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TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

Cellulase production by *Trichoderma*:

a crosstalk between the fungus and bacteria

Ausgeführt am Institut E166 für
Verfahrenstechnik, Umwelttechnik und technische Biowissenschaften
der Technischen Universität Wien

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1 Summary

In recent years research concerning interactions of fungi and bacteria increased, due to the awareness that these might greatly influence properties of both organisms, such as antibiotics resistance or enzyme production. Fungal-bacterial interactions are not limited to specific species but can be found in different taxonomic groups. We recently found that *Trichoderma* spp. might form associations with *Achromobacter* sp. (esp. *Trichoderma harzianum*) and *Burkholderia* spp. (esp. *Trichoderma reesei*). Up to this point there is not much known about the impact of these relations; however, they are of great interest because of the industrial and agricultural importance of *Trichoderma* spp.. The ability of *Trichoderma reesei* to secrete large amounts of cellulases has long been known, but as a result of a continuously growing demand for cellulases in industrial applications, further improved strains are required in order to maximize efficiency, yield and minimize costs of cellulase production. In this thesis we investigated the effect of naturally associated bacteria on secretion of cellulases by *Trichoderma*. For this purpose *T. reesei* strains were cured of bacteria by a mixture of imipenem and ceftazidime and the success of the procedure was proven by molecular techniques. The effect on cellulase production was studied by fermenting two high cellulase producers of *Trichoderma* with and without their naturally associated bacteria, namely *B. tropica* and *A. xylosoxidans*, for 4 days in liquid medium with microcrystalline cellulose or lactose as carbon source. Samples were analyzed with regard to biomass production, secreted protein concentration and endo- and exocellulase activity. The results of this thesis show that *T. reesei* can be cured from *B. tropica* without visibly affecting or harming the fungus. Furthermore naturally associated bacteria do have an effect on cellulase production; however this effect is not uniform, but depends on the involved species and the available carbon-source. Results also suggest that the ratio of bacterial to fungal biomass is essential for optimal and probably beneficial interaction.

2 Acknowledgements

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3 Abbreviations

A	ampere
APS	ammonium persulfate
BCA	bicinchoninic acid
bp	base pairs
CFU	colony forming units
CMC	carboxymethyl cellulose
COCY	*Camponotus cylindricus*
Ct	cycle-threshold
DNA	desoxyribonucleic acid
g	gram
HPLC	high performance liquid chromatography
kb	kilobase pairs
kDa	Kilo Dalton
l	liter
LB	lysogeny broth
M	molar
NaAc	sodium acetate
NaCl	sodium chloride
nm	nanometer
PCR	polymerase chain reaction
PDA	potato dextrose agar
pNP	para-nitrophenol
pNPL	para-nitrophenyl-β-lactoside
qPCR	quantitative polymerase chain reaction
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
T	temperature
TCA	trichloroacetic acid
TEMED	*N,N,N′,N′*-tetramethylethylendiamin
TSB	trypton soy broth
UV	ultraviolet radiation
V	volt
wt	wild type
YFP	yellow fluorescent protein

* = every strain marked with a '*' is cured of bacteria

4 Introduction

4.1 Bacterial – fungal interactions: breaking apart the stereotype

Historically, fungi and bacteria were mainly considered as independent organisms belonging to different domains with no noteworthy interaction, which led to studying these organisms separately. In 1928 Alexander Fleming's incidental discovery of penicillin through a *Penicillium* (Ascomycota, Fungi) contaminated *Staphylococcus* (Firmicutes, Bacteria) plate, led to pioneer projects with regard to the behavior of fungi in presence of bacteria (Fleming, 1929). This research however focused on antagonistic aspects of fungal-bacterial interactions, on the one hand because the high potential of antibiotics for improving health care was recognized and on the other hand because no other type of interaction was known or imaginable. Hence, for the past couple of decades bacterial-fungal interactions were falsely regarded to be of merely antibiotic or antagonistic nature. This was refuted in recent years by several studies showing that in many environments bacteria and fungi not only coexist but also interact on various levels. These interactions are not only very complex but also very extensive, and are not completely understood yet, which gives rise to a new exciting field of research. Current studies revealed the formation of physically and metabolically interdependent associations, which harbor properties that the individuals do not possess on their own (Frey-Klett et al., 2011). Therefore these interactions arouse great interest, especially with regard to their practical relevancies (Figure 1).

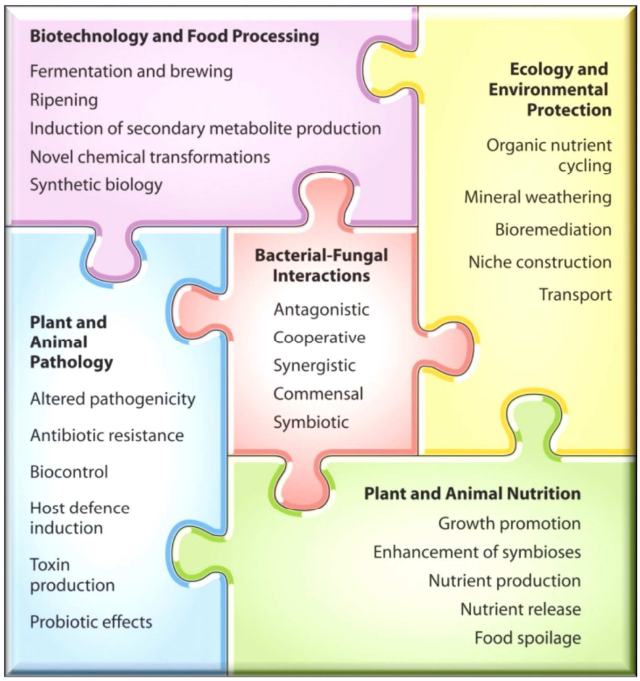

Figure 1: Relevance of bacterial-fungal interactions to various fields of study (Frey-Klett et al., 2011)

The complexity of these interactions starts at the level of physical association of bacteria with fungi, which is either planktonic, a mixed biofilm or an intrahyphal colonization. Planktonic association describes the rather disarranged form of physical interaction where bacteria are freely floating around or alongside of the fungal hyphae (Frey-Klett et al., 2011). The next, more intimate level is the mixed biofilm, which is formed between filamentous or nonfilamentous fungi and bacteria. In contrast to the planktonic association, organized communities, held together by an extracellular matrix, are created, leading to different physiological and physical properties than those of free-living cells. Mixed biofilm structures exist in two distinct forms, namely a truly mixed biofilm or a bacterial biofilm whose formation is supported by the fungus. Generally biofilms lead to an enormous decrease in susceptibility of bacteria to antibiotics compared to free floating cells, which proves to be exceptionally problematic in clinical environments, not only with regard to the high number of infections caused by biofilms but also with regard to significant problems with achieving proper sterility of medical devices (such as catheters)(Donlan and Costerton, 2002; Frey-Klett et al., 2011). A popular example of a clinically important mixed biofilm is the association of *Candida albicans* (Ascomycota, Fungi) with *Staphylococcus aureus* (Figure 2) (Harriott and Noverr, 2009).

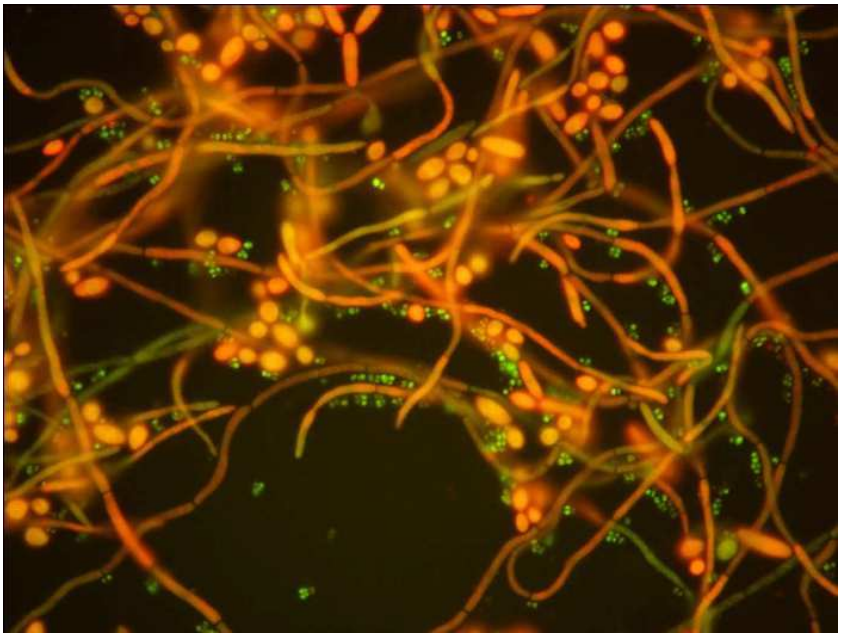

Figure 2: *Candida albicans* (orange) - *Staphylococcus aureus* (green) biofilm (from: http://www.advandx.com/wp-content/uploads/2014/05/CA-SA-Biofilm-1.jpg)

The highest level of intimacy, however, is reached when a symbiosis between bacteria and fungi is established, which might be of ecto- or endosymbiotic relationship. Ectosymbiosis is characterized by bacteria which are located externally of the fungal plasma membrane. The most popular example are cyanolichens, which is the universal term for a relationship between a heterotrophic fungus that requires a photoautotrophic symbiont to obtain fixed carbon (Fedrowitz et al., 2012). Typically the

photosynthetic cyanobacterium belongs to *Nostoc* spp. forming an ectosymbiotic relationship with Ascomycota fungi. Although *Nostoc* spp. (Cyanobacteria, Bacteria) usually tends to be ectosymbiotic, *N. punctiforme* and *Geosiphon pyriformis* (Glomeromycota, Fungi) have an endosymbiotic relationship, where *N. punctiforme* is located intracellularly in a special fungal 'bladder' that is morphologicalyl different from ordinary hyphae, as shown in Figure 3 (Frey-Klett et al., 2011; Kluge et al., 2002). This specialized structure is however rather uncommon and is not found in other fungi, that form endosymbiosis with bacteria, such as *Burkholderia* (Proteobacteria, Bacteria) and *Rhizopus* (Mucoromycotina, Fungi) *(*Partida-Martinez and Hertweck, 2005). Normally bacteria are located in the cytoplasm of hyphae and occasionally within the fungal spores.

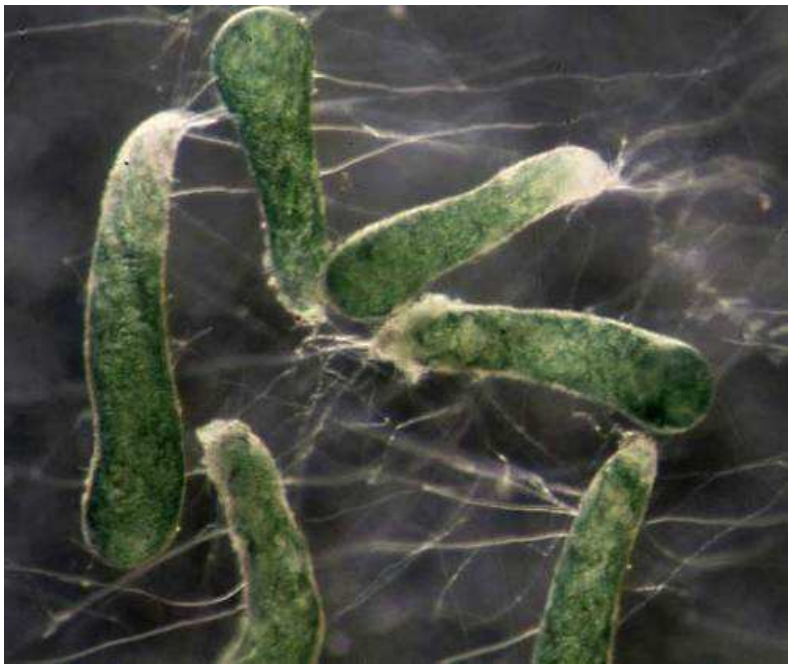

Figure 3: *Geosiphon - Nostoc* bladder (from: http://assets.slate.wvu.edu/resources/1254/1372530135_md.jpg)

Partly depending on the intimacy of physical association the existence of various interactions on the molecular level is known and greatly adds to the complexity of this partnership. The best known interaction is the already mentioned antibiosis, in which complex molecules are secreted in order to damage or even destroy the antagonist. This effect is caused by variable mechanisms, such as inhibition of cellular respiration, cell wall synthesis or destruction of cell membranes, depending on the type of the generated antibiotic. In comparison to antibiosis, signaling-based interactions exhibit a less apparent effect, which might be constructive (e.g. stimulation of hyphal growth) or destructive, contingent on the involved species (Frey-Klett et al., 2011). An interaction that was of great interest for this thesis is the cooperative metabolism, which is a derivative of trophic interaction. In trophic interaction bacteria and fungi often compete for nutrients, thus leading to reduced growth of one or both organisms. Yet, the mere existence of non-photosynthetic endobacteria, which are inside the fungal hypha, implies that not only competition for nutrients but also cooperation is present, as

bacteria must obtain life-essential substances from the fungal cytoplasm. In case of cooperative metabolism an exchange of metabolites is carried out by the degradation or formation of a molecule that cannot be produced by either partner on its own or the production of toxins by the fungus or bacterium is beneficial to its partner (Frey-Klett et al., 2011). This phenomenon was first found in the fungus *Rhizopus*, which causes rice seedling blight. Surprisingly the detrimental phytotoxin, rhizoxin, is not generated by *Rhizopus* itself but by its endosymbiotic bacteria described as *Burkholderia rhizoxinica* and *B. endofungorum* (Partida-Martinez et al., 2007a).These are only some of the various possibilities of molecular interaction, but they illustrate that every association of bacteria and fungi has consequences for both organisms, affecting among other things the development, physiology and survival of the organisms. One striking example of a bacterial-fungal interaction, which is essential for survival of the fungus was discovered by Partida-Martinez et al. (2007). They showed that curing *Rhizopus* of *Burkholderia* not only resulted in the loss of its ability to produce rhizoxin but the fungus also lost the ability to produce conidia, which is inevitably fatal to the fungus. This observation was proven by the reintroduction of *B. rhizoxinica*, upon which asexual reproduction of the fungus was re-established (Partida-Martinez et al., 2007b)

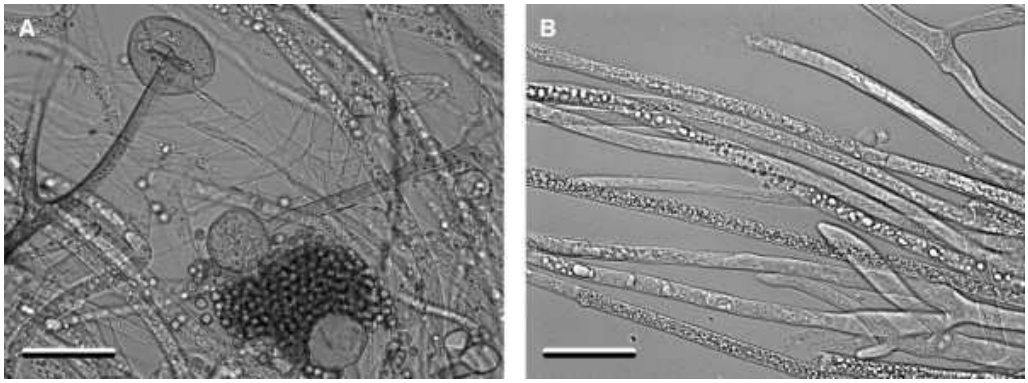

Figure 4: *Rhizopus microsporus* cultures. A) The wild-type of *R. microsporus* forming hypha and sporangia. B) Cured strain (no bacterial associates) which shows only hypha formation but no sporangia. (Partida-Martinez et al., 2007b)

As re-introduction was also carried out with GFP-labeled bacteria, the movement and localization of the endobacteria could be observed inside the fungus. Surprisingly *B. rhizoxinica* was not only found in the hyphae but also in the sporangia and spores of *Rhizopus* (Figure 5) (Partida-Martinez et al., 2007b). This remarkable example not only demonstrates that bacterial-fungal interactions might be highly beneficial but also essential for both partners.

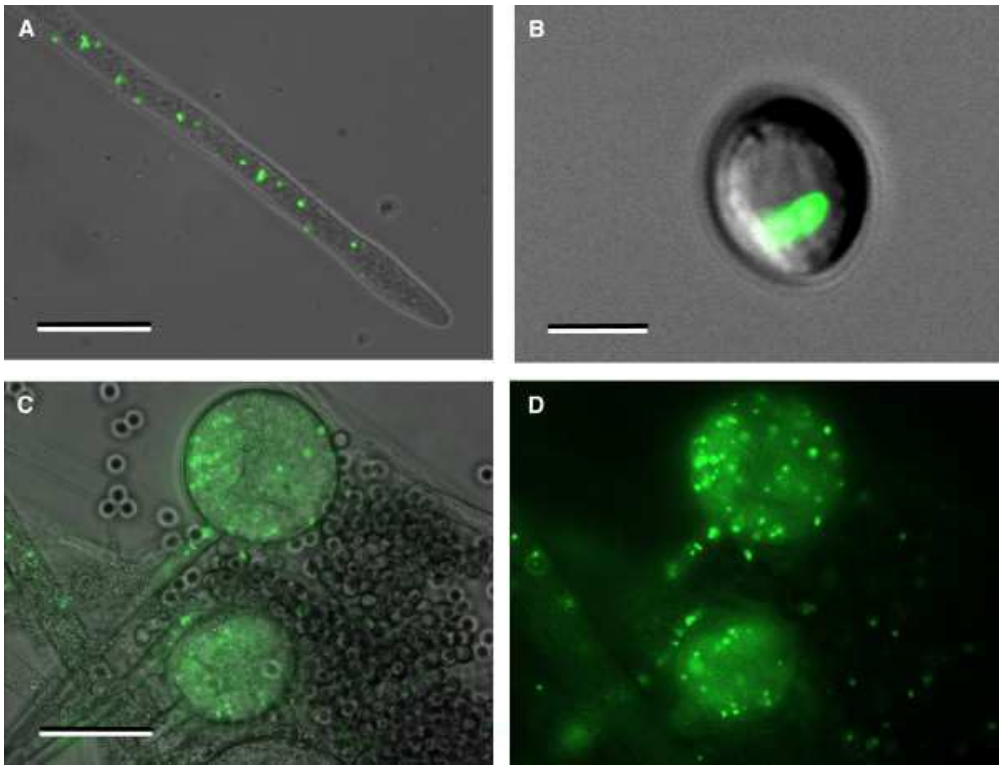

Figure 5: Confocal Laser Scanning Micrographs of *R. microsporus* after microinjection with GFP-labeled *B. rhizoxinica*. A: Bacteria migrating to tip of hypha (scale bar represents 25 µm). B: Vegetative spore with GFP-labeled endobacterium (scale bar represents 2 µm). C and D: Sporangiophores, sporangia, and spores formed after restitution of the symbiosis (white light and fluorescence mode, scale bar represents 30 µm) (Partida-Martinez et al., 2007b)

Considering that bacterial-fungal associations sometimes lead to a completely distinct behavior of the organisms and may even change their secretion profile, it is obvious that not only the organisms themselves are affected but such interactions also influence their surrounding environment. The impact on the surrounding environment is extremely versatile, so that only two representative examples will be given. Studies show that bacterial-fungal interactions play a major role in plant nutrition and in the protection against pathogenic fungi. The most common example of benefit in plant nutrition is mycorrhiza, a symbiosis of the root systems of many plants with mycorrhizal fungi, that enhances the plants access to water and minerals and supplies the fungi with carbohydrates released by the roots. This system is typically surrounded by a diversity of bacterial communities that support mycorrhiza formation and functionality and often provide it with nitrogen and phosphorus. Furthermore, several combinations of fungi with bacteria prove to be very effective fertilizers and biocontrol agents, e.g. *Trichoderma* (Hypocreales, Ascomycota, Fungi) with *Bacillus subtilis* (Firmicutes, Bacteria), which are highly effective against *Rhizoctonia solani (*teleomorph: *Thanatephorus* spp., Basidimycota, Fungi) (Hamdia Z. Ali and Kalaivani Nadarajah, 2013). A similar type of protection system can also be found in the attine ant garden, where the ants are dependent on their fungal associates, Lepiotaceae (Basidiomycota, Fungi) as they serve as their only source of nutrients for the larvae and queen and the ants supply the fungus with nutrients via plant material. However, this mutualistic relationship occasionally gets threatened by members of the genus

Escovopsis (Hypocreales, Ascomycota, Fungi), which preys directly on the fungal garden of the ants (Reynolds and Currie, 2004). In order to protect themselves of this predator, the ants support populations of actinomycete bacteria, such as species of *Streptomyces* (Actinobacteria, Bacteria), or *Pseudonocardia* (Actinobacteria, Bacteria)which produce specialized fungicides that selectively target *Escovopsis* (Figure 6)(Currie et al., 2006; Frey-Klett et al., 2011).

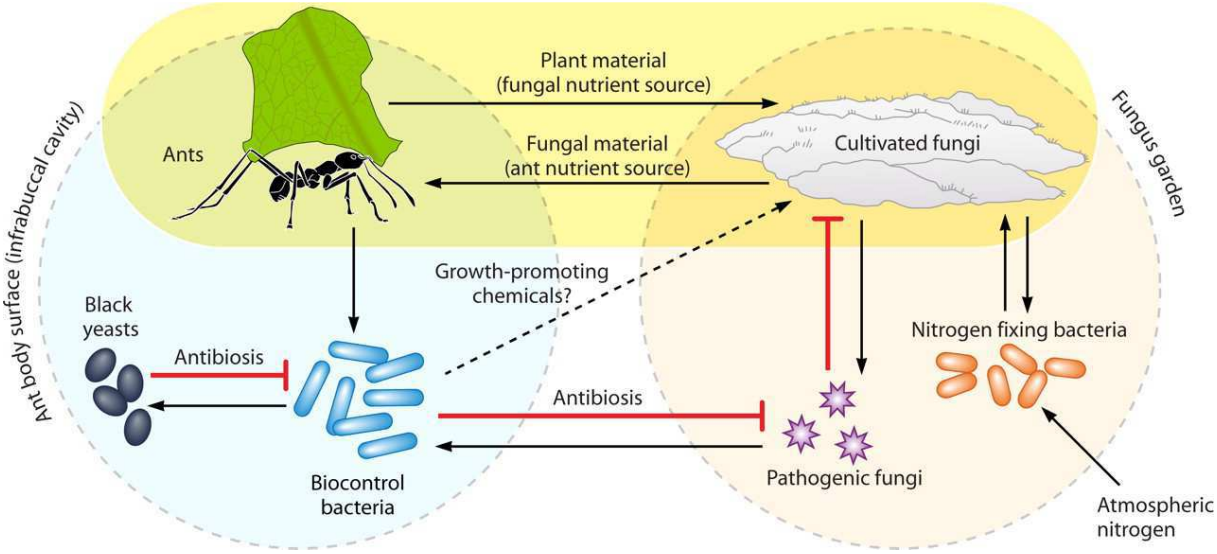

Figure 6: Bacterial-fungal interaction in attine ant garden (Frey-Klett et al., 2011).

All these alterations in behavior and benefits are especially interesting with regard to practical applications, that may they be industrial or environmental. One major focus lies on the bioremediation of pollutants, as soil pollution is a major problem nowadays, due to industrialization. Pollutants not only reduce bioavailability of essential nutrients for plants but also accumulate in food chains, which is most likely an important factor in a lot of human diseases. Cooperation between fungal and bacterial species has shown to degrade various pollutants, especially polycyclic aromatic hydrocarbons, more effective than either of the organisms on their own (Frey-Klett et al., 2011). All these recent findings highlight the great potential of bacterial-fungal interactions and of this new field of study.

4.2 *Trichoderma* – COCY – *Burkholderia*

Based on the findings of attine ants fungal gardens and due to its high advantages for all partners, Davidson et al. (2009) assumed that the mutualism between ants, fungi and bacteria is most likely not restricted to one ant taxon,. Hence a study on the microbial diversity in nests of wood-cutting ants *Camponotus (Colobopsis) cylindricus* (COCY, Arthropoda, Animalia), living in the rain forest of Borneo, was performed. To this end not only COCY-occupied but also artificial non-occupied and artificial by non-COCY species ant-colonized nests were sampled. Results of this study showed that

most COCY nests were statistically significantly associated with the two microbes: the fungi from the genus *Trichoderma* (Ascomycota, Fungi)and a nitrogen fixing bacterium *Burkholderia* . The co-occurrence of the two genera proved to be also significant as *Trichoderma* was present in 85.7 % of nests in which *Burkholderia* was detected, and *Burkholderia* in 75 % of nests in which *Trichoderma* was confirmed. Therefore this study revealed a possible bacterial-fungal interaction between *Trichoderma* and *Burkholderia*, which was unknown up to this point (Davidson et al., 2009). The samples of Davidson et al. (2009) were further investigated in the Microbiology laboratory of Vienna University of Technology (Vienna, Austria), which led to the discovery that the species *T. reesei* dominates the nests of COCY ants. Furthermore the co-occurrence of *Burkholderia* spp. with *Trichoderma* in those nests was verified by 16S rRNA sequence analysis, which provided evidence for at least eight putative *Burkholderia* taxa, whereas most isolates proved to be closely related to *B. tropica*, a nitrogen-fixing soil species (Atanasova L and Druzhinina I.S., personal communication).

4.3 *Trichoderma – Burkholderia*

The discovery of the association between *Trichoderma* and *Burkholderia*, specifically *T. reesei* with *B. tropica*, raised the questions whether this co-existence is limited to COCY nests or is generally present in this and other *Trichoderma* spp. Therefore a collection of DNA extracts of *T. reesei* and of genetically improved industrial (cellulase overproducers) mutants derived frm its most known strainQM 6a were tested for the presence of *Burkholderia* using *Burkholderia*-specific primers, GB-F and GB-N2 (Perin *et al.* 2006), which amplify part of the 16S rRNA locus. The results showed that almost all 'pure culture' wild-type strains of *T. reesei* and some mutants contained *Burkholderia*. Numerous wild-type strains including *T. reesei* QM 6a showed that they are actually associated with *B.* cf. *tropica,* leading to the conclusion that the *T. reesei – B. tropica* association is not by mere a COCY-related accident but is rather fundamental. Apart from *B.* cf. *tropica* also *B. fungorum* and putative new bacteria species were associated with various *Trichoderma* strains. Interestingly, the QM 6a mutants QM 9414 and NG-14 showed presence of *Burkholderia*, whereas the industrial cellulase producer RUT C-30 did not. Based on these findings the search for *Burkholderia* associates was extended to a random collection of other *Trichoderma* cultures, for which primarily DNA extracts from fresh, untreated fungal cultures were used. The obtained results showed that *B.* cf. *tropica* association is not restricted to *T. reesei* but may be also associated with *T. parareesei*, *T. atroviride* and *T. virens*, but only if the strains originated from tropical regions. Otherwise *T. atroviride* and *T. virens* contained predominantly *B.* cf. *fungorum*, *B.* cf. *cepacia* and putatively new *Burkholderia* phylotypes (Atanasova L and Druzhinina I.S., unpublished) (Figure 7). All these findings greatly suggest that one or most likely both organisms benefit from this association, but if, how and whether it is dependent on conditions or is generally mutualistic, is yet not known.

Another aspect worth considering is that *Burkholderia*, especially in the *B. tropica* – *T. reesei* association, might influence cellulase production by the fungus. This hypothesis is based on two facts: First of all on the discovery that only mutants with lower cellulase production levels, namely QM 9414 and NG-14, seem to be associated with *B. tropica*, while the prominent industrial strain RUT C-30 that secretes high levels of proteins, is not. This of course might be caused by several mutation steps, which had to be carried out to obtain this strain, thereby eradicating *B. tropica* in the process. Secondly, it is based on the precedent case of intimate relationship between *B. rhizoxinica* and *R. microsporus,* where e fungal survival seem to be controlled by the endobacterium, including toxin-production and asexual reproduction. As cellulase production is believed to be an essential mechanism for *Trichoderma* nutrition, especially *T. reesei*, to obtain nutrients from decaying wood, it seems plausible that *B. tropica* might be involved in the process.

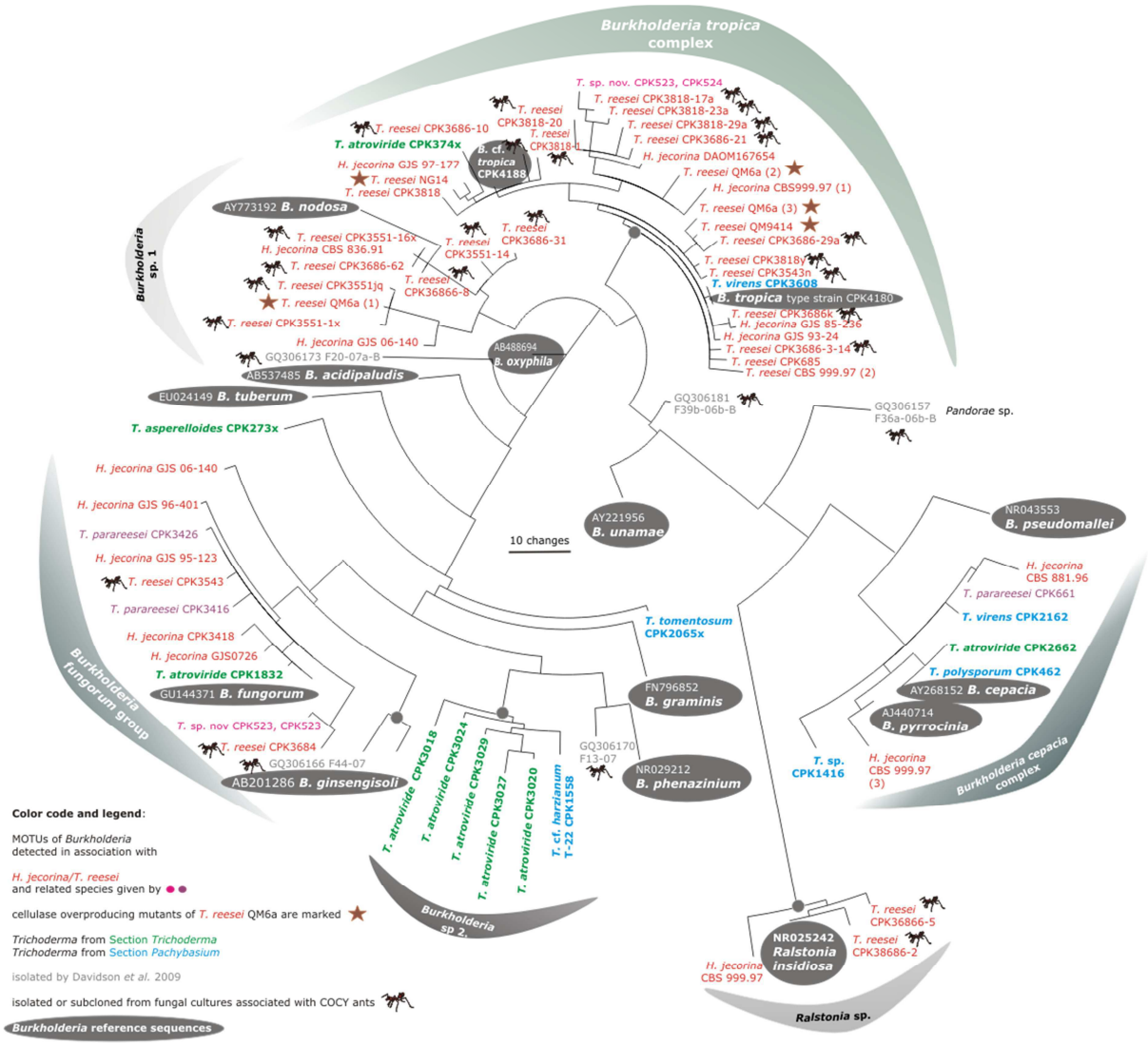

Figure 7: One of 100 maximum parsimony trees obtained of cf. *Burkholderia* 16S rRNA phylotypes recovered from different cultures of *Trichoderma*. Black cycles above nodes indicate statistical support in Bayesian phylogenetic analysis with posterior probabilities >0.94 (Atanasova L and Druzhinina I.S., unpublished)

4.4 The fungal genus *Trichoderma*

Species belonging to the genus *Trichoderma* (Hypocreales, Ascomycota) are among the most frequently found ascomycetes, being isolated from a high diversity of natural habitats, which demonstrates their adaptability to diverse ecological conditions. They are not only found in soil organic matter, on plant organs or mammalian tissues, but also in marine sites, highlighting their ubiquity (Mukherjee et al., 2013). One of *Trichoderma* spp.'s best known trait is its ability to prey on other fungi and/or secrete antifungal metabolites. This attribute makes various species to be perfect objects for biocontrol(Druzhinina et al., 2011). *Trichoderma* is able to sense the presence of other fungi and starts to grow towards it, while producing several enzymes which attack the target fungus. In addition to its antifungal properties, *Trichoderma* exhibits various direct effects on plants, such as inducing resistance in plants, promoting plant growth by establishing themselves in the plants rhizosphere, and solubilizing plant nutrients (Harman, 2006)(Figure 8). Especially strains of *Trichoderma harzianum* and *T. viride* show high antifungal potential. For example, *T. harzianum* has shown to be effective against brown spot disease of rice caused by *Bipolaris oryzae* (Ascomycota, Fungi) (Abdel-Fattah et al., 2007) or of tobacco by *Alternaria alternata* (Ascomycota, Fungi) (Gveroska and Ziberoski, 2012). Additionally studies show that *T.* cf. *harzianum*, e.g. strain T22, increases the yield of various plants, such as tomato and maize (Harman, 2008).

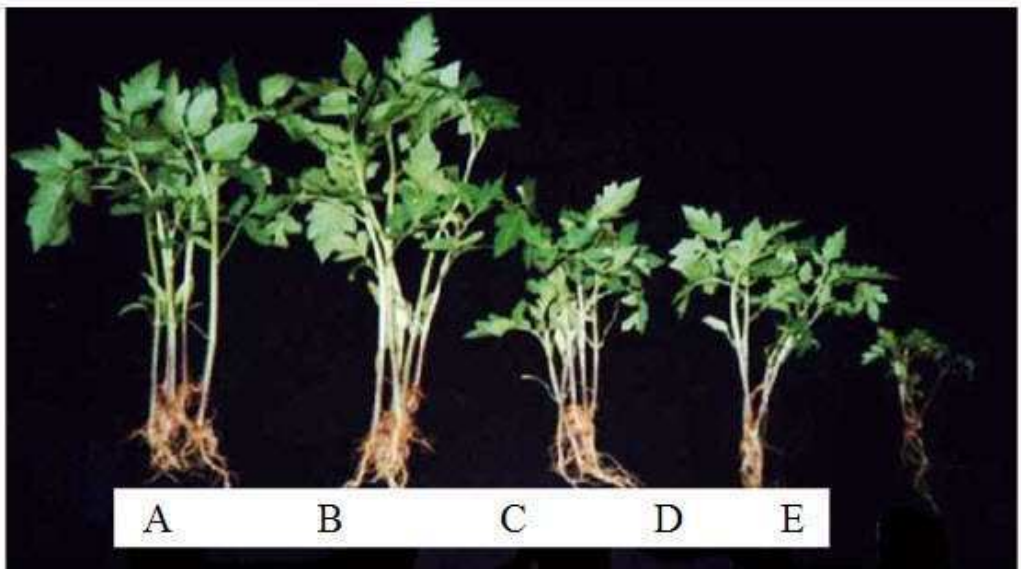

Figure 8: Tomato seedlings with various treatments. A-C: Trichoderma treated, D: Chemical fungicide treatment, E: no treatment(http://www.biosparkph.com/company-assessment/why-crops-using-biospark-trichoderma-grow-faster-than-other-crops)

Furthermore some species of *Trichoderma* have shown to be effective microorganisms for bioremediation of pollutants (Błaszczyk et al., 2014), especially heavy metals. Environmental pollution with heavy metals is particularly hazardous; being extremely toxic they not only have negative effects on flora and fauna but also contaminate groundwater and are taken up by plants, thus entering the food chain and cause health problems in humans. Heavy metals are continuously emitted into the environment as they are by- and/or waste products of various industrial processes. Their removal is challenging, since the procedure should neither introduce new toxins into the environment nor be expensive and laborious. With regard to these requirements research started to focus on bioremediation by fungi and found various fungi that effectively remove several heavy metals, including cadmium, nickel and lead. Fungi of the genus *Aspergillus* (Ascomycota, Fungi) seemed to be extraordinarily efficient, yet they did not comply with the requirement of not introducing other toxins into the environment. Consequently *Aspergillus* was not applicable, which motivated researchers to test numerous other fungal species known for their bioremediation activities, such as *Trichoderma* species. In a recent study Mohsenzadeh and Shahrokhi (2014) tested *T. asperellum*, *T. harzianum* and *T. tomentosum*, three non-pathogenic species, on their ability to remove cadmium ions under different conditions and learned that *T. asperellums* shows a cadmium removal efficiency of 76.17 %, designating it as potential agent against cadmium pollution (Mohsenzadeh and Shahrokhi, 2014).

Even though most *Trichoderma* species are considered to be non-pathogenic to humans, several cases of *Trichoderma* infections have been reported, normally in immunocompromised patients, which were often fatal. Especially *T. longibrachiatum* and *T. koningii* are of clinical importance (Kredics L, 2006). On the other hand recent studies target the utilization of high cellulase producers, like *T. reesei* RUT C-30, for the production of therapeutic proteins, by cloning constructs into the *cbh1* locus (Smith et al., 2014). The study of Smith et al. 2014 showed that the production of human α-galactosidase A is possible, but problems, such as activity loss, demand further research.

In industry *Trichoderma* species are utilized due to their capacity to produce several lytic enzymes, such as cellulases, hemicellulases and pectinases. These are applied to partially hydrolyze plant cell walls for animal feed, yielding a more digestible and nutritiously valuable product, which leads to augmented weight gain and higher milk generation. Furthermore the enzymes are used in food industry as additives, for example in the production of fruit and vegetable juices to increase maceration. Especially cellulases generated by *Trichoderma* are industrially important, which are not only applied in textile industry and in pulp and paper industry to modify fiber properties but also to degrade farm waste for the production of bioethanol (Błaszczyk et al., 2014; Schuster and Schmoll, 2010).

Next to enzymes, *Trichoderma* produces hydrophobins, small cysteine-rich proteins that are amphiphilic, which are only generated by filamentous fungi. These proteins are crucial for growth and development, as they self-assemble at a hydrophilic-hydrophobic interface such as water and air, forming a polymeric complex (inside: hydrophilic, outside: hydrophobic) that lowers the surface tension and allows for growth of aerial structures (Wösten and Scholtmeijer, 2015). Due to the ability to form this amphipathic film, hydrophobins are e.g. of great interest with regard to degradation of PET (polyethylene terephthalate), to enhance the efficiency of PET hydrolysis by microbial cutinases (Enrique Herrero Acero, 2011; Espino-Rammer et al., 2013). Recent studies showed that the application of hydrophobins indeed increases PET hydrolysis but best results are obtained by fusing cutinases with hydrophobins, leading to a 15-fold increase compared to the enzyme alone (Espino-Rammer et al., 2013; Ribitsch et al., 2015).

4.5 *Trichoderma reesei* – efficient cellulase producer

Lignocellulose -a complex polymer mainly consisting of lignin, cellulose and hemicellulose - is the most abundant renewable biomass on earth (Wong, 2009). The complexity of the substrate causes a number of difficulties with regard to its utilization as raw material for the production of simple sugars and their subsequent conversion to biofuels (Jun et al., 2011). Cellulose, a β-(1,4)-linked glucose polysaccharide provides plants with rigidity and structure as well as an increased resistance to degradation. Various types of cellulose degradation are additionally hampered by the presence of lignin and hemicellulose on the surface of cellulose. However, after pretreatment of lignocellulose, enzymatic degradation by cellulases proves to be the most effective and environment-friendly method to obtain simple, applicable sugars (Jun et al., 2011; Lee et al., 2014). In nature three main types of cellulases, namely endoglucanases, exoglucanases and β-glucosidases, exist that work together to degrade cellulase to glucose. Endoglucanases are needed to cleave internal bonds of the cellulose chain, generating numerous chain ends. Exoglucanases start from the chain ends and cleave cellobiose of the cellulose chains. Finally the β-1,4-glycosidic bond between the two glucose molecules of cellobiose is hydrolyzed by β-glucosidase, which makes glucose available (see Figure 9).

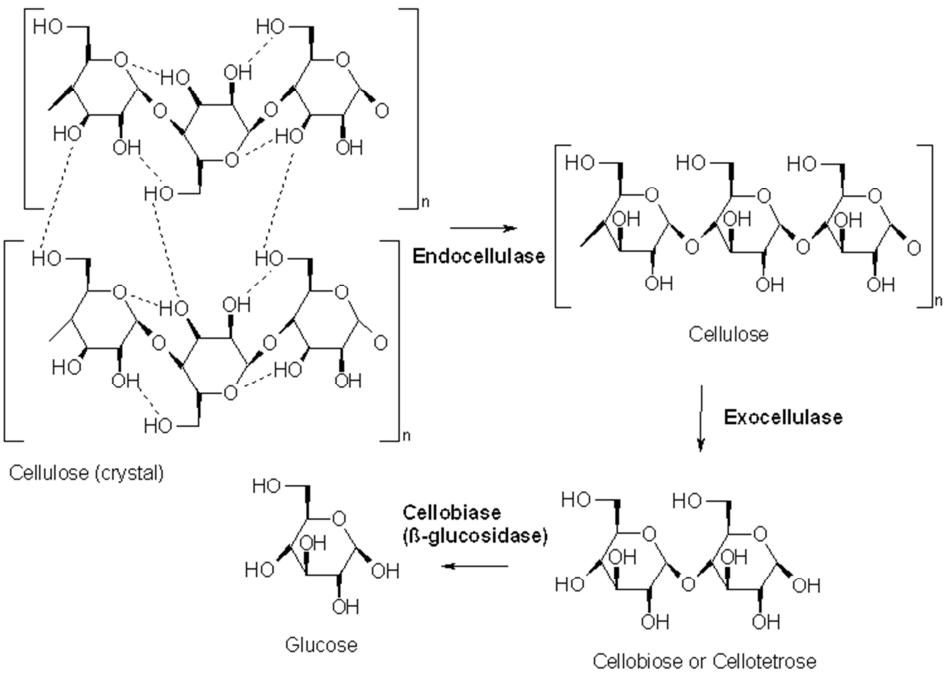

Figure 9: Three main types of cellulases and their involvement in cellulose degradation (from: http://en.wikipedia.org/wiki/Cellulase#/media/File:Types_of_Cellulase2.png(15.4..2015, 7:46)

Albeit *Trichoderma* exhibits many qualities that are of practical relevance, *Trichoderma* (in particular *T. reesei*) is probably best known for its capacity to secrete high amounts of cellulases. The oil shock in the 70's and the associated fuel crisis triggered extensive research in the field of cellulases, cellulase production and optimization of these enzymatic processes with special focus on the fungus *Trichoderma* reesei.The crisis exposed the need for alternative fuels, with focus on ethanol produced from sugar molecules from degraded cellulose, for which cellulases are required (Turner et al., 2007).

Trichoderma reesei QM 6a was first isolated from the Solomon Island during the World War II, where it destroyed the canvas of the tents of the US Army due to its high cellulolytic activity(Reese , 1950). After its first isolation *T. reesei* was never again found in nature as anamorph (asexual stage), only as its teleomorph (sexual stage) in that time named *Hypocrea jecorina*(Samuels et al., 1998). Nowadays mutant strains derived from the original QM 6a strain are used in industry for the production of cellulases, but even though *T. reesei*'s natural level of cellulase secretion is elevated compared to other fungi, it is too low to be economically feasible (Seidl et al., 2009). Hence research is constantly striving to improve *T. reesei* strains for increased cellulase production, which, in the past decades was mainly achieved by random mutagenesis. Through several steps of random mutation the hypercellulolytic strain *T. reesei* RUT C-30 was obtained (Seidl et al., 2008)(Figure 10), which is currently the most used strain in laboratory for cellulase production and serves as model organism to gain insight into the roots of cellulase production. The whole genome of RUT C-30 was sequenced and compared to the genome of its ancestral strain QM 6a (Koike et al., 2013), which revealed

serious genetic alterations, however none of these alteration could be directly attributed to the elevated level of cellulase secretion. Up to this point various factors, which are involved in regulation of cellulase production have been identified, still the complete mechanism is not fully understood yet (Poggi-Parodi et al., 2014). Getting full insight into the mechanism of this regulation would allow better targeted genetic improvement. As cellulase production, however, is also dependent on the provided environment, such as carbon source (induction of expression by e.g. cellulose, lactose)(Mach and Zeilinger, 2003; Seiboth et al., 2007) and temperature (for optimal growth of the fungus), other factors have to be considered to obtain the best yield of enzymes. *In situ* the provided environment is most likely influenced by naturally associated bacteria, such as *B. tropica* (see 4.3), changing the availability of nutrients. As these interactions are not characterized yet, it is highly speculative that these bacteria might affect cellulase secretion of *Trichoderma*, but it is worth considering, due to the facts stated in 4.3.

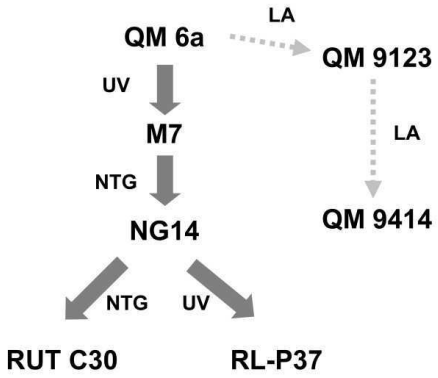

Figure 10: Lineage of mutants derived from QM 6a. Mutations were introduced by either irradiation with UV (ultraviolet) light or chemically affected by nitrosoguanidine (NTG) or linear accelerator (LA). (Seidl et al., 2008)

4.6 The bacterial genus *Burkholderia*

Burkholderia spp. are one of the most abundant and ecologically diverse bacterial genus in the ecosystem. The versatility of these gram-negative bacteria is not only manifested in their ecological ubiquity, but also in their morphological and metabolic diversity, leading to different behavior of the numerous species (Coenye and Vandamme, 2003; Compant et al., 2008). Due to these dissimilarities and the analysis of 16S rRNA and 21 conserved proteins, a recent study by Sawana et al. (2014) proposed a recognition of the two genera within the current genus *Burkholderia* comprising species that share conserved sequence indels, which are specific to the two genera,, into *Burkholderia* containing pathogens and *Paraburkholderia* comprising environmental species such as *B. fungorum* (Sawana et al., 2014). As the taxonomy and respective nomenclature were not officially altered up to this point, both, pathogenic and environmental species –particularly the object of this thesis *B. tropica* (according to suggested taxonomy belonging to *Paraburkholderia*)- will still be referred to as *Burkholderia*.

Probably the most commonand best studied species are a group of 17 closely related *Burkholderia* spp. belonging to a so-called *B. cepacia* complex (BCC), since they are primarily dangerous human and animal pathogens, being responsible for pulmonary infections in immunocompromised individuals, with a potentially lethal outcome (Mahenthiralingam et al., 2005; Sawana et al., 2014). Similarly other species related to the BCC exhibit high pathogenicity, such as *B. pseudomallei*, the causative agent for melioidosis (White, 2003), and *B. mallei*, a glanders causing species, which is regarded as potential biological warfare agent (Scholz et al., 2014). These clinical important *Burkholderia* species are not only critical because of their high pathogenic nature, but also due to their high resistance to various antibiotics, which is an obstacle in the treatment of infected individuals. This high resistance to antibiotics is not restricted to pathogenic *Burkholderia* species but can also be found in various other species. Apart from species that are pathogenic to animals including humans, there are several phytopathogens and endosymbionts of phytopathogenic fungi as well. For example, *B. plantarii* induces rice seedling blight (Azegami et al., 1987) and *B. gladioli* causes various plant diseases, such as bacterial soft rot in onions and grain rot in rice (Compant et al., 2008; Lee et al., 2005). Additionally some phytopathogenic fungi have specific *Burkholderia* species as endosymbionts, which was already presented by the endosymbiosis of *R. microsporus* with *B. rhizoxinica* (Partida-Martinez et al., 2007a; see 4.1). As presented in Table 1, relatively a few of these endosymbiotic relationships between phytopathogenic fungi and *Burkholderia* spp. are known up to this point.

Table 1: *Burkholderia* spp. associated with pathogenic fungi (Compant et al., 2008)

Burkholderia	Fungus	Host plant	Reference
B. fungorum	*Phanerochaete chrysosporium*	Trees	Coenye et al. (2001)
B. sordidicola	*Phanerochaete sordida*	Trees	Lim et al. (2003)
B. rhizoxinica	*Rhizopus microsporus*	Rice	Partida-Martinez et al. (2007)
B. endofungorum	*Rhizopus microsporus*	Rice	Partida-Martinez et al. (2007)

However, these species of *Burkholderia* are not only endosymbionts of fungi but might also be found in connection with plant-associated insects. For example the bean bug *Riptortus clavatus* (Arthropoda, Animalia) and rice bug *Leptocorisa chinensis* (Arthropoda, Animalia) contain *Burkholderia* spp. in crypts in the posterior region of the midgut (Kikuchi et al., 2005). Numerous bugs of which their symbiont was removed exhibit retarded growth, nymphal mortality and/or sterility (Compant et al., 2008; Fukatsu and Hosokawa, 2002) thus the endosymbiont seems to be essential, similar to the case of *R. microsporus*.

Although several species of the genus *Burkholderia* are themselves pathogens or hyperpathogens (= associated with pathogenic eukaryotes), the majority of *Burkholderia* species associated with plants are non-pathogenic, some even being mutualistic to their hosts (Compant et al., 2008). Interestingly, the highly animal pathogenic *B. cepacia* complex has been isolated from the rhizosphere of healthy maize, rice (*Oryza sativa)*, cotton (*Gossypium hirsutum)* and other plants (Compant et al., 2008; Parke and Gurian-Sherman, 2001; Ramette et al., 2005). Studies showed that the BCC produces antimicrobial compounds which protect plants or promotes plant growth and degrades a wide variety of natural and man-made organic compounds (see Figure 11) (Mahenthiralingam et al., 2005). Nonpathogenic species like *B. unamae* or *B. silvatlantica* (best known from Brazil) are associated with sugarcane (*Saccharum officinarum*) and maize (*Zea mays*) (Caballero-Mellado et al., 2004; Perin et al., 2006). One hypothesis suggests that the diversity of *Burkholderia* spp. in the rhizosphere of crop plants in different geographical regions may be caused by human activity and their practices (Compant et al., 2008).

Furthermore *Burkholderia* spp. might be endophytes or endosymbionts of benefical endophytic fungi. As endophytes fungi enter the plant's vegetative tissue and/or reproductive organs (Compant

et al., 2008). Several years ago an analysis of nitrogen-fixing bacteria associated with roots of crop plants, revealed several novel diazotrophic *Burkholderia* species, such as *B. vietnamensis* (Santos et al., 2001)and *B. tropica* (Reis et al., 2004). *Burkholderia* spp. is also often found in mycorrhizal fungi, for example *B. cepacia* was found to be an omnipresent inhabitant of three arbuscular fungi, namely *Glomus etunicatum (Glomeromycota, Fungi)*, *G. intraradices* and *G. mosseae* (Andrade et al., 1997). Obviously species belonging to the genus *Burkholderia* are able to establish intimate biotrophic associations with fungi, plants and insects. Most of these interactions are not fully understood yet, but are of great interest, especially with regard to symbiosis.

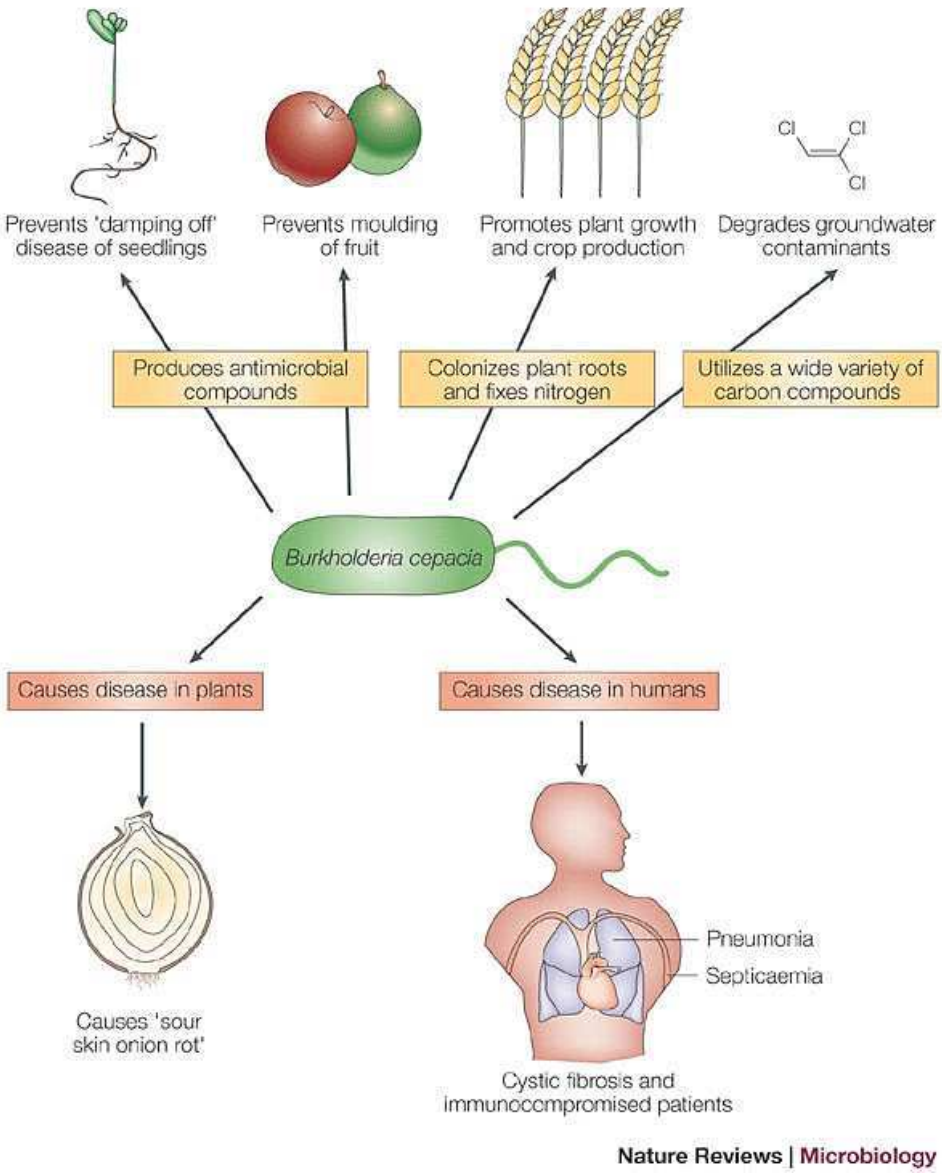

Figure 11: The two faces of the *Burkholderia cepacia* complex. Mostly known for its pathogenicity to humans, its highly beneficial attributes in interactions with other organisms are overlooked.

4.7 *Burkholderia tropica* – a nitrogen-fixing bacterium

B. tropica was first isolated and described in 2004 by Reis et al., when they analyzed the taxonomy of bacteria retrieved from sugarcane and maize grown in different geographical and climatic regions of Brazil, Mexico and South Africa. *B. tropica* is a nitrogen-fixing, plant-associated bacterium, whose optimal temperature of growth is at 30°C (Reis et al., 2004). Generally *B. tropica* is considered to be non-pathogenic to humans,however, in 2010 one case of infection of an infant with *B. tropica* was reported (Deris et al., 2010). The bacterium was not only found as an endophyte of sugarcane and maize plants but also in the rhizosphere of tomato, and showed a high level of mineral phosphate solubilization, thus providing the plant with phosphorus. It allowed the researchers to suggests that *B. tropica* promotes growth of associated plants (Caballero-Mellado et al., 2007). In 2013 a study by Tenorio-Salgado et al. was performed, evaluating antagonism of *B. tropica* to plant-pathogenic fungi. To this end the effect of 15 *B. tropica* strains on four plant-pathogenic fungi (*Sclerotium rolfsii* (Basidiomycota, Fungi), *Fusarium oxysporum* (Ascomycota, Fungi), *Fusarium culmorum, Colletotrichum gloeosporioides* (Ascomycota, Fungi)). Results showed that radial growth inhibition of the fungi by *B. tropica* was between 6-74%, depending on the *B. tropica* strain and the fungus (Figure 12) (Tenorio-Salgado et al., 2013).

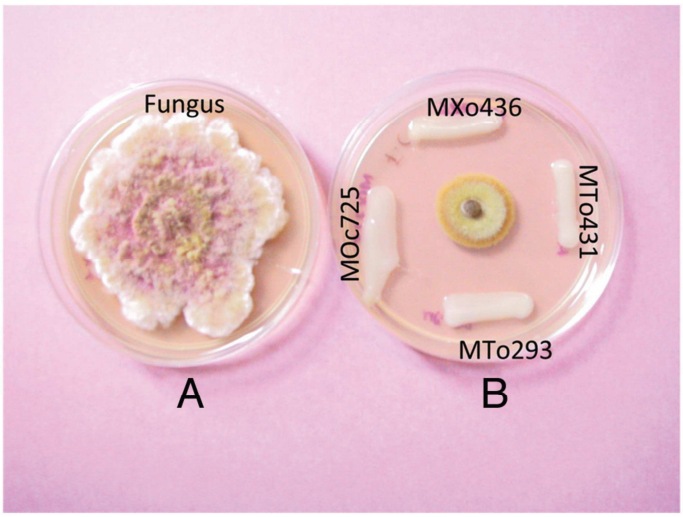

Figure 12: Antagonism assay of *B. tropica* strains against *F. culmorum* on PDA. A. Control with typical pink pigmentation. B. *F. culmorum* with four strains of *B. tropica*. Growth of the fungus is very limited and pigmentation is lost. (Tenorio-Salgado et al., 2013)

Further analysis showed that *B. tropica* generates volatile compounds which also inhibit growth of the tested plant-pathogenic fungi, and, in some cases, change the pigmentation of the mycelium, e.g. pink mycelium of *F. oxysporum* became white (Tenorio-Salgado et al., 2013).

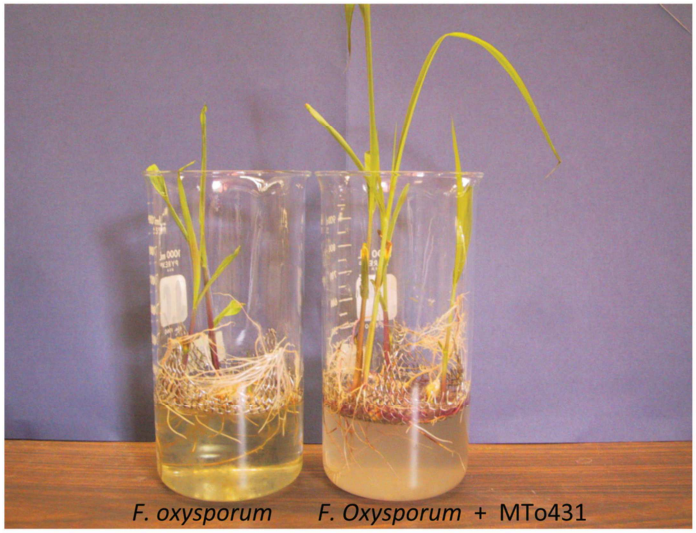

Figure 13: Maize infected with *Fusarium oxysporum*. Left side: Control. Growth of maize plant with *F. oxysporum*. Right side: *B. tropica* added. The maize plant is healthy and able to grow (Tenorio-Salgado et al., 2013).

B. tropica is most commonly found as endophyte, however, as mentioned in 4.2, it was also found in nests of COCY ants in the rain forest of Borneo and showed significant co-occurrence with *Trichoderma* (Davidson et al., 2009). In general hardly anything is known about *B. tropica* life cycle *in situ* yet, neither about its role and mechanism in association with plants nor about their relationship to ants and *Trichoderma*.

5 Aim

The aim of this thesis was to gain first insights into the relationship of naturally associated bacteria and *Trichoderma*, with focus on *T. reesei* - *B. tropica*.

Particularly the hypothesis that cellulase production of *Trichoderma* may be altered by associated bacteria was to be tested. Additionally the type of physical association of *B. tropica* with *T. reesei* was of high interest.

To achieve this aim the following tasks had to be carried out:

- Development of a method to cure *Trichoderma* strains from associated bacteria
- Selection of suitable model organisms
- Conducting pilot-experiments on the role of bacteria in cellulase production by *Trichoderma*
- Generation of a fluorescent *B. tropica* strain, which can further be used for determination of the localization of *B. tropica* in respect to a *Trichoderma* culture

6 Methods and Materials

6.1 Strains

Strains used in this thesis were obtained from TUCIM Collection of Industrially Microorganisms (Vienna University of technology) and are given in Table 2.

Table 2: Strains used in this thesis

TU CIM strain ID	Other strain IDs	Species	Notes
1683	QM 9414	*Trichoderma reesei*	Early cellulase overproducing mutant derived from *T. reesei* QM 6a
917	QM 6a		Wild type strain
4106	CBS 999.97		
	RUT C-30		Cellulase overproducing mutant derived from *T. reesei* QM 6a
4804		*T. harzianum*	
4742			
4455		*T. afroharzianum*	clean strain pretreated with chloramphenicol and imipenem (Mohammad Rahimi, Marica Grujic, Irina Druzhinina, personal communication).
4180	ATCC BAA-831	*Burkholderia tropica*	Ex-type strain
4802		*Achromobacter* cf. *xylosoxidans*	Isolated and provided by Mohammad Rahimi, Marica Grujic, Vienna University of Technology, Microbiology Group.

6.2 DNA extraction, PCR amplification and sequencing

DNA extraction was carried out by mechanically disrupting harvested mycelium using glass beads in combination with a grinding machine. The actual DNA isolation was performed by using QIAGEN DNeasy® Plant Mini Kit (Netherlands) and following the manufacturer's protocol. The concentration of the isolated DNA was measured using Nanodrop ND-1000 spectrophotometer at a wavelength of 260 nm.

Mycelium and bacterial cells were obtained in different ways, depending on the purpose of the DNA extraction. In order to prove that fungal strains were free from bacteria by PCR, mycelium from liquid cultivations was directly filtered (partly washed with sterile water) and used for subsequent steps.

For qPCR the strains were first pre-grown on PDA until sporulating. A conidial solution was prepared following the procedure described in 0. Of this spore solution ($4*10^7$ total cells /ml) three times 20 µl were pipetted onto a PDA plate covered with sterile cellophane. The plates were put on 28 °C wrapped with aluminium foil for 1-4 days respectively (see 6.3) and the mycelium was harvested by using a sterile spatula.

PCRs targeting various regions, namely rRNA gene cluster for fungi and bacteria were performed. For bacterial 16S rRNA the two primer pairs were used: the universal 16S primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP1 (5'-ACGGTTACCTTGTTACGACTT-3') (Weisburg et al., 1991), and a *Burkholderia* specific primer pair developed by Perin et al. (2006) as GB-F (5'-AGTAATACATCGGAACRTGT-3') and GB-N2 (5' – GCTCTTGCGTAGCAACTAG-3') Figure 14.

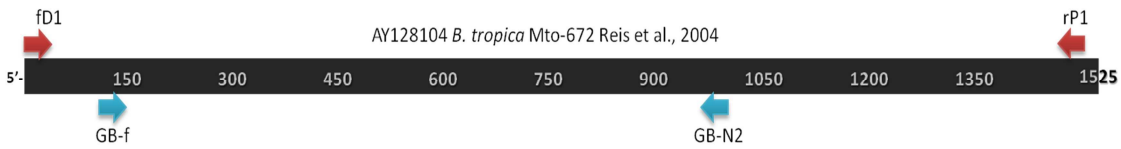

Figure 14: Positions of universal 16S rRNA primers (fD1, rP1) and *Burkholderia*-specific 16 S rRNA primers (GB-f, GB-N2) on the reference rRNA sequence of *B. tropica*.

For fungi the fragment of the house keeping gene encoding the translation elongation factor 1 alpha (*tef1)* spanning the area of exon 1 to exon 4, was amplified using EF1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and EF 2 (5'- GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') primer (O'Donnell et al., 1998).

The master mix was prepared by mixing Promega buffer with Promega nucleotide, $MgCl_2$ and primers and diluted with water, so that after addition of 5 µl of sample the following end concentrations were obtained: 1 M Promega GoTaq® Flexi Buffer ,3.0 $*10^{-3}$ M Promega $MgCl_2$,1.6 * 10^{-4} M Promega Nucleotide, 2.5 * 10^{-7} M of each primer forward and reverse, 0.001 U Promega GoTaq® DNA Polymerase. For the PCR with universal 16 S primers an end concentration of 0.02 U Promega GoTaq® DNA Polymerase was used. As negative control sterile distilled water was used instead of DNA. PCR amplification was carried out in the T3 Thermocycler Biometra® with the programs given in Table 3 .

Table 3: PCR ampflicfication programs for PCR with *tef1*, universal 16 S (fD1, rP1) and *Burkholderia* specific (GB-F, GB-N2) primers

Step	cycles	*tef1*		Universal 16 S (fD1, rP1)		*Burkholderia* specific (GB-F, GB-N2)	
		Time [min]	**T [°C]**	**Time [min]**	**T [°C]**	**Time [min]**	**T [°C]**
Initial denaturation		3	94	5	94	5	94
Denaturation	30	1	94	11 s	94	0.5	94
Annealing		1.5	56	0.13	56	0.75	60
Extension		2	72	0.16	74	1	72
Final extension		7	72	10	72	5	72

To analyze the amplified fragments native agarose gel electrophoresis was applied. A 1.5 % agarose-gel containing SYBR Safe (Invitrogen) was prepared, the pockets were filled with 10 µl of the PCR product, one pocket was loaded with 1 kb ladder (Fermentas, Germany). Electrophoresis was carried out at 80 V and 200 mA for 30 - 40 minutes. Gels were imaged under UV-light in GelDoc 2000 (Bio-Rad, USA). Amplicons were purified using a PCR purification kit (Qiagen, Germany) and sequenced at Eurofins MWG Operon (Ebersberg, Germany)

6.3 Obtaining of bacteria-free fungal strains and verification of the efficacy of curing

Fungal strains were pre-grown on PDA at 28°C for 7 days. A spore suspension for inoculation of the curing medium was prepared by adding spores with a sterile cotton swab to 10 mL of sterile physiological solution (0.9 % NaCl in distilled water) until transmission was reduced by 25% at 590 nm (using a standard turbidimeter of BIOLOG Inc., Hayward, CA, USA), resulting in a $4*10^7$ total cells /ml solution. The same procedure was followed to obtain a bacterial inoculation solution, which was used as control for determining the effectiveness of the used antibiotics.

As antibiotics a combination of imipenem (Sigma Aldrich) and ceftazidime (Sigma Aldrich) were used in a final concentration of 20 µg/ml of each. Antibiotics were chosen based on former studies (Deris et al., 2010; Thibault et al., 2004) on the effectiveness of different antibiotics against *Burkholderia* spp.

The curing process was carried out in 100 ml Erlenmeyer flask containing 30 ml of sterile liquid cultivation medium (Table 4) with added antibiotics. Lactose was used as carbon source, based on a

study of Reis *et al.* 2004, which shows that *B. tropica* is not able to oxidize lactose. For inoculation 1 ml of the prepared spore solution was added to each flask. The flasks were incubated in a shaker at 28 °C and 150 rpm for 5 days.

Table 4: Composition of the cultivation medium used to cure *Trichoderma* cultures from *Burkholderia* associates. The pH of the medium was adjusted to 5 by using 1 M NaOH.

Substance	g/L
KH_2PO_4	5
$(NH_4)_2SO_4$	1.4
Urea	0.3
$MgSO_4$ x 7 H_2O	0.3
$CaCl_2$	0.3
$FeSO_4$ x 7 H_2O	0.005
$MnSO_4$ x H_2O	0.0017
$ZnSO_4$ x 7 H_2O	0.0014
$CoCl_2$ x $2H_2O$	0.002
Peptone (casein)	1
Lactose	20

To quantify bacterial growth in association with *Trichoderma* cultures, DNA was extracted as described in 6.2. In order to see whether associated bacteria are viable, total DNA of each fungal strain was extracted after 24, 48, 72 and 96 hours of growth at 28°C in darkness. Of each strain three (triplicates) plates per time-point were prepared, to ascertain that a positive signal was not caused by a contamination. To set up a positive control the *T. reesei* QM 9414 strain was infected with *B. tropica* TU CIM 4180 (a $4*10^7$ total cells/ml solution of *B. tropica* TU CIM 4180 was diluted 1:10000, of this dilution 60 µL were added to 1 mL of QM 9414 $4*10^7$ total cells/ml).) After extraction DNA was diluted to 23 ng/µL, to obtain a standardized concentration, and further diluted 1:10 for qPCR

For qPCR a master mix was prepared as follows: per sample 12.5 µl iQ™ SYBR® Green Supermix (BioRad), 1 µl of forward and reverse primer respectively (final concentration in reaction mixture $2*10^{-7}$ M) and 8.5 µl sterile HPLC water were mixed. Of the master mix 23 µl were pipetted into each tube and 2 µl of diluted DNA (final concentration of 0.184 ng/ µl) were added. Triplicates were performed. The qPCR was performed in qTower (Analytik Jena), with the temperature profile given in Table 5. As primers *tef1* (targeting the fungal house keeping gene) and GB (*Burkholderia* specific 16 S

region) qPCR primers were used for each sample, respectively. The average of obtained cycle-threshold of the triplicates for each day of growth was formed, for both amplicons (*tef1* and GB) respectively, and the cycle-threshold ratio of *tef1* to *GB* was calculated.

Table 5: Temperature profile for qPCR run with *tef1* and *Burkholderia*-specific (GB) qPCR primers

Cycle	Step	Temperature [°C]	Time [s]
1	Inital denaturation	95	180
40	Deaturation	95	15
	Annealing	58	20
	Extension	72	20
1	Melting curve	65 - 95	6 s with ΔT 1 °C

6.4 Shake flasks fermentation of *Trichoderma* and *Burkholderia*

6.4.1 Preparation of the medium

Fermentation was carried out in 500 mL Erlenmeyer flasks with wide neck at 250 rpm and 28°C for 96 hours. Duplicates for each time point were sampled. As carbon-source 2 % lactose (Roth) or 2 % microcrystalline cellulose powder derived from wood (Serva) were used.

For each set of fermentation the medium was first prepared in a beaker of appropriate size, stirred with a magnetic stirrer until all components were completely dissolved (except for microcrystalline cellulose) and the pH was set to 5. Then 125 ml of homogenous medium were filled in each of 500 mL Erlenmeyer flask by using a measuring cylinder. The flasks were covered with cotton plaque and aluminium foil and autoclaved for 20 minutes at 121 °C.

Table 6: Composition of fermentation medium. The pH of the medium was adjusted to 5 by using 1 M NaOH.

Substance	g/L
KH_2PO_4	5
$(NH_4)_2SO_4$	1.4
Urea	0.3
$MgSO_4$ x 7 H_2O	0.3
$CaCl_2$	0.3
$FeSO_4$ x 7 H_2O	0.005
$MnSO_4$ x H_2O	0.0017
$ZnSO_4$ x 7 H_2O	0.0014

$CoCl_2$ x $2H_2O$	0.002
Peptone (casein)	1
C-source	20

6.4.2 Preparation of the microbial inoculum

Microbial strains were pre-grown on PDA at 28°C for 7 days. A highly concentrated conidial solution was prepared by adding between 5-8 ml of sterile physiological solution (6.3page 26) onto the plate and using a Drigalski spatula to transfer the spores in the solution. The spore solution was then filtered through glass-wool to remove mycelial debris. Defined volumes of this spore solution were then added stepwise to 10 ml of water (containing a drop of Tween 20) measuring the transmittance with a standard BIOLOG turbidimeter at 590 nm, until it reached 75 % (corresponding to total cells of $4*10^7$/ml). The total volume of conidial solution needed to obtain this decrease in transmittance was used to calculate the needed volume for inoculation.

$$V_{inoculum} = \left(\frac{V_{medium}\,(125\,ml) * 10^8}{4 * 10^7 total\ cells/ml} \right) * \ V_{transmittance\ 75\%}$$

The bacterial inoculum was prepared as described in 0and a final concentration of 600 CFU/ml was added to each flask.

6.4.3 Sampling

Samples were taken after 24, 48, 72 and 96 hours of fermentation. Flasks were shaken before transferring about 50 ml of fermentation broth that was immediately frozen before the analysis (see 6.5). The remaining fermentation broth was dead-autoclaved inside the flask.

6.5 Biomass measurement

6.5.1 Dry weight

Biomass of fermentation samples was determined via dry-weight for samples grown on lactose. To this 2 ml of homogenous fermentation broth were pipetted into the previously weighted 2 ml Eppendorf tubes. Per sample triplicates were performed. Next, the Eppendorf tubes with fermentation broth were centrifuged for 10 min at 4 °C and 5000 rpm. The supernatant was pipetted into fresh tubes, labeled according to the tarred Eppendorf tubes, which was used for further analysis. The pellet was washed by adding water, vortexing and centrifuging again at the same conditions. This step was performed twice, to remove soluble medium components from the biomass. The tarred Eppendorf tubes containing the washed biomass were put on 80 °C for 72 hours,

cooled down and weighed. The biomass was calculated by subtracting the tare (tubes) of the total weight of the tube with dry, washed biomass.

6.5.2 Intracellular protein concentration

The dynamics of the total intracellular protein concentration represent the measure of cell growth. To estimate this, 1 ml of homogenous fermentation broth was pipetted into an Eppendorf tube and centrifuged for 10 min at 4°C and 5000 rpm. The supernatant was discarded and 1 ml of 1 M NaOH was added to the pellet, vortexed and incubated for 3 hours with shaking at room-temperature. The obtained solution was diluted 10 times and the protein concentration was then determined by BCA-Assay, a biochemical assay for the determination of total protein concentration (see below).

6.6 Precipitation to remove lactose

Lactose, which interferes with reductive assays, was removed by a trichloroacetic acid (TCA)/acetone precipitation from fermentation samples with lactose as carbon source. The precipitated samples were afterwards used for BCA- and CMC-Assay (see below). The procedure was as follows: 0.5 ml of supernatant (containing the proteins) were mixed with 4 ml chilled (-20°C) acetone and 0.5 ml of chilled 100% TCA, in that exact order. Before and after the addition of TCA the mixture was carefully mixed on a bench vortexer. The mixture was put to -20 °C for one hour and then centrifuged for 15 min at 4 °C and 11,500 rpm. The supernatant was discarded and the protein pellet was washed 3 times with acetone. The washed pellet was air-dried and 0.5 ml of water with protease-inhibitor-cocktail (Roth) (1 µl inhibitor-cocktail/ml water) were added. The protease-inhibitor was added to the water since re-solubilization of the proteins had to be carried out at room-temperature, which takes some hours.

6.7 Bicinchoninic Acid Protein Assay (BCA)

The assay was carried out in 96 microwell plates using the commercial Pierce™ BCA Protein Assay Kit. First, 50 parts of BCA reagent A were mixed with 1 part of BCA reagent B, resulting in a green solution. Then 25 µl of the sample were pipetted into a well and 200 µl of prepared BCA solution was added. The microwell plate was incubated for 30 min at 37 °C, cooled down and color formation was measured in a Spektramax PLUS 384 microwell platereader at 562 nm. Of each sample triplicates were performed. For the blank the sample was substituted for distilled water. For each BCA solution a standard curve with bovine serum albumin (BSA) as standard was prepared, with eight concentrations ranging from 0.025 to 2 mg/ml.

6.8 Enzymatic assays (endo- and exocelluase activity assays)

Endocellulase activity was measured by CMC (carboxymethylcellulose)-assay (adapted from Ghose, 1987). For each sample duplicates and a blank were measured. In a glass tube 50 µl of sample were put and 200 µl of 1% CMC-solution in 50 mM sodium actetate buffer (pH 5) were added. The glass tubes were incubated for 30 min at 50°C in a water bath and the reaction was stopped by adding 250 µl of DNS-reagent. The mixture was then boiled for 5 min at 100 °C, cooled down and 2 ml of distilled water was added; the resulting solution was mixed on a benchtop vortexer. Color formation was measured at 540 nm in Spektramax PLUS 384in microplates. For the blank only the order of the addition of the substrate (1% CMC-solution) and stop reagent (DNS) was inverted, thus first adding DNS and then adding the CMC-solution. The blanks were not incubated but subsequent steps were identical with those of the test samples. For each new DNS-reagent a standard curve was prepared using 10, 8, 6, 4, 2, 1 and 0.5 mM glucose standards, respectively.

Exocellulase activity was determined via pNPL-assay using para-nitrophenol-β-D-lactoside (pNPL) (Megazyme). A solution of 2 mM pNPL in 50 mM sodium acetate buffer (pH 5) was prepared and used as substrate. The assay was carried out in 96 microwell-plates by adding 50 µl of substrate to 50 µl of sample and incubating it for 30 min at 50 °C. After incubation 100 µl of 2% Na_2CO_3 were added to the reaction mixture and after cooling down, the color formation was measured at 405 nm using the Spektramax PLUS 384 microplate reader. As blank the sample was replaced by distilled water.

A standard curve was prepared using defined amounts [nmol] of para-Nitrophenol (100 µl) and adding 100 µl of 2% Na_2CO_3. A single standard curve was used for all measurements, given in Figure 15.

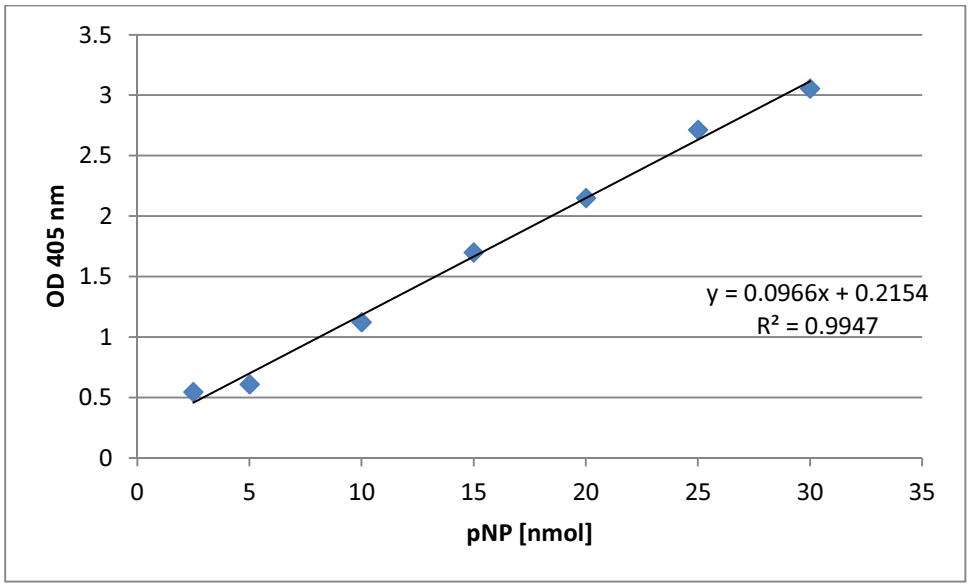

Figure 15: Standard curve for pNPL Assay

6.9 Zymogram

Zymogram optimization was carried out using samples with endocellulase activity. The quality of the zymogram was optimized mainly by changing incubation time and the concentration of the samples. The optimization process showed that some bands are only visible if the zymogram is incubated, whereas incubation will make strong bands leak to other lanes. In order to ensure that results were not biased by this 'leakage', two zymograms per sample set were carried out, namely one non-incubated and one incubated for 20 min at 50 °C.

For the zymograms sodium dodecyl sulfate polyacrylamid gels (SDS PAG) with an acrylamide concentration of 12% were prepared according to the recipe given in Table 7. In SDS-PAGE (sodium dodecyl sulfate polyacrylamid gel electrophoresis) proteins are denatured and their charge is masked by SDS, so that proteins are separated in the electrical field only according to their mass. The substrate for the endocellulases (carboxymethylcellulose (Roth)) was incorporated by using a 1.5 M Tris- buffer with 1% of dissolved CMC. All chemicals were mixed and the polymerization was started by adding APS and TEMED. The monomer solution was pipetted into a prepared vertical casting chamber up to ¾ and covered with isopropanol. After approximately 30 minutes the isopropanol was removed, and the residue was removed by washing with distilled water. The empty part of the casting chamber was dried using a filter paper, then the stacking gel (recipe in Table 7) was pipetted onto the separating gel and the comb was immediately placed in the chamber. After a polymerization time of approximately 15 minutes the comb was removed and the gel was taken out of the casting unit covered by the glass plates and put into the electrophoresis cell. The electrophoresis cell was filled with running buffer and the gel was loaded with 20 µl sample per pocket. The samples were not treated, only Laemmli buffer (BioRad) was added. The first pocket was loaded with PageRuler™ Prestained Protein Ladder (Thermo Scientific) as standard. The electrophoresis was run at 180 V until the blue dye of the samples buffer reached the positively charged end of the gel. The gel was removed from the glass plates and proteins were renaturated by washing the gel twice with a 2.5 % Triton X-100 solution for 10 min. Then the gel was washed three times with 50 mM NaAc-buffer (pH 5) for 5 min. One gel was incubated prior to staining at 50 °C for 20 min in 50 mM sodium acetate-buffer (pH 5). The buffer was removed and the gels were stained by incubating at room-temperature with 0.1 % CongoRed solution for 15 min with light shaking on 30 rpm. CongoRed was discarded, the gel was washed with water and decolorized with a 1 M NaCl-solution. The zymograms were scanned using GS-800 scanner (BioRad).

Table 7: Reagents needed for preparation of two SDS (sodium dodecyl sulfate) **polyacrylamidgels, which allow a separation of proteins according to their mass, due to SDS masking the native charge of the proteins.**

	Stacking gel	**12% Separating gel**
Analysis H_2O	3.74 ml	3.2 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v) (Roth)	1.125 ml	4 ml
0.5 M Tris – HCl buffer, pH 6.8	1.875 ml	
1.5M Tris-HCl (pH=8.8) with 1% CMC		2.6 ml
10% (w/v) SDS (Roth)	76 µl	0.1 ml
10% (w/v) ammonium persulfate (APS) (Roth)	50 µl	100 µl
TEMED (Roth)	15 µl	10 µl
Total volume	6.881 ml	10.01 ml

6.10 Transformation of *B. tropica* with mCherry and YFP plasmid

6.10.1 Transformation of *B. tropica* and pre-validation by PCR

The plasmid was provided by the research group of Teresa E. Pawlowska (Cornell University) in form of *E. coli* DH 5α- mCherry/YFP with gentamicin resistance (antibiotic marker). A midi-prep was carried out with these two *E. coli* strains. For this purpose the strains were pre-grown in 50 ml of Lysogeny broth (LB) medium at 37 °C and 170 rpm overnight. On the next day the culture was transferred to 50 ml falcons and centrifuged 5 000 × g at 4 °C for 10 minutes and the supernatant was discarded. The subsequent steps were carried out with the PureYield™ Plasmid Midiprep System (Promega, USA) following the manufactures protocol. The concentration of the isolated plasmid DNA was determined using Nanodrop ND-1000 spectrophotometer at 260 nm.

To obtain electrocompetent cells, *B. tropica* was pre-grown overnight in 25 ml Trypton-Soy- broth (TSB) at 30°C with shaking at 150/min. Per transformation a total of 6 ml of cell culture was pipetted into four microcentrifuge tubes and was centrifuged at room-temperature for 2 min at 16,000 g. The supernatant was discarded and the cell pellet was washed twice with 1 ml of 300 mM sucrose. The cell pellets were resuspended in a combined total of 100 µl of 300 mM sucrose.

The actual transformation was carried out by electroporation. First 750 ng of the vector were denatured for 5 min at 95 °C. The denatured vector was mixed with vector with 100 µl of *B. tropica* electrocompetent cells and incubated for at least 1 min on ice. The mixture was transferred to a pre-chilled electroporation cuvette (gap 0.2 cm, BioRad) and a pulse in BioRad MicroPulser (25 µF, 200 Ω, 2.5 kV) was applied. Immediately after the pulse 1 ml of TSB was added to the cells and the mixture was transferred to a glass tube for overnight cultivation at 30 °C and 115 rpm. The cells were then harvested, concentrated to 50 µl and plated on LB-medium containing gentamicin (Sigma Aldrich) and put on 30 °C. After 2 to 3 days the transformed colonies appeared. Those were picked and streaked onto fresh gentamicin-containing LB plates. After 2 days of growth a mini-prep with presumably transformed colonies was carried out and the obtained DNA was checked for the presence of the mCherry or YFP vectors by PCR (6.2) with primers targeting the mCherry (mCherry-fw: 5'-CGCTTCTCGTTGGGGTCTTT, mCherry-rev: 5'-TGAAGGGCGAGATCAAGCAG) or YFP (YFP-fw: 5'-CTACCCCGACCACATGAAGC, YFP-rev: 5'-AAAGACCCCAACGAGAAGCG) gene. The master mix was prepared as described in 6.2. The amplification was carried out with the same settings as for *tef1* (see 6.2), but with an annealing temperature of 59 °C. Transformants that were positive in the PCR were then checked using a confocal laser microscope.

6.10.2 Laser scanning confocal microscopy

The fluorescence of transformants that showed positive results in PCR with mCherry primers were checked using a laser scanning confocal microscope (LSM 510 Meta, Carl Zeiss, Göttingen, Germany). A concentrated suspension with water of the respective transformant was prepared and pipetted onto a microscope slide. The fluorescence signal of mCherry was detected with an excitation wavelength of 543 nm (HeNe laser), a 570 nm dichroic mirror, and an emission of 574–691 nm.

7 Results

7.1 *Trichoderma* strains are closely associated with bacteria at least *in vitro*

For the purpose of verifying that growth of *B. tropica* along with a *T. reesei* culture can be detected by qPCR with primers targeting a *Burkholderia*-specific region of 16S (GB primers), a cured strain of *T. reesei* QM 9414 was infected with *B. tropica* TU CIM 4180 and grown on PDA with cellophane (see 7.2). In course of this experiment it was observed that the mixed culture did not show any signs of antagonism, suggesting that the two microorganisms are naturally tolerant to one another. The results of the qPCR carried out with primers for the *Burkholderia*-specific 16S region (GB) and for the fungal *tef1* gene (Figure 16) confirm their ability to co-exist, showing that both, *B. tropica* and *T. reesei* are actually able to grow. However, compared to the cured *T. reesei* QM 9414 the cycle-threshold increases over time, thus less fungal DNA is present in the sample, which only displays that *B. tropica* is growing significantly faster than *T. reesei*, but not that *T. reesei* is inhibited in its growth. As a standardized concentration of DNA was used for the qPCR, all of the DNA present in a cured sample would come from the fungus (Ct of *tef1* is constant over 4 days of growth), whereas in the infected sample DNA comes from both organisms, thus leading to a shift in amplified product in case one of the organisms is growing faster than the other, as *B. tropica* does.

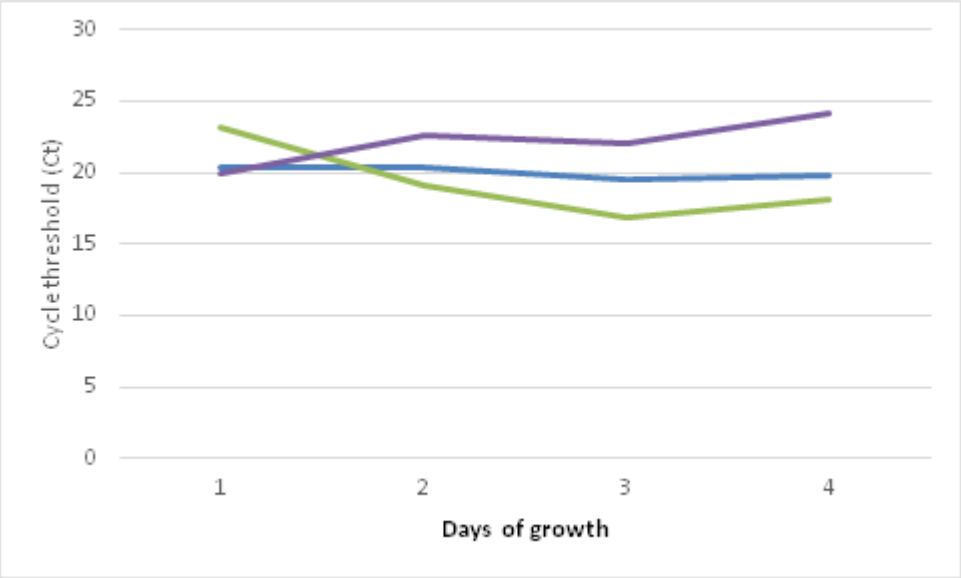

Figure 16: Results of qPCR carried out with *Burkholderia* specific (GB) and *tef1* primers of an infected (with *B. tropica*) *T. reesei* QM 9414. The lower the cycle threshold the more of the targeted region was amplified, thus corresponding to the growth of the bacterium (GB primer) or fungus (*tef1* primer). Results for qPCR with *tef1* primers for cured *T. reesei* QM 9414 (blue) and *T. reesei* QM 9414 infected with *B. tropica* TU CIM 4180 (violet), and with *Burkholderia* specific primers (GB) for *T. reesei* QM 9414 infected with *B. tropica* TU CIM 4180 (green).

A dual confrontation assay of *T. reesei* QM 9414 and *B. tropica* TU CIM 4180 on agar plates additionally supports this observation. In Figure 17 it can be seen that no antagonism is exhibited when the mycelium of *T. reesei* reaches the colony of *B. tropica*, however, the fungus seems to first grow around the colony and overgrows it only after longer incubation. Cultivation of mixed cultures (*B. tropica* with spores of *T. reesei*) further validates that neither *T. reesei* nor *B. tropica* are negatively influenced by the presence of the other, as both are able to propagate.

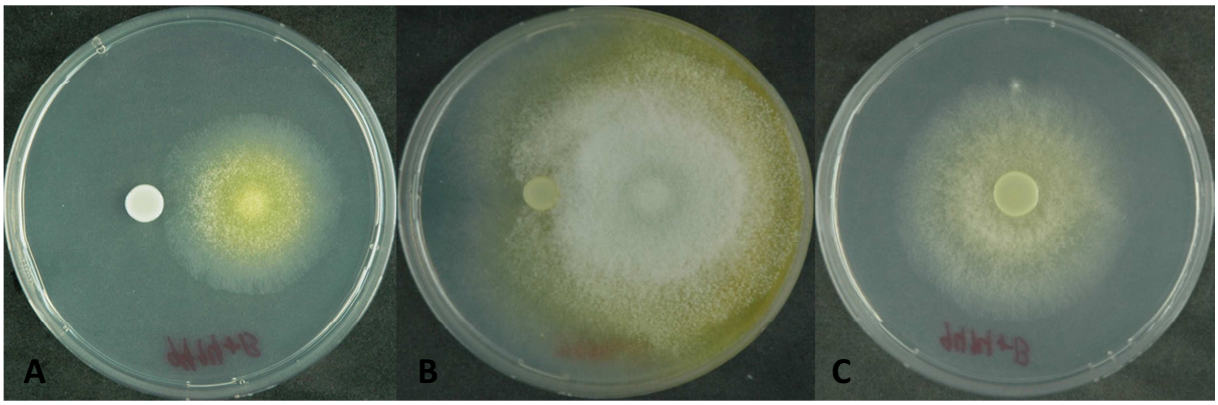

Figure 17: Behavior of *T. reesei* QM 9414 in presence of *B. tropica* TU CIM 4180 on PDA. A and B: Confrontation of *T. reesei* QM 9414 with *B. tropica* TU CIM 4180 after 48 and 84 hours of incubation at 28 °C. C: Growth of mixed culture (*T. reesei* QM 9414 and *B. tropica* 4180) after60 hours of growth. (Pictures provided by Mohammad Rahimi)

In contrast to *T. reesei* ability to co-exist with *B. tropica*, other fungi such as *Fusarium oxysporum* (several soil fungi tested, M. Rahimi, I. Druzhinina, pers. com) cannot grow completely towards the *B. tropica* colony and a clear antagonistic effect can be observed (Figure 18). Even after a longer period of incubation *F. oxysporum* does not grow around the *B. tropica* colony, which shows that the inhibitory effect of *B. tropica* is quite extensive. This growth inhibition of *Fusarium* by *B. tropica* displays that the ability of *T. reesei* and *B. tropica* to co-existent is rather exceptional.

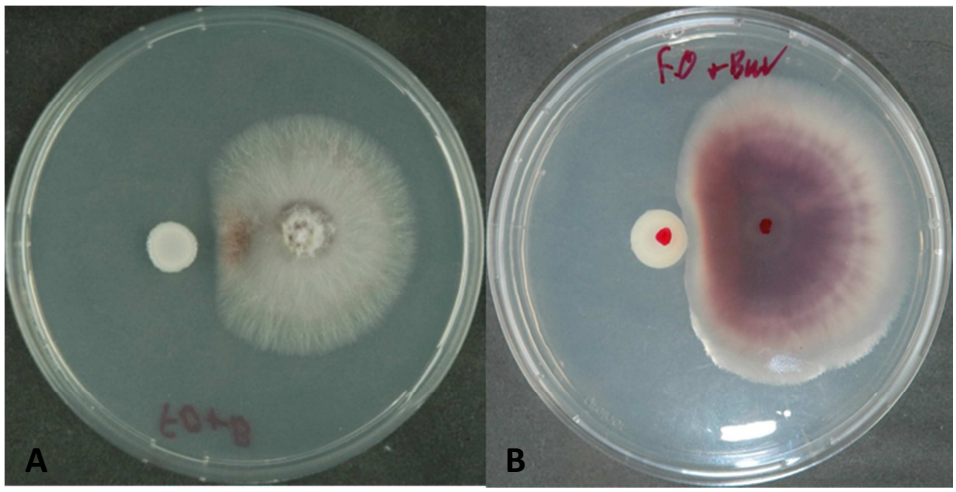

Figure 18: Behavior of *Fusarium oxysproum* in presence of *B. tropica* TU CIM 4180 on PDA. A and B: Confrontation of *Fusarium oxysporum* with *B. tropica* TU CIM 4180 after 48 and 84 hours of incubation at 28 °C. (Pictures provided by Mohammad Rahimi)

7.2 *Trichoderma* strains may be cured from associated bacteria in a species-specific way

7.3 *Trichoderma* strains may be cured from associated bacteria.

Independent of the mode of *Trichoderma* – bacteria interactions, antibiotics may be applied to break it. The goal was to achieve truly pure *Trichoderma* cultures, i.e. to eliminate the bacterial partner from the surface of or from the interior of the fungus. To do this, first the effectiveness and optimal concentration of the antibiotics had to be determined. For that purpose an experimental series with *T. reesei* QM 9414 was carried out, testing several concentrations of imipenem (Carbapenem, imi) and ceftazidime (Cephalosporin, cef) and a mixture of both (mix), as both antibiotics have proven to be effective against various *Burkholderia* species (Deris et al., 2010; Thibault et al., 2004). Applied concentrations of the antibitiocs are given in Table 8. As a control *T. reesei* QM 9414 was cultivated under the same conditions without antibiotics.

Table 8: Concentrations of applied antibiotics (Ceftazidime (cef), Imipenem (imi) and in the experimental series of *T. reesei* curing from *B. tropica*.

cef	imi
Individual [µg/mL]	
100	50
60	30
30	15
15	
Mixture [µg/mL]	
10	5
15	10
20	20

It was suspected that bacterial cells (dead or alive) might be attached to the hyphae. To test it one half of the mycelial sample obtained from the liquid cultivation with antibiotics was filtered and then washed three times with sterile water to remove not attached bacteria; the second part was used directly. Thus of each sample a 'washed' (w) and 'non-washed' (nw) DNA was extracted and tested for the presence of bacterial genetic material.

For the extracted total DNA of the presumably cured *Trichoderma* strains PCRs with universal 16 S rRNA primers (fD1, rP1), *Burkholderia*-specific primers targeting a fragment of 16 S rRNA (GB-F, GB-N2) and primers for the single-copy house keeping gene *tef1* (EF1, EF2) were carried out for each

sample. The amplification of the fungal genomic DNA locus encoding a fragment of the translation elongation factor 1-alpha (*tef1)* was used to determine the quality of the DNA extract by making sure that a single-copy fungal gene could be amplified. To determine the presence of the bacterial genetic material the amplification of the 16S rRNA fragment was performed with the universal bacterial 16 S rRNA primers (fD1, rP1) and GB primers that have shown specificity for *Burkholderia* genus and closely related taxa (Perin et al. 2006). In order to reinforce the amplification the PCR product resulting from each PCR reaction was used as template DNA for the subsequent amplification (nested PCR).

Figure 19 shows results from the initial (top row) and nested (bottom row) PCR for *Burkholderia* specific 16 S rRNA (GB-F, GB-N2) primers applied to DNA extracts from cultures grown with highest antibiotics pressure (Table 8). It is obvious that for the amplification of the *Burkholderia* specific 16 S fragment (GB primers) a nested PCR is necessary, as even the positive control (*B. tropica* TU CIM 4180 DNA) does not show a band in the initial PCR run. For each sample two different concentrations of DNA were tested by simply adding a final concentration of either 0.44 ng/µl (marked 1) or 0.7 ng/µl (marked 2) to the master mix, in order to determine whether the concentration of initial DNA influences the quality of amplification of the *Burkholderia* specific 16 S region with GB-F, GB-N2 primers . The nested PCR reveals some bands at the same height as the positive control at approximately 1000 bp. These PCR products can be seen for the samples where *T. reesei* QM 9414 was washed but no antibiotics applied (washed control '9414 c w' 1&2). Moreover the reaction was positive for the non-washed cultivation with 30 µg/ml imipenem '9414 imi 30 nw' 1&2 and for the washed cultivation with 100 µg/ml cefatzidime '9414 cef 100' 1&2. The only sample that does not show any band for both, washed and non-washed mycelia, is the cultivation with a mix of 20 µg/ml ('Mix 20') of each, imipenem and ceftazidime. To ensure that the absence of positive PCR product is not due to bad quality of the DNA, a PCR targeting fungal genomic single-copy *tef1* gene (see Figure 20) was performed. All samples show a PCR product of expected length around 900 bp thus proving that the DNA extract was of sufficient quality.

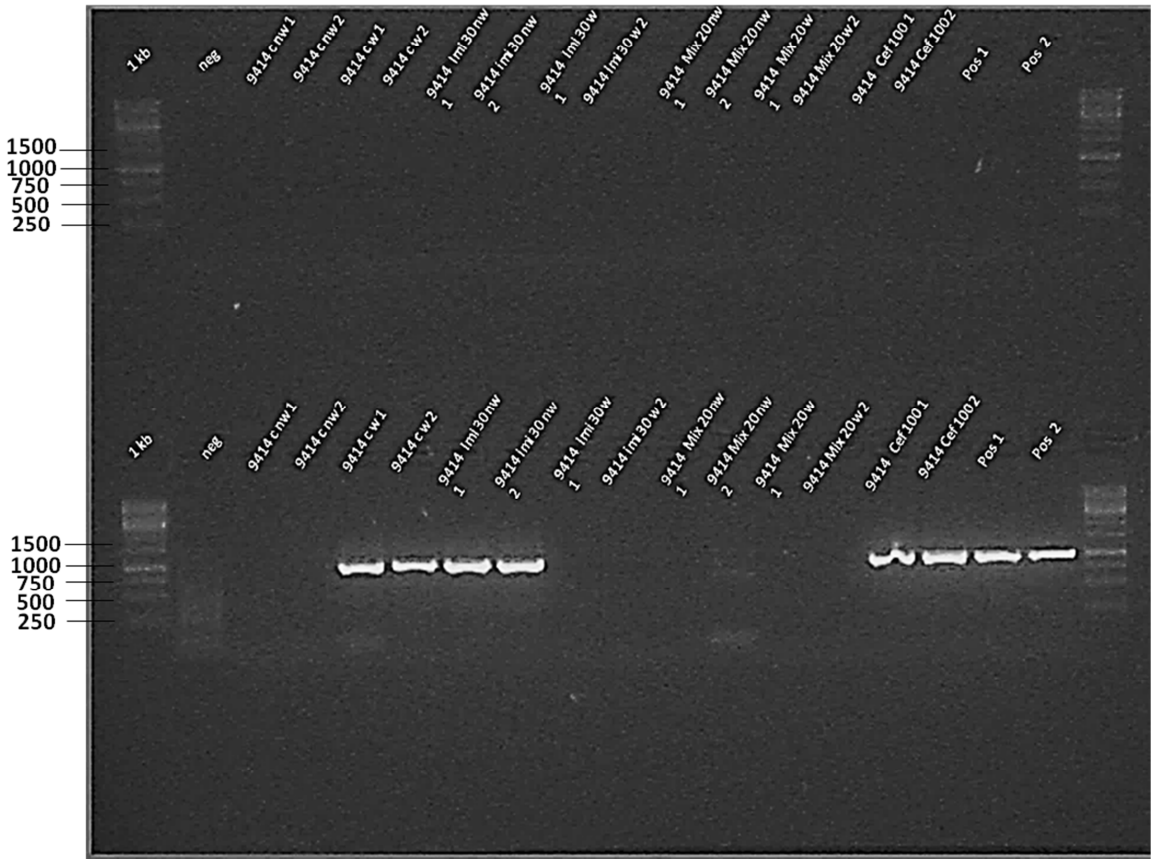

Figure 19: Results of PCR with *Burkholderia*-specific 16S rRNA (GB) primers. Top row shows results of the initial PCR, bottom row shows nested PCR. Mix denotes samples for which a mixture of imipenem and ceftazidime was used as indicated in Table 8. Numbers next to the antibiotic gives the concentration in µl/ml. *T. reesei* QM 9414 was cultivated under same conditions without antibiotics (marked 'c' for control). As positive control DNA of *B. tropica* TU CIM 4180 was used. Numbers 1 and 2 represent the final concentration of DNA used in the PCR, namely 0.44 (labeled 1) or 0.7 (labeled 2) ng/µl.

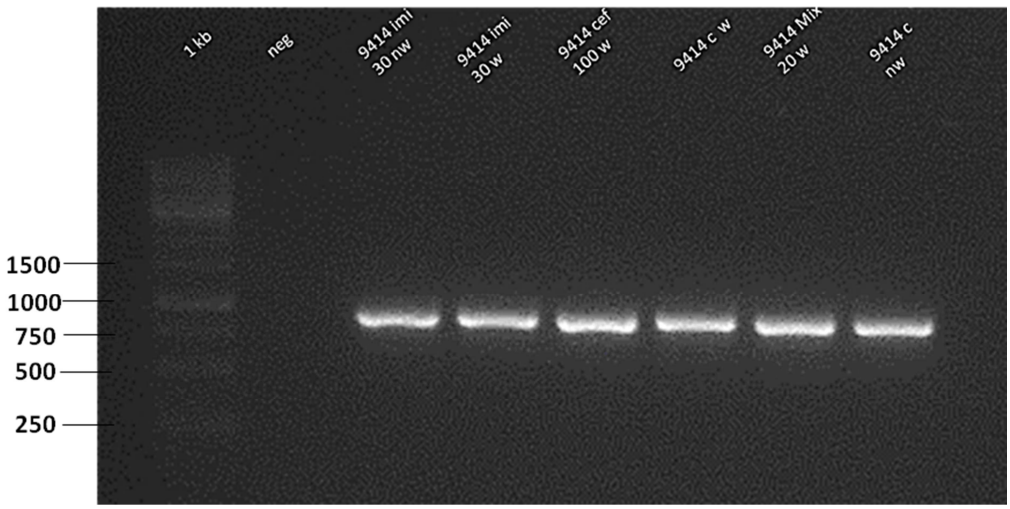

Figure 20: Results of the PCR targeting the fungal gDNA region *tef1* amplified in order to determine the quality of the total DNA extract.

By analyzing the results of those two PCRs, the following conclusions were drawn:

- the washing procedure applied is neither successful nor necessary, as non-washed sample shows a band and the washed one not. However for the other sample the pattern is reversed, and the washed sample is *Burkholderia*-positive.
- Ceftazidime alone is not effective to eliminate *Burkholderia* associates from *Trichoderma* cultures.
- A combination of imipenem and ceftazidime (20 µg/ml each) is effective, as no amplification of bacterial DNA was obtained neither for washed nor non-washed *Trichoderma* mycelia. Consequently this combination was used for further tests.
- The results of these experiments do not allow to speculate on the preferential either intracellular or extracellular location of associated bacteria as a random pattern was observed: a sample washed three times with sterile water was positive for *Burkholderia* , whereas the non-treated one was not. A contamination of the sterile water was excluded as it was used also for the treatment of the other samples. In case of intracellular located bacteria washing would be in general ineffective.

Despite that PCR tests for the presence of *Burkholderia* in/on *Trichoderma* mycelium were repeated, no unambiguous outcome was obtained. Thus another approach to determine whether a fungal culture is cured from bacterial (=is pure in strict sense), had to be developed. This method was based on the consideration that living bacteria proliferate at a rate higher compared to that of fungal cells, which should lead to an increase in *Burkholderia* specific-16S rRNA (GB) PCR product compared to fungal gDNA fragment *tef1.* Therefore a quantitative PCR (qPCR) was used. Consequently the total genomic DNA of the *Trichoderma* cultures was extracted after 24, 48, 72 and 96 hours of growth on PDA plates covered with cellophane. Afterwards a qPCR with *Burkholderia* specific primers (GB primers)- and *tef1* primers were carried out (for exact description of the method refer to 0). To verify this method a cured strain of *T. reesei* QM 9414 was infected with *B. tropica* and the same procedure was carried out. The ratio of the test culture *T. reesei* QM 9414 (in blue) does not increase but slightly decreases with time. It can be concluded that no viable bacteria are present in the sample cured with a mixture of 20 µg/ml imipenem and ceftazidime, respectively.

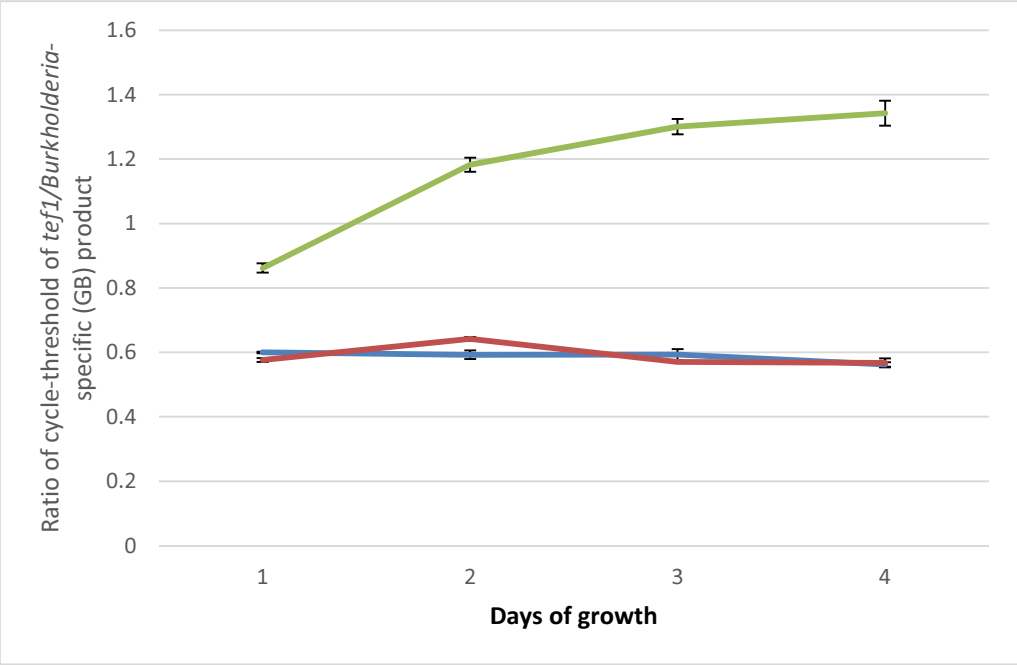

Figure 21: Results of qPCR shown as ratio of the average Ct (cycle-threshold) of fungal gDNA fragment *tef1* to bacterial 16S rRNA genes amplified with GB primers for each sample. Results for cured *T. reesei* QM 9414 (blue), *T. reesei* QM 9414 control (untreated, red) and *T. reesei* QM 9414 infected with *B. tropica* TU CIM 4180 (green) are shown.

All presumably cured strains and/or the controls were tested using this method. Controls in this case were completely untreated samples, thus neither antibiotics nor any other treatment was used. As most of the controls did not show any amplification of bacterial 16S rRNA (neither in PCR nor in qPCR), the treated samples were not tested via qPCR. As mentioned all untreated strains were first tested by PCR using 16S rRNA (16S, GB) and fungal *tef1* primer pairs. Figure 22 shows a non-nested and nested PCR carried out with universal 16 S primers (fD1, rP1) with *B. tropica* as positive control. Only the positive control shows a band in the non-nested PCR. The nested PCR reveals strong smearing for every sample, including the positive control, below the expected size of the PCR product but does not show any sharp bands, suggesting that the streaks are caused by a number of unspecific amplification events, rather than being evidence of the presence of bacteria. Additionally universal 16 S primers sometimes amplify mtDNA (mitochondrial DNA) of *Trichoderma*, which might cause false-positive results and smearing. Regardless of this result a *Burkholderia* specific 16S rRNA PCR was performed (with GB primers) as validation (see Figure 23). In general the PCR with primers targeting a *Burkholderia*-specific fragment of the 16 S rRNA (GB) confirms the results of the PCR with universal 16 S primers, with two exceptions. A band around 1000 bp (corresponding to length of the PCR product of GB primers) is visible for TU CIM 4106 (*T. reesei*) and TU CIM 4455 (*T. afroharzianum*). The PCR product was purified and sent for sequencing. The obtained sequences were checked using NCBI BlastN, showing 99 % similarity to *B. tropica* strains. The presence of *B. tropica* in TU CIM 4455 seemed to be a contamination of the applied DNA sample, as *B. tropica* was not present in

subsequently extracted DNA samples. The absence of bands of all the other samples suggests that they are free of *Burkholderia* and any other bacteria (see Figure 23).

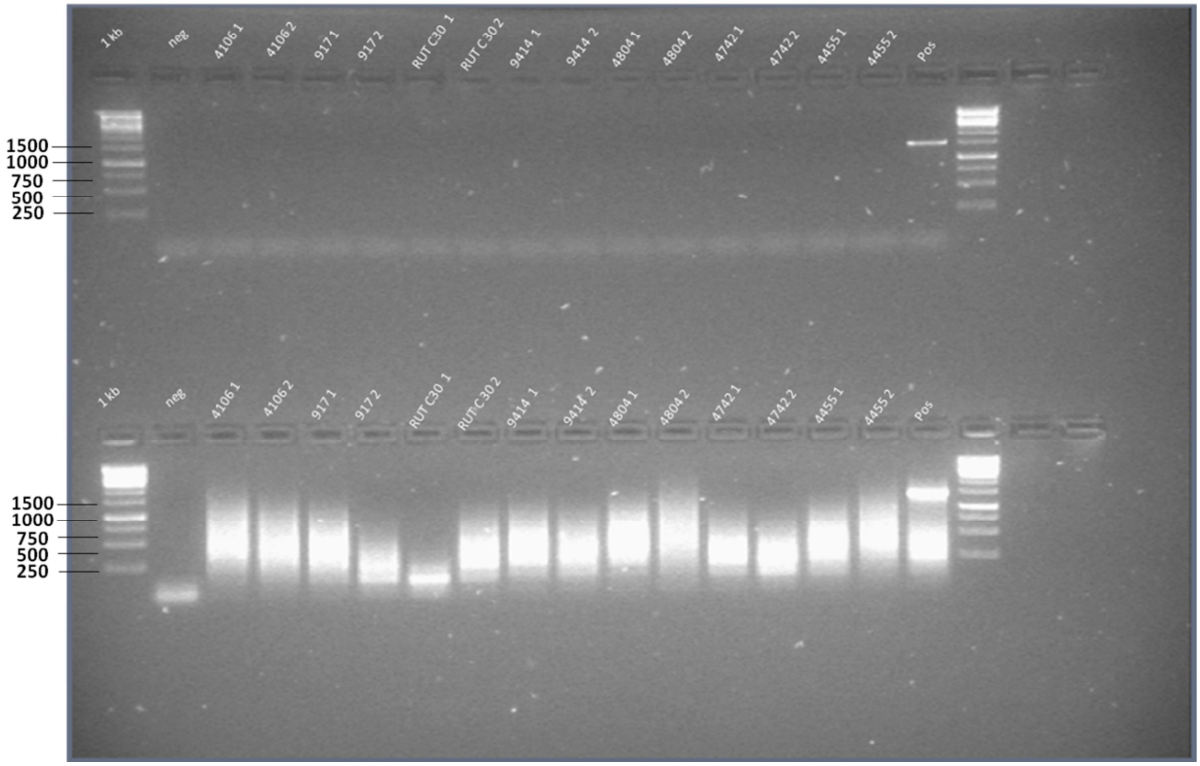

Figure 22: Results of the PCR with primers amplifying the bacterial 16S rRNA fragments (fD1and rP1 primers). Top row shows the non-nested and the bottom row the nested PCR. Numbers 1 and 2 next to strain ID numbers refer to the applied amount of DNA, thus 0.44 ng/µl or 0.7 ng/µl respectively. DNA of the pure culture of *B. tropica* TU CIM 4180 used as positive control.

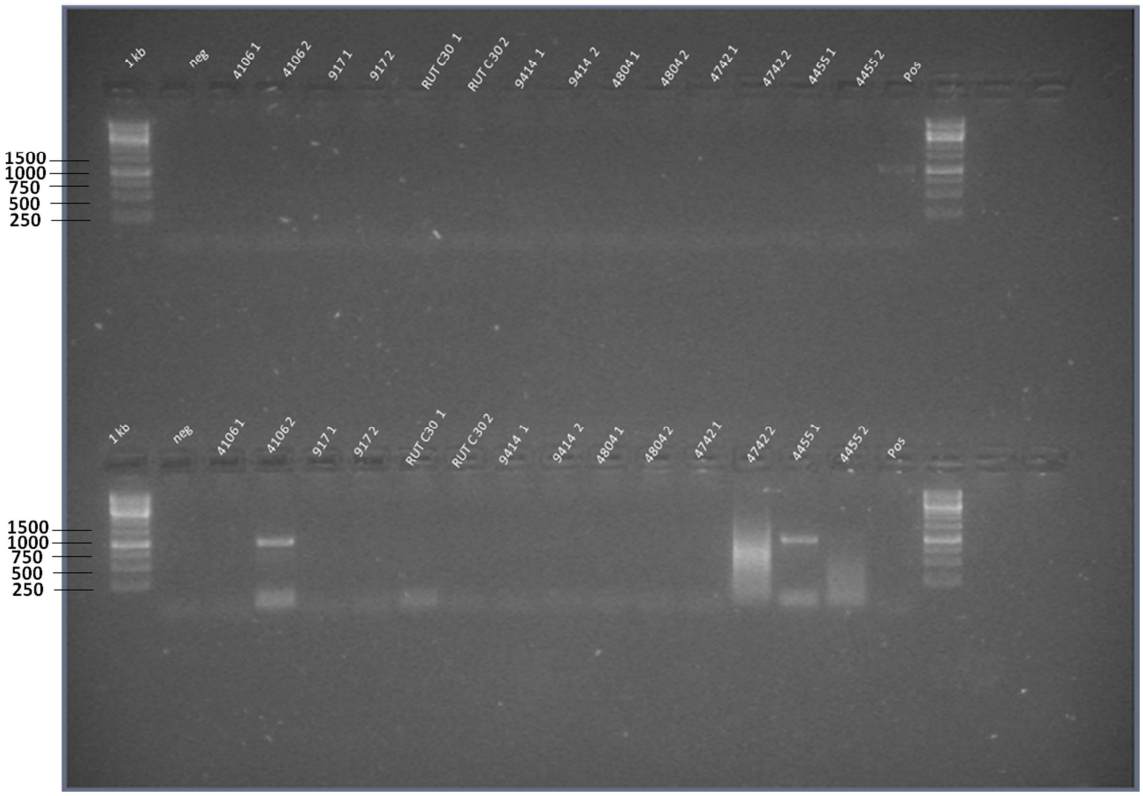

Figure 23: Results of the PCR amplifying the *Burkholderia*- specific 16 S rRNA fragment (GB-F, GB-N2) primers checking for the presence of *Burkholderia* . Top row shows the initial and the bottom row the nested PCR. Numbers 1 and 2 next to the strain ID numbers refer to the applied volume of DNA, 0.44 ng/µl or 0.7 ng/µl respectively. DNA of the pure culture of *B. tropica* TU CIM 4180 used as positive control (0.7 ng/µl). The positive control shows a band at around 1000 bp in the initial PCR, but does not show a band in the nested PCR. This is caused by exceeding the optimal initial DNA concentration in the PCR mixture.

T. reesei RUT C-30, a high cellulase producer strain used for further experiments, was also checked for the presence of possible bacterial associates by use of PCR (see Figure 22, Figure 23) and even though there was no evidence of *Burkholderia* being present, it was treated with antibiotics (combination of imipenem and ceftazidime) and checked by qPCR, to eliminate any possibility of *Burkholderia* being present but undetected. Results of this qPCR are displayed in Table 9, as negative control (neg) water was used and DNA of *B. tropica* TUCIM 4180 acted as positive control. Most samples do not show any amplification of 16S rRNA (GB primers, no Ct), and the few ones that do, have a cycle threshold that is higher than the one of the negative control, thus they can be considered to be negative. No ratios were calculated as it is not possible, thus *Burkholderia* is not present. The 'cured' *T. reesei* RUT C-30 will from here on be referred to as *T. reesei* RUT C-30*.

Table 9: Results of qPCR for 'cured' *T. reesei* RUT C-30 (from here on referred to as *T. reesei* RUT C-30*).

Sample name	Ct for GB	Ct for *tef1*	*ratio Ct (tef1*/GB)
RUT C-30* DAY 2/sample1	No Ct	17.23	Ct ratio calculation not possible
RUT C-30* DAY 2/sample1	No Ct	16.85	
RUT C-30* DAY 2/sample1	No Ct	17.36	
RUT C-30* DAY 2/sample2	No Ct	17.08	
RUT C-30* DAY 2/sample2	38.76	16.53	
RUT C-30* DAY 2/sample2	No Ct	16.86	
RUT C-30* DAY 3/sample1	No Ct	16.77	
RUT C-30* DAY 3/sample1	No Ct	17.31	
RUT C-30* DAY 3/sample1	36.24	16.94	
RUT C-30* DAY 3/sample2	35.77	17.51	
RUT C-30* DAY 3/sample2	37.25	17.75	
RUT C-30* DAY 3/sample2	37.46	17.31	
RUT C-30* DAY 4/sample1	No Ct	18.11	
RUT C-30* DAY 4/sample1	No Ct	18.18	
RUT C-30* DAY 4/sample1	No Ct	18.35	
RUT C-30* DAY 4/sample2	No Ct	17.95	
RUT C-30* DAY 4/sample2	No Ct	17.45	
RUT C-30* DAY 4/sample2	No Ct	18.18	
neg	34.88	30.07	
pos	25.15		

7.4 *B. tropica* has versatile effect on production of cellulases by *Trichoderma reesei*

In order to determine the role of *Burkholderia tropica* on secretion of cellulases by *Trichoderma reesei*, shake flask fermentations with two cellulase-inducing carbon sources, lactose and microcrystalline cellulose respectively, were performed as described in Materials and Methods. Samples of each cultivation were taken every 24 hours for 4 days of continuous fermentation and analyzed with regard to biomass production, total protein secretion and cellulase activity. For each carbon source the pure strains were cultivated separately, serving as control and in mixed cultures (*T. reesei* with added *B. tropica*) to evaluate the effect of *B. tropica* on metabolism of *T. reesei* in this system.

7.4.1 Growth of *Trichoderma* in presence of *Burkholderia* is strongly affected by the available carbon source

The growth of microorganisms may be quantified through the increase of respective biomass. However, the total microbial biomass could only be determined on the soluble carbon source (lactose in this case). Insoluble microcrystalline cellulose will bias dry-weight measurement. Therefore growth for samples cultivated on this carbon source was determined via the alternative approach that assumes that the increase of the intracellular protein content per milliliter is proportional to the increases of the number of cells per milliliter.

In Figure 24 the results for intracellular protein concentration [mg/ml], representing growth on microcrystalline cellulose, are displayed. *T. reesei* RUT C-30* (given in blue) shows continuous growth reaching its maximum after 96 hours of fermentation. Opposed to this, the propagation of *T. reesei* RUT C-30* is delayed in presence of *B. tropica* (shown in red). Consequently only a slight increase in growth can be observed after 72 and 96 hours of fermentation. This corresponds to the exhibited growth pattern by the sole *B. tropica*, showing that the bacterium is not able to grow on cellulose. Thus it can be concluded that in co-cultivation on microcrystalline cellulose *B. tropica* inhibits growth of *T. reesei* until bacteria are dying and the inhibitory effect with them.

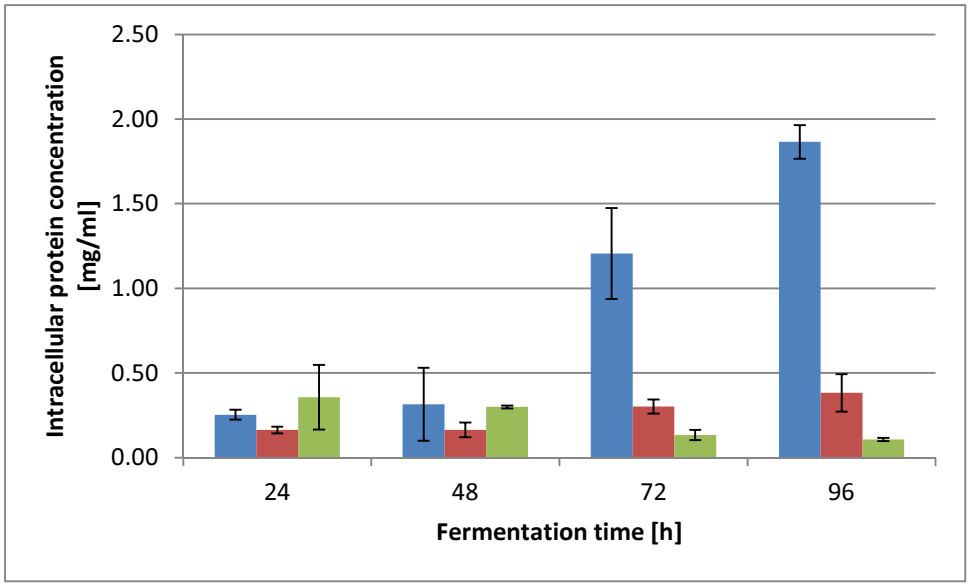

Figure 24: Growth of microorganisms on microcrystalline cellulose represented as a dynamics of intracellular protein content. Data for the growth of *T. reesei* RUT C-30* (in blue), co-cultivation (in red) and *B. tropica* (in green) are shown.

As mentioned above, the dry weight analysis may represent growth in case of a fermentation, which is performed on a soluble carbon source. In contrast to the obtained results for the cultivation on microrystalline cellulose, *T. reesei* RUT C-30* co-cultivated with *B. tropica* (in light blue) on the soluble carbon source lactose exhibits a higher production of biomass than the control (*T. reesei* RUT

C-30*, violet), as shown in Figure 25. It is obvious that biomass generation is significantly elevated throughout the fermentation (with the exception of the sample after 24 hours) in presence of *B. tropica*. The divergence is highest after 48 hours of fermentation. However, whether the increase in biomass is generated by fungal and/or bacterial cells, cannot be gathered from the data. Yet it can be concluded that this effect is based on the interaction between these two species, as results for the biomass production of *B. tropica* (displayed in orange) clearly show that *B. tropica* is not able to grow on lactose what is also in agreement with Reis et al. (2004). The minimal and delayed increase in biomass can be contributed to the fact that *B. tropica* can use peptone for its proliferation however no experiments to test it were performed. It has to be noted that the co-cultivation reaches a maximal biomass concentration of 18.35 g/L, which exceeds the theoretically possible amount of biomass. As a rule the conversion rate of carbon source to biomass does not exceed 50 % (C.P. Kubicek, personal communication), thus yielding a maximum of 10 g biomass per liter medium in case of an initial lactose concentration of 20 g/l. Additionally peptone (1 g/l) was in the fermentation medium, but the conversion rate is lower compared to lactose and the initial concentration is too low to lead to such an increase in theoretical maximal of biomass. In general a value with a deviation of more than 80 % of its theoretical value, indicates a mistake in analysis. The applied method of dry-weight determination is commonly used and was performed for every fermentation sample in the same way. As only the samples of co-cultivation show this excess of more than 80% of the theoretically possible value, it is rather improbable that this was caused by an inaccuracy in the analysis. Another possible explanation would be that a higher amount than 2 % of carbon source was added to the fermentation medium. This also can be excluded, since the liquid medium was prepared in one batch for all three cultivations. Standard deviations additionally show that the biological duplicates coincide, suggesting that the value is not an outlier. A most reasonable reason is that bacteria added to the biomass. In this case quantification of both microbes via qPCR would have validated or refuted this assumption, by showing the contribution of the microorganisms to growth, however during this thesis it was not feasible to perform. As there is no obvious explanation for the divergence in actual and theoretical value at present, the values have to be regarded with caution but should not be considered unreliable.

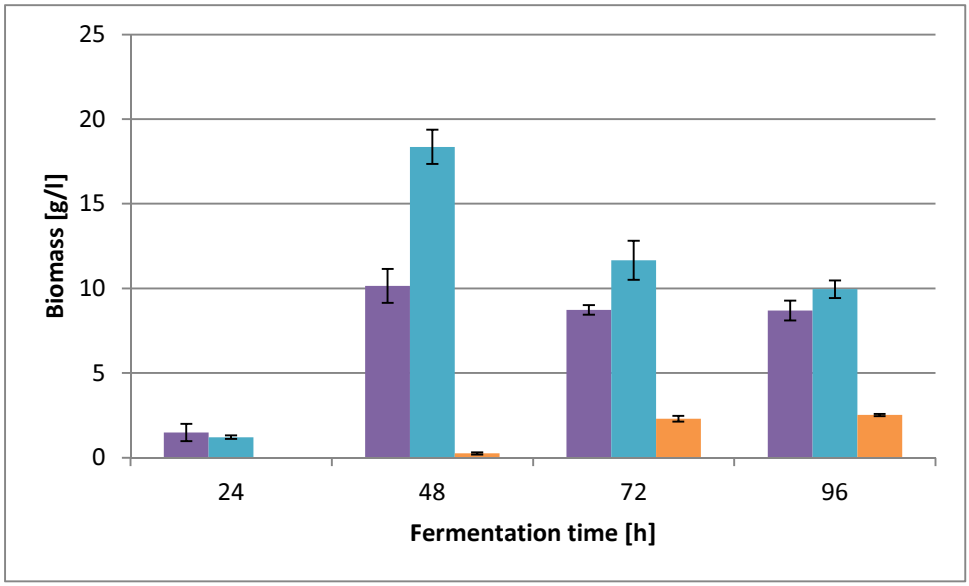

Figure 25: Growth of microorganisms on lactose determined via dry-weight [g/l]. Results for the growth of T. reesei RUT C-30* (in violet), co-cultivation (in light blue) and B. tropica (in orange) are displayed.

7.4.2 Secretion of extracellular proteins by *Trichoderma* is mediated by presence of *Burkholderia*.

When cultivated in non-catabolite repressing conditions *Trichoderma* usually secrets a diversity of extracellular proteins depending on the inductive property of the available carbon source(s). The amount of secreted proteins per ml fermentation broth was determined using BCA (Bicinchoninic acid) assay (Smith et al., 1985), which was chosen over Bradford Assay (Bradford, 1976) for several reasons, one of them being that the color formation of the Bradford reagent (Coomassie Brillant Blue G-250) greatly depends on the amino acid composition of the proteins (Compton and Jones, 1985). Especially for protein samples containing cellulases it has been shown to be inaccurate (Poggi-Parodi et al., 2014), yielding underestimated protein concentrations. As the principle of the BCA Assay is based on reduction (see Materials and Methods), lactose, being a reducing sugar, had to be removed prior to the assay. For that purpose a precipitation with trichloroacetic acid (TCA) and acetone was performed (see Materials and Methods).

Concentration of extracellular proteins for *Trichoderma* and/or *Burkholderia* cultivated on microrystalline cellulose can be seen in Figure 26 . The trend of secreted proteins is more or less consistent with the growth pattern (Figure 24), even though for the first 72 hours of fermentation protein secretion of all three cultivations is in the same range. Only after 96 hours there is a significant increase in extracellular protein concentration for *T. reesei* RUT C-30* (blue). Secretion of the co-cultivated sample (*T. reesei* RUT C-30* with *B. tropica*, in red) remains constant at a rather low level for 72 hours, but secretion is slightly elevated after 96 hours, which supports the hypothesis of

B. tropica having an inhibiting effect on the growth of *T. reesei* RUT C-30* on microcrystalline cellulose. Levels of extracellular protein fluctuate for *B. tropica* (green). As the bacterium is evidently not able to proliferate when grown on this carbon source (see Figure 24), it is most likely that quite a number of proteins are present in the fermentation broth due to cell lysis.

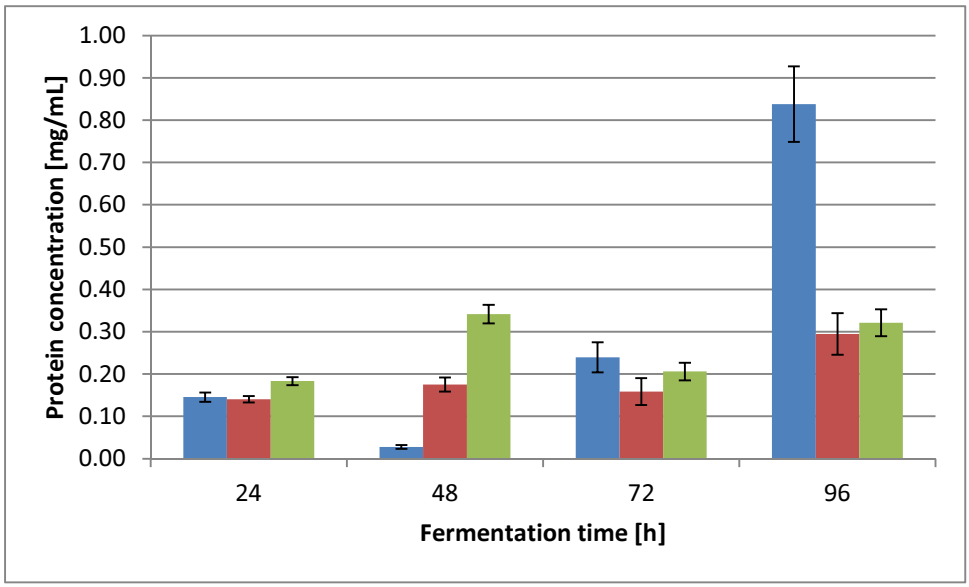

Figure 26: Amount of total secreted proteins [mg/ml] for cultivation of *T. reesei* and/or *B. tropica* on microcrystalline cellulose. Results for *T. reesei* RUT C-30* are shown in blue, co-cultivation in red and *B. tropica* in green.

Compared to extracellular protein concentration exhibited by samples fermented on microcrystalline cellulose, secretion levels on lactose are low. Highest secretion on lactose is exhibited by the co-cultivated sample (see Figure 27, in light blue) after 72 hours. In general the amount of secreted proteins represents the pattern of growth (shown in Figure 25) quite well, as the sole culture of *T. reesei* RUT-C30* is producing less biomass (in violet), as well as less extracellular proteins. *B. tropica* (orange) is hardly secreting any proteins, probably because limited utilizable resources are rather invested into its survival than in the production of non-essential proteins.

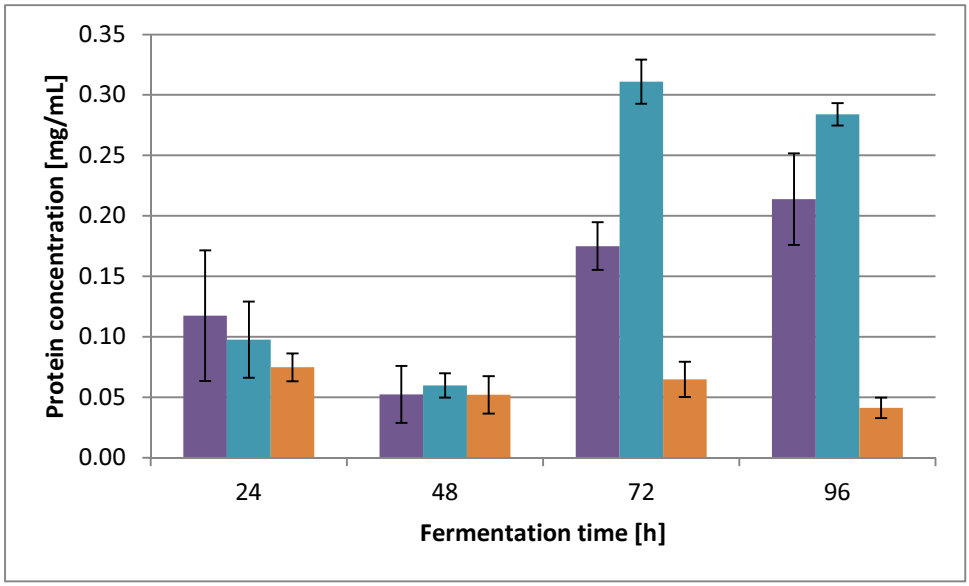

Figure 27: Amount of total secreted proteins [mg/ml] for cultivation of *T. reesei* and/or *B. tropica* on lactose. Results for *T. reesei* RUT C-30* are shown in violet, co-cultivation in light blue and *B. tropica* in orange.

7.4.3 *Burkholderia tropica* influences secretion of cellulases by *T. reesei* in a negative manner

Trichoderma spp. is known for its ability to secrete a diversity of cellulases, including endoglucanases, exoglucanases and β-glucosidases. For determination of cellulase activity two colorimetric assays were applied. A CMC (carboxymethylcellulose) assay, which is based on the measurement of generated glucose, reducing 3,5-dinitrosalicylic acid and leading to the formation of 3-amino-5-nitrosalicylic acid, which absorbs light at 540 nm. The second assay is based on the release of para-Nitrophenol (pNP) from the substrate para-Nitrophenol-β-D-lactoside through exocellulases. In an alkaline environment pNP is fully deprotonated and the formed amount of pNP can be measured at 405 nm.

In Figure 28 obtained endocellulase activity [U/ml] is displayed for all cultures on both carbon sources. Obviously only *T. reesei* RUT C-30* grown on microcrystalline cellulose (in blue) is able to produce significant amounts of (active) endocellulases. Secretion starts after 48 hours and increases continuously over the next 48 hours. Though endocellulase activity also increases on microcrystalline cellulose in co-cultivated samples (red) after 72 hours, the production is significantly decreased in comparison to the pure *T. reesei* strain. This observation again coincides with the assumed inhibitory effect of *B. tropica* on *T. reesei* in this system. Generation of endocellulases is delayed when compared to the control (*T. reesei* RUT-C30*), and most likely starts when no living bacteria are left.

As expected, *B. tropica* does not exhibit endocellulase activity on both carbon sources: microcrystalline cellulose (in green) or lactose (in orange). In general less endocellulases are secreted with lactose as carbon source. These results are supported by extracellular protein concentration data (see Figure 26, Figure 27), which show significantly lower secretion of total proteins on lactose. However endocellulase activity exhibited by the co-cultivation (shown in light blue) is higher than that of the pure *T. reesei* strain (in violet). Thus it seems as if endocellulase production is somewhat enhanced in presence of *B. tropica* when cultivated on lactose.

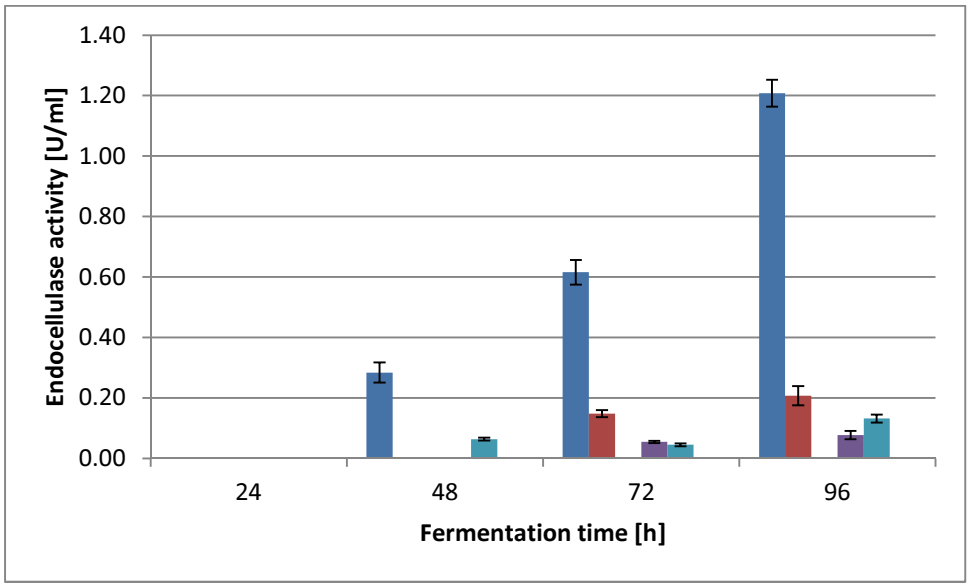

Figure 28: Activity of secreted endocellulases [U/ml] in culture filtrates of *T. reesei* and/or *B. tropica* measured by CMC assay. Activites for *T. reesei* RUT C-30* cultivated on microcrystalline cellulose (blue) or lactose (violet), co-cultivation (*T. reesei* RUT C-30* + *B. tropica*) on microcrystalline cellulose (red) or lactose (light blue) are given. All other cultivations showed no activity at any time point.

The results of the pNPL-Assay (Figure 29) show that production of exocellulases in both systems is rather limited. Only *T. reesei* RUT C-30* on microcrystalline cellulose (in blue) is generating considerable amounts of exocellulases. The onset of exocellulase production (after 72 h) is not simultaneous with the onset of endocellulase production (after 48 h). Although *T. reesei* RUT C-30* with added *B. tropica* did show some endocellulase activity after 72 hours (see Figure 28), no exocellulase activity could be observed. Again no cellulase activity was noted for *B. tropica* on either carbon-source. The minimal signal of *B. tropica* cultivated on lactose after 24 hours of fermentation most likely is an outlier, as standard deviation is higher than the actual value. Opposed to the results on microcrystalline cellulose, the co-cultivation (in light blue) exhibits exocellulase activity after 72

hours. The activity is significantly elevated compared to the control (*T. reesei* RUT C-30*, in violet), which itself does hardly produce any exocellulases.

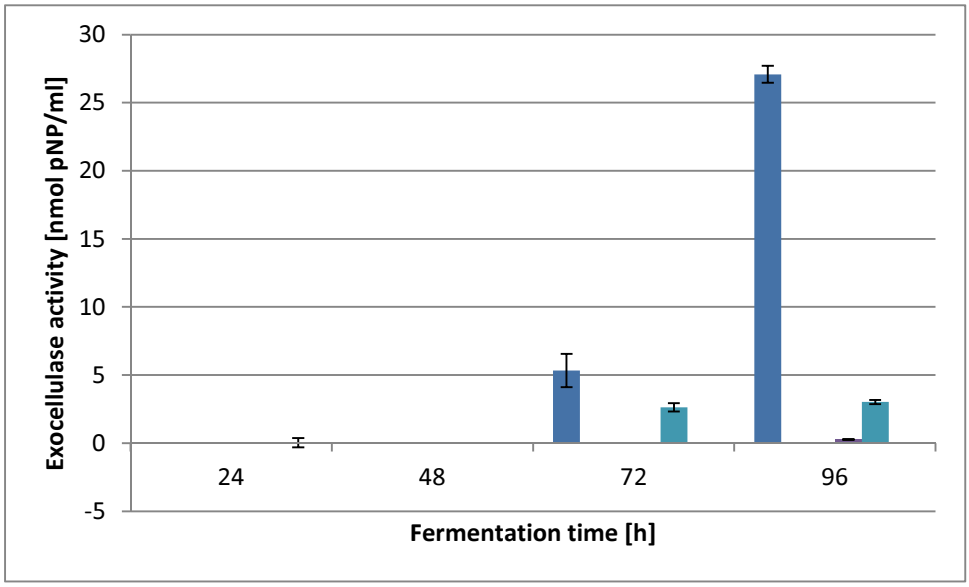

Figure 29: Activity of secreted exocellulases [nmol pNP/ml] in culture filtrates of *T. reesei* and/or *B. tropica* measured by pNPL-Assay. Activites for *T. reesei* RUT C-30* cultivated on microcrystalline cellulose (blue) or lactose (violet), co-cultivation (*T. reesei* RUT C-30* + *B. tropica*) on lactose (light blue), and *B. tropica* on lactose (orange) are shown. All other cultivations showed no activity at any time point.

Additional to the measurement of enzymatic activity described above, zymograms with CMC as substrate were performed, with the purpose of confirming the results of the CMC-Assay and to see whether bacteria influence the variety of produced endocellulases. Before zymograms for the samples were performed, the technique had to be optimized. To that end a number of pre-tests were carried out, leading to the decision that for each set of samples two zymograms had to be prepared, one without incubation of the gel (cellulase activity lower) to prevent strong bands mimicking activity on other lanes due to smearing and one incubated for 20 minutes at 50 °C to force the visualization of bands that are not visible (activity too low) when not incubated.

Figure 30 shows the zymograms for *T. reesei* RUT C-30* and/or *B. tropica* TU CIM 4180 cultivated on microcrystalline cellulose as described in 6.4. The results are in accordance with those of the CMC activity assay. Cellulase production by *T. reesei* RUT C-30* starts after 48 hours of fermentation and increases over time. There are only two bands, one above 170 kDa and one around 55 kDa, visible after 48 and 72 hours. After 96 hours an additional band around 23 kDa can be seen. In contrast to *T. reesei* RUT C-30* the co-cultivation with *B. tropica* is not showing bands, thus not producing endocellulases, coinciding with the results of the enzymatic assay. However, after 72 and 96 hours very faint bands are noticeable at above 170 kDa and 55 kDa, supporting the statement that *B. tropica* inhibits growth, most likely by inhibiting cellulase production on microcrystalline cellulose.

However these results may also show that as soon as bacterial cells are no longer viable (see above), cellulase production starts and the fungus becomes capable to grow. Whether this inhibitory phase caused by the bacterium later on influences growth and cellulase production was not yet investigated. The sole culture of *B. tropica* is not showing any cellulase activity, neither in the enzymatic assays nor in the zymograms.

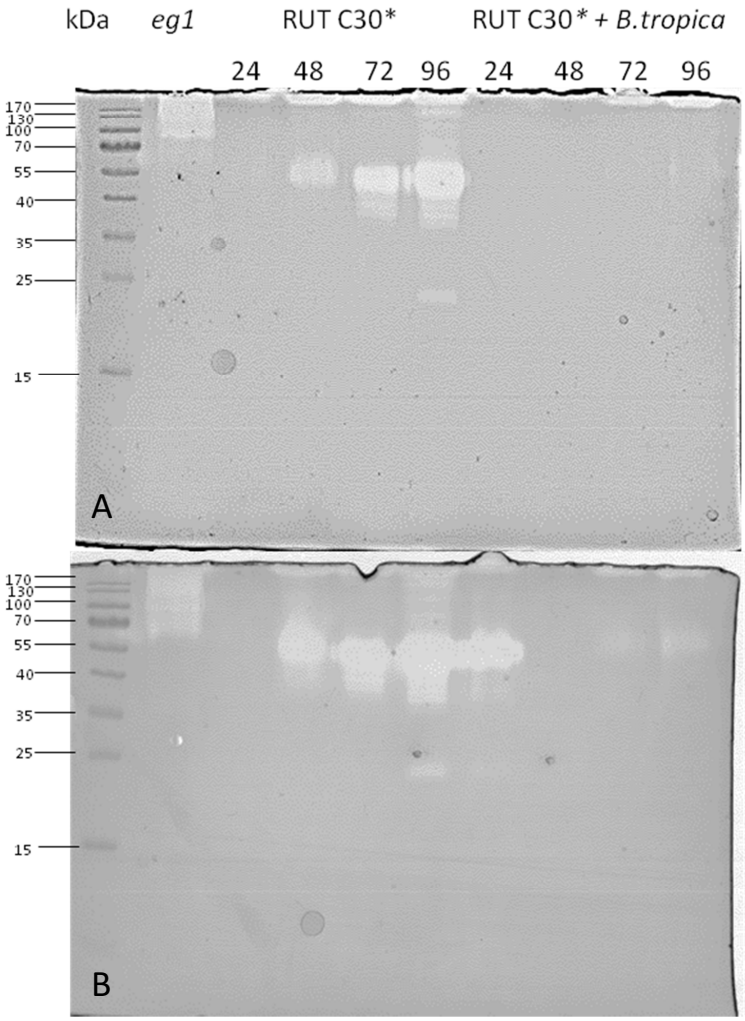

Figure 30: CMC-zymograms for *T. reesei* RUT C-30* and the co-cultivation (*T. reesei* RUT C-30* with *B. tropica*) cultivated on microcrystalline cellulose. A shows results for non-incubated and B for incubated gels. EG1 (endoglucanase 1) serves as control.

For samples grown on lactose the outcome of the zymograms concurs with the enzymatic assay, shown in Figure 31. Endocellulase secretion starts after 48 hours, for *T. reesei* RUT C-30* and *T. reesei* RUT-C-30* with *B. tropica*. The secreted endocellulase type seems to be different at 48 hours, as *T. reesei* RUT C-30* shows a band around approx. 45 kDa, whereas the co-cultivation shows one at 55 kDa. After 48 hours for *T. reesei* RUT C-30* this band disappears and is replaced by the band around 55 kDa. Additionally there are also bands above 170 kDa, at 25 kDa and a faint one way below 15 kDa, most likely around 10 kDa, as the marker protein of the ladder is not evident. These exact same bands are also visible for the co-cultivation, but an additional band around 170 kDa is also present. In general the higher endocellulase activity of the co-cultivation obtained by the enzymatic assay is also visible in the zymograms, showing broader bands for the co-cultivation than for *T. reesei* RUT C-30*. *B. tropica* does not show endocellulase activity, only after 96 hours two very faint bands (55 and 50 kDa) can be seen in the incubated (C) zymogram, suggesting that *B. tropica* produces minimal amounts of endocellulases when grown on lactose.

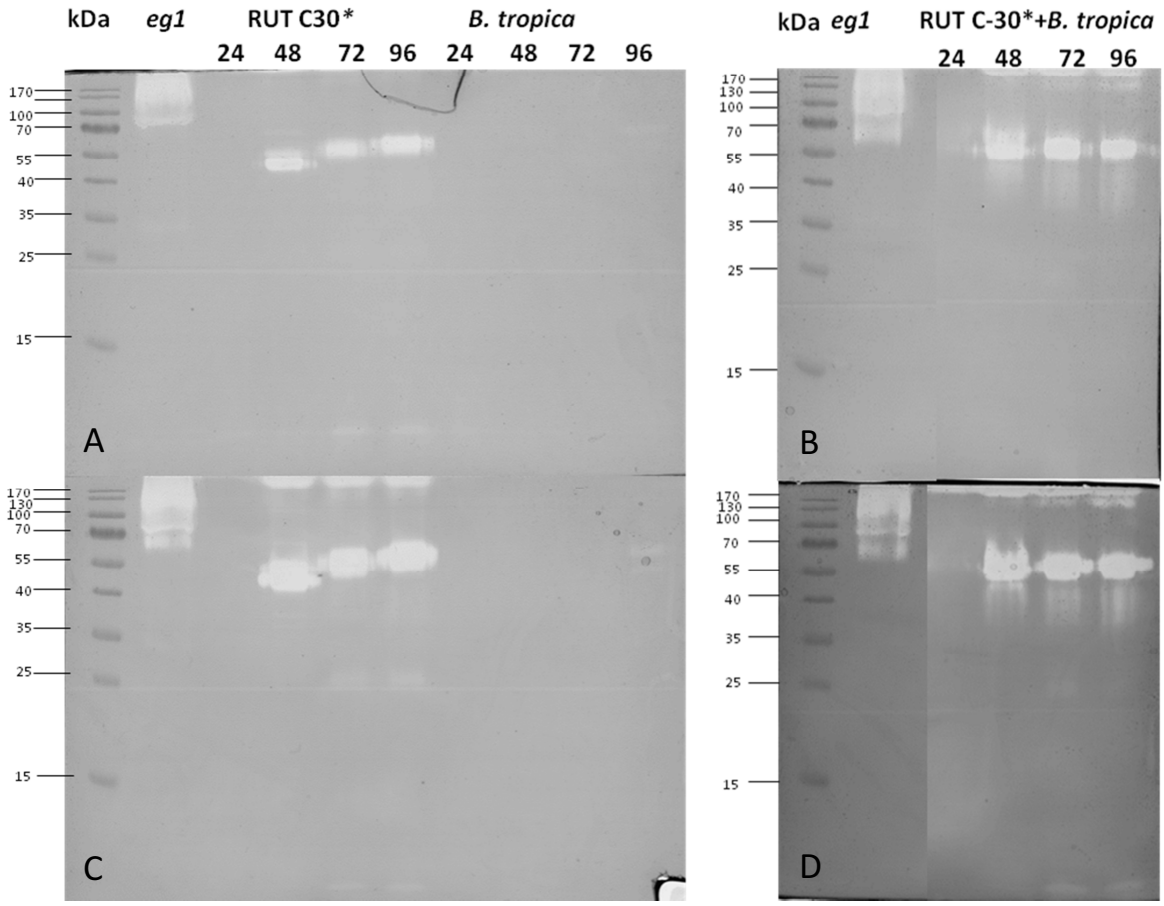

Figure 31: CMC-zymograms for *T. reesei* RUT C-30* , *B. tropica* and the co-cultivation (*T. reesei* RUT C-30* with *B. tropica*) cultivated on lactose. A and B show results for non-incubated gels. C and D show results for incubated gels. EG1 (endoglucanase 1) serves as control.

7.5 Cellulase production by *T. afroharzianum* may be influenced by associated bacteria

To determine whether the influence of naturally occurring bacteria on cellulase production by *Trichoderma* spp. depends on the involved species, a different system, namely *Trichoderma afroharzinanum* and its natural associate *Achromobacter xylosoxidans* (Proteobacteria, Bacteria) with lactose as carbon source was tested. Fermentations were carried out with pure cultures of *T. afroharzianum* (TU CIM 4455*) and *A. xylosoxidans* TU CIM 4802 acting as control, and three fungal-bacterial samples. These included the wild-type strain of *T. afroharzianum* TU CIM 4455 (4455 wt), which is naturally associated with *A. xylosoxidans* and the bacteria are still in the culture. For the other two combinations *B. tropica* TU CIM 4180 and *A. xylosxoidans* TU CIM 4802 were added to *T. afroharzianum* TU CIM 4455* respectively. Samples were taken every 24 hours for a total of 4 days and analyzed with regard to the biomass production, total protein secretion and cellulase activity.

7.5.1 Growth of *Trichoderma afroharzianum* is affected by the bacterial species and its concentration

Biomass [g/l] was determined via dry-weight measurement and the results are presented in Figure 32. The highest biomass values were observed when *T. afroharzianum* TU CIM 4455* (in blue) and it co-cultivated with *A. xylosoxidans* (in red) were assessed, however the co-cultivated samples exceeds biomass production of TU CIM 4455* at all time-points, except after 96 hours. For all samples (excluding *A. xylosoxidans*) the highest amount of biomass is produced after 48 hours of fermentation. As *T. afroharzianum* TU CIM 4455 wt is a mixed culture with *A. xylosoxidans* identical to the one used for the addition of the bacteria to the cured *T. afroharzianum* TU CIM 4455 strain, it was expected that the wild-type strain (in violet) would follow either *T. afroharzianum* TU CIM 4455*'s or the co-cultivated sample's trend. Interestingly, this is not the case. Biomass production of the wild-type strain is considerably lower than of the two other samples, only after 96 hours it is at the same level as the one of *T. afroharzianum* TU CIM 4455*. The obtained values for produced biomass exceed the theoretically possible value. This problem was discussed in 7.4.1. The sole culture of *A. xylosoxidans* TU CIM 4802 (green) is not able to grown on lactose and the amount of generated biomass remains stable. The addition of *B. tropica* to *T. afroharzianum* TU CIM 4455* (orange) shows a different effect than expected, thus good growth. Normally when 'foreign' bacteria are added to a fungal culture, a significant decrease in growth is observed as these two species are simply not compatible and act antagonistically.

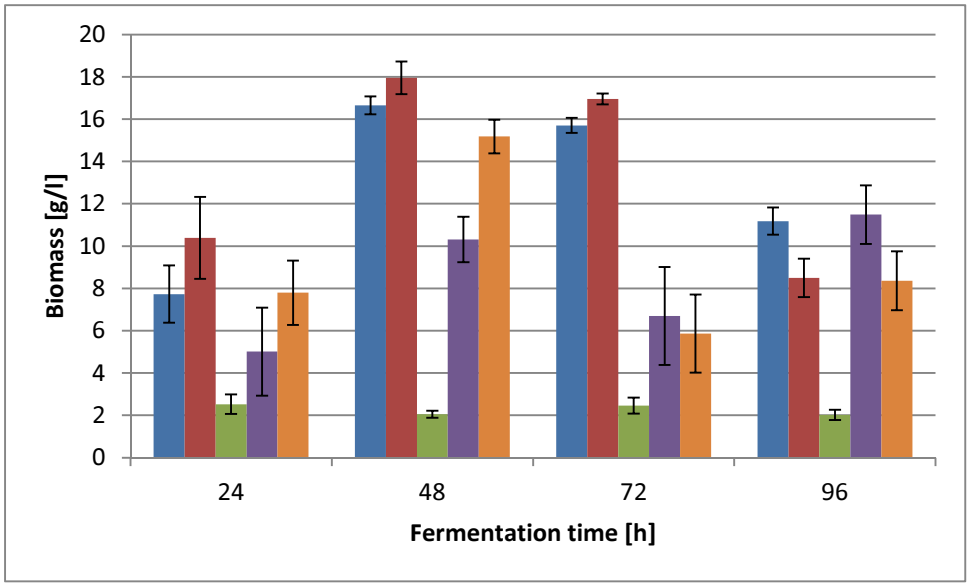

Figure 32: Growth of microorganisms on lactose determined via dry-weight [g/l]. Results for the growth of *T. afroharzianum* TU CIM 4455* (in blue), *T.afroharzianum* TU CIM 4455* with added A. xylosoxidans* (in red) / *B. tropica* (in orange), pure *A. xylosoxidans* (green) and wild-type of *T. afroharzianum* TU CIM 4455 wt (violet) are displayed.

7.5.2 Secretion of extracellular proteins by *Trichoderma afroharzianum* in presence of bacteria is indirectly proportional to growth

The analysis of the amount of secreted proteins was carried out after precipitation by BCA-assay, as described above. In Figure 33 the results of the protein concentration measurement are shown. Obviously protein secretion is behaving indirectly proportional to growth, as after 48 hours biomass production reaches its apex and whereas protein secretion is at its lowest point. For *T. afroharzianum* TU CIM 4455* (in blue) highest extracellular protein concentration was obtained after 96 hours. Though *T. afroharzianum* TU CIM 4455* generates way more biomass than *T. afroharzianum* TU CIM 4455 wt (in violet), its highest protein concentration is about 40 percent lower than that of the wild-type strain. The highest amount of proteins was obtained by *T. afroharzianum* TU CIM 4455 wt after 72 hours of fermentation. The co-cultiavtion of *T. afroharzianum* TU CIM 4455* with *A. xylosoxidans* (in red) rather follows the trend of the pure *T. afroharzianum* TU CIM 4455*, as already observed for growth. The highest amount of secreted proteins by the co-cultivation of *T. afroharzianum* TU CIM 4455* with *B. tropica* (orange) is greater than for both, the pure TU CIM 4455* and the co-cultivated TU CIM 4455* with *A. xylosoxidans* sample. *A. xylosoxidans* on its own does not exhibit considerable extracellular protein production. For some samples standard deviations are quite high, as the biological duplicates behaved differently, suggesting that the system is not completely stable.

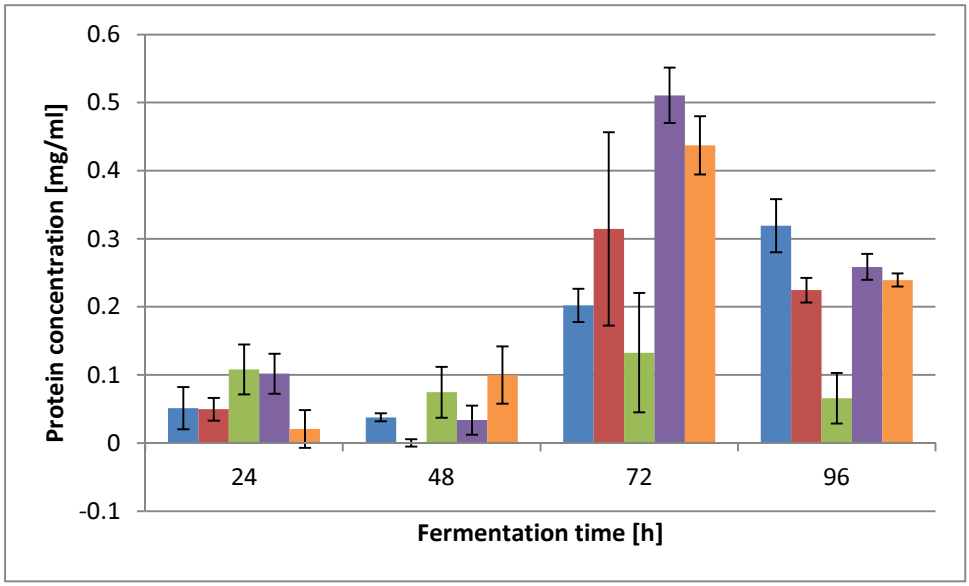

Figure 33: Amount of total secreted protein [mg/ml] determined by BCA-Assay. Results for the protein secretion of *T. afroharzianum* TU CIM 4455* (in blue), *T.afroharzianum* TU CIM 4455* with added *A. xylosoxidans* (in red) / *B. tropica* (in orange), pure *A. xylosoxidans* (green) and wild-type of *T. afroharzianum* TU CIM 4455 wt (violet) are displayed.

7.5.3 Cellulase secretion of *Trichoderma* is strongly affected by the initial concentration of naturally associated bacteria

Activities of endo- and exocellulases were determined by CMC- and pNPL-Assay, respectively. As shown in Figure 34 it seems as if the *T. afroharzianum* TU CIM 4455 strain does not produce endocellulases. Only minimal endocellulase activity can be observed for the wild-type strain (violet), but it has to be noted that standard deviation for the value of 72 hours is extremely high, which is the result of the differences between the biological duplicates.

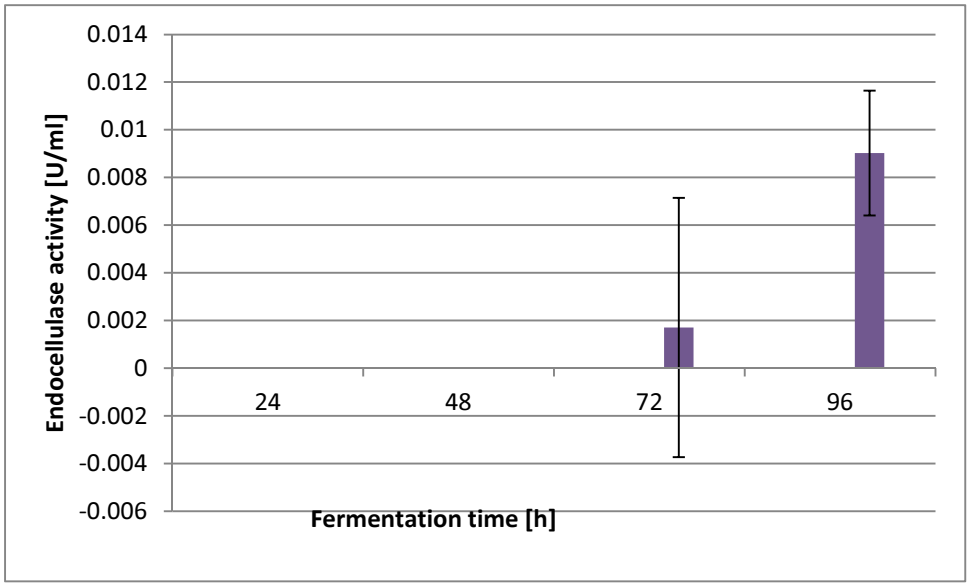

Figure 34: Activity of secreted endocellulases [U/ml] in culture filtrates of *T. afroharzianum* and/or bacteria measured by CMC assay. Results for endocellulase activity of *T. afroharzianum* TU CIM 4455 wt (violet) are shown. All other cultivations showed no activity at any time point.

In Figure 35 exocellulase activity [nmol pNP/ml] is presented. In general every sample, excluding *A. xylosoxidans* is producing exocellulases, which is surprising considering that almost no endocellulase activity was observed. The highest activity is reached by the wild-type of *T. afroharzianum* (TU CIM 4455 wt), exceeding the activity of *T. afroharzianum* TU CIM 4455* (blue) slightly. The co-cultivation of *T. afroharzianum* with *A. xylosoxidans* shows significantly lower activity than both, the wild-type and the cured strain. Thus it seems likely that *A. xylosoxidans* is only beneficial when present in a specific (yet unknown) concentration. The co-cultivation with *B. tropica* (orange) clearly shows that 'foreign' bacteria do inhibit some functions of *T. afroharzianum* TU CIM 4455*, as exocellulase activity, thus probably the secretion and/or production, is drastically decreased. However, interestingly this co-cultivation secreted more proteins than the co-cultivation with *A. xylosoxidans* and the cured *T. afroharzianum* strain.

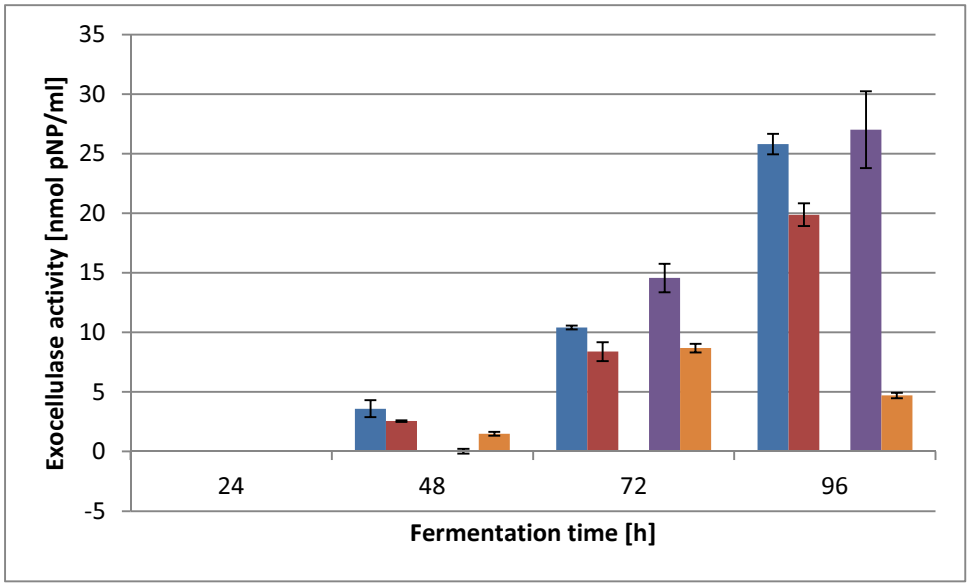

Figure 35: Activity of secreted exocellulases [nmol pNP/ml] in culture filtrates of *T. reesei* and/or *B. tropica* measured by pNPL-Assay. Activites for *T. afroharzianum* TU CIM 4455* (in blue), *T.afroharzianum* TU CIM 4455* with added *A. xylosoxidans* (in red) / *B. tropica* (in orange), and wild-type of *T. afroharzianum* TU CIM 4455 wt (violet) are displayed.

Especially as the CMC-Assay did not show endocellulase activity for almost all samples of this test-series, zymograms were needed to support these results. In Figure 36 zymograms for *T. afroharzianum* TU CIM 4455*, TU CIM 4455 wt and co-cultivation of TU CIM 4455* with *B. tropica* are displayed. The zymograms show that there is some endocellulase activity but it is rather minimal, so that the CMC-Assay was not sensitive enough to register it. *T. afroharzianum* TU CIM 4455* shows after 48 hours a streak from 55-37 kDa, with a main band that is slightly below 40 kDa. In addition weak bands at approx. 25 kDa and 20 kDa are present. After 72 h there are hardly any bands, just a faint band at 55 kDa and then around 96 h there is a broad but weak band from 55-40 kDa and a faint streak a until 20 kDa. Thus there aren't any strong bands, suggesting activity is very low, supporting the obtained results from the CMC-Assay. TU CIM 4455 wt, the only sample that showed minimal endocellulase activity, shows bands at 48 and 72 h. Both time-points have a band slightly above 55 kDa and one between 55 and 40 kDa (at approx. 45 kDa). The upper band at 48 hours is rather weak, whereas the lower is stronger. It is inverted for 72 hours, where the upper band is stronger. Due to these results minimal activity after 48 and 72 hours should be registered by the enzymatic assay, which is not the case. The assay shows activity after 72 and 96 hours. *T. afroharzianum* TU CIM 4455* with *B. tropica* shows only a really faint band between 55 and 35 kDa at 48 and 96 hours, thus *B. tropica* does obviously inhibit cellulase production.

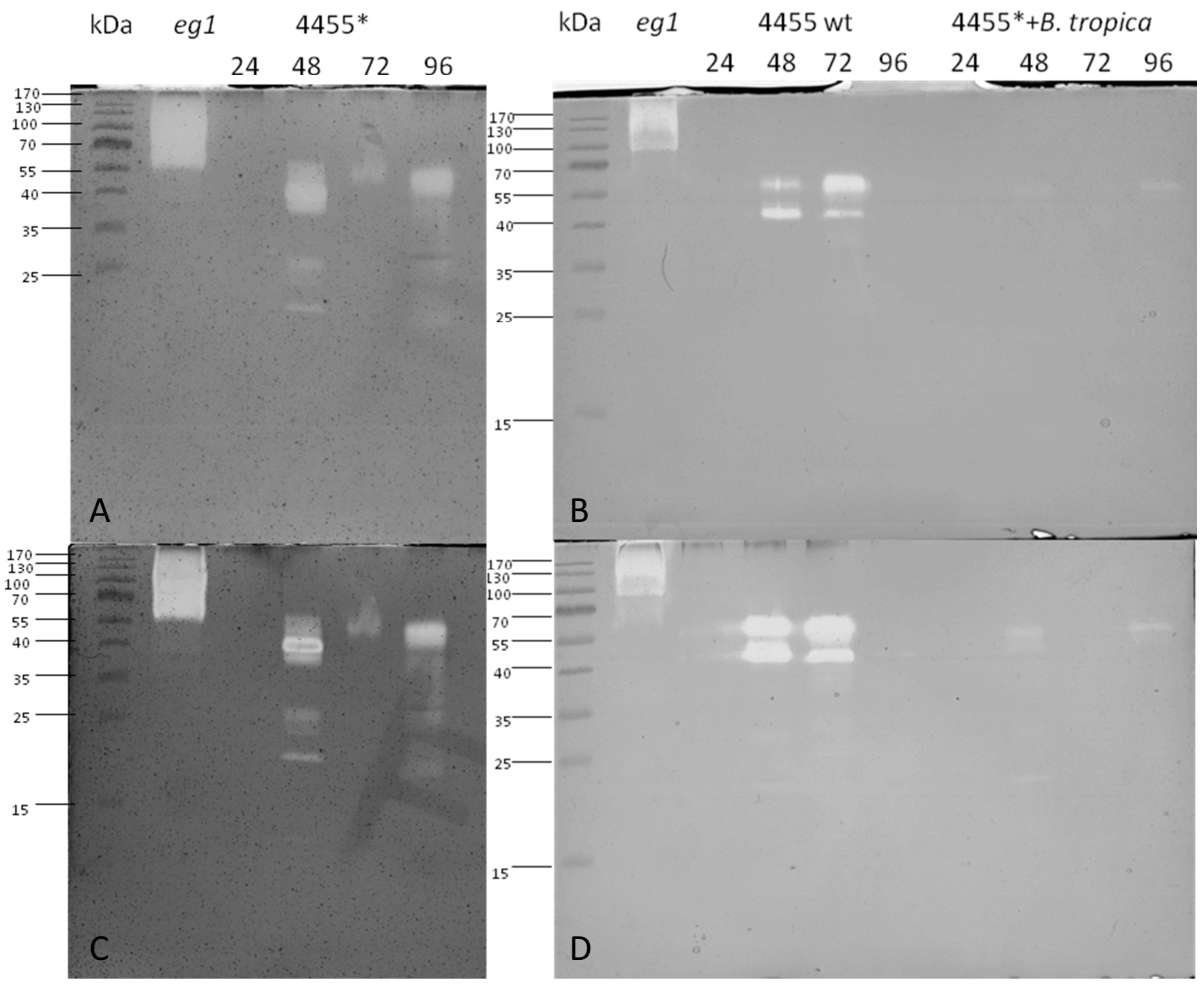

Figure 36: CMC-zymogram for *T. afroharzianum* TU CIM 4455*, *T. afroharzianum* TU CIM 4455 wt and the co-cultivation *T. afroharzianum* TU CIM 4455* with *B. tropica* cultivated on lactose. A and B show results for non-incubated gels. C and D show results for incubated gels. EG1 (endoglucanase 1) serves as control.

Figure 37 shows the zymograms for *T. afroharzianum* TU CIM 4455* with added *A. xylosoxidans* (Ach) and *A. xylosoxidans*. For the co-cultivation with *A. xylosoxidans* bands can be observed above 55 kDa and approx. at 50 kDa. Incubation reveals additional bands around 35 and 25 kDa. No bands are present after 72 hours suggesting that at this time-point there are no active endocellulases present, even though 24 hours earlier active enzymes were observed. After 96 hours there again is some minor activity, showing around 60 to 55 kDa. *A. xylosoxidans* does not show endocellulase activity at any time-point. The zymograms support the obtained results of the CMC-Assay by showing that bands are rather faint and that the assay is not sensitive enough to show the minimal activity.

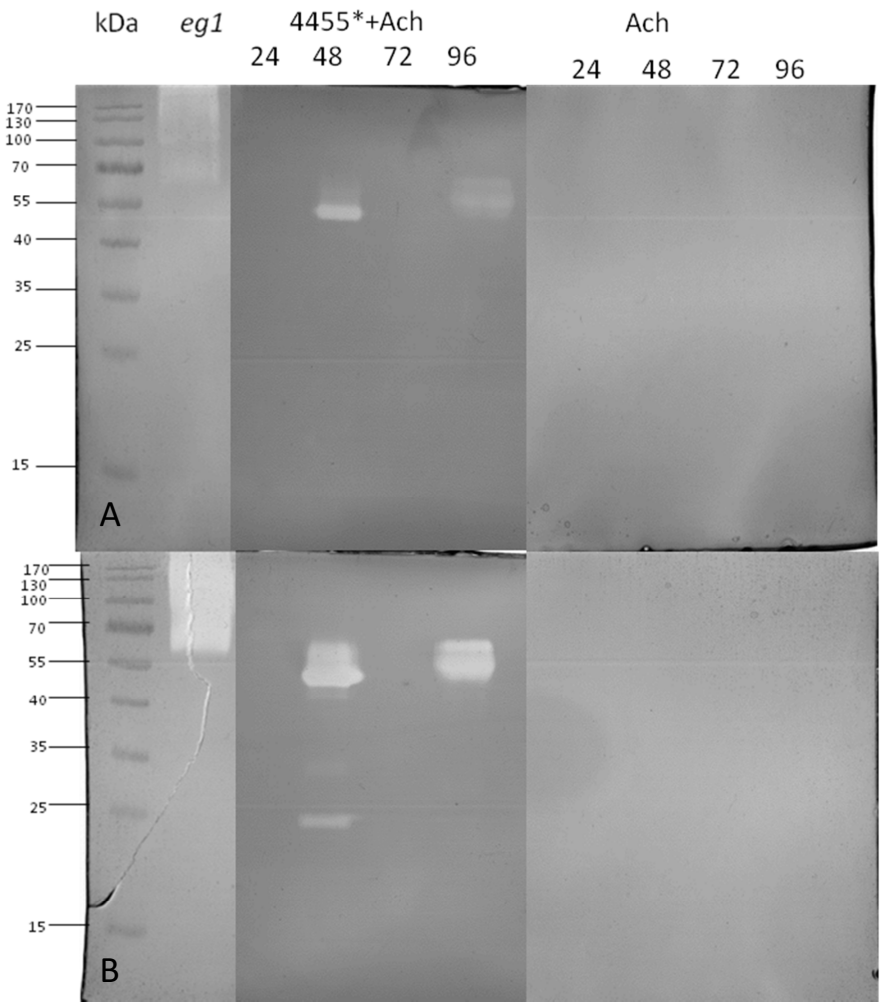

Figure 37: CMC-zymogram for *T. afroharzianum* TU CIM 4455* with *A. xylosoxidans* (Ach) and *A. xylosoxidans* (Ach) cultivated on lactose. A shows results for non-incubated and B for incubated gels. EG1 (endoglucanase 1) serves as control.

7.6 Generation of a tractable *B. tropica* strain

A transformation of *B. tropica* with mCherry and YFP vectors was carried out, in order to obtain a fluorescent *B. tropica* strain, to see its behavior toward *T. reesei*. Some colonies had a slight pink color, which strongly suggests the presence of mCherry plasmid. Presumably positive transformants were checked by primers targeting the mCherry or YFP gene. As positive control the respective plasmid was used. Figure 38 shows a result of the PCR performed with mCherry primers, testing two colonies with pink color for the presence of the mCherry gene. The PCR shows that the specificity of the primers is rather low (even at higher annealing temperatures), however these were the only primers that could be designed to pre-test the colonies before checking their ability to fluoresce, as there was no vector map available. The size of mCherry is 711 bp, thus a band slightly below 750 bp was to be expected. This band is visible for the positive control and for all samples even though it is rather faint. Due to the faintness of the bands a nested PCR with mCherry primers was performed (see Figure 39), which shows a clear and strong band around 750 bp for all samples.. As the purpose of the PCR was to pre-select colonies for further validation steps, it was primarily necessary to obtain a band around 750 bp, and the specificity was not the main target. The respective bands were excised of the gel, the PCR product was purified and samples were sent for sequencing. Obtained sequences were repeatedly too short to confirm that the band was indeed mCherry. Nevertheless, the two colonies, Y2 and M12 were then examined under the confocal laser microscope.

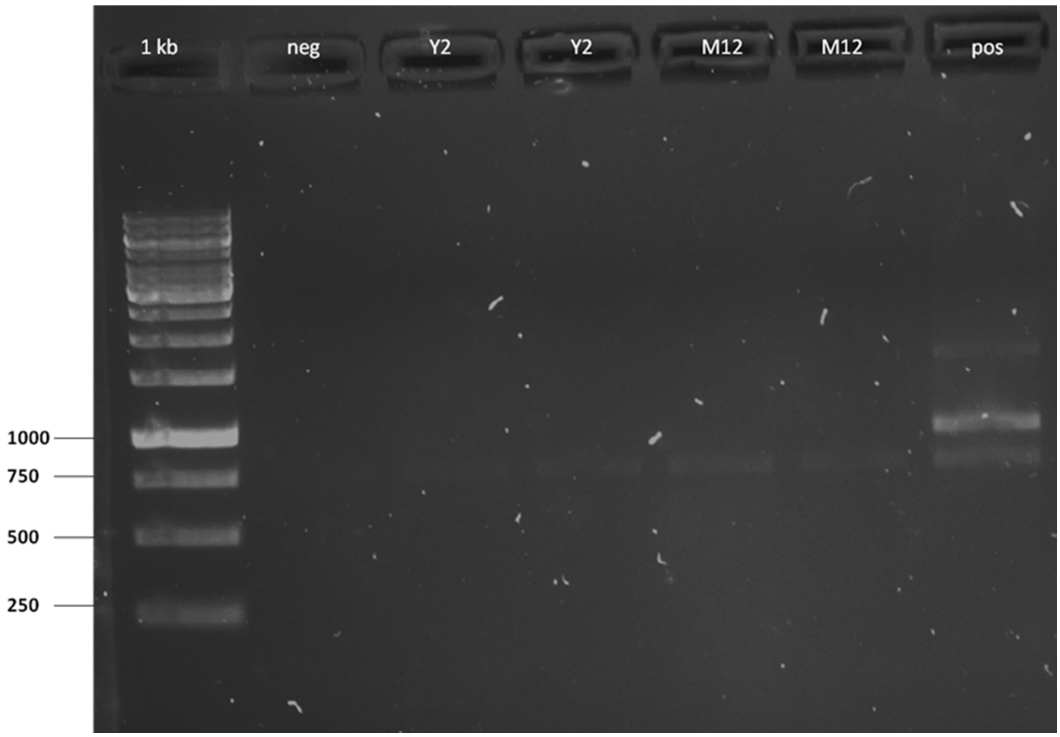

Figure 38: Results of the PCR made with mCherry primers on the DNA extracted from transformed *B. tropica* Y2 and M12 cultures. MCherry plasmid acted as positive control, water as negative control.

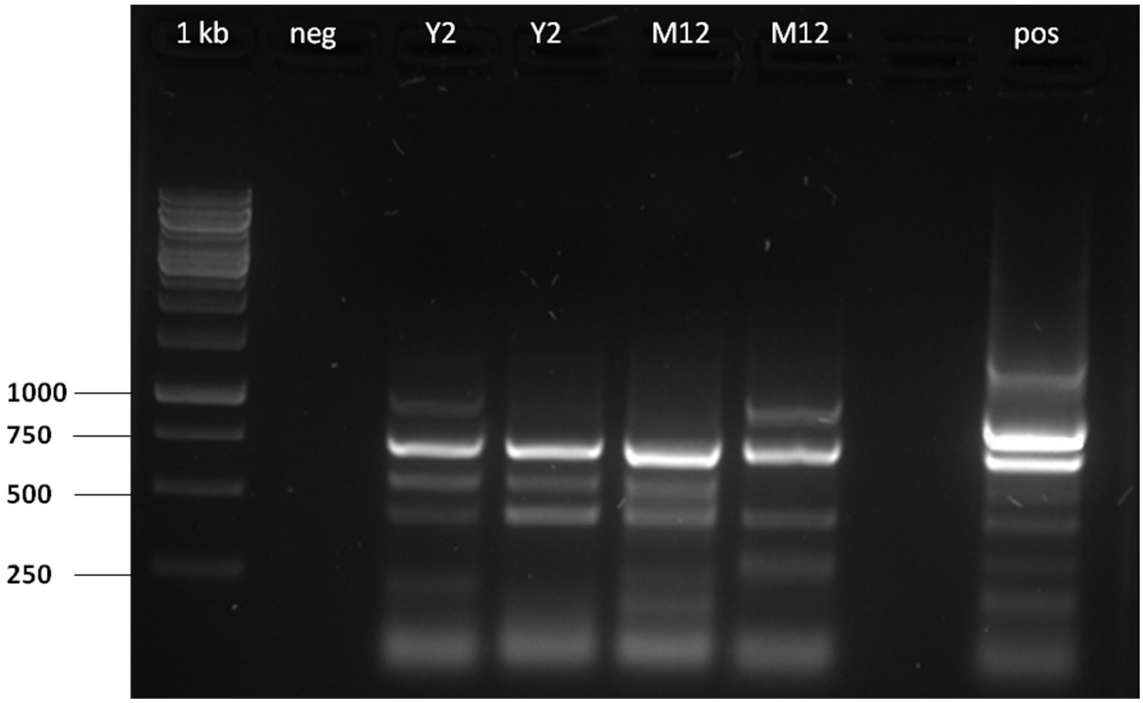

Figure 39: Results of the nested PCR carried out with mCherry primers on DNA extracted from transformed *B. tropica* Y2 and M12 cultures. MCherry plasmid acted as positive control, water as negative control.

In addition to testing whether *B. tropica* was successfully transformed, the identity of the transformed bacteria was validated by a PCR with *Burkholderia* specific primers (GB), as shown in Figure 40. The PCR proves that the transformed bacteria are indeed *B. tropica* by showing a band around 1000 bp. The positive control shows only a very faint band at 1000 bp, probably due to a suboptimal concentration of the DNA extracted from *B. tropica* TU CIM 4180.

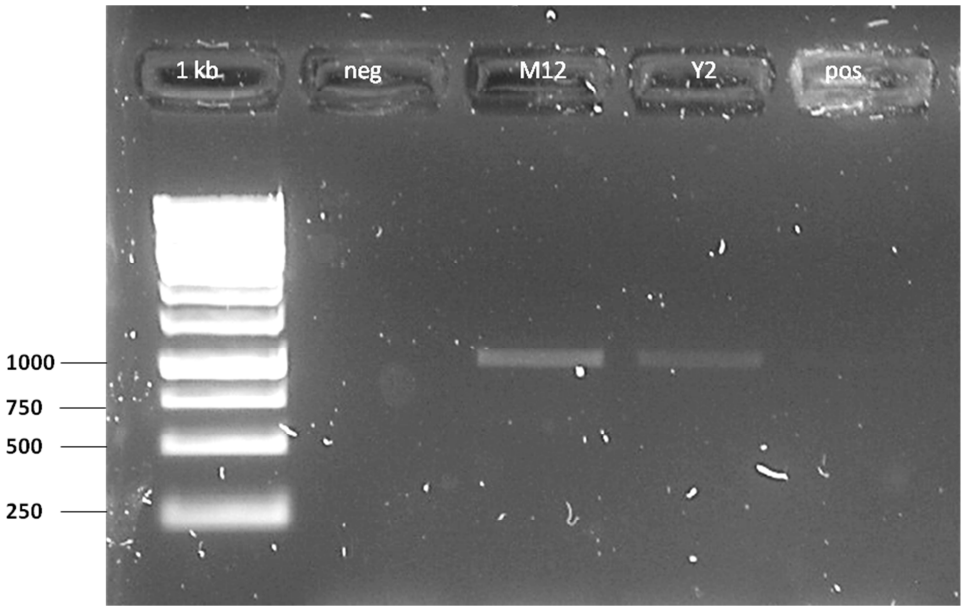

Figure 40: Results of PCR carried out with *Burkholderia* specific primer (GB-F, GB-N2) to validate that transformed bacteria M12 and Y2 are *B. tropica*. DNA of *B. tropica* was used as positive control.

In order to verify that the transformants also expressed the mCherry gene, the transformants Y2 and M12 were examined under the confocal laser microscope. The optimal excitation/emission wavelength of mCherry is 587 / 610 nm, but as no laser with this exact wavelength was available the red laser in this wavelength range was used. As positive control *E. coli* DH 5α- mCherry was used, to make sure that no signal was indeed due to lacking expression of mCherry by the transformants. Since the positive control did show fluorescence under the applied conditions (laser with wavelength that is not optimal), as presented in Figure 41the absence of a signal for a sample was denoted as unsuccessful transformation. The transformant Y2 did show a signal, however the number of fluorescent bacteria to non-fluorscent bacteria was very low, indicating that either only a few bacteria were successfully transformed or only very few of the transformed bacteria did actually express mCherry. As *B. tropica* does not have a gentamycin resistance, it seems more likely that the gene was not expressed. In comparison to Y2 the transformant M12 showed fluorescent to non-fluorescent ratio (see Figure 42 and Figure 43). Yet, the frequency and intensity of expressed mCherry seems to be a way lower than in *E. coli*, but the transformation was undoubtedly successful. As M12 shows fluorescence, it is recommended to use this transformant for further experiments.

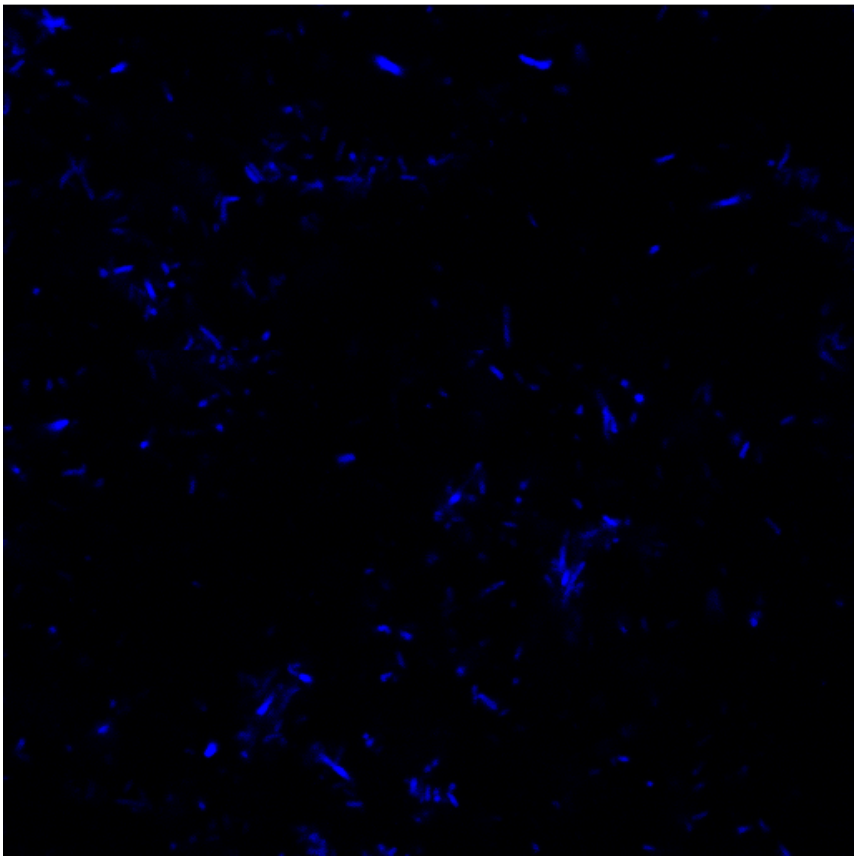

Figure 41: Picture taken under confocal laser microscope of *E. coli* DH 5α –mCherry

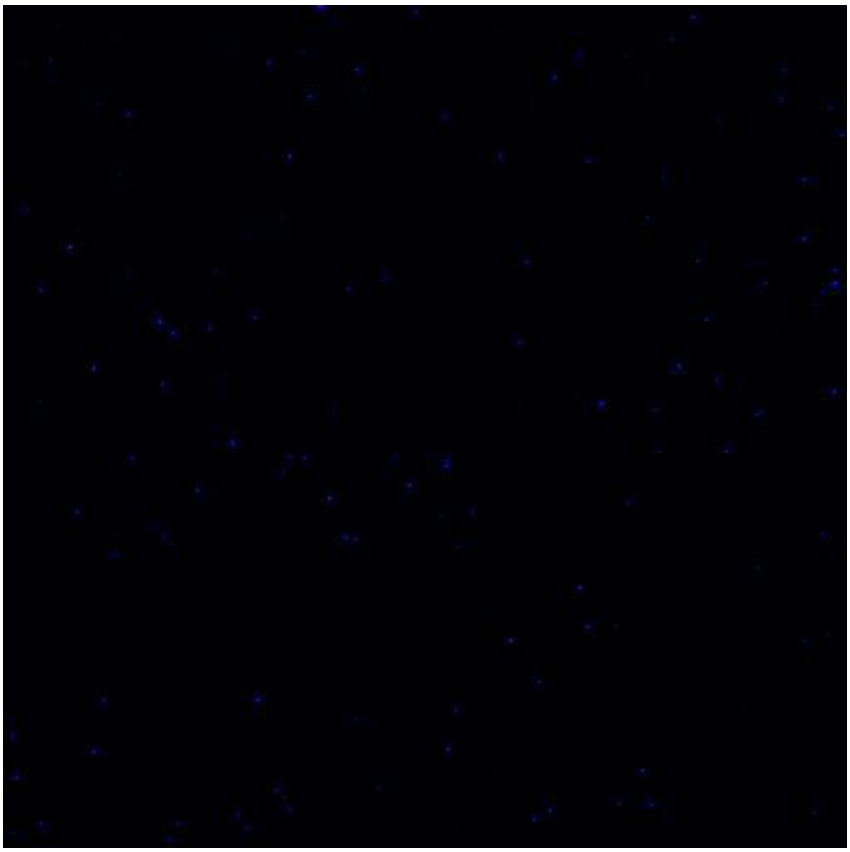

Figure 42: Picture taken under confocal laser microscope showing transformant M12.

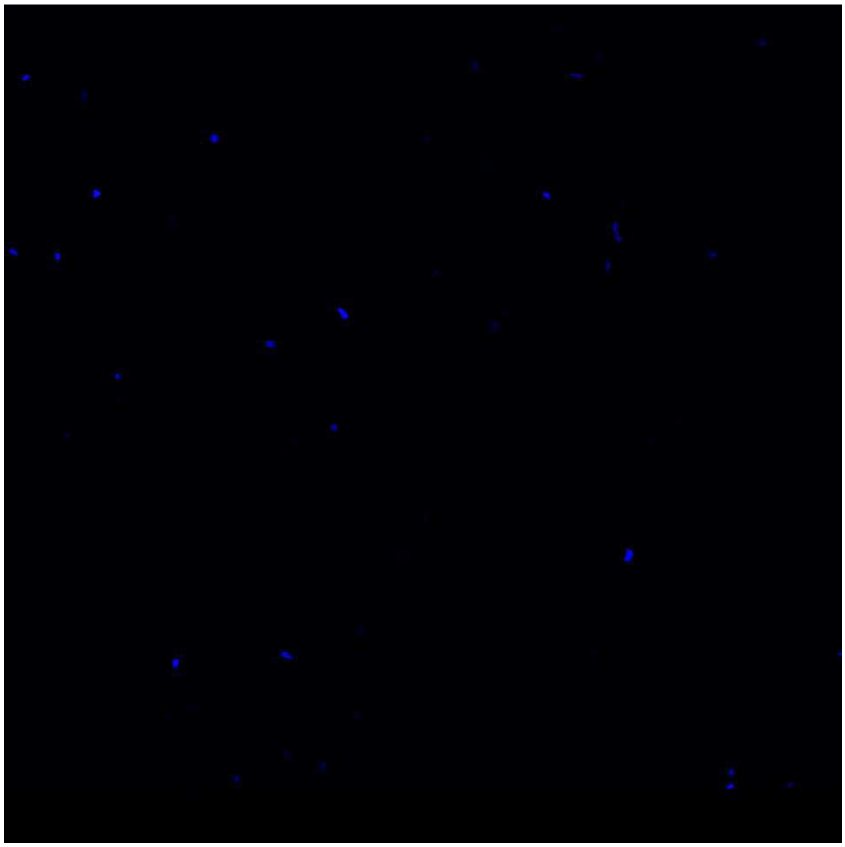

Figure 43: Picture taken under confocal laser microscope showing transformant M12

8 Discussion

In recent years the study of bacterial-fungal interactions gained importance, as it was discovered that the behavior and characteristics of the involved individuals in such an association, might considerably differ from those that the organism possesses on its own (Frey-Klett et al., 2011). The extent of these alterations depends upon the involved species and their physical associations with each other, especially endobacteria seem to greatly affect fungal behavior. The best known endobacterium is *Candidatus* Glomeribacter gigasporarum (Proteobacteria, Bacteria) which colonizes the cytoplasm of arbuscular mycorrhizal fungi (belonging to the phylum Glomeromycota) (Schüßler et al., 2001). The bacterium is nutritionally dependent on the fungal host and probably provides the fungus with essential factors in return (Desirò et al., 2014; Ghignone et al., 2012). Even though the fungal host is not obligate dependent on its endobacterium, removal of it leads to morphological changes and reduced proliferation of hyphae (Lumini et al., 2007; Mondo et al., 2012). In contrast to arbuscular mycorrhizal fungi, *Rhizopus microsporus* is obligately dependent on its endobacterium *Burkholderia rhizoxinica*, since the fungus is neither able to produce the toxin rhizoxin (produced by *B. rhizoxinica*), which is needed in order to prey on rice plants, nor to asexually reproduce in absence of the bacterium (Partida-Martinez and Hertweck, 2005; Partida-Martinez et al., 2007b). These findings suggest that associated bacteria, especially endobacteria, might influence the production and secretion of essential proteins and/or secondary metabolites and might additionally be important to maintain proliferation. In this thesis the impact of naturally associated bacteria on cellulase production by *Trichoderma* was evaluated, as cellulase production is an essential mechanism for this genus. Based on results of recent studies, which have shown that bacterial-fungal interactions might greatly influence the fungus protein secretion profile, it was assumed that the natural associated bacteria *B. tropica* and *A. xylosoxidans* might similarly affect cellulase secretion of *T. reesei* and *T. afroharzianum*, respectively.

8.1 The curing of *Trichoderma*

To test this hypothesis fungal strains had to be cured from the associated bacteria. The actual curing and verification process during this thesis was only carried out for *T. reesei*, whereas the applied *T. afroharzianum* strain was generously supplied by M. Rahimi (VUT, Microbiology group). Prior experiments showed that cleaning *T. reesei* of *B. tropica* was much more complex than expected, as *B. tropica* seems to possess the high antibiotic resistance common to *Burkholderia* spp. and high doses significantly harm the fungus, resulting in a hardly viable organism. With regard to these results the antibiotics imipenem and ceftazidime, which are known to be effective against several species of *Burkholderia* (Deris et al., 2010; Thibault et al., 2004) were tested in a different set up, namely a liquid culture with a carbon-source not appropriate by for the bacterium (lactose). Several

concentrations of both antibiotics were tested to find the optimal recipe, yielding unharmed and bacterial-free strains. The obtained results from this optimization revealed that a mixture of 20 µg/ml imipenem and ceftazidime is the best choice, as it was the only tested condition in which no bands in the rather ambiguous PCR with *Burkholderia*-specific primers (see below) and no harmful effects on the fungus were observed. Additionally the combination of imipenem and ceftazidime is more economic and universal, affecting both gram-positive and gram-negative bacteria, compared to the usage of imipenem alone. Not only the curing process itself, but also the verification postulated challenges. Simple PCRs with genus-specific primers (GB, specific for *Burkholderia*) and universal 16S primers (both targeting 16S rRNA gene cluster) provided ambiguous results, making it impossible to declare a strain cured and using it for further experiments. As all applied material was freshly obtained and all steps were repeated, leading again to vague results, biased results by contamination were excluded. This ambiguity might be caused by remnants of bacterial DNA or – in case of intracellular bacteria- by dead or living bacteria inside the hypha. As the localization of *B. tropica* in *Trichoderma* cultures is still unknown, this hypothesis is mere speculation, however based on these considerations another method was developed, which depends on the simple process of proliferation. Living organisms proliferate to secure their survival, especially bacteria have a high proliferation rate, thus the amount of bacterial gene product detected in a qPCR should increase when normalized to the gene product of the fungus (*tef1* (fungal single-copy house keeping gene)/*Burkholderia*-specific 16 S rRNA fragment (GB)), if bacteria are viable. The results of the *Burkholderia*-specific (GB) and *tef1* qPCR showed that the positive control (*T. reesei* QM 9414 infected with *B. tropica* TU CIM 4180) indeed revealed a continuous increase in the *tef1/GB* cycle-threshold ratio, whereas all other samples did either not show any increase, rather decrease over time, or not any amplification of the *Burkholderia*-specific 16 S fragment (GB primers). These results therefore prove that strains are free of bacteria. In addition to the verification of presumably cured fungal strains, this method also provided first evidence that *T. reesei* is able to grow alongside *B. tropica in vitro*, as the artificially infected *T. reesei* QM 9414 did neither show any sign of growth inhibition nor of antagonism. Confrontations of *B. tropica* TU CIM 4180 with *T. reesei* QM 9414 on PDA plates confirmed their ability to co-exist, whereas confronting *B. tropica* TU CIM 4180 with *Fusarium oxysporum* led to growth inhibition of the fungus, which coincides with the results of Tenorio-Salgado et al. 2013, who showed that *B. tropica* is inhibiting growth of 4 phytopathogenic fungi, including *F. oxysporum*. Furthermore cured *T. reesei* and *T. afroharzianum* strains were able to grow normally and to asexually reproduce in absence of their naturally associated bacterium, revealing that *B. tropica / A. xylosoxidans* is not essential for the survival of *T. reesei / T. afroharzianum,* respectively. Therefore these associations are not another rare example of obligate dependency of an individual organism on its associate, as shown by Partida-Martinez et al. (2007)

with the interaction of *R. microsporus* and *B. rhizoxinica*. However, whether the absence of the bacteria alter morphology or proliferation behavior of *T. reesei / T. afroharzianum* as it was observed for arbuscular mycorrhizal fungi in absence of *Candidatus* Glomeribacter gigasporarum (Lumini et al., 2007) was not yet studied, therefore partial dependency on the bacterium cannot be excluded.

8.2 Effect of bacteria on cellulase production by *Trichoderma*

For the determination of the effect of naturally associated bacteria on the cellulase production of *Trichoderma*, fermentations with two high cellulase producers, *T. reesei* RUT C-30 and *T. afroharzianum* TU CIM 4455, and their natural bacterial associates, *B. tropica* and *A. xylosoxidans*, in different combinations were carried out. The obtained results show various effects, which will be discussed in separately. Up to this point no studies were performed with regard to the effect of naturally associated bacteria on cellulase secretion of fungi, so that the obtained results cannot be compared.

8.2.1 *B. tropica* exhibits versatile effect on cellulase production of *T. reesei*

In order to determine the effect of *B. tropica* on cellulase production of *T. reesei*, fermentations with two cellulase-inducing carbon-sources, microcrystalline cellulose and lactose, were performed. Analysis of the samples showed that the addition of *B. tropica* results in reverse results for both carbon sources. *Burkholderia* tropica did not show any cellulase activity on either carbon source. Furthermore, as expected, both carbon sources did not promote growth of *B. tropica*, as the bacterium can neither use lactose nor microcrystalline cellulose, which is also reflected in its secretion of proteins. Remarkable differences can be seen between strains cultivated on microcrystalline cellulose and lactose, for both, the sole *T. reesei* RUT C-30* and the co-cultivated sample (*T. reesei* RUT C-30* with *B. tropica*). *T. reesei* grown on lactose showed significantly lower total protein secretion than *T. reesei* grown on microcrystalline cellulose, secreting a maximum of 0.21 mg/ml liquid medium, accounting for only 25 % of the maximum secretion on microcrystalline cellulose. This difference could also be observed in the exhibited endo- and exocellulase activity. Activites were significantly higher for *T. reesei* grown on microcrystalline cellulose reaching a maximum activity of 27.077 nmol pNP/ml and 1.21 U/ml exo- and endocellulase activity respecitively. Compared to that *T. reesei* on lactose showed minimal activities of 3.02 nmol/ml and 0.1314 U/ml respectively. Even though it is known that microcrystalline cellulose induces higher cellulase production than lactose, these activites are incredibly low for lactose, suggesting the cellulase induction didn't work properly. This phenomenon of lactose is known but cannot be explained yet. Co-cultivated samples show a versatile effect of *B. tropica* on the cellulase production

of *T. reesei*. Co-cultivated samples cultivated on lactose exhibit higher biomass production, protein secretion and cellulase activity than the control (*T. reesei* RUT C-30*), suggesting that *B. tropica* might increase biomass production and consequently total cellulase secretion. In contrast to that *B. tropica* inhibits *T. reesei* when grown on microcrystalline cellulose, so that *T. reesei* is hardly able to grow, which negatively influences protein secretion, thus cellulase activity. Compared to its control, the co-cultivated sample on microcrystalline cellulose shows no exocellulase activity and reaches a maximum endocellulase activity of 0.21 U/ml, being just 18 % of the maximum obtained activity. Results suggest that on this carbon source *B. tropica* actually inhibits cellulase production and just indirectly the growth of the fungus, as carbon is not utilizable in form of microcrystalline cellulose and has to be degraded by help of cellulases. It is important to mention that *T. reesei* RUT C-30, a mutant derived from *T. reesei* QM 6a, is not naturally associated with bacteria, however its ancestor is. This does not necessarily mean that the mutant lost its ability to interact with *B. tropica*, but it is most likely that bacteria were destroyed in the process of the several mutation steps. Still this strain was chosen for these experiments as pre-tests showed that cellulase secretion of other *T. reesei* strains was too low for the purpose of this thesis.

In summary the addition of *B. tropica* to *T. reesei* leads to higher biomass and cellulase production when grown on lactose but to drastically decreased biomass and cellulase production when grown on microcrystalline cellulose.

8.2.2 Cellulase production of *Trichoderma* may be increased by naturally associated bacteria

The above discussed results led to the question whether improvement in cellulase production of *Trichoderma* on specific carbon-sources, namely lactose, might indeed be caused by its naturally associated bacteria. Therefore cellulase production of another high cellulase producer, *Trichoderma afroharzianum* TU CIM 4455, which is naturally associated with *A. xylosoxidans*, on lactose was analyzed. The outcomes showed that cellulase secretion might indeed be increased by presence of its natural associate, however only, it seems, if the bacterium is present in an optimal concentration. This was displayed by comparing results of the *T. afroharzianum* TU CIM 4455 wild-type strain, which has *A. xylosoxidans* naturally in its culture, with the cured *T. afroharzianum* TU CIM 4455(*) strain to which *A. xylosoxidans* was added. The wild-type strain exhibited increased exocellulase activity compared to the control (*T. afroharzianum* TU CIM*) even though its maximum biomass concentration was lower than that of the control. In contrast to that, the artificial co-cultivation with *A. xylosoxidans* showed almost the same biomass concentration as the control, however slightly decreased cellulase activity. Co-cultivation of *T. afroharzianum* with a foreign bacterium (*B. tropica*) showed that even though the fungus was able to grow and to secrete proteins, cellulase production was significantly decreased. It could be speculated that the secreted proteins might contain various

antagonistic substances, to fight the bacterium, and vice versa. This is highly speculative as the composition of the secreted proteins was not analyzed. Interestingly, no endocellulase activity was observed for any strain except minimal for the wild-type strain. This result seemed implausible, but was verified by CMC-zymograms, which show no or very faint bands, whereas *T. reesei* cultivated on lactose showed much stronger bands, while hardly showing any activity in the CMC-assay. In spite of the lack of endocellulases *T. afroharzianum* produced high amounts of exocellulases, reaching a maximum activity that was just minimal lower than that of RUT C-30* grown on microcrystalline cellulose.

9 Conclusion

The results of this study show that *T. reesei* on one hand can co-exist with *B. tropica* but at the same time it can be cured of the bacteria without being visibly affected or harmed, being able to asexually reproduce and grow normally. Moreover the ability of *T. reesei* to grow alongside *B. tropica in vitro* without showing signs of antagonism demonstrates that the two species are indeed accustomed to each other and do not conceive their counterpart as enemy. It indicates that the bacterium is not essential to the fungus but might be neutral, beneficial and is unlikely parasitic. Still the exact mode of this interactions as well as localization of the bacterium in respect to *T. reesei* hyphae and spores is yet not known and no conclusions can be drawn based on the fungus ability to exist on its own.

The analysis of cellulase secretion showed that cellulase production might indeed be positively influenced by naturally associated bacteria, when cultivated on a suitable carbon source, such as lactose and only if bacteria are present in an optimal concentration. These results also proved that *T. reesei* and *T. afroharzianum* are able to co-exist with their naturally associated bacteria *in vitro* without being harmed or negatively influenced. Interestingly, however, *T. reesei* is inhibited by *B. tropica* when cultivated on the more complex microcrystalline cellulose, severely delaying the on-set of cellulase production and thus growth of the fungus. It seems that cells of the bacterium first need to reduce in their numbers, so that the fungus expression of cellulases starts. However, only a limited experimental work, so that the obtained results of the fermentations should be regarded as first insights into a complex system, rather than the confirmed scientific fact.

10 Outlook

As this project is in its initial stage, numerous questions are still to be answered. One of the most important ones at this stage might be the question about the exact localization of *B. tropica* in respect to the mycelium or spores of *T. reesei,* as this information could lead to significantly better understanding of this interaction. This should be achievable by application of the generated fluorescent *B. tropica* strain. To verify and clarify the obtained results further fermentations have to be carried out and additionally cellulase gene expression has to be examined, by qPCR. Many questions have to be still answered, such as: How and on what level does *B. tropica* influence *T. reesei* and vice versa? Is there an influence? Do the protein secretion and/or production profiles differ when they are associated?

Answering these questions would most likely also answer 'why' these two organisms co-occur *in situ* as observed in ant's nests. However this question cannot be answered by conducting a single experiment but will take various experiments that probably have to be designed with use of findings of previous experiments and this thesis. The extent of this project is enormous and clearly asks for years of research.

11 Reference

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12 Appendix

Buffers and media

- **0.1 % Congo red solution**

0.1 g Congo red (Sigma)
distilled water to 100 ml

- **DNS**

Solution A

8 g NaOH

5g DNSA (3,5-Dinitrosalicylic acid) (Sigma)

distilled water to 100 ml

Solution B

150 g KNa (Potassium sodium)-tartrate (Sigma)

distilled water to 250 mL

After the reagents of both solution completely dissolved, solution A was slowly mixed into solution B and filled with distilled water to 500 ml. The solution was stored at room-temperature.

- **LB (Lysogeny broth) plates (per l):**

10 g Peptone from casein (Roth)
5 g Yeast extract (Roth)
10 g Sodium chloride (Roth)
15 g Agar Agar (Roth)

- **LB (Lysogeny broth) (per l):**

10 g Peptone from casein
5 g Yeast extract
10 g Sodium chloride

- **TSB (per L):**

15 g Peptone (Roth)
5 g Peptone from soy (Roth)
5 g Sodium chloride

- **Running Buffer, 10x, pH 8.3**

30.3 g Tris (Roth)
144.0 g Glycine (Roth)
10.0 g SDS (Roth)

The reagents were dissolved in deionized water and after dissolving filled up to 1000 ml with deionized water. Afterwards the pH was adjusted with to 8.3 and the buffer stored at 4 °C.
Before usage the buffer was diluted 1:10 by mixing 100 ml of the stock solution with 900 ml of deionized water.

- **Tris – HCl buffer, 1.5M, pH 8.8 (separation gel buffer):**

90.75 g Tris
2 g SDS
350 ml deionized water

The pH was adjusted to 8.8 with hydrochloric acid and deionized water added to a final volume of 500 ml. The buffer was stored at 4 °C.

- **Tris – HCl buffer, 0.5 M, pH 6.8 (stacking gel buffer):**

30.25 g Tris
2 g SDS
350 ml deionized water

The pH was adjusted to 6.8 with hydrochloric acid. Deionized water was added to a total volume of 500 ml and the buffer stored at 4°C.

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                          *         20          *
4180- GB-f : ----------GGATAATACCGCATACGATCTACGGATG :  28
4455- GB-f : ----------------TACCGCATACGATCTACGGATG :  22
4106- GB-f : ---------------------------------------:   -
9414- GB-f : CGGCGAAGCCGGATTATACCGCATACGATCTACGGATG :  38
                                taccgcatacgatctacggatg

                40          *         60          *
4180- GB-f : AAAGCGGGGGATCTTCGGACCTCGCGCTATAGGGGCGG :  66
4455- GB-f : AAAGCGGGGGATCTTCGGACCTCGCGCTATAGGGGCGG :  60
4106- GB-f : ---------------------------------------:   -
9414- GB-f : AAAGCGGGGGATCTTCGGACCTCGCGCTATAGGGGCGG :  76
             aaagcgggggatcttcggacctcgcgctatagggggcgg

                  80          *        100          *
4180- GB-f : CCGATGGCGGATTAGCTAGTTGGTGAGGTAAAGGCTCA : 104
4455- GB-f : CCGATGGCGGATTAGCTAGTTGGTGAGGTAAAGGCTCA :  98
4106- GB-f : ------------------------------AAAAGCCTCA :  10
9414- GB-f : CCGATGGCGGATTAGCTAGTTGGTGAGGTAAAGGCTCA : 114
             ccgatggcggattagctagttggtgaggtAAAGgCTCA

                     120          *        140          *
4180- GB-f : CCAAGGCGACGATCCGTAGCTGGTCTGAGAGGACGACC : 142
4455- GB-f : CCAAGGCGACGATCCGTAGCTGGTCTGAGAGGACGACC : 136
4106- GB-f : CCAAGGCGACGATCCGTAGCTGGTCTGAGAGGACGACC :  48
9414- GB-f : CCAAGGCGACGATCCGTAGCTGGTCTGAGAGGACGACC : 152
             CCAAGGCGACGATCCGTAGCTGGTCTGAGAGGACGACC

                       160          *        180          *
4180- GB-f : AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG : 180
4455- GB-f : AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG : 174
4106- GB-f : AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG :  86
9414- GB-f : AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG : 190
             AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG

                         200          *        220
4180- GB-f : GGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAG : 218
4455- GB-f : GGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAG : 212
4106- GB-f : GGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAG : 124
9414- GB-f : GGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAG : 228
             GGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAG

                  *        240          *        260
4180- GB-f : CCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTC : 256
4455- GB-f : CCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTC : 250
4106- GB-f : CCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTC : 162
9414- GB-f : CCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTC : 266
             CCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTC
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                    *        280          *        300
4180- GB-f : GGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCCTGG : 294
4455- GB-f : GGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCCTGG : 288
4106- GB-f : GGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCCTGG : 200
9414- GB-f : GGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCCTGG : 304
             GGGTTGTAAAGCACTTTTGTCCGGAAAGAAATCCCTGG

                      *        320          *        340
4180- GB-f : TCCTAATATGGCCGGGGGATGACGGTACCGGAAGAATA : 332
4455- GB-f : TCCTAATATGGCCGGGGGATGACGGTACCGGAAGAATA : 326
4106- GB-f : TCCTAATATGGCCGGGGGATGACGGTACCGGAAGAATA : 238
9414- GB-f : TCCTAATATGGCCGGGGGATGACGGTACCGGAAGAATA : 342
             TCCTAATATGGCCGGGGGATGACGGTACCGGAAGAATA

                        *        360          *        380
4180- GB-f : AGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATAC : 370
4455- GB-f : AGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATAC : 364
4106- GB-f : AGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATAC : 276
9414- GB-f : AGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATAC : 380
             AGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATAC

                          *        400          *        4
4180- GB-f : GTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA : 408
4455- GB-f : GTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA : 402
4106- GB-f : GTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA : 314
9414- GB-f : GTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA : 418
             GTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA

             20          *        440          *
4180- GB-f : GCGTGCGCAGGCGGTGATGTAAGACCGATGTGAAATCC : 446
4455- GB-f : GCGTGCGCAGGCGGTGATGTAAGACCGATGTGAAATCC : 440
4106- GB-f : GCGTGCGCAGGCGGTGATGTAAGACCGATGTGAAATCC : 352
9414- GB-f : GCGTGCGCAGGCGGTGATGTAAGACCGATGTGAAATCC : 456
             GCGTGCGCAGGCGGTGATGTAAGACCGATGTGAAATCC

              460          *        480          *
4180- GB-f : CCGGGCTCAACCTGGGAACTGCATTGGTGACTGCATCG : 484
4455- GB-f : CCGGGCTCAACCTGGGAACTGCATTGGTGACTGCATCG : 478
4106- GB-f : CCGGGCTCAACCTGGGAACTGCATTGGTGACTGCATCG : 390
9414- GB-f : CCGGGCTCAACCTGGGAACTGCTTTGG----------- : 483
             CCGGGCTCAACCTGGGAACTGCaTTGGtgactgcatcg

                 500          *        520          *
4180- GB-f : CTTGAGTATGGCAGAGGGGGGTAGAATTCCACGTGTAG : 522
4455- GB-f : CTTGAGTATGGCAGAGGGGGGTAGAATTCCACGTGTAG : 516
4106- GB-f : CTTGAGTATGGCAGAGGGGGGTAGAATT---------- : 418
9414- GB-f : ---------------------------------------- :   -
             cttgagtatggcagagggggtagaatt
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                     540         *        560         *
4180- GB-f : CAGTGAAATGCGTAGAGATGTGGAGGAATACCGATGGC : 560
4455- GB-f : CAGTGAAATGCGTATAGATGTGGAGGAA---------- : 544
4106- GB-f : ---------------------------------------  :   -
9414- GB-f : ---------------------------------------  :   -

                        580         *        600
4180- GB-f : GAAGGCAGCCCCCTGGGTCAATACTGACGCTCATGCAC : 598
4455- GB-f : ---------------------------------------  :   -
4106- GB-f : ---------------------------------------  :   -
9414- GB-f : ---------------------------------------  :   -

                 *        620         *        640
4180- GB-f : GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA : 636
4455- GB-f : ---------------------------------------  :   -
4106- GB-f : ---------------------------------------  :   -
9414- GB-f : ---------------------------------------  :   -

                    *        660         *        680
4180- GB-f : GTCCACGCCCTAAACGATGTCAACTGGTTGTCGGGTCT : 674
4455- GB-f : ---------------------------------------  :   -
4106- GB-f : ---------------------------------------  :   -
9414- GB-f : ---------------------------------------  :   -

                      *        700         *        720
4180- GB-f : TCATTGACTTGGTAACGTAGCTAACGCGTGAAGTTGAC : 712
4455- GB-f : ---------------------------------------  :   -
4106- GB-f : ---------------------------------------  :   -
9414- GB-f : ---------------------------------------  :   -

                        *        740         *        760
4180- GB-f : CGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGG : 750
4455- GB-f : ---------------------------------------  :   -
4106- GB-f : ---------------------------------------  :   -
9414- GB-f : ---------------------------------------  :   -

                          *        780         *        8
4180- GB-f : AATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGA : 788
4455- GB-f : ---------------------------------------  :   -
4106- GB-f : ---------------------------------------  :   -
9414- GB-f : ---------------------------------------  :   -
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                 00          *        820          *
4180- GB-f : TTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTG : 826
4455- GB-f : ------------------------------------- :   -
4106- GB-f : ------------------------------------- :   -
9414- GB-f : ------------------------------------- :   -

                  840          *        860
4180- GB-f : ACATGTACGGAATTCCGCTGAGAGGTGG : 854
4455- GB-f : ----------------------------- :   -
4106- GB-f : ----------------------------- :   -
9414- GB-f : ----------------------------- :   -
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