FUNGAL BIOLOGY REVIEWS XXX (2016) 1–7

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/fbr

Review

Approaches to understanding protein hypersecretion in fungi

Morgann C. REILLY[a,b], *Jon K. MAGNUSON*[a,b], *Scott E. BAKER*[a,b,*]

[a]*Pacific Northwest National Laboratory, Richland, WA, USA*
[b]*DOE Joint BioEnergy Institute, Emeryville, CA, USA*

ARTICLE INFO

Article history:
Received 19 May 2016
Accepted 12 June 2016

Keywords:
Aspergillus
Neurospora
Protein secretion
Trichoderma

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 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 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 into glucose that is, in turn, fermented into the ethanol of the sake by *Saccharomyces cerevisiae*. Well over 100 y 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,

* *Corresponding author*. Pacific Northwest National Laboratory, Richland, WA, USA.
E-mail address: scott.baker@pnnl.gov (S. E. Baker).

http://dx.doi.org/10.1016/j.fbr.2016.06.002
1749-4613/

Takamine brought *A. 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 have 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, 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 important to a number of 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, which are subsequently converted into 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 *A. 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 procedures have been developed that 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 *A. 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 overexpressed, and one in particular, a gene named *prtT* 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 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, 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 hypersecreting fungal strains from a variety of species. One of the simplest and most effective screens to set up is the halo assay.

Please cite this article in press as: Reilly, M.C., et al., Approaches to understanding protein hypersecretion in fungi, Fungal Biology Reviews (2016), http://dx.doi.org/10.1016/j.fbr.2016.06.002