Production of Industrial Enzymes in *Trichoderma reesei*

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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

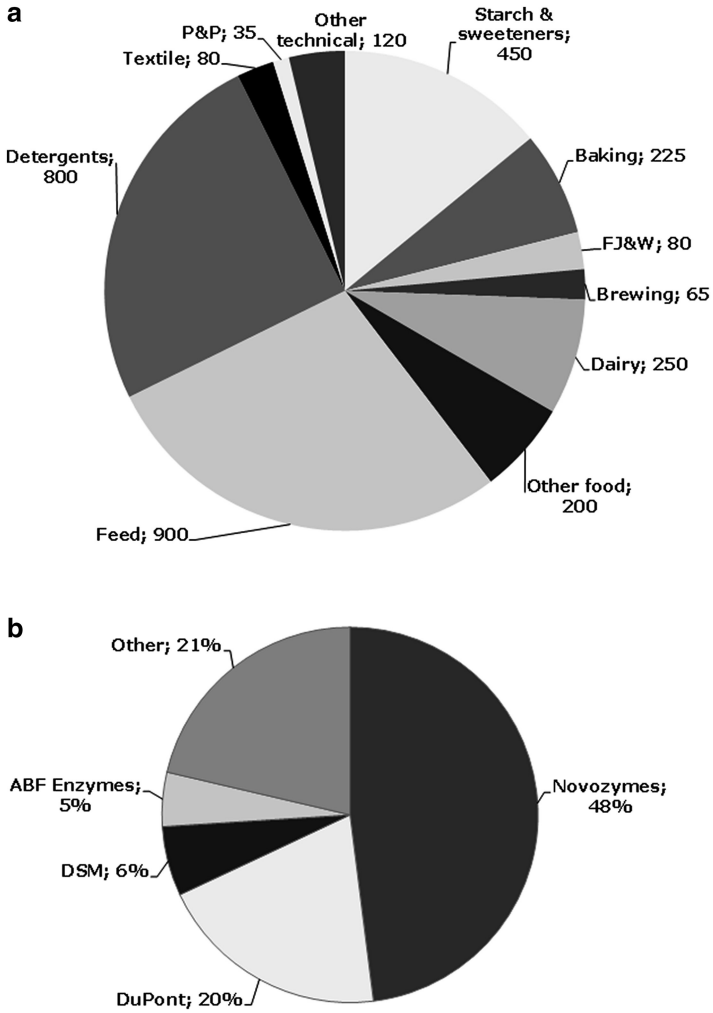

Fig. 1 (**a**) Estimation of industrial enzymes sale (€3.0 billion) per application segment. Starch & sweeteners, baking, FJ&W (fruit juice & wine), brewing and dairy segments belong to the food enzymes, and detergents, textile and P&P (paper & pulp) segments are classified as technical enzymes. (**b**)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

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[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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