

**Sugar as a Genetic Light Switch: A Sucrose-Inducible Expression System in
*Aspergillus niger***

Honors Thesis

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By

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Abstract

Mold is often condemned for its unwanted growth, such as on a cucumber in a lunchbox, in the corner of a shower, or in houseplant soil. However, this ability of mold to grow in a wide variety of environments makes it a powerful tool in modern biotechnology. Mold, or filamentous fungi, has diverse metabolite production which allows it to adapt to its surroundings. This capacity to express a vast range of products and stable growth makes it an ideal organism for large-scale production of metabolites. The filamentous fungi *Aspergillus niger*, specifically, produces an abundance of useful organic acids, enzymes, and proteins. The purpose of this research is to identify a method for increased yield of these target metabolites while also increasing the sustainability of the biofermentation process as a whole. The approach was implementing sucrose-inducible expression of target genes. In the presence of sucrose, *A. niger* could increase yield of target metabolites. Additionally, the sucrose could induce morphological changes that cause the fungi to grow around a sucrose-producing cyanobacteria, creating a lichen-mimicking system. This system could drastically decrease the inputs needed for biofermentation, increasing process sustainability. This research works to confirm the ability of a sucrose-inducible promoter (P_{fopA}) by increasing expression of a fluorescence protein in sucrose media. Using gene editing and fluorescence microscopy, this experiment demonstrated sucrose-inducible promotion of a target protein under sucrose culture conditions without compromising growth of the fungi. This knowledge suggests promising potential for gene editing techniques to increase *A. niger* production as well as creation of a synthetic lichen system for fungal production. These further developments would substantially improve sustainability and cost in current biofermentation industrial production processes.

Introduction

Filamentous fungi, also known as molds, have a negative reputation in the general public. They are associated with health issues, food spoilage, and growth is unfavorable. However, intentionally exploiting fungi growth in a controlled environment has led to the development of invaluable biofermentation practices for industrial applications. This is due to the fact that filamentous fungi have the ability to efficiently produce an extensive variety of valuable metabolites [1]. The adjustability of diverse products from filamentous fungi became an industrial focus after James Currie's experiments with *Aspergillus niger* in 1917. His experiments focused on controlling environmental factors such as nutrient availability, sugar concentration, and pH. This research illuminated the possibility to optimize environmental conditions to maximize citric acid production [2]. It is now estimated to be the method of production for 90% of worldwide citric acid [3]. Since this publication, *A. niger* has continued to be a powerhouse for industrial production because of its relative ease of genetic manipulation, high safety profile, and wide range of useful metabolic products. These products span a wide range of industries from food to pharmaceuticals to biofuel. The useful metabolites include a variety of organic acids, enzymes, and proteins [4,5]. These products are a result of both primary and secondary metabolism activation. Primary metabolites support essential cellular functions. Products of secondary metabolism are often formed from biosynthetic gene clusters (BGCs) and contribute to the diverse nature of the organism. BGCs are co-located and co-regulated genes on a chromosome that encode for proteins that share a secondary metabolite pathway. In nature, these pathways are activated under a corresponding environmental condition such as nutrient limitations or low pH conditions [6]. Therefore, like Currie proposed, *A. niger's* metabolism can be exploited by strictly controlling the culturing conditions. Along with optimizing these

conditions to the target product, newer methods of refining metabolite production using precise genetic and metabolic engineering techniques are arising [7]. The improvement of *A. niger* biofermentation processes and yield using these genetic engineering techniques is the focus of the following research. This research proposes using the addition of a native *A. niger* promoter to upregulate a target gene to increase production yield in the presence of sucrose, a common and effective carbon source for culturing. The β -fructofuranoside, also known as invertase, enzyme hydrolyses sucrose into glucose and fructose for the fungi to use as an energy source. Therefore, its promoter (PfpA) is induced in the presence of sucrose [8]. By creating a plasmid with the target metabolite's gene being promoted by PfpA, a sucrose-inducible system with an increased yield of this metabolite could be developed. To validate this hypothesis, a fluorescent protein was coupled with this sucrose-inducible promoter in order to quantify gene expression. Specifically, a yellow-green fluorescent protein with high photostability and intensity, LanYFP, more commonly referred to as mNeonGreen (mNG) [9]. Increased fluorescence intensity of this protein under sucrose conditions compared to other sugars suggests the success of a sucrose-inducible promoter to further improve current biofermentation processes.

Materials and Methods

Fungal Strain

The parent strain and control strain used for these experiments was *A. niger* MA70.15, which disables the non-homologous end-joining (NHEJ) pathway and favors homologous end-joining. This strain has the pyrG gene knocked out, meaning it cannot synthesize its own uracil or uridine, requiring supplemental addition of these nutrients. As opposed to strains created for

industrial production uses, MA70.15 was developed specifically for experimental manipulation and ease of genetic manipulation [10].

Plasmid Construct

Plasmids were constructed using modular cloning techniques. Namely, Golden Gate assembly using type IIS restriction enzymes [11, 12, 13]. Plasmids pModA-D and a backbone plasmid functioned as modular cloning vectors for hierarchical assembly. They carried the following elements: pModA - pgdpA promoter and hygromycin B (hygB) resistance gene, pModB - empty, pModC - empty, pModD - PfopA promoter and mNeonGreen (mNG) gene, Backbone - AMA1 (autonomously maintained in *Aspergillus*) [8,9,14,15,16]. This plasmid configuration is represented in Figure 1 (below). The final plasmid transformed into the *A. niger* parent strain functions as an episome in the fungal nucleus because of the backbone vector. The AMA1 backbone replicates extrachromosomally, so genome integration is avoided. However, the autosomally replicating fungal DNA element needs high selective pressure to evade being dropped from the nucleus. By integrating the hygB resistance gene with the constitutively expressed pgdpA promoter, hygB antifungal agent can be used to select transformed fungal colonies while maintaining this episome. The mNeonGreen protein driven by the PfopA promoter allows for quantification of sucrose-inducible protein expression.

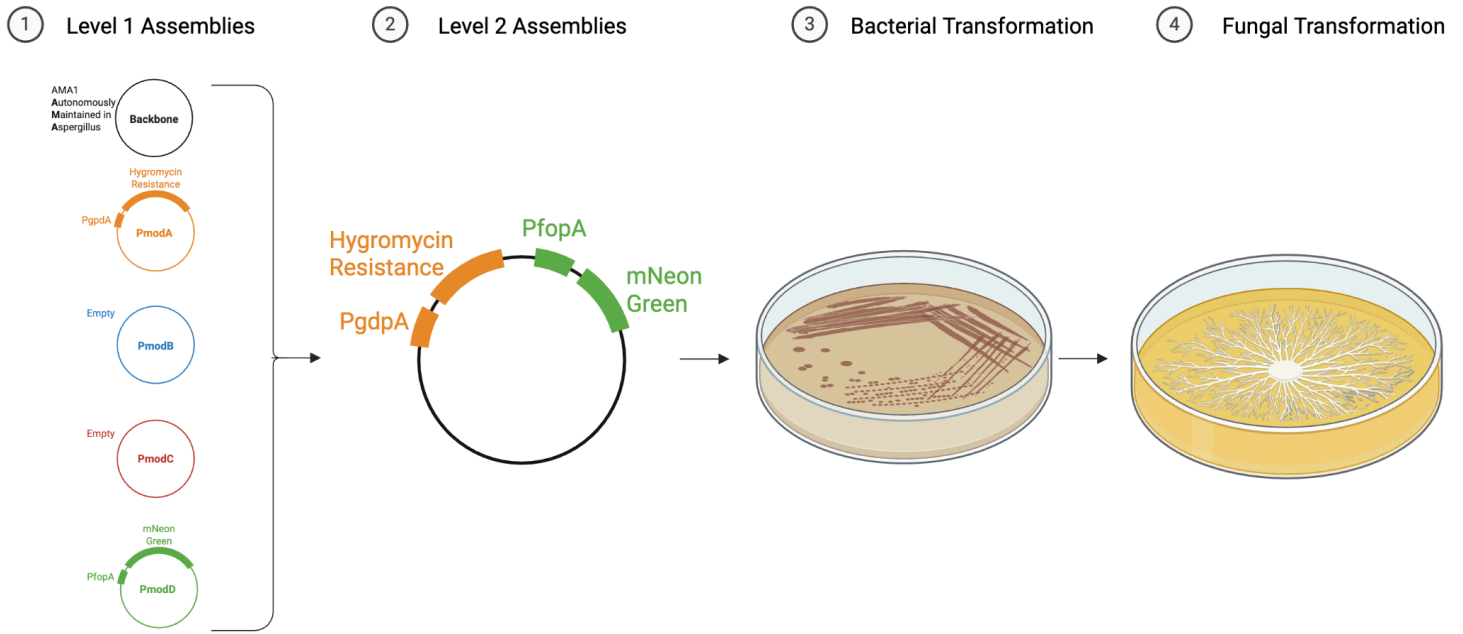


Figure 1. Transformed fungal strain construction pipeline. Level 1 assemblies constructed with BsaI restriction enzyme sites. Level 2 assemblies constructed with PaqCI restriction enzyme sites. Bacterial transformation used *E. coli* bacteria for growth with chloramphenicol resistance genes for selection of transformed colonies. Fungal transformation used hygromycin B resistance for selection of transformed colonies. Created in <https://BioRender.com>.

Transformation of *A. niger* MA70.15

Transformation of the assembled plasmid into *A. niger* MA70.15 followed standard fungal transformation protocols [17]. DNA is transformed into protoplasts, which are fungal cells with compromised cell walls. Heat-shock methods are used for DNA integration through these weakened walls. These cells are grown on nutrient-rich, antibiotic plates to select for transformed colonies. The PfopA strain was transformed with the target plasmid, while the knockout (control) strain went through the same transformation process, but without the addition of plasmid DNA.

Selection of Transformed Fungal Colonies

Hygromycin B resistance genes were integrated into the final episome in order to screen colonies for transformants. After fungal transformation, the fungi was plated on standard fungal complete medium with hygB at 100 $\mu\text{g}/\text{mL}$ [18]. These spores were collected and streaked onto a fresh complete medium containing 100 $\mu\text{g}/\text{mL}$, grown, and collected. A third streaking was done with the second set of spores on the same medium and new growth spores were collected. Each new screening on fresh hygB plates increases the likelihood of obtaining purely transformed samples. If fungi are left to grow on old plates for too long, selective pressure decreases and the episome may be dropped from the nucleus.

Spore Collection and Counting

Spores were collected by aspirating and dispensing a sterile water-and-Tween solution over fungal colonies with sporulation. This suspension was transferred to a sterile microcentrifuge tube and vortexed to homogenize it and break up aggregates. Spores were counted using a hemocytometer and diluted to 50 spores/ μL .

mNeonGreen Production Quantification

In order to test the sucrose-inducing ability of the system, inoculation in sucrose medium was compared with inoculation in glucose medium (control). The knockout strain was used for background fluorescence. The glucose-grown transformed strain indicated the basal expression level of the PfopA promoter. The sucrose-grown transformed strain demonstrated expression when the sucrose-inducible promoter was induced. For this experiment, spores were fixed to microscope coverslips. Before fixation, the coverslips were autoclaved for sterility. The

coverslips were soaked in a concanavalin A solution and incubated on a shaker at 30 °C to promote fungal cell attachment. After drying, 10 µL of the 50 spores/µL suspension was dispensed onto the coverslips and allowed to dry in a well plate. Then, 5 mL of either glucose- or sucrose-supplemented complete medium was added, and the coverslips were incubated for 24 hours in a 30 °C shaker. For the transformed strains, 50 µg/mL hygB was added to the media, while none was added to the untransformed knockout. Formaldehyde fixing solution was prepared in 3 mL portions, and the coverslips were transferred to their own well and fixed for at least 30 minutes. The coverslips were then transferred to a microscope slide, fungi facing down, and prepared for microscopy [19]. To set the threshold values, the knockout strain was observed. Dwell time and intensity range were refined so that the faintest possible fluorescence could be observed. These threshold values, as well as the magnification, were locked when taking the remaining microscopy images. Microscopy images were taken of the transformed strain after growth in glucose and sucrose media. To quantify the change in gene expression between these two sugars, the fluorescence images were analyzed using ImageJ [20]. The intensity of hyphal areas was segmented, excluding septa, which demonstrated increased fluorescence. For each hypha, the mean intensity of three small areas toward the hyphal tip was measured, mirroring Figure 2. The fluorescent microscopy images analyzed are provided in Figures A1 and A2 in the appendix.

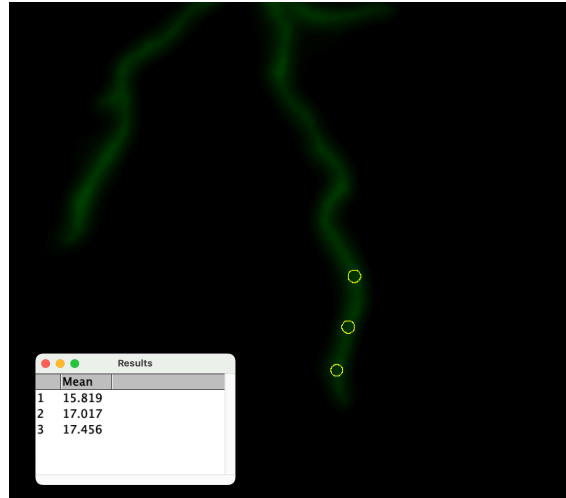


Figure 2. Image segmentation technique using imageJ to capture fluorescence values.

Fungal Growth Quantification

In order to examine the effect of the sucrose-inducible production system on fungal growth, fungal mass was investigated under different conditions. Culture tubes were weighed before any additions. Tubes were filled with either 5 mL of glucose or sucrose complete media. Tubes for transformed strains had 100 $\mu\text{g}/\text{mL}$ of hygB added. From the transformed and untransformed strains, 10 μL of the 50 spore/ μL dilution was added to glucose and sucrose cultures. They were incubated on a shaker at 37°C for a week. Fungal growth was separated from the media using centrifugation and media was discarded. Growth was dried in an oven for a week, until all liquid was evaporated. Culture tubes with fungal growth were weighed again to quantify and compare growth.

Results

Sucrose-inducible expression of mNeonGreen protein

While environmental optimization strategies have made great strides for the biofermentation industry, there is still opportunity for improvements in these processes. One promising avenue of genetic manipulation is to take advantage of the native promoters within *A. niger* [21]. By identifying and exploiting native promoters, significant increases in metabolite yields are possible. The PfopA promoter has previously demonstrated the ability to produce high levels of native β -glucosidase protein, used to break down biomass [8]. Research on another native promoter in *A. niger*, Pgas, has provided promising results for a low-pH-induced promoter [22]. These recent developments combine genetic engineering with optimization of the culturing environment and show promising results. The following research is utilizing this technique to produce high levels of mNeonGreen protein under sucrose media conditions. An expression cassette containing the mNeonGreen (mNG) gene under control of the PfopA sucrose-inducible promoter was constructed and co-transformed with the hygB resistance gene into *A. niger* MA70.15. The transformants were triple-screened on hygB plates. These transformants were fixed onto microscope cover slides and grown in either glucose or sucrose media before microscopy.

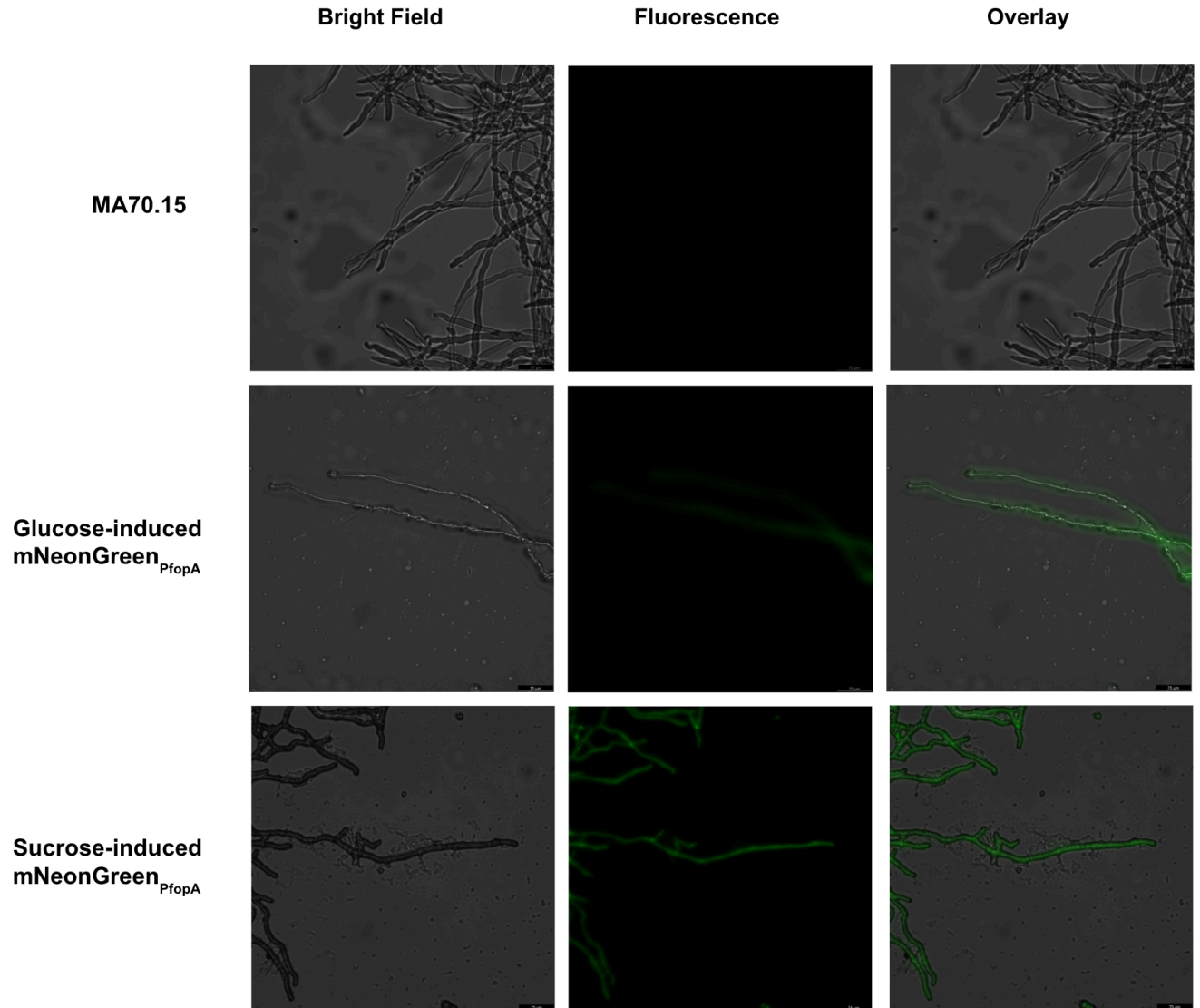


Figure 3. Fluorescent images of fungal mycelium. Top: *A. niger* MA70.15 strain grown in glucose. Middle: mNeonGreen with sucrose-inducible promoter strain grown in glucose. Bottom: mNeonGreen with sucrose-inducible promoter strain grown in sucrose.

Figure 3 (above) displays the fungal fluorescence under different conditions. The fluorescence intensity correlates to the strength of sucrose-inducible promotion of target protein. The transformed sucrose-inducible promoter strain inoculated with sucrose demonstrated an increase

in fluorescence compared to the glucose-grown sample, as seen visually in Figure 3 and statistically in Figure 4 (below).

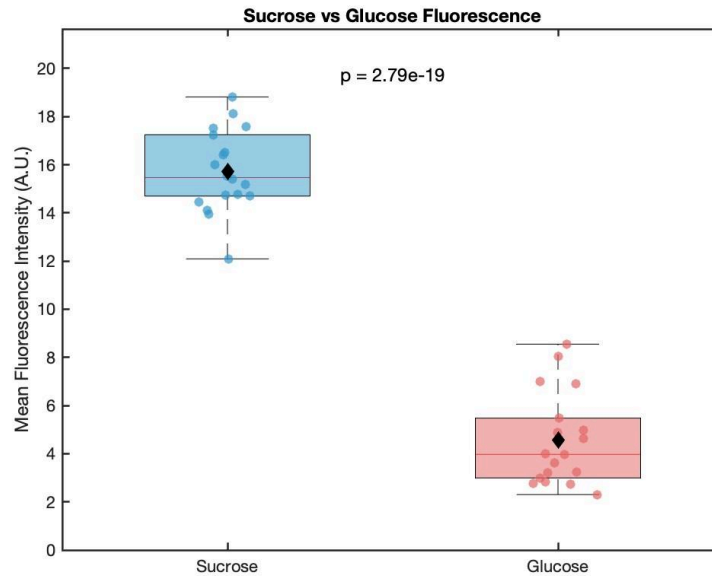


Figure 4. Mean fluorescence intensity of transformed fungi grown in glucose versus sucrose media conditions.

Created in MATLAB.

The transformed strain grown in sucrose has a statistically significant increase in fluorescence compared to those grown in the non-sucrose media (glucose). The mean intensity of the sucrose-grown fungi was 15.729 intensity units while the mean of the glucose-grown fungi was 4.559 intensity units. Both conditions had a standard deviation less than 2. The p-value of a two-sample t-test was less than 0.001, displaying a significant difference between the sucrose-grown and glucose-grown samples.

Effect of carbon source on mycelial growth

Metabolite production in *A. niger*'s relationship with fungal growth varies depending on the target product. For example, citric acid biosynthesis – this fungi's main industrial product – is triggered by metabolic imbalance and not directly related to increasing growth in the fungi. In

fact, growth is often stunted in high citric acid production conditions. For this reason, a majority of the citric acid production occurs during stationary phases of fungal growth [23]. In contrast, metabolic production of enzymes is often highly linked to hyphal growth where more growth corresponds to higher secretion. This is due to the fact that many of these enzymes have a function during active mycelial growth while products like citric acid are more closely associated with high stress conditions [24]. The overarching purpose of this research is to be able to interchange the gene being expressed by the PfopA promoter in the future. For this reason, knowing the impact on fungal growth may be a crucial factor depending on the gene. The growth of the knockout strain without hygB antibiotic was quantified in both sucrose and glucose cultures. The growth of the knockout strain without hygB antibiotic was quantified in both sucrose and glucose cultures.

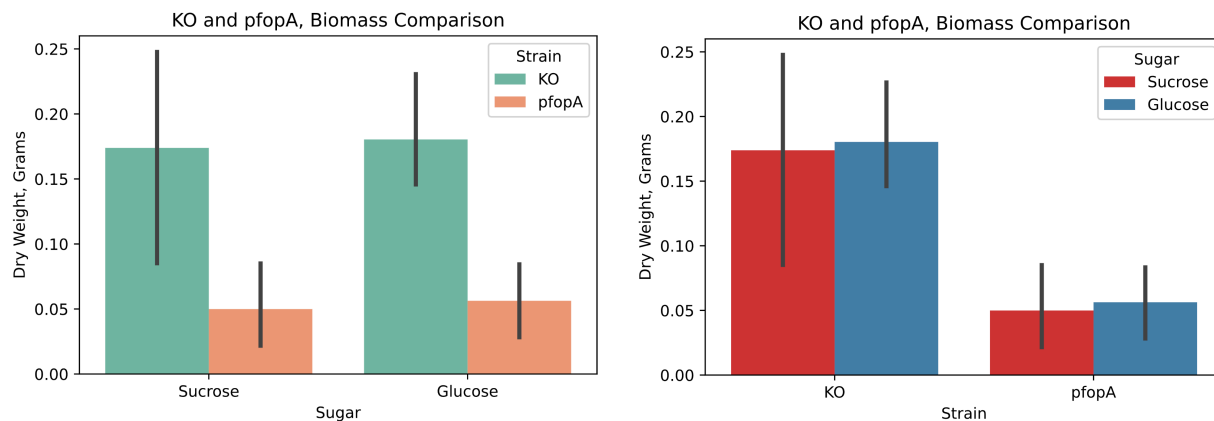


Figure 5. Effect of carbon source and sucrose-inducible promoter construct on fungal mycelium growth. Knockout (*A. niger* MA70.15) was grown in sucrose and glucose 1% (w/v) media without hygB. PfopA transformed strain was grown in sucrose and glucose 1% (w/v) and 100µg/mL hygB.

Growth experiment results demonstrate a decrease in the growth between the knockout strain and transformed strain with both sugars. Between the knockout and transformed strain, the sucrose had slightly lower growth than the glucose cultures, regardless of *hygB* additions.

Discussion

The purpose of this research was to combine environmental optimization techniques with gene editing in order to increase the efficiency of *A. niger* production of useful primary and secondary metabolites. Specifically, the upregulation of a target gene in the presence of sucrose-containing media was utilized. In order to confirm the upregulation of a target product, an mNeonGreen protein was coupled to the *PfopA* promoter. Results from the fluorescence experiments suggested high potential for this promoter to be able to optimize production of target products. When the transformed fungi were cultured with a non-sucrose media, basal level expression of the mNeonGreen protein was low. However, when cultured in sucrose-media the expression of the mNeonGreen protein was drastically improved as demonstrated through the increased fluorescence. These results suggest that sucrose-induction in this system can powerfully improve secretion of target metabolites. To further advance current biofermentation processes, in response to these results, this same construct could be created by swapping out the mNeonGreen gene for a variety of industrially relevant genes. Using this strain, there is a high potential for the yield of the product to be dramatically increased when cultured under sucrose conditions compared to current strains. In addition to the protein yield, growth of the fungi was tested. The results suggested a steep decrease in fungal growth when the transformed strain was grown under the same sugar conditions as the knockout. One likely explanation for this trend is the antibiotic effect on growth. During this experiment, the knockout strain media did not contain *hygB*

antibiotics because of its lack of resistance. If inoculated with hygB, the untransformed fungi would lack any protection against the hygB mechanism. Therefore, growth would be completely halted. For this reason, no hygB was added to these cultures, whereas the transformed fungi needed this hygB as a selective pressure to keep the target plasmid maintained in the nucleus. The addition of a hygromycin B resistance gene cassette expends extra energy for the cell to maintain [15]. This increases the metabolic burden on the fungi which could be leading to increased stress on the cells that takes away from the growth capabilities. These results suggest that selective pressure plays a large role in growth abilities. In addition to the comparison between transformed and untransformed, the carbon source impact was investigated. The growth trends were consistent across knockout and sucrose-inducible strains with respect to their sucrose vs. glucose response. Both strains displayed a slight decrease in growth in sucrose media compared to glucose. This could be a result of the difference in uptake between these carbon sources. Glucose is readily taken up in *A. niger* without the need for additional metabolic steps. It contains efficient glucose transporters that allow for easy utilization of this source [25]. In contrast, sucrose needs an extra metabolic step before it can be used as an energy source. Sucrose requires the production of the β -fructofuranosidase enzyme, or invertase, to break down sucrose into glucose and fructose. Then, the fungi can utilize these substrates [26]. This additional energy consuming step is a likely explanation for the decrease in growth between these two sugars. Overall, the growth and fluorescence results suggest a potential method for increased yield of *A. niger* products. However, this is not the only application that this work suggests. Another avenue of biofermentation improvement relates to the input of products. Filamentous fungi are heterotrophic, needing an external carbon source to grow. Traditional methods of biofermentation culturing use external carbon sources such as glucose, fructose, and sucrose [27]. It is also

common in large-scale fermentation to use byproduct streams or easily produced complex plant sugars such as starch, cellulose, or molasses. While the utilization of plant and waste streams have previously increased the sustainability and cost of biofermentation, there is still an increasing need for cheaper and more sustainable substrates [28]. This sucrose-inducible system suggests the ability to create a lichen-mimicking system as a substitute for current fungal substrates. Ideally, this system could completely replace the need for large carbon source inputs for biofermentation. A lichen-type system can be imitated using a photobiont (cyanobacteria) and a mycobiont (*A. niger*) where the mycobiont grows around and protects the photobiont which fixes and provides nutrients back to the fungi. Strains of cyanobacteria have been engineered to produce high levels of sucrose under their normal growth conditions. This growth only requires sunlight, water, carbon dioxide, and mineral nutrients such as iron and phosphorus [29]. The success of this sucrose-inducible system provides the opportunity to couple these sucrose-inducible fungal powers with the high sucrose production of cyanobacteria. Using this P_{fopA} promoter alongside a fungal morphology gene could create a synthetic lichen when grown in the presence of a sucrose-secreting cyanobacterial strain. The genes encoding for structural remodeling in fungi could be expressed in the presence of the cyanobacteria-produced sucrose, causing protective growth around the photobiont. By coupling these two organisms, a synthetic lichen could be created with minimal and sustainable inputs [30]. This potential application would significantly increase sustainability, decrease cost, and further increase yield in biofermentation processes.

Conclusion

In this research, a sucrose-inducible expression system was developed in *A. niger* MA70.15. This system was based on the sucrose-inducible promoter, P_{fopA}, and mNeonGreen fluorescence protein. The transformed strain demonstrated an increase in target gene expression in sucrose media cultures. Growth in antibiotic media decreased in the transformed strain, but allowed for closer strain purity. These results provide a method for increased yield in biofermentation as well as recombinant gene expression in *A. niger*. Additional work could explore the creation of a lichen-mimicking system to improve sustainability of biofermentation processes.

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Appendix

Glucose-induced mNeonGreen_{PfopA}

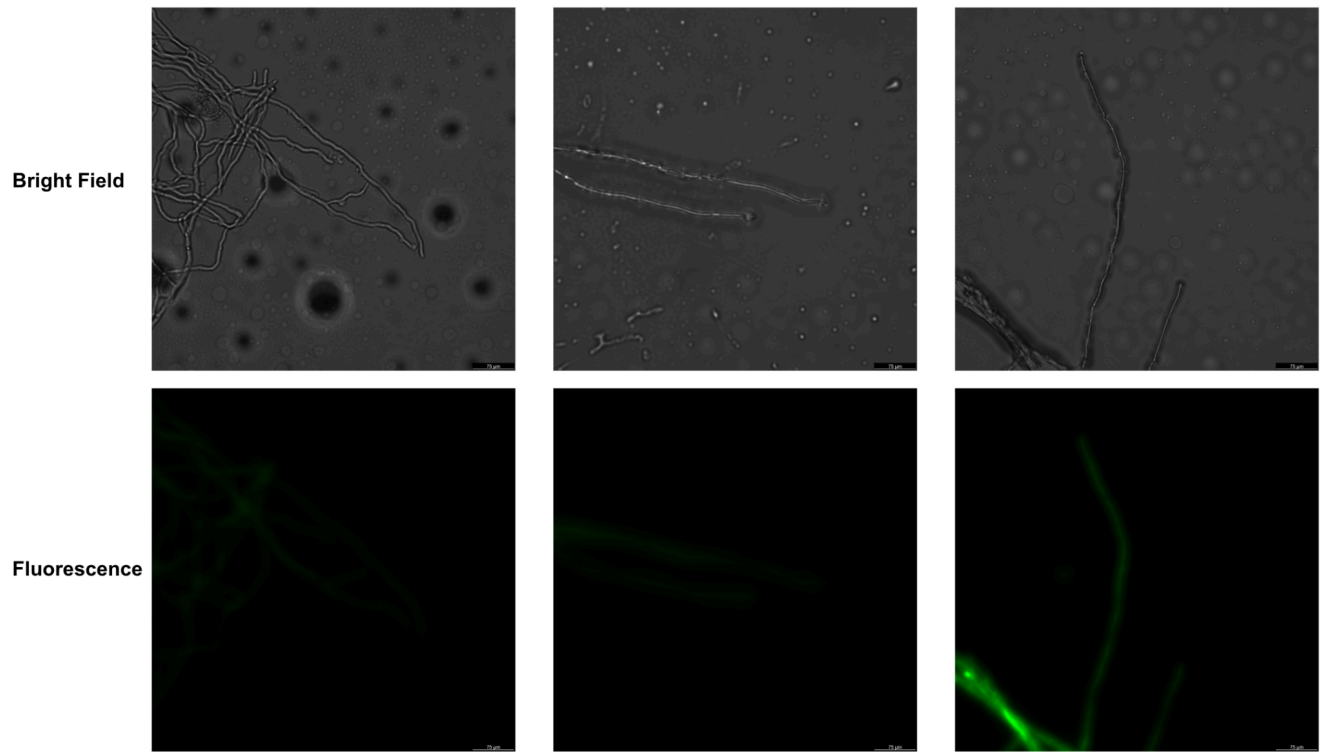


Figure A1. Fluorescent microscopy images of fungal mycelium from mNeonGreen with sucrose-inducible promoter strain grown in glucose. Used for fluorescence quantification in imageJ.

Sucrose-induced mNeonGreen_{PfopA}

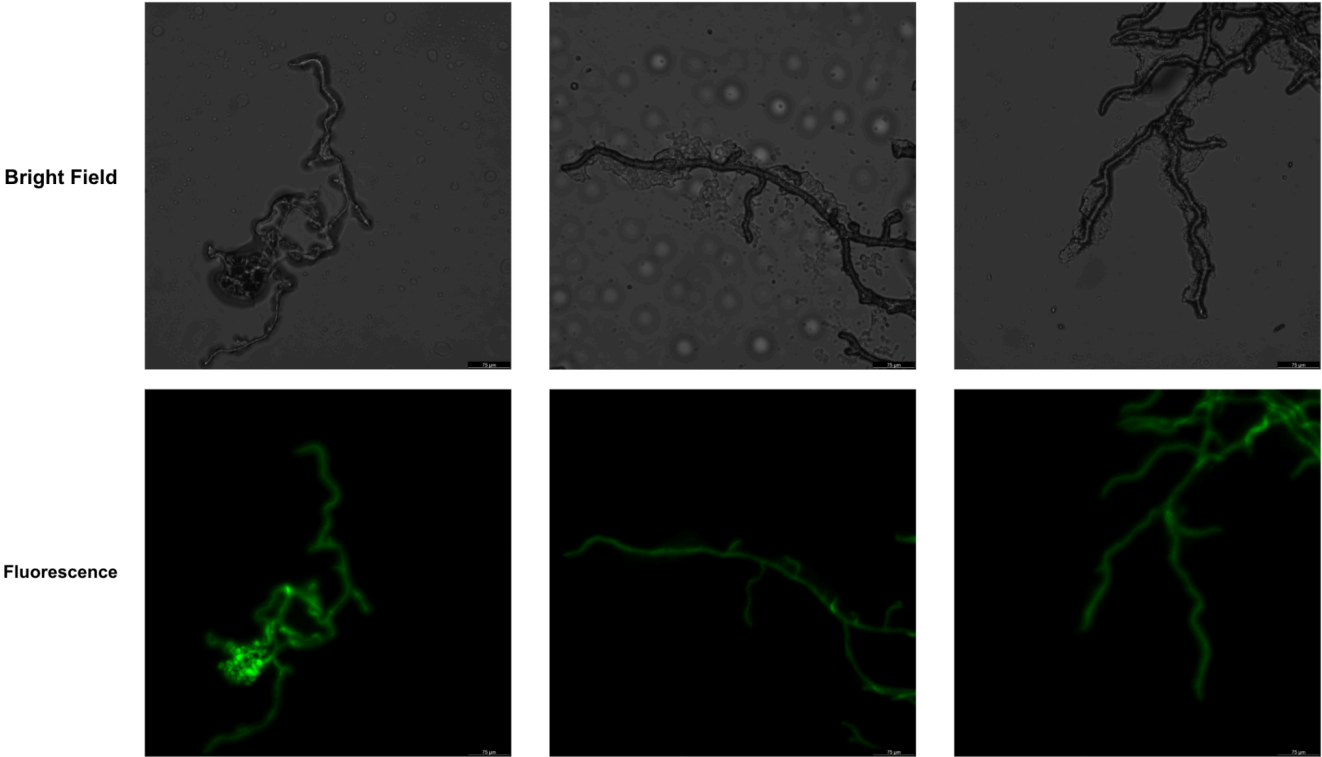


Figure A2. Fluorescent microscopy images of fungal mycelium from mNeonGreen with sucrose-inducible promoter strain grown in sucrose. Used for fluorescence quantification in imageJ.