Heterologous Protein Expression in *Hypocrea jecorina*: an Historical Perspective and New Developments

Arjun Singh¹, Larry E. Taylor II², Todd A. Vander Wall², Jeffrey Linger¹, Michael E. Himmel², Kara Podkaminer², William S. Adney², and Stephen R. Decker²

¹ National Bioenergy Center
National Renewable Energy Laboratory
15013 Denver West Parkway
Golden, Colorado 80401

² Biosciences Center
National Renewable Energy Laboratory
15013 Denver West Parkway
Golden, Colorado 80401

³ Corresponding Author: Stephen R Decker
Senior Scientist V
NREL
15013 Denver West Parkway
Golden, Colorado 80401
Steve.decker@nrel.gov
(303) 384-7759

Table of Contents

TABLE OF CONTENTS	2
ABSTRACT	3
KEYWORDS	3
1. INTRODUCTION	3
2. <i>HYPOCREA JECORINA</i> AS AN EXPRESSION HOST	5
2.1 HOST STRAINS FOR HETEROLOGOUS PROTEIN EXPRESSION	5
2.1.1 DELETION OF <i>CBH1</i> FROM THE GENOME OF <i>T. REESEI</i> RUT-C30	8
2.1.2 DELETION OF <i>CBH1</i> FROM THE GENOME OF <i>T. REESEI</i> QM6A	9
2.2 TRANSFORMATION AND SELECTION METHODS	9
2.2.1 PEG-MEDIATED DNA UPTAKE	10
2.2.2 AGROBACTERIUM-MEDIATED TRANSFORMATION	11
2.2.3 BIOLISTIC TRANSFORMATION	12
2.2.4 SPORE ELECTROPORATION	13
2.3 SELECTION MECHANISMS	13
2.3.1 NITROGEN UTILIZATION	14
2.3.2 AUXOTROPHIC MUTANT COMPLEMENTATION	15
2.3.3 ANTIBIOTIC RESISTANCE	18
2.4 CONTROL OF HETEROLOGOUS EXPRESSION AND PRODUCTION LEVELS	19
2.4.1 PROMOTER SELECTION	19
2.4.2 SITE OF INTEGRATION	20
2.4.3 EFFECT OF SIGNAL SEQUENCE	21
2.5 EXPRESSION OF RECOMBINANT CEL7A IN THE <i>CBH1</i>Δ STRAINS	23
3. CONCLUSIONS	25
4. ACKNOWLEDGMENTS	26

Abstract

Hypocrea jecorina, the sexual teleomorph of *Trichoderma reesei*, has long been favored as an industrial cellulase producer, first utilizing its native cellulase system and later augmented by the introduction of heterologous enzymatic activities or improved variants of native enzymes. Expression of heterologous proteins in *H. jecorina* was once considered difficult when the target was an improved variant of a native cellulase. Developments over the past nearly 30 years have produced strains, vectors, and selection mechanisms that have continued to simplify and streamline heterologous protein expression in this fungus. More recent developments in fungal molecular biology have pointed the way toward a fundamental transformation in the ease and efficiency of heterologous protein expression in this important industrial host. Here, 1) we provide an historical perspective on advances in *H. jecorina* molecular biology, 2) outline host strain engineering, transformation, selection, and expression strategies, 3) detail potential pitfalls when working with this organism, and 4) provide consolidated examples of successful cellulase expression outcomes from our laboratory.

Keywords

Trichoderma reesei, *Hypocrea jecorina*, QM6a, Rut-C30, *cbh1*, Cel7A, cellobiohydrolase, *cbh1* deletion, fungal transformation, cellulase expression systems

1. Introduction

Most cellulolytic enzymes used today in the biomass to biofuels or bioproducts industry are produced in the filamentous fungus, *Hypocrea jecorina* (Merino and Cherry, 2007). It is almost certain that for this industry, this organism will be the source for such hydrolytic enzymes in the foreseeable future. Originally isolated by Mary Mandels and Elwyn Reese

from rotting cotton goods brought to the U.S. Army Quarter Master Research and Development Center at Natick, Massachusetts from the Solomon Islands during World War II, *T. viride* QM6a (as it was originally called, the “QM” designation is derived from Quarter Master) was soon demonstrated to be a prolific cellulase producer. Later, the parent *T. viride* species was shown to be distinctly different from *T. viride* and so it was renamed *T. reesei* in honor of its discoverer. Much later, it was determined to be a sexual anamorph of a well-characterized fungus, *Hypocrea jecorina*, though much of the current literature continues the use of *T. reesei* (Kuhls *et al.*, 1996).

Beginning in the 1970s, several groups randomly mutagenized the parent QM6a strain, resulting in several hyper-producing strains including QM9414 (catabolite repressed, hyper-producer strain from Natick Labs) and RUT-C30 (catabolite derepressed, hyper-producer strain from Rutgers University) (Peterson and Nevalainen, 2012). The RUT-C30 strain formed the parent for all or nearly all commercial cellulase production strains of *T. reesei* (Seiboth *et al.*, 2011). A more detailed lineage of strains developed for increased productivity has already been published and so this topic will not be expounded upon here (Seiboth *et al.*, 2011). Although several genes encoding the hydrolytic enzymes from *H. jecorina* have been expressed in other organisms; for example, in yeasts (Boer *et al.*, 2000, Boonvitthya *et al.*, 2013, Den Haan *et al.*, 2007, Godbole *et al.*, 1999, Hong *et al.*, 2007, Mitsuishi *et al.*, 1990, Reinikainen *et al.*, 1992, Takada *et al.*, 1998), bacteria (Abdeljabbar *et al.*, 2012, Laymon *et al.*, 1996), and plants (Dai *et al.*, 1999, Liu *et al.*, 2004), the critical volumetric productivity levels required for cost effective cellulase deployment has been demonstrated only in fungi, with *H. jecorina* setting the bar at over 100 g/L for certain protein expression scenarios (Cherry and Fidantsef, 2003). Furthermore, consistent attainment of native-like specific activity (performance) characteristics for *H. jecorina* enzymes expressed in non-*H. jecorina* hosts has not been demonstrated.

There are several likely reasons for poor expression and/or activity levels observed for the heterologously expressed *H. jecorina* enzymes. One factor resides in differential protein glycosylation (Nevalainen and Peterson, 2014). It is known that protein glycosylation, both quantitative amounts and patterning, differs in yeast compared to filamentous fungi. Other

critical post-translational modifications that differ are protease activity and *N*-terminal processing of proteins. In addition, the Cel7A (cellobiohydrolase I from *H. jecorina*) fold is highly dependent upon di-sulfide bonds (specifically 10) for stability and many heterologous expression systems do not seem to be able to make the correct connections in this regard (Xu *et al.*, 2014). While functional expression of Cel7A has been demonstrated in non-native fungal host strains, results have been mixed regarding activity and stability. Regardless, it is apparent that to ensure that the functionality of heterologous or genetically improved hydrolytic enzymes is accurately evaluated for properties in an appropriate production strain for the biomass conversion industry, engineered genes should ultimately be expressed in that strain, which is very likely to be *H. jecorina*.

Expression of heterologous proteins in *H. jecorina* has been carried out for several decades, beginning in 1987 when Penttillä *et al.* reported a basic transformation protocol for this fungus (Penttila *et al.*, 1987). In this review, we will cover three main areas of *Hypocrea* molecular biology; 1) expression strains, 2) vector construction, and 3) selection protocols. Optimization of expression and biochemical characterization of the proteins will be left to other reviews, of which there are many. Recent reviews by Nevalainen *et al.* (Nevalainen *et al.*, 2005), and Kruszevska (Kruszevska, 1999) cover much of the general protein expression knowledge base in filamentous fungi today. We will focus on heterologous cellulases, particularly Cel7A and engineered variants.

2. *Hypocrea jecorina* as an Expression Host

Aside from proprietary industrial strains used to produce enzymes at very high titers, multiple research laboratories have transformed *T. reesei* to express a variety of proteins, including both native and heterologous cellulases. When evaluating a system of heterologous expression, several criteria must be considered: the host strain, the transformation mechanism, the selective pressure, and control of expression.

2.1 Host strains for heterologous protein expression

For general heterologous protein expression, simple random integration into any strain with a given selection is the general approach and has been carried out for decades. Several strains have been developed with specific traits useful for protein expression, such as increased protein production and secretion, decreased protease activity, or specific gene knockouts (Table 1). Utilization of some of these strains was limited, as they were proprietary to industry or developed in-house by various academic labs. Many are no longer readily available; however, the properties developed (hyper-production, gene knock-out, auxotrophic selection, low protease) are found in some more modern strains and the published methodology makes re-creation of these strains fairly straightforward for labs with reasonable understanding of the technology.

In the 1970s, the Natick lab began mutational studies on QM6a, as did Eveleigh and Montenecourt at Rutgers. Both groups developed a series of mutated strains, eventually leading to QM9414 and RUT-C30, respectively (Montenecourt and Eveleigh, 1979, Peterson and Nevalainen, 2012). The RUT-C30 strain secretes large amounts of cellulases and synthesis of these enzymes is not repressed by glucose (Montenecourt and Eveleigh, 1979, Tangnu *et al.*, 1981). It has been heavily engineered, resulting in large deletions of its genome, which is known to affect protein secretion and cellulase repression (Montenecourt and Eveleigh, 1979, Seidl *et al.*, 2008, Tangnu *et al.*, 1981). The QM6a strain produces a complete cellulase system when induced by cellulose, cellulose hydrolysis products such as cellobiose or cello-oligomers, or specific disaccharides, such as sophorose or lactose (Mandels *et al.*, 1962, Sternberg and Mandels, 1979); however, cellulase production is severely inhibited by growth on glucose (Mandels and Reese, 1957, Nisizawa *et al.*, 1972). While cellulase production can be repressed by growth on glucose (catabolite repression) in the parental QM6a strain and the hyper-producing QM9414 mutant strain of *H. jecorina*, the hyper-producing RUT-C30 strain is de-repressed and the native cellulase expression system is always “on,” making separation and subsequent characterization of the heterologous protein from the native proteins extremely difficult, though productivity in RUT-C30 is generally higher than most other strains.

In the 1980s and 1990s, several groups worked on developing *H. jecorina* strains for transformation, with VTT from Finland being the most prolific. Dozens of VTT-D-XXXXXXX strains were developed, with the VTT-D-79125 being the most commonly used starting point for further strain development; however, this strain and most of the others referenced in Table 1 no longer appear to be available from the VTT Culture Collection (<http://culturecollection.vtt.fi>). Properties of the VTT strains included hypercellulase activity/secretion, low protease, and gene knockout strains. VTT-D-79125 was also used to develop several ALKO-XXXX strains with similar properties, although very little information is available regarding these strains and the parent company, Alko, Ltd., which was, at the time, the Finnish government-owned alcoholic beverage supplier. In 1995, production of alcohol was separated from its distribution, creating Primalco, Ltd, which apparently maintained the ALKO strains. Later, Altia acquired Primalco and presumably the strains developed at Alko, Ltd as well. The authors have no idea if these strains still exist or are available.

Trichoderma strains deleted for cellulases are (apparently) widely used in industry; however, public information is very limited. Some publically disclosed deletion strains do exist, however. von Ossowski *et al.* used the *cel7a/cel7b*-double delete strain (ALKO 3414 from Primalco, Ltd.) to express mutant Cel7A proteins to better understand the exo-loop function in the protein (von Ossowski *et al.*, 2003). Stahlberg *et al.* also utilized a *cel7a*-delete strain, VTT-D-93201, to generate catalytically inactive mutants for crystallography studies. Several other groups have referenced using similar (or the same) strains; however, the details are not clear and the corresponding vectors are not readily available.

In response to a general lack of availability of strains and vectors, especially in the U.S., we recently developed transformation systems in QM6a and Rut-C30 specifically for studying *cbh1* mutations by knocking out the native *cbh1* gene in each strain. Deleting the native *cbh1* gene allowed Cel7A chimeras, improved mutant forms of Cel7A, and Cel7 enzymes from other sources to be expressed and evaluated without interference from the native Cel7A enzyme. QM6a was chosen because it is considered to be the “wild-type” strain of this fungus, having a presumably intact protein secretion, glycosylation, and post-

translational modification system; as well as possessing a completely sequenced and published genome (Martinez *et al.*, 2008). RUT-C30 was also chosen for our studies because it is an industrially relevant hyper-producer. Each of these strains has a single copy of the *cbh1* gene (Martinez *et al.*, 2008). The *cbh1* gene in each strain was knocked out by replacement with either *hph* (for RUT-C30) or *amdS* (for QM6a); the reciprocal marker was used for the subsequent transformation with heterologous *cbh1*. Whereas both systems are functional, knocking out the QM6a native *cbh1* by replacing it with *amdS* made subsequent selection of heterologous *cbh1* insertion much easier using hygromycin resistance. The inverse strategy was used in case of RUT-C30, which rendered that system slightly more difficult to use due to the relatively poor selectivity of *amdS* vs. hygromycin resistance (see below).

2.1.1 Deletion of *cbh1* from the genome of *T. reesei* Rut-C30

We replaced the DNA coding for the Cel7A protein (*cbh1*) with the *E. coli* Hygromycin B phosphotransferase gene (*hph*), removing the capacity of the resulting strain to produce endogenous Cel7A. In the deletion cassette (Figure 1A), *hph* is driven by the *H. jecorina* *pgk1* promoter and flanked by nucleotides homologous to 1492 bases upstream and downstream from the native *cbh1* sequence, providing homology on both sides of the Cel7A coding sequence in the genome, enhancing *cbh1* gene replacement frequency. The deletion cassette has *XbaI* endonuclease recognition sites at both ends, which permits the cassette to be excised from a carrier plasmid. A linear DNA fragment was then used to transform *H. jecorina* via PEG-mediated spheroplast DNA uptake and selection was carried out on 100 µg/mL hygromycin B plates (details in transformation section below). After several rounds of sporulation and selection on hygromycin B media, transformants were screened for Cel7A by western blot and yielded a homologous recombination frequency of ~5%, with 2/44 clones showing no Cel7A expression. After PCR confirmation that the native *cbh1* gene had been replaced by *hph*, AST871 was selected for use as a host strain for heterologous *cbh1* expression.

Figure 1 near here

2.1.2 Deletion of *cbh1* from the genome of *T. reesei* QM6a

The strategy used to delete *cbh1* from QM6a was similar to that used for RUT-C30. The *cbh1* gene was replaced with an expression cassette (Figure 1B) for the *Aspergillus nidulans* *amdS* gene, enabling growth on acetamide as the sole nitrogen source. This construct was designed to delete *cbh1* plus 1000 base pairs immediately preceding the *cbh1* start codon to ensure that there would not be an additional promoter to the one included in the expression cassette. This deletion cassette can be excised with the restriction endonuclease site (KpnI) present only at both ends of the cassette for use as a linear DNA fragment for transformation. Transformants were selected on acetamide plates and screened by western blot for Cel7A expression. A single transformant, AST1116, was demonstrated to be deleted for *cbh1* and was selected for further heterologous expression work.

Several aspects of *H. jecorina* as well as the protein of interest must be considered when deciding on an expression host strain. What is the purpose of expression? That is, is the goal to get the highest yield, to study native folding/processing/secretion, to compare variants of the same protein, or to study the interaction between native enzymes and the heterologous protein? Depending on the intent, host strains may be off-the-shelf or may require genetic manipulation prior to use. In general, host strains are available from major culture collections, such as ATCC or have been developed by various commercial, governmental, or academic labs (Table 1). The latter tend to be specific phenotypes such as low protease, high productivity, or specific gene knockouts, however availability to the general research community is mixed.

Table 1 near here

2.2 Transformation and selection methods

Getting DNA into the transformation host, having it integrate and express, and isolating heterologous clones is carried out using several methods. Transforming *H. jecorina* is similar to many other filamentous fungi. A primary concern is the need to ensure single genome transformation, requiring either spores or single cells (usually protoplasts) to be separated from multicellular hyphae. Protoplast DNA uptake, *Agrobacterium*-mediated

biolistics, and electroporation, approaches are all used. There are advantages and disadvantages to each system. The DNA can take several forms, including both circular or linearized plasmids; as well as PCR or restriction enzyme fragments. The older methodology of using multiple constructs, *i.e.*, one containing the selection marker and another the gene of interest is still a reasonable approach, although modern DNA synthesis and cloning methods allow much simpler and less expensive vector constructs, where multiple genes can be included on a single vector. Whereas circular plasmid DNA is also still viable, our experience is that linearized vectors provide much higher transformation efficiency. Others have reported transient expression with circular plasmids, as they apparently autonomously replicate for a period of time before being lost (Smith *et al.*, 1991). Another observation is that incubation of transformation plates under light enhances sporulation, which while generally well known (keeping bread in a dark breadbox), is frequently overlooked.

2.2.1 PEG-mediated DNA uptake

Historically, fungal protoplasts (complete removal of cell wall) have been used to passively uptake DNA in the presence of high levels of polyethylene glycol (PEG) of various high molecular weights. The earliest reports of *Hypocrea* transformation utilized PEG-mediated DNA uptake, as it had already been shown to work for other fungi. During PEG-mediated transformation, *Hypocrea jecorina* cell walls are digested with a cell lysing mix of enzymes in the presence of osmotic stabilizers to prevent the fragile cells from lysing due to osmotic differences. Heterologous DNA is introduced at fairly high levels and it is integrated into the genome. PEG-mediated transformation is fairly time consuming, as cell wall digestion must be done slowly and carefully to maintain cellular integrity. The starting cells should be freshly grown from frozen spore stocks and several wash steps are involved to remove spores and cell wall debris. It also requires fairly large amounts of DNA, typically 1 to 5 μ g for each transformation, and the efficiency is low. Additionally, the fragility of the protoplasts contributes to a high mortality, as they must deal with shear, osmotic, and thermal stresses that often lyse the fragile cells.

Briefly, DNA is propagated on a suitable vector, typically an *E. coli* plasmid, purified, and suspended at a concentration of several mg/mL. *Hypocrea jecorina* spores are grown in a rich medium until reasonable hyphae are formed. The hyphal mass is recovered by filtration, washed, and suspended in a protoplasting buffer with osmotic stabilizers such as sorbitol, KCl, or MgCl₂. A cell-wall lysing mixture is added, often a commercial *Trichoderma harzianum* prep (such as Novozyme 234) with high chitinase and β -glucanase activity. After digestion for several h to overnight, the undigested cell wall material is removed by filtration; the protoplasts are then separated by mild centrifugation and gently resuspended in 25-60% PEG + CaCl₂ + DNA. Unused protoplasts can be frozen at -70°C; however, the transformation efficiency decreases by 50% (Penttilä *et al.*, 1987). After incubation (room temp or 4°C), aliquots of the transformation mix is suspended in molten osmotically-stabilized top agar, gently mixed, and poured over a selection plate. After several days, colonies form, which are then picked, passed several times on selection medium, and then screened for the desired protein.

Obviously, many things can go wrong during the generation of the protoplasts and yield poor results. The time and high levels of DNA required make this method somewhat cumbersome and inefficient; however, it is a relatively simple procedure and can be accomplished by laboratories with limited resources.

2.2.2 Agrobacterium-mediated transformation

Agrobacterium tumefaciens is a soil microbe capable of transferring DNA directly into the cells of plants and fungi. Use of *Agrobacterium* requires a plasmid vector with specific transfer sequences bracketing the DNA for insertion. The vector-containing *Agrobacterium* cells are mixed with either *Hypocrea* protoplasts or conidia in a suitable medium, incubated for infection to occur, and then plated onto a selective medium capable of killing the *Agrobacterium* cells (Ma *et al.*, 2011, Meng *et al.*, 2013). *Agrobacterium*-mediated transformation efficiency is much higher (~10 to 50X) for protoplasts compared to conidia, although the process required to generate the protoplasts obviously complicates the method (Zhong *et al.*, 2007). Other complicating factors include the requirement for

separate transformation events for the bacteria and the fungi, and killing the bacterial cells post-transformation.

2.2.3 Biolistic transformation

Biolistics transformations in fungi is reportedly lower in efficiency than protoplast uptake (Hazell *et al.*, 2000), although Te'o et al reported approximately 35 to 40 transformants/ μ g of plasmid DNA (linear or circular) using *T. reesei* (Te'o *et al.*, 2002). In biolistics procedures, heterologous DNA is coated onto high-density microparticles (usually gold or tungsten), which are then “fired” into cells or spores using high-pressure helium. The DNA-coated particles are coated onto the underside of a rupture disk that is placed above the target cells or spores already spread onto a selective agar plate (Hazell *et al.*, 2000). The system between the rupture disk and target cells is placed under a vacuum or partial vacuum and the helium pressure above the rupture disk is increased until the disk ruptures, accelerating the particles towards the target cells. Rupture disks are available at different pressure ratings and this factor must be balanced with size and density of the microparticles, the level of vacuum, and the distance between the disk and target plate in order to achieve good transformation efficiency. In essence, the particles must be traveling with enough velocity to penetrate the spore coat, cell wall, cell membrane, and/or nuclear membrane (depending on target cell type), but not so fast that they exit the cell. This procedure is highly variable based on cell types, although conditions for many have been worked out (Te'o *et al.*, 2002).

The advantages of biolistics are primarily the ease of use and high co-transformation efficiency. Particles are relatively easy to prepare and the use of spores instead of protoplasts makes target cell preparation very simple. When multiple genes are coated onto the same microparticle, co-transformation frequency exceeds 90%, presumably because a successful insertion of a microparticle carries both genes, eliminating the need for separate uptake and transport events when other transformation mechanisms are used (Hazell *et al.*, 2000). The disadvantages are mainly the need for specialized equipment and low transformation efficiency, although the requirement to empirically work out the optimal parameters for new cell types may be another consideration.

2.2.4 Spore electroporation

Perhaps the most promising route of delivering DNA to *T. reesei* is through the use of spore electroporation for its ease, efficiency and reduced hands-on-time. Initial descriptions of methods to electroporate can be found in a patent application filed in 2008 by Danisco U.S., Inc. Genencor Division (Kim and Miasnikov, 2010). Additionally, this protocol was adapted and a direct comparison was recently made between protoplasting and electroporation. The results suggest similar efficiencies between the two methods but that electroporation was, in general, a simpler method (Schuster *et al.*, 2012a). It has been noted that transformation via electroporation at least using hygromycin-resistance cassettes leads to two distinct colony size distributions (Linger et al in preparation). Larger colonies tend to be true transformants and smaller colonies tend to be only transiently resistant to hygromycin. We note this finding as it reduces the likelihood of false-positive clone selection following electroporation.

In essence, spore-electroporation protocols involve harvesting fresh spores, washing, and suspending in a sorbitol solution. DNA is then added to the mix and electroporated. Spores are allowed ample time to recover and germinate prior to being plated onto selective medium. Regarding optimal electroporation conditions, it seems that this remains somewhat of a moving target. For example, in January of 2012, electroporation conditions were reported as 1.8 kV, 800 Ω , and 25 μ F (Schuster *et al.*, 2012a). However, in April of 2012 the same group used slightly different conditions 1.8 kV, 600 Ω 10 μ F (Schuster *et al.*, 2012b). While there is certainly success along this front, there lacks a clear publication surrounding the variables of *T. reesei* spore electroporation, and which conditions are likely to garner the highest transformation efficiency. Moving forward, an important data set will be the complete optimization of electroporation conditions surrounding numerous variables including: spore age, spore density, electroporation medium, voltage, internal resistance of the power supply, capacitance, DNA concentration, outgrowth medium, and outgrowth time.

2.3 Selection Mechanisms

Multiple selection mechanisms have been used with *H. jecorina*, with nitrogen utilization, auxotroph complementation, and antibiotic resistance being the most common. Although split marker systems, which require homologous recombination between separate (i.e. split) selection marker sections, have been used in yeast and filamentous fungi to enhance selection, this has not been demonstrated in *H. jecorina* (Kuck and Hoff, 2010, Steiger, 2013).

2.3.1 Nitrogen utilization

One of the most widely used selection mechanisms in *H. jecorina* transformation is growth on acetamide as a sole nitrogen source (Bailey *et al.*, 2002, Harkki *et al.*, 1991, Hazell *et al.*, 2000, Joutsjoki *et al.*, 1993b, Karhunen *et al.*, 1993, Miettinen-Oinonen *et al.*, 1997, Nykanen *et al.*, 1997, Penttila *et al.*, 1987, Saarelainen *et al.*, 1997, Saarelainen *et al.*, 1993, Schuster *et al.*, 2012a, Suominen *et al.*, 1993, Te'o *et al.*, 2000). Wild-type *H. jecorina* lacks the acetamidase gene (*amdS*) that cleaves acetamide into acetic acid and ammonium. The addition of an *amdS* gene (usually from *Aspergillus* spp.) enables *H. jecorina* to grow on acetamide as a sole nitrogen source, and as such, is one of the few non-antibiotic-based dominant selection mechanisms available for *H. jecorina* molecular biology. Selection is carried out using a minimal medium with a carbon source of choice and acetamide as the sole nitrogen source. Selection has been shown to be enhanced with CsCl and agarose (Penttila *et al.*, 1987).

2.3.1.1 Expression vector for *cbh1*Δ strain AST871

We recently developed an expression vector, shown in Figure 2, for expressing various genes in the RUT-C30-based deletion strain described above. As the deletion strain was already resistant to hygromycin B from the *cbh1* deletion, we used *Aspergillus nidulans* *amdS* as a secondary marker utilizing acetamide as the sole nitrogen source (Berges and Barreau, 1991). The expression of the *amdS* gene is controlled by the *T. reesei* *pgk1* promoter. This vector contains *cbh1* promoter followed by the transcription termination signal (*trpC*) from *A. nidulans*. The DNA encoding proteins to be expressed is inserted between the unique *NdeI* and *XbaI* endonuclease sites.

Figure 2 near here

2.3.2 Auxotrophic mutant complementation

Auxotrophic mutants lack the ability to synthesize a specific compound needed for viability. When cultured, the specific compound must be added to the medium in order to allow the cells to grow. As a selection method, auxotrophs are usually generated by spontaneous or random mutation, resulting in the inactivation of one or more genes in a specific biosynthetic pathway. The selection of auxotrophic mutants can be very tedious, as randomly mutagenized strains must be screened for growth in the presence and absence of the target compound on minimal media. Alternatively, a toxic analog can be used to poison cells still capable of synthesizing the target compound, allowing only auxotrophic mutants to grow on media containing the target compound and toxic analogue.

Once an auxotrophic strain is generated and confirmed, transformation proceeds by one of the aforementioned methods. A complementary gene that re-enables prototrophy (the ability to synthesize the needed compound) is co-transformed along with the target gene and cells are selected on minimal medium in the absence of the target compound. If a toxic analogue is available, it can be used to revert the strain auxotrophy by reselection on media containing the target compound and the toxic analogue. In this way, auxotrophy can be used as a means to recycle a selection marker, enabling multiple genes to be transformed into a single strain using the same selection marker. In *H. jecorina*, two compounds are the most widely targeted for auxotrophy; arginine and uridine.

Ornithine carbamoyl transferase (*argB*) is required for arginine synthesis (Penttilä *et al.*, 1987). For *argB* auxotrophs in *H. jecorina*, *A. nidulans argB* is often used to complement the strain back to prototrophy. Growth of the co-transformed auxotroph on minimal medium without arginine is the means of selection.

Uridine auxotrophs require uridine or uracil in the medium in order to grow, although uracil is used most frequently as it is inexpensive and convenient. Several mutations in the uridine biosynthesis pathway can cause uridine auxotrophy with orotidine 5'-monophosphate decarboxylase (*ura3/pyr4/pyrG*, depending on the organism) (Deane *et al.*,

1999, Gruber *et al.*, 1990, Smith *et al.*, 1991) or orate phosphorybosyltransferase (*ura5*) (Berges and Barreau, 1991) being the most common. Typically, *pyr4* from *Neurospora crassa* or *pyrA* from *Aspergillus niger* are used to select transformation on minimal *ura*(-) medium. Spontaneous mutations occur frequently in these genes, making it relatively easy to generate an auxotrophic strain, but also allowing reversion to occur, so strains must be screened carefully to ensure the target gene is present. Interruption or deletion of the gene, rather than simple mutation is preferred for this reason. Native *ura3/ura5* have high efficiency of complementation. More recently, Jørgensen *et. al.* developed a recyclable and targeted system using a *pyr2* auxotroph of a *tku70* delete strain of *H. jecorina*. The *tku70* deletion enhances targeted insertion, in this case into the *ade2* locus of the genome, providing the additional advantage of targeted insertions causing a red pigment formation in transformed cells(Jorgensen *et al.*, 2014).

5-fluoroorotic acid (5-FOA) is a metabolic analogue that, when cleaved by orotidine 5'-monophosphate decarboxylase in the uridine biosynthesis pathway, generates 5-fluorouracil, a toxin that kills the host cell (Steiger *et al.*, 2011). Inclusion of 5-FOA in uracil-containing media will kill strains with the complete uridine anabolic pathway, but allow spontaneous auxotrophic revertants to grow, thus allowing marker recycling in transformed strains, as long as the plasmid has integrated into the genome. Hartl and Seiboth utilized 5-FOA as a counter selection mechanism of a *pyr4* blaster cassette as a recyclable marker to sequentially delete glucokinase and hexokinase from *H. jecorina* (Hartl and Seiboth, 2005). To encourage marker excision during counter selection, repeat flanking regions are often included on either side of the marker. Direct repeats allow for seamless removal of the inserted sequence while inclusion of random repeats can leave extraneous DNA behind in the sequence.

One case has demonstrated the use of mannitol as a sole-carbon source allows for selection when hexokinase (*hvk1*) is deleted from the genome and then used as an autotrophic marker (Guangtao *et al.*, 2010). An advantage of this system is that the mannitol used as an osmotic stabilizer during *Agrobacterium*-mediated transformation of protoplasts is also the selection pressure.

2.3.2.1 Generation of a recyclable auxotroph with NHEJ

In practical terms, we have utilized *pyr4* deletion coupled with 5-FOA selection and removal of the *hygB* selection marker to establish a recyclable marker system in *H. jecorina*. To use the entire *pyr4* cassette as marker, the native copy was first deleted (Steiger *et al.*, 2011). A vector was constructed fusing the regions directly upstream and downstream of the cassette, preventing future homologous recombination at this site. This vector was transformed into the QM6a (*hygB::tmus53*) strain and colonies were selected in the presence of 5-FOA (Figure 3a). Confirmation of the *pyr4* deletion was confirmed both by PCR (Figure 3b) and sequencing.

Figure 3 near here

Initial efforts were unable to isolate a *pyr4* deficient strain from background growth in the presence of 5-FOA. Both in the untransformed control and the test plate, yellow mycelia and distinct colonies grew (Figure 4a, b). However, 5-FOA is active only at acidic pH. Thus, by lowering the pH, 5-FOA enters the cell and reduces background growth for a distinct selection (Figure 4c, d).

Figure 4 near here

In order to remove the *hygB* cassette at the *tmus53* locus and demonstrate the recyclability of the *pyr4* marker cassette, the following vector was transformed into the QM6a(*hygB::tmus53 Δpyr4*) strain. The first step inserted the *pyr4* cassette and the corresponding flanking regions at the *tmus53* locus through a selection on MA medium without uradine (Figure 5a).

Figure 5 near here

Subsequent loss of the *pyr4* cassette was the selected for by plating on MA with 5-FOA, looping out at the repeated *tmus53* DOWN regions (Figure 5b, top). The result is a clean deletion at the *tmus53* locus (Figure 5b, bottom). Deletion of the hygromycin marker cassette was confirmed by PCR (Figure 6) and sequencing. The resulting strain, QM6a($\Delta tmus53 \Delta pyr4$), is a uridine auxotroph that is sensitive to *hygB*, thus recycling both

markers for future use. This new strain allows gene deletions, promoter swaps, better expression control, reduced variability, and insertion of multiple genes with marker recycle.

Figure 6 near here

2.3.3 Antibiotic resistance

Two antibiotics are commonly used as selection markers in *H. jecorina*; phleomycin from *Streptoalloeus hindustanus* (Harkki *et al.*, 1991) and hygromycin (*hph*) from *E. coli* (Bailey *et al.*, 2002, Hazell *et al.*, 2000, Herrera-Estrella *et al.*, 1990, Mach *et al.*, 1994, Steiger *et al.*, 2011, Te'o *et al.*, 2002, Te'o *et al.*, 2000). We have also noticed that *Hypocrea jecorina* strains that we have tested appear to be poorly selected by Zeocin, the most common form of phleomycin. In our experience, hygromycin is more selective and results in very low background. We note that pyrithiamine (*ptrA*) (Kubodera *et al.*, 2002) has also been used.

2.3.3.1 Expression vector for *cbh1*Δ strain AST1116

We designed and constructed the expression vector, pTR50 (Figure 7) for expression of various cellobiohydrolases and other proteins in the *T. reesei* strain AST1116. This vector contains the *E. coli hph* gene whose expression is controlled by the *T. reesei pgk1* promoter, and thus provides for selection of transformants by conferring resistance to hygromycin B. It contains a *cbh1* promoter followed by *cbh1* coding sequence, without introns and *cbh2* transcription termination signals. The *NdeI/XbaI cbh1* coding sequence can be replaced with other genes for expression. The entire expression cassette can be excised by digestion with restriction endonuclease *XhoI* and used for transformation of AST1116. The transforming DNA fragment has homologies to *cbh1* 5' and 3' flanking regions to increase the frequency of integration of the expression constructs at the *cbh1* locus in the genome. Note that a large (2500 bp) *cbh1* promoter is used in this construct to provide for sufficient *cbh1* homology at the 5' end and that 1000 bp of the sequence immediately preceding the *cbh1* start codon has been deleted in AST1116 (Figure 7). This cassette was designed to be

used as a random-integration vector as it does not contain paired 5' and 3' targeting homology.

Figure 7 near here

2.4 Control of Heterologous Expression and Production Levels

Expression of heterologous proteins in filamentous fungi is a major obstacle in industrial enzyme production and many factors are involved. Koushki *et al.* has summarized these concerns in a mini-review (Koushki *et al.*, 2011); however, here we point out specific examples and applications concerning *H. jecorina* relevant to our work.

2.4.1 Promoter Selection

The primary means of expression control is the choice of promoter. Whereas the native *cbh1* promoter is inducible and the most commonly used for high expression levels, it has the distinct disadvantage of being tied to the rest of the cellulase system (Bailey *et al.*, 2002, de Faria *et al.*, 2002, Fang and Xia, 2013, Harkki *et al.*, 1991, Joutsjoki *et al.*, 1993b, Karhunen *et al.*, 1993, Miettinen-Oinonen *et al.*, 1997, Nykanen *et al.*, 1997). Similarly, the *cbh2*, *xyn2*, and *egl2* promoters have also been used individually and in series (Meng *et al.*, 2013, Miyauchi *et al.*, 2013, Miyauchi *et al.*, 2014). The *cbh1* promoter has also been shown to increase expression when multiple copies are used to drive expression (Liu *et al.*, 2008, Zhang *et al.*, 2010). Inducers of *cbh1* and other cellulase gene promoters invariably induce other cellulases and/or hemicellulases, resulting in a very complex mix of secreted proteins that often have similar or identical activities to that of the heterologous gene. To circumvent this, constitutive promoters such as *rp2* (He *et al.*, 2013), *pgk1* (Keranen and Penttila, 1995), *pki* (Keranen and Penttila, 1995, Mach *et al.*, 1994), *tef1* (Keranen and Penttila, 1995, Uzbass *et al.*, 2012), *gpd* (Penttila *et al.*, 1987, Zhang *et al.*, 2012), *cdna1* (Uzbass *et al.*, 2012), *pdc* (Li *et al.*, 2012, Wang *et al.*, 2014), and *eno* (Li *et al.*, 2012), have been used. There has also been work in modifying the *cbh1* promoter to remove the glucose repression effects, resulting in mutant *cbh1* promoters with high inducibility when grown on glucose (Liu *et al.*, 2008, Zou *et al.*, 2012). Deletion of the primary transcriptional

activating factor, *xyr1*, also has been shown to allow heterologous protein production in an essentially cellulase-free background when using the *cbh1* promoter (Uzbas *et al.*, 2012).

The use of the *cbh1* promoter has been shown to have its downsides. Qin *et al.* clearly showed that heterologous lipase production by the *cbh1* promoter was enhanced when Cel7A translation was inhibited by RNAi (Qin *et al.*, 2012). Expression of a heterologous endochitinase was proportionally reduced when multiple copies were inserted, presumably due to a lack of transcription and/or regulatory elements caused by multiple copies of the strong *cbh1* promoter (MargollesClark *et al.*, 1996).

The selection of constitutive promoters often leads to reduced levels of expression compared to a strong cellulose promoter. For research purposes; however, purification of target proteins from a cellulase free broth is often viewed as more desirable than working with a high cellulase background, even if the targeted protein is in higher titer. Historically, Nakari-Setälä *et al* reported in 1995 the development of a modified strain of *T. reesei* able to grow on glucose, where catabolic repression shuts down the normal cascade of cellulase expression, that produced targeted proteins using novel promoters (Nakari-Setälä and Penttilä, 1995). This early step in engineering promoter performance led the field toward engineered *T. reesei* strains that were able to secrete heterologous genes into the culture medium without the high background interference from other cellulases. Today, much of the work on promoter selection and engineering in *T. reesei* remains sequestered behind industrial firewalls.

2.4.2 Site of Integration

Protein production levels are also influenced by other factors, primarily site of genome integration and integrated gene copy number. Increased integrated copy number has been shown to lead to increased protein production in some cases (Bailey *et al.*, 2002, Levasseur *et al.*, 2006, Liu *et al.*, 2008, Miettinen-Oinonen and Suominen, 2002), but not in others (Miyachi *et al.*, 2013). The site of integration is also known to influence protein expression and can provide other advantages, such as visual confirmation of correct insertion by red pigment production when the *ade2* locus is targeted (Jorgensen *et al.*,

2014). Targeting heterologous genes to the *cbh1* locus has resulted in high expression levels; however, other factors, such as the use of the *cbh1* promoter has complicated a direct correlation in one case (Karhunen *et al.*, 1993). Another case clearly showed that targeted insertion of a glucoamylase gene from *Hormoconis resinae* into the *cbh1* locus yielded 3-fold higher production than the same gene randomly inserted in triplicate in the genome (Joutsjoki, 1994). This work was complicated by the use of gDNA for the targeted insertion and cDNA for the random insertion; however, both DNA forms gave folded and active protein, indicating that intron processing was carried out correctly for the heterologous gDNA. Saarelainen *et al.* used three strains of *T. reesei* as hosts for *xyn2* expression under its own promoter. Strains that had the *xyn2* gene targeted to the *cbh1* locus showed slightly lower *xyn2* expression levels; however, copy number in the random insertion transformants was proposed to be a probable cause for this effect (Saarelainen *et al.*, 1993). Whereas targeted insertion in *H. jecorina* has been facilitated by long sequence-identical arms flanking the heterologous gene followed by tedious analysis by Southern blotting or PCR, the development of Non-Homologous End-Joining (NHEJ) mutants has opened up this area by increasing the efficiency of targeting to >90%. The most common mechanism of generating NHEJ mutants is the deletion of either *tku70* or *mus53* (Guangtao *et al.*, 2009, Mustalahti *et al.*, 2013, Schuster *et al.*, 2012a, Steiger *et al.*, 2011).

In our work, we generated a NHEJ mutant through the deletion of *tmus53* in *H. jecorina* QM6a. The native copy of *tmus53* was replaced by a hygromycin B cassette as shown in Figure 8a. After selection on hygB, targeted insertions were screened by PCR, where a shorter PCR product indicates targeted insertion. (Figure 8b, c) Positive transformants were further purified and verified for the targeted deletion by sequencing.

Figure 8 near here

2.4.3 Effect of Signal Sequence

The choice of signal sequence (de Faria *et al.*, 2002) and fusion to known high-expression domains has also been demonstrated to increase yield in *H. jecorina*, although as with most studies, multiple factors, such as site of integration, copy number, and promoter

complicate the interpretation. One study using the same locus and single copy number of the same gene did show that signal sequence could affect protein production levels (Joutsjoki *et al.*, 1993a). Another study demonstrated that the GC content of the signal sequence could be altered to improved productivity without changing the amino acid sequence (Li *et al.*, 2007). Additional work in fusion proteins has indicated that fusion of heterologous protein sequences to native *H. jecorina* coding sequences can also increase expression yields (Nyyssonen *et al.*, 1993).

As with many filamentous fungi, protease production is a major concern for protein production in *H. jecorina* (Dienes *et al.*, 2007). Deletion of certain proteases has been shown to increase yields for several proteins (Zhang *et al.*, 2014); however, others are required for correct processing of native enzymes (Goller *et al.*, 1998). Moreover, it is generally known that lowering the culture pH increases proteolytic activity in *H. jecorina*, due to the activity characteristics of acidic aspartate proteases, although this effect is likely mediated by the presence of glycosylation on the target proteins (Eneyskaya *et al.*, 1999). Despite these examples, there is relatively little information available regarding the effects of protease inhibition or deletion on the expression and yield of heterologous proteins in *H. jecorina*.

Fusion proteins, in which a native *H. jecorina* protein or protein domain is genetically linked to the target gene, has been used to successfully express heterologous proteins. It has been shown to enable expression and increase protein yield; as well as to simplify purification. Mustalahti *et al.* recently showed that fusion of target proteins to the hydrophobin domain resulted in large segregated protein bodies that could be readily separated using a 2-phase aqueous extraction (Mustalahti *et al.*, 2013). Also, a bacterial xylanase gene (*Nonomuraea flexuosa xyn11A*) was expressed at high levels when fused to native *H. jecorina* Man5A core-hinge or Cel6A-CBM domains (Paloheimo *et al.*, 2003). While a direct comparison to the non-fusion gene was not carried out, the *Thermomonospora fusca tfxA* gene, 90% homology to *xyn11A*, expressed very poorly when used alone. Conversely, expression of a heterologous fungal xylanase (XynII from *Humicola grisea*) was reduced when fused to the Cel7A core-linker domain, even though the fusion

protein was shown to be processed correctly, resulting in a native-like XynII protein being produced (de Faria *et al.*, 2002).

Codon optimization is often cited as a means to increase expression of heterologous protein. Unfortunately, while *H. jecorina* genes have been optimized for expression in other hosts, there are very few examples of the inverse, especially when compared to the native gene sequence. Te'o *et al.* demonstrated dramatically increased functional expression of a *xynB* gene from a thermophilic bacterium after optimizing 20 codons for expression in *H. jecorina* and fusing the gene to a host carrier protein sequence (Bergquist *et al.*, 2004, Te'o *et al.*, 2000). When they expressed a heterologous fungal xylanase, codon optimization was not required, nor was fusion to a native carrier sequence (Bergquist *et al.*, 2002). More recently, Poldevin *et al.* expressed an optimized *Piromyces* gene in *H. jecorina*; however, they did not attempt expression of the native gene sequence (Poidevin *et al.*, 2009). Xin and Jia made similar claims using an *Orpinomyces* β -1-4 endoglucanase (Jin and Xia, 2011). Although protein expression was high using the codon-optimized gene, they did not test expression of the non-optimized gene.

Finally, whereas transient protein expression has been reported from non-integrated plasmids, this result is typically low expression and short-lived, resulting in rapid loss of the selection marker and subsequent loss of the strain.

2.5 Expression of recombinant Cel7A in the *cbh1* Δ strains

We demonstrated the utility of the two deletion strains, AST871 and AST1116, generated from Rut-C30 and QM6a, respectively, by transforming these strains with the appropriate expression vectors described above. As shown in Figure 9, the transformed strains, Rut-C30, and QM6a, produce Cel7A. We have also tested the deletion strains for synthesis of other cellulases (*cbh2* and *egl1*) and found that both deletion strains produce these enzymes at levels similar to the parent strains from which the deletion strains were derived.

Figure 9 near here

The Cel7A expression cassette in pTR50 has been designed such that integration of the expression system may be directed to *cbh1* locus by homologous recombination between the 5' and 3' regions of the *cbh1* region in the expression cassette and the corresponding regions in the AST1116 host strain. It is expected that the frequency of such events in AST1116 would be low. In approximately 5% of the transformants obtained in the experiments to delete the *cbh1* coding sequence from QM6a, the deletion cassette had integrated at the target and these constructs had 1.5 kb homology at 5' and 3' ends. We note that the frequency of homologous recombination, and thus of targeting, can be increased by deleting one of the genes, *e.g.*, *tku70*, that are required for the ectopic DNA integration by the non-homologous end joining (NHEJ) pathway (Guangtao *et al.*, 2009).

Even with the presence of the NHEJ pathway in AST1116, a transformant in which the integration event has occurred by homologous recombination at the *cbh1* locus can be distinguished from other transformants because of the characteristic phenotypes of these two classes of the transformants as shown in Figure 10. The parent strain does not utilize acetamide because it does not contain the *A. nidulans amdS* gene and it is sensitive to hygromycin B because it does not contain the *E. coli hph* gene. The deletion strain; however, utilizes acetamide, but is still sensitive to hygromycin B. If the expression cassette integrated at the *cbh1* locus by homologous recombination, this genomic region would have the structure shown in the bottom of the Figure 10. This transformant would now be resistant to hygromycin B and could no longer utilize acetamide as nitrogen source because the *amdS* gene would be lost. If the integration event happens elsewhere in the genome, the transformants would retain the expression of *amdS* and thus still be able to utilize acetamide. The acetamidase-expressing strains can be further distinguished by their sensitivity to the analogue fluoroacetamide (Hynes and Pateman, 1970, Steiger *et al.*, 2011).

Figure 10 near here

3. Conclusions

There are a large number of *Hypocrea jecorina* strains that have been used to express heterologous proteins for a variety of reasons. Many of these strains, particularly those that have specific genes or capabilities deleted or added, were never available to the public or are no longer used by academic labs. Modern molecular biology methodologies make it relatively straightforward for competent labs to recreate these strains and their associated vectors; however, it is incumbent upon these researchers to consider several criteria before embarking on this work. Specifically, they must consider the exact nature of the desired outcome, such as mutational studies of a specific protein, increased productivity, or structure/function studies of particular enzymes or enzyme classes. From this set of criteria, they can then select the appropriate host strain and tools to achieve that outcome. Host strain, selection markers, gene modification (codon optimization, fusion protein, promoter and signal sequence selection), copy number, insertion locus, and protease abatement all impact heterologous gene expression differently under variable circumstances. Some general guidelines emerge from the published record: proteins difficult to express in *H. jecorina* include those that are multi domain, require specific glycosylation, and/or are bacterial in origin. Note that exceptions exist, but these trends seem to hold.

To support biofuels research funded by the U.S. Department of Energy, we have developed expression hosts and the corresponding vectors for two *T. reesei* strains, the wild type strain, QM6a, and industrial “work horse” Rut C-30 specifically for expressing *cbh1* variants and mutations. We have replaced the *cbh1* genes in QM6a and in Rut C-30 with *A. nidulans amdS* and *E. coli* hygromycin B phosphotransferase genes, respectively; and developed expression vectors for inserting heterologous genes into these strains. The deletion strains are able to produce functional Cel7A proteins when DNA constructs encoding these proteins are reintroduced. These new *T. reesei* systems are suitable for production of 1) improved forms of cellulases by rational design or random mutagenesis, 2) diverse natural enzymes for phylogenetic studies, and 3) native enzymes with critical mutations designed to study biochemical modes of action.

4. Acknowledgments

This work was supported by the U.S. Department of Energy under Contract No. DE-AC36-08G028308 with the National Renewable Energy Laboratory. Funding for the work was provided by the DOE Office of Energy Efficiency and Renewable Energy, Bioenergy Technologies Office.

5. References

Abdeljabbar DM, Song HJ, Link AJ. *Trichoderma reesei* cellobiohydrolase II is associated with the outer membrane when overexpressed in *Escherichia coli*. *Biotechnology letters*. 2012;34:91-6.

Bailey MJ, Askolin S, Horhammer N, Tenkanen M, Linder M, Penttilä M, *et al*. Process technological effects of deletion and amplification of hydrophobins I and II in transformants of *Trichoderma reesei*. *Applied microbiology and biotechnology*. 2002;58:721-7.

Berges T, Barreau C. Isolation of uridine auxotrophs from *Trichoderma reesei* and efficient transformation with the cloned *ura3* and *ura5* genes. *Current genetics*. 1991;19:359-65.

Bergquist P, Te'o V, Gibbs M, Cziferszky A, de Faria FP, Azevedo M, *et al*. Expression of xylanase enzymes from thermophilic microorganisms in fungal hosts. *Extremophiles : life under extreme conditions*. 2002;6:177-84.

Bergquist PL, Te'o VS, Gibbs MD, Curach NC, Nevalainen KM. Recombinant enzymes from thermophilic micro-organisms expressed in fungal hosts. *Biochemical Society transactions*. 2004;32:293-7.

Boer H, Teeri TT, Koivula A. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. *Biotechnology and bioengineering*. 2000;69:486-94.

Boonvitthya N, Bozonnet S, Burapatana V, O'Donohue MJ, Chulalaksananukul W. Comparison of the heterologous expression of *Trichoderma reesei* endoglucanase II and cellobiohydrolase II in the yeasts *Pichia pastoris* and *Yarrowia lipolytica*. *Molecular biotechnology*. 2013;54:158-69.

Cherry JR, Fidantsef AL. Directed evolution of industrial enzymes: an update. *Current opinion in biotechnology*. 2003;14:438-43.

Dai Z, Hooker BS, Quesenberry RD, Gao J. Expression of *Trichoderma reesei* exo-cellobiohydrolase I in transgenic tobacco leaves and calli. *Applied biochemistry and biotechnology*. 1999;77-79:689-99.

de Faria FP, Te OV, Bergquist PL, Azevedo MO, Nevalainen KM. Expression and processing of a major xylanase (XYN2) from the thermophilic fungus *Humicola grisea* var. *thermoidea* in *Trichoderma reesei*. *Letters in applied microbiology*. 2002;34:119-23.

Deane EE, M. WJ, Peberdy JF. Transformation of *Trichoderma reesei* with a constitutively expressed heterologous fungal chitinase gene. *Enzyme and microbial technology*. 1999;24:419-24.

Den Haan R, McBride JE, Grange DCL, Lynd LR, Van Zyl WH. Functional expression of cellobiohydrolases in *Saccharomyces cerevisiae* towards one-step conversion of cellulose to ethanol. *Enzyme and microbial technology*. 2007;40:1291-9.

Dienes D, Borjesson J, Hagglund P, Tjerneld F, Liden G, Reczey K, *et al.* Identification of a trypsin-like serine protease from *Trichoderma reesei* QM9414. *Enzyme and microbial technology*. 2007;40:1087-94.

Eneyskaya EV, Kulminskaya AA, Savel'ev AN, Savel'eva NV, Shabalin KA, Neustroev KN. Acid protease from *Trichoderma reesei*: limited proteolysis of fungal carbohydrases. *Applied microbiology and biotechnology*. 1999;52:226-31.

Fang H, Xia L. High activity cellulase production by recombinant *Trichoderma reesei* ZU-02 with the enhanced cellobiohydrolase production. *Bioresource technology*. 2013;144:693-7.

Godbole S, Decker SR, Nieves RA, Adney WS, Vinzant TB, Baker JO, *et al.* Cloning and expression of *Trichoderma reesei* cellobiohydrolase I in *Pichia pastoris*. *Biotechnology progress*. 1999;15:828-33.

Goller SP, Schoisswohl D, Baron M, Parriche M, Kubicek CP. Role of endoproteolytic dibasic proprotein processing in maturation of secretory proteins in *Trichoderma reesei*. *Applied and environmental microbiology*. 1998;64:3202-8.

Gruber F, Visser J, Kubicek CP, de Graaff LH. The development of a heterologous transformation system for the cellulolytic fungus *Trichoderma reesei* based on a *pyrG*-negative mutant strain. *Current genetics*. 1990;18:71-6.

Guangtao Z, Hartl L, Schuster A, Polak S, Schmoll M, Wang T, *et al.* Gene targeting in a nonhomologous end joining deficient *Hypocrea jecorina*. *Journal of biotechnology*. 2009;139:146-51.

Guangtao Z, Seiboth B, Wen C, Yaohua Z, Xian L, Wang T. A novel carbon source-dependent genetic transformation system for the versatile cell factory *Hypocrea jecorina* (anamorph *Trichoderma reesei*). *FEMS Microbiological Letters*. 2010;303:26-32.

- Harkki A, Mantyla A, Penttila M, Muttillainen S, Buhler R, Suominen P, *et al.* Genetic engineering of *Trichoderma* to produce strains with novel cellulase profiles. *Enzyme and microbial technology*. 1991;13:227-33.
- Hartl L, Seiboth B. Sequential gene deletions in *Hypocrea jecorina* using a single blaster cassette. *Current genetics*. 2005;48:204-11.
- Hazell BW, Te'o VS, Bradner JR, Bergquist PL, Nevalainen KM. Rapid transformation of high cellulase-producing mutant strains of *Trichoderma reesei* by microprojectile bombardment. *Letters in applied microbiology*. 2000;30:282-6.
- He R, Zhang C, Guo W, Wang L, Zhang D, Chen S. Construction of a plasmid for heterologous protein expression with a constitutive promoter in *Trichoderma reesei*. *Plasmid*. 2013;70:425-9.
- Herrera-Estrella A, Goldman GH, Van Montagu M. High-efficiency transformation system for the biocontrol agents, *Trichoderma* spp. *Molecular microbiology*. 1990;4:839-43.
- Hong J, Tamaki H, Kumagai H. Cloning and functional expression of thermostable beta-glucosidase gene from *Thermoascus aurantiacus*. *Applied microbiology and biotechnology*. 2007;73:1331-9.
- Hynes MJ, Pateman JA. The genetic analysis of regulation of amidase synthesis in *Aspergillus nidulans*. II. Mutants resistant to fluoroacetamide. *Molecular and General Genetics*. 1970;108:107-16.
- Jin X, Xia L. Heterologous expression of an endo- β -1,4-glucanase gene from the anaerobic fungus *Orpinomyces* PC-2 in *Trichoderma reesei*. *World Journal of Microbiology and Biotechnology*. 2011;27:2913-20.
- Jorgensen MS, Skovlund DA, Johannesen PF, Mortensen UH. A novel platform for heterologous gene expression in *Trichoderma reesei* (Teleomorph *Hypocrea jecorina*). *Microbial cell factories*. 2014;13:33.
- Joutsjoki VV. Construction by one-step gene replacement of *Trichoderma reesei* strains that produce the glucoamylase P of *Hormoconis resinae*. *Current genetics*. 1994;26:422-9.
- Joutsjoki VV, Kuittinen M, Torkkeli TK, Suominen PL. Secretion of the *Hormoconis resinae* glucoamylase P enzyme from *Trichoderma reesei* directed by the natural and the *cbh1* gene secretion signal. *FEMS microbiology letters*. 1993a;112:281-6.
- Joutsjoki VV, Torkkeli TK, Nevalainen KM. Transformation of *Trichoderma reesei* with the *Hormoconis resinae* glucoamylase P (*gamP*) gene: production of a heterologous glucoamylase by *Trichoderma reesei*. *Current genetics*. 1993b;24:223-8.

Karhunen T, Mantyla A, Nevalainen KM, Suominen PL. High frequency one-step gene replacement in *Trichoderma reesei*. I. Endoglucanase I overproduction. *Molecular & general genetics* : MGG. 1993;241:515-22.

Keränen S, Penttilä M. Production of recombinant proteins in the filamentous fungus *Trichoderma reesei*. *Current opinion in biotechnology*. 1995;6:534-7.

Kim S, Miasnikov A. Method for introducing nucleic acids into fungal cells. Google Patents; 2010.

Koushki MM, Rouhani H, Farsi M. Genetic manipulation of fungal strains for the improvement of heterologous genes expression (a mini-review). *Afr J Biotechnol*. 2011;10:7939-48.

Kruszewska JS. Heterologous expression of genes in filamentous fungi. *Acta biochimica Polonica*. 1999;46:181-95.

Kubodera T, Yamashita N, Nishimura A. Transformation of *Aspergillus* sp. and *Trichoderma reesei* using the pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*. *Bioscience, biotechnology, and biochemistry*. 2002;66:404-6.

Kuck U, Hoff B. New tools for the genetic manipulation of filamentous fungi. *Applied microbiology and biotechnology*. 2010;86:51-62.

Kuhls K, Lieckfeldt E, Samuels GJ, Kovacs W, Meyer W, Petrini O, *et al*. Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93:7755-60.

Laymon RA, Adney WS, Mohagheghi A, Himmel ME, Thomas SR. Cloning and expression of full-length *Trichoderma reesei* cellobiohydrolase I cDNAs in *Escherichia coli*. *Applied biochemistry and biotechnology*. 1996;57-58:389-97.

Levasseur A, Saloheimo M, Navarro D, Andberg M, Monot F, Nakari-Setälä T, *et al*. Production of a chimeric enzyme tool associating the *Trichoderma reesei* swollenin with the *Aspergillus niger* feruloyl esterase A for release of ferulic acid. *Applied microbiology and biotechnology*. 2006;73:872-80.

Li J, Wang J, Wang S, Xing M, Yu S, Liu G. Achieving efficient protein expression in *Trichoderma reesei* by using strong constitutive promoters. *Microbial cell factories*. 2012;11:84.

Li XL, Skory CD, Ximenes EA, Jordan DB, Dien BS, Hughes SR, *et al*. Expression of an AT-rich xylanase gene from the anaerobic fungus *Orpinomyces* sp. strain PC-2 in and secretion of the heterologous enzyme by *Hypocrea jecorina*. *Applied microbiology and biotechnology*. 2007;74:1264-75.

- Liu M, Sun ZX, Zhu J, Xu T, Harman GE, Lorito M. Enhancing rice resistance to fungal pathogens by transformation with cell wall degrading enzyme genes from *Trichoderma atroviride*. Journal of Zhejiang University Science. 2004;5:133-6.
- Liu T, Wang T, Li X, Liu X. Improved heterologous gene expression in *Trichoderma reesei* by cellobiohydrolase I gene (*cbh1*) promoter optimization. Acta biochimica et biophysica Sinica (Shanghai). 2008;40:158-65.
- Ma L, Zhang J, Zou G, Wang C, Zhou Z. Improvement of cellulase activity in *Trichoderma reesei* by heterologous expression of a beta-glucosidase gene from *Penicillium decumbens*. Enzyme and microbial technology. 2011;49:366-71.
- Mach RL, Schindler M, Kubicek CP. Transformation of *Trichoderma reesei* based on hygromycin B resistance using homologous expression signals. Current genetics. 1994;25:567-70.
- Mandels M, Parrish FW, Reese ET. Sophorose as an inducer of cellulase in *Trichoderma viride*. Journal of Bacteriology 1962;83:400-8.
- Mandels M, Reese ET. Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. Journal of Bacteriology. 1957;73:269-78.
- MargollesClark E, Hayes CK, Harman GE, Penttila M. Improved production of *Trichoderma harzianum* endochitinase by expression in *Trichoderma reesei*. Applied and environmental microbiology. 1996;62:2145-51.
- Martinez D, Berka RM, Henrissat B, Saloheimo M, Arvas M, Baker SE, *et al.* Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). Nature biotechnology. 2008;26:553-60.
- Meng F, Wei D, Wang W. Heterologous protein expression in *Trichoderma reesei* using the *cbhII* promoter. Plasmid. 2013;70:272-6.
- Merino S, Cherry J. Progress and Challenges in Enzyme Development for Biomass Utilization. In: Olsson L, editor. Biofuels: Springer Berlin Heidelberg; 2007. p. 95-120.
- Miettinen-Oinonen A, Suominen P. Enhanced production of *Trichoderma reesei* endoglucanases and use of the new cellulase preparations in producing the stonewashed effect on denim fabric. Applied and environmental microbiology. 2002;68:3956-64.
- Miettinen-Oinonen A, Torkkeli T, Paloheimo M, Nevalainen H. Overexpression of the *Aspergillus niger* pH 2.5 acid phosphatase gene in a heterologous host *Trichoderma reesei*. Journal of biotechnology. 1997;58:13-20.
- Mitsuishi Y, Nitisinprasert S, Saloheimo M, Biese I, Reinikainen T, Claeysens M, *et al.* Site-directed mutagenesis of the putative catalytic residues of *Trichoderma reesei* cellobiohydrolase I and endoglucanase I. FEBS letters. 1990;275:135-8.

Miyauchi S, Te'o VS, Jr., Bergquist PL, Nevalainen KM. Expression of a bacterial xylanase in *Trichoderma reesei* under the *egl2* and *cbh2* glycosyl hydrolase gene promoters. *New biotechnology*. 2013;30:523-30.

Miyauchi S, Te'o VS, Nevalainen KM, Bergquist PL. Simultaneous expression of the bacterial *Dictyoglomus thermophilum xynB* gene under three different *Trichoderma reesei* promoters. *New biotechnology*. 2014;31:98-103.

Montenecourt BS, Eveleigh DE. Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. *Advances in Chemistry*. 1979;181:289-301.

Mustalahti E, Saloheimo M, Joensuu JJ. Intracellular protein production in *Trichoderma reesei* (*Hypocrea jecorina*) with hydrophobin fusion technology. *New biotechnology*. 2013;30:262-8.

Nakari-Setälä T, Penttilä M. Production of *Trichoderma reesei* cellulases on glucose-containing media. *Applied and environmental microbiology*. 1995;61:3650-5.

Nevalainen H, Peterson R. Making recombinant proteins in filamentous fungi- are we expecting too much? *Frontiers in Microbiology*. 2014;5.

Nevalainen KM, Te'o VS, Bergquist PL. Heterologous protein expression in filamentous fungi. *Trends in biotechnology*. 2005;23:468-74.

Nisizawa T, Suzuki H, Nisizawa K. Catabolite repression of cellulase formation in *Trichoderma viride*. *Journal of biochemistry*. 1972;71:999-1007.

Nykanen M, Saarelainen R, Raudaskoski M, Nevalainen K, Mikkonen A. Expression and Secretion of Barley Cysteine Endopeptidase B and Cellobiohydrolase I in *Trichoderma reesei*. *Applied and environmental microbiology*. 1997;63:4929-37.

Nyyssonen E, Penttilä M, Harkki A, Saloheimo A, Knowles JK, Keränen S. Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. *Bio/technology*. 1993;11:591-5.

Paloheimo M, Mäntylä A, Kallio J, Suominen P. High-yield production of a bacterial xylanase in the filamentous fungus *Trichoderma reesei* requires a carrier polypeptide with an intact domain structure. *Applied and environmental microbiology*. 2003;69:7073-82.

Penttilä M, Nevalainen H, Ratto M, Salminen E, Knowles J. A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene*. 1987;61:155-64.

Peterson R, Nevalainen H. *Trichoderma reesei* RUT-C30--thirty years of strain improvement. *Microbiology*. 2012;158:58-68.

Poidevin L, Levasseur A, Paes G, Navarro D, Heiss-Blanquet S, Asther M, *et al.* Heterologous production of the *Piromyces equi* cinnamoyl esterase in *Trichoderma reesei* for biotechnological applications. *Letters in applied microbiology*. 2009;49:673-8.

Qin LN, Cai FR, Dong XR, Huang ZB, Tao Y, Huang JZ, *et al.* Improved production of heterologous lipase in *Trichoderma reesei* by RNAi mediated gene silencing of an endogenic highly expressed gene. *Bioresource technology*. 2012;109:116-22.

Reinikainen T, Ruohonen L, Nevanen T, Laaksonen L, Kraulis P, Jones TA, *et al.* Investigation of the function of mutated cellulose-binding domains of *Trichoderma reesei* cellobiohydrolase I. *Proteins*. 1992;14:475-82.

Saarelainen R, Mantyla A, Nevalainen H, Suominen P. Expression of Barley Endopeptidase B in *Trichoderma reesei*. *Applied and environmental microbiology*. 1997;63:4938-40.

Saarelainen R, Paloheimo M, Fagerstrom R, Suominen PL, Nevalainen KM. Cloning, sequencing and enhanced expression of the *Trichoderma reesei* endoxylanase II (pl 9) gene xln2. *Molecular & general genetics : MGG*. 1993;241:497-503.

Schuster A, Bruno KS, Collett JR, Baker SE, Seiboth B, Kubicek CP, *et al.* A versatile toolkit for high throughput functional genomics with *Trichoderma reesei*. *Biotechnology for biofuels*. 2012a;5:1.

Schuster A, Tisch D, Seidl-Seiboth V, Kubicek CP, Schmoll M. Roles of protein kinase A and adenylate cyclase in light-modulated cellulase regulation in *Trichoderma reesei*. *Applied and environmental microbiology*. 2012b;78:2168-78.

Seiboth B, Ivanova C, Seidl-Seiboth V. *Trichoderma reesei*: A Fungal Enzyme Producer for Cellulosic Biofuels, Biofuel Production-Recent Developments and Prospects. In: Bernardes DMADS, editor.: InTech; 2011.

Seidl V, Gamauf C, Druzhinina IS, Seiboth B, Hartl L, Kubicek CP. The *Hypocrea jecorina* (*Trichoderma reesei*) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. *BMC genomics*. 2008;9:327.

Smith JL, Bayliss FT, Ward M. Sequence of the cloned *pyr4* gene of *Trichoderma reesei* and its use as a homologous selectable marker for transformation. *Current genetics*. 1991;19:27-33.

Steiger MG. Molecular tools in *Trichoderma* genetic studies. In: Mukherjee PK, Horwitz BA, Singh US, Mukherjee M, Schmoll M, editors. *Trichoderma: Biology and Applications*: CABI; 2013. p. 128-43.

Steiger MG, Vitikainen M, Uskonen P, Brunner K, Adam G, Pakula T, *et al.* Transformation system for *Hypocrea jecorina* (*Trichoderma reesei*) that favors homologous integration and employs reusable bidirectionally selectable markers. *Applied and environmental microbiology*. 2011;77:114-21.

Sternberg D, Mandels GR. Induction of cellulolytic enzymes in *Trichoderma reesei* by sophorose. *Journal of Bacteriology*. 1979;139:761-9.

Suominen PL, Mantyla AL, Karhunen T, Hakola S, Nevalainen H. High frequency one-step gene replacement in *Trichoderma reesei*. II. Effects of deletions of individual cellulase genes. *Molecular and General Genetics*. 1993;241:523-30.

Takada G, Kawaguchi T, Sumitani J, Arai M. Expression of *Aspergillus aculeatus* No. F-50 cellobiohydrolase I (*cbhI*) and beta-glucosidase 1 (*bgl1*) genes by *Saccharomyces cerevisiae*. *Bioscience, biotechnology, and biochemistry*. 1998;62:1615-8.

Tangnu S, Blanch HW, Wilke CR. Enhanced Production of cellulase, hemicellulase, and beta-glucosidase by *Trichoderma reesei* (Rut-C30). *Biotechnology and bioengineering*. 1981;33:1837-49.

Te'o VS, Bergquist PL, Nevalainen KM. Biolistic transformation of *Trichoderma reesei* using the Bio-Rad seven barrels Hepta Adaptor system. *Journal of microbiological methods*. 2002;51:393-9.

Te'o VS, Cziferszky AE, Bergquist PL, Nevalainen KM. Codon optimization of xylanase gene xynB from the thermophilic bacterium *Dictyoglomus thermophilum* for expression in the filamentous fungus *Trichoderma reesei*. *FEMS microbiology letters*. 2000;190:13-9.

Uzbas F, Sezerman U, Hartl L, Kubicek CP, Seiboth B. A homologous production system for *Trichoderma reesei* secreted proteins in a cellulase-free background. *Applied microbiology and biotechnology*. 2012;93:1601-8.

von Ossowski I, Stahlberg J, Koivula A, Piens K, Becker D, Boer H, *et al*. Engineering the exo-loop of *Trichoderma reesei* cellobiohydrolase, Cel7A. A comparison with *Phanerochaete chrysosporium* Cel7D. *Journal of molecular biology*. 2003;333:817-29.

Wang J, Zeng D, Mai G, Liu G, Yu S. Homologous constitutive expression of Xyn III in *Trichoderma reesei* QM9414 and its characterization. *Folia Microbiologica (Praha)*. 2014;59:229-33.

Xu L, Shen Y, Hou J, Peng B, Tang H, Bao X. Secretory pathway engineering enhances secretion of cellobiohydrolase I from *Trichoderma reesei* in *Saccharomyces cerevisiae*. *Journal of bioscience and bioengineering*. 2014;117:45-52.

Zhang G, Zhu Y, Wei D, Wang W. Enhanced production of heterologous proteins by the filamentous fungus *Trichoderma reesei* via disruption of the alkaline serine protease SPW combined with a pH control strategy. *Plasmid*. 2014;71:16-22.

Zhang J, Qu Y, Xiao P, Wang X, Wang T, He F. Improved biomass saccharification by *Trichoderma reesei* through heterologous expression of lacA gene from *Trametes* sp. AH28-2. *Journal of bioscience and bioengineering*. 2012;113:697-703.

Zhang J, Zhong Y, Zhao X, Wang T. Development of the cellulolytic fungus *Trichoderma reesei* strain with enhanced beta-glucosidase and filter paper activity using strong artificial cellobiohydrolase 1 promoter. *Bioresource technology*. 2010;101:9815-8.

Zhong YH, Wang XL, Wang TH, Jiang Q. *Agrobacterium*-mediated transformation (AMT) of *Trichoderma reesei* as an efficient tool for random insertional mutagenesis. *Applied microbiology and biotechnology*. 2007;73:1348-54.

Zou G, Shi S, Jiang Y, van den Brink J, de Vries RP, Chen L, *et al*. Construction of a cellulase hyper-expression system in *Trichoderma reesei* by promoter and enzyme engineering. *Microbial cell factories*. 2012;11:21.

Figure Legends

Figure 1. Cassettes used for *cbh1* deletion in (A) Rut-C30, (B) QM6a by homologous recombination into (C) the native *cbh1* locus.

Figure 2. Expression vector used in *H. jecorina* strain, AST871. The selection/expression cassette in this construct contains acetamidase gene (*amdS*) from *A. nidulans* for selection of transformants based on their ability to utilize acetamide as the sole nitrogen source. Note that AST871 is resistant to hygromycin B because this selection system was used to delete the *cbh1* coding sequence.

Figure 3. Deletion of native *pyr4* gene. (A) Schematic of *pyr4* deletion strategy. (B) Confirmation by PCR of targeted deletion. Shorter product length (Transformant 1) indicates targeted deletion.

Figure 4. Determining conditions for 5-FOA selection. MA plates with 5-FOA and 10mM uridine. (A) QM6a, initial pH 6.5. (B) *pyr4* transformation, initial pH 6.5. (Note: Colonies in (B) were picked before large mycelial mats were produced) (C) QM6a, initial pH 5.5. (D) *pyr4* deficient strain, initial pH 5.5.

Figure 5. *pyr4* as a bidirectional positive selectable marker. (A) Replacement of *hph* with *pyr4* cassette. (B) 5-FOA selection for loss of the *pyr4* cassette results in a clean deletion at the *mus53* locus.

Figure 6. Confirmation of clean deletion at *mus53*. Shorter PCR product indicates region has been deleted.

Figure 7. Expression vector for use in *cbh1Δ* strain AST1116. The selection of transformants in this system is based on the expression of bacterial hygromycin phosphotransferase gene (*hph*) conferring resistance to hygromycin B. The expression cassette is bound by 2,500 bp of sequence 5' to *cbh1* and 1,500 bp of sequence 3' to *cbh1*. This allows for integration of the expression cassette to the *cbh1* region by homologous recombination.

Figure 8. Deletion of *mus53* to reduce Non-homologous End Joining (NHEJ) . (A) Schematic of crossover. (B) Confirmation of deletion by PCR of selected transformants.

Figure 9. Cel7A expression in *cbh1Δ* strains transformed with *cbh1* expression constructs. α -cel7a immunoblot of a PAGE gel using culture supernatants. 1: Purified Cel7A; 2: Rut-C30; 3: *cbh1Δ* from Rut-C30; 4 & 5: Two independent transformants of *Rut-C30 cbh1Δ* with *cbh1* expression construct; 6: QM6a 7: *cbh1Δ* from QM6a; 8 & 9: Two independent transformants of QM6a *cbh1Δ* with *cbh1* expression construct.

Figure 10. Phenotypes of the parent (QM6a), *cbh1Δ* and α *cbh1*-targeted transformant. QM6a, (A); deletion strain derived from QM6a, AST1116, (B) and a transformant in which an expression system has been integrated at the *cbh1* locus (C).

Figure 1.

A) *cbh1Δ::hph* (RUT-C30)

B) *cbh1Δ::amdS* (QM6a)

C) Native locus

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

A.

B.

C.

Table 1. *Hypocrea jecorina* strains used in heterologous protein expression

Strain ID	Geno/Phenotype	Purpose	Source	Reference(s)
QM6a (VTT-D-74083T) (VTT-D-021262T) (IFO/NBRC31326) ¹	Wild-type	General protein expression	ATCC VTT VTT (NBRC)	(Kubodera <i>et al.</i> , 2002)
QM9414 (VTT-D-74075)	Hypersecreting mutant of QM6a	High yield general expression synergy studies with induced cellulase background	ATCC	(Bailey <i>et al.</i> , 2002, Dashtban and Qin, 2012, Fu <i>et al.</i> , 2010, Guangtao <i>et al.</i> , 2009, Mach <i>et al.</i> , 1994, Penttila <i>et al.</i> , 1987, Wang <i>et al.</i> , 2014, Zhong <i>et al.</i> , 2007)
RUT-C30 (VTT-D-86271)	Catabolite de-repressed, hypersecreting mutant of QM6a	High yield general expression Synergy studies with constitutive cellulase background	ATCC	(Bailey <i>et al.</i> , 2002, Hazell <i>et al.</i> , 2000, Joutsjoki <i>et al.</i> , 1993b, Kautto <i>et al.</i> , 2013, Murray <i>et al.</i> , 2004, Nykanen <i>et al.</i> , 1997, Peterson and Nevalainen, 2012, Saarelainen <i>et al.</i> , 1997, Salles <i>et al.</i> , 2007)
RUT-C30 M3	Protease deficient strain of RUT-C30	General protein expression	Shandong U	(Liu <i>et al.</i> , 2008)
CL847	High cellulase production mutant derived from QM9414	General protein expression		(Levasseur <i>et al.</i> , 2006)
TU-6 ²	Δ □□□□ derived from QM9414	<i>ura</i> auxotroph selection	TU Wein	(Gruber <i>et al.</i> , 1990, Guangtao <i>et al.</i> , 2009, Mach <i>et al.</i> , 1994)
TU-6H ²	Δ □□□□ <i>i</i> in TU-6	Hexokinase selection	Shandong U	(Guangtao <i>et al.</i> , 2010)
KU70.4 ²	Δ □□□□ $\tilde{}$ in TU-6	Non-homologous end-joining mutant	TU Wein Shandong U	(Guangtao <i>et al.</i> , 2009)
VTT-D-78085	β -G hyperproducer derived from QM9414		VTT	(Nevalainen <i>et al.</i> , 1980)
VTT-D-93201	Δ <i>cbh1</i> , <i>amdS</i> (+) derived from QM9414	Expression of Cel7A	VTT	(Reinikainen <i>et al.</i> , 1995, Stahlberg <i>et al.</i> , 1996)
VTT-D-79125	High cellulase production mutant	General expression, gene knockout studies	VTT	(Bailey and Nevalainen, 1981, Harkki <i>et al.</i> , 1991, Hazell <i>et al.</i> , 2000, Karhunen <i>et al.</i> , 1993, Mantyla <i>et</i>

	derived from QM9414			<i>al.</i> , 1992, Penttilä <i>et al.</i> , 1987, Saarelainen <i>et al.</i> , 1993, Suominen <i>et al.</i> , 1993a, b, Te'o <i>et al.</i> , 2002)
GC69	□□□□ in an RL-P37 background	<i>ura</i> auxotroph selection	Genencor	(Smith <i>et al.</i> , 1991)
PTUC1 ²	Δ□□□□ derived from QM9414	General protein expression	U Bordeaux	(Berges and Barreau, 1991)
VTT-D-87305 VTT-D-87307	<i>arg</i> (-) derived from VTT-D-79125	General protein expression	VTT	(Penttilä <i>et al.</i> , 1987)
HEP1	Protease-deficient, high enzyme secretion, derived from VTT-D-79125	General protein expression	VTT	(de Faria <i>et al.</i> , 2002, Te'o <i>et al.</i> , 2000)
ALKO3414 ³	<i>cbh1</i> (-), <i>cbh2</i> (-)	Expression of Cel7A, Cel6A	Primalco, Ltd.	(von Ossowski <i>et al.</i> , 2003)
ALKO2221 ³	Low aspartyl protease mutant of VTT-D-79125	Increased protein yield	Alko, Ltd	(Hazell <i>et al.</i> , 2000, Joutsjoki <i>et al.</i> , 1993b, Saarelainen <i>et al.</i> , 1997, Saarelainen <i>et al.</i> , 1993)
ALKO2721 ³	<i>TrpC</i> ⁺ into Δ <i>cbh2</i> in VTT-D-79125 background	General expression w/ <i>AmdS</i> selection	Alko, Ltd	(Saarelainen <i>et al.</i> , 1993)
ALKO3620	<i>cel5a</i> (-)	General protein expression		(Paloheimo <i>et al.</i> , 2003)
ALKO4468	<i>cel5a</i> (-) <i>cel7b</i> (-)	General protein expression		(Paloheimo <i>et al.</i> , 2003)
ZU-02 ²	Mutant (unclear phenotype) derived from ATCC 56764	General expression through <i>Agrobacterium</i> -mediated transformation	Zhejiang University	(Fang and Xia, 2013, Liming and Xueliang, 2004, Ma <i>et al.</i> , 2011)
AST871	Δ <i>cbh1</i> , <i>hph</i> ⁺ in Rut-C30	Expression of Cel7A in high cellulase producing strain	NREL	This study
AST1116	Δ <i>cbh1</i> , <i>amdS</i> ⁺ in QM6a	Expression of Cel7A in native strain	NREL	This study

1- NBRC31326 is QM6a deposited in the Japanese culture collection

2- Authors are unclear if these strains are available for distribution

3- Alko, Ltd. And Primalco, Ltd. no longer appear to be in business