# The use of Filamentous Fungi *Myceliophthora heterothallica* for Heterologous Protein Production

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# Abstract


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The goal of this research was the development of an expression system using a thermophilic host organism for heterologous protein production. *Mycothermus thermophilus*, *Myceliophthora thermophila* and *Myceliophthora heterothallica* were chosen as candidate host organisms due to their high production of extracellular proteins under cultured conditions and their ability to grow in wide range of pH. Of particular interest is *Myceliophthora heterothallica*, an organism largely unexplored as a host organism, which holds the potential of using sexual mating in strains development. Transformation of the host organism was done by means of polyethylene glycol mediated transformation using cotransformation with a selection plasmid containing the selectable marker *amdS* and an expression plasmid containing heterologous xylanase genes. Six different promoters were tested to drive the expression of xylanase genes of interest in order to assess the production of heterologous proteins under different conditions. These promoters included PglaA from *Aspergillus niger*, PgpdA, PpmoA, PcbdA, PagdA from *Myceliophthora thermophila*, and PagdB from *Myceliophthora heterothallica.* The level of expression in the transformants was estimated using levels of xylanase activity observed using both a xylanase spot assay and BCA assay. Production of a heterologous xylanase from *Aspergillus niger* were obtained with constructs containing PagdB from *Myceliophthora heterothallica* CBS375.69. Transformants containing the construct PagdB - *ANxynA* showed an 80 fold increase in xylanase activity 24 hours after induction by sucrose, over transformants who had been transformed with the selection plasmid alone. Yields remained low and the heterologous protein could only be detected by mass spectrometry rather than by SDS-PAGE. However this research demonstrates that *Myceliophthora heterothallica* has potential as host organism for heterologous protein production and provides not only a thermophilic host organism, but one with sexual mating system as well.

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# Abbreviations

| | |
|---|---|
| 5'FOA | 5-Fluoroorotic Acid |
| AMA1 | Autonomously replicating sequence from *Aspergillus nidulans* |
| AMFE | Association of Manufacturers and Formulators of Enzyme |
| ATP | Adenosine triphosphate |
| BCA | Bicinchoninic acid |
| bp | Basepair |
| DNA | Deoxyribonucleic acid |
| GFP | Green fluorescent protein |
| GH | Glycoside hydrolase |
| GUS | β-glucuronidase |
| JGI | Joint Genome Institute |
| Kb | Kilobases |
| KDa | Kilodaltons |
| LPMO | Lytic polysaccharide monooxygenase |
| MFS | Major Facilitator Super Family |
| NSRM | Non-selective regeneration media |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| RBB | Remazol Brilliant Blue |
| SDS-PAGE | Sodium dodecylsulfate-polyacrylamide gel electrophoresis |
| TDM | Trametes Define Medium |
| WT | Wildtype |

# 1 Introduction

## 1.1 Choice of filamentous fungi for heterologous protein production

The use of fungal enzymes in the food and wine [1, 2], biofuel, pulp and paper and agricultural industries [3] are widespread. Since most fungi naturally produce only small quantities of enzymes of interest [4, 5] efficient fungal expression systems are being developed. The genomes of fungal organisms are being mined in view of further exploiting their proteins for commercial uses. High levels of target proteins are needed for the characterization of these potentially commercially interesting enzymes. Fungal production systems are not only being used to produce heterologous fungal proteins, but also proteins from human, animal, plant and bacterial sources. In April 2014, the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) reported the commercialization of over 248 different enzymes, 50% of which were produced by filamentous fungi. Over 53% of the fungal enzymes were produced by various *Aspergillus* species. Another 23% were produced by various *Trichoderma* species. The mycoCLAP (https://mycoclap.fungalgenomics.ca/mycoCLAP/), which provides information on characterized lignocellulose-active proteins of fungal origin, also offers an overview of the host organisms that are used for the production of heterologous enzymes. As of spring of 2015, 79% of the heterologously produced proteins reported in mycoCLAP utilized an *Aspergillus* species as a host organism. An additional 14% of heterologous enzymes were produced by a *Trichoderma* species. It is important to realize, however, that a single host organism may not be suitable for all proteins of interest. Heterologous protein production may fail in a certain host, due to poor gene expression, improper protein folding, faulty glycosylation, sensitivity to proteases, or different codon preferences between host organism and the native organism of the gene of interest. In these cases, a second host organism can certainly be tried in hopes of a more favorable outcome.

In order to develop a new expression system, methods that have previously been tried, what has been successful, and what is feasible with the resources available are all issues which must be addressed. The choice of organism, method of transformation, method of selection of successful transformants, and choice of an efficient promoter for expression of the protein of interest, must all be decided upon.

### 1.1.1 Use of *Aspergillus* as a host organism

The *Aspergillus* genus comprises over two hundred species but only a few have been used as production organisms. Transformation and gene cloning into *Aspergillus* began in the early 1980's [6, 7]. Since then much work has gone into improving yields of both fungal and non-fungal heterologous proteins. The development of expression systems for *Aspergillus*, and factors affecting their expression and protein production have been reviewed by Lubbertozzi *et al.* [8]. The most common species for genetic manipulations to date are *A. niger* and *A. oryzae.* Recently, Culleton *et al.* [9] showed that *A. vadensis* has an advantage over *A. niger* for recombinant protein production, since it does not acidify its culture medium as it grows [10]. This is desirable for some heterologous enzymes which may be pH sensitive and may be rendered inactive in acidic media. It has been reported that *A. vadensis* produces a lower level of extracellular proteases than other *Aspergillus* species [10] resulting in superior levels of heterologous proteins compared to that of *A. niger* [11, 12]. In other species of *Aspergillus*, the problem of proteases and their effect on protein yields, are being addressed through the use of protease-deficient strains [13], disruption of protease genes [14] and pH control of protease activity in the extracellular environment[15]. Despite the high secreting potential of *Aspergillus,* efforts are being made to increase production past the currently capabilities. For example, Yoon et al. [16] mutated the *AoVPS10* gene in *A. oryzae*, which codes for a sorting receptor of vacuolar proteins, in order to obtain an increased production of bovine chymosin and human lysozyme proteins. Studies investigating the role of chaperone and foldase genes involved in the secretory pathway of *A. niger* have also been investigated [17-19] in order to devise new methods of increasing heterologous protein production.

### 1.1.2 Use of *Trichoderma* as a host organism

*Trichoderma reesei* is known for its innate capacity for production and secretion of high amounts of hydrolytic enzymes, specifically cellulases. The hyper-secreting mutant *T. reesei* RUT-C30 (ATCC 56765) is reported to be the strain most often used in academic research for production of homologous and heterologous proteins [20]. Peterson and Nevalainen [20] provided an in-depth review of the development of this strain over the course of thirty years, and its performance as a host for heterologous protein production. They concluded that although

titers of up to 100 g/L of a native mixture of cellulases can be produced from *Trichoderma*, production of heterologous protein remains at the hundreds of mg/L level [20]. Jorgensen *et al.* [21] provided a new expression system in *Trichoderma* where the *ade2* gene, involved in the biosynthetic pathway of purines, is replaced by a expression cassette containing a gene of interest. The transformants which lack *ade2* gene produce pink pigments that allow for reliable detection of transformants. To increase homologous recombination efficiency, their expression system also includes a mutation in the *tku70* gene which is involved in non-homologous end joining [21]. Many groups working with *Trichoderma* are expanding the list of effective native promoters, both constitutive and inducible [22-25]. Commonly used promoters such as *cbh1* are being optimized [26, 27] in order to increase expression. Dual promoters for co-expression of heterologous genes is also being investigated [28]. Protease-deficient strains are being developed [29] and silencing of highly expressed genes to improve output of heterologous proteins has been explored [30].

### 1.1.3 Use of *Penicillium* as a host organism

*Penicillium* is used commercially as a production organism for production of native proteins [31]. Its potential for production of heterologous proteins has been investigated since the 1990s [32]. *Penicillium chrysogenum* was first evaluated for heterologous protein production by Queener *et al.* [32] who used a isopenicillin N-synthetase gene promoter to express the *cefE* gene from *Streptomyces clavuligerus*. *P. chrysogenum* has also been used as a host organism by Graessle *et al.* [33] who fused a fungal xylanase gene to human tear lipocalin cDNA and placed it under the control of a native repressible acid phosphatase promoter. To further advance the use of *Penicillium funiculosum* as an industrial production organism, Belshaw *et al.* [34] evaluated a histone H4 promoter for the heterologous expression of a reporter bacterial β-glucuronidase (GUS) and a homologous xylanase genes. *Penicillium canescens* was used as a host organism by Abianova *et al.* [35] to express a heterologous laccase using an inducible β-galactosidase promoter. Recently, Teixeira *et al.* [36] used *P. canescens* as a host with the *gpdA* promoter from *Aspergillus nidulans,* in the hopes of finding another suitable promoter for heterologous protein production in this organism. Yields of over a thousand fold increase over natural levels [36] open the door to further studies for expression of heterologous genes in this species. Also in *P. canescens,* Vinetsksky *et al.* [37] introduced the *xlnR* gene from *A. niger* to investigate its

possible regulation of both the *axhA* promoter and *bgaS* promoter.

### 1.1.4 Use of *Myceliophthora* as a host organism

The thermophilic organism *Myceliophthora thermophila*, also known by the name of *Sporotrichum thermophile* or *Chrysosporium lucknowensense* C1, was developed a production organism by Dyadic (http://www.dyadic.com/), a producer and supplier of commercial enzymes. The C1 technology is now owned by DuPont Industrial Biosciences (http://biosciences.dupont.com/) following the sale of Dyadic's Industrial Technology to DuPont. The biotechnological potential of this organism has not gone unnoticed in academia. Many papers have been published in the last five years characterizing specific enzymes of *M. thermophila* [38-41]. The effect of enzyme mixtures on a variety of substrates have also been investigated [13, 42, 43]. Reviews covering genomic, transcriptomic and proteomic analysis of *M. thermophila*'s lignocellulolytic enzymes are available [40, 44, 45]. Comparisons between closely related strains and synergy between enzymes of *M. thermophila* and enzyme mixtures from other organisms have also been published [13, 46]. Improvements in the classification of *Myceliophthora* species have been made in order to provide clarification of the genetic diversity existing between members of this genus [46-48]. Many of these papers mention the biotechnological potential of non-proprietary strains of *M. thermophila* but the organism is not used as such in those works. Only recently has a paper been published, beyond those related to the establishment of C1 as patented platform organism for Dyadic [49, 50], showing gene disruption techniques for *M. thermophila* [51]. Much of this interest lies in the fact that *M. thermophila* is a thermophilic organism. The enzymes which thermophilic organisms produce are generally more thermostable than enzymes those produced by mesophilic organisms and hence valuable in industry [52].

### 1.1.5 Use of other filamentous fungi as production organism

The AMFE (http://www.amfep.org/ ) lists *Mucor javanicus* as a production organism for commercial production of a homologous lipase triacylglycerol. From the literature, it is *Mucor circinelloides,* a closely related species, which is used as a host for homologous and heterologous protein production [53-62].

A few members of the *Fusarium* genus have also been used for protein production.

Commercially, *Fusarium venenatum* is used to produce both homologous proteases involved in milk clotting, and a heterologous cellobiose dehydrogenase native to *Microdochium sp*. (AMFE, 2014). Laboratory strains used for heterologous protein production include *Fusarium oxysporum* for the expression of transalodase genes from *Saccharomyces cerevisiae* and *Pichia stipitis* [63, 64]. *Fusarium verticillioides* [65] and *Fusarium venenatum* are the host organisms used for glucoamylase production [66] as well as expression of a serine carboxypeptidase originating from *A. oryzae* [67]. *Fusarium graminearum A3/5* has been used for production of trypsin from *F. oxysporum* [68].

Ongoing research in laboratory settings is also being conducted using other filamentous fungi to find alternate suitable hosts for heterologous protein production. Some promising examples are listed in Table 1.

**Table 1. Host organisms under development**

| Host Organism | Donor Organism | Product | Ref |
|---|---|---|---|
| *Talaromyces cellulolyticus* | Pyrococcus sp. | Cellulase | [69] |
| *Ganoderma lucidum* | --- | Vector expression system developed | [70] |
| *Ashbya gossypii* | *Aspergillus niger* | β-galactosidase | [71] |
| *Cryptococcus neoformans* | *Magnaporthe oryzae chrysovirus 1 strain A* | MoCV1-A ORF4 | [72] |
| *Phanerochaete sordida YK-624* | *Pleurocybella porrigens* | Lectin | [73] |
| *Coprinopsis cinerea* | *Ganoderma sinense* | Immunomodulatory protein FIP-gsi | [74] |
| *Pseudozyma flocculosa* | *Ustaliga maydis; Pseudozymatsu kubaensis* | GFP reporter | [75] |
| *Lentinula edodes* | *Escherichia coli* | Intron1 GPD-GUS | [76] |
| *Hypholoma sublateritium* | *Agaricus bisporus* | GFP reporter | [77] |
| *Phanerochaete chrysosporium* | *Dichomitus squalens* | Manganese peroxidase | [78] |
| *Coprinus cinereus* | *Pleurotus ostreatus* | Manganese peroxidase | [79] |
| *Acremonium chrysogenum* | Bacterial | Cephalosporin C Acylase | [80] |
| *Acremonium chrysogenum* | Human | Thromobomodulin | [80] |
| *Podospora anserina* | Human | Ribosomal protein (*rig* gene) | [81] |

## 1.2 Methods for fungal transformation

Many methods are available for fungal transformation, but some fungal species may be more suited to a particular method. Other factors which may come into play when choosing a transformation method are the cost associated with the methodology used and the amount of transforming DNA needed. Finchman [82] gives an overview of the history of DNA mediated transformation and the use of protoplasts for fungal transformations. Case *et al.* [83] discuss the use of exogenous DNA and glucanase, the cell wall digesting enzyme, for the transformation of fungal protoplasts. According to Ruiz-Diez [84], transformation systems have now been developed in all phyla of fungi, although the approaches used remain diverse given that no one method is suitable for all fungal families [84].

Currently many enzyme or enzyme mixtures are commercially available for digesting the cell wall from filamentous fungi and exposing the protoplast. For many years, labs relied on Novozyme 234, a cell-wall-digesting enzyme mixture from *Trichoderma viride*, to carry out this task. Novozyme has rebranded their original product in favor of an enzyme mixture, VinoTaste® Pro (http://www.novozymes.com), containing both polygalacturonase and β-glucanase (exo-1,3). Also available from Clontech, Yatalase is an enzyme mixture prepared from cell culture supernatants of *Corynebacterium sp. OZ-21* (http://www.clontech.com). Their commercial preparation consists mainly of chitinase, chitobiase and β-1, 3-glucanase and is meant specifically for protoplast preparation from filamentous fungi. Zymo Research also offers an enzyme mixture, Zymolyase, which is capable of digesting both yeast and fungal cell walls. Their product is prepared from *Arthrobacter luteus* and consists of enzyme activities: β-1, 3-glucan laminaripentao-hydrolase and β-1, 3-glucanase (https://www.zymoresearch.com).

Low regeneration rates of fungal protoplasts are common. For example, regeneration rates of 10% or lower are reported in *N. crassa* [85]. Regeneration rates for all fungal protoplasts are affected by variables such as digestion time and temperature at the protoplast generation stage, as well as type and molarity of the osmotic stabilizers used to maintain the protoplast population [86].

### 1.2.1 Protoplast transformation by PEG and by lithium acetate

Exposure of the protoplast to polyethylene-glycol (PEG) changes the permeability of the

cell membrane and renders the cell amiable to the incorporation of exogenous DNA by endocytosis. The molecular mechanism by which this occurs in yeasts is explored by Kawai *et al.* [87]. They proposed that PEG is indispensable for the attachment of DNA to the cellular membrane thus increasing the uptake of the DNA by endocytosis and increasing transformation efficiency. PEG-mediated transformation remains a widely used method for fungal transformation due to the ease of the procedure and adaptability for processing several samples simultaneously [84, 88]. The use of lithium acetate in yeast and *E. coli* transformation was first described by Ito *et al.* [89] who found that the transformation efficiency was increased when combined with PEG. The use of lithium acetate for fungal transformation has been adapted for use in filamentous fungi including *N. crassa* [90], *Coprinus cireus* [91] and *Ustilago violacea* [92].

### 1.2.2 Electroporation

Electroporation involves the use of short pulses of high amplitude electric fields in order to cause short-term, reversible permeability of the cell membrane [84, 93]. This temporary alteration in permeability is what allows the uptake of exogenous DNA [84, 93]. The method can be applied to conidia as well as protoplasts [94, 95], although a cell wall weakening agent, such as a glucuronidase from *Helix pomatia* (http://www.sigmaaldrich.com), is required when using conidial preparations [96]. Trials in filamentous fungus began with *N. crassa* [94] and have become, according to Ruiz-Diez [84], one of the leading methods for fungal transformation. Many papers had been published in which the ideal field strength, capacitance, and pulse length were investigated [94, 96-98]. It appears that when the ideal settings are exceeded, a decrease in transformation efficiency occurs [98]. Ozeki *et al.* [93] performed a comparison of various transformation methods in *A. niger*, which included PEG mediated transformation, and both conidial and protoplast electroporation. They also assessed a variety of variables, including pretreatment of protoplast, and the use of integrative vectors versus non integrative plasmids. The highest transformation efficiencies were obtained with non integrative plasmid DNA using electroporation of pretreated conidia [93].

### 1.2.3 Agrobacterium mediated transformation (AMT)

*Agrobacterium tumefaciens*, a gram-negative plant pathogenic bacterium, transfers part of its DNA to the host. Until now, it had most often been used when more common methods of transformation had failed for a particular species of fungus, but seems to be picking up in popularity, judging by the number of published studies in which this method is used. Degroot *et al.* [99] were the first to demonstrate that this method, widely used for transformation in plants, could be applied to filamentous fungus. Michielse *et al.* [100] elaborated on the transfer mechanism involved in this system. A summary of fungal species in which this method had been applied is provided by this group, but it was made clear that the protocol must be optimized to obtain optimal transformation frequencies in each fungal species [100]. The types of optimization which appear to have the greatest effects on transformation frequencies include the length of co-cultivation between the fungal organism and the *A. tumefaciens*, as well as the ratio of fungal conidia to bacterium [101].

Early adopters of this method were Gouka *et al.* [102], who used *A. tumefaciens* for the transformation of *Aspergillus awamori* to express a heterologous *Fusarium solani pisi* cutinase gene. In 2004, Godio *et al.* [77] transformed the basidiomycete *Hypholoma sublateritium* using *A. tumefaciens.* Using agrobacterium mediated transformation (AMT), they induced expression with constructs driven by promoters solely from other basidiomycete species [77]. More recently, Ma *et al.* [103] introduced a β-glucosidase I gene from *Penicillium decumbens* into a *T. reesei* strain by AMT. Shortly thereafter, Lv *et al.* [104] developed a transformation system in *T. reesei* in using this method of transformation. Insertion of heterologous genes by AMT were shown to be stably inherited in *Flammulina velutipes* by Cho *et al.* [105]. Recently AMT has been used in a *ku70* deletion mutant of *M. thermophila* for targeted gene deletion [51].

### 1.2.4 Biolistic

The biolistic transformation method for the delivery of DNA into host cells involves the use of DNA coated with gold or tungsten. The targeting DNA is projected into host cells at high velocities using a helium gun particle delivery system. This method of transformation was first employed in *N. crassa* by Armaleo *et al.* in 1990 [106]. Transformation in other fungal organisms such as *Trichoderma sp.* [107-109], *Cryptococcus neoformans* [110] and *A. nidulans*

[111] followed. A study in *A. nidulans* by Barcellos *et al.* [112] revealed transformants produced by this method to be unstable. The authors observed that the level of mitotic instability was affected by the site of integration of the exogenous DNA into different sectors of host's chromosomal DNA [112]. In addition, a comparison of different transformation methods in *Aspergillus giganteus* by Meyer *et al.* [113] showed the biolistic method to be inefficient for this strain. Furthermore, the need for specialized equipment renders this method out of reach for some labs.

### 1.2.5 Use of shock waves for fungal transformation

The latest method for transformation of filamentous fungi is the use of underwater shock waves generated by a piezoelectric generator [114]. The generator produces waves by excitation of piezoelectric crystals arranged on a concave aluminum structure. When these waves are produced, they cause a transitory increase in cell membrane permeability and heterologous DNA can then be introduced into the cell [115, 116]. Magaña-Ortíz [114] was the first to attempt this type of transformation into filamentous fungus such as *A. niger, T. reesei, Phanerochaete chrysosporium* and *F. oxysporum.* Contrary to transformations using AMT, all four of these species showed high transformation efficiencies when underwater shock waves were used. Although the amount of DNA tested for this approach (50-200 µg/ml) showed very little effect on the number of transformants, the total amount of DNA needed for this method of transformation is substantially higher than that used for other methods [114]. A follow up to this first study showed that tandem shock waves, rather than single pulse shock waves increased the number of transformants depending on the delay between tandem pulses [117]. More work is needed to determine if the amount of heterologous material can be reduced or if DNA degeneration associated with this method will continue to necessitate high levels of transforming DNA. Additional advantages of this method include the ability to transform intact conidia without cell wall digestion, minimal species to species optimization, and high transformation frequencies. The disadvantages included high concentrations of transforming DNA, as well as the high initial cost of the shock wave generator.

## 1.3 Vectors for transforming DNA

### 1.3.1 Use of autonomously replicating vectors

The isolation of the AMA1 from *A. nidulans*, a chromosomal replicator sequence, has made it possible to construct autonomously replicating plasmids for fungal transformation resulting in high transformation frequencies [118-121]. Although transformants carrying the plasmids containing the 6.1 Kb AMA1 sequence were initially thought to be mitotically unstable [120], this was not found to be the case by Fierro *et al.* [122] in *P. chrysogenum* and *A. nidulans*. Studies in *P. chrysogenum* using truncated AMA1 sequences reveal that the deletion of a 0.6 Kb region present between two inverted repeats will decrease mitotic stability and copy number of the plasmid in the host organism [122]. Truncations of the inverted repeat present in the AMA1 sequence were found to negatively affect transformation efficiency and autonomous replication of the plasmid [122]. In a paper by Storms *et al.* [123], plasmids containing the AMA1 sequence without the central spacer resulted in transformation efficiencies 8 to 15 fold higher in *A. niger* than when an integrative plasmid without the AMA1 sequence was inserted into the same host.

The AMA1 sequence has been used in the transformation of a variety of fungal host organisms with mixed success. Bruckner *et al.* [124] introduced the AMA1 sequence into their vector for the transformation of *Gibberella fujikuroi* with only a 2-fold increase in efficiency and inefficient replication of the AMA1 carrying plasmid within the host organism. In *Zalerion arboricola,* Kelly *et al.* [125] saw a 3-fold increase in the transformation efficiency but no autonomous replication of the AMA1 carrying plasmid in the host. When the AMA1 sequence was used for transformation in *P. canescens* results were much more favourable, with a 2000-fold improvement in transformation efficiency and full ability of the plasmid to replicate autonomously in the host organism being reported [126]. The presence of AMA1 in *Penicillium nalgiovense* transforming plasmids was also beneficial as a 60-fold improvement transformation efficiency was observed compared to when an integrative plasmid containing the sequence was used [127]. Fierro *et al.* [122, 127] observed that in *P. nalgiovense*, a monomeric form of the AMA1 carrying plasmid was more common than in *P. chrysogenum* where multimeric forms prevailed. Furthermore, the mitotic stability of the monomeric AMA1 autonomously replicating plasmid in *P. nalgiovense* was reported to be superior to that observed in the closely related organism *P. chrysogenum* [127].

### 1.3.2 Integrative vectors: Use of integrating plasmids versus homologous recombination

In order to achieve homologous recombination in fungi, exogenous DNA must be flanked with regions homologous to the gene targeted for deletion or replacement. These integrative vectors, either linear or contained within a circular plasmid, can be assembled *in vitro* by overlap PCR or by ligation. A region of homology of up to 2 Kb on either side of the target gene is typically used [128] in order to obtain homologous recombination frequencies ranging from 0 %-30% [129, 130]. The length of the homologous sequence is known to affect homologous recombination rates, as does the locus at which recombination is attempted [129]. In filamentous fungi, homologous recombination can be improved by using non-homologous end joining mutant strains [129, 131]. As a result, end joining mutants in over 14 different species have created [132]. Despite the low transformation rates, gene replacement remains an interesting option for heterologous protein production, especially if the gene product targeted for replacement is known to be produced in high quantities. Its removal decreases the metabolic demands on the host organism, facilitating production and secretion of the heterologous protein [133].

An advantage of using integrating plasmids which are randomly inserted into the host genome is the possibility of multi-copy integration. Multi-copy integration has been demonstrated to increase expression of genes of interest until a certain number of copies are inserted [36, 134, 135]. However, if too many copies are introduced, a decrease in the general health of host organism occurs and the correlation between copy number and productivity ceases [136]. Multiple copies of the expression cassette in transformants do not always guarantee greater product yields. In a paper by Harkki *et al.* [137], the number of integrated expression plasmid in *Trichoderma* ranged from 1-10, but showed no correlation with the amount of heterologous mammalian protein secreted. Verdoes *et al.* [138] demonstrated that although there was an increase in GLA production by transformants carrying multiple copies of the *glaA* gene, final protein production was controlled at the transcriptional level in *A. niger*. The site of non-homologous integration seems to have a greater impact on yield than does copy number in fungi [136, 137, 139, 140]. In the study by Harkki *et al.* [137], transformants where cellulase production had been disrupted by the integrative plasmid showed increased yields of the target protein, demonstrating that the site of integration was crucial in affecting productivity. Since the method of transformation may affect copy number, it follows that the method employed for transformation can influence the final yields of a target protein[129]. PEG-mediated protoplast

transformation generates many more multi-copy integrations than an agrobacterium-mediated integration [129]. Early trials by shockwave integration revealed that most transformants result in single copy integration, with only a few transformants carrying two copies of the integrative DNA [114].

### 1.3.3 Cotransformation of plasmids

Cotransformation of two different plasmids into a single cell has been shown to work at high frequencies in *Schizosaccharomyces pombe* [141]. It has also demonstrated to be successful in *Trichoderma* [137], *Aspergillus* [137, 142-146] and *Penicillium* [147-149]. Reported rates of cotransformation varied widely with rates being reported as low as 10% in some trials [142] and as high as 80% in others [137]. It has been shown that each transformant integrates with varying copy numbers but also integrates randomly at different locations within the host genome [137]. Wernars *et al.* [146] demonstrated that the molar ratio of the two vectors as well as each of their concentrations affected the cotransformation frequency in the host organism. It has also been suggested by Miao *et al.* [150] that only a sub-population of host cells are rendered competent for transformation and that this subpopulation will be amiable to the uptake of both the selection and expression vectors. The fact that not all cells are competent for transformation has been confirmed by Pandit and Russo [151] who used a heterokaryotic strain of *N. crassa* to confirm uptake of exogenous DNA in only one of the two nuclei but not both.

## 1.4 Choice of selection marker

### 1.4.1 Dominant selectable markers

The easy identification of transformed versus non-transformed cells is reliant on a variety of selectable markers. Dominant selectable markers are those which confer resistance to a substance via transformational DNA. Some of these markers have been shown to be applicable across a large array of fungal species. An advantage to the use of dominant selectable markers is that very little genomic knowledge of the host organism is required. A list of antibiotic resistance markers, which falls into the category of dominant selectable markers, is provided in Table 2.

**Table 2. Dominant selectable markers**

| Dominant selectable marker | Gene name | Associated gene function | Source Organism | Ref |
|---|---|---|---|---|
| Benomyl | *bml* | β-tubulin structural gene | *Neurospora crassa* | [152] |
| Carboxin resistance | *cbxR* | succinate dehydrogenase iron-sulphursubunit | *Ustilago maydis* | [153] |
| Glufosinate resistance | *bar* | phosphinothricin acetyltransferase | *Streptomyces sp.* | [154] |
| Hygromycin | *hph* | hygromycin phosphotransferase | *Escherichia coli* | [155] |
| Neomycin / Geneticin | *neo* | aminoglycoside 3'-phosphotransferase | *Escherichia coli K12* | [156, 157] |
| Nourseothricin | *nat1* | nourseothricin acetyltransferase | *Streptomyces noursei* | [158] |
| Oligomycin | *oliC31* | ATP synthase (subunit 9) | *Apergillus nidulans* | [159] |
| Phleomycin / Bleomycin | *Sh ble* | glycopeptide binding protein | *Streptoalloteichus hindustanus* | [160, 161] |
| Pyrithiamine resistance | *ptrA* | mutated thiamine metabolism gene | *Apergillus oryzae* | [162] |
| Sulfonylurea resistance | *sur* | acetolactate synthase gene | *Magnaporthe grisea* | [163] |

### 1.4.2 Auxotrophic markers

Auxotrophic mutants requiring nutritional additives to thrive make ideal organisms for selection if they are amiable to complementation by a vector able to confer prototrophic properties to transformants. If nutritional auxotrophs for a species of interest have not yet been identified and isolated, then the process of creating and purifying such mutants may take a considerable amount of time. Creation of auxotrophic mutants by chemical or UV mutagenesis has the disadvantage of inducing random mutations throughout the genome, rather than solely in specific target genes, which may decrease the overall health of the organism [164]. Creating a gene knockout or disruption mutant for a specific gene requires knowledge of the pathway in which it is found in order to assure that no alternative pathways can compensate for the loss or disruption of the target gene. In addition, knowledge of the DNA sequence flanking the target gene is required. Nonetheless, many auxotrophy fungal strains, many of which are amino acid auxotrophs, are amiable to complementation which will revert the organism back to prototrophy. Some of the most common amino acid based selection markers are listed in Table 3.

Rather than a review of an exhaustive list of selection markers available, this section focuses on bidirectional selection markers since they can be used either as positive or negative selection method. Of particular interest is *amdS* selection, simply because many wild type fungi are unable to utilize acetamide as a source of nitrogen because they do not inherently possess a gene homologous to the *amdS* gene from *A. nidulans*. In other fungal strains, the *amdS* gene is present but expression is low, resulting in poor growth on acetamide [165]. Hence the wild type for these fungus can be utilized without the creation of a mutant is not necessary for *amdS* based selection [166]. A list of bidirectional markers is provided in Table 4.

**Table 3. Amino acid based selection markers**

| Selection method | Gene name | Associated gene function | Source Organism | Ref |
|---|---|---|---|---|
| Adenine prototrophy | *adeA* | Phosphoribosylaminoimidazolesuccino carboxamide synthase | *Aspergillus oryzae* | [167] |
| Adenine prototrophy | *adeB* | phosphoribosylaminoimidazole carboxylase | *Aspergillus oryzae* | [167] |
| Arginine prototrophy | *argB* | ornithine carbamoyltransferase | *Aspergillus nidulans* | [168] |
| Arginine prototrophy | *agaA* | arginase | *Aspergillus niger* | [169] |
| Leucine prototrophy | *leuA* | alpha-isopropylmalate isomerase | *Mucor circinelloides* | [170] |
| Leucine prototrophy | *leu1* | alpha-isopropylmalate isomerase | *Rhizopus niveus* | [171] |
| Methionine prototrophy | *met2-1* | homoserine O-acetyltransferase | *Muccor circinelloides* | [172] |
| Tryptophan prototrophy | *trp1* | tryptophan biosynthetic pathway gene | *Schizophyllum commune* | [173] |
| Tryptophan prototrophy | *trpC* | tryptophan biosynthetic pathway gene | *Penicillium chrysogenum* | [174] |

**Table 4. Bidirectional selection markers**

| Positive / negative selection method | Gene name | Associated gene function | Source Organism | Ref |
|---|---|---|---|---|
| Acetamide utilization / Fluoroacetamide sensitivity | *amdS* | acetamidase | *Apergillus nidulans* | [175] |
| Acetate utilization / Fluoroacetate sensitivity | *acuA* | acetyltransferase | *Ustilago maydis* | [176] |
| Inorganic sulfur utilization / Selenate resistance | *sC* | ATP sulfurylase | *Apergillus nidulans* | [177] |
| Nitrate utilisation/ Chlorate sensitivity | *niaD* | Nitrate reductase | *Apergillus nidulans* | [178] |
| Pyrimidine prototrophy / 5-fluorocytosine sensitivity | *Cdase* | cytosine deaminase | *Saccharomyces cerevisiae* | [179] |
| Uracil / uridine prototrophy / 5-fluoroorotic acid sensitivity | *ura3* | orotidine 5'-phosphate decarboxylase | *Saccharomyces cerevisiae* | [180] |
| Uracil / uridine prototrophy / 5-fluoroorotic acid sensitivity | *pyrG* | orotidine 5'-phosphate decarboxylase | *Aspergillus nidulans* | [181, 182] |
| Uridine prototrophy / 5-fluoroorotic acid sensitivity | *pyrE* | orotate phosphoribosyl transferase | *Thermus thermophilus* | [183] |
| Uridine prototrophy / 5-fluoroorotic acid sensitivity | *pyrF* | orotidine-5'-monophosphate decarboxylase | *Thermus thermophilus* | [183] |
| Uridine prototrophy / 5-fluoroorotic acid sensitivity | *pyr2* | orotate phosphoribosyl transferase | *Trichoderma reesei* | [21] |
| Uridine prototrophy / 5-fluoroorotic acid sensitivity | *pyr4* | orotidine-5'-monophosphate decarboxylase | *Neurospora crassa* | [184] |

Transforming vectors can be designed such that they carry both a marker for positive selection and a marker for negative selection. This system of dual selection markers was employed by Michielse *et al.* [100] in order to confirm whether their integrative vector was being inserted at the intended homologous site, or whether it was undergoing random integration. In this study, the *pyrG* gene in *Aspergillus awamori* was targeted for gene replacement by *amdS* through a homologous recombination strategy, in which the *amdS* gene provided a positive selection method. Flanking the gene replacement cassette, which consisted of the *amdS* gene and homologous DNA to target region, the hygromycin resistance gene, *hph*, was added as a negative selection marker. Transformants able to thrive on acetamide as the sole source of nitrogen but sensitive to hygromycin indicated homologous integration of the gene replacement cassette. Transformants with both the ability to grow on acetamide and having resistance to hygromycin demonstrated that random integration of the entire transforming vector had occurred. A similar dual selection marker system had been utilized by Takahashi *et al.* in *Aspergillus sojae* [185]. In this study, gene disruption by homologous recombination was confirmed using *pyrG* as a positive selection marker since the host strain was initially *pyrG*$^{-}$. A mutant *oliC31* gene encoding for a mutant form of subunit 9 of the $F_1F_O$-ATPase was used as a negative selection marker to weed out transformants which contained ectopic/ random integration of the transforming DNA since the presence of *oliC31* also confers resistance to oligomycin. More recently, Jorgensen *et al.* [21] used a different approach to dual selection in *T. reesei* to assure homologous integration of their expression cassette. Instead of positive and negative selection markers, they used a combination of positive selection and coloration of the transformed colony in order to confirm integration at the target locus. Transformants were selected on uridine deficient media since transformants revert to prototrophy due to the presence of a *pyr2* gene in the transforming vector. However it was the reddish colour of the transformants which insured proper homologous integration rather than ectopic integration. Unique in its phenotype, mutants of the *ade2* gene, chosen as the target locus for incorporation of the vector, produce colonies with a reddish appearance [21].

To overcome the limited number of functional selective markers for any given species, systems whereby the selective marker can be excised and recycled for the next round of transformation have been developed. Marker recycling systems have proven invaluable when multiple rounds of gene deletion/ gene disruption are required. Marker excision can be achieved

in a variety of ways, including the creation of self-excising selection cassettes, utilization of a *cre/loxP*, FLP/FRT or *βrec/six* recombination system. The *cre*/*loxP* sequences were originally derived from bacteriophage P1, the FLP/FRT sequences from a yeast plasmid whereas the *βrec/six* recombination system is a bacterial recombination system.

Design of a self-excising selectable marker cassette necessitates that the selectable marker be flanked on each side by tandem repeat sequences. Homologous recombination events between these two repeats will enable the excision of the selectable marker along with one of the repeats. Cells no longer containing the selectable gene marker can be counter-selected, thus the host organism is ready for the next round of transformation using the same selectable marker cassette. Marker recycling was first utilized by Alini *et al.* [186] in yeast whereby a *ura3* gene was excised in order enable the disruption of a second gene using the same selection marker. This method of marker recycling has been utilized in *T. reesei* by Hartl and Seiboth [187], who were able to excise a *pyr4* selection marker flanked by two direct repeats of a bleomycin gene. Similar excision strategies have been utilized in *Aspergillus* [188, 189].

Krappmann *et al.* [190] utilized a *cre* recombinase mediated approach for excision of a selectable marker cassette in *Aspergillus fumigatus*. This approach involved designing a selection cassette with *loxP* acceptor sites flanking both sides of the fragment slated for excision. Excision occurs only in the presence of *cre* recombinase whose expression was placed under the control of an inducible promoter on a separate plasmid. Counter selection was applied to select for successful excision events and the same selection marker could then be utilized for successive transformations. *cre/ loxP* marker excision has also been demonstrated to be a suitable bioengineering tool in other filamentous fungal organisms such as *N. crassa* [191].

Kopke *et al.* [192] utilized a FLP/*FRT* recombination system for marker recycling in *P. chrysogenum* and in *Sordaria macrospora*. Initially their approach closely resembled the *cre/loxP* recombinase system described above in that two separate plasmids were utilized for incision /excision events in *P. chrysogenum*. The first plasmid carried a nourseothricin resistance gene *nat1*, flanked on either side by FRT sequences. The gene coding for FLP recombinase, was then introduced via a second plasmid to induce excision of the *nat1* selection gene. The system was later amended to a one step process by creation a self-excising marker cassette in which the FRT sites flanked both the resistance gene marker and the *Pcflp* gene. An inducible promoter allowed for the control of the expression of the *Pcflp* recombinase gene and hence provided

control over the excision of the entire cassette. Counter selection provided a host organism in which no heterologous genes remained. The same marker excision system was then used in *S. macrospora* to demonstrate that the FLP/*FRT* recombinase system can be applied to other filamentous fungi.

Also available as a self-excising marker system is the *βrec/six* system first used by Hartmann *et al.* in *A. fumigatus* [193]. The self-excising cassette designed for this study included a *β-rec* recombinase-encoding gene placed between a xylanase inducible promoter and a *trpC* transcription terminator sequence, along with a dominant selectable marker *ptrA*. This cassette flanked on either side by recombinase-binding sites referred to as *six* sites. After a passage on xylose containing plates, the transformants, originally shown to contain the cassette in its entirety, was shown to have excised the selection marker. Szewczyk et al. [194, 195] successfully use this marker excision system in *N. crassa*.

On a different note, the importance of marker selection for heterologous protein production is demonstrated by Lubertozzi *et al.* [196]. They compared the expression levels in single copy number transformants, of isolates obtained either with *trpC*, *niaD* or *argB* selection [196]. Each was paired with both the constitutive promoter *gpdA* and the inducible promoter *alcA*. Results showed that transformants containing the same promoter but with different selection markers exhibited different levels of expression. For example, transformants obtained using *trpC* selection showed three times less expression than transformants obtained with *argB* selection with the same promoter and same plasmid copy number.

## 1.5 Promoters

The efficient production of heterologous proteins requires the use of strong promoters, either constitutive or inducible. Although these can be heterologous promoters rather than native promoters, they must be recognizable by the host organism to induce expression and protein production. If relatively little is known about the transcriptome of a specific species, using a promoter which has been shown to be functional across a large variety of organisms, such as the *gpdA* constitutive promoter from *A. nidulans* or the *glaA* inducible promoter from *A. niger* may increase the chances of obtaining adequate production of the protein of interest. The glyceraldehyde-3-phosphate dehydrogenase *(gpdA*) promoter from *A. nidulans* is one of the most widely used constitutive promoters for recombinant protein production in members of the

*Aspergillus* genus. [197]. Although PgpdA has successfully been used as a heterologous promoter in organisms such as *T. reesei* [21], the heterologous use of *gpdA* promoter has some limitations. Godio *et al.* [77] demonstrated that for the transformation of basidiomycete *Hypholoma sublateritium*, plasmids carrying promoters from other basidiomycetes showed good transformation efficiencies whereas plasmids carrying ascomycetes promoters did not.

Yields of heterologous proteins may be increased by selecting *gpgA* promoters native to the host organism rather than a heterologous *gpdA* promoter. This was demonstrated by Culleton *et al.* [197] who compared protein production in *A. vadensis* of an α-arabinofuranosidase from *F. oxysporum* either induced by the *A. nidulans gpdA* promoter or by five different native promoters, including a native *gpdA* promoter. Results showed a threefold increase in activity levels of the heterologous protein induced with the native *gpdA* promoter over that of the *gpdA* promoter from *A. nidulans*. Both native and heterologous *gpdA* promoters continue to be used to drive expression of heterologous proteins across a wide range of fungal organisms.

The maltose inducible *glaA* promoter from *A. niger* has mostly been used heterologously in closely related *Aspergillus* species. It has also successfully been used to drive expression of native wild type and mutant *WdCDC42* genes in the human pathogenic fungus *Wangiella dermatitidis* [198], demonstrating cross-genus recognition of the *glaA* promoter.

The cellulose-inducible native *cbh1* promoters have been used for heterologous protein production in *T. reesei*, *T. viride* and *M. thermophila* [27, 49, 199, 200]. In each of these studies native *cbh1* promoter sequences were utilized to drive expression of the protein of interest. Using *cbh1* to drive expression, yields of heterologous proteins were reported to be in the g/L range for production of an α-amylase protein from *A. oryzae* in *T. viride* [200], and for production of a human IgG antibody fused to a glucoamylase carrier in *M. thermophila* [49]. The production of a heterologous acid phosphatase from *A. niger* in *T. reesei* increased 240-fold in the host organism with a *cbhB* promoter compared to production in its native organism [199].

Increasing heterologous protein production can be achieved by duplication of promoter binding sites in the expression plasmids used for transformation. Liu *et al.* [201] designed integrative expression plasmids containing an increasing number of repeats of a region upstream of the *glaA* gene, coined region I, for transformation into *A. niger*. This region contains an activator protein binding site including the protein binding motif CCAAT, which is known to be essential for high expression of many fungal genes [201]. In this study, the number of repeats

integrated increased from two to eight, with an increase in expression of the heterologous gene clearly observed by Northern blot analysis [201]. Similarly, repeats of the upstream region of the *cbh1* promoter, including the CCAAT motif and an *ace2* cellulase activator binding site, were used in the construction of the expression plasmid for *T. reesei* [202]. An increase in activity of the heterologous β-glucuronidase (GUS) reporter protein was seen when 2 and 4 copies of the modified promoter region was used, but failed to increase further when six copies were used [202]. This limitation has previously been described by Verdoes *et al.* [140] who demonstrated that a depletion of the transcription factors negatively affected the production of both native glucoamylase and that of the heterologous GUS reporter protein when multiple copies of a *glaA* promoter region were used. To contravene this limitation, the use of two different promoters for the expression of a single heterologous protein has been suggested by Miyauchi *et al.* [203]. The promoter regions of both *egl2* and *cbh2* genes, including the binding sites for their distinct regulatory factors, have been integrated on separate expression plasmids to test their potential as efficient promoters for heterologous gene expression in *T. reesei*. Of their four vector designs, two included a secretion signal, one of two promoters under study and a gene coding for thermophilic xylanase enzyme. The other two vectors also included a cellulose -binding module and linker fragments in the hopes of increasing yields of the xylanase. Results indicate that all isolates transformed with the vectors containing *cbh2* promoters showed xylanase activity. Of the isolates transformed with vectors containing the *egl2* promoters however, only 30% showed activity if the vector design without the cellulose binding module and linker fragments was used. In isolates where the vector containing the cellulose-binding module and linker fragments was used, that number dropped to 25% of isolates showing xylanase activity. Large variations in the xylanase activity were reported with all vector designs. Their future plans include the integration of both promoters on a single plasmid in order to observe the effect on production of the heterologous protein [203]. Alternately, transcriptions factors can be overexpressed as was done by Valerious *et al* .[204] in *S. cerevisiae* in order to upregulate transcription of a HIS7 gene.

### 1.6 Sexual genetics in industrial organisms

Most of the production strains of filamentous fungi used in industry have long been considered asexual. Thus strain improvement by random mutagenesis using physical mutagenic agents such as X-rays, UV-rays and gamma rays have been used [205, 206]. Alternately

chemical mutagenic agents such as nitrous acid or ethyl methane sulfonate (EMS) can be employed [206, 207]. Unfortunately these mutagenic agents do not exclusively target loci which will generate the desired increase in production of the enzyme or other product of interest, but will also contribute to a general decline of overall strain health. As for strain improvement using recombinant technology, the process is lengthy, arduous, and often fails to increase production of a desired product. Increasingly, information about the presence two different mating types in ascomycete fungi is being brought forth. Thus, the assumptions which have been held with respect to their inability for sexual mating are being challenged.

Metzenberg and Glass [208] studied the sexual cycle of fungi using a heterothallic strain of *Neuroscora crassa* in which two opposite mating types, A and a, were identified. They observed that the crossing of two distinct mating types resulted in the formation of mitotic ascospores as well as the appearance of fruiting bodies. They also noted that although the sequences associated with the different mating types were found at the same chromosomal position, the DNA sequences themselves, called idiomorphs, were quite distinct from each other. In 2009, Seidl *et al.* [209] reported that the industrial workhorse *T. reesei QM6a*, considered asexual for over 50 years, in fact contains a MAT1-2 mating type locus. Furthermore, in looking at natural isolates of *H. jecorina*, they were able to identify a mating type counterpart, MAT1-1. They successfully induce sexual reproduction of *T. reesei QM6a* which resulted in the production of ascospores. They concluded that *T. reesei QM6a* is a sterile female unable to produce fruiting bodies, but still able to function as a male mating partner. The authors attribute the loss of female fertility, was attributed to years of subcultivation in laboratory settings [209]. In a review of the industrially relevant genus, *Aspergillus* [210], Bennett [210] discusses the finding of MAT1 and MAT2 loci in several *Aspergillus* species. The presence of genes necessary for development of ascocarps and other genes related to sexual reproduction in ascomycetes, leads the author to question whether heterothallic species of *Aspergillus* are more common than previously thought [210]. Recently, Bohm *et al.* [211] described conditions under which a sexual cycle in *Penicillium chrysogenum* was induced. They were able to confirm that recombination events had occurred during the sexual cycle, from both a molecular and phenotypic standpoint [211]. They found that the *MAT1-1–1* mating-type gene, in addition to controlling sexual identity, also played a role in the control of genes associated with hyphal morphology, formation of conidia, and most importantly from an industrial perspective, penicillin production [211].

Hutchinson *et al.* [212] not only identified both conserved and unique regions in *M. heterothallica* which play a role in mating capabilities of this species, but also but also compared its mating mechanism to that of other species within the Sordariales family. Furthermore, they determined that although optimal growth temperature for *M. heterothallica* is 45°C, a much lower temperature of 29°C is necessary for optimal ascocarp development.

## 1.7 Rationale

Given that *M. thermophila* has recently been the focus of much interest due to its high levels of secretion of a multitude of native enzymes, the lack of a greater number of publications where *Myceliophthora* is used as a host organism for heterologous protein production, is surprising. Only recently in a paper by Xu *et al.* [51], was *M. thermophila* ATCC 42464 employed to this end. However, the potential of closely related *M. heterothallica* as a host organism, which offers two different mating types and thus, the possibility of crossing two genetically engineered strains, remains unexplored so far.

The overall aim of this project was the development of an expression system using a thermophilic host organism for heterologous protein production. In addition, the choice organism was required to be a high producer of extracellular proteins under cultured conditions and a have wide ranging pH profile. *Mycothermus thermophilus*, also known as *Scytalidium thermophilum*, *Myceliophthora thermophila* and *Myceliophthora heterothallica* all met these requirements. Although all three of these organisms were selected as candidates for this project, of particular interest was *Myceliophthora heterothallica* which has the added advantage of being a sexual organism. Hence, this opened the door to the possibility of crossing engineered strains further down the line.

The sexual crossing in industrial strains of fungal organisms offers more than one advantage as a tool for strain improvement. First, industrial strains whose overall health has been weakened by iterative rounds of mutagenesis, can potentially be revived by a round of sexual crossing. Screening of progeny can yield isolates which retain the desired trait, such as high protein production, without the loss of function seen in the progenitors, which may have led, for example, to the need for nutritional additives. Furthermore, sexual crossing of two mating types can be set up between isolates with different desirable traits. Screening of the progeny may potentially to isolates containing both of these advantageous traits due to meiotic recombination.

In addition, Van den Brink *et al.* [46] demonstrated that when a cross was set up between two different mating types of *M. heterothallica,* selected progeny showed increased cellobiohydrolase activity than either of their parents. These examples demonstrate how recombination events, occurring across the organisms' entire genome, can provide a wide array of genetic variation in the progeny. The resulting F1 generation can then be screened for desired traits including increased protein production, specific regain of function, and presence of multiple desired traits in the select offspring.

# 2 Materials and Methods

## 2.1 Strain identification and maintenance

Both *Myceliophthora heterothallica* and *Mycothermus thermophilus* fungal strains used for this project were obtained from the Fungal Biodiversity Centre, KNAW-CBS. (*M. heterothallica*: CBS 375.69, CBS 202.75; *Mycothermus thermophilus*: CBS 627.91). *Myceliophthora thermophila* ATCC 42464 was obtained from the American Type Culture Collection. All strains were maintained on YPSS agar (0.4% (w/v) yeast extract, 1.5% (w/v) soluble starch, 0.1% (w/v) $K_2HPO_4$, 0.05% (w/v) $MgSO_4$, 1.5% (w/v) agar, pH 7.0) at 45°C. Spores were collected from week-old agar plates and resuspended in 0.02% (v/v) Tween 80/0.5% (v/v) saline solution.

## 2.2 Crossing of opposing mating types of *Myceliophthora heterothallica*

Crosses were set up between *Myceliophthora heterothallica* CBS 375.69 and *Myceliophthora heterothallica* CBS 202.75 on both YPSS agar plates and on Mycobroth agar plates (10g/l soytone, 40g/l D-glucose, 1ml/l trace element solution, pH adjusted to 5.0 with HCl). Plates were placed shielded from light and grown at 37°C for six days. On the sixth day of incubation a small amount of material was scraped from the area containing a dark line at the junction where the two strains met and observed under the microscope in order to verify presence of ascospores by microscopy.

## 2.3 Protoplast generation protocol

Protoplasts were generated using 1.0-2.0 g of mycelia from 18-24 hour cultures grown in 10x TDM (0.5 M $KH_2PO_4$, 1 M $MgSO_4$, 0.1 M $CaCl_2.2H_2O$, thiamine-HCl (1 mg/ml), L-asparagine monohydrate and trace element solution containing 2 mM ferrous sulphate, 1 mM cupric sulphate, 10 mM manganese sulphate monohydrate, 5 mM cobalt chloride hexahydrate ) [213] containing 2% (w/v) glucose as a carbon source. Mycelia were harvested by filtering through Miracloth (Calbiochem, San Diego, CA, USA) and washing with 2 volumes of 0.6 M MgSO4. Mycelia were transferred to a sterile 50 ml conical tube and resuspended in a solution containing 1.2 M $MgSO_4$, 10 mM $Na_2HPO_4$ , 10 mM $NaH_2PO_4$, and 0.375 g VinoTaste® Pro per gram of mycelia (http://www.novozymes.com) as a source of β-glucanase enzyme. After 3 hours at 30°C with shaking at 150 rpm, protoplasts were isolated by layering equal volumes of digested mycelial suspension and ice cold 0.6 M sorbitol, 0.1 M Tris-HCl pH 7.0. Tubes were centrifuged at 3000 *g* for 20 minutes at 4°C. Protoplasts, trapped at the interface, were harvested from the interface and resuspended in 1 ml of 1.2 M sorbitol.

## 2.4 Transformation protocol

Transformation was performed using 200 µl of protoplast suspension, 20 µl 0.4 M aurintricarboxylic acid, 4-5 µg of each plasmid DNA for cotransformation, and a 100 µl of 20% (v/v) polyethyleneglycol (PEG) solution containing 16.7 mM $CaCl_2$ and 3.3 mM Tris-HCl pH 7.5. The preparation was incubated at room temperature for 10 minutes followed by addition of 1.5 ml 60% (v/v) PEG solution containing 50 mM $CaCl_2$ and 10 mM Tris-HCl pH 7.5. After a 20-minute incubation at room temperature, 5 ml of 1.2 M sorbitol was added and the tubes were centrifuged at 3000 *g* for 10 minutes at 22°C. The supernatant was discarded and the pellet was resuspended in 1 ml of 1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5.

## 2.5 Assessment of sensitivity to antibiotics

Wells of a 24-well plate (Costar cat# 3524) were filled with 1 ml of 10x TDM containing 2% (w/v) glucose and were inoculated with $1x10^6$ spores/ml. Increasing concentrations of Hygromycin B and Geneticin ranging from 0 µg/ml to 10 µg/ml in gradual increments were prepared. 100µl of each of the dilutions were added to the wells. Plates were covered, placed in a

humidity chamber and in an incubator at 37°C. They were assessed for growth daily for one week.

### 2.6 Selection of transformants

Transformed protoplasts were plated on regeneration media containing 34% (w/v) sucrose, 2.0% (w/v) agar, 2.5% (v/v) stock solution A, 2.5% (v/v) stock solution B, and 0.1% (v/v) Hunter's trace element solution. Stock solution A contained 120 g $NaNO_3$, 10.4 g KCl, 30.4 g $KH_2PO_4$, and 22.5 ml of 4M KOH for a total volume of 500 ml in $ddH_2O$. Stock solution B contained 10.4 g $MgSO_4.7H_2O$ for a total volume of 500 ml $ddH_2O$. The Hunter's trace element solution contained 2.2 g $ZnSO_4$-$7H_2O$, 1.1 g $H_3BO_3$, 0.5 g $FeSO_4$-$7H_2O$, 0.17 g $CoCL_2$-$6H_2O$, 0.16 g $CuSO_4$-$5H_2O$, 0.5 g $MnCl_2$-$4H_2O$, 0.15 g $Na_2MoO_4$-$2H_2O$, and 5.0 g EDTA per 100 ml total volume. For selection of *amdS* transformants 0.7% (v/v) 1.5 M acetamide solution was added to the media. For selection of *pyrG*$^-$ transformants stock solution A without $NaNO_3$ was used and 20 mM uracil, 100 mM uridine, and 1.5 mg/ml 5-Fluoroorotic acid were added.

### 2.7 Vector for homologous recombination

A homologous recombination vector was constructed for *pyrG* gene replacement using *amdS* from *A. nidulans* as a selectable marker. About 1.3 Kb of 5' and 3' regions flanking *pyrG* of *M. heterothallica* CBS 375.69 were amplified by PCR. A repeat of the 3' region flanking *pyrG* was added to the construct for self-excision of the selection vector (Figure 1).

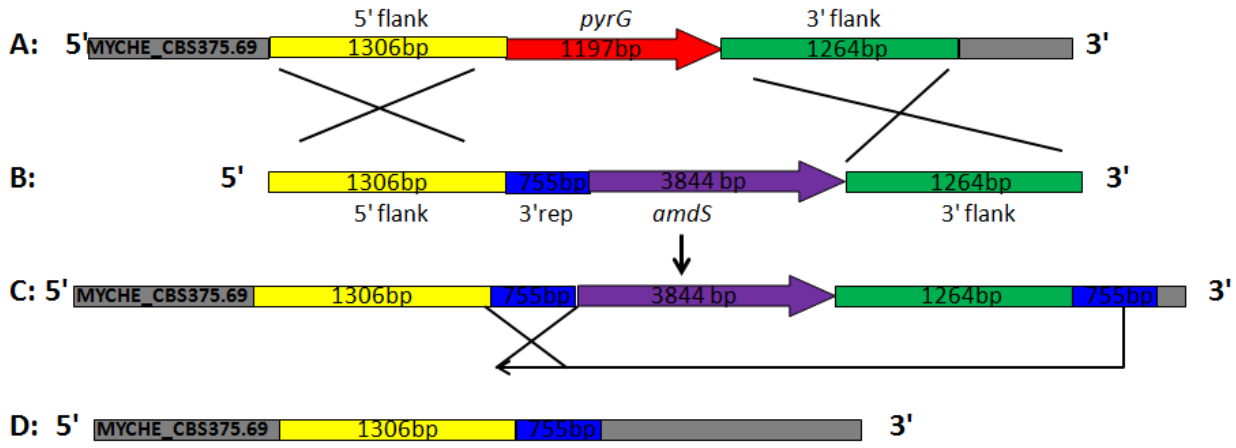



**Figure 1. Schematic representation *of M. heterothallica* CBS 375.69 *pyrG* replacement by *amdS* construct**

A. Representation of *pyrG* region of WT *M. heterothallica* CBS375.69 genome

B. *pyrG* replacement by *amdS* construct

C. Representation *M. heterothallica* after hypothetical homologous recombination of construct

D. Representation *M. heterothallica* after hypothetical self-excision event

## 2.8 Selection plasmid pGAMD

The plasmid used for positive selection of transformed organisms can be seen in Figure 2 and was provided by Dr. R. Storms from Concordia University, Department of Biology.

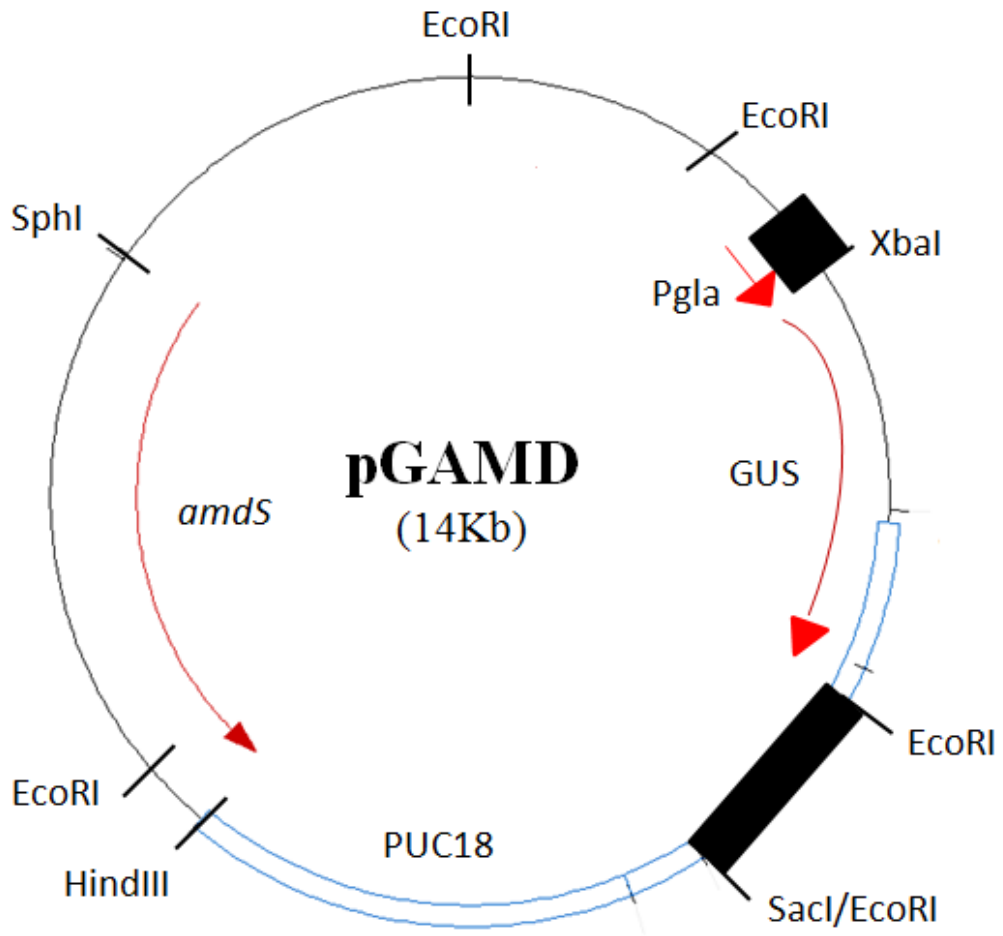



**Figure 2. Integrative selection plasmid containing the *amdS* gene from *A. nidulans***

Positive selection is based on ability of transformants in which pGAMD has been integrated to utilize acetamide as the sole source of nitrogen. The *amdS* gene is under control of its native promoter (courtesy of Dr. R. Storms).

## 2.9 Construction of expression vectors

### 2.9.1 Construction of expression cassettes

Plasmid ANIp7 [123] was as a template for the construction of expression vectors for transformation of *M. thermophila* and *M. heterothallica*. This plasmid initially contained the *glaA* promoter (PglaA) region from *A. niger* to drive expression of heterologous proteins. The promoter was replaced by promoter region of five different genes from *M. thermophila* and *M. heterothallica* (Table 5). Primers were designed to amplify the 1 Kb region upstream of the start codon from the genes listed above and to contain AatII and FseI restriction sites on the ends. Once band size was verified on gel, a PCR clean-up was performed using Qiagen PCR purification Kit (cat# 28104) and eluted with 35 µl of 10 mM Tris-HCl pH 8.0. A double digest with FseI and AatII (https://www.neb.ca/) was done at 37°C for two hours for both the amplified promoters and the original ANIp7 vector. Digested products were precipitated using one volume of sample: 0.1 volume of 3 M sodium acetate and two volumes of 95% (v/v) ethanol. Samples were resuspended in 10 mM Tris-HCl pH 8.0. Ligation was performed using an insert to vector molar ratio of 3.0 - 3.3: 1. Ligation reaction was performed overnight at 16°C using T4 ligase from NEB (Cat# M0202S). Ligated products were transformed into *E. coli* DH5α competent cell as per the NEB protocol. Plasmids containing the six different promoters are shown below (Figure 3).

**Table 5. Promoters used in expression cassettes**

| Organism | Gene model ID | Promoter name |
|---|---|---|
| *A. niger CBS 513.88* | An03g06550 | PglaA |
| *M. thermophila ATCC 42464* | MYCTH_2114025 | PpmoA |
| *M. thermophila ATCC 42464* | MYCTH_2311855 | PgpdA |
| *M. thermophila ATCC 42464* | MYCTH_111388 | PcbdA |
| *M. thermophila ATCC 42464* | MYCTH_2303065 | PagdA |
| *M. heterothallica CBS 375.69* | | PagdB |

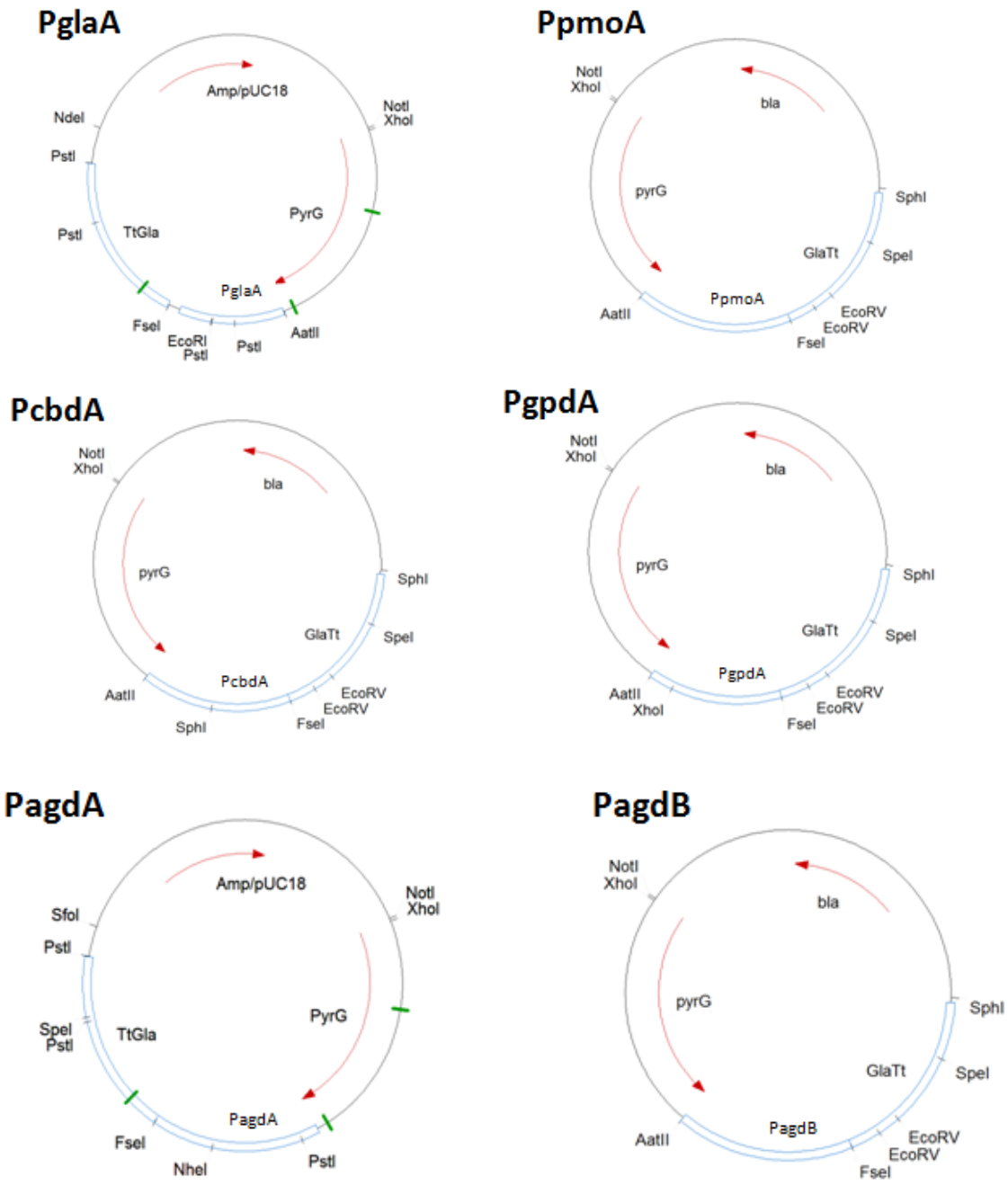



**Figure 3. Integrative plasmids containing six alternate promoters**

Expression plasmids each containing one of six promoters tested in order to drive the expression and subsequent production and excretion of heterologous proteins.

### 2.9.2 Ligation-independent cloning of target genes

For the insertion of heterologous genes of interest, xylanase genes from a variety of organisms were inserted into the plasmids containing promoters listed above using LIC cloning. Both annealing vector and insert were amplified using primers designed with identical LIC sequences.

The vector primer sequences used were:
Forward primer: GCCGTCCGTCGCCGTCCTTCACCGACCGCGACGGTG
Reverse primer: GCCGGTGTTTTGTTGCTGGGGAGGTTATGGCAGAAGGGGATTC

The insert primer sequences used were :
Forward primer: CCCCAGCAACAAAACACCGGCTCAGCAATGGTTCAGA
TCAAGGTAGC
Reverse primer: GAAGGACGGCGACGGACGGCTCTAGAGAGCATTTGCGATAGC

The various genes of interested inserted into the expression plasmid are identified in Table 6. Amplification products were treated overnight at 37°C with DpnI (https://www.neb.ca/) to get rid of all traces of the original plasmid by digesting methylated DNA but leaving PCR amplified DNA intact. Next PCR cleanup was performed using Millipore clean up filter plate (Cat# LSKMPCR10) (http://www.emdmillipore.com) to remove all traces of primers and unincorporated dNTP. The amplified vector and inserts were treated separately with T4 DNA polymerase exonuclease (New England Biolabs, Cat# M0203S) in a 20 µl reaction containing 300 ng of amplified DNA, 2 µl NEBuffer2, 0.8 µl 100 µM DTT, 2 µl 25 mM dTTP for the reaction mixture containing the vector or dATP for the reaction containing the amplified genes of interest, 1.25 µl of T4 Polymerase NEB (3 U/ml) and dH2O to 20 µl. The annealing reaction was performed at 22°C for 30 minutes, followed by inactivation of the exonuclease at 75°C for 20 minutes [214]. An annealing reaction was set up between the vector and GOI annealing in a 10 µl annealing reaction using 1:1 and a 2:1 insert to vector molar ratio in parallel with 100-150 ng total DNA. The reaction was incubated at room temperature for 30 minutes. The annealed reaction product was then used to transform *E. coli* DH5α competent cells.

**Table 6. Genes of interest inserted into expression plasmid**

| Organism | Gene model ID | Gene name |
|---|---|---|
| *A. niger NRRL3* | NRRL3_08708 | *ANxynA* |
| *A. niger CBS 513.88* | ASPNI_158107 | *ANxynB* |
| *T. terrestris NRRL 8126* | THITE_2107799 | *TtxynA* |
| *T. terrestris NRRL 8126* | THITE_2117649 | *TtxynB* |
| *T. terrestris NRRL 8126* | THITE_2118148 | *TtxynC* |
| *M. thermophila ATCC 42464* | MYCTH_99786 | *MtxynA* |
| *S. thermophilum CBS 625.91* | SCYTH2p4_007856 | *StxynA* |
| *P. herpotrichoides CBS 494.80* | Psehe2p4_001268 | *PhxynA* |

## 2.10 Screening and verification of positive clones

Screening for positive clones in order to verify the insertion of the alternate promoters in the expression plasmid was performed by colony PCR. Primers located within each of the promoters regions were used, except for verification of PagdA. For PagdA verification, the forward primer was positioned in *pyrG* in the expression plasmid and the reverse primer was positioned in TtglaA. The sequence of the primers used for identification of positive clones and expected band size can be seen in Table 7.

**Table 7. PCR primers used for verification of alternate promoter insertion**

| Promoter ID | Primer ID | Primer Sequence | Expected band size (bp) |
|---|---|---|---|
| PpmoA | 160F2 | AATTGACGTCCTGGCGAGGATGATCG | 1025 |
| | 180R | GCCGGTGTTTTGTTGCTGGGGACGTGATGTCGCTGCTCAG | |
| PgpdA | 172F2 | GACATCGGACGTCAGC | 862 |
| | 183R | GCCGGTGTTTTGTTGCTGGGGAGATTTCTGTGATGTGGGGAGG | |
| PcbdA | 162F2 | AATTGACGTCGGACCTTCGGAGGCG | 1015 |
| | 181R | GCCGGTGTTTTGTTGCTGGGGACTTGGATCGCAGAGACTGG | |
| PagdA | 668F | ACGGTGTCTGTATTTCCGGA | 1276 |
| | 647R | CTTACGAGAAAAGAGTTGGACTTTG | |
| PagdB | 168F2 | AATTGACGTCCCAATTGGTGCACGATG | 1067 |
| | 182R | GCCGGTGTTTTGTTGCTGGGGAGGTTAATTATGGCAGAAGGGG | |

Verification of proper insertion of the promoters was also confirmed using restriction enzymes. Digestions were performed and fragments run on an agarose gel to assure resulting fragments were of the proper size. The restriction enzymes selected and expected band sizes after digestion were as follows:

SfoI for PpmoA; expected bands 2086 bp and 3827 bp
XhoI for PgpdA; expected bands 4178 bp and 1539 bp
SphI for PcbdA; expected bands 1595 bp and 4281 bp
PstI for PagdA; expected bands 3932 bp, 1674 bp and 375 bp
SacI for PagdB; expected bands 1658 bp, 3773 bp and 497 bp

Screening for positive clones after LIC cloning was performed by colony PCR. The 668F forward primer was positioned in *pyrG* in the expression plasmid and the 647R reverse primer was positioned in TtglaA. The expected sizes of the bands for colony PCR screen with these primers can be seen in Table 8.

The primer sequences used for screening for positive clones after LIC cloning were:

668F ACGGTGTCTGTATTTCCGGA

647R CTTACGAGAAAAGAGTTGGACTTTG

**Table 8. Expected sizes for colony PCR screen after LIC Cloning for insertion GOI in expression plasmid**

| Promoter ID. | GOI | Gene only (bp) | Promoter only (bp) | LIC seq. (bp) | Expected size (bp) |
|---|---|---|---|---|---|
| **PglaA** | *ANxynA* | 984 | 744 | 43 | 1771 |
| | *TtxynA* | 687 | 744 | 43 | 1474 |
| | *TtxynB* | 1188 | 744 | 43 | 1975 |
| | *TtxynC* | 1110 | 744 | 43 | 1897 |
| **PpmoA** | *ANxynA* | 984 | 1274 | 43 | 2301 |
| | *TtxynA* | 687 | 1274 | 43 | 2004 |
| | *TtxynB* | 1188 | 1274 | 43 | 2505 |
| | *TtxynC* | 1110 | 1274 | 43 | 2427 |
| | *MtxynA* | 825 | 1274 | 43 | 2142 |
| | *ANxynB* | 765 | 1274 | 43 | 2082 |
| | *StxynA* | 1104 | 1274 | 43 | 2421 |
| | *PhxynA* | 1086 | 1274 | 43 | 2403 |
| **PcbdA** | *ANxynA* | 984 | 1247 | 43 | 2274 |
| | *TtxynA* | 687 | 1247 | 43 | 1977 |
| | *TtxynB* | 1188 | 1247 | 43 | 2478 |
| | *TtxynC* | 1110 | 1247 | 43 | 2400 |
| | *MtxynA* | 825 | 1247 | 43 | 2115 |
| | *ANxynB* | 765 | 1247 | 43 | 2055 |
| | *StxynA* | 1104 | 1247 | 43 | 2394 |
| | *PhxynA* | 1086 | 1247 | 43 | 2376 |
| **PagdA** | *ANxynA* | 984 | 1301 | 43 | 2328 |
| | *TtxynA* | 687 | 1301 | 43 | 2031 |
| | *TtxynB* | 1188 | 1301 | 43 | 2532 |
| | *TtxynC* | 1110 | 1301 | 43 | 2454 |
| | *MtxynA* | 825 | 1301 | 43 | 2169 |
| | *ANxynB* | 765 | 1301 | 43 | 2109 |
| | *StxynA* | 1104 | 1301 | 43 | 2448 |
| | *PhxynA* | 1086 | 1301 | 43 | 2430 |
| **PagdB** | *ANxynA* | 984 | 1289 | 43 | 2316 |
| | *TtxynA* | 687 | 1289 | 43 | 2019 |
| | *TtxynB* | 1188 | 1289 | 43 | 2520 |
| | *TtxynC* | 1110 | 1289 | 43 | 2442 |
| | *MtxynA* | 825 | 1289 | 43 | 2157 |
| | *ANxynB* | 765 | 1289 | 43 | 2097 |
| | *StxynA* | 1104 | 1289 | 43 | 2436 |
| | *PhxynA* | 1086 | 1289 | 43 | 2418 |

| | | | | | |
|---|---|---|---|---|---|
| **PgpdA** | *ANxynA* | 984 | 1171 | 43 | 2198 |
| | *TtxynA* | 687 | 1171 | 43 | 1901 |
| | *TtxynB* | 1188 | 1171 | 43 | 2402 |
| | *TtxynC* | 1110 | 1171 | 43 | 2324 |
| | *MtxynA* | 825 | 1171 | 43 | 2039 |
| | *ANxynB* | 765 | 1171 | 43 | 1979 |
| | *StxynA* | 1104 | 1171 | 43 | 2318 |
| | *PhxynA* | 1086 | 1171 | 43 | 2300 |

## 2.11 Verification of insertion of plasmids into fungal hosts

For verification of co-transformation of plasmids into the fungal host, gDNA was extracted from fungal isolates using QiaAmp DNA minikit (cat# 51304) from Qiagen. The extracted gDNA was used for PCR amplification. Internal *amdS* primers were used for verification of insertion of the selection plasmid and primers located in the promoter and terminator region of the gene of interest were used for verification of insertion of the expression plasmid.

Primers used for verification of PGAMD insertion:
21F: ACCGGAACAACCACGCTCGTG
22R: CAAAGCCGGTGCCGTCTTCTACG

For verification of expression plasmid insertion:
283F: TGCCCTCATCCCCATCCTTTAACTATAGC
133R: GATTCGTCGCCTAATGTCTCG

## 2.12 Screening of transformants expressing cloned genes

### 2.12.1 Media and set up for stationary cultures

Liquid cultures were grown in 10x TDM and a 2% (w/v) carbon source depending on the promoter (2% (w/v) sucrose and 10% (w/v) sucrose for PagdA and PagdB; 2% (w/v) carboxymethyl cellulose (CMC) for PcbdA and PpmoA; 2% (w/v) glucose for PgpdA; and 2% (w/v) maltose and 15% (w/v) maltose for PglaA). Cultures were prepared in a 96 well format with 250 µl liquid media / well. Wells were inoculated using sterile toothpick to transfer spores and mycelia from colonies on regeneration plates to the liquid culture plates. The plates were incubated at 37°C for 5 - 7 days in order to assure the appearance of mycelial mats atop of each well.

### 2.12.2 Spotting Assay protocol

Plates for spotting assay were prepared with 0.15% (w/v) Remazol Brilliant Blue R−D-Xylan,1.5% (w/v) agar (w/v), in 100 mM citrate pH 5.0 (RBB xylan plates). Positive controls

were prepared using an in-house xylanase. For the control a 5x serial dilution was done in 10 mM citrate buffer for final dilution of 3125X. Culture supernatants were centrifuged twice at 16000 *g* for 20 minutes at 4°C to precipitate spores and mycelia. Next, 3 µl of cleared supernatant from liquid cultures or control xylanase was spotted on the RBB xylan plates. Plates were incubated overnight at 37°C.

### 2.12.3 Shaking cultures

Liquid cultures were grown in 10x TDM containing 2% (w/v) carbon source depending on the promoter (2% (w/v) sucrose for PagdA and PagdB; 2% (w/v) CMC for PcbdA and PpmoA; 2% (w/v) glucose for PgpdA; and 2% (w/v) maltose PglaA). Cultures containing 25 ml of culture media were inoculated with $10^6$ spores per ml and incubated at 45°C with agitation at 220 rpm in 250 ml Erlenmeyer flasks. Cultures were grown for 24 to 96 hours or for 4 to 48 hours depending on the purpose of the assay.

### 2.12.4 Induction Experiment

For the induction experiment 25 ml liquid cultures flasks containing with 10x TDM and 2% (w/v) glucose were inoculated with $1x10^6$ spores/ml from *M. heterothallica* isolates #3 and #16 containing the PagdB - *ANxynA* construct. Cultures were grown overnight at 45°C and mycelia was washed in 10x TDM at 24 hours. I then divided the mycelial pellet into pellets of 1g each and added them to flasks containing 10x TDM containing 2% (w/v) sucrose and to fresh 10x TDM containing 2% (w/v) glucose. Samples of the supernatant were taken and centrifuged at 4 hours, 6 hours, 8 hours, 12 hours and 24 hours.

### 2.12.5 Protein determination

Total protein concentration in culture supernatant was determined using Bradford Reagent kit (Biorad Quickstart Bradford Protein Assay; Cat# 5000201).

### 2.12.6 BCA assay for determining reducing sugar release

Xylanase activity was measured by BCA assay using 0.4% (w/v) birch-wood xylan (Sigma-Aldrich; Cat# 95588) as the substrate. Activity at pH 5.5 was examined using 3 µl of extracellular supernatant was mixed with 10 µl of substrate. The mixture was incubation for 1

hour at 40°C. The reducing sugars produced in the reaction mixture were measured using a bicinchoninic acid-containing solution alongside xylose standards. Absorbance was measured by spectrophotometer at 562 nm. One unit of xylanase activity was defined as the quantity of enzyme required to liberate 1 μmol of xylose equivalent per minute at 40°C.

### 2.12.7 Concentration of samples and buffer exchange

Concentration of samples was performed using 10K Nanosep centrifugal tubes (Pall Corporation) for small volumes ≤1 ml. For concentrating 25 ml liquid culture supernatants Vivaspin (10,000 MWCO) 20 ml centrifugal concentrators were used for the first set of experiments. The buffer exchange was done using 10 mM citrate buffer. Concentration of subsequent large volume supernatant cultures were done by TCA precipitation. The TCA precipitation of samples was done using 4 volumes of cold 20% (v/v) Trichloroacetic acid with 20mM DTT in 80% (v/v) acetone. The samples were left to precipitate on ice for 60 minutes. The tubes were then centrifuged at 3200g for 30 minutes at 4° C. The supernatant was removed by decanting. The samples were rinsed using -20°C prechilled to 80% (v/v) acetone with 20mM DTT , vortexed and incubated at -20°C for 30 minutes. The protein was recovered by centrifugation at 3200g for 30 minutes at 4°C and the supernatant was removed by decanting. The pellet was air dried 5 minutes under a chemical hood and resuspended.

## 2.13 Protein detection

### 2.13.1 SDS-PAGE

SDS-PAGE was performed in 12% (v/v) polyacrylamide gel slabs. Samples containing 5 to 15 μg of protein was mixed with 10 μl sample loading buffer made from 450 μl Laemmli buffer (Bio_Rad #1610737), and 50 μl 3.5 M DTT. The samples were then denatured at 95°C for 5 minutes. The 20 μl of denatured sample was loaded into the wells. Samples were run into the gel for 50 minutes at 160 volts. Proteins were stained with 0.1% (w/v) Coomassie Brilliant blue or by silver nitrate depending on the initial amount of protein loaded.

### 2.13.2 Preparation of samples for mass spectrometer

Gel segments corresponding to size of expected recombinant protein were cut from SDS-

PAGE gel, chopped into 1 mm x 1 mm pieces and resuspended in 1% (v/v) acetic acid. Coomassie distaining was done by a series of washes in ammonium bicarbonate and acetonitrile. Samples were dehydrated at room temperature for 10 minutes and 10 mM DDT was added. Alkylation was done using iodoacetamide. Trypsin was added to each sample and the reaction mixture was incubated for 18 hours at 37 °C. Extraction solution containing 50% (v/v) acetonitrile, 1% (v/v) trifluoroacetic acid and 49% $H_2O$ was added to the samples and rounds of desalting was done using C18 ziptips™ (Millipore, Billerica, MA). Eluted peptides were dried in a SpeedVac and resuspended in a 60 µl solution containing 5% (v/v) acetonitrile and 0.1% (v/v) formic acid.

### 2.13.3 LC–MS/MS analysis

Samples were sent to Dr. M. Di Falco at Concordia University for LC-MS/MS analysis where 5 µl of digested peptide was loaded onto a PicoFrit column (New Objective, Woburn, MA) connected to a LTQ-Orbitrap Velos mass spectrometer (Thermo-Fisher, San Jose, CA). Peptide separation was done using a linear gradient generated by an Easy-LC II Nano-HPLC system (Thermo-Fisher).

# 3 Results

## 3.1 *Mycothermus thermophilus*

In order to confirm that *Mycothermus thermophilus* CBS 627.91 met the requirements of a suitable host organism, temperature and pH profiles were performed. Assays were done to assess the level of enzymatic activity in the extracellular media using culture supernatants. Results show that this organism grows optimally at 45°C with a temperature range between 22°C and 55°C. The pH range for this species on YPSS agar is between pH 4 and pH 11 with optimum at pH 8. The ability of *Mycothermus thermophilus* to thrive at alkaline conditions prompted us to attempt to develop a genetic transformation system for this organism. The liquid cultures showed more than 700 µg/ml of secreted protein after 72 hours when grown in 10x TDM containing 2% (w/v) barley at pH 8. Enzymatic assays show high levels of cellulase, xylanase and α-amylase activity (Figure 4). The results from these experiments validated the use of *Mycothermus thermophilus* CBS 627.91 as a potential host organism.

The organism was assessed for the ease with which it could be protoplasted in order to evaluated whether PEG-mediated transformation of protoplasts was possible for this species. I found protoplast generation with *Mycothermus thermophilus* to be problematic since only some of spores used for inoculation germinated. The remaining spores were swept up by the growing network of mycelia as the culture grew. These conidia were partially digested by the β-glucanase along with the mycelia and made the true protoplasts difficult to differentiate from the partially digested conidia. As an alternative, I attempted to digest the conidia alone. I started with the usual amount of 0.5 g of β-glucanase in 11 ml of conidial suspension in osmotic media, but spiked the solution with 0.5 g more β-glucanase after 4 hours and 8 hours of digestion. However this resulted in a mixed population of conidia at different stages of digestion, even after 24 hours of β-glucanase digestion. I used a variety of methods to rid the culture of ungerminated conidia including low speed centrifugation, filtering through glass wool and sub culturing at different concentrations in order to obtain a conidia-free culture. Ultimately the best method was a 1% (v/v) subculture, which yielded clean protoplast suspensions from which to work. Overall, this organism required additional manipulation and additional time as compared to the other candidates, before a clean preparation of protoplasts could be obtained.

Potential methods of selection were evaluated in order to find a suitable selection marker for detection of *Mycothermus thermophilus* transformants. Acetamide selection was the method of selection I originally had in mind for this species. Preliminary tests indicated that this was not possible since agar cultures using the wild-type strain showed no disadvantage when grown on acetamide as the sole source of nitrogen compared to growth using asparagine or $NaNO_3$ as the nitrogen source. Next, I considered *pyrG* selection for this organism. Unfortunately, I found that the protoplast regeneration media needed both starch and 0.1% yeast extract. Unfortunately, yeast extract was shown to provide the cultures with a source of uracil or uridine when I performed a control experiment using *A. niger*_N402 *pyrG*$^+$ and *A. niger*_N593 *pyrG*$^-$ strains. This eliminated the possibility of using positive *pyrG* selection after reintegration of *pyrG* on an expression vector even if a *pyrG*$^-$ strain could be generated. Although negative selection of *pyrG*$^+$ using 5' FOA remained an option, *pyrG* could not be used as a bidirectional selection marker. Given that this organism has very stringent nutritional requirements and was more difficult to protoplast, it was dropped as a potential host organism.

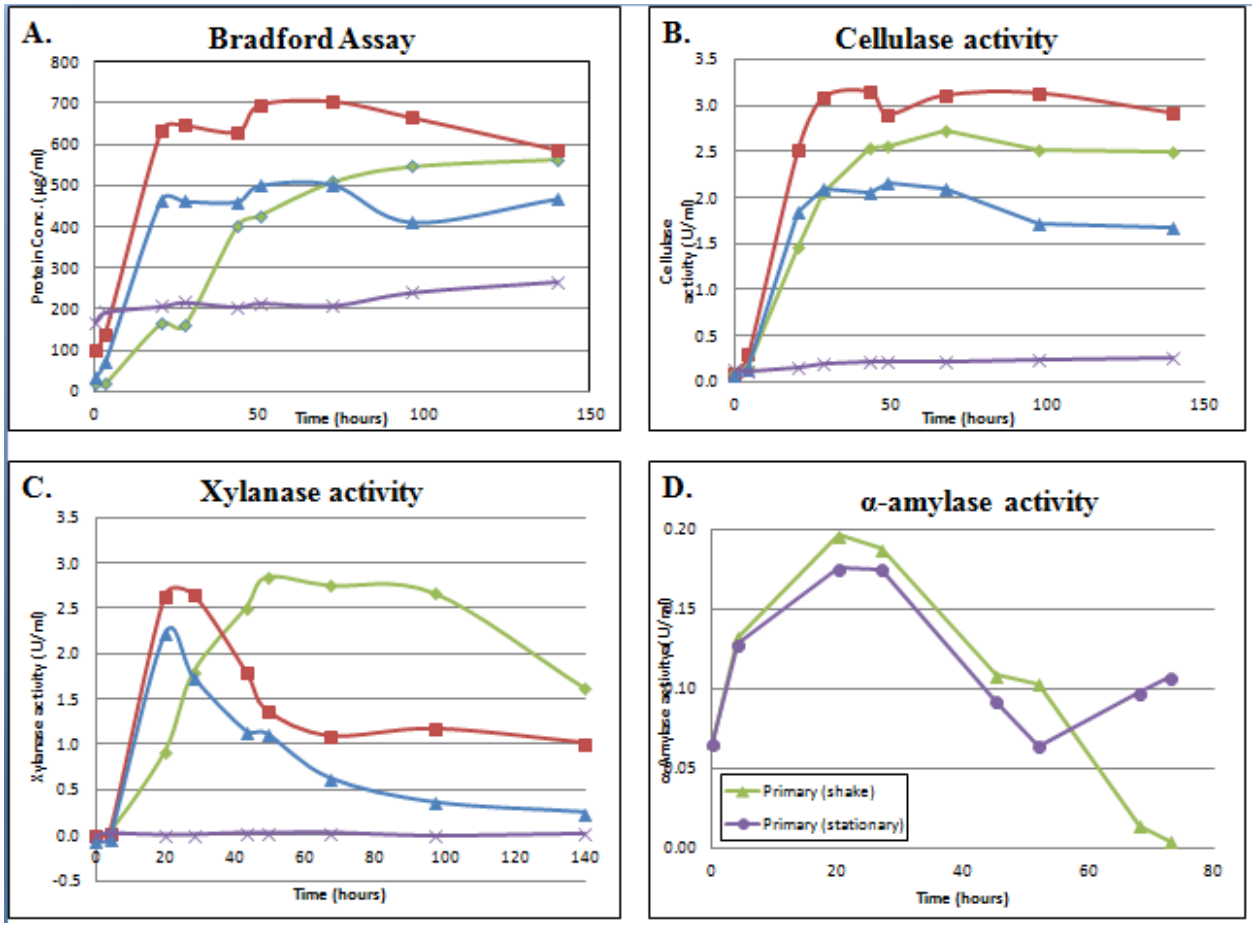



**Figure 4. Protein levels and enzymatic activity of *Mycothermus thermophilus* in liquid cultures**

A. Total protein concentration of *Mycothermus thermophilus* culture supernatant when grown in 10x TDM containing 2% (w/v) barley

B. Cellulase activity of culture supernatant of *Mycothermus thermophilus* culture supernatant when grown in 10x TDM containing 2% (w/v) barley

C. Xylanase activity of culture supernatant of *Mycothermus thermophilus* culture supernatant when grown in 10x TDM containing 2% (w/v) barley

D. α amylase activity of culture supernatant of *Mycothermus thermophilus* culture supernatant when grown in 10x TDM containing 2% (w/v) starch

For Figures A - C : Culture grown at pH 5.5, Culture grown at pH 8; Culture grown at pH 9; Culture grown at pH 10;

Assay buffer at pH 5.5 for all activity assays

## 3.2 *Myceliophthora heterothallica* and *Myceliophthora thermophila*

### 3.2.1 Growth profiles for *M . thermophila and M. heterothallica*

I evaluated *M. thermophila and M. heterothallica* as candidate host organisms by performing temperature and pH profiles and by performing assays using culture supernatants. Results showed that both of these organisms grow optimally at 45°C with a temperature range between 30°C and 50°C. The pH range at which I observed growth on agar plates was between pH 4 and pH 12 but with decreased growth above pH 11. Results of the temperature and pH profiles of *M. heterothallica* CBS 375.69 are shown in Figure 5 and Figure 6. I assayed cultured supernatants of both *M. heterothallica* and *M. heterothallica*. The amounts of secreted protein in *M. thermophila* culture supernatants is shown below (Figure 8) as are the levels of cellulase activity and xylanase activity in cultures grown in 10x TDM containing either 2% (w/v) barley or 2% (w/v) alfalfa. Results of assays done using culture supernatants show average levels of total extracellular proteins and average levels of xylanase and cellulase activity. These organisms were retained as potential hosts nonetheless since early protoplast generation trials were promising as were potential methods of transformation.

Since *M. heterothallica* is known to be a sexual species, crosses using strains of opposite mating types were performed in order to see whether ascocarps could be obtained. Results of the crosses between *M. heterothallica* CBS 375.69 and *M. heterothallica* CBS 202.75 on both YPSS agar plates and Mycobroth plates can be seen in Figures 7A and 7B. The formation of ascocarps can be seen at the junction line between the two strains. A small amount of material was scraped from the area containing dark lines at the junction of the two strains and placed on slides in order to visualize the ascocarps and ascospores under a microscope. Both of these structures, which can be seen in Figures 7C -7E, confirm the sexual nature of this species.

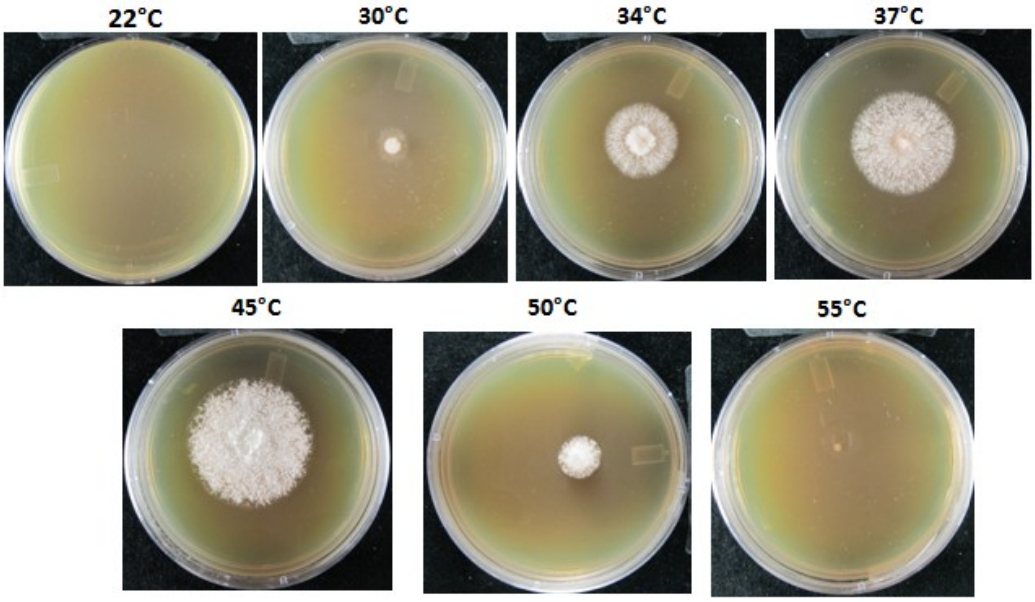



**Figure 5. Temperature profile of *M. heterothallica* CBS 375.69**

Growth of *M. heterothallica* CBS 375.69 at 48 hours post inoculation with 2x $10^4$ spores/plate on Mycobroth.

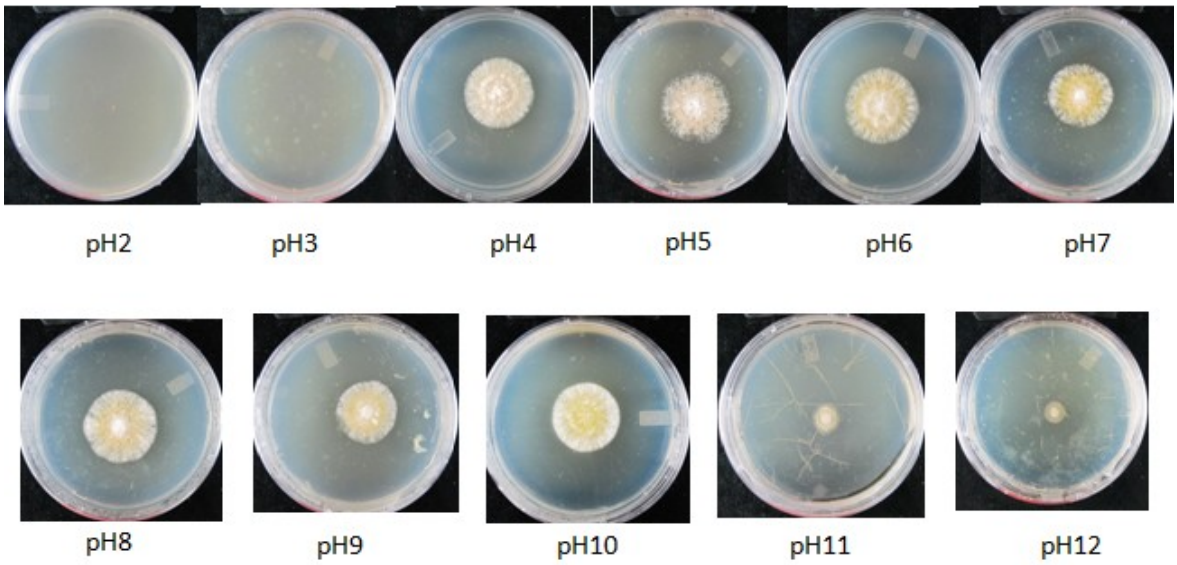



**Figure 6. pH profile for *M. heterothallica* CBS 375.69**

Growth of *M. heterothallica* CBS 375.69 at 48 hours post-inoculation with 1x $10^5$ spores/plate on YPSS at 45°C.

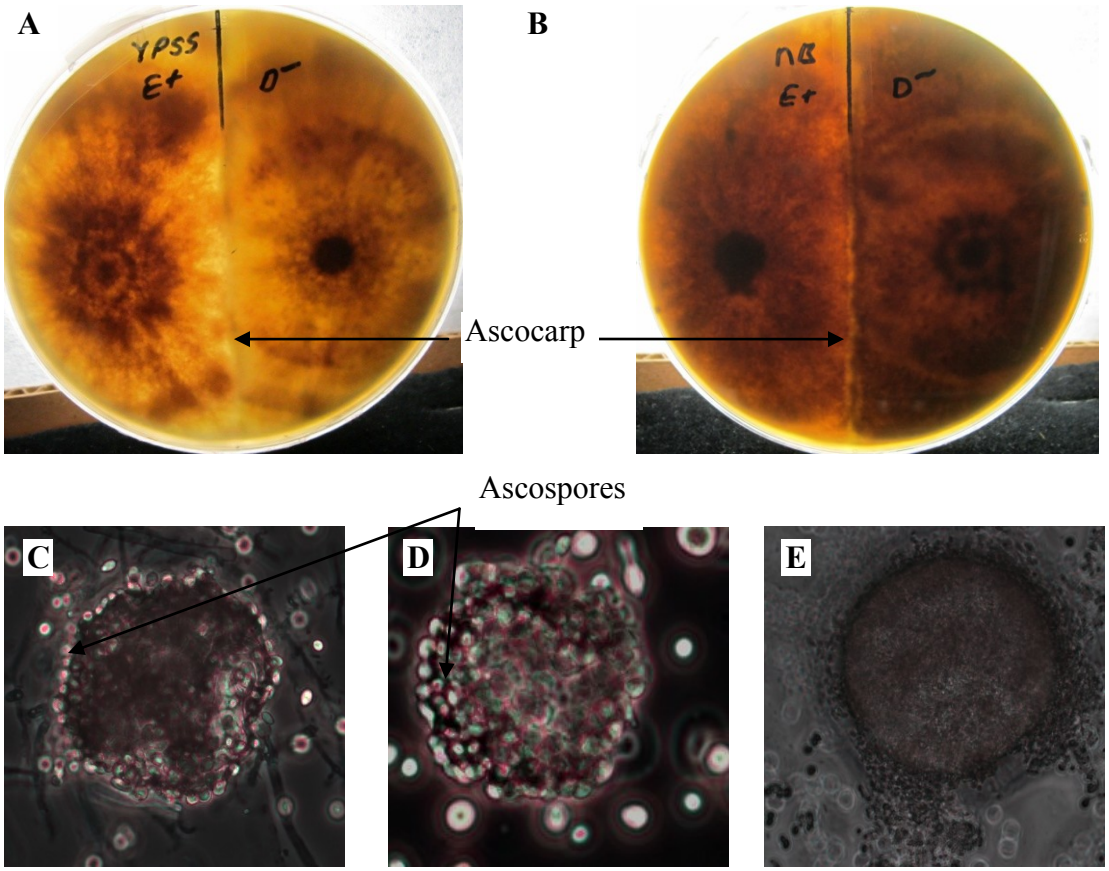



**Figure 7. Mating of *Myceliophthora heterothallica***

A. Cross between *Myceliophthora heterothallica CBS* 202.75 on left hand side of petri dish and *Myceliophthora heterothallica* CBS 375.69 on right hand side using YPSS agar plate.

B. Cross between *Myceliophthora heterothallica CBS* 202.75 on left hand side of petri dish and *Myceliophthora heterothallica* CBS 375.69 on right hand side using Mycobroth agar plate.

C. and D. Material collected from dark junction line between the two strains was resuspended in 10x TDM before being placed on a microscope slide; 400X magnification

E. Dry material collected from dark junction line between the two strains was placed directly on a microscope slide; 400X magnification

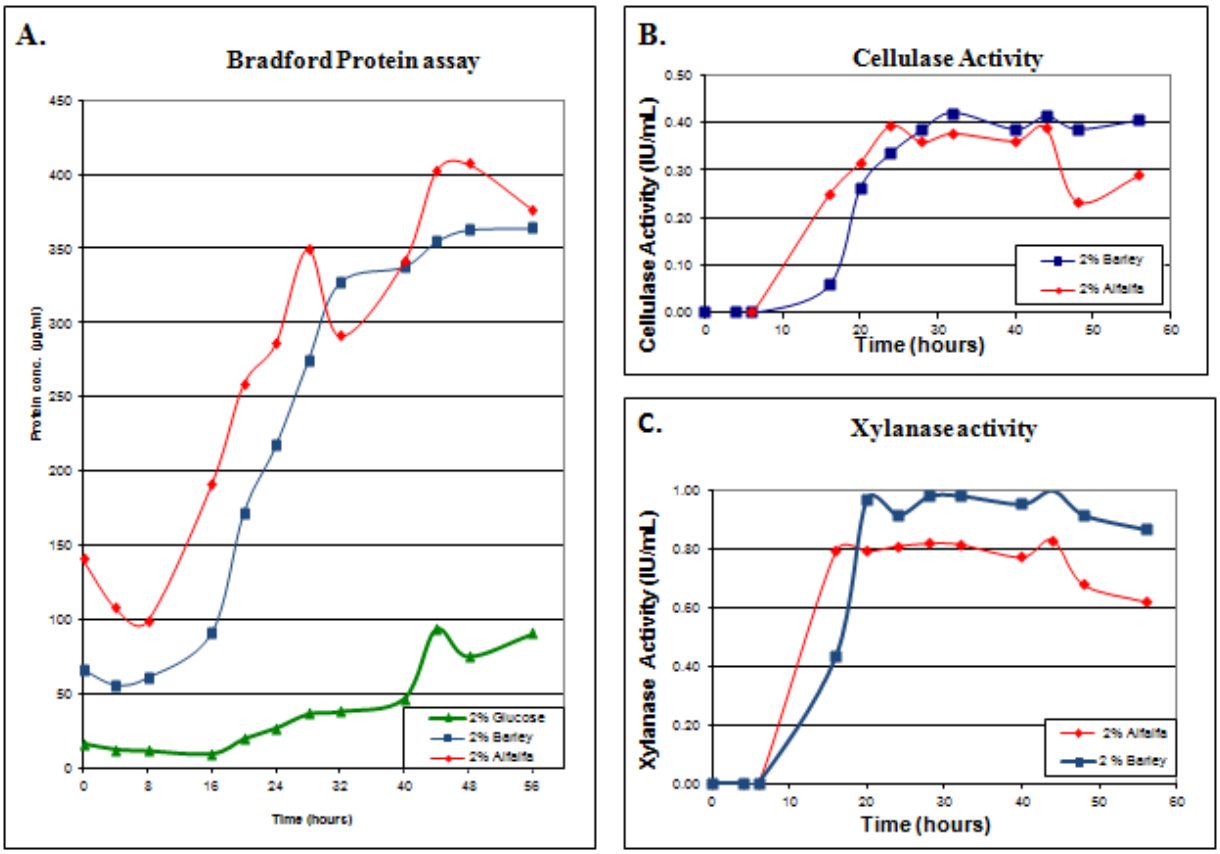



**Figure 8. Protein level and enzymatic activity of *M. thermophila* in liquid cultures**

A. Total protein concentration of *M .thermophila* culture supernatant when grown in 10x TDM containing 2% (w/v) barley, 2% (w/v) alfalfa or 2% (w/v) glucose

B. Cellulase activity of culture supernatant of *M .thermophila* culture supernatant when grown in 10x TDM containing 2% (w/v) barley or 2% (w/v) alfalfa

C. Xylanase activity of culture supernatant of *M .thermophila s* culture supernatant when grown in 10x TDM containing 2% (w/v) barley or 2% (w/v) alfalfa

### 3.2.2 Verification of insertion of alternate promoters into the expression plasmid

In order to verify that the PglaA promoter had been replaced by the alternate promoters in the expression plasmid, colony PCRs were performed. Results show amplification of fragments of sizes corresponding to the expected sizes for each of the alternate promoters indicating that the original PglaA promoter had been replaced as expected. Furthermore, digestions using restrictions enzymes which were performed in order to confirm the findings of the colony PCR. Different restriction enzymes were chosen for each of the constructs such that the expected fragments sizes differed between the expression plasmid containing PglaA and the expression plasmid containing each of the alternate promoters. Results from the colony PCR can be seen in Figure 9 and results of the digestions can be seen in Figure 10. These results show that the replacement of PglaA by alternate promoters in the expression plasmid had occured as intended.

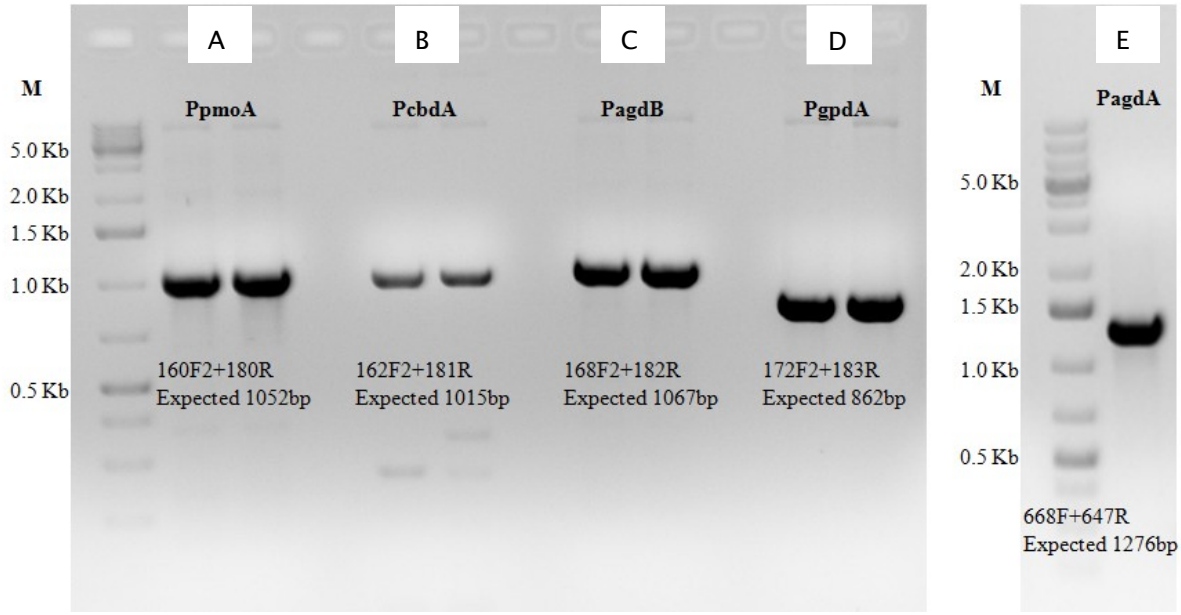



**Figure 9. Verification of proper replacement of PglaA by alternate promoters by colony PCR**

A. Amplification of fragment of expected size of 1052 bp indicates presence of PpmoA in expression plasmid

B. Amplification of fragment of expected size of 1015 bp indicates presence of PcbdA in expression plasmid

C. Amplification of fragment of expected size of 1067 bp indicates presence of PagdB in expression plasmid

D. Amplification of fragment of expected size of 862 bp indicates presence of PgpdA in expression plasmid

E. Amplification of fragment of expected size of 1276 bp indicates presence of PagdA in expression plasmid

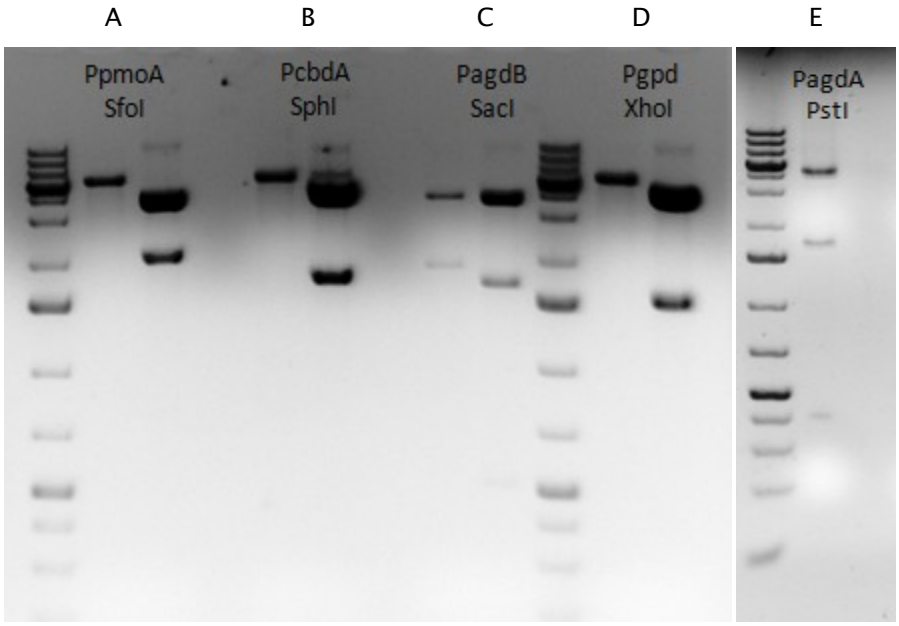



**Figure 10. Verification of insertion of alternate promoters in expression plasmid using restriction enzymes**

A. Enzyme SfoI cuts only once in expression plasmid containing PglaA resulting in one fragment of 5642 bp. The same enzyme cuts twice expression plasmid containing PpmoA resulting in fragments of expected sizes of 2086 bp and 3827 bp.

B. Enzyme SphI cuts only once in expression plasmid containing PglaA resulting in one fragment of 5642 bp. The same enzyme cuts twice in expression plasmid containing PcbdA resulting in fragments of expected sizes of 1595 bp and 4281 bp.

C. Enzyme SacI cuts twice in expression plasmid containing PglaA resulting in fragments of 1869 bp and 3773 bp. The same enzyme cuts three times in expression plasmid containing PagdB resulting in fragments of expected sizes of 1658 bp, 3773 bp and 497 bp.

D. Enzyme XhoI cuts only once in expression plasmid containing PglaA resulting in one fragment of 5642 bp. The same enzyme cuts twice in expression plasmid containing PgpdA resulting in fragments of expected sizes of 4178 bp and 1539 bp.

E. PstI cuts three times in expession plasmid containing PagdA resulting in fragments of expected sizes of 3932 bp, 1674 bp and 375 bp

### 3.2.3 Protoplast generation

In order to assess the ease with which protoplasts could be generated for *M. heterothallica,* as well as determining regeneration rates for this species, some preliminary tests were performed. A preparation of protoplast was obtained by digestion of mycelia from 18-hour cultures. For the digestion of 2.0 g of harvested mycelia, 0.5 g of β-glucanase was used. The above preparations were checked under a microscope every hour in order to access the progress of the digestion. The mycelia was found to be >90% digested after 2.5 - 3 hours at 30°C (Figure 11). The starting 2.0 g of mycelia generated between $10^7$ and $10^8$ protoplasts as determined by cell counts using a haemocytometer. I performed initial tests to evaluate to the regeneration rate of *M. heterothallica* protoplasts on non-selective regeneration media. Results of these tests showed regeneration rates ranging from 3.5% to 30.5% with an average regeneration rate of 18.48% based on the number of total protoplast plated. These numbers are based on regeneration of non-transformed protoplasts and are summarized below (Table 9). Despite relatively low regeneration rates, these results indicated that I would ultimate have enough protoplast able to regenerate for potential transformation.

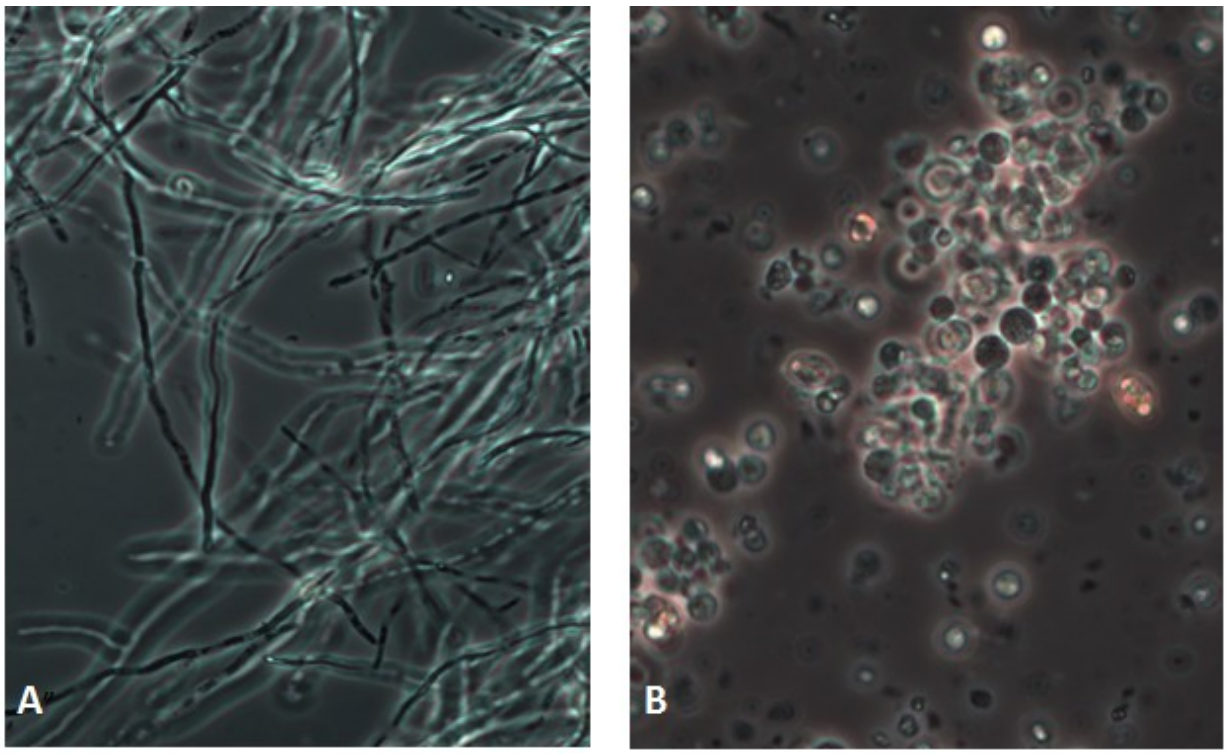



**Figure 11. *M. heterothallica* CBS 375.69 before and after β-glucanase digestion**

A. 18 hours *M. heterothallica* culture in 10x TDM containing 2% (w/v) glucose showing healthy mycelia

B. Generation of protoplast from *M. heterothallica* mycelia; culture after 3 hours digestion at 30°C with β-glucanase

**Table 9. Evaluation of regeneration rates for untransformed protoplasts of *M. heterothallica* on non-selective regeneration media**

| Experiment # | Regeneration rate on NSRM (%) |
|---|---|
| 1 | 24.1 |
| 2 | 27.4 |
| 3 | 30.3 |
| 4 | 8.98 |
| 5 | 3.47 |
| 6 | 21.06 |
| 7 | 14.04 |
| **Average regeneration rate (%)** | **18.48** |

### 3.2.4 Selection of transformants

As a method of selection transformants for *M. heterothallica* and *M. thermophila*, I considered the use of Hygromycin B or Geneticin for positive selection of transformants. I set up kill curves for *Myceliophthora heterothallica* with the antibiotics using 1 x$10^6$ spores/well and increasing dosses of the antibiotics up to 1 mg/ml. Results show that both *M. heterothallica* CBS 375.69 and *M. heterothallica* CBS 202.75 are most sensitive to Hygromycin B. Cultures containing 500 µg/ml or more of this antibiotic showed very little background growth even after a week in liquid culture. Kill curves using Geneticin showed that a full 1 mg/ml concentration was needed in order to control background levels of growth. A summary of results is shown in Table 10. Since the organisms were more sentsitive to Hygromycin B, this antibiotic would have been used for selection over Geneticin if a different option had not been available.

**Table 10. Summary of antibiotic kill curves for *M. heterothallica* CBS 375.69 and *M. heterothallica* CBS 202.75**

| **Hygromycin (µg/ml)** | **0.75** | **1.0** | **1.5** | **3.0** | **5.0** | **7.5** | **10.0** |
|---|---|---|---|---|---|---|---|
| *M. heterothallica* CBS 375.69 | + | + | + | + | - | - | - |
| *M. heterothallica* CBS 202.75 | + | + | + | + | - | - | - |

| **Geneticin (µg/ml)** | **0.75** | **1.0** | **1.5** | **3.0** | **5.0** | **7.5** | **10.0** |
|---|---|---|---|---|---|---|---|
| *M. heterothallica* CBS 375.69 | + | + | + | + | + | + | - |
| *M. heterothallica* CBS 202.75 | + | + | + | + | + | + | - |

\+ Growth observed with use of 100µl of concentration listed in table

\- No growth observed with use of 100µl of concentration listed in table

I also considered using *pyrG* as a selection marker for the selection *M. heterothallica* transformants. Hence, I designed gene replacement vector construct for the *pyrG* gene replacement using acetamide selection as I was considering using *pyrG* selection. When I introduced this linear vector into *M. heterothallica*, transformants were obtained only when acetamide selection alone was applied. Since $pyrG^-$ colonies were not expected to survive without uracil and / or uridine supplementations, both uracil and uridine were added to the acetamide plates. However their addition provided an additional source of nitrogen for the organism. This resulted in a high level of background on the acetamide plates that contained uracil and uridine, although two phenotypically different types of colonies were seen on the double selection plates (Figure 12). Since double selection left me with the inability to distinguish true homologous recombination transformants, negative selection was applied by cherry picking the few colonies which were whiter and fluffier in their appearance and transferring them onto 5'FOA 1 mg/ml plates. Alternately, I resuspended all colonies on the acetamide plus uracil/uridine plates and transferred to the 5'FOA plates with the presumption that only true transformants would survive. Neither of these methods resulted in colonies on the 5'FOA plates. Since I was able to obtain colonies through acetamide selection but not through 5'FOA selection, I assumed that ectopic integration of the vector had occurred rather than the desired gene replacement. Thus my efforts attempting to replace *pyrG* with *amdS* were unsuccessful.

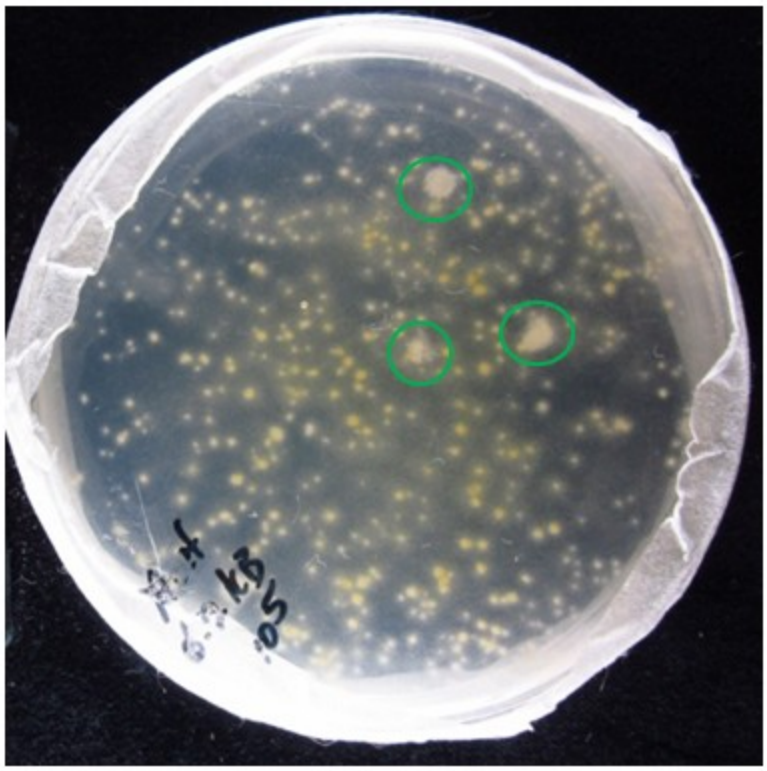


**Figure 12. Two phenotypically different colonies of *M. heterothallica* CBS 375.69 transformants acetamide selection plates**

Transformants growing on acetamide selection plate also containing uracil and uridine for the complementation of *pyrG*$^{-}$ isolates

Ultimately, selection on acetamide alone was shown to be the best method of selection for *Myceliophthora*. Figure 13 shows that *M. heterothallica* was unable to grow when acetamide was the only source of nitrogen provided, whereas the organism flourished when provided with sodium nitrate for nitrogen consumption.

I performed co-transformations using a selection plasmid containing *amdS* and a non-selectable expression plasmid for the expression of genes of interest. These co-transformations yielded transformants which could then be screened on the basis activity to verify integration of the expression plasmid. Positive selection on acetamide plates showed faint background and transformants were easily distinguishable in *M. heterothallica* at three days post transformation. *M. thermophila* showed higher background but I was able to distinguish transformants nonetheless (Figure 14). Thus, the choice of using *amdS* as a selection marker was made.

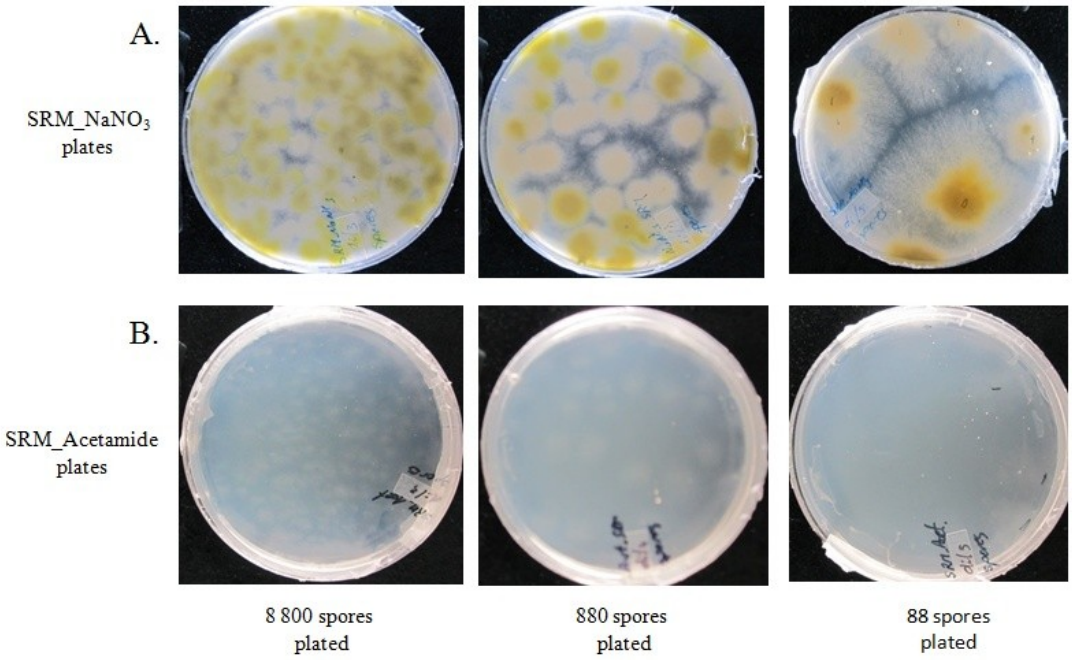



**Figure 13. *M. heterothallica* CBS 375.69 grown on agars containing different nitrogen sources**

A. *M. heterothallica* cultures with $NaNO_3$ as a nitrogen source

B. *M. heterothallica* cultures with acetamide as a nitrogen

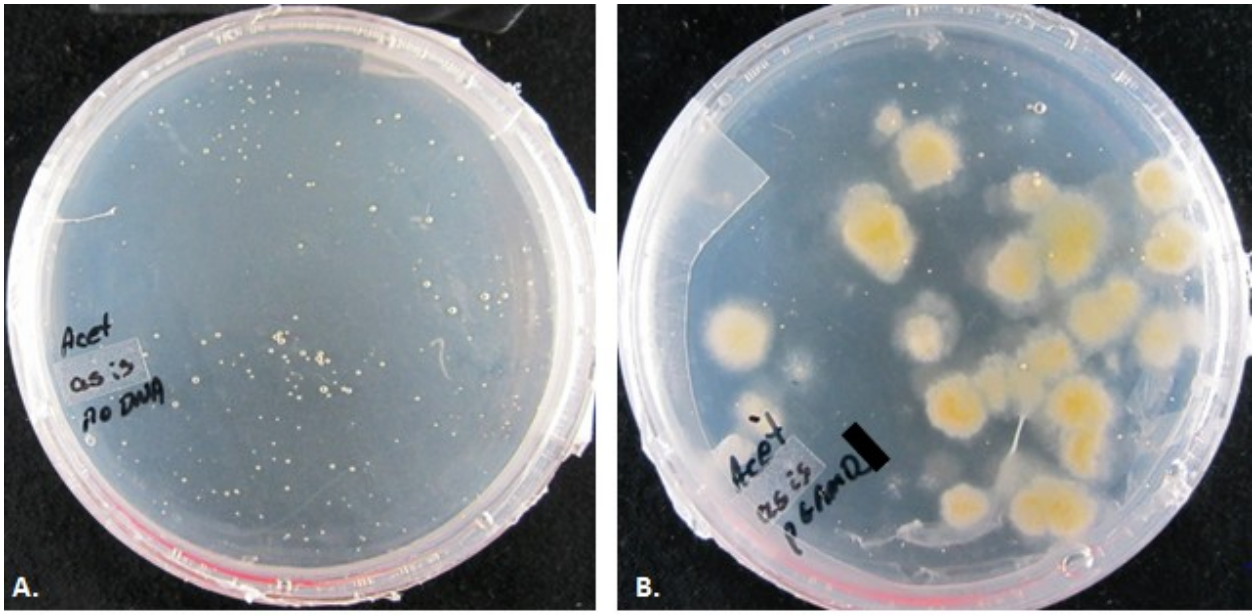



**Figure 14. *M. heterothallica* CBS 375.69 transformed protoplasts on selective regeneration plates**

A. *M. heterothallica* protoplasts transformed with no DNA and regenerated on acetamide plates

B. *M. heterothallica* protoplasts transformed with pGAMD and regenerated on acetamide plates

### 3.2.5 Transformation of *M. heterothallica* and *M. thermophila*

In my transformation experiments, regeneration rates on non-selective regeneration media (NSRM) decreased in protoplasts having undergone the transformation protocol as compared to those which did not undergo manipulation other than isolation. This reduction can be attributed to damage caused to the cell membrane of the protoplasts by the PEG solution and by loss of cells with each wash. Although *M. heterothallica* CBS 375.69 was the best performer among the strains tried, overall transformation efficiency remained low. Table 11 provides a summary of results for the strains used. The average transformation efficiency was calculated from the number of transformants obtained on selective regeneration media (SRM) per µg of the selection plasmid pGAMD used.

**Table 11. Regeneration rates and transformation efficiencies for *M. heterothallica* and *M. thermophila***

| | % Regeneration rate on NSRM | Transformation Efficiency (#Transformants/µg of selection plasmid) | # of Transformations Performed |
|---|---|---|---|
| ***M. heterothallica* CBS 375.75** | 6.15 ± 6.74 | 15.34 ± 10.06 | 14 |
| ***M. heterothallica* CBS 202.75** | 6.47 ± 5.39 | 0.99 ± 0.46 | 7 |
| ***M. thermophila* ATCC 42464** | n/a | 4.28 ± 2.38 | 7 |

Since uptake of the selection vector does not guarantee uptake of the non-selectable plasmid, the co-transformation rate was assessed using selected isolates from the acetamide selection plates. The gDNA was extracted using mycelia from these isolates and was used to test for both the presence of the *amdS* selection vector and for the presence of the gene of interest in the expression vector. Co-transformation rates as assessed by colony PCR are shown in Figure 15. This experiment was performed on three different batches of transformants. In all of the 24 transformants verified, both plasmids were confirmed by PCR to be present. Since these results showed that co-transformation rates approach 100% for this strain, I decided to screen transformants on the basis of xylanase activity only for the presence of the gene of interest.

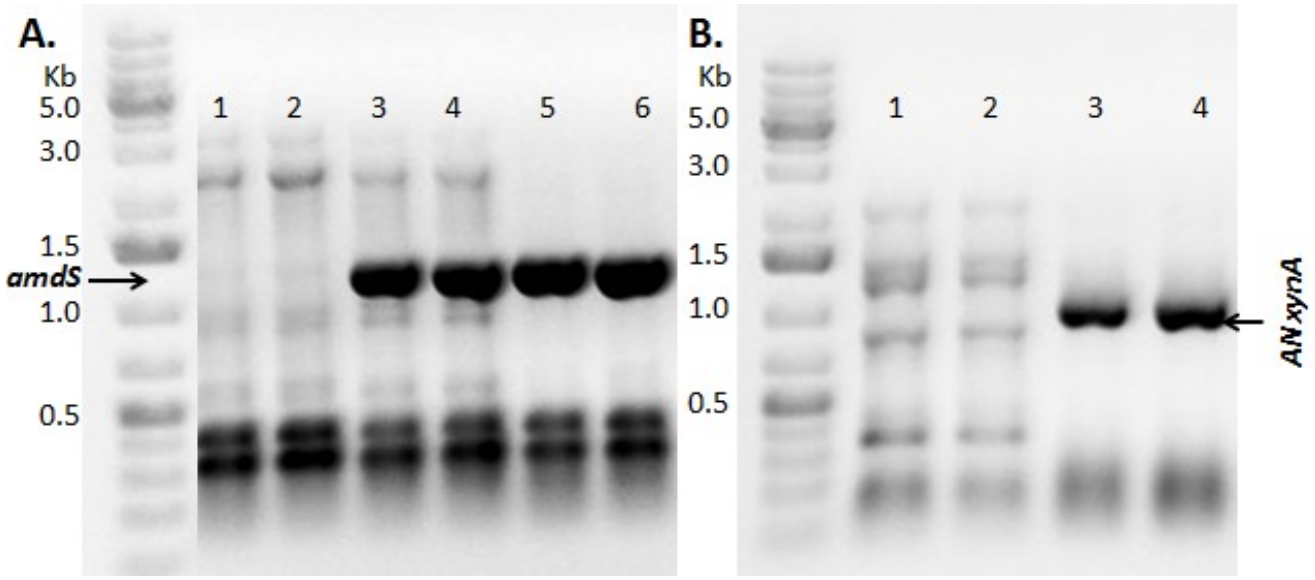



**Figure 15. Verification of plasmid insertions**

A. Verification of insertion of selection plasmid using *amdS* internal primers

Lanes 1&2 Non transformed *M. heterothallica* control

Lanes 3&4 *M. heterothallica* transformed using selection plasmid only

Lanes 5&6 *M. heterothallica* transformed using selection and expression plasmids

B. Verification of insertion of expression plasmid using primers flanking *ANxynA* on the expression plasmid

Lanes 1&2 Non transformed *M. heterothallica* control

Lanes 3&4 Verification of insertion of expression plasmid using primers flanking *ANxynA* on the expression plasmid

All pairs for each of the above verifications are technical replicates.

In order to verify the stability of the insertion, I passaged several transformants for three generations on acetamide selection plates. A second analysis by PCR amplification of the transforming plasmids confirmed the presence of both plasmids in all third-generation transformants as can be seen in Figure 16. Note that although one sample failed to show a band corresponding to the presence of *amdS* in Figure 16A section III, that lane is a technical replicate of lane 5, thus the presence of selection plasmid in that transformant is confirmed. In addition, that particular transformant continued to thrive on acetamide plates. I performed similar stability experiments on transformants in which xylanases from other organisms were introduced into *M. heterothallica*. Only one transformant, in which the presence of a xylanase from *M. thermophila* was initially confirmed, was no longer detected after three passages on acetamide selection plates (Figure 16). Overall, these results indicate that the insertion of the plasmids into the host genome are fairly stable.

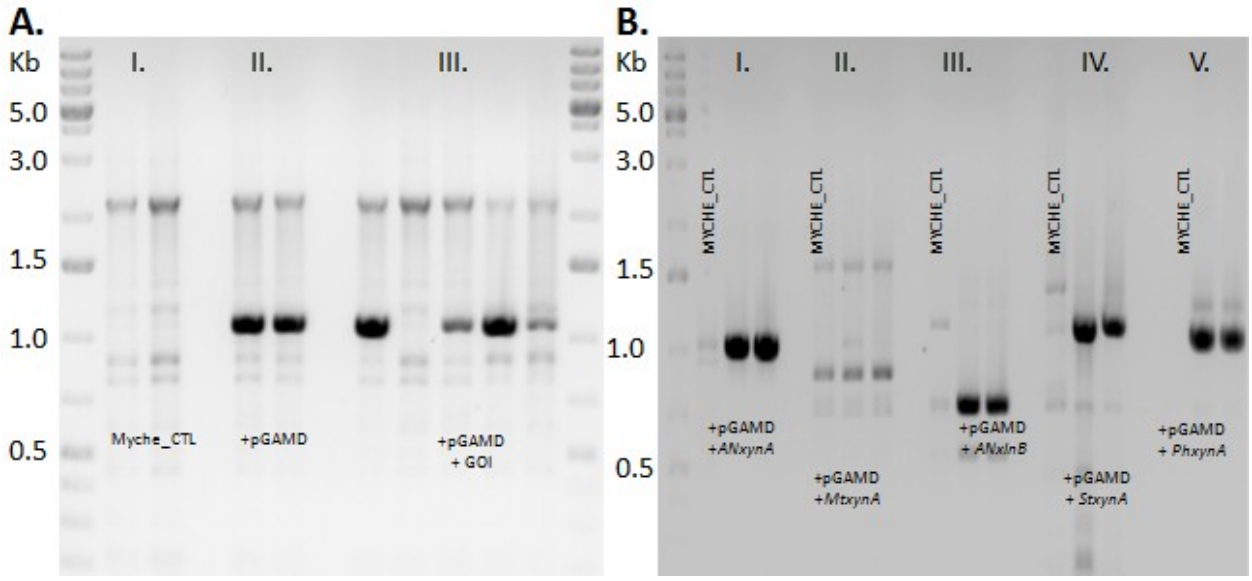



**Figure 16. Stability of insertion**

A. Verification of stability of selection plasmid using *amdS* after 3 passages on selective media

I. Technical replicates of non transformed *M. heterothallica* control

II. Technical replicates of *M. heterothallica* transformed using selection plasmid only

III. *M. heterothallica* transformed using selection and expression plasmids. The first two lanes are technical replicates, as are the third and fourth lane. The last sample in this category does not contain a technical replicate.

B. Verification of stability of expression plasmid using primers flanking genes of interest after 3 passages on selective media

I.-V. *M. heterothallica* transformed using selection plasmid and expression plasmids containing different genes of interest. The first position in the set is always a non-transformed control and the next two positions for each gene of interest are technical replicates of each other.

### 3.2.6 Use of the maltose inducible promoter PglaA from *A. niger* in the expression plasmid

Since the original Anip7 plasmid contained the glucoamylase promoter PglaA from *A. niger*, it was the first promoter to be tried in the expression cassettes which I used for the transformation of *M. heterothallica* CBS 202.75 and *M. heterothallica* CBS 375.69. In all of my initial plasmid constructs, the *glaA* promoter was fused to a different xylanase. The xylanases used in the expression plasmids were a *TtxynA* gene, a *TtxynB* gene, a *TtxynC* gene and a *ANxynA* gene. Co-transformation using each of these plasmids along with pGAMD for selection yielded variable numbers of transformants as shown in Table 12. It must be noted that for transformations with the plasmids containing a *T. terrestris* xylanase, I used a total of 21 - 29 µg of transforming DNA with a selection plasmid to expression plasmid ratio of 3:1. For all other transformations a 1:1 ratio between the two plasmids was respected with 5 µg of the selection plasmid and 5 µg of the expression plasmid being used. My goal was to see whether increasing amounts of transforming expression DNA would results in a higher yield of the protein of interest. Instead the number of selectable transformants seems to have decreased due to the change in the usual ratio, however the decrease may also be attributed to the change in *M. heterothallica* strain used for the transformation (See Table 12). Ultimately, the original 1:1 ration between the selection plasmid and expression plasmid was used for all subsequent experiments.

**Table 12. Summary of transformation trials using vectors containing PglaA**

| Organism used | Expression Construct used | Selection plasmid to Expression plasmid ratio | # of Transformants obtained |
|---|---|---|---|
| *M. heterothallica* CBS 375.69 | PglaA - *ANxynA* | 1:1 | 77 |
| *M. heterothallica* CBS 202.75 | PglaA - *TtxynA* | 1:3 | 6 |
| *M. heterothallica* CBS 202.75 | PglaA - *TtxynB* | 1:3 | 7 |
| *M. heterothallica* CBS 202.75 | PglaA - *TtxynC* | 1:3 | 5 |

To verify whether the isolates growing on the selection plates were able to produced the heterologous xylanase, I grew selected isolates using culture media containing the inducer. The isolates transformed with plasmids containing the *glaA* maltose inducible promoter were grown both in 10x TDM containing 2% (w/v) maltose and 15% (w/v) maltose. I used culture supernatants which were cleared by centrifugation to assess protein content by Bradford assay. Total protein levels remained below 100 µg/ml of total protein in both culture conditions. I assayed the supernatant from selected isolates for activity using both a xylanase spot assay and BCA xylanase assay. The xylanase spot assay failed to detect any candidates showing a greater level of xylanase activity than the control. These results were confirmed by BCA xylanase assay. In order to verify that the protein of interest was not been produced but simply inactive, I did a TCA precipitation of the protein in the supernatant. I then loaded 12 µg of each sample onto an SDS-PAGE gel and stained the gels using 0.1% (w/v) Coomassie Brilliant Blue. As shown below (Figure 17), the gels did not reveal bands corresponding to the size of the proteins of interest, thus confirming that the heterologous protein of interest was not present in the culture supernatants of isolates tested.

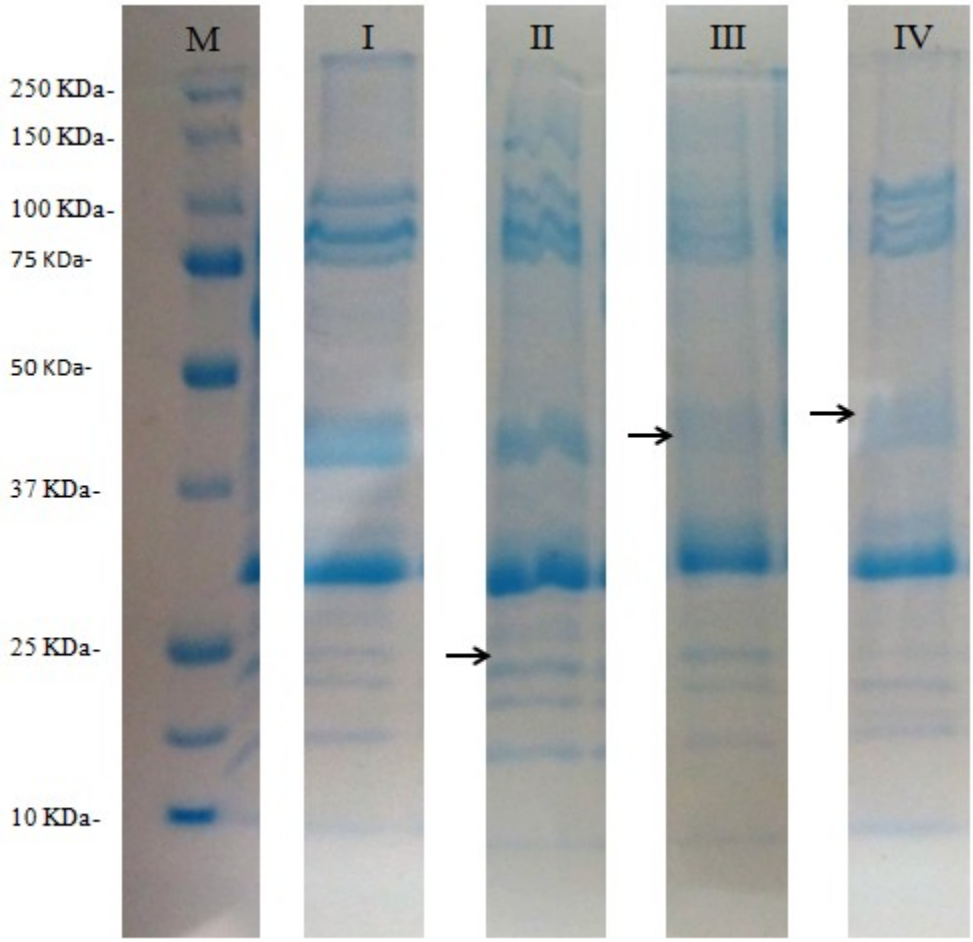



**Figure 17. SDS_PAGE: Supernatants of isolates transformed with vectors containing PglaA**

M. Molecular ladder showing protein bands of known sizes

I. *M. heterothallica* CBS 202.75 transformed with pGAMD

II. *M. heterothallica* CBS 202.75 transformed with pGAMD + PglaA - *MtxynA*; Arrow indicates position of expected band corresponding to size of heterologous protein of 24.34 KDa

III *M. heterothallica* CBS 202.75 transformed with pGAMD + PglaA - *MtxynB*; Arrow indicates position of expected band corresponding to size of heterologous protein of 41.05 KDa

IV. *M. heterothallica* CBS 202.75 transformed with pGAMD + PglaA - *MtxynC*; Arrow indicates position of expected band corresponding to size of heterologous protein of 42.20 KDa

### 3.2.7 Use of *Myceliophthora* promoters in the expression plasmid

In order to assess whether promoters within the same genus as the host organism would give rise to the production of proteins of interest, the next set of promoters which I considered were all from the genus *Myceliophthora.* PgpdA, the glyceraldehyde 3-phosphate dehydrogenase constitutive promoter from *M. thermophila* was among that set. Since *Myceliophthora* was originally selected as a host organism in part due to due to its ability to produce and secrete copious amounts of cellulases, it was imperative that I try using cellulose-inducible promoters as a tool for production of heterologous proteins. PpmoA, a monooxygenase promoter and PcbdA, a cellobiose dehydrogenase promoter, also both native to *M. thermophila* were hence selected. Both of the corresponding genes showed high levels of expression under any growth conditions in which cellulose was readily available such as CMC or Solka-Floc® [45]. Each of the above promoters was used to induce expression of a GH10 xylanase from *A. niger* (*ANxynA*). In addition, both *M. thermophila* and *M. heterothallica* were transformed using the PpmoA - *ANxynA* and PcbdA - *ANxynA* constructs along with the pGAMD selection plasmid. The last two promoters which I assessed were the GH13 α-glycosidase sucrose-inducible promoters from both *M. thermophila* and *M. heterothallica.* I transformed *M. heterothallica* CBS 375.69 using plasmids containing PagdA from *M. thermophila* to induce expression of five different heterologous xylanases. The expression plasmids used for transformation contained PagdA - *ANxynA*, PagdA - *MtxynA*, PagdA - *ANxynB*, PagdA - *StxynA*, or PagdA - *PhxynA* constructs. All transformations were performed using the pGAMD selection plasmid as well as one of the expression plasmids listed above. Expression of heterologous *ANxynA* and *MtxynA* was also attempted using α-glucosidase promoter from *M. heterothallica* (PagdB). I conducted transformation experiments using *M. thermophila* ATCC 42464, *M. heterothallica* CBS 375.69, and *M. heterothallica* CBS 202.75 as host organisms. The number of transformants obtained on acetamide selection plates from each of the transformation experiments can be seen in Table 13.

**Table 13. Number of transformants obtained on acetamide selection plates for each host strain**

| Expression plasmid construct | *M. thermophila* ATCC 42464 | *M. heterothallica* CBS 375.69 | *M. heterothallica* CBS 202.75 |
|---|---|---|---|
| **PgpdA - *ANxynA*** | 26 | 72 | n/a |
| **PpmoA - *ANxynA*** | 26 | 70 | n/a |
| **PcbdA - *ANxynA*** | 33 | 75 | n/a |
| **PagdA - *ANxynA*** | n/a | 56 | n/a |
| **PagdA - *MtxynA*** | n/a | 160 | n/a |
| **PagdA - *ANxynB*** | n/a | 128 | n/a |
| **PagdA - *StxynA*** | n/a | 71 | n/a |
| **PagdA - *PhxynA*** | n/a | 51 | n/a |
| **PagdB - *ANxynA*** | 28 | 25 | 3 |
| **PagdB - *MtxynA*** | 9 | 16 | 3 |

In order to test whether the transformants were able to produce the heterologous proteins when induced, selected isolates were grown using the appropriate culture media. For transformants obtained which contained the *gpdA* promoter in the expression plasmid, I grew selected isolates in stationary 10x TDM cultures containing 2% (w/v) glucose. Transformants which contained cellulose-inducible promoters PpmoA and PcbdA, were grown in 10x TDM containing 2% (w/v) CMC. As a control they were also grown in parallel in non-inducible culture media 10x TDM containing 2% (w/v) glucose. Both types of culture media for the PpmoA and PcbdA transformants contained wells where no growth was seen at all and may be due poor inoculations since isolates contained mostly mycelia with very little sporulation. To screen for production and secretion of heterologous xylanases in transformants containing the PagdA or PagdB constructs, isolates were grown in 10x TDM containing 2% (w/v) sucrose. For PagdB, the same isolates were grown in parallel in 10x TDM containing 10% (w/v) sucrose in order to determine whether higher activity levels of the heterologous proteins could be seen with higher concentration of the inducer in the media. Culture supernatants were sampled from day four to day seven post inoculation and centrifuged in order to obtain cleared supernatants free of spores or mycelia. Cleared supernatants were tested for xylanase activity both by xylanase spot assay and by BCA assay and total protein levels were assessed by Bradford assay. The number of isolates screened by xylanase spot assay for each transformation and the number of possible positive candidates obtained in each assay can be seen in Table 14.

**Table 14. Candidates detected by xylanase spot assay**

| | *M. thermophila* ATCC 42464 | | *M. heterothallica* CBS 375.69 | | *M. heterothallica* CBS 202.75 | |
|---|---|---|---|---|---|---|
| **Expression plasmid construct** | **Isolates screened** | **+ve candidates** | **Isolates screened** | **+ve candidates** | **Isolates screened** | **+ve candidates** |
| **PgpdA - *ANxynA*** | 20 | 0 | 24 | 6 | - | - |
| **PpmoA - *ANxynA*** | 24 | not distinguished from WT | 24 | not distinguished from WT | - | - |
| **PcbdA - *ANxynA*** | 24 | not distinguished from WT | 24 | not distinguished from WT | - | - |
| **PagdA - *ANxynA*** | - | - | 12 | 3 | - | - |
| **PagdA - *MtxynA*** | - | - | 12 | 2 | - | - |
| **PagdA - *ANxynB*** | - | - | 12 | 2 | - | - |
| **PagdA - *StxynA*** | - | - | 12 | 1 | - | - |
| **PagdA - *PhxynA*** | - | - | 12 | 2 | - | - |
| **PagdB - *ANxynA*** | 12 | 2 | 12 | 4 | 3 | 0 |
| **PagdB - *MtxynA*** | 9 | 1 | 12 | 0 | 3 | 0 |

To evaluate whether to transformed isolates produced increased amounts of total protein, Bradford protein assays were done using culture supernatants of the isolates. In culture supernatants from isolates containing the PgpdA - *ANxynA* total protein levels varied from 43 ng/µl to 139 ng/µl, which was no higher than protein level found in culture supernatants of control isolates. No positive candidates were identified using the xylanase spot assay for *M. thermophila,* however in *M. heterothallica* 6 cultures seemed to show increased activity (Figure 18). I was unconvinced by these results since one of the pGAMD only controls also showed the same level of xylanase activity as estimated by the size of the clearing zone on the RBB xylan plate. For this reason, I then regrew these 6 candidates in 10x TDM containing 2% (w/v) glucose and retested for activity. Results from the xylanase spot assay using 3µl of cleared supernatant showed no xylanase activity this time.

When 10x TDM containing 2% (w/v) CMC was used as a culture media for transformants containing PpmoA and PcbdA, very large clearing zones appeared on the RBB xylan plates in all wells in which growth had been observed. Since the clearing zone were so large, individuals samples were indistinguishable from each other. I then did a 1 in 25 dilution of the supernatants- and respotted fresh RBB xylan plates for easier visualization of results. Unfortunately, all samples including the controls showed equal xylanase activity making it impossible to differentiate transformants producing the *A. niger* xylanase *ANxynA* along with native xylanases from isolates producing only native xylanases (Figure 19). This holds true for isolates transformed with constructs containing either the PpmoA promoter or the PcbdA promoter. The samples in which no activity is seen, correlate to wells of the stationary culture which failed to grow. Since the xylanase spot assay was not sensitive enough to differentiate isolates producing the *A. niger* xylanase, I also screened using a BCA xylanase assay whereby activity was determined by comparing the total amount reducing sugars present in the reaction mixture before and after an incubation at 40°C in the presence of extracellular xylanases. One unit of xylanase activity was defined as the quantity of enzyme required to liberate 1 µmol of xylose equivalent per minute at 40°C. Xylanase activity of supernatants from transformants containing either PpmoA - *ANxynA* or PcbdA - *ANxynA* with the pGAMD selection plasmid were tested for increased activity compared to activity of supernatants from isolates transformed with pGAMD only. Culture conditions were identical for all groups. None of the selected PpmoA - *ANxynA* or PcbdA - *ANxynA* candidates showed increased xylanase activity when

assessed by BCA assay either.

In addition, I precipitated proteins by TCA and loaded the resuspended samples on an SDS-PAGE gel to verify whether the protein of interest could be located on the gel. The supernatants of the shake cultures from the 6 candidates were used. Although I intended to load 15 µg of protein per sample, this amount was not attainable for all samples since the total protein content in some of these cultures was so low. The gels did not reveal a band corresponding to the protein of interest.

In order to verify that the protein of interest was not present in amounts too low to be detected on an SDS-PAGE gel, I sent the supernatant samples to be analyzed by mass spectrometry. The GH10 xylanase from *A. niger* ANXynA was not detected in any of the samples by this method either.

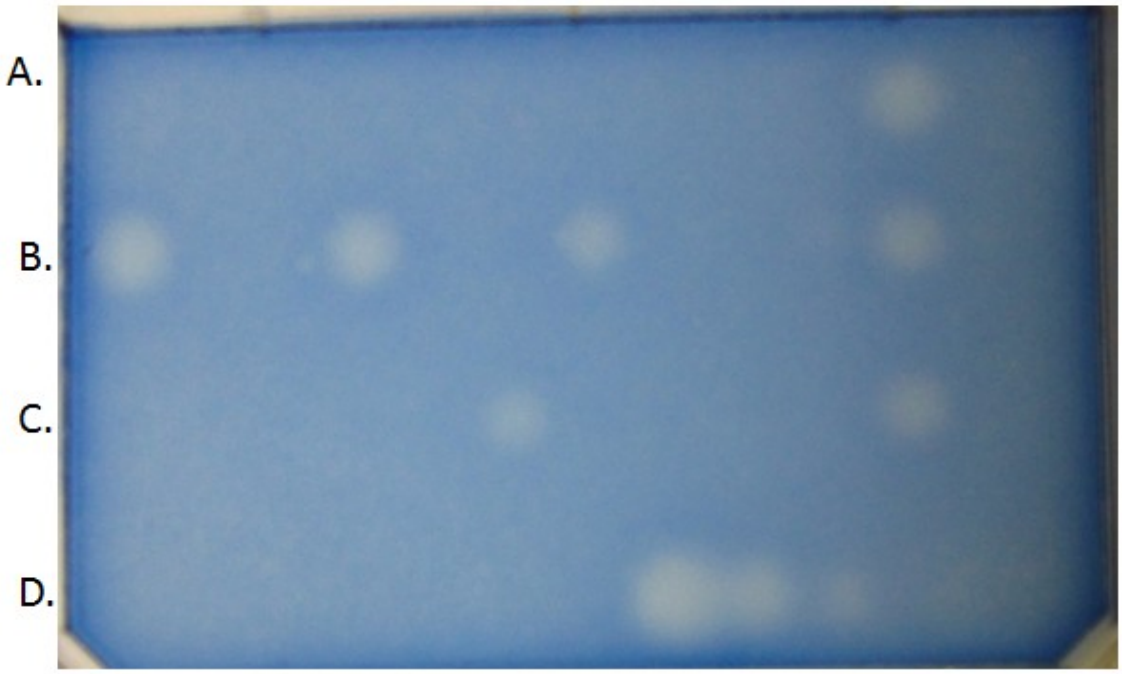



**Figure 18. Xylanase spot assay on RBB xylan plate: PgpdA transformants**

A. Supernatants from cultures of *Myceliophthora thermophila* isolates transformed with the selection plasmid only.

B.&C. Supernatants from cultures of *Myceliophthora thermophila* isolates transformed with the selection plasmid and with the PgpdA - *ANxynA* expression plasmid.

D. In house xylanase control with 1 in 5 serial dilutions

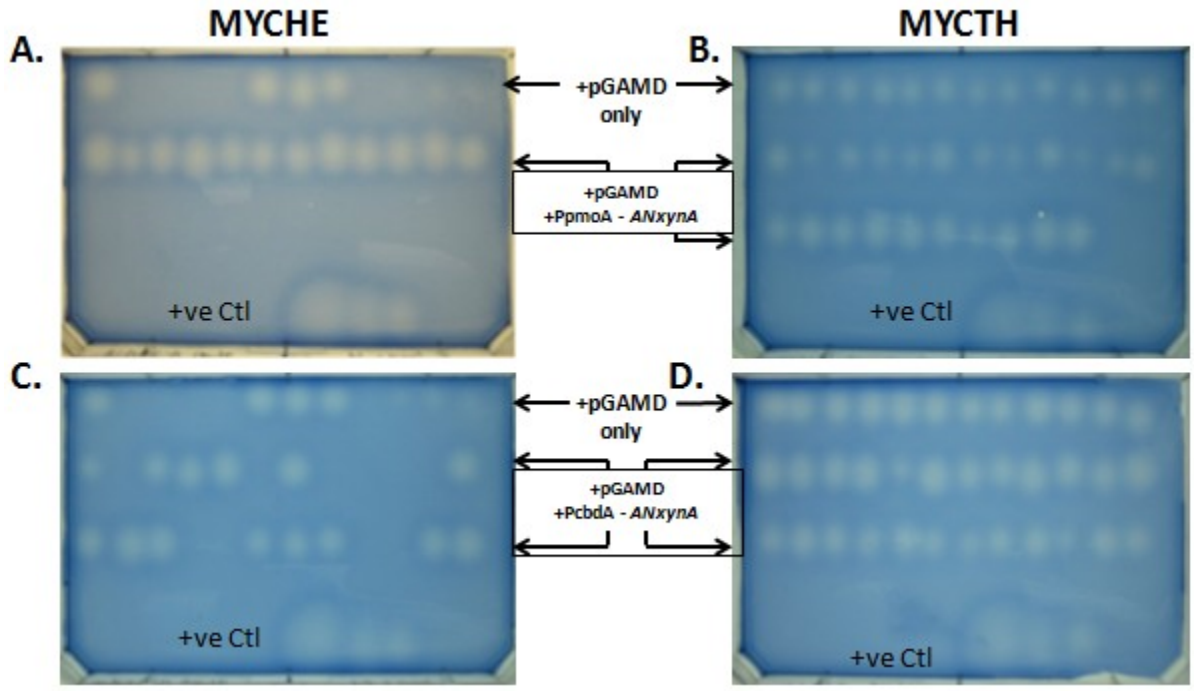



**Figure 19. Xylanase spot assay on RBB xylan plates: PpmoA and PcbdA transformants**

3µl of 1/25 diluted supernatants from cultures of isolates grown on 10x TDM containing 2% (w/v) CMC were spotted on RBB plates

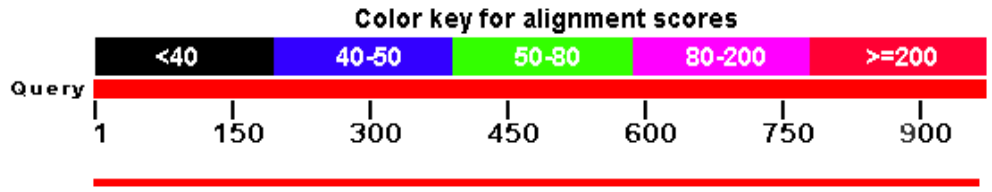
A. *M. heterothallica* CBS 375.69 PpmoA - *ANxynA* candidate transformants

B. *M. thermophila* CBS 375.69 PcbdA - *ANxynA* candidate transformants

C. *M. heterothallica* CBS 375.69 PpmoA - *ANxynA* candidate transformants

*D. M. thermophila* CBS 375.69 PcbdA - *ANxynA* candidate transformants

I also evaluated the GH13 α-glycosidase sucrose-inducible promoters from both *M. thermophila* and *M. heterothallica* since expression data showed high levels of α-glucosidase when induced by sucrose (Concordia University, Dr. Tsang's lab, unpublished data). I compared the 1 Kb region upstream of the start codon of GH13 α-glucosidase in *M. heterothallica* against the *M. thermophila* ATCC 42464 databases at JGI. This was done to assure that the promoter region from the intended GH13 α-glucosidase in *M. heterothallica* had been selected since both species contains many GH13s, two of which sit on chromosome 3. The greatest homology of the *M. heterothallica* 1 Kb α-glucosidase promoter region was with the promoter PagdA, the α-glucosidase residing in the telomeric region of chromosome 3. Results show 87% identity between the promoter regions of the two species. The alignment also shows 7% gap between the two sequences (Figure 20).




Myceliophthora thermophila ATCC 42464 chromosome 3, complete sequence

Sequence ID: gb|CP003004.1|Length: 5062665Number of Matches: 7

Related Information

Range 1: 4608 to 5532GenBankGraphicsNext MatchPrevious Match

| Score | Expect | Identities | Gaps |
|---|---|---|---|
| 1213 bits(1344) | 0.0 | 856/980(87%) | 75/980(7%) |

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Query  112   TACCTCATTGACACGTGCTGGTTA---CTTTGAATCGGGAACCGAGAGGGGAAGACCCGG  168
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Query  169   GGTTCGCATTCCCCATGGGGCCGCGAGCCATATCAGGGGATAAGTGGGGACTGCCCAACT  228
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Query  229   ATAATCCGTATACTACGGAGCGTATATGCGGATTACATACATACAAGACCCGGTCGTGTC  288
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Query  409   CTAACACGCGGTAGTGTAGAATTTGCTGTCGGCCCGACATAGGACCAATTAAAACGTAAG  468
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Sbjct  5147  ----CACGCGACAGTGTAGGATTTGCTGTCGGCCCGACATAGGACCAATCAAAACGTAAG  5092

Query  469   TTTGTCGGTCCGACAATAGACGAATCACACATGCAGCTTGCCAATAACTGACCCCTCTTG  528
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Query  529   TAGCGTCTCTGAATTGCCATTCCTGGTAGCGCCATTCC-CCAACCATTA------GAATA  581
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Query  702   GGTGCTATGAGAGGCCGTGGGTGGACTAGGCGTGGTGTTGCTCCAGCTGCGCCTGTACAT  761
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Sbjct  4627  AATTTCCATTCATTTTTTTT  4608
```

**Figure 20. Blast results of 1 Kb region upstream of GH13 α-glucosidase from *M. heterothallica* CBS375.69 against *M. thermophila* ATCC42464 database**

Homology between promoter region of GH13 MYCTH_2303065 and promoter region of GH13 α-glucosidase in *M. heterothallica* shows over 87% identity but contains gaps.

The xylanase spot assay performed to assess xylanase activity show clearing zones of increased diameter for a few transformants containing the PagdA promoter from *M. thermophila.* Results from the xylanase spot assays can be seen below (Figure 21). I also performed a BCA xylanase assay on the supernatants of the candidates having PagdA as a promoter. Disappointingly, the isolate having the largest clearing zone in the spotting assay did not show increased xylanase activity in the BCA assay.

The xylanase spot assay revealed candidates showing clearing zones of increased diameter for a number of isolates containing the PagdB promoter region from *M. heterothallica* as well. Selected results from the xylanase spot assays can be seen below (Figure 22). Based on day 2 to day 6 RBB xylan plate results, I selected candidates to be regrown in liquid shake cultures in 25 ml flask containing 10x TDM containing 10% (w/v) sucrose. Cleared supernatants were re-spotted on RBB xylan plates and used for a BCA assay in order to confirm increased xylanase activity in selected candidates from the spot assay. The *M. thermophila* transformant #9 containing PagdB - *ANxynA* which gave by far the largest clearing zone on the RBB xylan plate, also gave the highest values for xylanase activity in the BCA assay. For this sample 259 mU/ml of xylanase activity was detected compared to no detectable activity in samples containing only the selection vector. Increased xylanase activity in two *M. heterothallica* transformants, #3 and #16 containing PagdB - *ANxynA* was also confirmed.

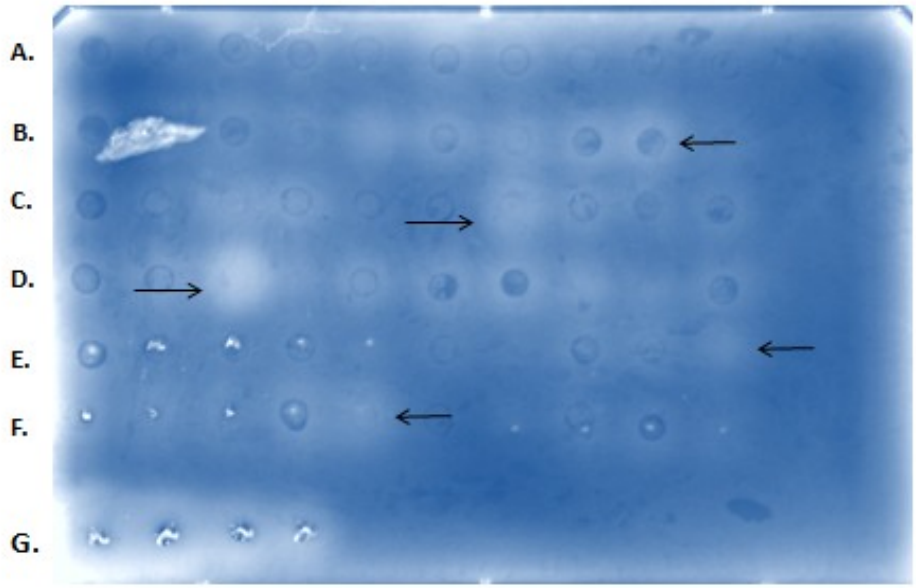


**Figure 21. Xylanase spot assay on RBB xylan plate: PagdA transformants**

3µl cleared supernatants from cultures of isolates grown on 10x TDM with 2% (w/v) sucrose and were spotted on RBB plates; Transformants contain expression plasmids with the GH13 α-glucosidase promoter (PagdA) from *M. thermophila*. The arrows indicate the isolates showing the most xylanase activity for each of the genes of interest.

A. *M. heterothallica* CBS 375.69 transformed with pGAMD only
B. *M. heterothallica* CBS 375.69 PagdA - *ANxynA* candidate transformants
C. *M. heterothallica* CBS 375.69 PagdA - *MtxynA* candidate transformants
D. *M. heterothallica* CBS 375.69 PagdA - *ANxynB* candidate transformants
E. *M. heterothallica* CBS 375.69 PagdA -*StxynA* candidate transformants
F. *M. heterothallica* CBS 375.69 PagdA -*PhxynA* candidate transformants
G. In house xylanase; positive control with 1/5 serial dilutions


**Figure 22. Xylanase spot assay on RBB xylan plate: PagdB transformants**

3µl cleared supernatants from cultures of isolates grown on 10x TDM containing 10% (w/v) sucrose and were spotted on RBB plates; Transformants contain expression plasmids with the GH13 α-glucosidase promoter (PagdB) from *M. heterothallica*.

A1 *M. thermophila* transformed with pGAMD only

A2 *M. thermophila* transformed with PagdB - *ANxynA*

A3 *M. thermophila* transformed with PagdB - *MtxynA*

B1 *M. heterothallica* CBS 375.69 transformed with pGAMD only

B2 *M. heterothallica* CBS 375.69 transformed with PagdB - *ANxynA*

B3 *M. heterothallica* CBS 375.69 transformed with PagdB - *MtxynA*

C1 *M. heterothallica* CBS 202.75 transformed with PagdB - *ANxynA* or PagdB - *MtxynA*

D1 In house xylanase; positive control with 1/5 serial dilutions

In order to confirm that this activity was truly induced by sucrose, I cleared supernatants were tested for xylanase activity both by spot assay and by BCA assay. As I expected, samples grown in glucose failed to show any activity. RBB xylan spot assay results clearly indicate that xylanase activity is induced by sucrose only in the transformants containing the PagdB - *ANxynA* plasmid but fails to be activated under transformants containing the selection plasmid only (Figure 23). The BCA xylan assays done with the same culture supernatants confirmed these results (Figure 24). At 24 hours post induction a 82.4 and a 77.8 fold increase in xylanase activity for isolates #16 and isolate #3 respectively, could be seen for the isolates containing PagdB - *ANxynA* construct compared to the control isolate transformed with only the selection vector. Although both the BCA and Xylanase spot assay clearly indicate that transformants produce the xylanase of interest, the protein remains elusive on an SDS-PAGE gel. I precipitated proteins in the supernatants of the RBB xylan candidates by TCA and loaded onto 12% (v/v) acrylamide SDS-PAGE gels. The heterologous protein from *A. niger* (ANXynA) had an expected size of 35.49 KDa and the xylanase from *M. thermophila* (MtXynA) of 24.21 KDa. Despite the sensitivity of silver nitrate staining, gels loaded with 2µg total protein per well, failed to reveal a band which could potentially be the heterologous proteins (Figure 25). Results of zymograms done using non denatured supernatants from confirmed transformants failed to expose the position of the protein. These results indicate that heterologous protein production induced with the α-glucosidase promoters remains low in the host organism *Myceliophthora*.

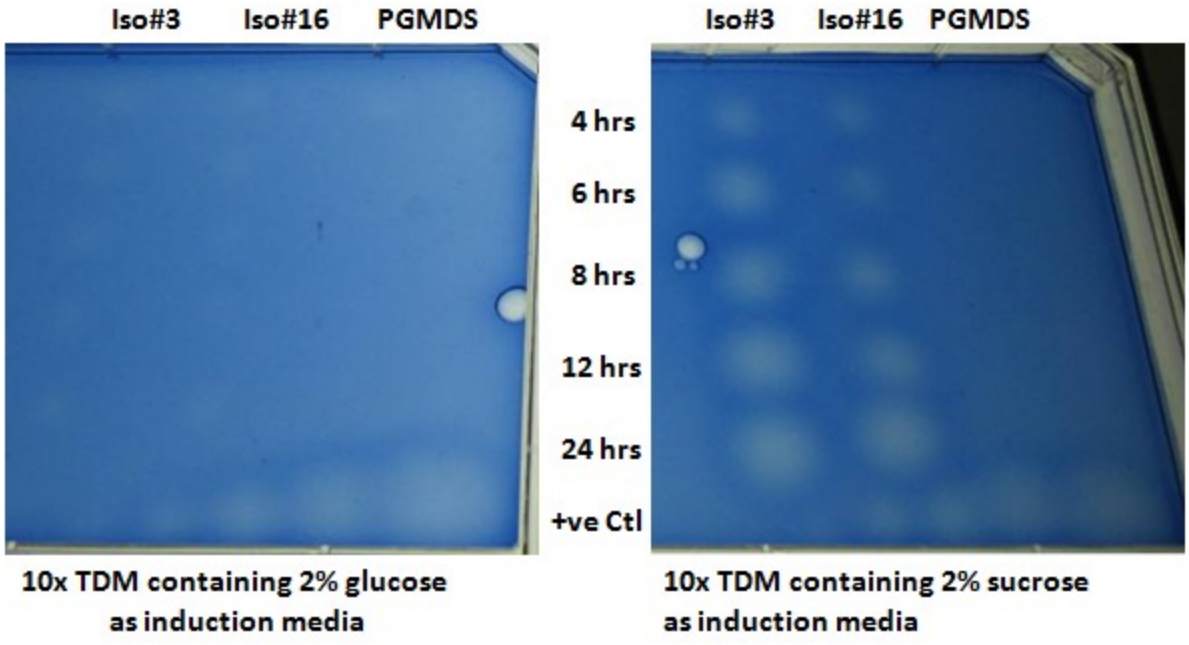


**Figure 23. Induction assay for *M. heterothallica* CBS 375.69 transformants in glucose versus sucrose**

*M. heterothallica* CBS 375.69 transformants containing with PGAMD + PagdB - *ANxynA* or PGMDS alone were grown 24 hours in 10x TDM containing 2% (w/v) glucose followed by induction in fresh 10x TDM containing 2% (w/v) glucose versus induction by 10x TDM containing 2% (w/v) sucrose.

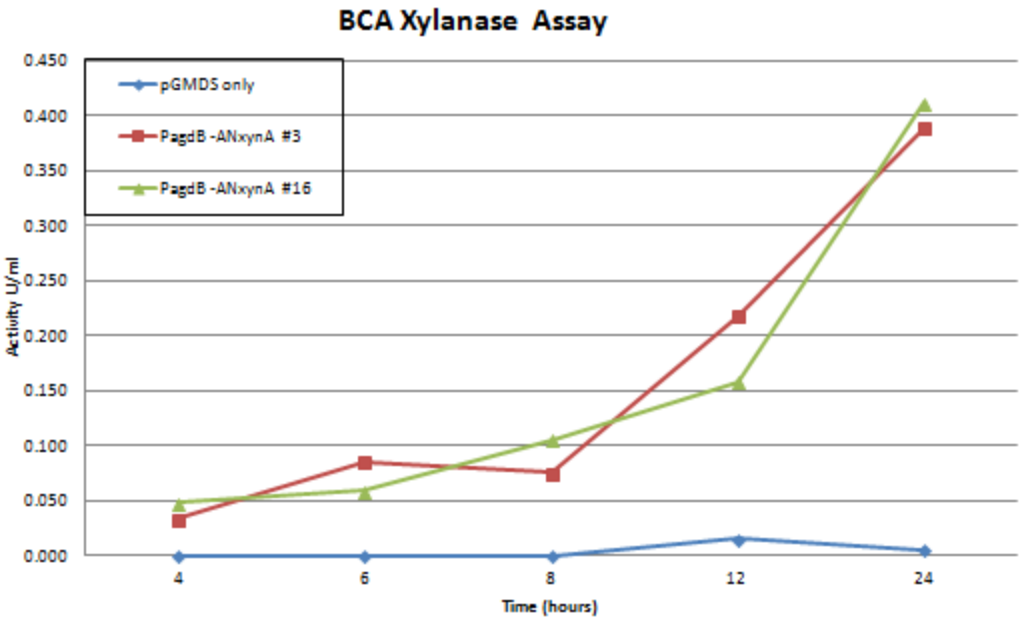


**Figure 24. BCA xylanase assay for *M. heterothallica* CBS 375.69 transformants following induction with different substrates**

PGAMD only control transformants show no xylanase activity 24 hours after induction with sucrose whereas transformants with PGAMD + PagdB - *ANxynA* show increased xylanase activity 24 hours after induction with glucose.

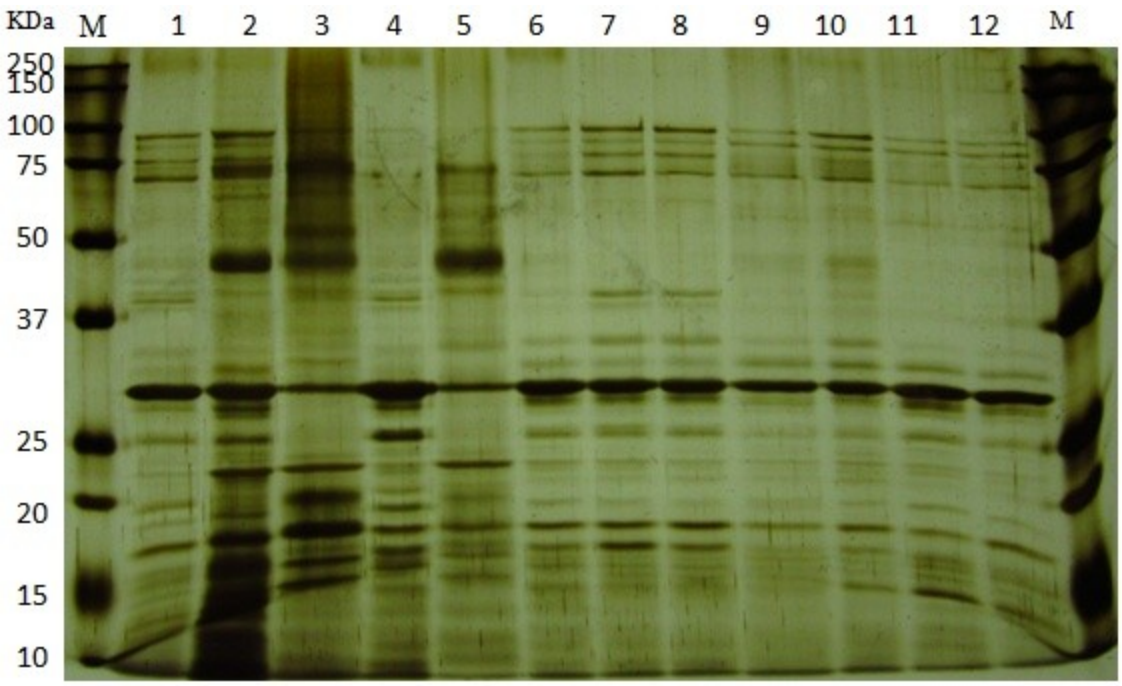


**Figure 25. SDS-PAGE: Supernatants of isolates transformed with vectors containing PagdB**

Lane 1. *M. thermophila* transformed with pGAMD only

Lanes 2 & 3. *M. thermophila* transformed with pGAMD + PagdB - *ANxynA*

Lanes 4 & 5. *M. thermophila* transformed with pGAMD + PagdB - *MtxynA*

Lane 6. *M. heterothallica* CBS 375.65 transformed with pGAMD only

Lanes 7 - 9. *M. heterothallica* CBS 375.65 transformed with pGAMD + PagdB - *ANxynA*

Lanes 10 - 12. *M. heterothallica* CBS 375.65 transformed with pGAMD + PagdB - *MtxynA*

Since the presence of the heterologous protein ANXynA was confirmed in *M. thermophila* and *M. heterothallica* by both spot assay and BCA assay, but could not be located on a gel, I selected one transformant for analysis by mass spectrometry. The supernatants from *M. heterothallica* isolate #16 at 4 hour post induction by sucrose as well as the glucose control were the samples chosen for this analysis. Supernatants from the same culture conditions but transformed with the pGMDS selection vector only were sent as a control as well. Results clearly show the presence of the heterologous xylanase ANXynA in the sucrose induction media for *M. heterothallica* isolate #16, but not in glucose. The isolate which had been transformed with the pGMDS selection vector only did not contain the heterologous protein under either the glucose or the sucrose induction conditions (Table 15). It must be noted that the peptides from the supernatants were searched against the *M. thermophila* database rather than *M. heterothallica* since the latter had not yet been fully sequenced and assembled but that peptides were recognized anyhow as the two organisms are so closely related.

**Table 15. Mass spectrometry results**

**A. Absence of heterologous ANXynA in *M. heterothallica* when induced by glucose**

| Description | Annotation | # Unique Peptides | Score | Coverage | # Peptides |
|---|---|---|---|---|---|
| Spoth2p4_001172 | Enolase | 17 | 93.59 | 35.16 | 15 |
| Spoth2p4_007238 | Putative endo-1,3(4)-beta-glucanase 2 | 18 | 10.28 | 5.20 | 3 |
| Spoth2p4_003720 | 1,3-beta-glucanosyltransferase gel1 | 9 | 42.61 | 23.57 | 9 |
| Spoth2p4_008301 | Chitinase | 8 | 5.30 | 4.71 | 2 |
| Spoth2p4_010931 | Ornithine carbamoyltransferase, mitochondrial | 6 | 13.17 | 15.32 | 4 |
| Spoth2p4_005075 | Hypothetical protein | 1 | 2.59 | 22.22 | 1 |
| Spoth2p4_005017 | Protein ecm33 | 9 | 29.13 | 17.91 | 7 |
| Spoth2p4_006544 | Chitinase | 9 | 3.06 | 3.29 | 1 |
| Spoth2p4_003453 | Fructose-bisphosphate aldolase | 6 | 26.64 | 13.46 | 4 |
| Spoth2p4_004533 | 1,3-beta-glucanosyltransferase gel4 | 10 | 27.19 | 12.20 | 6 |
| Spoth2p4_006438 | Exo-1,3-beta-glucanase | 12 | 24.66 | 12.69 | 8 |
| **Aspni_57436** | **endo-1,4-beta-xylanase** | **-** | **-** | **-** | **-** |
| Spoth2p4_010593 | Glucoamylase | 9 | 33.32 | 13.80 | 6 |
| Spoth2p4_005331 | Probable glucan endo-1,3-beta-glucosidase eglC | 10 | 51.09 | 17.04 | 8 |
| Spoth2p4_002025 | Probable aspartate-semialdehyde dehydrogenase | 5 | 14.46 | 17.13 | 5 |

**B. Presence of heterologous ANXynA in *M. heterothallica* when induced by sucrose**

| Description | Annotation | # Unique Peptides | Score | Coverage | # Peptides |
|---|---|---|---|---|---|
| Spoth2p4_001172 | Enolase | 17 | - | - | - |
| Spoth2p4_007238 | Putative endo-1,3(4)-beta-glucanase 2 | 18 | 135.62 | 24.72 | 17 |
| Spoth2p4_003720 | 1,3-beta-glucanosyltransferase gel1 | 9 | 18.88 | 14.10 | 4 |
| Spoth2p4_008301 | Chitinase | 8 | 38.93 | 20.35 | 6 |
| Spoth2p4_010931 | Ornithine carbamoyltransferase, mitochondrial | 6 | - | - | - |
| Spoth2p4_005075 | hypothetical protein | 1 | - | - | - |
| Spoth2p4_005017 | Protein ecm33 | 9 | 27.32 | 16.17 | 6 |
| Spoth2p4_006544 | Chitinase | 9 | 72.08 | 19.95 | 9 |
| Spoth2p4_003453 | Fructose-bisphosphate aldolase | 6 | | | |
| Spoth2p4_004533 | 1,3-beta-glucanosyltransferase gel4 | 10 | 42.95 | 19.14 | 10 |
| Spoth2p4_006438 | Exo-1,3-beta-glucanase | 12 | 53.07 | 16.92 | 11 |
| **Aspni_57436** | **endo-1,4-beta-xylanase** | **6** | **21.31** | **18.65** | **6** |
| Spoth2p4_010593 | Glucoamylase | 9 | 41.98 | 16.26 | 8 |
| Spoth2p4_005331 | Probable glucan endo-1,3-beta-glucosidase eglC | 10 | 47.16 | 17.28 | 9 |
| Spoth2p4_002025 | Probable aspartate-semialdehyde dehydrogenase | 5 | - | - | - |

# 4 Discussion

## 4.1 Choice of host organism

One of the requirements of a host organism is that it be fairly easy to culture, preferably with minimal nutritional requirements. Of particular interest are sources of carbon and nitrogen on which the organism can easily thrive. *M. thermophiles* growth media of choice is YPSS in which the carbon source is soluble starch. YPSS contains yeast extract which provides the organism with a source of nitrogen in the form of peptone. Unfortunately, yeast extract also seem provide a source of uracil or uridine when tested with *A. niger* N_402 and its *pyrG* ⁻ mutant N_593.The attempts I made to replace starch with another carbon source met with poor results. Both *M. thermophila* and *M. heterothallica* grow easily in 10x TDM containing 2% (w/v) glucose and requires no other rich nutritional additives.

Although I eventually was able to obtain a clean preparation of *M. thermophiles* protoplasts, the protocol adapted for this species required an extra day in the workflow over that of both *M. thermophila* and *M. heterothallica*. The generation of protoplasts in the later two species required no particular adaptation of the standard protocol used for transformation of *A. niger*. Spores will fully germinate under standard culture conditions and produce enough mycelial biomass for protoplast generation within 18 hours of inoculation.

Although the above aspects of *Mycothermus thermophilus* cultures are certainly deterrents for using *Mycothermus thermophilus* as a host organism, the final choice of *M. heterothallica* as a host organism was swayed by the fact that this organism has both positive and negative mating types [215]. The possibility of being able to cross two genetically engineered strains to obtain a double mutant was taken into considerations. This holds a great deal of importance since sexual crossing in currently used industrial strains of fungi is not common. Van den Brink *et al.* [46] showed that mating between two compatible mating types produced progeny with AFLP banding patterns showing varying degrees of mixing of the two parental strains. Furthermore, selected progeny showed increased enzymatic activity compared to the parental strains [46]. In addition, Hutchinson *et al*. demonstrated independent assortment of mating types using genetic markers when crossing *M. heterothallica* CBS strains 203.75 and 202.75 [212] . Roughly half of the progeny obtained from their crosses showed non-parental genotypes for each of the genetic markers. In keeping with this information, I alternated between

*M. heterothallica* CBS 375.69 a negative mating type, and *M. heterothallica* CBS 202.75, a positive mating type, for this body of work. Since *M. thermophila* has already shown to be a successful host organism for heterologous protein production by Dyadic, I used this species in parallel with *M. heterothallica*.

### 4.2 Method of transformation

Although I obtained transformants by PEG mediated transformation of protoplasts, low regeneration rates and low transformation frequencies in both species of *Myceliophthora* (Table 9 and 11) suggest that perhaps a different method would result a higher success rate. The use of agrobacterium mediated transformation has very recently been investigated for as a tool for genetic manipulation of *M. thermophila* by Xu *et al.* [51]. They report a 0.145% transformation frequency using a *ku70* deletion mutant which is more than three times the adjusted transformation frequency obtained here using PEG mediated transformation (Table 11). Moreover, they reported a rate of 97% for the successful deletion of *pyrG* in *ku70* deletion mutant when using AMT for gene disruption [51]. Considering that my attempts at replacing *pyrG* with *amdS* by PEG mediated transformation was unsuccessful, AMT certainly warrants consideration.

### 4.3 Method of selection

Initially my plan for a method of selection was to replace *pyrG* by *amdS* by homologous recombination. The linear construct designed included a 3' prime repeat region for the eventual excision of *amdS* (Figure 1). Transformants in which this disruption had been successful would initially thrive on acetamide as a nitrogen source and be resistant to 5' FOA. Once *amdS* was excised, this strategy would allow the use of both *pyrG* and/or *amdS* on an expression plasmid in a *pyrG*$^{-}$ mutant of *M. heterothallica.* Revertants could be selected on the basis of uracil and uridine auxotrophy or by their ability to utilize acetamide as a nitrogen source.

I based this strategy on a model which had previously been successful in *M. thermophila* and for which Visser *et al.* [49] had reported homologous recombination frequencies reaching 1 - 2% . In fact, even greater rates of homologous rates could be achieved in Δ*ku70* mutants but with a drastic decrease in cotransformation rates when Δ*ku70* strains were used [49]. Given the success of the strategy in their hands, the question as to why I was unable to generate similar

results must be addressed. First, my low regeneration rates are partially to blame. Average regeneration of non-transformed protoplasts on NSRM was almost three times that of regeneration of transformed protoplast on the same regeneration plates. Aside from cell loss at each wash step in the protocol, PEG treatment of the protoplast preparation may be an issue. Results from control transformations performed with no transforming DNA whatsoever show the same low regeneration rates as those performed with 5 - 10 µg of transforming DNA. Given the success rate of Ozeki *et al.* [93] discussed above, trials using electroporation as a method of transformation are an option. Beyond that, regeneration rates of non-transformed protoplasts may also be improved by adjusting the molarity of the protoplast resuspension buffer or decreasing digestion times in β-glucanase [86].

Although the low regeneration rates observed may indeed have contributed to my inability to obtain *pyrG* deleted mutants, I should still have obtained some transformants. I started with transformations with 200 µl of $1x10^7$ protoplasts/ml cell preparation. Supposing I had regeneration rates even of 1% and rates of homologous recombination frequency of 0.001%, I would expect to get roughly 200 colonies. So why were none obtained? Ultimately, the problem lies with the amount of transforming DNA. The individual fragments of the construct shown in Figure 1 were stitched together by overlap PCR. The PCR for the original fragments gave very clean strong bands. The nested PCR however yielded only very weak product of the expected size of 6.3 Kb. I tried three different pairs of nested PCR primers, with only one of them yielding any band at all. Multiple PCRs were done in order to pool the product before PCR clean up and gel extraction of the 6.3 Kb band, however even this gave low concentrations of below 50 ng/µl. The maximum volume of transforming DNA called for in the protocol was of 20 µl per transformation. Increasing the volume beyond that would mean upsetting the delicate osmotic balance in the transformation reaction and lower protoplast regeneration even more. If one considers that the transformation efficiency using 5 µg of selection plasmid was only of 15.34 transformants/µg, then 1 µg or less of a linear vector for homologous recombination was just not sufficient for gene disruption of *pyrG*. Perhaps a workflow using digestion and ligation would yield higher concentrations of the final vector and increase the chances of obtaining a *pyrG*$^-$ mutant.

Antibiotic resistance using Geneticin or Hygromycin B for positive selection of transformants were also methods which I considered. Although *Myceliophthora* was sensitive to

these antibiotics, a high concentration was needed in order to reduce background, especially when using Geneticin. Despite concerns about high levels of background and possible false positives, the use of Hygromycin B was a feasible option. The high costs of the antibiotic however, led to the pursuit of a different selection method.

Positive selection using *amdS* was clearly the best option. Transformants into which pGAMD had been integrated grew well within three days with little visible background (Figure 13 and Figure 14). Cotransformation with a non-selectable expression plasmid gave rates of cotransformation approaching 100% and this method was the one I ultimately deemed to be the most suitable approach.

### 4.4 Choice of promoters

For this study the first promoter I tried was the heterologous glucoamylase promoter from *A. niger* (PglaA) which is induced by the presence of starch in the culture media. Although transformants were shown by PCR to contain both the selection and expression vectors, screening results failed to detect an isolate showing xylanase activity due to the presence of heterologous protein ANXynA. Two methods were used to induce production of the protein. The first was by growing candidates in directly starch based media and alternatively I grew them in glucose first, then transferring mycelia to the starch based media. I performed spot assays to detect xylanase activity in the culture supernatants at regular intervals but no xylanase producing candidates were detected. Since RNA expression was not verified, it is not possible to determine if the heterologous *glaA* promoter is recognized. The presence of mRNA species corresponding to the gene of interest would indicate that gene expression is not to blame. Rather, the problem may lie with codon preferences of the host organism [133], improper glycosylation, or in the folding and maturation of the protein [216]. Ultimately, the exact reason why the heterologous protein was not produced is not relevant here since production and excretion into the extracellular media is the goal. Since the presence of the heterologous protein was not detected, other promoters were explored.

In part because recognition of the heterologous *glaA* promoter was unconfirmed in *M. heterothallica*, I then chose a *M. thermophila gpdA* promoter used to drive expression of the heterologous proteins in the expression plasmids. Although xylanase spot assay seemed to produce candidates by day 4, a control isolate carrying the selection plasmid only, showed a

similar clearing zone (Figure 18). In addition, the BCA assay done to confirm increase xylanase activity in the candidates, showed no difference between isolates transformed with pGAMD only and those co-transformed with both plasmids. In all likelihood, all of the isolates showing a clearing zone on the RBB xylan plate were simply samples in which clearing of the supernatant by centrifugation was incomplete. Either residual spores or bits of mycelia remained and were induced to produce native xylanase in response to the xylan present on the RBB plate. Thus the halo seen on the plate is not due to the presence of any heterologous xylanase in the supernatant. It is possible that the heterologous PgpdA from *A. nidulans* would have been a stronger promoter with which to drive expression of the heterologous protein.

As cellobiohydrolase I constitutes a large percentage of enzymes secreted by *M. thermophila*, its promoter, induced by cellulose, was used by Visser *et al.*[49] to drive the expression of recombinant proteins in their high cellulase (HC) C1 strain. In the present study, I introduced two different cellulose induced promoters, PpmoA and PcbdA, individually into the expression plasmid to drive the expression of the heterologous proteins. Seemingly, the cellulose based media used induced the production of many native genes. Not only were control isolates bearing only pGAMD indistinguishable from isolates having undergone cotransformation with the expression plasmid, but candidates producing the heterologous xylanase were not identifiable. Such high background levels are problematic with not only with respect to identification of candidates but also with purification of the protein of interest. The effect of a cellulose inducer on a wide array of native genes is not restricted *Myceliophthora*. Expression of native cellobiohydrolases, endo-β-glucanases and xylanases by *T. reesei* in cellulose based media is discussed by Li *et al.* [24] who advocate the development of a strong constitutive promoter instead of the frequently used *cbh1* promoter for precisely this reason. Furthermore, they point out that production of the protein of interest is subject to catabolic repression when inducible *cbh1* is used as a promoter [24] .

*M. thermophila C1* was also recently shown to possess lytic polysaccharide monooxygenase (LPMO) on which the organism relies on to degrade both cellulases and xylanases [217]. The promoter PpmoA was tried as a driver for production of the protein of interest, however no candidates were identified. Similarly to *cbh1*, this inducible promoter requires a cellulose inducer which will trigger production of too many enzymes, making the target difficult to identify.

The fusion of a tag to the protein of interest may be an interesting option in systems where heterologous protein production is masked by an multitude of confounding proteins triggered by the induction. Bergquist *et al.* [218] incorporated a 6x-His tag into their transforming vector for the production of a heterologous family 11 xylanase by *T. reesei*. In this study the promoter used was *cbh1* to drive expression but the purification of the target protein was facilitated by the presence of the His-tag.

Finally, I tested two α-glucosidase promoters in the expression vectors used to transform *M. thermophila* and *M. heterothallica* . The first of these two promoters is native to *M. thermophila* (PagdA), and the second a native *M. heterothallica* α-glucosidase promoter (PagdB) homologous to the first. The α-glucosidase promoters were selected since expression levels in *M. thermophila* of Spoth2_2303065 were extremely high in sucrose, but low in every other carbon source. Based on expression data, over 1500-fold expression over background levels are expected when induced by sucrose (Concordia University, Dr. Tsang's lab, unpublished data). Although both of these promoters generated candidates showing xylanase activity on the RBB xylan plates, only the isolates containing the PagdB constructs were confirmed to produce the heterologous protein of interest. Although at first thought it is tempting to advance that the PagdA promoter from *M. thermophila* was less a efficient promoter than that of *M. heterothallica* due to the slight variations between the two regions, it must be pointed out that the procedure for the validation of the candidates differed. The candidates transformed with vectors containing the PagdB promoter were regrown in 10% (w/v) sucrose rather than the initial 2% (w/v) sucrose. A closer look at the spot assay of the PagdA candidates (Figure 21) shows isolate #3 containing PagdA - *ANxynB* having a clearing zone of similar diameter and intensity to that of the *M. thermophila* isolate #9 containing PagdB - *ANxynB* constructs. It is possible that had this candidate been regrown in 10% (w/v) sucrose and retested in the same manner as the PagdB candidates, then the outcome may have been more positive. It should also be pointed out that none of the control isolates demonstrated any kind of xylanase activity, so it is unlikely that the cause of such a large and intense clearing zone be due to improperly cleared supernatants in this batch. Without further testing, my suspicion that both promoters are equally as efficient remains unsubstantiated.

## 4.5 Increasing yields of heterologous protein

Many methods have been used in order to increase heterologous production in fungal hosts. One possible method involves optimization of the media to either increase hyphal growth, control pH or to delay conidiation. Transport of extracellular proteins in fungi has been demonstrated to primarily take place at the hyphal tips [219-222] and so fungal morphology in culture can affect total yield of the target protein. Qin *et al.* [223] demonstrated that the deletion of *brlA* in *P. decumbens* not only suppressed conidiation but also resulted in increased levels of expression and activity of cellulases. These mutants also displayed increase branching as compared to the wild type. In *N. crassa* the use of hyper-branching strains has also shown to increase the amount of extracellular proteins produced [224]. Similarly, in a study by Bocking *et al.* [225], the production of glucoamylase was increased in *A. oryzae* mutants showing a highly branched morphology. Gyamerah *et al.* [226] found that they could increase the production of hen egg-white lysozyme in *A. niger* by controlling pH during growth. In effect, they found that by maintaining a constant pH of 4.0, the amount of proteases in culture were reduced thus positively increasing the yield of the intended protein [226]. Xu *et al* [227] observed that when the morphology of the submerged *A. niger* cultures changed from free mycelia to mycelial pellets, a decrease in the amount of extracellular protease activity was recorded. Along with the decrease in proteases, an increase in the amount of reporter GFP protein was noted [227]. With respect to my *M. thermophila* and *M. heterothallica* cultures, early conidiation was a factor and may have affected the amount of heterologous protein produced. Conidiation of in submerged liquid cultures when grown in either glucose or sucrose based media showed signs of conidiation sometime between 24 to 28 hours with budding seen at the ends of hyphae. By the end of 44 hours, my cultures were largely a collection of conidia mingled amongst mycelia in various stages of decay. I employed a few strategies to delay conidiation including reducing culturing temperatures from 45°C to 37°C and reducing inoculation concentrations from $1x10^6$ spores/ ml of culture to $1x10^5$ spores/ ml, with very little effect with respect to delay in the onset of conidiation. Moderate success was obtained by increasing sucrose concentrations in the culture media from 2% (w/v) to 10% (w/v) sucrose. In 10% (w/v) sucrose submerged liquid cultures, although conidia were still abundant, increased amounts of healthy mycelia were still present in the cultures at 48 hours and beyond. The increased health of the cultures 10% (w/v) sucrose was particularly visible in *M. heterothallica* CBS 375.69 as compared to *M. thermophila*. Although

manipulation of the culture media did have some success in delaying conidiation, the method employed by Qin *et al.* [223] of deletion of genes involved in the conidiation pathway would most likely have a greater effect on the yield of the heterologous proteins of interest.

Another widely used method of increasing heterologous protein production is the disruption of proteases and the use of protease deficient mutant strains. Yoon *et al.* [228] disrupted ten protease genes in *A. oryzae* which resulted in a 30% and 35% of recombinant human lysozyme (HLY) and bovine chymosin (CHY) production respectively. The use of *A. vadensis* has shown promise as a host for heterologous protein production due to its low levels of native proteases [10]. Although the mass spectrometry results obtained for the 4 hour supernatants of the PagdB - *ANxynA* constructs did not reveal high levels of proteases, it may be that later time points would have revealed proteases to be a factor affecting yields of the heterologous protein. Furthermore, it has been noted by Li *et al.* [24] that induction using cellulose based media may induce the production of extracellular proteases as well. It is likely that had the two cellulose inducible promoters tried in this body of work (PpmoA and PcbdA) had yielded identifiable candidates, then proteases may have in fact been a limiting factor in the amount of heterologous protein obtained.

Although cotransformation of the selection and expression plasmids has had good success in the scope of this project, the effect of adding the chosen mode of selection into the expression plasmid remains unexplored. I suspect that doing so would enable the amount of expression plasmid used for transformation to double without doubling the total amount of transforming DNA. In this way, the delicate balance of the transformation reaction is not upset. This method would serve to perhaps increase copy numbers of the expression plasmid integrated into the host. With cotransformation approaching 100%, a minimum of one copy of the selection plasmid and one copy of the expression plasmid is assumed. It would be interesting to assess if combining selection and expression constructs into the same plasmid, while maintaining the amount of total transforming DNA at 10 µg/ transformation reaction, would increase the number of multi copy integration of the expression construct. This in turn may lead to higher levels of production of the heterologous protein as demonstrated by others [36, 134-136].

Tsuboi *et al.* [229] employed yet a different approach in order to achieve a 30-fold increase in activity of a heterologously expressed GUS reporter in *A. oryzae*. In this study, improvements were made to the native enolase promoter (PenoA) by introducing multiple

tandem repeats of conserved elements in the promoter region of several amylolytic enzymes of this organism. The conserved areas targeted as repeat elements in their construct included both a starch responsive element known to bind to a transcription factor and the CCAAT box of the protein binding motif [229]. Since multiple repeats of the CCAAT box has also been shown to be an effective method of increasing heterologous protein production in both *A. niger* and *T. reesei*, a similar approach could potentially be used with the α-glucosidase promoters of *M. thermophila* and *M. heterothallica* once the core promoter region is identified in the selected promoters.

The 5' region flanking protein Mycth_2303065, the GH13 α-glucosidase from *M. thermophila* contains both a sugar-like transporter protein (Mycth_50903) and a putative transcription factor (Mycth_2303067). Designing an expression construct which includes the conserved domains of one or both of these elements could potentially affect the yield of heterologous protein. Mycth_2303067 contains two conserved domains one of which is a 33 bp Zn(2)Cys(6) DNA binding domain , and the other is identified as a 398 bp fungal transcription regulatory middle homology region. Mycth_50903 also contains two conserved domains. The first domain is identified as a Major Facilitator Super Family (MFS) and the other as a sugar porter sub-family of MFS. In order to identify which, if any, of these domains has the greatest effect on the production of the heterologous protein in the expression plasmids, a series of constructs containing these domains alone or in various combinations, need to be designed and tested.

Evidently a plethora of other promoters are available to drive expression of proteins of interest. It is possible that higher yields of heterologous proteins may be achieved using either novel promoters or ones commonly found in the literature. The use of Pcbh1 for over expression of native genes or *to* drive expression of heterologous proteins has had success in *T. reesei*, *T. viride, A. fumigatus, A. cellulolyticus* and *M. thermophila* [27, 49, 199, 200, 230, 231]. Visser *et al.* compared expression of *cbh1* by Northern analysis to that of two other genes before deciding on this promoter for their expression vectors designated for the high cellulase production strain[49]. In all probability, just as I observed when using the cellulose inducible promoters PpmoA and PcbdA, using Pcbh1 would induce expression of high levels of native enzymes and high levels of background can be expected [24].

The evaluation of other constitutive promoters has merits as well. The promoter of translational elongation factor (Pef -1α) in *M. thermophila* (JGIDB: Mycth_ 2298136 ), for

example, is a good candidate. This gene is highly expressed in all of carbon sources tested, including glucose. (unpublished data, Dr. A. Tsang's lab). Li *et al.* [24] had selected promoters from pyruvate decarboxylate (Ppdc )and enolase (Peno) as well as Pgpd in their search of a strong constitutive promoter for *T. reesei* . Their selection was based on expression levels of these genes in response to increasing levels of glucose.

The original objective of this work was to develop an expression system for heterologous protein production in a thermophilic fungal host organism for cases when cloning in *A. niger* fails to produce a protein of interest or when it is produced but inactive. For large part of this work, a xylanase from *A. niger* (ANXynA) was the enzyme I utilized as the target protein for heterologous protein production in *M. thermophila* and *M. heterothallica*. It was selected not because production is poor in its natural host but because it is known to be a highly robust enzyme with activity ranging from pH 2 to pH 9 with optimal activity at pH 5.0 (Courtesy of Annie Bellemare at Concordia University in Dr. A. Tsang's lab). Now that the *M. heterothallica* expression system has been shown work with this reliable enzyme, genes of interest which could not be expressed or produced in *A. niger* can be tried in *M. thermophila* with the assurance that although yields are low, the expression system is functional. To this end, I have prepared plasmids containing the constructs PagdB - GOI which are ready to be used for transformation in *M. heterothallica.* The heterologous genes of interest are xylanases from *M. thermophiles*, originally known as *Scytalidium thermophilum*, (StxynA) and a xylanase from *Pseudocercosporella herpotrichoides* (PhxynA). Although the *M. thermophila* expression system has also been validated with *xlnC,* the original interest in *M. heterothallica* due to its mating potential remains. For this reason, I would recommend that any follow-up work continue in *M. heterothallica.*

# 5 Conclusion

The filamentous fungus *M. heterothallica* is a feasible choice as a host for heterologous protein production. PEG mediated transformation of protoplasts has been shown to work but regeneration rates of transformed protoplasts remain low at 6.67%. Alternate methods of transformation should be considered including electroporation of conidia and agrobacterium mediated transformation. Although cotransformation of selection and expression plasmids has been extremely successful, adding *amdS* selection to the expression plasmids may increase copy number integration of the expression plasmid and may increase production of the heterologous protein of interest. A *pyrG* knock out strain was not produced but can be re-attempted if higher concentrations of transforming DNA can be generated to that end. Furthermore, it is recommended that the low regeneration rates be addressed before a subsequent attempt is made. Production of a heterologous protein has been achieved when expression is driven by the native α-glucosidase promoter but yields remain low. Modifications of the promoter by way of multiple repeats of the CCAAT box or by the integration of a conserved region of a *cis*-regulatory transcription factor should be considered. Alternately, other novel or common promoters can be tried both inducible and constitutive. Genes of interest whose expression and production have failed in *A. niger* can be inserted into *M. thermophila* via the developed expression plasmid. This would determine if a host organism in which pH and temperature profiles differs from that of *A. niger* is more favorable for the production of these enzymes of interest. Lastly, two mutant strains of opposing mating types can be crossed in the hopes of obtaining a double mutant.

Much work still remains to be done in order for *M. heterothallica* to be used as a host organism for heterologous protein production on a commercial scale. However, this body of work demonstrates the potential of this organism for such a purpose, with the added benefit over *M. thermophila* due to its sexual mating potential.

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