.

6 Microbial Communication as Inducer of Fungal Secondary Metabolite Gene Clusters

Recent studies have shown the complexity and diversity of fungal interactions with other species leading to the production of natural products. Given the complex environment in which most fungi live it is not surprising that simulation of such environments triggers silent SM gene clusters (Fig. 1) (Brakhage 2013). For example, the interaction between a fungus, *Aspergillus nidulans,* and a bacterium, *Streptomyces rapamycinicus*, showed how co-cultivation of organisms from the same habitat can lead to the discovery of new natural products. The bacterium was discovered from a collection of actinomycetes and shown to selectively activate silent PKS and NRPS gene clusters in the fungus (Schroeckh et al. 2009a). It was discovered that the intimate physical contact between these two organisms induced the production of the four metabolites orsellinic acid, the cathepsin K inhibitors F-9775A, F-9775B and lecanoric acid. Interestingly, the latter is already known from another fungus-bacterium interaction, better known as lichens, and thus likely plays a role in microbial communication (Stocker-Worgotter 2008). As lecanoric acid did not directly affect *S. rapamycinicus* the ecological function of the compound remains to be elucidated. It is conceivable that the compound affects other microorganisms associated with *A. nidulans* and/or *S. rapamycinicus*. Interestingly, *S. rapamycinicus* also activated a silent SM gene cluster in *A. fumigatus*. Transcriptome analyses of the fungus coculivated with the bacterium led to the discovery of the fumicycline gene cluster. The produced metabolites were isolated and named fumicycline A and B, the corresponding PKS of the SM gene cluster was named FccA (König et al. 2013). Especially *A. fumigatus* has proven to be a fruitful source for natural products derived from mixed fermentations. Zuck et al. (2011) co-cultured *A. fumigatus* with *Streptomyces peucetius* which led to formation of formyl xanthocillin analogues, named fumiformamide and *N,N'*-((1Z,3Z)-1,4-bis(4-methoxyphenyl)buta-1,3-diene-2,3-diyl)diformamide. Mixed fermentation of *A. fumigatus* with *Streptomyces bullii* resulted in the production of a multitude of new metabolites, among them ergosterol and seven metabolites belonging to the diketopiperazine alkaloids (Rateb et al. 2013). To shed light on the SMs for structuring microbial communities it is required to study interactions that are known to occur in nature. Such multispecies

interactions can be found in every habitat. Interactions of clinical relevance can be found in polymicrobial communities in human hosts where microbes form commensal, mutualistic, competitive, or antagonistic interactions with one another and with the host (Jabra-Rizk 2011). Microbial communities are also found in the lungs of cystic fibrosis (CF) patients which are characterized by a viscous mucus layer. In the lung sputum of CF patients *A. fumigatus* was often detected together with the Gram-negative bacterial opportunistic pathogen *Pseudomonas aeruginosa*. Moree et al. (2012) used imaging mass spectrometry to analyse the secreted metabolites of both organisms. Interestingly, they found that phenazine, produced by *P. aeruginosa*, was modified by the fungus to give derivatives such as other 1-hydroxyphenazine, 1-methoxyphenazine, phenazine-1-sulfate and phenazine dimers, with enhanced toxicity and the ability to induce fungal siderophore formation (Moree et al. 2012). Furthermore, Svahn et al. (2014) showed that the presence of bacteria-derived lipopolysaccharide, peptidoglycan, or lipoteichoic acid is sufficient to increase the secretion of gliotoxin by *A. fumigatus*. The influence of bacteria-derived metabolites on the production of gliotoxin suggests that fungi possess a so far uncharacterized detection system for such metabolites (Svahn et al. 2014). Previously, the concept that cell-wall components can trigger the formation of SMs was shown by Patterson and Bolis (1997). In this study, the cyanobacteria, *Scytomena ocellatum* was stimulated with cell-wall homogenates of the fungi *Penicillium notatum* and *Cylindrocladium spathiphylli*, leading to increased production of tolytoxin, an antifungal metabolite. Oh et al. (2007) observed that co-cultivation of *Emericella parvathecia,* a marine fungus, with the actinomycete *Salinispora arenicola* led to a 100-fold increased production of emericellamides A and B by the fungus. Both metabolites showed a slightly increased activity against methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria. A potent antibiotic against MRSA and vancomycin-resistant *Enterococcus faecium* was discovered with pestalone, a cocultivation-derived natural product. The SM was produced by mixed fermentation of *Pestalotia* sp. and an unidentified, likewise marine-derived gram-negative bacterium of the genus *Thalassopia* sp. (Cueto et al. 2001). Furthermore, interaction of the same bacterium with *Libertella* sp. led to the production of libertellenones A-D by the fungus. These compounds, however, did not exhibit antibiotic properties but an increased cytotoxic activity against a human adenocarcinoma cell line (Oh et al. 2005). Interestingly, this example illustrates that a single bacterium can induce biosynthesis pathways in different fungi. The same was also found for the interaction of *S. rapamycinicus* with *A. nidulans* and *A. fumigatus* described above. The bacterium *Bacillus subtilis* is also able to modify gene expression in fungi. When *P. chrysogenum* was co-cultured with this bacterium, several fungal gene clusters encoding PKSs and NRPSs were down-regulated. Moreover, the fungus showed up-regulation of two putative α-1,3-endoglucanase (mutanase) genes, which may enhance the activity of fungal antibiotics by degrading bacterial exopolysaccharides (Bajaj et al. 2014). Important to add is that unusual habitats such as environment with pollution, high acidity and heavy metal content also provide a source for pharmaceutical compounds. It is conceivable that these environments could critically increase the ability of microorganisms to create both defensive and offensive microbial interactions. For

example, the mixed fermentation of mine drainage-derived strains of *Sphingomonas* and *A. fumigatus* resulted in isolation of the new diketopiperazine disulfide glionitrin A (Park et al. 2009). Glionitrin A showed significant antibiotic activity against MRSA as well as increased cytotoxic activity against four human cancer cell lines.

In fact, mixed fermentation as source of new natural products was first used by Watanabe et al. (1982). The authors discovered formation of the antibacterial polyketide enacyloxin by *Gluconobacter* sp. W-315 during co-cultivation with the fungi *N. crassa* or *A. oryzae*. In the following, several unknown products were found by studying fungal interactions. Co-incubation of *Acremonium* sp. with *Mycogone rosea* led to formation of the new lipoaminopeptides acremostatins A, B and C (Degenkolb et al. 2002). A new alkaloid, designated as aspergicin, was discovered in mixed culture of two marine-derived mangrove epiphytic *Aspergillus* sp. (Zhu et al. 2011). The interaction of two other mangrove fungi, *Phomopsis* sp. K38 and *Alternaria* sp. E33, resulted in the biosynthesis of a so far unknown cyclic tetrapeptide, designated as cyclo-(L-leucyl-*trans*-4-hydroxy-L-prolyl-D-leucyl-*trans*-4-hydroxy-L-proline) (Li et al. 2014). A larger screening of mixed fungal cultures led to the identification of an unusual long-distance growth inhibition between *Trichophyton rubrum* and *Bionectria ochroleuca*. Five *de novo* induced compounds were found and one metabolite was successfully identified as 4″-hydroxysulfoxy-2,2″-dimethylthielavin P using analytical methods like LC-MS-based metabolomics (Bertrand et al. 2013).

In general, fungi and bacteria mutually stimulate the induction of silent gene clusters and in general the production of new secondary metabolites. A single bacterial species is even able to trigger the production of different metabolites when interacting with diverse fungal species. The habitats of these microbial communities, which form the basis of these interactions, are diverse as the species themselves.

7 Regulation of Natural Products by Chromatin Remodelling

In fungi, the biosynthesis of many natural products is strongly influenced by nutrients, environmental cues and interacting microorganisms sharing the same habitats (Brakhage 2013; Netzker et al. 2015). Recent findings indicate that the regulation of SM gene clusters largely depends on chromatin remodeling events. Chromatin is composed of genomic DNA wrapped around a histone octamer, which consists of the core histone proteins H2A, H2B, H3 and H4. This composition, however, can change due to chromatin regulation brought about by ATP-dependent remodeling of chromatin, methylation of genomic DNA or insertion of histone variants. The structure of chromatin is strictly controlled by post-translational modifications of histones preferably at their N-terminal tails. The presence or absence of functional groups bound to histone residues in the chromatin structure influences the accessibility of coding regions in the DNA and thereby have a major influence on

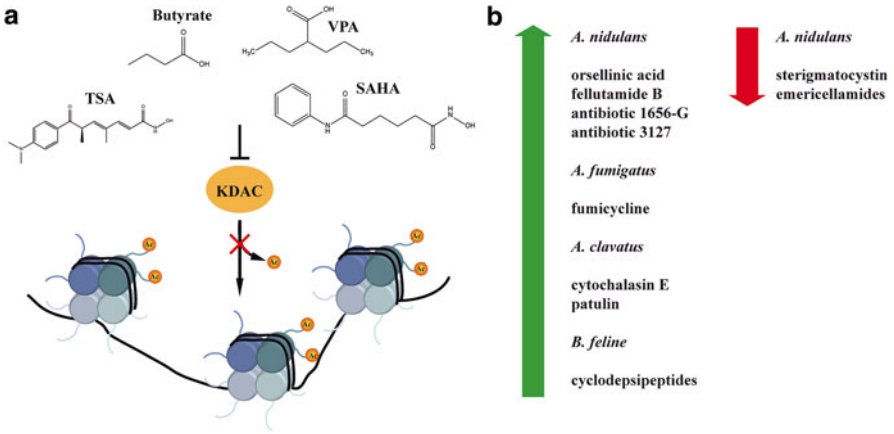

Fig. 3 The addition of KDAC inhibitors to the culture broth triggers the inactivation of a subset of enzymes which regulate the removal of acetyl groups from histones (**a**). This in turn can reduce (*red arrow*) or increase (*green arrow*) the production of natural products and can even lead to the identification of unknown metabolites (**b**)

DNA-dependent processes. Histones can be modified by acetylation, methylation, phosphorylation, sumoylation and ubiquitination (Gacek and Strauss 2012b; Brakhage 2013; Shilatifard 2006; Biran and Meshorer 2012). The proteins performing such modifications play an essential role in the regulation of transcription. Therefore, their selective activation or inhibition is a promising attempt to activate otherwise silent genomic regions.

A generally known modification to facilitate transcription is the acetylation of histones. Acetyl groups are added to lysine residues by lysine acetyltransferases (KATs, also known as HATs) comprising a diverse family of enzymes including, amongst others, the MYST-family, the p300/CBP-family, and the Gcn5-related acetyltransferases (GNATs) (Carrozza et al. 2003). Lysine deacetylases (KDACs, also HDACs) are enzymes that catalyze removal of acetyl groups from lysine residues of both histone and non-histone proteins. The inhibition of KDACs can lead to a subsequent hyperacetylation of the chromatin landscape and therefore to an opening of the nucleosomal structure. This in turn changes silent heterochromatic to euchromatic regions which can result in the activation of otherwise silent genomic loci (Grunstein 1997). Consistently, inhibitor studies for KDACs have successfully proven to alter SM formation in fungi (Fig. 3) (Zutz et al. 2013; Chung et al. 2013). Zutz and colleagues (2013) tested the effect of different KDAC inhibitors on the SM production of *A. clavatus*. The KDAC inhibitors valproic acid (VPA), trichostatin A (TSA) and butyrate had profound effects on SM accumulation and transcription of the corresponding biosynthesis genes. Addition of VPA, TSA and butyrate to the culture medium significantly increased the production of cytochalasin E and patulin. Furthermore, the amount of pseurotin A was significantly higher when *A. clavatus* was grown in the presence of TSA, butyrate and GlcNAc, the latter was used to simulate a bacterial fungal co-cultivation (Zutz et al. 2013).

In *A. nidulans*, supplementation of the cultivation media with the KDAC inhibitors, suberanilohydroxamic acid (SAHA), trichostatin A, or sodium butyrate led to a selective up-regulation of three fellutamides. This is the first report to show that *A. nidulans* is capable of producing these lipopeptide aldehydes, *i.e.*, fellutamides B and the structurally related antibiotics 1656-G and 3127. Interestingly, the formation of the mycotoxin sterigmatocystin and also of several emericellamides was reduced under the same conditions (Albright et al. 2015). A comparable protocol led to the production of several new products in the filamentous fungus *Beauveria felina*. Here, the addition of the KDAC inhibitor SAHA led to the isolation of several metabolites, including three new and five new cyclodepsipeptides (Chung et al. 2013).

In the previous chapter, we discussed the co-cultivation of the fungus *A. nidulans* with the bacterium *S. rapamycinicus*. This microbial interaction led to the induction of the otherwise silent orsellinic acid (*ors*) gene cluster (Schroeckh et al. 2009b). Interestingly, the addition of the KDAC inhibitor SAHA activated fungal orsellinic acid production without the need for co-incubation with *S. rapamycinicus*. Consistently, the addition of anacardic acid, a KAT inhibitor, omitted the induction of orsellinic acid, despite of the presence of the bacteria in the culture (Nützmann et al. 2011). This finding paved the way for the generation of a comprehensive KAT deletion library in *A. nidulans*, which led to the identification of GcnE as an essential mediator of the fungal-bacterial interaction. This GNAT-type KAT forms the catalytic subunit of the SAGA complex, a conserved multi-subunit complex also found in other eukaryotic organisms (Baker and Grant 2007; Govind et al. 2007). Furthermore, it was found that the specific acetylation of histone H3 lysine 9 and 14 is needed for the onset of the orsellinic acid gene cluster transcription and product formation (Nützmann et al. 2013). However, SAGA not only seems to play a role during interaction but also for the regulation of other well-known SMs such as penicillin (*pn*), sterigmatocystin (*stc*) and terrequinone A (Nützmann et al. 2011). The impact of chromatin remodeling on the interaction of two organisms demonstrated how these effectors modulate transcription upon a distinct stimulus. Until now, a variety of chromatin modifiers have been discovered which regulate natural product biosyntheses (Gacek and Strauss 2012a).

Because deletion of *gcnE* led to an altered natural product biosynthesis, it was conceivable that the systematic screening of the KAT deletion library of *A. nidulans* allowed for the identification of novel metabolites. Indeed, a drastically altered metabolic profile was detected in the Δ*nnaB* mutant strain, representing another GNAT-type KAT-encoding gene. Besides a number of orsellinic acid derivatives, the previously unknown pheofungins were discovered (Scherlach et al. 2001). In *A. parasiticus*, a correlation of active transcription and acetylation of histone H4 of aflatoxin biosynthesis genes was found correlating with the down-regulation of a MYST-type KAT under cluster-repressing conditions (Roze et al. 2007, 2011). Likewise, in *A. nidulans*, the SM gene clusters for *stc*, *pn*, *ors* and terrequinone showed acetylation of histone H4. Overexpression of the essential KAT Esa1 led to an increase of acetylation at the respective loci, emphasising that this MYST-type KAT is responsible for the modification (Soukup et al. 2012). The first correlation

between chromatin remodelling and SM gene cluster regulation was reported by Shwab et al. (2007). The authors showed that HdaA, a class II KDAC, plays a role in SM cluster regulation. Deletion of *hdaA* in *A. nidulans* not only led to reduced growth of the fungus during oxidative stress but also to a higher production of SMs such as *pn*, *stc* and terrequinone A (Tribus et al. 2005; Shwab et al. 2007). Consistently, HdaA has a significant impact on SMs produced in *A. fumigatus*, such as fumitremorgin B, pseurotin and gliotoxin. Surprising was the finding that gliotoxin production was down-regulated upon deletion of *hdaA* (Lee et al. 2009; Shwab et al. 2007). A major impact on development and SMs in *A. nidulans* was reported just recently for the KDAC RpdA. A knock-down strain with an inducible xylose promoter was generated in order to study the impact of RpdA on the fungus. Under repressing conditions this mutant showed developmental defects, such as reduced conidiation and radial growth (Albright et al. 2015). Interestingly, this KDAC also seems to represent a central regulator of SM biosynthesis, as upon repression the 100 least abundant SMs in the wild type increased over 60-fold. Among these metabolites were the cathepsin K inhibitors F9775A and B, alternariol and the antibiotic 3127. By contrast, the most abundant 100 compounds showed decreased expression of their biosynthesis genes by about 50 % under *rpdA*-repressing conditions. This included well-known metabolites, such as sterigmatocystin, emericellamides and the austinols, which were down-regulated compared to the wild type (Albright et al. 2015). Until now, quite a number of studies dealt with the regulation of SM gene clusters by acetylation of histones. However, also lysine methylation is regarded as one of the most complex modifications so far having diverse effects on gene transcription (Brakhage et al. 2008). In an initial study Bok et al. (2009) examined CclA, an ortholog of the *Saccharomyces cerevisiae* Bre2 protein, which is a member of the COMPASS complex. COMPASS is a conserved multi-subunit complex both facilitating and repressing transcription through methylation of lysine 4 of histone 3 (Mueller et al. 2006). The deletion of the *cclA* gene in *A. nidulans* highly reduced the H3K4 di- and trimethylation of the examined SM gene clusters. As a consequence, monodictyphenone, emodin and the anti-osteoporosis compounds F9775A and F9775B were identified (Bok et al. 2009). Similarly, the deletion of the *A. fumigatus* CclA orthologous genes also resulted in an altered SM profile; the production of gliotoxin as well as of some other metabolites was increased. The influence of a putative H3K4 demethylase, HdmA, was also tested in the same study. However, deletion of the respective gene did not influence the SM production in *A. fumigatus* (Palmer et al. 2013). Interestingly, deletion of a jumonji-type histone demethylase, KdmA, responsible for H3K36me3 demethylation in *A. nidulans*, was necessary for full expression of several SM biosynthesis genes (Gacek-Matthews et al. 2015). Reyes-Dominguez and colleagues (2010) studied lysine 9 methylation of histone H3 with respect to SM production. Upon growth arrest a reduced H3K9 methylation within the *stc* cluster borders was observed, which led to its activation. Intriguingly, this mark was associated with a protein responsible for heterochromatin formation, the heterochromatin protein A (HepA). Deletion of the respective gene led to the activation of the *stc* gene cluster (Reyes-Dominguez et al. 2010; Brakhage 2013).

8 Conclusion

Sequencing of fungal genomes and systematic genome mining concerning so far undiscovered SM metabolite gene clusters have uncovered the great potential of fungal species for SM biosynthesis. Until now, only for relatively few clusters the corresponding metabolites are known. Even less is known about the regulation of their production. Therefore, the discovery of the triggers activating or repressing SM biosynthesis is crucial to explore the full biosynthesis potential of fungal strains. It can be expected that this knowledge will lead to the discovery of novel drugs. In this article we also discussed some of the strategies allowing for the activation of silent SM gene clusters. Cultivation conditions simulating the natural environment of fungi led to the discovery of new natural products. Especially co-cultivation of distinct bacterial and fungal partners often caused the modulation of gene expression and the formation of novel molecules. Interestingly, even the addition of bacterial molecules such as LPS was apparently sufficient to trigger a response of the fungal partner. Another successful approach is to delete, overexpress or inhibit histone-modifying enzymes/genes. In line, approaches targeting KDACs led to some new and interesting products. Although these methods allow for activation of silent gene clusters without rationale prediction, they will not help to systematically activate distinct SM clusters in fungal genomes. This problem can be overcome by novel technologies of synthetic biology. One example is the expression of fungal SM gene clusters as polycistronic mRNA that encodes all required pathway-specific genes separated by 2A peptide sequences.

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Understanding the Mechanism of Carbon Catabolite Repression to Increase Protein Production in Filamentous Fungi

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1 Introduction

The production of proteins has become a branch of industry. The demand for enzymes exists because they are useable for many different applications. The most important question is which host is suitable for the production of a certain protein. On the one hand there are biological questions: Does a certain host already express the desired protein or is it possible to introduce a gene? How can the gene expression be increased and how difficult is the process of purification? On the other hand there are economic issues: How much does the substrate for the host or the inducer of gene expression cost? Filamentous fungi have proven to be very suitable hosts for protein production but there are still targets for optimization and one problem for protein production is carbon catabolite repression. This mechanism can reduce the yield and therefore make the whole process inefficient.

1.1 Carbon Catabolite Repression (CCR)

CCR is a mechanism to maintain metabolic efficiency of an organism by ensuring the utilization of favourable compounds. This regulatory tool can be found in all domains of life. CCR was extensively studied in *Saccharamyces cerevisiae,* where Mig1/2 constitute the key players of the mechanism (reviewed in Gancedo 1998).

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In case of filamentous fungi like *Neurospora crassa* and *Aspergillus nidulans* the main regulators of carbon triggered repression are Cre-1 (Ebbole 1998) and CreA (Dowzer and Kelly 1991), respectively. These factors were identified due to homologies with Mig1/2, but the mode of action is different. D-glucose is a very prominent activator of CCR in filamentous fungi, the availability of D-glucose (supplied from the culture medium) is going to lead to the transcriptional down-regulation of genes responsible for the utilization of alternative carbon sources. The signal for the onset of CCR might be D-glucose-6-phosphate. In *Trichoderma* reesei QM9414 the nuclear transport of Cre1 was demonstrated by Lichius and colleagues (2014). In germlings that were pre-cultured on cellulose the GFP-tagged Cre1 was translocated into the nucleus upon D-glucose addition. This was not the case when 6-deoxyglucose, a compound which cannot be phosphorylated, was added to de-repressed hyphae of *A. nidulans* (Brown et al. 2013). More evidence for this theory was supplied by a double knock-out in *T. reesei*. Kubicek and co-workers deleted two genes coding for hexose kinase orthologues, *glk1* and *hxk1*, which would lead to de-repression (Kubicek et al. 2009).

To get an idea of CCR we also have to take a look at the players that are going to promote the expression of otherwise CCR targeted genes: activators of transcription and their corresponding inducing molecules. Concerning filamentous fungi the main interest of protein production is focussed towards the lignocellulolytic enzymes. Exponents of these genera, *A. niger* and *T. reesei* are able to secrete large amounts of these industrially applicable proteins. Therefore, these species attract scientific attention due to their natural and potential properties.

1.2 First Studies and Discovery: Aspergillus nidulans and Neurospora crassa

In *A. nidulans* the lignocellulolytic machinery is positively regulated by XlnR and CCR is facilitated by CreA. XlnR is a binuclear zinc cluster protein and binds DNA motifs which are abundant in promoters of cellulase and hemicellulase genes (Stricker et al. 2008). On the other hand CreA binds 5′-SYGGRG-3′ DNA sequences and facilitates the D-glucose-triggered repression of genes (Kulmburg et al. 1993), one of the targets is also the *xlnR* gene (Tamayo et al. 2008). According to Roy and co-workers overexpression and several mutations of CreA had no influence on repression and de-repression (Roy et al. 2008). CreA itself is also target of CCR, which might hint to a feedback-loop mechanism (Strauss et al. 1999). The mode of action of CreA is still not fully understood. Experiments employing GFP-fusion proteins of CreA displayed a translocation from the cytosol into the nucleus when the fungus is transferred from inducing (e.g. D-xylose containing medium) to repressing conditions (D-glucose containing medium) (Brown et al. 2013). The kinase SnfA might be involved in the de-repression of genes under CCR. In a *ΔsnfA*-strain the fusion protein cannot be dislocated from the nucleus and the repression is preserved even in cellulose containing medium. So the questions to be addressed in

the future might be: Is a phosphorylation even needed to translocate CreA into the nucleus as in *S. cerevisiae* and which kinase is responsible? Is CCR a matter of nuclear CreA levels because it is equally distributed throughout induced hyphal cells, or are there other proteins involved? Facilitation of CCR by high nuclear CreA levels might hint to a competition for DNA-binding sites with inducing transcription factors. Furthermore, nucleosome positioning is also influenced by CreA, having an impact on chromatin status, and therefore, gene transcription (Mathieu et al. 2005). Besides CreA, there are also CreB, a deubiquinating enzyme (Lockington and Kelly 2001), CreC, a WD40-repeat protein (Lockington and Kelly 2002), and CreD (Boase and Kelly 2004), which might interact with an ubiquitin ligase (Polo and Di Fiore 2008). CreC prevents the proteolysis of CreB in absence of CCR (Todd et al. 2000) and CreB possibly prevents ubiquitin-dependent degradation of CreA. Interestingly, when a D-xylose-inducible and D-glucose-repressed endoxylanase from *Aspergillus oryzae* was cloned into *A. nidulans*, the gene became D-glucose-inducible (Kimura et al. 1998). Recent studies showed that there are other forms of CCR in *A. nidulans*: an acetate-dependent repression mediated by CreA unaffected by CreB and CreC (Georgakopoulos et al. 2012), and CreA-independent CCR of *rhaE* (Tamayo-Ramos et al. 2012).

N. crassa possesses 23 predicted cellulase- and 19 predicted hemicellulase-encoding genes (Amore et al. 2013). Cellulose induces both parts of the lignocellulytic machinery, xylan only induces hemicellulase-encoding genes. Some genes encoding hemicellulases are induced by Xlr-1, a zinc binuclear cluster protein (Sun et al. 2012). Cellulase expression is additionally induced by two other zinc binuclear cluster proteins, Clr-1 and Clr-2 (Coradetti et al. 2012), but full induction requires Xlr-1 (Sun et al. 2012). CCR is mediated by Cre-1, which binds to DNA motifs also occurring in promoter regions of genes coding for the lignocellulolytic machinery (e.g. promoter of *cbh-1*). The mode of action might be competition with factors inducing gene expression (Sun and Glass 2011). Xlr-1 is also a target of Cre-1 (Sun et al. 2012). A deletion of Cre-1 led to higher cellulase activity on cellobiose (Znameroski et al. 2012) and a constant cellulase gene expression independent of the supplied media (Sun and Glass 2011). Cre-1 does not target all hemicellulase-encoding genes and there occurs also CCR which does not depend on Cre-1 (Amore et al. 2013).

2 CCR in Industrially Used Filamentous Fungi

2.1 Aspergillus niger

A. niger has industrial application as a source of pectinases (Bussink et al. 1992; Delgado et al. 1993; Parenicova and Benen 2000) and xylanases (Gielkens and Visser 1997; van Peij et al. 1997). It can produce 25–30 g of glycoamylase per litre (Demain and Vaishnav 2009). The lignocellulolytic machinery is composed of six

cellulose-degrading, three xylan backbone-modifying, eight xylan accessory and five D-xylose metabolism enzymes (Mach-Aigner et al. 2012). So far *A. niger* is used to produce commercial enzyme cocktails (Berka et al. 1991), which consist of a very complex protein mix (Pel et al. 2007) compared to other industrially used, filamentous fungi.

The main positive regulator of the lignocelluloytic machinery is XlnR. It is a binuclear zinc finger protein (van Peij et al. 1998a), comparable to Gal4p of *S. cerevisiae* (Finn et al. 2006), which is specific for fungal transcription factors. 5′-GGCTAA-3′ is the consensus sequence bound by XlnR (Degraaff et al. 1994). Targets of XlnR control are genes coding for xylan degrading enzymes and D-xylose metabolic genes (de Groot et al. 2007; De Vries and Visser 1999; Hasper et al. 2000; van Peij et al. 1998b), endocellulases and cellobiohydrolases (Gielkens et al. 1999), and galactosidases (de Vries et al. 1999a). Overall, XlnR positively regulates 25 genes (Stricker et al. 2008). D-xylose is an important inducer of the carbohydrate-active enzymes (CAZYs) because the cellulolytic and xylanolytic machinery is co-regulated via this inducer molecule (Hasper et al. 2000; Gielkens et al. 1999; de Vries and Visser 2001). However, also lactose was found to be a good inducer of cellulase activity (Mrudula and Murugammal 2011).

In *A. niger* CreA not only mediates CCR but also represents a wide-domain regulator (Drysdale et al. 1993). CCR is not only activated by D-glucose (de Vries et al. 1999b) but also at higher concentrations of D-xylose (Mach-Aigner et al. 2012). One of the targets of CreA is XlnR, which is expressed on a basal level on any D-xylose concentration. In the same study Mach-Aigner and co-workers (Mach-Aigner et al. 2012), showed that in the CreA de-repressed strain NW283 (*creA^{d}4*) gene expression is independent of the D-xylose concentration. The mechanism behind this concentration-dependent regulation could not be identified so far. Another trait of this strain is the doubling of the hemicellulase transcript levels. Besides CreA, *A. niger* possesses three more *cre* genes: *creB*, *creC* and *creD*. These genes have homologs in *A. nidulans* and the function of these genes so far has been investigated only in this species. The identities according to BLAST are 70 %, 78 % and 78 %, respectively (accession numbers for *A. niger* enzymes: CreB XP_001389270, CreC AAF63188, CreD XP_001394284; accession numbers for *A. nidulans* proteins: CreB AAL04454, CreC AAF63188, CreD AAS10351; bait in each BLAST search was the *A. niger* enzyme), and functional domains are conserved. CreB is a deubiquinating enzyme (Lockington and Kelly 2001), CreC is a WD40 domain protein interacting and stabilizing CreB (Lockington and Kelly 2002; Todd et al. 2000), and CreD plays also a role in this ubiquitinating regulatory network (Boase and Kelly 2004). A strategy to exploit CCR for protein production could be to redesign strong promoters. This would most probably minimize the disadvantage of messing with the CreA influenced regulatory network, which happens in case CreA is deleted or non-functional. Chien-Huang et al. showed that the deletion of the CreA-binding motif in the *bgl2* promoter leads to an increased protein secretion and activity even in D-glucose containing medium (Li and Yan 2014), but the highest activity could be measured on bran. A combination of these approaches might be the modification of CreA-binding sites in promoters on one hand and directed mutation of CreA itself on the other hand.

2.2 *Trichoderma reesei*

Great research effort and interest is focussed on *T. reesei* (Saloheimo et al. 1994), which can be mainly accounted to its lignocellulolytic machinery and high secretory capacity (up to 100 g of protein per litre (Demain and Vaishnav 2009)). Its enzymes are biotechnologically applied in the food, and paper and pulp industry. The complexity of its CAZYs is reduced in comparison to other industrially applied ascomycetes like *A. oryzae* and *A. niger* (Martinez et al. 2008). The lignocellulolytic machinery is composed of ten cellulases (e.g. *cbh1* (El-Gogary et al. 1989)) and 16 hemicellulases (e.g. *xyn1* and *xyn2* (Törrönen et al. 1994)).

The expression of cellulase- and hemicellulase-encoding genes is regulated on the transcriptional level. On the one hand are positive regulators like Xyr1 (Stricker et al. 2006) and Ace2 (Aro et al. 2001), and on the other hand are repressors like Cre1 (Ilmen et al. 1996a) and Ace1 (Aro et al. 2003). Gene expression of CAZYs is increased when certain inducers, mono- or disaccharides, enter the cell, and these inducers are breakdown products of insoluble polymers. Therefore, a basal expression level is generally maintained by the fungus (El-Gogary et al. 1989; Carle-Urioste et al. 1997). Xyr1 is a central regulator of CAZY-encoding gene expression. It harbours a zinc binuclear cluster, which is typical for fungal transcription factors (Finn et al. 2006). Upon activation, Xyr1 might dislocate into the nucleus and bind to a 5′-GGC(A/T)$_3$-3′ motif (Furukawa et al. 2009). The arrangement of these motifs as inverted repeats has been shown to be functional *in vivo* (Rauscher et al. 2006). Ace2 is also a zinc binuclear cluster protein and plays a role in the expression of *xyn2*, by binding the xylanase-activating element and possibly interacting with Xyr1 (Stricker et al. 2008). The Hap2/3/5 complex contacts CCAAT-boxes leading to rearrangements in the chromatin structure (Mach et al. 1995; Zeilinger et al. 2003). Ace1, a repressor of cellulases and xylanases (Aro et al. 2003) binds to 5′-AGGCA-3′ sequences (Saloheimo et al. 2000) and therefore, might compete with Xyr1 for DNA-binding motifs (Rauscher et al. 2006).

CCR is mediated by Cre1 in *T. reesei* (Strauss et al. 1995). Further, Cre2, which is an orthologue of the *A. nidulans* CreB, influences the inducibility of the cellulolytic machinery on lactose and Avicel (Denton and Kelly 2011). Cre1 is a Cys2His2-type transcription factor (Dowzer and Kelly 1991; Ilmen et al. 1996a) binding 5′-SYGGRG-3′ motifs, which have been found e.g. in the *cbh1* (Ilmen et al. 1996b) and *xyn1* (Mach et al. 1996) promoters. Further consensus sequences were found in the promoters of *cbh2* and *egl1* (Ilmen et al. 1997). A comparative metabolic study in the *T. reesei* strain QM9414 revealed that 402 genes are under the influence of D-glucose, 123 genes are controlled by cellulose and 154 are regulated by sophorose (Castro et al. 2014a). Sophorose is the strongest inducer of the lignocellulolytic machinery in *T. reesei* (Mandels and Reese 1960; Mandels et al. 1962). Feeding of D-glucose leads to a decrease of *cre1* transcript by ten- to 20-fold suggesting the possibility of a feedback-loop (Ilmen et al. 1996a). Portnoy and co-workers (2011a) compared a *Δcre1*- and a QM9414 strain and found that 207 genes are under the influence of Cre1. 118 genes were repressed and 72 were induced. *Xyr1* is also a target of Cre1-dependent repression and its expression is rather de-repressed than

induced (Mach-Aigner et al. 2008). However, *xyr1* expression is also inducible by sophorose, and higher levels of Xyr1 correlate with higher expression of CAZYs (Derntl et al. 2013). In order to bind DNA more efficiently Cre1 has to be phosphorylated (Cziferszky et al. 2002), but the kinase facilitating this task has not been identified yet (Cziferszky et al. 2003). Furthermore, Cre1 mode of action cannot be only attributed to protein-DNA interaction, but there is also an indirect influence on gene expression by regulation of many other genes, e.g. regulation of transporters. It was reported that the influence of the inducer is stronger than the composition of the promoter (Castro et al. 2014b), independently of the number of Xyr1- and Cre1-binding sites. Rut-C30 is a *T. reesei* strain, which was generated by three rounds of random mutagenesis. This industrially important strain carries besides other genetic differences a 83 kb deletion and a truncated version of *cre1* (Seidl et al. 2008; Vitikainen et al. 2010). The comparison of a *Δcre1* strain, a strain with truncated *cre1,* and a wild-type strain QM6a directly cultivated in a bioreactor showed that even on D-glucose the amount of secreted protein is four times, the activities of CBHI and EGLI are ten times higher than in the wild-type. Keeping in mind that the activity is still four times lower compared to induced conditions (Nakari-Setälä et al. 2009). Only the strain bearing the truncated *cre1* showed *cbh1* transcripts detectable by Northern blot analysis. Another mutation of Cre1 was investigated by Porciuncula and co-workers: a single nucleotide polymorphism within the *cre1* gene in *T. reesei* strain PC-3–7 (Porciuncula et al. 2013). This mutation leads to a transition from threonine at position 78 to proline resulting in a lower binding affinity of Cre1 to the consensus sequence and a partial release of CCR on target genes.

In order to manipulate CCR for the means of protein production it is important to realize the drawbacks of different strategies. The response of the gene regulatory network is difficult to predict. Deleting the *cre1* gene was reported to lead to lower *xyr1* expression (Portnoy et al. 2011b), and an alteration of the gene expression patterns of all downstream genes. This seems to be a contradictory result to the premise that the deletion of a repressor should lead to a higher expression of its target. However, Cre1 is not only a repressor but plays also a role in chromatin remodelling. It orchestrates nucleosome positioning in induced and repressed conditions in the *cbh1* (Ries et al. 2014) and *cbh2* promoter (Zeilinger et al. 2003). Recent data from our group shows that the truncated form of Cre1 ($Cre1_{96}$) does not only lead to more open chromatin, but also to increased levels of *htf1* mRNA, a chromatin remodeller. Another consequence of this truncation is a higher level of *xyr1* transcripts, which is associated with higher levels of target genes under D-glucose conditions (Mello-de-Sousa et al. 2014). This leads to the question: how to avoid CCR as good as possible to guarantee efficient protein production.

2.3 *Penicillium chrysogenum*

P. chrysogenum is best known and industrially used to produce large quantities of penicillin N. Besides its *pen* genes it is also able to produce lignocellulolytic enzymes.

The *pen* genes *pcbAB*, *pcbC,* and *penDE* are organized as a cluster (Fierro et al. 1995) and the regulation of these genes is well understood. The promoter of *pcbAB* and *pcbC* is located between the two oppositely transcribed genes (Diez et al. 1990). The promoter harbours several different binding motifs: seven 5′-GCCARG-3′ motifs for PacC (pH regulator) binding (Suarez and Penalva 1996), six 5′-GATA-3′motifs organized in double sites for Nre (nitrogen catabolite repressor) binding (Haas and Marzluf 1995), six CCAAT-boxes, six 5′-SYGGRG-3′ consensus sequences for CreA binding (Cepeda-Garcia et al. 2014), and one 5′-TTAGTAA-3′ for Pta1 complex binding (Kosalkova et al. 2000, 2007). Additionally, other regulators like StuA (Sigl et al. 2011), LaeA (Keller et al. 2005), and VelA (Hoff et al. 2010), which generally play important roles in development also contribute to the regulation of the *pen* genes and might depict a link between morphology and secondary metabolite formation. Therefore, there seems to be no pathway-specific regulator but rather transcriptional regulation by wide-domain regulators (Martin et al. 2010). Industrially used strains of *P. chrysogenum* possess several copies of the *pen* gene cluster (Fierro et al. 1995) and lost some enzymes for the utilization of cellulose, sorbitol, and other carbon sources (Jami et al. 2010).

Focusing on CreA-mediated CCR, the creation of a deletion strain was not successful in *P. chrysogenum* (Cepeda-Garcia et al. 2014). But the mutation of the *pcbAB-pcbC* promoter indicated another approach to manipulate CCR. Deletion of CreA-sites closest to the respective gene had the strongest effect on removing CCR from the gene. Usually, D-glucose represses *pen* gene expression, but after the deletion of the three CreA-sites next to *pcbC* it was completely deregulated and expressed in D-glucose containing medium. A combination of removal of CreA-sites from the promoter and multiple gene insertions might be an applicable solution for native and heterologous protein production in *P. chrysogenum*.

Looking at the lignocellulolytic machinery of *P. chrysogenum, cbh1* from strain FS010 was isolated in 2007 (Hou et al. 2007). The promoter of the gene is 1.3 kb in length and harbours six CreA-binding sites. Sophorose, cellobiose, and cellulose induce strongest, D-glucose, fructose, and sorbitol repress the expression of *cbh1*. Lactose, D-xylose, and gentiobiose are able to induce the promoter moderately. Another expression system was proposed by Zadra and co-workers in 2000 (Zadra et al. 2000). They used the promoter of the endoxylanase *xylP,* which is under CreA-mediated CCR and harbours three XlnR-binding sites. The promoter can be easily induced by oat spelt xylan and D-xylose (Haas et al. 1993). Therefore, it might be a feasible approach to delete CreA-sites from the promoter and try to express native and heterologous proteins by using oat spelt xylan and D-xylose as inducers.

2.4 Other Fungi

Recent studies show that *Acremonium cellulolyticus* might also become an interesting host for the production of cellulases besides *T. reesei*. Comparison of the *A. cellulolyticus* strain CF-2612 with the *T. reesei* strain CDU-11, CF-2612 showed

twofold higher filter paper activity (FPase) and 16-fold higher β-glucosidase activity, while CDU-11 displayed twofold higher xylan-hydrolyzing activity (Fujii et al. 2009). In *A. cellulyticus* lactose is a strong inducer of *cbh1* (Hideno et al. 2013) and capable to induce cellulase expression overall. In combination with Solka-Floc powdered cellulose the effect is even more pronounced (Fang et al. 2008). CCR is also mediated via CreA in this filamentous fungus. The gene was identified via homology comparison with other filamentous fungi. Knock-out of creA led to higher FPase and xylanase activity on xylan, cellulose, and mixtures with D-glucose compared to the parental strain (Fujii et al. 2013). The same study also looked at the localization of the protein using a GFP-fusion construct. It was reported that CreA displays predominantly nuclear localization in D-glucose containing medium. Overall, the knowledge of *A. cellulyticus* is becoming more detailed, and it might become an important host of protein production in the future.

Besides, there is an increased research focus on *Trametes* spp. At the time the scientific attention is mostly on laccase properties and expression, and strain improvement via random mutagenesis. Further species of *Penicillium* are also being examined for their ability to secrete lignocellulolytic enzymes, e.g. *P. purpurogenum* and *P. decumbens*.

3 Outlook

Filamentous fungi are adequate hosts for the production of proteins and secondary metabolites. Advantages of fungi as cell factories are the established transformation techniques, their great secretory capacity including glycosylation patterns, the available information and research which has been performed, and most important, their ability to thrive on waste products. Altogether, this predestines them for biotechnological use.

There are many similarities between the different organisms we focussed on in this chapter but they also differ in very important ways, like their inducer molecules or the mode of action of the inducing and repressing transcription factors. After a long history of random mutagenesis it will be fascinating to see in which direction strain improvement is going to be developed. A promising approach might be the genetic modification of existing industrial strains via modified transcription factors and targeted alteration of promoters of genes of interest with the objective to avoid CCR.

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Inteins and Their Use in Protein Synthesis with Fungi

Skander Elleuche and Stefanie Pöggeler

1 Introduction

More than 20 years ago the first intein was identified as an in-frame insertion in the *VMA1* gene of the yeast *Saccharomyces cerevisiae*. The *S. cerevisiae VMA1* gene encodes a vacuolar membrane H^+-ATPase with a predicted molecular mass of 120 kDa. However, the size of the VMA1 protein, estimated from sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels was only 70 kDa. N- and C-terminal regions of the amino acid sequence were shown to be homologous to VMA1 proteins of other organisms but not a 454 amino-acid internal fragment. This insertion was found to be present in the *VMA1* transcript and translated into a precursor protein, but was not part of the mature VMA1 protein (Hirata et al. 1990; Kane et al. 1990). In analogy to RNA introns, the internal (or intervening) protein sequence was termed intein and the N- and C-terminal flanking external protein sequences were labeled N- and C-exteins, respectively. The post-translational process that removes the intein from the precursor and forms a peptide bond between the N- and C-exteins, was termed protein splicing (Perler et al. 1994) (Fig. 1a). Inteins can also be encoded by two separately transcribed and translated genes and are then called split inteins. The two parts, Int^N and Int^C, of the split intein self-associate and catalyze protein-splicing of the N- and C-exteins in *trans* (Fig. 1b). The accepted terminology for

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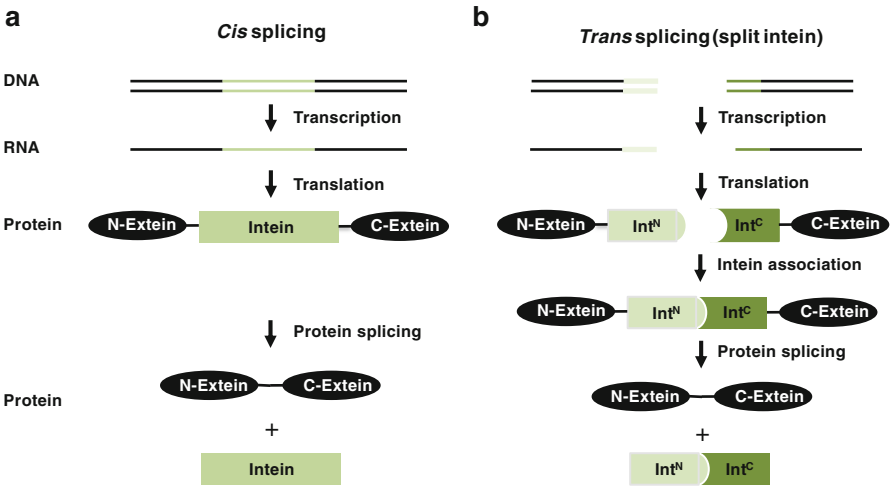

Fig. 1 Protein splicing of conventional contiguous inteins (**a**) and split inteins (**b**). The intein coding sequence is transcribed and translated to a nonfunctional protein precursor. Intein-catalyzed *cis*- and *trans*-splicing removes the inteins and joins the extein to a mature protein

inteins includes the three letter abbreviation of the species and the host gene name (e.g. the *S. cerevisiae* VMA1 intein is called *Sce* VMA1). Multiple inteins from one protein are numbered with Arabic numerals (Perler et al. 1994), when they are inserted at the same site in homologous genes they are considered intein alleles (Perler et al. 1997). The intein registry InBase at http://www.neb.com/neb/inteins.html, listed in 2010 more than 600 inteins in the genomes of Eubacteria, Archaea, and Eukarya, including 92 fungal inteins which have been identified in Chytrids, Zygomycetes, Ascomycetes and Basidiomycetes (Perler 2002). Inteins reside often in essential proteins involved in replication, DNA repair, transcription or in metabolism (Perler 2002; Shah and Muir 2014). In ascomyceteous yeasts, they are predominately found in homologs of the *S. cerevisiae VMA1* gene, whereas in filamentous ascomycetes they reside mostly in the *prp8* gene, encoding a pre-mRNA splicing factor. In rare cases, fungal inteins have been also identified in genes encoding glutamate synthases, chitin synthases, threonyl-tRNA synthetases, and subunits of DNA-directed RNA polymerases (Elleuche and Pöggeler 2009; Poulter et al. 2007).

1.1 Structure and Conserved Features of Inteins

Inteins can be grouped into minimal (mini) and large inteins. The latter have inserted a homing endonuclease (HE) within the intein splicing domain (Fig. 2). HEs make site-specific, double-stranded DNA breaks within intein-free alleles of their host gene and thereby promote lateral transfer between genomes in a recombination-dependent process known as "homing" (Belfort et al. 2005; Hafez

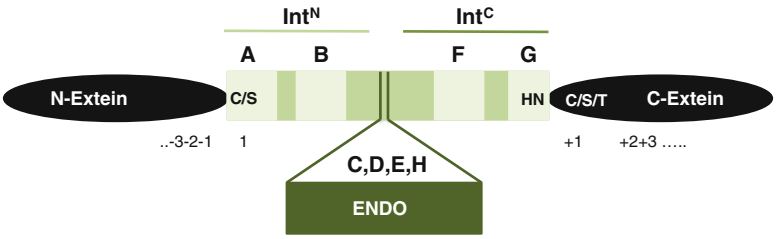

Fig. 2 Conserved sequence motifs and amino acids of mini and large inteins. The HE domain (ENDO) inserted between blocks A and F is found exclusively in large inteins. The first residue of the intein is labelled with 1. Amino acid residues of the N-extein are counted backwards with negative numbers those of the C-extein with positive numbers

and Hausner 2012). The HE domain splits the intein into the N- and C-terminal subdomains Int^N and Int^C, respectively. Although non-allelic inteins share only low sequence similarity (<40 %), both subdomains contain conserved blocks of amino acids, with blocks A and B in Int^N, and blocks F and G in Int^C (Perler et al. 1997; Volkmann and Mootz 2013). These domains can also be identified in mini-inteins and the two parts of split inteins, while the HE domain of large inteins has additional conserved blocks C, D, E, and H (Fig. 2). Splicing-efficient mini-inteins have been engineered from large inteins by deleting the central endonuclease domain. Thus, the HE domain is not involved in protein splicing (Chong and Xu 1997; Derbyshire et al. 1997; Shingledecker et al. 1998). Determination of the intein structure by NMR as well as X-ray crystallography revealed that Int^N and Int^C form a horseshoe-like fold comprised of two- or three-stranded β-sheets and loops together with two α-helices, termed the Hedgehog/Intein (HINT) domain (Ding et al. 2003; Hall et al. 1997; Klabunde et al. 1998; Koonin 1995; Oeemig et al. 2009, 2012; Perler 1998; Sun et al. 2005; Van Roey et al. 2007). Split inteins are rarely found in nature (Perler 2002). They are normally separated between motifs B and F, resulting in Int^N of ~100 amino acids, and Int^C of ~40 amino acids and adopt the same horseshoe-like fold when interacting in *trans* (Shah and Muir 2014; Wu et al. 1998). Contiguous inteins can be artificially split at the same position (Elleuche and Pöggeler 2007; Mills et al. 1998; Mootz and Muir 2002; Southworth et al. 1998).

In addition to the conserved blocks A, B, F and G, inteins have extremely conserved residues crucial for protein-splicing at their N- and C-termini. These include Ser or Cys at the first and His-Asn at the last two positions of the intein. Also the first amino acid of the C-extein is a conserved nucleophilic residue (Ser, Thr or Cys), whereas other residues of the exteins immediately flanking the intein are not highly conserved but required for optimal splicing efficiency (Amitai et al. 2009; Cheriyan et al. 2013; Mootz 2009; Oeemig et al. 2012; Perler 2002; Zettler et al. 2013) (Fig. 2).

1.2 *Splicing Mechanisms of Inteins*

Protein splicing is a rapid four step reaction resulting in the cleavage of two peptide bonds (between the exteins and the intein) and the formation of one peptide bond (between the two exteins). Inteins are grouped into three classes based on their sequence signature and splicing mechanism (Tori et al. 2010). Details of the chemical process involved in protein splicing have been comprehensively reviewed (Starokadomskyy 2007; Volkmann and Mootz 2013). Here, we will only briefly describe the canonical splicing mechanism of class 1 inteins and the principal differences to this pathway in the two alternative splicing processes of class 2 and class 3 inteins.

The standard class 1 intein displays all sequence features described in Fig. 2. The splicing involves four steps (Fig. 3). It starts with an initial N–S acyl-shift, if position 1 is a Cys with a thiol side chain or an N–O acyl-shift, if position 1 is a Ser with a hydroxyl side chain. After the first step, the intein residue at position 1 forms a (thio) ester bond at the N-extein-intein junction. In step 2, this linear (thio)ester intermediate undergoes a *trans* esterification reaction. The (thio)ester bond is attacked by the thiol- or hydroxyl-group of the Cys, Ser or Thr at position +1 of the C-extein. This

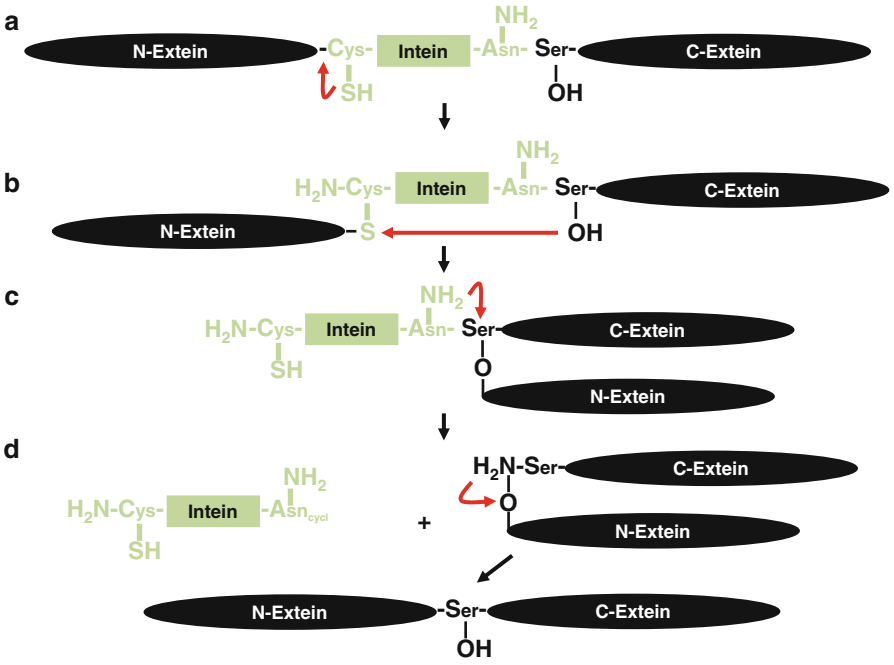

Fig. 3 Splicing mechanism of class 1 inteins. Intein splicing takes place in four reaction steps. (**a**) N-s acyl shift. (**b**) Transesterification. (**c**) Asn-cyclization. (**d**) N-O acyl shift

leads to a transfer of the N-extein to the side-chain of the first residue of the C-extein. A cyclization of the conserved, last Asn residue of the intein cleaves the peptide bond between the intein and the C-extein and releases the intein with a C-terminal succinimide ring (step 3). The exteins are linked by a (thio)ester bond. The peptide bond between the N- and C-extein is formed by an intein-independent acyl shift.

In addition to the standard protein splicing pathway, class 2 and class 3 inteins lack the nucleophilic residue Ser or Cys at position 1 and cannot perform the acyl shift that initiates the splicing reaction in class 1 inteins (Southworth et al. 2000; Tori et al. 2010). Class 2 inteins attack directly the peptide bond of the N-terminal splice site junction with nucleophilic side chain at postion +1 (Cys) of the C extein. The first two steps of the standard splice reaction are omitted. The branched intermediate is formed and the splicing proceeds with the Asn cyclization (Johnson et al. 2007; Southworth et al. 2000). Class 3 inteins carry a conserved Trp, Cys, and Thr in block B, F and G respectively. The Cys of block F attacks the peptide bond at the N-terminal splice site junction, forming a branched intermediate with a labile thioester linkage. The N-extein is then transferred by a *trans* esterification reaction to the residue at position +1 of the C-extein resulting in the formation of a standard branched intermediate as in class 1 inteins (Tori et al. 2010).

N- or C-terminal specific cleavage of the intein-extein junctions can also be achieved in the classical class 1 inteins by mutation of critical intein residues. N-terminal cleavage can be engineered by mutation of the last intein residue (Asn). This mutation abolishes steps 3 and 4 of the splicing reaction, while step 1 still occurs. The release of the N-extein is achieved by addition of an external nucleophile (water or thiol), which hydrolyzes the (thio)ester intermediate. C-terminal cleavage occurs when the conserved first residue of the intein is mutated. This abolishes steps 1, 2 and 4 of the splicing reaction, but Asn cyclization (step 3) still occurs and thereby separates the C-extein from the N-extein-intein portion. Controllable cleavage of modified *cis*-splicing inteins has been adapted for a wide range of useful applications in molecular biology and biotechnology (see below).

2 General Applications of Inteins

Post-translational modifications of peptide chains expand the functional and structural repertoire of the protein's primary structure. Therefore, inteins were modified in myriad ways for various basic and biotechnological applications. Most areas of intein applications are based on their natural or artificial ability to splice in *trans*, while only a few biotechnological fields using contiguous inteins were developed (Elleuche and Pöggeler 2010; Volkmann and Mootz 2013). However, the application of inteins is often restricted by their slow reaction kinetics, especially depending on the use of non-natural extein residues. Therefore, a major focus in intein research is on the detailed understanding of the influence of foreign extein sequences on protein splicing and the efficient assembly of split intein variants (Amitai et al. 2009). Remarkably, crystal structure determinations of artificially fused split-inteins

revealed that the N- and C-terminal fragments are profoundly entwined, enabling easy and rapid assembly of the single moieties (Shi and Muir 2005; Sorci et al. 2013). Nevertheless, the catalytic performance is highly dependent on the conservation of the extein residues at the splice site junctions. While the +1-amino acid residue is directly involved in the splicing reaction (see above), the -1-amino acid residue can profoundly influence the N/X acyl-shift reaction. A temperature-dependent splicing performance has also been evaluated for some inteins, showing that a switch to non-optimal conditions goes along with a prevalence of N- or C-terminal cleavage instead of protein splicing. Depending on their field of application, inteins with tailor-made properties, such as *cis*- or *trans* splicing, splicing induction and kinetics can be developed. The fastest splicing reactions have been investigated in several *trans*-splicing protein elements (Carvajal-Vallejos et al. 2012; Shah et al. 2012). Moreover, some inteins, like the *Nostoc punctiforme Npu* DnaE intein, exhibited an extremely high reaction rate at 37 °C both *in vitro* and *in vivo* making them useful for versatile biotechnological applications (Iwai et al. 2006; Zettler et al. 2009). Their practical utility and autocatalytic activity including all remarkable properties make inteins of biotechnological interest for various applications, such as expressed protein ligation, intein-mediated protein purification, peptide cyclization and segmental isotopic labelling (Elleuche and Pöggeler 2010; Vila-Perelló and Muir 2010).

2.1 Expressed Protein Ligation (EPL)

In vitro protein semi-synthesis can be achieved by cleaving an N-terminal extein to produce an α-thioester at the C-terminal flanking site of the extein-peptide using a mutated intein. The C-terminal thioester can be used to form a native peptide bond by condensation with an N-terminal cysteine residue in another peptide. The idea of expressed protein ligation (EPL) using recombinant peptides harbouring an activated thioester is based on the classical native chemical ligation (NCL) (Dawson and Kent 2000). While the latter method is limited by the size of the peptides, the production of recombinant proteins enables the directed linkage of macromolecules. EPL has been applied to connect proteins to non-canonical amino acids, photochemical cross linkers or further artificial moieties that cannot be connected by recombinant techniques alone (Volkmann and Mootz 2013).

To achieve a more flexible handling of synthetically synthesized peptides linked to recombinant proteins, semi-synthesis by protein *trans*-splicing was developed as advancement to EPL using contiguous inteins, thereby enabling the production of N- or C-terminally tagged peptides. Since the solid-phase peptide synthesis is limited to small proteins, artificially split intein variants were engineered to generate minimal sized intein variants with unnatural split sites. Modified variants of the *Npu* DnaE and *Ssp* GyrA inteins are capable of splicing in *trans* by spontaneous assembly of a six amino acid C-terminal intein part with its N-terminal partner (Appleby

et al. 2009; Oeemig et al. 2009). An active *trans* splicing intein with the split-site much closer to the N-terminus has been established by the Mootz group and contains only an 11 amino acid residue Int^N part (Ludwig et al. 2006). So far, the splicing efficiencies of these artificially split inteins are not as good as the performance of natural split inteins. Moreover, a disadvantage of natural split inteins is their size of around 30 to 40 amino acid residues, preventing them from an efficient utilization in protein semi-synthesis. Nevertheless, a remarkable tool has been recently established making use of the high reactivity and affinity of the natural split *Npu* DnaE-intein in intein-mediated protein purification coupled EPL-approach (Vila-Perelló et al. 2013). In this approach, a C-intein is immobilized on a column, while its Int^N-counterpart is fused to a protein of interest and produced in recombinant form in a heterologous host. Crude protein extracts including the fusion protein can be passed over the column, thereby assembling both intein-halves. After a washing step, induced intein cleavage separates the protein of interest and the activated α-thioester can directly be used to generate a semi-synthetic protein.

To investigate the structure and protein dynamics, Nuclear Magnetic Resonance (NMR) spectroscopy has been shown to be a method of choice. NMR makes use of the incorporation of heavy isotopes into proteins usually by expressing recombinant genes in bacterial cells growing in medium enriched with ^{2}H, ^{13}C or ^{15}N. However, this method is limited by a uniform distribution of labelled amino acids in large proteins. To generate segmentally labelled protein parts, EPL both using *cis*- or *trans*-splicing inteins has been applied, respectively (Volkmann and Iwai 2010; Züger and Iwai 2005).

2.2 Peptide Cyclization

A cyclic conformation of peptides usually goes along with an improved stability towards chemical, thermal and/or enzymatic degradation and often with an increased activity. A wide variety of organisms produce cyclic peptides for various reasons including cytotoxic or anti-bacterial compounds such as cyclosporin A, which is produced by peptide synthetases from the filamentous ascomycete *Tolypocladium inflatum* (Craik 2006; Survase et al. 2011).

Peptide cyclization has been achieved using both *cis*- and *trans*-splicing intein approaches (Xu and Evans 2001) (Fig. 4). The composition and position of the proximal termini is the bottleneck for efficient peptide cyclization. One method for intein-mediated cyclization of peptides makes use of the action of an intein that is fused with its N-terminus to a target protein and mutated at its C-terminus (Fig. 4a). Induction of N-terminal cleavage results in the release of the target protein with an activated C-terminal thioester. Moreover, the target protein contains a cysteine directly following the ATG-triplet encoded methionine at its N-terminus, which can be removed by an *E. coli* enzyme called methionyl-aminopeptidase. Afterwards, the cysteine at the N-terminus will react with the thioester to give a new peptide bond

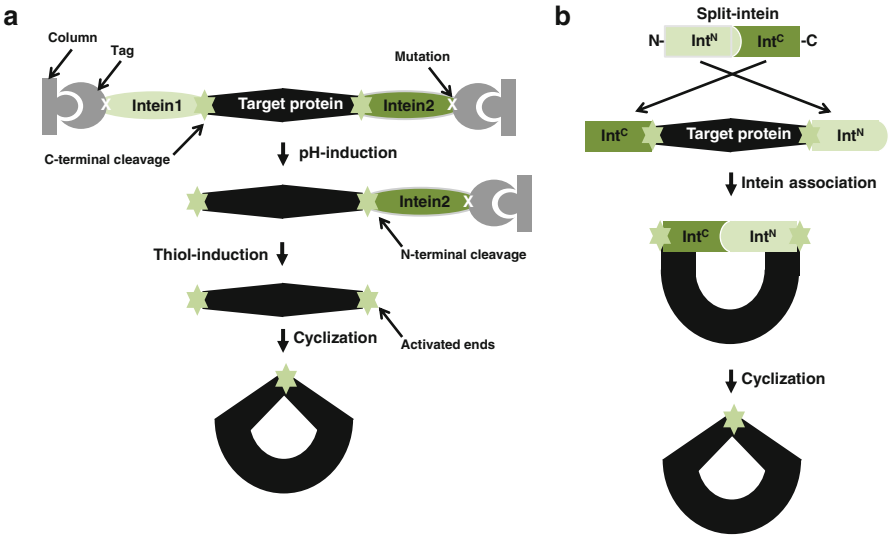

Fig. 4 Intein-mediated peptide circularization. (**a**) Principle of protein cyclization using two inteins. (**b**) Protein cyclization using the Splicing Intein-mediated Circular Ligation Of Peptides and ProteinS (SICLOPPS) method

in a circularized peptide. A further development of this method uses a second intein fused to the N-terminus of the target protein, allowing its release via C-terminal cleavage thereby generating a N-terminal cysteine as well, which reacts with the thioester at the C-terminus of the peptide (Fig. 4). A major disadvantage of this method is the possible formation of peptide polymerization instead of cyclization, which can be circumvented by utilizing a *trans*-splicing mediated protein cyclization (Fig. 4b). Splicing Intein-mediated Circular Ligation Of Peptides and ProteinS (SICLOPPS) is a method that has been developed using a *trans*-splicing intein with an inverted order of its two halves at a single protein of interest enabling a head to tail cyclization reaction (Fig. 4b). This method has been successfully applied to generate cyclic peptides composed of only eight amino acid residues and to screen large libraries of cyclic peptides for new potent therapeutic drugs (Cheriyan and Perler 2009; Tavassoli and Benkovic 2007).

2.3 *Temperature/Ligand-Induced Conditional Protein Splicing (CPS)*

In contrast to the utilization of contiguous inteins, it is possible to easy regulate the protein splicing performance of artificially split-intein variants *in vivo*, thereby facilitating the activation of proteins in an impulse-dependent manner. Conditional

protein splicing (CPS) enables the researcher to regulate the intein-mediated protein reaction. Using this method, the protein of interest can be activated *in vivo* or *in vitro* through control of the intein. Suchlike methods are very promising especially with regard to basic biological research field, because they act faster than traditional methods including promoter induction (Schwartz et al. 2007; Vila-Perelló and Muir 2010). A well-known CPS-strategy makes use of the rapamycin-induced heterodimerization of the mammalian proteins FKBP and FRB together with the artificially split *S. cerevisiae* VMA1 intein to trigger the ligation of two protein halves. The functionality of this induction system has been proven not only in single cells but in animals as well (Schwartz et al. 2007). To investigate the time-dependent formation of a specific protein in living cells, two non-functional parts of the protein of interest replace the natural exteins of a split intein. In addition, FKBP is fused to the N-intein and FRB to the C-intein near the assembly sites to allow efficient protein splicing only when the small molecule rapamycin interacts with FKBP and FRB.

An impressive temperature-dependent intein application has been recently developed to degrade plant cell wall material. The *Tth* DnaE-1 intein from the thermophilic bacterium *Thermus thermophilus* has been engineered to undergo heat-induced protein splicing. The *Tth* DnaE-1 intein has been inserted into the thermostable xylanase XynB from the bacterium *Dictyoglobus thermophilum* thereby impairing the catalytic xylanase activity of the precursor protein. A recombinant gene encoding the XynB-intein construct was integrated into transgenetic maize to improve the degradation of the lignocellulosic cell wall material. The expression of genes encoding active cell-wall degrading enzymes in plant feedstock usually reduces biomass yield and plant fertility, but the production of an inactive xylanase variant overcomes these problems. The compact structure of cellulosic and hemicellulosic components in harvested plant material can then be opened by heat-pretreatment methods, which also induces the splicing activity of the *Tth* DnaE-1 intein without degrading the thermostable xylanase XynB. After protein splicing, the active hemicellulose degrading enzyme is formed within the plant cell material and enables the degradation of lignocellulosic material from the inside (Shen et al. 2012).

Moreover, split-intein variants that spontaneously reassemble and splice *in trans* have also been useful for overcoming the size limitation of gene delivery vectors. The artificially split inteins from *Synechocystis* sp. PCC 6803 (*Ssp*) and from *Rhodothermus marinus* DnaB were used to produce a full-length dystrophin protein in a mouse model. The production of large dystrophin is hindered by the small packaging size of adeno-associated viral (AAV) vector systems, therefore a 6.3-kb open reading frame of the dystrophin gene was broken into two parts and each were fused to a split-intein sequence. The parallel delivery of two AAV-mediated constructs into a single target cell resulted in *trans*-splicing of the reassembled DnaB-intein and the production of functional dystrophin. The protein complemented Duchenne muscular dystrophy in the mouse model indicating an application of inteins in gene therapy (Li et al. 2008).

3 Applications Using Fungal Inteins

The majority of applications are based on the utilization of bacterial inteins. As indicated above, a lot of developments make use of the performance of protein *trans*-splicing, which are naturally occurring exclusively in (cyano-)bacterial species. Nevertheless, artificial split-inteins have been developed from fungal splicing elements, which display obvious properties making them attractive tools for versatile applications (Brenzel et al. 2006; Elleuche and Pöggeler 2007). Beside the yeast VMA1 intein, there are mainly the PRP8 inteins derived from several fungi, which were established in different systems.

3.1 The Classic: VMA1 Intein in Yeast

The *Sce* VMA1 intein was the first intein to be identified and was employed in multiple powerful technological applications. It was implemented in the first intein-mediated protein purification system developed by New England Biolabs (Chong et al. 1997). Later, intein-mediated protein purification tools have been advanced to work efficiently in fungal systems as well. Affinity-chromatography based on the interaction of a family 3 cellulose-binding module (CBM3) from the thermophilic bacterium *Clostridium thermocellum* was used to purify several recombinant model proteins produced in the methylotrophic yeast *Pichia pastoris*. Codon usage optimization of the gene encoding CBM3 enabled the production of an artificial fusion protein in the fungus at a high level. Furthermore, a modified variant of the *Sce* VMA1 intein allowing exclusively for N-terminal cleavage was embedded between the tag and the target protein. Induction of intein self-cleavage enabled the elution of tag-less target protein from a cellulose-containing column. This system was proposed to be applicable for large-scale industrial production processes of enzymes and pharmaceuticals (Wan et al. 2011).

The *Sce* VMA1 intein was also used for the production of human granulocyte macrophage colony stimulating factor (hGMCSF) in *E. coli* and in *P. pastoris* (Srinivasa Babu et al. 2008, 2009). The hGMCSF is a therapeutic cytokine, which is used in the treatment of leukaemia lowering the risk of infections. Purification and intein-mediated cleavage of the chitin-tag resulted in the production of freely released hGMCSF displaying comparable activities to the cytokine produced in mammalian cell cultures.

Among the most interesting applications of the *Sce* VMA1 intein is certainly the development of a series of conditional protein splicing tools, which are applicable in a variety of different cellular systems (Mootz and Muir 2002; Schwartz et al. 2007). The potential of an artificial split *Sce* VMA1 intein variant was also demonstrated in a very elegant light-dependent protein splicing system in yeast. The split intein moieties were each fused to a photodimerization system of the plant model *Arabidopsis thaliana*. Phytochrome B and phytochrome interacting factor 3 undergo reversible photo-dimerization when exposed to red light (660 nm) and disassemble at far-red

light (750 nm). The VMA intein halves were either fused to a maltose-binding protein or to a FLAG-tag enabling the researchers to demonstrate light-activated protein splicing *in vivo* in *S. cerevisiae* (Tyszkiewicz and Muir 2008). Moreover, a temperature-sensitive *Sce* VMA1 intein variant has been developed to investigate time-dependent gene function by precise control of gene expression *in vivo*. For this purpose, the VMA intein sequence was inserted into yeast Gal4 transcription activator resulting in inactivity as long as the intein was not spliced off. PCR-mediated mutagenesis enabled the detection of temperature-sensitive intein variants that were inactive at 30 °C and slightly active at 18 °C. The respective intein variant was then transferred into a transcription control system in *Drosophila melanogaster* to allow temperature-dependent production of green fluorescent protein (GFP). Moreover, it was proposed that this system is of potential use to detect lethal mutations, which can easily be tested for reversion by a temperature shift (Zeidler et al. 2004).

3.2 A Robust Mini-Intein from Different Penicillium Species

A homologous mini-intein, which is embedded in the spliceosomal machinery protein PRP8, has been identified in different species from the several fungal genera (Bokor et al. 2012; Butler et al. 2006; Perler 2002; Poulter et al. 2007). However, only a few of them have been tested experimentally (Elleuche et al. 2006, 2009; Liu and Yang 2004; Theodoro et al. 2011). The prototype PRP8 intein from the basidiomycetous yeast *Cryptococcus neoformans* and its counterpart from the filamentous ascomycete *Penicillium chrysogenum* have been studied in detail. Mutational studies led to the identification of conserved amino acid residues that are of relevance for protein splicing in the *Cne* PRP8 intein (Pearl et al. 2007a, b). The *Pch* PRP8 intein has been shown to be of impressive robustness towards amino acid deletions and intein-splitting thereby generating a series of *trans*-splicing variants. This mini-intein did not only tolerate modifications within the insertion region of an endonuclease, it also kept its capability of protein splicing when mutated at a position corresponding to a putative tongs-domain in a large intein from *A. fumigatus* (Elleuche and Pöggeler 2007; Elleuche et al. 2008). These features indicate that the *Penicillium* PRP8 inteins exhibit highly robust structures and folding abilities, indicating a potential for versatile biotechnological applications (Fig. 5).

The first *Penicillium* PRP8 intein that has been implemented in a biotechnological tool was derived from the plant pathogenic fungus *P. expansum*, which is the causal agent of the blue mould rot (Elleuche et al. 2006). The *Pex* PRP8 intein has been inserted in the GFP-encoding open reading frame without any conserved natural extein amino acid residues, except the first downstream extein residue Ser^{+1}, which is essential for protein splicing in *Penicillium* PRP8 inteins according to a model developed for an intein from *Mycobacterium tuberculosis* (Elleuche et al. 2006; Gangopadhyay et al. 2003). Denaturation followed by re-naturation of the GFP-intein fusion construct was mandatory for protein splicing, representing ideal pre-conditions to use this construct in a screening approach for inhibiting

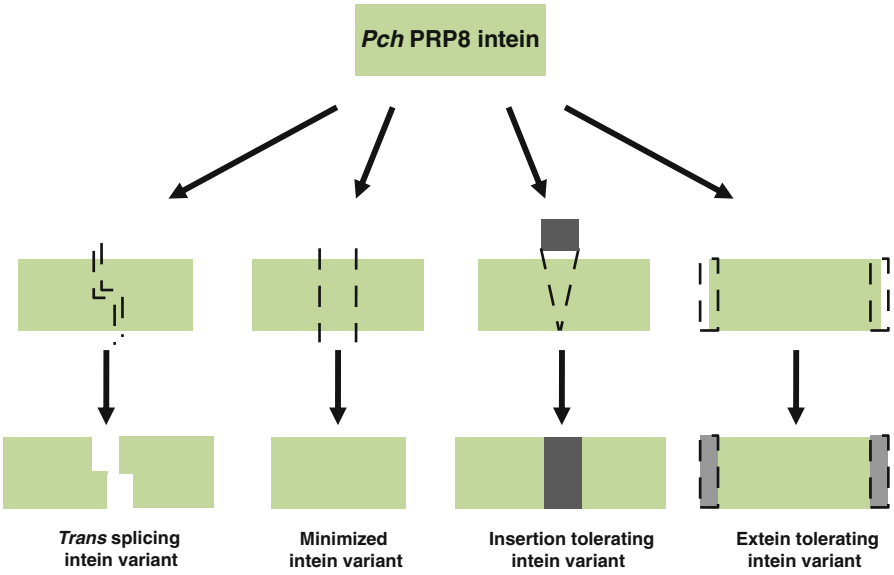

Fig. 5 Modifications of the *Pch* PRP8 intein for different molecular applications. The PRP8 intein is robust and tolerates splitting, amino acid deletions, insertions and linkage to foreign extein sequences

agents (Gangopadhyay et al. 2003). Since the PRP8 protein is a component of the essential spliceosomal machinery in eukaryotes, this intein might be an attractive drug target. Due to its destructive effect on harvested fruits, the filamentous fungus *P. expansum* is of economic importance, while some allelic PRP8 inteins, residing in human pathogens, are of medical significance (Liu and Yang 2004; Theodoro and Bagagli 2009).

The robustness and compactness of the *Pch* PRP8 intein has also been exploited to incorporate other biochemical activities into the intein without affecting its splicing ability, thereby generating a selectable marker with the property of self-excision (Ramsden et al. 2011). In this study, genetically selectable markers, such as imidazoleglycerol-phosphate dehydratase (HIS5), hygromycin B or aminoglycoside phosphotransferase and a transcriptional activator were inserted in place of the endonuclease domain of related large inteins. These inserted reporter proteins enabled the yeast *S. cerevisiae* to withstand toxic concentrations of hygromycin or to overcome histidine autotrophy. All intein constructs retained their splicing ability in *E. coli* and in *S. cerevisiae*, while tolerating insertions of up to 341 amino acids (Fig. 5). Moreover, the authors of this study coupled the HIS5-marked intein to GFP and integrated the construct into the yeast genome to fluorescently tag the calmodulin protein CMD1. Upon intein splicing, CMD1 was internally fused to GFP and the marked intein conferred histidine prototrophy to yeast. This method is advantageous over alternative approaches, because genomic integration and tagging occurs

in a single step, without the selective marker needs to be coupled to the tag and/or protein of interest (Ramsden et al. 2011).

Pch PRP8 intein has also been shown to tolerate several foreign extein sequences (Fig. 5). It has been used to generate peptides from functional and completely folded proteins to investigate the origin of peptides that are presented to the major histocompatibility complex class 1 and displayed at the cell surface (Farfán-Arribas et al. 2012). Moreover, the *Pch* PRP8 intein has been applied to incorporate the 21st amino acid selenocysteine (Sec) into proteins. Sec is encoded in various pro- and eukaryotic proteins by the UGA codon normally read as an opal stop codon (Driscoll and Copeland 2003). This special amino acid is incorporated in the so-called recording process, when the stem-loop structured selenocysteine insertion structure element (SECIS) follows the opal codon on the mRNA. In case of the mammalian thioredoxin reductase, the Sec introduction site is close to the C-terminus and the SECIS is found in the untranslated 3′-region of the mRNA. However, the incorporation of internal Sec into proteins is challenging and can be achieved by chemical conversion of a reactive serine for instance or by SECIS-mediated incorporation into a protein synthesized as two pieces followed by a protein ligation step. To circumvent this multi-step reaction approach, a SECIS element has recently been cached at the N-terminus of the *Pch* PRP8 protein. An UGA-codon preceding the intein sequence is translated to give Sec in a precursor protein including the translated intein during biosynthesis. Subsequently, the intein autocatalytically cleaves itself off the precursor and fuses the two exteins. The SECIS element is thereby excised together with the intein and a protein with a Sec incorporated at the target protein's inner part is produced. Using this invention a Sec amino acid can be incorporated at any position within a peptide chain (Arnér et al. 2009; Elleuche and Pöggeler 2010).

4 Applications in Fungi

Most of the intein applications such as intein-based purification methods of recombinant proteins, EPL, CPS and cyclization of peptides have been developed and established for non-fungal hosts (see above). However, in principle these applications can be adapted for fungi by construction of compatible expression plasmids, codon optimization of protein tags and bacterial inteins, or the utilization of fungal inteins. Recently, the site-specific *in vivo* protein cleavage by an intein-derived protease and the chemical functionalization of proteins through an intein-linked yeast display has been especially developed as intein-based applications for *S. cerevisiae*.

4.1 Intein-Derived Proteases for Site-Specific Protein Cleavage

Site-specific proteases such as the tobacco etch virus (TEV) protease are often used for the *in vitro* removal of affinity tags (Li et al. 2013), but can also be used *in vivo* to induce protein instability for the generation of conditional yeast mutants

(Jungbluth et al. 2010; Taxis and Knop 2012). An alternative intein-based cleavage system was recently developed by Volkmann et al. (2012). It utilizes the cyanobacterial *Ssp* DnaB S1 split-intein consisting of an 11 aa Int^N fragment and a 144 aa Int^C fragment (Sun et al. 2004). The short Int^N fragment was shown to serve as a recognition sequence for Int^C when fused between proteins. Through fragment complementation of *Ssp* DnaB S1 Int^N and Int^C, the fused proteins are cleaved before the Cys residue at position 1 of Int^N (Volkmann et al. 2009). By co-expressing a fusion protein consisting of the maltose binding protein, a 5 aa native N-extein sequence, Int^N and thioredoxin together with the His-tagged Int^C fragment under control of a galactose-inducible promoter, cleavage products corresponding to the expected sizes could be identified with specific antibodies. Thus, demonstrating that *Ssp* DnaB S1 Int^C can be used *in vivo* as a site-specific protease with a high degree of substrate specificity. Since the 11 aa recognition sequence originates from the center of the intein structure and has to form an anti-parallel β sheet to act as substrate for Int^C, cleavage of unintended proteins is almost impossible. In fact, negative effects on the cell viability of *S. cerevisiae* could not be determined after induction of Int^C expression (Volkmann et al. 2012).

4.2 Intein-Linked Yeast Surface Display

Yeast surface display is an efficient method for the directed evolution of proteins. The method identifies proteins from mutant libraries that bind a desired target protein with the highest affinity. First described by Boder and Wittrup (1997), the mutant protein is fused to the *S. cerevisiae* α-agglutinin Aga2p which is naturally anchored in the yeast cell wall by formation of disulfide bonds to the cell wall protein Aga1p. The localization at the cell surface allows the mutant protein to interact with its target in solution. Usually, the gene encoding the mutant protein is expressed from a yeast shuttle vector as an Aga2p fusion protein leading to the display of ~50,000 copies of the mutant protein on the surface of each cell (Gera et al. 2013). Screening of the library and the identification of the yeast clone that binds the target is achieved by magnetic sorting, Fluorescent Activated Cell Sorting (FACS) or other cell sorting techniques. Since its introduction, the yeast surface display has been used for a wide variety of protein engineering and characterization applications such as the mapping of functional protein epitopes, the identification of protein-protein interactions, as well as for the improvement of affinity, specificity and stability of proteins (Gai and Wittrup 2007; Gera et al. 2013).

Recently, Marshall et al. (2013) combined the intein-mediated EPL technique (see Sect. 2.1) with the yeast surface display to simultaneously release surface displayed proteins and conjugate them with chemical functionalities. Single chain antibodies (scFv) and GFP were used as model proteins and expressed as Aga2p fusion proteins. However, in contrast to the standard yeast surface display method, the proteins were fused to the N-terminus of Aga2p and separated by the modified *Mycobacterium xenopi Mxe* GyrA intein that undergoes nucleophile induced

N-terminal cleavage. Interestingly, the fusion of the intein had little or no impact on the expression or activity of the displayed proteins. After the spontaneous N–S acyl shift of the Cys at position 1 of the intein, the formed thioester is susceptible to nucleophilic attack. The release and functionalization was performed with two different nucleophiles, hydrazine azide and 2-mercaptoethanesulfonic acid (MESNA). While MESNA efficiently released the protein from the yeast surface and generated a thioester at the C-terminus of the released protein, hydrazine azide led to nonspecific reactions with other yeast proteins. The released protein thioesters were subsequently reacted with a cysteine alkene in an EPL reaction or immobilized on an azide-decorated surface after azide-alkyne cycloaddition. The intein-linked yeast surface-display provides thus a powerful tool to introduce numerous other useful chemical functionalities.

5 Conclusion

Various facets of the catalytic activity of *cis*- and *trans*-splicing inteins have been discussed in this article. Protein splicing can be exploited and applied in versatile approaches. A better understanding of the protein splicing mechanism and the discovery of new robust and fast splicing inteins or inteins with new biological functions will facilitate the applicability of intein-related technologies. For biotechnology applications in fungal hosts already established intein-based methods can be adapted to be suited to the fungal cell.

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Relevance of Signal Transduction Pathways for Efficient Gene Expression in Fungi

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1 Introduction

Efficient expression of performance genes is achieved by engineering of production organisms for optimized production. Basically, this can be regarded as an artificial adjustment of this organism to very specific requirements and conditions of growth. In nature, adjustment happens through evolution over millennia of optimization for dealing with challenging surroundings. The mechanisms fungi developed therefore are still operative and the regulatory pathways leading to gene regulation of transcription factors, their activation or modification represent an important, but often less studied resource for strain improvement.

Sensing its environment and adapting to the conditions is a crucial ability for every living organism (Bahn et al. 2007). Therefore, fungi possess a complex signal transduction network that allows them to sense and transmit a plethora of environmental cues inside the cell and to activate response pathways. Most cellular processes can be influenced by signals from outside including growth, sexual development, conidiation, production of enzymes and secondary metabolites. The natural environment of fungi provides different kinds and levels of nutrient sources like carbon, nitrogen, phosphor or sulphur sources and varying pH levels, along with pheromones, toxins and presents abiotic factors such as light and temperature, which are constantly monitored and interpreted for optimal adjustment.

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Several fungi are used for the production of enzymes, secondary metabolites and heterologous proteins. For industrial purposes high yields of these substances are crucial. Most signaling pathways influence gene expression of a great variety of targets, including hydrolytic enzymes, production of antibiotics as well as mycotoxins (Bahn et al. 2007; Brakhage 2013; Schmoll et al. 2014). Therefore it is important to consider the conditions under which production strains are grown—not only with respect to commonly surveyed parameters like carbon and nitrogen source, but also considering for example light pulses. Additionally, manipulating components of these pathways could increase production rates, if the mechanism of sensing and adapting to important cues is fully understood.

Signaling pathways do not act in an isolated manner. They transduce information on specific environmental cues, each one being tailored to an optimal response to a particular signal (Smith and Scott 2002). The combined and integrated output of different signaling pathways then results in a defined and optimized response to the environment—be it a tropical forest or a sterile steel fermentor.

One of the currently most important challenges in fungal research is the evaluation and exploitation of the potential of fungi to produce secondary metabolites, which may act as novel antibiotics. Therefore investigation of the regulation and activation of (silent) gene clusters is crucial (Yaegashi et al. 2014).

Similar to other secondary metabolites, the genes encoding the red pigment bikaverin (Wiemann et al. 2009) and gibberellin (Tudzynski 2005) are located in gene clusters. It has been shown that the gene sets encoding these secondary metabolites are regulated in response to different environmental signals such as light, nitrogen starvation or acidic pH (Avalos and Estrada 2010; Giordano and Domenech 1999; Rodriguez-Ortiz et al. 2009). The contribution of different signal transduction pathways transmitting the respective signals indicates a complex regulatory mechanism of secondary metabolite production in *Fusarium* sp. Additionally, encounter of other organisms in the environment also stimulates secondary metabolism and thus co-cultivation of fungal metabolite producers with such organisms represents a promising strategy for discovery of novel compounds (Brakhage and Schroeckh 2011).

Another challenge is the exploitation of fungi for their efficient enzyme systems, which can contribute to more energy efficient and less environmentally problematic chemical processes, and with their application for second generation biofuels fungal enzymes represent crucial factors for fighting climate change (Rubin 2008). Optimization of biotechnological processes to improve enzyme production has included diverse factors, most of which reflect an alteration of environmental conditions to mimic a situation where high amounts of enzymes are required for survival. Accordingly, both signal transduction pathways and their downstream output pathways have been modified (Bazafkan et al. 2014; Glass et al. 2013).

To sense a vast diversity of signals, many different receptors and signal transduction pathways have evolved, the relevance of which for gene expression systems we review in the following.

2 The Heterotrimeric G-Protein Pathway

Heterotrimeric G-protein signaling belongs to the crucial signaling pathways in eukaryotes and has been studied in detail in fungi (Li et al. 2007). Environmental signals from outside the fungal cell, like nutrient availability or pheromones are important for assignment of cellular resources to the appropriate processes. Such a process can be development, biosynthesis of secondary metabolites for protection or production of enzymes to enhance growth.

Therefore these external signals are sensed by G-protein coupled receptors (GPCR) and transmission of a signal is accomplished by heterotrimeric G-proteins (Neves et al. 2002). The so-called Regulators of G-protein signaling (RGS) influence signal transmission by this pathway (Yu 2006). Usually, the cAMP pathway is modulated as a consequence of sensing by the heterotrimeric G-protein pathway and regulates downstream processes (Lafon et al. 2006). The GPCRs are a very large family of transmembrane receptors that exhibit a great diversity in function as well as sequence. Several classes identified in mammals are also found in fungi (Krishnan et al. 2012). Their common feature is their structure that includes seven transmembrane domains and an interaction site with the heterotrimeric G-proteins. The heterotrimeric G-proteins consist of three subunits: Gα, Gβ and Gγ. Upon binding of a ligand to a GPCR this receptor is activated, leading to a conformational change which promotes the exchange of GDP with GTP in the α subunit of the associated heterotrimeric G-protein (Li et al. 2007). This leads to dissociation of the Gα and the Gβγ subunit. Both subunits can now activate downstream targets of the signaling pathway (Neves et al. 2002).

In *Trichoderma* species phylogenetic analyses revealed 14 classes of GPCRs (Gruber et al. 2013), whereas in *Aspergillus* species several putative GPCRS were identified that belong to nine different classes (Lafon et al. 2006).

Given their numbers in fungal genomes, GPCRs can sense and transmit a variety of signals, but only few ligands of GPCRs are currently known in fungi, including amino acids (Xue et al. 2006), glucose (Li and Borkovich 2006) or both (Brown et al. 2015) and oxylipins, which are involved in mycotoxin production and quorum sensing (Affeldt et al. 2012). Considering GPCRs as crucial targets for development of novel antifungals to fight the increasing threat by mycoses of immunocompromised patients, knowledge on the identity of their ligands is of utmost importance (Van Dijck 2009). In contrast, diverse cellular functions are known for GPCRs, which make them important factors in regulators of biotechnological processes. In *A. nidulans* GPCRs were found that are involved in the regulation of genes related mainly to secondary metabolism, sexual development, stress signaling, and amino acid metabolism (de Souza et al. 2013; Lafon et al. 2006). Interestingly, in this fungus, a metabolic shift upon deletion of GPCRs was observed and the lack of these GPCRs triggered stress responses, hence indicating a fine tuning mechanism of metabolic responses for adjustment to changing environmental conditions (de Souza et al. 2013). In *A. fumigatus* transcriptome analysis of deletions of the two putative G-protein-coupled receptors GprC and GprD revealed that in both mutants genes

involved in primary metabolism were down regulated, whereas transcript levels of several secondary metabolism gene clusters were elevated (Gehrke et al. 2010).

In *Neurospora crassa* the GPCR GPR-4 was found to be involved in carbon sensing. It interacts with the Gα subunit GNA-1 and upon ligand binding activates the cAMP signaling pathway (Li and Borkovich 2006). Interestingly, GPR-4 has no direct homologue in *T. reesei*, but synteny analysis of the corresponding locus revealed a deletion (Schmoll 2008). Nevertheless, it cannot be excluded that another GPCR has assumed a similar function in *T. reesei*.

A recent, comprehensive study of GPCRs in *Aspergillus flavus* revealed diverse functions in regulation of growth, development and response to nutrient sources in this fungus, which interestingly, did not correspond to classification of GPCRs (Affeldt et al. 2014). Consequently, it will be difficult to predict functions of novel GPCRs according to their structural classification.

Downstream of the GPCRs, G-protein α, β and γ subunits transmit the signal further. Most filamentous fungi possess three G-protein α subunits and a single Gβ and one Gγ subunit (Lafon et al. 2006; Li et al. 2007). In *N. crassa*, the function of Gα subunits has mainly been studied with respect to development and they have a profound influence on growth and sexual development, in particular female fertility (Won et al. 2012). Interestingly, so far no phenotype or function was found for GNA-2 and its homologues in fungi.

In *Aspergillus nidulans* known components of the heterotrimeric G-protein pathway are the three Gα subunits FadA, GanA and GanB (Chang et al. 2004; Yu et al. 1996), the Gβ subunit SfaD (Rosen et al. 1999) and the Gγ subunit GpgA (Lafon et al. 2006; Seo et al. 2005). Besides functions in development (Chang et al. 2004; Seo et al. 2005), also a role in triggering autolysis (Molnar et al. 2006) and sterigmatocystin biosynthesis (Shimizu and Keller 2001) was found for *A. nidulans*. With respect to enzyme production, mutant strains lacking *fadA*, *sfaD*, *gpgA* or *sfgA*, the regulator of FadA signaling, show elevated proteinase production after glucose depletion suggesting that FadA/SfaD/GpgA G-protein signaling inhibits proteinase production (Emri et al. 2008). Deletion of *ganB* and *rgsA*, the RGS-type regulator of GanB, resulted in decreased proteinase production (Molnar et al. 2006). Also functions in secondary metabolism are known for G-proteins in *Aspergilli* (Amare and Keller 2014). A function of G-protein subunits is also supported by the findings of regulation of toxin production and virulence in *Gibberella zeae* (Yu et al. 2008).

In *T. reesei*, heterotrimeric G-protein signaling was studied for its influence on enzyme production. The genome of *T. reesei* comprises three Gα subunits, GNA1, GNA2 and GNA3, one Gβ subunit, GNB1 and one Gγ subunit, GNG1 (Schmoll 2008). GNA1 positively influences cellulase transcript levels in light and negatively in darkness, while GNA3 exerts its positive influence only in light. In both cases, the influences reach a roughly tenfold increase. Nevertheless, constitutive activation of these Gα subunits did not abolish the requirement for an inducer, suggesting that they do transmit a signal crucial for enzyme production, but which is unlikely to be the presence of cellulose (Schmoll et al. 2009; Seibel et al. 2009). A further hint as to a broader function of GNA1 and GNA3 is the finding that both show a transcriptional feedback upon activation (Tisch et al. 2011a), which was shown to be carbon

source dependent for GNA1 (Seibel et al. 2009). In *T. reesei* also the Gβ and Gγ subunits GNB1 and GNG1 influence cellulase gene expression, albeit to a lesser extent than the Gα subunits (Tisch et al. 2011b).

In *Trichoderma atroviride* the heterotrimeric G-proteins are involved in regulation of production of chitinases and antifungal metabolites: In mutants lacking the Gα subunit Tga1 levels of chitinase were decreased. Also production of the major antifungal metabolite of *T. atroviride*, 6-pentyl-*α*-pyrone was reduced (Reithner et al. 2005), while amounts of peptaibols were elevated (Stoppacher et al. 2007). In Δ*tga3* mutants levels of extracellular chitinases were reduced as well, although transcript levels of the chitinase-encoding genes *nag1* and *ech42* were elevated. Studies revealed that in the absence of Tga3 chitinases are retained inside the cell, suggesting a role of Tga3 in chitinase transcription as well as secretion (Zeilinger et al. 2005). Silencing of the GPCR *gpr1* led to the inability of *T. atroviride* to react to the presence of host fungi with the expression of chitinases and proteases. Although the defect is similar to the one observed in mutants lacking *tga3*, no direct interaction between Tga3 and Gpr1 was observed and the ligand sensed during this process is not yet known (Omann et al. 2012).

In *T. atroviride*, a clear connection of the heterotrimeric G-protein pathway with light response was detected and in contrast to *T. reesei* with respect to secondary metabolism. Formation of atroviridin in *T. atroviride* is dependent on the presence of GNA3 (Komon-Zelazowska et al. 2007).

In summary, the heterotrimeric G-protein pathway can serve as an important target for strain improvement with biotechnological processes aimed at production of enzymes and secondary metabolites alike. As this pathway also significantly influences growth and development, undesired side effects of molecular alterations in terms of growth defects, loss of fertility or altered mycotoxin production should be considered.

3 cAMP Signaling

One major target of signaling pathways including the heterotrimeric G-protein pathway is the cAMP pathway with the secondary messenger cyclic AMP (cAMP) as core compound. cAMP is synthesized by adenylate cyclase and degraded by phosphodiesterase, hence forming a mechanism for fine tuning intracellular levels of cAMP. cAMP dependent protein kinase A is activated by cAMP and acts on downstream targets to modify transcriptional output (D'Souza and Heitman 2001; Gancedo 2013). Thereby, phosphodiesterase and protein kinase A act in a negative feedback loop (Hicks et al. 2005; Wang et al. 2005). Moreover, activation of PKA results in decreased cAMP levels through down regulation of adenylate cyclase (Vandamme et al. 2012). These intricate mechanisms for adjustment are in accordance with a function of adenylate cyclase and probably the whole cAMP pathway as coincidence detector to integrate diverse environmental signals as suggested previously (Hogan and Muhlschlegel 2011).

The cAMP pathway is involved in many processes in fungi such as differentiation, development, virulence, cell cycle progression and stress response. Moreover, this pathway is involved nutrient sensing.

cAMP signaling is involved in the regulation of secondary metabolites in several fungi (Yu and Keller 2005) and its evolution is assumed to have contributed to fungal divergence and niche adaptation (Guo et al. 2015). Species of *Fusarium* are important producers of secondary metabolites with applications in industry such as gibberellins, carotenoids or bikaverin (Avalos et al. 2007). In *Fusarium fujikuroi* adenylate cyclase is important for gibberellin production and pigmentation (Garcia-Martinez et al. 2012). In the fungal pathogen *Fusarium verticillioides* the cAMP signaling pathway is not only important for conidiation, plant infection and stress responses, but also for the production of bikaverin. However, the finding that fumonisin was produced at normal levels in mutants with defects in the cAMP pathway, shows also alternative routes of regulation (Choi and Xu 2010).

In *A. fumigatus*, a serious human pathogen, the cAMP dependent protein kinase A network is involved in the regulation of development and virulence (Liebmann et al. 2004). In the model fungus *A. nidulans*, AflR, an important regulator of secondary metabolism is regulated by protein kinase A at the transcriptional level as well as by posttranslational modification (Shimizu et al. 2003). As chromatin remodelling is also targeted by the cAMP pathway in *Fusarium* spp. (Guo et al. 2015), this pathway can be assumed to play a role in regulation of accessibility and hence activity of secondary metabolite gene clusters (Gacek and Strauss 2012).

In *Trichoderma* spp. the cAMP pathway was found to regulate growth, germination, mycoparasitism, carbon deprivation induced conidiation and secondary metabolism (Casas-Flores et al. 2006; Mukherjee et al. 2007; Schuster et al. 2012b). A concentration dependent influence of extracellularly added cAMP on cellulase production was reported already decades ago and was only observed in the presence of inducing compounds (Sestak and Farkas 1993). Investigation of the role of adenylate cyclase and protein kinase A in cellulase regulation in *T. reesei* showed that the cAMP pathway indeed has a distinct effect on cellulase transcript levels in light and darkness. Thereby, protein kinase A impacts light dependent complex formation in the *cbh2* promotor and is assumed to act on a regulator upstream of the cellulase transcription factor XYR1 (Schuster et al. 2012b).

In *Trichoderma virens* and *T. reesei* an interesting effect on the growth rate was observed in mutants lacking adenylate cyclase: growth of these mutants was severely retarded on plates while shake flask cultures showed relatively normal growth (Mukherjee et al. 2007; Schuster et al. 2012b).

Glycogen is an important storage carbohydrate and regulation of its intracellular levels is crucial for proper response to environmental conditions in terms of glycolytic flux, stress, starvation and fitness of an organism in general (Francois and Parrou 2001). Various signaling pathways contribute to regulation of glycogen levels with the cAMP pathway playing a major role (Wilson et al. 2010). In *N. crassa* the cAMP signaling pathway controls glycogen metabolism by regulating the glycogen synthase gene expression and phosphorylation (Freitas et al. 2010). As targets of the cAMP pathway in this respect, 17 transcription factors, which influence

glycogen levels in *N. crassa* come into consideration. Besides several previously uncharacterized regulators, also PacC, XlnR and NIT2, which are well known regulators of pH response, enzyme expression or nitrogen metabolism, were among these glycogen influencing transcription factors (Goncalves et al. 2011). Involvement of these transcription factors in regulation of glycogen metabolism suggests a complex mechanism for integrating nutritional and stress status for triggering an appropriate response with the cAMP pathway as central mediator. Accordingly, some of these transcription factors are targets of adenylate cyclase upon growth on cellulose in *T. reesei* (Tisch et al. 2014). The gene encoding the XlnR homologue, *xyr1*, is regulated by protein kinase A and adenylate cyclase also on lactose (Schuster et al. 2012b) and was found among the genes regulated by the photoreceptor ENV1 via the cAMP pathway (Tisch et al. 2014).

4 Kinases and Phosphatases

Phosphorylation is one of the key processes in transmitting extracellular signals and can activate or deactivate a function of a protein, alter its interaction properties (Mok et al. 2011; Schenk and Snaar-Jagalska 1999) or destine it for degradation (Nguyen et al. 2013).

In general, kinases and phosphatases are not frequently used as targets for strain improvement, although interesting results for example for enhanced cellulase production for the two kinases SCH9 and YAK1 in *T. reesei* (Lv et al. 2015) or a role in nutrient signaling for protein phosphatase 2A in yeast (Zabrocki et al. 2002) as well as diverse functions of protein kinases and phosphatases as detailed below are known.

In recent years, high throughput knock out procedures have been developed and adapted (for example Colot et al. 2006; Schuster et al. 2012a) that enabled communities to create whole genome knock out libraries. *N. crassa* is now the first filamentous fungus for which such a whole genome knock out library is available, for others, deletion sets for certain gene groups have been created.

Genome wide analysis of protein phosphatases in *N. crassa* revealed diverse phenotypes in development, growth or chemical sensitivity in all the mutants studied as well as a role in phosphorylation of p38 mitogen-activated protein kinase (MAPK) (Ghosh et al. 2014). A similar analysis of *N. crassa* protein kinases showed again various phenotypes in the same traits and that NCU02245 is necessary for chemotropy of mating partners. Additionally, NCU00406 (a p21 activated kinase) was found to be allelic with *velvet* (*vel1*) (Park et al. 2011), a regulator of development, secondary metabolism and cellulase gene expression (Bayram and Braus 2012; Karimi Aghcheh et al. 2014).

In *A. nidulans*, global analysis of protein phosphatases was performed with respect to carbon sensing and utilization and besides functions comparable to those in *N. crassa*, also a role in metabolic changes in response to glucose and alternative carbon sources as well as regulation of primary metabolism (de Assis et al. 2015).

Screening of protein kinases and phosphatases for hydrolytic enzyme production revealed the kinases SchA and SnfA as positive regulators of cellulose induced gene regulation in *A. nidulans* (Brown et al. 2013). In the pathogen *A. fumigatus*, protein phosphatases are important for iron assimilation as well as gliotoxin production and resistance (Winkelstroter et al. 2015).

4.1 MAP Kinases

The mitogen activated protein kinase (MAP kinase) pathway is one of the best studied signal transduction pathways conserved from yeast to humans (Widmann et al. 1999) and in fungi usually involves three to five cascades of three kinases: a MAP kinase kinase kinase (MAPKKK or MEKK), a MAP kinase kinase (MAPKK or MEK) and a MAP kinase (MAPK) (Furukawa and Hohmann 2013; Gustin et al. 1998; Turra et al. 2014). These kinases pass on a signal from an upstream sensor by being sequentially activated by a phosphorylation signal. The final activated MAPK then phosphorylates downstream components of the pathway, such as transcription factors (Chen and Thorner 2007). Additionally, regulation of MAPK signaling occurs via posttranslational modification and feedback cycles (Molina et al. 2010). As part of the signaling machinery, MAP kinase cascades show crosstalk with each other (Rodriguez-Pena et al. 2010; Saito 2010), which is regulated by phosphatases (Junttila et al. 2008) and with other signaling pathways such as the cAMP pathway (Gerits et al. 2008), the G-protein pathway (Cervantes et al. 2010) or others (Wang et al. 2011).

In filamentous fungi three major classes of MAPKs have been identified (Turra et al. 2014). They are homologs of the *Saccharomyces cerevisiae* MAPKs Hog1p, Slt2p and Fus3p (Gustin et al. 1998). They are involved in a variety functions, including protein production, conidiation, fruiting body development, circadian rhythm, stress response, polarized growth and cell wall integrity (de Paula et al. 2008; Ghosh et al. 2014; Turra et al. 2014; Widmann et al. 1999).

In *T. reesei* three putative MAP kinase cascades could be identified, involving three putative MAP kinases: TMK1, that is a homolog of *S. cerevisiae* Fus3p, TMK2, a homolog of *Sc*Slt2p and TMK3, a homolog of *Sc*Hog1p (Schmoll 2008). TMK2 and TMK3 are both involved in regulation of cell wall integrity and cellulase production. TMK2 has a negative effect on cellulase production, but interestingly, this effect is not reflected in transcript levels of cellulase genes or their regulators (Wang et al. 2014). Hence, TMK2 is likely involved in a posttranscriptional mechanism that acts on cellulase translation, secretion or stability. In contrast, TMK3 promotes cellulase production on a transcriptional level (Wang et al. 2013). Moreover, also an Ime2-like MAP kinase was found to negatively regulate cellulase gene expression in *T. reesei* despite positive regulation of *cre1* and *xyr1* transcript levels (Chen et al. 2015).

In *T. virens* the role of the *Sc*Fus3 homolog is unclear. Deletion of *tmkA* was reported to result in decreased cellulase and chitinase production compared to the

wildtype (Mukherjee et al. 2003). In contrast, deletion of *tvk1*, which corresponds to *tmkA* in a different *T. virens* strain led to increased chitinase and protease activities. Also glucanase activity was increased suggesting negative modulation of glucanase gene expression by Tvk1 (Mendoza-Mendoza et al. 2003). In accordance with these results *T. atroviride* Tmk1 negatively influences production of chitinases (Reithner et al. 2007).

In *N. crassa mik-1*, *mek-1* and *mak-1* encode MAPK homologues of the *S. cerevisiae* Slt2p pathway (Borkovich et al. 2004). They are required for vegetative growth and female sexual development. Furthermore, they were found to be involved in secondary metabolism as negative regulators of tyrosinase expression, which is required for required for l-DOPA melanin (eumelanin) biosynthesis (Park et al. 2008). *N. crassa* MAK-2 also contributes to regulation of secondary metabolism by impacting the isoprenylation pathway and participates in the phosphate signaling pathway (Gras et al. 2013). MAP kinases were also found to impact carbon and secondary metabolism in *Podospora anserina* (Bidard et al. 2012).

In *A. nidulans* deletion of the *Sc*Fus3 homolog *mpkB* leads to increased expression of the extracellular chitinase *chiB*, the β-1,3-endoglucanase *engA* and the proteinase *pepJ*, suggesting involvement of MpkB in regulation of hydrolytic enzyme expression (Kang et al. 2013).

Other functions of MAP kinases include the regulation of secondary metabolism in *A. nidulans* (Bayram et al. 2012) and *A. niger* (Priegnitz et al. 2015). The Fus3 MAPK pathway in *A. nidulans* controls not only sexual development and expression of hydrolytic enzymes, but also secondary metabolism. The *An*Fus3 MAP kinase signaling pathway consists of the MAP kinase MpkB (*An*Fus3), homolog of yeast Fus3, the upstream MAPKK MkkB (*An*Ste7) and MAPKKK SteC (*An*Ste11), homolog of yeast Ste11 and the adaptor SteD (*An*Ste50), homolog of yeast Ste50. Deletion of one of the components of this pathway led to reduced levels of the mycotoxin sterigmatocystin (Atoui et al. 2008; Bayram et al. 2012).

4.2 Casein Kinases

Casein kinase represent a further important group of kinases, that are best characterized in filamentous fungi with respect to their function in light response (Schafmeier and Diernfellner 2011). Casein kinase 2 was actually originally identified as being involved in carbohydrate metabolism (Al Quobaili and Montenarh 2012). Casein kinases 1 are expressed and active constitutively, but their targets are determined predominantly by other kinases and phosphatases. As a phosphate residue is required at the recognition site, other kinases or phosphatases can activate or deactivate a CK1 recognition site (Flotow et al. 1990; Gross and Anderson 1998). For the role of CK1 and CK2 in the circadian clock of *N. crassa*, cAMP dependent protein kinase A acts as a priming kinase (Huang et al. 2007).

In *T. reesei*, the casein kinase CKIIalpha2 was shown to be involved in regulation of glucose metabolism and chitinase expression (Wang et al. 2015). Casein kinase 2

regulatory subunits of *Penicillium oxalicum* positively influence transcript levels of cellulases and the three related transcription factors ClrB, XlnR and AmyR (Lei et al. 2014).

5 The Light Response Pathway

Light plays an important role in the life of most organisms as they adjust to the environmental changes in day and night (Rodriguez-Romero et al. 2010; Schmoll 2011). Even though fungi do not use light as a source of energy, they adapt to it by adjusting virtually all their metabolic processes including carbohydrate metabolism, fatty acid metabolism, carotenoid metabolism, sulfur metabolism and cAMP levels (Tisch and Schmoll 2010). Moreover, analysis of circadian rhythmicity of gene expression revealed that catabolic and anabolic processes are coordinated with dusk and dawn also in fungi (Sancar et al. 2015b) as well as a high potential for posttranscriptional regulation of metabolic processes (Hurley et al. 2014). Even in *S. cerevisiae*, which was believed to show no light response whatsoever, alterations in metabolic rhythms by visible light were shown which are attributed to unexpectedly photosensitive pathways (Robertson et al. 2013).

Most characterized photoreceptors in fungi react to blue light, although also red light responses are known (Idnurm and Heitman 2005; Idnurm et al. 2010). The best studied components of the light signaling machinery are the homologues of the *N. crassa* photoreceptors white collar-1 (WC-1) and white collar-2 (WC-2), which are both GATA-type transcription factors and can act as a complex or individually (for a review see Fuller et al. 2015). The third blue light photoreceptor in *N. crassa* is VVD, which acts as a universal break for light responses and adjusts gene regulation to different light intensities (Chen et al. 2009; Malzahn et al. 2010).

Analysis of light responses in *Trichoderma* spp. has a long tradition (Schmoll et al. 2010) and an influence of light on enzyme activities has been detected early (Nemcovie and Farkas 2001). In *T. reesei* light increases cellulase transcript levels and the blue light receptors BLR1 and BLR2 as well as the VVD ortholog ENV1 are involved in cellulase regulation (Castellanos et al. 2010; Schmoll et al. 2005). In agreement with this finding, complex formation within the *cbh2* promotor is altered in light (Schuster et al. 2012b). Moreover, cellulase production is modulated by BLR1, BLR2 and ENV1 (Gyalai-Korpos et al. 2010). Light dependent regulation of cellulase transcription and enzyme production was also found to occur in industrial production strains (unpublished results) and hence renders this pathway and its targets an interesting avenue for strategies in strain improvement.

Genome wide analysis of the targets of these photoreceptors revealed that particularly carbon metabolism is a target of light response in *T. reesei* (Tisch and Schmoll 2013) and this function is conserved in *N. crassa* (Schmoll et al. 2012). In *T. reesei* roughly 75 % of all genes encoding glycoside hydrolases are influenced by light in wild-type or mutant strains (Tisch et al. 2011b; Tisch and Schmoll 2013). Investigation of a potential link between light response and nutrient signaling

showed that the functions of the G-protein α subunits GNA1 and especially GNA3 are dependent on the light status (Schmoll et al. 2009; Seibel et al. 2009) and regulated by ENV1 (Tisch et al. 2011a). Thereby, ENV1 exerts part of its function by regulation of cAMP levels, which is assumed to be achieved through modulation of phosphodiesterase activity (Tisch et al. 2014). In turn, cAMP levels are known to be altered in light (Gresik et al. 1988), which in part may be attributed to light dependent activation of adenylate cyclase (Kolarova et al. 1992). Accordingly, regulation of cellulase gene expression including regulation of *xyr1* by adenylate cyclase and protein kinase A is dependent on the light status as well (Schuster et al. 2012b).

Interestingly, crosstalk between carbon metabolism and light response also impacts secondary metabolism: Sterigmatocystin biosynthesis levels in light and darkness were influenced in opposite ways on different concentrations of carbon source (Atoui et al. 2010). Also in the human pathogen *A. fumigatus*, growth, metabolism and stress response are subject to regulation by light (Fuller et al. 2013). Light further influences chromatin remodeling via the blue light photoreceptors (Sancar et al. 2015a), which is in accordance with a light dependent modulation of secondary metabolism (Brakhage 2013; Fischer 2008).

With respect to secondary metabolism, *N. crassa* VVD and its homologue in *F. fujikuroi* were found to influence carotenoid levels, albeit in opposite directions (Castrillo and Avalos 2014; Shrode et al. 2001). This difference may be due to the evolutionary conservation of a cystein at position 96 in *T. reesei* and Hypocreales including *F. fujikuroi*, which integrates response to oxidative stress and which differs in *N. crassa* and other fungi (Lokhandwala et al. 2015). In *T. atroviride* formation of atroviridin is regulated by light and photoreceptors as well as the Gα subunit GNA3 (Komon-Zelazowska et al. 2007). Light dependent gene expression was also observed for *Penicillium chrysogenum* with the bZIP transcription factor PcAtfA as regulator involved in this response (Wolfers et al. 2015). In *Alternaria alternata*, production of altertoxin (ATX) is strictly dependent on light and regulated by the photoreceptor LreA (Pruss et al. 2014).

Besides the blue light photoreceptors, also the Velvet family proteins and particularly VeA (Bayram and Braus 2012) are important factors in light dependent signal transduction. These proteins and their homologues play crucial roles in development and secondary metabolism (Amare and Keller 2014) in many fungi including *A. nidulans* (Bayram et al. 2008), *A. flavus* (Chang et al. 2013), *A. fumigatus* (Park et al. 2012b), *P. chrysogenum* (Kopke et al. 2013), *T. virens* (Mukherjee and Kenerley 2010) and *T. reesei* (Bazafkan et al. 2015). Interestingly, in *A. fumigatus*, in contrast to *A. nidulans*, gliotoxin production is not affected by VeA (Park et al. 2012a). In general, these studies showed that the effect of Velvet proteins on secondary metabolism is distinct for every component studied and can be positive or negative and might vary from species to species. The function of VEL1 in regulation of enzyme production has so far only been studied in *T. reesei*. In this fungus, VEL1 was found to be essential for cellulase gene expression on cellulose (Bazafkan et al. 2015) as well as on lactose and sophorose (Karimi Aghcheh et al. 2014).

A further signaling mechanism involved in light response is the COP9 signalosome (He et al. 2005; Zhou et al. 2012), which acts at the interface between signal

transduction, determination of protein fate and development (Braus et al. 2010). In *A. nidulans*, the deneddylase subunit of the COP9 signalosome, CsnE, was shown to be important for silencing of a secondary metabolite gene cluster and consequently, the COP9 signalosome can be a further target to be modified for discovery and enhanced production of novel antibiotics (Gerke et al. 2012).

6 pH Signaling

Most fungi prefer acidic environments as their habitats although they can tolerate a broad range of pH conditions (Arst and Penalva 2003). The first pH sensing system in fungi was identified in *A. nidulans*. It consists of palA, palB, palC, palF, palH and palI, components of the pH signal transduction pathway and the zinc finger transcription factor pacC (Denison 2000; Selvig and Alspaugh 2011).

Sensing of extracellular pH involves the plasma membrane complex including the seven transmembrane receptor PalH, which likely acts as pH sensor, the four transmembrane helper protein PalI and the positive-acting arrestin-like protein PalF (Herranz et al. 2005). PalF assists in localizing PalH to the plasma membrane. Upon exposure to alkaline pH PalF is phosphorylated and ubiquitinated in a palH dependent manner, resulting in activation of the pH signaling pathway (Hervas-Aguilar et al. 2010; Penalva et al. 2008). The transcription factor associated with this pathway, PacC is activated by proteolytic cleavage under alkaline or neutral conditions (Selvig and Alspaugh 2011).

Growth at different pH values is associated with significant alterations in gene regulation and influences metabolic pathways: In *A. nidulans*, xylanase expression is differentially regulated by pH and by PacC (MacCabe et al. 1998). Interestingly, in *T. virens*, PacC is important for biocontrol competence and more than 650 genes were regulated in response to pH, which represent functions in secondary metabolism and carbohydrate transport and metabolism among others (Trushina et al. 2013). In *T. reesei*, cellulase production is sensitive to pH and different buffer compositions (Juhasz et al. 2004), with optimal yields achieved around pH 2.8, which however may even inactivate the produced enzymes (Bazafkan et al. 2014; Mandels and Andreotti 1978). Nevertheless, sugar uptake is optimal at pH 5 (Sternberg and Mandels 1979). The *T. reesei* PacC homologue PAC1 turned out to be a negative regulator of cellulase gene expression as well as transcript levels of the transcription factor genes *xyr1* and *ace2* at neutral pH (He et al. 2014). In agreement with earlier findings, transcriptome analysis revealed several cellulase and hemicellulase encoding genes to be pH responsive, both positive and negative, but only few of them showed direct PAC1-mediated regulation (Hakkinen et al. 2015). In *N. crassa*, the pH signaling pathway and in particular PacC is involved in regulation of glycogen synthase and hence in glycogen accumulation (Cupertino et al. 2012).

Besides enzyme production, also production of secondary metabolites is responsive to different pH levels in fungi (Brakhage 2013; Penalva and Arst, 2002; Penalva et al. 2008). As efficient pH signaling and response is crucial for many functions

associated with virulence and pathogenicity, the pH response pathway was also suggested as a target for antifungal strategies (Cornet and Gaillardin 2014).

In *Aspergilli,* and other fungi production of antibiotics and toxins was determined to be modified by pH conditions. For example, the pH regulatory system controls transcription of *ipnA*, the gene encoding isopenicillin N synthetase (Espeso et al. 1993). Regulation by pH overrides carbon regulation of *ipnA* transcript levels. Growth under alkaline conditions, or mimicking these, can substantially increase penicillin production (Espeso and Penalva 1996; Espeso et al. 1993; Shah et al. 1991). A further model secondary metabolite, sterigmatocystin, is also regulated by pH in *A. nidulans* (Delgado-Virgen and Guzman-de-Pena 2009).

In the cereal pathogen *Fusarium graminearum* the mycotoxin trichothecene is only produced under acidic pH. *Tri* gene expression and toxin production is negatively regulated by the transcription factor Pac1 (Merhej et al. 2011). In *F. verticillioides* fumonisin biosynthesis is repressed by nitrogen as well as by alkaline pH. PAC1, which is required for growth under alkaline conditions, may act as a repressor of fumonisin biosynthesis (Flaherty et al. 2003).

7 Specific Aspects of Nitrogen Signaling

Alteration of nitrogen source and availability is a traditional approach to modify and improve production of secondary metabolites in fungi (for an overview see Tudzynski 2014). The central regulator of nitrogen metabolism, AreA is subject to regulation by multiple signaling pathways reporting quality and quantity of the nitrogen source in the environment (Caddick et al. 2006).

In filamentous fungi, nitrogen metabolite repression (NMR) is regulated by GATA-type transcription factors AreA and AreB. In *A. nidulans* expression of the transcription factor *areA* is modulated by the quality and availability of nitrogen sources (Morozov et al. 2001). AreA mainly acts as positive regulator (Michielse et al. 2014) and is required for expression of nitrogen-catabolic permeases and enzymes. Under nitrogen sufficient conditions AreA activity is low, whereas it increases under nitrogen limiting or starvation conditions. Its activity is regulated by several mechanisms that include transcriptional control, transcript stability and nuclear localization (Langdon et al. 1995; Morozov et al. 2000, 2001; Platt et al. 1996; Todd et al. 2005). The key determinant for AreA activity seems to be the intracellular level of glutamine (Margelis et al. 2001). The AreA ortholog in *N. crassa* is the positively acting GATA-factor Nit2 (Wong et al. 2008). The negative-acting *nmr1* regulatory gene appears to play a role in nitrogen catabolite repression (Young and Marzluf 1991).

AreA and AreB orthologs have been discovered in many other ascomycetes (Wong et al. 2008). In *Penicillium marneffei* AreA is necessary for utilization of non-preferred nitrogen sources (Bugeja et al. 2012). In *F. fujikuroi* three isoforms of AreB have been found that exhibit different subcellular localization under nitrogen repression indicating distinct functions. Under low nitrogen two of the AreB

isoforms colocalize with AreA in the nucleus. However, under high nitrogen conditions this localization is lost (Michielse et al. 2014).

In *A. nidulans* nitrogen availability and the presence of glutamine represent signals for regulation of AreA mediated gene regulation, which is adjusted by regulated mRNA stability and decay (Caddick et al. 2006; Morozov et al. 2001). The Ccr4-Caf1-Not complex and further factors involved in regulating transcript stability was shown as an important level of signal integration influenced by environmental factors (Morozov et al. 2010).

In *S. cerevisiae*, the TOR (target of rapamycin) signaling pathway is involved in nitrogen signaling (Beck and Hall 1999; Cardenas et al. 1999). However, analysis of this pathway in *A. nidulans* showed that rapamycin/sirolomus does not override nitrogen metabolite signaling. Nevertheless, a minor role in dependent gene regulation, particularly through *jipA* and *fprA* was observed (Fitzgibbon et al. 2005). Accordingly, partial derepression of some AreA target genes occurred upon rapamycin treatment in *F. fujikuroi* (Teichert et al. 2006).

Besides TOR signaling, also the MAPK pathway and in particular the HOG (high osmolarity glycerol) cascade is involved in nitrogen dependent gene regulation of secondary metabolism in *Fusarium proliferatum* (Kohut et al. 2009) and *A. fumigatus* (May et al. 2005). Moreover, a regulatory role of the G-protein α subunit FfG1 in regulation of secondary metabolism under different nitrogen availability conditions was shown (Studt et al. 2013). The velvet family protein VeA was found to be involved in regulation of growth on nitrate and a combinatorial role with AreA was suggested in *Fusarium oxysporum* (Lopez-Berges et al. 2014).

Although the relevance of the nitrogen source for fungal physiology and metabolism is best studied with respect to secondary metabolism, also an influence of the nitrogen source as well as the regulator AreA on cellulase regulation was detected (Lockington et al. 2002). Consequently, it can be expected that signal transduction pathways transferring nitrogen and carbon related signals are connected.

8 Specific Aspects of Carbon Signaling

Fungi are able to grow on wide variety of nutrient sources. In several fungi putative carbon-sensing GPCRs have been identified (Xue et al. 2008). In *S. cerevisiae* the G-protein coupled receptor Gpr1p is activated by glucose and mannose (Lorenz et al. 2000), with mannose acting as an antagonist (Lemaire et al. 2004). The signal is transmitted via a pathway involving the G-protein α subunit Gpa2p resulting in activation of adenylate cyclase and production of cAMP (Kraakman et al. 1999; Lorenz et al. 2000). Homologues of *Sc*Gpr1p include *Schizosaccharomyces pombe* Git3 (Welton and Hoffman 2000), *Candida albicans* Gpr1 (Miwa et al. 2004), and *N. crassa* GPR-4 (Li and Borkovich 2006). In *N. crassa* GPR-4 acts upstream of the heterotrimeric G-protein α subunit GNA-1. GPR-4 is implicated in glucose sensing as is required for the glucose-dependent transient increase in cAMP levels (Li and Borkovich 2006). Interestingly, in *T. reesei* the respective syntenic genomic locus

shows a gap (Schmoll 2008) and although GPCRs with similarity to GPR-4 were found, it remains to be shown whether one of them assumed the function of GPR-4. In *A. fumigatus* two GPCRs, GprC and GprD, similar to *Sc*Gpr1p were found. However, analysis of deletion strains of *gprC* and *gprD* indicates that GprC and GprD are not involved in glucose sensing (Gehrke et al. 2010).

Even though the function of these receptors may vary, homologues of fungal Gpr1 were found in many species and they can be assigned to three different classes (Van Dijck 2009). The first class includes GPCRs which are closely related to *Sc*Gpr1p. The second class contains GPCRs with close homology to the GprD receptor of *A. nidulans*. The third class consists of close homologs to *S. pombe* Stm1+ receptor. Homologs of this third class can be found, not only in fungi, but also in humans, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Arabidopsis thaliana*.

Interestingly, the carbon catabolite repressor CRE1/CreA homologue of *P. chrysogenum* is involved in regulation of penicillin biosynthesis. Similarly as with repression of enzyme production, CreA also exerts carbon catabolite repression on this process (Cepeda-Garcia et al. 2014). On the other hand, overlaps of the output of responses to carbon and nitrogen sources are also known for regulation of arginine catabolism by AreA, AreB and NmrA in *A. nidulans* (Macios et al. 2012). Consequently, crosstalk between nutrient signaling pathways has to be considered.

A special aspect of carbon signaling is the response to starvation. Carbon starvation significantly changes cellular growth and metabolism (Brown et al. 2014). Accordingly, hundreds of genes are induced representing a metabolic shift in *S. cerevisiae* (Wu et al. 2004) and filamentous fungi (Krohn et al. 2014; Xie et al. 2004).

In *N. crassa* glucose signaling and carbon catabolite repression are connected by the transcription factor VIB-1 (Xiong et al. 2014b), which was initially characterized as major regulator of responses to carbon and nitrogen starvation and involved in non self recognition (Dementhon et al. 2006). VIB-1 was subsequently found to regulate the hydrolytic enzyme regulator CLR-2. VIB-1 is essential for plant cell wall degradation, but this requirement can be circumvented by deletion of both *cre-1* and the transcription factor gene *col-26* (Xiong et al. 2014b).

Investigation of the changes in the proteome of *N. crassa* between growth under carbon starvation versus cellulose revealed induction of a subset of CAZymes indicating initiation of cellulose degradation and signaling. Although in this case the induction of gene transcription correlated well with protein abundance, this was not the case for many of the proteins downregulated upon growth on cellulose versus starvation (Coradetti et al. 2012; Xiong et al. 2014a). Hence, post-transcriptional regulation of protein abundance upon alterations of carbon source availability warrant further investigation, especially with respect to the signaling pathways involved. In *A. niger* the early response to growth on wheat straw involves the upregulation of many CAZyme genes, that are also active during carbon starvation, indicating a role in scouting (van Munster et al. 2014).

Phosphorylation was shown to play an important role in global signal transduction for regulation of plant cell wall degradation as well as for the response to

starvation (Xiong et al. 2014a) confirming significant involvement of kinases and phosphatases in the associated signaling. In *S. cerevisiae*, the TOR (target of rapamycin) pathway, casein kinases as well as the posttranslational modification of the protein phosphatases PP2A and PP1 via the PKA pathway are important for response to glucose. The Ras/cAMP pathway is suggested as a potential major route for glucose sensing, albeit the data leading to this interpretation would need further support (Brown et al. 2014). In *A. nidulans*, TOR signaling was shown to be associated with carbon starvation and many of the related starvation responses are dependent on the kinase AtmA, which regulates mitochondrial function and glucose uptake (Krohn et al. 2014).

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Sexual Development in Fungi and Its Uses in Gene Expression Systems

George D. Ashton and Paul S. Dyer

1 Introduction

Sexual reproduction has been widely studied in fungi since the earliest days of mycological research. The fact that the vast majority of fungal species are able to undergo sexual reproduction, combined with the diversity of sexual structures formed and mating systems, has attracted much interest. Most studies have been dedicated to studying fundamental process relating to sexual development—for example the morphological changes occurring, the genetic and molecular determinants of sexuality, and the evolutionary implications of sex as encompassed in the definitive text edited by Heitman et al. (2007). However, the sexual cycle in fungi can also be exploited for many practical purposes, which is often overlooked. This situation is likely to have arisen because many of the principal fungal species of biotechnological, agricultural or medical significance are unusual in that they are included in the approximate 20 % of fungi which lack a known sexual state and have been termed 'imperfect' (Dyer and O'Gorman 2011). Therefore fungal sexual reproduction has not been greatly exploited for practical purposes as this option has not been available for these key species. However, in the last decade it has been discovered that many of these supposed asexual species in fact have the potential for sexual reproduction if the correct mating partners and environmental conditions for sex can be identified (Houbraken and Dyer 2015; Dyer and O'Gorman 2012). Therefore there is renewed interest and opportunities for the exploitation of fungal sexual reproduction. In the present chapter an overview of sexual reproduction

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in fungi will first be provided, and then examples of how fungal sexual cycles can be exploited specifically to manipulate fungal gene expression, either by classical or molecular genetic means, will be presented.

2 Fungal Sexual Reproduction

As with other eukaryotic organisms, sexual reproduction in fungi involves the fusion of haploid nuclei to form a diploid zygote from which haploid cells are then produced via meiosis either immediately or following a period of mitotic cell division. Many fungi are also able to undergo asexual reproduction (Fig. 1). Fungi are unusual though in exhibiting a very wide range of different breeding systems to allow gamete fusion and zygote formation. Broadly, if a sexual state is present (also known as the 'perfect' state), reproduction may be by homothallic (self-fertility; only requires one individual) or heterothallic (obligate outbreeding; requires partners of compatible mating type to be present) means, although there are derived combinations of these such as pseudo-homothallism and many homothallic species

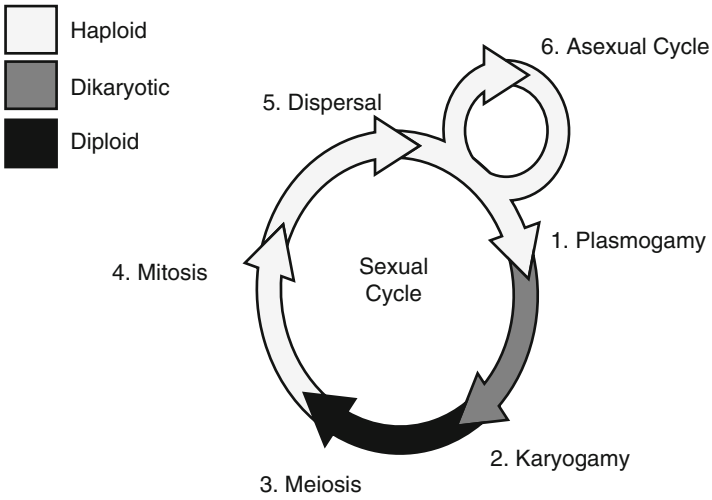

Fig. 1 The typical life cycle of a pezizomycete fungus. In heterothallic species when two complementary mating types meet, fusion between the two partners takes place, leading to the formation of dikaryotic ascogenous hyphae. This process is known as plasmogamy (1). Following this process, karyogamy, a process in which the nuclei fuse to form a diploid nucleus occurs (2). The diploid nucleus formed through karyogamy then undergoes meiosis to form 4 haploid nuclei (3). This is followed by mitosis of the four haploid nuclei to form eight ascospores, contained within a sexual structure (4). The ascospores are then dispersed and germinate to form mycelia (5) which may undergo an asexual cycle (6) or, upon meeting a complementary mating type, a sexual cycle. During the asexual cycle, haploid conidia are produced which are dispersed and undergo germination

retain the ability to outcross (i.e. are not restricted to self-fertilisation) if the right conditions are met (Dyer et al. 2016; Cavindera and Trail 2012). A key factor determining the sexual breeding system of specific fungal species is the presence of so-called 'mating-type' (*MAT*) genes, which determine the sexual identity of individual isolates. Depending on the type and structural organisation of *MAT* genes, isolates will exhibit either homothallic or heterothallic modes of sexual reproduction (Dyer et al. 2016).

2.1 Sexual Reproduction and MAT Genes in the Pezizomycotina

This chapter will focus primarily on the Pezizomycotina (the filamentous Ascomycotina), the largest sub-group in the fungal kingdom, which contains arguably the highest number of economically important species. In heterothallic species there are normally only two mating types present, by convention now termed *MAT1-1* and *MAT1-2* (although in some species older nomenclature is used, for example the mating types are known as *matA* and *mata* in *Neurospora crassa,* or *mat* + and *mat*− in the genus *Podospora*) (Dyer et al. 1992; Grognet et al. 2014). Furthermore, in some species individuals can either be male, female or hermaphrodites with regards to ability to form sexual structures (Debuchy et al. 2010). Therefore, not only must complementary mating types be present for sexual reproduction to occur, but isolates must also be complementary to each other with regards to acting as male/female for these latter species (Houbraken et al. 2015).

In pezizomycete fungi the sexual cycle begins with the fusion of fungi of complementary mating types in a process known as plasmogamy. Following plasmogamy and the formation of a dikaryotic ascus, karyogamy, the fusion of the two nuclei to form a diploid nucleus, occurs (Fig. 1). The diploid nucleus then undergoes meiosis to form four haploid nuclei, followed normally by one round of mitosis to form eight ascospores (although sometimes further mitoses can occur) which are contained within asci within a fruiting body whose structure depends on the particular sub-grouping of the Pezizomycotina. Examples of such sexual structures include: cleistothecia, a completely enclosed fruiting body of globose shape; perithecia, a flask shaped structure often with an open pore through which the ascospores are able to escape; and apothecia, an open-saucer shaped sexual structure where the sexual spores are freely exposed on the surface of the apothecia upon reaching maturity (Debuchy et al. 2010). The ascospores are then finally either forcefully ejected or passively dispersed from the fruiting body and under suitable conditions will germinate to grow as haploid mycelia (Fig. 1). The production of haploid progeny is a significant advantage where genetic analysis is concerned.

Many genes have been identified as being required for sexual reproduction in the Pezizomycotina. These genes have functions ranging from environmental sensing in order to determine when it is viable to undergo sexual reproduction, through to control of mating processes and pheromone signal transduction, and final development

of ascospores and sexual structures (Dyer and O'Gorman 2012). Of particular note are the *MAT* genes which determine the sexual identity of an individual, and which have recently also been shown to control the activity of many other genes involved with sexual development due to their activity as transcriptional activators (Dyer et al. 2016; Becker et al. 2015; Böhm et al. 2015). Thus, *MAT* genes are often considered as master regulators or orchestrators of sexual development. In the case of heterothallic pezizomycete species the mating type of an isolate is determined by the presence of gene(s) at a single *MAT* locus within the genome. By definition, *MAT1-1* isolates contain a *MAT1-1* locus, which includes at least a *MAT1-1-1* gene encoding a protein with an α1 domain, whereas *MAT1-2* isolates contain a *MAT1-2* locus, which includes at least a *MAT1-2-1* gene encoding a protein with a MATA_HMG domain (Dyer et al. 2016; Debuchy et al. 2010). Due to dissimilarity between the two *MAT* loci, they are referred to as idiomorphs as opposed to alleles in order to emphasize the differences in sequence (Metzenberg and Glass 1990), although recent phylogenetic analysis indicates that the α1 domain originally diverged from the MATA_HMG box domain family (Martin et al. 2010). By contrast, homothallic fungal species normally contain genes encoding α1 domain and MATA_HMG proteins within the same genome, and it appears that it is the presence of both such mating-type genes that confers the ability to self-fertilise (Paoletti et al. 2007).

2.2 *Sexual Reproduction and MAT Genes in the Basidomycotina*

The Basidiomycotina comprises the second largest sub-group in the fungal kingdom, containing over 30,000 species. Basidiomycetes can be monomorphic, where the growth state is either as a single-celled yeast growth form or a filamentous growth form, or dimorphic where the fungus can switch between these two growth forms (Fell et al. 2001). Sexual reproduction can occur within both monomorphic and dimorphic basidiomycete species. As with the Pezizomycotina, sexuality is again regulated by mating-type genes. However, greater complexity of *MAT* gene organisation is present in the Basidiomycotina. Briefly, heterothallic basidiomycete species normally have two distinct *MAT* loci, one of which contains genes encoding for homeodomain transcription factors, whilst the other contains genes encoding for pheromones and pheromone receptors. When these two loci are unlinked within the genome they are termed tetrapolar, a unique state seen in Basidiomycotina, whereas if the two loci are linked they are termed bipolar (Fraser and Heitman 2003). There can be several alternative alleles associated with each *MAT* locus which accounts for the fact that some model mushroom species may have thousands of different mating types. A full description is beyond the scope of the current chapter, and instead the reader is directed to relevant chapters in the 'Sex in Fungi' book by Heitman et al. (Heitman et al. 2007), which also includes discussion of sexual reproduction in zygomycete and chytridiomycete fungi.

2.3 Sexual Reproduction in Supposed 'Asexual' Species

Despite the many theoretical evolutionary advantages of sexual reproduction, approximately 20 % of all known fungal species are only known to reproduce by asexual means (Dyer and Paoletti 2005). However, even though a sexual state has not been observed, it does not necessarily mean that it does not exist and instead many fungi may exhibit 'cryptic sexuality' (Dyer and O'Gorman 2012). It may simply be the case that the fungus has not been exposed to the correct set of stimuli in order to reproduce sexually, and/or the correct mating partners have not been identified. Indeed, there has been accumulating evidence over recent years that many filamentous fungal species long considered to be purely asexual in fact have functional sexual cycles. This has been the case particularly in fungi of applied medical, biotechnological and agricultural importance where it would be advantageous to be able to alter gene expression. For example, a combination of molecular and classical microbiological techniques led to the pioneering discovery of a sexual cycle in the opportunistic pathogen *Aspergillus fumigatus* (O'Gorman et al. 2009). Soon afterwards a sexual state was described in the aflatoxin producing 'asexual' fungal species *A. parasiticus* and *A. flavus* (Dyer and O'Gorman 2012; Horn et al. 2009). Meanwhile, it was possible to trigger sexual reproduction in the industrial workhorse *Trichoderma reesei*, used as a producer of cellulolytic and hemicellulolytic enzymes, when an asexual production strain was crossed to certain female-fertile wild-type strains (Seidl et al. 2009). Most recently, a further example of discovery of a fungal sexual cycle was for *Penicillium chrysogenum*, a species of utmost medical importance as this fungus is used industrially as the source of penicillin (Böhm et al. 2013). A key advance for all of these species was the identification by molecular means of sexually compatible *MAT1-1* and *MAT1-2* isolates, in combination with discovery of suitable growth media and environmental conditions to trigger the sexual cycle (Houbraken et al. 2015). Tantalisingly, there have also been recent indications of possible sexuality in *A. niger*, a fungal species widely used in the biotechnology sector for metabolite production and various bioconversion processes. This species was thought to be entirely asexual and produce exclusively conidia for dispersal. However, Frisvad et al. (2014) have recently reported that certain strains of *A. niger* are able to produce sclerotia (a precursor to sexual reproduction) when grown on agar media containing raisins. This is the first step towards potentially exposing a sexual life cycle in this species.

The discovery of functional sexual cycles in such previously considered 'asexual' species suggests that there is potential, and good prospects in the future, for finding sexual states in many other economically important fungi (Dyer and O'Gorman 2011). This would allow the sexual cycle to be used as a valuable tool, either on its own or in conjunction with genetic manipulation techniques, in gene expression systems as will next be described.

3 Uses of Sexual Development in Gene Expression Studies

Once a sexual cycle has been found, it can be utilised in various ways to study gene expression and also be used to manipulate gene expression in a target species. The following section will provide details and examples of how fungal sexual reproduction can be exploited to identify genes of interest, enable strain development, verify gene function and also how *MAT* genes are emerging as potential master regulators for altering gene expression in traits of interest.

3.1 Genetic Basis of Traits and Identification of Genes of Interest

One very useful application of the sexual cycle is as an initial stage in gene expression studies to identify the genetic basis of a trait (character) of interest, for which little is known about at a genetic level. Through the use of crosses between two sexual partners that differ in the trait of interest it is possible to determine whether the trait is either monogenic (i.e. where a trait is determined by a single gene) or polygenic (where a trait is determined by several genes acting in conjunction with each other) in nature. Knowing this background will influence the design and approach to subsequent gene expression system analyses.

If a trait of interest is due to the effects of a single dominant gene, it would be expected that a 1:1 segregation pattern would be seen for that trait in the progeny (Fig. 2a). Whereas if the trait is determined by the action of several genes then patterns of inheritance showing increased complexity will be observed, manifesting itself by showing a continuous distribution of phenotypes between both parents used in the cross (Fig. 2b).

Once the genetic basis of a trait of interest has been determined, the sexual cycle can then further be used to try and locate and even identify individual genes of interest through the use of classical genetic methods, which have begun to be combined with next generation sequencing methodologies. These methods have been reviewed by Moore and Novak Frazer (2002) and Dyer and Darbyshir (2016) to which the reader is directed for more comprehensive details. But briefly, in the case of monogenic traits classical genetic methods include the use of map-based methods to identify a chromosomal region containing the gene of interest by mapping the segregation of markers linked to a particular phenotype (Foulongne-Oriol 2012). This approach ideally requires a highly-saturated genetic map to be present for a particular species, although this requirement is being superseded with the availability of whole genome sequences provided that linked markers are known within the genome. Where detailed genetic maps are not available then ‘bulk segregant analysis (BSA)’ provides an alternative method to try and identify a region of the genome containing the gene of interest (Michelmore et al. 1991). Here sexual crosses are set up between parents with or without the trait of interest. A suitable number of progeny (for

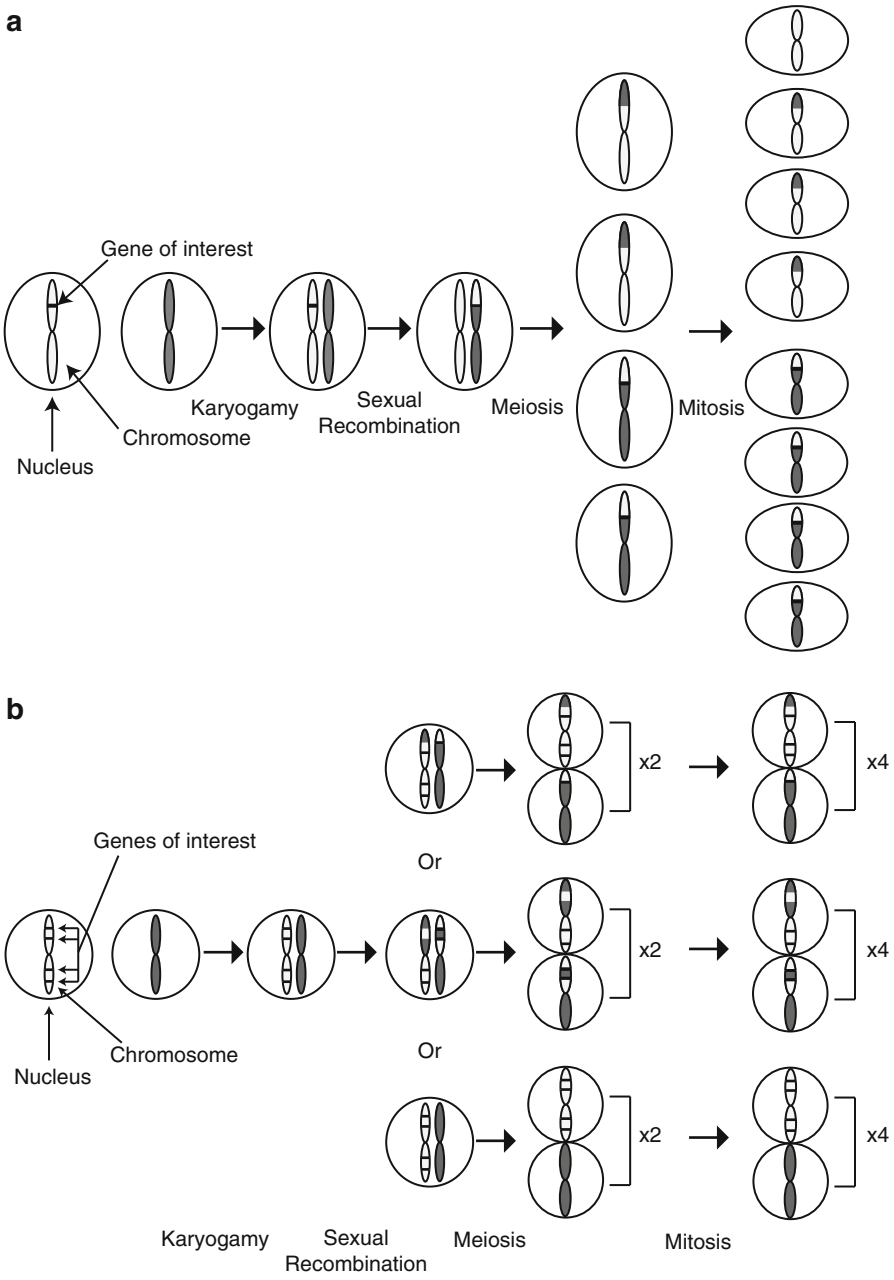

Fig. 2 Verification of gene function through use of a sexual cycle. Sexual recombination occurs once compatible nuclei have fused (karyogamy). This eventually leads to, through a round of meiosis followed by mitosis, eight ascospores being produced. Through analysis of the progeny the genetic basis of a trait of interest can be examined. (**a**) A 1:1 segregation pattern is expected if a trait has a monogenic basis. (**b**) A continuous distribution of the trait phenotype will normally be observed where a trait has a polygenic basis, dependent on how many genes of interest are present in the progeny following sexual recombination

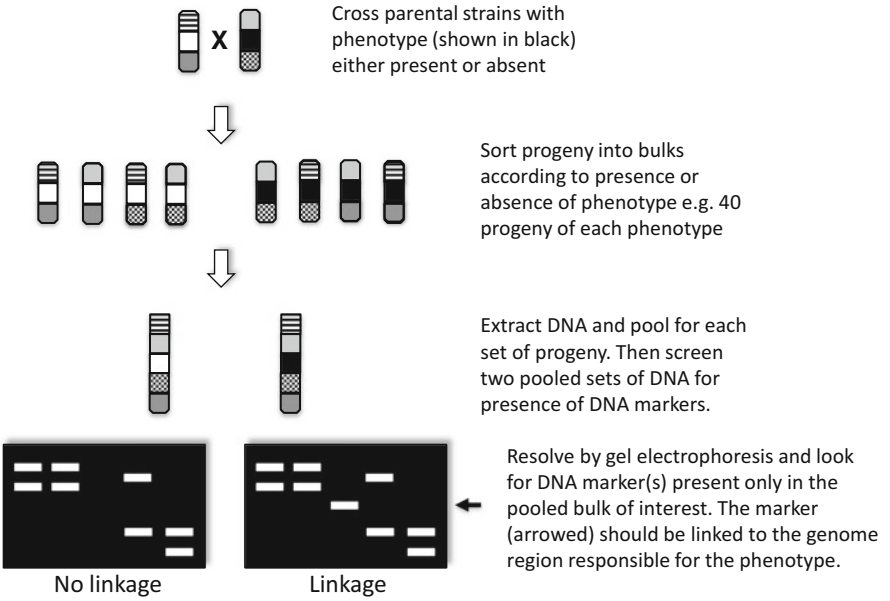

Fig. 3 Use of bulk segregant analysis to identify regions of the genome linked to a genetic trait of interest. See text in figure for explanation

example 80–120) are then split into two 'pools' according the presence or absence of the trait of interest. DNA is then extracted from all of the progeny isolates within each pool and mixed together. Although numerous genetic differences might be present between the parents, by combining recombinant offspring it would be predicted that the only differences between the pools would arise in regions of the genome linked to the gene conferring the trait of interest (Fig. 3). Originally the pooled sets of DNA were screened for the presence of DNA markers, such as RAPD-PCR fingerprints, that showed difference patterns between the pools and were therefore linked to the gene of interest (Fig. 3). But most recently the pools have been subjected to next-generation DNA sequencing to identify SNPs of interest (Pomraning et al. 2011; Nowrousian et al. 2012). BSA has proved a very valuable technique and has even been used to identify individual target genes responsible for a phenotype. But more commonly a region of the genome is identified, meaning that further chromosome walking or functional analysis of candidate genes is required. By contrast, where a trait of interest has been determined to have a polygenic basis the method of quantitative trait loci (QTL) analysis can be applied where genetic maps are available. The sexual cycle is used to produce a large number of progeny (commonly 80 or more), which are typed for the character of interest and the presence of molecular markers. QTL analysis programmes can then be used to determine both the number of effective genes contributing to a character and also can provide an indication of the genome location of such genes (Miles and Wayne 2008; Hall 2013). However, this technique alone very rarely identifies individual genes so further work is again needed in gene function studies.

There are numerous examples where sexual reproduction has been used to identify the genetic basis of traits of interest and then either mapping, BSA or QTL approaches have given further insights into gene function, as reviewed by Dyer and Darbyshir (2016). But particularly valuable insights have been gained regarding gene expression linked to resistance to fungicides, a major defence against fungal crop diseases in agriculture (Knight et al. 1997). Resistance to fungicides is unfortunately becoming increasingly common. Sometimes the genetic basis of resistance can be predicted, because resistance often evolves through the overexpression of genes encoding the target the fungicide acts on or through mutations in a gene conferring resistance to the fungicide and offers a competitive edge to those strains with the mutation (Hamamoto et al. 2000); but often the genetic basis of resistance is unknown. A recent example where the sexual cycle was used to identify genes involved in fungicide resistance was in the mould *Botrytis cinerea* (Kretschmer et al. 2009). In France and Germany, due to fungicide treatment, three *B. cinerea* strains with differing multiple drug resistance phenotypes are increasing in occurrence (MDR1, MDR2 and MDR3, respectively). These three strains all show increased resistance to a wide range of fungicides, although MDR3 shows the highest resistance levels and broadest spectrum of resistance. Through the use of a map-based cloning technique using sexual crosses, it was possible to determine that the phenotypes seen in both MDR1 and MDR2 are determined by one locus and that crosses of MDR1 and MDR2 can lead to production of strains with the MDR3 phenotype via genetic recombination. Studies were performed in order to identify the genes conferring these traits and it was determined that the phenotype of MDR1 is due to the overexpression of an ATP binding cassette (ABC) transporter *atrB* by a transcription factor *mrr1* (multidrug resistance regulator 1), whilst the phenotype of MDR2 is due to overexpression of the transporter gene *msfM2* (major facilitator superfamily transporter involved in MDR2) (Kretschmer et al. 2009). Other examples of gene identification methods being used in conjunction with the sexual cycle can be seen with regards to resistance to the fungicides prochloraz in the phytopathogen *Tapesia yallundae*, where either monogenic or polygenic inheritance was observed (Dyer et al. 2000), and resistance to benomyl (Ma and Michailides 2005) and separately temperature sensitivity in *Neurospora crassa* (Serna and Stadler 1978).

3.2 Strain Improvement

A second very useful application of the sexual cycle is to alter gene expression by setting up crosses between parental strains with traits of interest, and then screening progeny to select for those which show a corresponding change in phenotype linked to alterations in gene expression. This has most commonly been used in industrial strain improvement programmes. In this particular context the sexual cycle might also be used to restore the fitness of heavily mutated and damaged industrial strain through out-crossing with a complementary wild-type strain. Use of this method to

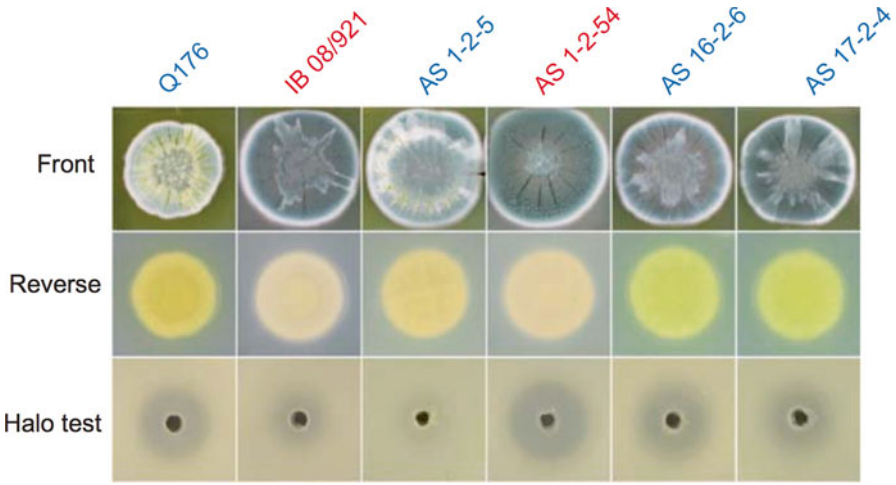

Fig. 4 Use of the sexual cycle for strain improvement and manipulation of gene expression in *Penicillium chrysogenum. Upper two panels* show colouration of colonies due to asexual sporulation and chrysogenin production in either parental (Q176, IB 08/921) or offspring (AS series) strains. *Lower panel* shows result of halo bioassay test, with clearing zone proportional to penicillin production. Reproduced from Böhm J, Hoff B, O'Gorman CM, Wolfers S, Klix V, Binger D et al. Sexual reproduction and mating-type-mediated strain development in the penicillin-producing fungus *Penicillium chrysogenum*. Proc Natl Acad Sci USA 2013; 110: 1476-1481 doi: 10.1073/pnas.1217943110 (Böhm et al. 2013)

restore fitness would ideally have little effect on the trait of interest because progeny that still exhibit high/low levels of the trait of interest can be selected for.

In typical strain improvement programmes strains with differing desirable traits are crossed together in order to select for progeny that have both desirable traits. For example, a strain that produces an industrially important secondary metabolite but exhibits slow growth can be crossed with a strain that produces very little of the secondary metabolite of interest, but which exhibits much faster growth rates. Once these crosses are performed, progeny can be selected that exhibit high secondary metabolite titres and faster growth rates. An example of strain improvement through sexual out-crossing can be seen in *P. chrysogenum* (Böhm et al. 2013). Strains Q176 and IB 08/921 were crossed in this study. The industrial precursor strain Q176 has high penicillin titre levels compared to wild type *P. chrysogenum* strains, and produces a yellow pigment, chrysogenin and pale green conidia. This is in contrast to the wild-type strain IB 08/921, which produces only low levels of penicillin, no chrysogenin and dark green conidia. The progeny from this cross showed varying characteristics from both parents. Progeny AS 1-2-54, however, was notable because it formed a clearing zone on bioassay plates, showing antibacterial capability, but lacked the ability to produce chrysogenin (Fig. 4). Chrysogenin is a contaminant in penicillin purification and therefore by selecting for progeny that retain the ability to produce high titres of penicillin but little to none chrysogenin, an improvement in

strain utility has been seen. A further example of where the sexual cycle might be used for strain development concerns the fungus *Penicillium roqueforti*, which is used as a starter culture in blue cheese production. A sexual cycle has very recently been described in this species by Swilaiman (2013) and Ropars et al. (2014), offering the exciting possibility of producing new variants of blue-veined cheese with altered flavor and growth characteristics.

A drawback of sexual reproduction for modifying gene expression and strain improvement is that this experimental approach can be slower than other methods of strain improvement such as mutagenesis. However, there are many benefits to using sexual reproduction for strain improvement that make up for this disadvantage. One important advantage is that recombination during the sexual cycle between the mating partners is genome wide, allowing considerable genetic diversity to be generated in the progeny. This could lead to unexpected beneficial effects on the trait of interest, such as some progeny with an increased titre of a secondary metabolite above the levels seen in either parent strain. This phenomenon is known as 'transgressive segregation' (Rieseberg et al. 1999), and has for example been observed in the aflatoxin producing *Aspergillus flavus*. In this species the mycotoxin cyclopiazonic acid (CPA) is produced that targets several organs in animals (Burdock and Flamm 2000). Through the use of sexual crosses it was found that progeny IC2193 showed over a four-fold increase in CPA production relative to the highest CPA producing parent strain IC278 (Olarte et al. 2012). A further advantage is that the sexual cycle is a natural process, unlike GM technologies which have faced consumer resistance, and the sexual cycle may therefore be used where gene manipulation techniques are unsuitable for a particular species.

One potential difficulty of using the sexual cycle for strain improvement is that industrial strains used in biotechnology may have accumulated considerable genetic damage due to previous mutagenesis treatment. Given that several hundred genes are likely to be required for sexual reproduction (Dyer and O'Gorman 2012) there may be a high risk that such strains would be infertile and unable to reproduce sexually. However, Seidl et al. (2009) found that strains RUT-C30 and QM9414 of *T. reesei*, heavily mutated for enhanced cellulase production, and TU-6, a uridine auxotrophic strain, were still able to complete a sexual cycle despite the damage arising from mutagenesis. Similarly, Böhm et al (2015) found that they were able to successfully cross an industrial strain of *P. chrysogenum* that had been subject to much industrial mutagenesis, so perhaps this risk is lower than might be predicted. Furthermore, if the industrial strain of interest has undergone many sub-cultures, then fertility can be reduced as a result of a 'slow decline' in fertility (Dyer and Paoletti 2005), although this might be counter-acted by use of a freezing technique as used to restore the sexual potential in *A. niger* (Frisvad et al. 2014). In addition, karyotype incompatibility might cause reduced fertility (Pöggeler et al. 2000; Dahlmann et al. (2015)

3.3 Verification of Gene Function

The next very useful application of the sexual cycle in gene expression studies is to verify that a particular gene has a particular function attributed that that gene. For example, an interesting trait may have been identified as being monogenic and through the use of classical or molecular genetic mapping techniques, a candidate gene has been identified. In order to prove that this gene is indeed responsible for the trait that is being seen, a sexual cross can be performed between two strains of a species that either possess or lack the gene (or allelic form of that gene). Ideally such crosses are made between parental isolates that are genetically similar, in order to limit the number of confounding genetic interactions. Once the cross has been performed, a suitable number of progeny (ideally at least 20–30, depending on ease of scoring the phenotype) can then be collected and analysed for the presence/absence of the gene (or allele) of interest, and it is then determined whether this correlates with the presence/absence of the trait of interest. If it is seen consistently that the trait of interest is absent when the gene (or allele) thought to be responsible for this trait is also absent, but present when the gene (or allele) is also present, then the role of the gene has been proven (Dyer and Darbyshir 2016). This approach has been used to investigate the role of genes involved with functions as divergent as conidial phenotype and antifungal resistance (Vitalini et al. 2004; Wood et al. 2001).

One other use of the sexual cycle in relation to gene function concerns its application for gene complementation studies. Oftentimes, when a genetic transformation technique has been used and a deletion mutant produced, it is a requirement that the mutant be complemented with the original gene to demonstrate restoration of function—thereby confirming gene function. This can prove difficult if selectable markers for the gene restoration are not available. However, as an alternative the sexual cycle can be used. A cross can be set up between a wild-type strain and the deletion mutant. If a consistent correlation between presence of the wild-type gene and restored trait and/or restoration of the parental genotype can be shown in the progeny, then the gene function can be confirmed (Dyer and Darbyshir 2016).

In a further example, it might be necessary to generate a strain with multiple gene deletions to provide evidence for the function and/or synergistic activity of these genes. Therefore, gene manipulation techniques would have to be used to introduce multiple successive gene deletions, which might prove problematic if a large number of serial transformations are required. However, the sexual cycle can provide an alternative. Crosses can be set up between complementary strains, again ideally genetically similar, containing gene deletions of interest. Once performed, progeny can be selected that have the desired gene combination and presence of the trait can be tested. This would provide evidence on the function of several genes. This crossing technique has been applied in the model fungus *A. nidulans* which despite being homothallic allows sexual outcrossing to be performed with near isogenic strains (Todd et al. 2007).

3.4 MAT Genes: Master Regulators?

A final intriguing way in which sexual reproduction might be used to modify gene expression relates to recent findings concerning the transcriptional activity of *MAT* genes. Accumulating evidence suggests that *MAT* genes may have many more functions than previously thought, possibly acting as a master regulator for many genome wide processes, as opposed to only being needed for sexual development (Dyer et al. 2016). Becker et al. (2015) through the use of chromatin immunoprecipitation combined with next generation sequencing (ChIP-seq), found evidence that the function of the *MAT1-1-1* mating-type gene is not solely restricted to sexual development in *P. chrysogenum*. A *MAT1-1-1* binding motif, of consensus sequence 'CTATTGAG', was discovered. Analysis of 254 genes that were targeted by *MAT1-1-1* revealed that a significant number of these genes were involved in roles other than sexual development such as primary and secondary metabolism, cell virulence, iron acquisition, and cell defense among other functions. Furthermore, enzymes and multidrug transporter genes that may have an impact on penicillin biosynthesis were found to be downregulated in a *MAT1-1-1* deletion mutant relative the wild type. This confirmed previous findings indicating that the *MAT1-1-1* gene could regulate processes as diverse as hyphal morphology and penicillin production (Böhm et al. 2013). The same group have recently reported that the *MAT1-2-1* gene of *P. chrysogenum* can also regulate a broad spectrum of cell activities beyond those simply involved with mating processes. It was observed that *MAT1-2-1* may play a role in light dependent asexual sporulation as well as in the germination of conidia (Böhm et al. 2015).

There is therefore the exciting prospect that controlled alteration of expression of sexual *MAT* genes might be used as a powerful technique to modify the expression of a broad range of target genes, which might be of commercial advantage given that *MAT* genes can alter primary and secondary metabolism. For example in the case of *P. chrysogenum*, it appears that penicillin biosynthesis is affected by *MAT1-1-1* gene expression (Becker et al. 2015; Böhm et al. 2013). Therefore, by altering *MAT* gene expression it may be possible to increase penicillin production in an industrial strain. It is even conceivable that the sexual cycle could be used to select for progeny with increased *MAT* gene expression conferring a favorable phenotype. Thus, further understanding of *MAT* gene function in fungi may make even more precise strain improvement possible through the use of sex or genetic manipulation techniques.

4 Conclusion

The sexual cycle of fungi is a very valuable tool with regards to genetic systems. Although many industrially and economically important fungal species remain asexual, the recent sexual revolution observed in many species of fungi (Dyer and

O'Gorman 2011) may lead to an increased use of the sexual cycle in gene manipulation and expression studies as sexual cycles continue to be discovered. This chapter has illustrated how the sexual cycle can be exploited in a series of different ways relating to gene expression studies. It is hoped that this will stimulate interest in further use of fungal sexual cycles. We argue that the combined use of genetic manipulation techniques together with fungal sexual reproduction offers greater scope for advance than the main current use of GM approaches alone, with the potential for effecting the transcription levels of many different genes and possibility of producing improved strains of industrial or economic importance.

There are also future so far unexplored opportunities for exploiting fungal sexual reproduction. It has been suggested that fungal sex hormones might be powerful regulators of developmental processes and linked gene expression (Dyer et al. 1992). The future isolation of fungal sex hormones offers the prospect that they might be used as novel antifungal agents, particularly in the agrochemical industry. They might also even be used to induce sexuality in presumed asexual species—the perfect stage therefore overcoming an imperfect future.

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Hybrid Infertility: The Dilemma or Opportunity of Applying Sexual Development to Improve *Trichoderma reesei* Industrial Strains

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Abbreviations

aCGH	Array-based comparative genomic hybridization
RTU	Return to euploid
SAN	Segmental aneuploidy
WCA	Whole chromosome aneuploidy

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1 Strain Improvement Programs Have Resulted in Multiple Chromosomal Alternations in the Genomes of *T. reesei* Hypersecreting Mutants

QM6a was first collected on the Solomon Islands during the Second World War (Mandels and Reese 1957). It was later renamed as *Trichoderma reesei* in honor of Elwyn T Reese, the lead investigator responsible for the identification of QM6a as a good producer of cellulases (Peterson and Nevalainen 2012; Mukherjee et al. 2013). QM6a had been considered to reproduce asexually for many years. To enhance cellulase production, several hypersecreting mutants were generated via random mutagenesis. For example, QM9414 was selected after two rounds of irradiation in a linear particle accelerator (Vitikainen et al. 2010). RUT-C30 was generated via three mutagenesis steps (UV, N-nitrosoguanidine and UV). These mutants produced several times more cellulase than QM6a. RUT-C30 also exhibits a carbon catabolite derepression phenotype, as the glucose repressor *cre1* gene was found encode a truncated protein with only one of the two zinc finger motifs in the wild-type CRE1 protein (Ilmen et al. 1996).

QM9414 and RUT-C30 not only displayed an increase in cellulase production but also acquired many genetic alterations via the random mutagenesis programs. Electrophoretic karyotyping and Southern hybridization revealed extensive alterations (i.e., deletions and rearrangements) on the chromosomes of QM6a and the hypersecreting mutants (Carter et al. 1992; Mantyla et al. 1992). Multiple genomic alternations have been identified in the genomes of RUT-C30, QM9414 and several other hypersecreting mutants via high-resolution array-based comparative genomic hybridization (aCGH) (Vitikainen et al. 2010) and comparative genome sequence sequencing (Peterson and Nevalainen 2012; Martinez et al. 2008; Seidl et al. 2008; Le Crom et al. 2009; Koike et al. 2013). For example, the results of an aCGH experiment revealed at least four and five translocation breakpoints in the genomes of QM9414 and RUT-C30, respectively (Vitikainen et al. 2010). An in-depth alignment of RUT-C30 and QM6a scaffold sequences suggested that the RUT-C30 genome might contain 11 translocations, 65 deletions, 455 nonhomologous regions and >2000 nucleotide mutations (Koike et al. 2013).

2 Breaking the Barriers of Sexual Crossing

QM6a is a haploid strain with a *MAT1-2* mating type locus. The opposite mating type locus was recently found in the *H. jecorina* CBS999.97 diploid, which is the only *T. reesei* wild isolate strain that is sexually competent under laboratory conditions (Seidl et al. 2009). CBS999.97 sexual development generates two sexually compatible haploids, CBS999.97(1-1) and CBS999.97(1-2), which harbor the *MAT1-1* and *MAT1-2* mating-type locus, respectively. Although QM6a is a sterile female, it can act as a male gamete to mate with CBS999.97(1-1). Sexual crossing of CBS999.97(1-1) with either CBS999.97(1-2) or QM6a produces fruiting bodies

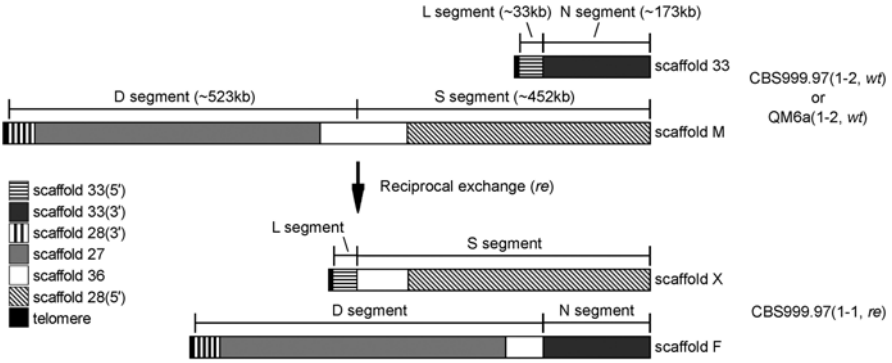

Fig. 1 Reciprocal exchange between scaffold M and scaffold 33. Scaffold M and scaffold 33 in CBS999.97(1-2, *wt*) and scaffold F and scaffold X in CBS999.97(1-1, *re*). Organization and length of the four segments (L, N, D and S) in these four scaffolds are indicated

containing asci with 16 linearly arranged ascospores (the sexual spores specific to ascomycetes) (Seidl et al. 2009). Using classical genetic and molecular biology approaches, we have recently shown that the 16 ascospores in an ascus are generated via meiosis followed by two rounds of postmeiotic mitosis (Chuang et al. 2015). Meiosis is a special type of cell division in sexually reproductive organisms that gives rise to genetic diversity via interhomolog recombination between non-sister homologous chromosomes. The identification of a QM6a mating partner has created new opportunities to further understand and improve this biotechnologically important fungus (Seidl et al. 2009).

3 Sexual Crossing of CBS999.97(1-1) with Either CBS999.97(1-2) or QM6a Frequently Produces Segmental Aneuploidy (SAN) Ascospores

Our results (Chuang et al. 2015) have also revealed that sexual crossing of wild type CBS999.97(1-1) with wild type CBS999.97(1-2) or QM6a produced three different types of ascospores: (1) Euploidy ascospores that can germinate to form mycelia and produce green conidia (i.e., asexual spores). CBS999.97(1-1), CBS999.97(1-2) and QM6a are also euploidy and produce green conidia. (2) Viable SAN ascospores that germinate to form mycelia but produce white conidia; their genomes contain a ~523 kb segmental duplication and a ~33 kb segmental deletion. The corresponding segments are referred to as the "D (duplicated)" segment and the "L (loss)" segment, respectively (Fig. 1). Notably, the *T. reesei* polyketide synthase 4 gene (*tpks4*) is located within the L segment. The PKS4 protein products are responsible for the green conidial pigmentation in the wild type strains (Atanasova et al. 2013). We also found that duplication of the D segment results in an increase in xylanase (but not cellulase) production in these viable SAN progeny. Intriguingly, there is a

carbohydrate-active enzyme (CAZyme) gene cluster in the D segment. This CAZyme gene cluster harbors a set of hemicellulases or xylanases (*abf1*, *bga1*, *cip2*, *cel74a* and *xyn3*) (Hakkinen et al. 2014). (3) Non-viable SAN ascospores, which are unable to germinate. Their genomes likely contain two L segments but lack the D segment, which causes meiotic-drive ascospore lethality. The D segments in the genomes of QM6a and CBS999.97(1-2) consist of the 5′ terminus of scaffold 36 (15 genes, ~52 kb), scaffold 27 (130 genes and ~431 kb) and the 3′ terminus of scaffold 28 (12 genes, ~40 kb). The D segment contains at least 113 annotated genes, including several putative essential genes such as an actin-like protein (protein ID 111468; http://genome.jgi-psf.org/Trire2).

These three types of ascospores are differentially distributed in three types of asci generated by sexual crossing of CBS999.97(1-1) with CBS999.97(1-1) or QM6a. The type I asci (~10 %) have 16 viable euploidy ascospores. The type II asci (~80 %) have eight viable euploidy ascospores, four viable SAN ascospores and four non-viable SAN ascospores. Finally, the type III asci (~10 %) have eight viable SAN ascospores, eight non-viable SAN ascospores, but they lack euploidy ascospore.

4 Identification of a Novel Chromosome Arrangement in CBS999.97(1-1)

Investigation of the genomes of QM6a and CBS999.97(1-2) revealed that the three scaffolds (36, 27 and 28) are contiguous segments and together form a larger scaffold “M”. Scaffold M is divided into the D segment and the “S” segment. Unlike the D segment, the S segment (~452 kb) is not duplicated in the viable SAN ascospores. The L segment is located at the 5′ portion of scaffold 33, whereas the 3′ portion (~173 kb) of scaffold 33 is referred to as the “N” segment (Fig. 1).

Notably, compared with the genomes of CBS999.97(1-2) and QM6a(1-2), a large chromosomal translocation or reciprocal exchange (*re*) was found at this region of the CBS999.97(1-1) genome. (1) The L segment connects with the S segment to form a new scaffold “X” in CBS999.97(1-1), and (2) the N segment physically links to the D segment, thereby forming another new scaffold “F” in CBS999.97(1-1) (Fig. 1). Accordingly, the two CBS999.97 haploid strains and QM6a are hereafter referred to as CBS999.97(1-1, *re*), CBS999.97(1-2, *wt* [*wild type*]) and QM6a(1-2, *wt*).

5 The Ancestral *T. reesei* Genome Contains Scaffold M and Scaffold 33

The sexually competent CBS999.97 diploid strain was isolated from a storage lake in French Guiana (Lieckfeldt et al. 2000). It was used to produce the CBS999.97(1-1, *re*) haploid strain and the CBS999.97(1-2, *wt*) haploid strain via sexual development

(Seidl et al. 2009). QM6a(1-2, *wt*) was isolated on the Solomon Islands during the Second World War (Mandels and Reese 1957). Several other non-CBS999.97 haploid strains were isolated from different geographical locations (Seidl et al. 2009). Using PCR and classic genetic approaches, we have shown that scaffold M and scaffold 33, but not scaffold X and scaffold F, were present in all nine non-CBS999.97 isolates examined (Chuang et al. 2015). These results indicate that the ancestral *T. reesei* genome likely contained scaffold M and scaffold 33 and that scaffold F and scaffold X evolved later in French Guiana via a chromosomal translocation between scaffold M and scaffold 33.

6 Chromosome Arrangement Is Responsible for the Production of SAN Ascospores

Our findings can be explained by classical genetics. The type I asci are "parental ditype" (PD). Eight ascospores harbor scaffold M and scaffold 33, whereas the other eight ascospores harbor scaffold F and scaffold X. The type II asci and the type III asci are "tetratype" (TT) and "non-parental ditype" (NPD), respectively. When meiotic interhomolog recombination (or crossover) fails to occur at the translocational breakpoints between these four scaffolds (M, 33, X and F), PD and NPD can be produced simply by random chromosome segregation during MI. In contrast, TT is likely generated via a single crossover between scaffold M and scaffold X or between scaffold 33 and scaffold F. NPD can also arise from two crossovers between two of these four scaffolds. As our single ascospore isolation experiments revealed more type II "TT" asci (>80 %) than type I "PD" asci (~10 %) and type III "NPD" asci (~10 %), we suggest that meiotic interhomolog recombination occurs at a high frequency between scaffold M and scaffold X or between scaffold 33 and scaffold F.

This hypothetic model had also been confirmed by sexual crossing experiments using two homozygous haploids. We first isolated two new haploid strains, CBS999.97(1-1, *wt*) and CBS999.97(1-2, *re*), from the type I asci generated from sexually crossing the two parental haploids, CBS999.97(1-1, *re*) and CBS999.97(1-2, *wt*). Sexually crossing CBS999.97(1-1, *re*) with CBS999.97(1-2, *re*), CBS999.97(1-1, *wt*) with CBS999.97(1-2, *wt*) or CBS999.97(1-1, *wt*) with QM6a (1-2, *wt*) generated asci with 16 viable euploidy. We conclude that genome heterozygosity caused by chromosome rearrangement is responsible for the production of viable and non-viable SAN ascospores (Chuang et al. 2015).

Our results are consistent with the classic chromosome speciation model proposed by Michael J. D. White in 1978, which states that chromosome rearrangement between populations can efficiently cause or contribute to heterozygote infertility (White 1978). Similarly, sexually crossing of two *Saccharomyces* strains (i.e., *S. cerevisiae* vs. *S. mikatae or S. paradoxus*) (Delneri et al. 2003) or two *Schizosaccharomyces* strains (i.e., *S. pombe* vs. *S. kombucha*) (Zanders et al. 2014) gave rise to viable hybrid diploids that efficiently completed meiosis, but fre-

quently generated non-viable spores. Chromosomal rearrangements (inversions) also contribute to reproduction isolation in two occasionally hybridizing North American *Drosophila* species, *D. pseudoobscura* and *D. persimilis* (Noor et al. 2001). *Cryptococcus neoformans* (a human fungal pathogen) meiosis generates SAN progeny with a large segmental duplication via telomere-telomere fusion and chromosomal translocation between two different chromosomes (Fraser et al. 2005; Ni et al. 2013). In these scenarios, chromosomal rearrangement and related recombination defects are the major cause of non-viable gametes. Intriguingly, it has been reported that two spore killer elements (*Sk-2* and *Sk-3*) are located near a chromosome rearrangement site in *Neurospora crassa* (Harvey et al. 2014). It remains unclear whether chromosome rearrangement can be a cause of meiotic drive. The molecular mechanism of spore killer in *Neurospora crassa* remains a mystery. Based on our *T. reesei* sexual crossing experiments described above, we hypothesized that most (if not all) of the non-viable gametes are SAN and that their infertility defects are likely caused by a loss of genes required for either fungal ascospore germination or for the formation of normal zygotes (e.g., *Drosophila*).

7 Mutagenesis Programs for Strain Improvement Resulted in Speciation

Genome sequencing and aCGH analyses have indicated that the genomes of RUT-C30 and QM9414 acquired multiple chromosomal changes (including translocations, deletions and nucleotide mutations) after multiple rounds of random mutagenesis. We found that RUT-C30 and QM9419 could mate with either CBS999.97(1-1, *re*) or CBS999.97(1-1, *wt*) and subsequently efficiently formed fruiting bodies and completed meiosis. However, the majority of asci have either no (70–80 %) or only four (20–30 %) viable ascospores (Fig. 2). These results indicate that multiple rounds of random mutagenesis apparently generated genetic barriers against the CBS999.97 wild isolate. Therefore, we hypothesize that high levels of ascospore lethality is due to hybrid infertility.

We noted a higher number of asci with 8 or 12 viable ascospores when CBS999.97(1-1, *wt*), but not CBS999.97(1-1, *re*), was used to mate with RUT-C30 or QM9414. In all cases, we found no asci containing 16 viable ascospores (Fig. 2). Compared with CBS999.97(1-1, *wt*), CBS999.97(1-1, *re*) has an additional chromosomal rearrangement between scaffold M and scaffold 33. It seems that an extra chromosome rearrangement in CBS999.97(1-1, *re*), compared with CBS999.97(1-1, *wt*), resulted in more severe hybrid infertility phenotypes. However, chromosome arrangement may not be the only cause of hybrid infertility. Other chromosomal alternations may also result in meiotic-drive lethality, e.g., deletions or nucleotide mutations in genes that specifically affect meiotic recombination (Hunter et al. 1996) or ascospore germination (Lambou et al. 2008; Strich et al. 2011).

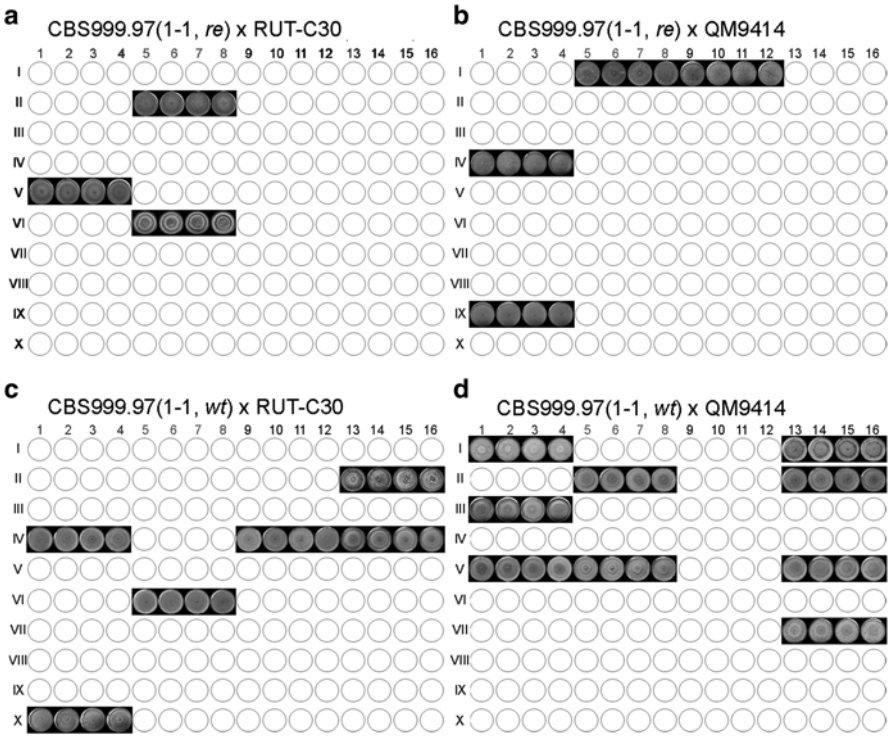

Fig. 2 Hexadecad dissection. CBS999.97(1-1, *re*) (**a**, **b**) and CBS999.97(1-1, *wt*) (**c**, **d**) were sexually crossed with RUT-C30 (**a**, **c**) or QM9414 (**b**, **d**). Sixteen ascospores from a hexadecad ascus were sequentially separated applying the yeast tetrad dissection method and then grown on individual 100-mm malt-extract agar plates. A single colony from one ascospore was isolated and transferred individually to a 60 mm potato dextrose agar plate to determine the spore viability, spore color and colony morphology. Sixteen single-ascospore colonies were aligned sequentially according to the ascospore order. The non-viable ascospores are indicated by a *black circle*

8 Conclusion

Our results suggest that speciation was artificially promoted via extensive random mutagenesis and selection regimes during strain improvements. RUT-C30 and QM9414, the two widely used industrial stains, are no longer the same species as QM6a and the CBS999.97 wild isolate. This supposition is consistent with the findings of comparative genome sequencing and aCGH experiments, which highlight many chromosomal alternations (i.e., translocations, deletions and nucleotide mutations) in these fungal genomes. According to the classic chromosome speciation model (White 1978), chromosome rearrangement and related meiotic-drive recombination can provide a simple and rapid method to create genetic barriers.

Because of their economic importance in the production of cellulolytic enzymes and recombinant proteins, these two industrial strains and related hypersecreting mutants have been extensively studied using genetic, molecular, biochemical, physiological, transcriptomic and proteomic approaches. Therefore, we suggest that they are ideal models to investigate the evolutionary process by which new biological species arise.

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Boosting Research and Industry by Providing Extensive Resources for Fungal Research

Kevin McCluskey

1 Living Microbe Collections Around the World Share Diverse Materials

Building upon the earliest laboratory and private collections, diverse fungal materials are maintained at every scale, from personal collections to International Biological Resource Centers, supporting academic research, pharmaceutical technology, and food and industrial biotechnology. The first organized living microbe collections were associated with food and alcoholic fermentation, although many such fermentations were open and relied upon environmental microbes to inoculate the substrate. With the demonstration of the germ theory (Pasteur et al. 1878), the ability to purify and characterize microbes led to multiplication of individual isolates, or strains, with reproducible and heritable characteristics.

Collections exist at a variety of scales and support unique and often non-overlapping research communities. This means that while collections have significant shared concerns, their main constituencies are often non-overlapping (Stromberg et al. 2013). Most of the largest collections support research targeted to human health and include mammalian cell lines as well as plasmids for transformation and protein expression in diverse host species. Such collections typically benefit from public support in addition to support generated by charging fees from clients (Smith et al. 2014). These public collections have persisted over many years and support the use of standardized materials (Janssens et al. 2010), often in the face of economic and legal challenges (Cressey 2014). To overcome some of these challenges, public living microbe collections have developed relationships to promote

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utilization and support of collections and to provide some opportunity to preserve collections faced with the lack of institutional support. This has not benefited private collections and many private collections were not maintained into the twenty-first century (Baker 2004). Renewed interest in living microbial collections, in the context of the Convention on Biological Diversity and to support the growing bioeconomy, has prompted the development of collection networks. Sharing data among collections has benefited from implementation of databases as well as the ability of data aggregators to provide global insight to collections (Verslyppe et al. 2014).

In the following sections, we will provide an overview of the impact of collections at every scale in basic and applied research. Diverse collections exist in many institutions and provide validated materials with clear provenance and legal foundation. Collections differ in the nature of the materials, the scale of holdings both with regard to number and also diversity, the type of materials including every type of microbe, and also in regard to the communities they support. Collection networks have evolved at multiple scales and promote the ability of collections to provide material for research and development. Collection groups also provide a means for coordination in the face of rapidly changing regulations governing the use of living microbial material in industry and biotechnology. Finally, the materials in the Fungal Genetics Stock Center have played a unique and central role in the development of gene manipulation and protein production in fungi both for research and for application. The plasmids and strains utilized have changed over many years, but the core characteristics have evolved very slowly and some of the earliest, pioneering work has had a continuing impact across diverse research areas and as technology has advanced.

1.1 Herbarium Collections

Historically, herbarium collections have emphasized plant specimens and fungal accessions were numerically small. This originates in the pre-molecular biology era when fungi were considered to be non-photosynthetic plants. Modern phylogenetics has shown that fungi are unique among the kingdoms of life and mycological herbaria have found new independence. While still emphasizing once-living specimens, mycological herbaria have the disadvantage that many fleshy fungi lose valuable morphological characters upon preservation. Microfungi may not have sufficient physical characters and suffer from the challenge of associating different stages of the same life-cycle with the same species level taxon.

1.2 Living Collections

Living collections typically emphasize biodiversity. The largest fungal collections in the world, such as the CBS collection have several isolates from each of thousands of taxa. This requires that each such collection have diverse expertise

appropriate for the diverse materials. Simply put, the expertise to grow yeast-like fungi does not translate into the expertise to grow and preserve macrofungi. This notwithstanding, the growing ability to cultivate diverse fungi has meant that holdings of living biodiversity collections have grown as the value of microbial diversity has been rediscovered. Collections of other organisms, from viruses to bacteria, invertebrates and even higher vertebrates, serve unique economic needs. Whether holding representatives of global biodiversity, serving an agricultural or biotechnological industry, these collections are tasked with preserving genetic diversity or characteristics and as such share key issues. Among these, genetic stability of materials as deposited is paramount and drives technological and data keeping practices. Much as convergent evolution shows the benefit of efficient morphologies or processes, living collections have found similar solutions to common problems such as genetic drift.

2 Networks of Collections Show Growth and Impact

Although their holdings may not overlap, living microbe collections have shared interests and must operate under a common framework (Dedeurwaerdere 2010). Networks provide a forum for interaction, discussion and presentation of shared concerns. Such networks take many forms depending on their genesis, intent, and support. Loosely based regional networks often cross national boundaries while formal national networks may have shared funding to support national emphasis on biotechnology goals. Current circumstances place microbe collections between two communities. These are natural history collections (such as herbaria) and living collections, such as arboretums, aquaria, research vertebrate colonies, and zoological preserves. The development of global, regional, and national networks promote the shared interests of living microbe collections and emphasizes the value and impact of the member collections.

2.1 Regional and National Networks of Living Microbe Collections

Regional networks have the benefit of sharing funding mechanisms, economic systems, and benefit from reduced tariffs and customs regulations. By way of contrast, the collections that make up loose regional or national networks are inherently in competition for resources often across diverse national funding agencies (Dedeurwaerdere 2010). The balance between benefits and competition can make networks fragile and some have relatively poor persistence.

In the USA, a network called the US Federation for Culture Collections (USFCC) existed for many years and supported diverse interests for living microbe resources.

As technologies developed, industrial culture collections were largely abandoned in the late 1990s and early 2000s (Baker 2004) and this eroded corporate support for the USFCC. The last formal meeting of the USFCC was held in 2001 and the last publication of the USFCC newsletter in 2002. Recent developments in the USA have led to the formation of a nascent United States Culture Collection Network which is supported by the US National Science Foundation in the expressed hope of turning competitors into collaborators (McCluskey et al. 2014a). This network is in its third year and has strategically partnered with the American Phytopathological Society, through its umbrella organization Scientific Societies to provide continuity and institutional management. Among the motivations for re-establishment of a network of US culture collections is the need for access to defined genotypes of plant pathogenic microbes (Kang et al. 2006) and that the existing support for microbe collections in the USA did not provide for this type of resource preservation or distribution.

2.2 Global Networks of Living Microbe Collections

The World Federation for Culture Collections (WFCC), formally chartered in 1972 with the simple mission "to establish an effective liaison between persons and organizations concerned with culture collections and between them and the users of cultures" (Anon 1972) continues to provide leadership and an authoritative voice for collection concerns at the international level. This is manifest through contributions to the Universal Postal Union in their definition of what is admissible in the post under Article 16 of the 2004 Bucharest Convention. The WFCC provides guidance on what constitutes perishable biological substances and this encourages the exchange of microbial culture in a non-active form, such as lyophilized cells or desiccated cells on a porous solid support (silica gel or filter paper disks). Additional impact of the WFCC comes through its engagement with international treaty organizations (see Sect. 3.1, below). The WFCC was originally chartered as an entity in the International Association of Microbiological Societies (IAMS) (Anon 1972). In 2015 the WFCC is a Commission within the International Union of Biological Sciences (IUBS) and a Federation recognized by the International Union of Microbiological Societies (IUMS). Further, the WFCC engages public entities such as the United Nations Educational, Scientific and Cultural Organization (UNESCO) through their programs in Basic and Engineering Sciences which established a program of Microbial Resource Centres (MIRCEN) as a network of 34 international culture collections. The early emphasis of this program on agricultural inoculants presaged such programs as the Phytobiome Initiative in the US which emphasize the value of microbial resources for modern sustainable agriculture (Leach 2015). Additionally, with support from UNESCO MIRCEN, the WFCC continues the World Data Centre for Micro-organisms (WDCM). Originally established in 1972 (Martin et al. 1972), the WDCM has gone from a print

publication through several iterations into a fully digital and interlinked online resource. Like many such trans-generational resources, the WDCM has relocated when the promoters retire and after many years careful nurturance under the forward looking attention of Dr. H. Sugawara first at RIKEN (Sugawara et al. 1996), and subsequently at the National Institute of Genetics (Miyazaki and Sugawara 2002), the WDCM is now housed in the Chinese Academy of Sciences Institute of Microbiology where the informatics team of Dr. J. MA bring their full resources to bear on this rich source of data and opportunity (Wu et al. 2013).

3 International Framework for Exchange of Genetic Materials

Living microbial germplasm is a vehicle for genetic information and as such is the target of international treaties and regulations. These seek to ensure that benefits from the use of genetic material return to the origin of the material. Prior to the modern implementation of these treaties, biological materials we considered to be the shared heritage of mankind (Uhlir 2011). These regulations came as biological sciences transitioned into biotechnology, but have not apparently impacted progress in this area. Between 1950 and the end of 1993 there were approximately 360 patents filed for "fungal gene expression" which equates to over 8 patents per year. Since the enactment of the Convention on Biological Diversity (CBD), approximately 340 similar patents were granted and this rate is double the rate prior to the enacting of the convention.

3.1 Convention on Biological Diversity and Nagoya Protocol

The CBD is a landmark international treaty that promotes the conservation of biological diversity and the sustainable use of its components. It entered into force in December of 1993 after having been ratified by 30 parties (who in this case correspond to countries). Although the USA never ratified the CBD, its dependence on local governments to enact legislation ensures that all utilization of genetic resources are governed by its guiding principles. As such, in order to collect any biological material outside one's own country, one must obtain prior informed consent (PIC) on mutually agreeable terms (MAT). These are organized through National Focal Points, which may be identified through the CBD online resources available at www.cbd.int. It is important to note that these requirements apply to all research, not just research with a commercial intent.

More recently these ideals have been codified in The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity. This protocol went into

force in October of 2014 and requires that utilization of genetic resources include benefits that return to the origin. Importantly, these benefits are acknowledged to include non-monetary benefits including training, technology transfer, and where appropriate funding to contribute to the preservation of biological diversity. The goals of the Nagoya Protocol are organized into an Access and Benefit-sharing Clearing-House to facilitate the implementation of the goals of the protocol on a very fine scale. This clearing-house function is available through a convenient web based interface hosted at the CBD site.

3.2 Material Transfer Agreements and Public Domain

While collections such as the FGSC have for many years maintained open sharing policies, even such collections have Material Transfer Agreements (MTA). For the FGSC, a click-through MTA stipulates that the requestor agrees to pay for the materials, to cite the FGSC as the source, and to accept the risk of using materials from the FGSC. Additional terms assert that the recipient not violate local or international laws in the use of materials from the collection. More broadly, many collections have used the Universal Biological Material Transfer Agreement (UBMTA) although this is more appropriate for peer-to-peer exchanges. The UBMTA assumes that once a strain (or plasmid) is deposited in a public collection, intellectual property rights go away and to simplify this, and to correspond with the requirements of the Nagoya Protocol on Access and Benefit Sharing, many collections are now adopting the TRUST code of conduct which avers that any rights owned by the depositor are intact and the user of materials from a collection need consider the depositors rights without reference to the intermediary collection. In this regard, collections are acting as an extension of the depositor.

4 Fungal Collections at Many Scales Promote Technological Development

Over many years there has been a natural trajectory of individual collections which often have their roots in an individual's private or institutional collection. These private or boutique collections grow as the interest in the materials grows and wane when the underlying materials are no longer of use or interest to the community that originally supported or benefitted from the materials. Examples of a declining community include the *Allomyces* collection organized by Olson (1984) and ultimately deposited into the Fungal Genetics Stock Center, or the broad collection of strains carrying unique mating type loci in *Schizophyllum* (Raper and Fowler 2004). At the opposite end of the spectrum is the collection of *Saccharomyces* strains organized by Robert K. Mortimer in the 1960s and supported for many years by the US

National Science Foundation. For this collection, the resources of an individual laboratory were not sufficient to support the tremendous explosion of research and the collection was transferred to the American Type Culture Collection (ATCC) where it was supplemented with deletion mutants and other resources (ATCC 2000). While significant resources are available for work with yeast, the semi-commercial management of these and other materials (gene deletion kits, etc.) has promoted peer-to peer exchanges over many years.

4.1 Laboratory, Institute, and Corporate Collections

Most universities and research institutes have multiple, laboratory specific collections of microbes. The best have organized central microbe collections and these may provide long term solutions to the material management plan requirements now common in grant proposals. Many corporate collections were destroyed in the late 1990s and early 2000s after the cost of preservation was seen to be disproportionate to the perceived benefit (Baker 2004). While the calculations that drove this change were straight forward and based upon the likelihood of identifying a natural isolate with novel, economically valuable metabolism, recent advances in understanding of regulation of secondary metabolite gene expression and the ability to conduct rapid and economic whole genome analyses has suggested that the value of these collections was greater than was revealed by the simple arithmetic used in their unsuccessful justification.

4.2 National Collections

According to the terms of the Convention on Biological Diversity (1992), most countries have established living microbe collections, referred to as "ex situ microbial germplasm repositories" in the CBD. These replace the wildlife refuges that are mandated to preserve macro flora and fauna in situ and reflect the observation that microbes may not be found in the same location at different times. National collections serve multiple functions. They provide legitimate access to validated materials with clear provenance. When these collections emphasize the biodiversity of the host country, they provide opportunities to license materials and as such are the antidote to bio-piracy. Examples of national collections are shown in Table 1. These examples include both large centralized collections and smaller topically-focused collections. In most cases other public collections are registered with the Global Catalog of Microorganisms and some countries have more than 50 collections of varying scale and emphasis. Some countries have diverse collections but do not have a central biodiversity collection.

Table 1 Examples of national microbe collections

Collection name	Country	Website
Belgian Coordinated Collections of Micro-organisms	Belgium	http://bccm.belspo.be/
China General Microbiological Culture Collection Center	China	http://www.cgmcc.net/
Collection de L'Institut Pasteur	France	https://www.pasteur.fr/en/research/crbip-biological-resource-center-institut-pasteur
Deutsche Sammlung von Mikroorganismen und Zellkulturen	Germany	http://www.dsmz.de/
Indian Type Culture Collection	India	http://www.iari.res.in/?option=com_content&id=1251&Itemid=1809
Iranian Biological Resources Center	Iran	http://ibrc.ir/Default.aspx?tabid=775
Japan Collection of Microorganisms	Japan	http://jcm.brc.riken.jp/en/
KCTC Korean Collection for Type Cultures	Korea	http://kctc.kribb.re.kr
Centraalbureau voor Schimmelcultures	Netherlands	http://www.cbs.knaw.nl/
All-Russian Collection of Microorganisms	Russia	http://www.vkm.ru/
CABI Genetic Resource Collection	UK	http://www.cabi.org/
American Type Culture Collection	USA	http://www.atcc.org
US Department of Agriculture ARS collection	USA	http://nrrl.ncaur.usda.gov/

4.3 International Depository Authorities

Because diverse patents for the use of microorganisms were anticipated, and granted, the World Trade Organization established International Depository Authorities (IDA) to serve as microbe repositories for the purposes of assuring that patented materials were available. Established under the Budapest Treaty of 1985 (WIPO 1977), these collections typically charge accession fees and provide materials for the duration of the patent. After the patent expires, materials may be transferred into the open collection. Most countries have an IDA and the US has the most with the ATCC being the largest. The USDA NRRL collection also acts as an IDA as does the National Center for Marine Algae and Microbiota. This latter IDA was established in 2013 and recognizes that algae have special requirements for maintenance and propagation not served by other collections.

5 Biodiversity Collections

Most fungal collections emphasize taxonomic and geographic diversity. Whether the materials are living or preserved, these collections provide a catalog of the diversity available in a particular region, or associated with a particular host category. Among the biodiversity collections worldwide, type collections are a special kind of collection typically associated with taxonomic characterization. Biodiversity collections are often not focused on just fungi and may include diverse taxa, with the associated research staff expertise. Some, like Korea or Belgium, have distributed networks of collections while others, like the USA, have centralized collections like the ATCC. The existence of centralized collections does not preclude the co-existence of smaller taxonomically or geographically focused collections and many such collections support individual research, industrial, or university activities. Diversity collections face the challenge of being required to have expertise across the range of materials and this is not always economically easy. The cost of maintaining diverse organisms needs to be balanced with the benefit of the access to living germplasm.

6 Genetic Collections

Genetic collections are unique in their histories and relationship with individual research communities. The US National Science Foundation (NSF) supported an array of organism-specific collections that grew to hold large numbers of closely related, genetically characterized strains. Since most collections of fungi emphasize biodiversity, it is accurate to say that the FGSC is the world's leading collection of genetically characterized fungi. The inverse of this claim is that the FGSC holds isolates of fewer than 100 species, and indeed has over 23,000 isolates of *Neurospora* including wild-collected, inbred wild-type, classical mutant, and genetically engineered strains. The Mortimer collection of Saccharomyces mutant strains was transitioned to the ATCC upon the retirement of the director and while these valuable strains are still available, most exchanges in the yeast community are peer-to-peer exchanges.

Most of the collections supported by the NFS over many years are genetic in emphasis and so there are several collections that house different materials, but which share material and data types. These data are typically different from information associated with bio-diversity collections. Among the NSF supported collections, the International Culture Collection of VA Mycorrhizal Fungi (INVAM) which holds diverse fungi utilized in research and agriculture while the *E. coli* Stock Center (http://cgsc.biology.yale.edu/), the *Bacillus* Stock Center (Zeigler 2013), and the FGSC hold genetic stocks of research microbes. Other NSF supported living collections include Drosophila genetic (Cook et al. 2010) and wild-type stocks (Richmond 2013), Mice (Crossland et al. 2014), Lemurs (Yoder 2013),

Chlamydomonas (Harris 2009), and even Salamanders (Baddar et al. 2015). Beyond the NSF support, the US National Institutes of Health supports collections holding human health related materials and they are beyond the scope of this review.

6.1 Molecular and Genetic Resources Are Increasingly Valuable

As recognition that genetic resources underlie the foundation of modern biotechnology, the Organization for Economic Cooperation and Development (OECD) recapitulated the UNESCO support for Microbial Resource Centers in their 2001 science and technology study on Biological Resource Centers and promoted the notion that the material held by culture collections, formally managed to high standards and with best practices comprised were "underpinning the future of life sciences and biotechnology" (OECD 2001). As emphasized by the Convention on Biological Diversity statement on Synthetic Biology (Diversity SotCoB 2015), genetically manipulated organisms may have unanticipated benefits and so collections housing genetically characterized resources, such as the FGSC, have an enhanced value.

6.2 Genome Resources

Culture Collections are a natural partner in genome studies of ex situ microbial resources. As partners in the Genomic Encyclopaedia of Bacteria and Archae, the DSMZ collection in Germany have seen the value of their holdings increase by being the reference genome strain for many species. Similarly, the FGSC has experienced tremendous growth as it has become the preferred repository for over 50 strains of diverse fungi as well as over 550 strains of *Neurospora* subject to various levels of DNA sequence characterization (McCluskey et al. 2011; Ellison et al. 2011). Similarly, the CBS collection in the Netherlands is expanding their holdings of strains subject to whole genome sequence analysis, as a complement to their DNA barcode sequence now available for most strains in the collection.

This genome information has the benefit of immediately making the strain more desirable, but since the strains chosen for genome characterization may or may not be the type strain for taxonomic purposes, they can cause conflicts in the literature. There is no adequate response to this concern and the impact of genome information on taxonomy is not yet resolved (Federhen 2014).

Similarly, the impact of freely available genome sequence on liabilities and responsibilities under the CBD has yet to be elaborated. Clearly, the CBD discusses the exchange of genetic information. With free international publication of whole genome sequence data, as is required by most public genome sequencing centers and publishers and according to common standards on sharing data (Foster and Sharp 2007), genome information can provide immediate access to gene diversity

Table 2 Public plasmid collections

Name/ acronym	Emphasis	Number of plasmids	Website URL
FGSC	Fungi	776	www.fgsc.net
Addgene	Diverse/mammalian	33,000	www.addgene.org
ATCC	Diverse	1,600	www.atcc.org
LMPB	Diverse/mammalian	2,176	bccm.belspo.be/about-us/bccm-lmbp
DNASU	Mammalian	200,000	dnasu.org/
DSMZ	Diverse	261	www.dsmz.de/
NBRP	*E. coli*	11,000	www.shigen.nig.ac.jp/ecoli/strain/
CBS NCCB	*E. coli*	465	www.cbs.knaw.nl/

valuable for developing products with novel characteristics. This is true beyond material in culture collections and includes the metagenomics and transcriptomic data produced environmental DNA and cDNA sequencing (Kuske et al. 2015).

6.3 *Resources for Gene Expression*

The FGSC collection is unique among public fungal collections in providing tools for molecular genetic manipulation of strains in addition to a diverse holding of strains for research. Among the tools that the FGSC holds and distributes are over 750 molecular clones or plasmids, gene libraries, and gene deletion tools and strains. Beyond the FGSC collection, tools for protein expression or characterization are typically focused on expression in bacteria, yeast, or higher eukaryotes and this emphasizes the value of a public collection such as the FGSC. Collections that hold and distribute tools for protein expression and manipulation include diverse resources, although only the FGSC collection is specifically intended to support research with filamentous fungi (Table 2). These data are representative, as many collections hold plasmids which are incidental to their main emphasis. The WDCM only lists 1,783 "vectors" among the holdings of worldwide culture collections (http://www.wfcc.info/ccinfo/statistics/, accessed 2/9/15) and this is clearly an underestimate of worldwide plasmid holdings by culture collections (Table 2).

6.4 *FGSC Plasmid Impact*

Beginning with the 1985 deposit of the *Neurospora* transposon Tad on plasmid pJR2 (Kinsey and Rambosek 1984), the FGSC collection includes 776 plasmids although 281 are Restriction Fragment Length Polymorphism mapping markers from *Fusarium* or *Magnaporthe* (Fig. 1). The most highly distributed plasmids in the FGSC collection include selectable markers for diverse fungi, protein tagging

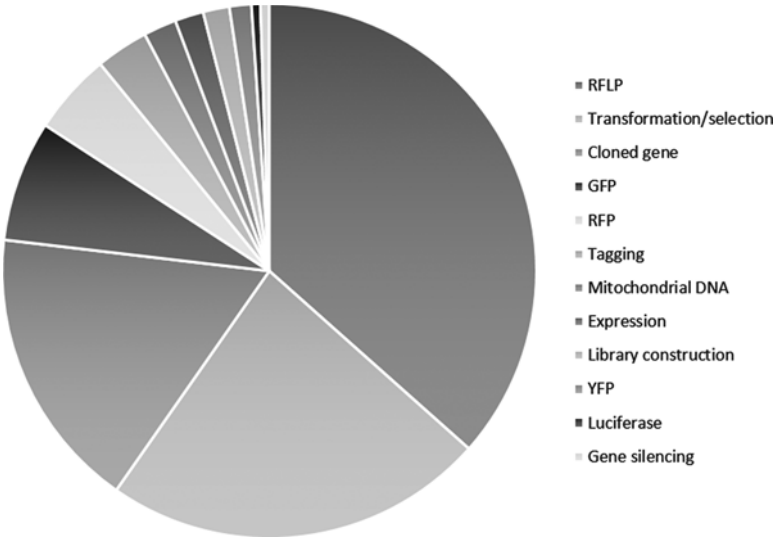

Fig. 1 Different types of plasmids make up the FGSC collection

vectors, and gene expression and silencing vectors (Table 3). The FGSC has distributed 4,507 individual plasmids to clients. Of all the plasmids in the FGSC collection, only 59 have been requested only once each. The most commonly distributed plasmid is the gene silencing plasmid, pSilent-1 (Nakayashiki et al. 2005), which has been widely used in the fungal research community, including plant pathogens (Kim et al. 2011), medical pathogens (Baldo et al. 2010), and model organisms (Flores-Alvarez et al. 2012). Among the most commonly used vectors, selectable markers were the most commonly requested categories of vectors. Tagging vectors, broadly defined as expressing a fluorescent protein or a tag for subsequent in vitro purification, are the next most commonly requested. Expression vectors, including those based on *gpdA* promoter, the *qa-2* promoter, and the *tet* inducible promoter have been widely requested and as a category have been distributed over 375 times.

Among vectors for selection of transformants, the diverse expression vector pBARGPE1 (Pall and Brunelli 1993) was one of the first to utilize the bar gene for selection of resistance to phosphinothricin (also known as glufosinate, ignite, or basta). This vector uses the *A. nidulans trpC* promoter and terminator and also has a multi cloning site conveniently between the *A. nidulans gpdA* promoter and the *trpC* terminator (Mattern et al. 1988) allowing expression of any fungal protein gene. Although not widely utilized in the post-genomics research era, this vector also carries lox, *Not*I, lox sequence (Elledge et al. 1991) allowing it to be linearized and ligated to lambda arms for packaging into phage lambda for expression screening, such as by antibody hybridization to membrane filters containing protein from a phage library plated on a lawn of *E. coli* cells (Young and Davis 1983). This vector has been distributed nearly 100 times, and continues to be popular, along with the variety of vectors made by this group (Brunelli and Pall 1993a, b; Pall and Brunelli

Table 3 Most commonly distributed plasmids from the FGSC collection

Plasmid name	Gene of interest	Function	Number of requests	Reference
pSilent-1	hph	Silencing	146	Nakayashiki et al. (2005)
pBC-Phleo	phleo	Selection	106	Silar (1995)
pCSN44	hph	Selection	101	Staben et al. (1989)
pBARGPE1	bar	Expression	91	Pall and Brunelli (1993)
pMF272	GFP	Tagging	76	Freitag and Selker (2005)
pRS426	*Ura3*	cassette	75	Christianson et al. (1992)
pCB1003	hph	Selection	72	Sweigard et al. (1997)
gGFP	GFP	Tagging	69	Maor et al. (1998)
pCB1004	hph	Selection	63	Sweigard et al. (1997)
pCSN43	hph	Selection	59	Staben et al. (1989)
pPK2	hph	Selection	56	Covert et al. (2001)
p3SR2	*amdS*	Selection	55	Wernars et al. (1985)
pBC-hygro	hph	Selection	52	Silar (1995)
pBARMTE1	bar	Library construction	51	Pall and Brunelli (1993)
pMYX2	ben	Expression	46	Campbell et al. (1994)
pMYX10	hph	Expression	45	Campbell et al. (1994)
pBARKS1	bar	Selection	43	Pall and Brunelli (1993)
ppyrG	*pyrG*	Selection	41	Turner et al. (1997)
pMT-BFP	BFP	Tagging	38	Toews et al. (2004)
cosmid An26	hph	Library construction	37	Taylor and Borgmann (1996)
pMT-mRFP1	mRFP1	Tagging	37	Toews et al. (2004)
pMF280	hH1-GFP	Tagging	35	Freitag et al. (2004)
pSD1	Geneticin	Selection	35	Nguyen et al. (2008)
pAO81	S-TAG	Tagging	34	Yang et al. (2004)
pFNO3	GFP	Tagging	34	Yang et al. (2004)
pMF309	Bml-GFP	Tagging	33	Freitag et al. (2004)
pMT-sGFP	sGFP	Tagging	33	Toews et al. (2004)
pRG3-AMA1-NotI	*pyr4*/AMA1	Library construction	33	Osherov and May (2000
pA-HYG OSCAR	hph	Selection	32	Paz et al. (2011)
p500	hph	Expression	31	Vogt et al. (2005)
pMF334	RFP	Tagging	31	Freitag and Selker (2005)
pMOcosX	hph	Library construction	31	Orbach (1994)
pXDRFP4	RFP	Tagging	31	Yang et al. (2004)
pD-Nat1	Nat	Selection	30	Kück and Hoff (2006)

(continued)

Table 3 (continued)

Plasmid name	Gene of interest	Function	Number of requests	Reference
pOSCAR	*ccdB*	Selection	30	Paz et al. (2011)
pBT6	*BmlR*	Selection	29	Orbach et al. (1986)
pCB1532	sur	Selection	27	Sweigard et al. (1997)
pDC1	*argB*	Selection	27	Aramayo et al. (1989)
pME2891	loxP-phleo^R	Selection	27	Krappmann et al. (2005)
pMG2254	M-cherry-TADH-URA3	Tagging	27	Gerami-Nejad et al. (2009)
p444	*ble*	Selection	26	Vogt et al. (2005)
pMF332	RFP	Tagging	25	Freitag and Selker (2005)
pSV50	*BmlR*	Selection	25	Vollmer and Yanofsky (1986)
pMF331	RFP	Tagging	24	Freitag and Selker (2005)
pBARGEM7-2	bar	Selection	23	Pall and Brunelli (1993)
pRF-HU2	hph/T-DNA	Selection	23	Frandsen et al. (2008)
pBARGRG1	bar		22	Pall and Brunelli (1994)
pCCG::C-Gly::HAT::FLAG	FLAG	Tagging	22	Honda and Selker (2009)
pCCG::N-GFP	GFP	Tagging	22	Honda and Selker (2008)
pGFP::hph::loxP	GFP	Tagging	22	Honda and Selker (2008)
p480	ble	Expression	21	Vogt et al. (2005)
pBM61	*his-3* target	Selection	21	Margolin et al. (2007)
pCB1636	hph	Selection	21	Sweigard et al. (1997)
pCCG::N-FLAG::HAT	FLAG	Tagging	21	Honda and Selker (2009)
pCSR1	*csr-1*	Selection	21	Bardiya and Shiu (2007)
pME2892	*niaD::cre*	Expression	21	Krappmann et al. (2005)
pYFP	YFP	Tagging	21	Bardiya et al. (2008)
ANEp7	AMA *pyrG*	Selection	20	Storms et al. (2005)
ANIp7	*pyrG*	Selection	20	Storms et al. (2005)
p3xFLAG::hph::loxP	FLAG	Tagging	20	Honda and Selker (2009)
p502	hph	Expression	20	Vogt et al. (2005)
pBM60	*his-3* target	Selection	20	Margolin et al. (2007)
pCB1635	bar	Selection	20	Sweigard et al. (1997)
pCCG::C-Gly::GFP	GFP	Tagging	20	Honda and Selker (2008)
pJH19	DsRedT4	Tagging	20	Toews et al. (2004)

Abbreviations: *bar* bialophys resistance, *ben/BmlR* benomyl resistance, *ble* Bleomycin resistance, *hph* Hygromycin resistance, *BFP* Blue fluorescent protein, *GFP* Green fluorescent protein, *RFP* Red fluorescent protein, *YFP* Yellow fluorescent protein, *sur* Sulfonylurea resistance, *nat* nourseothricin resistance, *phleo* phleomycin resistance

1994). Similarly, as a group, the library construction plasmids and cosmids, such as pMOcosX (Orbach 1994), pSV50 (Vollmer and Yanofsky 1986), and cosmidAN26 (Taylor and Borgmann 1996) have been widely utilized in a variety of fungal systems including *Neotyphodium* (Spiering et al. 2005), *Magnaporthe* (Sweigard et al. 1995), *Cryphonectria* (Churchill et al. 1990), and *Aspergillus oryzae* (Sakai et al. 2008), as well as for patents such as antihypercholesterolemic drugs (Hutchinson et al. 2004) including lovestatin (Hutchinson et al. 2002).

Vectors for protein localization and purification have found wide application. The entire suite of fluorescent proteins for *Neurospora* (Freitag et al. 2001, 2004; Honda and Selker 2008; Freitag and Selker 2005; Bardiya et al. 2008) as well as *Aspergillus* (Yang et al. 2004; Toews et al. 2004) and a broad suite of plant pathogenic fungi (Maor et al. 1998) have had a strong impact. These vectors have garnered thousands of citations and are used to study topics as diverse as organelle distribution (Harris et al. 2005), cell-cell interactions (Fleißner et al. 2005), gene silencing (Shiu et al. 2006), signal transduction (Park et al. 2008), and plant- microbe interactions (Liu et al. 2012). Similarly, the protein purification vectors (Honda and Selker 2009) have been distributed nearly 150 times, as a group and are utilized by many groups studying *Neurospora* biology (Dettmann et al. 2013; Lewis et al. 2010).

Among the most highly utilized expression systems, the combination of the *N. crassa cpc-1* promoter and the *A. nidulans trpC* terminator was used for the pMOcosX cosmid vector (Orbach 1994) to drive a bacterial hygromycin phosphotransferase (*hph*) gene for selection in Neurospora and later mobilized to the convenient cloning vector pCB-hygro (Silar 1995) and together they have been distributed to over 80 clients. Similarly, the expression of bacterial hph gene by the Aspergillus trpC promoter coupled to its own *trpC* terminator was developed for transformation of *A. nidulans* (Cullen et al. 1987) and later moved to the pCSN42 and pCSN44 vectors (Staben et al. 1989) among others. pCSN44 was used notably as the source of the hygromycin resistance cassette in the construction of the *N. crassa* gene deletion set (Colot et al. 2006) of which over 35 complete sets as well as over 10,000 individual strains have been distributed to clients in 31 countries. The use of strains from this set have been cited over 500 times (Google Scholar, February 13, 2015) making this one of the most successful fungal gene expression systems for research use. Other promoter/terminator combinations have been utilized for different purposes including constitutive (Nakano et al. 1993) and inducible (Campbell et al. 1994; Ohrnberger and Akins 1995) promoters but a complete description of promoter utilization falls outside the scope of the present chapter.

6.5 Host and Production Strains

Most fungal plasmids have a limited host range, with some being limited to Sordariomycetes and others Eurotiales or Dothidiomycetes. Limiting factors include the ability of the promoter and terminator used to drive the selectable marker, and

also the ability of a selectable marker to be useful. For example, hygromycin is widely used in the Sordariomycetes while complementation of nutritional auxotrophies (e.g., pPyrG) or expanded nutrient utilization (e.g. p3SR2) are more typically utilized for Aspergilli (Table 3). Differences in codon utilization are also foundational to ability of vectors to be used in a specific host system. Beginning with homologous donor DNA, *N. crassa* was the first filamentous fungus to be genetically transformed (Mishra and Tatum 1973). This was accomplished by introducing wild-type DNA to an inositol auxotroph and characterizing the inositol-independent progeny for stability and the Mendelian nature of inheritance of the acquired character. Transformation of *Neurospora* using recombinant DNA techniques followed soon, and took advantage of the ability to complement quinic acid mutants (Case et al. 1979) and in this and related cases, the inability of the host strain to grow without an externally provided copy of the relevant gene is the paramount concern. Early systems complemented Arginine (Gomi et al. 1987), Tryptophan (Vollmer and Yanofsky 1986), and Glutamate (Kinsey and Rambosek 1984) axotrophs, although complementation of pyrimidine auxotrophs (Turner et al. 1997) in *Aspergillus* and Histidine auxotrophs in *Neurospora* (Aramayo and Metzenberg 1996) are the most commonly used auxotrophs in the modern genetics era. *Aspergillus* strains carrying the *pyrG* mutation include most commonly studied species, largely because *pyrG* mutants can be generated directly by selection (Van Hartingsveldt et al. 1987; Oakley et al. 1987) although the specific characteristics of different alleles varies both among species and even within species. For example, the *pyrG*89 allele in *A. nidulans* is both pyrimidine requiring and also cold-sensitive (Oakley et al. 1987). In *Neurospora*, targeting to the *his-3* locus is widely utilized, but to avoid high background of ectopic mutants, the *his-3* allele 1-234-723 in FGSC 6103 is used.

Transformation using positive selection (antibiotics) or nutrient utilization was developed and meant that wild-type strains could be transformed with vectors originally developed for model organisms, such as benomyl (Orbach et al. 1986), or acetamide (Wernars et al. 1985). This ability to transform wild-type strains meant that many strains could be utilized as transformation recipient. Perhaps because of a perceived economic value, many of these strains were only deposited into public collections after the advent of next generation genome sequencing (McCluskey et al. 2014b). The FGSC collection, for example, now includes *Aspergilus niger* protein production strains (e.g., FGSC A1513 (Pel et al. 2007)) as well as strains of *Trichoderma* (FGSC 10290, aka RUTC30 (Koike et al. 2013)) and *Phycomyces* (Chaudhary et al. 2013). The FGSC is not explicitly an industrial production collection and the US Department of Agriculture NRRL collection holds a wide diversity of industrial production strains including diverse taxa utilized in fermentation, food, and chemical industry.

Recently the availability of strains for targeted transformation has meant that many species can benefit from nearly perfect gene replacements (Ninomiya et al. 2004). Because these generate strains that are genetically modified, the exchange of these strains is limited by US Department of Agriculture regulations and release of

such strains into the environment by the Cartegna protocol (Cartagena protocol 2000). The FGSC holds such strains, called ku strains by analogy to human genes, for a number of species including *N. crassa* (Ninomiya et al. 2004), *A. nidulans* (Szewczyk et al. 2006), *A. niger* (Chiang et al. 2011), *A. fumigatus* (Wagener et al. 2008; da Silva Ferreira et al. 2006), *A. parasiticus* (Wagener et al. 2008), and *Magnaporthe* (Villalba et al. 2008), although these latter strains would require a permit for interstate movement or importation.

6.6 Other Molecular Genetic Resources

Because the FGSC is both a culture collection in the classical sense, and a research reagent repository in the modern sense, its holdings go beyond strains and even plasmids. Arrayed mutant sets of a variety of fungi include over 13,000 *N. crassa* (Colot et al. 2006) gene deletion mutants, as well growing collections such as 3,300 *Candida albicans* (Noble et al. 2010; Gerami-Nejad et al. 2009), nearly 4,000 *Cryptococcus neoformans* (Liu et al. 2008), and several hundred *A. nidulans* (De Souza et al. 2013) gene deletion mutants. Complementing these are arrayed *Pichia pastoris* strains expressing cell wall degrading enzymes (Bauer et al. 2006) and more recently over 10,000 gene deletion cassettes for use in *A. nidulans*. These all are a reflection both of the modernity of the FGSC and also the deep relationship between the FGSC and its research community. Holding genome and gene libraries during the 1980's and 1990's the FGSC contributed to the advance into genomics by sharing libraries openly (McCluskey 2013). Some of these were condensed into chromosome-specific sets (Brody et al. 1991), or had gene locations published in the FGSC catalog (McCluskey and Plamann 2006), adding value and increasing efficiency of screening. Other libraries were used in the assembly of the *N. crassa* genome (Galagan et al. 2003) leapfrogging ahead of other well developed filamentous fungal model systems. Similarly, cDNA libraries were highly sought for many years, with over 265 individual aliquots shared with FGSC clients. Since the FGSC asked only a trivial fee, and the cost of obtaining libraries commercially was over $1,500 per library, this represents a value of nearly half of a million US Dollars. Of 590 libraries sent to FGSC clients, no ordered libraries have been requested since 2009 and no cDNA libraries since 2013. Even these recent requests for cDNA libraries were solely for use as template for direct amplification of cDNA from the library pool rather than as living reagents for experimental or bio-prospecting research. This change reflects larger changes in biotechnology and are a firm demonstration that living microbe collection have a unique perspective on developments in biological research. Similarly, the overwhelming reliance on gene deletion (Knock-out) mutant strains generated by molecular genetic technology is reflected in the observation that most strains distributed by the FGSC in recent years are genetically engineered (McCluskey and Wiest 2011).

7 Conclusion

Living microbe collections have supported diverse research into basic and applied microbiology for many decades. Among these, genetic collections such as the FGSC have transitioned to the molecular era by providing resources including gene libraries, plasmids, gene deletion cassettes, DNA, reagents, and arrayed gene deletion mutants. The FGSC is a pioneer in this regard and these diverse reagents have tested the resources at many collections as they seek to implement approaches pioneered at the FGSC. The value of molecular reagents has led to the formation of dedicated plasmid repositories including Addgene (Table 1). Depositing resources in public collections can double the number of citations to a shared resource (Furman and Stern 2011) and the wide utilization of resources in the FGSC collection is strong testimony to this fact. The impact of genome sequence has yet to be felt and many collections are diligently working to implement genome centric informatics. While naturally synergistic with biodiversity collections, the investment in genome sequencing (and of DNA extraction and purification) is more than some diversity collections can immediately embrace. As *Neurospora* led the classical genetics era (Perkins and Davis 2000), it is again demonstrating its value as a model, now for how a living collection can adapt and provide continuing value to a changing research community.Acknowledgements This is article number 15-418-B from the Kansas Agriculture Experiment Station.

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Systems Biological Applications for Fungal Gene Expression

Gunseli Bayram Akcapinar and Osman Ugur Sezerman

1 Introduction

Although Systems Biology is a relatively new field, it has been expanding rapidly. With the omics era, as the sequencing technologies advancing, the availability of complete genome sequences resulted in a huge amount of data which is needed to be analyzed and interpreted using a holistic approach provided by the field of Systems Biology (Kitano 2002). Parallel to the advancements in the computational power and with the development of powerful algorithms and software; we are gaining new insights to our understanding of the organisms, processes within the organism and the interactions of the organisms with their environment.

Fungi have been used by the industry for the production of various antibiotics, chemicals and enzymes. The qualifications of certain species of fungi, especially filamentous fungi, as microbial cell factories which are capable of secreting high levels of proteins made them attractive targets as recombinant protein production hosts (Nevalainen and Peterson 2014). This further led to the development of different gene expression systems for these filamentous fungi (Su et al. 2012). Moreover, novel gene expression platforms have been developed extensively for the yeasts such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha* (Cregg et al. 2000; Mattanovich et al. 2012).

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The holistic approach provided by systems biological studies are now being used to extend our understanding of the fundamental processes which control expression of the genes and the factors that affect heterologous protein production. Systems biology has provided an integrative approach to overcome the major bottlenecks of heterologous protein production in fungal hosts. These challenges include loss of expression; folding, aggregation, secretion of the protein product; posttranslational modifications such as glycosylation and proteolytic processing (Deshpande et al. 2008; Nevalainen and Peterson 2014; Puxbaum et al. 2015). Although these studies has begun in the recent years, they provide invaluable insights for the understanding of the metabolic networks and regulation of gene expression in fungi.

1.1 Construction of Fungal Knowledgebases for Metabolic Engineering in Fungi

In the recent years, genome scale metabolic models has been constructed for a number of filamentous fungi such as *Aspergillus niger*, *Trichoderma reesei*, *Neurospora crassa* for the model guided metabolic engineering (Vongsangnak et al. 2013; Dreyfuss et al. 2013; Pitkänen et al. 2014; Brandl and Andersen 2015). The metabolic fluxes are known to be regulated by gene expression, post-transcriptional and post-translational events. Changes in the transcriptome or proteome are often reflected in the changes in metabolome (Tan et al. 2009). The establishment of fungal knowledgebases based on fungal metabolic networks not only reduces the time for metabolic engineering since an *in silico* prediction and testing of the potential candidates decreases the number of *in vivo* targets for metabolic engineering, but also provides us with the information on the components of the metabolic pathways which could be involved in or important for protein expression. In this context, Deshpande et al. analyzed the N-linked and O-linked glycan synthesis pathways of twelve different medically, agriculturally, industrially important filamentous fungi. In this study, an integrated approach composed of comparative genomics and systems biology was used to form a knowledgebase for future engineering of the glycosylation pathways for recombinant protein production. They followed a systems biological approach to relate the results produced by the comparative genomics to the previously published high confidence information on the enzymes and fungal glycan structures in the literature. They have performed a comparison of the known information on glycosylation derived from the genome, proteome and glycome of the 12 filamentous fungi using *S. cerevisiae and S. pombe* as reference organisms and evaluated their findings in relation to the heterologous production of glycoproteins in filamentous fungi (Deshpande et al. 2008). The results of this integrative analysis revealed that N-glycosylation pathway in all filamentous fungi in the cytoplasm and endoplasmic reticulum was highly conserved and the high mannose pathway of *S. cerevisiae* was used at the final stages of the N-glycan synthesis in these filamentous fungi with the reduction in the level of glycan mannosylation. The extent of N-glycosylation in filamentous fungi was found to be not limited to mannosylation by the presence of highly specialized N-glycan structures with

galactofuranose residues, phosphodiesters, and other insufficiently trimmed structures. Although O-linked glycosylation was also found to be evolutionarily conserved in these filamentous fungi as in *S. cerevisiae* with the presence of several mannosyltransferases, the presence of highly variable and diverse O-linked glycans was also confirmed. As the technology develops further, as the quality of the available data increases, more elaborate and specific metabolic networks could be established in the knowledgebase which would further provide a powerful toolbox for the identification of unknown components and elaboration of underlying mechanisms of interaction between these components.

1.2 Systems Biological Studies for Analysis of Fungal Transcriptomes, Proteomes and Secretomes

Most of the systems biological studies on fungi currently focuses on the analysis of fungal omics data created by the comparison of genomes, transcriptomes, proteomes and secretomes of different mutants of a fungus or a fungus that is grown under different conditions such as in the presence of cellulose or under carbon starvation.

Dvir et al. focused their systems biological approach towards 5′ UTR regions in an attempt to identify the effects of sequence substitutions in these regions on the protein expression. It has been known that 5′ UTRs are important for control of the eukaryotic protein expression. They encode a repertoire of cis-regulatory elements. Most of these elements were known to alter the efficiency of translation whereas the others are known to have effects on translation, transcription or mRNA degradation thereby controlling the levels of protein produced by the host organism. Although these elements were identified, the rules by which these elements show their effects were not very well known. Dvir et al. investigated how the sequence substitutions in 5′-UTR sequence of an mRNA affects the protein expression in the yeast *Saccharomyces cerevisiae* by developing a predictive model which is capable of explaining the variation in the protein expression (Dvir et al. 2013). A library of mutants which has differences in the sequences in the ten base pairs, upstream of the translational start site of a yellow fluorescent protein as the fluorescent reporter in yeast was constructed. Over 2000 unique sequence variants were generated and the effects of these sequence variations on the protein abundance was measured through a high throughput sequencing methodology combined with fluorescence measurements. They have also included mcherry reporter under the control of *TEF2* promoter and used flow cytometry to measure yellow fluorescent protein produced by each mutant to mcherry fluorescence thereby assessing that the changes observed in the level of the proteins was due to the sequence differences in 5′ UTR region. Their results have proven that even a few base pairs change in the 5′ UTR region could lead to drastic differences in the protein levels. They have observed up to seven fold variation in the protein levels due to sequence variations. They have also shown that the protein abundance and mRNA levels are highly correlated by quantitative PCR. Their study also exhibited that mRNA secondary structure was another parameter which

was also important and highly correlated to protein abundance. Moreover, out of frame upstream AUGs which also belong to the cis regulatory elements were shown to attenuate protein expression thereby affecting translation. For the quantitative model generated based on the results of this research, a list of potential predictors such as mRNA secondary structure, in-frame and out of frame upstream AUGs, non-AUG start codons, predicted nucleosome occupancy, sequence composition statistics, and arbitrary k-mer sequences was formed which was followed by a cross validation strategy where the parameters were estimated from a training set formed from a subset of 5′ UTR variants and these were used to predict the protein levels of the other 5′ UTR sequence variants. A subset of features were predicted from the training set and a linear model was formed and then this model was used to predict the protein abundance of the test set and finally the experimental levels of protein abundance was compared to the model based calculations. For the influential feature selection and minimum number of features needed for robust prediction, a combined regression model was also generated. With these models almost 70 % of the variation in the protein expression could be explained. Their study provided a model which would form a basis for fine tuning of protein expression using sequence manipulations in the 5′ UTR region.

In a recent study, Cesbron et al. used White collar complex (WCC) and *frq* promoter of *Neurospora crassa* to obtain a natural light inducible gene expression system (Cesbron et al. 2015). WCC functions as the transcription activator and blue-light photoreceptor. The systems biological part of the study identified a large number of promoters which are triggered by a single short light pulse (LP) that activated WCC. In this process, it has been known that light activated WCC binds to a light responsive element (LRE) and activates *frq* promoter and activated WCC could rapidly become inactive by phosphorylation and in constant light, activated WCC goes through photoadaption with the small photoreceptor VVD. Based on this knowledge, the authors constructed a reporter system using luciferase with the *frq* and *vvd* promoters in *N. crassa* where they have induced these promoters with LP and analyzed the relative response of these promoters using a population approach for the biochemical analysis of a light induced promoter. Cesbron et al. found out that the *frq* promoter is refractory. Genome wide analysis of light induced transcription using Polymerase II Ser-5 ChIP-seq, revealed 71 promoters that were transiently and rapidly induced by low LP. The response of these promoters to high LP was found to vary over a broad range which suggested that the transcriptional refractoriness is widespread among light inducible promoters and dependent on the promoter architecture. This study also reveals the possibility of the transcriptional bursting and the following refractory period in *N. crassa* as a mechanism which might prevent potentially harmful overexpression of the induced genes. It has been known that secretion of proteins poses as another challenge for the efficient production of recombinant proteins in fungi. Liu et al. performed a genome scale analysis of the protein secretion system of *Aspergillus oryzae* (Liu et al. 2014). This study has provided a detailed analysis of the secretory machinery of *A. oryzae* which has been extensively used for the production of industrially important enzymes such as amylases, lipases, proteases based on the reported secretory model of the yeast *S. cerevisiae*. The secretory proteins were mainly identified by sequence comparisons

to the reference model *S. cerevisiae* and the additional secretory proteins were identified with reference to the reported components in other closely related fungi such as *Aspergillus niger* and *Aspergillus nidulans*. This comparative study was followed by the transcriptomic analysis of alpha amylase overproducing strains with varying secretory capacities. The transcriptional responses of these strains to various levels of alpha amylase production was evaluated using microarray chips. The transcriptomics analysis indicated that the expression of the genes involved in Endoplasmic Reticulum (ER) associated secretory processes, such as protein N-linked glycosylation, ER translocation and folding, signal peptide processing, ER to Golgi and the retrograde Golgi to ER vesicle trafficking, were significantly increased. On the other hand, expression of the genes involved in amino acid biosynthesis was found to be down-regulated. A total of 389 proteins were identified in the secretory machinery of this fungus. Based on the results of this study, secretome of the *A. oryzae* was established and the effects of amylase production on the secretome was analyzed. Systems biological approach to the analysis of the secretory pathway of *A. oryzae* in response to high levels of protein secretion, improved our understanding of the secretory capacity of this organism and provided a library of secretory proteins which could be engineered and manipulated further for homologous and heterologous production of proteins in *A. oryzae* in the future. Glycoside Hydrolases are an important group of enzymes produced by fungi. Some glycosyl hydrolases are involved in lignocellulosic biomass degradation. Improving the production of these enzymes presents a great potential for reducing costs in biofuel production. Therefore, a deeper understanding of the functions, interactions and regulation of these proteins at the transcriptional, translational and post-translational level is crucial. Systems biology could provide new insights for this understanding through combination and analysis of multi level omics data.

Within this context, Xiong et al. performed profiling of the proteome and phosphoproteome of *N. crassa* grown on cellulose, sucrose or under carbon starvation (Xiong et al. 2014). The profiling was performed using isobaric tags for relative and absolute quantification (iTRAQ) based LC-MS/MS analysis. Transcriptional part of the study revealed the occurrence of extensive post-transcriptional regulation events in response to the presence of the cellulosic substrates. Many of the genes involved in C-compound and carbohydrate metabolism were found to be upregulated when the organism was grown on Avicel. Moreover, a comparison of the phosphorylation levels under different conditions revealed several differentially phosphorylated amino acid residues. Two of these phosphorylation sites were identified to be on CLR1 which is a major transcriptional activator for the transcription of cellulase genes and CBT1, which is a cellobionic acid transporter. Mutational analysis of these phosphorylation sites in CLR1 and CBT1 revealed that only mutation of CBT1 phosphorylation site had an effect through the increase of its transport capacity. A quantitative analysis of the proteome and phosphoproteome of *N. crassa* provided extensive information on how the cellular machinery respond to carbon starvation and induction by cellulose which would in turn help us to have a deeper understanding of lignocellulose degrading enzyme production and might provide us with another knowledgebase for strain improvement.

Similar studies with transcriptional and proteomic profiling were performed with different organisms such as *Aspergillus fumigates* (Miao et al. 2015), *Myceliophthora thermophile* (Kolbusz et al. 2014), *Trichoderma harzianum* (Horta et al. 2014), *Trichoderma reesei* (Häkkinen et al. 2012) grown on different cellulosic substrates.

Another study was performed by van Munster et al. (2015) with *Aspergillus niger* which is used for the production of industrially important enzymes and organic acids, in an attempt to identify the functions of several Glycoside Hydrolases under carbon starvation conditions (van Munster et al. 2015). In the framework of this study, van Munster et al. used two deletion mutants (Δ*flbA* and Δ*brlA*, Orthologs of *BrlA*, a transcriptional activator and *FlbA*, a central player in the same regulatory network involved in asexual sporulation in *A. nidulans* were deleted in these mutants) which exhibit aconidial phenotype during growth on a plate and during submerged fermentations under carbon starvation conditions. During its life cycle either in the industrial fermentations or in its natural habitat, *A. niger* is known to experience carbon starvation. As a response to this condition, autolysis and sporulation was observed. It has been known that carbohydrate degrading enzymes are involved in these responses to carbon starvation especially during the cell wall remodelling. van Munster et al. employed a comparative systems biological approach to give an overview of the roles of carbohydrate degrading enzymes under carbon starvation conditions using transcriptomics, proteomics, enzyme activity and cell wall composition data. Understanding the underlying mechanisms of processes such as autolysis and sporulation and the proteins involved in the responses of the organism to carbon starvation is crucial not only to understand the life cycle of the organism but also to understand the bottlenecks of the industrial protein production so that this phenomenon could be used to further improve the industrial protein production by this host. As a result of comparative profiling of wild type and two deletion mutants van Munster et al. has shown that in all three strains, glycoside hydrolases are involved in cell wall recycling. In the deletion mutants, transcription of alpha mannosidase associated with glycosylation and mannanases which are involved in the degradation and modification of alpha-1,6-mannan in the cell walls of this organism were found to be increased.

Semi quantitative proteomic analysis of the culture filtrates of Δ*flbA* strain and wild type revealed a similar profile with a few differences such as the detection of increased quantities of endo-polygalactouronase PgalI and cellulose EglB in the deletion mutant during exponential growth and carbon starvation phases. During the exponential growth phase growth rate of Δ*flbA* strain and glycogen synthesis were found to be lower than the wild type and the other deletion mutant. Additionally CWI pathway transcription was found to be upregulated in Δ*flbA* mutant.

The proteomics analysis revealed the production of an increased number of proteins and carbohydrate degrading enzymes in Δ*flbA* strain in response to carbon starvation which makes it a suitable target for industrial strain improvement. For the Δ*brlA* strain, no significant differences were found in comparison to the wild type strain in terms of growth are, autolysis and cell wall composition but the transcriptomic profiling revealed a number of genes that code for carbohydrate active enzymes

that is strictly controlled by *BrlA* for expression which are probably important for mature conidiophore formation. The comparative analysis of proteome, transcriptome, carbohydrate active enzyme activity and cell wall profile of deletion mutants of *A. niger* with respect to wild type using a systems biological approach provided us with a deeper understanding of the changes in the cell wall during sporulation and revealed carbohydrate active enzyme coding genes which are controlled by *BrlA and* another set of enzymes strongly upregulated during sporulation. Understanding the fungal sporulation which requires a significant amount of carbon and energy is crucial for further manipulation of these processes for strain improvement.

Liu et al. (2013) employed a systems biological approach to understand the differences between the cellulase and hemicellulase hyper-producer strain of *Penicillium decumbens* 114 which is generated through repeated mutagenesis and screening as a result of long term strain improvement (JU-A10-T) and the parent strain which was isolated in 1979 (114-2) (Liu et al. 2013) and to understand the underlying molecular mechanisms behind the strain improvement which would in turn provide basis for the engineering of the strain for the further improvement of the lignocellulolytic enzyme production. For this purpose, a global comparison of the genomes of the wild type and the mutant strains were performed and the strains were found to share 9599 proteins (average amino acid identity, 99.55 %) which include 6699 proteins (average amino acid identity 100 % over the full length) which indicate that most of their proteins were homologous. The proteins that show less homology were analyzed and most of them were found to be associated with transcription factor activities. Approximately 30 % of the predicted transcription factors contained amino acid differences between the two strains. A comparison of the cellulase and hemicellulase genes revealed that the mutant strain did not contain three adjacent genes that encode for hemicellulase. A sequence comparison of the lignocelluloytic enzyme encoding genes identified 26 aminoacid differences in nonconserved regions of 16 enzymes between two strains. The secretome profiling of the two strains revealed that the secretion of lignocelluloytic enzymes was increased, whereas the amylase and protease secretion has shown a reverse trend in the mutant. Moreover, in the mutant, homolog *of T. reesei cre1* gene, *creA* which encode the main carbon catabolite repressor was found to have frameshift mutation. As a result the mutant strain exhibited a carbon catabolite repression resistant expression of lignocelluloytic enzymes. Further analysis of the promoter regions of 11 cellulase genes revealed 106 sequence differences. An analysis of the mutation sites for CreA binding has shown that the mutation in the promoter region of Cel7A had an effect on putative CreA binding site. Moreover, the sequence of the transcription factor *AmyR* which controls expression of amylase was found to be identical in both strains but the transcription was down regulated in the mutant. Deletion of this gene was found to decrease amylase activity and increase FPase activity. Both transcription and deletion data indicate that this transcription factor has a negative effect on cellulase expression in the parent strain. Moreover transcriptomic profiling indicated that the significantly up regulated genes in the mutant were the ones that encode extracellular polysaccharide hydrolases. Genes involved in pentose phosphate pathway, aminoacid biosynthesis, folding and ribosomal protein genes were upregulated in the mutant. System bio-

logical profiling of *P. decumbens* wild type and hyper producer mutant identified the mutations in the regulatory elements that are responsible for the hyperproduction, mutation in CreA as a cause for carbon catabolite repression resistant production, identified AmyR down regulation as a cause for hyper production in the mutant strain. Once the mechanisms for hyper production are understood, it would form a basis for the engineering and manipulation of the organism for either homologous or heterologous production of proteins.

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Fungal Biotechnology for Industrial Enzyme Production: Focus on (Hemi)cellulase Production Strategies, Advances and Challenges

Loreta Gudynaite-Savitch and Theresa C. White

1 Introduction

Enzymes found in nature have been used since ancient times in the production of foods, such as cheese, sourdough, beer, wine and vinegar, and in the manufacture of commodities such as leather, indigo and linen. All these processes relied on enzymes naturally produced in nature by a variety of microorganisms. The development of fermentation processes allowed large scale manufacture of enzymes as purified and well-defined preparations. Further improvements in manufacturing processes and enzyme properties followed the development of modern biotechnology. Random and directed mutagenesis, as well as modification of enzyme-producing strains, enabled higher protein productivity and improved enzymatic properties of industrially important enzymes.

At present, about 4000 enzymes are known. Approximately 200 enzymes of microbial origin are used commercially; however only a small number of these are produced on a truly industrial scale. Representative examples of these enzymes and their industrial applications are summarized in Table 1. The majority (about 75 %) of industrial enzymes are hydrolytic in action—carbohydrases, proteases and lipases—used for the degradation of various natural substances. Currently, proteases

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Table 1 Examples of enzymes used in various industrial fields and their applications (Davis and Boyer 2001; Kirk et al. 2002; Whitehurst and van Oort 2010; Li et al. 2012; Gupta et al. 2011; Demuner et al. 2011)

Industry	Field	Enzymes	Application/benefits for industrial process
Food processing	Dairy	Proteinases, peptidases	Accelerate cheese ripening
		Lipases	Cheese ripening, modification of milk fats
		Lysozymes	Nitrate replacer for washed-curd cheeses
		β-Galactosidases, lactases	Lactose removal in milk
		Proteases	Milk clotting, infant formulas, flavor
	Baking	Amylases	Increase bread volume and softness, improve crumb structure, color and flavor
		Xylanases	Dough conditioning: improvement of dough-handling properties, reduce water content in pasta
		Lipases, phospholipases	Increase dough strength and stability (emulsifier)
		Oxidoreductases	Increase gluten strength, increase dough volume, texture and stability
		Proteases	Modify gluten quality in biscuit, cookies and crackers
		Transglutaminases	Improve gluten strength in pasta
	Beverage (juice)	Pectinases	Degradation of pectin in fruit cell walls to decrease viscosity
		Amylases, glucoamylases	Starch breakdown for clarification of cloudy juice
		Laccases	Degradation of lignin for clarification of juice
	Meat and fish	Proteases, peptidases	Meat tenderization, production of fish protein hydrolyzates, viscosity reduction, skin removal and roe processing
		Lipases	Conversion of insoluble to soluble fatty acids, flavor formation in sausage products
		Transglutaminases	Flavor enhancing by glutamine to glutamic acid conversion
	Beer and wine	Amylases	Hydrolyzing starch to reduce viscosity (beer)
		Glucanases	Hydrolyzing glucans to reduced viscosity and improve filterability (beer)
		Cellulases, xylanases	Accelerate cell wall digestion in grains (beer)
		Pectinases	Breaking down pectin to accelerate pre-fermentation stage and enhance clarification (wine)

(continued)

Table 1 (continued)

Industry	Field	Enzymes	Application/benefits for industrial process
Animal feed		Phytases	Degrading phytic acid to release phosphorus
		Cellulases, xylanases	Degrading fibers to increase digestibility, improve silage production
		β-Glucanases	Removal of β-glucans, reducing viscosity and improving digestibility
Starch processing (including food, pharma and ethanol)		Amylases	Hydrolysis of starch to sugars in gelatinization process
		Glucoamylases, pullalanases	Saccharification of straight-chain oligosaccharides
		Glucose isomerases	Isomerization of glucose to fructose
		Cyclodextrin-Glucosyltransferases	Increasing the number of branch points in cyclodextrin production
Technical applications	Pulp and paper	Cellulases	Fiber modification (improving softness), de-inking in paper recycling
		Xylanases	Enhancing pulp bleaching process efficiency
		Lipases	Pitch control in pulping process
		Laccases	Bleaching and delignification
	Textile	Cellulases	Denim washing (biostoning), removing surface fuzziness (biopolishing)
		Amylases	Removal of starch coating (desizing)
		Pectinases	Destabilizing outer cell layer to improve fiber extraction (scouring)
		Laccases	Bleaching
		Peroxidases	Removal of excess dye
	Detergents	Proteases	Protein stain removal
		Lipases	Lipid stain removal
		Amylases	Starch stain removal
		Cellulases	Color clarification, softening, anti-redeposition (fiber modification)
	Biodiesel and bioethanol	Lipases	Transesterification of triglycerides (biodiesel)
		Cellulases	Cellulose hydrolysis in lignocellulosic ethanol production
		Xylanases	Viscosity reduction, degradation of hemicellulose
		Laccases	Digestion of lignin waste
	Leather	Proteases, lipases	Removal of unwanted proteins and fats during soaking and liming process

(continued)

Table 1 (continued)

Industry	Field	Enzymes	Application/benefits for industrial process
Organic synthesis		Acyl transferases (lipases, esterase, peptidases, aminases)	Formation of wide range of anime and ester bonds to obtain chiral building block in asymmetric synthesis, peptide synthesis
		Carbohydrate processing enzymes (glycosidases, oxidases)	Oligosaccharide synthesis
		Other hydrolytic enzymes (epoxidases, nitrilases)	Enatioselective hydrolysis, biodegradation of nitriles
		Reducing enzymes (alcohol dehydrogenases, lactate dehydrogenases)	Reduction of C=O, C=N bonds
Cosmetics and personal care		Amyloglucosidases, glucose oxidases, peroxidases	Antimicrobial activity in personal care products (e.g., toothpaste, mouthwashes)
		Oxidases, peroxidases	Hair dyeing
		Proteases	Gentle peeling effect in skin care products

are the dominant industrial enzyme because of their use in the detergent and food industries. The second largest group is the carbohydrate-degrading enzymes such as cellulases, xylanases, amylases, and pectinases. These enzymes are widely used in technical applications in the pulp and paper, textile, detergent and fuel alcohol industries as well as for starch, food, and feed processing. A large number of microorganisms, including bacteria, yeast and filamentous fungi, are used to produce different groups of enzymes used in industrial applications. Selection of particular strains and enzymes remains a tedious task, particularly when enzyme yields and production costs must be economically and commercially feasible. Meeting these criteria depends on number of factors including the nature of the substrate, industrial enzymatic reaction conditions and properties of particular enzymes.

Filamentous fungal cells are characterized by an extraordinary ability to secrete large amounts of proteins, including enzymes, metabolites, and organic acids into their growth medium making them very attractive for industrial enzyme production. Generally, carbohydrase enzymes, e.g., cellulases, xylanases, pectinases, are produced by submerged liquid cultures of filamentous fungi. *Trichoderma* spp. and *Aspergillus* spp. have been most widely used for production of these enzymes. In addition, the industrial importance of fungal cells is not limited to their wide range of secreted enzymes but also includes the development and commercialization of new products derived from genetically engineered fungal strains expressing modified homologous and/or heterologous enzymes.

2 Industrial Applications of Hydrolytic Enzymes: An Overview

2.1 Enzymes in Food Processing

Today's consumers demand higher quality of their foods in terms of taste, texture and natural flavor. As shown in Table 1, the application of wide variety of enzymes in the food industry ranges from texturizing and modification of nutritional substances to flavoring and product appearance. Food enzymes applied can be divided into two groups: (i) processing aid enzymes, which are applied upstream from the final product; and (ii) enzyme additives, which are included in the final product for improved nutritional or other qualities. The majority of food enzymes, including enzymes produced by filamentous fungi, are used as processing aids in food industry.

Carbohydrate degrading enzymes (cellulases, xylanases, pectinases) are used for extraction and clarification of fruit and vegetable juices to increase the yield of juices. They also improve cloud stability and texture and decrease viscosity of nectars and purees from tropical fruits. Fungal laccases, which are a class of polyphenol-oxidizing enzymes, aid in the cross-linking and removal of polyphenols in fruit juice, which improves clarification (Grassin and Coutel 2010).

A variety of hydrolytic enzymes—including proteases, lipases and carbohydrases—are widely used in baking industry. Wheat proteins, particularly glutens, are important factor in bread quality. In the dough, the gluten network determines the visco-elastic properties and gas retention of the dough during the bread-making process. Degradation of gluten proteins by proteases has an immediate effect on the covalent interactions in the gluten network; thus, various proteases are used to treat "bucky" dough for improving elasticity (van Oort 2010). In addition, lipases are used to degrade wheat lipids in replacement of traditional emulsifiers (e.g., DATEM and SSL) and provide the stabilization of gas cells in dough (van Oort 2010). Water-binding capacity and retention in the starch and hemicellulose fraction of the bread are critical for maintaining softness and elasticity of dough. Treatment with α-amylases and xylanases improves dough properties and final bread properties (Kirk et al. 2002).

Beer brewing is based on the action of enzymes activated during malting and fermentation. Malting of barley depends on seed germination and activation of barley seed enzymes that hydrolyze the starch reserve, cell wall polysaccharides and seed proteins. Application of exogenous enzymes such as amylases, glucanases, cellulases and xylanases significantly accelerates, or may even facilitate the bypass of, the malting process and increases the yield and quality of fermented products. Similarly, the application of hydrolytic enzymes in wine making improves color extraction, clarification, filtration, wine quality and stability (Gupta et al. 2011).

Use of enzymes, particularly amylases, in starch processing represents various segments of the food, pharmaceutical and technical industries and will be discussed in more details in Sect. 2.3.1.

2.2 *Enzymes in Feed Industry*

The use of enzymes in animal nutrition has an important role in current farming systems. Feed enzymes increase the digestibility of nutrients, leading to greater efficiency in feed utilization. Another benefit is positive impact on the environment by allowing better use of natural resources and reducing faecal nutrient release to the environment.

Phosphorus, an important macronutrient for animal growth, occurs naturally in many feed ingredients but only parts of it is available to monogastric animals because phosphorus in bound in phytic acid. Inorganic phosphorus supplements are produced from phosphate rock in an energy consuming process and the release of undigested phosphorus into the environment via livestock manure leads to nutrient enrichment of water bodies. Addition of phytase enzymes hydrolyses the phytic acid and releases phosphorus in feed, reducing the need for inorganic phosphorus supplementation as well as decreasing the amount of phosphorus release in manure (Jegannathan and Nielsen 2013). Due to the positive effects on phosphorus uptake and enhanced bioavailabilities of Ca, Zn and Fe from feed (Lei and Stahl 2000), phytases have grown to become the largest enzyme segment in the animal feed industry. Phytases from *Aspergillus niger* and *Aspergillus oryzae* are most commonly used, but other fungal phytases with 4–50 fold higher specific activities have also been identified (Lassen et al. 2001). More effective phytase enzymes have been developed by site directed mutagenesis (Tomschy et al. 2000). Thermal stability of phytases is an important property in order to apply this enzymes in pelleted feed products, were the enzymes must to survive high temperatures (above 80 °C) during pelleting. One of the first rationally-designed, thermally-stabilized *Aspergillus* phytase was constructed using a "consensus phytase" approach based on homologies among various phytases (Lehman et al. 2000).

Non-starch polysaccharide fibers such as xylans and cellulose are present in cereal cell walls and are an energy-rich constituent of animal feed. However, their complex polymer structure makes them indigestible to monogastric animals. Addition of non-starch polysaccharide degrading enzymes (xylanases, cellulases, β-glucanases) increases the dietary value of animal feed, thereby reducing the feed requirement per unit of animal product. Moreover, pre-digestion of feed with hydrolytic enzymes reduces the level of organic material released to the land (Li et al. 2012). Cellulases, xylanases and pectinases are also used to improve silage production for cattle feeding. This process generally includes heat treatment to inactivate potential viral and microbial contaminants. Therefore, application of thermostable cellulases in feedstock production has the potential to facilitate combination of heat treatment and feed transformation in a single step.

Similarly, proteases are applied to increase the digestibility of feedstock proteins, e.g., in poultry feed. Enzymatic treatment substantially reduces the amount of non-protein nitrogen supplement required in animal diets and decreases the emission of ammonia, nitrous oxide and excretion of urea into the environment (Jegannathan and Nielsen 2013).

Overall, enzymatic degradation of complex components in the feed increases the energy and nutrient value of feed and reduces emission of organic pollutants into the environment. Thus the global market for feed enzymes is a promising and growing segment of the enzyme industry.

2.3 Technical and Other Applications of Enzymes

Technical enzymes are typically used as bulk enzymes in detergent, textile, pulp and paper, organic synthesis, starch processing and biofuel industries. Commercially available enzymes used in these areas include amylases, proteases, lipases, cellulases, xylanases, and catalases, etc. Among these, α-amylases are the most versatile enzymes in the industrial sector.

2.3.1 Amylases

Amylases are of great significance in present day biotechnology with applications ranging from the conversion of starch to sugar syrups for food industry, the production of cyclodextrins for the pharmaceutical industry to the technical applications in textile, bioethanol and pulp and paper industries. Amylases are classified as α- and β-amylases according to the anomeric type of sugars produced by the enzyme reaction. α-Amylases are widely distributed secretory enzymes and represent one of the most important forms of industrial amylases. Although α-amylases are universally distributed throughout the animal, plant and microbial kingdoms, the enzymes from fungal and bacterial sources are dominant in industrial applications.

For decades, enzymes such as barley malt and microbial α-amylases have been widely used in the baking industry. Supplementation of flour with α-amylase enhances the rate of fermentation and reduces the viscosity of dough, resulting in increased volume and improved texture, and also generates additional sugar in the dough, which improves taste, crust color and toasting qualities (Gupta et al. 2003). Fungal α-amylases were first approved as bread additives in the US in 1955 and in the UK in 1963 (Gupta et al. 2003). Supplementation of flour with exogenous fungal α-amylases is presently used all over the world in the bread-making industry.

Despite the long history of the use of amylases in the brewing and baking industries, the major food industry market for α-amylases lies in the production of starch hydrolysates such as glucose and fructose, e.g., high fructose corn syrups. Because of their high sweetening properties, these syrups are used in high quantities in the beverage industry. In contrast to the brewing and baking industries, where amylases of intermediate temperature stability are used, the syrup production process requires the use of highly thermostable α-amylases for starch liquefaction (van der Maarel 2010). Similarly, the production of bioethanol for biofuels from starch, e.g., corn ethanol, involves the same liquefaction process and requires thermostable amylase enzymes (Bothast and Schlicher 2005).

Cyclodextrins are cyclic α,1-4 linked oligosaccharides mainly consisting of 6, 7, or 8 glucose residues (α-, β-, or γ-cyclodextrin, respectively). Cyclic rings of cyclodextrins are arranged in such manner, that central cavity is hydrophobic. This enables cyclodextrins to form inclusion complexes with a variety of hydrophobic guest molecules leading to various applications of cyclodextrins in analytical chemistry, agriculture, biotechnology, pharmacy, food and cosmetics. For the industrial production of cyclodextrins, starch is first liquefied by a heat-stable α-amylase and then the cyclization occur with a cyclodextrin glycosyltransferase from *Bacillus macerans* (van der Maarel et al. 2002).

Modern textile production processes involves a considerable strain on the warp yarn during weaving; thus, a starch protective layer (sizer) is applied to the yarn to prevent breaking. The de-sizing of starch-sized textiles is achieved by the application of α-amylases, which selectively remove the size and do not attack the textile fibres. After enzymatic treatment, the starch which has been cleaved into water soluble dextrins can be easily removed by washing.

The use of α-amylases in the pulp and paper industry is in the modification of starches for coated paper. As for textiles, sizing of paper is performed to protect the paper against mechanical damage during processing. It also improves the quality of finished paper. However, the viscosity of natural starch is too high for paper sizing and it is adjusted by partially degrading the polysaccharide with α-amylases (Gupta et al. 2003).

2.3.2 Other Carbohydrases in Industrial Applications

Enzymes in Textile Industry Raw cotton undergoes various processes such as scouring, bleaching, polishing and dyeing before it is converted into fabric. These processes consume large amounts of energy, water and chemicals, making the textile industry a resource-consuming and polluting industry (Santos et al. 2007). Therefore, new enzymatic activities have been introduced in textile processing and in the manufacturing of denim. One of the most energy- and water-intensive steps in the processing of cotton is the scouring step—the removal of remaining cell-wall components from cellulose fibers at high temperatures and under strong alkaline conditions. However, a pectinase enzyme-based process, which can be performed under much lower temperatures and with less water, can replace the harsher chemical process (Tzanov et al. 2001). Following the introduction of pectinases for scouring, enzymes have now been introduced into most of the major steps in the manufacturing of cotton textiles. For example, cellulases (endoglucanases) have been successfully used for the biostoning of denim, replacing pumice stone and reducing damage of fibers. Cellulase preparations rich in endoglucanases are also used for biopolishing and biofinishing for enhanced look and feel of fabrics by reducing the tendency for pill formation and providing a cleaner surface structure with less fuzz through the partial detachment of microfibrils on the surface of fabric (Gupta et al. 2011). Although both enzyme applications involve the same enzymatic activity in modification of cellulose fibers, the conditions for each application are

different and require enzymes active in either neutral pH or acidic pH for biostoning and biopolishing, respectively.

Enzymes in Pulp and Paper Industry The traditional pulp and paper production process is based on chemical and mechanical processing of wood or recycled material and creates considerable pressure on the environment. The introduction of carbohydrase enzymes into biomechanical pulping processes resulted in substantial energy savings (20–40 %) during subsequent refining and improvements in handsheet strength properties (Bhat 2000). The most important enzymes used in biomechanical pulp and paper processing are cellulases, laccases, xylanases and lipases. While cellulases and lipases are primarily applied in the processing of paper, xylanases and laccases are more commonly used in the bleaching and delignification processes. The key target of cellulase application is the softening and modification of fibers prior mechanical processing. Cellulases in deinking process also soften the recycled pulp and facilitate the release of ink, saving on processing time and deinking chemicals (Demuner et al. 2011). Lipases are used for controlling formation of pitch (an insoluble deposit from lipophilic materials present in wood) and presently replace the conventional application of cleaning agents and talc. In the paper bleaching process, xylanases are used to degrade xylan and facilitate lignin removal, while laccases, one class of lignin-degrading enzymes, replace the chlorine and alkali chemical treatment (Jegannathan and Nielsen 2013). Pulp and paper processing are usually performed at high temperatures and alkaline conditions; thus, great efforts are applied to increase thermostability and alkali-tolerance of the enzymes used in this industry (Demuner et al. 2011).

Enzymes in Biofuel Production Fossil fuel depletion, growing energy prices and environmental concerns of fossil fuel usage have made biodiesel and bioethanol attractive alternative fuels. Conventional biodiesel production from vegetable oil and alcohol consumes high amounts of heat and chemicals, while side reactions lead to soap formation. Immobilized lipase can degrade triglycerides and is a promising alternative biocatalyst in the transesterification process for biodiesel production leading to energy and chemical savings as well as avoiding soap formation (Noureddini et al. 2005).

Enzymatic saccharification of lignocellulosic material such as agricultural and forestry residues by cellulases for bioethanol production is perhaps the most popular application currently being investigated and developed. Bioconversion of lignocellulosic material to ethanol is performed in multistep processes: pre-treatment (mechanical, chemical or biological), enzymatic hydrolysis of biopolymers to fermentable sugars, fermentation of sugars to ethanol followed by separation/purification of final product. However, lignocellulosic feedstocks are not easily broken down into sugar monomers which can be used for ethanol fermentation. While most of the hemicellulose can be hydrolyzed by physical and/or chemical pretreatment (e.g., steam explosion in the presence of dilute sulfuric acid, or pretreatment with alkali or an organic solvent), degradation of cellulose is typically done by enzymatic hydrolysis which requires high amounts and high activities of cellulolytic enzymes.

So far, the enzymatic hydrolysis of pretreated lignocellulosic feedstocks is an inefficient step in the production of cellulosic ethanol and its cost constitutes one of the major barriers to commercial viability. Improvement of biological enzyme production systems as well as genetic engineering of cellulolytic enzyme activities remain very important and still very challenging tasks for the development of efficient biocatalyst process in cellulosic ethanol production.

Trichoderma reesei is one of the most widely used species of filamentous fungi for the production of cellulolytic enzymes for lignocellulosic biomass degradation. It produces all enzymes required for full cellulose hydrolysis and it is one of the most efficient cellulase degraders known. The term cellulase broadly refers to enzymes that catalyze the hydrolysis of the beta-1,4-glucosidic bonds in the cellulose polymer. The catalytic mechanism involves the synergistic actions of endoglucanases, cellobiohydrolases and beta-glucosidases. Cellobiohydrolases act on opposing ends of cellulose chains, while the endoglucanases act at internal locations in the cellulose. The primary product of these enzymes is cellobiose, which is further hydrolyzed to glucose by one or more beta-glucosidases. Thus, in contrast to many other industrial enzyme applications, conversion of cellulose-to-ethanol requires production of well-balanced multi-enzyme cocktails optimized to specific feedstock.

Enzymes in the Detergent Industry The main advantage of enzyme application in detergents (e.g., laundry, dishwashing) is the requirement of much milder conditions than with enzyme-free detergents. Proteases, lipases and amylases are used for removal of respective stains/residues, while cellulases in laundry detergents can modify cellulose fibrils leading to improvement of color brightness, softening of fabric and facilitation of dirt removal by other enzymes. Due to energy saving initiatives in many households, development of new-generation detergent enzymes is directed towards improving enzymatic activities at lower temperatures and alkaline pH. In addition, compatibility of enzymes with detergent components also represents a challenging task in biotechnology for improved enzyme development.

3 Fungal Biotechnology for Improved Industrial Enzyme Production

Although the advantages of replacing chemical treatments with enzymes are often compelling from an environmental viewpoint, enzymes must compete economically with often inexpensive traditional chemical and mechanical processes. The commercial competitiveness of enzymes in given industrial enzymatic reaction process largely depends on:

- Enzyme properties, such as enzyme specificity to the substrate, catalytic activity and effectiveness in industrial process conditions;
- The cost of enzyme production which is directly dependent on the enzyme yields obtained from the microorganism fermentation process.

In the following sections we will discuss the biotechnological approaches used in development of novel or improved enzymes for industrial applications as well as fungal strains with increased enzyme production and secretion capacity.

3.1 Improvement of Enzyme Properties

Unfortunately, many naturally occurring enzymes are not suitable for industrial applications because of their poor selectivity to the substrate. For example, some family 10 xylanases display cellulase activity along with xylanase activity, and are less preferred in applications where only hemicellulase degradation is required (e.g., pulp and paper or baking industry) (van Oort 2010). Textile applications such as biopolishing require endoglucanase activity. However, not all fungal endoglucanases are highly active and preferred in these applications. Endoglucanases from glycoside hydrolase families 5, 12 and 45 have found the most utility for biostoning and biopolishing applications (Calixte et al. 2012; Samanta et al. 2012).

Other important parameters to consider when selecting suitable enzymes are industrial process conditions, particularly pH and temperature. The range of pH and temperature conditions in various industrial enzyme application processes are shown in Fig. 1. Fungal enzymes usually display their highest catalytic activities at slightly acidic pH and moderate temperature, while bacterial enzymes exhibit activities under a broader range of pH and temperature conditions. Although, commercial production of amylases is carried out by both bacterial and filamentous fungal cultures, bacterial α-amylases are generally preferred for starch liquefaction at high temperatures due to their high temperature stability. However, discoveries of new

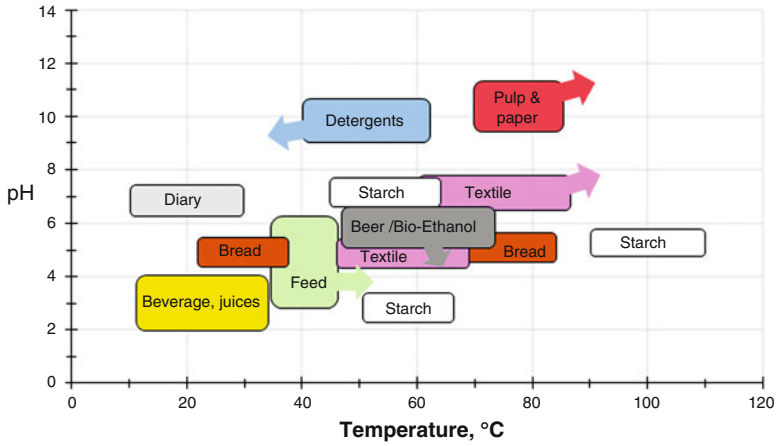

Fig. 1 Approximate pH and temperature requirements for various industrial enzyme applications. Certain tendencies for the development of new enzymes are indicated with *arrows*

fungal enzymes with higher thermal stability opens new opportunities in development of novel amylases for industrial starch processing (Aquino et al. 2003; Peixoto-Nogueira et al. 2008).

Enzyme performance in industrial lignocellulosic biomass hydrolysis is also affected by temperature and pH. Many biomass pretreatment methods are based on high temperature and acidic conditions to make biomass accessible for enzyme hydrolysis. Cellulases perform well at pH 4–5, but activity decreases significantly at lower pH (Bhatia et al. 2002). Thus, the pretreated biomass must be pH adjusted before enzymatic treatment. To achieve higher enzyme activities currently hydrolysis is performed at 50 °C. However, when reaching high temperatures, protein stability is affected, particularly taking in account that the lignocellulosic biomass hydrolysis process typically runs for the duration of several days. Therefore, the development of thermostable and acid tolerant enzymes for biomass degradation is also very attractive approach for improvement of hydrolysis process and reduction of amount of enzyme required. On the other hand, using alkaline chemicals such as dilute sodium hydroxide, aqueous ammonia and lime to remove lignin prior enzymatic hydrolysis process has long been known to improve cellulose digestibility (rev. Zhang and Shahbazi 2011), thus, enzymes with improved activity and stability at higher pH are also of interest.

Low enzyme stability in process streams containing variety of enzyme inhibitors, organic solvents or detergents results in slow enzymatic reaction rates. For example, in cellulosic ethanol production enzyme performance is reduced during lignocellulosic biomass hydrolysis by interaction with lignin or lignin–carbohydrate complexes (Berlin et al. 2006). Moreover, all enzymes are prone to substrate or product inhibition resulting in loss of enzymatic activity or even activation of reverse enzymatic reaction. The intermediate and end-products of lignocellulose hydrolysis generally are not removed. This results in product inhibition of cellulolytic enzymes and significant decrease in the rate of reaction as cellulose hydrolysis proceeds (Tolan 2002). For example, cellobiohydrolases and endoglucanases are inhibited by cellobiose (Murphy et al. 2013), making beta-glucosidases important for avoiding product inhibition through conversion of cellobiose to glucose. On the other hand, beta-glucosidases are inhibited by their end-product, glucose (Xiao et al. 2004). Other than inhibiting beta-glucosidase reaction by occupying the active site, glucose can also take part in the reverse transglucosidase reaction using the active site capacity in non-hydrolyzing action and resulting in the production of unwanted products such as cellotriose and cellobiose (Saloheimo et al. 2002).

Today's biotechnology and recombinant DNA technologies significantly contributed in developing of economically feasible industrial enzyme applications. In many cases, enzymes that perform under unnatural conditions, such as elevated temperatures, non-optimal pH or presence of inhibitors, were created. Several enzyme engineering approaches, such as rational design mutagenesis, gene shuffling, directed evolution and metagenome mining, have been developed over the past decades and are extensively applied in the development of novel enzymatic activities and properties.

3.1.1 Enzyme Mining

While filamentous fungi naturally produce a rich variety of hydrolase, oxidase, and esterase enzymes, these enzymes cocktails and their constituent enzymes are often not optimally suited for industrial applications. For example, the individual enzymes may not be optimally active or stable at the temperature and pH of the application, or there may be small molecules added or generated during the process that reduce or inhibit enzyme activity. Further, enzyme cocktails produced from a single organism may be lacking a key activity or factor that can enhance the performance of the enzyme cocktail.

For applications which require enzyme activity at high or low temperature or high or low pH, researchers have isolated and cloned enzymes from organisms found in extreme environments. Thermophilic fungi have long been recognized as rich sources of hydrolytic enzymes with industrial utility: proteases, lipases, amylases, cellulases and hemicellulases (rev. Maheshwari et al. 2000). These enzymes have found utility in a number of industrial applications where stability of enzymes in high process temperature is required, including pulp and paper processing, textiles processing, detergents, grain processing, brewing, baking, animal feeds, and biomass conversion to fermentable sugars. Thermophilic and/or thermostable cellulases and hemicellulases have been isolated from a number of thermophilic fungi such as *Myceliopthora thermophila*, *Chaetomium thermophilum*, *Talaromyces emersonii*, *Humicola grisea*, *Humicola insolens*, *Melanocarpus albomyces*, and *Thermoascus aurantiacus* (rev. Li et al. 2011). These organisms produce a rich array of cellobiohydrolases, endoglucansaes, beta-glucosidases and xylanases (rev. Li et al. 2011; rev. Maheshwari et al. 2000).

A systematic classification of glycosyl hydrolyases, including as cellulases, hemicellulases, and amylases, based on conservation of three-dimensional structure, amino acid sequences, and catalytic mechanism was developed in the mid-1990s (Henrissat 1991). The classification of thousands of glycosyl hydrolase sequences into over 150 GH families can be found at the Carbohydrate Active Enzymes database, www.cazy.org. Such classification has enabled the use of conserved regions of amino acid sequences to develop degenerate primers for the amplification of genes encoding Glycosyl Hydrolase (GH) enzymes from known fungal species as well as from metagenomic libraries. For example, this approach was used successfully to clone genes encoding cellulases from GH Families 6, 7 and 45 from 16 thermophilic fungal species (Busk and Lange 2013). Cellulase-encoding genes have also been successfully identified in metagenomic libraries constructed from environmental samples as well as from the microbiomes of insects and ruminants (rev. Ilmberger and Streit 2010). Similarly, screening metagenomic soil or herbivorous animals' rumen libraries have proven successful in exploring diversity of novel beta-glucosidases (Lammirato et al. 2010; Bao et al. 2012). Heterologous expression of these enzymes in *T. reesei* improved glucose yields during lignocellulytic biomass hydrolysis (Dashtban and Qin 2012).

3.1.2 Enzyme Engineering

Despite the enormous diversity of microbes and their enzymes, large-scale production of these enzymes at the levels required for industrial applications is often not economically feasible because either the native organism cannot be cultured effectively at large scale or produces the desired enzyme in a complex protein cocktail, or the enzymes cannot be expressed effectively from a heterologous host. Therefore, it can be more desirable to engineer enzymes that can be highly expressed from their native or heterologous host organisms for improved activity and/or stability under the desired industrial conditions. The earliest work in the engineering of fungal enzymes for industrial applications was conducted using site-directed mutagenesis methods, usually guided by sequence and structural comparisons of the target enzyme with homologues from other sources that may exhibit one or more of the desired attributes that is desired in the target enzyme.

As a result of their small size (around 200 amino acids), highly conserved compact structure, and sequence similarity, GH family 11 xylanases are perfect candidates for enzyme engineering. An elegant example of the use of directed mutagenesis employing swapping of stretches of amino acids, in combination with site-directed mutagenesis of specific amino acids, is the work of Sung et al. (1998). By swapping the first 33 amino acids of *T. reesei* xylanase 2 with the homologous region of *Thermobifida fusca* and then subsequently narrowing the length of the substituted region, three key mutations in that region of *T. reesei* xylanase 2 (N10H, Y27M, an N29L) were identified that enhanced the thermostability, thermophilicity (temperature range) and alkalphilicity (pH range) of the enzyme (Sung et al. 1998). Employing similar strategies to other regions of the protein, additional mutations beneficial for increasing the operating temperature and pH ranges *T. reesei* xylanase 2 were identified at positions 11, 40, 75, 104, 105, 125, 129, 132, 135,144, 157 161, 162, 165, and a disulfide positioned between 99 and 118 (Sung 2001; 2003; 2007). Several of these *T. reesei* xylanase 2 variants were developed into products used to reduce chemical usage in the bleaching of Kraft pulp, as well as in the processing of grains to sugar and alcohol. A similar strategy of swapping regions of diversity between the thermophilic *T. fusca* xylanase A and a Family 11 xylanase from *Streptomyces olivaceoviridis* (SoxB) identified mutations at positions 2, 4, 32 and 33 to improve the thermostability of the SoxB xylanase (Zhang et al. 2014b).

In parallel efforts, Sung et al. improved the thermostability of *T. reesei* xylanase 2 via the introduction of cysteine residues at key positions to form disulfide bonds that stabilized the structure of the Family 11 xylanase. Stabilizing disulfide bonds were introduced between positions 110 and 154, 108 and 158 of *T. reesei* xylanase 2 (Wakarchuk et al. 1994; Sung and Tolan 2000). The improved thermostability conferred by the 110–154 disulfide, in combination with a N44D mutation to lower the pH optimum of the enzyme, make these variants of *T. reesei* xylanase 2 well suited for animal feed pelleting applications; the thermostability prevents the enzyme from denaturing during the pelleting process and the increased activity at lower pH enables the enzyme to function in the digestive tract of swine and poultry. *T. reesei* xylanase 2 (xyl11B) continues to be a target for enzyme engineering. A

combination of Look-Through Mutagenesis (LTM™) and Combinatorial Beneficial Mutagensis (CBM™) identified four additional mutations that can enhance the thermostability of this enzyme: N71D, Y73G, T95G, and Y96Q (Hokanson et al. 2011).

The two cellobiohydrolases from *T. reesei*, as well as homologous enzymes from other fungi, have been subjected to site-directed mutagenesis in order to remove N-glycosylation sites (Adney et al. 2001), improve thermostability (Goedebeguur et al. 2005; Aehle et al. 2006; Bott et al. 2014), or increase specific activity (Aehle et al. 2006). However, because of the large size of the fungal cellulases (over 400 amino acids), and their relatively poor expression in non-fungal systems, large improvements in these properties were difficult because of the limited sequence space that could be explored using site-directed mutagenesis methods.

Directed Evolution, which combines the generation of large libraries of enzymes variants produced by one or more random mutagenesis methods with high throughput expression and screening methods, has enabled a much greater exploration of sequence space for fungal cellulases. Libraries of enzyme variants can be produced by one or more of the following methods: error-prone PCR, in vitro gene shuffling; in vivo gene shuffling, and site-saturation mutagenesis at a few, several or all positions within the enzyme's amino acid sequence (rev. Sen et al. 2007; Chica et al. 2005; Eijsink et al. 2005).

In order to be able to screen the enzyme libraries for variants with the desired attributes, the variants need to be cloned and expressed from an appropriate screening host. For fungal enzymes, both traditional yeasts (*Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*) and non-traditional yeasts (*Pichia pastoris*, *Yarrowia lipolytica*) yeasts have been used (rev. Lambertz et al. 2014; Boonvitthya et al. 2013). Often, fungal enzymes expressed from yeast hosts are hyperglycosylated and this can affect the folding and activity of the expressed enzyme. Thus, it is important to confirm any improvements identified when using a yeast expression hosts for Directed Evolution of fungal enzymes by expression back in a filamentous fungal host. For some fungal enzymes, such as *T. reesei* cellobiohydrolase I, a suitable yeast host that can produce that enzyme with the same activity as produced from the native host has yet to be identified. In order to overcome this limitations, high-throughput transformation systems for filamentous fungi have been developed (Punt et al. 2001; Van den Berg et al. 2008).

Well-designed assays are key to the successful identification of improved enzymes, particularly in the case of stochastic methods (e.g., directed evolution). Although soluble chromogenic and fluorogenic substrates have been developed for detecting the activity of GH enzymes and may be used in some screening assays, the performance of a GH enzyme on these artificial substrates often does not correlate to activity on a native or technical substrate such as cellulose or xylan. Instances where improvement on one substrate did not correlate to improvement on another have been documented (Teeri et al. 1998; Voutilainen et al. 2010; Kurasin and Väljäme 2011).

Because the substrates that fungal enzymes act upon in industrial applications are often polymeric and insoluble, development of high-throughput activity assays using such substrates has been difficult. Early efforts in the Directed Evolution of

glycosyl hydrolases took advantage of soluble, colorimetric or fluorogenic substrates for high throughput screening. For example, such surrogate substrates were used by several groups to identify thermostable variants of *T.reesei* CBH1 (Goedebeguur et al. 2005; Day et al. 2004) and of *T. reesei* CBH2 (St-Pierre et al. 2008; Tomashek et al. 2010; Lavigne et al. 2010; Masri et al. 2011). Often these substrates were incorporated into agar medium and used as a quick and easy method to screen out transformants that were expressing inactive variants, as evidenced by the absence of a colorless halo or clearing zone around the colony, thus reducing the size of the library to be screened with more complex assays. With the growing need to identify enzyme variants useful for the hydrolysis of lignocellulosic substrates, microplate assays incorporating pretreated lignocellose were developed and utilized to identify useful cellulase variants (Lavigne et al. 2009; Aehle et al. 2010; Dhawan and Segraves 2011; Ang et al. 2013; Montalibet et al. 2013; Song et al. 2010). Most recently, an ultra-high throughput microfluidic cellulose hydrolysis assay has been developed in which release glucose is converted via an enzyme-coupled reaction to a fluorescent compound that can be detected on a chip (Ostafe et al. 2014).

The application of Directed Evolution methods to cellulases and xylanases has led to the development of numerous variants with improved properties. Global site-saturation mutagenesis has been used to identify variants of *T. reesei* CBH1 with reduced end-product inhibition (Stege et al. 2012) and variants of *T. reesei* CBH2 with increased specific activity (Lyon et al. 2014). A combination of error-prone PCR, site-saturation mutagenesis, and recombination has led to the development of *T. reesei* CBH2 variants with improvements in thermostability, thermoactivity (St-Pierre et al. 2008; Aehle et al. 2006, 2010), increased activity on beta-glucan (Tomashek et al. 2010), reduced glucose inhibition (Lavigne et al. 2009), and reduced inactivation by lignin (Lavigne et al. 2010; Cascao-Pereira et al. 2009). Similar approaches have identified variants of *T. reesei* CBH1 with improved activity on lignocellulose, reduced inactivation by lignin, and reduced inhibition by lignocellulose hydrolysates (Montalibet et al. 2013), of *T. reesei* EG2 with improved activity (Masri et al. 2011), of *Myceliophthora thermophila* CBH2 and EG1b with improvements in a number of biophysical and biochemical properties (Dhawan and Segraves 2011; Ang et al. 2013). A SCHEMA approach has been used to shuffle structural domains of CBH2 enzymes from *T. reesei*, *Humicola insolens* and *Chaetomium thermophilum* (Heinzelman et al. 2006) to produce variants with improve thermostability. GH Family 11 xylanases with improved performance in the degradation of lignocellulosis substrates have been developed using a combination of error-prone PCR, DNA shuffling and site saturation mutagenesis (Song et al. 2012).

Among the cellulose degrading enzymes fungal beta-glucosidases attracted special attention from enzyme development perspectives (rev. Sorensen et al. 2013; rev. Tiwari et al. 2013). During industrial lignocellulosic substrate hydrolysis process the intermediate and end-products generally are not removed resulting in product inhibition of cellulolytic enzymes and significant decrease in the rate of reaction as cellulose hydrolysis proceeds (Tolan 2002). For example, cellobiohydrolases and endoglucanases are inhibited by cellobiose (Murphy et al. 2013),

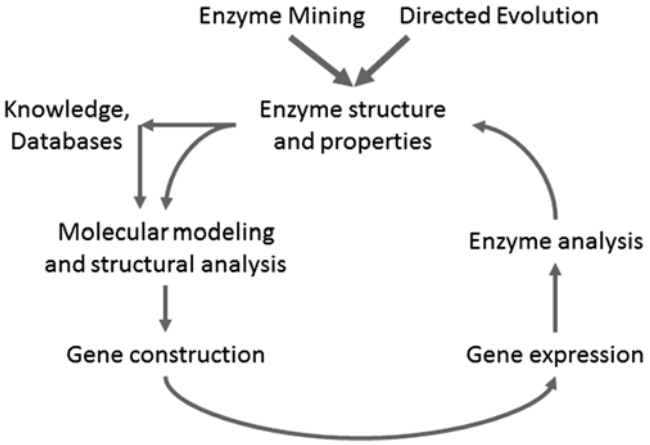

Fig. 2 The enzyme engineering cycle. The process starts with the isolation and characterisation of the enzyme (native or mutated). New and existing database information is analysed to produce a possible improvement strategies. Enzyme is modified by site-directed mutagenesis, isolated and characterised. The results, successful or unsuccessful, are added to the database, and the process repeated until the required result is obtained

making beta-glucosidases important for avoiding product inhibition through conversion of cellobiose to glucose. On the other hand, beta-glucosidases are inhibited by their end-product, glucose (Xiao et al. 2004). Other than inhibiting beta-glucosidase reaction by occupying the active site, glucose can also take part in the reverse transglucosidase reaction using the active site capacity in non-hydrolyzing action and resulting in the production of unwanted products such as cellotriose and cellobiose (Saloheimo et al. 2002). Thus, significant efforts using DNA shuffling or error-prone PCR are directed toward the improvement of this enzyme activity, thermostability and catalytic reaction properties (Scott et al. 2010; Liu et al. 2009; Pei et al. 2011; Hardiman et al. 2010). With increasing knowledge from directed evolution studies and characterization of beta-glucosidases from variety of other microorganisms, rational design and bioinformatics based strategies to perform more advanced mutagenesis are becoming more viable and successful in enzyme improvement (Lee et al. 2012, Fig. 2).

3.1.3 Optimized Enzyme Cocktails

For industrial applications that benefit primarily from a single enzyme activity, such as pulp and paper or starch processing, identification of useful enzymes from natural diversity or protein engineering studies will lead directly to candidates enzymes for commercial development. When the industrial application requires the synergistic interaction of two or more enzymes, it is necessary to screen for useful enzymes in the presence of their synergistic partners. This has been a particularly relevant

issue for the development of highly effective cellulase systems for the conversion of lignocellulosic substrates to fermentable sugars.

By combining isolated enzymes with various activities with complete cellulase cocktails produced by fungi such as *T. reesei* or with artificial cocktails containing mixtures of selected cellulase enzymes, a number of "accessory enzyme activities" have been identified that enhance the cellulolytic activity of the parent cocktail. These accessory enzyme activities include polysaccharide monooxygneases that were formerly classified as GH61 endoglucanases but are now classified in GH Auxilliary Activity Family 9 (Merino and Cherry 2007; Harris et al. 2010; Scott et al. 2009), swollenin, a non-hydrolytic protein hypothesize to increase available surface area of cellulose substrates (Scott et al. 2009; Huang et al. 2009), GH Family 16 enzymes (Hill et al. 2013), and CIP1, a protein that is co-expressed with cellulases by *T. reesei* (Scott et al. 2009). Enzymes known to play a role in hemicelluloses and lignin degradation, including GH Family 10 xylanases (Vlasenko et al. 2011), acetyl xylan esterase (Iyer et al. 2013), ferulic acid esterase (Han et al. 2012), and cellobiose dehydrogenase (Sweeney et al. 2010), have also shown benefit for improved enzymatic hydrolysis.

Finally, for industrial applications requiring the synergistic interaction of multiple enzymes, it is also important to not only ensure that the enzyme cocktail has all of the necessary activities, but also to ensure that the relative proportions of these activities is optimal. Significant efforts have been dedicated to optimizing the proportions of cellulase enzymes in enzyme mixtures used the hydrolysis of lignocellulose substrates (Hill et al. 2008; Gusakov et al. 2008; Baker et al. 1998), for animal feed applications (Bedford et al. 1995, 2006), and for textiles processing (Hill et al. 2012; Foody et al. 1999; Miettinen-Oinonen et al. 1996; Hahn et al. 1998).

3.2 Modifications of Enzyme Production Hosts

Although many microbial species found in nature produce a variety of enzymes potentially suitable for specific industrial application, not all microorganisms can be cultivated in laboratory conditions and only few of them can be grown in industrial scale fermentation for enzyme production. Selection of an ideal host organism is essential because each organism not only has its own unique features but also has advantages and disadvantages for a specific application. In particular, yeast and filamentous fungi are being developed as excellent enzyme production systems. In addition to high protein secretion capacity, they have rigid cell walls making them resistant to shear stress and allowing them to grow to high cell densities. They also can grow and produce proteins in simple, chemically defined media, which makes the production costs much lower compared to other eukaryotic cell systems. Moreover, the development of basic research and genetic engineering technologies focusing on yeast and filamentous fungi offers multiple opportunities to manipulate these organisms toward generation of improved industrial enzyme production strains.

3.2.1 Filamentous Fungi as Enzyme Production Hosts

The ability of filamentous fungi to grow on rather simple and inexpensive substrates as well as their capacity to produce and secrete a wide range of commercially interesting enzymes attracted considerable interest to exploit them as production organisms in biotechnology. Due to their exceptional high secretion capacity, filamentous fungi become indispensable for the production of fungal and non-fungal origin enzymes. Currently, asexually reproducing *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei* strains dominate the markets as homologous and heterologous enzyme productions hosts. These strains were extensively modified to accommodate high protein production requirements in harsh fermentation conditions. With the development of fungal genetic engineering technologies, many new fungal species belonging to different genera such as *Penicillium*, *Aspergillus*, *Trichoderma*, *Rhyzopus*, *Fusarium*, *Myceliophthora*, *Agaricus*, *Chrysosporium*, are gaining more interest as a novel enzyme production strains (rev. Ward 2012).

Development of Industrial Enzyme Production Strains

Several decades of development of *Aspergillus* and *Trichoderma* industrial enzyme production strains achieved significant improvements in enzyme productivity. Before the advent of modern biotechnological approaches, many of strains producing high volume/low cost enzymes were generated by classical mutagenesis using UV light and/or mutagenic chemicals such as nitrosoguanidine (NTG) (Montenecourt and Everleigh 1977). Although the genome sequences of these highly productive fungal strains are currently available (http://genome.jgi.doe.gov/TrireRUTC30_1/TrireRUTC30_1.home.html; http://genome.jgi.doe.gov/Aspni_NRRL3_1/Aspni_NRRL3_1.home.html), the genetic bases of mutations leading to the improvement of protein productivity are still poorly understood. The complexity of these strains, such as *Trichoderma reesei* industrial enzyme production strain RUT-C30, underlay within the large number of various genomic rearrangements, small and large deletions as well as point mutations (Seidl et al. 2008). Among identified mutations probably the most significant change affecting hydrolytic enzyme production is the truncation and at least partial loss-of-function of the carbon catabolite repressor *cre1* resulting in derepression of hydrolytic enzyme gene expression (Ilmén et al. 1996; Nakari-Setälä et al. 2009). However, recent findings indicated that the remaining truncated *cre1* in Rut-C30 is still transcribed into mRNA, that its putative gene product (Cre1-96) is still able to bind DNA (Mello-de-Sousa et al. 2014). Moreover, it was proposed that truncated Cre1 may contribute indirectly to a more open chromatin status by regulating a chromatin remodeler and act as positive regulator for hydrolytic gene expression (Mello-de-Sousa et al. 2014). The role of other identified mutations in industrial enzyme production strains, such as *T. reesei* RUT-C30, remains to be untangled. Some of described changes in addition to higher hydrolytic enzyme production also affect cellular morphology, metabolism and organelle structure such as endoplasmic reticulum (Peterson and Nevalainen 2012).

Targeted strain modifications could enable further improvements of industrial enzyme production. The progress of strain development is highly dependent on advancement of basic knowledge of gene regulation, protein quality control and secretion mechanisms (discussed in Sect. 3.3).

Fungal Transformation Technologies

Two key challenges in industrial protein production are that not every component of the enzyme cocktail produced is relevant to the application and that the relevant activities are not necessarily expressed in optimal ratios to maximize synergism of their activities (discussed in Sects. 3.1.1 and 3.1.2). The development of transformation techniques for filamentous fungi offered more targeted and powerful approach for genetic engineering of filamentous fungi to increase productivity, optimize component ratios, and minimize unwanted by-product formation. Followed by the successful development of protoplast-mediated transformation of the yeast *Saccharomyces cerevisiae*, the use of protoplasts for transformation has been extended to filamentous fungi (Fincham 1989; Ruiz-Diez 2002). In order to improve transformation efficiency the progress has been made in establishing and optimizing alternative transformation methods such as electroporation, biolistic transformation (White and Hindle 2000; Hazell et al. 2001; Ruiz-Diez 2002) and *Agrobacterium*-mediated transformation (Michielse et al. 2005a, b). Common to all four techniques is the necessity to optimise every method for the fungal strain and often only one or two of these methods can be applied to a particular species (Meyer et al. 2003). The type of integration and integration copy-number should also be taken into account when selecting a transformation method for filamentous fungi. For example, biolistic and protoplast transformation often result in multicopy integration events, which might be suitable for improved single protein production, while *Agrobacterium*-mediated transformation mainly results in single-copy integration of transgene and can be used for gene targeting (Meyer 2008). In addition, gene targeting frequency can be affected by transformation method: biolistic transformation is less suitable for targeted integration events compared to protoplast transformation (Iogen, unpublished data). *Agrobacterium*-mediated transformation has been shown to have highest gene targeting frequency possibly via delivery of single-stranded DNA and promoting homologous recombination in host cell (Michielse et al. 2005a).

Gene Targeting in Filamentous Fungi

Targeted gene integration to a specific genomic locus is a strategy to improve and optimize transgene expression. Moreover, genetic deletion to eliminate the expression of unwanted components is a valuable approach for optimization of enzyme cocktails and redirection of secretion machinery to targeted enzyme production. The process of DNA integration into the genome during transformation is predominantly determined by the DNA repair and recombination machinery of the host. In eukaryotes, two main recombination pathways have been identified: homologous

recombination (HR) involving interaction between homologous sequences and non-homologous end joining (NHEJ) involving direct ligation of the strand ends independent of DNA homology. Both DNA repair mechanisms are conserved and operate in a wide range of organisms. However, the ratio of HR/NHEJ varies in different species. In yeast, DNA sharing homology with the genome preferably integrates by homologous recombination, while non-homologous recombination events are more frequent in filamentous fungi. Recent studies in filamentous fungi revealed that by deleting components of the NHEJ-pathway, the random integration of DNA fragment can be strongly reduced. It was demonstrated that in *Neurospora crassa* and *Aspergillus* strains deleted for either Ku70 or Ku80 homologs encoding NHEJ component, the frequency of gene targeting during transformation significantly increased (Ninomiya et al. 2004; Krapmann et al. 2006; Nayak et al. 2006). On the other hand, the protein machinery involved in homologous recombination (Rad51, Rad52 and Rad54) is absolute requirement for correct targeted T-DNA integration in *Saccharomyces cerevisiae* (van Attikum and Hooykaas 2003). Low activity of homologous recombination in filamentous fungi points to the existence of rate-limiting steps in this pathway. The importance of Rad54 function in all stages of homologous recombination (Heyer et al. 2006) together with the reduced rates of gene targeting in *mus25* (Rad54 homolog) knockout mutants of *Neurospora crassa* (Ishibashi et al. 2006) suggests that Rad54-like activity could be a major rate-limiting factor in gene targeting in eukaryotes. It is important to stress that the Ku proteins are essential to maintain telomere length in yeast and plants and are necessary for chromosome stability in mammals (Boulton and Jackson 1996; Bailey et al. 1999; Bundock et al. 2002). Congruently, phenotypic analysis of fungal strains defective in NHEJ demonstrated that these strains have higher susceptibility to various toxins and irradiation (Ninomiya et al. 2004; Meyer et al. 2007) and thus, might not be suitable for industrial fermentation. Restoring the wild type NHEJ phenotype by out-recombination of disrupting fragment or transiently silenced NHEJ genes could be an attractive option to bypass this problem. A high throughput toolkit in QM9414 strain of *T. reesei* defective in NHEJ was recently developed by Schuster et al (2012). Furthermore, the NHEJ-defect was successfully removed by crossing out the generated mutant with sexually competent strain derived from parental QM9414 strain (Schuster et al. 2012). At the current stage, the application of these technologies for industrial enzyme production strains might be complicated due to the high genetic variability of these strains often developed through random mutagenesis, limited knowledge of genetic changes present in Rut-C30 derivatives, and absence of sexually competent isogenic crossing partners.

RNA-Based Technologies in Filamentous Fungi

In some cases, the requirements for targeted gene disruption are less stringent and the modification of gene/protein expression levels might be achieved using knockdown mutants. On the other hand, some essential genes cannot be completely deleted or deletion may result in impaired growth and protein production abilities in fermentation. An alternative strategy circumventing DNA-based approaches and gene

targeting employs gene silencing induced by antisense RNA and RNA interference (RNAi) technologies (rev. Meyer 2008). The advantage of such genetic modification strategies is that the transformation of the construct resulting in gene silencing does not require the targeting into the desired gene locus; thus, it is not dependent on transcriptional activity and chromatin structure of targeted locus. In addition, the organism can be transformed with the construct leading to the simultaneous silencing of two or more unrelated genes located at different chromosomal loci. An inducible promoter may also be employed for condition dependent gene silencing. Although full knockout phenotypes were only rarely observed, the improvement of this method remains an open field to explore. With increasing basic knowledge of RNA-mediated gene silencing in filamentous fungi (rev. Chang et al. 2012; rev. Billmyre et al. 2013), using RNAi tools to customize industrial fungal strains for genetic engineering targeted to the production of specific products becomes more viable.

3.2.2 Heterologous Enzyme Expression Hosts

Yeast as Enzyme Expression Hosts

As heterologous protein production hosts, yeasts combine ease of genetic manipulations, fermentation of microorganisms and capability to secrete foreign proteins which are post-translationally modified according to a general eukaryotic scheme. Their rapid growth in high-density fermentation, simple and inexpensive cultivation media and relatively high protein secretion capacity make them attractive hosts for industrial enzyme production. On the other hand, secretory expression of heterologous proteins in yeasts is subjected to several bottlenecks such as limited enzyme yield in fermentation and non-native enzyme glycosylation patterns often affecting enzyme activity.

The conventional yeast system used for heterologous protein production is based on baker's yeast *Saccharomyces cerevisiae*. More recently, non-conventional yeast expression systems were developed, including *Schizosaccharomyces pombe* (Giga-Hama and Kumagai 1999), *Pichia methanolica* (Raymond et al. 1998), *Pichia pastoris* (Ilgen et al. 2005), *Hansenula polymorpha* (Gellissen 2000), *Kluyveromyces lactis* (Hollenberg and Gellissen 1997), and *Yarrowia lipolytica* (Madzack et al. 2005). The initial selection of suitable host is based on screening for protein-host compatibility, codon optimization and selection of promoter/secretion signal peptide. However, many heterologous proteins are still secreted at comparatively low levels often due to incompatibility of host-protein post-translational modification and processing machinery. The current strategies for yeast strain engineering as heterologous enzyme expression hosts focus on : (i) engineering of protein folding and quality control system in the endoplasmic reticulum, (ii) engineering of intracellular protein trafficking pathways, (iii) minimization of post-secretory proteolytic degradation, and (iv) engineering of post-translational glycosylation (rev. Idiris et al. 2010).

Despite the inability of yeasts to compete with filamentous fungi for protein production and secretion capacity, they still remain attractive hosts for specific applications. In contrast to filamentous fungi that produce multi-enzyme cocktails in response to inducing carbon source, yeast systems can produce mono-component secreted protein products making them suitable for industrial applications requiring a single enzyme or a defined enzyme supplement to a multi-enzyme cocktail. Due to the ease of genetic manipulations, rapid growth rates and well developed high throughput screening methodologies, yeasts are also valuable hosts for development and screening of improved enzymes. The main disadvantage of yeast expression systems for such applications is different protein glycosylation patterns often affecting enzyme activity. For example, beta-glucosidases (Cel3A), cellobiohydrolases II (Cel6A) and several endoglucanases can be successfully expressed, modified and secreted by *Saccharomyces cerevisiae*. These enzymes retain their activity even when differentially glycosylated in yeast. High throughput screening of enzyme variants in yeast hosts resulted in development of multiple novel enzymes with improved enzymatic properties such as improved catalytic efficiency, pH profile, thermostability or resistance to product inhibition (discussed in Sect. 3.1.2). In contrast, cellobiohydrolase I (Cel7A) expression and screening in yeast systems so far is limited by low productivity and differential glycosylation pattern affecting enzyme activity (unpublished data).

Asparagine (N)-linked protein glycosylation is present in all domains of life and is characterized by the high structural diversity of N-linked glycans found among different species. Yeast N-glycosylation has the high-mannose content compared to filamentous fungi that often affects heterologous protein properties and activity. To overcome this disadvantage for human therapeutic glycoprotein production, humanizing of yeast glycosylation systems has become the main strategy (rev. Wildt and Gerngross 2005; rev. De Pourcq et al. 2010). However, development of such strains requires multi-step modifications of entire glycosylation system and affects yeast native protein processing resulting in "sick" strains with low protein productivity yields below 2 g/L (Potgieter et al. 2010; Ye et al. 2011; Berdichevsky et al. 2011) making them less suitable for high-scale low-cost industrial enzyme production.

Filamentous Fungi as Hosts for Heterologous Protein Production

Recent advances in fungal genetic engineering, genomics and biodiversity studies offered the possibility to exploit filamentous fungi as heterologous enzyme production hosts. However, among recognized disadvantages is (i) their ability to produce large amounts of proteases which could degrade heterologous proteins, (ii) different protein glycosylation patterns, and iii) lower production levels of non-fungal proteins compared to those of homologous proteins, likely due to limitations at transcription, translation, post-translational modifications and/or protein secretion levels. The bottlenecks, strategies and challenges in improvement of homologous and heterologous enzyme production by filamentous fungi are largely common and will be discussed in Sect. 3.3.

Protease Deletion

In nature, filamentous fungi must compete with each other and with other microbes and organisms for nutrients; as such, proteases comprise a significant part of the secreted enzyme cocktails that they produce. Both *Aspergillus* and *Trichoderma*, which are commonly used for industrial enzyme production, produce and secrete into growth medium a wide range of proteases—acid proteases, serine proteases, cysteine proteases, alkaline proteases and metallo proteases (rev. van den Hombergh et al. 1997; Haab et al. 1990; rev. Peterson and Nevalainen 2012; Pel et al. 2007). Protease production reduces the yield of secreted proteins from filamentous fungi, particularly for heterologous protein. Thus, significant efforts have been made to reduce or eliminate protease production from filamentous fungal hosts.

The spectrum of proteases varies between different species as well as with nutrient availability and pH conditions in the growth environment. Protease activity increases particularly when there is a limitation of available nitrogen. At a culture pH of 5 or lower, aspartic proteases are the most active and/or abundant. Thus, operational strategies to reduce protease activity by maintaining the culture pH at or above 5.0 and avoiding nitrogen limitation have been used successfully to eliminate acid protease activity (Haab et al. 1990; Eneyskaya et al. 1999).

Depending upon the stability of the desired secreted product(s) to different pH regimes, such strategies may not prove effective given that the filamentous host can still produce neutral and alkaline proteases. Therefore, fungal strains deleted in one or more proteases have been developed. For example, strains of *Myceliophthora thermophila* containing deletion of up to four protease genes have been reported to reduce the proteolysis of the major secreted cellulase CBH1a (Szabo et al. 2013). *Trichoderma reesei* strains deficient in the production of alkaline serine protease (Zhang et al. 2014a), or deficient in the production of one or more subtilisin-type serine protease, one or more aspartic acid proteases, and /or a trypsin-like serine protease, show improved expression and yields of heterologous proteins (Maiyuran et al. 2011). Similarly, improved production of heterologous proteins was observed in *Aspergillus oryzae* strains possessing deletion of an alkaline protease gene (Lehmbeck 1997). *Aspergillus* strains deficient in the production of aspartic acid proteases have also been reported to enable increased production of heterologous proteins (Wang 2006; Clarkson et al. 2006).

3.3 Biotechnology for Improved Protein Production and Secretion in Filamentous Fungi

3.3.1 Transcriptional Regulation of Carbohydrolytic Enzyme Expression

Enzyme production and secretion from filamentous fungi is a cornerstone of commercially feasible industrial enzyme technology. Of the fungi used for enzyme production, *Trichoderma reesei* strains are particularly prominent due to their ability to

secrete large amounts of proteins. High productivity of fungal strains is dependent upon multiple factors. The fermentation feed type and rate will determine which secreted enzymes are produced by acting through the transcription factors and their cognate promoters that are either activated or repressed under certain conditions. The expression and production of polysaccharide degrading enzymes by filamentous fungi is regulated mainly at the transcriptional level in response to available carbon sources with full repression of these genes in the presence of glucose. Under glucose-limiting conditions, expression of genes encoding carbohydrolytic enzymes is de-repressed. Full activation of enzyme production requires the presence of an inducing carbohydrate. In most cases the regulatory systems respond to characteristic low-molecular weight molecules liberated from a given substrate through the action of small amounts of constitutively secreted enzymes. E.g. cellulases are induced by β-linked disaccharides such as cellobiose, sophorose, gentiobiose or lactose. Full activation of hemicellulase-encoding gene transcription is dependent on the presence of xylan and hemicellulase-derived carbohydrates such as xylose or xylobiose. Similarly, amylolytic enzyme (amylases, glucoamylases) expression and production is induced in the presence of starch or starch-derived disaccharide maltose.

Transcriptional Regulation of (Hemi)cellulolytic Enzyme Production

The transcription factor xylanase regulator (homologs identified in various fungal species, e.g., *Trichoderma* Xyr1, *Aspergilli* XlnR) controls the expression of all major hemicellulase and cellulase genes (rev. Stricker et al. 2008). XlnR was first identified as a positively acting regulator of the expression of (hemi)cellulolytic genes in *A. niger* (van Peij et al. 1998; Gielkens et al. 1999), followed by identification of *T. reesei* homolog Xyr1 (Stricker et al. 2006). *Trichoderma* is so far is the best (hemi)cellulase production host for industrial enzyme application; the discovery of general regulator Xyr1 attracted a high level of interest from academic and industrial researchers seeking improvement of (hemi)cellulolytic enzyme production.

Xylanase regulators belong to the class III zinc binuclear cluster protein family found exclusively in fungi and bind GGSTAR response elements in the promoters of target genes as monomers, homodimers or heterodimers (Stricker et al. 2008). Other functional domains such as nuclear localization signal, activation and glucose inhibition domains, of these proteins are predicted from homology with *Aspergillus niger* XlnR characterized by loss-of-function mutations and rational design mutagenesis analyses (Hasper et al. 2004). In addition, spontaneously mutated *T. reesei* Xyr1 protein revealed that an A824V point mutation in the putative glucose inhibition domain causes de-repression of hemicellulase genes in the absence of hemicellulase inducing carbohydrates and the presence of glucose (Derntl et al. 2013). However, cellulase gene expression was not affected by this point mutation and the presence of cellulase inducers was still required (Iogen, unpublished data).

In the differential regulation of cellulase and hemicellulase genes, *T. reesei* Xyr1 interacts with other identified activators and repressors, Ace2, Ace1, respectively (Aro et al. 2001; Saloheimo et al. 2000), CCAAT binding copmplex Hap2/3/5 (Wilson 2009) and yet-to-be identified inducer-specific and/or gene-specific regulators. Despite the commonality of being general (hemi)cellulase transcriptional activators, *Aspergillus* XlnR and *Trichoderma* Xyr1 induction mechanisms are different. For example, in *A. niger* single cis-acting elements were found in the 5′ upstream regulatory regions of the XlnR regulated genes, while in *T. reesei* only double sites of the binding motif proved to be functional. In *T. reesei* interplay between Xyr1 and Ace2 including heterodimerization and phosphorylation has a key role in regulation of *xyn2* gene expression, however, an *ace2* orthologue could not be identified in the *A. niger* genome (rev. Stricker et al. 2008). Along with the specific (hemi)cellulase transcriptional regulators, hydrolytic enzyme expression is also regulated by the cross-talk between other transcriptional regulation pathways, including nitrogen and pH response as well as protein secretion stress (rev. Aro et al. 2001). Sulphur metabolism (Gremel et al. 2008), light signalling machinery (Castellanos et al. 2010; Gyalai-Korpos et al. 2010) and regulatory pathways involved in chromatin remodeling of secondary metabolism genes (Seiboth et al. 2012) were also reported to be implicated in regulation of (hemi)cellulase gene expression in *Trichoderma*.

Indirect repression of (hemi)cellulolytic genes in the presence of glucose also occurs through direct repression of xylanase regulator by Cre protein (Carbon catabolite repressor) (Tamayo et al. 2008; Mach-Aigner et al. 2008). As discussed in Sect. 3.2.1 industrial enzyme production strain *T. reesei* RUT-C30 and its derivatives are deficient in Cre-protein function. These strains are widely used for industrial (hemi)cellulase production for textile, bioethanol and pulp and paper applications. Despite de-repression of (hemi)cellulolytic genes, production of enzymes is still dependent on the presence of corresponding inducers in fermentation media: addition of xylose or glucose dimers is required for hemicellulase or cellulase production, respectively. Lowering the cost of cellulase production for renewable biofuel applications may be achieved in part by eliminating the requirement for cellulase-inducing carbohydrates in enzyme production fermentation by replacing conventional glucose based feed containing cellulase inducers with plant biomass hydrolysates after pre-treatment where mainly pentose sugars (xylose, arabinose) are present. In Rut-C30 derived strains, improved cellulase production without cellulase inducers was achieved by overexpressing *xyr1* under beta-xylosidase (*bxl1*), strong xylose inducible, promoter (Fig. 3; Gudynaite-Savitch et al. 2009). However, inducer independent enzyme production was not achieved as the addition of hemicellulase inducers was necessary (unpublished data). In these fermentation conditions modified strain produced both, cellulases and hemicellulases. Simultaneous hemicellulase induction and secretion to growth media partially takes-up capacity of protein secretion and at the same time reduces the proportion of cellulases in the total secreted protein resulting in lower cellulase activity per protein produced (unpublished data). On the other hand, such strains are also not useful for hemicellulase production targeted to textile and pulp and paper applications

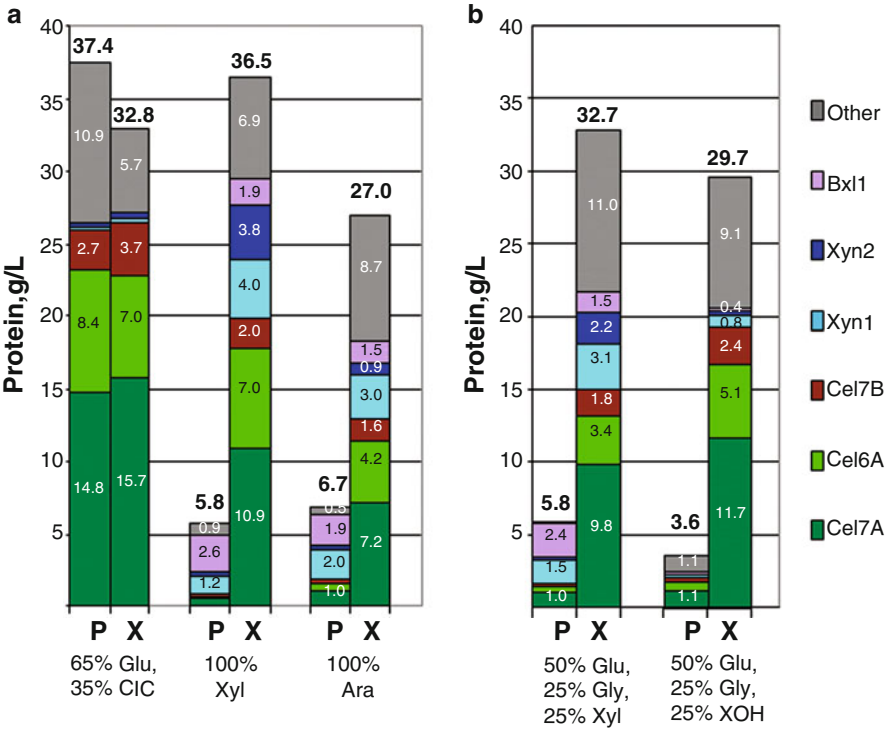

Fig. 3 The amount of total protein and the abundance of major (hemi)cellulases in *T. reesei* industrial enzyme production strains (RUT-C30 derivatives: P—parental, X—overexpressing *xyr1* under control of *bxl1* (beta-xylosidase) promoter) produced in 14 L fed-batch fermentation with mixed carbohydrates. Fermentation filtrate was collected after ~165 h and amount of each hemi/cellulase was determined by ELISA. The amount of total protein is indicated on each bar. Carbohydrates used as feed for each fermentation indicated below each bar: Glu—glucose, Xyl—Xylose, Ara—arabinose, Gly—glycerol, XOH—Xylitol, CIC—cellulase inducing carbohydrates (mix of glucose dimers) (modified from Gudynaite-Savitch et al. 2009)

since the majority of secreted protein is composed of cellulases (Fig. 3). Thus, production of targeted hydrolytic enzymes that is independent of carbon source is highly desirable for industrial enzyme production.

One obvious approach is the overexpression of Xyr1 under a strong constitutive promoter in glucose repression deficient strains. Wang et al. (2013) reported enhanced cellulase production by *T. reesei* Rut-C30 strain expressing xyr1 under constitutive *pbc* (pyruvate decarboxylase) promoter. After 5 days of cultivation in batch fermentation conditions with cellulose the total secreted protein amount was 64 % higher compared to parental Rut-C30 strain. The knock-down of *ace1* in the background of P*pbc-xyr1* expression increased total secreted protein by 45 % compared to Rut-C30 with constitutive *xyr1* expression. A significant increase in carboxymethyl cellulase activity (CMCase 10–30-fold) was also reported when the

above strains were grown on glucose (Wang et al. 2013). However, the maximum protein productivity reached in batch culture was at about 1.6 mg/mL (Wang et al. 2013) while optimized pilot scale fed-batch fermentations of Rut-C30 can produce about 30–40 mg/mL of total secreted protein. Thus, protein yields in batch fermentations are limited by factors other than secretion capacity and are therefore not a good system for studies aimed at identification of factors that limit secretory capacity. Depletion of carbon source at the end of batch fermentation can also affect experimental results since starvation, changes in pH, oxidative stress and other factors may induce other signaling pathways possibly involved in hydrolytic gene regulation. When tested in 14 L pilot scale fed-batch fermentation with glucose as carbon source, constitutive expression of *xyr1* from a constitutive phosphoglycerate kinase (*pgk*) promoter in Rut-C30 derived industrial strains did not increase cellulase production compared to the parental strain (Iogen, unpublished data). Moreover, when the native *xyr1* promoter was replaced with constitutive promoters, such as promoters from the histone 3 and basic-leucine zipper transcription factor genes, attempts to achieve improved cellulase expression in the absence of cellulase inducers also failed. In contrast, the production of cellulases in the presence of cellulase inducers decreased compared to parental strain with wild type *xyr1* gene (Iogen, unpublished data) indicating that induced high native *xyr1* expression levels are hard to achieve by constitutive expression of this gene. Also, it is possible that regulated post-translational modification of Xyr1 is required for inducer-dependent full activation of (hemi)cellulase-encoding gene transcription. Xylose triggered reversible phosphorylation of *Aspergillus* XlnR (Noguchi et al. 2011) and putative *T. reesei* Xyr1 phosphorylation sites identified *in silico* (R.L. Mach, personal communication) support that hypothesis. In vivo studies are required to fully elucidate the role of Xyr1 functional domains and post-translational modification in regulatory activity of this transcription factor.

Although evidence exists that Xyr1 is essential for expression of all major (hemi) cellulase-encoding genes (Stricker et al. 2006), Xyr1 binding to the regulated gene promoters was mainly demonstrated by in vitro studies (Furukawa et al. 2008; Stricker et al. 2008; Ling et al. 2009). Reported slight variations in the nucleotide sequence of XRE (Xyr1 response elements) and the composition of surrounding promoter sequences might result in different affinity of Xyr1 and/or Xyr1-protein complexes with other transcription regulators to specific elements found in different promoters of hemi/cellulase-encoding genes. The development of highly sensitive in vivo footprinting technique for identification of cis elements (Gorsche et al. 2014) facilitating high throughput analysis of regulatory elements on various (hemi) cellulase gene promoters may provide more insight to Xyr1 promoter specificity and regulatory mechanisms.

Overall, fine tuning of Xyr1 promoter-binding properties and specificity via protein modification along with manipulation of XRE on regulated gene promoters may offer the opportunity for targeted enzyme expression in the absence of specific inducers.

Recombinant Genes for Industrial Enzyme Production

Selection of a suitable promoter is one of the key components in developing strategies for fungal strain modification for industrial enzyme production. The choice of promoter depends on whether moderate enzyme production is required to balance optimal enzyme cocktail performance or maximum expression levels are necessary for mono-component enzyme production. It is also important to align the transcriptional regulation mode of given promoter with fermentation conditions, particularly the carbon source. Although in some applications constitutive expression of protein might be desired, the strength of such promoters are far from being competitive with the strength of inducible promoters, particularly when high-yields and low-cost industrial enzyme production is required. In addition, inducible secreted protein expression is coordinated with increased expression of genes involved in protein folding and secretion as observed by transcriptomics profiling of *T. reesei* Rut-C30 derived strain enzyme production fermentation (unpublished data). Thus, in most cases, high production of industrial enzymes may be achieved by expressing the target protein from strong inducible promoter.

Despite the large number of inducible promoters driving secreted protein expression in filamentous fungi, the choice of promoters used in biotechnological applications is limited. The most prominent strong inducible promoter which is widely used for recombinant industrial protein production in *T. reesei* is the cellobiohydrolase I (*cbh1*) promoter (Miettinen-Oinonen et al. 2005; Mäntylä et al. 2007; Paloheimo et al. 2007). In highly productive strains, such as Rut-C30, the abundance of CBH1 reaches up to 50–60 % of total secreted protein (Enari and Niku-Paavola 1987). However, expression of a recombinant protein from the *cbh1* promoter introduced into such strain does not reach native CBH1 levels and, furthermore, can reduce the CBH1 content in the total secreted protein. Possible competition for transcriptional regulators and/or limitation of protein secretion capacity are responsible for observed effect.

For example, the dose of native *T. reesei* beta-glucosidase for efficient cellulose conversion in bioethanol production process is estimated to be at the level of 20–30 % of total enzyme, while the wild type *T. reesei* beta-glucosidase expression level is only up to 1–2 % of total secreted protein. The strong inducible cellobiohydrolase I and II (*cbh1*, *cbh2*) promoters currently used to over-express beta-glucosidase in turn reduce cellobiohydrolase expression and overall cellulase activity (Rahman et al. 2009a). Certain individual cellulases, such as endoglucanase 3 (or Cel12A) from *T. reesei*, has not been shown to contribute significantly to the hydrolysis of lignocellulosic substrates, however, this enzyme is still relatively highly expressed (Iogen, unpublished data; Rahman et al. 2009a). Direct replacement of the gene encoding *T. reesei* endoglucase 3 with one or more copies of beta-glucosidase under control of the endoglucanase 3 promoter could be a viable strategy for over-expression of beta-glucosidase. Non-cellulase inducible promoters for beta-glucosidase over-expression such as xylanase 1 or 3 (Rahman et al. 2009b; Nakazawa et al. 2012) require the presence of xylose as an inducer. This in turn induces hemicellulase production,

the ballast enzymes taking-up the capacity of secretion machinery and reducing the proportion of cellulase enzymes in the total secreted protein.

Another important consideration for recombinant protein production strategies is selection of secretion signal peptide. Secretion signals generally range from 15 to 50 amino acids in length. They can be divided into a positively charged N-terminus, a hydrophobic central region, and a polar C-terminus. Although the amino acid sequences of secretion signal peptides can vary greatly between the secreted enzyme of a given organisms as well as between related secreted enzymes among different organisms, secretion signal peptides are conserved in their function and are often interchangeable between species and/or secreted proteins. For example, the *T. reesei* hydrophobin secretion signal peptide was successfully used for efficient secretion of heterologous proteins from *Pichia pastoris* (Kottmeier et al. 2011). The *T. reesei* xylanase 2 secretion signal peptide is successfully used for enhanced production of beta-glucosidase, endoglucanase, mannanase and laccase from *T. reesei* (White and Hindle 2000; White et al. 2005). The secretion signal peptides of *T. reesei* endoglucanase 2 and cellobiohydrolase 2 were reported to drive secretion of bacterial xylanase from *Trichoderma* (Miyauchi et al. 2013). Thus, different signal peptides are capable of directing the secretion of homologous and heterologous proteins, but they do not necessarily have the same efficiency and successful secretion of target protein may not necessarily mean that system is optimized. Unfortunately, our current knowledge about the efficiency of different secretion signal peptides is limited. In our studies, we attempted to overexpress endoglucanase Cel45A from *T. reesei* under control of *cbh1* promoter for enzyme production with higher biopolishing activity. The secretion of this protein was driven by either the xylanase 2 or native Cel45A secretion signal peptide (Calixte et al. 2012). Despite comparable extracellular Cel45A protein production, strains secreting Cel45A driven by native secretion signal accumulated about 10-fold higher amounts of intracellular Cel45A protein compared to parental strains and strains secreting Cel45A under control of xylanase 2 secretion signal (Iogen, unpublished data). The accumulation of high amounts of intracellular protein is usually indication of secretion bottle necks which is followed by activation of unfolded protein response pathways and down-regulation of secreted protein expression (Pakula et al. 2003; Saloheimo and Pakula 2012; Kautto et al. 2013). The analysis of secretion signal sequences revealed that Cel45A signal is Type I, used for secretion by signal particle recognition, SPR-dependent and SPR-independent pathways, while xylanase 2 secretion signal is Type II targeted for secretion by SPR-independent pathway only. Thus, the optimized compatibility between the secretion signal and mature protein sequence targeted to the secretion via specific pathway should also be considered when aiming for high secretion of target protein and mitigation of recombinant protein secretion stress.

In conclusion, multiple factors involved in the induction and transcriptional regulation of secreted enzyme gene expression have been identified. However, the full regulatory network still remains untangled and the efforts continue to identify novel factors that regulate hydrolytic enzyme gene expression so, further improvements of fungal enzyme production strains can be made. Moreover, transcriptional control

is also tightly regulated by feedback loop signaling from protein secretion machinery (Aro et al. 2001) and improved knowledge of this regulation is necessary for fine tuning of recombinant protein production.

3.3.2 Production and Secretion of Industrial Enzymes

The secretion of enzymes into the extracellular growth medium is another important factor in enzyme production by filamentous fungi. It depends on the particular secretion signal sequence of the protein and the capacity of the secretion apparatus of the cell. In many cases, high enzyme productivity appears to be limited at the level of secretion (Pakula et al. 2003). *T. reesei* strains generated by classical mutation/selection and/or genetic engineering can secrete up to 100 g/L of extracellular protein. These improvements in productivity have resulted a shift in basic carbon metabolism from cell growth to enzyme production. However, a number of these strains are unable to sustain high productivity phase for more than 90 h in both fed-batch and continuous culture operations (Dufresne et al. 2011).

Protein Secretion and Unfolded Protein Response

Multiple research reports demonstrate that protein over-expression can result in intracellular protein accumulation and activation of the Unfolded Protein Response (UPR) (Arvas et al. 2006). The UPR response is well characterized in yeast and mammalian cells and is an evolutionary conserved mechanism in all eukaryotes (rev. Moore and Hollien 2012; rev. Hollien 2013). The basic response includes an initial transient inhibition of ribosomal biogenesis and protein synthesis to temporarily stop production of new proteins, followed by transcriptional induction of chaperone genes that promote protein folding, and finally, induction and activation of the ER-associated degradation (ERAD) system for proteasome-dependent degradation of misfolded proteins (rev. Zhang and Kaufman 2008; rev. Lai et al. 2007, rev. Moore and Hollien 2012). If these responses fail to restore ER homeostasis and misfolded protein continues to accumulate in the ER, the UPR activates cell destructive pathways such as apoptosis and cell death (Boyce and Yuan 2006). In yeast, 135 and 155 genes have been identified as being transcriptionally up-regulated and down-regulated by the UPR, respectively (Arvas et al. 2006). The UPR has also been studied in filamentous fungi where the response to secretion stress shares several features in common with UPR in the yeast *S. cerevisiae* (Saloheimo et al. 2003; Arvas et al. 2006; Guillemette et al. 2007). Unlike yeast, filamentous fungi such as *Trichoderma* and *Aspergillus*, also exhibit transcriptional down regulation of genes encoding secreted proteins (Repression under Secretion Stress, ReSS) (Pakula et al. 2003; Arvas et al. 2006). Although the main pathways of the UPR are known, the mechanisms of UPR-associated transcriptional regulation and protein secretion control have not been fully explored.

In contrast to *Saccharomyces cerevisiae*, modifications of UPR pathway in filamentous fungi with attempts to achieve improved protein productivity have achieved only limited success. Both the yeast and filamentous fungal UPRs utilize a regulatory mechanism at the level of mRNA splicing of the transcription factor Hac1 that changes the open reading frame of the gene in such a way that an activation domain becomes connected to the protein (Mori et al. 2000; Saloheimo et al. 2003; Whyteside et al. 2011). The constitutive induction of UPR and increased production of heterologous proteins in *Aspergillus niger* can be achieved by overexpression of activated form of HacA (Hac1 homolog) (Valkonen et al. 2003); however, overall increased protein productivity using this genetic modification strategy is not reported. Similarly, the overexpression of protein disulfide isomerase (PDI), which assists in the maturation and folding of secretory proteins in eukaryotes, did not increase yields of heterologous protein expressed from *Aspergillus* (Ngiam et al. 2000) or production of cellulases from *Trichoderma* (Iogen, unpublished data), suggesting that PDI itself is not likely to be a limiting factor in protein folding and secretion. Despite the activation of UPR pathways and Hac1 post-transcriptional splicing, overexpression of other two components of UPR, *ire1* and *ptc2*, in *Trichoderma reesei* did not result in increased heterologous protein production (Valkonen et al. 2004). The most significant improvement so far is reported by Jacobs et al. (2009). They integrated transcriptomics and proteomics data obtained from three different *Aspergillus niger* strains over-expressing homologous protease, homologous hydrolase and heterologous lipase cultivated in strictly controlled fermentation conditions. The results indicated that protein degradation and protein folding play an important role in determining protein titres for production strains (Jacobs et al. 2009). Their studies demonstrated that simultaneous knock-out of *doaA*, a factor required for ubiquitin-mediated proteolysis, and overexpression of *sttC* gene, involved in glycosylation of secretory proteins, increased production of a model heterologous protein, GUS (β-glucuronidase), from *Aspergillus niger* (Jacobs et al. 2009). However, further investigations are required to determine whether these modifications can overall improve protein productivity in *Aspergillus* and other industrial fungal strains.

Is Epigenetics Involved in Regulation of Enzyme Production?

As discussed above, strategies that seek to maximise secretion capacity for improved protein production from filamentous fungi can result in the induction of secretion stress leading to activation of UPR, ERAD and ReSS pathways.

Many studies reported the activation of histone modifiers and locus specific chromatin remodelling on cytoprotective UPR regulated gene promoters upon the induction of the ER stress response in yeast and mammalian cells (e.g., Schröder et al. 2004; Donati et al. 2006; Bartoszewski et al. 2008). The mechanisms involved in the UPR-induced down-regulation of genes encoding secreted proteins in filamentous fungi are still largely unknown; however, DNA methylation and histone modifications in the regulation of gene expression have been reported (Selker et al. 2002;

Cheng et al. 2003). Multiple lines of evidence for chromatin remodeling regulatory mechanisms controlling secondary metabolite, development and virulence gene expression exist (rev. Brakhage and Schroeckh 2011; Studt et al. 2013; Connolly et al. 2013). The LaeA was identified as a putative protein methyltransferase capable of regulating secondary metabolite genes at the level of histone modifications in *Aspergillus* (Bok and Keller 2004). Later, it was discovered that an LaeA ortholog in *T. reesei*, Lae1, has an effect on (hemi)cellulase gene expression, although this regulation is not directly associated with H3K4 and H3K9 methylation at cellulase and hemicellulase loci (Seiboth et al. 2012).

Other studies indicate that (hemi)cellulase protein expression is subject to epigenetic regulation by chromatin remodelling and/or DNA methylation. Active heterochromatin associate protein complex Hap2/3/5 binding elements (CCAAT) are present in major hemi/cellulase-encoding gene promoters. Deletion of this binding element from the promoters of *xyn1* and *xyn2* leads to a general increase of gene expression, irrespective of the carbon source used for cultivation (Würleitner et al. 2003; Rauscher et al. 2006), while deletion of the CCAAT element from the *cbh2* promoter led to a strong decrease in *cbh2* expression (Zeilinger et al. 2003). The investigation of chromatin structure in the *cbh2* promoter revealed that nucleosome positioning was dependent upon the carbon source and the presence of Cre1, a catabolite-repressor protein that represses cellulase gene expression in the presence of glucose (Zeilinger et al. 2003).

Severe secretion stress induced by high production of cellulases in some industrial *T. reesei* enzyme production strains (e.g., Rut-C30 derivatives developed by random mutagenesis) leads to the complete loss of protein secretion coupled with higher biomass formation and the development of cellulase non-producing (*cel*−) phenotype, which can reach up to 100 % of the whole cell population in the bioreactor (Fig. 4b, c; Dufresne et al. 2011). This phenotype is heritable and *cel*− isolates do not produce cellulase even when introduced into fresh media with cellulase-inducing conditions (Iogen, unpublished data). The *cel*− cells possess a different morphology from the cellulase-producing cells: hyphal cells are less bulbous and more elongated. An intermediate phenotype (*cel*+/−) can be transiently isolated from mid to late stages of continuous and fed-batch fermentations. When grown on solid media containing acid swollen cellulose (ASC), these isolates form significantly smaller clearing zones compared to cellulase producing parental strains (Iogen, unpublished data). During serial passages to fresh ASC media, *cel*+/− isolates slowly lose their ability to secrete cellulases, indicating irreversible development to a full *cel*− phenotype and suggesting that phenotype formation is the result of epigenetic rather than genetic mechanisms. In addition, whole genome sequencing of three stable *cel*− isolates and their *cel*+ progenitors revealed the absence of genetic mutations unique to the *cel*− strains (Iogen, unpublished data). Possibly, secretion stress and epigenetic control mechanisms are interrelated in vivo and newly epigenetic gene regulation mechanisms can be stably inherited to progeny cells.

The epigenetic signals can be maintained by cis- or trans-epigenetic mechanisms: (i) Cis-epigenetic mechanisms are regulated by changes in chromatin structure and

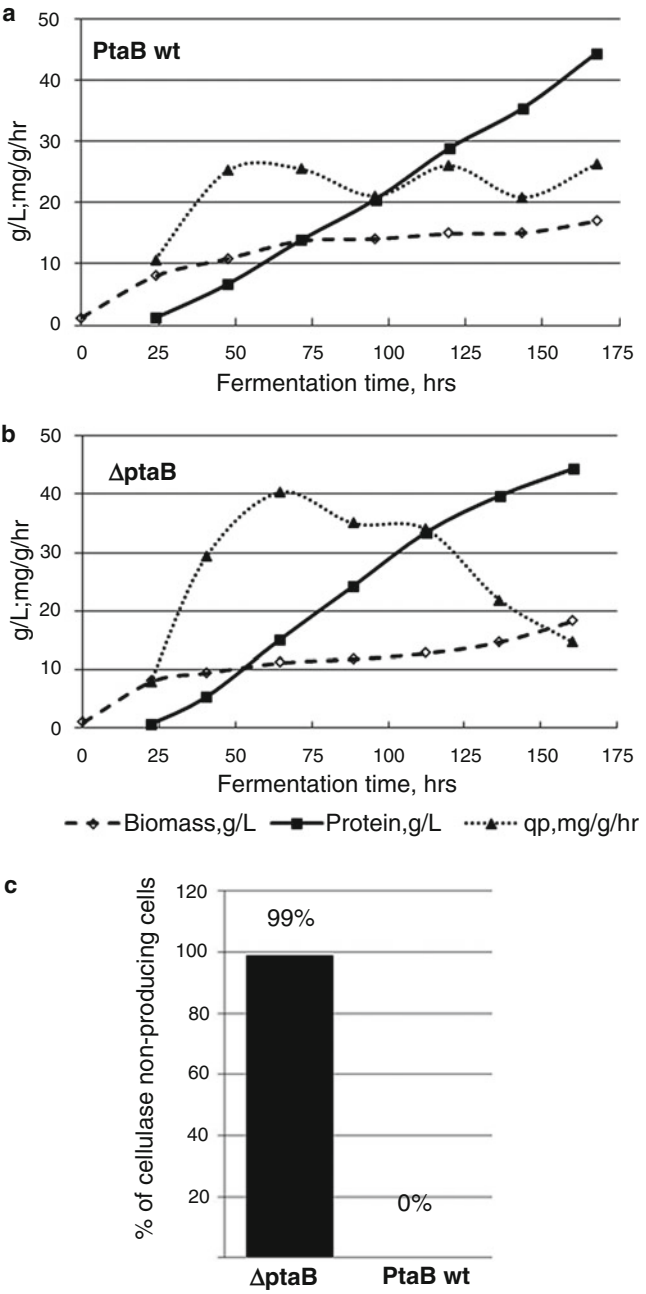

Fig. 4 Fermentation profile of industrial T. *reesei* strains—*ptaB* deletion (**a**) and wild type (**b**). Biomass and protein productivity is expressed as gram of biomass or protein, respectively, per 1 L of fermentation volume; qp—specific protein productivity expressed as mg or protein produced per gram of biomass per 1 h. (**c**) Accumulation of inherited cellulase non-producing phenotype at the end of fermentation (124 h) in *ptaB* deletion and wild type strains (modified from Dufresne et al. 2011)

are physically associated and inherited with chromosomes on which they act (Mendelian inheritance); while (ii) Trans-epigenetics mechanisms are caused by specific proteins such as autoregulated transcription factors or prions. These signals are self-propagating and maintained through the feedback loops and networks of proteins and transcription factors and have cytosolic inheritance type (non-Mendelian inheritance) (rev. Bonasio et al. 2010). Preliminary studies using protoplast fusion of *cel*− isolates with their *cel*+ progenitors suggest cis-epigenetic mechanism of *cel*− inheritance (unpublished data) rather than a trans-epigenetic inheritance mechanism involving prions or modified transcription factors.

Whole genome sequencing and SNP (single nucleotide polymorphism) analysis of several *T. reesei* industrial enzyme production strains generated by random mutagenesis (Rut-C30 derivatives) led to the identification of a critical protein factor involved the loss of cellulase production capacity at the end of fermentation (Dufresne et al. 2011). The *Aspergillus* homologue of this protein was described as the product of the *ptaB* gene (putative transcriptional activator) involved in the suppression of mutations in *areA*, the principal regulator of nitrogen metabolism in *Aspergillus* (Conlon et al. 2001). The mutation or deletion of the *ptaB* gene in *T. reesei* resulted in the increased protein production during the early–mid stages of fermentation; however, at the end of fermentation, productivity dropped significantly followed by the accumulation of stable *cel*− phenotypes (Fig. 4; Dufresne et al. 2011). These findings suggest that the PtaB protein may play a role in the epigenetic control of gene expression in response to secretion stress via unknown signaling pathways.

In conclusion, the expression of hydrolytic enzymes from filamentous fungi may also be controlled by chromatin remodeling at specific genomic loci induced in response to protein secretion stress. The signals activating epigenetic changes are largely unknown and identification of essential signaling pathway components may contribute to the development of genetic engineering strategies for improved industrial enzyme production from filamentous fungi.

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On the Safety of Filamentous Fungi with Special Emphasis on *Trichoderma reesei* and Products Made by Recombinant Means

Anna Gryshyna, Liisa Kautto, Robyn Peterson, and Helena Nevalainen

1 Introduction

Filamentous fungi and their products are routinely utilised in industry for a wide range of applications, including the manufacture and processing of food products (cheese, soy sauce, miso, fruit juice, baked goods, brewed beverages, sweeteners), other consumer products (textiles, leather, laundry detergents), animal feed, and industrial goods (pulp and paper, biofuels). While a number of fungi such as *Aspergillus*, *Guehomyces*, *Mucor*, *Neurospora*, *Rhizopus*, and *Zygosaccharomyces* are used for fermentation of food (microbial food cultures, MFCs; Bourdichon et al. 2012), in the majority of cases, the processing occurs by hydrolytic enzymes produced by fungi. Filamentous fungi that dominate the markets as production hosts are *Aspergillus niger*, *Aspergillus oryzae* and *Trichoderma reesei* (Nevalainen et al. 2005). The main types of industrially-relevant fungal enzymes feature amylases, amyloglucosidases, proteases, cellulases, lipases, xylanases, pectinases, phytases and glucose isomerases (Novozymes Fact Sheet 2013; GENECOR 2014; Li et al. 2012). Almost 75 % of industrial enzymes are hydrolases of which the majority is produced by recombinant means (reviewed in Adrio and Demain 2010).

Before genetic engineering became a common technology, regulations concerning the use of fungi in industry focused simply on the safety of the specific fungal species utilised, and its natural products such as enzymes and antibiotics. In recent years, the landscape has changed considerably and the principal use of fungi in

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industry now is as production hosts for recombinant proteins, either homologous or heterologous. In this light, national regulations for their acceptance require additional evidence for the safety of the recombinant product made, natural host (gene donor), and the production host. On top of this, the manufacturing process is regulated by Good Manufacturing Practice (GMP) and ISO (International Organization for Standardization) management system standards. It is important to note that usually, the industrially produced and used enzyme preparations are not purified for the main activity and thus contain several other enzymes (side activities) and metabolites. Following from this, metabolites and other potential allergens produced along with the target product also must be identified and their safety evaluated, and circumstances that instigate their production, defined. In addition, separation of the product from the recombinant host must be verified, particularly in countries with restricting laws concerning the use of genetically modified organisms (GMOs).

Enzymes used in food are classified as food additives and processing aids. From the regulatory point of view, distinction between processing aids and additives is crucial as the respective regulations differ significantly between different countries, including the EU Member States. In general, processing aids are defined as compounds used during the food manufacturing process, but do not have a function in the final product. Technical enzymes are typically used in large amounts (bulk) in detergent, textile, pulp and paper industries, organic synthesis and manufacture of biofuels. Nevertheless, their safety will equally require assessment.

The key in evaluating safety of a product is the assessment of the production strain. The primary areas of consideration in establishing the safety of filamentous fungi and their products are presented below with a particular focus on *Trichoderma reesei*. Once an unidentified, nameless fungus in the jungles of the Pacific, *T. reesei* has since risen to a prominent industrial organism and a safe production host for various hydrolytic enzymes. The issues involved in the acceptance of *T. reesei* and its products for human use are similarly applicable to other fungi, including the widely exploited *Aspergillus* strains. Regulations and practices in the European Union, United States of America and Australia are presented as examples typical of many developed nations with special reference to products manufactured by recombinant means, especially for food applications. The main regulatory bodies worldwide include Food and Drug Administration (USA) and European Food Safety Authority (EU).

Paradoxically, filamentous fungi can also cause disease in humans, plants and other forms of life. What, then, are the determinants that allow particular fungal species and their products to be deemed safe for industrial exploitation?

2 Identification and Safety Evaluation of the Production Organism

Establishing a confident identification of a fungal species and its general safety in the environment are the essential first steps prior to industrial use and dictate the necessity for further testing. In this context, the term “safe strain lineage” has been

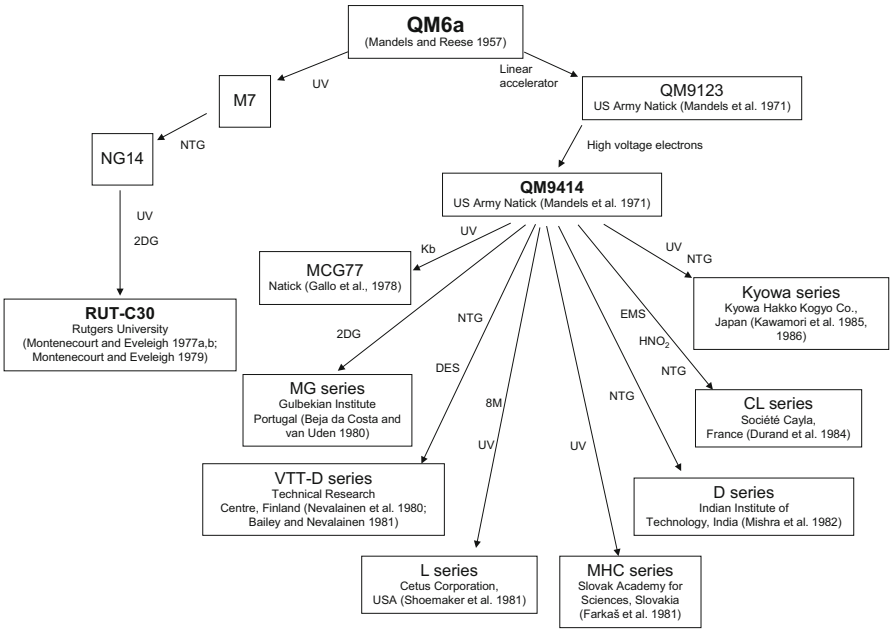

Fig. 1 Genealogy of *T. reesei* strains produced by various mutagenesis and screening programs worldwide. Modified from Nevalainen et al. (1994). *Abbreviations*: *UV* ultraviolet light, *NTG* nitrosoguanidine, *2DG* 2-deoxyglucose, *Kb* Kabamycin, *DES* diethyl sulphate, *8M* 8-methoxypsoralen, *EMS* ethylmethane sulfate, HNO_2 nitrous oxide

introduced. This refers to a group of related strains all of which have been derived by genetic modifications from a single isolate, *i.e.* parental strain, which has been thoroughly characterised and shown to be non-toxigenic and non-pathogenic before the modifications to improve enzyme production or function were initiated. Therefore, further assessment by extensive programs such as toxicology tests in animals is not required and the strain can be used as a starting point for further development (Safety evaluation of technical enzyme products with regards to the REACH legislation 2009).

Within the context of *T. reesei*, the parental strain, named QM6a (ATTC 13631; Mandels and Reese 1957) from which the majority of currently used industrial strains have been derived (Fig. 1), was first isolated by the US Army as the cause of tent canvas rot in the Solomon Islands in the Second World War. The fungus was initially identified as *T. viride* by morphological means but then found to be a distinct species within the *T. longibrachiatum* group and consequently named *T. reesei*, after Elwyn Reese who was one of the first researchers to study it (Simmons 1977). Molecular taxonomy approach has confirmed this assignment (reviewed in Lickfeldt et al. 1998). *T. reesei* has a sexually reproducing form *Hypocrea jecorina* which also appears in the literature and sometimes in place of *T. reesei* (*e.g.* Hartl and Seiboth 2005; Martinez et al. 2008; Seidl et al. 2009; Steiger et al. 2011).

Recently, the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy organised a vote on the preferred name and the resulting recommendation was to use the anamorph-related (asexual) name *T. reesei* (International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy 2012). The subcommission also provides a protocol and phylogenetic markers for the identification of *Trichoderma* species.

There has been no documented evidence of *T. reesei* causing disease or allergy in healthy humans. Other *Trichoderma* species have been associated with infections in immunocompromised patients, for example *T. longibrachiatum*, *T. harzianum*, *T. koningii*, *T. pseudokoningii*, *T. citrinovirde*, and *T. viride* were identified from infections of a peritoneal dialysis patient, immunocompromised transplant recipients, leukemia patients after bone marrow transplantation, in brain abscesses and in HIV patients (reviewed in Schuster and Schmoll 2010; Hatvani et al. 2014; De Miguel et al. 2005; Vesper et al. 2006; Kredics et al. 2003; Druzhinina et al. 2008). An allergic response in humans to *Trichoderma* species including *T. viride* and *T. citrinoviride* has also been demonstrated by the identification of mould-specific IgE antibodies (Pennanen et al. 2007; Koivikko et al. 1991). However, *T. reesei* has not been associated with human disease or allergy for over 40 years leading to the current classification of the species as Biosafety Level 1. This group includes well-characterised agents not known to consistently cause disease in healthy adult humans, which are of minimal potential hazard to laboratory personnel and the environment (Centers for Disease Control and Prevention: Biosafety 2013). This information further supports the safety of *T. reesei* as a production host.

Extensive mutagenesis and screening programmes using chemical and physical agents have been carried out worldwide resulting in a wide range of *T. reesei* strains of industrial and academic importance (Fig. 1). High protein-secreting mutant *T. reesei* strains have been used in industry since the early 1970s thus fulfilling the requirement for a history of safe commercial use. Currently, selected high protein-producing mutant strains are also used as expression hosts for recombinant proteins. Following a number of petitions for including *T. reesei* to the list of microorganisms that may be used as recipients for genetic material, and be exempt for full notification and reporting under the Toxic Substances Act, EPA (Environmental Protection Agency) has proposed adding the wild type *T. reesei* QM6a and its derivatives to the list on the basis that they do not present an unreasonable risk to human health or the environment provided that particular criteria for the genetic material and physical containment are met (Microorganisms 2012). The exemption is restricted to the use of submerged standard fermentation conditions and operations where no solid plant material or insoluble substrate is present in the fermentation broth. This ruling stems from the case where *T. reesei* was shown to produce a potentially toxic secondary metabolite, paracelsin, under the circumstances described above and discussed further in Sect. 4 (Brückner and Graf 1983).

Sequencing of the genome of the wild type QM6a (Martinez et al. 2008), the high protein-secreting hypercellulolytic strain Rut-C30 (ATTC 56765; Montenecourt and Eveleigh 1977a) and its ancestor NG14 (Seidl et al. 2008) have revealed many changes in the genome content such as deletion of an 85 kb genomic fragment from

Rut-C30 and NG14. Also, many point mutations have been identified (Le Crom et al. 2009). Genome sequencing has also revealed metabolic pathways for the synthesis of various secondary metabolites (Mukherjee et al. 2012), such as peptaibols and other compounds with unknown biological activity. With regard to this data and differently to the US, *T. reesei* is not exempted from full notification in the EU. Consequently, *T. reesei* is not included in the European Food and Safety Agency list of Qualified Presumption of Safety microorganisms and therefore risk assessment applications should include toxicological data (EFSA 2013).

3 Safety and Functions of Introduced Sequences

Genetically modified microorganisms (GMMs) are defined as microorganisms in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination (Directive 2001). Genes encoding recombinant products (*e.g.* enzymes) are typically introduced into the host strains using expression vectors. An expression vector is a plasmid that carries an expression cassette of which the essential components include a gene promoter driving the expression, a gene encoding the desired product, and a transcription terminator. In addition, a selectable transformation marker is added to the expression cassette.

The gene of interest is usually placed under the control of a promoter and terminator that are native to the expression host. An example of a strong promoter predominantly used in *Trichoderma reesei* is provided by the *cbh1* promoter driving expression of the gene encoding the main cellulase enzyme CBHI (Cel7A) that confers about 60 % of the total secreted protein (Margeot et al. 2009; Markov et al. 2005). Examples of mainstream selection markers are *hph1* (bacterial gene encoding resistance to the antibiotic hygromycin), *amdS* (*Aspergillus* gene that enables growth on acetamide as the sole nitrogen source) and *pyr4* (an auxotrophic marker). Often a plasmid carrying the expression cassette also contains the ampicillin resistance gene functional in *E. coli* that is used for the amplification of the recombinant DNA. Mainstream filamentous fungi do not contain extrachromosomally maintained nuclear replicating plasmids, thus the expression cassette needs to be integrated into the fungal genome (Timberlake 1991). Generally, well-characterised, commercially-available plasmids are used as a base for constructing specific expression vectors (Olempska-Beer et al. 2006).

Safety regulations regarding genetic modification of fungi vary to a certain extent depending on the country concerned. However, broad guidelines in the US, European Union and Australia state that the function of all genetic material in the vector must be known and well-characterised, and there can be no sequences present that could result in a phenotype harmful to humans or the environment through production of toxins, allergens or pathogenicity factors. Sequence and function of the DNA to be inserted and its position on the vector must be clearly stated, and the gene donor should not be a pathogenic organism. It is also recom-

mended that the vector and insert are as small as possible in size, thereby restricting the potential for inclusion of unnecessary and harmful material. Common methods of introducing exogenous DNA into the filamentous fungi include protoplast transformation, electroporation and biolistic transformation (reviewed in Ruiz-Díez 2002).

Potential for subsequent transfer of the vector to another organism from the production host (original recipient) should be averted by elimination of transfer functions and, if possible, achieving stable integration of the DNA insert into the chromosome of the expression host. In particular, antibiotic or heavy metal resistance genes used in the selection purposes should not be allowed to transfer to other organisms (Office of the Gene Technology Regulator 2000).

In *T. reesei*, DNA-mediated transformation relies on a small number of markers which generally limits the number of genetic manipulations that can be performed on a particular strain thus also limiting the rounds of manipulations that can be carried out. To circumvent this problem, molecular techniques allowing recycling of transformation markers have been developed recently. Some examples are the inducible Cre/loxP recombination system and spontaneous direct repeat recombination (Seidl and Seiboth 2010).

In the USA, the Environmental Protection Agency further regulates the use of new "intergeneric" microorganisms under section 5 of the Toxic Substances Control Act (TSCA). The EPA considers intergeneric microorganisms to be those formed from organisms of different genera or microorganisms formed with chemically synthesised genes originating from a different genus. Persons intending to manufacture or import intergeneric microorganisms for commercial purposes in the US must submit a Microbial Commercial Activity Notice (MCAN) to the EPA at least 90 days before such manufacture or import (Microbial Commercial Activities Notification Requirements. 40 CFR 725 (d) 2011).

4 Safety of the Recombinant Products (Enzymes)

The major known safety risk associated to an active enzyme is respiratory allergy; in case of proteases the minor risk involves skin/eye irritation. Even though adverse effects due to the catalytic activity of new enzymes including recombinant products are not likely, the products still require assessment. Verifying the safety of a recombinant enzyme may involve toxicology tests on animals and referral to any documented reports of associated human allergies or disease.

All safety studies are conducted in accordance with internationally accepted guidelines of the Organization for Economic Cooperation and Development (OECD), Joint FAO/WHO Expert Committee on Food Additives (JECFA) (General Specifications and Considerations for Enzyme Preparations Used in Food Processing 2006), and EC guidelines (Directive 2004/10/EC; Regulation (EC) No 1272/2008 2008).

4.1 Allergenic Properties

Risks of allergic reactions in workers handling enzymes have been discussed at great length in the scientific literature, within industry, among stakeholders, and even by general press. In 1998, the AMFEP (Association of Manufacturers and Formulators of Enzyme Products) Working Group on Consumer Allergy Risk from Enzyme Residues in Food concluded that there are no scientific indications that small amounts of enzymes in bread and other food can sensitise or induce allergy reactions in consumers, and that enzyme residues in foods do not represent an unacceptable risk (Association of Manufacturers of Fermentation Enzyme Products 1998). Allergy testing features inhalation toxicity, irritation/corrosion to eye and skin and skin sensitisation studies (OECD Guidelines for Testing of Chemicals 2014).

4.2 Toxic Properties

While allergenic properties of enzymes are intrinsic to the structure of the enzyme protein (and possibly other proteins present in the enzyme preparation), concerns on toxic effects are largely focused on by-products and contaminants that might be present in the enzyme preparation and thereby be transferred to food (Spök 2006). In general, all new enzymes should be analysed for toxins that might be reasonably expected to be present using chemical, biochemical, or biological methods.

To determine toxic effects of an enzyme preparation, an acute oral toxicity test and 91-day oral toxicology test are performed. Acute toxicity informs about the amount of substance required to create a toxic effect in one dose. The repeated dose 91-day oral toxicology test is used to evaluate chronic toxic effects, primarily on various organ systems, associated with known microbial toxins, to establish a 'no observed adverse effect level' (NOAEL) (OECD Guidelines for Testing of Chemicals 2014). The NOAEL should provide at least a 100-fold margin of safety for human consumption, calculated using standard methods (Klaassen 2013).

4.3 Mutagenic Properties

Mutagenic evaluation of enzyme preparation features a bacterial gene mutation test (Ames test, '*Salmonella typhimurium* Reverse Mutation Assay') (OECD Guidelines for Testing of Chemicals 2014; Ames et al. 1975). In addition, there are *in vitro* mammalian cytogenicity tests for chromosome aberration in cultured mammalian cells, and *in vivo* tests in mice to detect damage to the chromosomes or the mitotic apparatus (OECD Guidelines for Testing of Chemicals 2014). With regard to the mutagenic evaluation tests reported by the members of Enzyme Technical

Association (ETA), Pariza and Johnson (2001) concluded that testing enzyme preparations from traditional and genetically modified microorganisms for genotoxicity is unnecessary for safety evaluation; nevertheless, this practice continues, driven largely by regulatory requirements.

4.4 National Regulations

Specifically in the USA, any substance that is intentionally added to food is a food additive that is subject to premarket review and approval by FDA (U.S. Food and Drug Administration 2011). The types of data to be included into a food additive petition are described in Federal Regulation 21 CFR 171.1(c) (Petitions 2013). Petition (application) for an enzyme preparation should contain information about the identity of the enzyme, its proposed use, intended technical effects, method of analysis of the presence of the enzyme in the food and a full report of all safety investigations. An excellent detailed description of the food additive approval process in the US is provided by Rulis and Levitt (2009). The FDA accepts a decision tree (Fig. 2) for evaluating enzymes as proposed by Pariza and Johnson (2001).

Exemptions from full testing requirements may be justifiable if the production strain of an already tested and approved enzyme preparation is substituted by a genetically modified strain (Fig. 2). An enzyme preparation may even be accepted without specific toxicological testing if the production organism has a long history of safety in food use, and belongs to a species where no toxins are produced, and if the particular strain is of well documented origin, *i.e*. from safe strain lineage (Fig. 2).

Protein engineering of enzymes by altering their amino acid sequence may improve their process performance in changing the pH optimum, increasing the thermal stability, and stabilising against oxidation. Such engineered enzymes are unlikely to be toxic and thus no additional tests are required if the natural form of the enzyme has been evaluated (Fig. 2) (Pariza and Johnson 2001).

Section 201(s) of the FDA Federal Food, Drug, and Cosmetic Act exempts the use of a substance that is generally recognised as safe (GRAS) from the definition of a food additive. According to the FDA regulations, the use of a food substance may be GRAS either through scientific procedures based upon published studies, or for a substance used in food before 1958, through experience based on common use in food (Gaynor and How 2006). GRAS is issued for a specific use of a substance rather than for substance itself (Eligibility for Classification as Generally Recognized as Safe. 21 CFR 170.30 2012). GRAS notices for enzymes contain essentially the same information as a food additive petition, showing that the enzyme is safe under the intended conditions of use. Additional requirement for GRAS notifications is the public availability of the data and general recognition and acceptance of a safety conclusion based on those data (Rulis and Levitt 2009). Once a substance receives GRAS status it is not subject to the premarket review and approval by FDA.

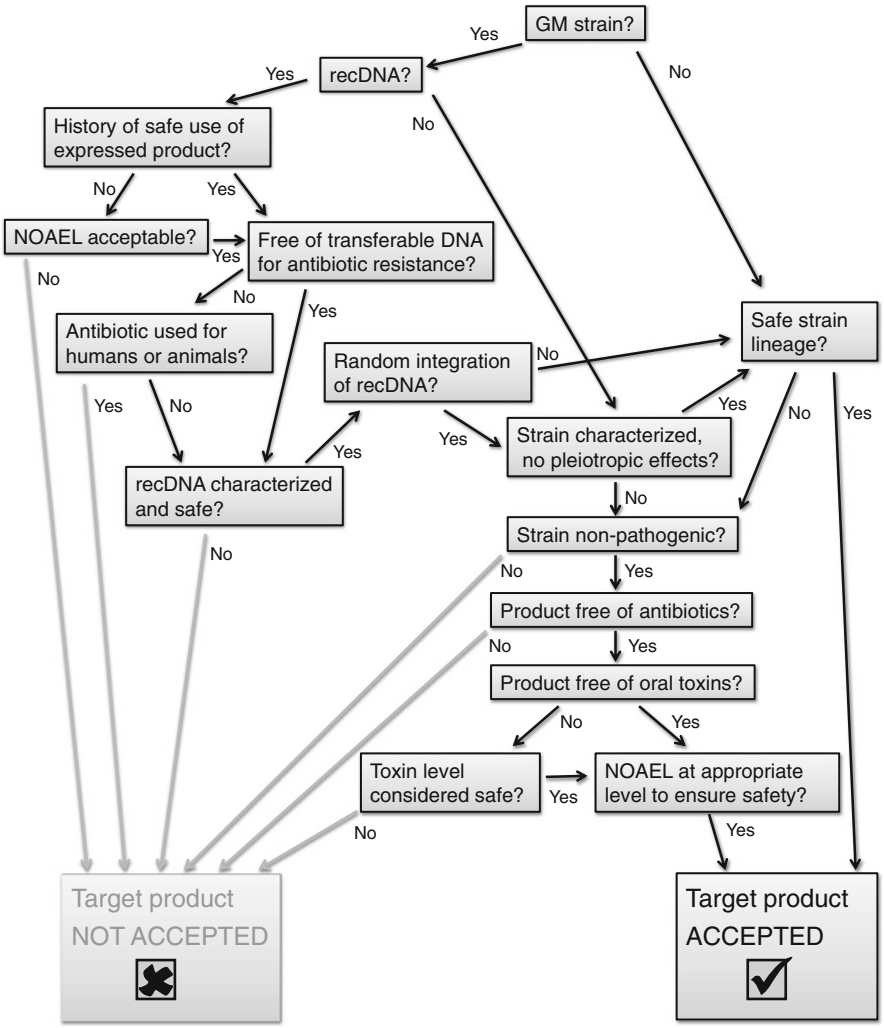

Fig. 2 Decision tree for evaluating the safety of microbial enzymes (modified from Pariza and Johnson 2001)

In Australia, food enzymes are considered to be processing aids and their use is regulated by Food Standards Australia New Zealand (FSANZ) (Australian Government ComLaw 2014). FSANZ carries out safety assessments on a case-by-case basis, which means each new genetic modification is assessed individually for its potential impact on the safety of the food. In the case of GM food, the primary purpose is: (i) to identify new or altered hazards associated with the food as a result of the genetic modification; (ii) to assess whether there is any risk associated with any identified hazards under the intended conditions of use; and (iii) to determine if

any new conditions of use are needed to enable safe use of the food (Food Standards Australia New Zealand 2005).

The production of food and feed enzymes in the European Union is regulated by the European Food Safety Authority. In order to harmonise national rules on the marketing and use of food enzymes, the EU published a Food Improvement Agent Package (FIAP) on 31 December 2008 (Regulation (EC) No 1331/2008 2008). The Package consists of four new regulations providing a common authorization procedure for food additives, food enzymes and food flavorings, plus individual regulations on enzymes, additives and flavorings. This covers only enzymes that are added to food to perform a technical function in manufacturing, processing, preparation, treatment, packaging, and transport or storage of such food, including enzymes used as processing aids. The Regulation (Regulation (EC) No 1331/2008; Regulation (EC) No 1332/2008) requires preparation of a Community List of Approved Enzymes, and specifies conditions for inclusion in the List (safe when used, technical need for the use and no misleading of the consumer). Data required for assessment of food enzymes are laid down in Regulation No 234/2011 (Commission Regulation (EU) No 234/2011 2011). Members of AMFEP have commercialised at least 20 enzymes produced by *T. reesei* by April 2014 (Table 1; Association of Manufacturers and Formulators of Enzyme Products 2014). For more reading, the regulation and safety assessment of food substances in various countries are reviewed in great detail by Magnuson et al. (2013).

5 Safety of the Manufacturing Process

Establishing the basic safety of a fungal strain and target product is not sufficient to allow industrial production until the large scale manufacturing process itself has been evaluated. For example, Directive 2009/41/EC (2009) regulates research and industrial work involving genetically modified microorganisms and requires that any "contained use" of genetically modified microorganisms to produce an enzyme must be notified. Here, contained use is defined as research, industrial and storage activities involving GMMs in which the contact with the population and the environment is limited (Fontana 2010). Genetically modified microorganisms are assigned in four classes of contained use with regards to their effects on human health and the environment, which might arise from such use. For example, *T. reesei* belongs to Class 1 and therefore represents no or negligible risk to human health and the environment. Due to the safe nature of *T. reesei*, inactivation of the organism in contaminated material and waste is optional (Directive 2009/41/EC 2009). Class 4 is reserved for highly dangerous human or animal pathogens which are highly transmissible and for which there is no prophylaxis.

In Australia, the Office of the Gene Technology Regulator (OGTR) oversees the development and environmental release of GM organisms under the Gene Technology Act 2000. According to OGTR, dealing with *T. reesei* and its GMO strains are classified as exempt and do not require a specified level of containment.

Table 1 Examples of enzymes expressed in *T. reesei* and commercialised by AMFEP members

Enzyme activity	Donor organism	IUBMB number	Application
Amylase (alpha)	*Aspergillus* sp.	3.2.1.1	Food/tech
Catalase	*Aspergillus* sp.	1.11.1.6	Tech
Cellulase	NA	3.2.1.4	Food/feed/tech
Cellulase	*Staphylotrichum* sp.	3.2.1.4	Tech
Cellulase	*Trichoderma* sp.	3.2.1.4	Food/feed/tech
Glucanase (endo-1,3(4)-beta)	NA	3.2.1.6	Food/feed/tech
Glucanase (endo-1,3(4)-beta)	*Trichoderma* sp.	3.2.1.6	Food/feed/tech
Glucoamylase or Amyloglucosidase	*Trichoderma* sp.	3.2.1.3	Food/tech
Glucosidase (alpha)	*Aspergillus* sp.	3.2.1.20	Food
Glucosidase (beta)	*Trichoderma* sp.	3.2.1.21	Tech
Laccase	*Thielavia* sp.	1.10.3.2	Tech
Mannanase (endo-1,4-beta)	*Trichoderma* sp.	3.2.1.78	Feed/tech
Pectin methylesterase or Pectinesterase	*Aspergillus* sp.	3.1.1.11	Food/feed/tech
Phospholipase A2	*Aspergillus* sp.	3.1.1.4	Food/feed/tech
Phospholipase B	*Aspergillus* sp.	3.1.1.5	Food/feed
Phytase[a]	*Aspergillus* sp.	3.1.3.x	Feed
Phytase[a]	*Buttiauxella* sp.	3.1.3.x	Food/feed/tech
Polygalacturonase or Pectinase	*Aspergillus* sp.	3.2.1.15	Food/feed/tech
Protease (incl. milk clotting enzymes)	*Trichoderma* sp.	3.4.2.x.xx[b]	Food/tech
Xylanase	*Actinomadura* sp.	3.2.1.8	Feed
Xylanase	NA	3.2.1.8	Food/feed
Xylanase	*Trichoderma* sp.	3.2.1.8	Food/feed/tech
Xylanase	*Aspergillus* sp.	3.2.1.8	Food

IUBMB—refers to the enzymes nomenclature of the International Union of Biochemistry and Molecular Biology (2014)
Tech refers to enzymes for technical applications such as use in the detergent or leather industry
NA not applicable
[a]Includes 3-, 4- and 6-phytase
[b]There is no general IUB number for proteases. All these enzymes fall under the 3.4.2.x category

The only legislative requirement for exempt dealing is that it must not involve an intentional release of a GMO into the environment (Australian Government ComLaw 2001).

Guidelines for the fermentation conditions and factory setting must meet safe industry practice guidelines specified by the relevant authorities of the country as well as mandatory current good manufacturing practice (cGMP) certification; meeting ISO standards is voluntary. There are also strict guidelines concerning separation of the production organism from the target product; the maximum acceptable detection limit for the organism is typically 1 CFU/g of product.

Enzymes used in food processing are either not to be carried over to food or should be inactivated during cooking or baking. Thus, total viable counts for bacteria and fungi are usually determined in enzyme preparations and the presence of coliforms, *Salmonella* sp., *E. coli*, and pathogenic microorganisms is investigated (Pariza and Johnson 2001).

Changes in bioprocessing conditions such as pH, temperature, medium ingredients and the purification process might affect the nature and quantity of by-products, including possible toxins. Following from this, certain restrictive guidelines addressing the production process using a particular organism may be put in place when the organism has been found to produce toxic metabolites under certain conditions. This question recently arose with *T. reesei* when a potentially toxic secondary metabolite, paracelsin, was found to be produced by the fungus only when grown on solid plant material or insoluble substrates (*Trichoderma reesei*; Proposed Significant New Use Rule 2012). Paracelsin has been shown to have hemolytic activity on human erythrocytes and cytotoxicity to Gram-positive bacteria, so inclusion of the enzyme preparation in any product processed or consumed by humans raised serious concerns (Brückner and Graf 1983). As a result, EPA recently restricted the acceptance of *T. reesei* strains for safe industrial use to cases in which cultivation was carried out under standardised conditions. Accepted processes involve submerged fermentation in which no solid plant material or insoluble substrate is present in the fermentation broth. EPA is also proposing to require the use of specified inactivation procedure prior to disposal of the waste that reduces the viable microbial population by at least six logs in liquid and solid wastes (*Trichoderma reesei*; Proposed Significant New Use Rule 2012). In addition, a Significant New Use Rule was proposed by EPA in 2012 for genetically modified *T. reesei* (MCAN J-10-2). Any entity wishing to use *T. reesei* under the "new use" conditions must first file a Significant New Use Notice (SNUN) with EPA. A SNUN allows EPA to review the health and safety implications of the proposed use of the organism under those conditions. At present, EPA has issued 16 MCANs and one SNUN for different *T. reesei* strains (U.S. Environmental Protection Agency 1998).

The placing on the market of GM food and feed products in the EU is regulated by Regulation No 1829/2003 (2003) addressing genetically modified food and feed. According to these regulations, genetically modified food and feed should only be authorised for placing on the market after scientific evaluation of the highest possible standard. Therefore, the testing for any risks they may present for human and animal health and the environment should be undertaken under the responsibility of EFSA.

6 Concluding Remarks

Filamentous fungi such as *T. reesei* and *Aspergillus niger var. awamori* are most commonly used in industry for the production of recombinant enzymes which are predominantly applied in the food, animal feed, detergent, textile and other

industries. The global enzyme market is estimated be worth $7.1 billion in 2018 (BCC Research 2014). The largest market sectors comprise food and beverage enzymes and technical enzymes both expected to reach sales of over $1 billion in 2015. Faster market growth is seen in the Asia/Pacific and other developing regions which underlines the importance of having in place adequate, globally accepted regulations concerning the production organism, safety of the recombinant products and the manufacturing process. Future regulations will be facing an added challenge to deal with production organisms and products made using synthetic biology approaches.

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Applications and Benefits of Thermophilic Microorganisms and Their Enzymes for Industrial Biotechnology

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1 Thermophilic Microorganisms

Environmental temperature is the factor that most influences the growth of microorganisms because it acts directly on the structure and the function of biomolecules and on the maintenance of integrity of cellular structures. Geothermally stable environments have exerted selective pressure on microorganisms, selecting the thermotolerant and those that require high temperatures to survive. Those latter ones are classified into moderate thermophilic organisms with growth range from a minimum of 20 °C up to a maximum of 60 °C and with optimal growth in temperatures above 40 °C. They include the prokaryotic domains Bacteria and Archaea and also eukaryotic organisms in domain Eukarya (represented mainly by filamentous fungi). Hyperthermophilic microorganisms are able to grow at temperatures between 65 and 110 °C. They contain several representatives of Bacteria and Archaea but do not include eukaryotes due to their limited membrane system adaptation in this temperature range (Vieille and Zeikus 2001).

Thermophilic bacteria have been reported in the orders Bacillales, Clostridiales and Thermoanaerobacteriales. These organisms can be found in different environments and are classified into acidophiles, neutrophiles, alkalophiles and meso-

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philes; aerobes, facultative anaerobes, and strict anaerobes, chemoorganotrophs and chemolithotrophs.

Among the hyperthermophilic bacteria, there are more than 70 species, 29 genera included in 10 orders, but most of them are included in the Archaea (Huber et al. 1986). Some examples of bacteria are *Aquiflex pyrophilus*, *A. profundus*, *Fervidobacterium pennavorans*, *Thermotoga maritima*, *T. neapolitana* and *Thermocrinis rubber* (Hedlund et al. 2012; Huber et al. 1986). Among them, *T. maritima* and *A. pyrophilus* exhibit the highest growth at temperatures of 90 and 95 °C. In the Domain Archaea, the genera *Archaeoglobus*, *Ferroglobus*, *Pyrobaculum*, *Pyrodictium*, *Pyrococcus*, *Pyrobolus*, *Sulfolobus*, *Thermoproteus*, *Thermococcus*, *Desulforococcus* are the most prominent with growth above 80 °C. *P. fumari* and *P. occultum* grow at the highest temperature described for living cells 113 and 121 °C, respectively (Berlemont and Gerday 2011).

Among the Kingdom Fungi, only moderately thermophilic species are described, distributed in different taxonomic groups with the majority of them included in the phyla Zygomycota and Ascomycota (Salar and Aneja 2007), with only one Basidiomycota species known (de Oliveira et al. 2015). Maheshwari et al. (2000) suggested that from the 50,000 fungi described until 2000, only 18 genera were considered truly thermophilic (*Canariomyces*, *Chaetomium*, *Coonemeria*, *Corynascus*, *Dactylomyces*, *Malbranchea*, *Melanocarpus*, *Myceliophthora*, *Myriococcum*, *Paecilomyces*, *Remersonia*, *Rhizomucor*, *Scytalidium*, *Stilbella*, *Talaromyces*, *Thermoascus*, *Thermomyces* e *Thielavia*) comprising a total of 30 species. However, in the light of recent changes in fungal taxonomy, the One fungus = One name movement and the new species described since 2000, thermophilic fungi may comprise a group of 20 genera (*Acremonium*, *Arthrinium*, *Canariomyces*, *Chaetomium*, *Humicola*, *Malbranchea*, *Melanocarpus*, *Myceliophthora*, *Myriococcum*, *Rasamsonia*, *Remersonia*, *Rhizomucor*, *Scytalidium*, *Sordaria*, *Thermoascus*, *Thermomyces*, *Thermomucor*, *Thermophymatospora* and *Thielavia*) summing up 44 species (de Oliveira et al. 2015).

Few yeasts species have been reported as being either thermophilic or thermotolerant e.g., *Candida bovina* (=*Torulopsis bovina*), *C. sloofii*, *C. thermophila* (=*Hansenulla augusta*), *Kazachstania telluris* (=*Arxiozyma telluris*) e *Torulopsis pinfolopesii*. Some species of *Kluyveromyces* and *Saccharomyces* are considered thermotolerant with growth at temperatures between 40 and 45 °C. Both *C. parapsilosis* and *S. telluris* are considered thermotolerant strains because they are able to grow at 40 °C although the maximum growth occurs in mesophilic temperatures (Arthur and Watson 1976; Shin et al. 2001). However, thermophilic properties in yeasts remain to be studied in detail.

2 Thermostable Proteins

The tolerance of a microorganism to temperatures above the mesophilic range depends on the membrane, on proteins and on DNA adaptations. However, for this adaptation no new molecular machinery was required, since thermophiles have very

similar molecules to mesophiles, although some differences in the sequence, structure, dynamics and thermodynamic properties can be observed among psychrophilic, mesophilic and thermophilic proteins (Niehaus et al. 1999). The understanding of the mechanism by which thermophilic proteins maintain stability at elevated temperatures will be a big advance for the production of thermophilic enzymes with improved catalytic properties for industrial application (Ruller et al. 2008).

The thermostability of proteins resulted from intrinsic characteristics and is directly associated with the molecular structure resulting in increased rigidity and resistance to unfolding. Additionally, extrinsic factors that help stabilize the proteins in a specific environment, such as solutes, metal binding, molecular chaperones and the substrate have been shown (Bruins et al. 2001).

The native protein is maintained by a delicate balance of non-covalent forces such as hydrogen bridges, ion pairing, hydrophobic interactions and the Van der Waals force. Studies have shown that the adaptation of proteins to high temperature depends on the rigidity/flexibility balance of the molecule. A higher intrinsic rigidity of the thermophilic protein requires a high temperature to promote the thermal motion and the flexibility is essential for catalytic activity.

The main mechanism of intrinsic thermostability of the protein, related to molecular rigidity, is the "hydrophobic effect". Hydrophobic residues are "seized" into the molecule, preventing contact with water, hence directing the folding of the protein which results in a native structure of the molecule with low tendency to unfolding. These residues are better conserved in thermostable proteins than in their mesophilic counterparts from mesophilic organisms. A number of thermostable proteins described have a high content of hydrophobic amino acids and aromatic residues (Scandurra et al. 1998).

The number of hydrogen bonds, salt bridges and the packing of external residues is strongly correlated to the thermostability of thermophilic and mesophilic proteins. Also, the secondary structures of thermostable proteins tend to have a higher number of β-sheet and α-helices than occurring in mesophilic proteins (Szilágyi and Závodszky 2000).

Despite evidence of various structural features of the proteins correlated to thermostability, there isn't a consensus on which of them would be really definitive for this property yet.

2.1 Thermostable Enzymes

Thermostable enzymes have become the focus of biotechnological interest because they are more tolerant to the conditions in industrial processes and storage. The production of thermostable enzymes has grown through advances in isolating a large number of thermophilic microorganisms of the Archaea, Bacteria and Eukarya from different ecological niches and their enzymes have been characterized (Bhalla et al. 2014; Hung et al. 2011; Qiao et al. 2014).

The advantage of the use of thermostable enzymes is the possibility of conducting biotechnological processes at elevated temperatures and thus reducing the risk of contamination by mesophilic microorganisms, decreasing the viscosity of the reaction medium, increasing the bioavailability and solubility of organic compounds, increasing the diffusion coefficient of substrates and products resulting in higher reaction rates (Kumar and Nussinov 2001).

Although it is expected that thermophilic organisms produce more thermostable enzymes than their mesophilic counterparts, it has been shown that the thermophilic characteristic of the microorganism is not the unique condition for the production of thermostable enzymes (Ibrahim et al. 2014). Leite et al. (2007) studied the β-glucosidases from the mesophilic yeast *Aureobasidium pullulans* and from the thermophilic fungus *T. aurantiacus* and concluded that the enzyme produced by the mesophilic strain was more thermostable than that produced by the thermophilic one and the proteins showed a higher level of glycosylation. Extracellular enzymes from filamentous fungi usually present a high glycosylation level and this has been a factor associated with thermostability (Martins et al. 2013).

2.1.1 Thermostable Glycoside Hydrolases

The enzymes market had a turnover of around USD 4411.6 million in 2013 and is expected to increase to USD 7652.0 million by 2020. Around 20 % of the world enzyme market refers to glycoside hydrolases (GHs) that catalyze the release of monosaccharide from cellulose, hemicellulose, pectin and starch (Bajpai 2014). Table 1 contains a summary of the biocatalytic conversions and industrial applications for some thermostable glycoside hydrolases produced by fungi.

The nomenclature of glycoside hydrolases follows the pattern of Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB 1999) taking into account the substrate specificity and the molecular mechanism. The classification of GHs in families, proposed a few years ago, is based on similarities in their amino acid sequences and the type of three-dimensional folding (Cantarel et al. 2009; Damásio et al. 2014). This system is composed of 133 families cataloged in the database CAZy (*Carbohydrate-Active enZYmes*; http://www.cazy.org/Glycoside-Hydrolases.html).

Traditionally, many GHs with application in commercial bioprocesses such as cellulases, xylanases, pectinases and amylases have been produced from mesophilic yeasts, filamentous fungi and bacteria. The fungi of the genera *Aspergillus*, *Trichoderma*, *Humicola*, *Penicillium*, *Fusarium* and *Phanerochaete*, are the most frequently used in the industrial production of xylanases and cellulases (Lynd et al. 2002) and *A. niger*, *A. awamori* and *Rhizopus oryzae* for the production of amylase (Singhania et al. 2010). Pectinolytic enzymes are produced by *A. niger*, *T. harzianum*, *Rhizopus* sp. *A. niger* stands out for the industrial production of enzymes, since it is generally recognized as a safe microorganism (GRAS). However, these enzymes usually act in a mesophilic temperature range and could not be used in processes with high temperature conditions (Sahnoun et al. 2012).

Table 1 Bioconversion reactions and applications of thermostable hydrolyse

Enzyme	Temperature (°C)	Applications
Amylases	50–60	Starch hydrolysis and dextrin production
Cellulases	45–95	Detergent additive and biomass saccharification for ethanol production
Xylanase	45–105	Pulp and paper industry and biomass saccharification for ethanol production
Lichenase (B-1,3; 1,3-Glucanase)	70	Brewing industry
Laccases	70	Pulp and paper industry and biomass saccharification for ethanol production
Chitinases	70	Oligosaccharides production
β-Mannanase	80	Biomass saccharification for ethanol production and Oligosaccharides production
Phytase	60	Additive in monogastric feed
Lipase	70	Dairy products, detergent additive, biodiesel production, leather industry and pharmaceutical and fine chemical's industries
Protease	65–85	Baking, brewing, detergents, leather industry
Oxidoredutase	70–75	Active alcohols and prochiral ketones in pharmaceutical and fine chemical's industries

Advances in structural biology have shown a total of 100 GH families that have at least one member with a three-dimensional structure available in the PDB. Due to the increasing data about the structure of GH and the fact that the structure of these proteins is usually better conserved than their sequences, the strategies of comparison and thus, grouping or organization from different families into clans were adapted in: GH-A to GH-N (CAZy; http://www.cazy.org/), with most of these clans comprising two to three families, with the exception of the clan GH-A which covers 19 families (http://www.cazy.org/Glycoside-Hydrolases.html) (Fushinobu et al. 2013; Henrissat and Bairoch 1996).

Even when comprised in the same genome, GHs generally present a diverse range of multimodal settings. The polypeptides associated with the hydrolysis of plant cell walls commonly contain a catalytic domain and a carbohydrate-binding module (CBM). CBMs are small domains with specific affinity to carbohydrates and, therefore, act to achieve the catalytic portion of the enzyme to its cognate substrate (Shoseyov et al. 2006). Recently, Qiao et al. (2014) demonstrated that the CBM associated with thermostable xylanases has no catalytic function but has great importance in the thermostability of the cellulase from hyperthermophilic archaea. The presence of a multi-domain has been shown to improve the deconstruction of lignocellulose above 90 °C by thermostable cellulase (Graham et al. 2011).

With respect to the mechanism of catalysis and despite the complex structural diversity of hydrolytic enzymes, all glycoside hydrolases, except members of Family 4, are separated into two distinct classes. The first class catalyzes the hydro-

lysis of glycosidic linkages by a unique displacement mechanism displacement with the inversion of the anomeric center configuration and the second class catalyzes a double displacement mechanism with retention of the stereochemical configuration of the anomeric carbon (Collins et al. 2005). The reversing mechanism operates via direct displacement of the group releasing the water molecule while the retaining mechanism, operates through the double displacement involving the formation of an intermediate (enzyme-carbohydrate). Despite these differences, it is important to emphasize that both classes use a mechanism of acid catalysis, in which two amino acid residues are involved at the active site of the enzymes. In GHs, an inversion acid residue acts as an acid and another as a general base while retaining the GHs. A residue acts as a nucleophile and a leaving group (Collins et al. 2005).

In general, the availability of cellulases, xylanases and pectinases for industrial applications is restricted by their poor stability or low relative activity at higher temperatures or pH range. In this sense, the thermostable GHs of fungal origin are the main focus of research because the hydrolysis of complex carbohydrates such as cellulose and hemicellulose is nowadays an important way of obtaining alternative fuels (Dodd and Cann 2009).

The industrial processes for the deconstruction of lignocellulose by thermostable enzymes, which maintain stability, structural and functional integrity above 55 °C, have big advantages over their mesophilic counterparts. The increased stability of proteins allows the extension of the periods of hydrolysis of backbone polymer into monomers such as glucose or pentose decreasing the amount of enzyme required (Viikari et al. 2007). Thermostable enzymes provide better compatibility with the thermal pre-treatments applied to reduce the crystallinity of cellulose and facilitate the access of the enzymes to polymers (Szijártó et al. 2008). In thermophilic conditions, the risk of contamination of the reaction media by mesophilic microorganisms is decreased and the storage time without loss of enzyme activity is increased (Haki and Rakshit 2003).

Thermostable Xylanases

The hemicellulose fraction consists of highly branched heteropolysaccharide. The sugar residues found in hemicellulose include pentoses (D-xylose, L-arabinose), hexoses (D-galactose, L-galactose, D-mannose, L-raminose, L-fucose) and uronic acids (D-galacturonic acid) (Fig. 1). These sugar residues are modified by acetylation and methylation. The classification of hemicelluloses depends on the type of monomers united to form heteropolymers and are called xylans, mannans, galactans or arabinans (Deutschmann and Dekker 2012).

The xylanolytic complex includes endo-1,4-β-D-xylanase (EC 3.2.1.8) that hydrolyzes the β-1,4-glycosidic bonds within of the xylan backbone producing xylo-oligosaccharides which are converted into xylose units by 1,4-β-D-xylosidase (EC 3.2.1.37). To complete hydrolysis of the xylan molecule, accessory enzymes that remove substituent side chains (branching points) are needed such as: the α-L-arabinofuranosidase (EC 3.2.1.55), which hydrolyses the terminal nonreducing ara-

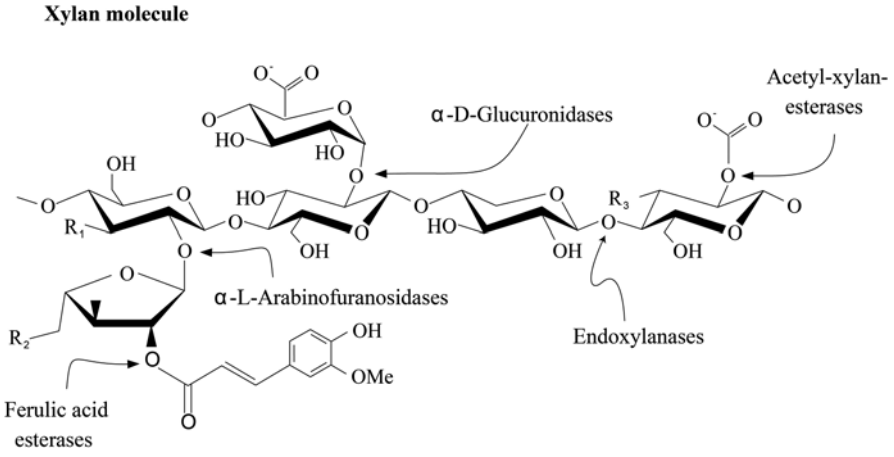

Fig. 1 General chemical structures of xylans and hemicellulose actions

binofuranose, arabinogalactan and arabinoxylan groups; the α-D-glucuronidase (EC 3.2.1.139) that hydrolyzes the 1,2-linked α-glycosidic between xylose and glucuronic acid; acetyl xylan esterase (EC 3.1.1.72) and the feruloyl esterase (EC 3.1.1.73) that remove acetyl and phenolic groups, respectively; mannan endo-1,4-β-mannanases (EC 3.2.1.78); the 1,4-β-mannosidases (EC 3.2.1.25); and endo-arabinan 1,5-α-L-arabinosidases (EC 3.2.1.990 (Shallom and Shoham 2003).

The xylanolytic enzymes with high molar mass (>30 kDa) are included in the GH 10 family, and those with molar mass less than 30 kDa, in the GH11 family. Family 10 includes the xylanases with highest thermostability (Dominguez et al. 1995).

Endo- and exo-xylanases have been widely applied in biotechnological processes such as biobleaching of paper pulp, animal feed production, clarifying of fruit juices, bread making (Kulkarni et al. 1999), production of packaging films based on xylan (Hansen and Plackett 2008) and production of biodegradable surfactants (Damez et al. 2007) (Fig. 2).

Alkali-thermostable xylanase from *B. pumilus* ASH was applied for bio-scouring of jute fabric at 55 °C for increasing whiteness and brightness of fabric by up to 10.2 % and also for decreasing the yellowness by 5.57 % compared to conventional processes (Garg et al. 2013).

Catalysis at a high temperature could be advantageous in the bioconversion of xylan from lignocellulosic materials into xylitol. A thermal pre-treatment is often included to enhance the degradability of lignocellulose. It improves the enzyme penetration for hemicellulase conversions and for an efficient hydrolysis of xylan into xylose monomers. The subsequent step is the conversion into xylitol or another product with added value such as organic acids, flavoring and pigments. Use of thermophilic xylanase allows the enzymatic hydrolysis to occur simultaneously with the heating step, without the need for the pre-cool step (Turner et al. 2007). Recently, these enzymes have also been highlighted with regard to the production

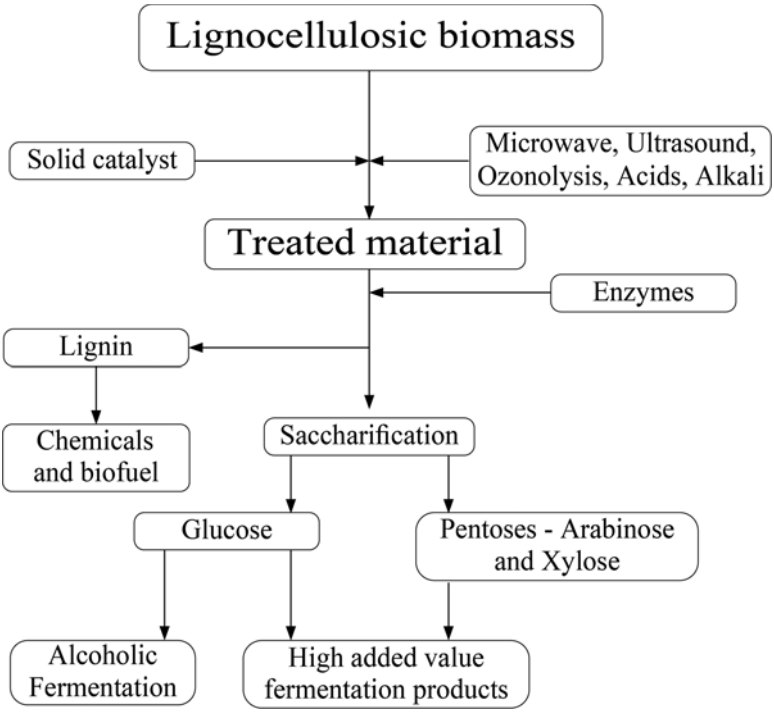

Fig. 2 Pathways of deconstruction and products from lignocellulosic material

of bioactive xylooligosaccharides (XOs) that are considered prebiotics (Rustiguel et al. 2010). Teng et al. (2010) obtained a high yield of XOs using corncobs submitted to steam explosion pretreatment followed by hydrolysis using thermostable xylanase from *Paecilomyces themophila* J18, at 70 °C.

Nowadays, the main aim of biomass saccharification is to produce second generation ethanol. It is believed that such biomass-based biofuels will increase one hundred-fold during the next 10 years. The full utilization of lignocellulosic biomass, including pentose sugars, derived from the hydrolysis of hemicellulose and associated with the transformation of glucose to ethanol, has been the proposal of biorefineries (Kim and Kim 2014).

Several studies have reported the production of thermostable xylanase from thermophilic and hyperthermophilic organisms, prokaryotes and eukaryotes whose activities have ranged from 60 to 100 °C (Bhalla et al. 2014; Palackal et al. 2004; Qiao et al. 2014; Rättö et al. 1994; Ruttersmith et al. 1992). Among thermophilic bacteria, *B. amyloliquefaciens*, *B. circulans*, *Streptomyces* sp., *Thermoactonomyces thalophilus* produce xylanases with high heat tolerance whose activities occur around 80 °C (Breccia et al. 1998; Dhillon and Khanna 2000; Kohli et al. 2001; Simpson et al. 1991). Xylanases produced by hyperthermophilic, namely *Thermotoga* sp., *Dictyoglomus sp.*, *T. marítima*, *T. neapolitana*, *T. themarum*, *Thermoanaerobacterium saccharolyticum*, have optimal temperature for activity

around 90 °C (Cannio et al. 2004; Leuschner and Antranikian 1995; Saul et al. 1994; Winterhalter and Liebl 1995) while xylanase from *P. furiosus* and from *S. solfataricus*, act at 100 °C and 105 °C, respectively (Haki and Rakshit 2003).

Thermophilic fungi, such as *Thermomyces thermophiles*, *T. lanuginosus*, *Thermoascus aurantiacus*, *Rhizomucor miehei*, *Thermomucor indaticae-seudadicae*, produce thermostable xylanases with action from 50 up to 80 °C (Damaso et al. 2003; Lee et al. 2009; Maalej et al. 2009; Saha 2002; Zhang et al. 2011).

Thermostable Cellulases

Cellulose is about 33 % of the dry weight of the plant. It is a linear, non-branched homopolysaccharide with 10–15 thousand units of D-glucoses linked by β (1-4)-glycosidic bonds. Along the polymer there are predominantly ordered regions (crystalline) interwoven with disordered regions (amorphous) (Stone and Scallan 1965) (Fig. 3).

The ordered crystalline region prevents water penetration and hinders the action of cellulases providing structural and chemical stabilities and, consequently, recalcitrance. Amorphous regions of the cellulose molecules, located on the surface, are more susceptible to enzymatic hydrolysis. Thus, cellulases have to adsorb to the surface of the pulp prior to catalyzing the hydrolysis, promoting the molecular disorder of the crystalline regions and exposing the cellulose chains (Coughlan 1985). Recalcitrance of cellulose has been overcome with pretreatments based mainly on heating the material before enzymatic hydrolysis. These pretreatments allow using thermostable cellulases to avoid the cooling step and so reducing time and cost (Moretti et al. 2014).

Two types of cellulases are distinguished on the basis of the ability to recognize or not recognize the terminal portion of the glucose chains: the exo-cellulases or cellobiohydrolases (CBH) and endo-cellulase or endoglucanase (EG). The endoglucanase or endo 1,4 β-glucanase (EC 3.2.1.4) recognize and hydrolyze the amorphous regions in the polymer internally at random, decreasing the polymerization degree of the molecule and at the same time, providing for the substrate for cellobiohydrolases. The exoglucanase or cellobiohydrolase (EC 3.2.1.91) recognize the ends of the chain and act removing cellobiose units. Two types of CBH are distinguished: those that act from the reducing end (CBHI) and those that act from the non-reducing end (CBH II). Some studies, however, have questioned the existence of the exo and endo types and suggest that all cellulases are endo type and proposed a classification used for glycoside hydrolases based on the hydrolysis, namely processive and non-processive cellulase types (Henrissat et al. 1995).

The processive cellulases have continuous threading of a single carbohydrate chain through the catalytic site of the enzyme, yielding disaccharides in successive cleavages. CBHs with a tunnel-shaped active site entrance are generally considered to be processive enzymes that slide along polysaccharide chains continuously. The non-processive cellulases with an open cleft-like active site do not exert continuous

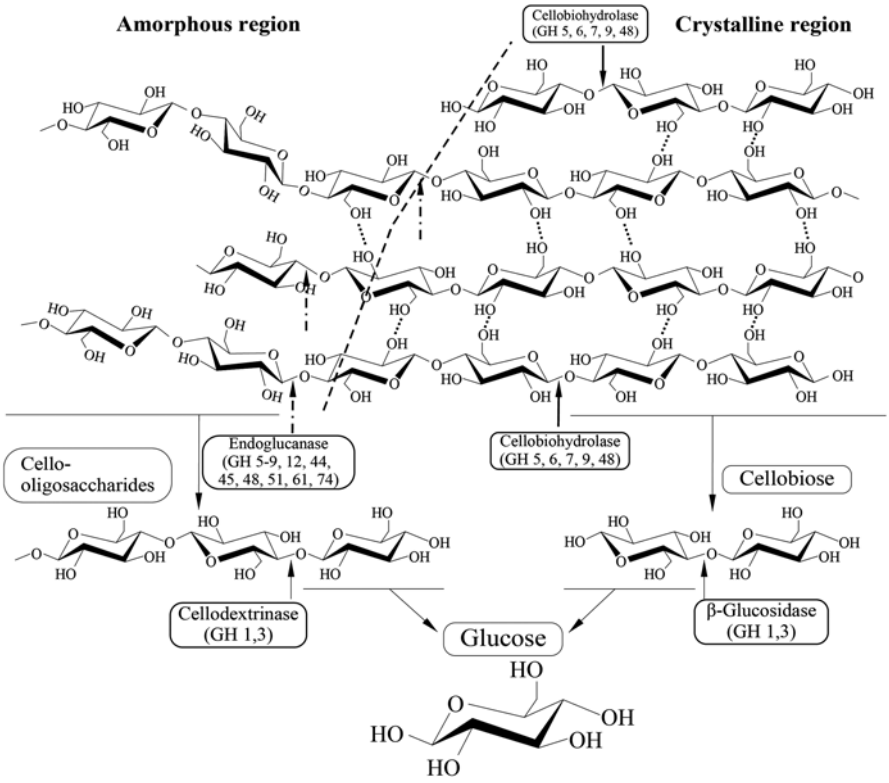

Fig. 3 Schematic representation of cellulose and the cellulolytic complex, highlighting the sites of higher activities of cellulolytic enzymes

action and the active site is disconnected from the substrate after each hydrolysis which is reinitiated at a new site. Based on this classification, CHB could be classified as an endo-processive type while the EG would be a non-processive endo (Várnai et al. 2014).

Another important enzyme of the cellulolytic group is the β-glucosidase (cellobiases) or 1.4-β-glucosidase (EC 3.2.1.21) that hydrolyzes cellobiose and other short cellooligosaccharides releasing glucose. Some of them are also involved in the reactions of transglycosylations of β glycosidic bonds in glucose conjugates (Qi et al. 2008).

Besides the classical cellulases, the swollenins and the expansin-like protein were found to play an important role in the degradation of crystalline cellulose. It is believed that these proteins are involved in the disruption of hydrogen bonds between cellulose microfibrils or cellulose and other polysaccharides from the cell wall causing the sliding of cellulose or expansion of the cell walls fibers (Whitney et al. 2000).

The cellulose degradation involves an oxidative enzyme class, the lytic polysaccharide monooxigenases (LPMOs) that oxidize one of the two new chain ends,

introducing a C1 or C4 oxidation at the released reducing or non-reducing end, respectively. LPMOs are widespread in fungal genomes and, recently, a new class, namely auxiliary redox protein (AAs) was created in the Carbohydrate-Active Enzymes (CAZy) database to accommodate the LPMOs (Horn et al. 2012).

In industrial processes, cellulolytic enzymes have been employed in the extraction of pigments and flavor compounds in fruit juice and wine production; as additive of detergents for washing jeans; in the pretreatment of biomass to improve the nutritional quality of forage for animal feed; in the textile industry in the polishing process of cotton fibers and for saccharification of lignocellulosic residues to obtain reducing sugar (Ando et al. 2002; Baffi et al. 2013). This latter is one of the emerging applications of the cellulolytic enzyme complex, wherein the lignocellulosic raw material source units are converted to glucose by enzymatic action for the production of second generation ethanol. However, other products with higher added value can also be obtained from glucose derived from cellulosic material (Olofsson et al. 2008).

The interest in the use of cellulases to produce fermentable sugars from cellulosic wastes at present is focusing on biofuel production such as biogas, bioethanol, biodiesel and fuel cells. Bioethanol is the most common renewable fuel today, being the "Energy for the Future: around the world. The ethanol is commonly derived from starch in US or sucrose sugar cane in Brazil and Latin America. The use of whole biomass to obtain alcohol-based fuels requires an efficient conversion of lignocellulosic material into fermentable pentose and hexose sugars.

Although cellulosic ethanol has already been established as an alternative biofuel in this decade, the estimated economic cost of cellulase enzymes used for lignocellulosic ethanol ranges from 25 to 50 % of the total production cost with a value around 0.11 USD per liter of ethanol. The main reasons are the low yield of enzymatic hydrolysis and low stability of enzymes marketed today. They tolerate neither the climatic conditions of tropical countries nor the conditions in industrial application (Dias et al. 2012; Moretti et al. 2014).

Thermal stability of several commercial cellulase preparations is an important parameter for the success of the process. The best known commercial enzymes Celluclast®, Accellerase 1000® and Spezyme®, studied by Pribowo et al. (2012), retained 85 % of their initial β-glucosidase, Cel7A and endoglucanase activities at 50 °C, but little or no activity was retained at higher temperatures. However, the industries have been developing cellulases with higher thermal stability and especially stable at industrially relevant conditions. The research is focused on new producer microorganisms and techniques for engineering proteins. Many thermostable cellulolytic enzymes of both eukaryotic and prokaryotic origin are known to date (Table 2).

Thermostable Pectinases

Pectic substances are complex glycosidic macromolecules of high molecular weight present in the primary cell wall and are the main components of the middle lamella, an extracellular thin adhesive layer the cells walls of two adjoining cells together.

Table 2 Cell wall plant degrading enzyme from thermophilic and thermotolerant microorganisms

Organisms	Enzyme	Optimal temperature (°C)	References
Clostridium abosum	Xylanase	75	Rani and Nand (2000)
Thermotoga maritima MSB8	Endo-Xylanase B	92	Jiang et al. (2005)
Thermotoga neapolitana	Xylanase	95	Bok et al. (1998)
Thermotoga neapolitana	Xylanase	85	Bok et al. (1998)
Thermotoga neapolitana	Xylanase	102	Zverlov et al. (1996)
Thermotoga thermarum	Xylanase	80	Sunna et al. (1997)
Thermotoga sp. strain FjSS3-B1	Xylanase	105	Ruttersmith and Daniel (1991)
Thermotoga sp. strain FjSS3-B1	Xylanase	*115*	Simpson et al. (1991)
Caldicellulosiruptor kronotskyensis	Xylanase	*75*	Qiao et al. (2014)
Geobacillus sp.	Xylanase	70	Bhalla et al. (2014)
Thermoanaerobacterium saccharolyticum NTOU1	Xylanase	63	Hung et al. (2011)
Streptomyces sp. strain S38	Xylanase	60	Georis et al. (2000)
Thermobifida halotolerans YIM 90462^T	Xylanase	80	Zhang et al. (2012a, b)
Scytalidium thermophilum	Xylanase	65	Arifoğlu and Ögel (2000)
Fusarium proliferatum	Xylanase	55	Saha (2002)
Thermoascus aurantiacus	Xylanase	50	Kalogeris et al. (1998)
Thermoascus lanuginosus-SSBP	Xylanase	70-75	Lin et al. (1999)
Thermoascus lanuginosus (*W*/*M*)	Xylanase	60, 70	Bakalova et al. (2002)
Malbranchea cinnamomea	Xylanase	*80*	Fan et al. (2014)
Laetiporus sulphureus	Xylanase	80	Lee et al. (2009)
Myceliophthora thermophila. M.7.7	Xylanase	60	Moretti et al. (2012)
Aspergillus fumigatus M.7.1	Xylanase	65	Moretti et al. (2012)
Thermotoga maritima MSB8	Endoglucanase	95	Bronnenmeier et al. (1995)
Thermotoga neapolitana	Endo-cellulase	106	Bok et al. (1998)
Clostridium stercorarium	Avicelase I	75–80	Riedel et al. (1998)
Fervidobacterium nodosum	Endo-glucanase	80	Wang et al. (2010)
Bacillus halodurans CAS 1	Endoglucanase	80	Annamalai et al. (2013)
Geobacillus sp.	Endo-glucanase	75	Rastogi et al. (2010)

(continued)

Table 2 (continued)

Organisms	Enzyme	Optimal temperature (°C)	References
Neocallimastix patriciarum J1	Exo-glucanase	70	Wang et al. (2013)
Acidothermus cellulolyticus	Endo-glucanase	80	Zhang et al. (2012)
Myceliophthora thermophila. M.7.7	Endo-glucanase	70	Moretti et al. (2012)
Aspergillus fumigatus M.7.1	Endo-glucanase	70	Moretti et al. (2012)
Thermoascus aurantiacus	β-glucosidase	75	Leite et al. (2007)
Aureobasidium pullulans	β-glucosidase	80	Leite et al. (2008)
Thermomucor indicae-seudaticae N31	β-glucosidase	75	Pereira et al. (2014)
Bacillus halodurans M29	Exo-Polygalacturonase	80	Mei et al. (2013)
Bacillus licheniformis KIBGE-IB21	Polygalacturonase	45	Rehman et al. (2015)
Bacillus pumilus	Polygalacturonase	50	Sharma and Satyanarayana (2006)
Aspergillus carbonarius	Endo-Polygalacturonase I	55	Anjana Devi and Appu Rao (1996)
Aspergillus carbonarius	Endo-Polygalacturonase II	50	Anjana Devi and Appu Rao (1996)
Aspergillus carbonarius	Endo-Polygalacturonase III	55	Anjana Devi and Appu Rao (1996)
Aspergillus niger HFD5A-1	Polygalacturonase	45	Ibrahim et al. (2014)
Aspergillus giganteus	Exo-Polygalacturonase	55	Pedrolli et al. (2008)
Aspergillus awamori	Polygalacturonase	50	Dey et al. (2014)
Penicillium frequentans	Exo-Polygalacturonase II	50	Chellegatti et al. (2002)
Penicillium frequentans	Exo-Polygalacturonase I and III	50	Barense et al. (2001)
Thermoascus aurantiacus	Endo-Polygalacturonase	60–65	Martins et al. (2007)
Acremonium cellulolyticus	Endo-Polygalacturonase	50	Gao et al. (2014)

They are responsible for the structural integrity and cohesion of plant tissues (Pilnik and Rombouts 1981). Three major groups of pectic polysaccharides are recognized, each containing D-galacturonic acid to a greater or lesser extent: homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII). HG is a linear polymer composed of D-galacturonic acid that can be acylated and/or esterified with methyl groups (Jayani et al. 2005); RG I is the compound of rhamnose-galacturonic acid disaccharide (Willats et al. 2006) and RGII homogalacturonan is a chain of galacturonic residues, with side chains attached to them (Willats et al. 2006) (Fig. 4). The pectic polysaccharides have been used as bioactive food ingredients as a supplement in children's food and as a thickening agent in the jellies industry.

Pectinases are a group of enzymes that catalyze the degradation of pectic substances by depolymerization reaction (hydrolases and lyases) and by de-esterification reactions (esterases). The main ones are namely Protopectinases A and B, Endopolygalacturonases (EC3.2.1.15—EndoPG) hydrolyze in a random fashion glycosidic α-1,4 bonds along the polygalacturonan network, Exopolygalacturonase (EC 3.2.1.67—ExoPG) hydrolyze at the non-reducing end of the polymer, generating a monosaccharide galacturonic acid. Endopolygalacturonase lyase (EC 4.2.2.2—EndoPL) Exopolygalacturonase lyase (EC 4.2.2.9—ExoPL) cleaves sequencial α-1,4 bonds at the end of pectin, releasing unsaturated products from the reducing end of polymer, and Pectin methyl esterase (EC 3.1.1.11—PME) catalyzes the hydrolysis of the methyl esters of galacturonic acid (Pedrolli et al. 2009).

The protopectinases of type A react with the region of polygalacturonic acid and of type B recognize the region that connects the galacturonic acid chains to other constituents of plant cell wall. The EndoPG catalyze the hydrolytic cleavage of 1,4-β glycosidic bonds in the internal chain of pectin and Exo-PG cleave the 1,4-β glycosidic bonds of the non-reducing end of the chain (Alkorta et al. 1998).

Microbial pectinases account for 25 % of the global food enzymes sales and are used extensively for fruit juice extraction and clarification, refinement of vegetable fibers, degumming of natural fibers, curing of coffee, cocoa and tobacco and also for waste-water treatment. In some applications, it can be more efficient to use thermostable enzymes, particularly when pectins are poorly soluble at ambient temperature (Jayani et al. 2005).

One of the most common applications of pectinases is in fruit processing for various purposes like musts, juices, pastes and purées. In maceration for grape juice extraction, incubation of the fruits at 60–65 °C promotes the plasmolysis of the membrane and cell wall disruptions facilitating the release of the liquid and the anthocyanins responsible for the color of juices. In the extraction of grape juice for wine production, the macerated fruit is treated at 80 °C to facilitate maceration and denature oxidases which cause loss of color of the wine during storage. In the extraction of the "pulp wash", the orange pulp (mixture of pulp and seeds), resulting from the first sieving juice, is heated at 90 °C to denature fruit pectin esterase which causes problems of coagulation of pectin. In addition to the mentioned functions, the heat treatment also has the purpose of pasteurizing the juices, aimed at reducing the microbial contaminants, particularly yeast. In all these cited cases, the material

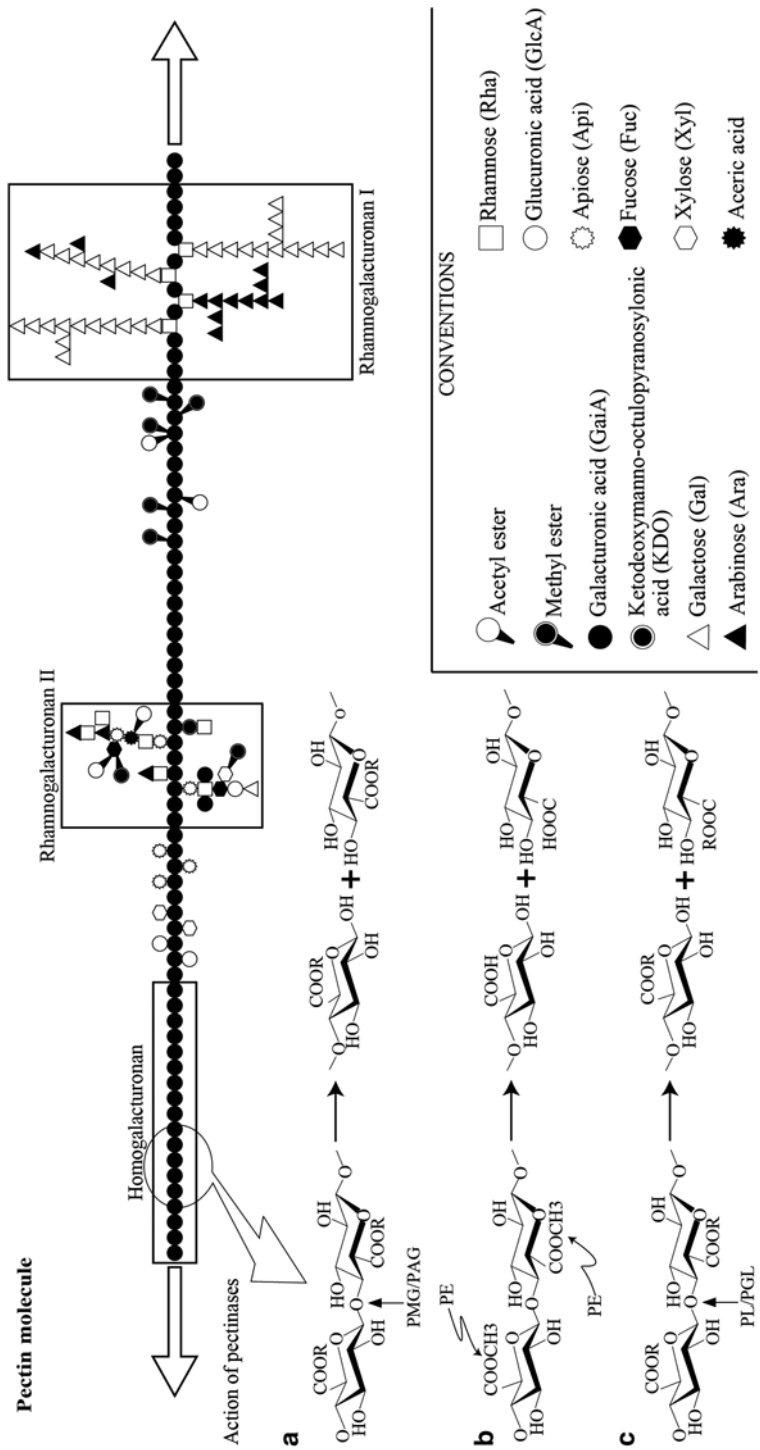

Fig. 4 Schematic representations of structure of pectin molecule and action of pectinases. (**a**) *PMG* polymethylgalacturonases and *PG* endopolygalacturonases (EC 3.2.1.15) create galacturonic acid residue from esterified galacturonic acid. R=CH_3 for PMG and H for PG; (**b**) *PE* pectinesterase (EC 3.1.1.11) catalyzes the hydrolysis of the methyl esters of galacturonic acid; (**c**) *PL* pectin lyase (EC 4.2.2.10) breaks links between esterified galacturonic acid and residues, *PGL* polygalacturonase lyase. R=H for PGL and CH_3 for PL

must be heat treated and subsequently cooled to 50 °C for treatment with commercial pectinases which are thermolabile (Lea 1995). The use of thermostable pectinases avoids the cooling step and so it could reduce the time and cost of processes (Zhang et al. 2011).

The extraction of sugar beet, responsible for 29 % of world sugar production, requires depectinization to increase the extraction efficiency, since more than 30 % of the dry weight of the beet corresponds to pectin. Industrially, this extraction is carried out at temperatures of about 70 °C and, thus, a simultaneous depectinization and extraction using thermostable pectinase would be economically interesting (Singh et al. 1999)

The obtaining of new products from conventional raw materials treated enzymatically has also been a proposal by the food industry. Study about thermostable pectinase treatment potato flours demonstrated that the pectinase changed the physical, thermal, and pasting properties of starch with improve of physical functionalities of potato flours and allow that the pectinase-treated potato flour could expand the diversity of dehydrated potato products, which could contribute to the improval of physical functionalities of potato flours and allow that the pectinase-treated potato flour could expand the diversity of dehydrated potato products, which could contribute to the improved functionalities required for existing or emerging potato-based food systems (Van Hung et al. 2006).

Thermostable pectinase would be very useful also in the degradation of pectin waste from processing plant material industry, reducing BOD and COD (Kapoor et al. 2000). Recently, pectinase from *P. echinulatum*, associated with a cellulolitic enzyme complex, improved sugar cane bagasse saccharification, suggesting a new application for these enzymes (Delabona et al. 2013).

Thermostable bacterial pectinases have been reported, such as alkaline polygalacturonases of *Bacillus* sp. MG-cp-2, *Bacillus* sp. DT7 and the *Bacillus* sp. KSM-P7 (Kapoor et al. 2000; Kashyap et al. 2001; Kobayashi et al. 1999); pectate lyase from *Thermoareobacter italicus* sp.nov. (Kozianowski et al. 1997), polygalacturonase from *Clostridium thermosulfurogenes* and exo-polygalacturonase de *T. maritime* (Parisot et al. 2003) and *B. halodurans* (Mei et al. 2013).

Few pectinolytic thermophilic fungi have been isolated (Maheshwari et al. 2000). Inamdar (1987) studied 40 thermophilic fungi, of which only seven genera were capable of growing in liquid medium containing pectin and the majority did not produce detectable polygalacturonase activity in the media.

The thermophilic *Thermoascus aurantiacus* produced considerable amounts of pectinase in media based of citrus peel (Martins et al. 2002) which showed optimal activity at 70 °C and stability at 60 °C for 2 h.

Kumar and Palanivelu (1999) as well as Puchart and colleagues (1999) reported the production of polygalacturonase by *T.lanuginosus*, whose optimum temperature was 70 °C when in crude solution and 60 °C after purification (Kumar and Palanivelu 1999). Martin et al. (2010) described Exo-polygalacturonase (Exo-PG) from *T. indicae-seudadicae* with optimum temperature at 65 °C and Exo-PG and pectate lyase from *P. viridicatum* with activity at 60 °C was reported (Ferreira et al. 2010; Gomes et al. 2009). A thermostable polygalacturonase from thermophilic *Myceliophthora thermophile* showed optimal activity at 55 °C (Moretti et al. 2014).

2.2 *Microbial System for Cloning and the Heterologous Expression of Thermostable Proteins*

Alternatives to improve the thermostability of enzymes are the recombinant proteins technologies and the genetic modification of proteins (Stephens et al. 2014). However, these techniques are only possible when the structural determinants related to the thermostability of the protein is known and, thus, the isolation of thermophilic strains producing thermostable enzymes is a promising path for supplying protein models and genes important for advancing enzymatic technology.

The genetic engineering of microorganisms has been used in order to create new systems for enzyme production aiming at an increase of the expression efficiency and activity as well as changes in the characteristics of the enzymes. A number of articles have been published on genes coding for cellulase, xylanase and pectinases of prokaryotic and eukaryotic origin, which were expressed in different systems, such as *Escherichia coli*, *Pichia pastoris*, *Saccharomyces cerevisiae and Aspergillus nidulans*. Microbial systems for the expression of heterologous proteins are attractive due to fast growth on low cost substrates; they are well-characterized genetically and physiologically and the availability of a large number of cloning vectors and host strain are commercially available (Ben Mabrouk et al. 2013).

Changes in the intrinsic properties of molecules, which allow an increase in the thermal stability, tolerance to changes in pH and other denaturation conditions could be achieved. Thereby, techniques of random mutation, directed evolution epPCR (error-prone PCR), site-directed mutagenesis and DNA shuffling were applied (Castro and Pereira Jr 2010; Fenel et al. 2004; McHunu et al. 2009; Trevizano et al. 2012; Turunen et al. 2002). Big companies in the field of enzyme production have also invested in this technology (Lynd et al. 2002).

In general, the best host system and promoter for obtaining heterologous proteins depend on the target protein itself and also on the objective of the heterologous expression. Many bacterial systems are not capable of introducing proper post-translational modifications, such as glycosylation and sulphonation, which may be critical depending on the target protein. Alternative hosts, such as yeast and filamentous fungi, are available for the expression of heterologous proteins (Cain et al. 2014). The next section will discuss the main types of hosts and promoters widely used for production of heterologous proteins.

2.2.1 Bacteria as Heterologous Expression System

For production of heterologous proteins, in prokaryotic systems, has been used strains of *E. coli*; *B. megaterium*, *B. subtilis*, *B. brevis* (Udaka and Yamagata 1993) and *Caulobacter crescentus* (Awram and Smit 1998). The other less common systems such as *Staphylococcus carnosus* (Hansson et al. 2002) *Pseudomonas fluorescens* (Schneider et al. 2005), *Ralstonia eutropha* (Barnard et al. 2004), *Streptomyces* species (Brawner 1994), methylotrophic bacteria like *Methylobacterium extorquens*

(Gutiérrez et al. 2005), cyanobacterium such as *Anabaena* sp. (Desplancq et al. 2005), have been studied. These systems are still underutilized due to the need for further studies on the regulation of gene expression and metabolism, and also the lack of the appropriate vector and promoter systems being commercially available. Recently, *Rhodobacter* (Katzke et al. 2010) and acid lactic bacteria (Cain et al. 2014) were evaluated for heterologous protein obtainment.

Despite its ease of application, the prokaryotic system has some disadvantages. *E. coli* cultures accumulate the lipopolysaccharide endotoxin (LPS) which are pyrogenic in humans and other mammals, limiting this system for obtaining proteins for therapeutic purposes (Petsch and Anspach 2000). Also, being a prokaryotic system, *E. coli* is not able to modify proteins post-translationally through glycosylation and does not secrete heterologous glycosylated proteins from eukaryotic origin which are converted into insoluble aggregates that accumulate in the cytoplasm requiring additional steps of cell lyses for extraction and purification (Georgiou and Segatori 2005). Some small polypeptides of around 10 kDa can be degraded in the *E. coli* cytoplasm by endogenous proteases (Hammarström et al. 2002). Moreover, the correct formation of disulfide bonds, the absence of specific chaperonins for correct folding and poor solubility may occur (Peroutka Iii et al. 2011; Sahdev et al. 2008). The use of *E. coli* BL21 and K12 strains and their derivatives that are deficient in *ompT* and *lon* proteases reduces the degradation of the expressed heterologous eukaryotic proteins (Phillips et al. 1984) (Table 3).

Other methods have been developed to address the secretion of heterologous proteins, such as slow translation by reducing the temperature of expression (Hammarström et al. 2002), mRNA concentration decreased by reducing the concentration of the inducer (Huang et al. 1994), optimization of the promoter and the manipulation of induction conditions (Qing et al. 2004), co-expression of target proteins with specific molecular chaperonins (de Marco and De Marco 2004) and the expression of fused protein (Ahn et al. 2007). Commercial fusion partners, called tags, including *HIS* (histidine) *MBP* (maltose binding protein) and *GST* (glutathione S-transferase) have shown increased solubility (Song et al. 2011). Another factor to consider is that, for each type of selected strain, there is an expression vector for the process of gene insertion into the vector and its subsequent spread in strains of *E. coli*. The pET-28a (5.4 Kb) expression system, for example, represents one of the most common commercial expression vectors of the *E. coli* pET system (Novagen 2003).

2.2.2 Fungi as a Heterologous Expression System

Among the eukaryotic systems used for heterologous expression of proteins, the yeasts have the best cost-benefit ratio for protein production on a large scale due to their unicellular characteristic. Genes encoding cellulolytic enzymes and xylan-degrading bacterial and fungal sources have been cloned and expressed in *S. cerevisiae* and *P. pastoris* (Lynd et al. 2002; Parachin et al. 2009; Torres and Moraes 2002).

Table 3 Some *E. coli* strains most frequently used for heterologous protein production and their key features

E. coli strain	Derivations	Properties	References
AD494	K-12	*trx*B mutant, disulfide bond formation in the cytoplasm is enhanced	Derman et al. (1993)
BL21 BL21(DE3)[a]; BL21 trxB[b]; BL21 Codon Plus-RP[c] and Plus-RIL[d]	B834 and or BL21	Both Deficient in *lon* and *ompT* proteases; with *hsd*SB mutation prevent the loss of plasmid.[a] lacUV5 promoter drives the expression of the T7 RNAP.[b] *trx*B mutant, disulfide bond formation in the cytoplasm is enhanced.[c–d] Enhances the expression of eukaryotic proteins that contain codons rarely used in *E. coli*: AGG, AGA, CCC; AGG, AGA, CCC, respectively	Daegelen et al. (2009)
BLR	BL21	recA mutant; stabilizes tandem repeats; deficient in *lon* and *ompT* proteases	Terpe 2006
C41; C43	BL21	Mutant and double mutant, respectively, designed to withstand the expression of toxic proteins	Miroux and Walker (1996)
HMS174		*rec*A mutant. It has a positive effect on plasmid stability	Campbell et al. (1978), Marisch et al. (2013)
Origami; Origami B	K-12	*trxB*/*gor* mutant; disulfide bond formation in the cytoplasm is enhanced; deficient in *lon* and *ompT* proteases	Derman et al. (1993)
Rosetta; Rosetta-gami[a]	BL21	Both enhances the expression of eukaryotic proteins that contain codons rarely used in *E. coli*: AUA, AGG, AGA, CGG, CUA, CCC, and GGA; deficient in *lon* and *ompT* proteases.[a] *trxB*/*gor* mutant, disulfide bond formation in the cytoplasm is enhanced	Terpe (2006)

S. cerevisiae has been widely studied, its genome had been fully sequenced, with 3000 genes characterized, and it presents important parameters for the expression of extracellular proteins such as well-established genetic, physiology and post-transcriptional processes. In addition, yeasts also produce chaperonins that assist the folding of heterologous proteins, especially those with large numbers of disulfide bridges (Demain and Vaishnav 2009). It is classified as a safe organism (GRAS—Generally Regarded as Safe), which is essential for the production of biopharmaceuticals using genetic engineering (Ostergaard et al. 2000).

Several types of vectors have been developed for expression in *S. cerevisiae*, including artificial chromosomes, YACs better known as *Yeast Artificial Chromosomes* (*YACs*)) (Romanos et al. 1992). Genetic manipulations of this cell and changes in metabolic pathways have increased the yield of many heterologous proteins as the first recombinant hepatitis B vaccine (Gellissen and Hollenberg 1997). The cellulases CBH I, CBH II, CBH IIb were expressed in *S. cerevisiae* and easily secreted to the medium (Ilmen et al. 2011).

Heterologous expression in *S. cerevisiae* has a well-established protocol and a very efficient transformation rate of homologous recombination *in vivo*. Furthermore, it is one of the few eukaryotic organisms having a natural multicopy plasmid, the 2μ, which allows the construction of vectors for gene transfer and expression systems . One of the known vectors is pYES2 plasmid has a multicopy episomal vector of *S. cerevisiae* (Invitrogen 2008).

Another alternative and versatile system for heterologous protein expression is *P. pastoris*, a methylotrophic yeast capable of growth on a medium containing methanol as carbon source (Potvin et al. 2012). The *P. pastoris* genome is relatively simple to manipulate and commercial expression kits are available. This expression system has a strong promoter derived from the alcohol oxidase (*AOX*) gene of *P. pastoris* and is regulated by methanol with induction in the presence of and repression in the absence of this alcohol (Torres and Moraes 2002) or the gene of glyceraldehyde-3-phosphate dehydrogenase (GAP), with a constitutive strong promoter (Potvin et al. 2012). Two genes *AOX*1 and *AOX*2 encode functional enzymes, the first one being responsible for up to 95 % of the total amount of alcohol oxidase expressed due to the strength of its promoter and, thus, it is preferred for the expression of protein (Macauley-Patrick et al. 2005).

This system has the advantage of little secretion of the native protein so that the heterologous protein is the major secreted protein which makes the purification process more simple (Cereghino and Cregg 2000). Similarly to *S. cerevisiae*, *P. pastoris* has the ability to perform post-translational modifications such as the formation of disulfide bonds, sequence modifications and proteolytic processing (Hellwig et al. 2001) and it does not produce endotoxin (Açik 2009). However, *P. pastoris* also has some disadvantages, such as no native glycosylation, high potential for proteolysis, a requirement of a high level of oxygenation of the medium and the excretion of toxic intermediate oxidative catabolism of methanol, as formaldehyde, formic acid and hydrogen peroxide (Gao and Shi 2013) and, also, methanol handling problems due to its high toxicity and inflammatory power (Potvin et al. 2012).

The vectors of *P. pastoris* have an expression cassette composed of the promoter and the terminator region of the *AOX*1 gene transcription. In addition it has a selection mark, being frequently the most used histidinol dehydrogenase gene (*HIS*4). This mark allows the selection of prototrophic *His*+ transformants from a host strain *his*4. One of the most commonly used vectors is pPIC9 plasmid (Catalog Number K1710-01, Publication Number MAN0000012 in http://www.lifetechnologies.com/order/catalog/product/K171001-Invitrogen)

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a constitutive promoter isolated (Waterham et al. 1997) and it has been used to express heterologous proteins in many cells using glucose or glycerol as substrate, without the problems associated with the process of induction of expression with methanol that occur for the *AOX*1 promoter. Unlike the *AOX*1 system, using the GAP promoter, the synthesis of biomass and protein occurs simultaneously and is directly correlated with the number of pGAP-regulated genes (Vassileva et al. 2001).

Many heterologous proteins have been successfully expressed in the *P. pastoris* system pGAP with significant changes in expression levels based on the properties

of the expressed protein. An advantage of the GAP promoter with respect to the *AOX*1 promoter is that, being constitutive, it is not necessary to change one culture medium to another to induce expression. However, among the disadvantages is, that the use of the GAP promoter is suitable only for genes whose product is not deleterious to the cell; the level of expression from the *AOX*1 promoter is usually increased when methanol is added to the culture; and growth rate limiting in fermenters, the same phenomenon is not observed with the GAP promoter (Mitrovic et al. 2014).

The use of filamentous fungi as host for heterologous protein expression has the advantages due to their ability to secrete high quantity of protein by the hyphae tip, besides, the eukaryotic cell apparatus allow an efficient folding and post-translational modifications as the glycosylation that is important for thermostability of extracellular protein. Some filamentous fungi are GRAS (generally regarded as safe) by Food and Drug Administration in USA or are listed in "Qualified Presumption of Safety" (QPS) for a premarket safety assessment by European Food Safety Authority (EFSA) and can be used for food and medicine application (Bourdichon et al. 2012).

The polymerase chain reaction (PCR) product, circular plasmids, and restriction endonuclease-treated linear plasmids have been used as the DNA source for transformation of filamentous fungi (Wang and Xia 2011).

The transformation in multicellular filamentous fungi required the development of specific techniques compared to bacteria *Escherichia coli* and unicellular fungi as *P. pastoris* and *S. cerevisiae*. The *N. crassa*, *A. nidulans*, *A. fumigatus* and *T. reesei* have been the filamentous fungi used for this proposition (Sugui et al. 2005). With few exceptions, in eukaryotic organisms the presence of a chromosomal replicator on a circular vector molecule is not sufficient to confer on it the ability to persist and replicates extra chromosomally. Therefore, DNA fragments were isolated from genomes of filamentous fungi to provide extra chromosomal maintenance and replication of the plasmids. The sequences most frequently used are the effective plasmid replicators *AMA*1 (autonomous maintenance in *Aspergillus*) and the transformation enhancers (*ANS*1 or MATEs) important to initiate the replication, vector rearrangement and multimerization and chromosomal integration (Aleksenko and Clutterbuck 1997). The *AMA*1 vectors consists of two palindromes inverted repeats separated by a central spacer (Gems et al. 1991). The plasmids may be genome-integrating which maintaining in the host cell is due to the replication and segregation to be synchronized with the fungal chromosomal replication. The non-integrating plasmid may contains sequences necessary to replicate autonomously independent of the chromosomal replication (Gems et al. 1991).

The transformation techniques used for filamentous fungi are PEG-mediated protoplast fusion, *Agrobacterium tumefaciens*-mediated transformation (ATMT), electroporation and biolistic transformation.

The transformation by PEG-Mediated protoplast fusion in filamentous fungi is a simple method and requires three steps: preparation of protoplasts that are competent for DNA uptake; mixing the DNA and the protoplasts which allows DNA adsorption and uptake and spreading of the protoplast/DNA mixture onto appropriate agar plates for regeneration of the cell wall. The limiting step is the high concentrations of protoplasts necessary for transformation efficiency (Liu and Friesen

2012). The protoplast can be obtained from the treatment of hyphae with wall lysing enzymes in conditions of protoplast stabilizing as presence of osmotic buffer containing sorbitol, KCl or $MgSO_4$ (Balasubramanian et al. 2003). A concentration of 60 % of PEG6000 is recommended for the transformation of the circular plasmid while 25 % PEG6000 for the linearized one (Wang and Xia 2011).

The electroporation procedure uses a high-amplitude electric pulse for a short duration to induce the reversible permeabilization with small pores along the membrane surface, permitting the uptake of exogenous DNA (Ruiz-Díez 2002). Although the optimal electrical conditions are variable according the filamentous fungi, for most species, general electrical parameters are 25 μF capacitance, 200–800 Ω resistance, and 2–15 kV/cm field strength (Sánchez and Aguirre 1996). The pretreatment of conidia to form protoplasts could be required for electroporation (Ozeki et al. 1994; Schuster et al. 2012). This technique has been used in combination with the protoplast/PEG method for the transformation of *A. nidulans* (Sánchez and Aguirre 1996), *N. crassa* (Chakraborty et al. 1991), *A. niger* (Ozeki et al. 1994), and *A. fumigatus* (Brown et al. 1998) and *Mucor circinelloides* (Gutiérrez et al. 2011).

The Agrobacterium tumefaciens-mediated transformation (ATMT) common in transformation of plants can transfer the Ti plasmid to the filamentous fungi in a similar way (Bundock et al. 1995). The transformation of the *A. giganteus* ATMT has been demonstrated to be more efficient than the PEG-mediated protoplast transformation (Meyer et al. 2003).

The Biolistic (biological ballistics) has been applied as a method for incorporation of plasmid DNA into intact, thick-walled fungal cells (Klein et al. 1987). It is based on Tungsten particles that coat with DNA and accelerated to a high speed to bombard fungal spores or the hyphae. This does not require the use of protoplasts.

The inducible promoter glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) that was homologous to the expression host or by the constitutive *A. nidulans* was reported to be functional in industrially used for *Penicillium*, *Aspergillus Trichoderma and Clonostachys* species (Keränen and Penttilä 1995; Radzio and Kück 1997). The *cbh1* is a very strong inducible promoter that has been used for the expression of extracellular enzymes in *T. reesei*, *P. funiculosum*, *A. aculeatus* and *Melanocarpus albomyces* (Goodey 1993). The *gpdA* promoter was also successfully used for expressing a laccase gene with a construct similar to that used for the *cbh* genes, as well as for the expression of other genes in *A. carbonarius* (Zoglowek et al. 2015).

The most widely used system is *A. nidulans* with the expression and secretion vector pEXPYR. This vector, when integrated into the genome of *A. nidulans* and induced by maltose, leads to over expression of the target protein with high accumulation in the extracellular medium, facilitating the collection and of recombinant proteins (Damásio et al. 2011; Kück and Hoff 2010; Squina et al. 2009).

The expression of different proteins in a single host system has been developed using expression in *A. nidulans*. The flanking marker orotidine carboxylase 5′-phosphate (pyrG+) with repetitive elements MATE1 (Aleksenko and Clutterbuck 1997) facilitates the homologous recombination and excision of this marker selection. The PyrG-strains can be selected in the presence of 5-FOA. An excision check mark

enables the transformation series of vectors pEXPYR containing different proteins. *A. niger* could be used as a heterologous protein expression host too, however the efficient promoter needs to be evaluated. The β-glucosidase II gene (*bgl*II) promoter elements did not contain the CREA and it was free from carbon catabolite repression and was described as the potential to be an expression vector in this fungus (Segato et al. 2012).

3 Conclusions

Although several thermophilic microorganisms have been isolated and identified, little knowledge about the physiology of this group is available. It is known that thermophilic microorganisms produce extracellular enzymes with tolerance to high temperature, however, mesophilic organisms have also shown the ability to produce enzymes with considerable thermostability indicating that it is still necessary to find out much more about the genetic and structural features of these proteins.

The thermostable enzymes have been applied in a number of industrial processes for the production and processing of food, in the textile industry, in paper production and biofuels production, but there is still great potential for further applications to be found. For this reason, the production of native or genetically modified enzymes is an expanding market.

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ERRATUM

Gene Expression Systems in Fungi: Advancements and Applications

Monika Schmoll and Christoph Dattenböck

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In the contents of front matter page viii a part title has been included between chapter 18 and 19 as below:

Part III – Challenges in industrial application.

Also, title of Chapter 8 is changed from Fungi Gene Expression Systems: Applications and Advancements to Gene Expression in Filamentous Fungi: Advantages and Disadvantages Compared to Other Systems.

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