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Research review paper

Heterologous protein expression in *Hypocrea jecorina*: A historical perspective and new developments

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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 a 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.

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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, *Trichoderma 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 *Trichoderma 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 de-repressed, 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 Pentillä 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 Kruszewska (Kruszewska, 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.

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.

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

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