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Production of Extremophilic Bacterial Cellulase Enzymes in *Aspergillus Niger*

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Production of Extremophilic Bacterial Cellulase Enzymes in *Aspergillus Niger*

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Abstract

Enzymes can be used to catalyze a myriad of chemical reactions and are a cornerstone in the biotechnology industry. Enzymes have a wide range of uses, ranging from medicine with the production of pharmaceuticals to energy were they are applied to biofuel production. However, it is difficult to produce large quantities of enzymes, especially if they are non-native to the production host. Fortunately, filamentous fungi, such as *Aspergillus niger*, are broadly used in industry and show great potential for use a heterologous enzyme production hosts. Here, we present work outlining an effort to engineer *A. niger* to produce thermophilic bacterial cellulases relevant to lignocellulosic biofuel production.

ACKNOWLEDGMENTS

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NOMENCLATURE

DOE	Department of Energy
SNL	Sandia National Laboratories
JBEI	Joint BioEnergy Institute
IL	Ionic Liquid
orf	open reading frame
GFP	green fluorescent protein

1. INTRODUCTION

1.1 Problem Statement

Lignocellulosic biofuels hold great promise for dramatically reducing the US dependence on foreign oil. These technologies aim to convert sugars derived from plant biomass feedstocks into biofuels. Typically, plant feedstocks are chemically pretreated to gain access to the sugar polymers, which are then enzymatically hydrolyzed using cellulase and hemicellulase cocktails and fermented into biofuel. Current biofuel bioprocessing configurations operate under mesophilic conditions, but there is a push toward thermophilic conditions for a variety of reasons, including reduced contamination, more efficient fuel recovery, better enzyme kinetics, higher metabolic rates (higher productivity), etc, all of which would significantly reduce costs for biofuel production.

Companies, such as Green Biologics Ltd, have already begun developing thermophilic biofuel production hosts, but have yet to complete the thermophilic bioprocessing configuration by developing a compatible thermophilic cellulase cocktail, which severely limits the commercial potential of this technology. Current commercial cellulase cocktails are derived from filamentous fungi that produce very high titers of these enzymes, a characteristic that is essential for commercial viability. Unfortunately, these cocktails are inactive at high temperatures. Thermophilic bacteria produce highly stable thermophilic cellulase enzymes and are excellent alternatives to the fungal enzymes. However, bacteria generally produce low titers of these enzymes (an order of magnitude lower than fungi) and are therefore themselves not commercially viable production hosts. To overcome this barrier, we developed the industrial filamentous fungus *Aspergillus niger* into an expression host for thermophilic bacterial cellulases.

1.2 Technical Approach and Leading Edge Nature of Work

Expression of bacterial enzymes in filamentous fungi has historically proven difficult because these enzymes are quite foreign to fungi, which leads to poor expression and inhibitory modifications. We have devised a multipronged approach to overcome these barriers, including construction of an expression system built off of the most highly secreted enzyme in *A. niger* and extensive engineering of the bacteria genes to make them more compatible with fungi.
The ultimate goal of this work is to develop a thermophilic cellulase cocktail that can function under more extreme temperatures than current commercial cocktails derived from filamentous fungi commonly used in industry, such as *Trichoderma reesei* and *A. niger*, allowing it to be integrated into more efficient thermophilic bioprocessing configurations for lignocellulosic biofuel production. Thermophilic bacterial enzymes tend to be more stable than fungal enzymes, making them a good choice for source enzymes to develop a thermophilic cellulase cocktail. As an added bonus, the higher stability of thermophilic bacterial enzymes allows them to tolerate inhibitory compounds that may be produced during biomass pretreatment. For example, thermophilic bacterial cellulase enzymes are more active at both higher temperatures and at higher concentrations of ionic liquids (ILs) than enzymes from the filamentous fungi *T. reesei* [1,2]. Ionic liquids have shown great promise in biomass pretreatment, and having enzymes that are

stable in ILs significantly advances the potential of ILs being integrated into a commercial biofuel bioprocessing configuration. This example just highlights the utility of thermophilic cellulase enzymes, and shows their potential value in the biofuels market is multifaceted. Work done at the Joint BioEnergy Institute (JBEI) has produced a list of many thermophilic cellulase enzymes from each of the three classes of cellulases: β-glucosidases, cellobiohydrolases, and endoglucanases that have been successfully incorporated into a thermophilic and IL-tolerant cellulase cocktail (**Figure 1**) [1]. However, these enzymes are currently produced in *E. coli* at very low titers. To enable further R&D and to demonstrate commercial viability these enzymes must be ported into an industrial production host capable of high titer production, which was the aim of this LDRD project. The work described here has laid the foundation for a new Fungal Biotechnology group recently established at JBEI that continue advancing *A. niger* as a heterologous enzyme production host.

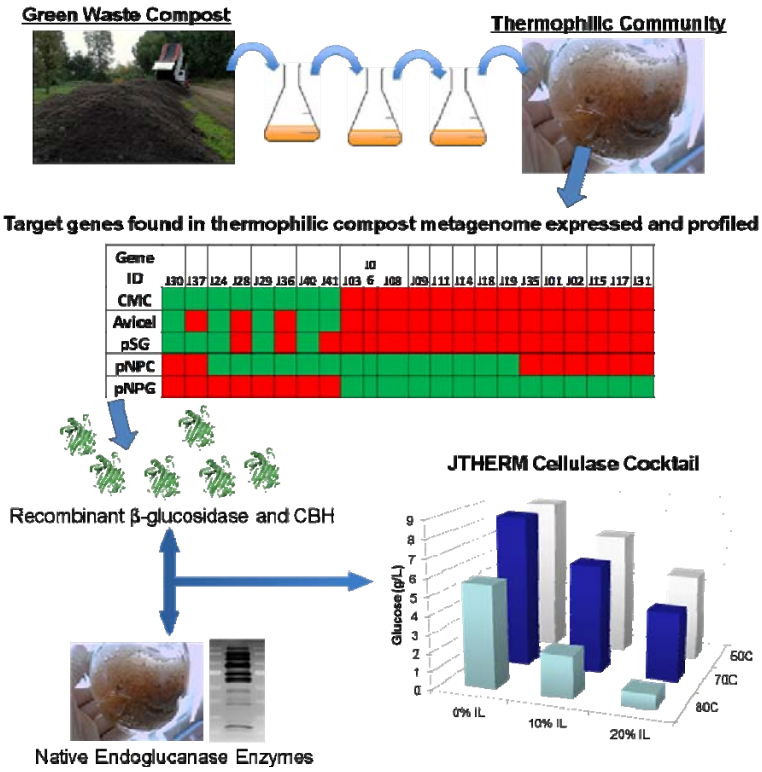

Figure 1. Thermophilic bacterial enzymes isolated from green-waste compost microbial communities at JBEI were used to construct the Il-tolerant cellulase cocktail JTherm.

The first step in the development of a cellulase cocktail is to produce the relevant enzymes at high titer in an industrial host organism. We attempted to express several thermophilic bacterial cellulase enzymes in a commercial fungal host, which are known to produce high titers of enzymes. Using a fungal host has the added benefit of allowing the bacterial enzymes to be easily integrated into the current cellulase industry; the thermophilic enzymes could be used either as a standalone thermophilic cellulase cocktail or blended with fungal cellulase enzymes, making a broad range mesophilic/thermophilic cellulase cocktail. Aspergillus is a genus of filamentous fungi that has been used industrially for over a hundred years, and it is the leading genus for fermentation-derived enzyme production[3]. Over the last two decades this genus has

been used to express heterologous proteins derived from a variety of different organisms, including bacteria and humans [4]. After *T. reesei*, Aspergillus species are one of the primary sources of enzymes for commercial cellulase cocktails used in the lignocellulosic biofuels industry [5]. The fact that Aspergillus has well developed expression systems and is currently used to produce cellulases, makes this genus a good target to express the thermophilic bacterial cellulases. *A. niger*, is a species commonly used for heterologous expression and a suitable host to express thermophilic bacterial enzymes. A variety of molecular tools have been developed in *A. niger*, facilitating transformation and integration of DNA constructs designed for heterologous protein expression. The glucoamylase gene (*glaA*) promoter has been widely used in expression constructs over the last two decades and a variety of modifications have been made over the years to increase expression from this promoter and stabilize the heterologous proteins[4]. This wealth of information makes *glaA*-based expression constructs a good choice to express thermophilic bacterial enzymes and was the route taken in this study.

Figure 2. *Aspergillus niger* chlamydoconida protruding from solid growth media.

Attempts have been made to express high titers of extremophilic bacterial enzymes in other fungi, and lessons learned from those attempts were used to guide efforts in this proposal, including optimizing codon usage and modification of the bacterial genes to enhance their encoded protein's secretion and recovery [6,7]. In addition, it is important to establish how the expression of bacterial genes in *A. niger* impacts their function. Efforts were made in this study to design expression constructs that facilitate the purification and characterization of the thermophilic bacterial cellulase genes expressed in *A. niger* so that they could easily be compared to their counterparts expressed in *in vitro* or in *E. coli*. Even with a well designed expression system, it is hard to predict whether any protein will express in a new production host. To mitigate this issue, ~30 genes covering each of the three classes of cellulase enzymes derived from several different thermophilic bacteria were screened to ensure a sufficient number would actively express to cover each class of cellulase enzyme, and important step toward the development of a thermophilic cellulase cocktail. Production of thermophilic cellulases in *A. niger* will help transition promising research in the laboratory into commercially viable products with a high potential value in the burgeoning alternative energy market.

2. *A. NIGER* HETEROLOGOUS EXPRESSION SYSTEM: DESIGN AND VALIDATION

2.1 Design and Assembly of the *A. niger* Expression Construct

The first step in the development of a heterologous expression system is to assemble the genetic components required to build an expression vector. As mentioned earlier, the glucoamylase gene *glaA* is an excellent target for these parts, and was used to source the promoter and signal sequence for the expression construct. The *glaA* signal sequence was included into the vector to target the heterologous proteins for secretion, a step that dramatically simplifies their recovery. The *glaA* terminator could be used as well, but its inclusion in the construct could bias integration into the native *glaA* locus. Since it is unclear how important position effects are in *A. niger*, the decision was made to set up an expression construct capable of random integration so that the construct could land in multiple loci within the genome and the most amenable location could be selected via activity of the heterologous enzyme. Therefore, the terminator region of the *A. nidulans* tryptophan biosynthesis pathway gene trpC was chosen to prevent targeted integration into the *glaA* locus in *A. niger*[8]. The open reading frame (orf) of each of the thermophilic bacterial cellulase genes codon optimized for *A. niger* is then inserted between these regulatory elements to build a complete expression unit. In addition, Strep and 8xHis-tags were inserted at the 3'end of each gene. These tags should allow the cellulase proteins to be easily detected and purified from the native *A. niger* proteins, simplifying the quantitation of their expression levels and activity. **Figure 3** outlines the basic setup of the construct. To enable rapid cloning in *E. coli*, the *A. niger* sequences were inserted into an *E. coli* bluescript plasmid containing the *E. coli* hph gene for resistance to hygromyosin, making an *E. coli/A. niger* shuttle vector[9].

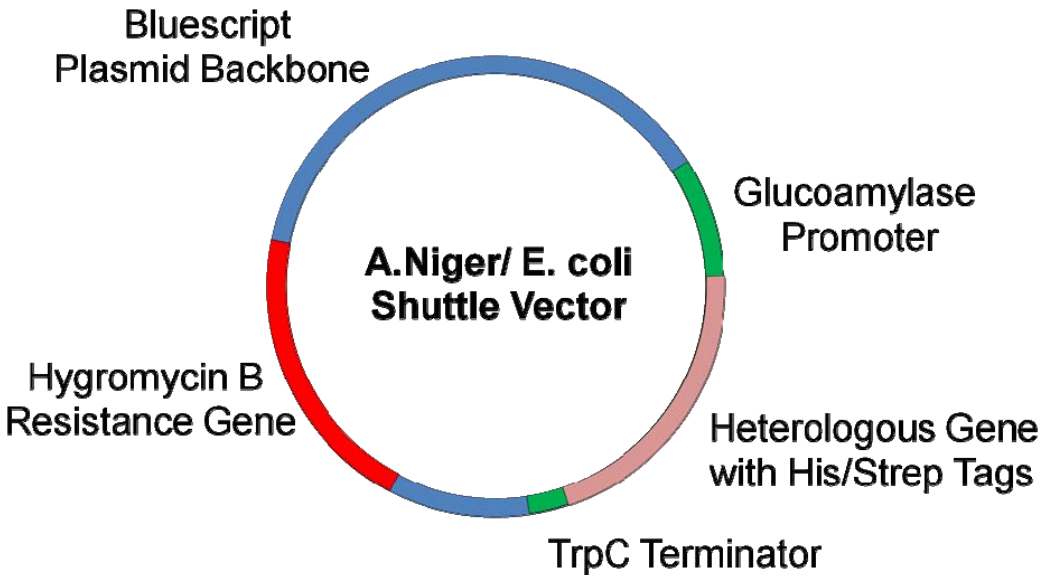

Figure 3. Expression construct for heterologous protein expression in *A. niger*.

2.2 Functional Validation of the *A. niger* Expression Construct

Once the *A. niger* expression vector was assembled, several control genes were cloned into it: green fluorescent protein (GFP), and three cellulases from other filamentous fungi (*Thermoascus aurantiacus* and *T. reesei*; **Table 1**). The control genes were selected to help determine the functionality of the expression construct and to assess whether there were any issues associated with expression bacterial proteins in *A. niger*. The GFP was selected to enable easy monitoring of the protein both within the cell and in the secreted fraction. The three fungal cellulases were selected to determine whether there is any preference for genes from organisms more similar to the *A. niger* host. In addition, the *T. aurantiacus* enzymes are thermophilic, so they can help assess whether there are any issues with expressing thermophilic enzymes[10].

The GFP expression construct was the first tested and results were promising. Three independent transformants were obtained and two of the three exhibited GFP expression in the fungal hyphe, confirming that the expression construct was capable of inducing heterologous protein expression in *A. niger* (**Figure 4**). The GFP appeared to be concentrated at the hyphal tips; this phenotype would be expected for actively secreted proteins as secretion occurs at the hyphal tips in *A. niger*[11]. GFP was also observed in vesicle-like bodies further back in the hyphe. These structures moved around but did not progress to the hyphal tips, suggesting that they were effectively trapped within the hyphe. In *A. niger*, hyphe elongate via cellular division at the hyphal tips so the observation of these GPF bodies in the hyphe suggest that this fraction of GFP failed to be secreted or was produced after the tip had move forward with a new cell and the GFP was unable to be transferred into the forward cell for secretion[12]. Attempts were made to detect GFP in the culture supernatant but were unsuccessful. This is not surprising as other reports have indicated that GFP is degraded by extracellular proteases in *A. niger*[13]. Therefore, absence of GFP in the supernatant does not conclusively indicate a failure to secrete it.

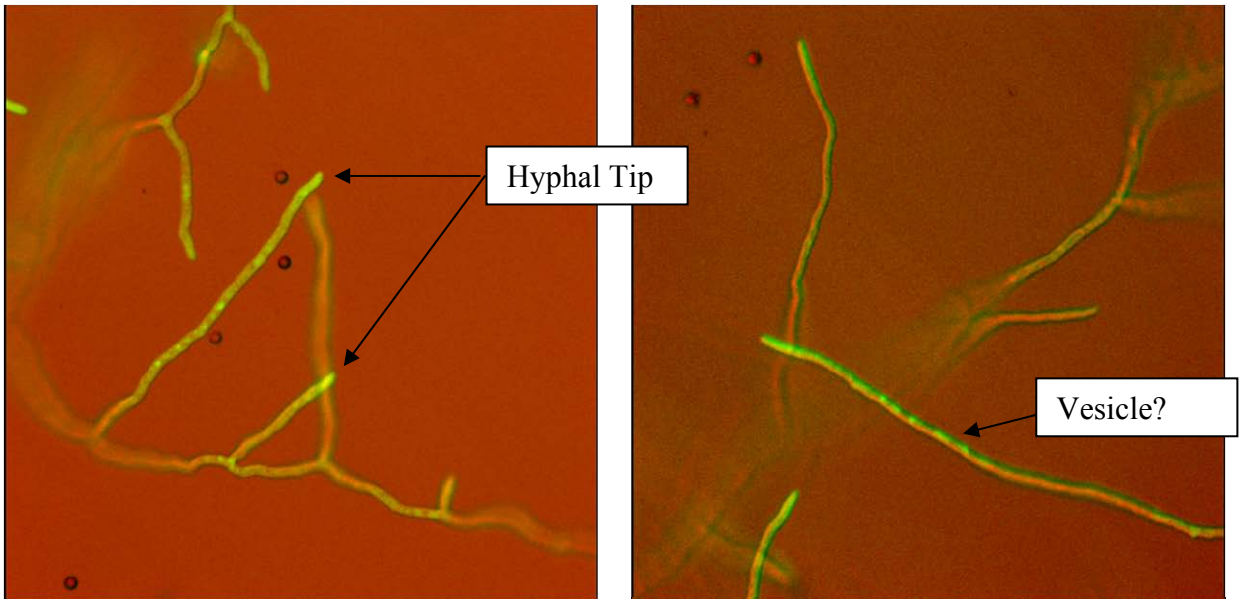

Figure 4. Detection of GFP expression in *A. niger* using confocal microscopy.

A better gauge of protein secretion comes from the three control fungal cellulase genes. All three enzymes were transformed into *A. niger* and expression was detected for all three, indicating that the expression construct not only works but can successfully target heterologous proteins for secretion. Attempts were made to purify these enzymes to establish expression levels but they all failed. This indicates that there is an issue with the Strep and His peptide tags used, suggesting that they we either inaccessible or degraded by the same extracellular proteases that may have degraded GFP. Anecdotally, the observed activity levels in the enzymes assays used were quite low compared to similar enzymes expressed in *E. coli*, suggesting that while these enzymes were expressed, their titers are likely to be in the sub mg/L scale. This issue with the purification tags makes quantitation difficult, but will be at least partially addressed in the next section.

3. EXPRESSION OF BACTERIAL CELLULASES IN *A. NIGER*

3.1 Assessment of Control Bacterial Genes in *A. niger*

The analysis of *A. niger* strains harboring the four control genes in the previous section clearly demonstrated that the expression construct functions as designed to express and secret heterologous proteins. Table 1 lists the thermophilic bacterial cellulases that were to be target for expression in *A. niger*. These genes were all bacterial in origin and only recently identified at JBEI from a thermophilic enrichment culture of a compost microbial community. Since these genes have not been well characterized, two additional well characterized bacterial genes, Csac and A5IL97, were added to the list to enable more precise comparisons between genes expressed in *A. niger* and *E. coli*[14]. These genes, as well as the aforementioned control genes, were analyzed for N-linked and O-linked glycosylation using the NetNGlyc 1.0 Server and NetOGlyc 4.0 Server, respectively. Various predicted glycosylation sites were detected on some but not all of the proteins. Since it is unclear how gylcosylation would impact expression of these enzymes, it was decided to first express the native proteins before attempting to modify them to remove the sites. Most if not all of the proteins expressed so no further action was taken in this arena. One modification that was made was to codon optimize each gene using the Genscript platform (see their website for details). Each gene was analyzed by Genscript and the nucleotide sequence was modified to achieve maximal expression efficiency, primarily based on codon usage frequency in *A. niger*. These codon optimized genes were then synthesized and cloned into the *glaA*-based expression vector in *E. coli*.

Each of the control bacterial cellulases was successfully cloned into the expression construct and transformed into *A. niger*. The control genes Csac and A5IL97 were analyzed first to ensure the system was functioning properly. **Figure 5** illustrates that cellulase activity was detected for both enzymes using Azo-carboxymethylcellulose (Azo-CMC) and p-nitrophenyl-glucopyranoside (pNPG) colormetric substrates for Casc and A5IL97, respectively. These substrates were also used to detect the activity of the other bacterial cellulase genes in this study.

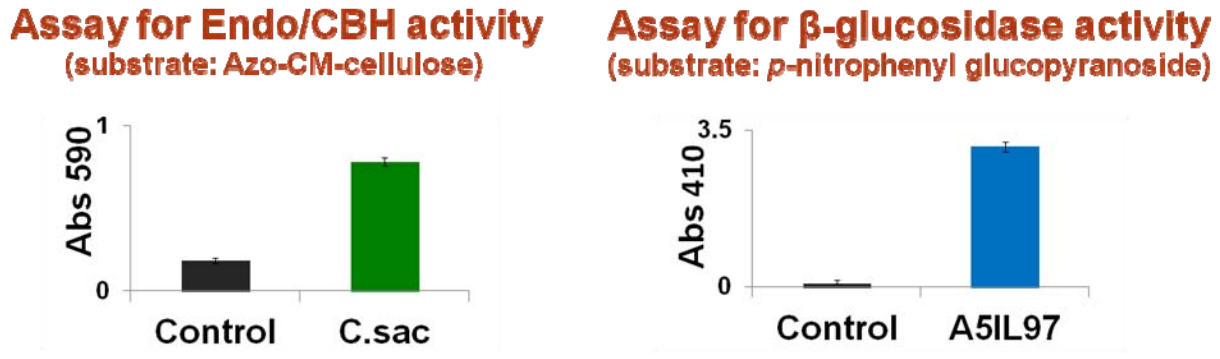

Figure 5. Example activity assays for endogluconases/cellobiohydrolases and β-glucosidases, with a GFP strain as a negative control.

Table 1. Genes for Heterologous Expression in *A. niger*.

Gene	Activity	Opt Temp (°C)	Source Organism	Expression
GFP	GFP F64L S65T +control	37	*Aequorea victoria*	Yes
Csac	cellobiohydrolase +control	70	*Caldicellulosiruptor saccharolyticus*	Yes
A5IL97	beta-glucosidase +control	70	*Thermotoga petrophila*	Yes
EG1	Endoglucanase +control	70	*Thermoascus aurantiacus*	Yes
BGL1	Beta-glucosidase +control	70	*Thermoascus aurantiacus*	Yes
CBH1	Cellobiohydrolase +control	65	*Trichoderma reesei* strain L27	Yes
J01	Beta-glucosidase	70	Bacteria	No
J02	Beta-glucosidase	85	Bacteria	Yes
J03	Beta-glucosidase	70	Bacteria	Yes
J06	Beta-glucosidase	65	Bacteria	Yes
J07	Beta-glucosidase	70	Bacteria	Yes
J08	Beta-glucosidase	85	Bacteria	Yes
J09	Beta-glucosidase	70	Bacteria	No
J11	Beta-glucosidase	70	Bacteria	No
J14	Beta-glucosidase	70	Bacteria	Yes
J15	Beta-glucosidase	70	Bacteria	No
J16	Beta-glucosidase	70	Bacteria	No
J17	Beta-glucosidase	70	Bacteria	Yes
J18	Beta-glucosidase	70	Bacteria	No
J19	Beta-glucosidase	70	Bacteria	No
J24	Endo/Exo-glucanase	70	Bacteria	Yes
J26	Endoglucanase	70	Bacteria	Yes
J28	Endoglucanase	70	Bacteria	No
J29	Endo/Exo-glucanase	70	Bacteria	Yes
J30	Endoglucanase	70	Bacteria	Yes
J31	Beta-glucosidase	70	Bacteria	N/A
J35	Beta-glucosidase	70	Bacteria	No
J36	Endoglucanase	70	Bacteria	Yes
Prumi	Endoglucanase	60	Prevotella ruminicola	Yes
Pho3	Endo/cellobiohydrolase	70	Pyrococcus horikoshii	No
Cel9A	Endoglucanase	70	Alicyclobacillus Acidocaldarius	Yes
Cel5A	Endoglucanase	70	Thermotoga Maritima	Yes
JMB19063	Beta-glucosidase	70	Bacteria	Yes

In addition, the proteins were analyzed by SDS-PAGE and a clear band was detected for A5IL97 but not for Csac (**Figure 6,** Csac not shown). Later experimentation conducted on Csac outside the scope of this project indicated that there are at least three isoforms of Csac produced in *A. niger*, two of which are high molecular weight and likely glycosylated, yet all three remain

active. The distribution of this protein into multiple fractions therefore made it difficult to resolve using SDS-PAGE. However, the A5IL97 protein expressed well relative to the native *A. niger* proteins, and appeared to be one of the most abundant proteins in the culture supernatant, second only to the native glycoamylase.

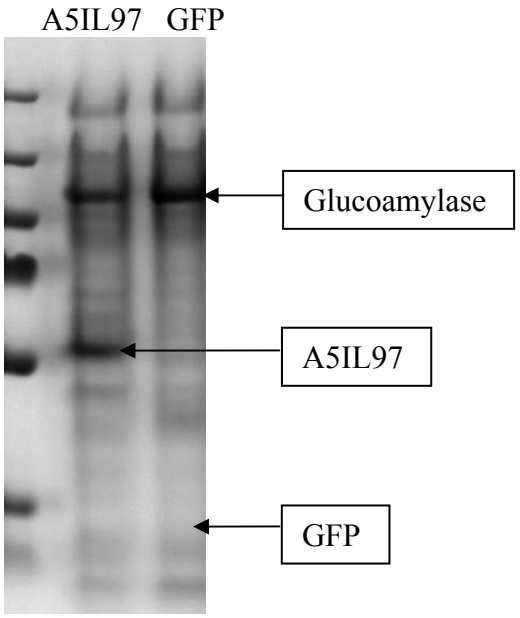

Figure 6. SDS PAGE gel of culture supernatant from A5IL97 and GFP *A. Niger* strains. A5IL97 was secreted at about 20mg/ml and no GFP secretion was detected.

A comparison of the activity of the *A. niger* expressed A5IL97 protein to the same protein expressed and purified in *E. coli* indicated that this protein is at a concentration of approximately 20 mg/L in the *A. niger* culture after 5 days, which is about ten times the level of expression per volume as in *E. coli*. The standard cure in **Figure 7** was used to estimate concentration.

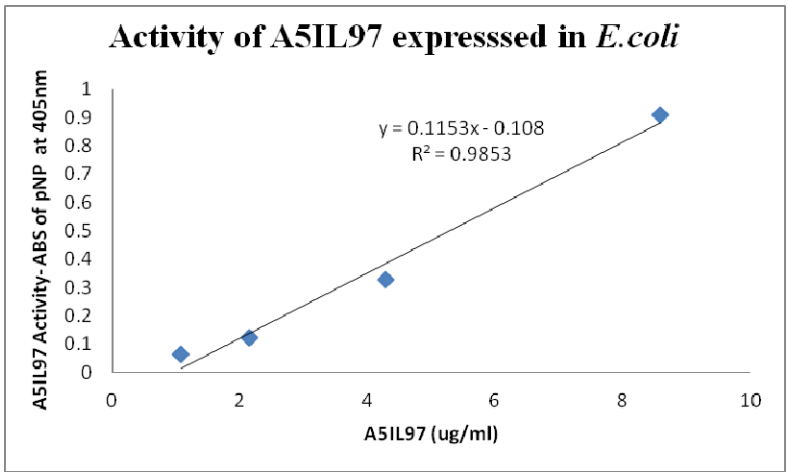

Figure 7. A5IL97 expressed in purified from *E. coli* was used to estimate the concentration of A5IL97 expressed from *A. niger* by comparing the activity of each enzyme on pNPG.

The final and most important assessment that needs to be made on the *A. niger* expressed bacterial enzymes is to determine whether there has been a shift in enzyme functionality compared to the *E. coli* version. **Figure 8** illustrates the temperature, pH, and IL-tolerance activity profiles of A5IL97 expressed from each organism. In essence, the two enzymes behave virtually identical, suggesting that both systems produce an equivalent enzyme. The pH and IL-tolerance profiles are a bit different, but most of this difference is likely due to residual pH 6.2 buffer from the *A. niger* culture that was difficult to remove completely. The same analysis was done with two other enzymes, Csac and J36, and those results appear to also indicate that proteins expressed in *E. coli* and *A. niger* behave similarly.

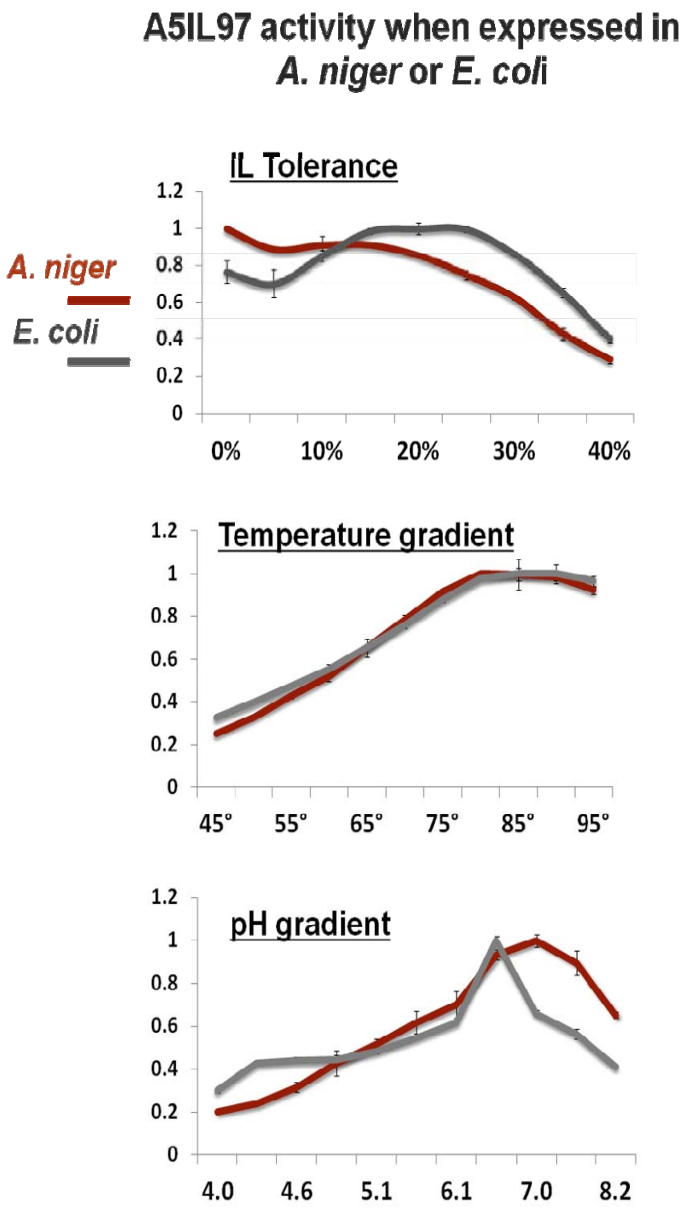

Figure 8. Activity profiles of A5IL97 expressed in E. *coli* and in *A. niger*.

3.2 Expression of Compost Community-derived Bacterial Cellulases

The analysis of A5IL97 and Csac enzyme activity support the idea that bacterial cellulases can be successfully and functionally expressed in *A. niger*. In addition to the control bacterial genes, there were 27 other candidate genes isolated from a compost microbial community at JBEI that are good candidates for the development of industrial cellulase cocktails, and are therefore prime targets for expression in a compatible industrial host such and *A. niger*. Each of the 27 codon optimized cellulase genes was successfully cloned into the glaA-based expression construct. Attempts were then made to transform the vector into *A. niger*, and all except for the J31-harboring vector were successful. Of the 26 constructs transformed into *A. niger*, 16 showed signs of expression. For each cellulase gene, 10 transformants were tested for activity, and often several of them showed no activity. This is a consequence of random integration of the construct into the genome, making it susceptible to position effects, both positive and negative. Typically, researchers in the *A. niger* community will screen around 40 transformants, a number that is often sufficient to obtain a transformant that lands in a locus amenable to gene expression. Due to the large number of genes to be screened, the number was reduced to 10 in this study. For the 10 cellulase genes that did not exhibit activity upon transformation, this number may be insufficient and more transformants need to be screened before it can be confidently determined to have not expressed. Due to the inability to purify these enzymes, their expression levels were not determined.

3.3 Generation and Analysis of an *A. niger* Protease-deficient Mutant

A. niger secretes multiple extracellular proteases, with PEPA being the most abundant[15]. It has been shown that reduction in PEPA, either through direct knock-out or knock-out of its regulator *prtT*, can reduce the level of extracellular protease activity[16]. This reduction could be beneficial to heterologous protein expression and secretion by 1) reducing the extent of proteolytic degradation of the heterologous protein, and 2) preventing the degradation of affinity tags on the heterologous protein, enabling it's purification. For these reasons, an attempt was made to knock-out *prtT*, a gene encoding a member of the fungal-specific Zn2Cys6-binuclear cluster protein family that is likely to function as a transcription factor that positively regulates proteases such as *pepA*[16].

Figure 9 outlines the strategy to knock-out *prtT* using a gene conversion strategy where *prtT* is replaced with the prythiamine resistance gene *ptrA*.

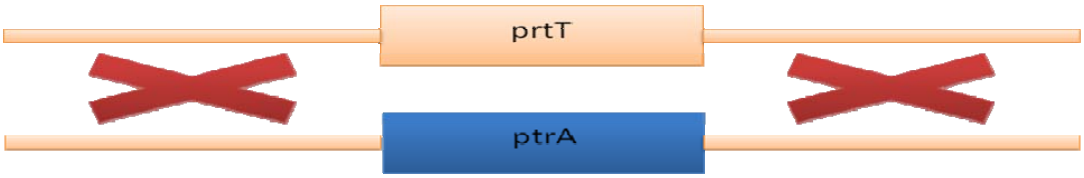

Figure 9. Homologous recombination knockout strategy for the protease regulator gene *prtT* in *A. niger* using a prythiamine resistance gene *ptrA*.

The knock-out occurs via a double homologous recombination event between each of two flanking sequences of a *ptrA* construct that are identical the 5' and 3' flanking sequences of the *prtT* gene and the endogenous locus in the *A. niger* genome[17]. Selection of the knock-out can them be made by selection on growth media containing prythiamine. This approach resulted in the generation of a *prtT* knock-out mutant completely lacking the *prtT* coding sequence, thus preventing the chance of reversion.

To establish the effect of this mutant on expression of heterologous genes, the Csac control gene expression construct was transformed into both wild-type and the *prtT* – strain, and the expression levels of multiple transformants was compared. Results indicate that the expression of Csac can be higher in the mutant than in wild-type, as the transformant with the highest expression levels, as indicated by enzyme activity, was obtained in the *prtT*- background (**Figure 10**). In addition, it was easier to obtain transformants with relatively high levels of expression in the *prtT*- background compared to wildtype.

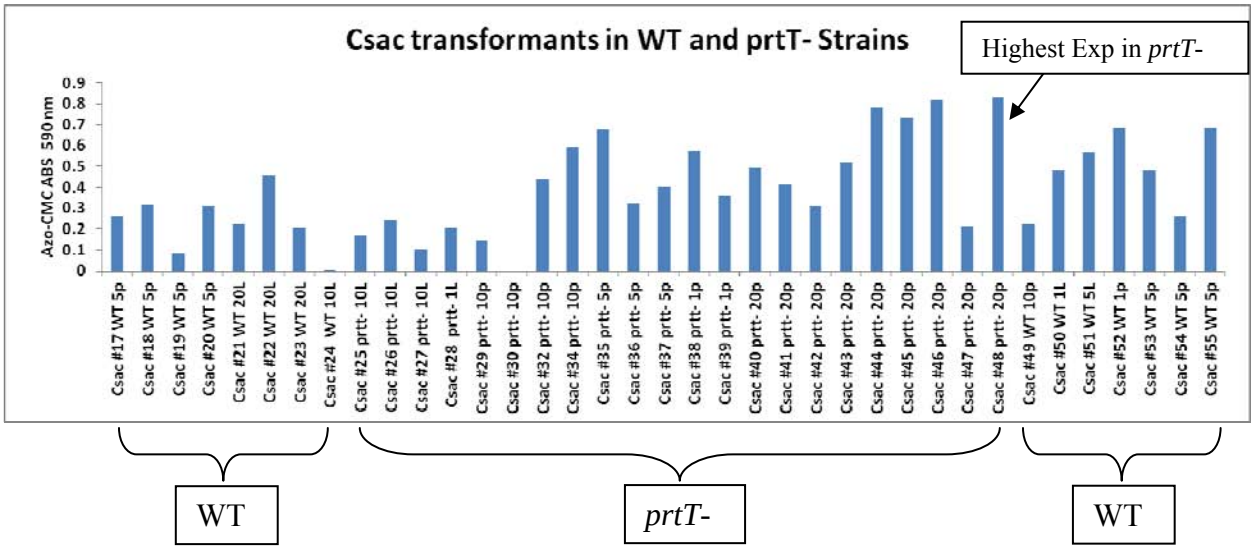

Figure 10. Transformation of Csac into wild-type and *prtT*- mutant *A. niger* strains.

4. CONCLUSIONS

The ability to produce high titers of industrially relevant enzymes is an important cornerstone of biotechnology, and is especially important in the lignocellulosic biofuels industry. This industry is currently reliant on the enzymatic breakdown of plant biomass to produce a pool of carbon that can be readily converted into biofuel by a biocatalyst such as an engineered microorganism. The primary route of choice is conversion of glucose derived from the plant cell wall polymer cellulose into a range of biofuels via fermentation in *E. coli* or yeast[18]. The liberation of glucose from plant biomass is one of the largest bottlenecks in the process and much work has gone into developing more efficient technologies to alleviate this bottleneck, including the development of thermophilic cellulase cocktails. This study's main goal was to establish whether the filamentous fungi *A. niger* was a viable production host for lignocellulose deconstructing enzymes, a goal that was accomplished and, for the most part, suggests that it is a promising candidate for the industrial production of these enzymes.

A heterologous expression construct based on the gene encoding the highly expressed and secreted glucoamylase enzyme was successfully designed and validated using several control genes. One interesting observation from the analysis of GFP was that not all the heterologous protein appears to be secreted. This indicates that future engineering of this organism to tailor it for heterologous expression should involve 1) identifying the key genetic components within the native complement of genes that aid secretion of proteins so that they can be incorporated into expression constructs and 2) identifying the systemic bottlenecks that prevent functionally produced enzymes from being secreted. Another observation was that the fungal cellulase genes used as control enzymes in this study did not express well in general, and in fact often expressed more poorly than the bacterial enzymes tested in this study, based on a comparison of enzymatic activity. Although the sample size was small, this suggests that there is no innate bias toward heterologous enzymes derived from more evolutionary close relatives and that more primary characteristics of any particular enzyme, such as primary amino acid sequence, dictate the success or failure of any given protein to express.

One critical question that this study addressed was whether enzymes form thermophilic bacteria would be suitable for expression in *A. niger*. There are few reports of heterologous thermophilic enzyme expression in *A. niger* so it was unclear whether this particular class of enzyme would be amenable to the host[19]. Overall, 65% of the bacterial enzymes tested (18/28) showed some level of expression, a success rate that was similar to that observed in screen of similar enzymes conducted at JBEI in *E. coli*. In addition, enzymes that express well in *E. coli*, such as Csac and A5IL97, also expressed well in *A. niger*, while ones that were difficult to express in *E. coli* were amongst the enzymes where activity was not detected in *A. niger*. There results suggest suggesting that successful expression in *E. coli* is a good benchmark or indicator of the likelihood for successful expression in *A. niger*. In addition, the enzymes expressed in *A. niger* appear to be quite similar to those produced in *E. coli*. Activity profiles of enzymes produced in each host were remarkably similar. Therefore concerns of host-specific inhibitory modifications appear to be unwarranted, at least for this set of enzymes.

One of the major drawbacks of using filamentous fungi for heterologous enzyme production is that they are prolific producers of extracellular proteases[20]. Fortunately, many of these proteases

are known in *A. niger*, and this system has the tools to knock them out. In this study it was demonstrated that mutants deficient in extracellular proteases are more amenable to the isolation of highly active transformants expressing heterologous enzymes. Further work needs to be done to assess the level of protease reduction and whether the loss of protease activity will facilitate the purification of heterologous proteins labeled with peptide affinity purification tags.

In summary, *A. niger* is s suitable host for the production of bacterial enzymes, even those derived from thermophiles, with optimal enzyme activity as high as 95°C. The host in this study was unmodified and yet was able to express titers of some bacterial enzymes at levels higher than observed in *E. coli*. Many modifications are described in the literature that can be used to increase heterologous enzyme production in *A. niger*, and therefore this organism has much potential to be developed into a production host that can outperform the standard lab workhorse *E. coli* my many orders of magnitude, and establish itself as a gold standard for industrial production of industrially relevant enzymes.

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