Approaches to understanding protein hypersecretion in fungi

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Abstract

Fungi are well known for secreting high levels of proteins. A number of strategies have been used to characterize and maximize protein secretion for industrial purposes. In this review, we highlight three different ascomycetes and focus on a specific protein production example for each. *Aspergillus niger* has been utilized as a production host for amylases and multiple molecular genetic approaches have been applied to increase secretion in this organism.

Saccharification of plant biomass is an integral part of biofuel production and classical genetic and genomic approaches have been used in *Trichoderma reesei* to understand and manipulate the pathways controlling secretion of plant cell wall degrading enzymes. Finally, *Neurospora crassa*, a model filamentous ascomycete has been exploited as a model to understand a wide range of biological processes including protein secretion.

1. Introduction

We all live in the fungal digestive tract. Fungal growth is dependent on the transport of biochemical building blocks from the extracellular environment into the growing hyphae. Biochemical building blocks are released from complex polymeric substrates by the action of enzymes secreted by the fungal hyphae. The breadth of enzymes are produced by fungi to digest a wide variety of substrates is impressive and this ability to catalytically decompose their surroundings has value not only for the fungus, but also for industry and medicine. Enzymes from fungi and other microbes have a multiplicity of uses, ranging from food and beverage preparation, to boosting the power of laundry detergents, to sugar release from plant biomass and for these reasons, fungi are valuable enzyme producing cell factories. However, fungi and other microbes do not “want” to secrete more carbon and nitrogen via enzymes than they take in, therefore, secretion of these enzymes is tightly regulated.

Various fungi have been domesticated over the last several centuries. For example, fungal enzymes are essential to the koji processes used for sake and soy sauce production.

Aspergillus oryzae is a well characterized koji fungus whose enzymes saccharify rice starch in to glucose that is, in turn, fermented into the ethanol of the sake by *Saccharomyces cerevisiae*.

Well over 100 years ago, the koji process served as an inspiration for one of the first biotechnology patents wherein fungal enzymes were used as digestive aids. A Japanese scientist, Takamine brought *Aspergillus oryzae* to the US and formed Takamine Corp which produced a digestive aid called Takamine diaste. Enzymatic digestive aids are still in use today, with even some brands of infant dried cereal containing fungal enzymes for enhanced digestion.

Over time, applications that utilize fungal enzymes has grown to a much larger number of industrial applications, from food and beverage production to the digestion of plant biomass in bioenergy and bioproducts applications. As new enzyme activities are discovered and characterized, more even more applications are developed and deployed by industry. It is not

surprising that the enzyme production industry continues to grow year after year. Thus, it is of paramount importance to a number of important industries to maximize the secretion of enzymes. Both basic and industrial research efforts are focused on the control of enzyme secretion by filamentous fungi. In this review we will discuss three “case studies” in the area of fungal protein hyperproduction.

2. From genetics to genomics: Amylase production in *Aspergillus niger*

Production of starch hydrolyzing amylases (mainly α -amylase, EC 3.2.1.1 and glucoamylase, EC 3.2.1.3) by *Aspergillus niger* and closely related species has been in practice in industry for several decades (Souza, 2010). The corn products industry is dependent on these enzymes to convert starch to dextrins and glucose and thence on to a myriad of products from ethanol to corn syrups to acidulants. Early studies pinpointed a zygomycete, *Mucor indicus* (originally called *Amylomyces rouxii* by Calmette) and *Aspergillus oryzae* as organisms responsible for the amylolytic activity in certain Chinese and Japanese fermented food processes, respectively (Calmette, 1892; Takamine, 1914). By the mid 1940s a screen of 367 fungi – mainly *Penicillium* and *Aspergillus* species – by a research team associated with the fermentation division of the USDA Northern Region Research Laboratory in Peoria, Illinois, identified *A. niger* strain NRRL 337 (now classified as the close *A. niger* relative, *Aspergillus acidus* and prior to that, *Aspergillus foetidus*) as a high amylase producer (Le Mense et al., 1947). Additional amylase strains of *A. niger* and close relatives were identified and later, mutagenesis screens were performed to isolate mutants with increased glucoamylase production (Armbruster, 1961; Baker, 2006).

While *A. niger* strains have been put to work as citric acid and enzyme production organisms, it has also been a model system for basic and applied biology and genomics research programs (Andersen et al., 2011; Baker and Bennett, 2008; Baker, 2006; Karaffa and Kubicek, 2003; Pel et al., 2007). At present, traditional genetic crossing in *A. niger* has not been described which limits the ability to map and recombine mutants for removal of deleterious background

mutations. Parasexual recombination is an alternative and has been utilized to isolate an improved strain of *A. niger* from a cross of a high enzyme yielding strain with a related strain that was lower yielding but had improved fermentation characteristics (Ball et al., 1978; Bodie et al., 1994; Bos et al., 1988; Debets et al., 1993; Loera and Córdova, 2003; Montiel-González et al., 2002). Parasexual genetics has not been widely utilized in strain improvement programs but may become more widespread as the cost of sequencing has dropped considerably making parasexual genetic enabled bulk segregant analysis more tractable (Niu et al., 2016).

While classical mutagenesis and screening allow a whole organism approach, reverse genetic approaches have been developed which offer a gene-centric method for understanding biological processes including protein secretion; indeed, *A. niger* was among the first filamentous fungi for which transformation protocols were developed (Buxton et al., 1985; Goosen et al., 1987; Kelly and Hynes, 1985; Van Hartingsveldt et al., 1987). The development of transformation led to the ability to make gene fusions between glucoamylase (*glaA*) and non-*A. niger* proteins to better understand how *Aspergillus niger* could be used for heterologous gene expression (Roberts et al., 1992). In another molecular genetic study, researchers fused a truncated glucoamylase gene to a gene encoding hen egg-white lysozyme (HEWL) and secretion was enhanced by at least 10-fold indicating the importance of native amino acid sequences in secretion (Jeenes et al., 1993). Heterologously expressed proteins are subject to proteolytic degradation by secreted and mycelial proteases (Archer et al., 1992).

Isolation of mutants with decreased production of secreted proteases has been used as a strategy to increase the amount and stability of secreted proteins. Beginning with the deletion of *pepA* in *Aspergillus awamorii*, reverse genetic approaches have been utilized to delete specific proteases (Berka et al., 1990). Classical mutants generated by UV mutagenesis were screened for protease deficient phenotypes leading to the isolation of *prt* genes (Mattern et al., 1992). Some of these genes have been cloned and deleted or over-expressed, and one in particular, a

gene named *pvtT* encoding a protein involved in the regulation of protease expression, when deleted has a dramatic protease deficient phenotype (Punt et al., 2008).

In another series of molecular genetic studies, mutant strains with multiple copies of the glucoamylase gene stably integrated into a chromosome were selected (Verdoes et al., 1993). In this study, up to 200 copies of the glucoamylase gene were integrated into a individual single strain and in some cases this caused instability at the insertion locus (Verdoes et al., 1994b). Subsequent research indicated that the level of glucoamylase protein and enzymatic activity was strongly correlated with expression (i.e. transcript) level, to a point - beyond a certain number of gene copies, transcript level plateaued indicating that the high number of copies of the gene promoter eventually titrated the transcription factor (Verdoes et al., 1994a; Verdoes et al., 1994b). In short, the level of transcription factor protein was a bottleneck to production of the target protein whose expression it controlled.

3. Cellulase secretion, classical genetics, mutant screens and genomics in *Trichoderma reesei*

Classical genetic approaches utilizing mutagenesis and screening resulted in generation of numerous protein hyper-secreting fungal strains from a variety of species. One of the simplest and most effective screens to set up is the halo assay. In these screens fungal spores are mutagenized and spread on plates that have an opaque substrate, which is cleared upon action of the desired enzyme activity. Thus it is simply a visual screening for an increased halo size that results in the identification of mutants whose phenotype is increased enzyme secretion. Indeed, multiple rounds of mutagenesis and screening have resulted in very productive strains that are used in industry today.

The challenge of the classical genetic approach is the fact that the molecular nature of the mutation leading to the increased secretion phenotype is not immediately obvious. However, with lower cost and higher throughput DNA sequencing and the relatively small genome size for many fungi, it is now possible to simply sequence the genome of the high secreting strains and

compare this to the wild type reference genome sequence to identify all of the mutations present, and generate a list of gene candidates potentially associated with the phenotype (Baker, 2009; Koike et al., 2013; Le Crom et al., 2009; Lichius et al., 2015; McCluskey et al., 2011; Niu et al., 2016; Pomraning et al., 2011; Smith et al., 2008). One of the most attractive systems in which to employ this approach is *Trichoderma reesei* whose genome was published in 2008 (Martinez et al., 2008). The *T. reesei* wild type reference strain Qm6a was isolated in the 1940s the cellulase system was described over the next few decades (Mandels et al., 1962; Mandels and Reese, 1957, 1960). Beginning in the 1970s strains were mutagenized and selected for high secretion of cellulase enzymes (for example, (Mandels et al., 1971; Montenecourt and Eveleigh, 1977)). Researchers reported in 2009 that they could perform genetic crossing of *T. reesei* strain Qm6a (which turned out to be female sterile) with a wild type strain of the opposite mating type (Seidl et al., 2009). Because of its utility as a cellulase producer and the many high cellulase secreting mutant lineages generated from Qm6a, the ability to cross made it possible that genetic mapping approaches could be utilized to more clearly delineate the genetic factors underlying high cellulase secretion (Schuster et al., 2012; Seidl and Seiboth, 2010). Moving this system even closer to enhanced genetic analysis was the discovery of a mutation in the *T. reesei* ortholog of the *Neurospora crassa* gene MAP kinase scaffold protein encoding gene, *ham-5* that causes strain Qm6a female sterility (Linke et al., 2015). Another *T. reesei* genetic study, wherein one of the crossing partners had a reciprocally exchanged chromosome resulted in segmented aneuploidy progeny with the duplicated segment containin genes involved in hemicellulose degradation (Chuang et al., 2015).

A number of *T. reesei* Qm6a lineages were developed that later became the starting point for industrial production strains (for example, (Mandels et al., 1971; Montenecourt and Eveleigh, 1977; Peterson and Nevalainen, 2012). Thus, generating genome sequences for mutant strains of the lineages that were derived from Qm6a is a tractable method for identifying and

characterizing genes whose protein products are involved in protein hyperproduction. In one of the first fungal resequencing projects, a parent strain of several industrial strains, *T. reesei* Rut-C30, and one of its parental strains, were sequenced and compared to the wild type parental Qm6a (Koike et al., 2013; Le Crom et al., 2009). Surprisingly, there were a relatively small number of mutations in the Rut-C30 and other mutant strain genomes; mutagenesis is a scalpel rather than a sledgehammer (Koike et al., 2013; Le Crom et al., 2009; Nitta et al., 2012; Vitikainen et al., 2010). One of the most important mutated genes in Rut-C30 encodes the master regulator of carbon catabolite repression, CRE1 (Ilmen et al., 1996). Another *T. reesei* strain, PC-3-7, was resequenced in Japan also revealed and mutation in *cre1* and a beta-glucosidase regulator encoding gene, *bglr* (Nitta et al., 2012). There are other mutations found in *T. reesei* Rut-C30 including a large (~85Kb, 29 genes) deletion and a frameshift in the gene encoding glucosidase II alpha subunit enzyme that results in altered an pattern of protein glycosylation (Geysens et al., 2005; Seidl et al., 2008). In addition to resequencing of hyperproductive strains, strains which produce little or no cellulose are also of interest. The genome of *T. reesei* strain, QM9136, was resequenced and a mutation in the gene encoding the transcriptional regulator XYR1 was shown to underly its lack of cellulase secretion (Lichius et al., 2015).

4. Secretion in a model fungal organism, *Neurospora crassa*

Another filamentous fungus, *Neurospora crassa*, has been a model genetic system for the understanding of a number of biological processes. Recently, a whole genome gene deletion strain library was generated. The strain library has been utilized in recent years for increasing our understanding of factors involved in secretion of enzymes particularly those involved in biomass degradation. The synthesis of secreted proteins in filamentous fungi has been presumed to be similar to the single-celled yeast, *Saccharomyces cerevisiae* (Idiris et al., 2010; Shoji et al., 2014). Translated transcripts intended for secretion are translocated into the endoplasmic reticulum (ER) where the proteins undergo folding and early glycosylation steps.

The proteins are then transported via vesicles to the Golgi where they can undergo additional glycosylation. Mature proteins are then secreted to one of several destinations: to the endosome for eventual cycling into the vacuole, directly to the vacuole, to the plasma membrane for interaction with exocytic machinery, or (in a step specific to filamentous fungi) to the Spitzenkörper. This latter is a site of vesicle accumulation at the hyphal tip, primarily composed of Golgi-derived vesicles intended for exocytosis but also some endocytic vesicles (reviewed in (Riquelme, 2013). Interestingly, new cellular structures specific to *N. crassa* and likely other filamentous fungi continue to be identified, including specific prevacuolar compartments that were recently described by Bowman (Bowman et al., 2015).

Historically, the hyphal tip was thought of as the primary, if not sole, region of protein secretion in filamentous fungi. As a result, the structure and organization of organelles within the first hyphal segment of *N. crassa* has been well-described. Four regions in the apical (Region I) and sup-apical (Regions II-IV) of mature leading hyphae were identified according to the distribution of the Spitzenkörper, nuclei, mitochondria, and vesicle structures (Riquelme et al., 2002). A later study identified similar regions within germlings (Araujo-Palomares et al., 2007).

It has only been more recently that researchers have begun to explore protein secretion at regions distal to the hyphal tip. This change in approach was summarized by Read, who outlined the growing evidence for hyphal growth and exocytosis at regions beyond the tip of filamentous fungi, including: intercalary growth, thickening of the secondary wall, septum formation, and sub-apical branch initiation (Read, 2011). Specifically in *Neurospora*, recent work has found evidence of a protein secretion route that occurs behind the apex and is independent of the Spitzenkörper (Fajardo-Somera et al., 2013). As interest in regions distal to the leading edge of a fungal colony have increased, studies in which proteins typically found in specific organelles – endoplasmic reticulum, vacuole, nuclei, mitochondria – are tagged with fluorescent markers have emphasized how different the internal structures of *N. crassa* hyphae are either at the tip compared to distal from it (Bowman et al., 2009).

The most recent observations on protein secretion in *N. crassa* have resulted from investigations exploring its lignocellulolytic capabilities. Decreasing the production cost of enzymes needed for the commercial degradation of cellulose requires an understanding of enzyme synthesis and secretion. This includes defining the regulatory networks that underly enzyme production. *Neurospora crassa* was an obvious choice for these studies given the many genetic and genomic tools that have been developed for this model organism over the decades as well as its inherent lignolytic capabilities . The first systems analysis of lignocellulose degradation by *N. crassa* proposed a model that included a dramatic upregulation of cellulose, hemicellulose, and transporter genes (Tian et al., 2009). Of those genes that showed increased expression in response to crystalline cellulose, greater than 50% are predicted to enter the secretory pathway (Coradetti et al., 2012). This same trend was maintained as the transcriptional response of *N. crassa* to a variety of lignocellulosic substrates has been explored (Benz et al., 2014; Cai et al., 2015; Sun et al., 2012). Importantly, upregulation of one of these transcription factors resulted in a cellulosic response in the absence of a cellulosic inducer, allowing for isolation of cellulolytic enzymes from culture supernatant (Coradetti et al., 2013). This massive upregulation of proteins transversing the secretory pathway has led to new studies of protein secretion in *N. crassa*, beginning with analyses of known and predicted glycolytic proteins found in culture secretome and expanding to include mechanisms of protein production (Phillips et al., 2011; Tian et al., 2009).

An abundance of unfolded or misfolded proteins in the lumen of the ER can induce the Unfolded Protein Response (UPR). Conserved in eukaryotes, this stress response aims to balance the amount of protein entering the secretory pathway with the protein-folding capacity of the ER (reviewed in (Heimel, 2015)). Interestingly, the ability of fungi to secrete large quantities of protein appears linked to an upregulation of the UPR. This begins with the activation of Ire1, which subsequently splices the mRNA of the transcription factor Hac1, allowing for its translation and a subsequent upregulation of genes resulting in remodeling of the secretory

pathway. In *Neurospora*, the cellulosic response includes an increase in both overall *hac-1* transcription levels as well as the ratio of spliced transcript compared to non-spliced transcript (Benz et al., 2014). This is consistent with increased expression of genes associated with UPR and indicates ER stress when exposed to crystalline cellulose. Interestingly, while *hac-1* and the UPR are essential for growth on cellulose, it is not required when *N. crassa* is exposed to a hemicellulose such as xylan (Montenegro-Montero et al., 2015). The work of Fan et al., provides preliminary evidence for the overlap and complicated interactions of *Neurospora*'s responses to cellulosic substrates and ER stress (Fan et al., 2015).

Recent work has linked hyper-production of secreted proteins in *Neurospora* with the Sterol Regulatory Element Binding Protein (SREBP) pathway: deletion of components of the SREBP pathway can result in a two-fold increase in protein secretion under cellulolytic conditions (Reilly et al., 2015). This pathway has been studied in *Schizosaccharomyces pombe* (Hughes et al., 2005; Hughes et al., 2009) and *Aspergillus fumigatus* (Blatzer et al., 2011; Chung et al., 2014; Willger et al., 2012; Willger et al., 2008) where it has been linked to ER-associated protein degradation and adaptation to hypoxic conditions. In mammalian cells the SREBP transcription factors are involved in sterol biosynthesis (Osborne and Espenshade, 2009), suggesting role in hyper-production of secreted proteins in *N. crassa* may be related to lipid make-up of cellular membranes. It is interesting that two pathways associated with the regulation of lipids, UPR and SREBP (reviewed in (Lagace and Ridgway, 2013; Raychaudhuri et al., 2012), have now been linked to protein production in filamentous fungi. In the future, the *Neurospora* system can be used to explore the relationship between lipid metabolism and secreted protein production.

5. Summary and conclusions

Understanding and exploiting protein expression in fungi remains a crucial endeavor of fundamental scientific and practical importance. Fungi are key producers of enzymes that add to our every day quality of life. They also produce cellulases, hemicellulases and ligninases crucial to the sustainable production of fuels and chemicals to enable continued economic development.

We reviewed three critical aspects of protein secretion: genome structure, regulation and the secretion pathway. At the level of the genome, copy number is a critical aspect of hypersecretion of proteins. Protein production increases with gene copy number but is limited by level of transcription factor driving gene expression. Gene regulation is another key component, as indicated by classical mutagenesis studies in *T. reesei* which show that mutations to *cre1*, *xyr1* and *bglr* influence cellulase secretion. Finally, the secretion pathway itself is key, as UPR and the cell's secretion machinery play important roles in the level of protein output from the cell.

The historical developments discussed in the fungal protein production systems reviewed above demonstrate that traditional methods of isolation and improvement of protein production hosts, such as mutagenesis and screening, and reverse genetics to demonstrate the action of specific genes, will continue to have value for generation of improved strains in the future. The impact of the advances of the genomics era is that these standard approaches have become more powerful and the rate of progress has increased dramatically. Much remains to be learned about effective and efficient protein production in general and heterologous protein production in particular, but the tools we have at our disposal today are accelerating progress in this area. Importantly, this combination of traditional and advanced approaches will also enable the development of new and better fungal protein production hosts.

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