Marshall Plan Scholarship

Final Report

"Uncovering the induction of cellulase and ligninase secretion by the fungus *Thermoascus aurantiacus* for a cost-effective conversion of plant biomass to biofuels and other goods"

submitted by:

Marina Jecmenica, MSc

Supervisor University of Vienna:	**Prof. Dr. Andreas Richter**
Supervisors Joint BioEnergy Institute:	**Steven W. Singer, PhD**
	Raphael Gabriel, MSc

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Zusammenfassung

Derzeit basiert unsere Wirtschaft auf begrenzt-vorhandenen Ressourcen fossiler Brennstoffe, die Treibhausgase freisetzen und damit den Klimawandel und die damit verbundenen Risiken für die Gesellschaft und die Biodiversität fördern. Nicht essbare pflanzliche Biomasse, welche in Biokraftstoffe und bio-basierte Chemikalien umgewandelt werden kann, könnte als vielseitige, kostengünstige und erneuerbare Alternative zu fossilen Brennstoffen genutzt werden. Die Umwandlung von Biomasse in Kraftstoffe erfordert allerdings spezifische Enzyme (Cellulasen und Ligninasen), die komplexere Pflanzenbiomasse in kleinere chemische Bausteine, wie zum Beispiel Glucose, aufspalten können, welche wiederum zur Herstellung einer Vielzahl wertvoller chemischer Produkte verwendet werden können. Jene notwendigen Enzyme werden von filamentösen Pilzen produziert, die ausgezeichnete Enzyme für verschiedene Anwendungen im hohen Maßstab produzieren. Da genetische Transformationssysteme für die meisten dieser Pilze noch nicht etabliert sind, werden derzeit nur wenige für die industrielle Enzymproduktion eingesetzt. Das Fehlen eines effizienten Transformationsprotokolls gilt auch für den thermophilen Fadenpilz *Thermoascus aurantiacus*. Dieser Pilz produziert wichtige Cellulasen und Xylanasen, welche hitzebeständiger sind als derzeit verwendete Enzyme. Durch den Ersatz derzeit verwendeter Enzyme mit Hitze beständigen Alternativen, können die Kosten für den Abbauprozess von Pflanzenbiomasse reduziert werden, wodurch die Verfügbarkeit von Biokraftstoffen wettbewerbsfähiger im Vergleich zu fossilen Brennstoffen werden kann.

Hier zeigen wir die erste erfolgreiche Etablierung eines durch *Agrobacterium tumefaciens*-vermittelten Transformationsprotokolls für den filamentösen Pilz *Thermoascus aurantiacus* auf. Darüber hinaus führte die erfolgreiche Überexpression von *xlnR*, einem positiven transkriptionellen Xylanase Regulator, zu einer Erhöhung der Xylanase-Aktivität um 500% in transformierten Stämmen im Vergleich zum Wildtyp und bringt uns damit einen weiteren Schritt näher zur Verwendung von *T. aurantiacus* für biotechnologische Anwendungen. Derzeitige Bemühungen konzentrieren sich auf die weitere Optimierung von *T. aurantiacus* Stämmen für eine höhere Produktion endogener und heterologer Enzyme und die Etablierung dieses Organismus als Modellsystem für Pilzgenetik und Zellbiologie.

Abstract

Currently, our economy is based on finite fossil fuel resources, which release greenhouse gases, promoting climate change and its associated risks to society and biodiversity. Non-edible plant biomass, which can be converted into biofuels and bio-based chemicals, has the potential to be used as a versatile, inexpensive and renewable alternative to fossil fuels. Biomass conversion to biofuel requires specific enzymes (cellulases and ligninases) that can deconstruct more complex plant biomass into smaller chemical building blocks used in the production of a wide range of valuable bio-based fuel and chemical products. The necessary enzymes are produced by filamentous fungi, which are excellent producers of enzymes for various applications. Since genetic transformation systems for most of them are not yet established, only a few fungi are currently used for industrial enzyme production. The absence of an efficient transformation protocol is also the case for the thermophilic filamentous fungus *Thermoascus aurantiacus*. This fungus secretes cellulases and xylanases, which have been found to be more heat resistant than currently used enzymes. Replacement of the currently utilized enzymes can reduce the cost of the plant biomass deconstruction process, making the cost and availability of biofuels more competitive.

Here we report on the successful establishment of an *Agrobacterium tumefaciens*- mediated transformation protocol for *Thermoascus aurantiacus*. In addition, successful overexpression of *xlnR*, a positive transcriptional xylanase regulator, led to a 500% increase in xylanase activity in the mutant strain and thus, advanced the use of *T. aurantiacus* for biotechnological applications. Current efforts focus on further optimizing *T. aurantiacus* for higher production of native and heterologous enzymes and to establish this organism as a model system for fungal genetics and cell biology.

Introduction

Fossil fuels

Our daily lives strongly depend on limited fossil fuel resources and nowadays we are already starting to recognize the consequences of a fossil fuel-based society from the past decades. We see the amount of carbon dioxide (CO_2) in our atmosphere annually increasing coming along with elevated average earth surface temperatures and an anthropogenically-induced rapidly changing climate (IPCC 2014). Today, climate change is considered to be one of the greatest dangers to human society and its fast progression is undeniably an outcome of continuous anthropogenic greenhouse gas emissions (Alley et al. 2003). Pronounced periods of heat, yearly decreasing ice cover and raising sea levels together with more frequent extreme weather events are already visible and evidently will continue to become more likely until the point, where certain areas will become less and less inhabitable as it is predicted for low-lying coastal areas such as, for example, many of the atolls in the tropical Pacific Ocean (Storlazzi et al. 2015, IPCC 2014, IPCC Special Report 2018). Those are only some of the effects of our progressively changing climate. If we continue to support economic growth with fossil fuels as the main energy source as in previous decades, it will result in even more pronounced global changes (IPCC Special Report 2018). That is why finding clean, renewable, but also economically viable alternatives represent a key step in addressing the effects of global warming and all its inherent implications. So far, various promising alternatives have been developed: Hydropower, solar, geothermal, wind and many more are already being utilized as renewable energy sources (Panwar et al. 2011). Many of them are still too expensive in practice and it will be important that future alternatives become economically more competitive to fossil fuels.

Biomass as a promising alternative

Facing the impacts of climate change scenarios in the near future, many scientists explore the applicability of plant biomass as sustainable and "green" alternative to fossil fuels.

Lignocellulosic biomass is the most abundant organic material on the planet (Popper et al. 2011). This non-edible plant biomass is a renewable and a highly abundant by-product of forestry, agriculture, the paper and food industry (Carroll and Somerville 2019). Making use of a waste product and converting it into "green" energy could be the key to our energy crisis and thus, investing into biofuel research will be a crucial step for starting the energy change.

Plant biomass conversion into simple sugars is more versatile than conventional renewable energy sources and thus offers the opportunity to create new and economic opportunities for rural areas (de Jong et al. 2012).

Plant biomass itself mainly consists of long-chain sugar polymers (cellulose) and a wood-forming bio-polymer (lignin). 70% of the plant cell walls consist of cellulose (β-1,4-linked d-glucose), hemicelluloses and pectin (Jorgensen et al. 2017, Glass et al. 2013). Cellulose is a highly stable polymer and the structural component, responsible for mechanical strength. These polymers can be deconstructed by simple enzymes (cellulases and ligninases) into smaller chemical building blocks such as simple sugars (mainly glucose). Subsequently, those can be further processed into biofuels, bioplastics and other valuable products using bacteria and yeasts, making plant biomass more versatile than conventional renewable energy sources (de Jong et al. 2012). However, enzymatic plant biomass degradation is still very expensive and the cost-efficient removal of cellulose into free sugars is currently the biggest obstacle preventing biofuels to become economically competitive to fossil fuels. Therefore, cellullases and related biomass degrading enzymes are the key factor and bottleneck at the same time for the successful production of biofuels (Klein-Marcuschamer et al. 2011).

The role of ascomycete fungi

The natural ability of filamentous fungi to efficiently degrade pant biomass makes this group of organisms highly interesting for the production of biofuels and other biotechnological purposes (Kubicek et al. 2009). Ecologically, the ability of ascomycete fungi to efficiently decompose organic matter contributes significantly to the global carbon cycle. For the successful degradation of plant derived polysaccharides fungi release a tremendous amount of carbohydrate-active enzymes (CAZymes), which enable them to access simple sugars to cover their energy demand (Glass et al. 2013). This set of enzymes (secretome) usually depends on the specific carbohydrates present in the environment, but it also varies across fungal species. While *Aspergillus* species cover a wide range of pectin degrading enzymes (Berka et al. 2011; Kubicek et al. 2011), *Trichoderma reesei* is well known for its efficient degradation of cellulose (Martens-Uzunova and Schaap 2009). The actual secretion of enzymes, however, is highly controlled by transcriptional regulators ensuring efficient and economic release and acquisition of resources. The successful degradation of plant biomass to simple sugars by filamentous fungi makes them a major target for the biotechnological industry, however, to reduce the costs of biomass degradation into valuable

sugars there is still a high demand for novel fungal strains with advanced features (Berka et al. 2011; McClendon et al. 2012; Blumer-Schuette et al. 2013). So far, ascomycete fungi represent the main source of commercially available cellulases with *Trichoderma reesei* (*Hypocrea jecorina*) being the most important industrial strain for enzyme production, producing up to 100 g L^{-1}, so far (Le Crom et al. 2009).

Usually, cellulases are produced commercially by hypersecretory fungal strains, which are generated by decades of undirected mutagenesis (UV radiation and other mutagens). Robust strains with desirable phenotypes can take up to 30 years. Recently, a *Penicillium oxalicum* strain was advanced within six months by genetic engineering, resulting in equal enzyme production as an industrial strain of the same species, which was generated within 30 years of random mutagenesis (Yao et al. 2015). Increasing knowledge about genetic regulation of CAZymes in fungi together with new biotechnological tools and methods are urgently necessary to generate novel genetically stable fungal hyper-secretory strains that will help reducing the overall cost of cellulose break-down.

Thermoascus aurantiacus

As mentioned, ascomycete fungi represent the main source of ligno-cellulosic enzymes that are from industrial relevance with *Trichoderma reesei* still representing the most important species (Glass et al. 2013). So far, little attention has been drawn to the thermophilic ascomycete fungus *Thermoascus aurantiacus*, although the advantages of thermostable enzymes for the industrial breakdown of cellulose are widely recognized (Berka et al. 2011; Xu et al. 2015). *T. aurantiacus* natively secretes a high amount of cellulases (> 2 g L^{-1}) and thus, genetic modifications of the right enzymatic regulators might easily lead to the secretion of industrially relevant levels of enzymes (McClendon et al. 2012; Schuerg et al. 2017). Hugo Miehe was the first to describe *Thermoascus aurantiacus* in 1907 and he classified it to belong to the order of *Eurotiales*. *T. aurantiacus* was found in various terrestrial habitats ranging from different soil habitats and compost to agricultural residues such as self-heating hay piles (Hugo Miehe, 1907) all around the globe. It was described to have an optimum growth temperature around 48-50 °C, whereas no growth occurs at temperatures below 30 °C (Schuerg et al. 2017). Unsurprisingly, this elevated temperature profile could also be observed for its cellulase activity. McClendon et al. (2012) showed that *T. aurantiacus* enzymes were able to perform saccharification reactions up to temperatures of 70 °C,

largely exceeding the enzyme activity of another thermophilic fungus *Thielavia terrestris* and of the commercial Cellic Ctec2 enzyme cocktail above these temperatures.

Higher temperatures during the saccharification of pre-treated biomass result in higher conversion rates during shorter incubation times. The viscous plant biomass solution shows improved flow behavior at elevated temperatures and at the same time, the risk of contamination by other microorganisms for fermentable sugars will likely decrease with increasing reaction temperatures. Another advantage is, that bio-reactors would not need to be cooled to this high extent anymore, which on a long term significantly reduces cooling costs. All these factors lead to a more economic degradation of cellulose into free sugars when thermostable enzymes are being used (Berka et al. 2011; Blumer-Schuette et al. 2013; Schuerg et al. 2017). Recent research efforts aim to make enzymes from currently used fungi more heat-stable.

Schuerg et al. 2017 identified a high degree of similarities regarding the most important regulatory genes for cellulase production in the genome of *T. aurantiacus* compared to other ascomycete fungi (Figure 1, Schuerg et al. 2017). Among those, an important transcriptional repressor for the expression of cellulases in the presence of easily available sugars, cre-1, was found. At the same time, homologs of the transcriptional activators *xlnR*, *clr-1* and *clr-2* were identified, which are known to promote the cellulase secretion in *Trichoderma reesei* and others. Knock out and overexpression of those regulators represent promising initial targets for first transformation attempts in *T. aurantiacus*.

Transcription Factor	***Thermoascus aurantiacus* protein ID (Mycocosm)**	**Proposed Function**	**Closest Homolog (GenBank)**
Cre-1 (Zn finger)	Theau_44762	Carbon catabolite repression	*Rasamsonia emersonii* CBS 393.64 (78%)
XlnR (Zn finger)	Theau_38177	Regulation of cellulase/xylanase gene expression	*Rasamsonia emersonii* CBS 393.64 (72%)
Clr-1 (Zn finger)	Theau_37408	Induction of cellulase genes	*Aspergillus fumigatus* Af293 (71%)
Clr-2 (Zn finger)	Theau_50720	Induction of cellulase genes	*Aspergillus lentulus* (56%)
ClbR (Zn finger)	Theau_41281	Induction of cellulase genes	*Byssochlamys spectabilis* No. 5 (74%)

Figure 1: Homologs of the most relevant regulators for cellulase production in ascomycete fungi identified in the genome of *Thermoascus aurantiacus* (ATCC 26904) by Schuerg et al. 2017.

Aim of the project

The overall goal of this project is to develop an efficient genetic transformation protocol for *Thermoascus aurantiacus* (ATCC 26904) and with this, to set the key stone for industrial application for this particular thermophilic fungus. While establishing a successful protocol, previously identified potential regulatory elements in the *T. aurantiacus* genome (*creA, xlnR, clr-1, clr-2*) will be targeted to produce higher enzyme activities. Ideally, this strategy will lead to high-cellulase producing strains, however, most certainly this approach will shed light into the complex gene regulation for the secretion of cellulolytic enzymes in *Thermoascus aurantiacus*, a still very poorly studied organism with great potential for the biotechnological industry.

Previous experiments indicated that protoplastation as transformation technique was not successful, and therefore, the new attempt will include establishing a protocol based on *Agrobacterium tumefaciens*- mediated transformation (Figure 2). Various conditions will be tested and potential candidates will be confirmed via DNA extraction and PCRs designed to specifically target the amplification of the potentially integrated constructs.

Finally, successfully modified *Thermoascus aurantiacus* strains will be screened for the desired (growth) phenotype and conclusions will be made regarding the importance of targeted genes in *T. aurantiacus* enzyme secretion. Transformants will be compared to the wild type in terms of enzyme secretion using enzyme assays, Brandford Protein assay and SDS PAGE. This study will be performed in the Microbial and Enzyme Discovery group at the Joint BioEnergy Institute (JBEI) in Emeryville, California, which is managed by the Lawrence Berkeley National Laboratory (LBNL).

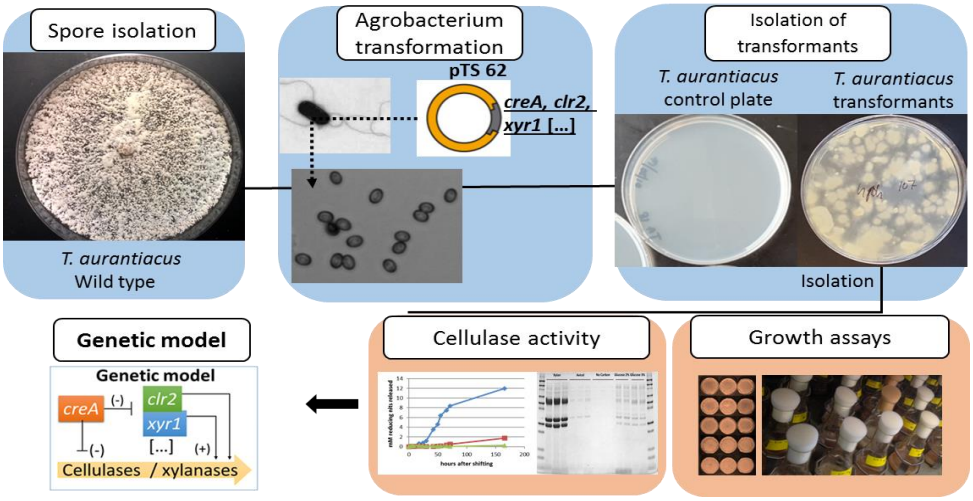

Figure 2: Overview of the workflow: Spores of *T. aurantiacus* will be isolated and transformed using the natural transformation capabilities of *Agrobacterium tumefaciens*. This bacterium will insert copies of cellulase regulators or the *hph* gene into the fungal genome to either overexpress or delete these regulators. Successful transformants will be isolated by resistance to hygromycin and verified via PCR. Growth assays and cellulase activity assays will show changes in fungal phenotypes to establish a genetic model of cellulase regulation in *T. aurantiacus*.

Material and Methods

Agrobacterium tumefaciens- mediated transformation

As previous attempts to transform *T. aurantiacus* protoplasts were not successful, we decided to develop an *Agrobacterium tumefaciens*- mediated transformation protocol for *Thermoascus aurantiacus*. Using the natural transformation capabilities of *Agrobacterium tumefaciens* is a very common and effective technique to generate desired mutants of filamentous fungi (Fang et al. 2006, Duarte et al. 2007). *A. tumefaciens* is a gram negative soil bacterium that causes tumor growth at wound sites of infected plants. It has the natural ability to transfer a DNA segment (T-DNA) from its Ti plasmid into plants, where it integrates into random positions of the chromosomes. The DNA transfer itself is triggered by the expression of virulence genes in *Agrobacterium*, which is being induced by a chemical compound acetosyringone (AS) secreted by wounded plant cells (Hoekema et al. 1983). Thus, it is evident that the acetosyringone concentration used, will likely influence the transformation efficiency. Various publications on *Agrobacterium tumefaciens*- mediated transformation suggest an optimal AS concentration of 200 µM (Figure 3) and therefore, we started our experiments with this concentration.

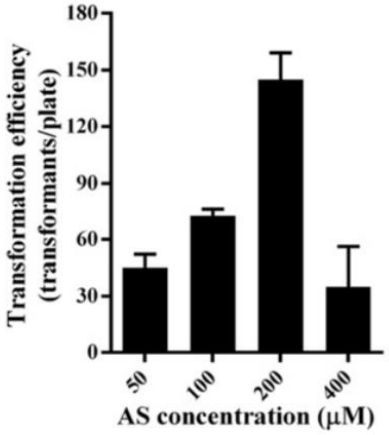

Figure 3: Bar chart from Xu et al. 2015. Number of transformants received after using different acetosyringone (AS) concentrations [50, 100, 200, 400 µM] in the induction medium for the transformation of *Myceliophthora thermophila*, a thermophilic fungus. The highest number of transformed colonies can be found for treatments with 200 µM AS concentration.

Agrobacterium tumefaciens*-mediated transformation for *Rhodosporidium toruloides

After various attempts of protoplast transformation could not produce any transformed *Thermoascus aurantiacus* colonies, different *Agrobacterium tumefaciens*- mediated transformation protocols were tested and subsequently optimized for *Thermoascus aurantiacus*.

A successful protocol for the transformation of *Aspergillus niger* was provided by colleagues of the Pacific Northwest National Laboratory (PNNL). The protocol could be reproduced for *A. niger*, which was used as a positive control during transformation attempts (Figure 4), however, this protocol repeatedly failed to produce *T. aurantiacus* mutants. Therefore, another protocol was tested, which was provided by colleagues from JBEI. This protocol is optimized for *Rhodosporidium toruloides* (red yeast), which served as our positive control for further experiments and that was stepwise modified for the transformation of *Thermoascus aurantiacus*.

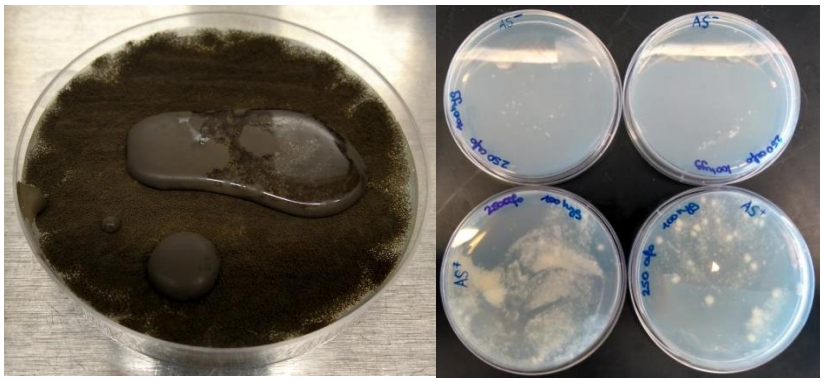

Figure 4: *Aspergillus niger*. Transformation using the protocol provided by Pacific North West National Laboratory (PNNL). Successful transformation of the positive control *A. niger* (treatments with and without addition of acetosyringone), no colonies could be obtained for *T. aurantiacus* using this protocol.

In brief, single-cell colonies of *Agrobacterium tumefaciens* carrying the correct plasmids were transferred to 10 ml liquid LB_{kan} medium and grown an OD of 1 was reached. At this point, the LB_{kan} medium was replaced by the induction medium containing 200 µM of acetosyringone (IM: salts, phosphor buffer, MES-buffer, glucose, thiamine, acetosyringone, water), in which cells were incubated for 24 hours at 250 rpm until they could be used for transformation purposes. In the meantime, *Rhodosporidium toruloides* (APA 2687) was streaked onto YPD plates and three days later single-cell colonies were transferred into 1 ml YPD liquid medium. Cells were diluted to obtain *R. toruloides* cultures of an OD between 0.8 and 1 at the 24 h *A. tumefaciens* bench mark. 1 ml of *R. toruloides* cells were merged with 1 ml of *A. tumefaciens* cell solution and incubated

for 5 minutes at room temperature. After centrifugation (4000 rpm for 20 min), the cell mixture was re-suspended in 1 ml induction medium. Cells were spread onto Millipore membranes (HAWP 0.45 µm) using a vacuum system to remove the liquid residue and the membranes containing the bacterial and yeast cells were placed onto induction media plates (IM plates: salts, phosphor buffer, MES-buffer, glucose, thiamine, acetosyringone, water + 1.5% agar). These plates were stored at 26 °C for four days and another three days at room temperature until *Rhodosporidium toruloides* cells could be harvested off the co-incubation membranes using 200 µl of cefotaxime containing YPD. Subsequently, *Rhodosporidium toruloides* cells were plated onto selection media YPD_{hyg} (hygromycin 50 µg ml^{-1}) and incubated for 2-3 days at 30 °C. Mutant colonies were expected after 48 hours and were confirmed via checking PCRs. For each transformation attempt a negative control (containing *R. toruloides* cells to which no induced bacteria were added) was performed. On the basis of this protocol, different parameters and conditions were optimized for *T. aurantiacus*.

Prior to fungal transformations, *Agrobacterium tumefaciens* strains were generated as follows: The pTS57 vector (Pgdp::P::gfp::T::TxlnR; Ptef1::hph::TtrpC), containing a GFP-drop out cassette for type II restriction enzyme mediated Golden Gate Cloning, was used as a basis for construction of further vectors driving the expression of a gene of interest (*xlnR*, *clr-1*, *clr-2*) with the constitutive *Thermoascus aurantiacus gdp* promotor and *xlnR* terminator. Successful transformants, in which the *gfp* was replaced by the gene of interest, were expected to show no green-fluorescing properties and could be selected under a blue-screen. Hygromycin was chosen as the selection marker for all expression vectors. Vectors were isolated from *E. coli* overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen ™) and transformed into *A. tumefaciens* strain EHA 105 through electroporation. For this reason, electro-competent cells of *A. tumefaciens* (EHA 105) were thawed on ice and 50 µl of the cell suspension were incubated with around 10 µl (containing ~1 µg DNA) plasmid DNA for 2 minutes. The cell-DNA mixture was transferred to a pre-cooled 0.2 cm BioRad electroporation cuvette. Each cuvette was transferred to the electroporator (at 25 µF, 400 ohms, 2.5 kV) separately and a single electrical pulse (field strength of 12.5 kV cm^{-1}) was applied. Immediately following the pulse, cells were re-suspended in 1 ml of LB broth and transferred to a 15 ml round bottom tube, in which they were incubated at 30 °C for three hours (on a 250 rpm shaker). After three hours, cells were streaked out on LBkan (kanamycin 50 µg ml^{-1}) plates and incubated at 30 °C. After 2-3 days, transformed colonies were visible and could be picked to obtain

over-night cultures. Correct integration of desired plasmids was re-checked once more via restriction digest and afterwards could be used for the actual transformation of *T. aurantiacus*.

Optimization of *Thermoascus aurantiacus* transformation

The transformation protocol for *Rhodosporidium toruloides* resulted, besides successful transformation of APA 2687 (was used as the positive control within each transformation treatment), also in two *h1 gfp* containing *Thermoascus aurantiacus* mutant strains. Therefore, we decided to use this protocol as a basis for the following optimization of *Thermoascus aurantiacus* transformations (Figure 5).

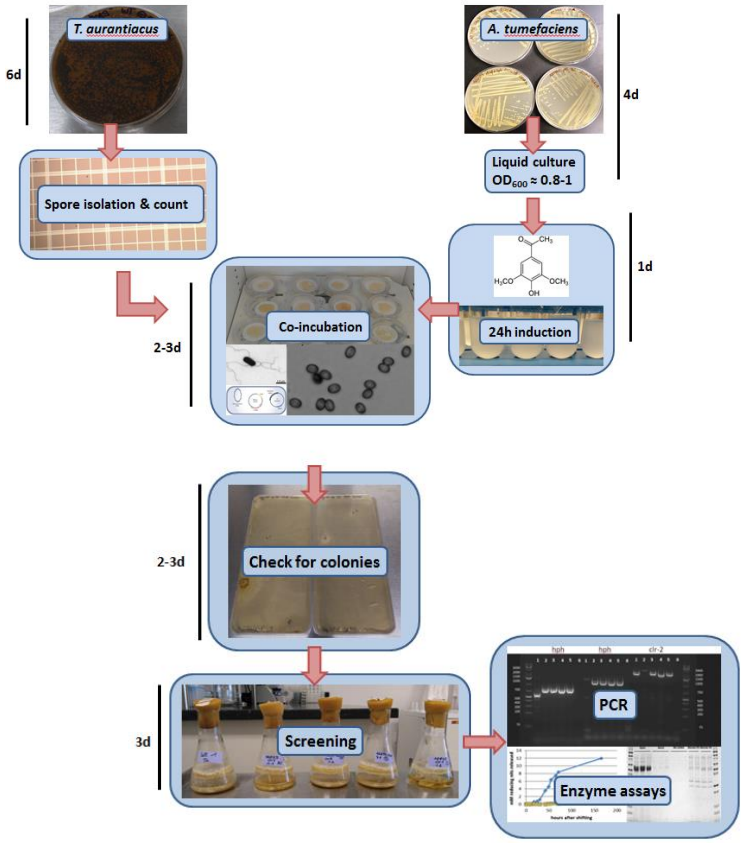

Figure 5: Schematic overview on *Agrobacterium tumefaciens*-mediated transformation for *Thermoascus aurantiacus* on the basis of a *Rhodosporidium toruloides* (APA 2687) transformation protocol. The key steps include: preparation of bacterial cells and fungal spores, the co-incubation of both, the selection on hygromycin containing PDA plates and the screening for successful mutant strains for the correct integration of desired fragments.

Further transformation experiments were performed with the aim to detect the optimal conditions for the efficient generation of *Thermoascus aurantiacus* mutant strains. Variable conditions to consider included choosing an appropriate selection marker, determining the optimal ratio of fungal spores to bacterial cells during co-incubation, as well as the co-incubation time and

temperature. In addition, the pH of the induction medium was optimized and different membranes, on which the co-incubation was performed, were tested for the most efficient transformation success. All those variables had to be tested in separate transformation experiments to be able to elucidate the effect that each of them has on the number of mutants.

Strains and culture conditions

Thermoascus aurantiacus strain ATCC 26904 was obtained from the American Type Culture Collection and was usually grown on Teknova potato dextrose agar (GSS TKNP0047PK, Potato Dextrose Agar Plates. 100 mm 20 Plates per S) for transformation purposes. PDA plates were inoculated with ascospores and incubated for two days at 45 °C and for another four days at 50°C until they were harvested in dH_2O (alternatively in Tween 0.8%) by filtration through Miracloth for subsequent transformation purposes.

Agrobacterium tumefaciens strain EHA105 was streaked onto Luria-Bertani medium plates containing 50 µg ml^{-1} kanamycin (LBkan, Figure 6). After two days, 2-3 *Agrobacterium* colonies carrying the right plasmids were picked and inoculated in 10ml of liquid LBkan (to select against the loss of the plasmid) medium and incubated at 30 °C overnight. For the actual transformation, bacterial liquid culture was diluted to an OD_{600} 0.5 and incubated for another 90 minutes, in order to obtain the bacterial cells in their exponential growth phase and an OD_{600} of 0.8-1 for the co-incubation with fungal spores.

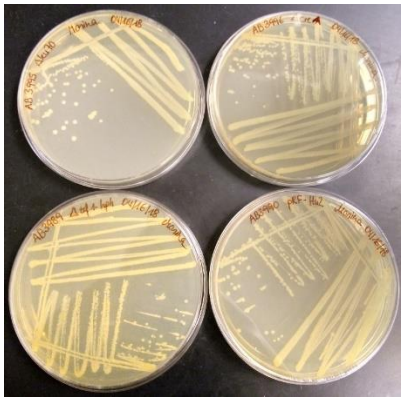

Figure 6: *Agrobacterium tumefaciens* single colonies on Luria-Betrani medium. Each plate contained bacterial cells carrying a different vector: *hph*-cassette, *creA*-KO, *clr-1* and *clr-2*-OE. After three days, single colonies were transferred to LB_{kan} containing liquid media and incubated at 30 °C and 200 rpm for 24 hours.

Marker selection for *Thermoascus aurantiacus* (ATCC 26904)

For a successful transformation, an appropriate selection marker for the determining successfully transformed mutants was chosen. Acetamide represents a nitrogen source that cannot be metabolized by many fungi, and therefore the *amds* gene can be used to isolate transformants in media containing no other nitrogen source. In a first attempt, *T. aurantiacus* was grown on a minimal medium containing acetamide as the only nitrogen source by transferring equal amounts of spores onto the minimal medium (Vogels salts, 2% sucrose, 1.5% agar and 25 mM acetamide/ammonium nitrate/no nitrogen; McClendon et al. 2012) selection plates. A nitrogen depleted medium (N^-) and one containing NH_4NO_3 medium were used as a growth reference.

Simultaneously, the resistance towards hygromycin was tested. Again, a 6-day old spore solution was prepared and equal volumes were transferred to the center of each hygromycin containing potato dextrose plate (PDA) plate. As a reference, *T. aurantiacus* growth was compared between PDA plates without hygromycin (WT hyg 0) and plates with either 10 µg ml^{-1} (WT hyg10) or 50 µg ml^{-1} (WT hyg 50) of hygromycin added (Figure 4). Finally, hygromycin was chosen as the appropriate selection marker for future transformations.

Ratio of fungal spores to bacterial cells

The early phase of protocol optimization was spent testing different concentrations of fungal spores that are being transformed and modifying the amount of *A. tumefaciens* cells that have been added during transformation.

Spores were counted using a metallized, bright-line hemacytometer (Hausser Scientific, http://hausserscientific.com/products/reichert_bright_line.html). 10 µl of a 1:100 diluted spore solution were loaded on each side of the hemacytometer and all spores within the counting grid were assessed (Figure 7). The mean of both counts was calculated and the number of spores per milliliter was calculated using following equation:

cells counted [mean] x dilution factor x 10.000 = ___ cells / ml

As the original protocol was optimized for yeast and optical density (OD) measurements were used as a basis for determining cell concentrations, different dilutions of fungal spores had to be tested to enhance transformation efficiencies for the filamentous fungus *T. aurantiacus*. Therefore, different transformation set-ups were prepared to contain either $1x10^6$, $5x10^7$ or $1x10^8$ fungal spores that were merged with by the protocol suggested amount of bacterial cells. In another

experiment, different volumes of bacterial cells (OD_{600} 0.8-1, 1 ml = 1x, 3 ml = 3x or 4.5 ml = 4.5x) were added to equal amounts of fungal spores ($1x10^8$).

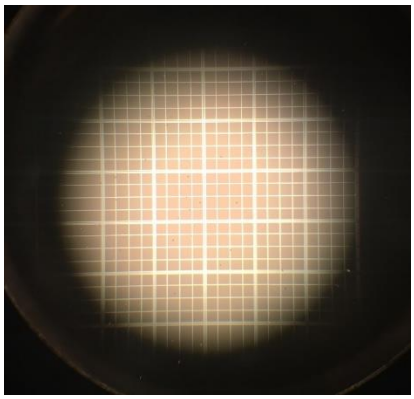

Figure 7: Bright-Line hemacytometer (Hausser Scientific): Part of counting grid for determining fungal spore concentration. 10 µl of fungal spores were counted in duplicates and the mean value was used to obtain spore numbers per ml.

Co-incubation time and temperature

Fungal spores and bacterial cells were fused together and co-incubated on a membrane according to the transformation protocol (4 days at 26 °C and another 3 days at room temperature) for *Rhodosporidium toruloides*. However, to optimize the protocol for *Thermoascus aurantiacus* both parameters, temperature and incubation time, were modified. Preliminary tests, but also related literature have suggested a much shorter incubation time for filamentous fungi. On the basis of the transformation protocol for *Aspergillus awamori* (from PNNL) and the publication of Xu et al. 2015, we tested co-incubation periods of either two or three days (Figure 8).

In a separate set-up, different temperatures for the two or three-day co-culture time were tested. Induction media plates carrying the membranes, on which the bacteria and spore mixture were applied, were co-cultured at either room temperature (20-22 °C), 26 °C or 28 °C for either two or three days, respectively.

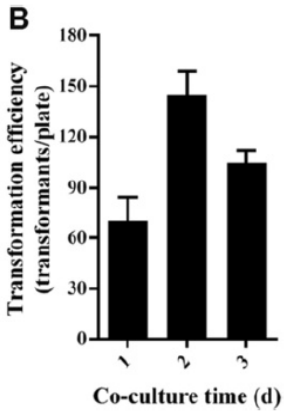

Figure 8: Bar chart from Xu et al. 2015. Number of *Myceliophthora thermophila* transformants received after co-culture time of either one, two or three days. The highest number of transformants was achieved after two days of co-incubation.

pH of induction medium

As mentioned, before *A. tumefaciens* can be applied onto the membranes containing the fungal spores, the expression of virulence genes needs to be induced by culturing bacterial cells in a medium containing the chemical compound acetosyringone (200 µM) for 24 hours prior to transformation. Subsequently, induction media agar plates are used during co-culture. To test whether the pH of the induction medium influences the amount of resulting mutants, we designed an experiment, in which we modified the pH of the induction medium with either HCl or KOH relative to the control pH of around 5.7. Different volumes of 3 M HCl and 3 M KOH were added to the medium prior to sterile filtration, to end up with a final pH of 5, 5.5 and 6. The same experiment included the control treatment, in which the pH was not modified (pH 5.7). All membranes were incubated for either two or three days at 26 °C.

Co-culture membranes

To investigate the effect of the membrane material that is used for the co-cultivation of fungi and bacteria on the number of mutants, we performed two transformations, which only differed in the membranes used for co-culture. Here we tested transformation efficiencies after co-incubation on cellulose or nylon membranes, respectively.

Verification of mutant strains

The DNA of potential mutants was isolated using the Maxwell® RSC Plant DNA Kit (Promega©). Primers for PCR reactions and sequencing were designed to bind the promotor (*tef1*) and terminator (*trpC)* region of the hygromycin cassette and the *gpd* promoter and *xlnR* terminator of the sequence of interest.

To screen for enzyme secretion of *T. aurantiacus* transformants isolates were cultured in a medium containing suitable carbon sources such as sugars, peptone, cellulose, xylan, starch and whole plant biomass (Figure 9). Shift experiments were performed to compare the protein content among mutant and wild type strains, respectively. Supernatants of these cultures were used for Bradford and DNS assays to measure protein content and enzymatic activity, respectively.

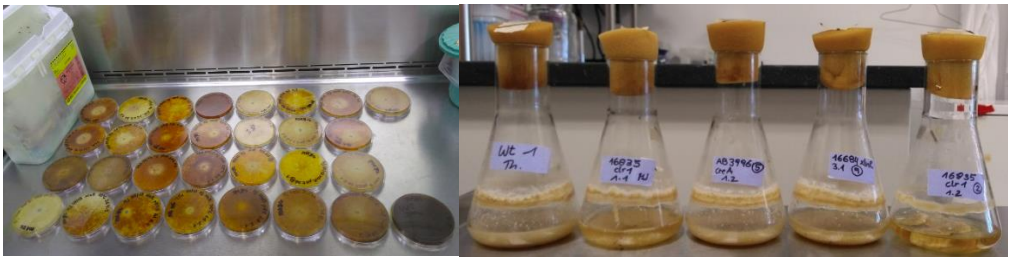

Figure 9: Screening of potential mutants. Biomass was scraped off the plates for DNA extraction and subsequent PCR verification. Spores were inoculated in 50 ml culture tubes containing glucose (1.5 %) and biomass was shifted after three days to starvation media. Supernatants of these cultures were used for Bradford Protein and DNS assays.

Results and Discussion

A selection marker for *T. aurantiacus* was chosen for future transformation experiments. Figure 10 clearly shows *T. aurantiacus* growth on minimal medium containing acetamide (25 mM) as the only nitrogen source, and thus, acetamide was ruled out as a potential selection marker for isolation of potential mutants. Thus, an alternative selection marker was used for mutant detection.

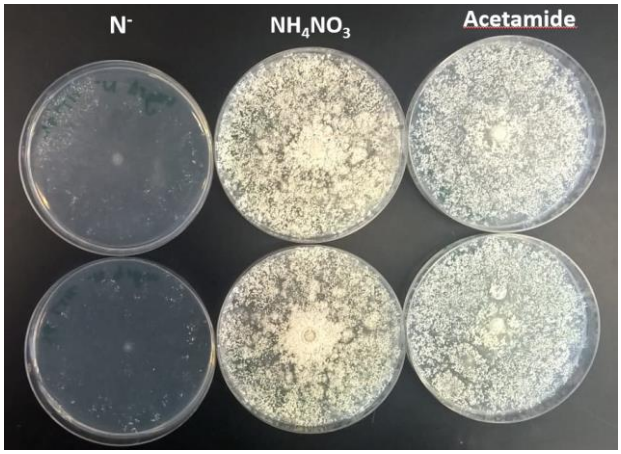

Figure 10: Experiment on acetamide sensitivity in *Thermoascus aurantiacus* (ATCC 26904). *T. auratiacus* growth after 5 days on a nitrogen depleted minimal medium (McClendon MM, McClendon et al. 2012) (N^-), a NH4NO3 containing minimal medium (NH_4NO_3) and on a acetamide medium (25 mM).

As mentioned, to determine the appropriate concentration of hygromycin for the selection of mutants, growth experiments were performed. Figure 11 shows the growth curve of *Thermoascus aurantiacus* (ATCC 26904) in the presence of different concentrations of hygromycin (0, 10, 50 µg ml^{-1}). In comparison, growth experiments were also performed with a *Δku70* strain of *T. aurantiacus* carrying a hygromycin resistance marker. Hygromycin concentrations for *Δku70* growth experiments ranged from 0 to 200 µg ml^{-1} (0, 10, 50, 100, 200 µg ml^{-1}). No growth was observed for the wild type strain at hygromycin concentrations of 50 µg ml^{-1}, whereas the *Δku70* strain could grow at hygromycin concentrations of up to 200 µg ml^{-1}. On the basis of this experiment, a final hygromycin concentration of 80 µg ml^{-1} was chosen for the selection of mutants for all transformation experiments.

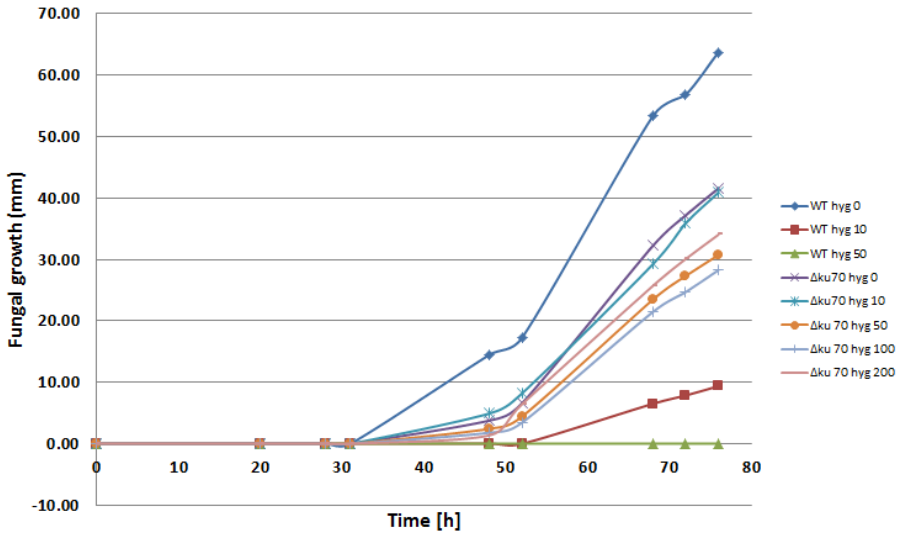

Figure 11: Growth experiments with *Thermoascus aurantiacus* (ATCC 26904) and the *Δku70* strain in the presence of different concentrations of hygromycin. Growth curves of the *wt* strain are depicted by dark blue, red and green lines (0, 10 and 50 µg ml^{-1}) and all remaining lines represent growth rates of the *Δku70* strain (0, 10, 50, 100 and 200 µg ml^{-1}). *Wt* strains show no growth above 50 µg ml^{-1}.

Previously constructed overexpression and knock-out vectors were transformed into *Agrobacterium tumefaciens* strains via electroporation and the isolated vectors were verified via multi-cut restriction enzyme digests. Correct fragment lengths were obtained for all constructs and the fragment patterns can be seen in the gel-electrophoresis picture of figure 12. All transformations experiments were performed with *A. tumefaciens* strains, in which the presence of correct plasmid vectors was verified.

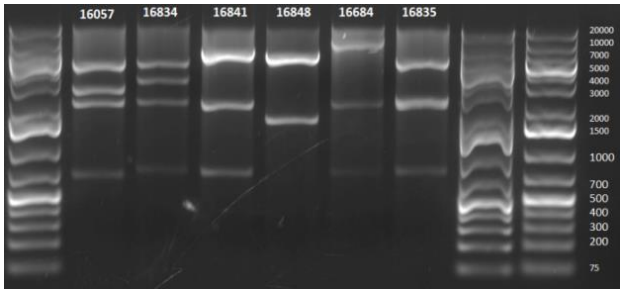

Figure 12: Gel-electrophoresis of multiple-cut restriction digest of isolated plasmid constructs out of successfully transformed *A. tumefaciens*. 16057 (not used), 16834 (*clr-2*), 16841 (*hph* only), 16684 (*xlnR*), 16835 (*clr-1*).

The following paragraph shows results of the very first transformation experiments. Initially, only very few mutants could be generated, however, on the basis of these results conditions were tested and optimized for further experiments.

Figure 13 show correct fragments lengths and sequencing results for the first successful mutant strains generated for *Thermoascus aurantiacus*. The *hph* resistance cassette could be verified via PCR and sequencing for three isolates. All three isolates are showing the correct fragment length of 800 base pairs for the *hph* gene. A fragment band was obtained for the *wild type* using primers for the *hph* gene, however, it clearly shows a smaller size and thus, might have been generated due to internal homologies with the designed primers. The hygromycin resistant *Δku70* strain was used as the positive control for this PCR and showed a clear fragment at 800 base pairs. At this point, no successful integration of the *clr-2* overexpression cassette could be verified, however, two *h1-gfp* strains were generated by this experiment (data not shown). Out of three mutants that were obtained during this experiment, two came from treatments, in which we used 3 times the suggested concentration of *A. tumefaciens* cells. Therefore, all following experiments were performed with enhanced *A. tumefaciens* concentrations, but also with a spore concentration of 10^8. The ratio of bacterial cells to fungal spores is considered to be one of the most critical steps for the successful and efficient transformation of filamentous fungi and thus, for each species has to be determined empirically (Michielse et al. 2008).

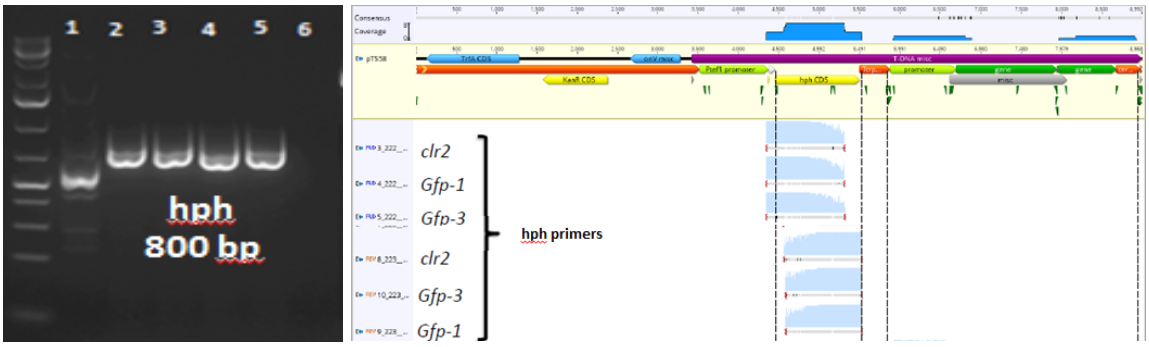

Figure 13: Verification of the first *Thermoascus aurantiacus* mutants. Gel electrophoresis shows the successful integration of *hph* cassette (1= Wild type; 2= *Δku70*; 3= h_1-*gfp*_1x, 4= h_1-*gfp*_3x, 5= *clr*-2_3x) in three mutants and sequencing results showing successful integration of the *hph* gene.

The following transformation was performed to test whether differences in the numbers of mutants could be obtained by using different membranes for the co-cultivation of *Agrobacterium* cells and *T. aurantiacus* spores. The co-incubation on nylon filters produced no transformants, therefore we

continued to use cellulose membranes for future experiments. Six mutants successfully integrated the *hph* gene, whereas two of them (mutant 3 and 4, see figure 14) also incorporated the *clr-1* overexpression cassette into their genome. One potential *ΔcreA* strain was generated and to check whether mutants show an enhanced enzyme secretion, we performed a Bradford Protein assay (Figure 15). Results of the Bradford Protein assay can be seen in figure 15, which shows the protein concentration in µg ml^{-1} of the supernatants of potential *T. aurantiacus* mutants in comparison to the wild type control. The wild type was cultured in triplicates and the mean value and standard deviation of all three supernatant protein concentrations is depicted in Figure 15. For this measurement we also included mutants, which only had the *hph* gene included to be able to make conclusions on the fitness costs that comes along with the transformation.

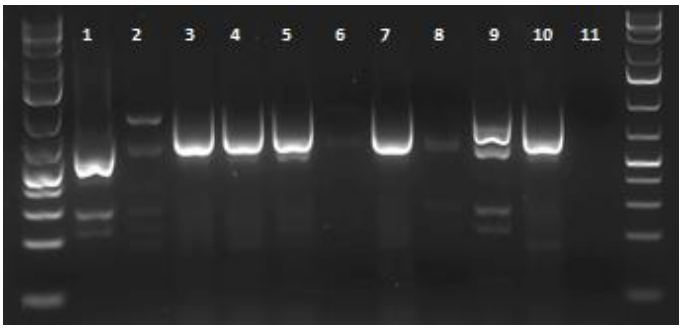

Figure 14: PCR verification of *T.aurantiacus* mutants. Gel electrophoresis showing the amplification of the *hph* gene (1= Wild type; 2= *clr-1*; 3= *clr-1*; 4= *clr-1*; 5= *creA*; 6= *creA*; 7= *hph* only; 8= *hph* only; 9= *hph* only; 10= *hph* only; 11= negative control). Bands at position 3, 4, 5, 7 and 10 show a correct band size of around 800 base pairs.

Compared to the wild type all generated mutants showed a reduced performance in terms of protein secretion. Especially, successfully integrated *clr-1* overexpression mutants showed a reduced growth and did not exhibit the desired phenotype. A reduced protein content could also be shown for the mutants that only received the *hph* gene and can be explained by the fact that random integration might interfere with growth relevant genes. Multiple copies of the *hph* gene can integrate at different positions of the *T. aurantiacus* genome and thereby affect growth relevant genes. For the *creA* knock outs homologous flanks of around 1000 bp were designed up- and downstream of the *hph* gene to promote targeted integrations by the homologous recombination DNA repair pathway. However, in *T. aurantiacus* the non-homologous end joining repair pathway occurs to a significantly higher extent and thus, a high number of *creA* mutants needs to be

screened for the correct phenotype. All other strains most likely contain the hygromycin cassette at random positions in the genome.

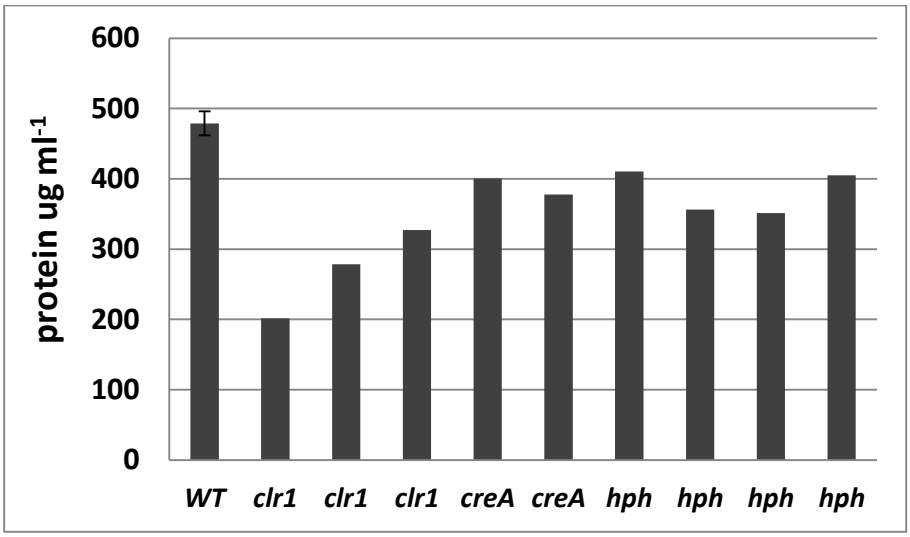

Figure 15: Bradford Protein assay. Protein content of *T. aurantiacus* supernatants after 3 days of growth in minimal medium in µg ml^{-1}. The wild type was measured in triplicates.

As mentioned, following experiments included variations in the temperature during co-incubation and variations in the pH of the induction medium. Both parameters were modified in separated experiments and results can be seen in figure 16 and figure 17. To ensure a high degree of comparability between treatments, all remaining parameters were kept equal and thus, both transformation attempts were performed with the same strains of *A. tumefaciens* carrying the *xlnR* overexpression cassettes as well as the same set of *T. aurantiacus* spores derived from the same culture plates. For pH modification treatments the highest number of transformants was achieved at a pH of 5 with up to 11 isolates, representing the highest number of colonies compared to all other transformation attempts. All remaining treatments (pH 5.5, 6 and the control 5.7) resulted in lower amounts of transformed colonies relative to the pH 5 treatment.

In addition to pH variations, two different incubation periods were chosen (two and three days). Overall, the incubation period of three days resulted in a slightly enhanced number of colonies within the pH 5 treatment as compared to a two-day incubation. However, for the remaining treatments no such trend could be observed and therefore, the less time consuming experimental design of two day-incubation was chosen for future transformation experiments.

Looking at the different co-incubation temperatures in figure 16, we observed the highest amount of *xlnR* mutants at a temperature of 28 °C (up to four per plate). Room temperature and 26 °C

treatments produced no colonies during the three-day co-incubation treatment and generally lower colony numbers than treatments in which *A. tumefaciens* cells and fungal spores were incubated for two days. Compared to other transformation methods (e.g. protoplast transformation), *A. tumefaciens*- mediated transformation protocols require all conditions to be "optimal" for both organisms that are involved (*A. tumefaciens* and the respective fungus). A shift of incubation temperature towards 28 °C compared to the original protocol favors the thermophile *T. aurantiacus*, while not interfering with the transformation capabilities of *A. tumefaciens*.

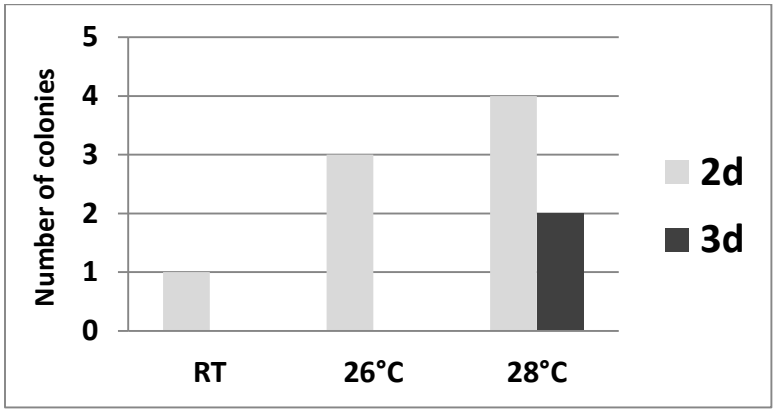

Figure 16: Variations in co-cultivation temperature conditions (RT= room temperature, 26 °C and 28 °C) and its effect on transformation efficiency (= number of colonies). Light grey bars indicate the number of colonies after two days and dark grey bars after three days of co-incubation.

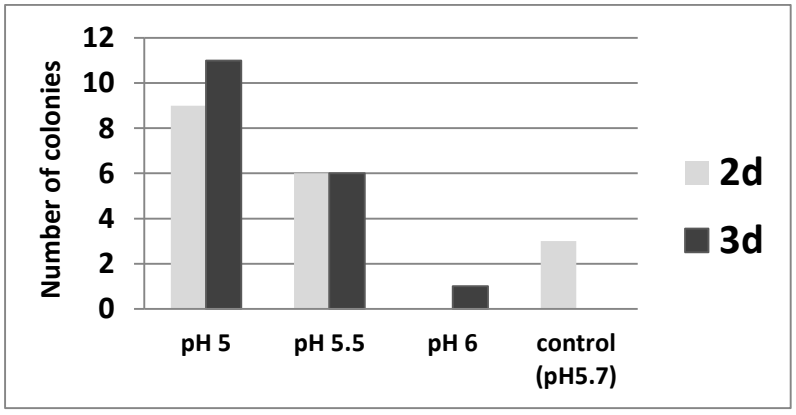

Figure 17: Variations in pH conditions of the induction medium (pH 5, pH 5.5, pH 6 and control pH 5.7) and its effect on transformation efficiency (= number of colonies). Light grey bars indicate the number of colonies after two days and dark grey bars after three days of co-incubation.

Once again, a PCR was performed to check for the insertion of the *hph* gene (800 bp) and the *xlnR* overexpression cassette (3600 bp) for strains taRG014 to taRG029, see figure 18. Out of them isolates were successfully transformed with the *hph* gene and nine of them contained the over-

expression cassette in their genomes. To check for the desired phenotype, a preliminary screen was performed and the xylanase activity and protein content of the supernatants was measured and compared relative to the wild type (Figure 19). The xylanase activity of most of the tested mutant strains is significantly higher than for the wild type (% relative of *WT*). However, the overall protein concentration mostly stayed within levels comparable to the wild type or slightly exceeded them (% relative of *WT*). Out of these results, the best four mutant strains were taken (taRG028, taRG023, taRG020, taRG005) and a shift experiment was performed with culture triplicates. After, three days in a 1.5 % glucose medium, equal biomass amounts of each mutant strains were transferred to starvation media for another 2-3 days. The results of the xylanase activity and protein content measurements are depicted in Figure 19. *XlnR* mutant strain taRG020 showed a xylanase activity of up to 500 % higher than the wild type and all the remaining strains also showed increased activity values between 100 and 200 % more relative to the wild type. For all tested mutants the supernatant protein content did not significantly differ from the overall protein in the wild type supernatants (Figure 19).

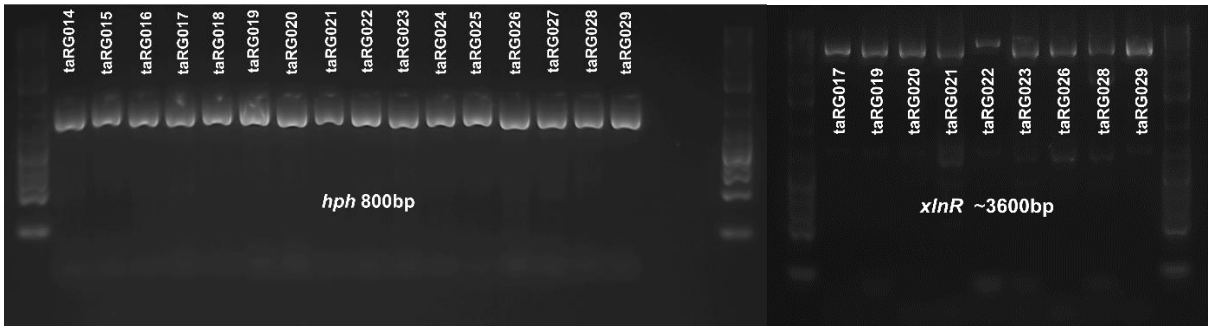

Figure 18: PCR verification of *T. aurantiacus* mutants. Gel electrophoresis showing the amplification of the *hph* gene (800 bp) and the *xlnR* overexpression cassette (3600 bp).

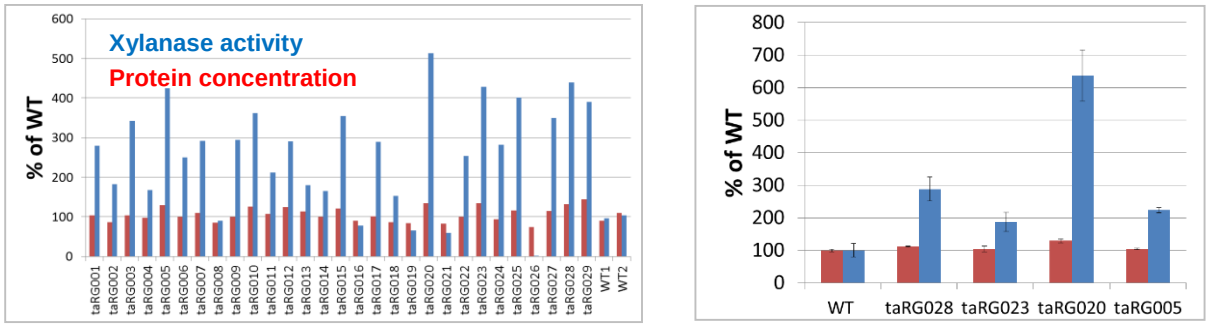

Figure 19: Xylanase activity and protein content of *T. aurantiacus* mutant culture supernatants. On the left *xlnR* transformants screen for xylanase activity and protein secretion and on the right shift experiment with culture triplicates with the four best strains discovered in the preliminary screen.

First attempts of *Agrobacterium tumefaciens*- mediated transformation for filamentous fungi have been described 1998 by de Groot et al. and ever since then are a commonly used tool that allow an efficient transfer of genetic material between prokaryotic and eukaryotic cells. In some cases, *Agrobacterium tumefaciens*-mediated transfomations show even higher success rates than direct DNA transfers (de Boer et al. 2013). Especially for *Thermoascus aurantiacus*, where common approaches, such as protoplast transformation, failed to produce mutants, *Agrobacterium tumefaciens*-mediated transformation is an easy and efficient alternative. For the optimization of the *Thermoascus aurantiacus* transformation protocol, co-incubation temperature and induction medium pH had the most pronounced effect on transformation efficiency, whereas the increase in co-culture time to three days was not affecting the number of colonies significantly. Interestingly, 48 hours of co-incubation was shown to be the optimal co-incubation time for fungal spores and bacterial cells resulting in the maximum number of generated colonies in many different fungal species (Mullins et al. 2001; Rho et al. 2001; Yang et al. 2015). However, this factor should always be empirically tested if applied to another species of fungi.

The random integration, but also different copy numbers of the overexpressed genes led to variations in spore production, but also to differences in pigmentation of isolated mutants when compared to the wild type. Random integrations usually represent a problem when affecting growth-relevant genes resulting in unexpected phenotypes, however, this study represents a first step to test gene expression and to establish *T. aurantiacus* as a new platform for the production of thermostable cellulases.

Conclusion

A first step towards the development of *Thermoascus aurantiacus* as a new platform for the production of industrially-relevant plant biomass degrading enzymes has been made and an efficient and reproducible protocol for genetic engineering in this particular thermophilic filamentous fungus was established.

During protocol development, the modification of co-incubation temperature and the pH of the induction medium resulted in the highest increase of transformant colonies and thus, were essential factors to consider for the establishment of a first genetic engineering protocol for *Thermoascus*

aurantiacus. In addition, co-culture membranes, but also the ratio of fungal spores to *A. tumefaciens* cells were sensitive parameters determining the transformation success in this study.
Additionally, we now have a first insight into the genetic regulation of plant biomass degrading enzymes in *Thermoascus aurantiacus*, with *xlnR* being a highly relevant transcriptional activator for the up-regulation of xylanase activity as previously reported for other filamentous fungi. The xylanase activity was increased by up to 500 % for mutant strains relative to the wild type showing the great potential for this species for industrial applications within this sector. The overexpression of *clr-1*, however, did not yet fully reveal its function within the CAZyme regulation in *T. aurantiacus*. The few strains generated by this study showed growth deficits relative to the wild type and thus, further overexpression, but also *clr-1* knock out strains will be necessary to elucidate its exact roles in *T. aurantiacus* enzyme secretion. These different phenotypes, however, might be a result of the random integration of the overexpressed genes, which might integrate at positions relevant for fungal growth and development. We observed differences in spore production and pigmentation of *T. aurantiacus* isolates, which is another very promising transcriptional activator, *clr-2*, could not yet be genetically engineered for this fungus, however, further over-expressions and knock-outs will be targeted within future experiments.
Conclusively, this study was able to generate the first protocol for genetic engineering and giving preliminary data on genetic CAZyme regulation in *Thermoascus aurantiacus*. However, further experiments and genetic modifications will be necessary to establish *Thermoascus aurantiacus* as a genetic model system and produce industrially relevant production strains.

Outlook

As mentioned, with this work provides the first step towards the development of *Thermoascus aurantiacus* as an industrially relevant strain for cellulase production. With a reproducible transformation protocol being established, genetic engineering can be easily performed and further targets for an enhancement of plant biomass degrading enzymes can be modified. Combining all optimized conditions in each experiment (optimal co-incubation temperature and pH of induction medium), will most likely result in even higher numbers of transformants and higher transformation efficiencies.
CreA knock out strains are currently being screened for the desired phenotype to elucidate the role of this gene in the CAZyme metabolism. In general, knock out cassettes for the transcriptional

activators would help to shed light into their exact role within this particular fungal species and will result in a better understanding of genetic regulation of cellulases and xylanases in *T. aurantiacus*. Meanwhile, also other sets of genes will be examined such as for example Lytic Polysaccharide Monooxygenases (LPMO), which are known to promote the degradation by destabilizing complex cellulose polymers and increasing their accessibility for other relevant plant biomass degrading enzymes.

Further experiments aim to produce a *creA* knock out strain carrying a different resistance marker, such as for example nourseothricin. This will allow the generation of a *ΔcreaA* X *xlnR* strain by sexual crossing and thus, potentially lead to a hypersecretion strain possibly relevant for industrial purposes. A successful protocol for sexual crossing in *T. aurantiacus* is already established.

Developing the CRISPR/Cas9 technology within this organism will additionally enable easy and efficient engineering. This will also enable targeted deletions within *T. aurantiacus* and provide a better insight into genetic regulation of enzyme secretion.

Further experiments and genetic modifications will be necessary to establish *Thermoascus aurantiacus* as a genetic model system and produce industrially relevant production strains.

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I would like to thank Steven Singer for kindly accepting me in his lab and helping me with all necessary organizational steps for my stay at the Joint BioEnergy Institute. I learned a lot during our lab meetings, felt included into the scientific community from the beginning on and really enjoyed being a member of his research team.

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