EPFL

Thèse n° 10 804

An optimized high throughput platform, from genetic transformation to fermentation, to screen for secreted laccases in *Aspergillus niger*

Présentée le 29 octobre 2021

Faculté des sciences de base
Laboratoire de biotechnologie computationnelle des systèmes
Programme doctoral en chimie et génie chimique

pour l'obtention du grade de Docteur ès Sciences

par

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2021

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I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor in Philosophy.

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Acknowledgments

I would like to thank my directors, Vassily Hatzimanikatis and Blake Simmons, for helping me navigate the graduate school years and allowing me to have a smooth experience within the two most reputable institutes of Europe and the United States. I want to personally thank Blake Simmons for supporting every new idea that I had that shaped this thesis, for every training that I wanted to engage in, and for helping me fund all my research at the Joint BioEnergy Institute (JBEI).

I want to thank my managers, for helping me with my scientific questions and supporting me through my Ph.D., and my co-workers at JBEI for their collaborations on many projects and for helping me to design experiments. I want to thank in particular Megan Garber for taking me on the journey of our Podcast, The Secret Life of a Graduate Student, with whom I had a lot of fun interviewing scientists across the world. I also want to thank Nurgul Kaplan, for helping me to design my robotic platform experience. She made this collaboration a bliss and made me want to create more robotic platforms just to continue our collaboration.

Also, I am very grateful that the jury members, Florence Mingardon, Carolina Barcelona, and Jeremy Luterbacher, and the president, Kevin Sivula, accepted the participation in the evaluation of this thesis.

Obviously, I want to thank my friends and family from California to France going by New York, Canada, the United Kingdom, Spain, and Switzerland, to always be here for me, and for supporting me during my graduate school. Particularly my mother, Sylvie de Thomasson, who understood that my research across the globe was important to me and made me very happy despite the 12 hours of flight and 9 hours of jetlag that distant us. Thank you for always picking up the phone to listen to me during the most challenging time and reminding me of how proud you are, even if you cannot always understand my research.

Finally, I extend a big thank you to Sam Curran, for telling me that I could do a Ph.D. in the first place and being such a good mentor during all my graduate school years. Thank you for proofreading every piece of paper and email that I wrote in English

and debating so many times over experiments, such as on what was the best control and hypothesis to answer. Mostly, thank you for being a wonderful supportive husband and father.

I'm dedicating this thesis to my daughter, Georgia Sylvie Marcia Curran. I hope she will find a role model to pursue in her mother, like I found in mine, and never give up her dreams.

Abstract

Many challenges are faced in the conversion of biomass into advanced biofuels, one of which is finding the correct organism for the job. The filamentous fungus *Aspergillus niger* has been chosen as a biocatalyst for cellulose, hemicellulose, and lignin degradation because it can secrete numerous hydrolytic enzymes, such as lignin-degrading enzymes and, in particular, laccase enzymes (1). However, low transformation efficiency has hindered efforts to unlock the full potential of this organism.

This thesis presents a novel method to efficiently edit the genome of *A. niger* that overcomes several of the current challenges encountered when using CRISPR/Cas9 (2). We designed a genetic construct that is efficient and precise concerning knockout efficiency and phenotype control and demonstrated its utility for genetic knockouts, integrations, and alterations. Using the new CRISPR/Cas9 toolbox, we developed a high throughput platform to transform *A. niger* using robotics. We then transformed a library of 81 laccases that could be potentially secreted by *A. niger* in two different strain backgrounds and obtained six new laccases secreted by *A. niger*. We engineered a strain that increased total protein secretion fourfold and extracellular laccase activity threefold. We are currently applying for a patent for this discovery. We characterized each secreted enzyme by downscaling in micro-culture to study their optimum efficiency (e.g., media, pH, and fermentation time). These discoveries bring us significantly closer to generating strains that can produce a cocktail of laccases to unlock recalcitrant biomass for downstream processing. Additionally, the methods we developed will enable the rapid building and testing of genetic variants in *A. niger* for metabolic engineering, synthetic biology, and many other applications.

Thesis Summary

This thesis reports the research on the development of a platform from genetic to fermentation to mine for laccase enzyme secretion using a non-model organism, *Aspergillus niger*. This thesis is divided into five main chapters:

- Chapter 1 is an overall introduction from the beginning of microbiology and biotechnology to CRISPR/Cas9 innovation. The history of microbiology and biotechnology research shows how it shapes our current society and continues so with the ethical questions of the use of CRISPR.
- Chapter 2 is a review on laccase enzymes, from characterization to industrial applications, and the lignin valorization for biofuels and bioproduct production published in *Biotechnology Advances*.
- Chapter 3 and Chapter 4 are about the development of a new approach Cas9-based to engineer the genome of the non-model organism *A. niger* published in PLoS One and *Thermoascus aurantiacus* published in Biotechnology Biofuels. The development of a new Cas9 system for *A. niger* helped to reduce the workload and to accelerate the edition of the genome. This third part of the thesis was the groundwork of the final part, the development of a strain library.
- Chapter 5 of this thesis contains a description of the development of a strain library of *A. niger* to express and secrete laccase enzymes. We engineered a genetic robotic platform, using the new Cas9 system, to integrate 81 laccase enzymes in two different strain backgrounds of *A. niger*. The result obtained is newly-secreted laccase enzyme by *A. niger*, and a new engineered *A. niger* strain with increased overall protein secretion, which we demonstrated with one of the laccase enzymes from the library.

This thesis contains conclusions about the development of new systems, such as CRISPR/Cas9 and robotic platforms in non-model organisms to fast-track the discovery of new enzymes (e.g., laccases in this thesis) for the development of affordable biofuels and bioproducts.

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Chapter 1: Introduction

1.1. History

1.1.1. Microbiology and Biotechnology

Microbiology is the science of observing microorganisms, and Antoni van Leeuwenhoek was a pioneer of this branch of science. The first time that microorganisms were observed was at the end of the seventeenth century when Antoni van Leeuwenhoek built a single-lens microscope and observed what he called at that time animalcules (3). What Antoni van Leeuwenhoek was actually observing were bacteria, refuting the idea of spontaneous generation. He was also a pioneer in the reproduction theory when he observed 30 different sperm species and suggested that the male sperm fertilize the ovary of the female (4).

After the establishment of microbiology by Antoni van Leeuwenhoek, it took a century for Louis Pasteur to make important discoveries in microbiology and biotechnology. First, Louis Pasteur studied the role of microbiology in food processing by observing dairy and wine fermentation processes (5). Then in 1865, Louis Pasteur developed a method to preserve food by killing microorganisms, the “pasteurization” (6). Later, Louis Pasteur hypothesized the existence of infectious diseases caused by microorganisms. This led his research to the first vaccine to treat chicken cholera (*Pasteurella multocida*) and to the dawn of the immune system discovery (7).

Since then, researchers have been studying the existence of life on a micro-scale. The field of microbiology was expanding rapidly, and new types of organisms were discovered, which started to complex the classification of all living organisms without a proper taxonomy to catalog them. First Robert Whittaker proposed in 1969 to group living life in Kingdoms as the largest category, where he classified *Fungi*, *Animalia*, *Plantae*, and *Protista* as being within the *Eukarya* group and the fifth Kingdom the Prokarya where all organisms lacking a nuclear membrane were grouped (8).

In 1990, Carl Woese found that Kingdoms were not enough to differentiate life, but those Kingdoms belonged to a bigger category, the domain. Carl Woese created the first phylogenetic tree of three branches (9). The three domains are Eubacteria (e.g., *Escherichia coli*, the most studied organism), Eukarya (e.g., *Saccharomyces cerevisiae*, the most used organism in food processing, *Aspergillus niger, which* we focused on in this thesis, and us, *Homo Sapiens*), and Archaea (e.g., *Thermococcus gammatoleras*, the most radiation-resistant organism that is known today). Since the discovery of phylogeny, researchers have been also focusing on classifying the entire life on earth, making the task tremendous and meticulous with ~1.8 million named species to catalog (10).

Biotechnology is the science of using living organisms to make products, and without naming it then, humans started to study biotechnology 14,400 years ago northeastern of Jordan where a community of Natufian hunter-gatherers used wild einkorn to make bread (11).

Some researchers debate about the beginning of biotechnology. Some say that it started with the invention of fire, and others say it started with the breadmaking process (12, 13). However, a great breakthrough happened in the 20th century thanks to the development of the biotechnological process of penicillin during the Second World War. It started in 1928 with the discovery of antibiotics by the physician and Nobel Prize Sir Alexander Fleming. He wrote, "*When I woke up just after dawn on September 28, 1928, I certainly didn't plan to revolutionize all medicine by discovering the world's first antibiotic, or bacteria killer*." Alexander Fleming found that bacteria were fighting each other using molecules that he named antibiotics (English "anti," 19reek *bios* "life," and 19reek *biotikos* "fit for life"). Later, Alexander Fleming found the penicillin antibiotic from the mold *Penicillium notatum*, when he noticed the death of the bacteria next to the mold (13). During the Second World War, researchers worked diligently to mass-produce this antibiotic. This work will revolutionize a second time the biotechnology field, by making this one a new asset to the pharmaceuticals industry (14). Norman Heatley then showed a similar concept when problem-solving vaccine development and mass-production for COVID-19.

Today, we count four major disciplines under biotechnology: medical, agriculture, industrial, and environmental biotechnology. In this thesis, we are crossing those last three major domains of biotechnology.

1.1.2. Biofuel

Since 1850, the global economy has been primarily based on the use of coal, fossil fuels, and natural gas. The abundance and low cost of these resources have enabled the rapid industrialization of the planet, and have increased economic growth, industrial productivity, and revolutionized the transportation sector (15). These positive outcomes have come at a heavy price, in 1967, Dr. Syukuro Manabe and Dr. Richard Wetherald reported on the direct consequences of increased levels of atmospheric CO_2 on the rise of planetary surface temperatures (16). They correlated fossil fuel combustion to CO_2 emissions and therefore linked the consumption of fossil fuels to climate change, which at this time was still an unknown effect. Since then, an overwhelming amount of scientific evidence points to the seemingly inevitable rise of planetary temperatures that may have dire consequences (17). The development of alternative, low carbon solutions capable of maintaining economic growth and quality of life that mitigate the risk of climate change is of paramount importance.

The climate changed became evident to the population, who is asking that new alternative energies should be investigated to eventually replace fossil fuels, coal, and natural gas. Moreover, the population is aware that the energy found on earth is a limited source of energy, and one day, we will face a penury (18). Coming to this conclusion, politics, industrials, and scientists are well aware that investment should be made to find alternative energy. Thanks to this awareness, scientists developed different kinds of sustainable energy, such as solar panels, wind turbines, ocean turbines, and biofuels (19). Today, scientists are still working on the development of those technologies to make them competitive, accessible, and greener. In this study, we will study one of the challenges to optimize biofuels to make them more competitive and greener than fossil fuels, and we will suggest new perspectives for further scientific investigation.

The JBEI has been founded in 2007 by Jay Keasling with the support of the Lawrence Berkeley National Laboratory and the Department of Energy to create a plant-based biofuel to replace fossil fuels (20). JBEI has divided its research center into four main areas to make biofuel and bioproducts from biomass. The first sector of discovery is the Feedstock division, where plants are engineered to optimize their fermentable sugar content yield and that is easy to extract (21). The second area of research is the deconstruction division that bears its name from the deconstruction of the plants into fermentable sugars. This sector is focusing on the optimization of each carbon source deconstructed to identify the maximum potential of the plant. The deconstruction division makes a “no waste carbon source theory” the priority for the economic viability of the biofuel process (22). Leading the insitute to the other domain of research, the fuel synthesis and bioproducts division. This division is majorly focusing on the development of new microbe strains to produce biofuels and bioproducts using the carbon source from the plants (Figure 1.1) (23). The final area of research is the Technology department, where researchers are developing for example robotic platform and high throughput method to fastrak the research on biofuels and bioproducts. The overall institute mission will enable the researchers to not only make important microbiology and biotechnology discoveries but also bring into the industrial sector a solution to preserve the environment (24).

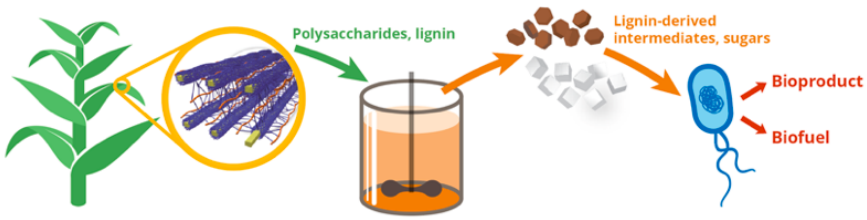

Figure 1.1: JBEI mission.

1.1.3. *Escherichia coli* and *Saccharomyces cerevisiae*

Before moving on to the main studied organism of this thesis, *Aspergillus niger*, let us introduce the major studied organisms that without those discoveries, it would not have been possible to do this thesis work. *Escherichia coli* was first isolated by a German bacteriologist, Theodore von Escherich from the human colon in 1885 (25). This bacterium has been used as a model organism, mostly since 1940, because it is recognized as non-pathogenic, versatile, and it can grow rapidly on different nutrient sources and can be easily isolated. This organism enabled researchers to find the most important keys that help us understand the biology of today, such as decoding the genetic code, transcription, translation, and replication (26), and it continues today with CRISPR/Cas9 (27). With this knowledge advancement in biology, other organisms such as *A. niger* can beneficiate similar tools to exploit and unlock knowledge to study their organism.

Saccharomyces cerevisiae is also well-studied organism and belongs to the same kingdom as *A. niger*, the fungi, but in two different classes, making those two organisms physiologically very different. Although they share enough similar genetic information to have been able to exploit the research found on *S. cerevisiae* to *A. niger* and vice versa (28) (29). Today, *S. cerevisiae* is a well-known studied organism because it has been used by bakers and brewers for centuries. Even though microbiology was not established when the first bread makers were using *S. cerevisiae*, it naturally became one of the most researched since 1935 when Winge developed a genetic tool to study it (30). Since then, *S. cerevisiae* has been used as a model organism for fermentation in food processing and genetic discovery in eukaryotic cells, and therefore, research findings about it are applicable to human genetics (31). Indeed, after the first sequence of the whole genome of *S. cerevisiae* was done, Giaever et al. deleted every single gene, generating a library of genes associated with functioning (32). Since this finding, researchers of *A. niger* are looking to expand the same library, and the CRISPR/Cas9 revolution made this increasingly possible (33).

1.1.4. *Aspergillus niger*

Until 1969, researchers were classifying fungi and plants in the same belonging kingdom. Then Robert H. Whittaker found that because of their difference in their mode of nutrition (plants use photosynthesis, and fungi secrete degrading enzymes), the classification was wrong, and fungi have their own distinct kingdom (34). The fungi kingdom found roots in ancient times with the evidence that researchers found the oldest fungi fossil *Ourasphaira giraldae* in Arctic Canada dating with Uranium-lead as 1,013 ±25 million years ago (Ma) (35). Today, fungi are known to be the most diverse living organism on earth (36), and the largest one, which raises the hypothesis about the propagation of life on earth thanks to this organism (37).

A. niger belongs to the fungi kingdom, the Ascomycota division, Eurotiomycetes class, Eurotiales order, the Trichocomaceae family, and finally the *Aspergillus* genus. It was isolated and classified first by a French botanist researcher Leon van Tieghem in 1867 (38). *A. niger* is mostly known to be found as a mold on fruits, peanuts, and onions because its sexual spores are volatile and like to grow on rich carbon sources. The first researcher to study the citric acid production by *Penicillium* mold was C. Wehmer in 1893 (39). But it is only in 1917, that the researcher James Curry, found a way to successfully cultivate *Aspergillus niger* to secrete citric acid (40). J. Curries found the unique elements of fermentation, such as the inorganic salt requirement, the general equation of metabolism, and finally, the reaction of the medium in which *A. niger* could produce citric acid successfully. The major economic aspect of this biotechnology process was the use of molasses as a carbon source instead of refined sugar. Until then, the production of citric acid was produced by unripe citrus fruits, which made the process and compound expensive, $1.25 /lb. Two years later, the Pfizer company patented the biotechnological process to produce citric acid in high quantity and high yield using *A. niger*, reducing the price of citric acid to $0.20 /lb (41). Because of the reduced price of citric acid using filamentous fungi, *A. niger* became the first well-known organism in the biotechnology industry in 1919. In 2007 scientists estimated the production of citric at 1.6 billion kg/year (42). After citric acid production, *A. niger* also was found to be useful due to the production of gluconic acid, a

compound that is necessary for food additives like citric acid and supplementing iron- and calcium-deficient diets (43).

It was only at the end of the 20th century that *A. niger* became also interesting for its production of hydrolytic enzymes and researchers engineered strains to produce homologous enzymes at the industrial level (44). As a high-capacity secretor *A. niger* produces majorly the glucoamylase enzyme (GlaA). Glucoamylase enzyme that hydrolyzes the degradation of terminal linked alpha-D-glucose residues from non-reducing ends of beta-D-glucose chains (45). The promoter (PglaA), a well-studied promoter due to the hypersecretion of GlaA, is a constitutive promoter (46). After the introduction of genetic tools to modify the genome of *A. niger*, researchers discover its potential as a heterologous enzyme secretion host with varying success depending on the tested genes (47).

With the development of sequencing methods, *A. niger ATCC v4.0* has been fully sequenced by the Joint Genome Institute (JGI) (48). Moreover, this type of strain is generally recognized as safe (GRAS), allowing researchers to work safely with this organism (49). Note that not all *Aspergillus* species are safe (e.g., *A. fumigatus*) and not all strains of *A. niger* are also recognized safe by GRAS. This is why in this study, we used the strain *A. niger ATCC v4.0* because it has been fully sequenced and is safe to work on.

1.2. Genome Editing

1.2.1. Transformation in filamentous fungi

Native filamentous fungi may not secrete enough of the targeted enzymes needed for industrial purposes, and therefore genetic engineering of these strains is an important strategic aspect (50). The first genetic engineering success was obtained in 1973 (51) on *Neurospora crassa* to rescue inositol dependence and demonstrated that these fungi can uptake and use exogenous deoxyribonucleic acid (DNA). Since that initial report, there have been several efforts to increase protein production and secretion, reduce pathogenicity, and elucidate gene function in several

filamentous fungi (52, 53). Although these approaches have demonstrated that significant impacts can be achieved concerning recombinant protein expression, they are typically laborious, inefficient, and complex and require significant fiscal resources. These challenges can be directly attributed to the lack of a robust genetic toolbox that researchers can use to edit these strains on demand for any given protein that is desired to be expressed at high titers.

The integration of exogenous DNA via homologous recombination (HR) has been widely applied (54, 55). The efficiency varies depending on filamentous fungi and the locus of the targeted gene (56). Adjusting the length of the HR arm and the use of transformation techniques depending on the host are required, but in general, HR in filamentous fungi was less efficient than in yeast (57). Several strategies have been employed to increase the efficiency of HR, such as engineering the RAD52 HR protein (58) or removing the *Ku* genes responsible for non-homologous end-joining (NHEJ) (54). Although deleting the *Ku* improved the efficiency of the transformation to nearly 100% (54), it can also result in genome instability because NHEJ remained the main pathway of repair in filamentous fungi compared to HR (59, 60).

Successful mutants of gene engineering are selected by the incorporation of a selectable marker at the interested locus. These markers include resistance markers such as hygromycin phosphorylase or nutritional markers, such as the orotidine 5'-phosphate carboxylase *pyrG*, that can complement a deficient strain (61). However, selectable markers are single-use and multiple mutations require multiple markers. The repertoire of markers is limited to <20 unique genes (62,63). However, the use of multiple markers can lead to genomic instability and can interfere with primary or engineered metabolism. On an industrial scale, the use of markers (even a single marker) can lead to heterogeneity of cultures and introduces additional costs (61). This is challenging because rewiring the primary metabolism and other processes often requires many mutations (64).

The use of transient rescue markers provides a powerful approach to the genetic engineering of strains that ultimately become marker-free (65). The rescue marker approach follows the traditional integration of a nutritional marker, such as *pyrG*, at the

chosen locus followed by selection for *pyrG*+ transformants. The *pyrG* insert is flanked by direct repeats so that in the presence of a counter-selectin agent such as 5-FOA, the host itself eliminates the marker with a second HR step. This technique has been applied in *A. niger* targeting *lacA* and *glaA* (66), *A. nidulans* (67, 68), *A. oryzae* (69), and *A. fumigatus* (70). Nevertheless, HR efficiency in *Ku*+ backgrounds remains low.

1.2.2. CRISPR/Cas9

The targeting of double-stranded breaks (DSBs) to the site of DNA integration is well known to increase the efficiency of HR (71–74). Early attempts to target coupled nucleases with modular zinc finger proteins (ZFPs) or transcription-activator-like proteins (TALs) increased HR efficiency but relied on complex protein design and production. These techniques have since been eclipsed by the efficiency and reliability of the CRISPR/Cas9 system (Figure 1.3). In nature, CRISPR/Cas9 functions as an adaptive bacterial immune system against phage and viruses (75). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) are arrays of repeated sequences in the bacterial DNA. The DNA strands between repeats are called “spacers” and are generally between 20 and 40 base pairs in length. These regions correspond to viral DNA sequences and participate in the microbial adaptive defense mechanism against phage and viruses. The endonuclease Cas9, the RNA transcribed from CRISPRs, cuts the target DNA sequences that are complementary to the CRISPR RNA. CRISPR/Cas9 was revealed as a rapid, facile technique for targeting of DSBs by engineering the CRISPR RNA into a single unit, termed the sgRNA (76). After the DSBs of DNA, the cells need a way to repair the DNA, and if Cas9 cuts constantly, the gene will eventually mutate itself to avoid repeated cuts, which will generate an altered mutation. If donor DNA is provided during the transformation, then the cell will most likely use it to repair the cut, generating into a sequence integration that could be a gene insertion, for example. In addition to facilitating HR, CRISPR/Cas9 can be used to introduce deletions, alteration, and point mutants without necessarily introducing donor DNA (77).

This technology is cheaper, faster, and more efficient than any previously-developed technologies for editing genomes. Over the past 9 years, since Jenifer Doudna and Emmanuelle Charpentier's publication on CRISPR/Cas9, the paper has already been cited more than 12,000 times (compared to the TALs paper only 2000 times for the last 9 years of research, and paper molecular structure of nucleic acids paper from Watson and Crick 13,000 times over the last 60 years). On top of that, it has been easily adapted to most organisms, even eukaryotic cells, such as the human genome (78). The applications for using CRISPR/Cas9 are beyond gene editing, but it is also used to study gene function, disease modeling, epigenome editing, genome imaging, and even gene therapy, which was difficult to do in the past (79).

Such potential within this technology comes with a great moral challenge. Genome editing has never been as accessible as it is today, and people can purchase CRISPR/Cas9 kits to do genome editing at home. This discovery will propel the research to unlock faster genome mysteries, human genome included. When applied wisely, this technology can help resolve difficult scientific questions, such as how to cure or treat Alzheimer's disease (80), and when combined with computation, researchers can even tackle future disease by developing fast vaccine solutions that contain a programmable antiviral protein called Cas13, for example (81). Although it is one thing to use CRISPR/Cas9 to help to cure or prevent disease, it shouldn't be used without clear ethical consortium prior experiments. Now is a good time to take a step back regarding CRISPR/Cas9 potential, and not use it at any questions asked, without pending every outcome possibility, especially regarding the human genome potential. For example, a group of researchers in China claimed they delivered the first CRISPR babies. The scientific community never allowed the publication of the research because it is unethical and too early to assess such research because modifying the human genome and creating life with it is still forbidden. Using CRISPR/Cas9 on animals in the laboratory in a controlled environment is safe and ethically questionable. However, changing the DNA of humans at its embryonic stage is still unethical today. Ethic research and discussions within the scientific community needs to be achieved to make such research (82), until then the scientific community

adopted a moratorium on germline editing (83). Another consequence is the opinion of the public regarding CRISPR/Cas9 that could backfire any positive research used with CRISPR/Cas9 because it can be misunderstood and misused by a small group of researchers (84).

1.2.3. CRISPR/Cas9 and filamentous fungi

CRISPR/Cas9 has been demonstrated in several filamentous fungi (e.g., *A. niger*, *A. oryzae*, *A. fumigatus*, and *Neurospora crassa*) (53, 85, 86). The promoter to express *cas9* varies depending on the fungus studied. Constitutive promoters, such as *tef1* (87), or *gpdA* (88), but inducible promoters have been used to avoid unwanted off-target effects (89), like the starch-inducible *amyB* promoter in *A. oryzae* (90). Similarly, multiple approaches have been used to express the sgRNA, either *in vivo* with constitutive promoters (53) or *in vitro* (87). The addition of a hammerhead ribozyme to the 5' end of the sgRNA can increase efficiency (86, 91). CRISPR/Cas9 induction of DSBs increased integration of the auxotrophic *pyrG* marker by HR to nearly 100% (86). Rescue markers were also adapted to CRISPR/Cas9 using the *pyrG* vs 5-FOA method in *A. fumigatus* (92) and the amdS method in *P. chrysogenum* (93), and a cre-recombinase dependent rescue marker in *N. crassa*, *A. niger*, and *Metarhizium anisopliae* (88, 94).

Despite these successes, the CRISPR/Cas9 system is not free of off-target effects, due to the mismatch of the sgRNA in the genome (95). Deletions, point mutations, and integrations of donor DNA (cDNA) at the wrong locus can occur with negative phenotypic effects. This phenomenon has been studied in the filamentous fungus *Ustilago maydis* (89). To reduce the frequency of off-target effects, researchers have been developing algorithms to predict the best protospacer sequence, such as CHOPCHOP.com (96) or CRISPOR (97). Other approaches have focused on improving efficiency by engineering the Cas9 itself, such as the use of truncated sgRNA with high-fidelity SpCas9 (98), or the replacement of Cas9 by two Cas9 nickases (99), and engineering of Cas9 for a heightened threshold for selectivity (100). In this thesis, we

established a new approach to edit the genome of *A. niger* using CRISPR/Cas9 and a twist in how to use rescue markers to facilitate lab work to screen for positive mutants (2). Moreover, we downscaled the transformation platform to use robots and make the method less time-consuming than previous classical protocol and high throughput using 96 well plates.

Chapter 2: Review of advances in the development of laccases for the valorization of lignin to enable the production of lignocellulosic biofuels and bioproducts.

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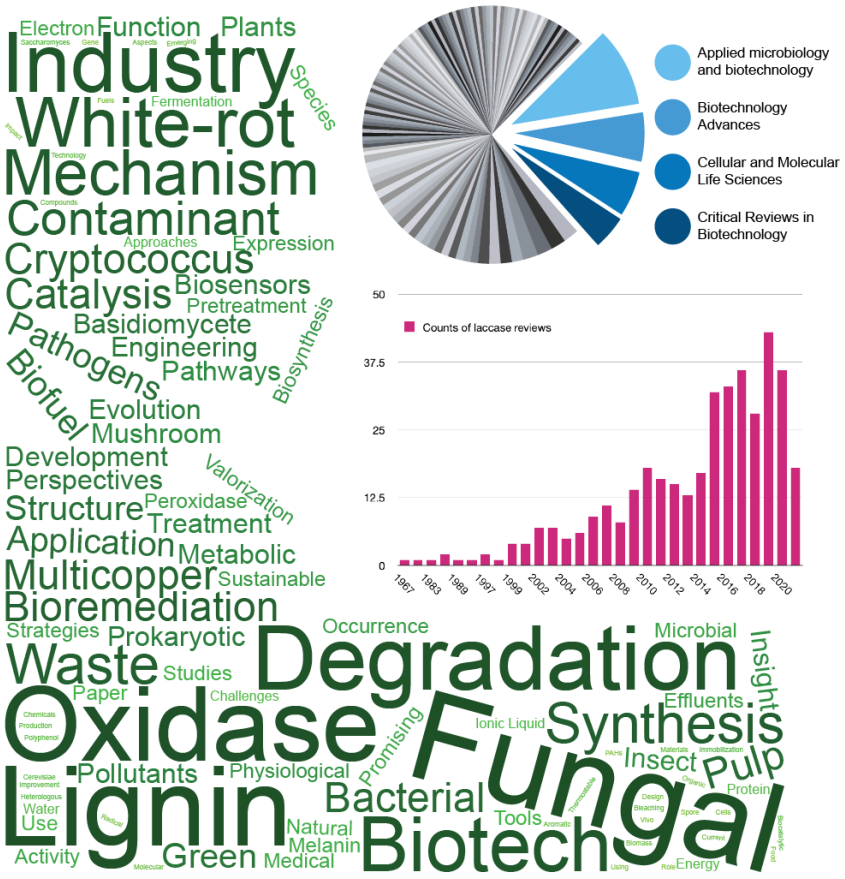
Abstract

Development and deployment of commercial biorefineries based on conversion of lignocellulosic biomass into biofuels and bioproducts faces many challenges that must be addressed before they are commercially viable. One of the biggest challenges faced is the efficient and scalable valorization of lignin, one of the three major components of the plant cell wall. Lignin is the most abundant aromatic biopolymer on earth, and its presence hinders the extraction of cellulose and hemicellulose that is essential to biochemical conversion of lignocellulose to fuels and chemicals. There has been a significant amount of work over the past 20 years that has sought to develop innovative processes designed to extract and recycle lignin into valuable compounds and help reduce the overall costs of the biorefinery process. Due to the complex matrix of lignin, which is essential for plant survival, the development of a reliable and efficient lignin conversion technology has been difficult to achieve. One approach that has received significant interest relies on the use of enzymes, notably laccases, a class of multi-copper green oxidative enzymes that catalyze bond breaking in lignin to produce smaller oligomers. In this review, we first assess the different innovations of lignin valorization using laccases within the context of a biorefinery process, and then assess the latest economical advances that these innovations offered. Finally, we review laccase characterization and optimization, as well as the prospects and bottlenecks of this class of enzymes within the industrial and biorefining sectors.



Abstract Figure 2.0.: Green word cloud: Word cloud generated from the titles of the latest 100 review papers on laccase in PubMed. The size of the words is proportional to the number of times the word figures in the review titles. Blue pie chart: Proportion of journal editors publishing on laccase review of the latest 200 review researches in Pubmed. Pink graph: The number of reviews published about laccase figuring in PubMed since 1967.

2.1. Lignin valorization

2.1.1. From Plants to Biofuels and Bioproducts

Plants use photosynthesis to capture and convert solar energy and CO_2 into the stored energy deposited in plant cell walls (101). Renewable plant biomass, such as the lignocellulose located in plant cell walls, has long been envisioned as a sustainable feedstock for production of biofuels and bioproducts, and its widespread adoption is predicted to significantly reduce CO_2 emissions in the transportation sector (102). In comparison with starch and sugar crops as the carbon source for biofuels and bioproducts, lignocellulosic biomass offers several significant advantages. Lignocellulose is a non-food biomass that accounts for 15 to 40% by weight of most terrestrial plants and is therefore an abundant source of energy. Additionally, it can add value to agro-based and forestry wastes, and it is a carbon-neutral substrate that does not compete with food crops (103,104). Lignocellulosic biomass is primarily composed of three natural polymers: cellulose, hemicellulose and lignin (105). Cellulose consists of glucose monomers which, when extracted and depolymerized, can be fermented into biofuels and other valuable bioproducts (106). Hydrolysis of hemicellulose generates pentoses and hexoses that can also be used as a feedstock for biofuels and bioproducts fermentation (63). Lignin is an energy dense, three-dimensional amorphous polymer and its presence hinders the efficient extraction of cellulose and hemicellulose, which increases the costs associated with the conversion of lignocellulose into biofuels and bioproducts (107). Valorization of lignin could unlock significant

economic value for biofuels and bioproducts and is essential for building an economically viable biofuels industry (108). Biological valorizing of lignin requires first depolymerizing it to fragments amenable to uptake and conversion by engineered host organisms. Consequently, the development of technologies to reduce biomass recalcitrance caused by the inherent heterogeneity of lignin has been the focus of intensive research (109). There is particular interest in engineering enhanced feedstocks with altered lignin composition, technologies that enhance the efficiency of biomass deconstruction and lignin extraction, and microbes that could convert all of the lignocellulosic intermediates generated into biofuels and other valuable bioproducts.

Effective and affordable lignin depolymerizing enzymes, such as laccases and peroxidases, are an essential step in transforming lignin into bioavailable substrates that can be converted into biofuels and bioproducts by microorganisms. In the first part of this review, we will discuss the different methods employed for lignocellulose depolymerization and the importance of lignin valorization for biofuels and bioproducts production in each. Secondly, we will address the potential of laccase enzymes for the depolymerization of lignin, its recent advances and bottlenecks towards its commercialization within biorefineries and other relevant industrial sectors.

2.1.2. Lignin

The first scientist to discover the presence of lignin in plants was the botanist A. P. de Candolle in 1819, which was 19 years before A. Payer discovered cellulose in 1838 (110). de Candolle named lignin after the word *lignum* (Latin for wood), due to its fibrous properties and the insolubility of lignin in water and alcohol (111). Lignin is critical to plant health and growth, providing mechanical support, impermeability, disease and pest resistance, and water and nutrient transport (112). It is the second most abundant biopolymer on earth, after cellulose, and composes 20-30 % of the dry biomass (113). Lignin is primarily composed of syringly (S type unit), guaiacyl (G type unit), and hydroxyphenol (H type unit) phenylpropanoid monolignols (Figure 2.1.), but other

monolignols can be present in smaller amount depending on the type of the plants, such as caffeyl alcohol, flavonoid tricin, hydroxybenzaldehydes, dihydro-hydroxycinnamyl alcohol, and acylated monolignols (114). The biosynthesis of monolignol starts in the cytosol from the amino acid phenylalanine (115). Then the monolignol is attached to a glucose molecule which confers water-solubility and can be transported through the plant cell wall (116). The S:G:H ratio varies between plants and genotypes, giving rise to the many different forms of lignin (117). For example G units are more abundant than H units in softwood, while in hardwood, G and S units are both similarly present (118). Another characteristic of this complex biopolymer is the variety of its linkages that occurs during lignin polymerisation. The predominant linkage present in lignin is β-O-4 aryl ether linkage, present at 50% in softwood vs 60% in hardwood (119). Other important linkages present in lignin are β-5 (phenylcoumaran) linkage, but others are also found in small various amount, such as, β-β (resinol) linkage, 5–5′ (biphenyl) linkage, and 4-*O*-5 (diaryl ether) linkage (Figure 2.1.) (120). The diversity of bonds makes lignin among the most complex biomolecules and leads to its recalcitrance (121).

Lignin biosynthesis in plants is activated by oxidative enzymes such as laccases and peroxidases (122). Laccases catalyze the O_2-dependent radical oxidation of monolignols, allowing the polymerization of lignin. Similarly, peroxidases use H_2O_2 to oxidize monolignols. It is only after the deposition of the polysaccharides, cellulose and hemicellulose, that the coupling of the radicalized monolignols occurs, initiating lignin polymerisation (123). In this process, the polysaccharides act like a framework of the plant cell wall, and lignin reinforces the structure by providing a highly engineered complex support that forms a type of composite material that enables structural support (124).

What is the path from this highly complex molecule to valuable biofuels and bioproducts? The three major steps are: (1) selection and growth of bioenergy crops, (2) deconstruction of those crops into targeted intermediates, such as sugars, monomers and oligomers of lignin, and (3) conversion of those intermediates into biofuels and bioproducts using microorganisms (125).

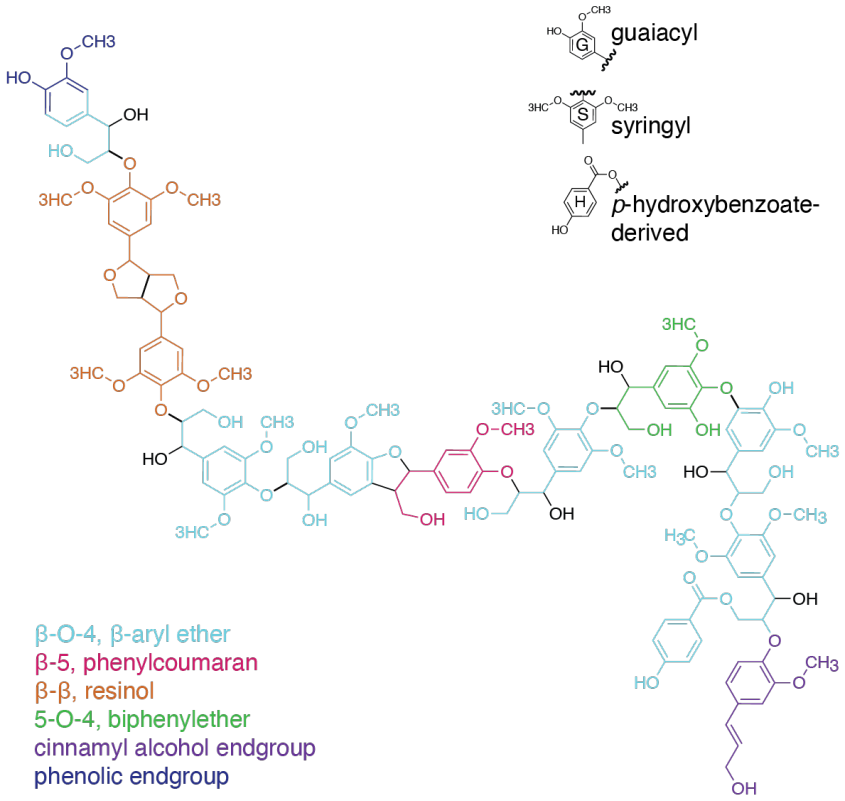


Figure 2.1.: Lignin structure, components and linkages from a wild type poplar, adapted from Stewart et al., 2009 (126).

2.1.3. Feedstock

The choice of the bioenergy crop is a key element since it impacts the entire conversion downstream processes needed. Many bioenergy crops have been studied such as switchgrass (*Panicum virgatum*), sorghum (*Sorghum spp*), and poplar (*Populus spp*) (Figure 2.2.a) (127). Each plant species has different lignin

structure (linkages) and content (S:G:H ratios) (128). One of the strategies to reduce the downstream costs, is to engineer the lignin biosynthesis by the plant and so reduce the biomass recalcitrance by modifying the lignin content and composition (129). The genetic engineering of the lignin biosynthesis pathway to reduce lignin content has direct consequences on the overall growth and development of the plant. Lignin reduction can lead to problems such as reduced plant viability, lower biomass content, and altered secondary metabolism (130). Due to the unpredictability of the results, this strategy is laborious. However, other approaches have been developed to increase the cell-wall degradability, by influencing the lignin composition rather than the content, resulting in more promising outcomes. Altering the composition of S:G:H shows that it affects the biomass digestibility characteristics. For example, a high S-lignin content can increase the enzymatic hydrolysis of the biomass, because it presents a lower polymerisation degree, which is the number of monolignol units within the macromolecule lignin (131). A similar approach has been used to reduce the degree of polymerization of lignin, by expressing in the lignifying tissue a bacterial hydroxycinnamoyl-CoA hydratase-lyase, reducing the biomass recalcitrance and enhancing the conversion efficiency (132). However, the beneficial genetic transformation of lignin has been mostly applied in laboratories under ideal conditions for the plants but only few studies have been realized in the field, where conditions make plants more vulnerable to the environment (133).

2.1.4. Pretreatment

After the choice of the bioenergy crops, comes the growth of the plants, and the harvest and pretreatment of the biomass. Cellulose and hemicellulose extraction are protected by lignin. Lignin is tightly crisscrossed all around the cell wall, impairing the interaction of enzymes with cellulose and hemicellulose, which decrease the yield of biofuels and bioproducts production (134). Pretreatment is an important step that increases enzyme accessibility by solubilizing and potentially depolymerizing and

extracting lignin. Some of the recent advances in biomass pretreatment for this purpose include the use of hydrothermolysis (135) ionic liquids (ILs) (136) or organic solvents (137) and many others… (134). Hydrothermolysis is a pretreatment method used in lignocellulose, that utilizes the pressure of liquid water at high temperatures (138). At high temperature, the water goes into the cell wall structure of the biomass, hydrates the cellulose, solubilizes the hemicellulose, and moderately removes lignin (139). Recent studies applied on pine wood shows that 35% of lignin was dissolved at 240°C after hydrothermolysis pretreatment (140). Due to the complexity of lignocellulosic biomass, it is difficult to develop a single pretreatment method. Within the different methods to thermochemically extract lignin, there also exist pyrolysis and microwave methods (134). It generally requires a combination of robust and techno-economical processes (141).

While ILs research started from a hardly noticed breakthrough by P. Walden in 1914, it is today subject to extensive research due to its numerous uses in different industrial applications (142). Walden was looking for a liquid salt at room temperature, and discovered that ethylammonium nitrate has a melting point of 12°C (143). ILs boast low vapor pressures and flammability, high electrical conductivity, high thermal stability and high solvation strength. ILs can be used as a solvent to dissolve cellulose (144), but ILs can also stabilize the hydroxyl group present in lignin, accelerating the cleavage process of the carbon-oxygen bonds, and they attack high electron deficient protonated carbon-oxygen bonds by acting as nucleophiles (145). The longer the pretreatment using ILs is, the better the downstream delignification efficiency is (146). After the ILs pretreatment, ILs can be isolated from the reaction, and recycled, decreasing costs (Figure 2.2. b) (147). Although the pretreatment process is one of the keys to delignification, it can be cost prohibitive and further advances are needed before a viable commercial approach can be realized (148). Moreover, certain ILs can inhibit enzyme activity either by destabilizing the enzyme structure or by other inhibitory processes if not fully removed (149). For chemical and catalytic applications for lignin treatment, there are also different methods than ILs pretreatment, such as acid, base and metallic catalysts and oxidative lignin using hydrogen peroxide for example (134).

After pretreatment, the biomass is typically separated in two output streams - a liquid and solid phase. The liquid phase, mostly constituted of cellulose and hemicellulose, can undergo direct enzymatic hydrolysis and be used to feed the downstream process of microbial conversion into biofuel and bioproducts. The solid phase, containing most of the lignin and some residual ionic liquid, goes through a different phase of depolymerization, to convert and depolymerize lignin into carbon sources or in ILs. ILs are isolated and recovered to be reused for the pretreatment (Figure 2.2.b) (150).

2.1.5. Enzymatic Depolymerization

The next step in lignin deconstruction is to use enzymes or microbes that can depolymerize the lignin (Figure 2.2. b). Enzymatic lignin depolymerization typically requires a mixture of oxidative enzymes, such laccases and peroxidases (151), esterases and etherases (152,153), cellobiose oxidizing enzymes, arylalcoholoxidases, and aryl alcohol dehydrogenases (154). Several of the enzymes known to be involved in lignin depolymerization are secreted by fungi in the *Basidiomycetes* and *Ascomycetes* divisions of the fungi kingdom (151). As an example of a *Basidiomycete*, *Phenerochaete chrysosporium*, known as white rot fungi, can grow directly on wood and has been the object of extensive research for its lignocellulosic enzyme secretion proficiency (155). In 1980, it was estimated that there are ~1700 fungal species capable of degrading lignin (156).

Laccases, mostly secreted by wood-rotting fungi, are oxidoreductase enzymes with low redox potential that can only oxidize directly phenolic lignin units (157), and nonphenolic subunits using a mediator (158). In the second part of this review, we will further develop the role of laccase in other industrial applications, their mechanism of degrading lignin, and their prospects and bottlenecks towards industrial processes.

Peroxidases, a less-studied class of enzymes that includes lignin peroxidase (LiP), manganese peroxide (MnP) and versatile peroxidases (VP) were reported in 1980 in *P. chrysosporium* (154).

Peroxidases have high redox potential, particularly LiP with redox potentials measured at 1.2 V at pH 3, making this enzyme capable of oxidizing substrates that no other peroxidases would be able to (159). Similar to the general peroxidase mechanism, LiP, MnP and VP contain a heme-iron in their active site that mediates catalysis. The heme-iron is first oxidized by hydrogen peroxide. Electrons are then shuttled from lignin through soluble mediators such as the phenolic veratryl alcohol, or, in the case of MnP, Manganese(II). Thus, lignin is oxidized in order to reduce hydrogen peroxide (152). The synergy between laccases and peroxidases is still nascent and further study is needed. In nature, fungi are capable of producing more than one peroxidase and accessory oxidases at a time, such as glucose oxidase or alcohol oxidase, that are known to degrade lignin (160).

A more recently investigated hypothesized that the copper oxidoreductase enzyme, named polysaccharide monooxygenase (LPMO), catalyzes oxidative cleavage of glycosidic linkage thereby breaking down cellulose and enhancing biomass degradation (161). LPMOs are usually used in a cocktail of enzymes, with cellulases and xylanases, to increase the saccharification of lignocellulose for the production of biofuels (162).

While these lignin degrading enzymes are effective in their native ecosystems, we lack the tools and expression hosts to produce them in high quantities at low costs. For example, although *Escherichia coli* and *Saccharomyces cerevisiae* have the potential to secrete lignin degrading enzymes (163), they typically produce very low yields of soluble, active enzyme, and the high cost of downstream processes to recover and purify the enzyme makes using these organisms prohibitively expensive (164). Moreover, enzyme depolymerization technology can be enhanced after ILs pretreatment. There is therefore a particular interest in the development of thermostable enzymes with higher tolerance for ILs (165).

2.1.6. Microbe Engineering

Successful lignin depolymerization yields small oligomers and monomers that can be fed into microbial biosynthesis platforms, such as microbial production of biofuels and bioproducts. Bacterial fermentation of glucose and xylose has been studied to obtain high productivity, yield and high ethanol conversion (166). Today, efforts are focusing on lignin valorization, including the screening for microbes that are capable of tolerating the toxicity of some aromatic monolignol and have the catabolic potential to degrade monolignols to simpler compounds that can enter primary metabolism. An additional challenge of microbe engineering is the necessity of funneling and converting numerous different monolignols into a single product e.g. biofuel. Thus, significant effort is being invested in engineering anabolic pathways.

Rhodococcus opacus and *Pseudomonas putida* are the most studied bacteria for lignin valorization (109). Due to *R. opacus* tolerance to toxic compounds, its conversion of monolignol into lipids (a biofuel precursor), and its many different catabolic pathways, this organism has been subject to many researches. *R. opacus* has allowed the identification of aromatic catabolic pathways and substrate transporter, but also a development of a genetic toolbox to optimize catabolic pathways and transcriptional controls to create a cost-effective fermentation strain (167). *R. opacus* accumulates up to 87% (dry w/w) in lipids, which can be converted into biodiesel (168). *P. putida* presents a high tolerance to environmental stress, and it is also capable of depolymerising high molecular weight of lignin, and then can catabolize them, making this organism a great choice for biofuels application (169). *P. putida* is also well known for its capability to degrade aromatic compounds (170). *P. putida* remains under intensive investigation to unlock its complex metabolic pathways that allow it to degrade aromatic compounds such as found in lignin (171). There is a clear need for more expansive studies on lignin valorization using microorganisms; better understanding of metabolic pathways for lignocellulose biomass conversion, more productive bacteria to increase the yield of biofuels and bioproducts, and finally, coupled with the upstream process, a better adaption of the microbes to the

depolymerization process by making them more ILs tolerant (Figure 2.2.b) (172).

The affordable, sustainable and scalable production of biofuels and bioproducts requires the efficient conversion of as much carbon in the plants as possible into these desired outputs. While the production and conversion of fermentable sugars has received the most significant attention to date, the efficient deconstruction and conversion of lignin is needed in order for the enterprise to be viable. This fact has been long-been recognized but has not received significant attention within the scientific community until the past decade or so. While there have been several breakthroughs over this time, there is still no proven and commercially viable approach to lignin conversion. In the second part of this review, we are focusing on the development of a particular technology, laccase enzymes, to degrade lignin. We are reviewing why laccase enzymes are central to this effort and how scientists have developed more efficient laccases at a reduced cost, and how they are still reinventing solutions to push the limit of this technology.

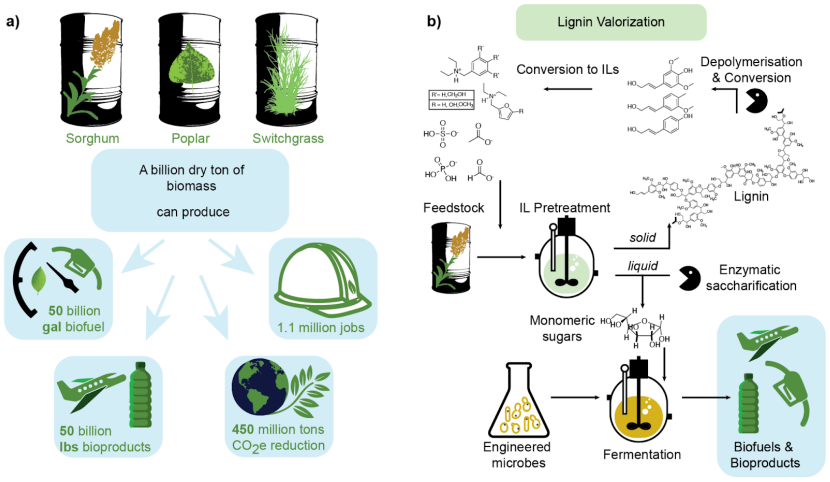


Figure 2.2.: a) Economical value of biofuels of second generation from sorghum, poplar and switchgrass biomass (127). b) A suggested biorefinery process from feedstock pretreatment to fermentation, and reconversion of lignin into ILs. Image adapted from Socha et al. 2014 (150).

2.2. Laccase industrialization

2.2.1. Laccase in multiple kingdoms of life

Laccases (polyphenoloxidase; EC 1.10.3.2) are highly versatile multi-copper oxidases that can catalyze a range of bond types in lignin, leading to depolymerization of lignin into fragments amenable to biological uptake and conversion (173). In 1983 Yoshida identified the enzyme responsible for lignin degradation, a laccase from the tree *Rhus vernicifera* (174). Today, laccases are well known to be distributed within the Fungi, Plantae, and Bacteria kingdoms, and there are recent reports they also exist in insects

(175). In Part 1 of this review, we explained the important role of laccases in plants, where, in the vascular tissue of the plants, they oxidize monolignols and cause lignification (176). This section will focus on the second function of laccases - catalysis of lignin degradation. Fungal laccases are potentially more wide-spread and active in lignin degradation. It has been estimated that almost 1700 wood-rotting Basidiomycetes are present in North America (156). Basidiomycetes play an important role in the ecosystem, as they are responsible for the recycling of carbon removed from the atmosphere by autotrophic organisms, such as plants and bacteria. Moreover, by growing on older trees, Basidiomycetes weaken them, expediting their death and providing nutrients for new trees to grow (177). The Basidiomycetes contain well-studied laccase-producing fungal species, such as *Trametes species*, *Trametes versicolor*, *Cerrena unicolor*, *Agaricus bisporus* and *Phanerochaete chrysosporium* (178,179). Fungi are particularly interesting for their ability to secrete highly active enzymes at high concentrations. For example, LccH laccase from the white-rot Basidiomycete, *Hexagonia hirta* MSF2, reached a LccH activity of 5671.3 $U.mL^{-1}$ after phenol induction (180).

Bacterial laccases are also being investigated for being produced intracellularly and extracellularly, with stable enzymes within a wide range of pH and temperature, e.g. *Bacillus subtilis* Cot A laccase reaches its maximum of activity at 75 °C, and has a half-life of 4h at 80 °C (181). *Pseudomonas aeruginosa* ADN04 produced 46 $U.mL^{-1}$ after media optimization (182). Other examples of bacterial laccase producers include *Bacillus spp* (very well studied and characterized), *E. coli* and *Campylobacter jejuni* (less studied since they present a lower enzymatic activity than *B. subtilis)* (183), *Pseudomonas putida*, *Streptomyces antibioticus*, and more (181).

Such a biodiversity of laccase producing species is promising in terms of prospecting for a diverse set of laccases that can be optimized for process-specific enzymes for efficient lignin degradation. To date, there are hundreds of studies in which laccases from across all kingdoms have been produced and characterized (184). However, for industrial purposes, it is difficult to optimize fermentation conditions for each species that produce laccases. Therefore, heterologous expression in tractable

hosts has been the objective of much research (185). The development of genetic tools has allowed laccases to be identified, expressed and characterized in model organisms (186). The most used organisms for heterologous expression that have been studied for laccase characterization are *E.coli*, *S. cerevisiae*, *Pichia pastoris*, and *Yarrowia lipolytica* (187). Filamentous fungi, such as *Aspergillus oryzae*, *Aspergillus niger* and *Trichoderma atroviride*, are alternative host organisms to study for production and secretion of laccases, because they offer advantages such as high enzyme secretion levels, fast growth in liquid culture and availability of new genome-editing tools (2,187).

2.2.2. Characterization of laccases

Laccases belong to the cupredoxin superfamily, among other oxidases such as manganese oxidase (183). Due to their broader substrate specificity, laccases have been characterized as a subgroup of enzymes, sharing a signature that distinguishes them from other multi-copper oxidase enzymes (188). This signature is composed of four conserved segments of length ranging from 8 to 24 amino acids. These conserved regions contain the genetic information of its active center, encoding the copper ligand coordination and maintenance of the three-dimensional folded protein (189).

2.2.2.1 Structure and mechanism of laccases

Laccases have a typical molecular mass between 50 to 70 kDa, but some laccases have masses as large as 140 kDa, depending on the organism and degree to which the protein is glycosylated (190). Fungal laccases contain an N-terminal secretion signal peptide of 20-22 amino acids. Overall the laccase protein can be glycosylated between 10-25%, based on increases in mass (178). In general, the laccase structure is composed of three cupredoxin domains having β-barrel symmetry (191).

The laccase catalytic site contains three to four copper atoms (Cu^{2+}) (152). Fungal laccases typically have four copper atoms, with the Cu^{2+} at the surface of the structure designated as the first active site (T1), because it can interact directly with the substrate, which can be either lignin or a mediator. Three other coppers tend to form a cluster, with one comprising the second active site (T2) and the two other coppers making up the third active site (T3) (192). Laccases function by relaying electrons between these coppers to catalyze a sequential of four electrons that substrate oxidation with concomitant reduction of molecular oxygen to two molecules of water H_2O (Figure 2.3.a) (193).

The T1 copper is the most solvent-accessible copper, and it is the site where the substrate binds and one electron is transferred from the substrate to the laccase active center (Figure 2.4.). The T1 copper has an optical absorption at 600 nm, and is thus named the "Blue" copper ion. It is directly coordinated to two histidines and a thiol group of a cysteine, forming a trigonal structure (194). Depending on the organism, there may also be a methionine, phenylalanine or a leucine coordinated to the copper at T1 (Fig. 3.b) (195). The maximum redox potential of laccase is determined by the capacity of its T1 active site potential, meaning that the ionization potential of a compound cannot exceed the T1 redox potential (196).

Following this initial electron transfer event, the electron is transferred from T1 to the buried T2/T3 cluster (12 Å) by the Cys-His pathway where the T2 and T3 sites are only separated by a distance of 4 Å (193). T2 is a mononuclear copper with no detectable absorption, and experiments show that removing T2 decreases the redox capacity of laccases. The T2 copper is linked by histidines and a hydroxyl group. T3 is a pair of binuclear coppers approximately 4 Å apart and that can be detected at a wavelength of 330 nm (197). Each copper in the T3 site is linked by three histidines (Figure 2.3.b).

In the laccase oxidized "resting" form, the two T3 coppers are linked together by a hydroxyl group. If reduced, then the T3 coppers separate (Figure 2.3.a) (198). Overall, the activity of laccases is tightly linked to the pH of the solution. Substrate oxidation by the T1 active site is improved at high pH. However, the transfer of electrons to T2/T3 is reduced at high pH due to the

hydroxide anion binding to its active site (196). These effects tend to balance each other out and optimal activity is generally observed in the pH range between 3.0 and 5.5 for fungal laccases (199).

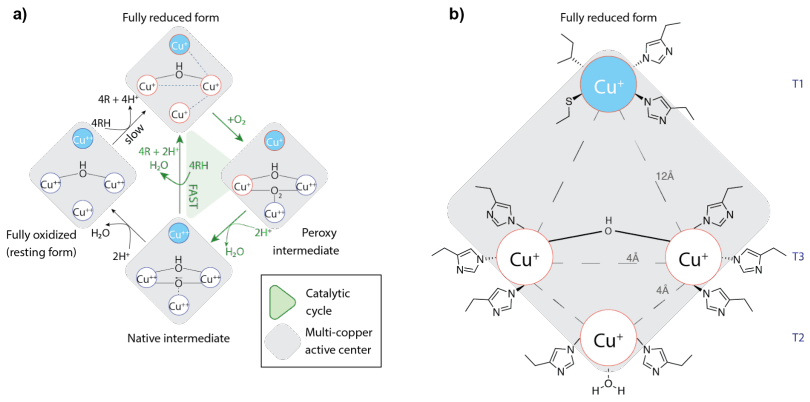


Figure 2.3.: a) Active multi-copper center of laccase, and mechanism from reduction to oxidation. Image adapted from Wong 2009 (200). b) Example of the active center structure of the white-rot fungi *Rigidoporus lignosus*, adapted from Garavaglia 2004 (201).

2.2.2.2. Activity on various substrates

2.2.2.2.1. Phenolic substrate

Phenols are oxidized to phenoxy free radicals by direct interaction with laccases, which results in either radical-coupling-based polymerization or radical rearrangement. In these reactions,

the laccase generates either dead-end products (202,203) or uses the phenolic substrate as a mediator in other laccase catalyzed reactions (204,205). Several publications have described the ability of naturally occurring phenolic compounds from lignin to act as redox mediators (206–208). Most laccases have optimal activity toward phenolic compounds at acidic to neutral pH (209–212) and laccases have not been shown to catalyze lignin depolymerization under alkaline conditions (199,213,214). Laccases present a diversity of binding modes depending on the substrate (179), and protein engineering efforts have been carried out to understand and obtain tailor-made biocatalysts presenting the desired properties (179,215). For functional diversity, a few engineered laccases with increased activity toward phenolic compounds such as 2,6-dimethoxyphenol, guaiacol at alkaline conditions (pH ≥ 8.0) have been reported (216). At alkaline pH, oxidation of phenolic compounds (either a phenolic mediator or a phenolic lignin dimer) to a phenoxy radical is favored by the presence of the phenolate form (204). At high pH, laccase activity can be inhibited by the abundance of hydroxide anion due to competitive binding to T2/T3 coppers (196). Toward enhanced activity at alkaline pH, more understanding of the electron transfer mechanism and T2/T3-catalyzed molecular oxygen reduction to water at the molecular level is needed for laccase engineering.

2.2.2.2.2. ABTS substrate

The use of mediators, such as 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS), helps to reduce the steric hindrance between the substrate and laccase. Substrates such as lignin and other larger phenolic compounds can have difficulties accessing the active site of a laccase due to their size. Mediators like ABTS, being smaller compounds, can be oxidized directly by laccase and then the oxidized mediator diffuses to the larger substrate where it is reduced by the substrate (192). A classic assay used to detect any kinetic reaction of laccase activity utilizes ABTS as a substrate and oxidation of ABTS as the readout. ABTS acts as an electron donor during the oxidation reaction by laccase, reducing its form into a radical cation $ABTS^{\cdot}$ that absorbs light at 734 nm and

turns the reaction to a blue color, the higher the laccase activity is, the faster the blue color appears (217).

The mechanism of action starts when ABTS gives its electron to the T1 active site of laccase. Large substrates, such as ABTS, have an unique way to bind to the T1 active site, by delocalizing a density of electrons on multiple atoms. ABTS forms hydrogen bonds and π-π interactions with the one of the T1 histidines (179). Then the electron gets transferred all across in its center active site at the T3 active site, and then to the T2 active site. When T2 receives the electrons, it sequentially reduces a molecule of dioxygen O_2 in two molecules of water H_2O (193).

2.2.2.3. Assay in development: ligNIMS

Knowing that the β-*O*-4 linkage represents 50 to 60% of most of the linkage in lignin, a team of researchers developed a specific activity assay for lignin degrading enzymes (LDEs) and this specific linkage using nanostructure-initiator mass spectrometry (NIMS) (218). They synthesized two β-aryl ether substrates, one phenolic lignin like model compound and one non-phenolic lignin like model compound, both attached to a NIMS-tag to be able to run the experiments through the NIMS. They studied two different enzymes, laccase enzyme and MnP enzyme, and quantified β-*O*-4 bond breaking and measured the reaction kinetics for these enzymes on both substrates . To analyze the laccase mechanism, Deng et al. used two different assay approaches, one assay used the phenolic substrate without the use of a mediator and one assay used the non-phenolic substrate with the mediator 1-hydroxybenzotriazole (HOBt)(Figure 2.4.). This assay provides distinct advantages over colorimetric assays like ABTS oxidation by allowing researchers to directly measure the types of laccase catalyzed bond breaking reactions required to enzymatically depolymerize lignin to defined breakdown products. Developing technology to be able to characterize laccase depolymerization per enzymes helped to understand the variety of mechanisms of action that enzymes can offer. While laccases grew interest in the scientific community, it also expanded its possibilities of action in industry.

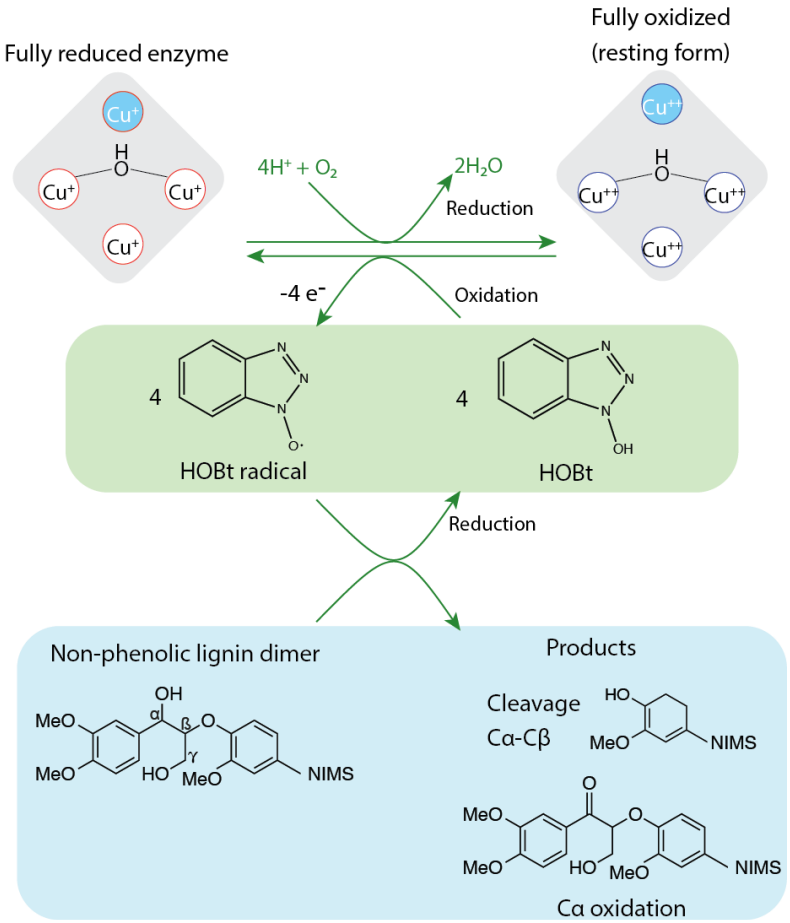


Figure 2.4.: Reaction mechanism of laccase oxidation using HOBt intermediaries to degrade lignin (218) (219).

2.2.3. Laccase, a solution for many different industries

Laccases are promising biocatalysts for degrading lignin into fragments amenable to being upgraded to valuable biofuels and bioproducts but find many additional industrial applications. Due to their broad substrate specificity, and their being naturally a green chemistry, laccases are being used in the food (220), pulping and bleaching (221), pharmaceutical and biosensors industries (222) and are being used for bioremediation (178) and in water treatment technologies (Figure 2.5.) (223) in environmental industries.

Phenolic byproducts are present in a variety of food industries, such as fermentation of sugarcane molasses, brewing and olive oil (220). The presence of phenolic compounds in beverages affects their taste, color and overall quality. Laccases demonstrated high efficiency of removing the phenolic compounds responsible for these deleterious effects compared to other chemical treatments, and the use of laccases also demonstrated great enhancement of the color stability of the drink after treatment, particularly in the red wine industry where the color is very important (224). In industrial baking, laccases have been used to increase the stability and strength of the machinery of the dough. Because laccase plays an oxidizing role in the dough, it improves the absorption and distribution of water, and so increases the overall volume and softness of the bread (225). A CU1 laccase from *Trametes maxima* has been tested in the bread, and the physicochemical properties of the bread were enhanced, such as the solubility of the arabinoxylans, the proteins and phenols content, the hardness was reduced, and the height and color amplified (226).

Over the last century, industrialisation and urbanisation led to a rise in pollution of the ecosystem and increased contamination of water. The broad activity of the laccases provides a sustainable solution to biotransform polluants molecules in water and to degrade organic compounds (178). Pollutants that can be degraded by laccases include plastics, herbicides, fertilizers, nonylphenol and bisphenol A and chlorinated paraffin phthalates. Additionally,

laccases can degrade environmental contaminants from pharmaceuticals, including pain killers, antibiotics, hormones and endocrine-disrupting chemicals (227,228). Laccases work in these cases by catalyzing degradation of these toxins to less harmful compounds. For example, laccase was shown to catalyze degradation of bisphenol A into Beta hydroxybutyric acid (229), chloramphenicol into chloramphenicol aldehyde (230), and others (231). In the northwestern Mexico groundwater, researchers evaluated the biotransformation of Bisphenol A, ethynylestradiol, triclosan and nonylphenol catalyzed by a laccase cocktail and obtained 55 to 93% biodegradation (232).

Another major industrial application of laccase is found in the pulping and paper industry. Pulping is a process where lignocellulose, and especially cellulose, is extracted from the wood. The cellulose is the main ingredient to make the pulp, which is then used to make paper. White liquor, containing sodium hydroxide and sodium sulfide in water, and steam are added to separate the cellulose from lignin. After digestion, the lignin is dissolved and concentrated through the Kraft process and separated from the pulp. The Kraft pulping process consists of lignin depolymerization, condensation and extraction. The pulp, commonly referred to as black liquor, contains concentrated lignin, which after filtration and chemical transformation is precipitated and purified (233). Every step in this process makes the pulp whiter and brighter. This process has several inefficiencies such as the generation of toxic byproducts and the use of large amounts of water, aspects which can be improved for enhanced environmental and economic advantage (234). For example, chlorophenols are used as bleaching agents and since 1999 have been known as harmful substances and listed as environmental pollutants by the United States Environmental Protection Agency (US EPA) (235). Green chemistries based on using enzymes such as laccases offer a more sustainable replacement to answer the challenges of the pulp and paper industry (236). The use of laccase in the pulp and paper industry could permit the replacement of chemicals such as chlorine dioxide, hydrogen peroxide, ozone, hydrosulfite and alkaline extraction (237), making the pulping process a biopulping, the bleaching process a biobleaching etc… and detoxifying the paper mill effluents. Moreover, laccases demonstrated an increase in

brightness after lignin digestion, and an enhancement after biobleaching of up to 12% consistency of the pulp, using laccase from *Trametes villosa* with HOBt, violuric acid (VA), and ABTS as mediators (221). A laccase from *Bacillus sp.* showed, after biobleaching and deinking of Old Newsprint (ONP), a brightness improvement of 13% and whiteness enhancement of 106% compared to the traditional chemical method (238).

Biosensors are analytical devices that permit the monitoring of chemicals such as phenolic compounds by converting physical or chemical signals into an electric signal that is amplified and processed to be analyzed and directly monitored. The most commonly used biosensors today are glucose biosensors that monitor blood glucose concentration in diabetics (239). To detect analytes of interest, biosensors require the use of enzymes to convert analytes of interest to detectable compounds. Laccase-based biosensors are designed to be fast, online and *in situ* devices for monitoring reactions with phenolic compounds. Phenol biosensors have applications in the food industry, environmental analyses, and pharmaceutical industry (235). Immobilisation of laccase, meaning the capture of enzymes in a physical support, allowed the development of biosensors for phenolic compounds determination (240). Immobilization methods include covalent binding, adsorption, cross-linking, encapsulation and entrapment (241). The substrate molecule reacts with the immobilized laccase and is converted to a product that issues an amperometric, voltammetric, potentiometric or conductometric signal, that is amplified and analyzed by the electronics of the biosensor (235).

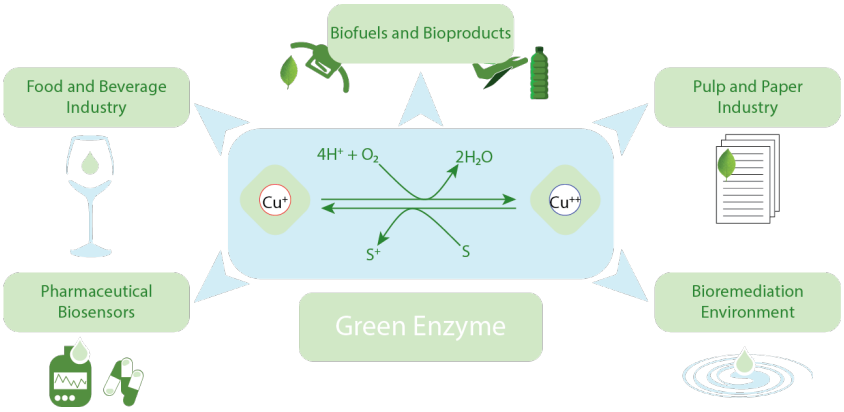


Figure 2.5.: Laccase enzyme, a green chemistry in the service of several industries.

2.2.4. Challenges in application of laccases

Laccases have been the subject of numerous studies for the last twenty years, resulting in their use in many applications with various goals (Figure 2.6.). As climate change raises concerns, it gives industry new challenges, such as combating pollution and applying the rule of "reduce-reuse-recycle" (220). Being an eco-friendly, swiss-knife applicant, laccases offer promising solutions due to their versatile mode of action, but not at a cheap price. First of all, one of the major drawbacks is that there is no current technology to recycle laccases in industry, and without reusing the enzyme, it impairs drastically the economic viability of the process (236). Indeed, large scale industrial applications using laccases remain arduous. The full viable commercialization of laccase would require a high production and purity level for an affordable price per ton of enzyme, and cheap intensive mediators (such as ABTS), that help to increase the laccase redox potential (221). The cost of production is highly increased by the downstream process, for this

reason it is important to focus on the development of affordable purification methods (242). Another industrial disadvantage is the need of *in vitro* enzymes rather than *in vivo*, the activity potential is lowered after extraction of the enzymes, due to the instability and insolubility of the protein in the chosen substrate e.g. solvents (243,244). Laccase-based biosensors are facing additional challenges due to the enzyme immobilization. In fact, the costs carriers and immobilization are high due to the isolation, purification and recovery cost of the enzyme, but also because of enzyme instability after immobilization and sometimes loss of catalytic properties (235). Finally, the current available literature on laccases is heterogeneous, such as the investigation of their chemical properties and their characterization, resulting in the limit of their possible comparison and interaction (245). Today, most oxidoreductase enzymes are currently under active development for being affordable commercially by developing better purification platform processes and increasing their stability and their choice of substrate.

2.2.5. Development and prospect of laccase industrialization

Despite all the bottlenecks and challenges presented, research on laccases has resulted in great innovations over the last few years and researchers continue to generate new innovations for optimal exploitation of laccases (Figure 2.6.). A techno-economic analysis of the industrialization of lignin degrading enzymes such as laccase shows that more effort is required to lower the price of enzymes for biofuels production (246). Thanks to the development of new approaches in computational, biophysical and biochemical fields, our ability to engineer enzymes for improved stability and performance has greatly improved. This enables custom optimization of enzymes and their functionality for different applications (247,248). The development of bioinformatic tools are useful for metagenomic analysis and identification of new enzymes with new functionalities from non-model organisms (249). As an example, recent promising research using directed evolution

resulted in the adaptation of the Lcc9 laccase from *Coprinopsis cinerea*, expressed by *P. pastoris,* to function optimally at pH between 8.0 - 8.5 (199). The development of biosensors has led to innovative methods of enzyme immobilization, such as adsorption, entrapment, covalent-binding, encapsulation and self-immobilization, showed encouraging results to reuse laccase enzymes but the activity of the recovered enzyme after immobilization is still not always consistent (241). The development of novel biotechnologies has allowed researchers to discover new enzymes for lignocellulose bioconversion, by exploring the biodiversity of extreme habitats endowing the advantageous characteristics, such as this highly functional thermostable TtLMCO1 laccase from *Thermothelomyces thermophila* that has a wide spectrum of substrate specificity and has been successfully expressed in *Pichia pastoris* (250). In order to reduce the production costs, homologous and heterologous hosts are being investigated to secrete laccases enzymes, such as *Aspergillus sp*. which is a well-known industrial host that can produce more than several $g.L^{-1}$ and could potentially reduce the purification downstream process considerably (251). It is possible to derivatize lignin into mediators that can reduce the cost of using traditional mediators that are used today to improve the redox potential of laccases (247). Moreover, during the laccase bleaching stage, mediators undergo decomposition which limits their turnover efficiency (192).

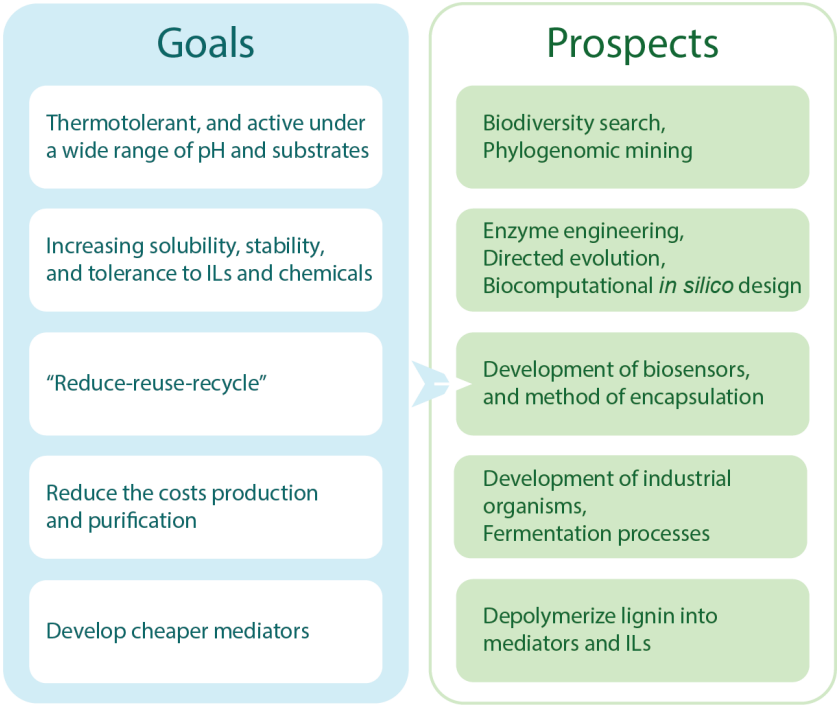


Figure 2.6.: Goals and prospects of laccase industrialization.

2.3. Conclusion

The efficient and affordable valorization of lignin into biofuels and bioproducts remains one of the most significant challenges facing commercial biorefineries and the bioenergy enterprise. While studied for decades, there remain significant gaps in our fundamental understanding of how lignin is broken down by biological systems. In particular, the lack of any robust and efficient biochemical methods (e.g, enzymes) capable of rapidly breaking

down lignin into targeted intermediates and final products limits our ability to valorize lignin. Laccase enzymes are among the most studied class of lignin degrading enzymes and present a compelling suite of characteristics that may be key to solving these challenges. Over the last 20 years, there have been several reviews published about laccase characterization and their applications in different domains of industry. New technologies promote the potential of the enzyme and industrialisation processes. However, laccases are still too expensive to be used and produced industrially, there is a need to constantly develop better tools and innovate, to make laccase an affordable industrial enzyme and to get one step closer towards affordable biofuels and bioproducts to replace fossil fuel economically and durably. Here we have presented some potential approaches based on laccases that provide a roadmap for the scientific community to work towards achieving the true potential of lignin.

Chapter 3: A new approach to Cas9-based genome editing in *Aspergillus niger* that is precise, efficient and selectable

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Abstract

Aspergillus niger and other filamentous fungi are widely used in industry, but efficient genetic engineering of these hosts remains nascent. For example, while molecular genetic tools have been developed, including CRISPR/Cas9, facile genome engineering of *A. niger* remains challenging. To address these challenges, we have developed a simple Cas9-based gene targeting method that provides selectable, iterative, and ultimately marker-free generation of genomic deletions and insertions. This method leverages locus-specific "pop-out" recombination to suppress off-target integrations. We demonstrated the effectiveness of this method by targeting the phenotypic marker *albA* and validated it by targeting the *glaA* and *mstC* loci. After two selection steps, we observed 100% gene editing efficiency across all three loci. This method greatly reduces the effort required to engineer the *A. niger* genome and overcomes low Cas9 transformations efficiency by eliminating the need for extensive screening. This method represents a significant addition to the *A. niger* genome engineering toolbox and could be adapted for use in other organisms. It is expected that this method will impact several areas of industrial biotechnology, such as the development of new strains for the secretion of heterologous enzymes and the discovery and optimization of metabolic pathways.

3.1. Introduction

The recombinant production of enzymes at high titers using various hosts, such as filamentous fungi, is an important aspect affecting costs for many commercial applications today, including pharmaceuticals (252)], food processing [(253)], biofuels [(254)], and detergents. Despite the widespread deployment of these fungal strains in industry, the genetic toolbox by which they can be efficiently optimized for any given application, such as improved recombinant protein production from gene expression, remains challenging and time consuming [(255)]. One of the industrial approaches to the conversion of starches and polysaccharides into

monomers suitable for subsequent bioconversion into biofuels relies on the use of hydrolytic enzymes, such as amylases, cellulases, and hemicellulases that are naturally found in fungi and bacteria [(151,256)]. In order for recombinant enzymes of this type to be produced at the commercial scale, they must be produced at high titers and yields in order to reduce costs. While these enzymes could be produced by the filamentous fungi in which they are found in naturally or in recombinant hosts, these fungi may not secrete enough of the targeted enzymes needed and therefore genetic engineering and optimization of these strains is an important component of commercial viability [(50)].

Aspergillus niger is a filamentous ascomycete fungus utilized industrially for the production of citric acid and for its ability to produce and secrete high levels of endogenous and recombinant enzymes [(55)]. It is generally recognized as safe at the commercial scale, its genome is sequenced and it is amenable to standard genetic modification techniques [(257)]. The genomic integration of exogenous DNA via homologous recombination (HR) has been widely applied in *A. niger* and other filamentous fungi [(55)]. Typically, genes are replaced with a "fixing template" containing a selectable marker, thereby permitting selection of the integration event. The *pyrG* gene, encoding encodes orotidine-5′-monophosphate decarboxylase, an intermediate in the pyrimidine pathway forming uridine monophosphate, is both positively and negatively selectable; the integration of *pyrG* can be selected for by culturing in the absence of uracil/uridine while the absence of *pyrG* can be selected for in the presence of 5-fluoroorotic acid (5-FOA) [(258,259)]. *pyrG* converts 5-FOA into fluoroorotidine monophosphate which is subsequently converted into fluorodeoxyuridine by ribonuclease reductase. Fluorodeoxyuridine is a suicide inhibitor of the thymidylate synthase and therefore inhibits DNA synthesis and leads to cell death. 5-FOA is non-toxic in the absence of *pyrG*. The positive/negative selection of *pyrG* can be exploited to permit iterative targeting by selecting for the "pop-out" excision of *pyrG* via HR after integration [(65)].

Targeting double stranded breaks (DSBs) to the site of DNA integration is known to increase the efficiency of HR [(71,73,74,260)]. Originally a bacterial defense system, the now-ubiquitous CRISPR/Cas9 (Clustered Regularly Interspaced Short

Palindromic Repeats; CRISPR associated protein 9) was engineered for rapid targeting of DSBs [(261)]. In this system, a small guide RNA (sgRNA) targets the Cas9 endonuclease to its complementary DNA. In addition to facilitating HR, CRISPR/Cas9 can be used to introduce deletions and point mutations without necessarily introducing foreign DNA [(77,262)]. CRISPR/Cas9 was previously demonstrated to be effective in several filamentous fungi, e.g. *A. niger*, *A. oryzae*, *A. fumigatus*, and *Neurospora crassa* [(53,85,86)].

Nevertheless, this method requires extensive screening as off-target integrations, mediated by non-homologous end-joining (NHEJ), lead to an overwhelming rate of false positives [(85)]. Several strategies have been employed to increase the efficiency of HR, including the adjustment of length of the HR arms [(263)], engineering the RAD52 HR protein [(58)], or knocking out the *Ku70* genes responsible for NHEJ [(54)]. Complete disruption of NHEJ can lead to genomic instability and increases the risk of DNA damage [(264)]. Therefore, high-efficiency specific gene editing in *A. niger* and other filamentous fungi remains a significant challenge. Editing efficiency has been reported to be from anywhere between 1 and 100% efficient depending on the CRISPR/Cas9 setup and the target locus [(85)]. Targeting non-phenotypic genes requires laborious sequencing of transformants.

To address these challenges, we have developed reusable, transiently-selectable donor DNA for a specific integration system. After validating this methodology using the phenotypic marker *albA,* we sequentially targeted two genes likely to improve heterologous enzyme production. We replaced *glaA* (glucoamylase) with the *Thermotoga petrophila* β-glucosidase designated A5IL97 [(265)]. We then interrupted the sugar transporter *mstC* [(266)] and observed 100% efficiency of the desired mutations at all three loci using positive and negative selection pressure. This approach allows for the efficient engineering of *A. niger* and eliminates the need for screening hundreds of transformants. To the best of our knowledge, this is the first published report on this new Cas9 approach and applying it in *A. niger* (or any fungi) and significantly reduces the time required for the screening of positive mutants at high efficiencies.

3.2. Results

Our approach relies on the induction of a genomic DSB with a targetable Cas9/sgRNA complex, incorporation of a selectable marker via HR, and selection of *pyrG*-containing mutants by culturing in the absence of uracil/uridine. To validate this approach, we targeted *albA,* a polyketide synthase responsible for the production of a black spore pigment [(267)]. When *albA* is disrupted, colonies present a white rather than black spore phenotype, providing a convenient and commonly used selection technique.

We generated a fixing template, construct DNA 006 (cDNA006), with 1,500 bp homology arms for targeting *albA* (Figure 3.1.). cDNA006 contains a 5' stop codon repeat for disrupting translation and the *pyrG* gene. To generate a "recyclable" marker system, *pyrG* was flanked with direct repeat sequences [(65)]. Upon exposure to 5-FOA, transformants containing *pyrG* should undergo "pop-out" recombination to remove the marker, thereby permitting additional rounds of gene targeting using *pyrG* selection.

While some methods contain the fixing template and sgRNA on the same plasmid as Cas9, this necessitates additional cloning steps when targeting new genes and leads to off target effects due to constitutive expression [(85)]. We therefore opted for *in vitro* preparation of the sgRNA and fixing template (**see Materials and method**). cDNA006, an *albA*-targeting small guide RNA (sgRNA001) and plasmid pFC332, containing a constitutively expressed *A. niger* codon-optimized Cas9, were simultaneously transformed into ATCC 1015 *pyrG*-. Transformants were plated onto minimal media without uracil/uridine and with 300 µg/mL hygromycin to select for the integration of *pyrG* and the maintenance of pFC332, respectively. After 4 days, 79% of the colonies had the white spore phenotype, indicating successful targeting of *albA* (Figure 3.1.). We then isolated black and white colonies and re-streaked them on minimal media containing uracil/uridine and 5-FOA, to select for the "pop-out" recombination of *pyrG* (Figure 3.1.A, step 2). These colonies were then re-plated on MMA + uracil. Sequencing the specific locus revealed that the 100% of the black colonies were free of mutations at the *albA* locus, while 100% of the white colonies contained the integrated stop

codon exact protospacer location of the sgRNA (Figure 3.1.) (Supplemental Figure 3.1.).

We observed efficient, selectable gene deletion with successful excision of *pyrG*. Nevertheless, 21% of colonies did not have mutations at the *albA* locus but survived on MMA + hygromycin without uracil/uridine supplied (Figure 3.1.B, 3.1.C), indicating NHEJ-mediated off-target integration of the fixing template [(268)]. While NHEJ-mediated repair can be suppressed by knocking out genes in the NHEJ pathway, this can lead to genomic instability and mutagenic sensitivity [(264)]. Therefore, we sought to engineer a fixing template to screen positive mutations at the correct integration locus.

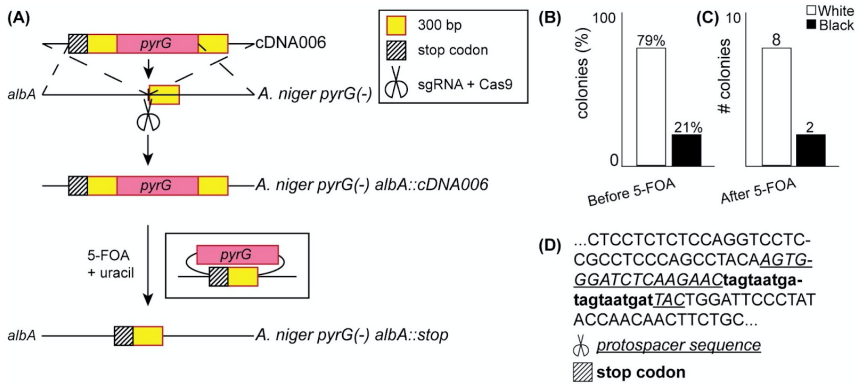

Figure 3.1.: Design and application of cDNA006 for disruption of *albA*.
(A) of cDNA006 contains *pyrG* flanked by 300bp repeats at 1000 bp homology arms to *albA*. After integration, *pyrG* is excised by homologous recombination in the presence of 5-FOA. (B) and (C) Phenotypes obtained before 5-FOA and after 5-FOA. (D) Representative sequence showing the integration of the stop codon at the *albA* locus in a white colony.

3.2.1. Developing a specific pop-out marker

We designed a fixing template (cDNA008) that will excise *pyrG* when it is specifically integrated at the *albA* locus (Figure 3.2.A). Rather than inserting a stop codon, cDNA008 was designed to delete 1000 bp of *albA* to disrupt the gene. Like cDNA006, cDNA008 contains the *pyrG* gene. A 300 bp cassette was placed in front of the *pyrG* gene that are homologous to the 3' region of *albA.* After integration and exposure to 5-FOA, *pyrG* should undergo pop out recombination if it is correctly integrated into the *albA* locus. HR loses efficiency as the distance between homologous sequences increases [(269)]. Therefore, HR-mediated excision of *pyrG* will be inefficient for off-target integrations, and cells with off-target integrations should die in the presence of 5-FOA.

After transformation of Cas9, sgRNA001, and cDNA008, 71% of the colonies had the white spore phenotype (Figure 3.2.B). 7 white and 3 black colonies were re-streaked on plates containing 5-FOA. The white colonies survived on plates containing 5-FOA, while there was no detectable growth of the black colonies after one week (Figure 3.2.C) (Supplemental Figure 3.2.). PCR amplification of the *albA* locus at each stage showed (#2) the integration of *pyrG*, and (#3) the pop-out recombination of *pyrG* and deletion of 1000bp of *albA* (Figure 3.2.D). Sequencing the *albA* locus of all mutants confirmed the integration of *pyrG* and subsequent recombination upon 5-FOA treatment. Therefore, on the 10 analyzed colonies, we observed 100% of correct *albA* locus modifications after treatment with 5-FOA, suggesting the method suppresses off-target integrations (Supplemental Figures 3.2 and 3.3).

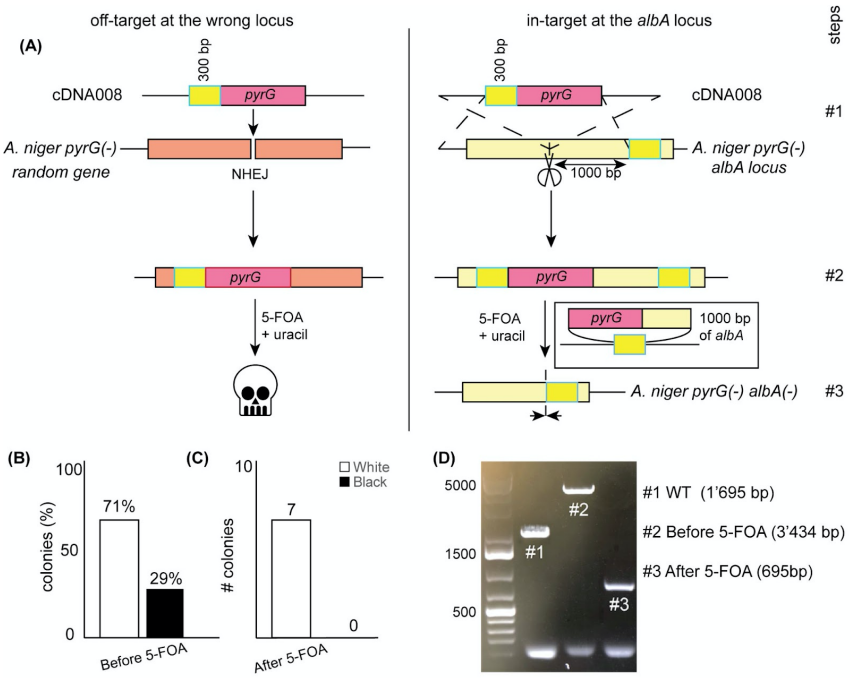

Figure 3.2. Design and application of a specific construct cDNA008 for disruption of *albA*.
(A) Design of cDNA008 construct inserted at the *albA* locus to delete 1,000 bp making *A. niger pyrG*⁻ *albA*⁻, use of sgRNA001. (B) and (C) Results obtained before 5-FOA and after 5-FOA. (D). PCR amplification of the *albA* gene in wild type (WT) strain #1, before 5-FOA insertion of *pyrG* at the *albA* locus #2, and deletion of 1000 bp of *albA* after 5-FOA #3.

3.2.2. Targeting a non-phenotypic gene

After demonstrating the feasibility of our method at the *albA* locus, we then targeted the non-phenotypic gene *glaA*, and replaced it with another gene, *A5IL97*, in a single procedure. The *glaA* gene encodes the glucoamylase enzyme, a natural highly secreted enzyme of *A. niger* [(270), which has a strong promoter,

P_{glaA} [(271)], that can be used to produce heterologous enzymes [(266)]. As a proof of concept, we used the gene that encodes for the β-glucosidase *A5IL97* that has been previously shown to be secreted by *A. niger* [(266)]. We designed a construct, cDNA009, to target the *glaA* locus. cDNA009 resembles the cDNA008 with the addition of the open reading frame (ORF) for A5IL97 (Figure 3.3.A). After transformation, 10 colonies were isolated on MMA selecting for the integration of *pyrG*. After PCR amplification at the *glaA* locus, only 8 colonies of the 10 selected on MMA had integration of the *pyrG* marker at the locus. After 5-FOA selection, only the 8 colonies containing previously *pyrG* survived on 5-FOA. Sequencing of 5-FOA resistant mutants confirmed 100% efficient deletion of *glaA*, integration of A5IL97 and the *pyrG* marker was removed at the locus (Table 3.1.) (Supplemental Figure 3.4.).

As 5-FOA exposure led to the excision of *pyrG* and the genotype *A. niger ΔglaA/*P_{glaA}*-A5IL97 pyrG*$^-$, this method is inherently recyclable. After successfully replacing *glaA* with A5IL97, we verified the iterative nature of this method by targeted disruption of a second gene, *mstC*, in this strain (Figure 3.3.B). *mstC* encodes a glucose transporter that, once disrupted, has been identified to enhance the P_{glaA} for heterologous enzyme production [(266)]. With an off-target suppressing construct, we targeted *mstC* and observed 100% deletion after 5-FOA (Table 1), making the strain *A. niger pyrG*$^-$ *ΔglaA/*P_{glaA}*-A5IL97 ΔmstC* (Supplemental Figure 3.5.).

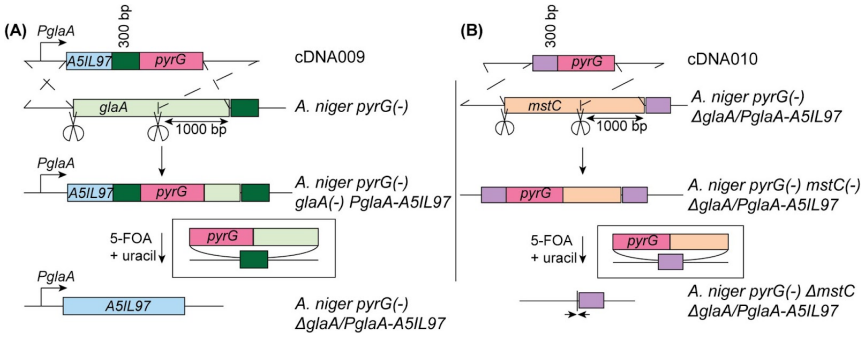

Figure 3.3. Design and application of cDNA009 (*glaA* locus) and cDNA010 (*mstC* locus).
(A) cDNA009 construct inserted at the *glaA* locus to insert *A5IL97* gene making *A. niger pyrG - ΔglaA/P*$_{glaA}$*-A5IL97*, use of sgRNA002 and sgRNA003. (B) Design of cDNA010 construct inserted at the *mstC* locus to delete *mstC* on the *A. niger pyrG - ΔglaA/P*$_{glaA}$*-A5IL97*, resulting in the strain *A. niger pyrG - ΔglaA/P*$_{glaA}$*-A5IL97 ΔmstC*.

Gene targeting	Constructs	sgRNA	Method	Before 5-FOA	After 5-FOA
albA $^{-}$ Codon stop insertion	cDNA006 X-pyrG-X	sgRNA001	Non-selective	19 white colonies 5 black colonies (79% white colonies)	8 white colonies 2 black colonies
albA $^{-}$ Deletion of 1000 bp	cDNA008 X-pyrG	sgRNA001	Selective	20 white colonies 8 black colonies (71% white colonies)	7 white colonies 0 black colonies
ΔglaA Gene replacement with A5IL97	cDNA009 A5IL97-X-pyrG	sgRNA002 sgRNA003	Selective	8 colonies with *pyrG* at the locus 2 colonies without *pyrG* at the locus	8 on target 0 off target
ΔmstC Gene deletion	cDNA010 X-pyrG	sgRNA004 sgRNA005	Selective	7 colonies with *pyrG* at the locus 3 colonies without *pyrG* at the locus	7 on target 0 off target

Table 3.1.: Efficiency obtained before and after selection of 5-FOA by PCR amplification at the mutated locus and sequence verified.

3.3. Conclusion

We have designed and demonstrated a technique that efficiently edits the genome of *A. niger* based on CRISPR/Cas9. We targeted the non-phenotypic genes *glaA* and *mstC* on the same strain and obtained 100% efficiency after selection on 5-FOA. Despite the 100% efficiency observed at these three different loci using the method, there is no guarantee that 100% efficiency will be observed for all loci. Many factors influence the probability of

genomic modification, including the essentiality and accessibility of a gene [(272)]. The originality of this technique is in the design of the construct which leads to a simple counter selectable method for in-target integration, allowing us to tolerate loss of efficiency due to the organism, the gene target [(273)], the choice of the sgRNA or the way in which it is delivered (*in vitro* or *in vivo*, choice of the promoter), and the Cas9 expression method. It should be noted that other off-target effects, such as the generation of point mutants caused by Cas9, are not suppressed. The method presented here should overcome limitations in genome editing in filamentous fungi such as low efficiency editing for some loci and the time required to screen mutants when the gene in target is not phenotypic. The described method is a worthwhile addition to the tools available for genome editing in filamentous fungi such as the use of short recombination arms [(274)], and reduction of off-target effects by knockout of the NHEJ protein *KusA* [(275)].

We used the Cas9 plasmid under a constitutive promoter but not with the sgRNA on the plasmid to reduce the risk of off-target effects [(262,276)] and facilitate the preparation of the sgRNA for the transformation. For our purposes *in vitro* sgRNA preparation was sufficient for 100% gene editing, which is in line with other reports demonstrating the efficiency of *in vitro* sgRNA [(89,268)]. The choice of the sgRNA is crucial for the Cas9 targeting efficiency. A simple test *in vitro* with Cas9 can demonstrate the efficiency of each individual sgRNA (see **Methods**). Looking forward, *in vitro* sgRNA preparation may be the easiest method for testing many sgRNAs without the need for extensive sub cloning [(268).

The primary focus of this study was to reduce the workload of screening for positive mutants and to generate a recyclable rescue marker for iterative mutation, which we have demonstrated. This method can be adopted to generate point mutants by incorporating the mutation in the fixing template. In this study we only used the auxotrophic marker *pyrG* vs 5-FOA, but there are more rescue markers available that have not been tested, such as *amdS*. This method may be applied to multiplex genome engineering in the same recyclable, specific manner. Many of the pre-existing CRISPR/Cas9 methods work in multiple filamentous fungi [(85)]. While we have only tested these methods on *A. niger*, these methods may likely be applied to other species. In conclusion,

this novel method greatly simplifies genome editing in *A. niger* and will enable the rapid generation of genomic mutants and libraries for the investigation of biology and further improve the use of *A. niger* as an important heterologous production host.

3.4. Materials and methods

3.4.1. Reagents

All chemicals were purchased from Sigma unless otherwise noted.

3.4.2. Strains

The strains used in this paper are listed in Table 3.2. The genome sequence of strain ATCC 1015 v4.0 is accessible from the Joint Genome Institute (JGI).

Name	Genotype	Source	Access
JBEI-14377	*ATCC 1015 pyrG* $^{-}$	[(267)	https://registry.jbei.org/folders/1399
JBEI-099147	*ATCC 1015 pyrG* $^{-}$ *albA* $^{-}$	This study.	https://registry.jbei.org/folders/1399
JBEI-099148	*ATCC 1015 pyrG* $^{-}$ *albA* $^{-}$	This study.	https://registry.jbei.org/folders/1399
JBEI-099149	*ATCC 1015 pyrG* $^{-}$ *ΔglaA/*P_{glaA}*-A5IL97*	This study.	https://registry.jbei.org/folders/1399
JBEI-099151	*ATCC 1015 pyrG* $^{-}$ *ΔmstC ΔglaA/* P_{glaA}*-A5IL97*	This study.	https://registry.jbei.org/folders/1399

Table 3.2.: *A. niger* strains used in this study and their accession information.

3.4.3. Plasmids

This study builds off of pre-existing Cas9 expression of the pFC332 shuttle plasmids for *A. niger* [(86)]. The plasmids express an *A. niger* codon optimized Cas9 under expression of the TEF-1 promoter. These contain the *A. nidulans* AMA1 replication cassette which mediates replication in multiple species of filamentous fungi [(277)]. The plasmid contains an hygromycin (*hph*) resistance marker for the selection of the plasmid. All plasmids were re-sequenced before proceeding further. Each transformation has been executed with a positive control, using two plasmids pFC330 (*pyrG* marker) and pFC332 (*hph* marker), and a negative control, using water.

3.4.4. Construction of sgRNA

All of the sgRNA used, except for the *albA* sgRNA [(86)], were designed using the CRISPOR algorithm [(97)] and chosen to minimize off-target mismatches (Table 3.3.). Once the sgRNA were chosen using the CRISPOR algorithm, they were prepared and tested *in vitro* using the Guide-it sgRNA Screening Kit (Takara). After the sgRNA were validated *in vitro*, they were amplified for transformation using the GeneART gRNA synthesis (Thermo Fisher). The concentration of sgRNA obtained after purification was ~10 µg/µL (Nanodrop). 20 µg sgRNA were used for each transformation to reach an optimal efficiency.

Gene targeting	Sequencing	name	Source
albA	AGTGGGATCTCAAGAACTAC	sgRNA001	[(86)
glaA 5'	CTGTGCAGACGAGGCCGCTC	sgRNA002	CRISPOR.tefor.net
glaA 3'	TCTACACGAAGGAAAGACCA	sgRNA003	CRISPOR.tefor.net
mstC 5'	TCCGCGTTGTATGAATCCAC	sgRNA004	CRISPOR.tefor.net
mstC 3'	GTGCCAGGCAGCCTGACCGG	sgRNA005	CRISPOR.tefor.net

Table 3.3.: Sequence of sgRNAs with original source.

3.4.5. Donor DNA

3.4.5.1. DNA Design

Each donor DNA (cDNA) contained the *pyrG* gene and was flanked with 1000 bp or 1500 bp HR arms for efficient integration [(54)].

Strains	Plasmid	Amplicon	Gene target	Homology arms (bp)	Selectable marker	Sequence
JBEI-099138	pIIk034	cDNA006	*albA*	1500	*pyrG*	https://registry.jbei.org/folders/1399
JBEI-099142	pIIk036	cDNA008	*albA*	1000	*pyrG*	https://registry.jbei.org/folders/1399
JBEI-099144	pIIk038	cDNA009	*glaA*	1000	*pyrG*	https://registry.jbei.org/folders/1399
JBEI-099146	pIIk039	cDNA010	*mstC*	1000	*pyrG*	https://registry.jbei.org/folders/1399

Table 3.4.: cDNA features and their accession information.

3.4.5.2. DNA Preparation

The preparation of the donor cDNA was performed via PCR cloning from the genome of *A. niger* or purchased from Genscript (https://www.genscript.com/) (Table 3.4.). The cDNA was integrated into the plasmid pUC57, transformed into DH10b competent cells (New England Biolabs, NEB) and selected on LB with 100 µg/mL carbenicillin plates. The resulting plasmids (Table 3.4.) were sequence verified by Quintara (https://www.quintarabio.com/). The plasmids were used as the template to generate linear cDNAs by PCR amplification using Phusion Hot Start II (Thermo Fisher) and their respective primers (Supplemental Table 3.1.). The four cDNAs PCR products were purified and concentrated to 1 µg/µL and 10 µg was used per transformation as described below.

3.4.6. Transformation

Before transformation, *A. niger* was prepared for a protoplast-mediated transformation (PMT) [(57)], which consist of degrading the cell wall using VinoTaste Pro. After simultaneous transformation of the plasmid containing Cas9, the sgRNA *in vitro*, and the donor DNA into *A. niger pyrG* $^{-}$, the mixture was incubated on ice for 20 minutes in a transformation solution (25% polyethylene glycol (6,000), 50 mM $CaCl_2$, and 10 mM Tris HCl, pH 8.0). The mixture was plated on a 1% glucose minimal media containing agar and 1M sorbitol (MMA) + 300 µg/mL hygromycin, and the plates were incubated at 30°C. After transformation, the colonies were isolated on plates containing MMA + 300 µg/mL hygromycin. After visible growth but before the appearance of the first spores, the colonies were scooped out and isolated on slants containing only MMA. The Cas9 plasmid is lost in the absence of selective pressure (hygromycin). Once the colonies in the slants formed spores, the spores were isolated on plates containing MMA + 1.3 mg/mL 5-FOA + 1.2 mg/mL uracil. If the colonies were growing, they were re-isolated using MMA + 1.3 mg/mL 5-FOA + 1.2 mg/mL uracil plates again, then before the appearance of the first spores the colonies were scooped out and placed on slants containing MMA + 1.2 mg/mL uracil/uridine. For each transformation a minimum of 10 colonies were isolated, transformed on 5-FOA then re-isolated for analysis by PCR and sequencing (Supplemental Figure 3.6.). To determine the efficacy of 5-FOA, the colonies were lysed and analyzed before and after exposure to 5-FOA. Note that if the *pyrG* marker needs to be recycled, it is recommended that the fungi recover between experiments. Also, manipulation of spores often leads to contamination and requires great care during the transformation [(28)]. The detail protocol "Transformation *Aspergillus niger* using Cas9, AMA1 vector, *pyrG* rescue marker and sgRNA *in vitro*" is available on protocols.io.

3.4.7. Lysis

20 µL spores were harvested in 0.1 % of tween buffer and mixed in 500 µL a solution containing 400 mM of Tris-HCl pH 8.0, 60 mM of ethylene diaminetetraacetic acid (EDTA) pH 8.0, 150 mM

NaCl and 1% (v/v) sodium dodecyl sulfate (SDS). After incubation at room temperature for 10 minutes, 100 µL of a second solution containing 2 M potassium acetate, and 7.6% glacial acetic at pH 4.8 was added to the mixture. After centrifugation at 10,000 rpm, the supernatant containing the DNA was cleaned using isopropyl alcohol followed by 70 % ethanol (EtOH). The ethanol was evaporated in a rotavapor (Vacufuge Plus Eppendorf) and the DNA was resuspended into 50 µL dH_2O. The detail protocol "Lysis *Aspergillus niger*, extracting and purifying DNA" is available on protocols.io.

3.4.8. PCR

Every transformation was analyzed by PCR (AB Applied Biosystems/Veriti 96 well Thermal Cycler) before 5-FOA and after 5-FOA (Supplemental Figures 3.1 3.3 3.4 3.5). We used LongAmp Taq DNA polymerase purchased from NEB and the primers synthesized by Integrated DNA Technology (IDT) (S2-S3 Tables). The protocol followed was provided by NEB.

In the following Chapter 4, the author of this thesis, Laure Leynaud-Kieffer Curran, only contributed partially:

- I designed and constructed the ATMT plasmids
- I performed the experiment CRISPR/Cas9 establishment
- I read and approved the final manuscript

Chapter 4: Development of genetic tools for the thermostable cellulase and hemicellulase-producing filamentous fungus *Thermoascus aurantiacus*

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Abstract

Fungal enzymes are vital for various industrial applications such as the conversion of plant biomass to biofuels and bio-based chemicals. In recent years, there is increasing interest in using enzymes from thermophilic fungi, which often have higher reaction rates and thermal tolerance compared to currently used fungal enzymes. The thermophilic filamentous fungus *Thermoascus aurantiacus* produces highly thermostable plant cell wall degrading enzymes. However, no genetic tools have yet been developed for this fungus, which prevents strain engineering efforts. The goal of this study was to develop strain engineering tools such as a transformation system, the CRISPR/Cas9 system and a sexual crossing protocol to aid strain engineering for improved enzyme production.

Here we report on first-time *Agrobacterium tumefaciens*-mediated transformation of *T. aurantiacus* using the *hph* marker gene, conferring resistance to hygromycin B. The newly developed transformation protocol was optimized in efficiency and used to integrate an expression cassette of the transcriptional xylanase regulator *xlnR*, which led up to 500% increased xylanase activity of those strains. Furthermore, the CRISPR/Cas9 gene editing system was established in this fungus, and two different gRNAs were tested to delete the *pyrG* orthologue with 10% and 35% deletion efficiency, respectively. Lastly, a sexual crossing protocol was established using a hygromycin B- and a 5-fluororotic acid-resistant parent strain. Crossing and isolation of progeny on selective media was shown to be completed in a week.

Thus, the necessary genetic tools have been established, and as a proof of concept, we generated high xylanase expressing fungal strains. These tools can now be used individually or in

combination to further improve thermostable enzyme production of this fungus for cost-efficient biofuel production.

4.1. Introduction

Due to the potentially deleterious impacts of climate change, which is mainly caused by the use of fossil resources, great efforts have been made to explore the applicability of lignocellulosic plant biomass as a sustainable alternative to fossil fuels. Lignocellulosic biomass is the most abundant organic material on earth, consisting primarily of the sugar polymers cellulose and hemicellulose and the aromatic polymer lignin (278) (279). These sugar polymers can be deconstructed by enzymes (cellulases and hemicellulases) into simple sugars that can be further converted into biofuels and other bio-based products using metabolically engineered bacterial and fungal hosts, which reduces our dependence on finite fossil resources (280). The cost-efficient deconstruction of lignocellulose is currently the biggest obstacle preventing biofuels from becoming competitive to fossil fuels.

Filamentous fungi are efficient lignocellulose degraders, possessing an arsenal of secreted enzymes that digest cellulose and hemicellulose (281). These organisms have evolved an elaborated sensing system to detect the components of lignocellulosic biomass and fine-tune expression of cellulase and hemicellulase genes (282). Therefore, filamentous fungi are the most important industrial cellulase producers (283,284).

Recently, there is increased interest in establishing thermophilic organisms that secrete thermostable enzymes for the conversion of plant biomass to biofuels (285–287). The thermophilic fungi *Thielavia terrestris* and *Myceliophthora thermophila* produced enzymes that were more active across all temperatures tested and released more sugars from pretreated plant biomass than the enzymes of the mesophiles *Trichoderma reesei* and *Chaetomium globosum (288)*. In a separate study, enzymes from another thermophilic fungus, *Thermoascus aurantiacus*, demonstrated a higher level of sugar release from ionic liquid pretreated switchgrass than *T. terrestris* enzymes and showed activities comparable to the commercial enzymatic mixture CTec2 (289).

T. aurantiacus is a homothallic fungus that grows optimally at 50°C. Induction experiments indicated that both cellulases and xylanases were induced by controlled feeding with xylose, suggesting that the regulatory systems for enzyme expression in *T. aurantiacus* had similarities to the regulatory system in *Aspergillus niger* (290). These initial results make *T. aurantiacus* an intriguing host for thermostable enzyme production. Improving enzyme production and investigating regulation of cellulase and xylanase expression in *T. aurantiacus* is limited by the absence of genetic tools for this promising fungus.

Efficient strain engineering requires genetically tractable hosts. Several methods have been established to genetically engineer filamentous fungi such as protoplast transformation, electroporation, biolistics and *Agrobacterium tumefaciens* mediated transformation (ATMT) (55). ATMT relies on the ability of the plant pathogen *A. tumefaciens* to inject DNA into plant cells and other eukaryotic cells. In this manner, various genetic modifications have been made in fungal genomes, including applying CRISPR/Cas9-based gene editing systems (291). The initial development of the CRISPR/Cas9 system for filamentous fungi often involved the deletion of counter-selectable marker genes such as *pyrG (292)* and *amdS (293)*, which allows the fungus to grow in the presence of otherwise toxic 5-fluororotic acid or fluoroacetamide, respectively. Sexual crossing is another versatile tool, which accelerates strain engineering through combining desired phenotypes, mapping genomic loci, removing undesired mutations and generating genetically uniform fungal homokaryons (294) (295). Notably, sexual crossing is not possible with a variety of industrially highly relevant fungi, and a sexual cycle was only recently established for the classic cellulase producer *T. reesei*, however, not including the industrial strains such as Rut-C30 *(296)* (297) (298).

Genetic tools have been successfully applied to generate high enzyme secreting strains. A *Penicillium oxalicum* strain with strongly increased cellulase production was generated through overexpression of *clrB* and deletion of the cellulase repressors *creA* and *bglR* (299). This strain displayed equal enzyme production as the industrial cellulase hypersecreting *P. oxalicum* strain JU-A10-T, which was generated through classical mutagenesis. Increases in

cellulase and xylanase secretion were also achieved through overexpression of x*lnR* and *clrB* and deletion of *creA* in this fungus (300). Similarly, a *M. thermophila* cellulase hypersecreting strain was recently generated by deleting four genes through CRISPR/Cas9-based editing (293). These examples show the extraordinary potential of genetic strain engineering strategies based on the knowledge of cellulase gene regulation.

Notably, regulation of enzyme coding genes can vary substantially among related fungal species. The transcriptional activators for cellulolytic genes are ClrB in *A. niger* and *P. oxalicum* and Clr-2 in *Neurospora crassa (300,301)*. ClrA is another transcriptional regulator, whose deletion in *A. niger* had a minor effect on plant biomass deconstruction compared to the deletion of ClrB, while deletion of its orthologue Clr-1 in *N. crassa* led to strongly impaired cellulase production and severe growth defects on cellulose and cellobiose (302) (303). The transcription factor XlnR and its orthologues are involved in regulation of xylanolytic genes in *A. niger, P. oxalicum*, *T. reesei* and *N. crassa* (300,304,305). In *A. niger*, XlnR is also involved in the activation of cellulolytic genes (279,303). In *T. reesei*, the *xlnR* homolog *xyr-1* is the most important regulator of cellulases and xylanases, and its deletion leads to severe growth defects on cellulose (306). These results make those genes attractive targets for strain engineering purposes.

Development of genetic tools to improve the regulation of plant cell wall degrading enzymes in filamentous fungi provides a pathway to engineer a wider variety of hypersecreting fungal strains. The goal of this study was to (1) develop genetic tools, namely an ATMT based transformation system, the CRISPR/Cas9 system and a sexual crossing protocol, for *T. aurantiacus* and (2) employ those tools to manipulate the xylanase regulator *xlnR* for a proof of principle study for strain engineering of xylanase secretion. Here we report on successful establishment of those objectives: an ATMT procedure was established, which was then used to implement the CRISPR/Cas9 system in *T. aurantiacus* by inactivating the native *pyrG* gene through mutations caused by the Cas9 endonuclease. Lastly, a sexual crossing protocol has been developed for this fungus, allowing rapid combination of genetic modifications within a week. As a proof of concept, we generated

high xylanase secreting strains via integration of a *xlnR* cassette into the fungal genome with ATMT, displaying the applicability of the developed methods for generating high enzyme secreting *T. aurantiacus* strains for cost-efficient biofuel production.

4.2. Results

4.2.1. Agrobacterium tumefaciens mediated transformation system development for T. aurantiacus

Various transformation protocols have been developed for filamentous fungi, such as protoplast generation, electroporation, ATMT and nanoparticle-based methods such as biolistics (55). Attempts to transform *T. aurantiacus* by protoplastation and electroporation were unsuccessful (data not shown). Therefore, ATMT was chosen for the transformation of *T. aurantiacus.*

T. aurantiacus is a homothallic fungus and was reported to only produce ascospores for propagation through self-crossing (307); no conidiospores have been observed for this species. ATMT involves the co-cultivation of the bacteria with germinating spores of the fungus. We therefore first determined optimal culture conditions for *T. aurantiacus* ascospore production by testing the growth media PDA, Vogel's minimal medium and YPD (data not shown). Spore production was found to be as follows: PDA > Vogel's minimal medium > YPD. Since PDA yielded the largest number of spores, it was chosen for the following experiments. In the next step, we tested different pre-culture conditions for optimal spore production and germination rates. The most efficient spore germination was found when spores were harvested from PDA plates grown for 2 days at 50°C and 3 to 4 days at 45°C (Figure 4.1.a). However, a higher spore yield was obtained from plates, on which *T. aurantiacus* was grown for 4 days at 45°C (~7 * 10^8 spores per plate, see Figure 4.1.b). Therefore, the latter incubation time was chosen to harvest spores for ATMT.

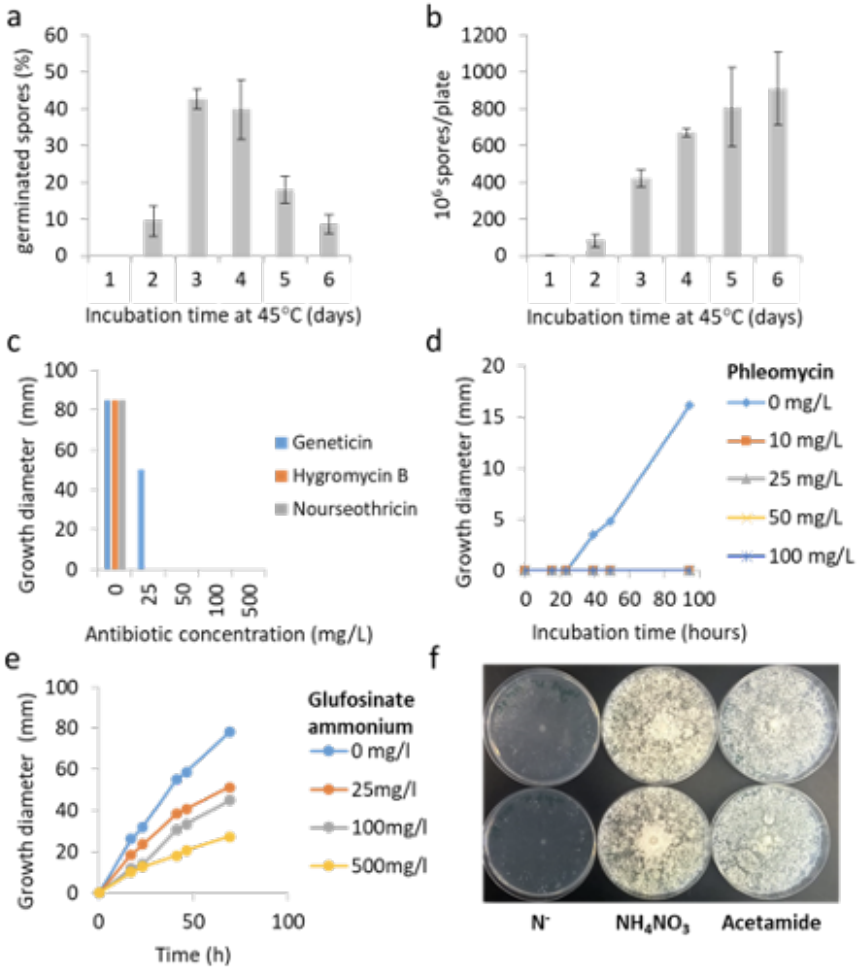


Figure 4.1: Ascospore production and antibiotic susceptibility of *T. aurantiacus*: (a) Germination rates were assessed from spores of fungal cultures incubated at 50°C for 2 days and then 45°C for 1-6 days. At the indicated time, spores were scraped from 3 replicate plates for each day; germination was detected via randomized counts of spore suspensions. (b) The total amount of produced spores was calculated with a hemocytometer. Growth tests of *T. aurantiacus* on different selection markers: (c) hygromycin B,

nourseothricin and geneticin (PDA medium), (d) phleomycin (Vogel's minimal medium) and (e) glufosinate ammonium (Vogel's minimal medium). (f) *T. aurantiacus* is able to grow on acetamide (Vogel's minimal medium with no nitrogen added, ammonia nitrate and acetamide from left to right). Two replicate plates were used for all assays. Note that all antibiotics or acetamide were separately sterile filtered and added to the media after autoclaving. Two biological replicates were used for each test.

Transformation of fungi usually involves the selection of transformants via antibiotic resistance markers (55,57). Commonly used antibiotic resistance genes confer resistance to hygromycin B, nourseothricin, glufosinate ammonium, geneticin and phleomycin (55,308). Alternatively, acetamide can be used as a nitrogen source to isolate successful transformants through integration of an acetamidase gene (*amdS*), since not all species possess this essential enzyme for acetamide utilization (309). To test the potential application of these selection systems for *T. aurantiacus*, the basic resistance level of the wild type strain against the above-mentioned antibiotics was determined. In addition, we tested if the fungus can grow using acetamide as the sole nitrogen source. Strong growth inhibition was observed on plates containing hygromycin B, nourseothricin, geneticin and phleomycin, while the fungus was able to grow on Vogel's medium supplied with glufosinate ammonium (Figure 4.1.c-e). *T. aurantiacus* showed robust growth on minimal media plates supplemented with acetamide as the sole nitrogen source (Figure 4.1.f), which was consistent with the presence of a putative *amdS* gene in the *T. aurantiacus* genome (https://mycocosm.jgi.doe.gov/Theau2/Theau2.home.html).

For the first approach to establish ATMT, the Golden Gate compatible plasmid pTS57 (Table 4.1.) was constructed to mediate ectopic integrations of genes of interest into the fungal genome and to allow selection using hygromycin B resistance (Figure 4.2.a). In the pTS57 plasmid, the *hph* gene is driven by the native *T. aurantiacus tef-1* promoter and there is a cloning site for genes of interest expressed by the native *T. aurantiacus gpd* promoter.

Name	Bacterial marker	fungal marker	Parent Plasmid	Insert	ICE Repository ID
pTS57	Kan	*hph*	NA	*gfp*	JBx_076696
pTS67	Kan	*hph*	pTS57	*xlnR*	JBx_076157
pJP1	Kan	*hph*	pTS57, JP36_1	*Cas9, gRNA 1*	JBx_102720
pJP3	Kan	*hph*	pTS57, JP36_3	*Cas9, gRNA 3*	JBx_102886

Table 4.1: List of plasmids used in this study.

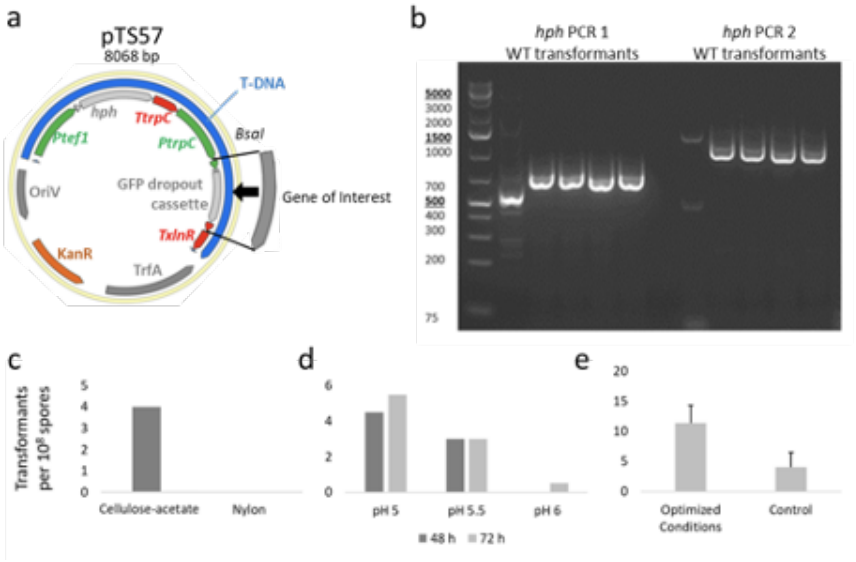


Figure 4.2.: (a) The ATMT plasmid pTS57 was designed for efficient insertion of genes of interest and screening through Golden Gate Cloning upon replacement with a GFP-drop-out cassette. The gene of interest is expressed with the native *T. aurantiacus gpd*-promotor and *xlnR* terminator, the *hph* gene is expressed with the native *T. aurantiacus tef-1* promotor and *trpC* terminator. (b) PCR analysis to verify the *hph* integration into *T. aurantiacus* via ATMT. Optimization of the ATMT procedure for (c) membrane used, and (d) incubation time and pH. (e) A combination of optimized pH and temperature was tested regarding transformation rates. (c: 1 biological replicate, d: 2 biological replicates, and e: 3 biological replicates. Error bars indicate the standard deviation of 3 biological replicates).

A previously developed ATMT protocol for *Rhodosporidium toruloides* (310) was modified for transformation of *T. aurantiacus* (for details see the Material and Methods section). Briefly, 10^8 fungal spores were mixed with 2 ml of an induced *A. tumefaciens* culture of OD_{600} of 1 carrying the plasmid pTS57 and incubated on a filter for 48 h on induction agar containing acetosyringone. After

incubation, the spores were washed off the filters and spread on hygromycin B PDA containing cefotaxime to remove remaining bacteria. The grown fungal colonies were isolated after 2 days of incubation. In the initial experiment, four transformants were obtained from cellulose acetate filters using 10^8 spores while no transformants were obtained when using a nylon filter (Figure 4.2.c). The presence of the hygromycin B resistance gene *hph* in all four strains was PCR verified (Table 4.3.) through two different primer sets (Figure 4.2.b, Table 4.2.). Thus, this initial transformation approach was successful; however, transformation frequency was low.

PCR		name	Sequence
hph1	FWD	RG1	CTCGGAGGGCGAAGAATCTC
	REV	RG2	ATTTGTGTACGCCCGACAGT
hph2	FWD	TS222	CGTAGTACCTGAGCACCCCTCTGAGCTCTT
	REV	TS223	CCATTTGTCTCAACTCCGGAGCTGACATCGA
pyrE	FWD	RG75	GACGGTTTCTATACAGTCTTTTCAG
	REV	RG76	CCCCCGATGTTACTCCGC
pyrG	FWD	LLK683	TTCTTACTACAACTTGGCAACCTTC
	REV	LLK686	ACAAGCCAAATTACCAGCAGAATAC

Table 4.2.: List of primers used in this study

The influence of the pH of the induction medium, the time of co-cultivation of the fungus and the bacteria, and the cultivation

temperature was tested in order to further optimize the transformation protocol. We found that reducing the pH of the induction medium from 5.5 to 5 yielded on average 1-2 more transformants per 10^8 spores, while varying the incubation time (48 vs 72 h) had virtually no effect on the number of transformants obtained (Figure 4.2.d). The temperature test indicated that increasing the temperature from 26°C to 28°C led to slightly higher transformation rates (data not shown). The combination of changing the induction medium pH to 5 and raising the incubation temperature to 28°C led to the isolation of ~2.5 times more colonies compared to the initial conditions of pH 5.5 and 26°C (Figure 4.2.e).

4.2.2. Genomic integration of *xlnR* expression cassettes lead to increased xylanase secretion

After establishing the ATMT procedure of *T. aurantiacus* ascospores, we used the method to demonstrate a proof of concept approach for the expression of a gene of interest in *T. aurantiacus*. Previous work had demonstrated that a continuous xylose feed induced both cellulase and xylanase activities in *T. aurantiacus*, raising the question of the involved transcriptional regulators (290). In *T. reesei*, the transcription factor Xyr1 acts as an activator for xylanases and cellulases. This regulatory function is conserved for the respective homologs in different ascomycete species (306,311,312). A *xyr1* homolog, named *xlnR,* had been identified in the *T. aurantiacus* genome in an earlier study (287). To test the function of this regulator, we cloned the *xlnR* open reading frame into pTS67, where the gene is expressed by the native *T. aurantiacus gpd* promoter (Table 4.1.). The plasmid was transformed into the wild type reference strain by using the established ATMT protocol. 29 hygromycin B resistant transformants were obtained. For a subset of 16 isolates, the presence of the resistance gene within the genome was verified by PCR analysis using an *hph*-specific primer pair (data not shown). To test the effect of the newly integrated construct on xylanase activity, the 29 transformants and the wild type recipient strain were

cultured in liquid media containing Avicel cellulose, a substrate that poorly induces xylanase, as the sole carbon source and xylanase activity was determined after 3 days of cultivation. For 24 out of the 29 isolates, a >50% increase in xylanase activity was observed, and 10 transformants out of this group demonstrated a >300% increase with one transformant displaying a 500% increase (Figure 4.3.a). The secretion of elevated amounts of xylanase by the transformants was also tested under non-inducing conditions. A shift experiment was performed with 4 isolates that displayed the highest amount of xylanase activity during incubations on Avicel cellulose. These strains and the wild type were grown in glucose medium first and equal amounts of fungal biomass were then shifted to carbohydrate-free medium. We found a 6-fold increase in xylanase activity compared to wild type in these isolates (Figure 4.3.b). This proof-of-concept test indicated that enzyme secretion of this fungus could be successfully manipulated with the established ATMT procedure.

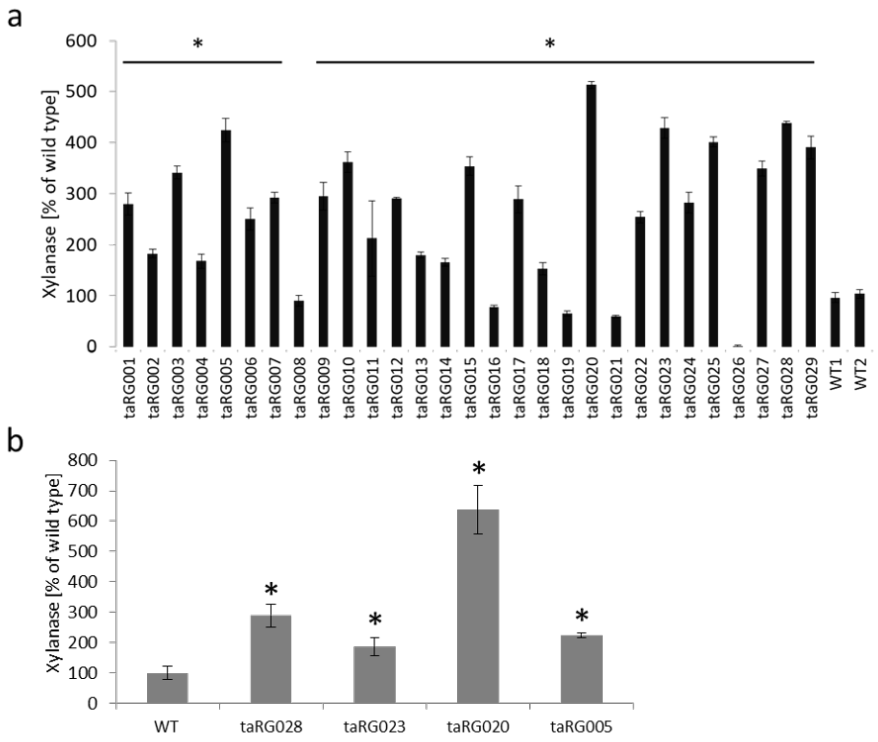


Figure 4.3.: Xylanase activity of the *T. aurantiacus* strains transformed with a *Pgpd*::*xlnR* construct. (a) The DNS Assay was used to screen 29 transformants grown in Avicel medium for xylanase. (b) From a subset of the mutants tested in (a), a subset of 4 mutants displaying the highest xylanase activity was used for a shift experiment. The mutants were grown in McClendon's medium supplemented with soy meal peptone and glucose for 48 hours and equal amounts of mycelium were shifted to starvation medium for 72 h and xylanase activity was measured. (a) bars represent one biological replicate and the error bars are the standard deviation of 3 technical replicates, the horizontal bars with the asterisk indicate statistical significant difference to the wild type strains (pval < 0.05), (B) bars and standard deviation are derived from three biological replicates, the asterisk indicate statistical significant difference to the wild type strains (pval < 0.05).

4.2.3. Development of a sexual crossing protocol for *T. aurantiacus*

T. aurantiacus is a self-fertile, homothallic fungus, which completes its sexual life cycle without a crossing partner. However, other homothallic species, such as the model fungus *Sordaria macrospora*, are often able to outcross (313). In these cases, the basis for outcrossing is the formation of heterokaryotic mycelia via vegetative hyphal fusion of genetically compatible strains. Completion of the sexual cycle of these heterokaryons gives rise to genetically recombinant progeny. In order to test if outcrossing occurs in *T. aurantiacus* and to establish a crossing protocol, two strains with different selectable markers were employed. The hygromycin B resistant *T. aurantiacus* strain taRG008 (Figure 4.3.a) carrying the *xlnR* expression cassette described above was chosen as one of the crossing partners. For the second crossing partner, UV mutagenesis of *T. aurantiacus* ascospores was performed to isolate mutants that were uracil auxotrophs and resistant to 5-fluoroorotic acid (5-FOA). Metabolism of 5-FOA by wild type fungi generates the toxic intermediate fluorodeoxyuridine. 5-FOA therefore selects for mutants with non-functional *pyrG*, which encodes for orotidine 5'-phosphate decarboxylase and *pyrE*, which encodes for orotate phosphoribosyltransferase (314,315). UV mutagenesis yielded two 5-FOA resistant strains (FOAR1 and FOAR2) that were isolated on 5-FOA minimal medium plates containing uracil. Subsequent sequencing of the *pyrE* gene region identified causative mutations for 5-FOA resistance (Figure 4.4.a). An insertion of 190 bp was found in FOAR1, which turned out to be a duplication of a part in the *pyrE* gene sequence while FOAR2 had a 1 bp insertion in *pyrE*, which created a frameshift mutation for both strains. FOAR2 was chosen as the partner to be crossed with the hygromycin B resistant strain taRG008 (Figure 4.3.a). Recombinant progeny was expected to harbour both resistances that could be easily screened for on media supplemented with hygromycin B, uracil and 5-FOA. The plate set-up for fungal crossings is shown in (Supplemental Figure 4.1.). Briefly, 2 fungal strains were inoculated

on a PDA plate supplemented with uracil in alternating fashion to maximize the possibility to form a contact interface. From this interface that was expected to contain the crossed spores of both strains, the mycelium was scraped off the surface with a spatula and eluted in water. The spores were released through vortexing and filtered. Different dilutions were made and spread onto squared agar plates containing hygromycin B, 5-FOA and uracil to yield only very few (< 10) growing colonies, which simplified the isolation. Six progeny colonies (P1-6) were randomly isolated for further analysis on the selective plates. Genomic DNA was extracted from those colonies and was used to verify the integration of the *hph* gene cassette that was passed on from parent strain taRG008 (Figure 4.4.b, left gel) as well as the *pyrE* mutation of the parent strain FOAR2 (Figure 4.4.c). The wild type, FOAR1, FOAR2, the *xlnR*/*hph* expressing strain taRG008 and one progeny isolate (P1) were included as controls for both PCRs (Figure 4.4.b, right gel). The PCR amplification of the *hph* gene and Sanger sequencing of the *pyrE* PCR confirmed that both modifications were only present in the progeny isolates (Figure 4.4.c).

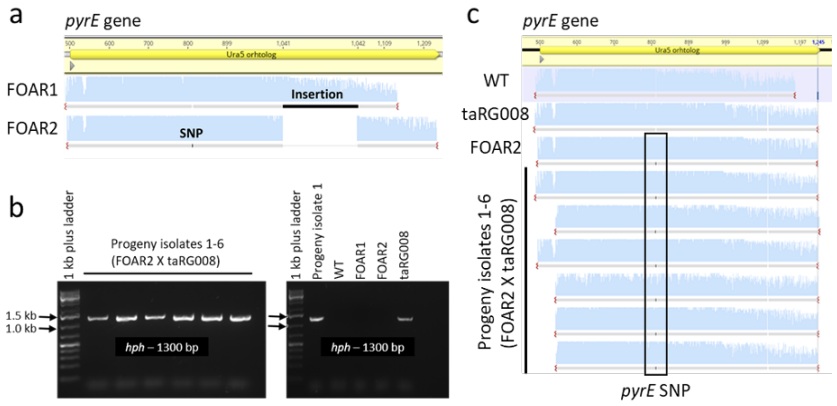


Figure 4.4.: Testing sexual outcrossing in *T. aurantiacus*: (a) Sequencing data of the *pyrE* gene of two 5-FOA resistant isolates (FOAR1-2) were aligned to the *pyrE* reference sequence (primers are listed in Table 4.2.). FOAR2 was crossed with the hygromycin B resistant strain taRG008 (*hph* strain). The progeny of this cross

was analyzed through (b) PCR amplification of the *hph* gene (wild type, FOAR strains and taRG008 were included as controls) (c) Sequencing data of the *pyrE* gene sequence of the crossed strains in (b) were aligned to the native *pyrE* reference sequence. The sequence analysis was performed with Geneious version 11.1 (Biomatters). This analysis indicated that only the progeny isolates displayed genomic integrations of the *hph* gene and the *pyrE* mutation from FOAR2.

4.2.4. Development of a CRISPR/Cas9 protocol for gene deletion in *T. aurantiacus*

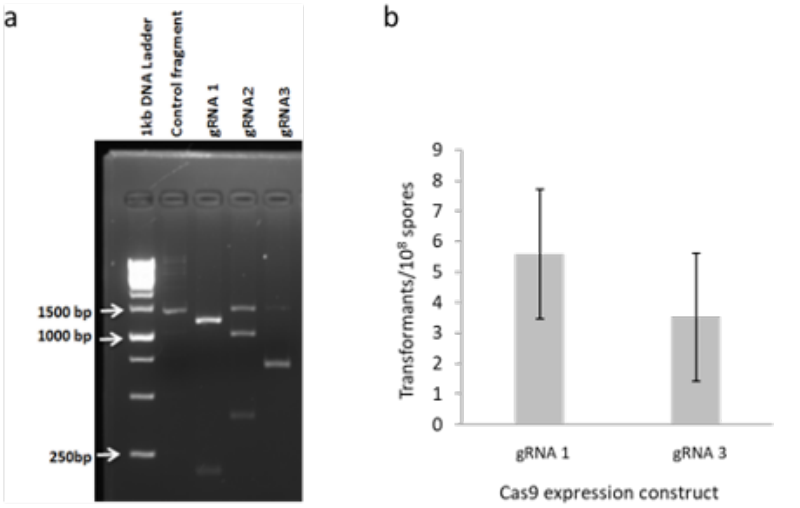
CRIPSR/Cas9 is a powerful genome editing tool consisting of an RNA-guided endonuclease (Cas9) and one or multiple guide RNAs (gRNAs) for targeting one or several genomic loci at the same time (74). Cas9 can be introduced into the fungal cell in the form of DNA, RNA or a protein-RNA complex. Unlike other commonly used transformation strategies, ATMT only allows transformation of DNA fragments into the fungal cell. Therefore, ATMT-mediated Cas9 introduction into fungal cells relies on genomic integration of the Cas9 and gRNA expression cassettes (316) (317). To apply Cas9-based editing in *T. aurantiacus*, an AMA1 plasmid-based expression approach from Nødvig (86) was chosen and modified for ATMT. In the present study, Cas9 and gRNA expression cassettes were amplified from AMA1 based Cas9 plasmids generated in the study mentioned before and integrated into the ATMT plasmid pTS57, generating a new series of plasmids (pJP1, pJP3, Table 4.1.). These ATMT compatible Cas9 plasmids were then used to integrate an expression cassette of the Cas9 gene, the gRNA and the *hph* marker into the fungal genome.

To demonstrate the CRISPR/Cas9 gene editing approach in *T. aurantiacus*, the *pyrG* gene was chosen as a target for gene inactivation. Disruption of *pyrG* causes uracil auxotrophy but also confers resistance to 5-flouroorotic acid (5-FOA), which allows screening of *pyrG* mutants. Three different gRNAs targeting the *pyrG* gene in *T. aurantiacus* were designed using CRISPOR web-

tool (311) (Table 4.3.) and first tested *in vitro* through performing a Cas9 cleavage assay. Accordingly, purified Cas9 protein, a *pyrG* PCR product and one *in vitro* transcribed gRNA per reaction were incubated to facilitate *pyrG*-DNA cleavage mediated by the Cas9 ribonucleoprotein complex. Each reaction was then analyzed through agarose gel electrophoresis to visualize the cleaved DNA fragments. The cleavage efficiency was found to be as follows: gRNA 1 > gRNA 3 > gRNA 2 (Figure 4.5.a). Therefore, gRNA 1, gRNA 3 and the Cas9 gene were cloned into the ATMT plasmid pTS57, yielding the plasmids pJP1 and pJP3, respectively. Transformations of *T. aurantiacus* ascospores using those plasmids were performed by ATMT. The selection for positive transformants was performed through screening for hygromycin B resistance (Figure 4.5.b) and, on average, approximately 5.5 (pJP1) and 3.5 (pJP3) transformants per 10^8 spores were obtained. A subset of 20 of each of these transformants were randomly picked and further screened for 5-FOA resistance on Vogel's MM supplemented with uracil and 5-FOA. Three (pJP1) and 12 (pJP3) out of 20 transformed *T. aurantiacus* isolates displayed 5-FOA resistance (Supplemental Figure 4.2.a, Figure 4.5.c). Sequencing of a subset of isolates from transformations with pJP1 and pJP3 revealed mutations within the protospacer targeting sequence of the *pyrG* gene, confirming that Cas9 cleavage led to base deletions and mismatches next to the PAM sequence, which caused a frameshift in the *pyrG* gene in all sequenced strains and thus 5-FOA resistance of the respective strains (S. Fig. 2b). The deletion efficiency was then calculated based on the fraction of *T. aurantiacus* transformants isolated on hygromycin B medium after the ATMT transformation that also had a mutation in the *pyrG* gene: gRNA 1 displayed a deletion efficiency of 10% while gRNA 3 displayed a deletion efficiency of 35% (Figure 4.5.c). Thus, the Cas9 system successfully introduced mutations in the *pyrG* gene, and selection on 5-FOA turned out to be effective to screen for those mutants.

Target locus	**ID**	**Protospacer sequence (5'-3')**	**PAM (5'-3')**
pyrG	gRNA 1	CTTTTGCGCGCGAGCGCCGT	AGG
pyrG	gRNA 2	GAGTCTTCCTGCACAGGCCT	GGG
pyrG	gRNA 3	TCGGCGCCCGACTTCCCCTA	CGG

Table 4.3.: List of protospacers and PAM sequences used in this study



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Construct name	gRNA version	Grown on hyg B	Subset tested on 5-FOA	Grown on 5-FOA	Sequenced strains	*pyrG* mutations	Deletion efficiency
pJP1	gRNA 1	95	20	3	3	2	10%
pJP3	gRNA 3	60	20	12	7	7	35%

Figure 4.5.: CRISPR/Cas9 development in *T. aurantiacus*: (a) *in vitro* Cas9 cleavage assay: Agarose gel depicting the uncleaved control fragment and the Cas9 cleavage of the target *pyrG* sequence with gRNA 1, 2 and 3. (b) Transformation efficiency per 10^8 spores with gRNA 1 and 3 containing vectors, selected on hygromycin B uracil plates. Each bar displays the mean and standard deviations from 17 biological replicates. (c) Deletion efficiencies of both gRNAs targeting Cas9 to the *pyrG* gene in *T. aurantiacus*.

4.3. Discussion

In this study, we have established a variety of genetic tools to engineer *T. aurantiacus*. These tools include an ATMT method for transformation, a sexual crossing protocol and a Cas9-based method for gene editing. While genetic tools have been developed for a number of mesophilic filamentous fungi, there are limited

genetic tools for thermophilic fungi, so development of genetic tools for *T. aurantiacus* represents the first step towards establishing this fungus as a production platform for thermostable enzymes.

ATMT was a successful approach to transform *T. aurantiacus*; however, the process is more time-consuming than other frequently used transformation approaches and limits the extent of engineering possibilities. Developing protoplast transformation or electroporation protocols for *T. aurantiacus* will accelerate and expand engineering. ATMT was previously established for conidiospores of the thermophilic fungus *M. thermophila* (292) and generated up to 145 transformants per 10^5 spores. Thus, the transformation rates reached for *T. aurantiacus* ascospores in this study (10 per 10^8 spores) were significantly lower. The ATMT procedure was then used to genomically integrate an expression cassette of the transcriptional regulator *xlnR* (Theau_38177). Transformants carrying the *xlnR* construct exhibited high variability of xylanase activity in the culture supernatants, which would be consistent with random integrations of the cassettes in unknown genomic regions and variable numbers of genetic copies inserted into the genome. Nevertheless, up to 500% increased xylanase activity was observed compared to the wild type in strains carrying the *xlnR* construct. Therefore, the *T. aurantiacus xlnR* appears to have a comparable function to its homologs in the closely related *Aspergillus spp.* and *P. oxalicum*, which regulate xylanase gene expression (300) (303) (312). Notably, cellulases and xylanases secretion of *A. niger* in the presence of D-xylose was linked to phosphorylation of XlnR, which mediates the induction of the respective genes in the presence of this carbon source (279) (305). *T. aurantiacus* is closely related to *A. niger* and was found to produce high amounts of cellulases and xylanases during D-xylose fed-batch conditions, which might be mediated by XlnR as well (290).

Furthermore, the ATMT method enabled the successful establishment of the CRIPSR/Cas9 system in *T. aurantiacus*. The gene editing system relied on the non-homologous end joining (NHEJ) repair pathway to generate mutations in the *pyrG* gene, which has been previously demonstrated in *M. thermophila* for the *amdS* gene (293). Additionally, the ability to generate protoplasts for *M. thermophila* led to the introduction of multiple plasmids, which

permitted deletions of genes using homology-directed repair (HDR) mechanisms with a *ku70* deletion strain (292). Nonetheless, the CRISPR/Cas9 system can now be used to modify and investigate the role of other well-known regulators, such as *creA*, *clrA*, *clbR* and *amyR* in a multiplexed manner to further uncover cellulase and xylanase regulation in *T. aurantiacus (303)* (308,312,318,319). Moreover, other genes related to carbon catabolite repression and secretion of other carbohydrate active enzymes might be vital targets for understanding and engineering CAZyme secretion in *T. aurantiacus* (320,321). Finally, recyclable markers such as *pyrG* allow to delete target genes with a high efficiency and then remove the marker through a loop-out mechanism by adding homology repeats (2).

The demonstration of sexual crossing between two strains of *T. aurantiacus* reveals an important advantage for this fungus as a potential platform for producing thermostable enzymes. Crossing under laboratory conditions is a valuable genetic tool only available for a limited number of species, such as the model fungi *N. crassa (322)*, *A. nidulans (68)* and very recently also *T. reesei (297)*, but is lacking for several industrially-relevant fungi with unknown teleomorphs such as *A. niger* and *A. oryzae (296)* (298). *M. thermophila* is not capable of self-crossing and does not cross with close relative *Myceliophthora heterothallica*, which has been experimentally demonstrated to have a sexual life cycle (323,324). An additional advantage of the homothallic *T. aurantiacus* is that crossing does not require strains with different mating types as in heterothallic fungi like *T. reesei (297)* or *P. chrysogenum (325)*. Ascospores are the only means of propagation in *T. aurantiacus* and are produced in as little as 4-5 days. Since ascospores originate from a single nucleus, the resulting progeny are always homokaryotic, allowing for quick and simple purification of originally heterokaryotic transformants. Additionally, the sexual crossing of *T. aurantiacus* was demonstrated on conventional fungal media such as PDA. Since crossed transformants were isolated within a week, it appears that the crossing procedure with this fungus is substantially faster and easier than procedures used for other fungi such as *N. crassa* or *A. nidulans (326)*.

In summary, the developments demonstrated in this paper will enable rapid stacking of genetic modifications into new strains

for subsequent strain tests. We expect these developments and further improvements of the genetic transformation procedure to turn *T. aurantiacus* into a novel host for studying plant cell wall deconstruction, sexual biology and cell biology. In addition, these protocols provide the basis for developing *T. aurantiacus* as a host for numerous biotechnological applications.

4.4. Conclusion

The methods generated in this study will enable to substantially expand the use of *T. aurantiacus* in both applied and fundamental studies. *T. aurantiacus* is an intriguing host for cellulase production due to the extraordinary thermostability of its cellulases, the high enzyme titers secreted by the wild type and, since it is a homothallic fungus, the possibility to rapidly cross strains carrying different mutations into homokaryotic progeny in substantially shorter time frames than currently used industrial fungi, thereby enhancing strain engineering. With further development regarding the transformation system, CRISPR/Cas9, and the crossing protocol, it will be possible to generate genetically modified strains that can be crossed to combine desired mutations. This will enable high CAZyme production with *T. aurantiacus* through deleting or overexpressing regulators and other genes known to impact CAZyme production in related filamentous fungi.

4.5. Materials and methods

4.5.1. Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

4.5.2. Strains and culture conditions

T. aurantiacus ATCC® 26904™ was obtained from the American Type Culture Collection and grown on TEKNOVA potato dextrose agar (PDA) plates to obtain ascospores for transformation purposes. The PDA plates were inoculated with ascospores and incubated for two days at 50 °C before they were transferred to 45 °C for another four days. This shift was performed due to elevated evaporation of PDA plates at 50 °C. The plates were covered with a glass beaker to reduce drying, and plastic containers filled with distilled H_2O provided a moist atmosphere. Cultivation of the uracil auxotroph strains generated in this study was performed on solid Vogel's minimal medium containing Vogel's salts solution, 2 % sucrose and 1.5 % bacto agar supplemented with 1 g/L uracil and 1 g/L 5-FOA as indicated.

Agrobacterium tumefaciens strain EHA105 was grown Luria-Bertani (LB) medium plates (supplemented with kanamycin at 50 µg ml^{-1} when culturing transformed strains harboring plasmids for the fungal transformations). After two days, 2-3 *A. tumefaciens* colonies carrying the desired plasmids were inoculated in 10 ml of liquid LB medium at 30°C supplemented with kanamycin as described above.

4.5.3. Antibiotic resistance plate tests of *T. aurantiacus*

For all plate tests, counted *T. aurantiacus* spores were placed in the center of 9 mm agar plates containing the desired antibiotic. These plates were then incubated at 45°C, and fungal growth was measured after 72 h (hygromycin B, geneticin, nourseothricin, 5-fluoroortic acid [5-FOA] and 5-fluoroacetamide [5-FAA]) or as indicated (glufosinate ammonium and phleomycin). The fungal mycelium diameter was measured with a vernier caliper from two sides and averaged. Antibiotic concentrations were added as indicated or 1.3 mg/ml for 5-FOA and 5-FAA. All antibiotics were sterile filtered separately and added after sufficient cooling of the agar. The following media compositions were used: PDA for hygromycin B, geneticin and nourseothricin, Vogel's minimal

medium with 2% sucrose for glufosinate ammonium, phleomycin (pH 8), 5-FOA and 5-FAA.

4.5.4. Ascospore production and germination rate tests

PDA plates (Sigma-1879V) were inoculated in 3 biological replicates for each time point and ascospores were incubated at 50°C for 2 days and 1 – 6-das at 45°C. Spores were harvested through scraping off the surface with a cell spreader tow times and filtering through miracloth. These spores were counted with a hemocytometer, diluted appropriately, spread on a new plate and incubated for 16 hours at 45°C. These spores were then randomly imaged with a Leica-DM4000B microscope, and the germination rate was calculated via counting germinated versus non germinated spores with ImageJ (327). A minimum of 385 spores was counted for each day, except for day 3, where almost no spores were present.

4.5.5. Plasmid design and cloning strategy

The base vector pTS57 (P*gpd*::P::*gfp*::T::T*xlnR*; P*tef1*::*hph*::T*trpC*) was used to generate all further vectors (Table 4.1). pTS57 expresses the gene of interest with the *T. aurantiacus gpd* promotor and *xlnR* terminator, which flank a *gfp*-dropout cassette that is recognized by *E. coli*. This cassette has two *BsaI* restriction sites at either end and allows genes of interest to be inserted through Golden Gate Cloning. *E. coli* transformants harboring the plasmid with the integrated gene of interest can then be identified through loss of *gfp* fluorescence on a blue-screen. Additionally, pTS57 contains the hygromycin B phosphotransferase (*hph*) expressed by the native *T. aurantiacus tef-1* promotor and *trpC* terminator. Plasmids were isolated after assembly and electroporation into MEGAX DH10B T1R Electrocomp Cells (Thermo Fisher Scientific) with the QIAprep Spin Miniprep Kit

(Qiagen) and transformed into *A. tumefaciens* strain EHA105 through electroporation. The plasmid pTS67 (Table 4.1) was also used, which was derived from pTS57 by the above-mentioned procedure to constitutively express the transcription factor *xlnR*.

ATMT compatible CRISPR/Cas9 plasmids were designed to target the *pyrG* gene in the target host *T. aurantiacus*. The target sequences were obtained from JGI mycocoms. All plasmid maps were designed using the software Geneious 11.1.2 (https://www.geneious.com). The gRNAs used in this study were designed using the CRISPOR algorithm (328) (http://crispor.tefor.net) to obtain predicted guide sequences for PAMs in the target gene. Three different gRNA sequences (protospacers) with no predicted off-targets were chosen and tested *in vitro* for correct cleavage of the target sequence by Cas9 endonuclease before performing *in vivo* transformation experiments (86).

All steps for the gRNA synthesis were followed according to the GeneArt Precision gRNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, United States). Then, the *in vitro* Cas9 cleavage was performed using a previously amplified target *pyrG* amplicon following the steps of the Guide-it™ sgRNA In Vitro Transcription and Screening Systems User Manual (Takara Bio USA, Inc., Mountain View, CA, United States).

The two gRNAs with the highest cleavage efficiency (gRNA1 & 3) were inserted into vector pFC334 and then combined with the Cas9 gene from pFC332 via USER Cloning as described by Nødvig et al. (86). The resulting plasmids were named JP36_1 (gRNA1) and JP36_3 (gRNA3). Those plasmids were then used as templates to insert their gRNA-cas9-expression-cassette into the ATMT vector pTS57 through Gibson Assembly yielding. the ATMT compatible Cas9 plasmids pJP1 (gRNA1) and pJP3 (gRNA3).

4.5.6. ATMT transformation procedure

T. aurantiacus ascospore preparation and *A. tumefaciens* cultivation were performed as described above. The solid and liquid

A. tumefaciens induction medium contained 200 µM acetosyringone (induction medium: salts, phosphor buffer, MES-buffer, glucose, thiamine, acetosyringone and water, see (310)). All reagents were sterile filtered with Corning filter systems or small filters and a sterile syringe. The pH of the induction medium was adjusted to pH = 5.

A modified version of the ATMT procedure for *Rhodosporidium toluroides* was used (310). Briefly, ascospores of *T. aurantiacus* were harvested from 6-day old PDA plates and counted with a hemocytometer. *A. tumefaciens* EHA105 was grown overnight in 10 ml of liquid LB medium containing 50 µg/ml kanamycin. From this culture, a new liquid LB-kanamycin culture was generated with optical density at 600 nm (OD_{600}) of 0.5 that was grown to OD_{600}=1 and then pelleted, washed three times with induction medium, resuspended in induction medium and incubated for further 24 hours. Freshly harvested fungal spores and *A. tumefaciens* cell cultivated in induction medium overnight were filtered onto a 0.45 µm cellulose acetate membrane (0.45 µm MCE Membrane, MF-Millipore) and incubated on induction medium agar plates for 2 days. The spores and cells were washed off with a wash solution containing 200 µg/ml of cefotaxime and were spread on PDA plates containing 200 µg/ml of cefotaxime and 50 µg/ml of hygromycin B with subsequent incubation for 3 days at 45 °C. Colonies were isolated and grown on a fresh PDA plates containing 200 µg/ml of cefotaxime and 50 µg/ml of hygromycin B to remove untransformed spores through harvesting ascospores from the proximate region for generating cryostocks and performing further strain tests. For Cas9 tests, those colonies were then screened for 5-FOA resistance due to CRISPR-mediated mutations in *pyrG* on Vogel's minimal medium containing 2% sucrose, 1 mg/ml 5-FOA and 1 mg/ml uracil.

4.5.7. Strain tests and screening of transformants

For cellulase and xylanase activity tests, strains isolated from hygromycin B PDA plates were used to inoculate McClendon's

medium,0.8% SMP and a carbon source as indicated (Avicel cellulose or no carbon added). For enzyme assays, 0.8 ml of the culture broth was filtered through a spin filter column (Mini Spin Column, EconoSpin). The enzyme assays were performed on a Biomek FX through a DNS method. The first step involved manually adding 75 µl of 1% w/v Beechwood xylan (Megazyme) solution to a 96 well PCR plate (FLAT 96 WELL PCR PLATE, VWR) and 5 µl of enzyme solution. The Biomek FX was used to add DNS reagent to the PCR plates. Upon incubation of these plates at 95 °C, the plate content was transferred with Biomek FX to a flat bottom 96 well plates, and the absorbance was measured at 540 nm. D-glucose was used as a standard for the CMCase assay and D-xylose for the xylanase assay. Uracil auxotrophic strains were isolated on 5-FOA agar as described above and inoculated in PD broth containing 1 g/L uracil.

For strain verification, the mycelium DNA was extracted with the Maxwell RSC Plant DNA Kit (Promega) on the Maxwell RSC Instrument (Promega) according to the manual. One modification involved bead beating of intact mycelium with 300 µl extraction buffer. The concentration of the isolated DNA was measured with NanoDrop 2000 and used for PCR verifications of successful transformation. All sequencing verification was performed through Sanger sequencing.

4.5.8. Sexual crossings

The mutant *T. aurantiacus* strains taRG008 (hygromycin B resistant) and FOAR2 (5-FOA resistant) were first grown individually as described above. A PDA-uracil petri dish was divided in four quarters and spore suspensions from taRG008 and FOAR2 were spotted on the middle of each quarter in an alternating fashion (Supplemental Figure 4.1.). Incubation was performed at 45 °C for six days. Once a lawn of ascospores was produced, spores were scraped off at the interface of the two crossing strains with a sterile spatula, transferred into 750 µL sterile H_2O, vortexed, and filtered through a sterile filter tip with miracloth. A dilution was prepared and plated onto a 12x12 cm square plate with Vogel's minimal medium supplemented with 1 g/L 5-FOA, 1 g/L uracil and 50 µg/mL hygromycin B in triplicates. After incubation at 45 °C for three days,

growth was visible. Randomly picked colonies were isolated and grown on the same media as the isolation plates. Genomic DNA was extracted from isolated colonies and used for PCR-based verification purposes.

Chapter 5: An optimized high throughput platform, from genetic transformation to fermentation, to screen for secreted laccases in *Aspergillus niger*

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Abstract

To develop affordable biofuels and bioproducts from plant biomass, it is imperative to optimize the conversion of every carbon source available, such as cellulose, hemicellulose, and lignin. The degradation of the recalcitrant biopolymer's lignin presents multiple challenges that could be overcome using lignin-degrading enzymes, such as laccases. Laccases are multicopper oxidizing enzymes that can degrade non-phenolic compounds such as subunits of lignin, using mediators like ABTS. The actual challenge to use laccase in bioindustry is its expensive price due to its low productivity and activity level, and it's highly substrate-specific. We chose *Aspergillus niger* to mine for new laccase secreted enzymes, due to the capacity of this filamentous fungi to produce high levels of secreted recombinant enzymes and its low-cost purification downstream process. In this paper, we proposed a list of Agarimycetes laccase enzymes that we integrated into the genome of *A. niger* using an optimized high throughput transformation platform. We analyze each strain using a 10 mM ABTS on a solid plate, one positive secreted laccase, named in this paper Lac26, was further analyzed in liquid culture with ABTS. We then engineered the strain of *A. niger* by deleting two genes, *mstC* and *prtT*, and we obtained a higher yield of overall secreted enzymes. Using the biochemical assay ABTS from a fermentation in liquid culture, we determined the optimum pH and fermentation day point to obtain a maximum yield of Lac26 secretion.

5.1. Introduction

Plant biomass has long been used as a source of energy. Using photosynthesis, plants can convert solar energy, water, nutrients, and CO_2 into organic compounds (carbohydrates, proteins, and lipids) and oxygen (329). With the rise of CO_2 in the atmosphere contributing to climate change, this conversion is of particular interest to scientists (330). One of the foci in this research is to develop tools to decompose the cell wall, extract each component, and convert them into sugars that can be used for value-added biofuels and bioproducts. Carbohydrates, such as cellulose and hemicellulose in plant biomass, are stored in cell walls. Cellulose and hemicellulose have been used for fermentation in the development of affordable renewable energy (e.g., biofuels and bioproducts) (331, 332). Lignin, a complex biopolymer, is also a major component of the cell wall and helps maintain the fibers and vessels and resist weather, disease, and pests. Without it, plants would not be able to properly develop and survive in normal conditions (333). Although lignin is an abundant and attractive energy source that could reduce the cost of biofuels and bioproducts, its structural complexity renders it recalcitrant to many degradation techniques (1). Tools in development to degrade lignin are, for example, ionic liquid, high temperature/ pressure treatment, and lignin-degrading enzymes (LDEs) (334). LDEs are especially attractive because they are safe, versatile (used in a wide range of pH and temperature), and environmentally friendly (245). The different types of LDEs found in nature are laccases, lignin peroxidases, and manganese peroxidases. In this study, we focus our attention on a particular LDE, laccase.

Laccases are multicopper oxidase enzymes that can oxidize phenolic compounds by conducting one-electron oxidation, or non-phenolic compounds such as subunits of lignin using mediators like 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (158). ABTS is among the most widely used chemical compounds to study antioxidant activity. With laccase, it acts as a substrate and only requires oxygen to be converted. After oxidation ABTS turns into its free radical cation ABTS+ and, the compound turns green and can be detected via absorbance λ=420 nm (335). ABTS is relatively easy to use, and the results obtained are reproducible, making this

assay one of the most used in literature (336). Today, more complex and precise techniques are being developed to directly observe the enzyme process on subunits of lignin (e.g., ligNIMS). ligNIMS has been developed to measure the lignin depolymerization on one of the major bonds forming the biopolymer, β-*O*-4 linkage, using nanostructure-initiator mass spectrometry (NIMS) (218).

Laccases have high value due to their use in bioproduct and biofuel production, as well as the bioremediation, pulp and paper, and food and beverage industries (337). Despite their demand in different industries, their cost of production remains high, their degradation efficiency is low and they are still too dependent on specific substrates (245). To address those different challenges, different areas of laccase optimization are being investigated, such as genome mining for new laccase enzymes, testing and developing different substrates, enzyme engineering, and host engineering (1).

One of the biggest challenges in laccase discovery is choosing the production host. Laccases are widely produced by a variety of fungi and found in plants, bacteria, and insects (178). In nature, the great producers of laccases are *Trametes species*, Agaricomycetes that can directly grow on wood (338, 339). However, *Trametes* are not the most suitable host for industrial production applications, due to difficulties involve in enzyme purification (340). Therefore, laccases are mostly analyzed by heterologous expressions in yeast such as the methanotroph *Pichia pastoris (341–343)*. However, the choice of yeast as a host presents disadvantages, such as hyperglycosylation of the recombinant proteins impacting themselves (344). Filamentous fungi like *A. niger* are more widely used in industry for their capability to produce and secrete recombinant protein and chemicals at higher secretion levels (345). *A. niger* is GRAS and has been used in the biotechnology industry for the citric acid fermentation invention since 1917 (346). *A. niger* can use a wide variety of carbon sources to produce large amounts of homologous and heterologous enzymes such as glucoamylase and proteases (347). One of the downsides of using *A. niger* was the difficulty to engineer its genome (55). Recent developments in genetic tools, such as CRISPR/Cas9 made it easier and efficient to transform *A.*

niger (348), (2). Additionally, the development of high throughput methods and robotics greatly expedited strain engineering in filamentous fungi (349).

In this paper, we leveraged the natural protein secretion ability and recent advances in strain engineering to screen a library of laccases in *A. niger*. We optimized a high throughput robotic platform in combination with an optimized "pop-out" construct that ensures a quick, efficient, and easy analysis of our strain library. Using this method, we transformed 81 laccase constructs in two different optimized *A. niger* strains, one with only the replacement of *glaA* by the laccase genes, and another one with the additional deletions of the sugar transporter gene, *mstC*, and the transcription factor gene, *prtT*. To test the secretion capacity of each strain we used ABTS solid-state method, and for further kinetic analysis, we optimized a downscale fermentation protocol using *A. niger* and a ABTS liquid culture protocol. To the best of our knowledge, this is the first time that a high-throughput method has been published on *A. niger* to mine for new enzymes heterologous secretion.

5.2. Results

5.2.1. Library of enzymes, publication analysis

After the analysis of the 81 chosen enzymes composing our library of laccases, we found that 31 % of those enzymes have been studied and their sequences published. Among those published enzymes 30% have been characterized, 7% have been metagenomic studied and their phylogeny relatives have been analyzed, 21% have been heterologous expressed and one strain has been analyzed in fermentation conditions, the rest have only their sequence published. Among the ones that have been overexpressed, 67% have been transformed in *P. pastoris*, 1% in *E. coli*, 1% in *S. cerevisiae*, and 1% in *Aspergillus oryzae* (Supplemental Figure 5.1., Supplemental Table 5.1). From the entire transformed library, we codon optimized each laccase sequence to match with the *A. niger* genome and build a construct DNA for each laccase to be integrated at the *glaA* loci. Then we transformed in *A. niger* ATCC 1015, we obtained multiple clones of expressed lacases enzymes, but only one secreted enzyme from

A. niger, Lac26. This laccase, Lac26, is a hybrid laccase strain that has been shuffled in by HR in *S. cerevisiae* (350).

5.2.2. Strain optimization

Strain engineering is an essential asset to improve homologous and heterologous enzymes production (351). Different tools for engineering *A. niger* strains have been developed, such as chemical or ultraviolet (UV) mutagenesis, targeted gene deletion to improve secretion pathway, promoter choice, number of copies of the gene of interest, and codon optimization (345). In this study, we optimized an *A. niger* strain by deleting three different genes. The first one is the glucoamylase gene, *glaA*. Glucoamylase is one of the major secreted enzymes by *A. niger*. Researchers managed to obtain 30 g/L of glucoamylase enzymes after strain and fermentation optimization (352). However, in this study, we are not focusing on the glucoamylase enzyme itself, but, rather, on its constitutive promoter, P*glaA*. The choice of the promoter is particularly important for the regulation of protein synthesis. Among the different promoters, there are the constitutive, inducible, and tunable ones (353). P*glaA* is a strong constitutive promoter induced by maltose that has been used in numerous studies for homologous and heterologous secretion (345). To simplify the downstream process of screening, we chose the use of the constitutive promoter P*glaA*. We replaced the gene of *glaA* with each laccase gene from the library, to obtain a strong constitutive transcription of the different laccases. To optimize furthermore the strain, we envisioned the deletion of two additional genes, a low-affinity sugar transporter gene *mstC*, and transcription factor gene *prtT*. *mstC* deletion has been associated with the increase of recombinant enzyme secretion driven by the P*glaA* (266). An ionic liquid-tolerant heterologous enzyme beta-glucosidase, *A5IL97*, had its secretion level increased fourfold when promoted by P*glaA* in *A. niger* (266). *prtT, a* transcription factor gene, is an important gene that encodes a transcriptase that catalyzes the formation of RNA from DNA of four different protease genes, *pepA*, *pepB*, *pepD*, and *pepF* (354). The deletion of *prtT* resulted in the reduction of the expression of the four proteases. Proteases are essential to the effective functioning of the cells by catalyzing the breakdown of proteins into

polypeptides and amino acids during protein catabolism (355). In a recent study, researchers deleted *prtT* and observed a 1.8-fold increase compared to the mother strain of the heterologous protein cutinase, and a 77% increase in the stability of the protein in the culture filtrate (356). In this study, we deleted *mstC* and *prtT*, and replaced *glaA* with laccase genes to increase the regulation of laccase synthesis. After re-transforming the entire library into the optimized background with *mstC* and *prtT* additionally deleted, we obtained additional secreted enzymes from the Trametes species. However, only Lac26 was producing spores in both backgrounds and could be further analyzed (Table 5.1).

Name	Genotype	Source	Access
JBEI-14377	*ATCC 1015 pyrG-*	((357))	https://registry.jbei.org/folders/1399
JBx_104590	*ATCC 1015 ΔmstC ΔprtT pyrG-*	In this study	https://registry.jbei.org/folders/1399
JBx_150690	*ATCC 1015 ΔglaA/PglaA-Lac26 pyrG-*	In this study	https://registry.jbei.org/folders/1399
JBx_150691	*ATCC 1015 ΔglaA/PglaA-Lac26 ΔmstC ΔprtT pyrG-*	In this study	https://registry.jbei.org/folders/1399

Table 5.1: Strains list used and developed in this study.

5.2.3. Transformation optimization

To perform 81 transformations to integrate the laccase library in two different backgrounds, we optimized then downscaled a transformation protocol (2). Starting from a previously disclosed high throughput protocol method (349), we optimized the protocol to increase the number of colonies per plate by tenfold (Supplemental Figure 5.2). The difference is found in the concentration of $CaCl_2$ that is 10-times more concentrated in our protocol. Then we downscaled the transformation protocol, allowing the transformation of 96 strains at a time using the robotic platform.

5.3.4. Results of the transformation

5.2.4.1. ABTS solid-state results

After the transformation of the entire laccase library in the Aspergillus ATCC 1015 *ΔglaA* background, the ABTS assay on solid plates (MMA + 10 mM ABTS) resulted in only one secreted laccase enzyme, Aspergillus ATCC 1015 *ΔglaA/PglaA*-Lac26 (Table 5.1, Figure 5.1). After re-transforming the entire library in the optimized background Aspergillus ATCC 1015 *ΔglaA ΔmstC ΔprtT*, the ABTS assay on solid plate resulted in additional secreted laccase strains all located in the Trametes species branch, Lac16, Lac21, Lac22, Lac23 and Lac25 (Supplemental Table 5.1). However, only Aspergillus ATCC 1015 *ΔglaA/PglaA-Lac26 ΔmstC ΔprtT* resulted in healthy spore production and could be further investigated in liquid culture. We hypothesized that the production of laccase on a constitutive promoter P*glaA* was toxic for *A. niger*, impairing the spore production. We suggest changing the laccase library and, instead, using an inducible promoter, such as the TET-On promoter that shows promising results with *A. niger* (353). Moreover, on solid plate no fundamental differences of laccases production have been observed between the two different strains, *ΔglaA/PglaA*-Lac26 and *ΔglaA/PglaA-Lac26 ΔmstC ΔprtT.* For a more accurate analysis, we investigated the production yield of laccase secretion using liquid fermentation and analyzed the media with a liquid ABTS assay on a plate reader.

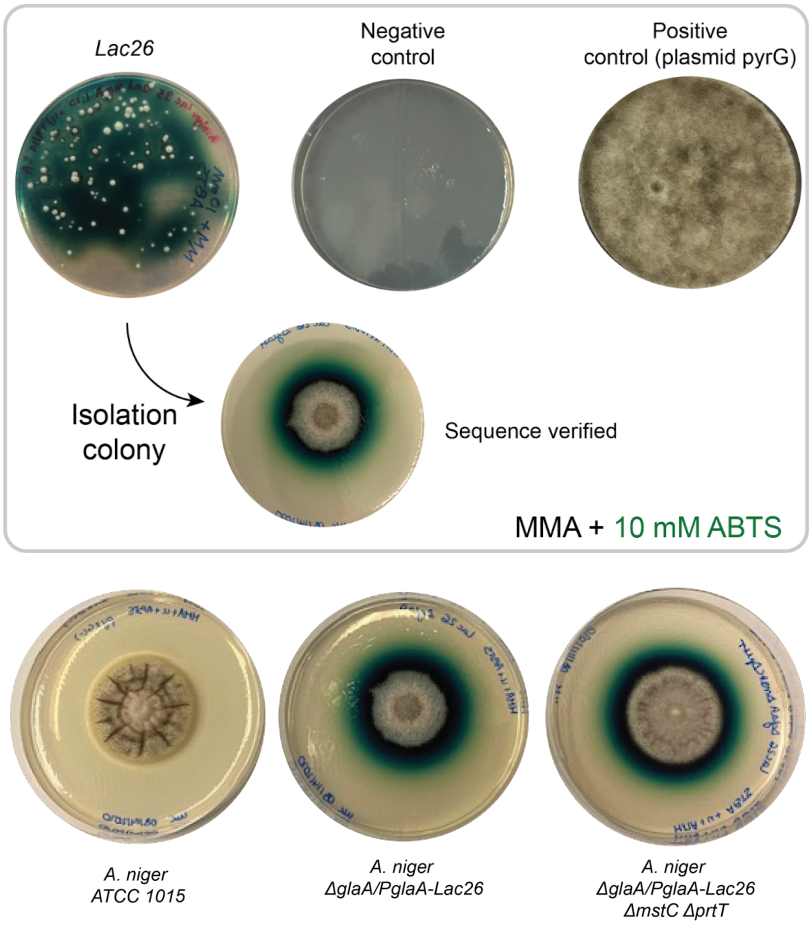


Figure 5.1: (Top) Results of the transformation with *A. niger ΔglaA/PglaA-Lac26* with positive and negative control. After an isolating colony we, can measure the halo around the colony from biomass to ABTS. (Bottom) Differences between *A. niger* ATCC 1015, *A. niger ΔglaA/PglaA-Lac26* and *A. niger* ATCC 1015 *ΔglaA/PglaA-Lac26 ΔmstC ΔprtT* on MMA + 10uM of ABTS.

5.2.4.2. ABTS liquid culture results

From the transformation, we continued to analyze further the two strains *A. niger ΔglaA/PglaA*-Lac26 and the optimized background *A. niger ΔglaA/PglaA-Lac26 ΔmstC ΔprtT*. We cultured those two strains in 5 mL of Minimal Media (see Materials and method) in quadruplets using a 24 well plate, and compared them over different sets of days of fermentation and at different pH of ABTS assay. As a result, we obtained a higher yield of overall production after 6 days at 800 rpm and 30°C, and a stronger activity with ABTS assay at pH 5.5, and overall, the optimized background always resulted in a higher secretion yield (Figure 5.2). The overall protein production measured with the Bradford assay, resulted in 0.106±0.022 mg/mL of the *A. niger ΔglaA/PglaA-Lac26 ΔmstC ΔprtT* after 6 days of inoculation, 2.7 times more than the non-optimized background *A. niger ΔglaA/PglaA*-Lac26. Past 6 days of incubation time resulted in a very viscous media with almost no more media to analyze. The ABTS assay after 6 days of incubation at a 5.5 pH resulted in 0.53±0.028 mU/L of the *A. niger ΔglaA/PglaA-Lac26 ΔmstC ΔprtT* while after 6 days of incubation, the protein secretion of the strain *A. niger ΔglaA/PglaA-Lac26* resulted better at pH 3.5 with 0.45±0.062 mU/L then at other pH. This result can be explained by the fact that the pH of the media after 5 days of fermentation is low (pH 3.5), rending the remaining enzyme more stable at low pH rather than higher pH. Morevover, there is less active protease at a lower pH in the media, which decrease the chance of enzyme degradation.

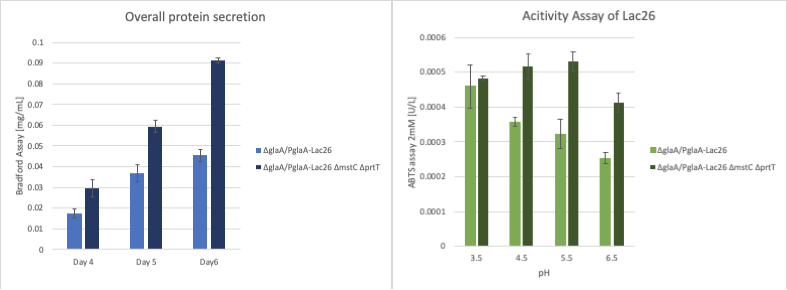


Figure 5.2: (Left) Bradford assay to measure the overall protein secretion per biomass of the two Lac26 strains at different points of fermentation time (Right) ABTS assay to measure the activity per biomass of two Lac26 strains at different pH .

5.2.4.3. Secretome analysis

The secretome was analyzed after 5 days of incubation and concentration of the media. We performed an overall analysis of the secreted protein from *A. niger* and targeted analysis for *Lac26*. The results of the secretome analysis confirmed that both strains, *A. niger ΔglaA/PglaA*-Lac26 and *A. niger ΔglaA/PglaA-Lac26 ΔmstC ΔprtT*, secreted Lac26. We found partial sequences of *glaA* in both strains due to the use of the P*glaA* and the signal peptide of *glaA*. In the strains for *A. niger ΔglaA/PglaA-Lac26 ΔmstC ΔprtT*, we did not find *pepA*, *pepB*, *pepD*, and *pepF* like expected after deleting *prtT*.

5.3. Conclusion

From downscaling the transformation platform to the fermentation using *A. niger*, we demonstrated, thanks to CRISPR/Cas9 and the approach developed in Chapter 3, that we are now capable of fast-tracked enzyme discovery with *A. niger*.

In this paper, to transform the entire laccase library into the genome of *A. niger*, we also downscaled and optimized the transformation protocol (from Chapter 3) making this protocol high throughput using the robotic platform. Moreover, we downscaled

the fermentation of *A. niger* into 5mL to use 24well plate, and the biochemical ABTS assay to use 96 well plate reader format.

Then we integrated an entire library of laccase genes in two different backgrounds, one WT background and one optimized background. The optimal background, which is the deletion of the sugar transporter, *mstC*, and the transcription factor, *prtT*, showed an increase of 4-fold of the overall protein secretion and 3-fold of the laccase enzymatic assay compared to the WT background. The optimal background resulted in additional secreted laccase enzymes from the *Trametes species*, it will be interesting to study further those enzymes using a different promoter to avoid the presume toxicity of laccase on *A. niger*. It will also be interesting to test more laccase enzymes from Trametes species and using biocomputational analysis to understand why this species has such an affinity with *A. niger* secretory pathway compared to other species that were not as successful.

After the fermentation of *A. niger* to produce Lac26 enzyme, we tried to purify the enzyme for further analysis. However, we have not been successful. We tried nickel beads column extraction and Western Blot. The hypothesis is that the His-Tag is either clipped from the acidity of the media after fermentation or it is buried in the 3D structure of the protein or due to glycosylation of *A. niger*, the His-Tag is inaccessible. More experimental tests need to be done to purify the protein Lac26.

5.4. Materials and Method

5.4.1. Library of laccase enzymes

The goal is to begin the process of understanding the enzymatic depolymerization of lignin produced from various ionic liquid pretreated biomass processes. The gene library used in this research is a set of diverse laccase enzymes that should provide a complete picture of lignin depolymerization into defined fermentable lignin fragments (Supplemental Figure 5.1, Supplemental Table 5.1). The enzymes coded for by the genes being synthesized for this research are hypothesized to be important for catalyzing lignin depolymerization.

For optimization of the laccase secretion, each laccase sequence from the library was codon-optimized for *A. niger* and a signal peptide from the glucoamylase was added at the N-terminus (358, 359). Each construct was designed to be inserted at the glucoamylase promoter, PglaA, and to replace the glucoamylase, glaA gene. To facilitate protein purification, a His-tag was added at the C-terminus of the protein before the *TrpC* terminator. The strain used for the transformation is *pyrG(-)* auxotroph, resulting in the lack of orotidine 5'-phosphate decarboxylase production, impairing the uridine pathway production. However if the fermentation media contain uridine or uracil, the strain can grow without it the *pyrG* gene. Following the method developed in Chapter 3, we used the *pyrG*/5-FOA counter selection method to replace *glaA* by every laccase gene at the *glaA* locus (Figure 5.3).

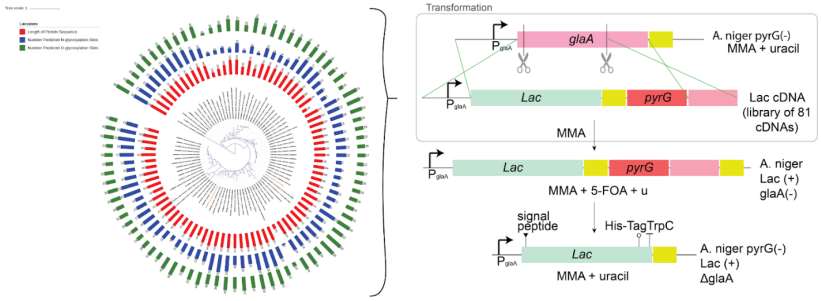


Figure 5.3: Right, laccase enzyme library codon-optimized for *A. niger*. Left, transformation method employed to integrate each laccase enzyme in *A. niger* at the *glaA* locus.

5.4.2. High throughput transformation method

5.4.2.1. Protoplast preparation

The protoplast preparation has been previously described in this paper (2). Only 2 mL of 10^7 protoplasts/mL washed by the 1M Sorbitol, 50 mM Tris-HCl pH 8.0 and 500 mM $CaCl_2$ (STC) buffer was used for 96 transformations at a time.

5.4.2.2. Ribonucleoprotein Cas9-gRNA (RNP) complex preparation

RNP complexes were provided by Integrated DNA Technology company (IDT) using a high-fidelity endonuclease variant (HIFI) Cas9 and two sgRNAs to cleave the deletion gene on both its extremities. After preparation following IDT guidance, the RNP complexes were maintained on ice until transformation.

5.4.2.3. Transformation

The following optimized platform consist of: 10uL of protoplast, mixed with 5uL of RNP complex, 4uL of 1 M Sorbitol, 50 mM Tris-HCl pH 8.0 (ST) buffer and 10 uL of 25% of 4000 Polyethylene glycol (PEG), 500 mM $CaCl_2$, 10mM Tris-HCl, pH 7.5 (PEG transformation) buffer. The mixture was placed on ice for 20 minutes. After an additional 57uL of PEG buffer is added, and placed at room temperature (RT) for 5 minutes. Finally, 113uL of ST Buffer is added to the mixture and placed on MMA + u + ABTS (10 mM) plates at 30C for a week (Figure 5.4). The entire transformation process has been optimized to be used in a Biomek FXP and the protocol adapted for a robotic platform (Supplemental Figure 5.3).

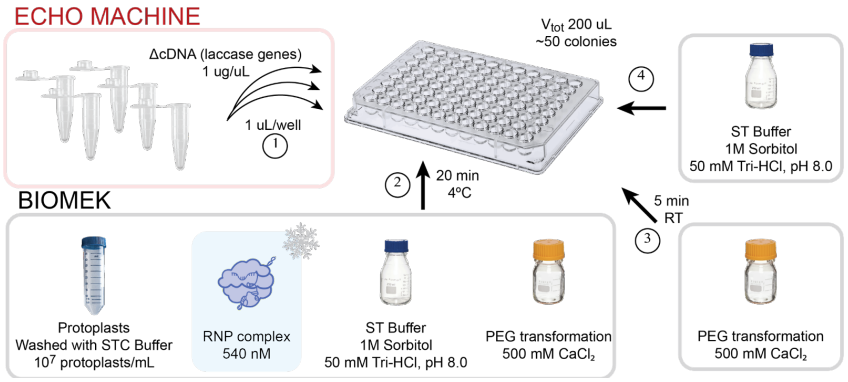


Figure 5.4: *A. niger* downscale protocol for genome transformation. After transformation, each was plated on a minimal media agar plate without uracil, to use the pyrG(-) auxotrophy resistant mutant.

5.4.2.4. After transformation

Each transformation produced roughly 50 colonies that were then re-streak on MMA + 5FOA+ uracil, to lose the pyrG marker. The colonies that survived on 5-FOA were then replated on MMA + ABTS + uracil. Each positive secreted laccase strain was lysed and then sequence verified (Table 5.2).

PCR	Name	Sequence
Forward	LLK719	GTGCAGATGAGGTTTGGCTATAAATTG
Reverse	LLK720	GCTCTGCTAAGCTATTCTTCTCTTC

Table 5.2: List of primers used in this study. The forward primer is located in the PglaA and the reverse primer in the TrpC locus, both are common to every laccase in the library.

5.4.3. ABTS assay

5.4.3.1. Strains

Four strains were analyzed during ABTS and Bradford analysis and are listed in Table 5.1. The strains were all transformed from the *A. niger* ATCC 1015 v4.0, and the genome is available from the Joint Genome Institute (JGI) (357).

5.4.3.2. ABTS solid assay

Media on solid plate

Minimal media agar, MMA, (10 g/L of Glucose, 50 mL/L of 20X Nitrate Salts stock solution, 1 mL/L of 1000X Trace elements stock solutions, 1 mL/L of 1000X Vitamin stock solution at pH 6.5 using potassium hydroxide (KOH)) with 18 g/L granulated agar and 1 M of Sorbitol and 1.12 g/L of uracil. To test the activity of ABTS, 10 mM were added to the media after autoclaving.

Preparation of the spores and incubation time

5 days after solid-state growth at 30°C, the biomass was harvested using 0.4% of the Tween-20 buffer. The spores were filtered through miracloth and washed before being concentrated at 1.75 $x10^8$ cells/mL, and 2 uL of each biomass (3.5 $x10^5$ cells) was plated in the middle of the plate. The plates were incubated at 30°C for 2 days and at RT for an additional 5 days.

5.4.3.3. ABTS liquid assay

Media in liquid culture

Minimal media, MM, containing 10% glucose, with 3 g/L of Synthetic Complete (SC) media (Sunrise Science 1459-100) and 1.12 g/L of uracil. We cultured 5 mL in quadruplets per strain using a 10 ml 24 Well Deep Well Plate.

Preparation of the spores and incubation time

5 days after solid-state growth at 30°C, the biomass was harvested using 0.4% of Tween-20 buffer. The spores were filtered through miracloth and washed before being concentrated, and 20,000 spores/mL were inoculated in the liquid culture for 6 days at 800 rpm in 30°C.

After 5 days of incubation

The biomass is filtered, and the remaining media is analyzed via Bradford for overall protein secretion and ABTS for specific laccase activity analysis. The ABTS solution contained 2 uM of ABTS in 100 mM of sodium acetate at pH 5.5. Using a plate reader, the absorbance was measured over 5 minutes. 10 uL of fresh media were added to 190 uL of ABTS solution. To measure the activity of laccase enzyme, we used the following formula:

$$Activity\ [U/L] = {}^{\Delta A * Vt}/_{Vs * d * \varepsilon}$$

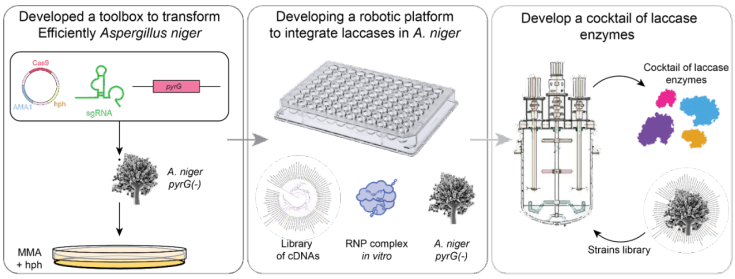
where ΔA is the difference absorbance measure over 5 minutes, Vt is the total volume (200uL), Vs is the volume of the substrate (20uL), d is the path of the light (0.56 cm) and ε is the extension coefficient (29300 $M^{-1}.cm^{-1}$). 1 U/L represents the amount of laccase required to oxidize 1umol of ABTS/min. This formula assumes that the substrate volume Vs represents pure laccase enzyme, it is why the obtained results are relative measurements and not absolute.

Analysis of the Secretome

The media was concentrated up to 200-times using 10kDa amicon tubes. The concentrated protein was washed with 100 mM of ammonium bicarbonate and cleaned up of any PEG residues using a detergent kit (HiPPR Detergent Removal Spin Column Kit, from Thermo Fisher Scientific). The samples were run through a mass spectrometer to analyze the protein content in the Secretome.

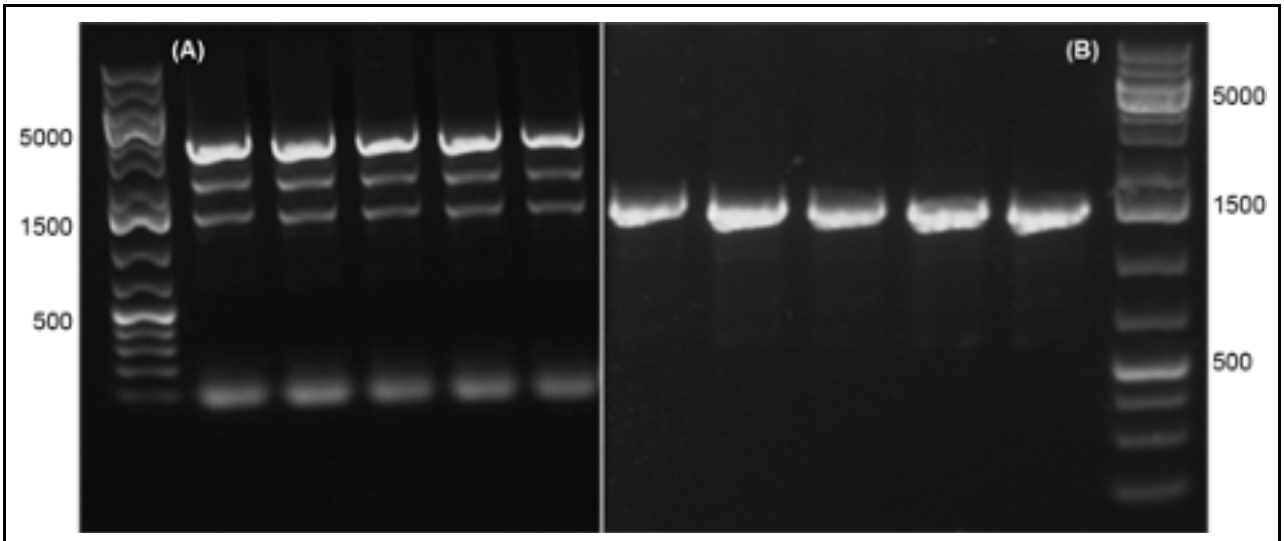
Conclusion of the Thesis

Thanks to the development of technology in the biotechnology industry, such as CRISPR/Cas9 or robotic platforms, new non-model organisms are becoming easier to work with, in this particular case *A. niger*. In this thesis, we demonstrated from genome editing to downscale fermentation, how such powerful tools can shape a new way to mine for enzyme secretion. The development of a new type of construct to counter-select only positive integrated locus in *A. niger* reduced considerably the workload of the downstream work and defined the groundwork of Chapter 5. Then the downscale transformation platform and the downscale fermentation allowed to mine an entire library of laccase in *A. niger* and strain engineer it. It is the first time that such a combination of technologies has been reported in the related literature using this organism. This should open a new frontier of enzyme discovery with *A. niger* as well as a faster way to do synthetic biology, and/or investigate metabolic engineering using this organism.



Conclusion Figure: Review of the overall Ph.D. phases. From left to right, first development of the new Cas9 construct, then of the robotic platform development with laccase library integration, and finally future prospect of scaling up fermentation to develop a cocktail of laccase enzymes.

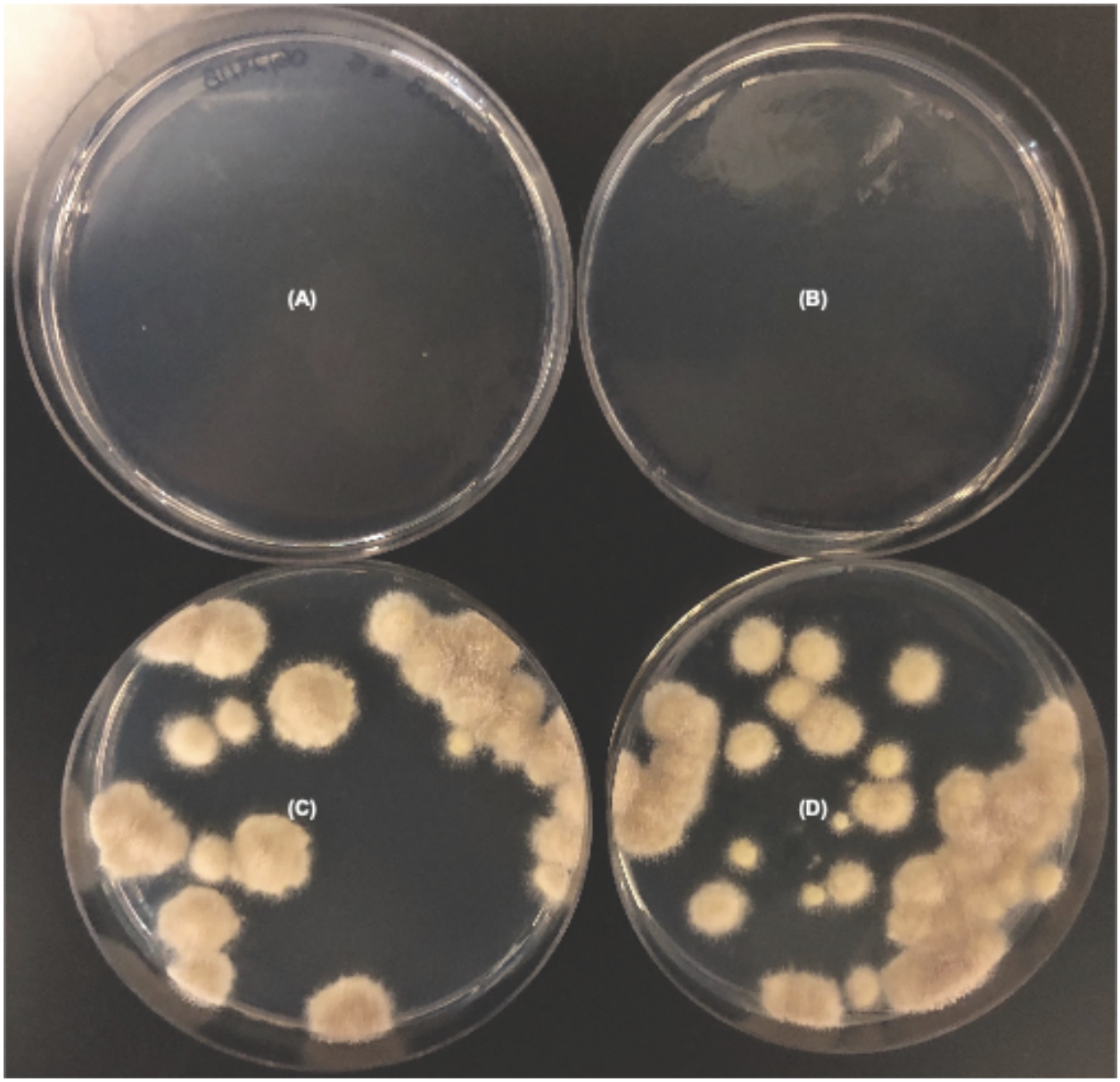
Appendices



Supplemental Figure 3.1.: Representative cDNA006 PCR before and after 5-FOA (A) cDNA006 before 5-FOA, 5 colonies after transformation PCR amplification with 350/590, 3'125 bp. (B) After 5-FOA, 5 white colonies undergone *pyrG* excision, 1'386 bp, using both 1 kb Plus Ladder (Thermo Fisher/ 1 kb Plus ready-to-use).

cDNA	Primers		Sequence	Length
cDNA006	LLK554	Forward	CCGACAGACTTGGCGAAG	4’758 bp
	LLK555	Reverse	CCTGGCTCATTGGGGCCAA	
cDNA008	LLK554	Forward	CCGACAGACTTGGCGAAG	4’258 bp
	LLK582	Reverse	AAACGTAGACATCACCAGCC	
cDNA009	LLK587	Forward	CAAGTATATGATGCGGTAGTGGAATCT	6’324 bp
	LLK588	Reverse	GGCTATGCATTGAATGACAGTG	
cDNA010	LLK595	Forward	CGAAGAAGATTCCAGGAACG	4’239 bp
	LLK596	Reverse	CACCAAGTAAGGTTCGTATAT	

Supplemental Table 3.1: Primers cDNA preparation.

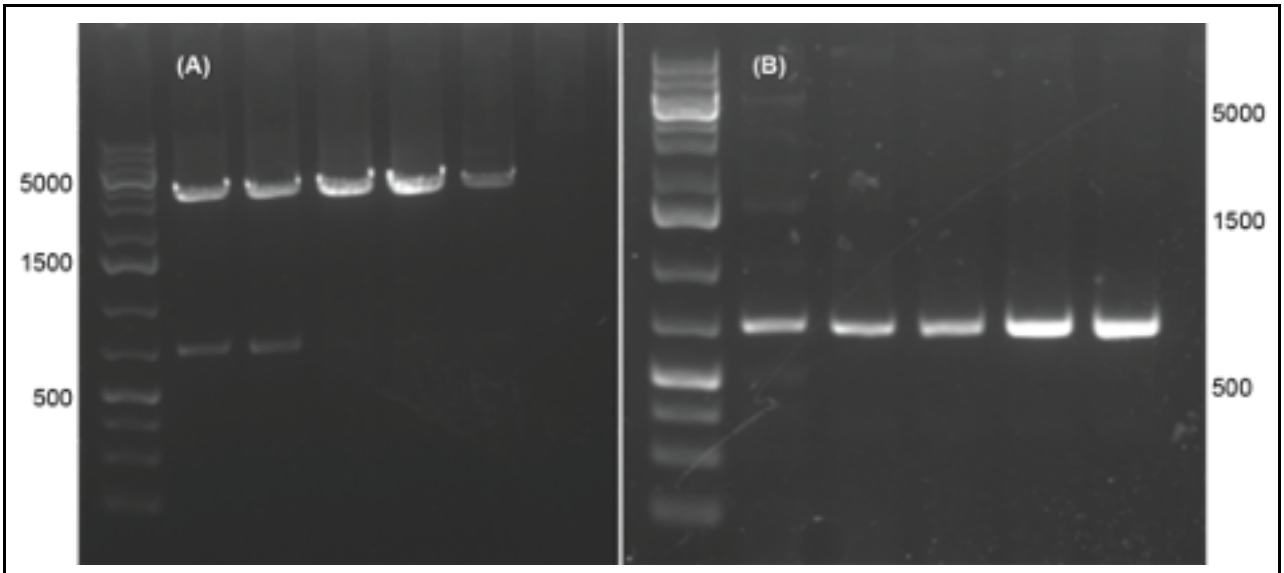


Supplemental Figure 3.2.: 5-FOA plates of cDNA008 transformation. (A) First black colony after re-streaking on 5-FOA. (B) Second black colony after re-streaking on 5-FOA (C) First white colony after re-streaking on 5-FOA. (B) Second white colony after re-streaking on 5-FOA.

Primers Name	Sequence
350	ATGCGAAGCTGACCAATGACAAGAC
589	ATGAAGATGGAGGATTCGAG
590	ACTGTGACACCAATGATTAG
603	CTCTTCTTTACTCTGATAGCTTGAC
604	GATCAGACCAGTAGGTAAACTAG

608	AGTTTCCAGAAGGGTTTTTGTG
609	CAAGCTATCAGAGTAAAGAAGAGG
624	ATTCAGGTACTCACCGTCCACATC
627	TCTTCTAAGTTCCTGCTCACTCTCC
629	GCGAAGAAGATACCTGGAAGGTC

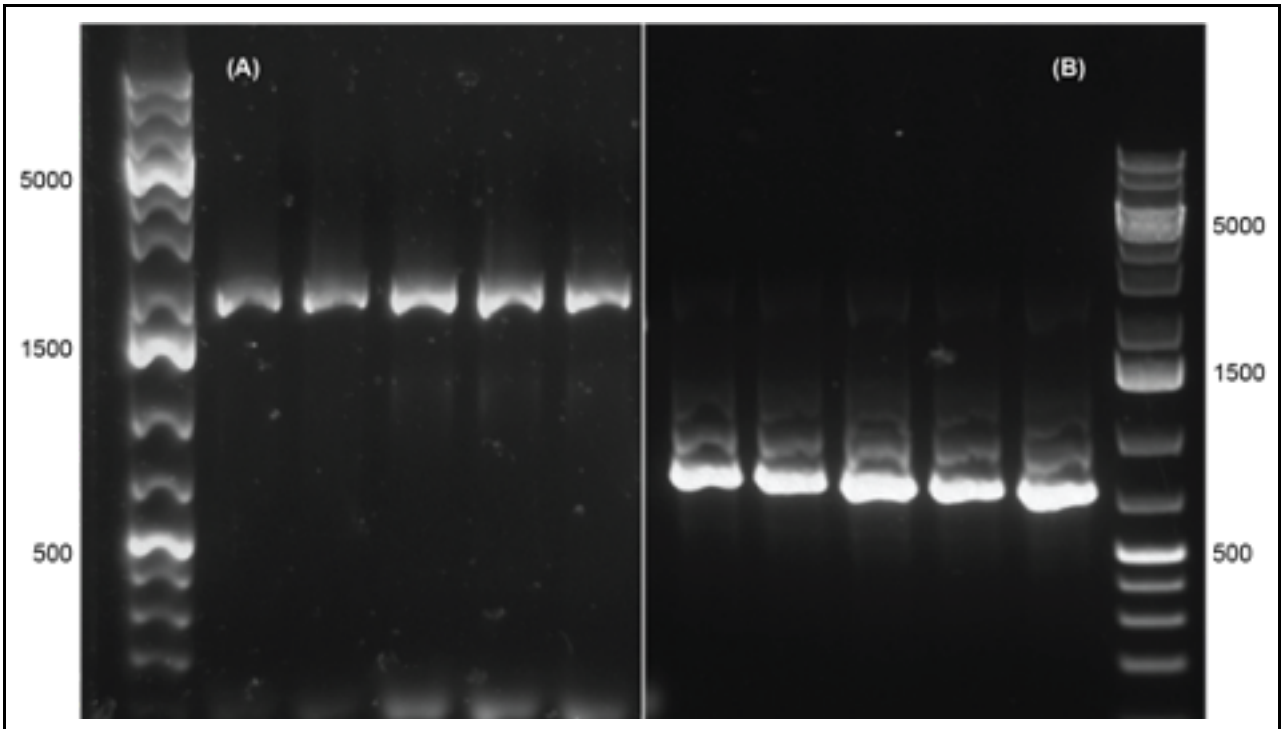
Supplemental Table 3.2.: Primers B5FOA and A5FOA. Primers used for the amplification of amplicons before exposure of 5-FOA (B5FOA), after exposure of 5-FOA (A5FOA) and WT, to verify the length and the sequence (Supplemental Figures 5: Amplicons B5FOA and A5FOA).



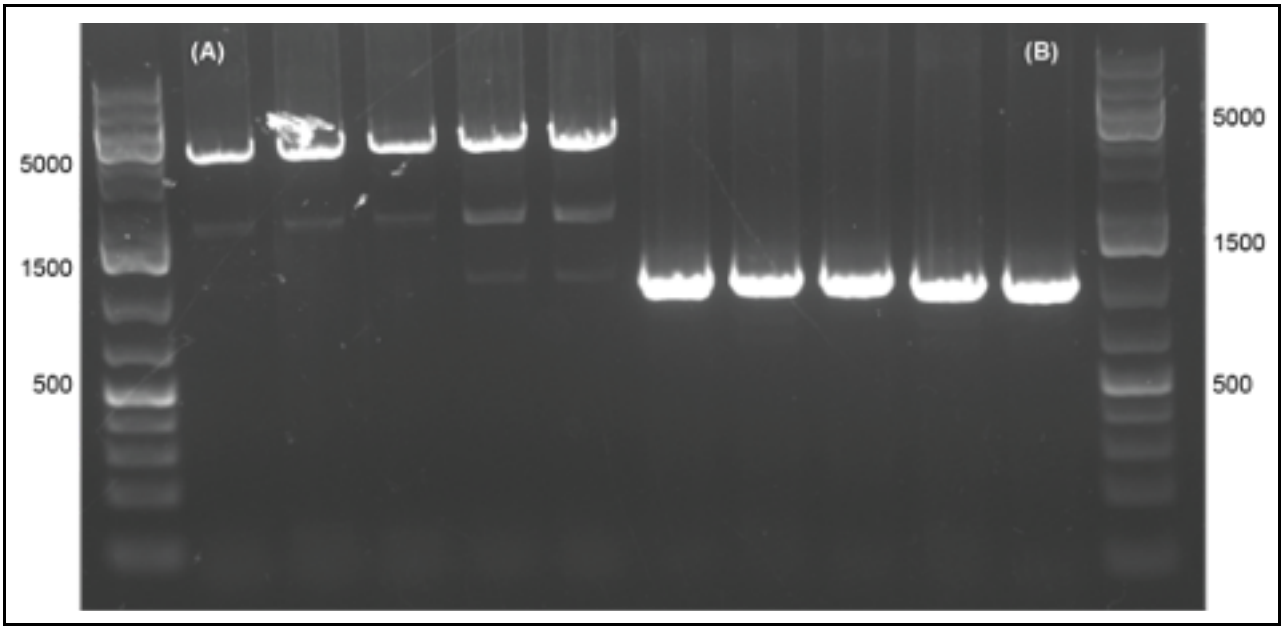
Supplemental Figure 3.3: Representative cDNA008 PCR before and after 5-FOA (A) cDNA008 before 5-FOA, 5 colonies after transformation PCR amplification with 629/631, 3'434 bp. (B) After 5-FOA, 5 white colonies undergone pyrG excision, 695 bp. 1 kb Plus Ladder (Thermo Fisher/ 1 kb Plus ready-to-use).

construct–strain *A. niger*	Primers	T[C]	B-5FOA	A-5FOA	WT
cDNA006–albA (-)	350/590	55	3'125 bp	1'386 bp	1'367 bp
cDNA008–albA (-)	629/631	55	3'434 bp	695 bp	1'695 bp
cDNA009–ΔglaA/PglaA-A5IL97	603/604	54	3'458 bp	719 bp	0 bp
cDNA010 - ΔmstC	624/627	58	3'753 bp	1'024 bp	3'213 bp
*cDNA009_**010 ΔmstC**/ ΔglaA/PglaA-A5IL97*	624/627	58	3'753 bp	1'024 bp	3'213 bp
β -glucosidase-A5IL97	608/609	55	1'713 bp	1'713 bp	0 bp

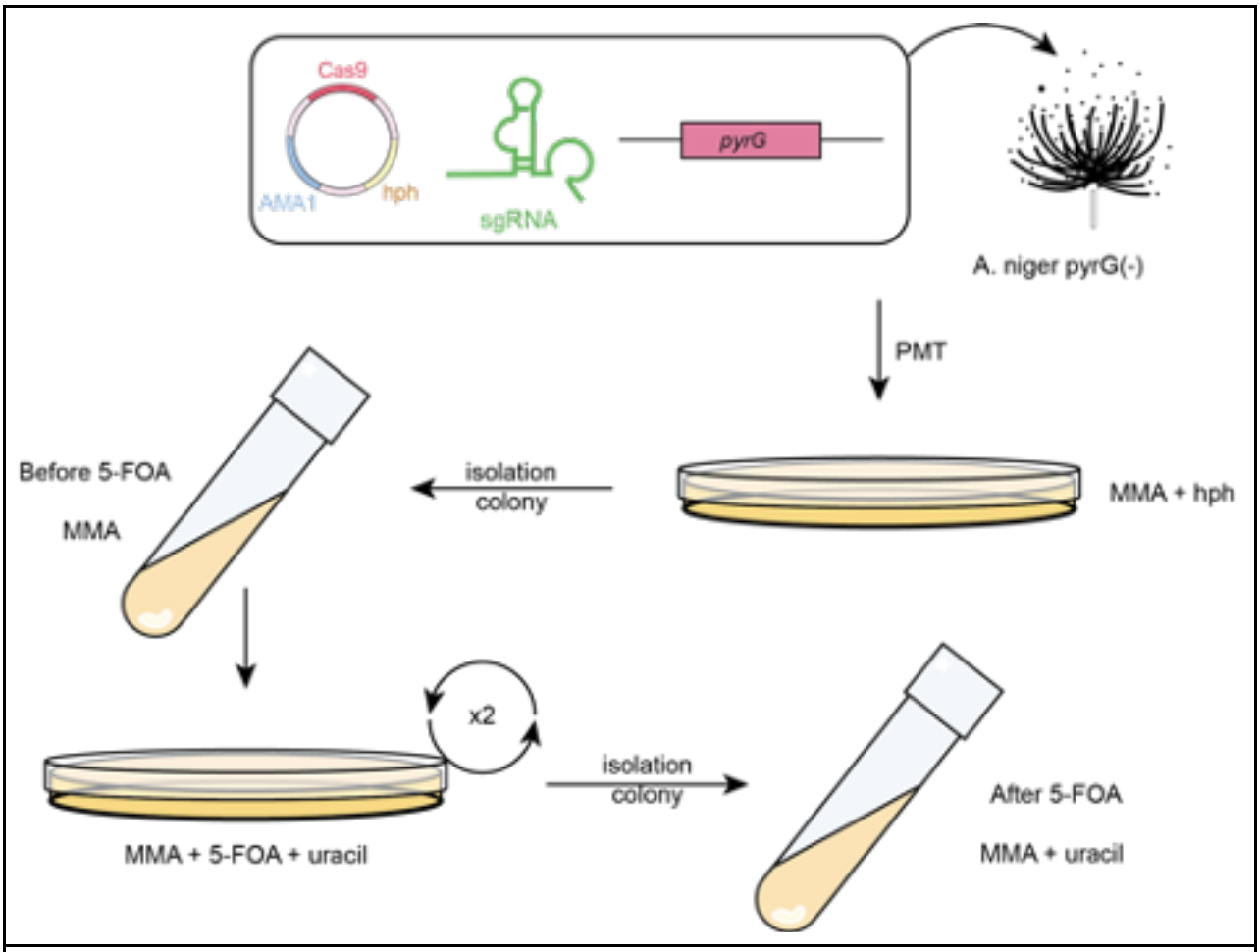
Supplemental Table 3.3: Amplicons B5FOA and A5FOA. Amplification of amplicons before exposure of 5-FOA (B5FOA), after exposure of 5-FOA (A5FOA) and WT, to verify the length and the sequence.



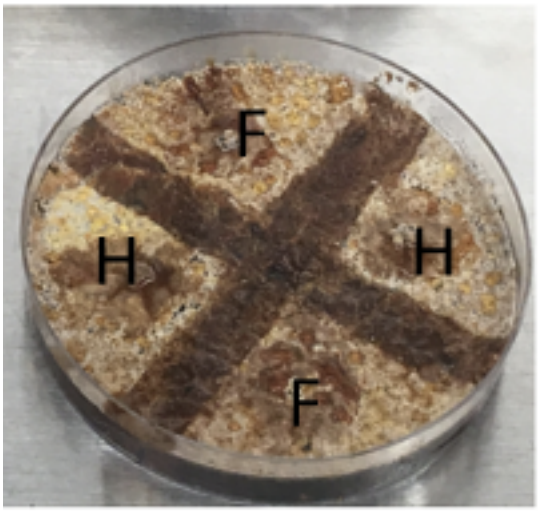
Supplemental Figure 3.4: Representative β-glucosidase (A5IL97) PCR and cDNA009 PCR after 5-FOA (A) Amplification of the A5IL97 cassette of five colonies after transformation PCR with 608/609, 1'713 bp. (B) cDNA009 after 5-FOA of five colonies undergone pyrG excision, 719 bp. 1 kb Plus Ladder (Thermo Fisher/ 1 kb Plus ready-to-use).



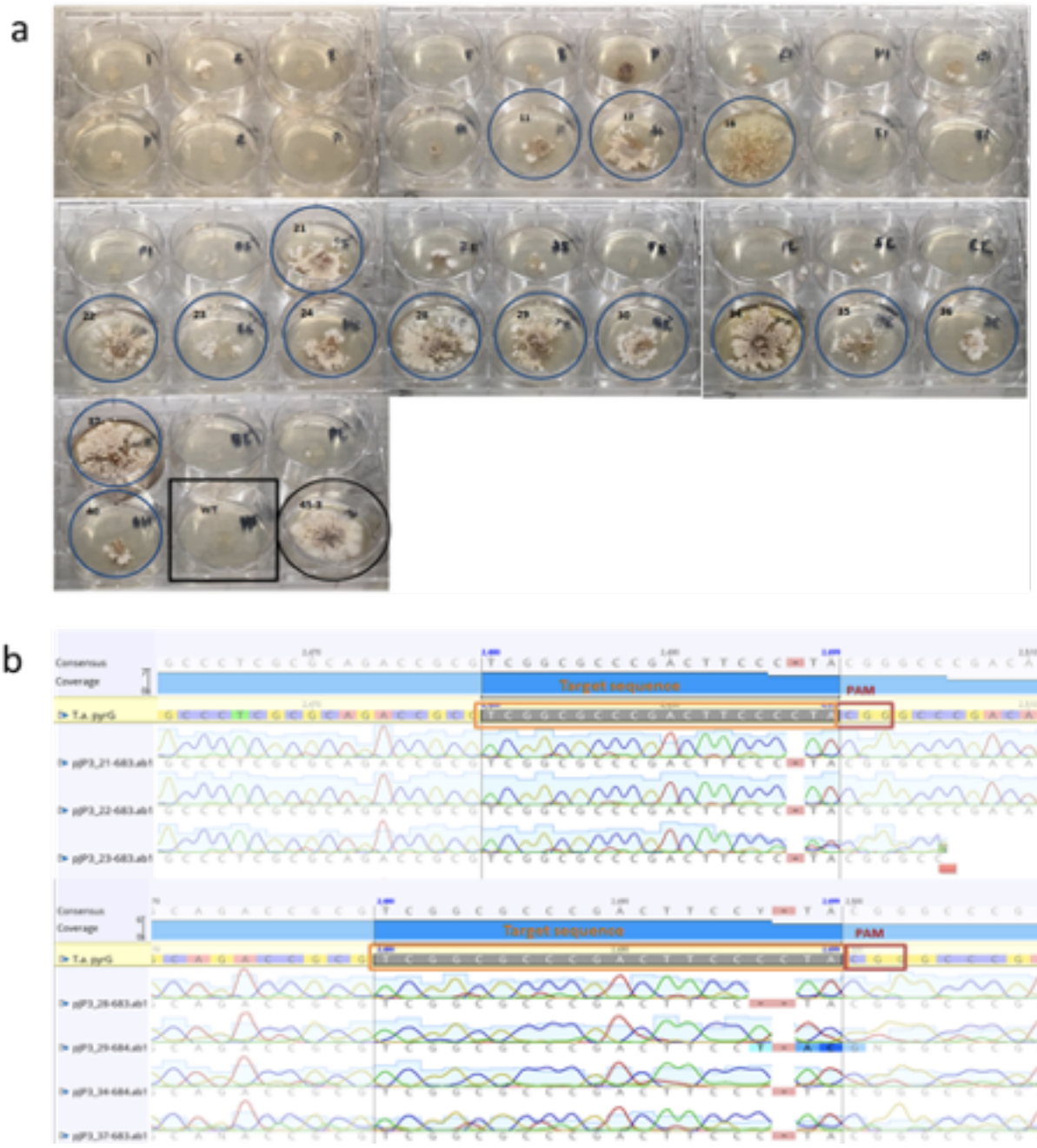
Supplemental Figure 3.5: Representative cDNA010 PCR before and after 5-FOA. (A) cDNA010 before 5-FOA, five colonies after transformation PCR amplification with 624/627, 3'753 bp. (B) After 5-FOA five colonies underwent pyrG excision, 1'024 bp. 1 kb Plus Ladder (Thermo Fisher/ 1 kb Plus ready-to-use).



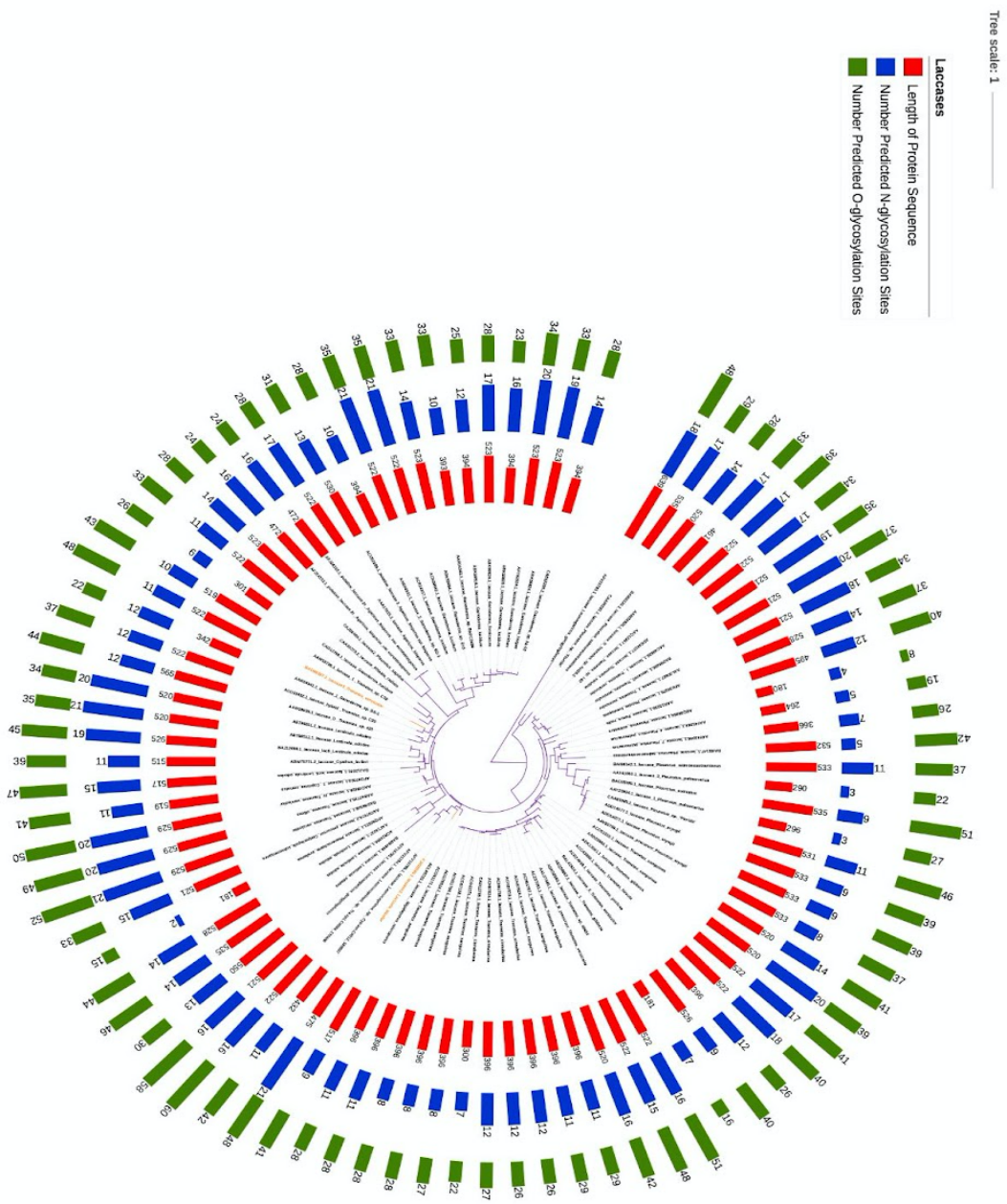
Supplemental Figure 3.6: **Transformation**. Schematic depiction of the process used for PMT transformation of *A. niger* using *pyrG* (-) auxotrophic marker.



Supplemental Figure 4.1: Outcrossing of *T. aurantiacus*. Image of the plate setup for strain crossings: the two parent strains were plated in alternating fashion (F: 5-FOA resistant parent strain FOAR2, and H: hygromycin B resistant parent strain taRG008).



Supplemental Figure 4.2: Screening for *pyrG* deletion strains on 5-FOA uracil medium. (a) A subset of 20 colonies from ATMT transformations using pJP1 (gRNA 1) and pJP3 (gRNA 3) were selected for 5-FOA resistance each. The wild type (black square frame) and FOAR2 as a 5-FOA resistant positive control (black round frame) were included. pJP1 colony 11, 12 and 16 as well as pJP3 colony 21, 22–24, 28–30, 34–37, and 40 were positive transformants on the selection medium and were used for Sanger sequencing verification procedures. (b) Sanger sequencing results for *T. aurantiacus* pJP3 transformants revealing deletions and mismatches through Cas9 cleavage next to the PAM sequence (framed in red) in the *pyrG* target sequence (framed in orange). The sequence analysis was performed with Geneious version 11.1 (Biomatters).



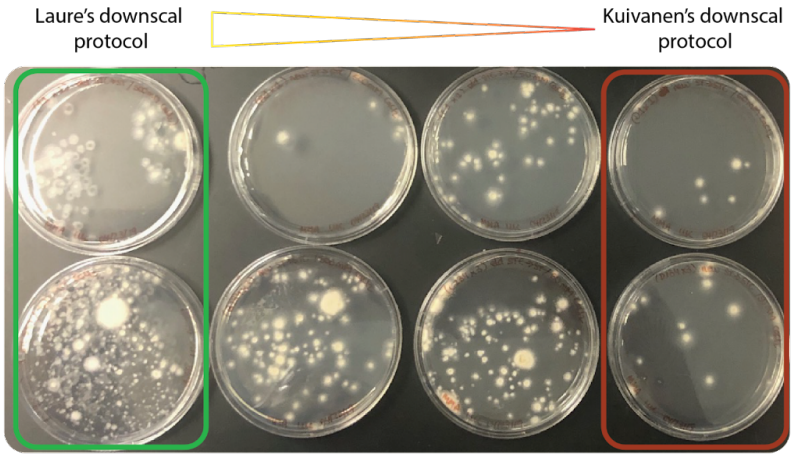
Supplemental Figure 5.1: Laccase library.

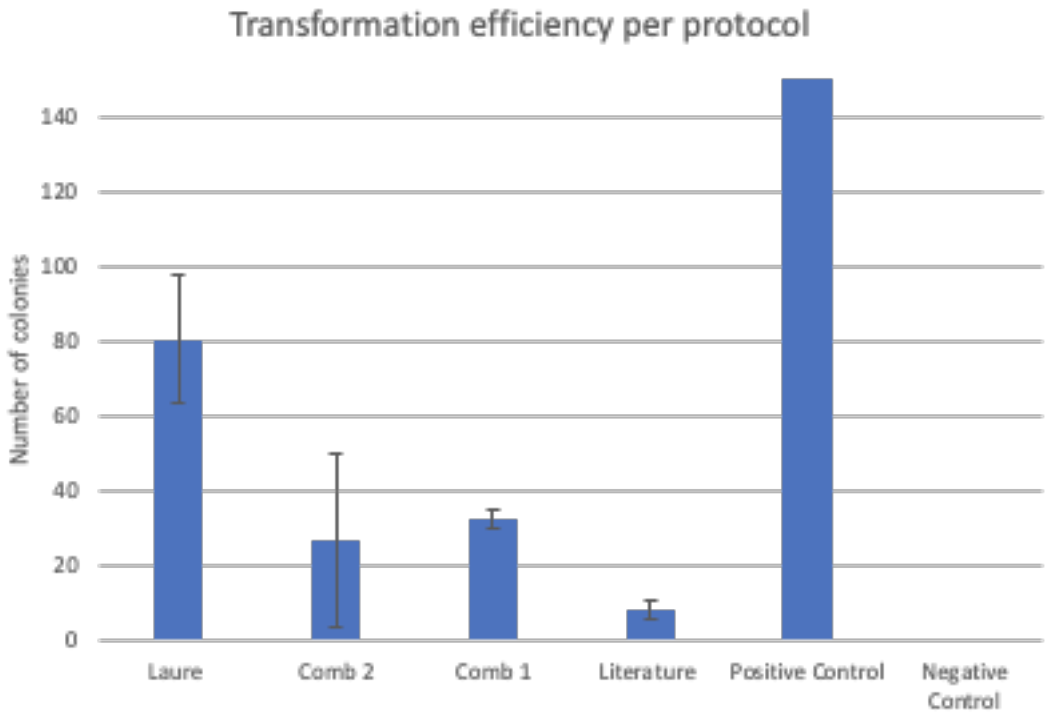
user ID	GenBank ID	Oganism Origin
Lac77	AEQ38864.1	Pleurotus ostreatus
Lac33	AEQ38867.1	Trametes gibbosa
Lac69	AFQ90521.1	Phanerochaete avellanea
Lac74	AAY41064.1	Pleurotus pulmonarius
Lac73	AAY41063.1	Pleurotus pulmonarius
Lac72	AAY23994.1	Pleurotus pulmonarius
Lac34	CAD12769.1	Trametes cinnabarina
Lac59	CAD12768.1	Trametes cinnabarina
Lac48	AAR04341.1	Ganoderma sp. BS-1
Lac75	AAY41066.1	Pleurotus pulmonarius
Lac50	AAR04343.1	Ganoderma sp. BAFC2488
Lac49	AAR04342.1	Ganoderma sp. BS-1
Lac51	ABK59818.1	Ganoderma lucidum
Lac52	ABK59820.1	Ganoderma lucidum
Lac60	CAE52290.2	Ganoderma sp. kk-02
Lac16	ACG61151.1	Trametes sanguinea
Lac17	ACG61171.1	Trametes sanguinea
Lac18	ACG75924.1	Trametes sanguinea
Lac19	ACH61787.1	Trametes sanguinea
Lac20	ACH61788.1	Trametes cinnabarina
Lac21	ACH61790.1	Trametes sanguinea
Lac22	ACH87819.1	Trametes cinnabarina
Lac23	ACH87820.1	Trametes sanguinea
Lac24	ACH87824.1	Trametes cinnabarina
Lac27	ACO57128.1	Trametes sanguinea
Lac28	ACR24938.1	Trametes punicea
Lac46	AFV15795.1	Leucocoprinus sp.
Lac13	AAW28935.1	Trametes sp.
Lac40	AFJ24332.1	Agaricus bisporus
Lac86	FJ432086.1	Laccaria bicolor
Lac68	AAR13230.1	Panus rudis
Lac47	ABW75771.2	Cyathus bulleri
Lac67	BAJ12091.1	Lentinula edodes

Lac85	ACL93333.1	Rigidoporus microporus
Lac10	AAM10738.1	Trametes sp.
Lac44	AFD97050.1	Coprinus comatus
Lac12	AAR20864.1	Trametes sanguinea
Lac25	ACO51010.1	Trametes sanguinea
Lac29	ACZ37081.1	Trametes sanguinea
Lac63	AET86511.1	Lentinula edodes
Lac64	AET86512.1	Lentinula edodes
Lac79	BAI66139.1	Pleurotus salmoneostramineus
Lac01	AAC49828.1	Trametes versicolor
Lac41	AFV15785.1	Leucoagaricus gongylophorus
Lac43	AAC97074.2	Gelatoporia subvermispora
Lac06	BAD98307.1	Trametes versicolor
Lac04	AAL07440.1	Trametes versicolor
Lac11	AAQ12267.1	Trametes sp.
Lac15	ACC43989.1	Trametes hirsuta
Lac26	ACO53432.1	Trametes sp.
Lac30	ADE44157.1	Trametes velutina
Lac31	ADK13091.1	Trametes gibbosa
Lac32	ADK55593.1	Trametes sp.
Lac37	AAA17035.1	Agaricus bisporus
Lac45	AFV15794.1	Leucocoprinus sp.
Lac55	ACN38062.1	Ganoderma lucidum
Lac56	ACR24357.1	Ganoderma lucidum
Lac70	CAA36379.2	Phlebia radiata
Lac53	ABK59824.1	Ganoderma tsugae
Lac54	ABK59826.1	Ganoderma fornicatum
Lac57	ADK55594.1	Ganoderma sp.
Lac58	AFY52524.1	Ganoderma lucidum
Lac84	CAJ00406.1	Pleurotus sapidus
Lac08	BAL42810.1	Trametes versicolor
Lac66	BAJ12090.1	Lentinula edodes
Lac61	AAF13037.1	Lentinula edodes
Lac71	AFK30375.1	Phlebia brevispora
Lac07	BAD98308.1	Trametes versicolor

Lac09	AAB47735.2	Trametes villosa
Lac38	ACU52699.1	Agaricus bisporus var. bisporus
Lac83	CAA80305.1	Pleurotus ostreatus
Lac81	BAI66147.1	Pleurotus salmoneostramineus
Lac35	AAV85769.1	Pleurotus eryngii
Lac76	ADD14077.1	Pleurotus eryngii
Lac80	BAI66142.1	Pleurotus salmoneostramineus
Lac62	ACR24356.1	Lentinula edodes
Lac78	BAC65099.1	Pleurotus ostreatus
Lac82	CAA06291.1	Pleurotus ostreatus
Lac65	BAB84356.1	Lentinula edodes
Lac14	AAW28939.1	Trametes sp.
Lac87	AN9170.2	Aspergillus nidulans
Lac89	AN0878.2	Aspergillus nidulans
Lac42	AFV15793.1	Leucoagaricus gongylophorus
Lac88	AN5397.2	Aspergillus nidulans

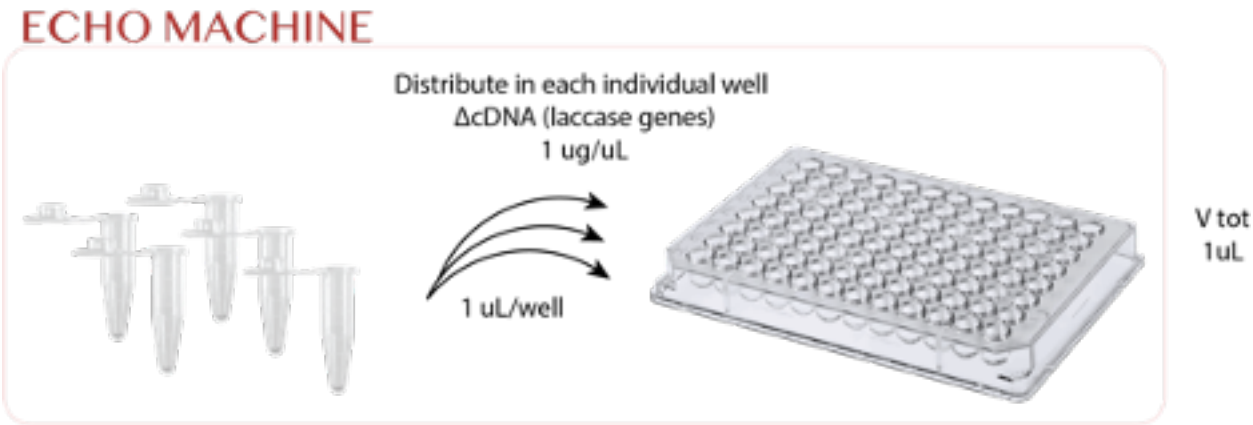
Supplemental Table 5.1: Laccase library.



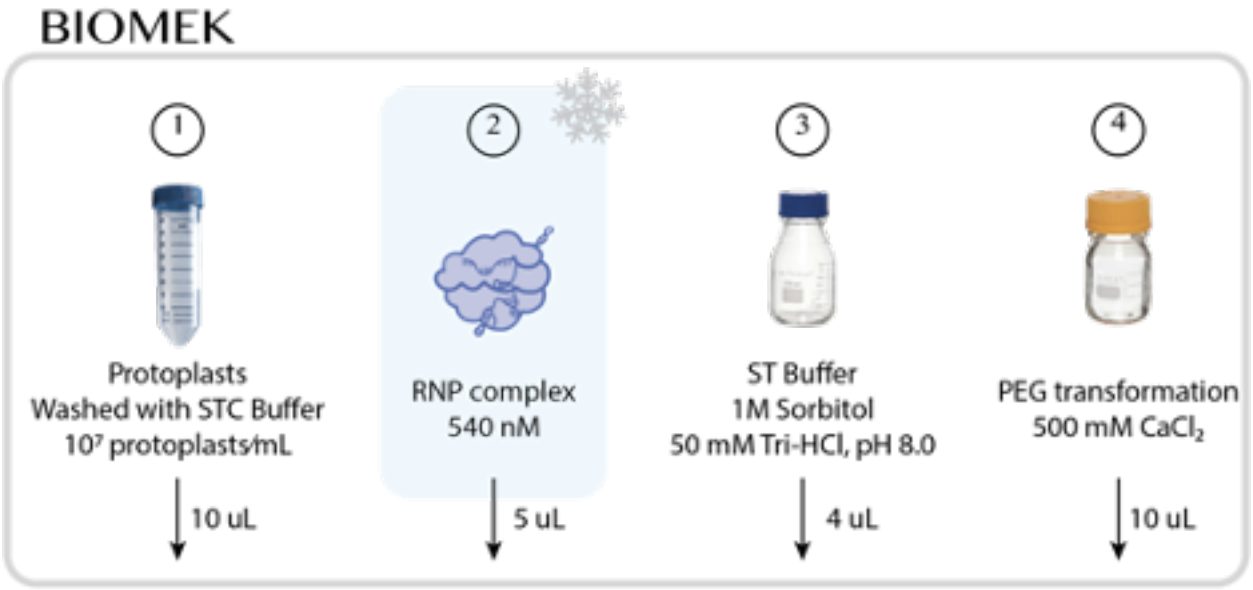


Supplemental Figure 5.2: Kuivanen optimization plates. Laure's protocol is the optimal protocol developed in this study, comb 2 and comb 1 are the different protocol combinations of Kuivanen (349) protocol and the one obtained in this study.

Steps 1
Prepare the 96 well plate with 1ug of DNA template (This will be your sample plate-Nunc plate)



Steps 2
Prepare freshly <1 mL of *A. niger* protoplast (x2) (keep on ice)
Prepare freshly <1 mL of RNP complex (keep on ice)
Prepare ST Buffer and PEG transformation in the reservoir using flame and tape the top to protect for contamination until the very end.



Steps 3

At the Biomek computer, open the Biomek software.

Project -> Open: “Fungal Growth,” observe now at the bottom left of the screen that the project is “Fungal Growth.”

Click on the folder -> Open the Method HTP_Aniger_transformation. Once the computer asks the password, click “Cancel” (unless you want to be able to modify the method).

Click first on “Finish” at the bottom of the code before running it, and observe if there is any Errors in the code. If not and ready, then click on the “Play” (green triangle). Follow the instructions.

Check if there are any big bubbles during the purge

Then browse to charge the csv file and continue.

variable_name	value	src_pos	src_well_proto	src_well_rnp	src_well_st	src_well_peg	dest	dest_well	src_pos_peg_st	src_well_peg_two	src_well_st_two
sample_num	96	reagent	1	2	3	4	sample	1	reagent_peg_st	1	2
		reagent	1	2	3	4	sample	13	reagent_peg_st	1	2
		reagent	1	2	3	4	sample	25	reagent_peg_st	1	2
		reagent	1	2	3	4	sample	37	reagent_peg_st	1	2
		reagent	1	2	3	4	sample	49	reagent_peg_st	1	2
		reagent	1	2	3	4	sample	61	reagent_peg_st	1	2
		reagent	1	2	3	4	sample	73	reagent_peg_st	1	2
		reagent	1	2	3	4	sample	85	reagent_peg_st	1	2
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		reagent	1	2	3	4	sample	14	reagent_peg_st	1	2
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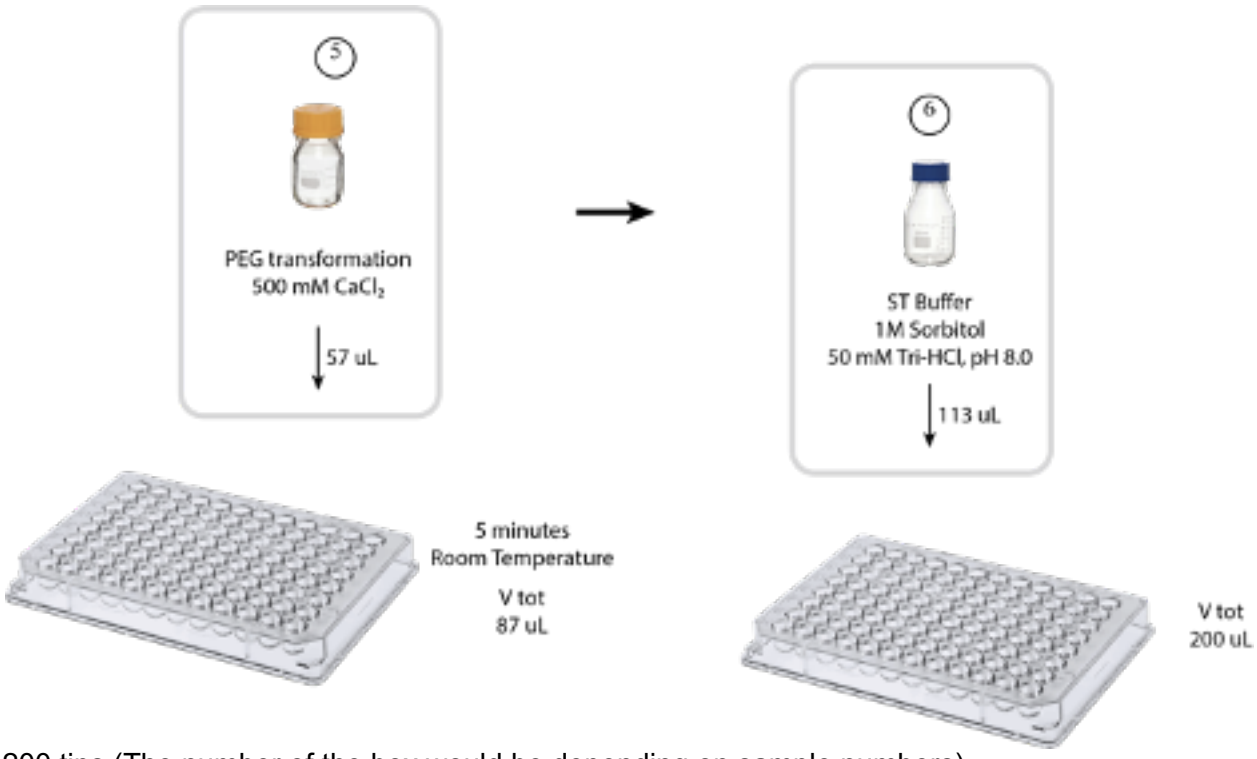
		reagent	1	2	3	4	sample	84	reagent_peg_st	1	2
		reagent	1	2	3	4	sample	96	reagent_peg_st	1	2

Steps 4
First Instrument Setup:
filter 50 tips (The number of the box would be depending on sample numbers)
Sample plate: Nunc plate with lid- 1 microliter DNA sample in it
Empty Nunc plate just as a holder for moving lid back and forth between reagent transfers.
Reagent Reservoir Well 1: Protoplast, Well 2: RNP, Well 3:ST Buffer, Well 4: PEG

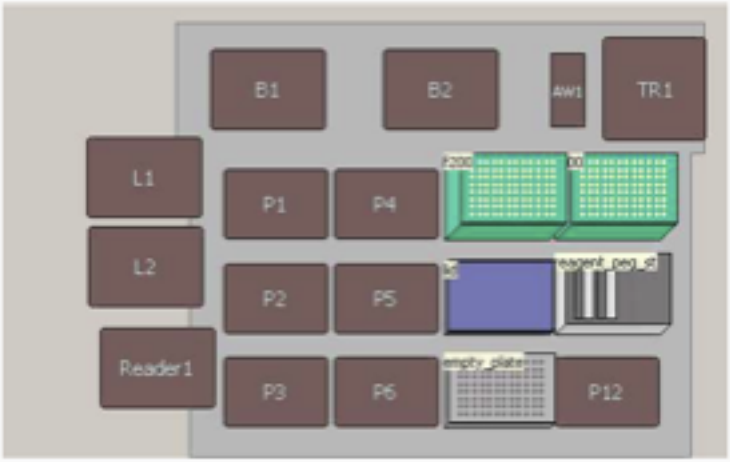
Once you set up the deck based on the prompt, click on “OK” to start the liquid handling transfers.

Steps 5
At user pause, take the sample plate and keep it at 4° Celsius for 20 minutes. After 20 minutes, place the sample plate back at “P8” deck location. Click on “OK” to continue the method.

Steps 6
Instrument set up:



filter 200 tips (The number of the box would be depending on sample numbers)
Sample plate: Nunc plate with lid- 30 microliter (DNA, Protoplast, RNP, ST, PEG)
Empty Nunc plate just as a holder for moving lid back and forth between reagent transfers.
Reagent Reservoir Well 1: PEG, Well 2: ST Buffer



Once you set up the deck based on the prompt, click on “OK” to continue the liquid handling transfers.

Supplemental Figure 5.3: Transformation for robotic protocol using Biomek FXP Robotic Machine.

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Index

PCR, polymerase chain reaction
PAM, protospacer adjacent motif
HR, homologous recombination
S, syringly type unit
G, guaiacyl type unit
H, hydroxyphenol type unit
ILs, ionic liquids
LiP, Lignin peroxidase
MnP, manganese peroxide
VP, versatile peroxidase
LPMOs, polysaccharide monooxygenases
cDNA, construct DNA
ABTS, 2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid)
HOBt, hydroxybenzotriazole
ATMT, A Tumefaciens-mediated Transformation
DNA, deoxyribonucleic acid
UV, ultraviolet
STC, Sorbitol, Tris-HCl and $CaCl_2$ buffer
RNP, ribonucleoprotein
IDT, Integrated DNA Technology
HIFI, High-fidelity endonuclease variant
ST, Sorbitol, Tris-HCl buffer
PEG, Polyethylene glycol
RT, room temperature
KOH, Potassium hydroxide
SC, synthetic complete media

Laure Curran

Professional Summary

Resourceful Graduate Researcher with a track record of leading large and complex projects, from genome engineering to fermentation. Experienced in down-scaling complex biological engineering, culture, and assay.

Skills

- CRISPR/Cas9 in prokaryotes/eukaryotes
- High throughput robotics (Biomek FXP)
- Biochemical assay development (ABTS, pNPG)
- Collaboration and team leadership
- Chemical and Biological process engineering
- Fermentation optimization (filamentous fungi)

Work History

Graduate Student Researcher, 03/2017 to Current
Joint BioEnergy Institute, Simmons Lab – Emeryville, CA

- Developed selectable high efficiency of CRISPR/Cas9 tool for filamentous fungi *Aspergillus niger* and *Thermoascus aurantiacus*.
- Developed libraries of protein secretion strains in *A. niger*.
- Down scaled transformation, culture, and biochemical assay. Up scaled fermentation for enzyme production.

Research Assistant, 07/2016 to 02/2017
Joint BioEnergy Institute, Simmons Lab – Emeryville, CA

- Developed CRISPR/Cas9 transformation protocol for *A. niger*.
- Fermentation optimization media for enzyme production with *A. niger*.
- Optimization 2L fermentation for methyl ketone production with *E. coli*.

Internship Student, 09/2015 to 02/2016
Joint BioEnergy Institute, Keasling Lab – Emeryville, CA

- Investigated guide RNA secondary structure determinants of CRISPR/Cas9 genome editing efficiency.
- Performed genome editing in *E. coli* using CRISPR/Cas9.
- Analyzed data with Python and ImageJ.

Internship Student, 07/2014 to 08/2014
Syngenta – Monthey, Switzerland

- Optimized processes for building-scale energy conservation.
- Generated alternative strategies to reduce water, steam and electricity use of an entire chemical process line.
- Collaborated with technicians and staff scientists.

Education

Doctor of Philosophy: Chemical Engineering and Biotechnology, Current
Swiss Federal Institute of Technology (EPFL) – Lausanne, Switzerland

- Sponsored by the Lawrence Berkeley National Laboratory at the Joint BioEnergy Institute.
- Co-mentored in the Simmons and Hatzimanikatis Laboratories.

Master of Science: Chemical Engineering and Biotechnology, 02/2016
Swiss Federal Institute of Technology (EPFL) – Lausanne, Switzerland

- Minored in Management of Technology and Entrepreneurship. Received Scholarship of EPFL.

Bachelor of Science: Chemistry and Chemical Engineering, 07/2013
Swiss Federal Institute of Technology (EPFL) – Lausanne, Switzerland

- Minored in Mathematics. Received Scholarship of EPFL.

Publications

- **Leynaud-Kieffer, L.M.C.**, et al. A new approach to Cas9-based genome editing in *Aspergillus niger* that is precise, efficient and selectable. Plos One (2019).
- Gabriel, R., Prinz, J., Jecmenica, M., Romero-Vazquez, C., Chou, P., Harth, S., Floerl, L., **Leynaud Kieffer Curran L.M.C.**, Oostlander, A., Matz, L., Fritsche, S., Gorman, J., Schuerg, T., Fleißner, A., and Singer, S.W. Development of genetic tools for the thermophilic filamentous fungus *Thermoascus aurantiacus*. Biotechnol Biofuels 13, 167 (2020).
- **Leynaud Kieffer Curran, L.M.C.**, et al. Review: Lignin valorization development and laccase industrialization for biofuel and bioproducts. Biotechnology Advances (2021).
- **Leynaud Kieffer Curran, L.M.C.**, et al. An optimized high throughput platform, from genetic transformation to fermentation, to screen for secreted laccase in *Aspergillus niger*. (2021) Manuscript in process.

Certifications & Conferences

- Yeast Genetics and Genomics, Cold Spring Harbor Laboratory, 2019.
- SIMB conference, Poster presentation, Denver, Colorado, 2017.
- RAFT conference, Poster presentation, Bonita Spring, Florida 2017.
- DOE Genomic Program (GSP) meeting, Poster presentation, Washington DC, 2018.
- GSA Fungal Genetic conference, Poster presentation, Monterey, California 2019.
- ECFG15, European Conference on Fungal Genetics, Speaker, Roma, Italy 2020.

Personal Achievements

- Co-founder and co-host of the Podcast Secrete Life of a Graduate Student, 2019-2020.
- Elite Marathoner Qualification at the Sacramento Marathon 2019.
- Junior Plus & Instructor in Windsurfing at the Berkeley Cal Sailing Club.
- Language: French, English and German.