Uncovering the determinants of cellulase hyperproduction in *Thermoascus aurantiacus* using omics methods

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Abstract

Enzymes from cellulolytic fungi are excellent converters of plant biomass derived polysaccharides to simple sugars. Employing enzymes from the thermophilic ascomycete fungus *Thermoascus aurantiacus* under elevated processing temperature would confer several advantages such as: increased reaction rates, decreased contamination risk, utilization of waste heat, lowering viscosity at high solids loading and overcoming end-product inhibition. The goal of the internship was to quantify the increase in cellulase and xylanase transcript gene expression in *T. aurantiacus* and determine the factors that promote high levels of transcription and protein production.

The growth physiology of *T. aurantiacus* was investigated regarding temperature, pH, the selection markers Phleomycin and Basta, and ascospore production and spore germination kinetics were investigated. The culture medium for *T. aurantiacus* was optimized in regard to mineral salts, N source and C source. A fermenter run at 2L scale was employed to gain valuable knowledge of cultivating this fungus in bioreactors. Moreover, mutant strains were generated with UV and EMS mutagenesis and screened for enhanced cellulase production. Transcript sequencing was performed on a Illumina MiSeq platform with a custom RNA-seq pipeline at JBEI. Additional enzyme and protein quantification assays revealed the composition and activity of crude enzyme mixes, secreted by the fungus when cultured with inducing carbon sources.

Future work with random and targeted mutagenesis is intended to establish cellulase hyperproducing and carbon catabolite repression insensitive de-repressed strains and establish a sexual crossing protocol. Additionally, further fermenter runs will employ the knowledge generated in this project to successfully culture at 2L scale and further increase protein titers at this scale.

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1. Introduction

1.1. Scientific background and relevance

Due to various risks of relying on fossil fuels as an energy source, there is an increasing need to find renewable, carbon neutral and sustainable alternatives. Cellulosic plant biomass is a promising candidate accordingly. Its enzymatic breakdown to simple sugars and their conversion to fuels through microbes offers a cost efficient and scalable opportunity for biofuel production. The objective of this project is to develop a thermophilic fungal platform for the industrial production of cellulase enzymes. Using thermostable cellulase mixtures has several advantages, such as increased reaction rates, lowering the contamination risk, utilization of waste heat, lowering viscosity at high solids loading and overcoming end-product inhibition. The ascomycete fungus *Trichoderma reesei* (*Hypocrea jecorina*) was initially recognized as a potent cellulase producer[1]. The development of certain strains, which produce up to 50-100 g/L of cellulase enzymes has enabled the establishment of the cellulosic ethanol industry[2]. Current efforts focus on the improvement of thermostable cellulase production[3].

An alternative approach is to develop thermophilic fungi as platforms for cellulase production[4,5]. Cellulytic thermophilic fungi are known for more than a century. However their potential for industrial plant biomass deconstruction has been investigated rather recently. Genome analysis of *Thielavia terrestris*, *Myceliophthora thermophila* and *Rasamsonia emersonii* revealed the potential of these fungi for commercial cellulase production. The *M. thermophila* C1 strain has been successfully engineered into commercial cellulase producer[6].

Another promising fungus for plant biomass deconstruction is the ascomycete *Thermoascus aurantiacus*, which belongs to the order of the *Eurotiales*. It was first isolated in 1907 from self-heating hay[7]. Later on, additional strains were isolated from other habitats such as soil, compost, bark, wood chips and peanuts all over the world. The species was assigned to the new genus *Thermoascus* (thermos [greek] = hot, ascus [greek] = tube) and species *aurantiacus* (lat., orange) due to the color of the fungal fruiting bodies (Figure 1.1. A). The growth optimum was determined to be at 50°C with no growth happening below 30°C. The fruiting bodies consist of cleistothecia, which cover the eight ascospores containing asci. *T. aurantiacus* produces large amounts of these ascospores, which seem to be the primary form of propagation, while the majority of molds rely on asexual conidiospores. Cystoid structures were also discovered in *T. aurantiacus*. These were

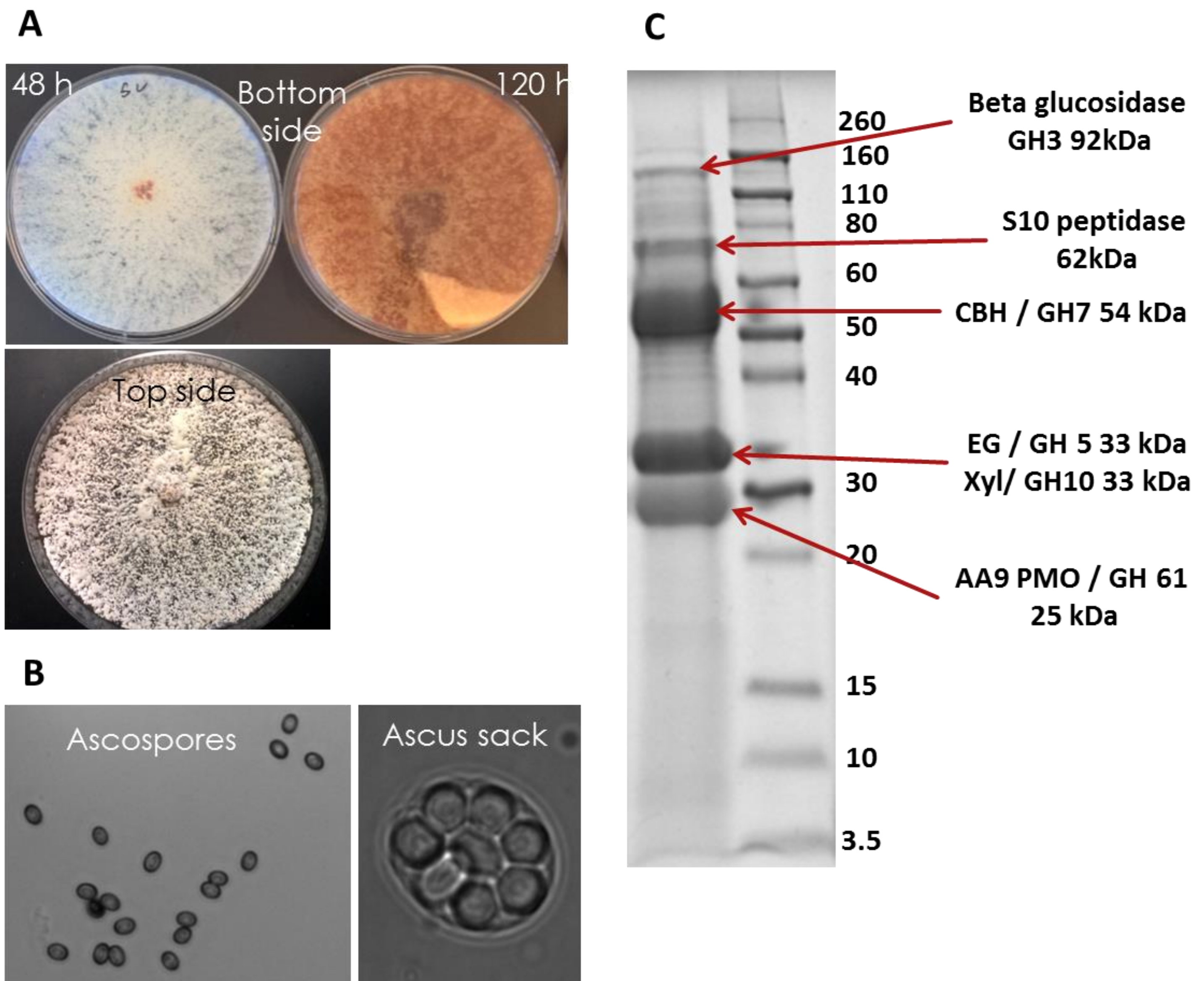


Figure 1.1: (A) Development of *T. aurantiacus* on PDA plates. (B) Ascospores free (left, 40x air objective) and contained in an ascus sack (right, 63x oil objective). (C) SDS PAGE with major cellulase bands. Medium: mycelium incubated in beech wood xylan, soy meal peptone and McClendon`s salts for 96h (15 ul loaded). CBH = cellobiohydrolase, EG = endoglucanase, Xyl = xylanase, PMO = lytic polysaccharide monooxygenase.

considered to be chlamydospores, an asexual propagation structure. We were also able to observe these structures during our experiments. However, germination of these chlamydospores has never been observed.

Previous work with the wild type strain ATCC 26904 revealed that it produces high levels of cellulases (> 1g/l). It secretes a remarkably simple cellulase mixture, consisting of four primary cellulases: (beta-glucosidase (GH3), ~92 kDa; cellobiohydrolase (GH7), ~54 kDa; endoglucanase (GH5), ~33 kDa; polysaccharide monooxygenase (GH61), ~25kDa A xylanase (GH10) was also

identified that overlapped with the GH5 protein at ~33 kDa) (Figure 1.1. C). The cellulases are still active at high temperatures (70°C) compared to a commercial enzyme mix (CTec2, Novozymes)[8]. Moreover, *T. aurantiacus* has a high xylanase activity and a remarkably fast growth speed compared to other thermophilic fungi. These features make strain optimization and improving culture conditions of this fungus for plant biomass saccharification a promising avenue.

1.2. Project goal and strategy

1.2.1. Project goal

In this project, strain improvement of *T. aurantiacus* by random and targeted mutagenesis will be combined with optimized cultivation conditions to hyperproduce cellulase enzymes from *T. aurantiacus.* Broths from these *T. aurantiacus* cultivations will be used to hydrolyze pretreated corn stover (ionic liquid, alkaline and acid pretreatment) at 5-10% biomass loading at 50-65°C to establish yields and rates of glucose release. The overarching goal is to establish a *T. aurantiacus* strain that produces >20 g/L of protein and a cellulase mixture capable of releasing >80% glucose from acid-pretreated corn stover at 65°C. This project is a coordinated effort of the Lawrence Berkley National Lab (LBNL) and the Fungal Biotechnology Group at Pacific Northwest National Laboratory (PNNL).

1.2.2. Goal of the internship

The goal of the internship was to determine the factors that promote high levels of cellulase gene transcription expression and protein production in *T. aurantiacus* ATCC 26904. Transcriptomics under cellulase hyperproducing conditions greatly facilitates the identification of candidate genes for sexual crossings and targeted mutagenesis to increase enzyme production. Transcript sequencing was performed on a MiSeq platform with a custom RNA-seq pipeline at JBEI. Additional enzyme assays revealed the composition and activity of enzyme mixes secreted by the fungus.

1.2.3. Strategy

Strategy 1: Exploring the growth physiology of *T. aurantiacus* and media optimization

In various plate and shake flask assays, the growth response of *T. aurantiacus* towards temperature, pH, commonly used selection markers, mineral salt solutions and nitrogen sources was determined. Special emphasize was also put on the identification of cellulase inducers in

shake flask shift experiments. Moreover, the spore germination kinetics and ascospore production was studied, since inoculation of liquid media with spores turned out to be superior compared to inoculation with agar plugs.

Strategy 2: Strain Optimization of *T. aurantiacus* for cellulase hyperproduction

Chemical and UV mutagenesis was employed to create strains secreting higher levels of cellulase on cellulose-agar plates and to detect strains in the presence 2-deoxy-glucose, which display no down regulation of cellulase production in the face of a catabolite repressor[9,10].

Strategy 3: Pilot-scale enzyme production from *T. aurantiacus*

One optimized strain from strategy 2 and the wild type (WT) strain were cultivated and monitored in bioreactors at the 2L scale. Scale up to 20L and 300L bioreactors will be dependent on obtaining hyperproduction strains and test if hyperproduction of enzymes is reproducible at this scale and if the proportions of individual enzymes are consistent at bench and pilot scales. Parallel techno economic modelling will evaluate the impact of cellulase hyperproduction by *T. aurantiacus* in a biorefinery.

Strategy 4: Differential gene expression analysis under cellulase inducing conditions

Complementary to the growth and activity assays of strategy 1 to 3, an RNA-seq test run was included to investigate the transcriptional network and its activation with the cellulase and xylanase inducer beech wood xylan. This analysis provides additional information on the temporal gene expression profile under cellulase gene inducing conditions to pinpoint the most interesting sampling time points. Based on this information, further RNA-seq runs with different inducers will reveal promotors and regulators that may be targeted via genetic engineering. The Illumina transcript libraries were prepared and subsequently sequenced on a MiSeq platform at the Joint BioEnergy Institute and analyzed using a custom pipeline for RNA-seq analysis developed at JBEI. The transcript analysis will be supported by enzyme assays using purified substrates and saccharifications performed on pretreated corn stover.

2. Material and Methods

2.1. Strain and culture maintenance SOP

In all experiments, *T. aurantiacus* strain ATCC 26904 was employed and cultured on PDA plates. Wild type and mutant cultures of this strain were first incubated for 48h at 50°C and were then transferred to 45°C. This trade off combines the faster growth at 50°C (Figure 3.1) with reduced drying at 45°C for longer storage. All plates were covered with glass ware at both temperatures to reduce drying. Additional water filled plastic containers in the incubators kept the atmosphere moist.

2.2. Spore isolation and counting SOP

A total of 5ml autoclaved water was poured on a plate and the spores were scraped off with a cell spreader. The suspension was added to a 15 ml tube and the volume adjusted to 5ml. The suspension was filtered with P1000 tips, containing cheesecloth or miracloth. A suitable dilution (usually 1:100) was generated. Spores are counted with a counting chamber. Spores were spread with autoclaved glass beads by adding 300ul of a 2 * 10^7 spores/ml concentrated stock, which yield a sufficient density. Brightfield pictures were taken with a Leica-DM4000B microscope using a 40x air objective. No coverslip was placed on the agar, since spores and hyphae can be better distinguished from another that way (Figure 2.1.).

2.3. Shift experiments with shake flasks SOP

TA was grown in 250 ml baffled shake flasks with 50ml medium on a rotary shaker. The cultivation was performed at 50°C and 180 rpm. All flasks were sealed with foam stoppers to prevent excessive evaporation. We used 6 – 8 days old ascospores at a concentration of 10^6/ml as an inoculum if not noted otherwise. This yields more homogenous cultures compared to using agar plugs. All cultures were grown as a pre-culture in glucose and shifted to a new flask containing carbon sources of interest or no carbon source after 48 hours. The mycelium was filtered with glass fiber filters and once rinsed with roughly 50 ml 1x McClendon salts to get rid of residual glucose. The entire pellet was shifted to the new medium and incubated for further 4 – 6 days.

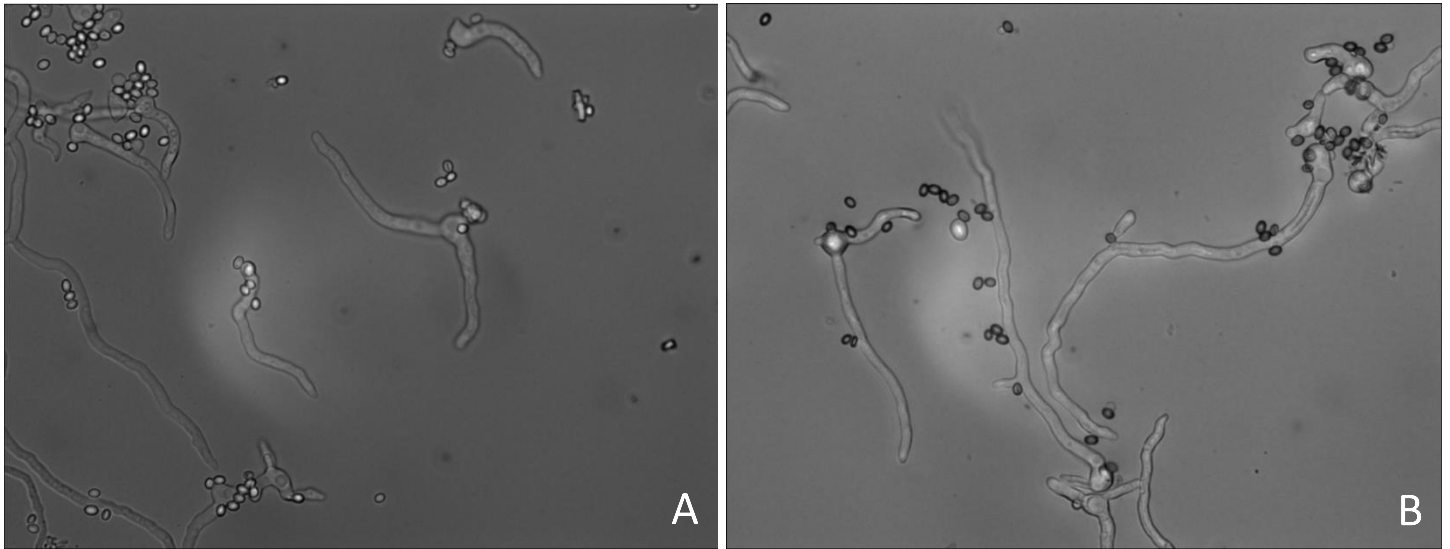


Figure 2.1: Image quality for counting germinating versus non-germinating spores (A) with coverslip and (B) without coverslip.

2.4. The effect of temperature on growth speed

Mycelium from a 6 day old plate was excised with an inverted P1000 tip and placed in the center of 4 PDA plates. Two plates each were incubated at 45°C and 50°C, respectively, for 2 days. The growth was measured with an electronic caliper.

2.5. The pH optimum

The experimental set-up was planned according to a pH study with different filamentous fungi[11]. Dextrose plates or liquid cultures were set up to three pH: 4.0, 5.5, 7.0 (33 mM phosphate buffer each) and an unbuffered control at pH 5.0 (3 mM Phosphate buffer). Liquid media were inoculated with spores according to our SOP (chapter 2.3.). Plates were inoculated with an inverted P1000 tip excised agar plug from a PDA plate.

Plates (duplicates)**:** 2% glucose, 1.5% Agar, 33mM PB, 1x McClendon salts (old trace elements), 0.8% Fluka soy meal peptone (SMP)

Liquid media (triplicates): 50ml, 2% glucose, 1x McClendon salts (new trace elements), 0.8% Fluka SMP

On the plates, the growth front was used as an indicator for pH sensitivity. For liquid media, the total biomass weight (submersed mycelium + mycelium ring) was used. After 4 days, the

whole cultures consisting of floating mycelium and the mycelium ring at the liquid front were filtered on previously weighed Whatman filter paper and once washed with sterile water to flush out salts and potential residual sugars. The filters were dried for 48h and weighed.

2.6. Basta and Phleomycin sensitivity

Basta plates (duplicates): 2 % Dextrose, 1x Vogel salts solution pH 5.0 without 25mM Ammonia nitrate, 1.5% ultra-pure Agar.
Phleomycin plates (singles): 2% glucose, 1x Vogels salts with 25mM Ammonia nitrate, 1.5% Agar and pH set to 8.

We added Basta and Phleomycin after cooling the agar sufficiently down to be sure to not denature it. The Basta / Phleomycin was first added to 50 ml tubes and afterwards, 25ml of agar was added. After mixing, the agar was poured into plates and 5 ul *T. aurantiacus* spores were used as an inoculum in the center of the plate. The front of hyphae was always monitored and the diameter measured periodically to determine the sensitivity.

2.7. Spore germination kinetics and ascospore production

PDA plates were inoculated with agar plugs and incubated for 2 days at 50°C. Thereafter, the plates were shifted to 45°C. Three plates were taken for spore isolation daily, for 3 days after until 8 days after inoculation. The spores were isolated according to our spore isolation and counting SOP (chapter 2.2). A total spore count was calculated. Spores from each isolated plate were spread on a new plate as described and counted after 16h incubation at 45°C. The germination rate was calculated via counting germinated versus non germinated spores with ImageJ. A minimum of 385 spores was counted for each day, except for day 3, where almost no spores were present.

2.8. The effect of spore density on mycelium morphology

Liquid cultures in 250ml shake flasks were inoculated with different amounts of ascospores to investigate the amount and morphology of propagules formed in the medium. The medium consisted of 2% glucose, 0.8% Fluka soy meal peptone and 1x McClendon salts (pH 5.0). The medium was inoculated with ascospores from 6 day old *T. aurantiacus* plate. Different dilutions of spores were established: 10^1 / 10^2 / 10^3 / 10^4 / 10^5 / 10^6 / 10^7 spores per ml. The cultures were incubated at 50°C and 180 rpm.

2.9. Mineral salt solution and nitrogen source

In order to find the optimal medium for TA cultivation and the optimal N source, we cultivated *T. aurantiacus* in McClendon`s and Vogel`s with soy meal peptone (SMP) or ammonia nitrate. For this purpose, a 50x Vogel`s stock was prepared without ammonia nitrate. The N source was added separately. Mineral salts were diluted to 1x and media were set up with 1 % glucose and either 0.8% SMP or 25mM ammonia nitrate. All media were inoculated according to our SOP (2.3) and incubated for 4 days. All cultures were filtered and dried (Figure 3.6. B).

2.10. Optimizing Medium composition: New McClendon`s salts trace elements

In order to test whether an old McClendon`s trace element stock with precipitates or a freshly prepared one yield higher protein titers, we tested both in a shift experiment with beech wood xylan.

Pre-culture: 2% glucose, 0.8% SMP and the respective McClendon salts. The inoculation and shifting happened according to our shift experiment SOP (chapter 2.3).

Shift medium: 2% xylan, 0.8% SMP and the respective McClendon salts.

The sample filtering was done as described in the shift experiment SOP (chapter 2.3). For protein quantification, the Bradford assay was performed (Biorad®) according to the Bradford assay protocol.

2.11. Optimizing Medium composition: Fluka versus Amresco soy meal peptone

Triplicate media were set up to evaluate which soy peptone promotes higher cellulase titers. This shake flask shift experiment was done according to our SOP (chapter 2.3.).

Pre-culture: 2% glucose, 1x McClendon (new trace elements) pH = 5.0., 0.8% SMP (either Fluka or Amresco)

Shift medium: 2% beech wood xylan, 1x McClendon (new trace elements) pH = 5.0., 0.8% SMP (either Fluka or Amresco). Shift cultures were incubated for 96h.

The sample filtering was done as described in the shift experiment SOP (chapter 2.3). For protein quantification, the Bradford assay was performed (Biorad®) according to the Bradford assay protocol. On the SDS PAGE, 15 ul supernatant and 4x loading dye (Biorad®) was applied (= virtually 11.25 ul pure supernatant) and 5 ul Novex sharp prestained marker (Thermo Fischer).

2.12. Inducers of Cellulase secretion

Shift experiment (xylan, Avicel, glucose and no carbon)

Duplicate media were set up to evaluate which inducer (beech wood xylan, Avicel compared to glucose and no carbon) promotes higher cellulase titers. This shake flask shift experiment was done according to our SOP (chapter 2.3.).

Pre-culture: 1% glucose, 1x McClendon (old trace elements) pH = 5.0., 0.8% SMP

Shift medium: 1% beech wood xylan, 1x McClendon (old trace elements) pH = 5.0., 0.8% SMP (either Fluka or Amresco). Shift cultures were incubated for 75h. A time course of samples was taken twice a day starting at 19 hours after shifting.

The sample filtering was done as described in the shift experiment SOP (chapter 2.3). For protein profiling, a SDS PAGE was performed. Accordingly, 10 ul supernatant and 4x loading dye (Biorad®) was applied (= virtually 7.75 ul pure supernatant) and 5 ul Novex sharp prestained marker (Thermo Fischer).

2.13. Flask experiments without shifting

In order to save time, these above mentioned inducers were evaluated by cultivating *T. aurantiacus* together with glucose and the inducer in the same new trace elements) pH = 5.0., 0.8% SMP and 0.1% xylose, xylobiose or MXP; or 1% arabinoxylan or xyloglucan.

The sample filtering was done as described in the shift experiment SOP (chapter 2.3). For protein quantification, the Bradford assay was performed (Biorad®) according to the Bradford assay protocol. On the SDS PAGE, 15 ul supernatant and 4x loading dye (Biorad®) was applied (= virtually 11.25 ul pure supernatant) and 5 ul Novex sharp prestained marker (Thermo Fischer).

2.14. Shake flask shift experiment with optimized medium

Triplicate media were set up to evaluate which inducer (beech wood xylan, Avicel compared to 2 and 3% glucose and no carbon) promotes higher cellulase titers. This shake flask shift experiment was done according to our SOP (chapter 2.3.).

Pre-culture: 2% glucose, 1x McClendon (new trace elements) pH = 5.25, 0.8% SMP (Fluka)

Shift medium: One of the following C source: 2% beech wood xylan; 2% Avicel, 2% glucose; 3 % glucose; no carbon 1x McClendon (new trace elements) pH = 5.25., 0.8% SMP (Fluka).

The sample filtering was done as described in the shift experiment SOP (chapter 2.3). For protein quantification, the Bradford assay was performed (Biorad®) according to the Bradford assay protocol. On the SDS PAGE, 7 ul supernatant and 4x loading dye (Biorad®) was applied (= virtually 5.25ul pure supernatant) and 5 ul Novex sharp prestained marker (Thermo Fischer). For the xylanase assay, the supernatants were diluted 1:40 and 5ul were used according to our group protocol (Appendix).

2.15. Survival 2-deoxy-glucose sensitivity of **T. aurantiacus**

Ascospores were harvested according to our spore isolations SOP (chapter 2.2.). These spores were plated on plates with 2-deoxy-glucose (DOG). Glucose-DOG plates: 2% glucose and 1x Vogel`s salts with 25mM ammonia nitrate and DOG (0.2% - 0.001% and a control). CMC – DOG plates: 2% CMC, 1x Vogel`s salts with 25mM ammonia nitrate and DOG (0.2% - 0.001% and a control). The plates were incubated for 16h at 50°C. The microscopy, spore counting and calculation of the germination rate was done as described in chapter 2.3.

2.16. Survival rate of TA ascospores after EMS and UV treatment

UV treatment

All UV mutagenesis treatments were performed on our fisher scientific fb-uvxl-1000 UV cross linker. The default settings are irradiation with 265nm and 120 mJ/cm^2. The isolated spores were diluted with 4 parts water and placed in the crosslinked in a standard petri dish or a smaller petri dish for 20 seconds. The spores were plated on 12 x 12 cm 2% CMC and 0.001% DOG plates.

EMS treatment

Isolated spores were mixed with 10 % EMS (v/v) and incubated for 30 min at 30 degrees Celsius. These spores were directly used for plating and the EMS was not washed. In the combined UV EMS treatment, previously UV mutagenized spores were mixed with EMS as described before. We plated 300 ul of spores on 12 x 12 cm 2% CMC and 0.001% DOG plates using a suspension with a concentration of 3 x 10^7 spores per ml.

2.17. Screening of mutagenized **T. aurantiacus** *strains for cellulase hyperproducers*

Protein secretion in glucose medium:

Liquid culture media with 50 ml 2% glucose, 0.8% soy meal peptone and 1x McClendon salts were inoculated with 2 agar plugs excised with an inverted P1000 tip from mutant and WT cultures. Some mutants grew slowly and required a substantial amount of time to sufficiently develop. The cultures were incubated for three days. After one day, 20 ml 3% glucose 0.8% soy meal peptone and 1x McClendon were added to the cultures to keep the fungi de-repressed. The same was repeated on day two with 1 ml concentrated glucose (60 %) and 1 ml concentrated SMP.

The sample filtering was done as described in the shift experiment SOP (chapter 2.3). For protein quantification, the Bradford assay was performed (Biorad®) according to the Bradford assay protocol. On the SDS PAGE, 15 ul supernatant and 4x loading dye (Biorad®) was applied (= virtually 11.25 ul pure supernatant) and 5 ul Novex sharp prestained marker (Thermo Fischer).

Protein secretion in xylan medium (shift experiment)

Pre-culture: 2% glucose, 1x McClendon (new trace elements) pH = 5.25, 0.8% SMP (Fluka), inoculated with 2 agar plugs excised with P1000 tip.

Shift medium: 2% beech wood xylan, 1x McClendon (new trace elements) pH = 5.25., 0.8% SMP (Fluka).

The shifting was done via pouring all cultures into 50 ml falcon tubes and centrifuging them at 5250 rpm for 15 min at room temperature. The supernatant was discarded and the mycelium was shifted to the flasks by adding xylan medium to the Mycelium and adding the entire falcon tube to the shake flask.

The sample filtering was done as described in the shift experiment SOP (chapter 2.3). For protein quantification, the Bradford assay was performed (Biorad®) according to the Bradford assay protocol. On the SDS PAGE, 5 ul supernatant and 4x loading dye (Biorad®) was applied (= virtually 3.75 ul pure supernatant) and 5 ul Novex sharp prestained marker (Thermo Fischer).

2.18. *2 L bioreactor enzyme production with* T. aurantiacus

The fungal inocula were first grown in a glucose medium to reach sufficient biomass (liquid pre-culture) or applied in form of ascospores, harvested according to our spore isolation SOP (chapter 2.2.). The cellulase production medium (beech wood xylan) was supplied after sufficient glucose was consumed.

Pre-culture medium

The glucose medium consisted of 3 % glucose (w/v), 0.8% Fluka soy meal peptone (w/v) and 1x McClendon salts solutions with 33 mM phosphate buffer, (new trace elements) pH set to 5.0 before autoclaving for reactors 1,2 and 3 and was accidentally kept at around 5.9 in case of reactor 4.

Reactor 1 (shift and fed batch)

Shift medium: 2% beech wood xylan (w/v), 0.8% Fluka soy meal peptone (w/v) and 1x McClendon salts solutions with 33 mM phosphate buffer, 1x trace elements and pH set to 5.25 before autoclaving, since the pH drops to ~after the autoclaving (1.5l).

Reactor 2 and 4 (shift and fed-batch)

Shift medium: 2% beech wood xylan (w/v), 0.8% Fluka soy meal peptone (w/v) and 1x McClendon salts solutions with 33 mM phosphate buffer, 1x trace elements and pH set to 5.25 before autoclaving (0.75l).

Fed batch medium: 4% beech wood xylan (w/v), 1.6% Fluka soy meal peptone (w/v) and 1x McClendon salts solutions with 33 mM phosphate buffer, 2x trace elements and pH set to 5.25 before autoclaving (0.75l).

Reactor 3 (fed-batch w/ two pulse additions; no shift)

Pulse medium 1 and 2: 4% beech wood xylan (w/v), 1.6% Fluka soy meal peptone (w/v) and 1x McClendon salts solutions with 33 mM phosphate buffer, 2x trace elements and pH set to 5.25 before autoclaving (0.5l).

All cultures were incubated at 50°C. Filtered room air was continuously supplied. The turbine pillar rotation was set to a maximum of 300 rpm. The pH of the pre-culture media dropped to 4.8 after autoclaving in case of reactors 1, 2 and 3. Since TA has problems at growing at too low temperatures, we set the pH to 5.5 with ammonia hydroxide. Based on calculations, we added 52 g of the 50% ammonia hydroxide to reactor 1, which was 13.46 g of total ammonia added (0.89 % w/v in the medium). Therefore, reactors 1, 2 and 3 received a substantial additional amount of nitrogen.

Inocula

Reactors 1, 2 and 4 were inoculated with spores, isolated from PDA plates. All spore solutions were directly inserted with a syringe into the pre-culture glucose medium. Reactor 3 was inoculated with a 4 day old pre-grown shake flask liquid TA culture in 2% glucose medium. The culture was inserted into the pre-culture glucose medium with a syringe.

SDS PAGE

The SDS PAGE was done with purchased 15 well Biorad gels (8 – 16% gradient) at 140V for 40 min. For loading, a 4x loading dye from Biorad was used. The gel was loaded with 7.5 ul protein extract and 5 ul Novex sharp pre-stained protein standard. The staining was done with the SimplyBlue safe stain (Invitrogen) for 2h and de-stained with pure water.

Bradford assay

The Bradford Assay was done according to the Biorad protocol. In short, the reagent was diluted with 4 parts water. In each well of a 96 well plate, 5 ul of BSA standard (0– 1000 ug/ml) was added and 250 ul of the diluted reagent. For each sample, virtually 1 ul medium was added (5 ul, 1: 5 diluted) was pipetted in triplicates.

DNS - xylanase activity

For the DNS, 2 ul of medium were used. The xylan medium mixture was incubated in a 96 well plate for 30 min at 50 degree Celsius. All samples were pipetted in triplicates. A xylose and

glucose standard was used (0 – 6.25 mM). The procedure was done as described in DNS Enzyme assay (Appendix).

2.19. *Transcriptional response of T. aurantiacus towards beech wood xylan*

Spores from three plates of *T. aurantiacus* 26904 were isolated according to our SOP (chapter 2.2.) from a 7 day old plate and 4 replicates of liquid cultures for xylan and no carbon were inoculated and shifted according to our SOP (2.3.). Cultures were grown under conditions of permanent light.

Pre-culture (8 flasks): 2% glucose, 1x McClendon (new trace elements) pH = 5.25, 0.8% SMP (Fluka).

Shift medium (2 x 4 flasks): 2% beech wood xylan or no carbon, 1x McClendon (new trace elements) pH = 5.25., 0.25mM ammonia nitrate.

From the shift cultures, xylan and no carbon samples were taken 1, 4, 6 and 8 hours after shifting. The sampling procedure involved taking an aliquot of 8 ml culture and filtering and washing it on a Whatman paper. The sample was immediately peeled off and frozen in liquid N. Right after sampling, all samples were ground with a mortar and pistil in liquid N and resuspended in Trizol. RNA was isolated according to the following protocol (Appendix, RNA Extraction Protocol). RNA quantity was determined on a NanoDrop and RIN values were calculated on a Bioanalyzer (RNA 6000 kit). The quality of the samples was suitable for Miseq (RIN values > 8). Samples from all cultures and time points were taken for cDNA synthesis. All steps were done according to the TruSeq LT sample protocol from Illumina. The library was constructed from 6 xylan and 6 no carbon samples.

2.20. *Induction of protein production and cellulase activity in T. aurantiacus*

Pre-culture: 2% glucose, 1x McClendon (new trace elements) pH = 5.25, 0.8% SMP (Fluka), inoculated with ascospores.

Shift medium: 2% beech wood xylan, 1x McClendon (new trace elements) pH = 5.25., 0.25mM ammonia nitrate.

The sample filtering was done via centrifugation in filter spin columns. For protein quantification, the Bradford assay was performed (Biorad®) according to the Bradford assay protocol. On the SDS PAGE, 15 ul supernatant and 4x loading dye (Biorad®) was applied (= virtually 11.25 ul pure supernatant) and 5 ul Novex sharp prestained marker (Thermo Fischer). For the xylanase assay, the supernatants were diluted 1:5 and 5ul were used according to our group protocol (Appendix). For the CMCase assay, undiluted 20 ul were used according to the same protocol.

3. Results

3.1. Growth physiology of T. aurantiacus

The experiments on the growth physiology of *T. aurantiacus* are summarized in this chapter.

3.1.1. The effect of temperature on growth speed

Due to harsh evaporation of PDA plates of TA at 50°C, we chose to shift freshly inoculated plates after 2 days to 45°C. We wanted to assess the difference in growth by inoculating two sets of plates at both temperatures for 2 days. The growth was measured continuously. Between 30 and 40 hours, the plates at 50°C already reached the maximum plate diameter, while the plates incubated at 45°C required around 45h (Figure 3.1.).

3.1.2. The pH optimum

For finding the optimal pH growth range of *T. aurantiacus*, we cultured ascospores in liquid cultures and agar plugs on plates at pH: 4.0, 5.5, 7.0 and an unbuffered control. In the plate experiment, there was now big difference among pH 5.5, pH 7.0 and the unbuffered control plates. Only pH 4.0 grew slower (Figure 3.2). The same trend could be observed in the liquid culture experiment. There, the pH 7.0 culture has the highest average this time. For future pH studies, it might be better to only filter the submersed mycelium and not the ring. In the pH 4.0 liquid cultures, there is little submersed mycelium present and this biomass weight would be much lower then.

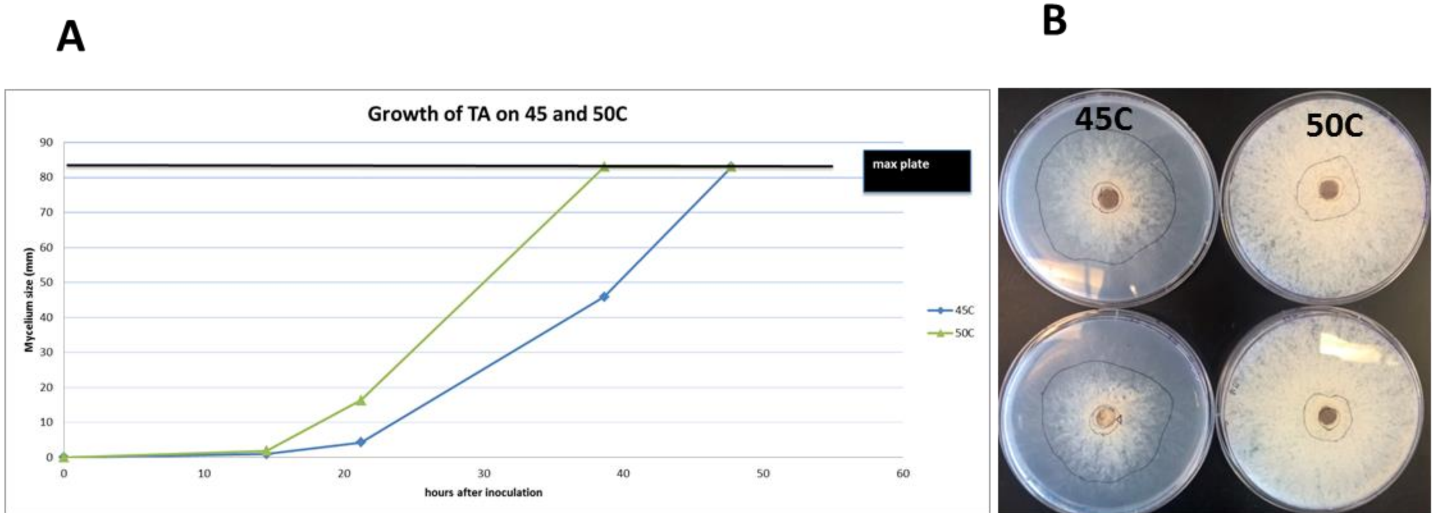


Figure 3.1: Growth of *T. aurantiacus* at 45 and 50°C. (A) Growth kinetics of TA during 2 days incubation at the respective temperature (n =2). (B) Images of the plates after 2 days incubation. The brown round structure in the center is the agar inoculum.

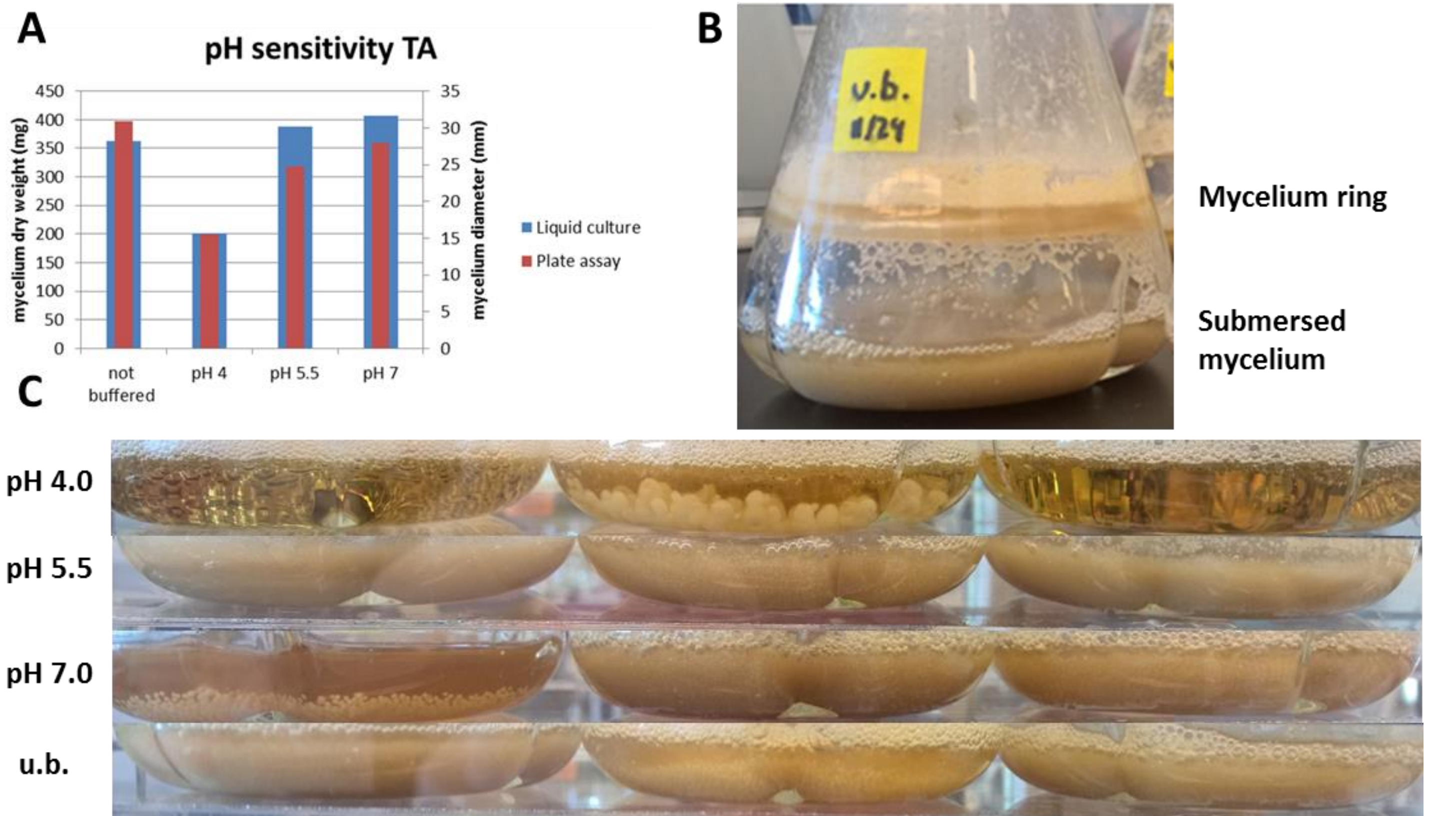


Figure 3.2: growth of *T. aurantiacus* at four different pH: 4.0, 5.5, 7.0 and unbuffered control: pH =5.0. (A) Comparison of biomass in liquid cultures after 4 days (blue bars, n = 3) and growth on plates after 24h (red bars, n = 2). (B) Mycelium ring and floating mycelium of a control flask. (C) Images of all submersed mycelia. Different pellet sizes are visible and pH 4.0 media are empty except for one flask.

3.1.3. Basta and Phleomycin sensitivity

In order to perform the targeted mutagenesis of *T. aurantiacus*, we were testing two selection markers: Basta (Phosphinothricin) and Phleomycin. Ascospores were used as an inoculum, since agar plugs are biased regarding the amount of mycelium transferred. *T. aurantiacus* had a very low sensitivity towards Basta. However the fungus is more sensitive towards Phleomycin. It has to be noted, that the fungus has a slow growth on the Phleomycin control plates. The plate medium might not be suitable to facilitate recovery and

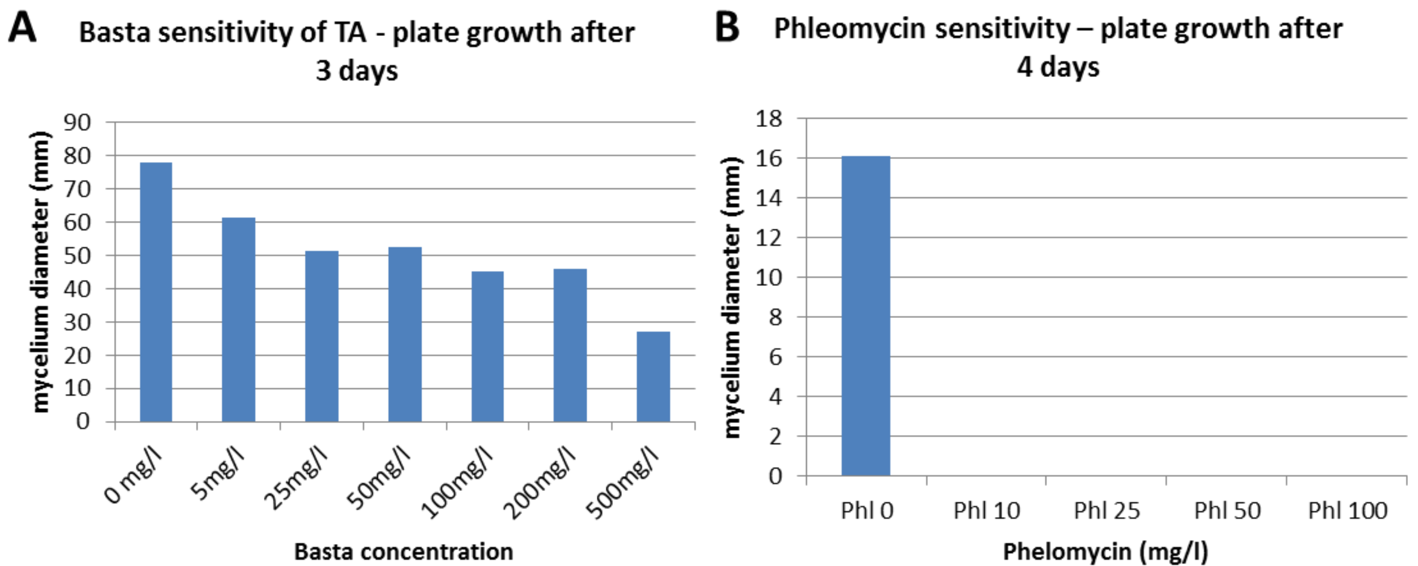


Figure 3.3: Growth of *T. aurantiacus* on plates containing different concentrations of (A) Basta or (B) Phleomycin.

growth since transformation via protoplasting causes great stress to the fungal cell.

3.1.4. Spore germination kinetics and ascospore production

Inoculating liquid media with ascospores has several advantages compared to using agar plugs. The resulting cultures display much less variability while agar plugs often result in more variation. Therefore, using ascospores yields more reproducible data. To this end, we wanted to assess how old a fungal culture on a PDA plates needs to be, to produce spores with a maximal germination rate. PDA plates were inoculated and after 3 to 8 days, spores were isolated and

plated on fresh plates. After incubation for 16 h at 45 C the germination rates were determined using the ImageJ software and microscopic images. The maximum germination rate lied between day 5 and 6 with a sharp drop at day 6 (Figure 3.4 A). The total amount of spores harvested increases till day 7 and seems to reach a plateau (Figure 3.5). This might not necessarily indicate that additional spores are produced. We observed that the amount of intact

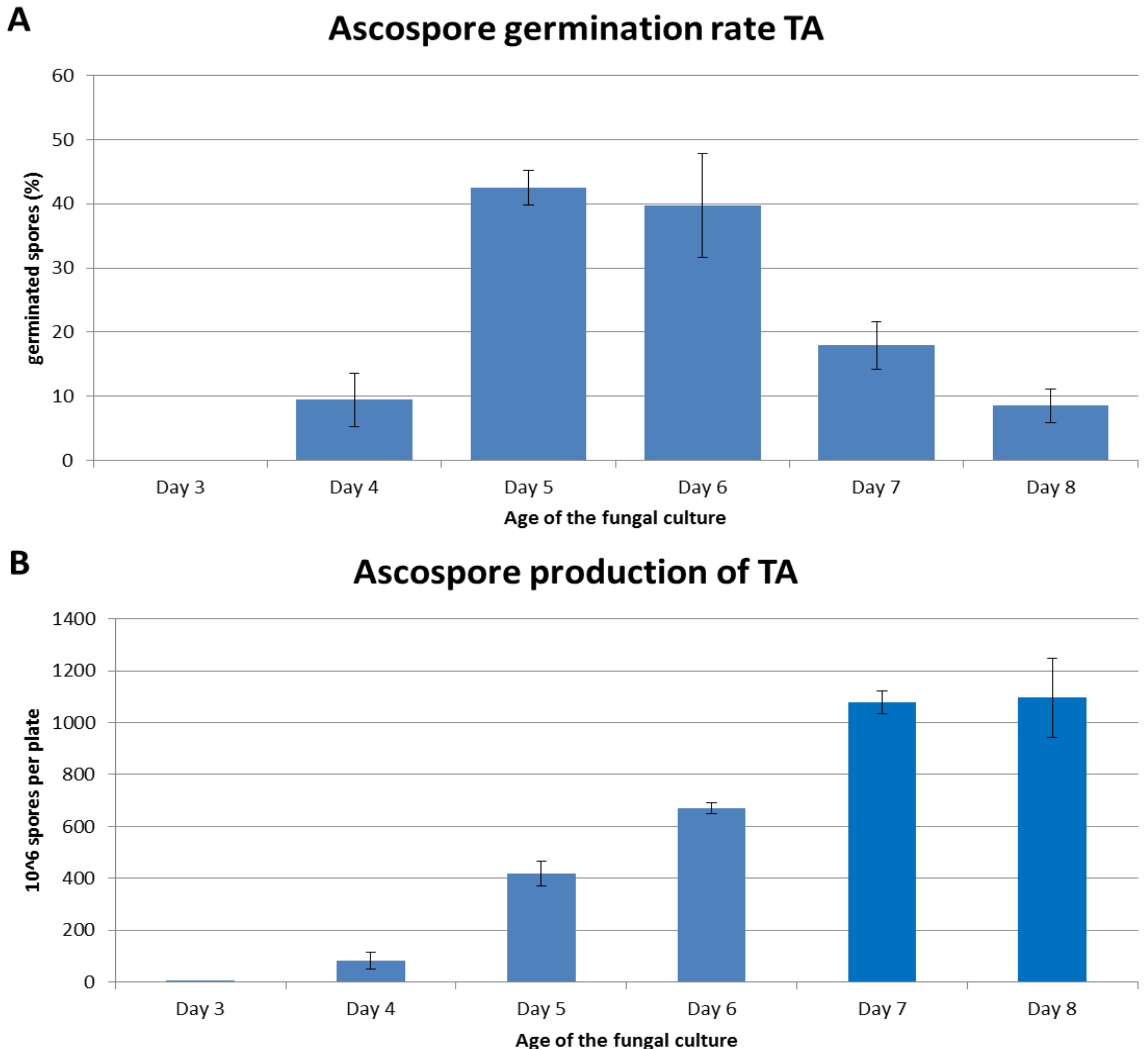


Figure 3.4: Spore germination kinetics of TA in a time course of 8 days. Spores were spread on new plates and taken to microscopy after 16h at 45 degree Celsius. (A) TA spore germination rates are shown (n =3). (B) Total spore count of an entire plate (n = 3).

asci declines on later time points, which implies that the spores are more easily released.

3.1.5. The effect of spore density on mycelial morphology

For our liquid media experiments, we always used 10^6 spores/ml. This is a suitable amount for 50ml shake flasks. However for bioreactors, a lot of spores would be needed this way. Using a lower spore titer might be still sufficient to have suitable biomass and propagule morphology. We hypothesized that the number of propagules rises with the spore inoculum and that the diameter of each propagule decreases when more spores are added. Many propagules with low diameter are favorable, since they have a bigger surface area and therefore more capabilities to take up nutrients and secrete proteins. Accordingly, a higher spore titer might be beneficial.

The spore titer seems to have huge implications for the propagule morphology (Figure 3.5). Flasks containing 10^1 to 10^4 spores/ml have no propagules in the medium, the 10^5 spores/ml dilution contains few but huge propagules and the higher spore concentration have tiny dense propagules. The overall biomass in the 10^7 spores/ml medium appeared to be higher than in the standard 10^6 spores/ml medium. Also the mycelium ring is influenced by the spore titer. In case of the highest concentration, the ring grew so heavy that it slipped into the liquid medium. Propagule morphology is therefore determined by the amount of germinating spores present. The same is true for the mycelium ring formation.

3.2. Media optimization for cellulase hyperproduction

Our efforts for generating a high cellulase secretion liquid medium for *T. aurantiacus* are summarized in this chapter.

3.2.1. Medium composition: Mineral salt solution and nitrogen source

Mineral salt solution and N source have important implications for fungal growth in liquid cultures. Pervious work on *T. aurantiacus* at JBEI employed the McClendon salt solution, which was developed by a former researcher[8]. Another widely used salt solution are the Vogel`s salts. There are differences in the composition of both media. Vogel's salts contains Bo and Mo compared to McClendon salts and additional citric acid to prevent precipitation of Phosphate with Mg and Ca. McClendon salts in contrast contains Co and has no citrate added, which is why we split it in three components: a 50x component A (Mg, Ca, Na), a 50x component B (Phosphate buffer) and 1000x Trace elements. We compared the growth of *T. aurantiacus* in both media

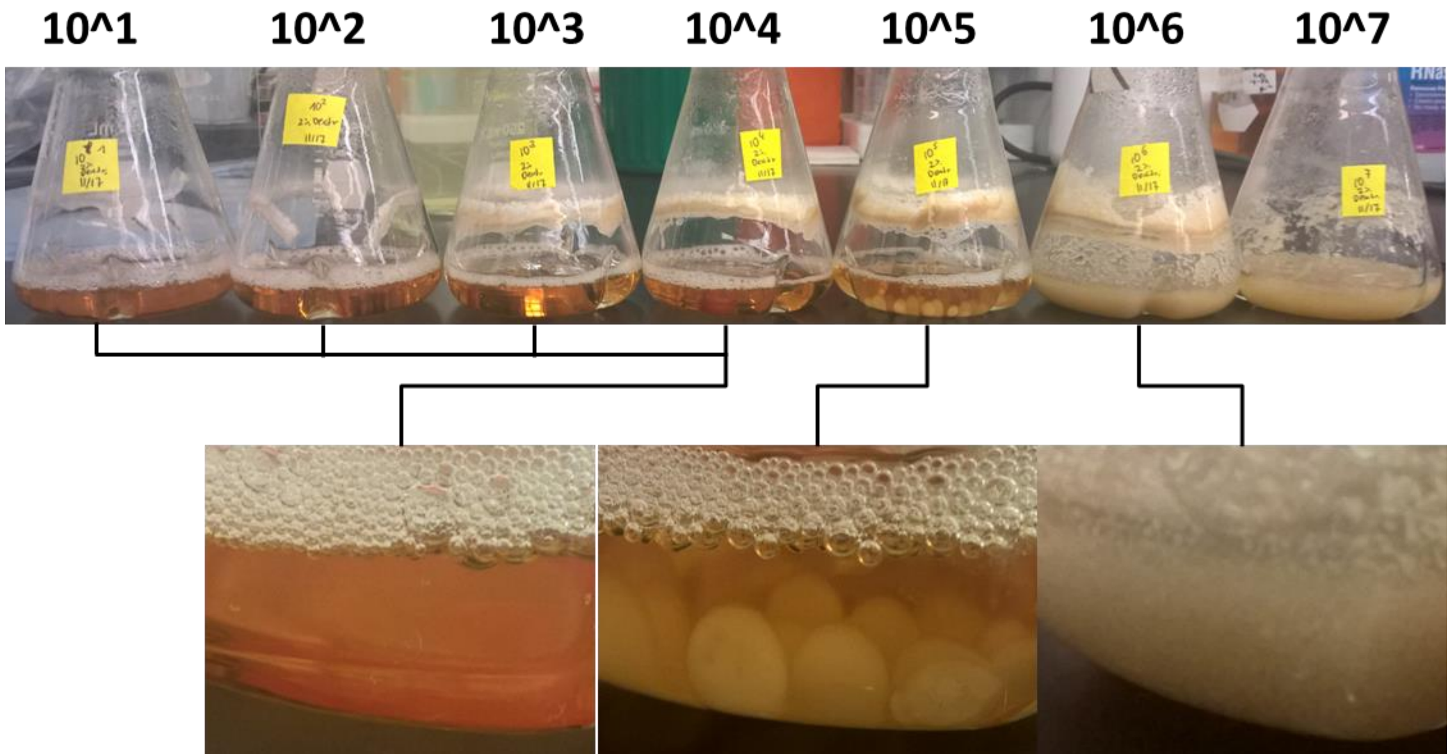
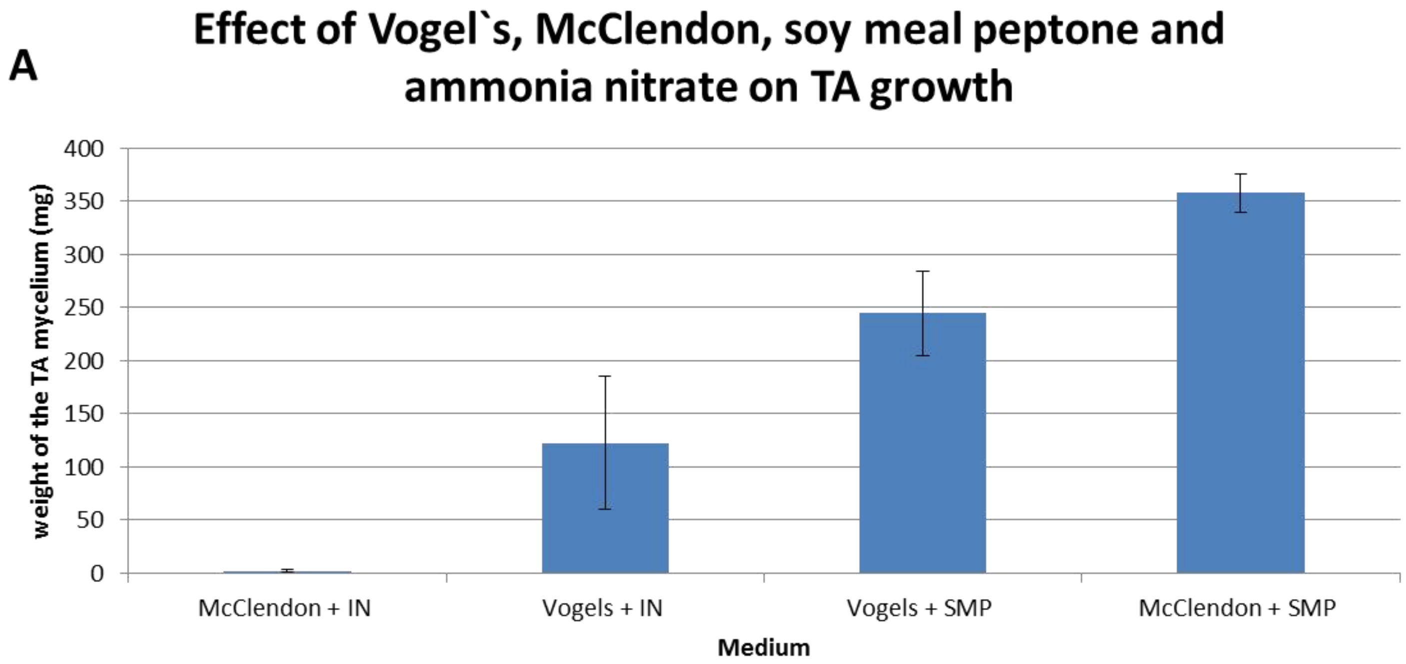


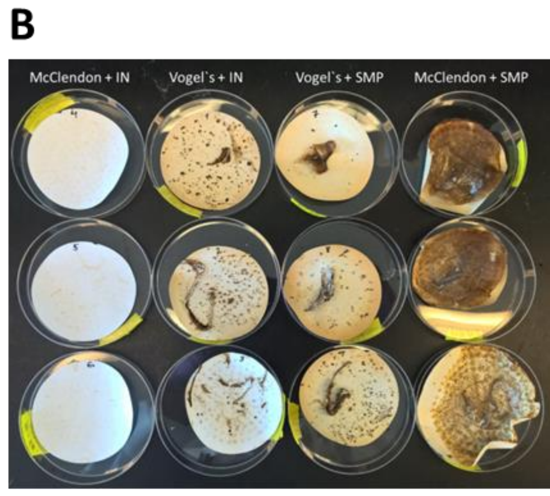
Figure 3.5: Inoculation of liquid media with different ascospore titers. Propagule morphology is displayed in the bottom images. Flasks containing 10^1 to 10^4 spores / ml have no propagules in the medium, the 10^5 spores / ml dilution contains few but huge propagules and the higher spore concentration have tiny dense propagules.

types by adding either ammonia nitrate or soy meal peptone (SMP). The biomass gain in all 4 combinations was measured. The highest biomass increment was measured when SMP was added. McClendon`s salts and SMP had the highest biomass while McClendon`s and ammonia nitrate the lowest (Figure 3.6. A). This indicated that *T. aurantiacus* grows well on organic N sources. Also, the only media which exhibited protein secretion were the SMP containing media, probably due to faster growth and corresponding catabolite de-repression due to glucose consumption (Figure 3.6. D).

3.2.2. Medium composition: New McClendon`s salts trace elements

In previous experiments, we aimed at improving cellulase and protein secretion of *T. aurantiacus* by optimizing the growth medium. McClendon`s salts together with SMP were determined to be the best growth medium in shake flasks.





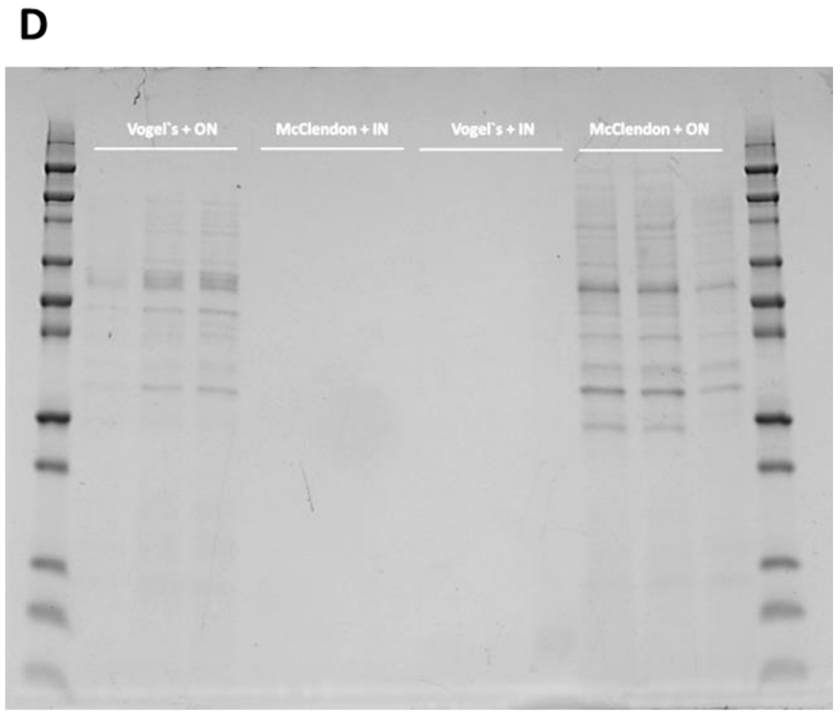


Figure 3.6: Comparison of the *T. aurantiacus* growth in Vogel`s salts, McClendon salts, soy meal peptone (SMP) and ammonia nitrate (IN) media. (A) Growth in the respective media (n=3 for all media). (B) Overview image of the filters from the respective culture medium. (C) Image of the fungal cultures from the respective medium before the filtering (ON = SMP, IN = ammonia nitrate). (D) SDS PAGE from the filtered medium of each liquid culture.

For all media, we used an already prepared 1000x McClendon's trace element solution with a green color (old TE) and green yellow precipitates for several experiments. Once these elements became scarce, we set up a new trace element solution according to the protocol of the old stock. The new stock (new TE) had a blue color and did not show any signs of precipitation. We compared the growth of the fungus in media with both trace elements to see, how vital the trapped nutrients in the precipitates are. In the shake flasks and on the filters, the cultures of the

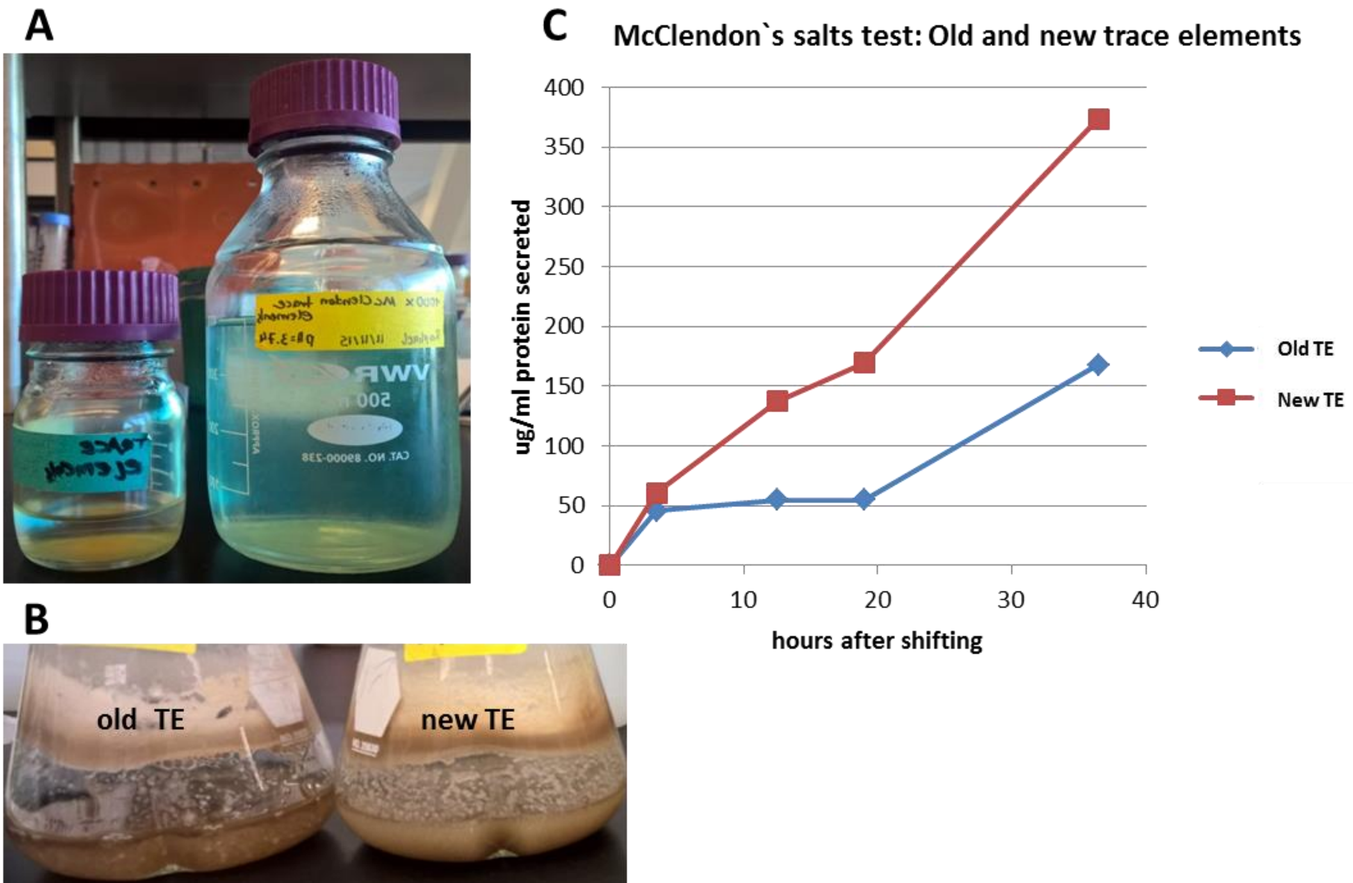


Figure 3.7: McClendon salts test with (A, left bottle) an old batch of trace element solution (green TE) with precipitation and (A, right bottle) a freshly prepared one (blue TE). (B) Pre-cultures before shifting. The new trace elements lead to more biomass growth. (C) Bradford of samples taken after shifting to beech wood xylan medium. Only the time course till 37h is shown, since the 90h time point thereafter displayed heavy protein degradation (not shown).

old trace elements clearly had lower biomass (Figure 3.7). The two trace element solutions lead to differences in growth and protein secretion rates. Only the time points until 37h are shown, because the next and last time point at 90h already displayed heavy protein degradation on a SDS PAGE gel (not shown). For all further experiments, we employed the new trace elements.

3.2.3. Inducers Medium composition: Soy peptone brand Fluka versus Amresco

With the new trace elements, we achieved higher protein titers. Moreover SMP turned out to be the superior N source compared to ammonia nitrate. Currently, we employed SMP from two different companies: SMP from Fluka and Amresco. In another shake flask shift experiment, we compared both SMPs. The peptones exhibited no significant difference in protein secretion 72h after the shift (Figure 3.8.). For all future experiments, we decided to stick with one peptone and

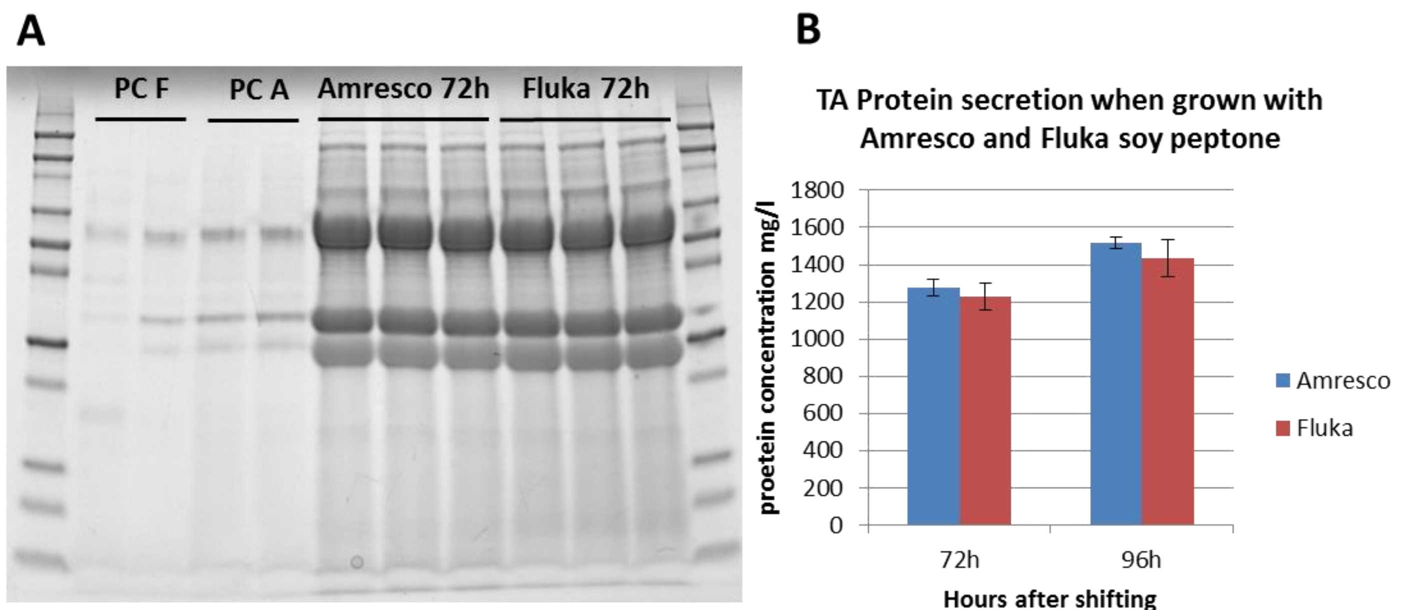


Figure 3.8: Comparison of Fluka and Amresco SMP in TA cellulase secretion. (A) SDS PAGE profiles of *T. aurantiacus* pre-cultures (PC F and PC A) and shifted cultures to 2% beechwood xylan. The shifted cultures exhibit substantial protein secretion into the medium with the prominent complex carbon degrading bands. Virtually 11.25ul of medium were loaded. (B) Bradford assay of filtered supernatant samples. No significant differences were observed.

chose the freshly ordered Fluka SMP.

3.2.4. Inducers of Cellulase secretion

In order to identify suitable inducers for *T. aurantiacus* cellulase secretion, we conducted shake flask shift experiments. The initial shift experiment revealed beech wood xylan as a strong inducer of TA cellulases compared to crystalline Avicel cellulose (Figure 3.9.). The fact that Avicel in combination with SMP as a N source fails to induce cellulases was surprising. The same trend was also observed in other experiments not shown here. As shown in Figure 26.B, the addition of

ammonia nitrate before autoclaving leads to higher protein secretion. We propose a pretreatment mechanism during autoclaving with ammonia nitrate.

Additional inducers were tested for stimulating cellulase secretion of TA (Figure 3.10.). These inducers were the low molecular weight sugars xylose, xylobiose and the non metabolizable methyl-xylopyranosid (MXP) at a concentration of 0.1%. In addition, wheat straw arabinoxylan

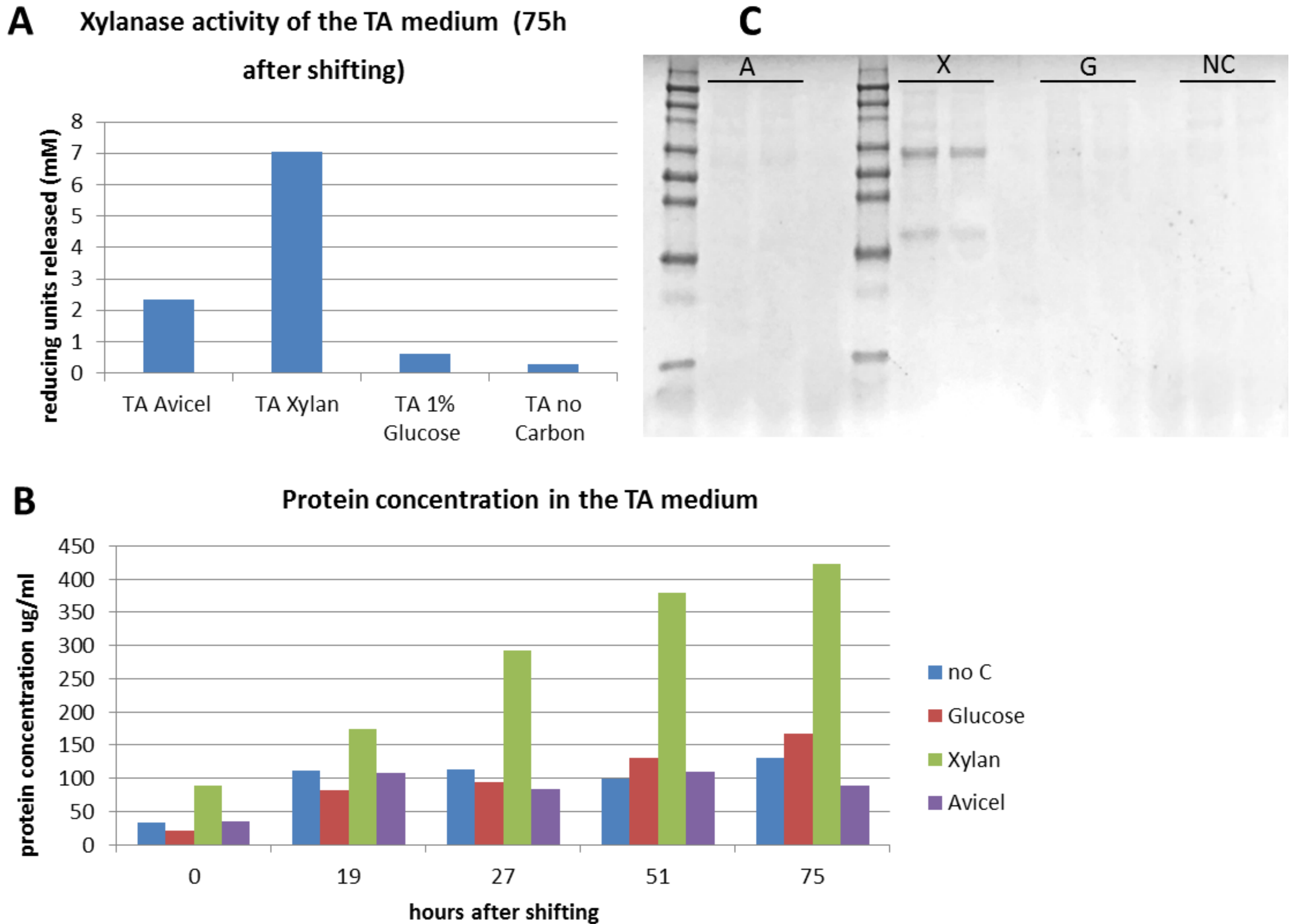


Figure 3.9: Preliminary shift experiment: Inducing effects of beech wood xylan and Avicel cellulose on *T. aurantiacus* cellulase secretion. (A) Xylanase activity of TA supernatant. (B) Protein concentration after 75h measured with the Bradford assay (n=2). (C) SDS PAGE profile after 43h (10 ul loaded).

and xylogucan were also tested at a concentration of 1%. The Fungi were grown in the presence of 2% glucose, 0.8% SMP and the inducer. MXP is an interesting inducer in this regard, since it can be applied in very low amounts and stimulates cellulase secretion very well. Xyloglucan might be also interesting. It has to be supplied in higher amounts though and is also quite expensive. MXP in combination with a catabolite de-repressed mutant strain of *T. aurantiacus*

might be sufficient to secrete continuous amounts of cellulases in the presence of glucose. Xylose and xylobiose are most likely consumed too fast to act as suitable inducers.

3.2.5. Shake flask shift experiment with optimized medium

In the previous experiments we discovered that McClendon minimal salts with the new trace elements stock and SMP as an N source promotes biomass production and protein secretion in *T. aurantiacus*. Moreover beech wood xylan turned out to be a potent inducer of cellulase secretion in this fungus, while Avicel crystalline cellulose did not act as an inducer. Since all previous shift experiments, which intended to investigate the inducing effect of Avicel and xylan and the repressing effect of glucose, were set up with the old trace element solution, we started

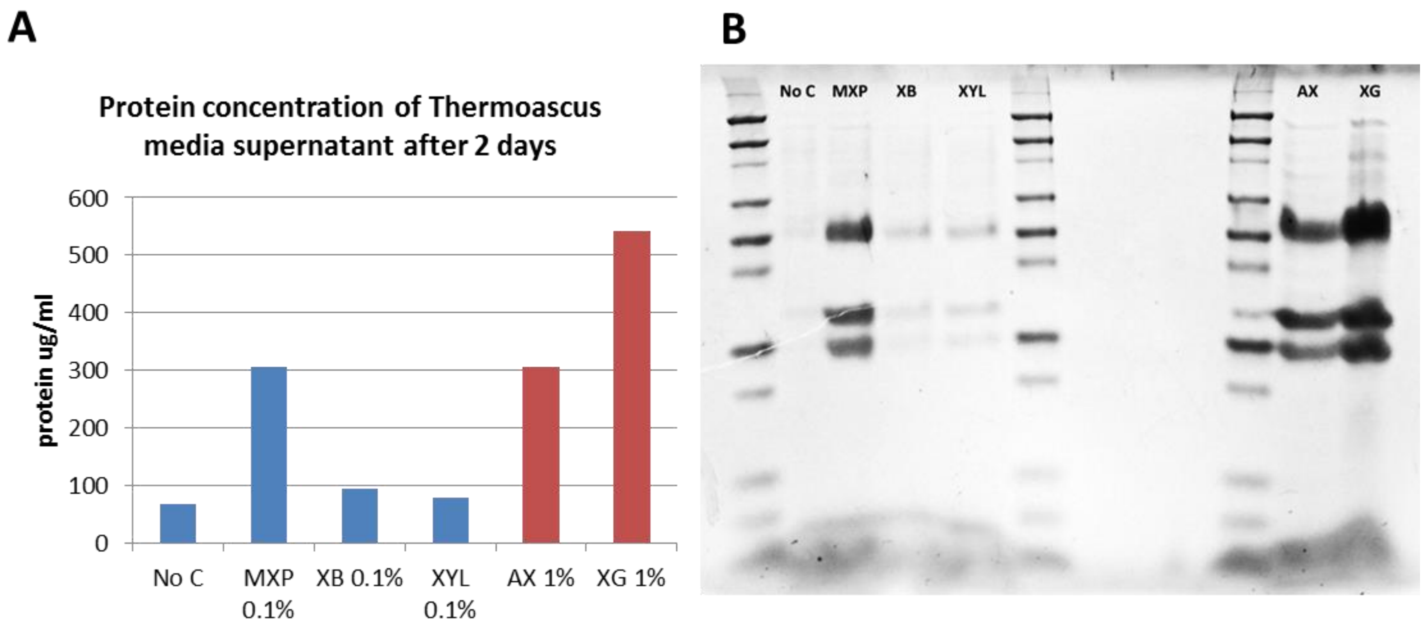


Figure 3.10: Stimulation of different C sources on *T. aurantiacus* cellulase secretion: (A) Bradford assay (n=1). (B) SDS PAGE of 48h supernatant (15ul undiluted). No C = no carbon, MXP = methyl-xylopyranosid, XB = xylobiose, XYL = xylose, AX = wheat straw arabinoxylan, XG = xyloglucane.

another shift experiment with the new trace elements (Figure 3.11.). Moreover, we wanted confirmation whether Avicel still shows no inducing effect. The values of protein concentration were corrected for the water evaporation at each time point. The xylanase activity of the xylan cultures was very high and reached a release of 12mM reducing unit at the last time point despite a 1:40 dilution of the supernatant (5 ul employed). The xylanase rates were not corrected for the water loss, since the activity rates in the DNS assay were shown to be non-linear in regard to protein concentration (data not shown).

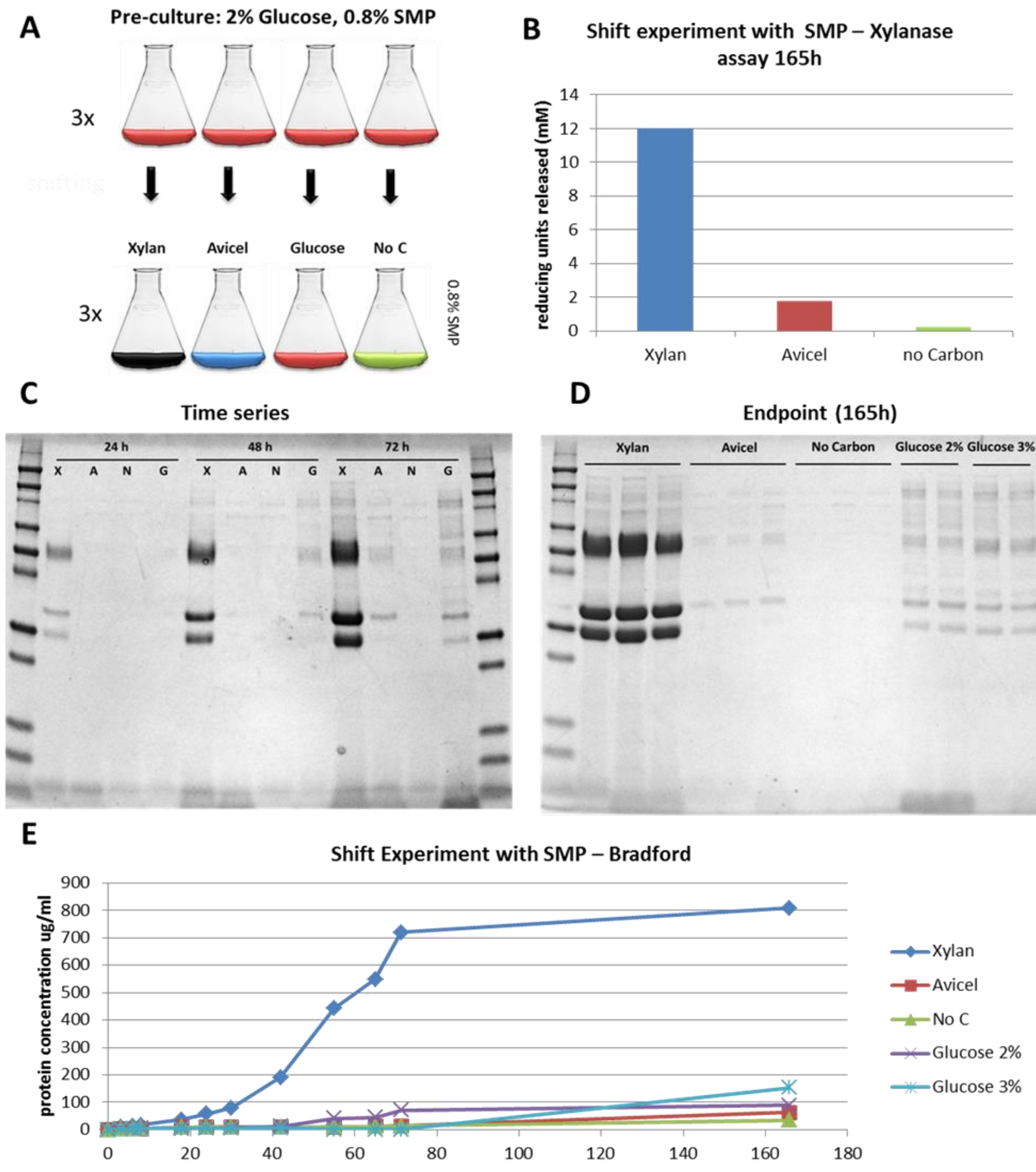


Figure 3.11: Inducer shift experiment with xlan, Avicel, glucose and no carbon with SMP as an N source. (A) Experiment setup. (B) Xylanase assay of last time point (5 ul 1:40 diluted supernatant loaded, n = 3). (C, D) Time course and end point samples visualized on a SDS PAGE: X = xylan, A = Avicel, N = no C, G = 2% glucose (5 ul undiluted supernatant loaded). (E) Bradford of assay of sampling time course. The protein concentration of each time point was corrected for the water evaporation at 50°C (n = 3).

Moreover, this experiment confirms again, that Avicel cellulose in combination with SMP as an N source fails to induce cellulase secretion in our fungal strain. The glucose cultures exhibit cellulase secretion at latter time points. At these time points, the glucose is certainly consumed and the mycelium becomes catabolite de-repressed. The biomass of these cultures is likely higher than of any other culture and proportionally more cellulases are secreted than in the no carbon and Avicel cultures. Xylan again exhibits by far the highest protein secretion.

3.3. Strain Optimization for cellulase hyperproduction

We employed random mutagenesis with UV radiation and Ethylmethansulfonate (EMS) on *T. aurantiacus* ascospores with subsequent plating on CMC and 2-deoxy-glucose (DOG) to select for catabolite de-repressed mutants. These mutants were compared with wild type *T. aurantiacus* for protein secretion and biomass gain to identify potential catabolite de-repressed strains. These screening experiments were done in glucose medium (Figure 3.15.) and xylan medium (Figure 3.16.).

3.3.1. 2-deoxy-glucose sensitivity of **T. aurantiacus**

Before selecting for catabolite de-repression with the not metabolizable glucose analog DOG, we first needed to assess if there is a concentration at which this compound becomes toxic to the fungus. We tested this in the presence of glucose, where the fungus is catabolite repressed and repression of cellulase genes through DOG should not cause any harm. At high concentrations however, DOG might interfere with glucose metabolism and cause reduced growth. At a concentration of 0.01%, DOG already reduced growth of WT in the presence of glucose, indicating a slight toxic effect at that concentration (Figure 3.12. A). Besides evaluating the effect of DOG on fungal growth, we wanted to determine the concentration, at which DOG is inhibitory for the growth of the WT on 2% CMC plates. We determined, that a concentration of 0.001% DOG still allows strongly retards germination after 16h, while 0.025 % is already inhibitory (Figure 3.12 B). Taken together, we chose 0.001% DOG as the concentration for our screening plates. At this concentration, the WT grows in a strongly reduced manner and the concentration is not yet toxic to the fungus. We did not choose the concentration of 0.01%, since mutagenized spores exhibit a per se lower germination and growth rate. Too stringent conditions would reduce the chance of gaining sufficient mutants.

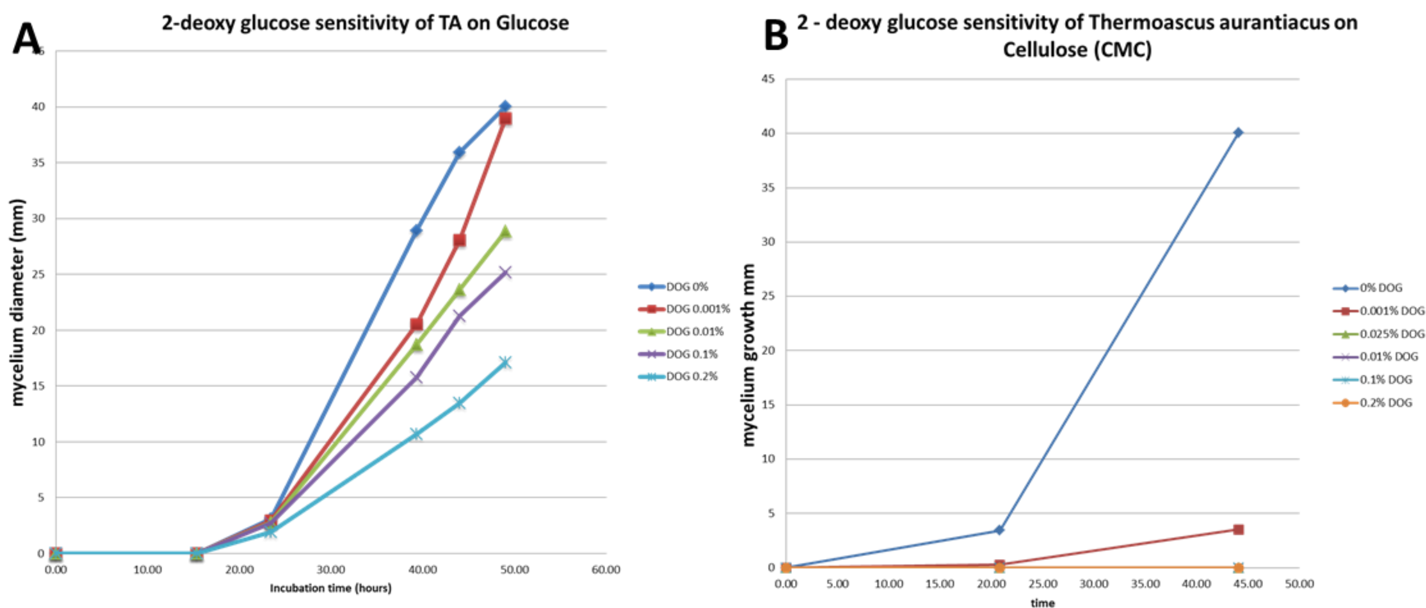


Figure 3.12: Sensitivity of T. aurantiacus towards 2-deoxy glucose on plates, (A) when grown in the presence of 2% glucose or (B) on 2% CMC plates.

3.3.2. Survival rate of TA ascospores after EMS and UV treatment

Besides investigating the basic germination rate of *T. aurantiacus*, we were also interested in measuring the survival rate of spores after EMS and / or UV treatment. This information is valuable to us to know how many spores we have to spread on our plates in screening assays to compensate for the kill rate. It can also be used as a rough proxy for the degree of mutagenesis (Figure 3.12). The average germination rate of day 6 old spores was set as the basic survival rate of ascospores (100% survival rate). The kill rate was calculated as the reduction of the survival rate due to mutagenesis treatment. As determined in previous experiments, the UV treatment has a very strong effect on the survival rate of ascospores. After 30 seconds, the survival rate droped to 5 - 8% (data not shown). We used 20 seconds UV treatment, which turned out to be a tradeoff for generating sufficient mutations and keeping the survival rate high enough. The 30 minutes EMS treatments lead to a higher germination rate than the UV treatment and presumably less cell damage. This can be inferred from figure 3.13. B, where EMS treated spores produced longer germinating hyphae than UV treated ones. The EMS + UV treatment lead to no germination after 16 hours spore incubation. A total of 13 mutant strains were isolated after plating UV or EMS treated spores on 0.001% DOG plates (Figure 3.14). These strains exhibited a reduced sensitivity towards DOG and are therefore potentially catabolite de-repressed.

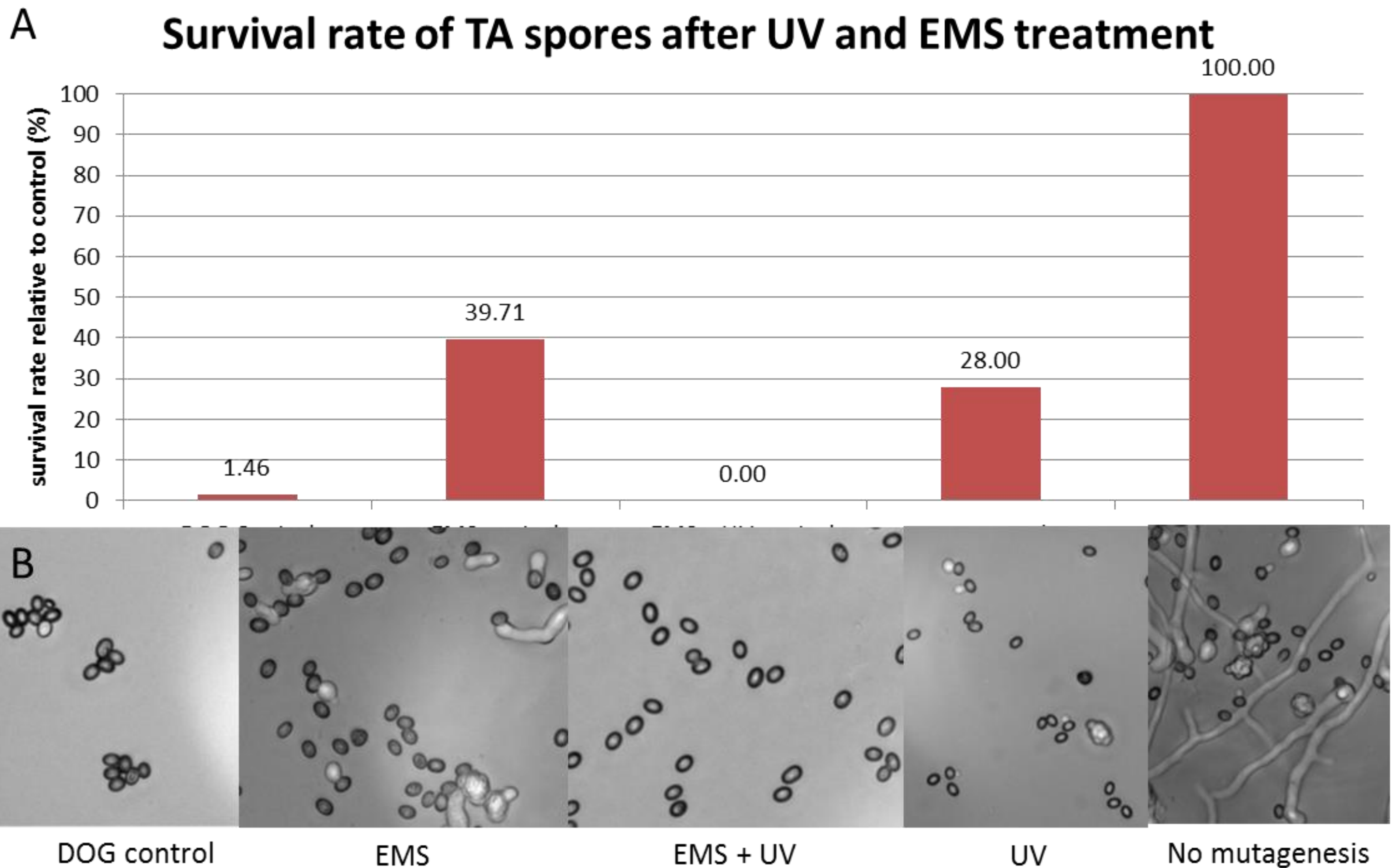


Figure 3.13: Survival rate of *T. aurantiacus* spores after combinations of UV, EMS and UV + EMS. The survival rate is normalized for the germination rate of not mutagenized spores on PDA plates. (A) Survival rates of three mutagenesis conditions (UV, EMS and UV + EMS). UV treatments in were done for 20 seconds. The DOG control resembles non-mutagenized TA spores on 0.001% 2-deoxy-glucose and 2 % CMC. B) Representative microscopic images of the conditions in A.

3.3.3. Screening of cellulase hyperproducers

All mutant strains were tested for superior protein secretion capabilities compared to wild type. Frist, all strains and wild type were grown in glucose medium to check for signs of de-repression without a cellulase inducer present. The next step was to check whether the mutants did not lose their ability to grow on xylan. At last, the mutants were cultured in the presence of glucose and MXP to see if they secrete more protein than the wild type does.

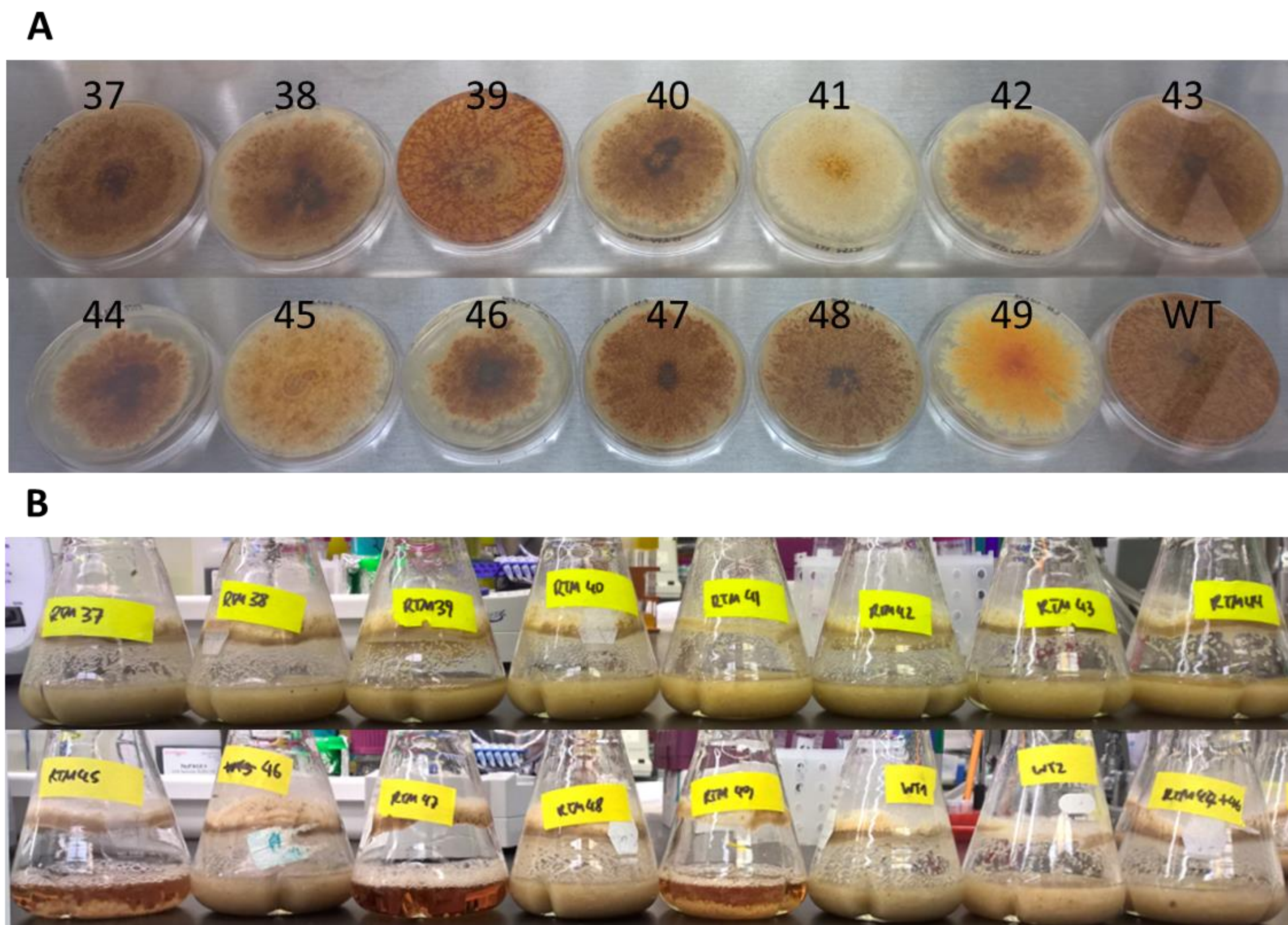


Figure 3.14: Images of mutagenized *T. aurantiacus* strains. (A) PDA plate grown mutant strains (37 – 49) and wild type which served as an inoculum. (B) Liquid glucose cultures at day 3 before the harvest.

Protein secretion in glucose medium

These strains and the wild were incubated for 3 days in glucose and SMP (Figure 3.15.). Additional glucose was supplied on the first two days to prevent de-repression of the wild type. Several mutants grew much slower than the wild type, while other mutant strains grew similar to the WT strains. The Bradford assay revealed that some mutant supernatants had higher protein titers than the WT. This has to be taken with caution since SMP was the N source. Mutants which would consume SMP slower would have higher Bradford values. In the SDS PAGE it is apparent, that the WT cultures secreted the highest amount of cellulases compared to all mutants.

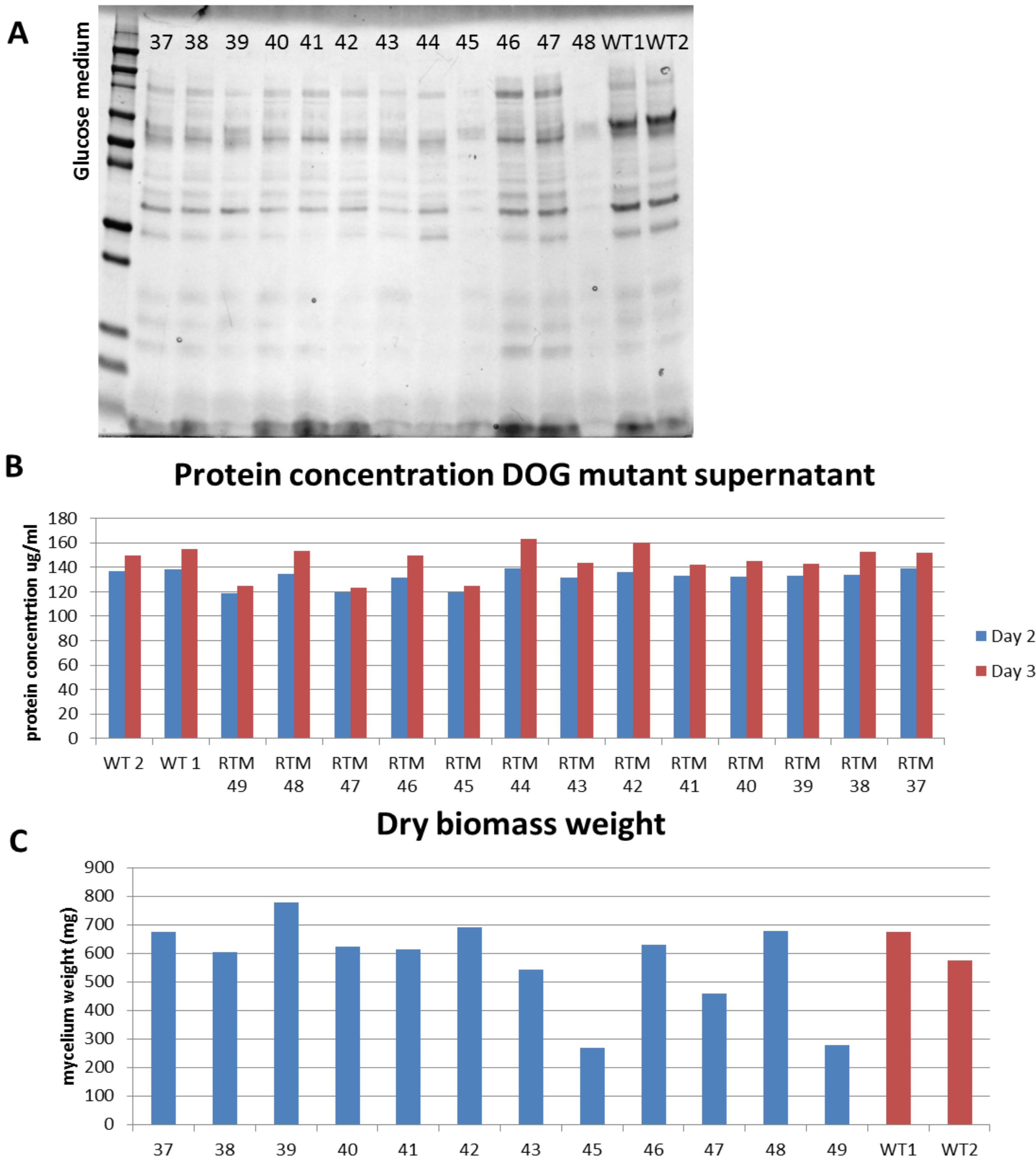


Figure 3.15: Protein secretion and biomass of DOG mutants versus wild type in glucose medium. A) SDS PAGE of supernatants in glucose medium. Virtually 11.25 ul of undiluted medium were loaded onto the gel. Numbers indicate mutants (RTM 37 – 49). B) Protein concentration was measured with Bradford. C) Dry biomass of filtered and washed mycelia.

This is not corrected for biomass, however we see that there is no substantial higher cellulase secretion in the mutant strains. The DOG resistance of these strains towards 0.001% might not be enough to select for catabolite de-repression.

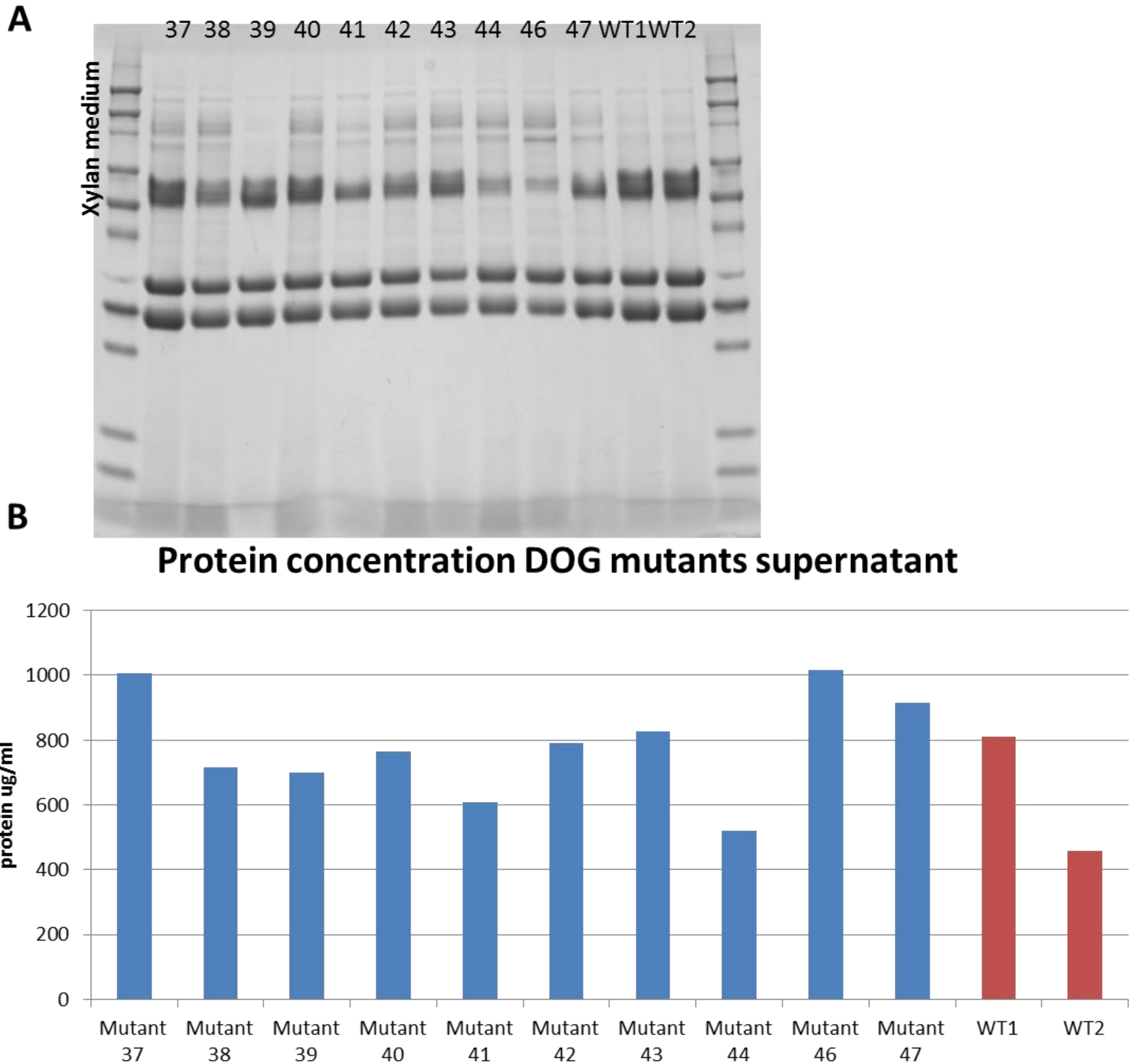


Figure 3.16: Protein secretion and biomass of DOG mutants versus wild type in xylan medium after shifting. A) SDS PAGE of supernatants in 2% xylan medium. Virtually 3.75 ul of undiluted medium were loaded onto the gel. Numbers indicate mutants (RTM 37 – 47). B) Protein concentration was measured with Bradford.

Protein secretion in xylan medium

All mutants except RTM 49, which had a weak growth in the first experiment, were grown for two days in glucose and the shifted to xylan. Most mutants show decent amounts of protein secretion (Figure 3.16). The Bradford values might not reflect the actual amount of secretion so well, because SMP was used as an N source and the SDS PAGE reveals, that the WT secretes plenty of cellulase unlike the Bradford results suggest. In general, the mutants are still able to grow well in xylan medium. Based on Bradford and SDS PAGE, mutant 37 might secrete more protein than WT does.

3.4. 2 L bioreactor enzyme production with T. aurantiacus

Our project goal is to generate a mutant strain of *T. aurantiacus*, which produces protein titers of 20g/l and has a high saccharification potential. The enzyme production is intended to happen at the 300 L scale and even higher volume fermenters. All previous experiments were conducted in 250 ml shake flasks, which does not mimic conditions present in the bioreactors. Therefore we started a bioreactor experiment at the 2 L scale with collaborators at the Advanced Biofuels and Bioprocess Development Unit (ABPDU, LBNL) to gain experience with culturing our *T. aurantiacus* at this scale. During this experiment (Fungal AOP project) three wild type strains and one mutant strain (RTM 12) were cultivated in 2 L fermenters each. All cultures were grown in glucose medium as pre-cultures. Thereafter, a beech wood xylan medium was supplied. The process parameters were continuously surveyed. Media samples were taken every 4 – 8 hours for the duration of the experiment and analyzed for protein concentration, protein profile and cellulose activity. The final culture filtrate was used for cellulose saccharification.

We employed four reactors for this experiment with three different treatments: Reactor 1 (shift and batch), Reactor 2 and 4 (shift and fed-batch) and Reactor 3 (fed-batch w/ two pulse additions; no shift). Reactor 4 was stopped after 70 hours since a bacterial contamination was detected. The process parameters are presented below. Representative images of the reactors are displayed in figure 3.17. The most intense mycelium ring formation happened in Reactor 3 (mycelium inoculum), which had the lowest starting volume.

3.4.1. Culture growth parameters

The growth parameters glucose consumption, optical density, and mycelium dry weights are summarized in Figure 3.18. The glucose consumption rates reveal that Reactor 3 took up the glucose very fast since mycelium was the inoculum. Reactor 2 (spores, WT) was the slowest

consumer. The addition of concentrated trace elements and SMP to this reactor helped to re-induce glucose uptake again when a plateau was reached. However, the consumption was not complete and another plateau was reached thereafter. This hints that additional supply of these nutrients can boost glucose consumption and might also increase xylan degradation and therefore cellulase secretion. The final mycelium dry weight measurements reveal that Reactor 2 had the highest biomass. This is not surprising, since the other reactors had signs of lysis, indicated by low Oxygen consumption rates and parallel increase in pH. Reactor 2 was the only one, which was harvested before this increase in dissolved oxygen (DO) and pH happened.

Top: early time point; Bottom: late time point
Reactor: 1 2 3
Reactor 4, Top: inoculated
Bottom: before inoculation

Figure 3.17: Images of the fermenter an cultures at early and late time points. Reactor 4 was stopped early, since it contained a contamination. A blank medium before inoculation is displayed in the bottom right image.

Reactor 1 (shift and batch, wild type)

This reactor got inoculated with wild type spores. The whole culture was shifted to the xylan medium via centrifugation after 48 hours. The process parameters are displayed in Figure 3.19. We propose partly lysing of the mycelium at around the 140 hour time point, since the DO% and pH rose.

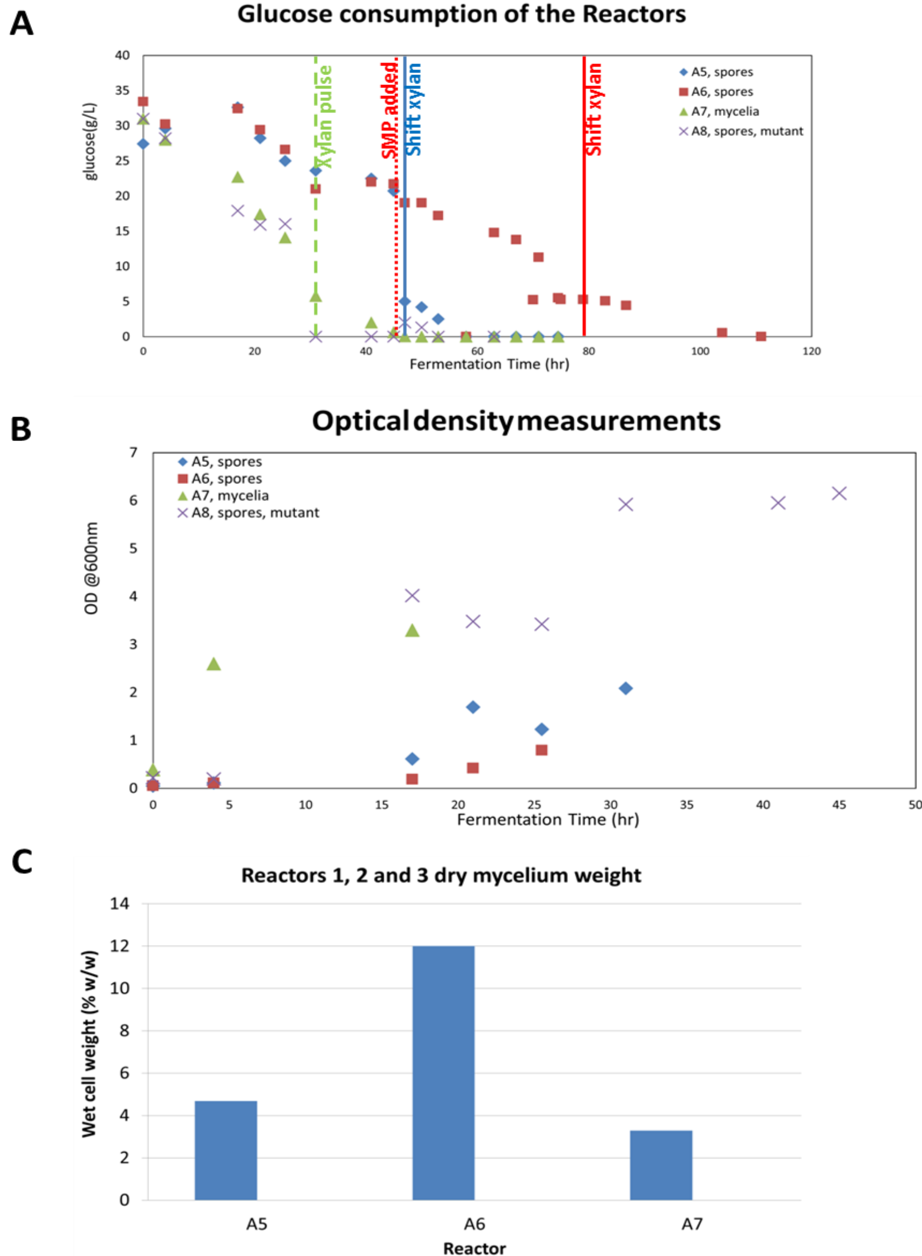


Figure 3.18: Growth indicators of *T. aurantiacus* mycelium. (A) Glucose consumption of the reactors (A5 = Reactor 1, A6 = Reactor 2, A7 = Reactor 3, A8 = Reactor 4). Colored lines indicate addition of xylan: solid lines indicate mycelium shift via centrifugation and re-suspension in xylan, dashed lines indicate addition of xylan without shifting, the dotted red line displays the addition of concentrated trace elements and soy peptone to Reactor 2. (B) OD measurements. (C) Mycelium dry weight.

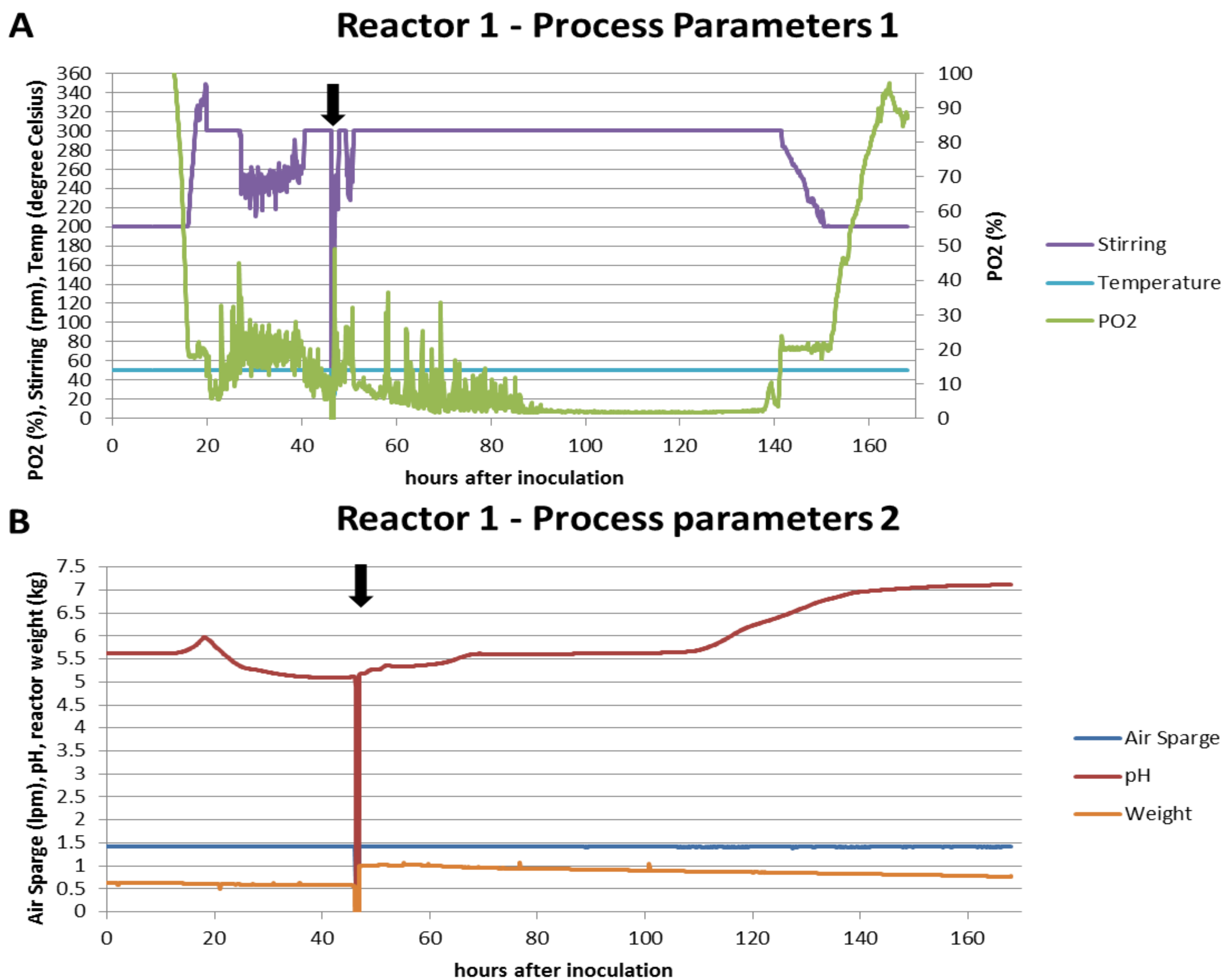


Figure 3.19: Process parameters of Reactor 1. (A) Stirring magnitude, temperature and dissolved Oxygen saturation. (B) Air sparge, pH and reactor weight. Solid black arrows indicate shifting and resuspension in xylan medium. Harsh drops of lines are due to disconnecting of the reactors for xylan medium addition.

Reactor 2 and 4 (shift and fed-batch, wild type)

These reactors got inoculated with wild type spores (Reactor 2) and mutant spores (Reactor 4). Reactor 4 had a contamination growing. The process parameters of Reactor 2 are displayed in Figure 3.20. The culture got shifted later than Reactor 1 at 78 h, since this culture consumed the glucose much slower. Additional concentrated trace elements and SMP was added only to this culture after 49 hours. The fed batch was started after 129 hours and lasted till 180 hours

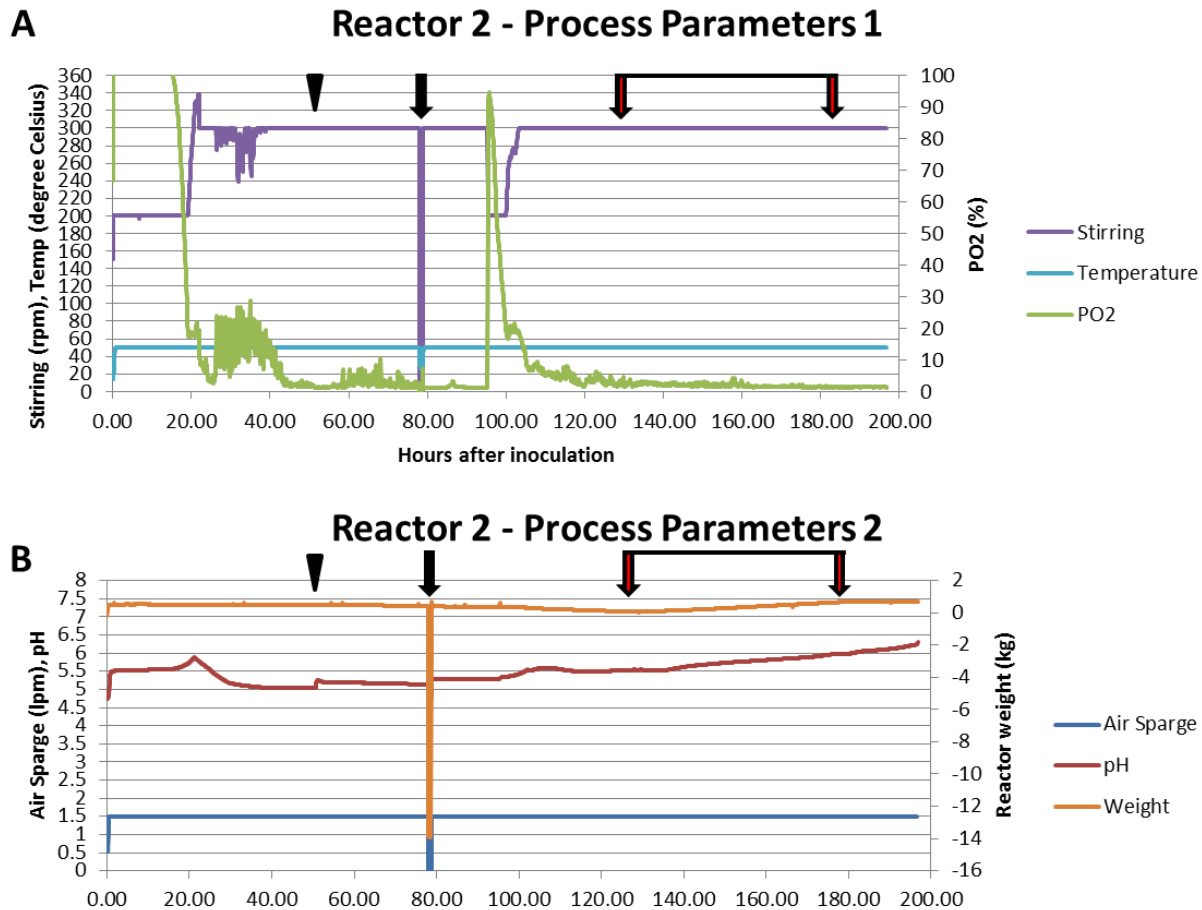


Figure 3.20: Process parameters of Reactor 2. (A) Stirring magnitude, temperature and dissolved Oxygen saturation. (B) Air sparge, pH and reactor weight. Solid black arrows indicate shifting and resuspension in xylan medium. Arrows with red fill connected by a black line indicate the span of continuous xylan medium feed at a speed of 10 ml / hour. The black triangle marks the supply of additional trace elements and soy peptone. Harsh drops of lines are due to disconnecting of the reactors for xylan medium addition.

after inoculation at a rate of 10 ml / hour. Moreover a failure during the shift happened: the air supply was not reattached and the medium was without oxygen supply until the next morning. The DO spike before 100 hours occurred when the supply was reattached.

Reactor 3 (fed-batch w/ two pulse additions; no shift, wild type)

This reactor was inoculated with pre-grown wild type mycelium. Additional medium was pulse fed after 33 hours (Figure 3.21.). A fed batch was started after 79 hours and lasted until 129 hours post inoculation at a rate of 10 ml / hour. Same as for Reactor 2, we propose lysing of the culture, since the DO also went up here after 150 hours, the pH increased as well and the dry mycelium weight was comparably low as in Reactor 1.

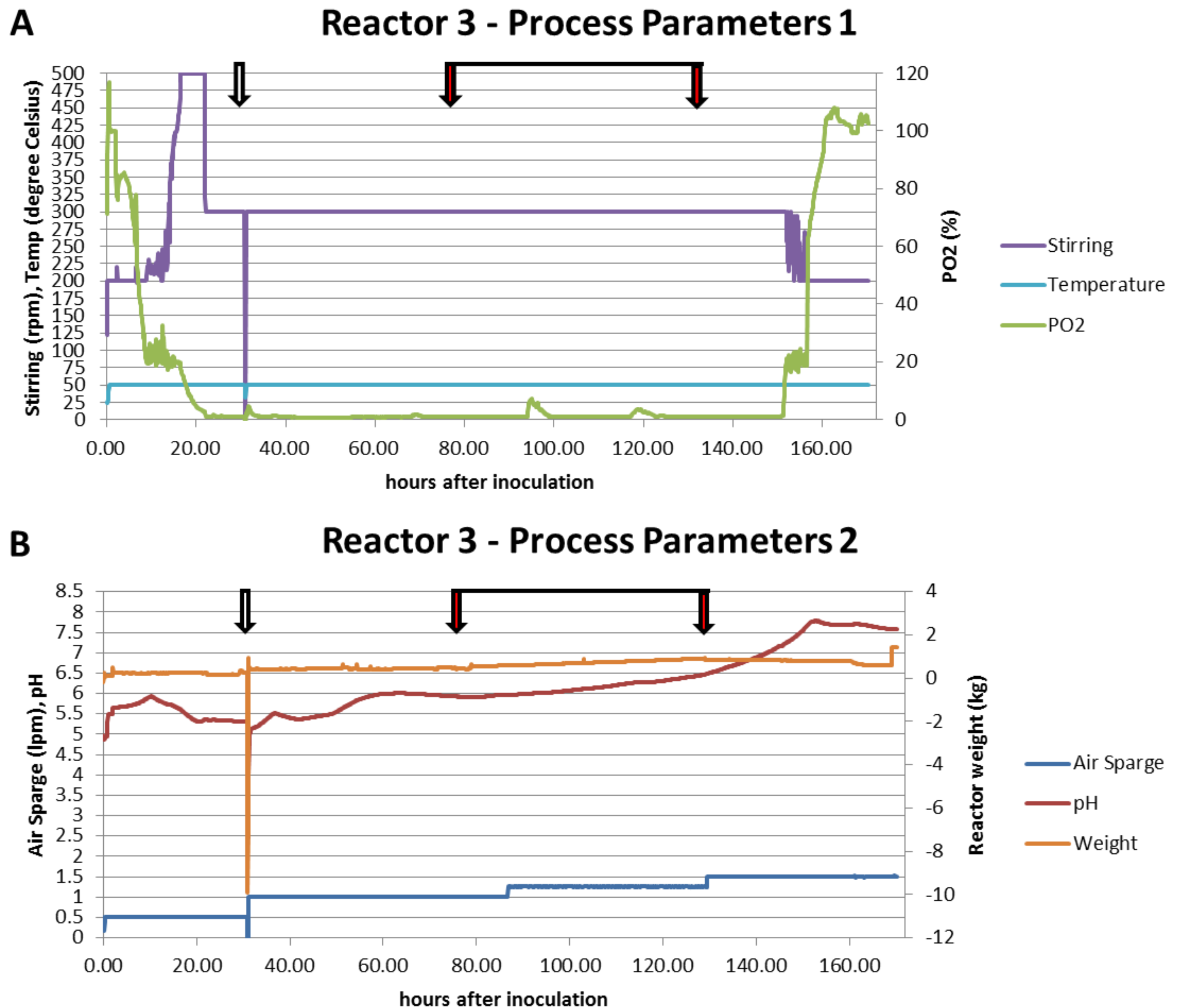
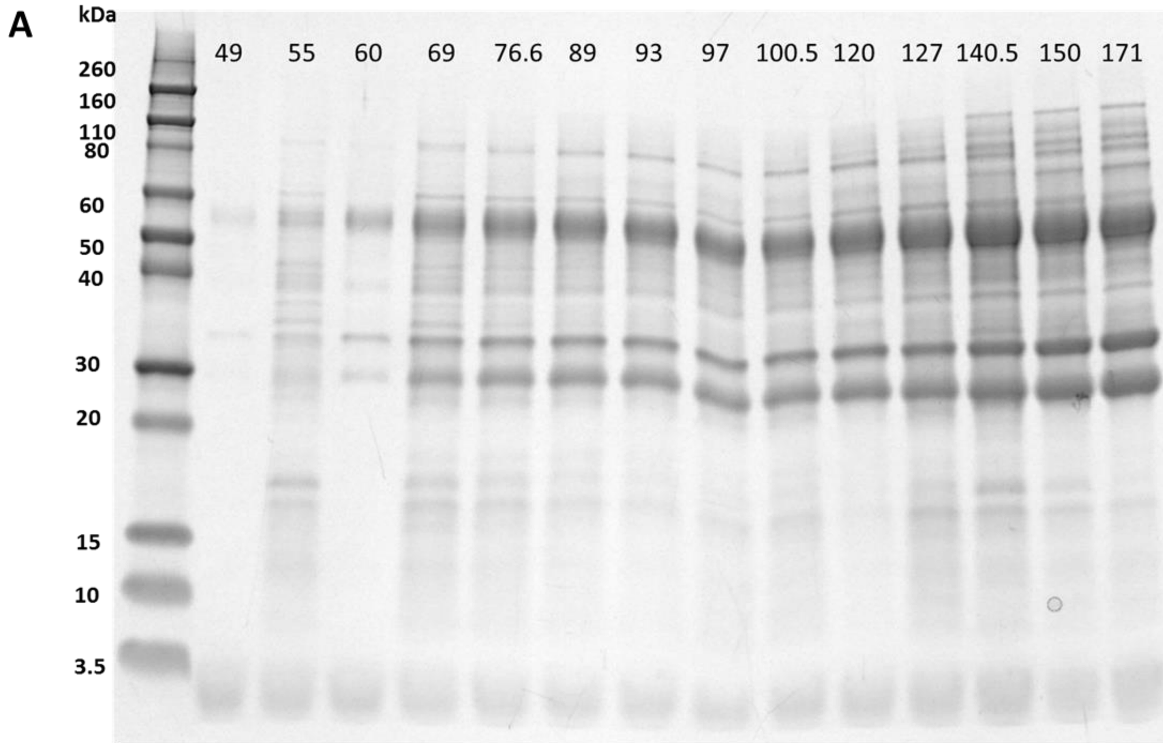


Figure 3.21: Process parameters of Reactor 3. (A) Stirring magnitude, temperature and dissolved Oxygen saturation. (B) Air sparge, pH and reactor weight. Arrows with white filling indicate pulse feeding of xylan medium. Arrows with red fill connected by a black line indicate the span of continuous xylan medium feed at a speed of 10 ml / hour. Harsh drops of lines are due to disconnecting of the reactors for xylan medium addition.

3.4.2. Protein secretion and cellulase activity

Reactor 1 (shift and fed batch, wild type)

The amount and profile of secreted protein and the cellulase rates are displayed below.



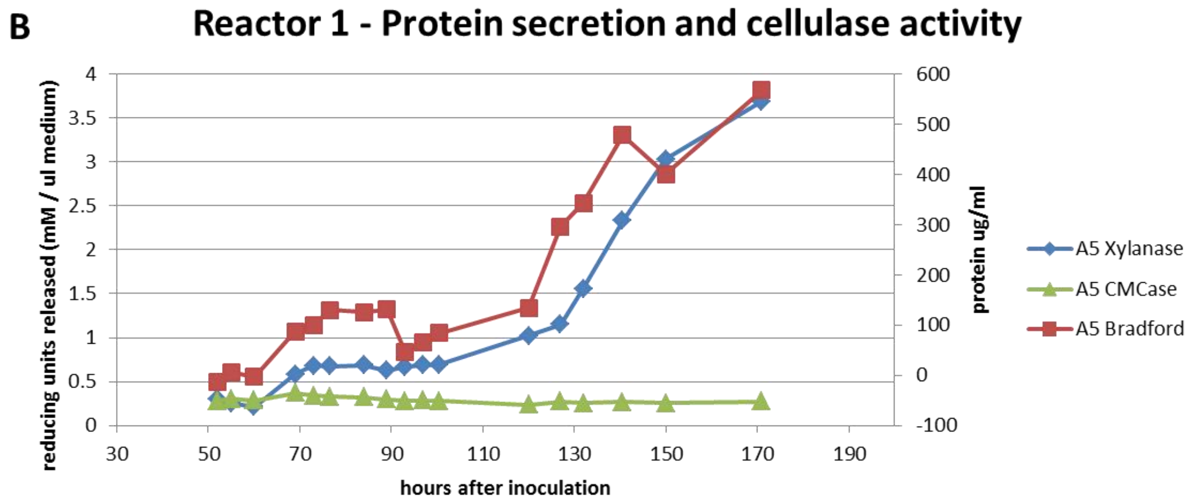
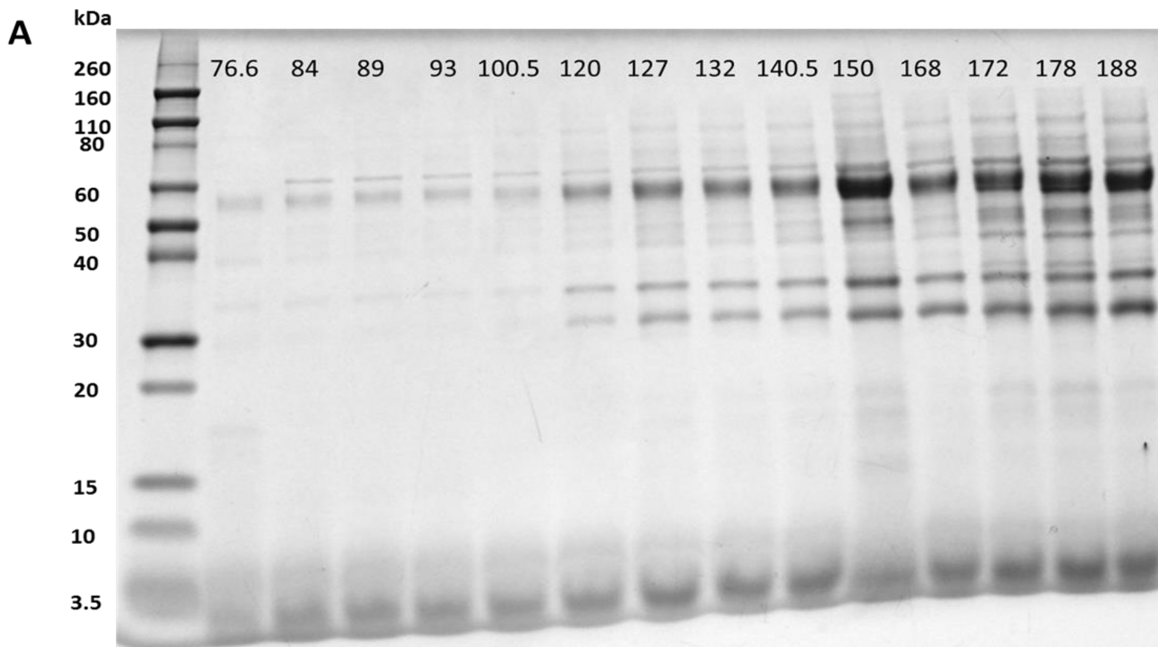


Figure 3.22: Protein secretion and cellulase activity analysis of reactor 1. (A) SDS PAGE: 7.5 ul medium were loaded onto the gel. (B) Protein amounts, CMCase and xylanase activity of Reactor 1. Bradford: 1 ul medium was used, CMCase and xylanase: 2 ul medium were used, the rates were normalized to 1 ul medium.

Reactor 2 (shift and fed-batch, wild type)



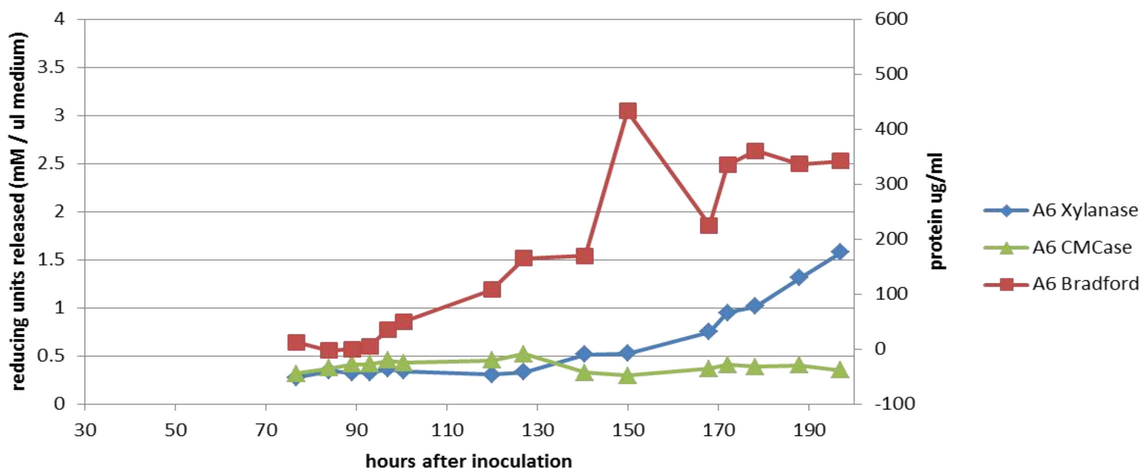
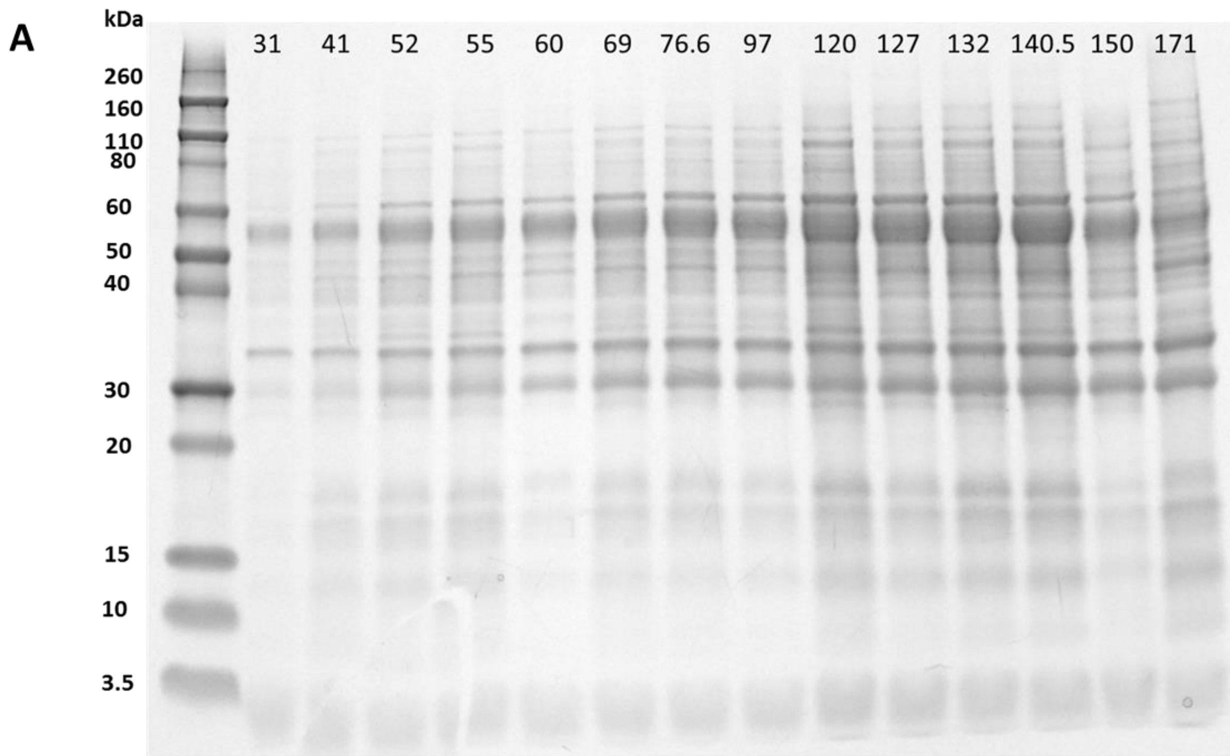


Figure 3.23: Protein secretion and cellulase activity analysis of reactor 2. (A) SDS PAGE: 7.5 ul medium were loaded onto the gel. (B) Protein amounts, CMCase and xylanase activity of Reactor 2. Bradford: 1 ul medium was used, CMCase and xylanase: 2 ul medium were used, the rates were normalized to 1 ul

Reactor 3 (fed-batch w/ two pulse additions; no shift, wild type)



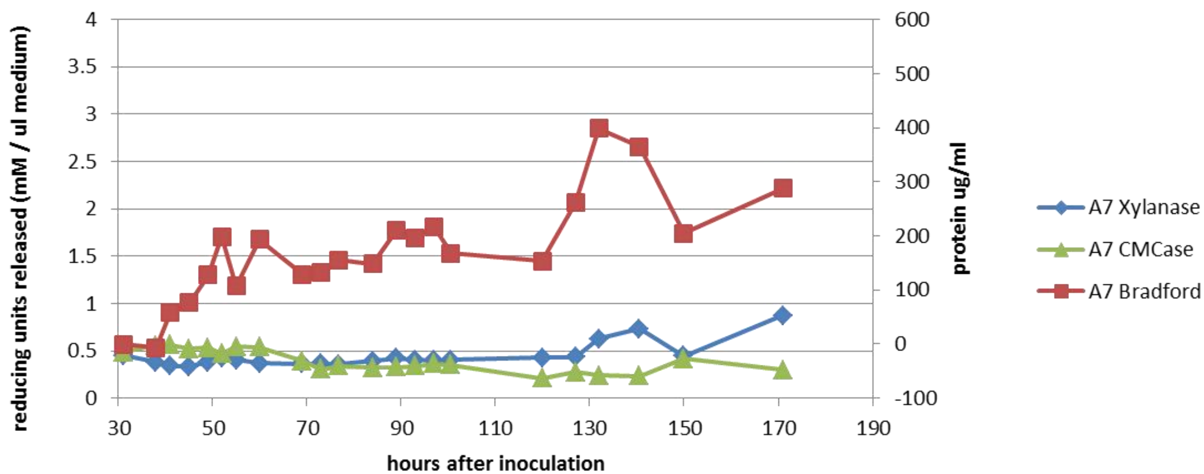


Figure 3.24: Protein secretion and cellulase activity analysis of reactor 3. (A) SDS PAGE: 7.5 ul medium were loaded onto the gel. (B) Protein amounts, CMCase and xylanase activity of Reactor 3. Bradford: 1 ul medium was used, CMCase and xylanase: 2 ul medium were used, the rates were normalized to 1 ul medium.

3.4.3. Saccharification rates

The saccharification rates of the three reactors are shown below and compared with supernatant from a shake flask experiment. In the saccharification assay also reactor 1 had the best performance (Figure 3.25. A). As a control, we used frozen, filtered supernatant from a shake flask experiment, which had high protein titers and no protein degradation. The saccharification rates of the shake flask supernatant were not so much higher. Fresh supernatant from shake flasks might have resulted in a higher conversion rate though.

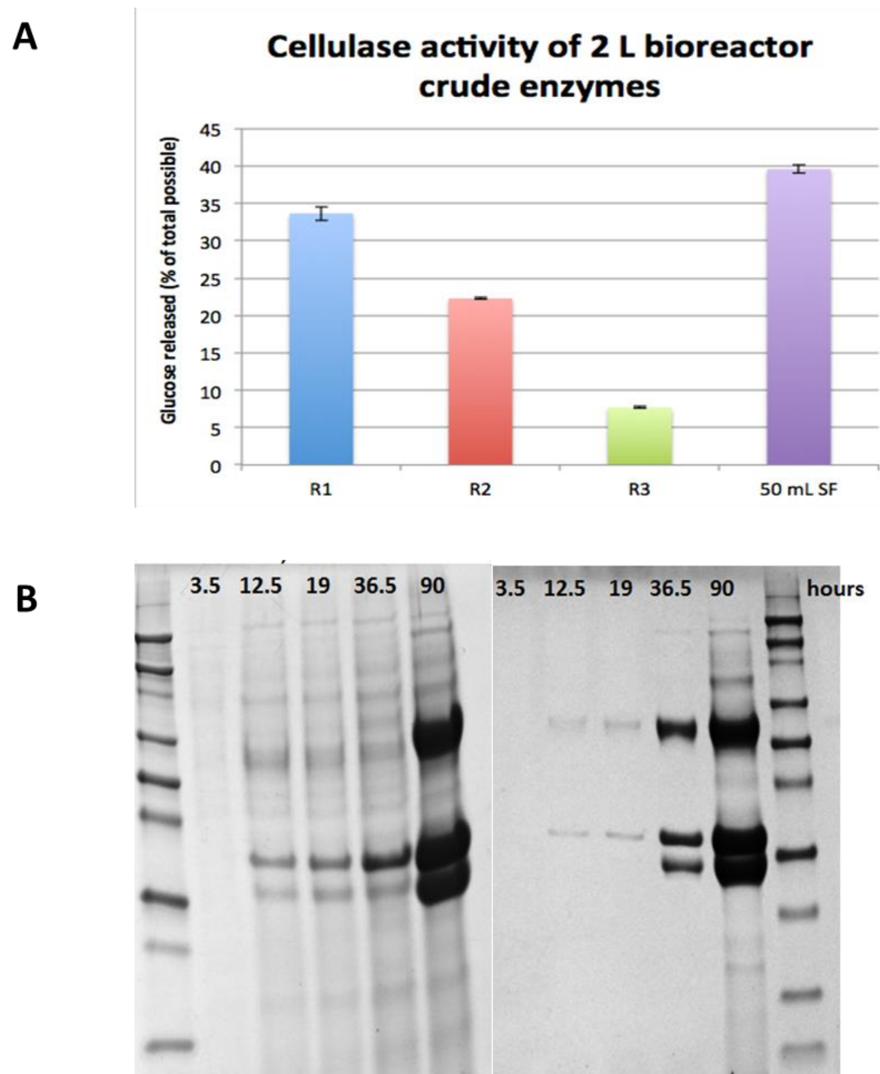


Figure 3.25: Saccharification assay of crystalline cellulose with the reactor broth filtrates, compared to shake flask broths. (A) Saccharification rates of triplicate assays with the respective reactor medium. The 50 ml shake flask medium is used as a control. Shake flask media usually have higher cellulase titers than the reactor media filtrates. (B) The protein profile from the pooled shake flask media of A is displayed (5 ul medium was loaded onto this gel). The shake flask media were taken from a frozen stock, which is why the rates are not much higher than Reactor 1 medium.

3.5. Cellulase induction in T. aurantiacus

Besides determining factors, which promote high cellulase titers in *T. aurantiacus*, we also aimed to investigate the induction response of the fungus on a transcriptional level. In parallel, the quantity and activity of secreted cellulase enzymes was measured when grown in the presence of different inducers.

3.5.1. Induction of protein production and cellulase activity in T. aurantiacus

Complementary to the transcript sequencing run, we tested the induction potential of several C sources in regard to stimulation of cellulase secretion and activity. Suitable inducers will be targets for further transcript sequencing runs in the future. The inducers used in this shift experiment were beech wood xylan, Avicel, xylobiose, xylose, MXP compared to glucose and no carbon. As N source in the shift medium, ammonia nitrate was used, this exerts a minimal influence on the cellulase response compared to SMP. Pre-cultures were grown with SMP to facilitate sufficient biomass gain.

Xylan (2%) and MXP (0.1%) lead to high levels of protein secretion, while xylobiose (0.1%) and xylose (0.1%) again failed to induce cellulase expression (Figure 3.26.). This is in accordance with the previous experiments. Interestingly, Avicel lead to high levels of protein secretion as well, while in all previous experiments with SMP as an N source, no induction occurred. We hypothesized that autoclaving with ammonia nitrate acts as a pretreatment mechanism, which modifies Avicel into an inducer for this fungus. We tested protein secretion rates by adding ammonia nitrate before and after autoclaving (Figure 3.26 B, red bars). Autoclaving Avicel with ammonia nitrate seems to promote protein secretion. The xylanase activity was highest in the MXP medium followed by the xylan medium. The Avicel medium has lower rates, probably due to low xylanase secretion. The glucose cultures with ammonia nitrate are fully repressed and lack all major cellulases. With SMP as an N source, glucose cultures tend to express low levels of cellulases after 48h (Figure 3.11 C). The CMCase activity is high in Avicel, xylan and MXP cultures. On the SDS PAGE, the presence of the major cellulase bands is in accordance with the Bradford and the DNS assays. Signs of protein degradation can be detected for the CBH1 bands of the xylan samples already after 24h. The protein degradation was visible on all samples after 48h and huge degradation happened at the 72h time point (Figure Appendix 1.).

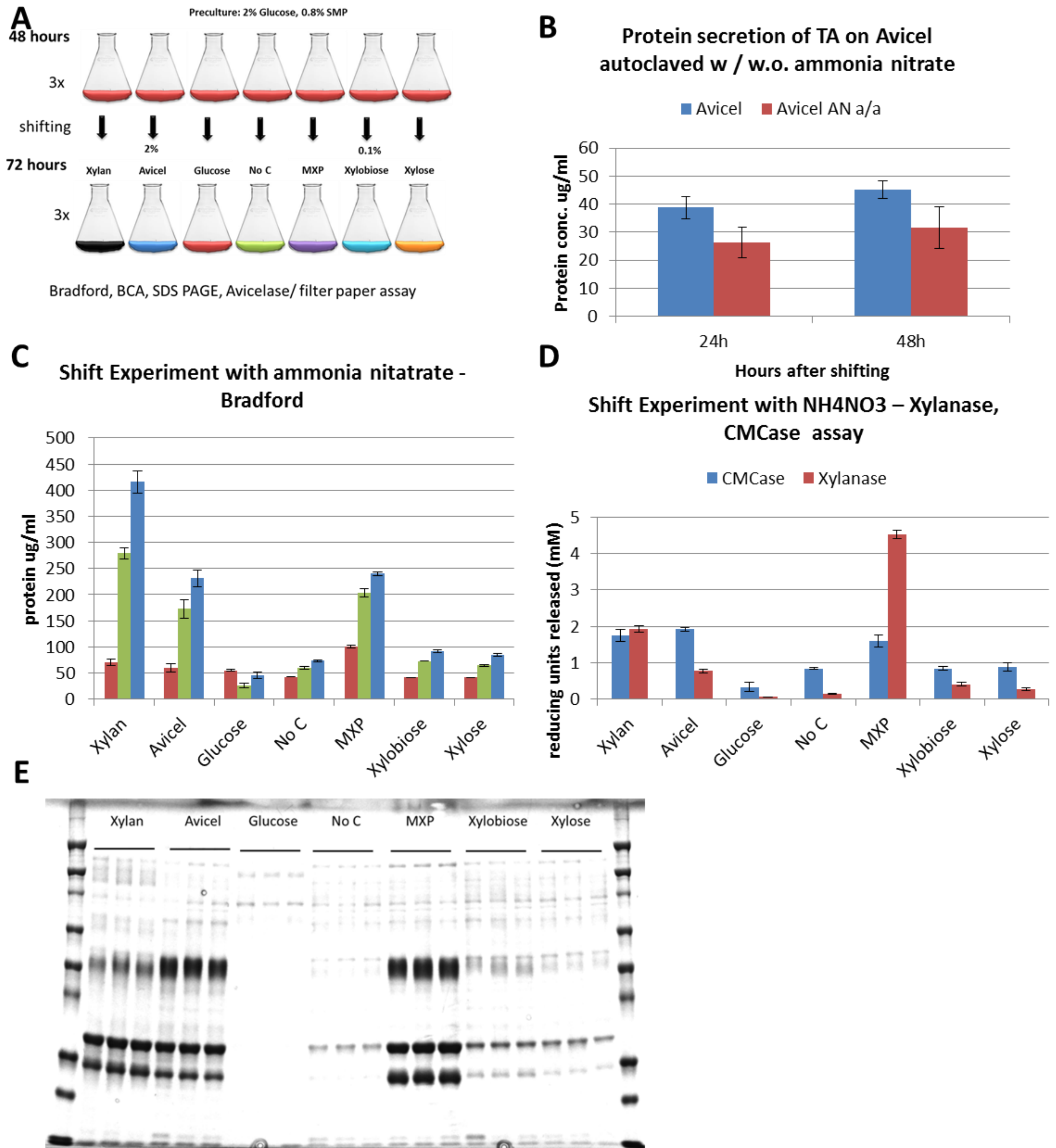
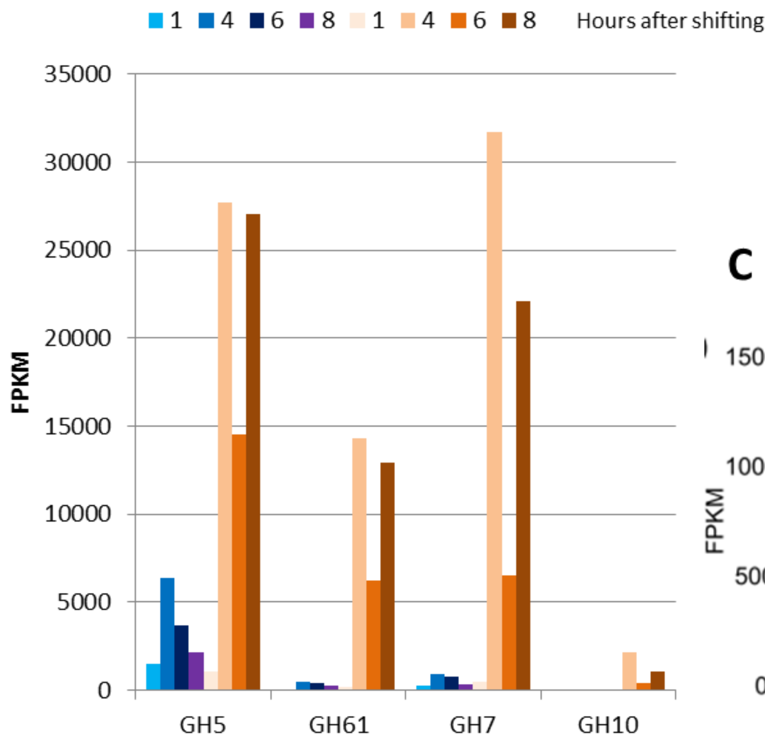


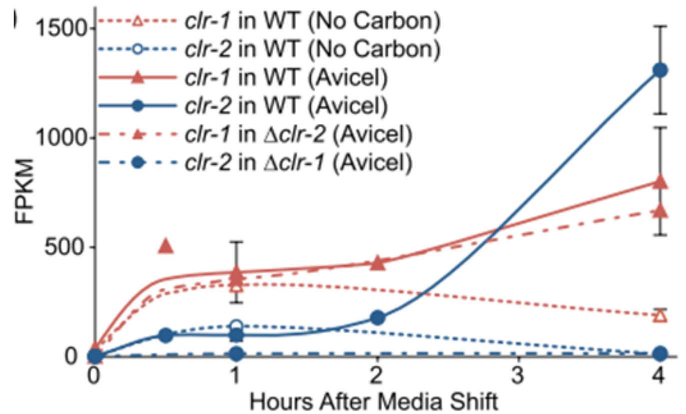
Figure 3.26: Inducer shift experiment with beech wood xylan, Avicel, MXP, xylobiose, xylose, glucose and no carbon with ammonia nitrate as an N source in the shift medium. (A) Experimental set-up. (B) Potential inducing effect of Avicel when autoclaved with ammonia nitrate (AN a/a = ammonia nitrate added after autoclaving) (C) Bradford of assay of sampling time course (red 24h, green 48h, blue 72h; n = 3). (D) Xylanase and CMCase assay of the 48h time point (xylanase 5 ul 1:5 diluted; CMCase 20ul undiluted, n = 3). (E) 24h time point samples visualized on a SDS PAGE: (15 ul supernatant loaded with 4x loading dye = virtual11.25ul undiluted supernatant).

Ammonia nitrate in the medium seems to be a poor nitrogen source and *T. aurantiacus* seems to use proteases to degrade its own enzymes to counteract N starvation.

3.5.2. Transcriptional response of **T. aurantiacus** *towards beech wood xylan*

The induction patterns of the glucohydrolases GH3 (beta-glucosidase), GH5 (endoglucanase), GH61 (polysaccharide monooxygenase), GH7 (cellobiohydrolase) and GH10 (xylanase) in the no carbon and xylan cultures are displayed in Figure 3.27.A and Figure 3.28. These cellulases are strongly expressed, with the relative strongest expression of the xylanase (Figure 3.28. A,C). The increase in transcript number of the transcription factors Clr1, Clr2, ClrB, Cre1 and XlnR and the S10 peptidase are shown after the shift to the no carbon medium (blue bars) and the xylan medium (brown bars) at 1, 4, 6 and 8h after shifting. The induction of the Clr1 and Clr2 genes in *T. aurantiacus* are compared with the upregulation of these two genes in *N. crassa* in Avicel medium (Figure 3.27.C). It has to be noted, that the values from the time point 3 (6h after shifting) in the xylan cultures are consistently lower than the other time points. All samples from this time point stem from the same culture. Thus, this culture might be compromised in a way, other than the remaining cultures.





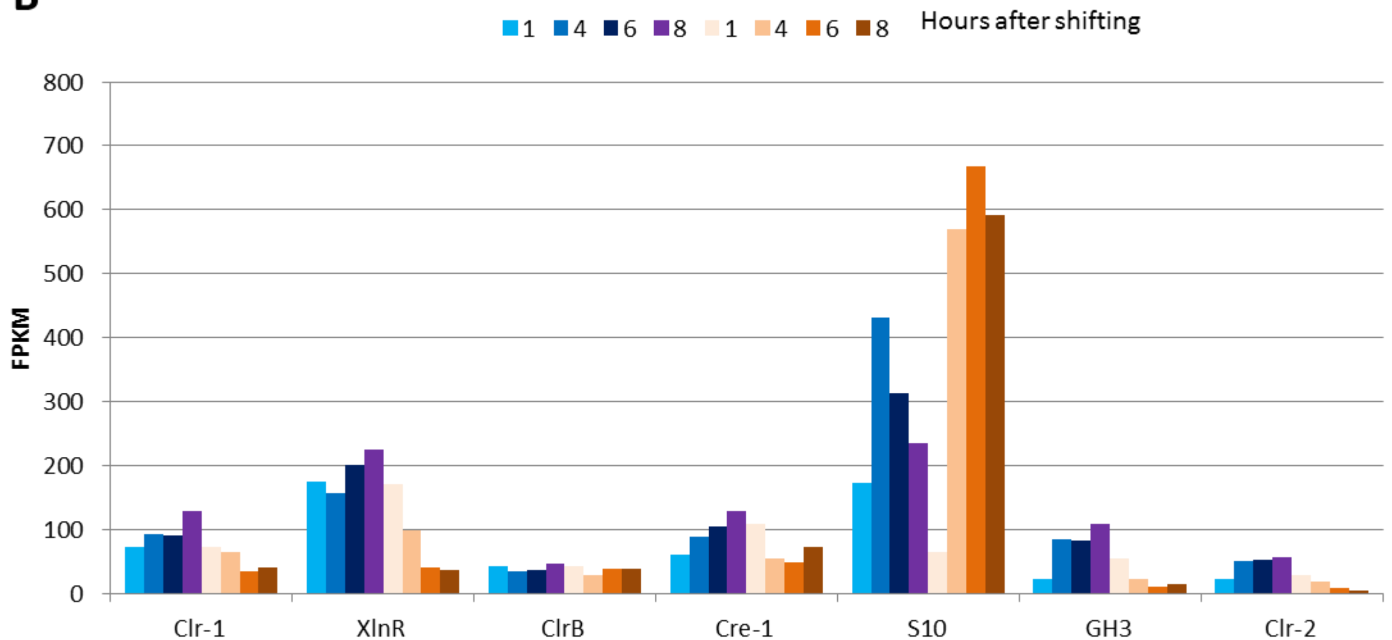


Figure 3.27: Transcript sequencing experiment: (A, B) Transcript upregulation of selected cellulases and transcription factors in T. aurantiacus in FPKM, 1, 4, 6 and 8 hours after shifting to either no carbon medium (blue) or beech wood xylan medium (brown). (C) Increase of the Clr-1 and 2 genes in N. crassa after shifting to no carbon medium or cellulase inducing medium (Avicel); Coradetti et al. 2012, PNAS.

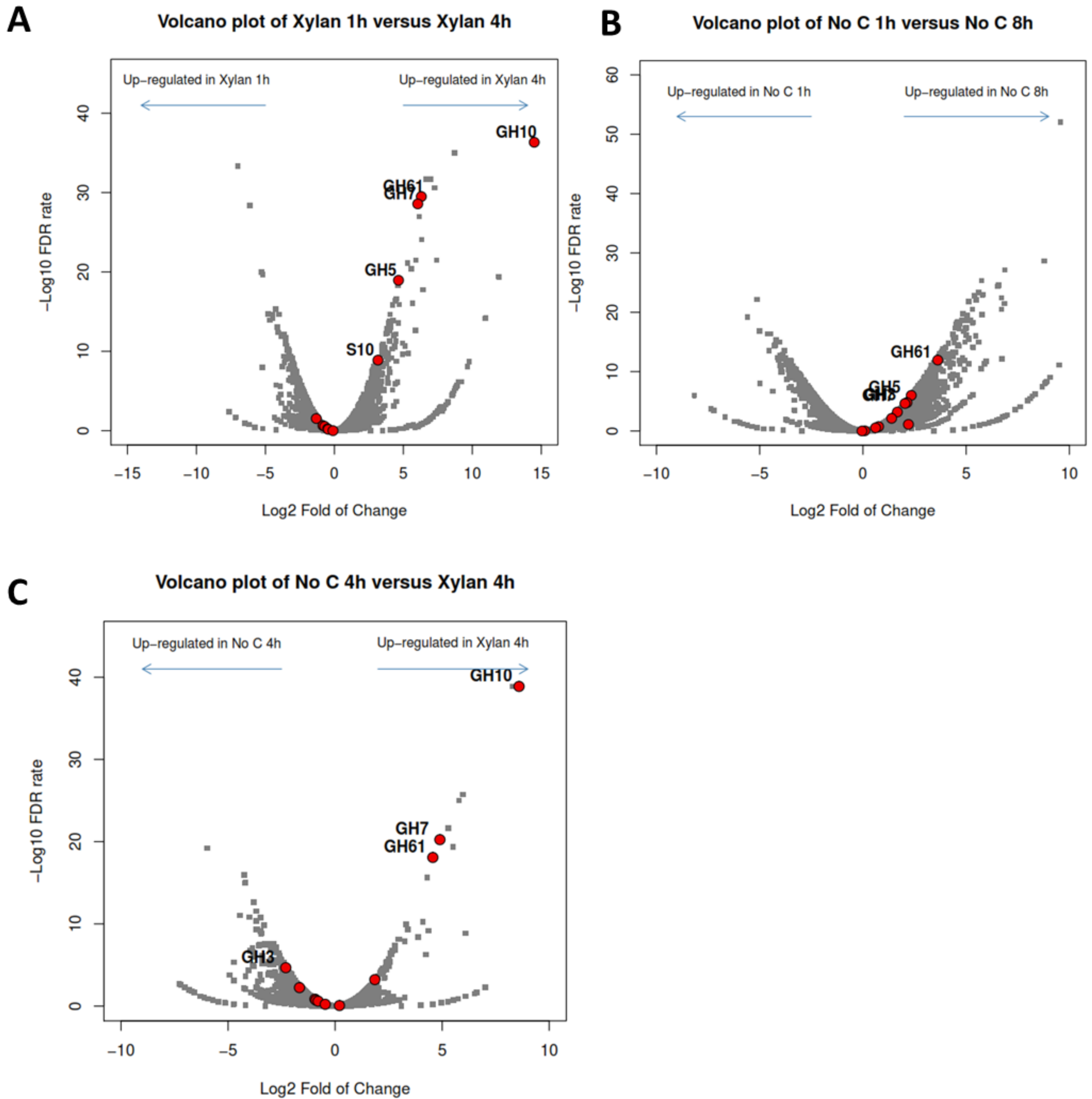


Figure 3.28: Transcript sequencing experiment volcano plots: upregulation of selected genes in xylan cultures after 4/8 hours after shifting (plotted by Yu-Wei Wu).

4. Discussion

Growth physiology of *T. aurantiacus*

The tests on the growth behavior confirmed again that *T. aurantiacus* strain 26904 grows well at 50°C and moderate pH around 5 to 7. This strain is very sensitive to Phleomycin, though the agar medium does not support the growth of the fungus very well. This might be due to the high pH of 8.0. Changing the medium composition might turn Phleomycin in a suitable selection marker, once the control plates exhibit better growth. The Basta resistance of this fungus (up to 1000 ug / ml) was confirmed in several plate experiments.

The ascospore germination kinetics revealed an interesting trend: after 5 to 6 days, the germination rate reaches maximum and levels off rapidly at day 7. This kinetic was observed in two additional experiments. Different results were obtained in a study, where *T. aurantiacus* ascospores were harvested from slant cultures on yeast glucose agar over a time frame of 40 days at 47.5°C[12]. The maximum germination rate in this study (10%, 28 days) is much lower than in our experiment (42.5%, 5 days; Figure 3.4 A) and at a much later time point. Culturing on PDA plates might be the cause of the differences. Another fascinating aspect is the influence of spore titer on propagule morphology. Our experiments suggest that high spore titers are more beneficial to have plenty of small propagules, which all together comprise a high surface area. The cause for this difference in growth morphology is not clear yet. It could be a physical mechanism that accumulates most spores in the flask area, in which the liquid is spinning at the glass wall, where a dense ring will form and only the remaining spores will germinate in the medium. Only spores that cannot attach to the glass wall will reside in the liquid and germinate. With increasing spore titers, the mycelial ring obviously saturates at some point, leading to increased amount of submerged propagule. The higher the amount of submerged propagule, the higher the competition for nutrients and dissolved oxygen, which ultimately leads to smaller sized propagule particles. Another explanation might be a reduced germination rate in the liquid media versus a higher rate at the moistened glass surface area within the flask. Only few spores will germinate in the liquid while plenty of spores with a faster growth than in the liquid will germinate at the moistened glass surface and produce the ring. Therefore, only a small fraction of spores germinates in the liquid medium and high titers are needed to increase this fraction. This finding could be of relevance for shake flask and fermenter experiments. Understanding the underlying mechanisms will ultimately allow to control the propagule morphology.

Media optimization for cellulase hyperproduction

Our efforts for generating a medium which promotes higher titers of *T. aurantiacus* cellulases revealed that McClendon salts containing 1000x McClendon trace elements without precipitation and SMP as an N source promotes highest biomass production. The most promising C source turned out to be beech wood xylan, which induced all major cellulases and the xylanase. Another promising inducer is MXP, which also yields high titers of all major cellulases. However, this inducer cannot be metabolized and will starve the fungus without appropriate C source. Once a transformation protocol is established and a suitable catabolite de-repressed strain is generated, the most promising medium combination will be a low glucose medium with MXP as an inducer. This might lead to continuous secretion and high titers of cellulases. Additional suitable inducers were also xyloglucan and wheat bran.

Strain optimization of *T. aurantiacus* for cellulase hyperproduction

Historically, high cellulase secreting fungal strains were generated by random mutagenesis and sexual crossing of mutant strains. Genetic engineering of targeted gene modification is a promising tool for selectively improving traits without conferring deleterious mutants caused by random mutagenesis[13–15]. Our random mutagenesis efforts did not yet lead to the isolation of a clearly catabolite de-repressed strain. The isolation procedure by plating mutagenized spores on CMC-DOG plates can be improved by using a more stringent DOG concentration to select more strongly for these mutants. Moreover, performing the screening also on xylan-DOG plates might be beneficial, since this will also select for mutants, which grow very well on xylan. *T. aurantiacus* ascospores are highly sensitive towards UV. Mutagenesis with EMS does lead to healthier spores as indicated by longer hyphal growth after plating. Further improvement is feasible in the screening assays. Using SMP for growing mutant strains in glucose and inducer media does not allow an accurate quantification with photometric protein assays such as Bradford, since some mutants grow slowly. These mutants consume less SMP compared to the WT. In order to obtain values for protein secretion per mg biomass, a different N source in the inducing medium needs to be employed. We already tested medium containing 89 mM Glycine and 10 mM Asparagine (= 0.8% org N). This molarity of both amino acids is compatible with the Bradford assay. Moreover, the biomass of the mutants and WT can be adjusted during shifting to have the same amount of inoculum.

Transformation of *T. aurantiacus* was not achieved yet. Successful protoplasts were obtained though (not shown). Another possibility would be the transformation via

Agrobacterium tumefaciens, which was successfully employed for transforming the thermophilic fungus *Myceliophthora thermophila*[16].

2 L bioreactor enzyme production with *T. aurantiacus*

Reactor set-up: The most productive reactor, based on activity and protein secretion, was reactor 1 (shift and batch, wild type). This reactor set up however, with the shift of the mycelium in between, is not feasible for further upscaling. A suitable alternative might be to culture *T. aurantiacus* in a glucose low-xylan medium as a pre-culture to induce right away, once the glucose concentration becomes low. Afterwards a xylan medium will be added. The set-up of Reactor 3 (fed-batch w/ two pulse additions; no shift, wild type) was very different from the other reactors. It had the smallest pre-culture volume (0.5l) and consumed the glucose very fast. This setup is very favorable for future bioreactor conditions since no shifting is required and the cellulase production starts already after 30 hours. The main challenge here is that mycelium ring formation is higher than in the other reactors. This can be circumvented by using a start volume of 0.75l instead, which avoids splashing through the turbine. In the future, we want to avoid shifting, because it increases the chance of getting contaminations, technical problems and it seems to cause stress to the fungus. In the future, we need to set up the fermenter runs to allow for mass balance analyses to account for medium losses due to evaporation or other factors.

Media and process improvement:

The overall yields of cellulases and their activity were lower in this experiment compared to previous shake flask experiments (288 – 568 ug/ml in the reactors compared to roughly 800 ug/ml in shake flasks corrected for evaporation, Figure 3.11 E). The differences are also apparent on SDS PAGE gels. We plan to decrease the initial 3% glucose of the pre-culture medium to 2% in the future and add the remaining amount together with SMP in concentrated form when the sugar concentration drops. The overall glucose consumption was lower than expected in all reactors. Sufficient biomass is necessary to allow the fungus to secret high titers of cellulases. However, there might be a biomass optimum and it is not clear whether more biomass always improves cellulase secretion.

We argue that the high stirring rates in our set up might have caused stress to the fungal cultures. We employed a Rushton turbine impeller. However, a helical impeller might cause less stress to the culture and allow for better mixing. In the future, at least one culture should be

stirred with this type of impeller at much lower rates. Reducing stirring to 200 rpm might be sufficient as well.

Cellulase induction in *T. aurantiacus*

As mentioned above, xylan and MXP are potent inducers of cellulases of this fungus. Moreover xyloglucan and wheat straw also turned out as valuable inducers. The induction of Avicel when autoclaved with ammonia nitrate is an interesting phenomenon. Accordingly, Avicel might be a suitable inducer for *T. aurantiacus*, despite ammonia nitrate being an inappropriate N source when aiming for high cellulase titers. The N source is an important prerequisite for high cellulase titers. We argue that SMP is hydrolyzed by an exogenous peptidase and taken up for growth and cellulase production. In case of ammonia nitrate however, N starvation happens due to poor utilization of this N compound and the protease activity is increased to utilize the secretome as an N source. Another explanation is that SMP maybe is just inducing stronger due to plant derived inducers, which might be in it. The sooner or later both, SMP and ammonia nitrate cultures should be N starved. In case of SMP, a smaller amount of peptidase might be sufficient in order to counterbalance starvation, due to the fact that there is much more extracellular protein around to feed on.

The transcript sequencing test run revealed, that already after 1h, several glucohydrolases (GH3, GH5, GH61, GH7 and GH10) are already fully induced. None of the transcription factors was upregulated under both conditions. The S10 peptidase increased considerably in transcript number at 4 hours in the xylan and no carbon cultures (Figure 3.27.). The cellulases GH5, GH61 and GH7 were significantly upregulated in the xylan cultures. Interestingly, these cellulases were much higher upregulated compared to the GH10 xylanase regarding FPKM. Nevertheless, the xylanase is the relatively highest upregulated hydrolase in the xylan cultures after 4h compared to the 1h time point, followed by GH61, GH7, GH5 and finally S10 (Figure 3.28.A). The upregulation of these gene transcripts is reflected by the high protein amounts of the respective bands in the SDS PAGE. Only GH3 is downregulated in the xylan cultures, which makes sense, since xylan degradation does not result in significant amounts of cellobiose. In the No carbon medium, GH61 is highest upregulated after 8h compared to 1h (Figure 3.28.B). The GH 10 is also the relatively highest upregulated gene when comparing the no carbon and xylan cultures at the 4 hour time point (Figure 3.28.C).

The cellulase upregulating transcription factors Clr1 and Clr2 were not upregulated, since these regulators are expected to be triggered by cellulose degradation products. In *N. crassa*, these regulators were induced in Avicel cultures compared to no carbon media, reaching high

FPKM values after 4h[17] (Figure 3.27.C). It remains to be investigated, whether cellulose induces these genes in *T. aurantiacus* in the same way. However, the regulator XlnR was also not upregulated. In *Aspergillus spp.* and *T. reesi*, this regulator is responsible for the upregulation of cellulases and hemicellulases[18]. Thus, this regulator seems not to be induced in *T. aurantiacus* by xylan either. The same is true for CblR, which is a conserved transcriptional inducer in cellulytic ascomycete fungi[19]. As expected, the catabolite repressor protein Cre-1 is downregulated in both media, since no high amounts of monosaccharides are present. The regulation pattern indicates that other transcription factors and signaling pathways than XlnR, CblR, Clr1 and Clr2 are involved in the induction process of the cellulases and the xylanases. The detection of these unknown transcriptional regulators will be in the focus of deeper transcriptome analysis with the ultimate goal to unravel the induction mechanism of xylan on *T. aurantiacus* cellulases. Moreover, future experiments might also aim to capture a 30min time point after shifting to have a time point, where not all carbon degrading enzymes are fully upregulated.

5. Outlook

Future research will focus on further increasing the protein titers of *T. aurantiacus' secreted* cellulases. Upcoming fermenter runs on a 2L scale will employ the lessons learned for improving the culturing of this fungal strain. During the first week of March 2016, the second fermenter run took place and protein concentrations of 3.2 g/L were achieved. Therefore, the second run was more successful compared to the first one summarized in this report. Additional attempts to transform *T. aurantiacus* will be taken to finally obtain a catabolite de-repressed strain, capable of secreting higher titers while feeding it with a low cost carbon source. Plasmid construct for Cre1 knockouts, the gene responsible for catabolite repression in many ascomycetes, were already designed. Moreover, additional transcript sequencing runs with additional biological replicates obtained from xylan and no carbon cultures and new sampling of mycelia shifted to MXP, Avicel and glucose will further elucidate the transcriptional response of this fungus to different carbon sources.

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Appendix

50X Vogel's salts

From Microbial Genetics Bulletin 13:42-43, 1956.

Can be obtained online at the FGSC *N. crassa* protocols site.

In 700 mL of distilled water, dissolve successively with stirring at room temperature. **You must completely dissolve each component before adding the next one!**

Ingredient	Amount	Notes
Na_3 citrate, 5 1/2 H_2O	150 grams	(NOTE, you may substitute 125 g Na_3 Citrate 2 H_2O)
KH_2PO_4, anhydrous	250 grams	
NH_4NO_3, anhydrous	100 grams	
$MgSO_4$, 7 H_2O	10 grams	

$CaCl_2$, 2 H_2O	5 grams	**Dissolve $CaCl_2$ in 50 mL of distilled water first before adding to the solution**
Trace Element Solution (see below)	5 ml	
Biotin Solution (see below)	2.5 ml	

The resulting total volume is about 1.00 liter. Chloroform (2 ml) is added as a preservative. Store at room temperature.

Trace Element Solution

The trace element solution (containing citric acid as a solubilizing agent) is made up as follows: In 95 ml. distilled water, dissolve successively with stirring at room temperature:

Ingredient	**Amount**
Citric acid, 1 H_2O	5 grams (if anhydrous add 4.57 g instead)
$ZnSO_4$, 7 H_2O	5 grams
$Fe(NH_4)_2(SO_4)_2$, 6 H_2O	1 gram
$CuSO_4$, 5 H_2O	0.25 grams
$MnSO_4$, 1 H_2O	0.05 grams
H_3BO_3, anhydrous	0.05 grams

Na_2MoO_4, 2 H_2O	0.05 grams

The resulting total volume is about 100 ml. Chloroform (1 ml.) is added as a preservative, and the trace element solution is stored at 4°C.

Biotin Solution

The biotin solution is prepared by dissolving 5.0 mg biotin in 50 ml distilled water. The solution obtained is dispensed in test tubes and stored at -20°C.

McClendon Salts

Name: Timo Schuerg

Modified from: Shara McClendon et al 2012

Date Modified: October 18th 2015

Thermophilic Fungi Minimal Medium

Component I: 1 x McClendon salts

20 ml of 50 x McClendon salts A

20 ml of 50 x McClendon salts B

800 ml of DI-H_2O

Adjust pH to 5.0 and bring volume to 1 L.

Note: McClendon salts A form a brown precipitate. Make sure to mix well before use in order to suspend precipitate.

Component II: Organic nitrogen source

Weigh soy meal peptone (N454, Amresco, Ohio) directly into culture flask to a final concentration of 0.8 % (w/v). For example, to prepare 50 mL culture medium, add 0.8 g of soy meal peptone to a tared 250 mL Erlenmeyer flask. Alternatively make up a bigger batch in a bottle, autoclave, mix by stirring, and divide to sterile flasks under the hood.

Component III: Carbon source

Weigh biomass and add to culture flask, typically we use 1% (w/v) biomass loading. For example, to prepare 50 mL culture medium, add 0.5 g biomass (pretreated switchgrass, MCC, CMC, etc) to the 250 mL Erlenmeyer flask.

Procedure

1. Prepare Component I in a beaker and mix by stirring

1. Add Component II (nitrogen source) to culture flask.

2. Add Component III (carbon source/biomass) to culture flask.

3. Add 50 mL Component I to a 250 ml baffled culture flask.

4. Autoclave and allow to cool before inoculating with fungus.

5. For *Thermoascus aurantiacus* growth, incubate fungus at 50°C with shaking at 150-180 rpm

50 x McClendon salts A (- Phosphate)

	per liter	Final concentration in 1 x
Nanopure H_2O	600 ml	
NaCl	100 g	34 mM
1M $MgCl_2 \cdot 6H_2O$	125 ml	2.5 mM
1M $CaCl_2 \cdot 2H_2O$	35 ml	0.7 mM
Trace elements*	50 ml	
Bring volume to 1L		

50 x McClendon slats B (Phosphate salts)

	per liter	Final concentration in 1 x
Nanopure H_2O	600 ml	
KH_2PO_4 (monobasic)	150 g	22 mM
K_2HPO_4 (dibasic)	100 g	11 mM
Bring volume to 1L and pH to 5.0		

NOTE: When preparing the 50 x salts solution, add each component one at a time and wait until it is completely dissolved before adding the next component. Liquid stocks of magnesium chloride and calcium chloride should be used to avoid precipitation.

<u>*1000x Trace elements (Stock in Jen's Deli Fridge):</u> ddH_2O, 100 ml; $ZnSO_4\bullet 7H_2O$, 0.15g; $MnCl_2\bullet 4H_2O$, 0.79g; $FeSO_4\bullet 7H_2O$, 0.11g; $CoCl_2\bullet 6H_2O$, 0.02g; $CuSO_4\bullet 7H_2O$, 0.64g. Add H_2SO_4 and adjust pH to 1 if necessary to get Fe and Cu into solution.

Cellulase induction in T. aurantiacus – Time course

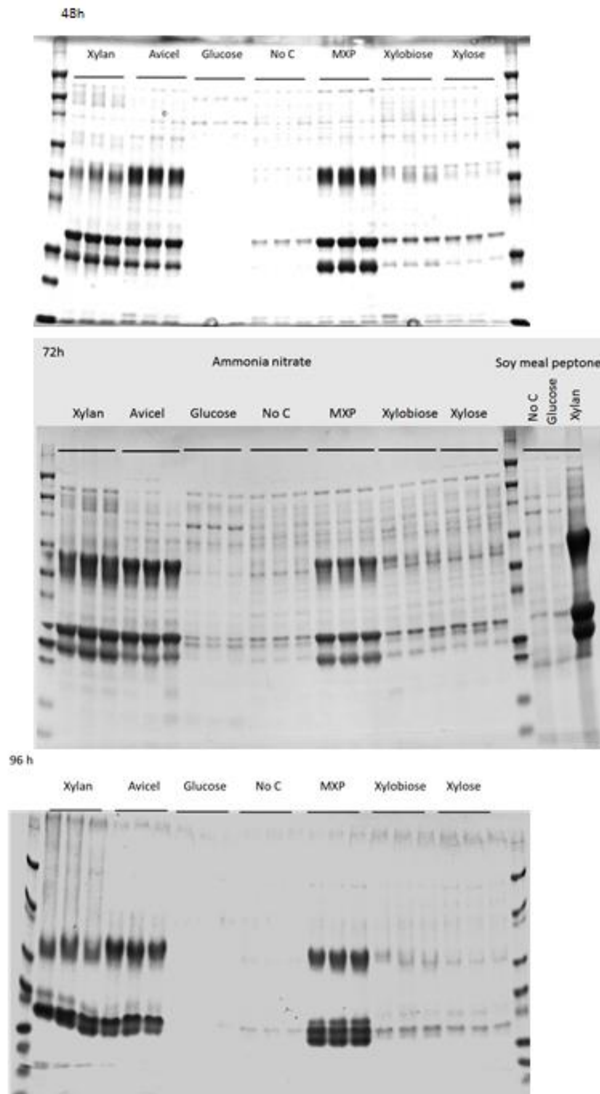

Figure Appendix 1: Inducer shift experiment with beech wood Xlan, Avicel, MXP, Xylobiose, Xylose, Glucose and no Carbon with ammonia nitrate as a N source in the shift medium. (A) 48h (B) 72h and (C) 96h time point samples visualized on a SDS PAGE: (15 ul supernatant loaded with 4x loading dye = virtual11.25ul undiluted supernatant).

RNA Extraction protocol

Total RNA Extraction

1. harvest mycelia though miracloth or filter. Lyohilize the tissue over night in lyophilizer
2. Ground the tissue with sterilized toothpick or mortal/pestle (large volume). Adjust the total amount of mycelia = 100ul equivalent
3. Add 1ml of Trizol (Life Technologies). Mix well with mycelial tissues using toothpick
   *You can stop the extraction at this step and keep samples at -80C
4. Add 200ul of PCI (phenol:chloroform:IAA)
5. Mix well with tube stand and leave for couple min at RT
6. Spin at 12000rpm for 15 min at 4C or cold room
7. Transfer aqueous layer to new tube, ~400ul
8. Add the equal volume of PCI and mix well again
9. Repeat 2 times of step 6-8.
   *At the final PCI step, you need to carefully transfer upper aqueous layer, ~300ul. This should be equal volume of all samples = makes HUGE differences of final RNA concentrations and quality (wavy bands) for Northern
10. Add 500ul of isopropanol and mix well, leave at RT for 5 min
11. Spin at 12000rpm for 10 min at 4C
12. Decant supernatant and add 1ml of 70% EtOH/DEPC
13. Spin at 12000rpm for 5 min at 4C
14. Decant supernatant and air-dry for 5 min
15. Add 40 ul of DEPC-H2O and incubate at 65C for ~5min until completely dissolve the pellet.

DNS enzyme assay

Materials

1. DNS Solution
   - 10 g DNS
     16 g NaOH
     Ad. 300 ml dH_2O
     Heat slightly (take about 3 h to dissolve)
   - 300 g sodium potassium tartrate
     Ad. 300 ml dH_2O

Heat slightly

- Combine

  Ad. 1 L dH_2O

2. Buffer 1M MES ph 6.5
3. 2% CarboxyMethylCellulose (CMC) (from Evelyn D)
4. 2% Xylan (from Evelyn D)
5. 50 mM Glucose (from Evelyn D)
6. 50 mM Xylose (from Evelyn D)

Assay

1. Each reaction (Enzyme/supernatant/fraction volume has to be determined)

2% CMC (or Xylan)	40 ul
Buffer	4 ul
Enzyme	1 - 36 ul
H_2O	Ad. 80 ul

Standard curve

2% CMC (or Xylan)	40 ul	40 ul	40 ul	40 ul	40 ul	40 ul
Buffer 4 ul	4 ul	4 ul	4 ul	4 ul	4 ul	4 ul
50 mM Glucose (or Xylose)	10 ul	8 ul	6 ul	4 ul	2 ul	0 ul
H_2O	26 ul	28 ul	30 ul	32 ul	34 ul	36 ul

2. Seal plate
3. 30 min @ 50°C

Reaction	80 ul
DNS Solution	80 ul
End-volume	160 ul

4. Seal plate
5. 5 min @95°C
6. Transfer 100 ul into clear reading plate
7. Read absorbance @540 nm.