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MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Poly(1,4-butylene adipate-*co*-terephthalate) degradation by the black fungus *Knufia chersonesos*"

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2018 / Vienna 2018

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 863
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Biologische Chemie
Betreut von / Supervisor:	Assoz. Prof. Mark Manuel Somoza, BSc MSc PhD

1 Acknowledgments

The present work was carried out at the Austrian center of biological resources and applied life science (ACBR) in the Department of Biotechnology (DBT) at the University of Natural Resources and Life Sciences, Vienna in cooperation with the department of Agrobiotechnology of the IFA-Tulln, Tulln and der Donau, Austria.

Firstly, I would like thank Assoc. Prof. Dr. Katja Sterflinger for the supervision of my master's thesis and the possibility to conduct the major part of my practical work in her group, at the Austrian center of biological resources and applied life science (ACBR).

Special thanks shall also be given to the co-supervisor of my thesis, Dr. Donatella Tesei, MSc, whose FWF Hertha-Firnberg project supported this research work. She supported me with advice and active help over the course of my thesis. I could always seek her advice, when I was uncertain about further proceeding or problems regarding my work. I have to thank her for enabling me to work with this fascinating organisms and delve deeper into the topic of extremotolerant fungi by supervising my thesis. I wish Donatella all the best for her personal future and a successful further research in this field.

Additionally, I would like to acknowledge the Austrian Science Fund (Fonds zur Förderung der wissenschaftlichen Forschung (FWF), Project T872-B22, Hertha-Firnberg and the Extremophile Center (AG Sterflinger) for funding this research work, along with BASF SE for providing the PBAT polymer test substances.

Furthermore, thanks are due to Univ.Prof. Dipl.-Ing. Dr.techn. Georg Gübitz and Mag. Dr. Doris Ribitsch for granting the access to analysis possibilities at the Department of agrobiotechnology, IFA-Tulln.

Special thanks are also due to Felice Quartinello, MSc and Klemens Kremser for continued support with analyses and documentation work.

Additionally, I have to thank all my co-workers for good cooperation and intake into their working group.

The supervision of my external master's thesis by Assoz. Prof. Mark Manuel Somoza, BSc MSc PhD in the name of the Universität Wien is thankfully acknowledged.

Gratitude shall also be given to my parents, who enabled me to attend studies of chemistry and biochemistry at university by their kind support. Thanks are also due to my personal friends and Vanessa who supported me at the practical work and documentation of the thesis.

I wish for the reader of this work to find interesting knowledge and that it is for you, as it was for me, an enrichment.

My thanks are due to you, who made this thesis possible!
Christoph Unterberger

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3 Introduction

3.1 Black fungi

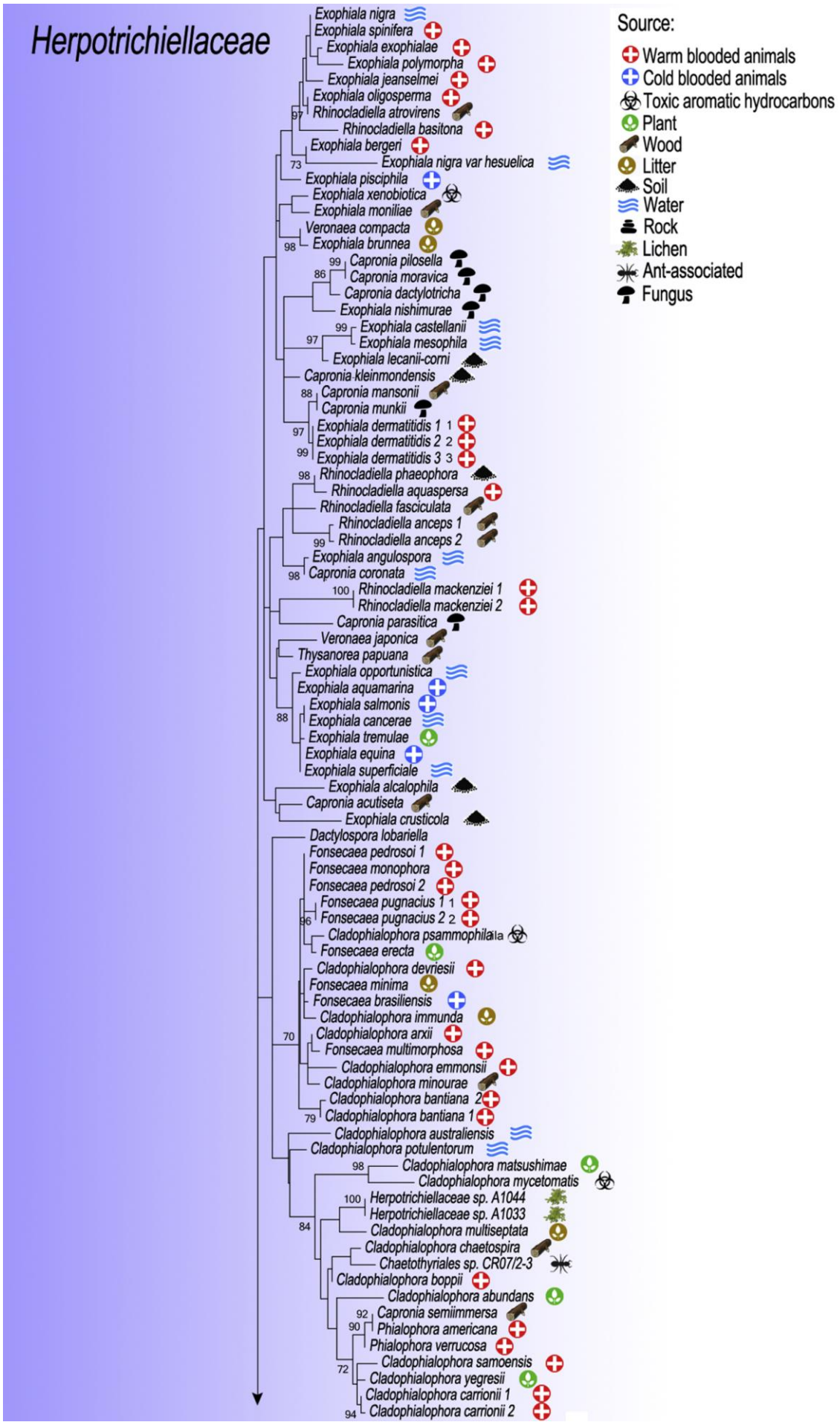
Black fungi are a heterogeneous group of ascomycetes regarding taxonomy and phylogeny, which share a common morphology (Figure 1). In literature members of this group are referred to as black yeasts, meristematic-, microcolonial-, or dematiaceous fungi, used with an overlapping meaning.[1–3]

The term 'black yeasts' is describing a group of fungi that are different in taxonomy and phylogeny, but share the common morphological features, such as a melanised cell wall and that the daughter cells are formed by yeast-like multilateral or polar budding. In addition to yeast-like growth, most black yeasts exhibit a mycelial growth mode and generation of conidia from simple phialides, phialides with collarettes, annelated phialides, on rhachides or undifferentiated conidigenous cells. The formed conidia can be unseptated or have up to three transversal septa.[1,4]

The term 'meristematic fungi' is used to describe a group of fungi that forms aggregates of thick-walled and melanised cells enlarging themselves until reproduction by isodiametrical division. The offspring is separated by breaking apart from the aggregate or by endogenous conidiogenesis with subsequent disruption of parental cell wall. The formation of blastic conidia from undifferentiated cells and yeast-like budding are also found within meristematic fungi.[2,4]

The term 'microcolonial fungi' refers to the growth pattern of some black fungi growing on mineral substrates. On hard substrates black fungi form black cauliflower-like colonies with 1 mm in diameter, consisting of a densely aggregation of cells with thick cell walls. While maintaining this growth mode, different species of black fungi are not distinguishable solely considering their morphology.[2,5]

The group 'dematiaceous fungi' is solely characterised by dark pigmentation as common feature and therefore includes black yeasts, meristematic-, and microcolonial fungi.[1]


Herpotrichiellaceae
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Cold blooded animals
Toxic aromatic hydrocarbons
Plant
Wood
Litter
Soil
Water
Rock
Lichen
Ant-associated
Fungus
Exophiala nigra
Exophiala spinifera
Exophiala exophialae
Exophiala polymorpha
Exophiala jeanselmei
Exophiala oligosperma
Rhinocladiella atrovirens
Rhinocladiella basitona
Exophiala bergeri
Exophiala nigra var hesuelica
Exophiala pisciphila
Exophiala xenobiotica
Exophiala moniliae
Veronaea compacta
Exophiala brunnea
Capronia pilosella
Capronia moravica
Capronia dactylotricha
Exophiala nishimurae
Exophiala castellanii
Exophiala mesophila
Exophiala lecanii-corni
Capronia kleinmondensis
Capronia mansonii
Capronia munkii
Exophiala dermatitidis 1
Exophiala dermatitidis 2
Exophiala dermatitidis 3
Rhinocladiella phaeophora
Rhinocladiella aquaspersa
Rhinocladiella fasciculata
Rhinocladiella anceps 1
Rhinocladiella anceps 2
Exophiala angulospora
Capronia coronata
Rhinocladiella mackenziei 1
Rhinocladiella mackenziei 2
Capronia parasitica
Veronaea japonica
Thysanorea papuana
Exophiala opportunistica
Exophiala aquamarina
Exophiala salmonis
Exophiala cancerae
Exophiala tremulae
Exophiala equina
Exophiala superficiale
Exophiala alcalophila
Capronia acutiseta
Exophiala crusticola
Dactylospora lobariella
Fonsecaea pedrosoi 1
Fonsecaea monophora
Fonsecaea pedrosoi 2
Fonsecaea pugnacius 1
Fonsecaea pugnacius 2
Cladophialophora psammophila
Fonsecaea erecta
Cladophialophora devriesii
Fonsecaea minima
Fonsecaea brasiliensis
Cladophialophora immunda
Cladophialophora arxii
Fonsecaea multimorphosa
Cladophialophora emmonsii
Cladophialophora minourae
Cladophialophora bantiana 2
Cladophialophora bantiana 1
Cladophialophora australiensis
Cladophialophora potulentorum
Cladophialophora matsushimae
Cladophialophora mycetomatis
Herpotrichiellaceae sp. A1044
Herpotrichiellaceae sp. A1033
Cladophialophora multiseptata
Cladophialophora chaetospira
Chaetothyriales sp. CR07/2-3
Cladophialophora boppii
Cladophialophora abundans
Capronia semiimmersa
Phialophora americana
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Cladophialophora carrionii 2

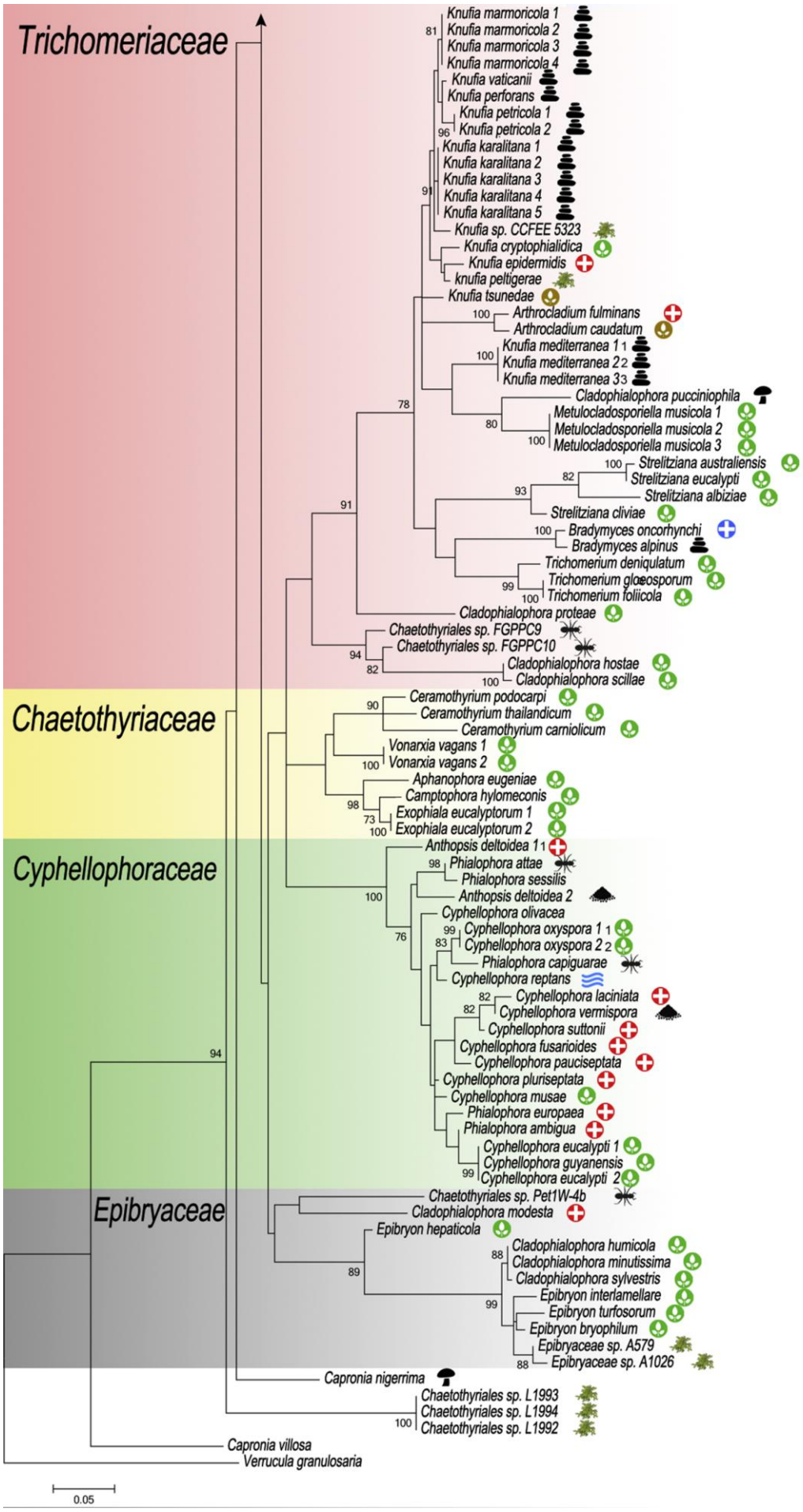
Trichomeriaceae
Chaetothyriaceae
Cyphellophoraceae
Epibryaceae
Knufia marmoricola 1
Knufia marmoricola 2
Knufia marmoricola 3
Knufia marmoricola 4
Knufia vaticanii
Knufia perforans
Knufia petricola 1
Knufia petricola 2
Knufia karalitana 1
Knufia karalitana 2
Knufia karalitana 3
Knufia karalitana 4
Knufia karalitana 5
Knufia sp. CCFEE 5323
Knufia cryptophialidica
Knufia epidermidis
knufia peltigerae
Knufia tsunedae
Arthrocladium fulminans
Arthrocladium caudatum
Knufia mediterranea 1 1
Knufia mediterranea 2 2
Knufia mediterranea 3 3
Cladophialophora pucciniophila
Metulocladosporiella musicola 1
Metulocladosporiella musicola 2
Metulocladosporiella musicola 3
Strelitziana australiensis
Strelitziana eucalypti
Strelitziana albiziae
Strelitziana cliviae
Bradymyces oncorhynchi
Bradymyces alpinus
Trichomerium deniqulatum
Trichomerium glaeosporum
Trichomerium foliicola
Cladophialophora proteae
Chaetothyriales sp. FGPPC9
Chaetothyriales sp. FGPPC10
Cladophialophora hostae
Cladophialophora scillae
Ceramothyrium podocarpi
Ceramothyrium thailandicum
Ceramothyrium carniolicum
Vonarxia vagans 1
Vonarxia vagans 2
Aphanophora eugeniae
Camptophora hylomeconis
Exophiala eucalyptorum 1
Exophiala eucalyptorum 2
Anthopsis deltoidea 1 1
Phialophora attae
Phialophora sessilis
Anthopsis deltoidea 2
Cyphellophora olivacea
Cyphellophora oxyspora 1 1
Cyphellophora oxyspora 2 2
Phialophora capiguarae
Cyphellophora reptans
Cyphellophora laciniata
Cyphellophora vermispora
Cyphellophora suttonii
Cyphellophora fusarioides
Cyphellophora pauciseptata
Cyphellophora pluriseptata
Cyphellophora musae
Phialophora europaea
Phialophora ambigua
Cyphellophora eucalypti 1
Cyphellophora guyanensis
Cyphellophora eucalypti 2
Chaetothyriales sp. Pet1W-4b
Cladophialophora modesta
Epibryon hepaticola
Cladophialophora humicola
Cladophialophora minutissima
Cladophialophora sylvestris
Epibryon interlamellare
Epibryon turfosorum
Epibryon bryophilum
Epibryaceae sp. A579
Epibryaceae sp. A1026
Capronia nigerrima
Chaetothyriales sp. L1993
Chaetothyriales sp. L1994
Chaetothyriales sp. L1992
Capronia villosa
Verrucula granulosaria
0.05

Figure 1: Phylogenetic tree of the Chaetothyriales, which harbours obligatory melanised fungi. The phylogenetic tree is based on an Maximum Likelihood analysis of 172 large subunit ribosomal DNA sequences with statistical bootstrapping procedure involving 500 replicates. Only Bootstrap values above 70 % are shown. Family boundaries are shown in coloured blocks.[6]

Black fungi are amidst the most stress-resistant eukaryotes known and described as polyextremotolerant or extremophilic.[7–9] The stress-resistance includes UV-B- and ionising radiation, complete desiccation and rehydration, high temperatures and temperature variations, osmotic stresses and extreme pH conditions.[10–16] Such combination of stress-resistance properties is achieved by physiological and morphological adaptations as the two major mechanisms that reduce environmental stresses on the fungus.

Physiological adaptations for stress-resistance are taken by formation of thick and multilayered cell walls, secretion of an extracellular polysaccharide (EPS) capsule, melanisation and incrustation of the cell wall, and intracellular accumulation of polyols and trehalose.[2,4,11,12,14,17–19]

Especially melanin is a key factor for survival of black fungi. Melanin is a polymeric pigment, containing conjugated moieties (e.g. aromatic rings) for electronic resonance, whose structure, due to its diversity and complexity, remain yet mostly unknown.[3] In fungi, melanins are prevalently biosynthesised by polymerisation of 1,8-dihydroxynaphthalene (DHN) in melanosomes and contained on the cell surface or released in the extracellular space. A few fungi synthesise melanin via L-3,4-dihyroxyphenylalanine (L-dopa), in a pathway that resembles mammalian melanin biosynthesis and that use either L-dopa or tyrosine as precursor molecule. Several species use both pathways, others such as *Cryptococcus neoformans* rely only on the L-dopa pathway.[3,20]

It has been shown that melanin provides UV-protection by absorbance over the whole UV-visible electromagnetic spectrum due to its complex-disordered structure.[3,19,21–23] Additionally, melanin provides tolerance towards ionising radiation, through the absorption and dissipation of radiation energy and the quenching of free radical species.[3,19] Further, black fungi being able to absorb UV-visible or gamma radiation and transduce it into metabolic energy have been reported.[3,10,24,25] In cold habitats melanin is used to absorb solar radiation and dissipate the absorbed energy in form of heat, which is essential for ectothermic organisms relying on external sources of heat to maintain temperature homeostasis.[3,23,26] Hence, a correlation between latitudinal distribution and yeast

pigmentation was found, with a dark pigmented yeasts being less probable to be found in tropic reagions.[23] Protection against heat stress by melanin was also reported for several melanised fungi. The mechanism, however, is still unknown, but hypothesised to be associated with quenching of heat-induced reactive oxygen species and buffering of heat influx.[3,27–29] The adaptation to dry conditions and desiccation protection was also associated with melanin, which increases the cells ability to absorb and retain water due to its hygroscopic character and changes the porosity of the fungal cell wall.[3,19,30] In addition, melanin pigmentation of the fungal cell wall offers extra mechanical strength to the fungal hyphae.[31,32] Thus, allowing rock inhabiting black fungi to grow through crevices into deeper rock layers, further protecting it from UV radiation.[33,34] Osmotic and hydrostatic pressure resistances are also improved by melanisation strengthening the fungal cell wall and altering cell wall permeability.[3,35] The enhanced biomechanical strength of the melanised cell walls also attributes to the ability of some black fungi to develop pathogenic behaviour as their hyphae can penetrate host tissues and melanin acts as non-specific armour against the hosts immune response.[3,36,37] The antioxidant effect of melanin also contributes to the virulence ability of black fungi as it increases the resistance towards digestion upon phagocytosis.[3]

Morphological adaptations to reduce environmental stresses are taken by isodiametric growth, resulting in distinctive microcolonial, cauliflower-like architecture of the colonies. The fungi are minimising the surface-to-volume ratio, hence the evaporation surface is at a minimum. In addition, primarily asexual reproduction takes place reducing the energy required, which is important in hostile oligotroph environments.[1,4]

It is attributable to the oligotrophic lifestyle of black fungi together with their high resistance to environmental stresses that these organisms are abundant and wide spread in nature.[3,9] As the most abundant natural habitat, rock surfaces are colonised by oligotrophic black fungi in all climatic zones.[9,16] Even in the most hostile environments on earth, such as Antarctic dry valleys, the Atacama desert, or high alpine habitats in the Himalayas, black fungi can be found colonising bare rock surfaces.[5,16]

In addition to their global distribution, some black fungi can show opportunistic pathogenicity in chronic infections to the respiratory tract and skin (e.g. chromoblastomycosis, phaeohyphomycosis) in human and animal hosts.[38] Even pathogenicity by invading and living in neural tissue of immune competent patients can be found within the group of black fungi.[38,39] The shown pathogenicity is partly possible due to

the polyextremotolerance exhibited by black fungi. Although, it is barely thought of as this, the skin and body of a warm-blooded animal represent an extreme environment for microorganisms with elevated temperatures and salt levels, as well as mechanical stresses.[8] The strength of the thick melanised cell walls enables black fungi to better withstand these mechanical stresses and their hyphae to penetrate host tissues.[36,37] Therefore, black fungi with suitable preadaptations or exaptations might represent potential medically important fungi, when switching from colonising environmental niches to human bodies.[8,40]

3.2 Fungi and biotechnology

Biotechnology in combination with fungi is used since primeval ages to produce products of daily use, such as bread and alcoholic beverages. These applications make use of the fungal primary metabolism pathways. Other than that, the fungal secondary metabolism pathways are used to generate bioactive molecules, for example antibiotics. With the development of genetic engineering the biotechnological importance of fungi rose even further, since *Saccharomyces cerevisiae* or even filamentous fungi can be genetically engineered to produce a desired product. Fungi, as all other Eukaryotes, are able to apply posttranslational modification (e.g. glycosylation) on their gene products. Therefore, fungal production systems enable the production of functional eukaryotic proteins, which is not possible in bacterial expression.[41]

A metabolic product, which is biotechnologically produced with the help of fungi, is citric acid. The production of citric acid on an industrial scale for the food industry is conducted with *Aspergillus niger*. Fungi are also used to produce vitamins, such as Riboflavin (vitamin B_2) by the fungus *Ashbya possypii*. In this case the wild type strain is already producing increased amounts of vitamin B_2 and the yield is further improved by genetic engineering of the fungus. A strong promotor is used to enhance the expression of a gene product, which is the limiting factor in riboflavin biosynthesis of the wild type strain.[41] The first antibiotic Penicillin G was produced with the fungus *Penicillium notatum*. Instead of physiological optimisation to maximise antibiotic production yields, fungal production strains are improved by mutagenesis and selection or by metabolic engineering to generate optimal yields and the possibility of easy product purification.[41] Today a wide variety of antibiotics in form of Penicillins to Cephalosporins produced by fungi is available and a recent study characterised secondary metabolism gene clusters in *Penicillium* species with a potential use to produce novel bioactive compounds.[42–44]

3.2.1 Biotechnological applications of black fungi

Unlike other fungal species already used for biotechnological applications, black fungi have been scientifically neglected for a long time as rare and exotic fungi.[45] However, because of their highly specialised lifestyle regarding tolerance to radiation, low-, high- or varying temperature, desiccation, and osmotic stresses, black fungi are a potential source of proteins highly adapted to these adverse conditions.[10–16,46]

The best known example of a black fungi with industrial importance is *Aureobasidium pullulans*, due to its capability to produce the linear storage maltotriose polysaccharide pullulan.[41,47] Pullulan is commercially used as edible, biodegradable, and oxygen-impermeable food coating and wrapping material, as food ingredient, and in medical applications.[47–49] Besides pullulan, *A. pullulans* produces the starch debranching enzyme pullulanase, which is industrially used in the production of glucose- and maltose syrups from starch, and other important enzymes such as fructofuranosidase, xylanase, and glucoamylase.[47,50]

In addition to that, black fungi could be a source of novel bioactive molecules and compounds as was previously shown.[51–53] *Exophiala pisciphila*, a marine black yeast dwelling in a suggested symbiotic relationship with the sponge *Mycale adhaerens*, was identified as the producer of a novel antibiotic compound. The molecule exophilin A exhibits an antibiotic activity against Gram-positive bacteria. Its structure shows no similarities to other reported bioactive substances isolated from the same environment.[51] In another study, fungal inhabitants of solar salterns were investigated for their production of bioactive metabolites regarding antibacterial activity. The halophilic black yeast *Hortaea werneckii* and *Trimmatostroma salinum* were found to have increased hemolytic activities as a representation of cytotoxic compounds in increased salt environments in their acetone- and methanolic extracts.[52]

Melanin itself, a common feature shared by all black fungi, has an antioxidant activity. This was shown by testing the resistance of the black fungi *Wangiella dermatitidis* and *Alternaria alternata* and their non-melanised mutants against the oxidants permanganate, hypochlorite, and H_2O_2 with *in vivo* titration.[53]

Another potential application of black fungi in biotechnology could be in the field of pollutant bioremediation or degradation of waste polymers.[54–57] The ability to degrade toluene as sole carbon- and energy source was reported for *Cladophialophora immunda*,

Exophiala xenobiotica, *Exophiala lecanii-corni*, and *Exophiala sideris*. *Exophiala mesophila* was able to grow on toluene and hexadecane. Several other black fungi (e.g. *Exophiala oligosperma*, *Exophiala jeanselmei*, *Pseudallescheria boydii*) were able to degrade hexadecane. These compounds are a representation of different relevant environmental pollutants in the soil with the possibility to be degraded by fungi.[54,55] In another study *Exophiala jeanselmei* was reported to grow on gaseous styrene as sole carbon and energy source.[56,57] Styrene is a bulk chemical, which is used for the production of synthetic polymers. Hence, emissions of styrene into the environment regularly occur around styrene or polystyrene manufacturing plants.[56,58]

The destabilisation of polymers, such as polyvinyl chlorides (PVCs), by migration of plasticisers to the surface and subsequent colonisation with black fungi has also been reported. These reports refer to black fungi attacking polymers of cultural heritage, but a similar process can be expected with waste PVCs at landfills.[59–61]

3.3 Polymers

Polymers are a class of materials, built up by linkage of smaller repeating monomer units.[62,63] Depending on their chemical composition and molecular weight distribution, polymers have a wide range of properties and various applications.[64] Naturally occurring polymers, such as cellulose, hemicellulose, lignin, chitin, and starch, are abundantly found in nature.[62,63,65,66] Other polymers, which are technically built up of natural occurring materials, are named synthetic polymers.[62,63] Synthetic polymeric materials are indispensable in our present world because of their numerous beneficial properties as flexibility, toughness, and ease of fabrication. The development in polymer science over the last decades has led to polymers that are durable, long-lasting, resistant to environmental influences, and recalcitrant to microbial attacks.[67] The resistance to microbial degradation stems from their excessive molecular weight, high number of aromatic rings, unusual bonds, and halogen substitutions.[62,68]

Especially polyesters find various applications in a wide variety of areas, such as food packaging, textile production, and tissue engineering.[69–72] Though these properties are initially beneficial in short- to mid-term usage of the polymeric materials, the accumulation in the environment through absence of microbial degradation leads to environmental- and waste management problems. In order to counteract these problems, a scientific focus was

set on the development of biodegradable polymers and the identification of microorganisms and related enzymes able to conduct polymer degradation.[67,73]

3.3.1 Polymer degradation

Polymers are removed from the environment by photo-induced-, thermal-, and chemical degradation, consisting of solvolysis, ozonolysis, and oxidation. Degradation through galvanic action and biological degradation also take part in the removal of polymers.[74] The ability to degrade polymeric substances is an important source of nutrients for various microorganisms as natural polymers are omnipresent in nature.[75] As polymeric molecules cannot pass through cellular membranes in consequence of their extensive size, depolymerisation must occur first in order to degrade the polymer through biological means. This initial depolymerisation is achieved by a variety of physical, chemical, and biological processes.[62,63] The biodegradability of a polymer is strongly dependent on its chemical composition and the provision of nutrients for the degrading microbiota. Therefore, natural polymers and polymers consisting of natural monomers exhibit a greater biodegradability compared to synthetic polymers.[66,76,77] In aerobic conditions waste polymers are broken down by heterotrophic microorganisms. Both, fungi and bacteria, are involved in the natural degradation of natural and synthetic polymers. In anaerobic conditions bacterial degradation prevails and methane is produced as byproduct.[67,73,76,78]

The degradation of polymers is conducted by breaking down the polymeric macromolecules by biochemical transformation. Bio-degradation of polymers is described by the reduction of the mean molecular weight of the polymeric molecules by the catalytic activity of microbial enzymes. Hence, resulting in an alteration of the physical properties of the polymer, such as loss of mechanical strength and surface properties.[66,79]

The process of bio-degradation is occurring in different sequential phases encompassing bio-deterioration, bio-fragmentation, assimilation, and mineralisation. In bio-deterioration, the physical and chemical properties of the polymer are altered. Bio-fragmentation is the cleavage of the polymer by extracellular enzymes into smaller subunits. Following these phases, the monomer units are assimilated into the degrading microorganisms and metabolised into CO_2, H_2O and, if anaerobic conditions prevail, CH_4. With completion of the last step the biological remineralisation of polymers is accomplished.[66,80]

3.3.2 Polyester degradation

Hydrolysis is the prevailing reaction in terms of enzymatic polymer cleavage. Monomers connected via glycosidic bonds, ester-, and peptide linkages can all be cleaved through a led nucleophilic attack on the carbonyl carbon. The polymer-degrading enzymes mediating this reaction are excreted in the surrounding milieu to allow direct contact with their polymeric substrates. Non-specific hydrolysing enzymes would be a hazard for the degrading fungi and bacteria, which external layers consist of hydrolysable polysaccharides. Therefore, most fungal and bacterial hydrolases are largely specific regarding the chemical structure of their substrates. Nevertheless, a number of hydrolases are able to degrade synthetic polymers as polyesters as well, either due to the low substrate specificity of the degrading enzymes or to modification by genetic engineering. The degradation process is aided in case of synthetic polymers, whose structure is easily accessible by the enzyme.[81]

Several secreted microbial cleavage enzymes are easily able to degrade aliphatic polyesters. However, aliphatic polyesters exhibit thermal and mechanical properties, which most often do not suit the specifications required by various potential sectors of applications. Therefore, aromatic polyesters, which show worse biodegradation behaviours than aliphatic polyesters, are used for these applications instead.[72,82,83]

A variety of different enzymes with the ability to degrade polyesters have already been reported.[72,84–89] Polyethylene terephthalate (PET) was shown to be degradable by cutinases, lipases, serine-esterases and nitrobenzyl-esterases originated from several different organisms.[84,88] Polycaprolactone (PCL) could be degraded by a cutinases from *Fusarium* species.[85] Emulsified poly(butylene succinate-*co*-adipate (PBSA) was degraded by an enzyme with significant similarity to cutinases from other fungi secreted by *Paraphoma*-related fungus.[86] TtcutA, a cutinase from *Thielavia terrestris*, was able to degrade polyethylene terephthalate (PET), polycaprolactone (PCL), and polybutylene succinate (PBS), while maintaining stability to extremely high concentrations of various solvents.[87] The random aromatic-aliphatic copolyester from 1,4-butandiol, terephthalic acid, and adipic acid (PBAT) was shown to be degraded by a hydrolase excreted by *Thermomonospora fusca*.[89]

3.3.2.1 Enzymatic degradation mechanism

The main degrading enzymes cutinases, esterases and lipases all belong to the carboxylesterase enzyme class (EC 3.1.1).[72] The reactivity of these enzymes is based on a common catalytic triad consisting of a serine, aspartate, or cysteine as nucleophile, a

histidine as catalytic base, and aspartate or glutamate as catalytic acid.[90] The serine, being used as nucleophile, is found in a penta peptide structure the 'nucleophile elbow' consisting of the peptides Gly-X-Ser-X-Gly or Ala-X-Ser-X-Gly. The 'nucleophile elbow' forms a sharp turn in the peptide chain that is easily approached by the substrate or the hydrolytic water molecule and forms an oxyanion binding site that stabilises the negatively charged transitions state that occurs in the catalytic triad during the hydrolysis of the substrate. The protein, surrounding the active site, is in a very close structure called α/β hydrolase fold, evolved to maintain the position of the conserved peptides in the catalytic triad.[72,91,92] Additionally, lipases form a 'lid' domain, which increases the hydrolase activity of the enzymes in presence of hydrophobic surfaces found in emulsions or particles. The lid recognises the surface by opening due to adsorption onto the interface, which leads to the mechanism of 'interfacial activation' increasing the enzyme activity.[72,93,94] This feature allows lipases to maintain an increased degradation activity, when adsorbing onto hydrophobic polymers.[95]

3.3.3 Polyester functionalisation

Bio-based and degradable polyesters do not only rely on economic competitiveness to compete with fossil-based polymers, but also bring new possibilities of chemical and functional properties to polymers. By exploiting the selectivity and efficiency of catalytic enzymes' ability of polyesters hydrolysis, modifications and functionalisations could be introduced into bio-based polyesters, while retaining the bulk properties.[96–99]

3.3.4 Poly(1,4-butylene adipate-*co*-terephthalate) (PBAT)

Aliphatic polyesters are degraded by secreted cleavage enzymes, however aromatic polyesters prevail in polymer usage owing to their superior properties, such as thermal stability, durability and tensile strength, in comparison with aliphatic polyesters.[72,82] Consequently, copolyesters of aromatic and aliphatic compounds have been developed, which are bio-degradable while retaining the beneficial properties of aromatic polyesters.[72,82,100]

One of the developed aliphatic-aromatic copolyesters currently on the market is poly(1,4-butylene adipate-*co*-terephthalate) (PBAT).[72] PBAT is random copolymer consisting of butylene adipate and butylene terephthalate monomers (Figure 2). In technical production PBAT is prepared by melt polycondensation of 1,4-butandiol, dimethylterephthalate, and

adipic acid, catalysed by tetrabutylorthotitanate.[101,102] In random or statistical copolymers, the chemical structure of the polymer is purely dependent on the statistical usage of different monomers by the polymer elongation reaction, upon polymer production. Hence, the probability of finding a specific monomer at a given location is independent from the nature of the adjacent monomer units in this polymer site, as opposed to alternating or block copolymers.[103]

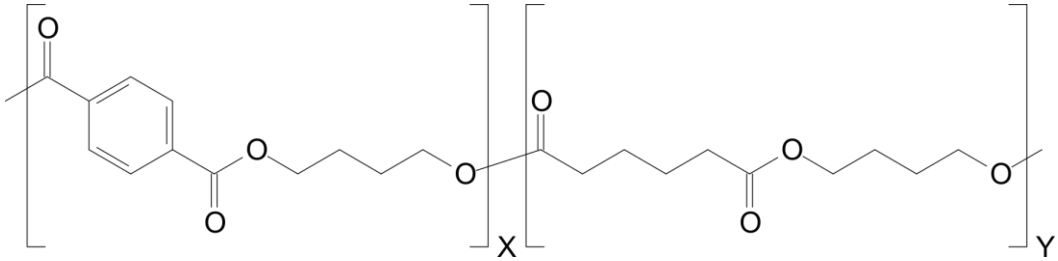

Figure 2: Chemical structure of poly(1,4-butylene adipate-co-terephthalate) (PBAT) consisting of butyleneterephthalate (X) and butyleneadipate (Y).

PBAT is owing its bio-degradability to the butyleneadipate and its mechanical properties and stability to the butyleneterephthalate, resulting in high elongation properties up to 700 %, but a relatively low tensile strength of 32 MPa.[101,104] Due to the similarity of these properties to that of low-density polyethylene (LDPE), this material is in application for food packaging as container and lamination material and as agricultural equipment like mulch films.[72,105,106]

The degradability of PBAT has been reported as a mineralisation rate of 67.3 ± 2.8 % in manure compost, 44.9 ± 2.6 % in food compost, and 33.9 ± 1.5 % in yard compost after a 45 day period, respectively.[107] This remineralisation rates show a similar pattern to the degradation of cellulose under comparable conditions. In the process of PBAT degradation, hydrolase cleavage or chemical scission of ester linkages is necessary to reduce the polymer molecule size into size ranges, where bio-assimilation and metabolisation by microorganisms can occur. Overall PBAT degradation is strongly influenced by microbial activity present in the environment. The hydrolysis of the polyester is expedited on the ester linkages in aliphatic and amorphous regions of the polymer. Aromatic and crystalline regions of the polyester are less susceptible to hydrolisation.[107]

Different compositions of PBAT defined by their adipate : terephthalate copolymer ratio can be acquired by melt polycondensation of the monomers in appropriate reaction

mixtures.[108,109] These PBAT compositions exhibit varying mechanical properties, degradability, and crystallinity behaviour, further researched in this thesis.[108,110,111]

3.4 *Knufia chersonesos*

Knufia chersonesos (synonym *Knufia petricola*; former *Phaeococcomyces chersonesos*, *Sarcinomyces petricola*) is a species belonging to the black fungi morphological group of ascomycetes, which main features are an extensive melanin pigmentation of the cells, no recognisable sporulation, and a high resistance to a broad range of environmental stresses.[112] The fungal genus *Knufia*, order *Chaetothyriales*, family *Trichomeriaceae*, class *Eurotiomycetes*, is a clade relatively limited in size, mostly consisting of extremotolerant species colonising bare rock surfaces (Figure 3, Figure 4).[112] Other *Knufia* species inhabiting plants and insects, as well as isolations from clinical samples have been reported.[113–115]

K. chersonesos was purposed as a combination of *P. chersonesos* and *S. petricola*, which turned out to be conspecific as molecular analysis showed identical internal transcribed spacer (ITS) sequences.[115,116]

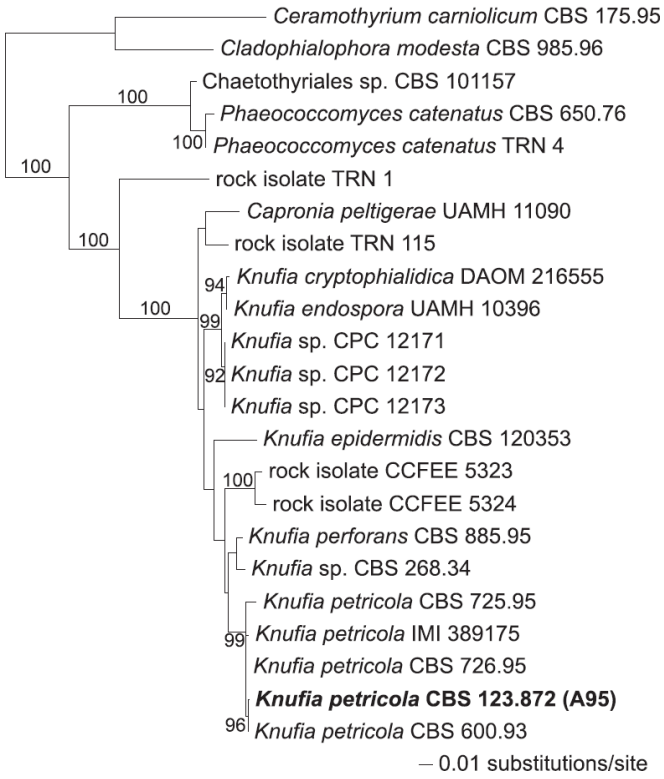

Figure 3: Phylogenetic placement of *Knufia chersonesos* (synonym *Knufia petricola*). The phylogenetic tree is based on Maximum Likelihood analysis of a five-gene dataset (nuLSU, nuSSU, mtSSU, ITS, *RPB*1). Only branches with a bootstrap values above 70 % are indicated in the graph. The taxon name is followed by the strain number.[117]

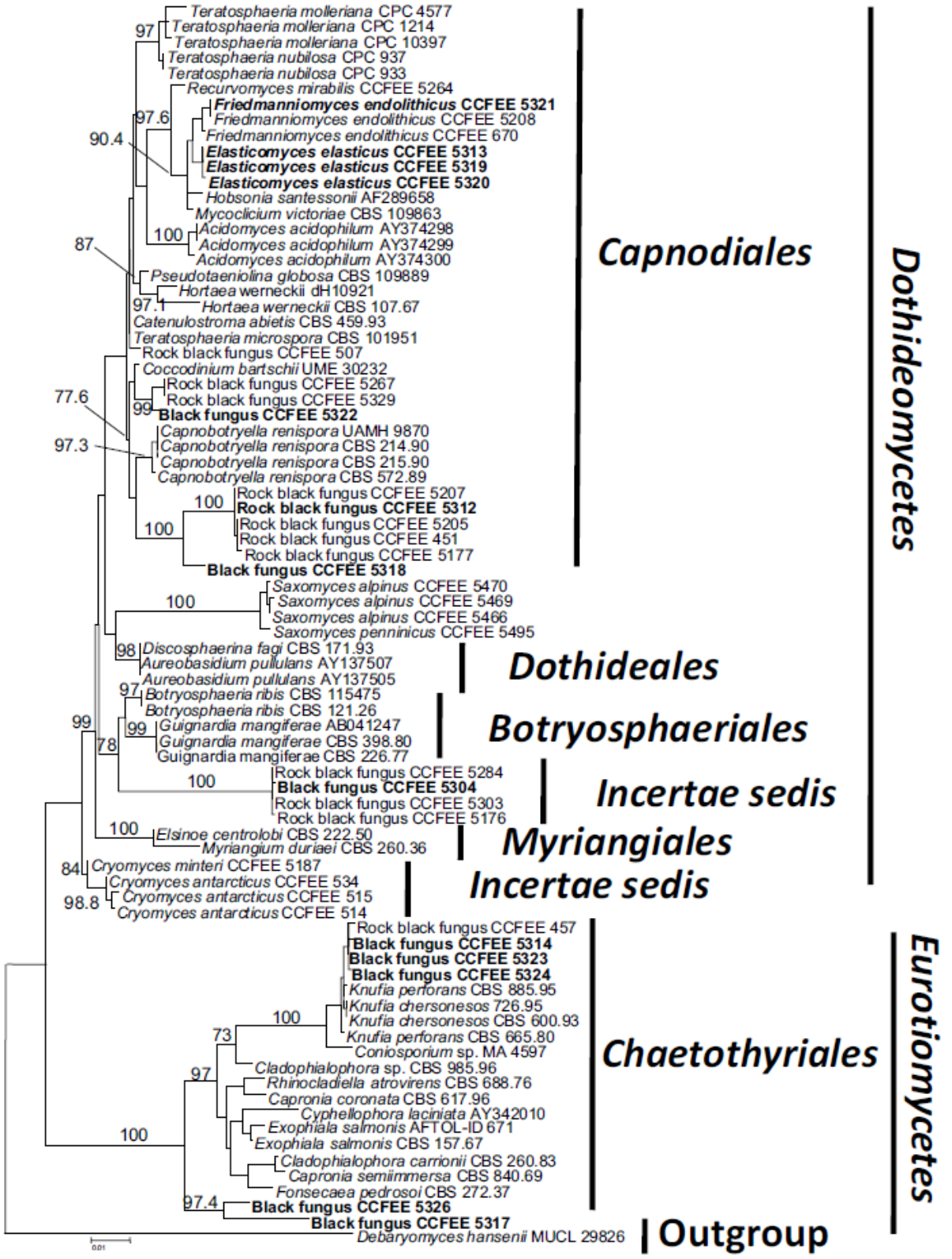

Figure 4: Overview of the phylogenetic placement of *Knufia chersonesos*, *Chaetothyriales*, in the black meristematic fungi. The phylogenetic tree is based on Maximum Likelihood analysis of small subunit ribosomal DNA data. Bootstrap values are a result of 1000 pseudoreplicates. Only branches with a bootstrap values above 70 % are indicated in the graph.[118]

The first reported isolation of *K. chersonesos* was done from marble in Chersonesos, Crimea, Ukraine.[116] However, *K. chersonesos* is widely distributed from Mediterranean basins (Figure 5, 1) to the Arctic regions.[11,119,120] This species has solely been found colonising bare rock surfaces and was therefore the first fungus having its natural ecological niche on stone without exhibiting lichenisation.[119]

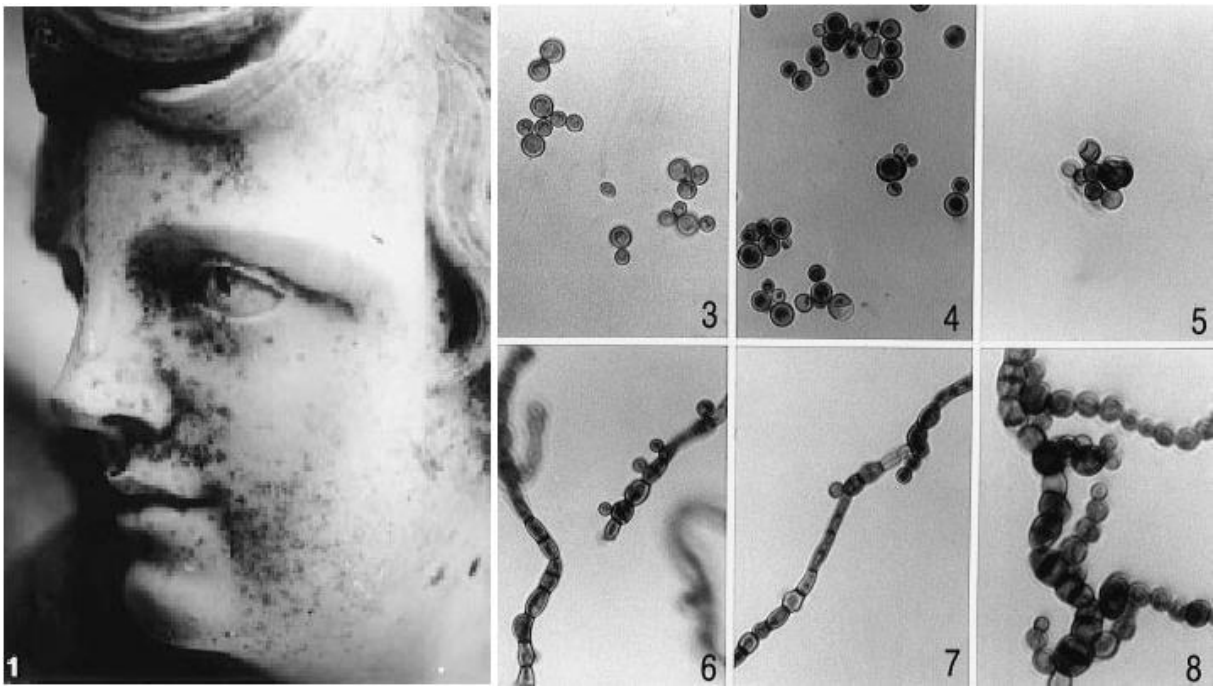

Figure 5: *Knufia chersonesos* (former *S. petricola*). (1) Colonisation of a marble statue exposed to outdoor conditions in Messina, Italy. (3,4) Cells undergoing multilateral budding. (5) Cells subdivided by septa. (6,7) Hyphal formation in 2 month old cultures. (8) Hyphae with meristematic development.[119]

3.4.1 Morphology of *K. chersonesos*

Microscopically, *Knufia chersonesos* shows a conversion in growth mode, from a yeast-like cell growth with multilateral budding cells to meristematic growth (Figure 5, 3-8). Furthermore, the conversion eventually leads to cells undergoing endogenous reproduction or arising after meristematic swelling of the hyphal cells.[119] The thallus is composed of melanised and meristematically dividing clusters of cells, which occasionally branch into short torulose hyphae (Figure 6, D-E). Singular *K. chersonesos* cells are about 5 µm in diameter, but old cells reach up to 15 µm when they are full of melanin granules located at the exterior fungal cell wall (Figure 6, F-I).[117,121,122] Yeast-like growth is mostly observed during the exponential growth phase, however it is not predominant (Figure 6, J).[117]

Macroscopically, the group of black fungi is unusually undifferentiated in terms of morphology (Figure 6, A-D).[1,123] However, morphological observations are, in combination with phylogenetic analyses, a premise for taxonomic classification.[124,125] The colonies of *K. chersonesos* are entirely black, thick, cerebriform, growing vertically rather than along the agar surface, branched in large lobes, and reaching 2.5 to 3.5 cm in diameter after 3 weeks growth (Figure 6, A). The lobes are of irregular shape and length. The surface of the colonies is rough, shiny, and wet (Figure 6, B, C).[117]

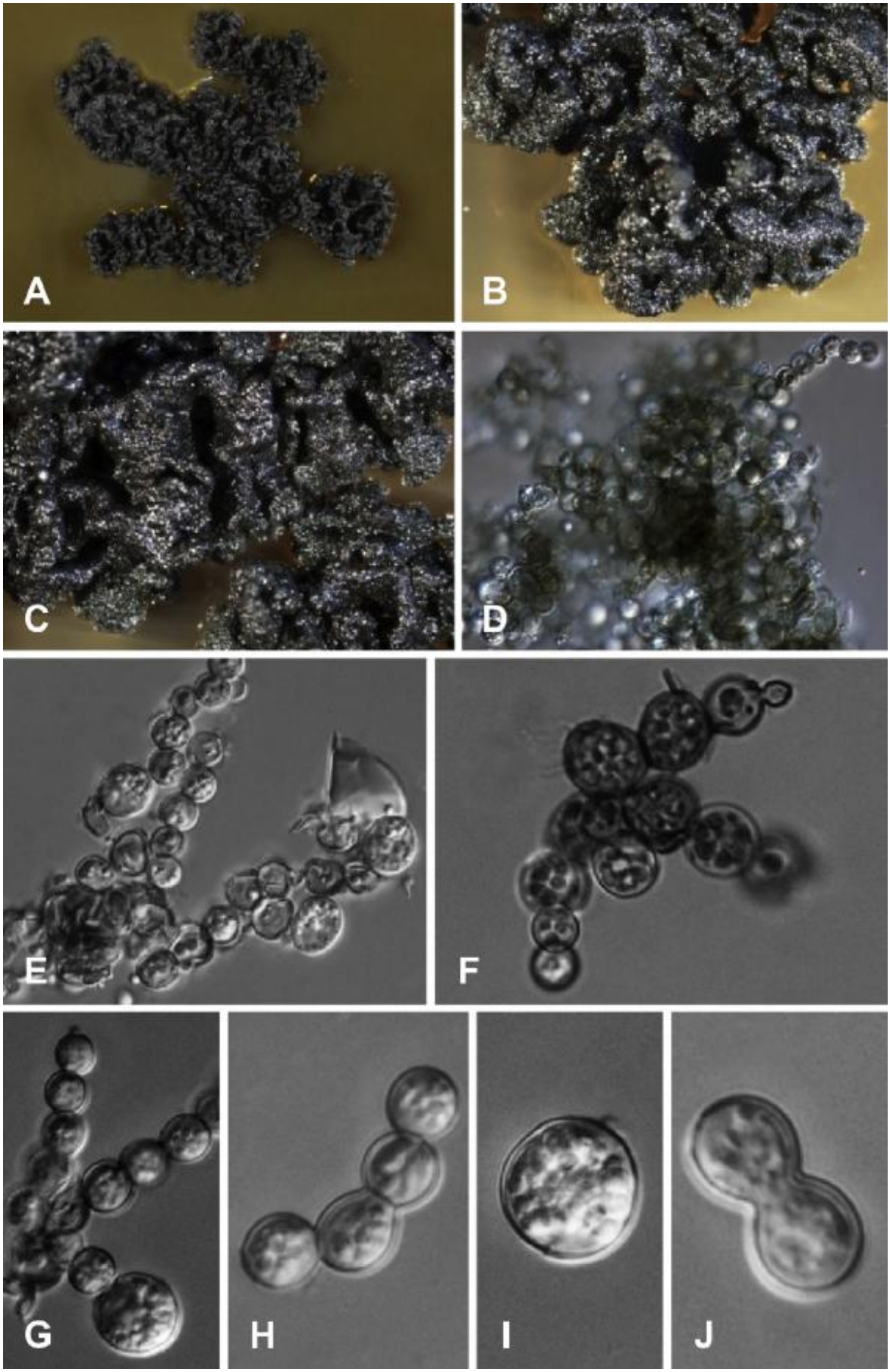

Figure 6: Macroscopic- (A-C) and microscopic morphology (D-J) of *K. chersonesos* (synonym *K. petricola*) growing on malt extract agar (MEA) at 25 °C.[117]

3.4.2 Non-melanised pink mutants of *K. chersonesos*

Pink mutants (Figure 7, right) of *K. chersonesos* (Figure 7, left) have been observed under laboratory condition, upon regular cultivation of the wild type strain, thus making *K. chersonesos* the only species among black fungi known to have a non-melanised spontaneous mutant.[124] As reported by Nai *et al.*[(124)], these mutations are spontaneous, constitutive and result in stable pink phenotypes, which are maintained over cell subculturing.[124] The altered pigmentation of the mutant probably stems from an affected synthesis of melanised cell wall.[124] Hence, the carotenoids abundant in the internal of the fungal cell are no longer overlaid by melanin in the cell wall, which is resulting in this phenotype.[11,124] The wild type phenotype is partially restored for some cases, if the non-melanised mutant strain is exposed to ultraviolet radiation, low temperatures while growth, and chloride anions in a dose dependent manner.[124] Overall, the non-melanised mutant is mostly identical to its parent strain, apart from the obvious difference in pigmentation, regarding colony morphology, growth temperature optimum, nutritional metabolism, ultraviolet radiation stress, and karyotype.[124] The growth rate of *K. chersonesos* wild type and its non-melanised pink mutant is comparable as well.[124] Slight differences are present at the pH growth optimum, resistance towards drugs and tolerance to osmotic stress, with the mutant surprisingly having a higher resilience to osmotic stresses.[124]

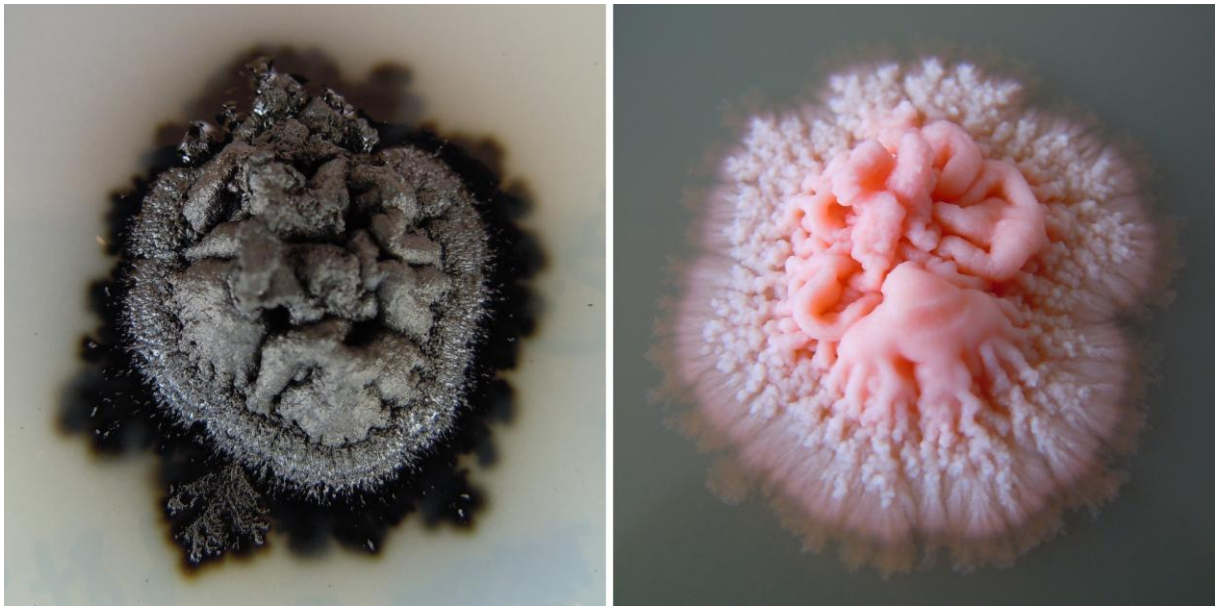

Figure 7: *Knufia chersonesos* wild type (left) and its non-melanised pink mutant (right) growing on 2 % MEA at 20 °C.[126]

3.4.3 Physiology of *K. chersonesos*

K. chersonesos is relatively fast growing compared to other rock-inhabiting black fungi. The generation time of the wild type strain was found to be 13.0 h in malt extract broth (MEB) medium and 17.4 h in A95-specific medium (ASM). The non-melanised mutant strains analysed by Nai *et al.*[124] exhibited generation times of 12.6 h, 13.8 h, and 16.0 h in malt extract broth (MEB) medium, respectively.[117,124] This species exhibits halotolerance, prototrophy, oligotrophy, and has its growth optimum at a pH of 5, determined by phenotype micro array (PM) according to Bochner.[117,124,127] The nutrient metabolism of *K. chersonesos* is used in a rather selective manner. The activity of the used metabolic pathways is concomitant with the utilisation of carbon sources. In a recent study focusing on *K. chersonesos* metabolism, only 33 % of the metabolic pathways were found to be active and supporting growth, which accords with the exhibited oligotrophy.[124] This species is instead less selective with the utilisation of nitrogen- and phosphor sources, where 75 % and 50 % of the pathways were found to be active and to support growth, respectively.[124] Due to its natural niche on sun exposed rock surfaces, *K. chersonesos* has acquired adaptations to recover from complete desiccation, withstand high temperatures, and UV radiation.[11] Ongoing studies additionally suggest that its set of adaptations enables *K. chersonesos* to cope with concentrations of ozone otherwise considered harmful for animal- and plant tissues, as well as with microgravity, among other stresses (Tesei D, unpublished data).[112] The differences in physiology of the non-melanised pink mutant compared to its parental species *K. chersonesos* wild type known so far, are a slightly increased tolerance towards osmotic stress and effectiveness of β-lactame antibiotics and aminoglycosides.[124] Further investigations, however, need to be carried out in order to fully elucidate the physiology of both strains and the impact of melanin and carotenoids on stress tolerance.

3.4.4 *K. chersonesos* as model organism

K. chersonesos is a promising model organism for rock-inhabiting black fungi and their resilience to environmental stresses based on the features comprehensively described in this chapter.[112,124] This non-pathogenic environmental strain, exhibiting high mycosporine content and pigmentation, is relatively fast growing compared to other black fungi and easily handled in the laboratory.[11,121,124,128] Additionally, the clade *K. chersonesos* belongs to, is an early diverging lineage from which the symbiotic lichenisation lifestyle has evolved.[124,125] It

houses many pathogenic ascomycetes including *Exophiala dermatitidis*, which is an intensively studied pathogenic black fungus.[17,39,125,129–131] Furthermore, the spontaneously occurring natural non-melanised mutant, which exhibits nearly perfect similarity to the wild type, is representing an excellent candidate for studying the effect of melanin on growth and stress resistance.[112,124] Recently, the genomes of both the wild type and the non-melanised *K. chersonesos* strains were sequenced, which will aid an improved understanding of the biochemical pathways involved in the cell physiology, as well as in stress resistance even further.[112] It should also enable an insight into the differences determined by the spontaneously occurring mutation, most likely concerning a deficiency in melanin synthesis.[112,124] The model organism *K. chersonesos* might enable researchers to assess a multitude of mechanisms underlying black fungi. Namely, the ubiquitous distribution and colonisation of unfavourable niches, stress resilience, colonisation of material surfaces, symbiotic interactions between co-occurring autotrophic organisms, and the benefit of pigmentation in form of melanisation and mycosporine content.[124]

3.4.5 Use of *K. chersonesos* for polymer degradation

Some fungal species are known for their ability to degrade aromatic compounds. The metabolic pathways for the degradation of such materials stem from the bioremediation of lignin. This bioremediation is carried out by depolymerisation and usage of lignin as nutrient source, which is best understood for white rot basidiomycetes.[117,132] The utilisation of hydrocarbons as sole source of nutrients has been reported for members of the black fungi group. Namely, *Exophiala* spp. and *Cladophialophora* spp. were found to grow solely on hydrocarbons.[124,133–135] Despite, assimilation of hydrocarbons by black fungi seems to be predominantly restricted to the *Herpotrichiellaceae* family[135], both of these fungal genera belong to, *Knufia* spp. have been isolated from hydrocarbon-polluted environments.[55] Hence, indicating a resistance towards or biodegradation of hydrocarbon compounds by *Knufia* spp., which is an appealing trait for bioremediation purposes.[55,124] Growth on a variety of hydrocarbon compounds has been reported, with especially monoaromatic substances, such as p-hydroxybenzoic acid and p-/m-hydroxyphenylactetic acid in *K. chersonesos*.[117] Therefore this species is not only able to tolerate, but also utilise carbon source otherwise recalcitrant to microbial degradation.[117] The hyphal growth is slowed or halted by aromatic hydrocarbons in species that, unlike *K. chersonesos*, cannot utilise these

compounds as an alternative carbon source, as also shown in other ascomycetes not belonging to the black fungi group.[117,136]

The metabolic adaptation to utilise aromatic hydrocarbons as carbon source would enable *K. chersonesos* to act as bioremediant for aromatic polyesters, through enzyme-mediated degradation of polyesters. It was then shown, that *K. chersonesos* is indeed able to degrade aliphatic-aromatic copolyesters (Tesei D, Quartinello F, Ribitsch D, Gübitz G, and Sterflinger K, unpublished data). This makes this species and the extracellular enzymes it uses for degradation, interesting candidates for biodegradation of synthetic polymers and therefore for biotechnological applications.[112]

3.5 Analysis techniques

Different analysis techniques are applied to analyse proteins secreted by *Knufia chersonesos* into the surrounding media and to monitor the progression of poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) degradation.

3.5.1 Hydrolase activity assay

The hydrolase activity assay is performed to characterise the activity of hydrolase enzymes, such as esterases, cutinases, and lipases.[72,137–140] In the present work it is used to assess the overall hydrolase activity of the various enzymes secreted into the medium by the cultured fungi.

The procedure commonly used to investigate the activity of hydrolases relies on p-nitrophenyl esters. For the ester compound aliphatic esters of various lengths can be utilised.[140,141] Short aliphatic esters, such as acetate and butyrate, are soluble in aqueous buffers and used to investigate esterases. Longer aliphatic esters, such as laurate, palmitate, and oleate, enable the investigation of lipases, but require emulsifying agents to allow solubilisation. The hydrolase catalysed release of p-nitrophenol from the ester (Figure 8) can be measured spectroscopically, due to an increase of absorbance at around 410 nm by p-nitrophenol. Through the time dependent increase of absorbance, enzyme activity can be determined.[140]

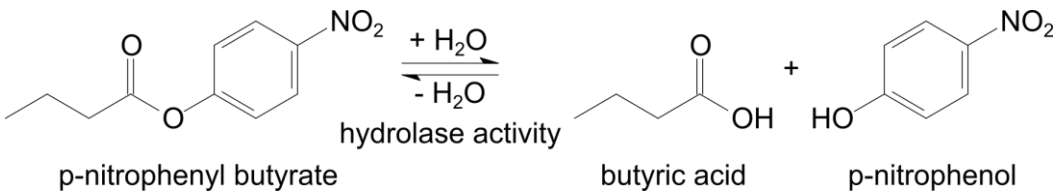

Figure 8: Hydrolysis reaction of p-nitrophenyl butyrate to butyric acid and p-nitrophenol catalysed by the hydrolase activity of the tested sample.

This method of hydrolase activity characterisation is advantageous, as it just requires an ultraviolet-visible spectrophotometer, the reagent is commercially available and inexpensive, the measurements can be scaled up to a 96-well format, and kinetic measurements can be conducted.[140,142]

There are general limitations of this method, however. Non-specific esterase activity, mediated by non-specific esterases, non-enzymatic proteins, and proteases can hydrolyse p-nitrophenyl esters as well, which applies especially for short aliphatic esters. Hence, if the esterase activity of a particular enzyme is sought to be determined, the purified enzyme, devoid of these contaminants, is suited best for characterisation with this method.[140,143–146] Furthermore, measurements cannot be conducted in acidic pH, as this substantially alters the absorbance of p-nitrophenol, which may not be favourable for certain lipases.[140,147] These limitations, however, do not affect the hydrolase activity assay in the present thesis, as the hydrolase activity of the secretome as a whole is sought to be determined and the neutral pH within the assay is maintained by a pH 7 buffer solution.[139]

The assay is conducted as described by Quartinello *et al.*, which was adopted from Biundo *et al.* and Lehner *et al.*.[72,138,139]

3.5.2 High-performance liquid chromatography (HPLC)

The high-performance liquid chromatography (HPLC) is a separation method utilising a liquid mobile phase and, depending on the technique of separation, a solid or liquid stationary phase. Various techniques can be used to separate mixtures of substances chromatographically. Adsorption chromatography utilises a liquid-solid concept and is best used for the separation of stereoisomeric molecules or non-polar aliphatic hydrocarbons and alcohols. Partition chromatography uses a liquid-liquid concept and therefore includes normal- and reversed-phase chromatography separation techniques. With the latter being used in this thesis and the most used chromatographical method overall. Furthermore,

chromatography based on the principal of ion exchange-, size exclusion-, or affinity separation is utilised in analysis.[148]

The main difference between high-performance liquid chromatography and conventional liquid chromatography is the reduced size of column particles or diameter of wall-coated open-tubular columns. This decreases the height equivalent of a theoretical plate and therefore increases the theoretical plate number, which proportionally enhances the efficiency of the separation column. However, to maintain practicable flow rates of minimum 1 ml/min for particle sizes between 3 to 10 µm, used in HPLC systems, pressures up to 15 MPa have to be applied. Naturally, such pressures are not applicable in conventional liquid chromatography systems. As a consequence, the instrumental setup of a HPLC system is adapted to these requirements. The HPLC system is consisting of a reservoir for mobile phase solvents, system of pumps, sample injection system, separation column, detector, and an optional fraction collector (Figure 9).[148]

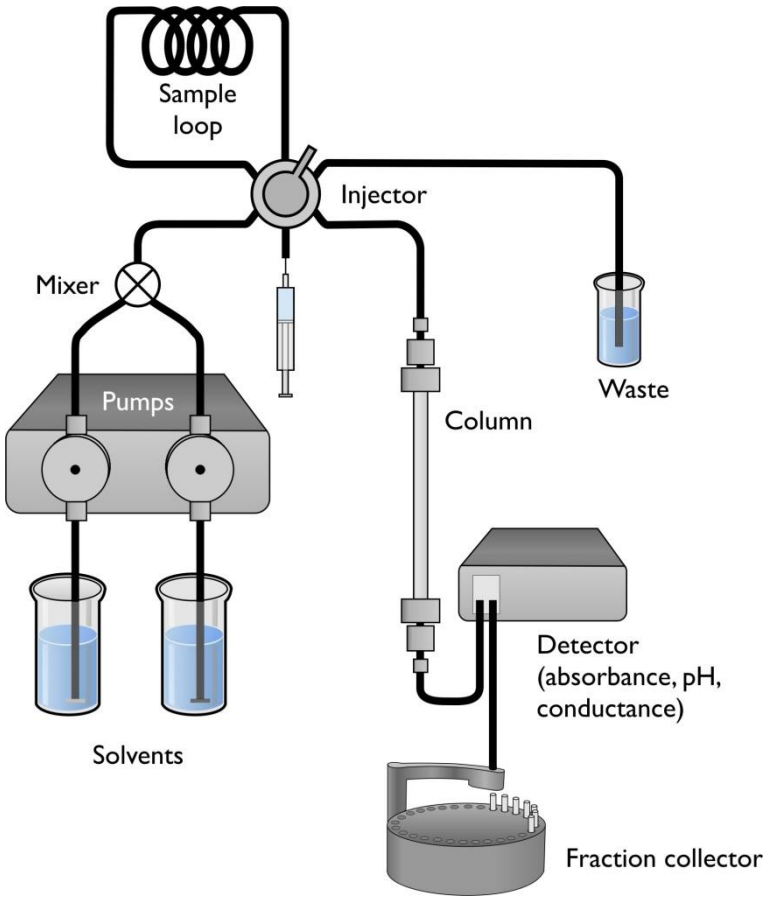

Figure 9: Instrumental setup of a high-performance liquid chromatography (HPLC) system.[149]

To withstand the high pressure applied on the HPLC system, separation columns are out of polished high-grade steel or thick-walled glass with a diameter ranging from 1 to 4.5 mm. Columns filled with 3 to 10 µm particles reach theoretical plate numbers of 50 000 to 100 000. In partition chromatography, liquids immobilised or chemically bound onto silica gel or aluminium oxide particles are used as stationary phase. Reversed-phase chromatography utilises a combination of a relatively non-polar stationary phase and polar mobile phase, which is best used for the separation of substances with high solubility in non-polar solvents. Aside from the stationary phase, the composition of the mobile phase can be used to optimise separation efficiency. In the isocratic method, the composition of the mobile phase is held constant over the course of the HPLC run. Better separation efficiency is usually achieved with the gradient elution method. In this method the composition of the mobile phase consisting of two different solvents with different physical properties, like polarity, is altered over the course of the HPLC run, in order to improve elution of substances with longer retention times. The detection of the eluted substances is realised in two possible methods. The first method is to measure physical properties of the mobile phase solvent, which are altered by the presence of elute, like refraction index and conductivity. The second method makes use of the properties of the eluted substance, such as absorbance, fluorescence, and diffusion current at an electrode. The ultraviolet-absorbance detector is the detection system most in use for chromatography.[148]

3.5.2.1 Applications of partition HPLC

The applications of HPLC are numerous and widespread. HPLC with reversed-phase chromatography is regularly the first technique considered when approaching a new analysis problem. The C_{18}-reversed stationary phase is standardised and the retention behaviour and selectivity is altered by the choice of the mobile phase composition, which can be varied further by usage of isocratic or gradient elution mode. The reversed-phase HPLC can be applied in all fields, in which not highly polar molecules are analysed. That includes applications in pharmaceutics, biochemistry, forensics, medicine, chemical industry, food industry, and environmental analyses of pollutants.[148]

3.5.2.2 Observation of PBAT degradation with partition HPLC

HPLC-related methods have already been established to monitor polyesters degrading into water soluble mono- and oligomers by enzymatic means.[88,139,150]

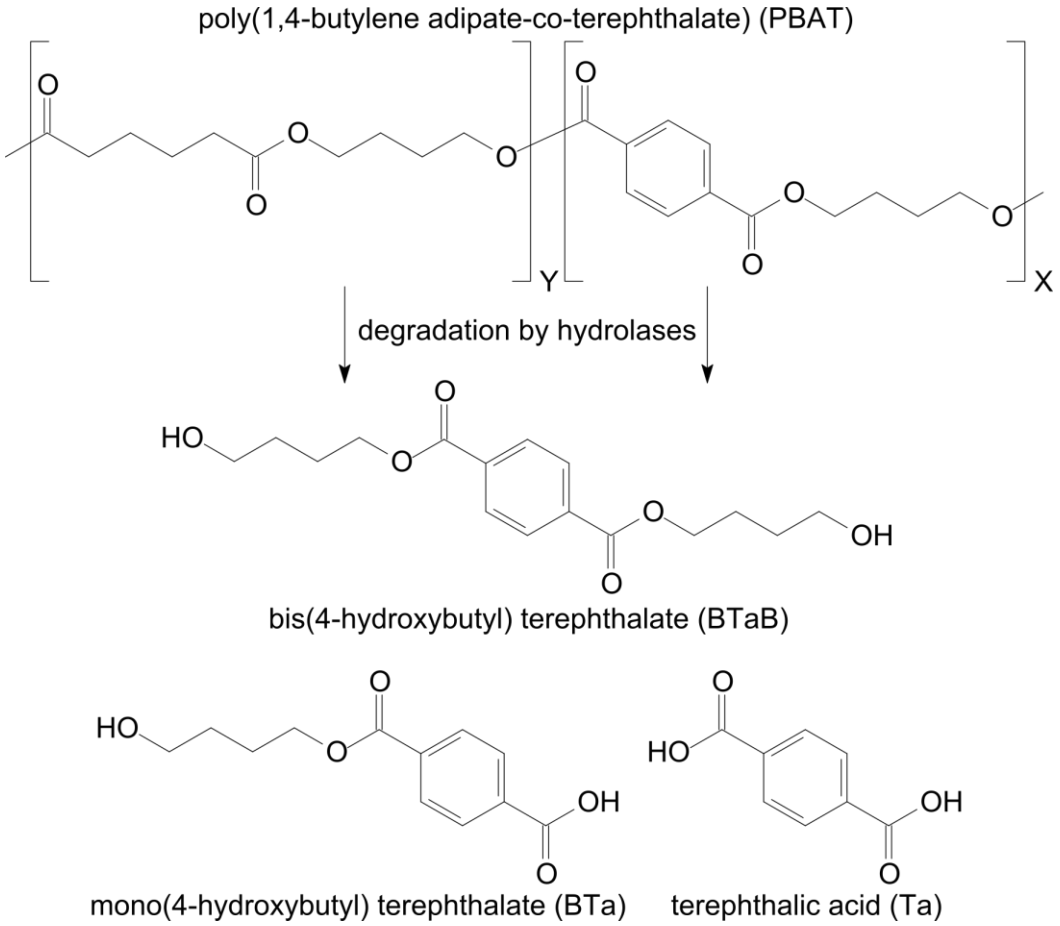

Figure 10: Monomeric hydrolase degradation products of poly(1,4-butylene adipate-*co*-terephthalate) (PBAT), which include a terephthalic acid subunit.

All possible monomers (Figure 10) of the degraded polymer poly(1,4-butylene adipate-*co*-terephthalate) (PBAT), which include a terephthalic acid [terephthalic acid (Ta), mono(4-hydroxybutyl) terephthalate (BTa), and bis(4-hydroxybutyl) terephthalate (BTaB)], are detectable with ultraviolet-visible spectrometry at 241 nm, as shown in Figure 12.
The HPLC analysis is conducted as described by Quartinello *et al.*.[139]

3.5.3 Proteomics

Investigations of the secretome of *K. chersonesos* are undertaken by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed up in future with shotgun-proteomics analysis using mass spectrometry.

3.5.3.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The separation of protein mixtures, like the secretome of *K. chersonesos*, can be accomplished with gel electrophoresis (GE). Polyacrylamide (PA), casted between two glass plates, is used to restrain proteins depending on their sequence length, when an electrical field is applied for segregation. However, the relation between size, charge and molecular mass of a protein is mainly dependent on the prevailing pH and temperature. Hence, separation of proteins in an electric field is not readily applicable for native proteins. To circumvent this issue, the anionic detergence sodium dodecyl sulfate (SDS) is utilised to overcompensate for the inherent charges of the native protein. When the native proteins are linearised by denaturation, sodium dodecyl sulfate undergoes attachment to the protein with a constant charge to molecular mass ratio, which enables the separation in an electric field based on the protein size. This method is advantageous, because the negatively charged proteins are soluble and do not aggregate, highly charged proteins exhibit high migration velocities in an electric field, and all the proteins are uniformly charged and migrate into the same direction in the electric field. The separated proteins can be visualised with pre- (e.g. SYPRO Ruby staining) or post-staining methods (e.g. silver staining). For the present work, silver staining was used as the method of choice. In the silver staining procedure, the silver ions of an applied silver nitrate solution are reduced to metallic silver by proteins and therefore creating visible bands in the close proximity of protein accumulation.[148]

SDS-PAGE is used in the present thesis to get insight in the protein banding pattern of the *K. chersonesos* secretome, depending on growth in different media and in presence or absence of different PBAT polymers.

3.5.3.2 Mass spectrometry

In mass spectrometry (MS) analysed substrates are vaporised, ionised with electron or ion radiation, accelerated and separated in an electric or magnetic field based on the molecule ratio of mass and charge (m/z). There are numerous applications of mass spectrometry in different research fields, which include the chemical structure determination of organic molecules, quantitative and qualitative analyses of organic and inorganic compounds in complex substance mixtures, determination of element isotope ratios, and investigations of structure and composition of material surfaces. The technical setup of a mass spectrometer consists of an injection system, ion source, a system to accelerate the analysed ions,

separation- or analysis system, and a detector coupled with an output software (Figure 11).[148]

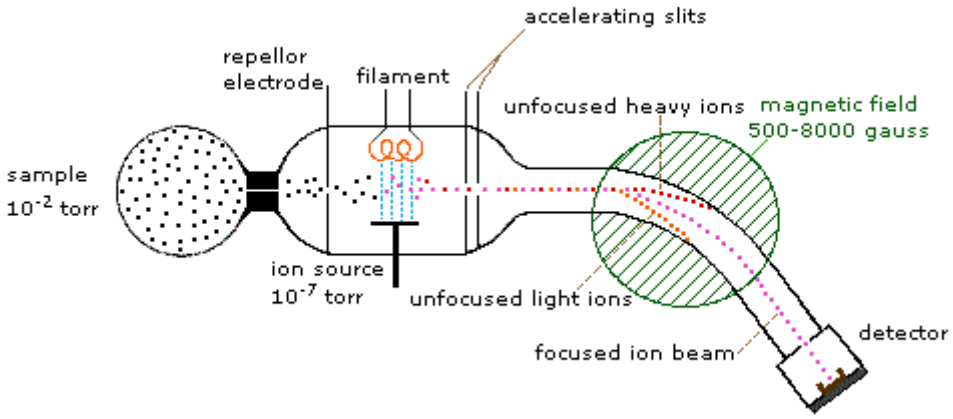

Figure 11: Instrumental setup of a mass spectrometry system.[151]

A multitude of possible technical solutions are available for the ion source- and analysis system.[148] Electron spray ionisation (ESI) and ion trap mass analysis is used in the present thesis.

Electro spray ionisation (ESI) is, beside matrix-assisted laser desorption ionisation (MALDI), a method conducting ionisation in a way that allows for the analysis of proteins with mass spectrometry. Conventional ionising systems are unable to evaporate and ionise proteins for mass spectrometric analyses. Electro spray ionisation is mostly used as an online ionisation method coupled with liquid chromatography. To accomplish evaporation, the mobile phase of the pre-separation with liquid chromatography including the solved analytes is nebulised at the electro spray interface.[148] The droplets are charged by a strong electric field and dried by heated nebulising gases (e.g. nitrogen) until they shrink, undergo fission, burst, and evaporate.[148,152] Organic modifiers are added to the mobile phase, which are hydrophobic yet water miscible additives, to improve the mass spectrometry signal response. Acetonitrile and methanol are used as organic modifiers for ESI-MS.[153] Additionally, high concentrations of acetonitrile vapour at the electrospray ionisation site suppress background ions in mass spectrometry analyses and enhance the detection performance for peptides.[154] The ionisation of the analytes is achieved by addition of organic compounds, such as trifluoroacetic acid (TFA), morpholine, acetic acid, and formic acid, and salt additives, such as ammonium acetate, ammonium formate, ammonium bicarbonate, and triethyl acetate, to the mobile phase in millimolar concentrations prior to evaporation.

The organic compounds and salt additives charge the analyte by protonation or adduction of organic ions upon evaporation of the solvent.[148,153,155–157] In positive ionisation mode of ESI the amino acid side chains of arginine, histidine, and lysine are charged. These gentle methods of ionisation are possible under atmospheric pressure and highly charge large molecules with many functional groups, such as proteins, which allows the analysis using mass spectrometers with limited mass analysis ranges.[148]

The ion trap analysis method utilises ring electrodes powered by a combination of alternating- and direct currents to generate a quadrupolar electric field and trap ions in orbital trajectories in the mass analyser. Through continuous shifts in force of the electric field, emitted from the ring electrode, ions of a certain mass are subsequently ejected from the ion trap and led in the detector. In an ion trap additional fragmentations of the analyte can occur via collisions, due to the prolonged time (µs to ms) ions are held in the ion trap.[148]

3.5.3.3 Shotgun-proteomics

Shotgun or bottom-up proteomics cover techniques aiming for identification and quantification of all proteins in a certain proteome. This effort encompasses protein expression, cellular localisation, protein interactions, post-translational modifications, and protein turnover in a time dependent manner. The term 'bottom-up' proteomics refers to the analysis of proteins based on partial peptides, released from the whole protein by a sort of proteolysis.[158] If this procedure is performed for a mixture of proteins, it is referred to as 'shotgun'-proteomics.[158–161] In a shotgun-proteomics analysis, the mixture of proteins is subjected proteolytic digestion into peptides, fractioned in liquid chromatography, and analysed with a coupled mass spectrometry system. The identification of the analysed proteins is then achieved by comparison of the obtained peptide mass spectra to theoretical mass spectra from databases. These theoretical mass spectra are calculated for a selected amount of proteins, which are fragmented at the same protease cleavage sites (motifs), the used proteases (i.e. Trypsin, LysC) are specific for. The identified proteins are related to each other by comparison of their peptide sequences. Protein groups can be established based on peptide sequences being unique for a particular protein or shared by group of proteins.[158]

The bottom-up proteomics approach circumvents most of the limitations the top-down proteomics approach – which relies on the analysis of whole proteins – faces regarding protein fractionation, protein ionisation, and protein fragmentation in gas phase, by relying on the analysis of peptides. Shotgun-proteomics is used in many different fields of biology

encompassing applications for proteome profiling, protein quantification, protein modifications, and protein-protein interactions.[158]

4 Aim of the study

Two main objectives are pursued in the frame of this master's thesis work.

- The biological degradability of different poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) compositions by *K. chersonesos* is studied.
- The secretome of *K. chersonesos* upon degradation of poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) is investigated for degrading enzymes.

Namely, the copolymer compositions 50:50 PBAT, 60:40 PBAT, and 90:10 PBAT, with the numbers describing the adipate : terephthalate ratio in the copolyester, are studied for biodegradability in milled form in liquid culture of *K. chersonesos*. With the *K. chersonesos*, a model organism for black yeasts, which has a naturally occurring non-melanised mutant, the influence of a functional melanin production and therefore melanised cell wall on the degradation can be elucidated. An additional questionable aspect of the degradation procedure is, if the polyester degrading enzymes of *K. chersonesos* are secreted constitutively or have to be induced by the presence of PBAT.

The degradation of PBAT is ought to be experimentally verified by the observation of its monomeric degradation products through HPLC analysis and the enzymatic hydrolase activity of the secretome investigated with an assay as indicator for the ability to degrade polyesters. Furthermore, the secretome of *K. chersonesos* is ought to be examined for the individual proteins it is composed of with a SDS-PAGE analysis.

5 Materials, methods, and procedures

5.1 Cultivation of fungal strains

5.1.1 Strains

Two fungal strains were used.

- *Knufia chersonesos* wild type, MA 5789
- *Knufia chersonesos* non-melanised natural mutant, MA 5790

The environmental strain *Knufia chersonesos* wild type, MA 5789 had previously been isolated from red sandstone in Ny London, Svalbard, Norway. The strain *Knufia chersonesos* non-melanised mutant, MA 5790 emerged spontaneously under laboratory conditions.[112] Both strains were obtained from the culture collection of the Austrian Center of Biotechnological Resources and Applied Mycology (ACBR) of the University of Natural Resources and Life Sciences, Vienna, Austria.

5.1.2 Generation of fungal biomass

Knufia chersonesos wild type and mutant were inoculated on 2 % malt extract agar (MEA, Table 1) plates in triplicates by direct transfer of biomass or by spreading 100 µl of the fungal biomass suspended in 0.9 % NaCl solution to generate biomass for following experiments. The cultivation took place at 21 °C, which is the strains temperature optimum when growing on 2 % MEA (Tesei D, unpublished data), in 94 mm Petri dishes sealed with Parafilm.[124] All experiments, described in this thesis, were carried out using fungal cultures with comparable growth periods since inoculation and therefore in the same fungal growth phase, which makes the experiments comparable.

Table 1: Composition of 2 % malt extract agar (MEA).

Substance	β / [g/l]
malt extract	20.0
D(+)-glucose * H_2O	20.0
agar	20.0
bacteriological peptone	1.0

5.1.3 Washing of PBAT

The washing solutions (Table 2) were filtered with a 0.2 µm PES sterile filter (VWR, Radnor, Pennsylvania, United States). The milled PBAT was washed for 15 minutes with Triton X-100 washing solution, 15 minutes with Na_2CO_3 washing solution, and 3 times 15 minutes with sterile dH_2O under constant stirring. After each washing step, the PBAT particles in dispersion were left to settle down before the solution was discarded. The PBAT was thereafter dried in 55mm Petri dishes in the laminar flow hood, sealed with Parafilm once dry and stored at room temperature (21 °C). This procedure was conducted for every milled PBAT that was used for liquid culture experiments.

Table 2: Washing solutions for milled PBAT. Each solution was filled up to final volume with dH_2O.

Washing solution	β(substance) / [g/l]
Triton X-100	5.0
Na_2CO_3	10.6

5.1.4 PBAT degradation by fungal cultures

In order to assess the best experimental conditions (e.g. with special regard to the concentration of the secreted proteins and the abundance of melanin in the supernatant), pre-experiments were carried out using the media compositions as shown in Table 3, on the basis of which the media ME 2% (referred to as optimal media) and ME 0.2 % -Glu (referred to as minimal media) were chosen as the most favourable ones.

Table 3: Composition of malt extract medium variations.

	β / [g/l]		
Substance	**ME 2 %**	**ME 2 % -Glu**	**ME 0.2 % -Glu**
malt extract	20.0	20.0	2.0
D(+)-Glucose	20.0	0.0	0.0
bacteriological peptone	1.0	1.0	0.1

The blends 50:50 PBAT (EcoFlex Blend C1200, 68818836WO, 500 µm maximum particle size, BASF, Ludwigshafen am Rhein, Germany), 60:40 PBAT, 90:10 PBAT, with the ratios describing the aliphatic : aromatic monomer composition of the polyester, were investigated for degradation by *K. chersonesos*. Thereby the biological degradability of different PBAT compositions by *K. chersonesos* can be elucidated with regard to influence of the fungal cell wall melanisation and nutrient availability. With the presence or absence of PBAT in the

experiments a possible PBAT mediated induction of hydrolase enzyme secretion in *K. chersonesos* can be determined.

A total of 80 mg milled, washed, and dried PBAT were weighed into 0.5 ml micro tubes and 50 mg biomass of *K. chersonesos* wild type and/or mutant were weighed into 1.5 ml micro tubes. 1 ml of the according growth medium was pipetted into each tube containing the fungal biomass and the biomass was broken into small clumps through pipetting repeatedly with cut off tips.

The experiments were conducted in 3 different conditions, namely negative control, control, and treatment cultures. The compositions for the experimental conditions are shown in Table 4. All experiments were conducted in 6-well-plates with lid (CytoOne, Star Lab, Hamburg Germany). 7 ml of the growth medium were added into each well of the 6-well-plate. For the treatment cultures each well was inoculated with the 50 mg biomass suspended in 1 ml of the according growth medium and 80 mg milled PBAT was added. In the control cultures no milled PBAT was added into the wells. In the negative control cultures the wells were not inoculated with fungal biomass. All the experiments were performed using 3 different wells as biological replicates. The cultures were grown on an orbital shaker at 100 rpm, for 15 days at room temperature (i.e. 21°C).

Table 4: Composition of the negative control-, control-, and treatment experimental conditions.

Components	Negative control cultures	Control cultures	Treatment cultures
PBAT, milled / [mg]	80	0	80
fungal biomass / [mg]	0	50	50
growth medium / [ml]	8	8	8

At the end of the experiment, each culture supernatant was collected in a 15 ml tube and centrifuged at 4700 rpm with the 75003607 rotor in Hereaus Megafuge 40R (Thermo Fisher Scientific, Waltham, Massachusetts, United States) for 10 minutes to pellet remaining biomass and polymer. The culture supernatant was split into aliquots of 100 µl for hydrolase activity assay, 500 µl for PBAT degradation analysis with HPLC, and 1000 µl for protein quantification and SDS-PAGE.

5.2 Protein concentration

Prior to SDS-PAGE, the culture supernatant devoid of cell debris and polymer residues was concentrated using the Vivaspin 500, PES tubes with a 5 kDa cut-off value (Sartorius,

Göttingen, Germany). Preliminary to the supernatant concentration of the control and treatment experimental conditions investigating the degradation of 50:50 PBAT, 40 µl protease inhibitor stock solution (1 tablet of cOmplete, Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, Missouri, United States) dissolved in 2 ml H_2O) were added per 1 ml supernatant. After an initial membrane washing step with 50µl mQ H_2O at 15000 rcf for 5 minutes, 500 µl supernatant were concentrated to a volume of 50 µl by centrifugation with the F45-24-11 rotor in Centrifuge 5415R (Eppendorf, Hamburg, Germany) at 15000 rcf for 40 – 130 minutes. Longer centrifugation times were required for samples with higher protein- or melanin content. The concentrated samples were stored at -20 °C.

5.3 Protein quantification

Protein quantification was carried out with the Qubit Protein Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, United States) for the measurement interval of 0.0125 – 5 µg/µl. The used working solution consisted of 99.5 % Qubit protein buffer and 0.5 % Qubit protein reagent and was kept protected from light. For the calibration, 10 µl Qubit protein standard - with concentrations equal to 0 ng/µl, 200 ng/µl, and 400 ng/µl protein - were added to 190 µl working solution. For the sample measurement 1 – 20 µl of the sample solution were added to 199 – 180 µl Working Solution, in order to stay within the linear interval of the Qubit Protein Assay Kit. If the high melanin content of a sample, stemming from *K. chersonesos* wild type cultures, hampered the determination of the protein concentration through interference, the sample was diluted 1/5 or 1/10 with the according medium. The samples and the standards were prepared in Qubit assay tubes, vortexed, incubated at room temperature (21 °C) for 15 minutes and measured with the Qubit Fluorometer 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) in 3 technical replicates.

The protein concentration in the concentrated and the culture supernatant was calculated as shown in Formula 1 and Formula 2.

Formula 1: Calculation of protein concentration in the concentrated supernatant.

$$\beta(protein)_{conc.} = f_{dilution} * \bar{\beta}(protein)_{techn.rep.}$$

$\beta(protein)_{conc.}$ protein concentration in the concentrated supernatant in [µg/µl]

$f_{dilution}$ dilution factor = 1, 5, or 10 depending on sample dilution

$\bar{\beta}(protein)_{techn.rep.}$ mean protein concentration of the measured technical replicates in [µg/µl]

Formula 2: Calculation of protein concentration in the culture supernatant.

$$\beta(protein) = f_{conc.} * \beta(protein)_{conc.}$$

$\beta(protein)$ protein concentration in the culture supernatant in [µg/µl]

$f_{conc.}$ concentration factor = 10

$\beta(protein)_{conc.}$ protein concentration in the concentrated supernatant in [µg/µl]

5.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

5.4.1 Preparation of the gel

The stacking- and resolving gel solutions were prepared according to Table 5. After the addition of tetramethyl ethylene diamine (TEMED) and ammonium persulfate (APS), the resolving gel solution was pipetted into the gel casting frame (Hoefer, Holliston, Massachusetts, United States) and left to polymerise for 45 minutes with a layer of water-saturated isobutanol on top. Following polymerization, the gel surface was washed with 1x sodium dodecyl sulfate (SDS)-running buffer (Table 6) to remove traces of isobutanol. The stacking gel solution - added with TEMED and APS - was thereafter pipetted on top of the resolving gel and left to polymerise for 45 minutes with a comb on top to seal the casting frame and to create wells for the loading of the samples. After the completion of polymerisation the comb was removed and the gel was placed in the electrophoresis chamber, subsequently filled up with 1x SDS-running buffer.

Table 5: Components of the stacking- and resolving gel solutions.

Components	Stacking gel / [ml]	Resolving gel / [ml]
mQ H_2O	5.7	8.2
acrylamide, 40 %	1.7	6.6
tris-buffer, 0.5 M, pH 6.8	2.5	-
tris-buffer, 1.5 M, pH 8.8	-	5
SDS, 10 %	0.1	0.2
TEMED	0.01	0.01
APS, 10 %	0.05	0.1

Table 6: Components of the 1x SDS-running buffer solved in mQ H_2O.

Components	β / [g/l]
tris(hydroxymethyl)aminomethane (tris)	2.9
gylcine	14.4
SDS	1

5.4.2 Sample preparation

A definite sample volume, with a protein content of 1 - 5 µg, which was kept constant for a whole gel, was diluted with mQ H_2O to 7.5 µl and added to 7.5 µl of 2x SDS-sample buffer (Table 7) to 15 µl finale volume. The samples were denaturated in a Thermomixer (Eppendorf, Hamburg, Germany) for 2 minutes at 85 °C and 600 rpm and subsequently loaded into the gel wells. 2 µl of Page Ruler Plus Prestained protein ladder, 10 to 250 kDa, (referred to as stained marker; Thermo Fisher Scientific, Waltham, Massachusetts, United States) or Page Ruler Unstained Protein Ladder (referred to as prestained marker; Thermo Fisher Scientific, Waltham, Massachusetts, United States) were loaded as reference.

Table 7: Composition of 2x SDS-sample buffer.

Components	V / [ml]
tris-buffer, 0.5 M, pH 6.8	10
glycerol	8
SDS, 10 %	16
bromophenol-blue, 0.1 %	2
mQ H_2O	4

5.4.3 Electrophoresis conditions

The gels were run in vertical configuration at 150 V and 35 mA using the Invitrogen PowerEase500 power supply (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The run was manually ended once the front line reached the bottom of the gel.

5.4.4 Gel staining

The protein bands were visualised with the silver staining method. Gels used to analyse the experiment investigating the 50:50 PBAT degradation were additionally stained with the SYPRO reagent prior to silver staining in order to circumvent melanin interference with the silver staining method for samples with the wild type strain.

5.4.4.1 Silver staining

All steps were conducted on a rotary shaker and with the staining solutions shown in Table 8. The gel was stored in fixing solution overnight at 4 °C and thereafter placed in incubation solution for 30 minutes. The gel was then washed 3 times 5 minutes each with mQ H_2O, subsequently incubated in pre-cooled Silver solution at 4 °C for 20 minutes and afterwards briefly rinsed with mQ H_2O. The bands of the samples and the protein marker made were visualised by gel incubation in the development solution. Subsequently to stopping the development of the gel with stop solution by incubation for 10 minutes, the gel image was acquired using the Epson Perfection V800 Photo scanner (Epson, Suwa, Nagano, Japan).

Table 8: Silver staining solutions. The solutions were solved in or filled up with mQ H_2O to final volume. The solutions are stored at room temperature with the exception of silver- and development solution, which are prepared freshly for each staining process.

Fixing solution	
ethanol, 96 %	400 ml/l
acetic acid, 96 %	100 ml/l

Silver solution	
$AgNO_3$	1 g/l
formaldehyde, 37 %	100 µl/l

Incubation solution	
ethanol, 96 %	300 ml/l
Na-acetate	68 g/l
$Na_2S_2O_3$ * 5 H_2O	2 g/l
glutaraldehyde, 50 %	2.5 ml/l

Development solution	
Na_2CO_3	25 g/l
formaldehyde, 37 %	100 µl/l

Stop solution	
EDTA	14.6 g/l

5.4.4.2 SYPRO staining

To conduct protein band staining with the SYPRO fluorescent dye, the gel was washed 3 times 10 minutes each with mQ H_2O and incubated for 180 minutes at room temperature (i.e. 21 °C) with 50 ml SYPRO Ruby Protein Gel Stain (Thermo Fisher Scientific, Waltham, Massachusetts, United States), while being protected from light and gently moved on the orbital shaker. Subsequently, the gel was rinsed with SYPRO washing solution (Table 9) and washed for 30 minutes under the same conditions as the incubation, according to manufacturer recommendations. Thereafter, the gel was scanned in fluorescence scan mode for SYPRO Ruby with an excitation wavelength of 410 nm and an emission wavelength of 610 nm with Typhoon FLA 9500 (GE Healthcare, Chicago, Illinois, United States).

Table 9: Composition SYPRO washing solution solved in mQ H_2O.

Components	
methanol	100 ml/l
acetic acid	70 ml/l

5.5 PBAT degradation analysis

The culture supernatants were prepared for analysis with HPLC as following. A total of 500 µl of the supernatant was admixed with 500 µl cold methanol (-20 °C) and acidified with 1 M HCl to pH 3.5. The precipitated proteins, cellular debris and interfering substances (e.g. melanin) were pelleted via centrifugation with the FA-45-48-11 rotor in Centrifuge 5427 R (Eppendorf, Hamburg, Germany) for 15 minutes at 12700 rpm and 4°C. 400 µl of the supernatant were passed through a 0.45 µm sterile filter and transferred into HPLC vials, which were sealed with HPLC membrane caps. The samples were analysed by a LC-MS system consisting of a Dionex Ultimate 3000 Pump generating a flow rate of 0.4 ml/min, coupled with a X-Terra column, and using a nonlinear gradient (Table 10).

Table 10: Non-linear eluent gradient used in HPLC for PBAT monomer analysis.[72,139]

Time / [min]	H_2O / [%]	Methanol / [%]	Formic acid / [%]
0	75	15	10
13	75	15	10
30	50	40	10
35	0	90	10
46	75	15	10
60	75	15	10

The polymer degradation products (i.e. PBAT monomers) were detected at 241 nm via UV-spectroscopy. For the sample quantification the standards 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 mM of terephthalic acid (Ta), mono(4-hydroxybutyl) terephthalate (BTa) and bis(4-hydroxybutyl) terephthalate (BTaB) were used (Figure 12) and the lowest standard of 0.001 mM was set as cut-off value with lower measured concentrations being treated as undetectable.

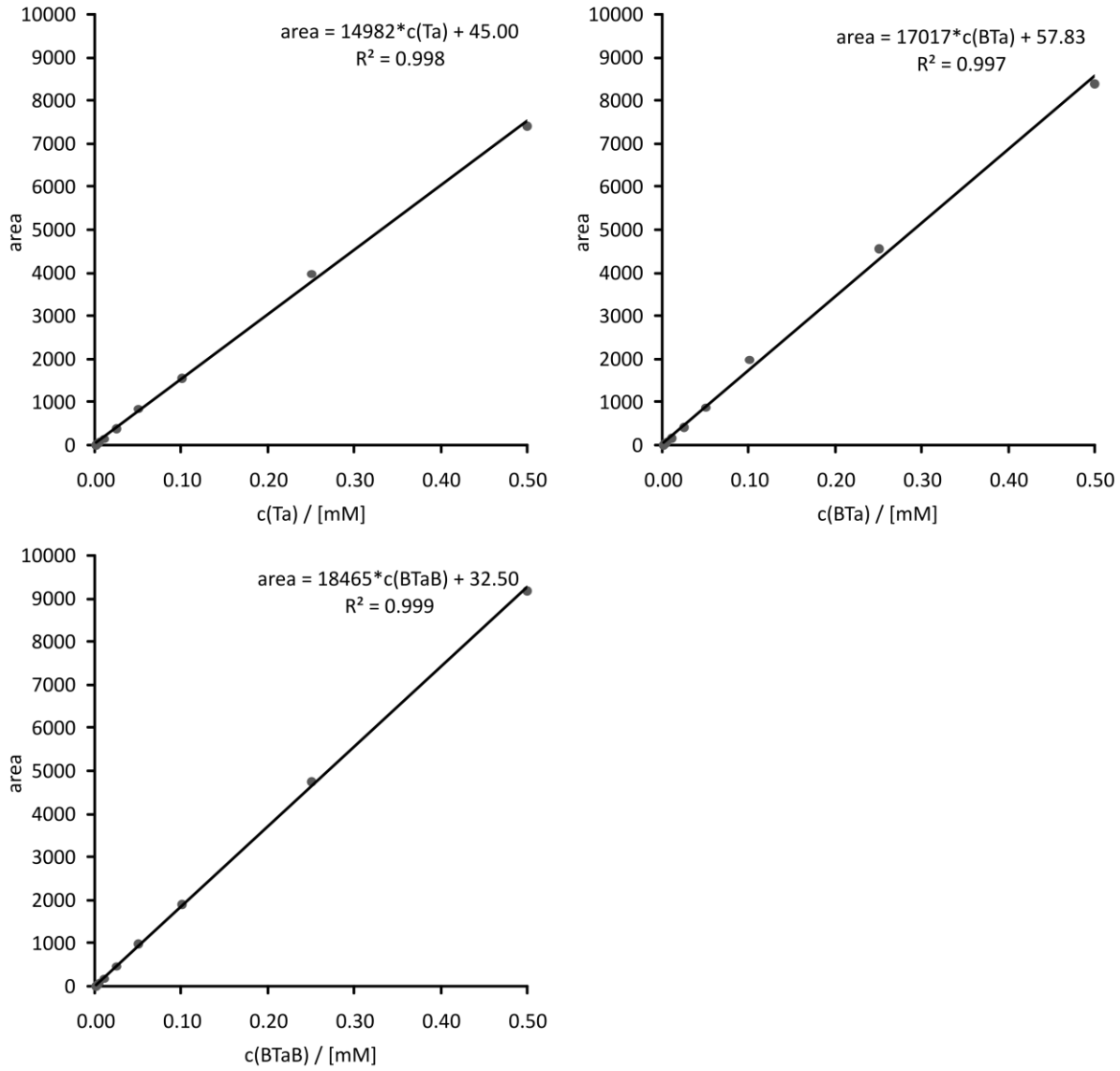

Figure 12: Linear correlation of terephthalic acid- (c(Ta), top left), mono(4-hydroxybutyl) terephthalate- (c(BTa), top right), and bis(4-hydroxybutyl) terephthalate concentration (c(BTaB), bottom left) to the corresponding peak area, calculated from the signal over time, detected by UV-spectroscopy at 241 nm.

The concentrations of the PBAT degradation products in the culture supernatant were calculated based on the according slope and intercept from the monomer concentration-area-correlation (Figure 12) as shown in Formula 3, Formula 4, and Formula 5.

Formula 3: Calculation of terephthalic acid (Ta) concentration in culture supernatant.

$$\beta(Ta) = \frac{area_{Ta} - y_{Ta}}{x_{Ta}} * \frac{MW(Ta)}{V_l}$$

$\beta(Ta)$	concentration of terephthalic acid in the culture supernatant in [mg/ml]
$area_{Ta}$	area of the terephthalic acid peak determined in HPLC analysis
y_{Ta}	c(Ta)-area-correlation intercept = 45.00
x_{Ta}	c(Ta)-area-correlation slope = 14982 [1/mM]
$MW(Ta)$	molecular weight of terephthalic acid = 166.13 [g/mol]
V_l	millilitre per litre = 1000 [ml/l]

Formula 4: Calculation of mono(4-hydroxybutyl) terephthalate (BTa) concentration in culture supernatant.

$$\beta(BTa) = \frac{area_{BTa} - y_{BTa}}{x_{BTa}} * \frac{MW(BTa)}{V_l}$$

$\beta(BTa)$	concentration of mono(4-hydroxybutyl) terephthalate in the culture supernatant in [mg/ml]
$area_{BTa}$	area of the mono(4-hydroxybutyl) terephthalate peak determined in HPLC analysis
y_{BTa}	c(BTa)-area-correlation intercept = 57.83
x_{BTa}	c(BTa)-area-correlation slope = 17017 [1/mM]
$MW(BTa)$	molecular weight of mono(4-hydroxybutyl) terephthalate = 238.24 [g/mol]
V_l	millilitre per litre = 1000 [ml/l]

Formula 5: Calculation of bis(4-hydroxybutyl) terephthalate (BTaB) concentration in culture supernatant.

$$\beta(BTaB) = \frac{area_{BTaB} - y_{BTaB}}{x_{BTaB}} * \frac{MW(BTaB)}{V_l}$$

$\beta(BTaB)$	concentration of bis(4-hydroxybutyl) terephthalate in the culture supernatant in [mg/ml]
$area_{BTaB}$	area of the bis(4-hydroxybutyl) terephthalate peak determined in HPLC analysis
y_{BTaB}	c(BTaB)-area-correlation intercept = 32.50
x_{BTaB}	c(BTaB)-area-correlation slope = 18465 [1/mM]
$MW(BTaB)$	molecular weight of bis(4-hydroxybutyl) terephthalate = 310.35 [g/mol]
V_l	millilitre per litre = 1000 [ml/l]

5.6 Hydrolase activity assay

The hydrolase activity in the culture supernatant was determined with an Infinite M200 Pro microplate Reader (TECAN, Manenndorf, Switzerland). The assay was performed in a 96-Well-Plate and for each sample in triplicates. 20 µl of the sample supernatant solution were pipetted into each well and 200 µl of 116 µM p-nitrophenyl butyrate in 0.1 M K_3PO_4-buffer pH 7, prepared fresh shortly before use and kept on ice, were added to each well shortly before the measurement. The absorbencies were acquired at 405 nm, for 25 flashes, and 30 consecutive kinetic cycles of measurement with a minimum 5 seconds delay between measurements.

The hydrolase activity of the culture supernatant was calculated as shown in Formula 6 and Formula 7.

Formula 6: Calculation of the absorbance slope.

$$slope(absorbance) = t_{minute} * F_{slope}\,[(ABS - \overline{ABS}_{blank}), (t)]$$

$slope(absorbance)$	slope of the measured absorbance in [ABS/min]
t_{minute}	seconds per minute = 60 [s/min]
$F_{slope}\,[(Y), (X)]$	slope function of Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, Washington, United States) yielding slope of the measured absorbance in [ABS/s]
ABS	absorbance at a given time point
$\overline{ABS}_{blank}$	mean absorbance of the blank at a given time point
t	measurement time point in [s]

Formula 7: Calculation of the hydrolase activity in the culture supernatant.

$$activity(hydrolases) = \frac{slope(absorbance)}{\varepsilon * d} * \frac{V_f}{V_e}$$

$activity(hydrolases)$	hydrolase activity in [U/ml] or [µmol/min*ml]
$slope(absorbance)$	slope of the measured absorbance in [ABS/min]
ε	molar extinction coefficient of p-nitrophenol = 8.5 [ml/µmol*cm]
d	optical path length through assay solution = 0.592 [cm]
V_f	final volume of assay solution = 0.22 [ml]
V_e	volume of enzyme solution = 0.02 [ml]

5.7 Sample preparation for the shotgun-proteomics analysis

5.7.1 Protein extraction from culture supernatant

The secretome, the set of proteins secreted in the culture media, was isolated and purified from interfering compounds by precipitation. Prior to precipitation, protease inhibitors were added to the culture supernatants in a concentration equal to 40 µl per 1 ml sample (1 tablet of cOmplete, Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, Missouri, United States) dissolved in 2 ml H_2O). The supernatants were thereafter concentrated using Vivaspin membrane tubes 500, PES with a 5 kDa cut-off value (Sartorius, Göttingen, Germany). After an initial washing step with mQ H_2O at 15 000 rcf for 5 minutes, the supernatant was concentrated by a factor of 10 by centrifugation with the F45-24-11 rotor in Centrifuge 5415R (Eppendorf, Hamburg, Germany) at 15000 rcf. To precipitate the proteins secreted in the culture supernatant, 5 times the volume of precipitation solution (0.1 M ammonium acetate in methanol, -20 °C) was added and the proteins were precipitated overnight at -20 °C. The precipitated proteins were pelleted by centrifugation at 7500 g with the F15S-6x100 6091549 rotor with 15 ml tube inserts in Hereaus Megafuge 40R (Thermo Fisher Scientific, Waltham, Massachusetts, United States) for 30 minutes at 4 °C. The supernatant was discarded and the pellet was washed with 2 ml methanol, -20 °C and subsequently with 2 ml acetone, -20 °C to remove co-precipitated contaminants. The protein pellet was incubated in each of the washing solutions for 15 minutes at -20 °C before centrifugation at 7500 g with the F15S-6x100 6091549 rotor with 15 ml tube inserts in Hereaus Megafuge 40R (Thermo Fisher Scientific, Waltham, Massachusetts, United States) for 20 minutes at 4 °C. The solvent was evaporated at room temperature (21 °C) by loosening the tube caps. Thereafter, the pellet was redissolved in 250 µl DiGE lysis buffer (Table 11) by orbital shaking of the tubes for 60 minutes and stored at -20 °C.

Table 11: Composition DiGE lysis buffer solved in mQ H_2O.[162,163]

Substance	β / [g/l]
tris	3.6
urea	420
thio urea	152
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	40

The extracted proteins were quantified with the Qubit Protein Assay Kit as already described. The proteins were furthermore subjected to a SDS-PAGE analysis to verify the

successful protein extraction in regards to size diversity, presence of proteins in the sizes most interesting for the investigation of fungal hydrolases (i.e. 30 to 70 kDa[66,72,168–171,83,85,88,138,164–167]), and protein degradation. The analysis via SDS-PAGE was conducted as described in the according chapter.

5.7.2 Sample preparation for mass spectrometry

To prepare the samples for shotgun-proteomics, several steps were performed for disulfide bridge reduction, alkylation of the nascent sulfhydryl groups, and protein digestion.

The sample volume containing 20 µg protein was admixed with 100 µl 100 mM dithiothreitol (DTT) and diluted to 200 µl by addition of 8 M urea in 50 mM tris buffer in order to obtain a final concentration of 50 mM DTT. The disulfide bridges were reduced by incubation for 60 minutes at 37 °C with the Thermomixer (Eppendorf, Hamburg, Germany). Before transferring the reduced lysate fully into an Amicon Ultra 0.5 ml centrifugal filter (Merck Millipore, Burlington, Massachusetts, United States) with a cut-off value of 10 kDa, the filter was washed with 200 µl 8 M urea in 50 mM tris buffer by centrifugation at 14 000 rcf for 20 minutes with the FA-45-24-11 rotor in Centrifuge 5424 (Eppendorf, Hamburg, Germany). After addition of 200 µl 8 M urea in 50 mM tris buffer, the sample was concentrated by centrifugation at 14 000 rcf for 20 minutes with the FA-45-24-11 rotor in Centrifuge 5424 (Eppendorf, Hamburg, Germany).

To conduct sulfhydryl alkylation, 100 µl 150 mM iodoacetoamide (IAA) in 8 M urea in 50 mM tris buffer were added to the concentrated sample, incubated at room temperature (21 °C) and protected from light for 30 minutes under orbital shaking at 550 rpm with the Thermomixer (Eppendorf, Hamburg, Germany). The sample was again concentrated and washed 3 times with 200 µl 50 mM ammonium hydrogen carbonate (ABC) buffer by centrifugation at 14 000 rcf for 20 minutes with the FA-45-24-11 rotor in Centrifuge 5424 (Eppendorf, Hamburg, Germany).

The centrifugal filter was transferred into a new Eppendorf tube with 50 µl 50 mM ABC buffer on the bottom to maintain humidity levels, 100 µl 0.1 µg/µl Trypsin/LysC protease mix (Promega, Madison, Wisconsin, United States) were added to the sample and the proteins were digested for 14 hours at 37 °C in the Thermomixer (Eppendorf, Hamburg, Germany). Thereafter, the peptides were eluted and washed out of the filter with 3 times 50 µl water Optima LC/MS W6-1 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) by

centrifugation at 14 000 rcf for 20 minutes with the FA-45-24-11 rotor in Centrifuge 5424 (Eppendorf, Hamburg, Germany).

The peptide samples were desalted with a Pierce C18 Spin Column (Thermo Fisher Scientific, Waltham, Massachusetts, United States) according to the protocol provided by the manufacturer.[172] The purified peptides were dissolved in 0.1 % trifluoroacetic acid (TFA) prior to mass spectrometry analysis.

5.8 Statistics

The protein quantification and the hydrolase activity measurements were conducted in 3 technical replicates and with 3 biological replicates. The PBAT degradation product measurements were conducted with 3 biological replicates. Potential outliers were assessed with the Grubbs' test, $\alpha = 0.01$. The statistical analysis was conducted with IBM SPSS Statistics 24 (IBM, Armonk, New York, United States) via one-away analysis of variance (ANOVA) with the Tukey's honest significance difference (HSD) post-hoc test, $\alpha = 0.05$.

6 Results

The experiments presented in this chapter were performed in 2 % ME and 0.2 % ME without glucose inoculated with *Knufia chersonesos* wild type and mutant, respectively. For the treatment cultures, milled 50:50, 60:40, or 90:10 PBAT was added to the fungal cultures. In the control cultures no polymer was added. The culture supernatant was processed for protein quantification with Qubit, hydrolase activity assay, HPLC measurement for PBAT degradation products, and secretome analysis with SDS-PAGE.

The aim of these experiments was the elucidation of the process of PBAT degradation by *K. chersonesos*. With the experimental conditions chosen, the process can be investigated with consideration of various factors influencing PBAT degradation, such as melanisation of the fungal cell wall, chemical composition of PBAT, and nutrient availability in the media.

The data shown in the bar graphs (Figure 13, Figure 15, Figure 17, Figure 19, Figure 21, Figure 23, Figure 25 - Figure 27) are the mean values of the measurements (grey bars) ± standard deviation (error bars). The protein concentration measurements and hydrolase activity assays were conducted in 3 biological and 3 technical replicates. The HPLC measurements were conducted in 3 biological replicates and the non-biologically induced degradation was assessed in 3 replicates. No outliers were assessed with Grubbs' test, $\alpha = 0.01$. The statistical analysis was conducted with one-away analysis of variance (ANOVA) with the Tukey's honest significance difference (HSD) post-hoc test, $\alpha = 0.05$. The statistical significance of the differences between means is indicated by letters (a, b, c). Groups of means showing no significant differences share a specific letter, while means distinguished from each other by a significant difference are denoted with different letters. Different subscripted numbers alongside these letters indicate not related statistical tests within the same graph. The mean concentrations of PBAT degradation products of treatment conditions were only assigned with letters, if they were significantly different from the negative control measurements (Figure 25) and larger than the cut off value of 0.00017 mg/ml (Ta), 0.00024 mg/ml (BTa), 0.00031 mg/ml (BTaB) representing the concentrations of the lowest standard used for the quantification.

In the SDS-PAGE analyses (Figure 14, Figure 16, Figure 18, Figure 20, Figure 22, Figure 24) the amount of proteins loaded per fractionation lane was not varied within a particular experiment owing to consistency. In some experimental setups the secreted amount of proteins was so low, however, that maximising the loaded protein amount was paramount

to visualise the protein bands in the gel. This issue is discussed in more detail for the experiments (Figure 20, Figure 24) it refers to.

6.1 Fungal-mediated degradation of 50:50 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT)

In the experiment 2 % ME was inoculated with *K. chersonesos* wild type and mutant, respectively. 50:50 PBAT was added in the treatment cultures to investigate the polymer degradation mediated by these fungal strains. The culture supernatant was analysed with protein quantification, hydrolase activity-, and HPLC measurement for PBAT degradation products. 3 biological replicates were conducted per experimental condition.

Analysis of *K. chersonesos* grown in 2% ME with (treatment condition) and without (control condition) 50:50 PBAT

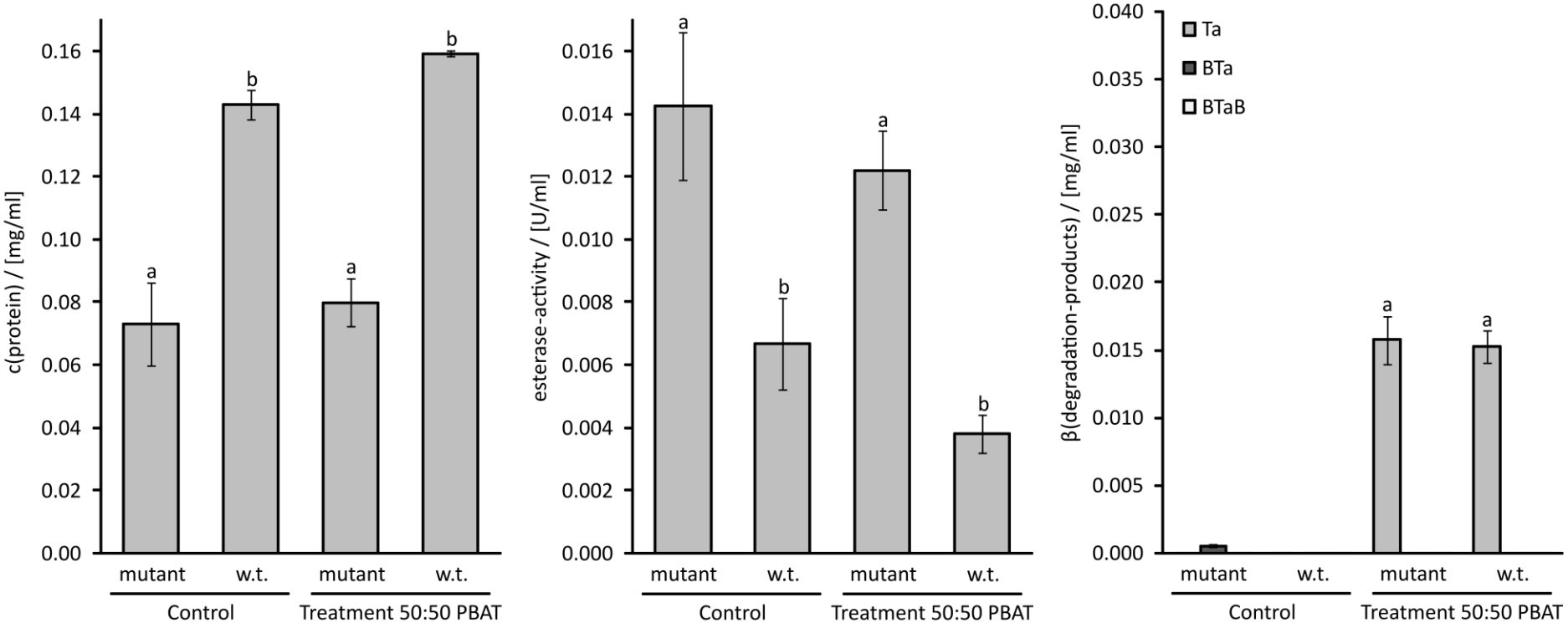

Figure 13: Protein concentration (left), hydrolase activity (middle), and concentration of the PBAT degradation products terephthalic acid (Ta), mono(4-hydroxybutyl) terephthalate (BTa), and bis(4-hydroxybutyl) terephthalate (BTaB) (right) detected in the culture supernatant of *K. chersonesos* wildtype (w.t.) and mutant, grown in 2 % ME for 15 days. Treatment condition: media added with 50:50 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT, EcoFlex Blend C1200, 68818836WO, 500 µm maximum particle size; BASF, Ludwigshafen am Rhein, Germany); Control condition: no polymer added to the media.

Protein concentrations (mean ± standard deviation) are equal to 0.073 ± 0.013 (mutant, control), 0.1431 ± 0.0047 (w.t., control), 0.0800 ± 0.0075 (mutant, treatment), and 0.15933 ± 0.00088 mg/ml (w.t., treatment). The values of hydrolase activity are equal to 0.0143 ± 0.0024 (mutant, control), 0.0067 ± 0.0015 (w.t., control), 0.0122 ± 0.0013 (mutant, treatment), and 0.0038 ± 0.0006 U/ml (w.t., treatment). Terephthalic acid concentrations are equal to 0.0157 ± 0.0018 (mutant, treatment), 0.0152 ± 0.0012 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control). Mono(4-hydroxybutyl) terephthalate concentrations are equal to 0.00054 ± 0.00013 mg/ml (mutant, control) and were not detected in the other conditions. Bis(4-hydroxybutyl) terephthalate concentrations were not detected in any of the conditions.

Furthermore, the cultures supernatants were concentrated and the secretome was analysed with SDS-PAGE and subsequent SYPRO- and silver staining to investigate variations in protein secretion.

Secretomics of *K. chersonesos* grown in 2% ME with (treatment condition) and without (control condition) 50:50 PBAT

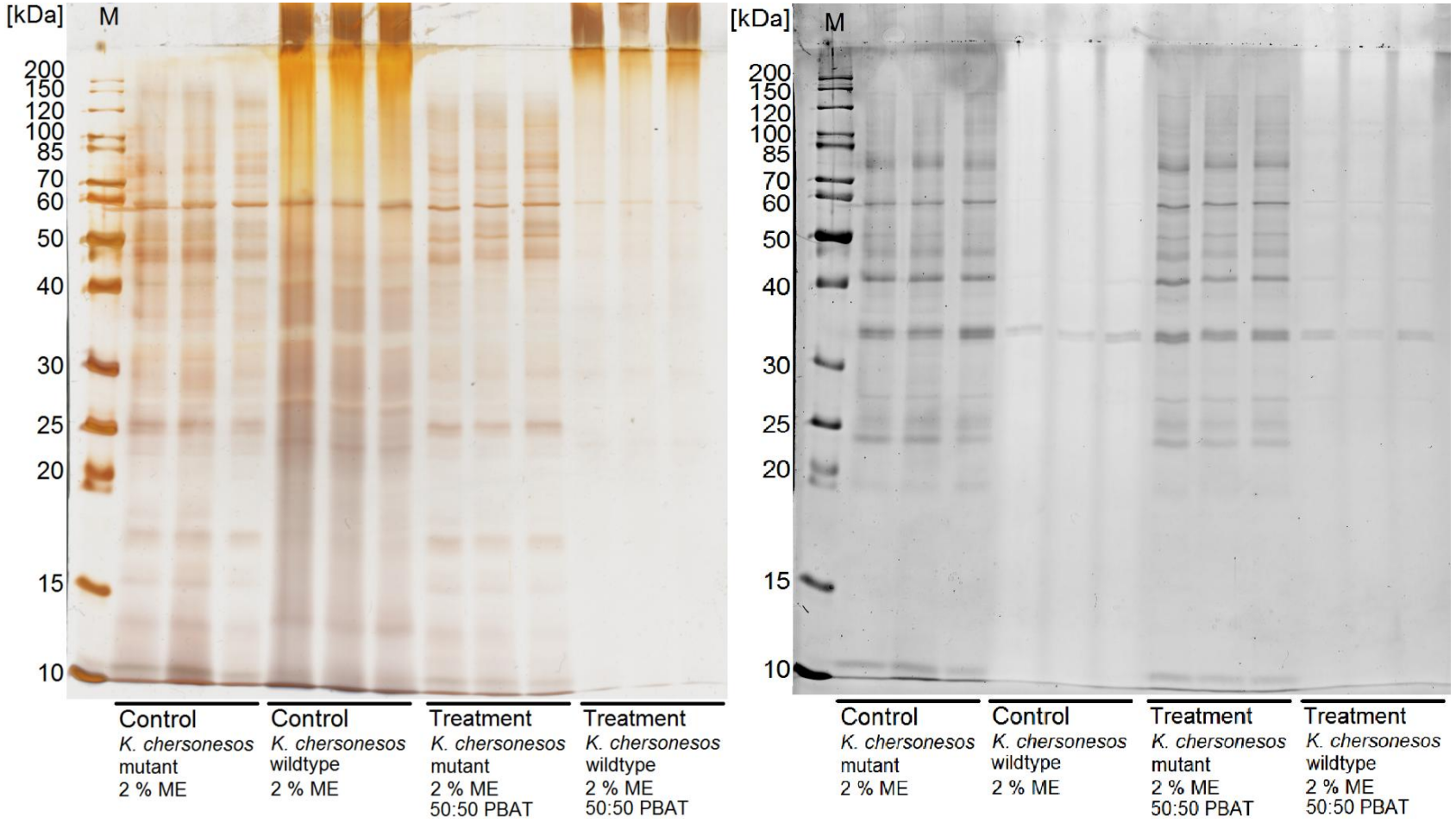

Figure 14: SDS-PAGE analysis of *K. chersonesos* wildtype (w.t.) and mutant, grown in 2 % ME for 15 days (Control) and treated with 50:50 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT, EcoFlex Blend C1200, 68818836WO, 500 µm maximum particle size; BASF, Ludwigshafen am Rhein, Germany) (Treatment). The unstained marker (M) was run as reference. 1.5 µg proteins were fractionated per lane and their visualisation was accomplished with silver staining (left) and SYPRO staining (right).

Silver staining of the gel resulted in the detection of proteins in the range of 10 to 140 kDa. The protein samples of *K. chersonesos* wild type show a high content of melanin, which is spread alongside the protein fractionation and cumbers the detection of proteins. Due to melanin interference, only the most distinct protein bands are detected at about 58, 47, 40, 26, 23, 13, and 11 kDa in the *K. chersonesos* wild type samples. No staining is present in the range of 41, 35, and 27 kDa. When comparing Control- and Treatment conditions of *K. chersonesos* wild type, the samples of the Treatment conditions are generally less stained but most distinctive protein bands are present in both conditions. Based on the staining intensity the melanin content is lower in the samples of *K. chersonesos* wild type treated with 50:50 PBAT. When comparing Control- and Treatment conditions of *K. chersonesos* mutant, a protein band at about 50 kDa is more distinct in Treatment conditions. Otherwise these conditions are largely identical.

SYPRO staining of the gel resulted in the detection of proteins in the range of 11 to 140 kDa. As shown above in the silver stained gel, the protein samples of *K. chersonesos* wild type show a high content of melanin, which is spread alongside the protein fractionation and cumbers the detection of proteins. Due to melanin interference, only the most distinct protein bands are detected at about 58, 40, 35, 34, and 27 kDa in the *K. chersonesos* wild type samples and show no difference in protein band occurrence depending on the conditions. When comparing Control- and Treatment conditions of *K. chersonesos* wild type, the protein bands at about 58 and 27 kDa are more distinct in Treatment conditions. Based on the staining intensity the melanin content is lower in the samples of *K. chersonesos* wild type treated with 50:50 PBAT. When comparing Control- and Treatment conditions of *K. chersonesos* mutant, the protein bands at about 58 and 40 kDa are more distinct in Treatment conditions.

For the second part of this experiment to investigate PBAT degradation with lower nutrient availability to the fungus, 0.2 % ME without glucose was used for the inoculation with *K. chersonesos* wild type and mutant instead.

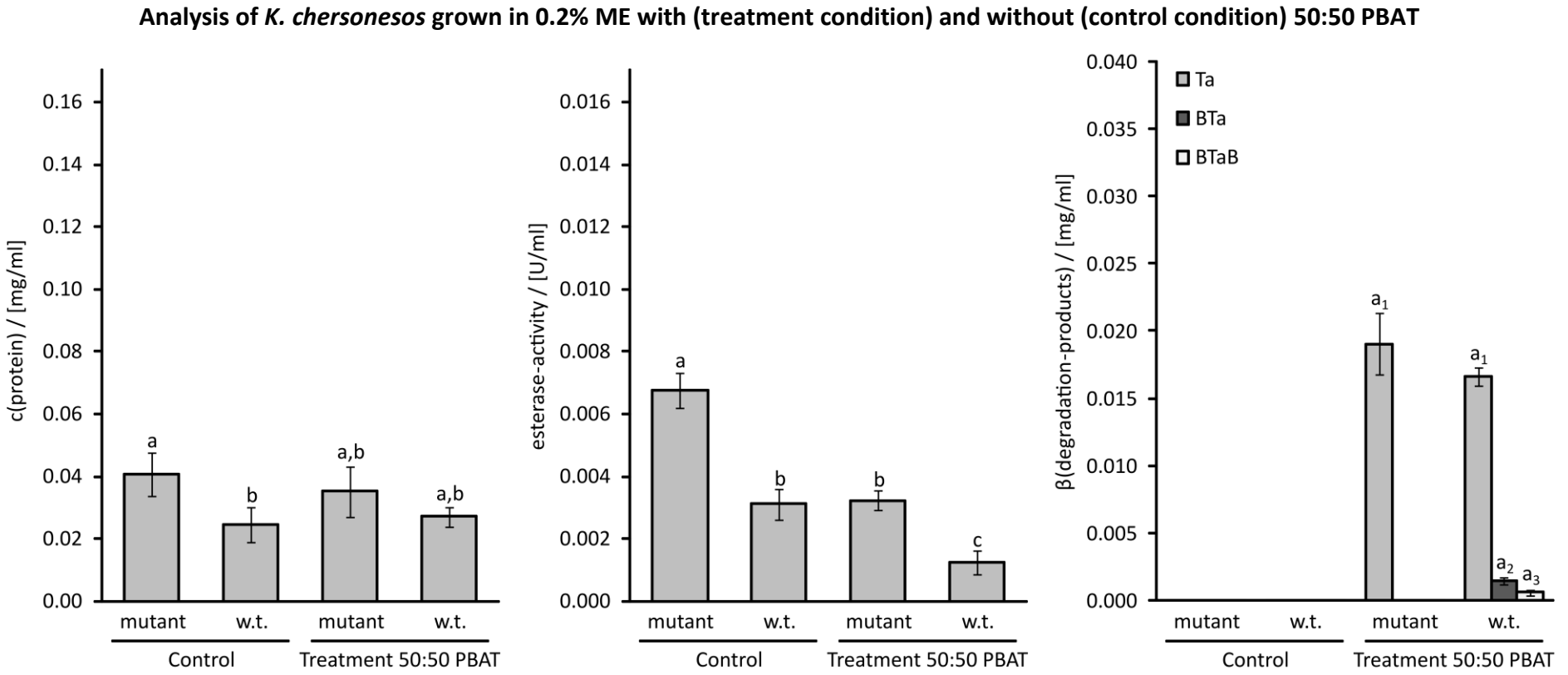

Figure 15: Protein concentration (left), hydrolase activity (middle), and concentration of the PBAT degradation products terephthalic acid (Ta), mono(4-hydroxybutyl) terephthalate (BTa), and bis(4-hydroxybutyl) terephthalate (BTaB) (right) detected in the culture supernatant of *K. chersonesos* wildtype (w.t.) and mutant, grown in 0.2 % ME without glucose for 15 days. Treatment condition: media added with 50:50 poly(1,4-butylene adipate-co-terephthalate) (PBAT, EcoFlex Blend C1200, 68818836WO, 500 µm maximum particle size; BASF, Ludwigshafen am Rhein, Germany); Control condition: no polymer added to the media.

Protein concentrations (mean ± standard deviation) are equal to 0.0407 ± 0.0069 (mutant, control), 0.0245 ± 0.0054 (w.t., control), 0.0351 ± 0.0081 (mutant, treatment), and 0.0271 ± 0.0031 mg/ml (w.t., treatment). The values of hydrolase activity are equal to 0.00677 ± 0.00056 (mutant, control), 0.00312 ± 0.00050 (w.t., control), 0.00322 ± 0.00031 (mutant, treatment), and 0.00123 ± 0.00038 U/ml (w.t., treatment). Terephthalic acid concentrations are equal to 0.0191 ± 0.0023 (mutant, treatment), 0.01670 ± 0.00067 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control). Mono(4-hydroxybutyl) terephthalate concentrations are equal to 0.00149 ± 0.00029 mg/ml (w.t., treatment) and were not detected in the other conditions. Bis(4-hydroxybutyl) terephthalate concentrations are equal to 0.00064 ± 0.00020 mg/ml (w.t., treatment) and were not detected in the other conditions.

As before, the cultures supernatants were concentrated and the secretome was analysed with SDS-PAGE and subsequent SYPRO- and silver staining to investigate variations in protein secretion.

Secretomics of *K. chersonesos* grown in 0.2% ME with (treatment condition) and without (control condition) 50:50 PBAT

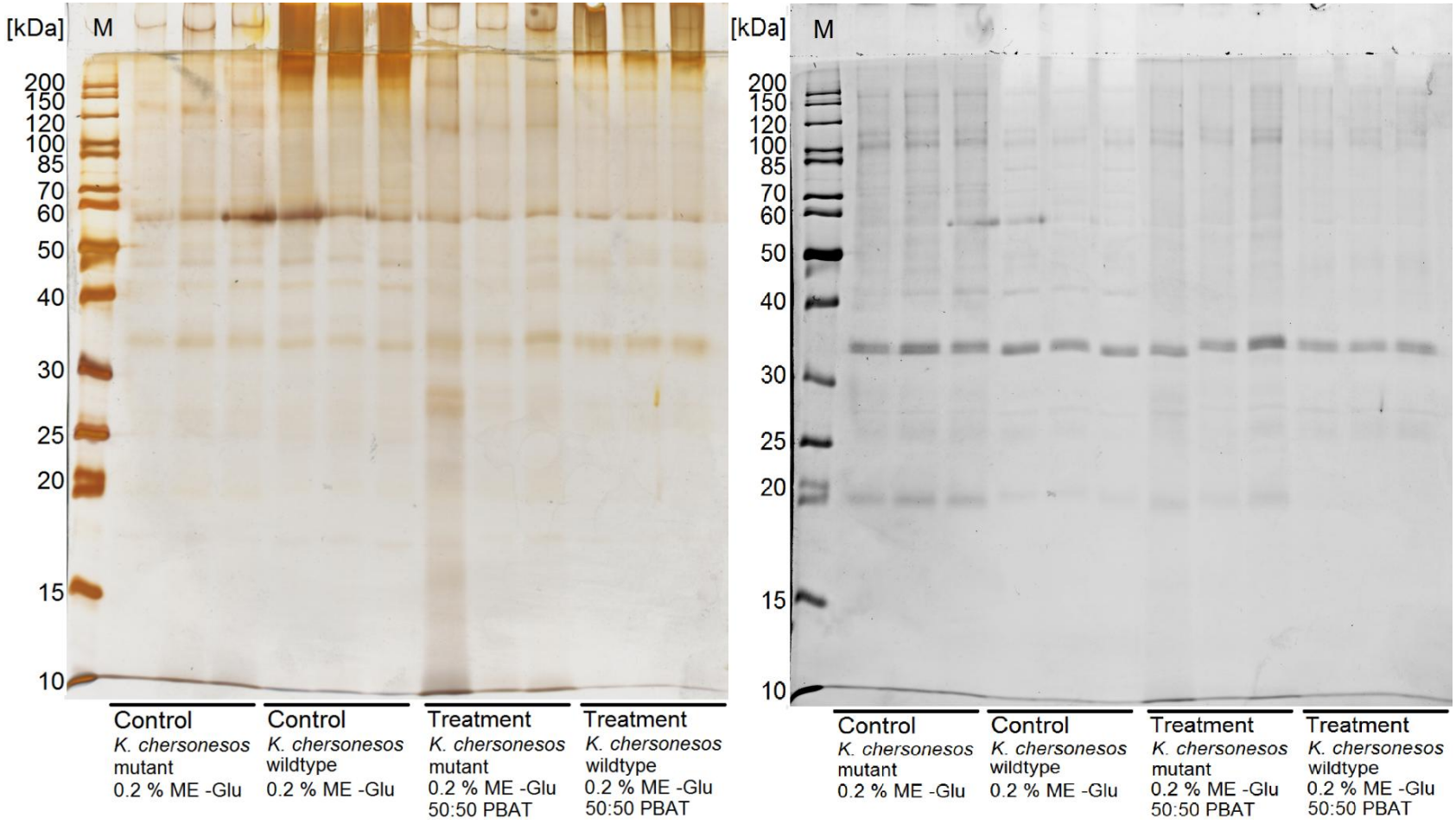

Figure 16: SDS-PAGE analysis of *K. chersonesos* wildtype (w.t.) and mutant, grown in 0.2 % ME without glucose for 15 days (Control) and treated with 50:50 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT, EcoFlex Blend C1200, 68818836WO, 500 µm maximum particle size; BASF, Ludwigshafen am Rhein, Germany) (Treatment). The unstained marker (M) was run as reference. 1.5 µg proteins were fractionated per lane and their visualisation was accomplished with silver staining (left) and SYPRO staining (right).

Silver staining of the gel resulted in the detection of proteins in the range of 17 to 175 kDa. In the protein samples of the *K. chersonesos* wild type cultures a content of melanin is present, but low enough that it remains in the stacking gel. When comparing Control- and Treatment conditions of *K. chersonesos* wild type, the protein bands at about 135, 60, and 42 kDa are more distinct in Control conditions. When comparing Control- and Treatment conditions of *K. chersonesos* mutant, the protein bands at about 175, 110, 28, and 27 kDa are more distinct in Treatment conditions and the protein bands at about 135, 60, 47 kDa are more distinct in Control conditions. When comparing Treatment conditions of *K. chersonesos* wild type and mutant, the protein bands at about 42, 28, and 27 kDa are more distinct for *K. chersonesos* mutant.

SYPRO staining of the gel resulted in the detection of proteins in the range of 20 to 175 kDa. In the protein samples of the *K. chersonesos* wild type cultures a content of melanin is present, but low enough that it remains in the stacking gel. When comparing Control- and Treatment conditions of *K. chersonesos* wild type, the protein bands at about 80, 73, 42, and 20 kDa are more distinct in Control conditions. When comparing Control- and Treatment conditions of *K. chersonesos* mutant, the protein bands at about 175, 80, 75, 73, and 42 kDa are more distinct in Control conditions. When comparing Treatment conditions of *K. chersonesos* wild type and mutant, the protein band at about 20 kDa is more distinct for *K. chersonesos* mutant.

6.2 Fungal-mediated degradation of 60:40 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT)

In the second experiment 2 % ME was inoculated with *K. chersonesos* wild type or mutant. 60:40 PBAT was added in the treatment cultures to investigate the degradation of another polymer composition. The culture supernatant did undergo protein quantification, hydrolase activity-, and HPLC measurement for PBAT degradation products. 3 biological replicates were conducted per experimental condition.

Analysis of *K. chersonesos* grown in 2% ME with (treatment condition) and without (control condition) 60:40 PBAT

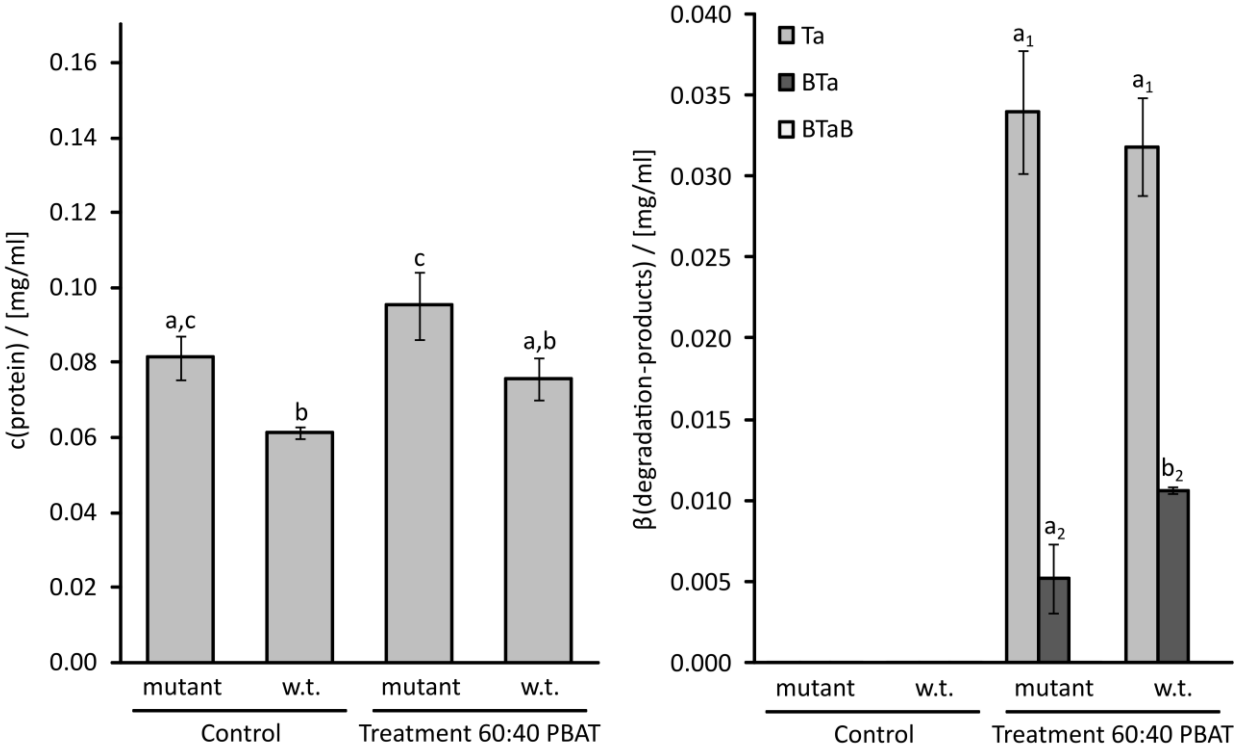

Figure 17: Protein concentration (left) and concentration of the PBAT degradation products terephthalic acid (Ta), mono(4-hydroxybutyl) terephthalate (BTa), and bis(4-hydroxybutyl) terephthalate (BTaB) (right) detected in the culture supernatant of *K. chersonesos* wildtype (w.t.) and mutant, grown in 2 % ME for 15 days. Treatment condition: media added with 60:40 poly(1,4-butylene adipate-co-terephthalate) (PBAT; BASF, Ludwigshafen am Rhein, Germany); Control condition: no polymer added to the media.

Protein concentrations (mean ± standard deviation) are equal to 0.0812 ± 0.0057 (mutant, control), 0.0613 ± 0.0016 (w.t., control), 0.0951 ± 0.0090 (mutant, treatment), and 0.0757 ± 0.0057 mg/ml (w.t., treatment). Terephthalic acid concentrations are equal to 0.0340 ± 0.0038 (mutant, treatment), 0.0318 ± 0.0030 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control). Mono(4-hydroxybutyl) terephthalate concentrations are equal to 0.0052 ± 0.0022 (mutant, treatment), 0.01067 ± 0.00018 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control). Bis(4-hydroxybutyl) terephthalate concentrations were not detected in any of the conditions.

The secretome in the cultures supernatants was analysed with SDS-PAGE and subsequent silver staining to investigate variations in protein secretion, despite the interference of melanin with the staining method.

Secretomics of *K. chersonesos* grown in 2% ME with (treatment condition) and without (control condition) 60:40 PBAT

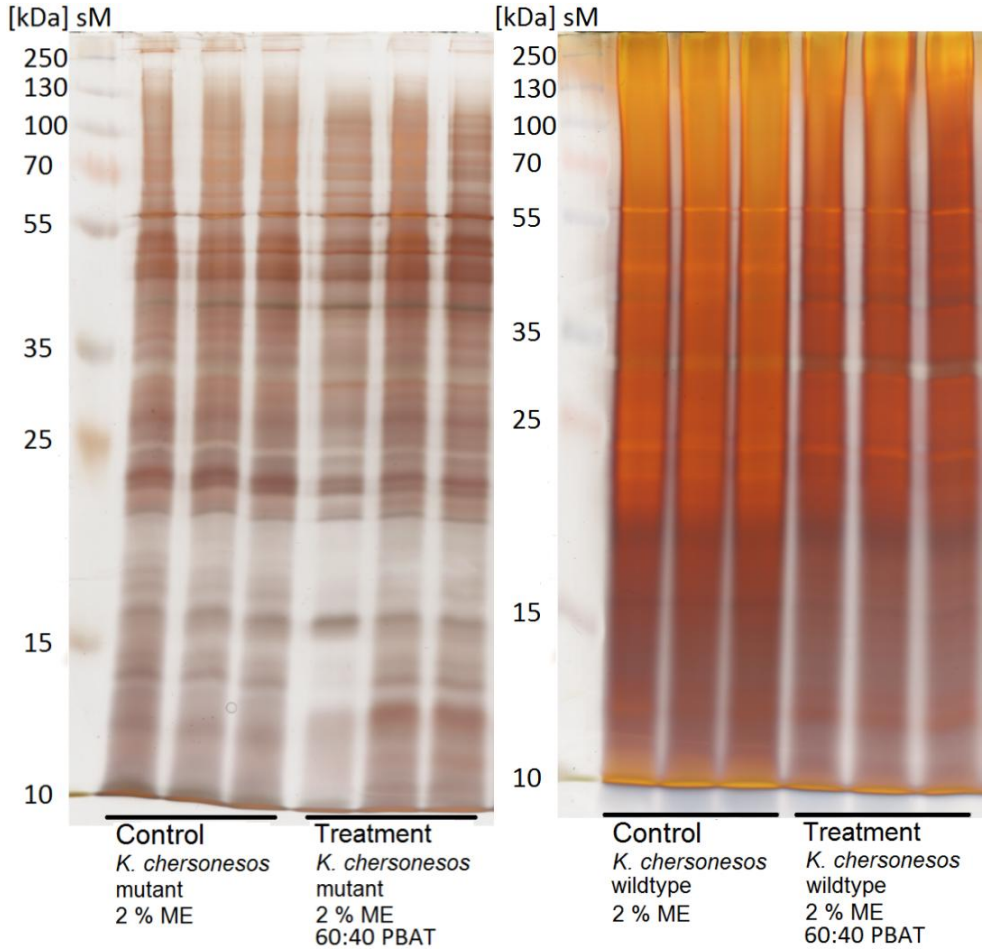

Figure 18: SDS-PAGE analysis of *K. chersonesos* wildtype (w.t.) and mutant, grown in 2 % ME for 15 days (Control) and treated with 60:40 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT; BASF, Ludwigshafen am Rhein, Germany) (Treatment). The stained marker (sM) was run as reference. 4.5 µg proteins were fractionated per lane and their visualisation was accomplished with silver staining.

In this experiment it was possible to load a higher amount of proteins (4.5 µg) per fractionation lane compared to the experiments with 50:50 PBAT (1.5 µg), due to the higher concentration of proteins in the supernatant. Proteins were detected in the range of 10 to >250 kDa. The protein samples of *K. chersonesos* wild type show a high content of melanin, which is spread alongside the protein fractionation and cumbers the detection of proteins. Due to melanin interference, only the most distinct protein bands are detected at about 57, 50, 45, 40, 24, 23, 20, 16, 13, and 12 kDa in the *K. chersonesos* wild type samples. Less staining is present in the range of 30 and 21 kDa. When comparing Control- and Treatment conditions of *K. chersonesos* wild type, the protein bands at about 50 and 40 kDa are more distinct in Treatment conditions and the protein bands at about 23 and 20 kDa are more distinct in Control conditions. When comparing Control- and Treatment conditions of *K. chersonesos* mutant, the protein bands at about 40 and 13 kDa are more distinct in Treatment conditions and the protein band at about 12 kDa are more distinct in Control conditions. The comparison of Treatment conditions of K. chersonesos wild type and mutant is difficult, due to the high interference of melanin with the staining method. Based on the protein bands present in the *K. chersonesos* wild type samples the banding pattern and intensity is largely identical.

The second part of this experiment, for the investigation of PBAT degradation with lower nutrient availability to the fungus, was conducted with 0.2 % ME without glucose for the inoculation with *K. chersonesos* wild type and mutant instead.

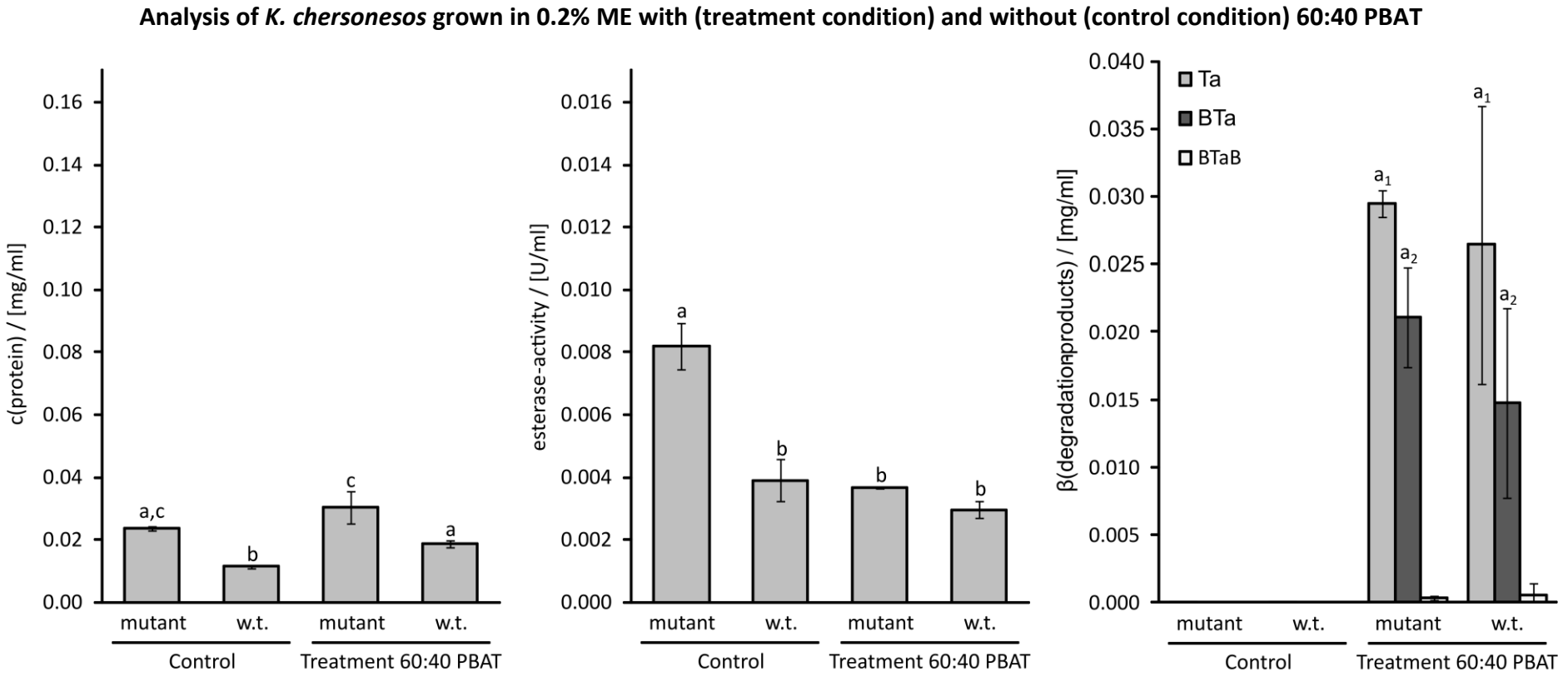

Figure 19: Protein concentration (left), hydrolase activity (middle), and concentration of the PBAT degradation products terephthalic acid (Ta), mono(4-hydroxybutyl) terephthalate (BTa), and bis(4-hydroxybutyl) terephthalate (BTaB) (right) detected in the culture supernatant of *K. chersonesos* wildtype (w.t.) and mutant, grown in 0.2 % ME without glucose for 15 days. Treatment condition: media added with 60:40 poly(1,4-butylene adipate-co-terephthalate) (PBAT; BASF, Ludwigshafen am Rhein, Germany); Control condition: no polymer added to the media.

Protein concentrations (mean ± standard deviation) are equal to 0.02382 ± 0.00066 (mutant, control), 0.01133 ± 0.00058 (w.t., control), 0.0303 ± 0.0052 (mutant, treatment), and 0.0187 ± 0.0012 mg/ml (w.t., treatment). The values of hydrolase activity are equal to 0.00818 ± 0.00074 (mutant, control), 0.00390 ± 0.00067 (w.t., control), 0.00366 ± 0.00003 (mutant, treatment), and 0.00296 ± 0.00026 U/ml (w.t., treatment). Terephthalic acid concentrations are equal to 0.02955 ± 0.00098 (mutant, treatment), 0.026 ± 0.010 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control). Mono(4-hydroxybutyl) terephthalate concentrations are equal to 0.0211 ± 0.0037 (mutant, treatment), 0.0148 ± 0.0070 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control). Bis(4-hydroxybutyl) terephthalate concentrations are equal to 0.00038 ± 0.00016 (mutant, treatment), 0.00062 ± 0.00084 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control).

As before, the cultures supernatants were concentrated and the secretome was analysed with SDS-PAGE and subsequent silver staining to investigate variations in protein secretion.

Secretomics of *K. chersonesos* grown in 0.2% ME with (treatment condition) and without (control condition) 60:40 PBAT

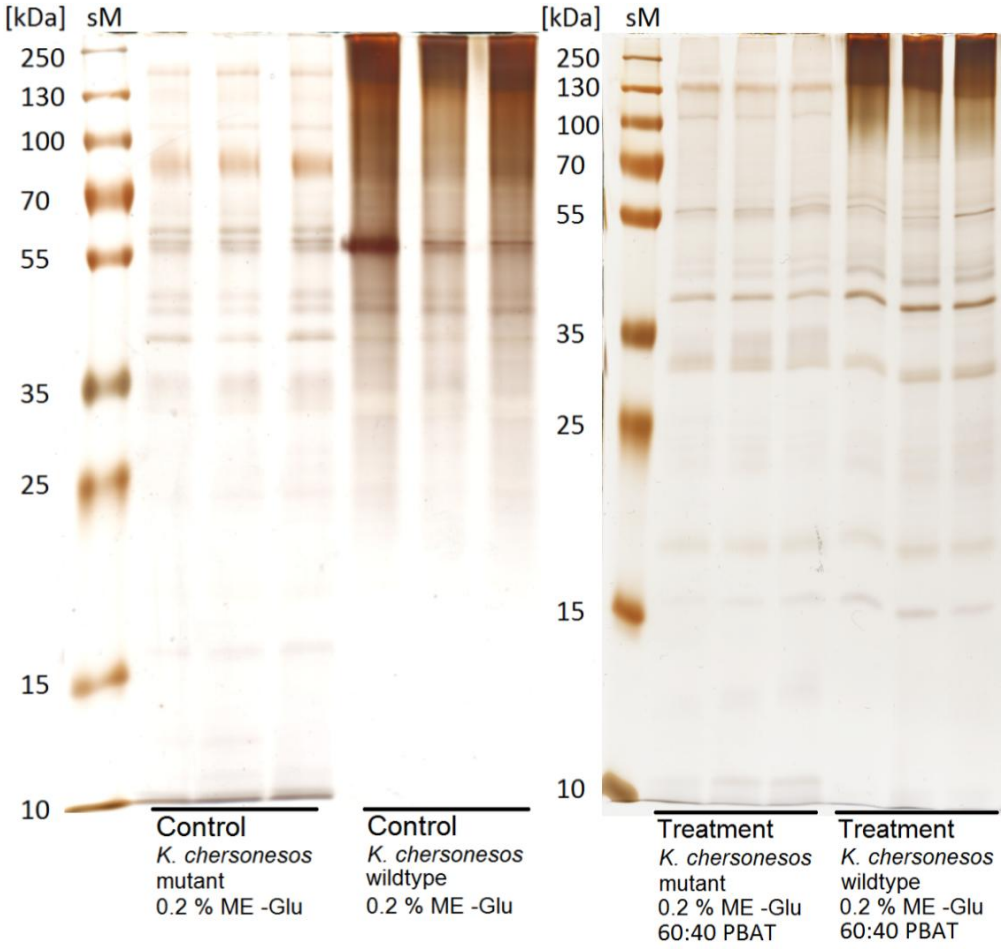

Figure 20: SDS-PAGE analysis of *K. chersonesos* wildtype (w.t.) and mutant, grown in 0.2 % ME without glucose for 15 days (Control) and treated with 60:40 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT; BASF, Ludwigshafen am Rhein, Germany) (Treatment). The stained marker (sM) was run as reference. 1 µg (Control) and 1.16 µg (Treatment) proteins were fractionated per lane and their visualisation was accomplished with silver staining.

In this experiment it was necessary to load a lower amount of proteins (1 µg for control, 1.16 µg for treatment) per fractionation lane compared to the experiments with 50:50 PBAT (1.5 µg), due to the lower concentration of proteins in the supernatant. The protein amount loaded onto the gel was higher for the treatment samples to allow the visualisation of protein bands. Whenever possible, the same protein amount was used for treatment and control within the same experimental set to allow comparison. Due to the very low protein concentrations detected in 0.2% ME, however, loading a higher protein amount was paramount in order to visualise protein bands. Proteins were detected in the range of 11 to 200 kDa. The protein samples of *K. chersonesos* wild type show a high content of melanin, which is spread alongside the protein fractionation and cumbers the detection of proteins, but most of the protein bands are visible despite melanin interference. When comparing Control- and Treatment conditions of *K. chersonesos* wild type, the protein bands at about 40, 30, 20, and 15 kDa are more distinct in Treatment conditions. Based on the staining intensity the melanin content is lower in the samples of *K. chersonesos* wild type treated with 60:40 PBAT. When comparing Control- and Treatment conditions of *K. chersonesos* mutant, the protein bands at about 140, 30, and 20 kDa is more distinct are Treatment conditions and the protein bands at about 200, 85, 65, 50, and 57 kDa are more distinct in Control conditions. When comparing Treatment conditions of *K. chersonesos* wild type and mutant, the protein bands at about 110 and 11 kDa are more distinct for *K. chersonesos* mutant and the protein band at about 40 kDa is more distinct for *K. chersonesos* wild type.

6.3 Fungal-mediated degradation of 90:10 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT)

In the third experiment 2 % ME was inoculated with *K. chersonesos* wild type and mutant, respectively. 90:10 PBAT was added in the treatment cultures to investigate the degradation of a third PBAT composition mediated by these fungal strains. Protein quantification and hydrolase activity measurements were conducted to investigate the culture supernatant. HPLC measurements were applied to detect PBAT degradation products in the culture supernatant. 3 biological replicates were conducted per experimental condition.

Analysis of *K. chersonesos* grown in 2% ME with (treatment condition) and without (control condition) 90:10 PBAT

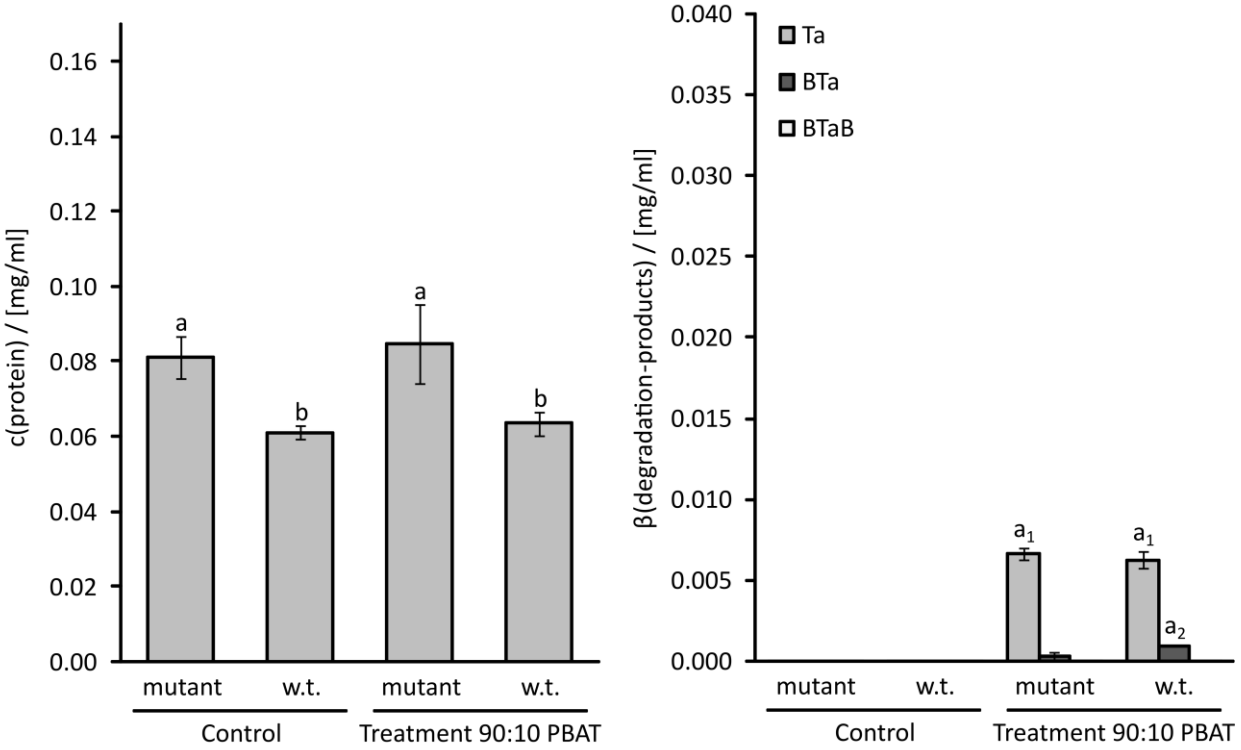

Figure 21: Protein concentration (left) and concentration of the PBAT degradation products terephthalic acid (Ta), mono(4-hydroxybutyl) terephthalate (BTa), and bis(4-hydroxybutyl) terephthalate (BTaB) (right) detected in the culture supernatant of *K. chersonesos* wildtype (w.t.) and mutant, grown in 2 % ME for 15 days. Treatment condition: media added with 90:10 poly(1,4-butylene adipate-co-terephthalate) (PBAT; BASF, Ludwigshafen am Rhein, Germany); Control condition: no polymer added to the media.

Protein concentrations (mean ± standard deviation) are equal to 0.0812 ± 0.0057 (mutant, control), 0.0613 ± 0.0016 (w.t., control), 0.085 ± 0.010 (mutant, treatment), and 0.0636 ± 0.0032 mg/ml (w.t., treatment). Terephthalic acid concentrations are equal to 0.00666 ± 0.00038 (mutant, treatment), 0.00629 ± 0.00050 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control). Mono(4-hydroxybutyl) terephthalate concentrations are equal to 0.00033 ± 0.00024 (mutant, treatment), 0.00100 ± 0.00004 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control). Bis(4-hydroxybutyl) terephthalate concentrations were not detected in any of the conditions.

The secretome was separated by SDS-PAGE and the protein bands were subsequently visualised by silver staining to investigate variations in protein secretion, despite interference of melanin with the staining method.

Secretomics of *K. chersonesos* grown in 2% ME with (treatment condition) and without (control condition) 90:10 PBAT

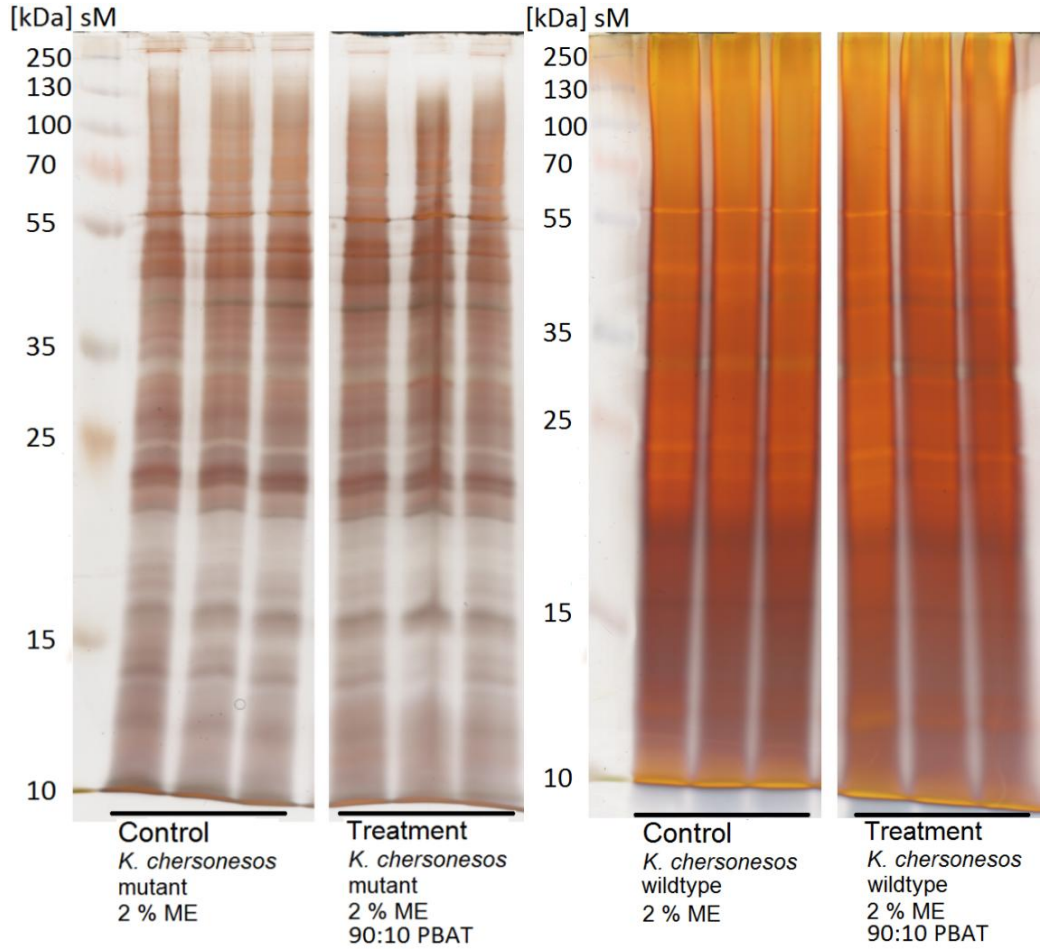

Figure 22: SDS-PAGE analysis of *K. chersonesos* wildtype (w.t.) and mutant, grown in 2 % ME for 15 days (Control) and treated with 90:10 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT; BASF, Ludwigshafen am Rhein, Germany) (Treatment). The stained marker (sM) was run as reference. 4.5 µg proteins were fractionated per lane and their visualisation was accomplished with silver staining. The Treatment conditions were run on the same gel as the according marker and the Control conditions, respectively, but 3 lanes between Control- and Treatment conditions were removed for clarity.

In this experiment it was possible to load a higher amount of proteins (4.5 µg) per fractionation lane compared to the experiments with 50:50 PBAT (1.5 µg), due to the higher concentration of proteins in the supernatant. Proteins were detected in the range of 10 to >250 kDa. The protein samples of *K. chersonesos* wild type show a high content of melanin, which is spread alongside the protein fractionation and cumbers the detection of proteins, but most of the protein bands are visible, despite melanin interference. When comparing Control- and Treatment conditions of *K. chersonesos* wild type, the protein bands at about 40, 23, and 13 kDa are more distinct in Treatment condition. When comparing Control- and Treatment conditions of *K. chersonesos* mutant, the protein bands at about 40 and 23 kDa are more distinct in Treatment conditions. The comparison of Treatment conditions of *K. chersonesos* wild type and mutant is difficult, due to the high interference of melanin with the staining method. Based on the protein bands present in the *K. chersonesos* wild type samples the banding pattern and intensity is largely identical.

The second part of this experiment was conducted for the investigation of PBAT degradation with lower nutrient availability to the fungus. 0.2 % ME without glucose was used for the inoculation with *K. chersonesos* wild type and mutant instead.

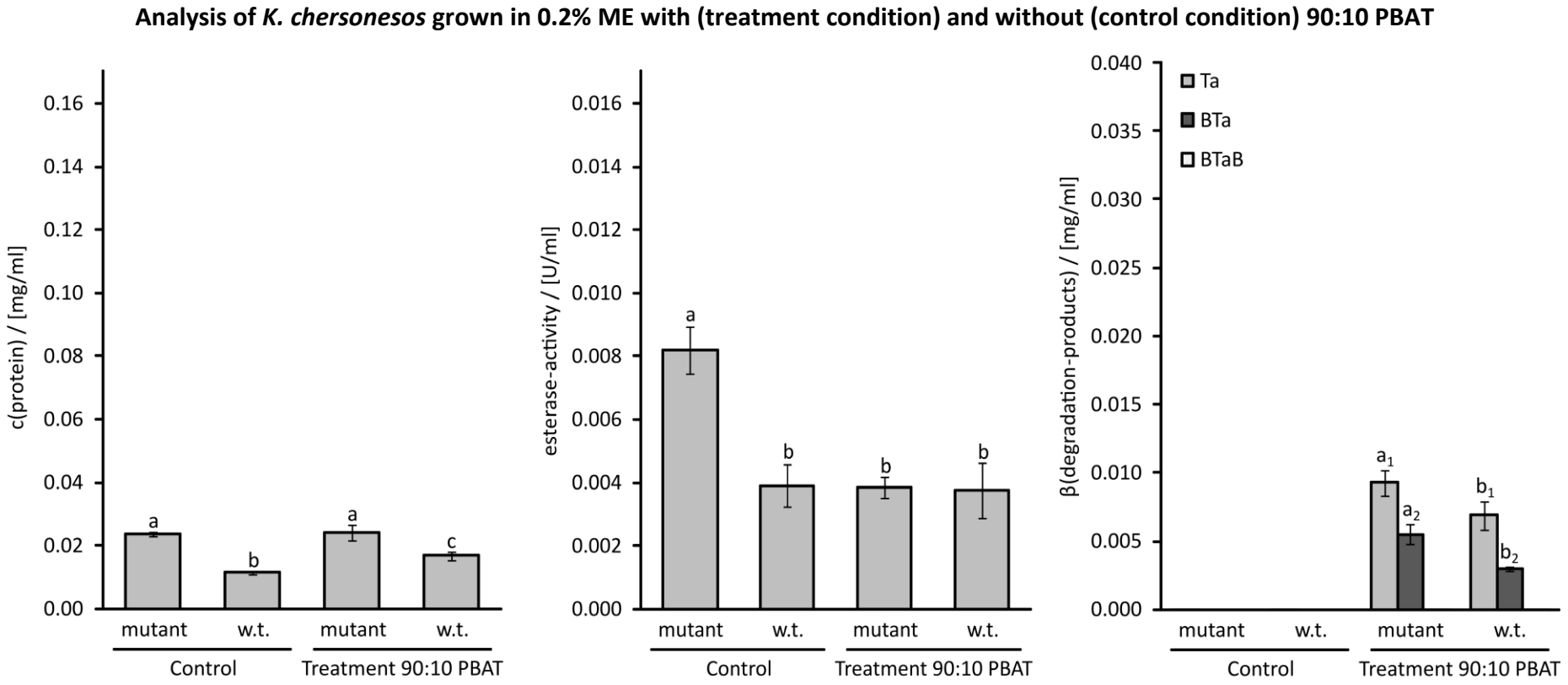

Figure 23: Protein concentration (left), hydrolase activity (middle), and concentration of the PBAT degradation products terephthalic acid (Ta), mono(4-hydroxybutyl) terephthalate (BTa), and bis(4-hydroxybutyl) terephthalate (BTaB) (right) detected in the culture supernatant of *K. chersonesos* wildtype (w.t.) and mutant, grown in 0.2 % ME without glucose for 15 days. Treatment condition: media added with 90:10 poly(1,4-butylene adipate-co-terephthalate) (PBAT; BASF, Ludwigshafen am Rhein, Germany); Control condition: no polymer added to the media.

Protein concentrations (mean ± standard deviation) are equal to 0.02382 ± 0.00066 (mutant, control), 0.01133 ± 0.00066 (w.t., control), 0.0240 ± 0.0025 (mutant, treatment), and 0.0167 ± 0.0013 mg/ml (w.t., treatment). The values of hydrolase activity are equal to 0.00818 ± 0.00074 (mutant, control), 0.00390 ± 0.00067 (w.t., control), 0.00385 ± 0.00033 (mutant, treatment), and 0.00376 ± 0.00087 U/ml (w.t., treatment). Terephthalic acid concentrations are equal to 0.00928 ± 0.00093 (mutant, treatment), 0.0069 ± 0.0011 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control). Mono(4-hydroxybutyl) terephthalate concentrations are equal to 0.00548 ± 0.00071 (mutant, treatment), 0.00298 ± 0.00014 mg/ml (w.t., treatment), and were not detected in both control conditions (mutant, control and w.t., control). Bis(4-hydroxybutyl) terephthalate concentrations were not detected in any of the conditions.

As for the experiments with optimal media, the cultures supernatants were concentrated and the secretome was analysed with SDS-PAGE and subsequent silver staining to investigate variations in protein secretion.

Secretomics of *K. chersonesos* grown in 0.2% ME with (treatment condition) and without (control condition) 90:10 PBAT

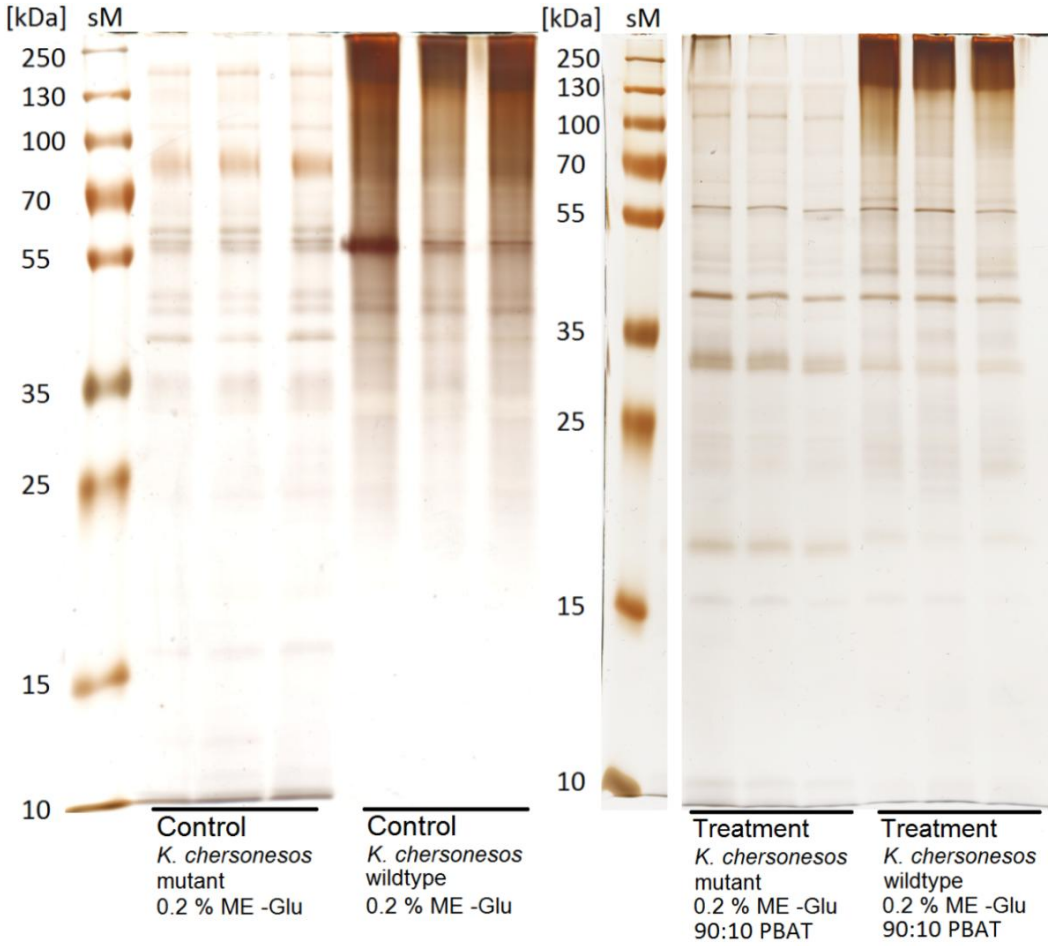

Figure 24: SDS-PAGE analysis of *K. chersonesos* wildtype (w.t.) and mutant, grown in 0.2 % ME without glucose for 15 days (Control) and treated with 90:10 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT; BASF, Ludwigshafen am Rhein, Germany) (Treatment). The stained marker (sM) was run as reference. 1 µg (Control) and 1.16 µg (Treatment) proteins were fractionated per lane and their visualisation was accomplished with silver staining. Both Treatment conditions were run on the same gel as the according marker, but 6 lanes between the marker and Treatment conditions were removed for clarity.

In this experiment it was necessary to load a lower amount of proteins (1 µg for control, 1.16 µg for treatment) per fractionation lane compared to the experiments with 50:50 PBAT (1.5 µg), due to the lower concentration of proteins in the supernatant. As discussed before, the discrepancy in protein amount between the control and treatment samples stems from maximising the amount of loaded proteins within an particular gel to allow the visualisation of protein bands. The comparability of the different samples suffers, this was paramount in order to visualise protein bands, however. Proteins were detected in the range of 11 to 200 kDa. The protein samples of *K. chersonesos* wild type show a high content of melanin, which is spread alongside the protein fractionation and cumbers the detection of proteins, but most of the protein bands are visible despite melanin interference. When comparing Control- and Treatment conditions of *K. chersonesos* wild type, the protein bands at about 40, 30, 17, and 15 kDa are more distinct in Treatment conditions. Based on the staining intensity the melanin content is lower in the samples of *K. chersonesos* wild type treated with 90:10 PBAT. When comparing Control- and Treatment conditions of *K. chersonesos* mutant, the protein bands at about 40 and 17 kDa is more distinct are Treatment conditions and the protein bands at about 200, 85, 63, 57, and 13 kDa are more distinct in Control conditions. When comparing Treatment conditions of *K. chersonesos* wild type and mutant, the protein bands at about 110, 17, and 11 kDa are more distinct for *K. chersonesos* mutant and the protein bands at about 60, 45, 35, and 18 kDa are more distinct for *K. chersonesos* wild type.

6.4 Negative controls

The experiments were performed by addition of 50:50, 60:40, and 90:10 PBAT to 2 % ME and 0.2 % ME without glucose for 15 days, respectively. To conduct negative control experiments, the media were not inoculated with fungal biomass. The objective of these experiments was the assessment of a possible non-biologically induced degradation of the investigated PBAT compositions by components of the media or other factors such as thermal- or radiation mediated processes over the 15 days period.

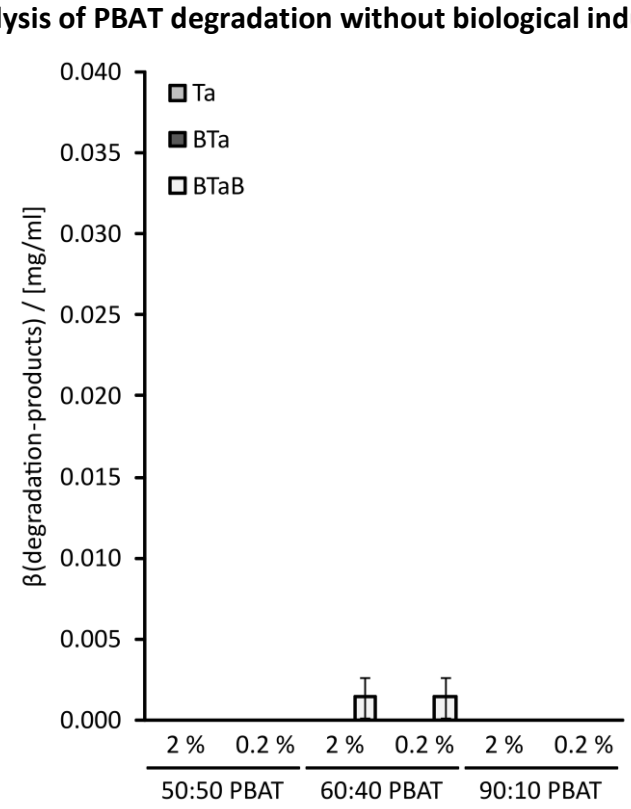

Figure 25: Concentration of the PBAT degradation products terephthalic acid (Ta), mono(4-hydroxybutyl) terephthalate (BTa), and bis(4-hydroxybutyl) terephthalate (BTaB) in 2 % ME and 0.2 % ME without glucose, added with 50:50, 60:40, and 90:10 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT; BASF, Ludwigshafen am Rhein, Germany) for 15 days, respectively.

Terephthalic acid concentrations and mono(4-hydroxybutyl) terephthalate concentrations were not detected in any of the conditions. Bis(4-hydroxybutyl) terephthalate concentrations are equal to 0.0014 ± 0.0012 (2 %, 60:40 PBAT), 0.0014 ± 0.0012 mg/ml (0.2 %, 60:40 PBAT), and were not detected in both conditions with 50:50 PBAT (2 %, 50:50 PBAT and 0.2 %, 50:50 PBAT) and in both conditions with 90:10 PBAT (2 %, 90:10 PBAT

and 0.2 %, 90:10 PBAT). The occurrence of the PBAT degradation product bis(4-hydroxybutyl) terephthalate in both negative control experiments conducted with 60:40 PBAT indicates an unexpected non-biologically induced degradation of 60:40 PBAT, which cannot be observed for the 50:50 and 90:10 PBAT polymers.

6.5 Factors influencing the degradability of PBAT

To compare the degradability of PBAT in the treatment cultures, depending on the chemical composition of the polymer, the availability of nutrients to the fungus, and the cell wall melanisation of *K. chersonesos*, statistical analyses were performed.

The data shown in Figure 26 and Figure 27 are the added molar concentrations of the PBAT degradation products terephthalic acid, mono(4-hydroxybutyl) terephthalate, and bis(4-hydroxybutyl) terephthalate detected in the culture supernatant arithmetically averaged over all treatment conditions sharing the determined parameters. In Figure 26 the results of the treatment conditions with the same culture media and PBAT composition were pooled together. Whereas, in Figure 27 the treatment results sharing media, PBAT, and *K. chersonesos* strain were averaged.

The statistical analysis of the PBAT composition influencing degradability (Figure 26, left) was conducted with one-away analysis of variance (ANOVA) with the Tukey's honest significance difference (HSD) post-hoc test, $\alpha = 0.05$. The statistical analysis of the influence of nutrient availability on PBAT degradation (Figure 26, right) was conducted with one-away analysis of variance (ANOVA) with a post-hoc t-test pairwise comparison, $\alpha = 0.05$, comparing the PBAT degradation product concentrations for each PBAT composition in optimal (2 % ME) and minimal media (0.2 % ME).

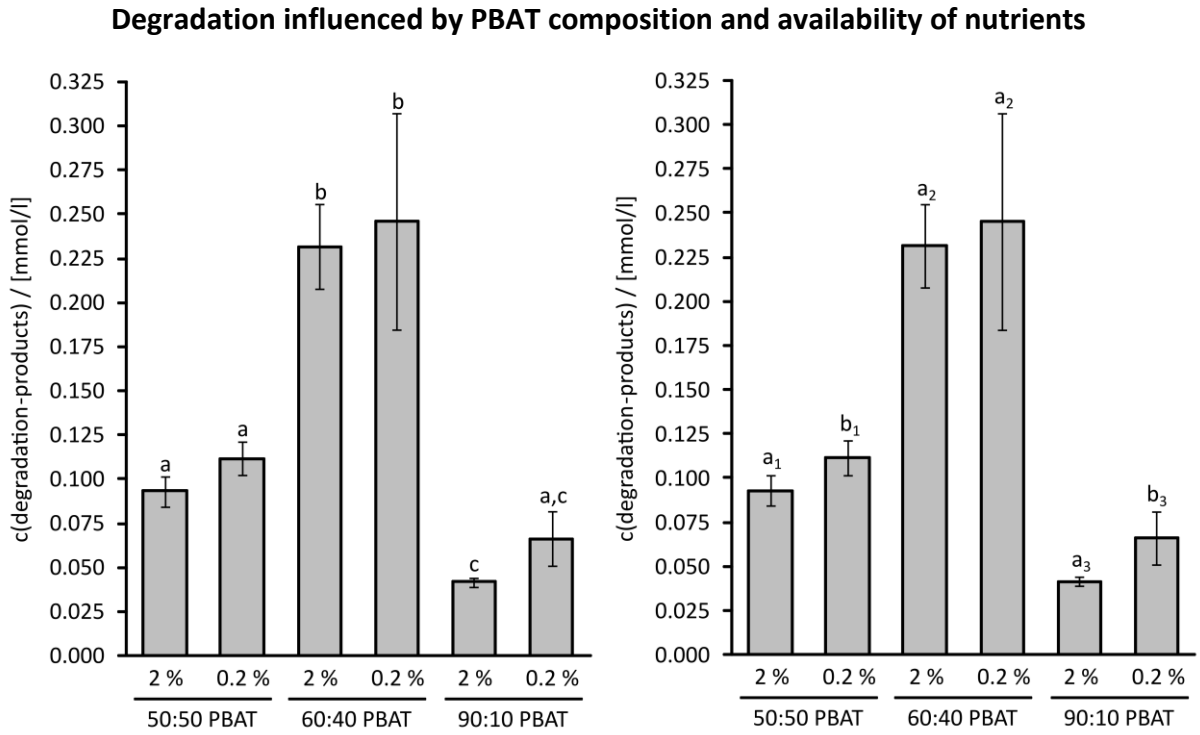

Figure 26: Molar concentrations of all PBAT degradation products terephthalic acid, mono(4-hydroxybutyl) terephthalate, and bis(4-hydroxybutyl) terephthalate detected in the culture supernatant of *K. chersonesos* grown in 2 % ME and 0.2 % ME without glucose, in presence of 50:50, 60:40, and 90:10 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT), respectively. The degradability of the different PBAT compositions was compared by an ANOVA with Tukey's HSD post-hoc test, α = 0.05 (left graph). The comparison of the PBAT degradability depending on the nutrient availability was performed by an ANOVA with a post-hoc t-test pairwise comparison, α = 0.05 (right graph).

PBAT degradation product molar concentrations (mean ± standard deviation) for both graphs are equal to 0.0932 ± 0.0085 (2 %, 50:50 PBAT), 0.1118 ± 0.0095 (0.2 %, 50:50 PBAT), 0.232 ± 0.024 (2 %, 60:40 PBAT), 0.246 ± 0.061 (0.2 %, 60:40 PBAT), 0.0418 ± 0.0028 (2 %, 90:10 PBAT), and 0.066 ± 0.015 mmol/l (0.2 %, 90:10 PBAT).

The influence of the *K. chersonesos* cell wall melanisation on the ability to degrade PBAT was assessed with another statistical analysis, comparing the PBAT degradation product molar concentrations in treatment cultures for the wild type and mutant strains. The statistical analysis of the influence of nutrient availability on PBAT degradation (Figure 27) was conducted with one-away analysis of variance (ANOVA) with a post-hoc t-test pairwise comparison, α = 0.05.

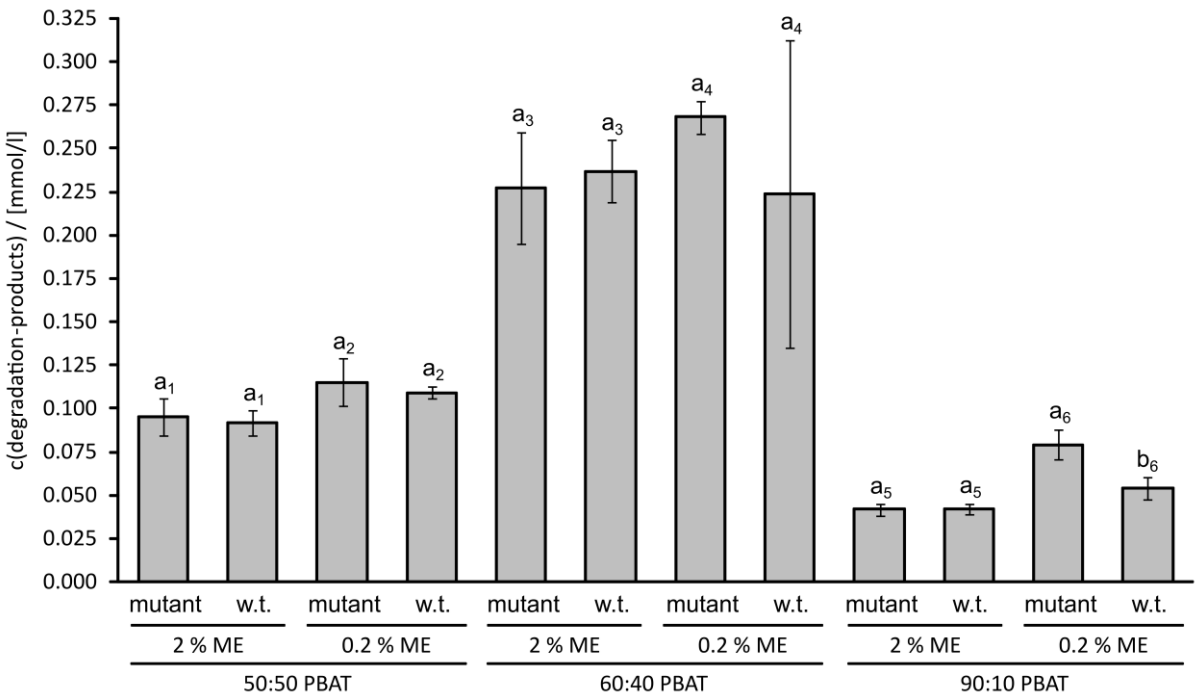

Figure 27: Concentrations of all PBAT degradation products terephthalic acid, mono(4-hydroxybutyl) terephthalate, and bis(4-hydroxybutyl) terephthalate detected in the culture supernatant of *K. chersonesos* wild type (w.t.) and mutant grown in 2 % ME and 0.2 % ME without glucose, in presence of 50:50, 60:40, and 90:10 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT), respectively.

PBAT degradation product molar concentrations (mean ± standard deviation) are equal to 0.095 ± 0.011 (mutant, 2 %, 50:50 PBAT), 0.0917 ± 0.0074 (w.t., 2 %, 50:50 PBAT), 0.115 ± 0.014 (mutant, 0.2 %, 50:50 PBAT), 0.1089 ± 0.0032 (w.t., 0.2 %, 50:50 PBAT), 0.227 ± 0.032 (mutant, 2 %, 60:40 PBAT), 0.237 ± 0.018 (w.t., 2 %, 60:40 PBAT), 0.2678 ± 0.0092 (mutant, 0.2 %, 60:40 PBAT), 0.224 ± 0.089 (w.t., 0.2 %, 60:40 PBAT), 0.0415 ± 0.0033 (mutant, 2 %, 90:10 PBAT), 0.0421 ± 0.0029 (w.t., 2 %, 90:10 PBAT), 0.0789 ± 0.0086 (mutant, 0.2 %, 90:10 PBAT), and 0.0538 ± 0.0062 mmol/l (w.t., 0.2 %, 90:10 PBAT).

7 Discussion

The black fungus *Knufia chersonesos* is able to degrade milled poly(1,4-butylene adipate-*co*-terephthalate) as it is experimentally shown in the extent of this thesis. The factors potentially influencing the degradability of PBAT, such as the chemical composition of the polymer, the content of nutrients in the medium, and the role of fungal melanin, are investigated.

7.1 Choice of experimental conditions

As preparation for the conducted experiments with *K. chersonesos* and poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) the best media for fungal growth with the accompanied protein secretion and avoidance of interfering melanin in the medium has been determined. The results of feasibility studies are shown in "9.2 Data" (Table 12 – Table 16).

In the experimental conditions with optimal media (2 % malt extract media, Figure 34) and those with sub-optimal media (2 % malt extract media without glucose, Figure 32), a relatively high protein secretion, hydrolase activity, and concentration of PBAT degradation products is observed in the culture supernatant. However, these conditions additionally feature a high melanin content in wild type culture supernatant, which renders the analyses cumbersome.

In the experimental conditions with minimal media (0.2 % malt extract medium without glucose, Figure 33), a low melanin content, compared with optimal and sub-optimal media conditions, is observed in the wild type culture supernatant. However, the fungal biomass growth, the accompanied protein secretion, and the hydrolase activity measured in the supernatant are decreased, but not the concentration of PBAT degradation products in treatment conditions.

None of the tested media is able to combine a high protein secretion with a low melanin content in the supernatant. Therefore, the experimental conditions with 2 % malt extract medium and 0.2 % malt extract medium without glucose have been chosen to be used for the poly(1,4-butylene adipate-*co*-terephthalate) degradation experiments in order to use both optimal and minimal media. This choice enables the assessment of the fungal proteome and the response to the presence of PBAT with regards to nutrient availability.

7.2 PBAT influence on the release of melanin into the culture medium

Interestingly, a lower content of melanin is observed for all experimental conditions, where *K. chersonesos* wild type is treated with 50:50 PBAT (Figure 28, Figure 29, and Figure 32 – Figure 34) compared to the according control conditions of *K. chersonesos* wild type. No such effect is exhibited in presence of the 60:40 and 90:10 compositions of PBAT (Figure 30, Figure 31). However, this observance is not based on photometrical measurements, but rather on visual observations. The analysed protein secretion is not significantly affected by the treatment with 50:50 PBAT (Figure 13, Figure 15). A positive correlation of this effect can be observed for the hydrolase activity of the supernatant in minimal media (Figure 15), however not in optimal media (Figure 13). Unfortunately, the cause of the melanin content reduction in the supernatant remains elusive, as it is not associated with aromatic PBAT degradation products present in the medium, which might hamper the growth of *K. chersonesos*.[136] The concentration of terephthalic acid is highest in *K. chersonesos* wild type cultures treated with 60:40 PBAT (Figure 17, Figure 19), where no reduction of melanin content is observed between control and treatment conditions.

7.3 Hydrolase activity

7.3.1 Hindrance to hydrolase activity determination

The hydrolase activity in the culture supernatant of the experimental conditions with 60:40 PBAT (Figure 17) and 90:10 PBAT (Figure 21) in 2 % malt extract medium has not been possible to assess using hydrolase activity assay. The absorptions measured with the plate reader instrument fluctuate around the starting values over the course of the kinetic measurement. As there is no increase of absorption over time, hydrolase activity can not be experimentally verified in the supernatant. In contrast, the blank with only substrate and buffer added to the well, remains constant as it is intended to. Hence, the components interfering with the hydrolase activity assay should originate from the samples themselves and are not caused by the used reagents and the instrument. Despite no hydrolase activity can be detected by means of the activity assay, the presence of terephthalic acid and mono(4-hydroxybutyl) terephthalate, as products of PBAT degradation, has been detected in the supernatant with HPLC analyses. As a consequence, a significant hydrolase activity is expected to prevail in the culture supernatant.

7.3.2 Secretion of hydrolases by *K. chersonesos*

When the hydrolase activity measured in the culture supernatant, is compared between the according control and treatment conditions (Figure 13, Figure 15, Figure 19, and Figure 23), it can be observed that the treatment of the *K. chersonesos* cultures with PBAT does not increase the hydrolase activity in the supernatant. The hydrolase activity is even decreased in minimal media for *K. chersonesos* wild type in the presence of 50:50 PBAT and for the mutant strain in the presence of any PBAT composition compared to the control conditions, respectively. The protein secretion shows no significant decrease from control to treatment conditions, however. These data suggest that the hydrolases of *K. chersonesos* are secreted constitutively and are not up regulated in the presence of PBAT as a potential nutrient source.

7.3.3 Influence of a melanised cell wall on hydrolase secretion

Furthermore, if the hydrolase activity in the culture supernatant of *K. chersonesos* wild type and *K. chersonesos* mutant for each growth condition are compared (Figure 13, Figure 15, Figure 19, and Figure 23), it can be observed that the supernatant of the wild type strain exhibits a significantly lower hydrolase activity. As the main difference between these two fungal strains is the melanisation of their cell wall, the distinction in secretion of enzymes with hydrolase activity should be attributable to mainly this feature, which might hinder an equal secretion of exoenzymes. For certain conditions, namely in treatment conditions with 60:40 and 90:10 PBAT in 0.2 % malt extract medium without glucose, the hydrolase activity between wild type and mutant strain is comparable. In this particular case, the low amount of nutrients might be the limiting factor rather than the melanin content of the fungal cell wall.

7.4 PBAT degradation

7.4.1 Degradability of PBAT depending on the chemical composition

K. chersonesos is able to degrade PBAT to varying degrees, depending on the chemical composition of PBAT and the experimental conditions. With the data from HPLC measurements of the PBAT degradation products (Figure 13, Figure 15, Figure 17, Figure 19, Figure 21, and Figure 23), a conclusion for the differential degradability of the experimentally investigated PBAT compositions by *K. chersonesos* can be drawn. By

comparison of the added molar concentrations of all detected aromatic PBAT degradation products (Figure 26, left), as indicator on how much polymer was degraded in a 15 days period, 60:40 PBAT is the best degraded polyester composition in both optimal and minimal media. In optimal media the degradation rate of 50:50 PBAT is 40 % and 90:10 PBAT is 18 % of the degradation rate of 60:40 PBAT over a 15 day period. In minimal media the degradation rate of 50:50 PBAT is 46 % and 90:10 PBAT is 27 % of the degradation rate of 60:40 PBAT.

The higher rate of polyester degradation for 60:40 PBAT is significant, even without the non-biologically induced degradation. The PBAT degradation product bis(4-hydroxybutyl) terephthalate, which has been detected in both negative control experiments (Figure 25) conducted with 60:40 PBAT, indicates a non-biologically induced degradation of only this PBAT composition. For the 50:50 and 90:10 PBAT no such polyester degradation can be observed. The 60:40 PBAT degradation, not induced by *K. chersonesos*, may be caused by an inherent enzymatic activity of components in the culture media or by thermal- and radiation mediated processes.

The comparability of the degradation of PBAT blends with different chemical compositions solely based on the abundance of the aromatic PBAT degradation products terephthalic acid, mono(4-hydroxybutyl) terephthalate, and bis(4-hydroxybutyl) terephthalate is limited, though. The reason for this limitation of comparability is the fact that the release of aromatic degradation products is dependent on the composition of PBAT. In a complete hydrolysis or a theoretical hydrolysis independent from the polyester structure, the release of aliphatic and aromatic compounds from PBAT would be in the same ratios as the composition of the polyester. In such a case, due to molecular structure of the polymer blends, the release of aromatic degradation products from 90:10 and 60:40 PBAT would be 80 % or 20 % lower than that of 50:50 PBAT, respectively. The ratios are describing the adipate : terephthalate composition of PBAT.[109]

The hydrolysis of aliphatic-aromatic copolyesters starts in aliphatic and amorphous regions of the polymer and then proceeds to the more aromatic and crystalline regions.[106,107] The beginning of the copolyester degradation process can be followed by the measurement of the aromatic PBAT degradation products in a comparable manner.

Additionally, for the comparison of different experimental conditions with identical chemical composition of PBAT, the abundance of the aromatic PBAT degradation products is a adequate indicator for polyester degradation.

7.4.2 Degradability of PBAT depending on the experimental conditions

The comparison of PBAT degradation data, considering the experimental conditions (Figure 26, right), yields the conclusion that *K. chersonesos* degrades 50:50 PBAT at 20 % and 90:10 PBAT at 59 % higher rates in minimal media than in optimal media, respectively. For the 60:40 composition of PBAT no significant alteration of the PBAT degradation can be verified in regards of experimental conditions.

When analysing the data to investigate for an influence of the cell wall melanisation in the *K. chersonesos* strain on the degradation rate of PBAT, no significant pattern occurs (Figure 27). Solely at the degradation of 90:10 PBAT in minimal media, *K. chersonesos* mutant exhibits a 47 % higher degradation rate than the wild type strain.

7.5 SDS-PAGE analyses of the secretome

The protein diversity secreted by *K. chersonesos* into the surrounding medium is overall not influenced by the treatment with PBAT (Figure 14, Figure 16, Figure 18, Figure 20, Figure 22, and Figure 24), but by the nutrient content of the culture medium. In both the control and treatment conditions the secretomes of *K. chersonesos* show a greater diversity in optimal media (2% malt extract medium) than in minimal media (0.2 % malt extract medium without glucose) independent of the method of visualisation.

Also, the results of the experiments with 50:50 PBAT (Figure 14, Figure 16) show, compared with the experimental conditions with 60:40 and 90:10 PBAT (Figure 18, Figure 20, Figure 22, and Figure 24), that the protease inhibitor, added before sample concentration, reduces the protein degradation of the exoenzymes. Hence less protein fragment bands are present in the range 10 – 25 kDa.

7.5.1 Differential protein secretion in the *K. chersonesos*

The protein size of hydrolase enzymes, including cutinases, esterases, and lipases, generally lies between 30 and 70 kDa, as it has been reported in various sources.[66,72,168–171,83,85,88,138,164–167] Therefore, this range is given increased attention in the unspecific visualisation of protein bands realised with silver- and SYPRO staining techniques.

In the experimental conditions with 50:50 PBAT in 2 % malt extract medium (Figure 14) with silver staining the band at about 50 kDa is more distinct in treatment conditions for *K. chersonesos* mutant. With SYPRO staining the band at about 40 kDa is more distinct in treatment conditions for *K. chersonesos* mutant, the band at about 27 kDa is only more distinct in the wild type strain, and the band at about 58 kDa is more distinct in both strains compared to the control conditions. In 0.2 % malt extract medium without glucose (Figure 16) with silver staining the band at about 27 and 28 kDa is more distinct in treatment conditions for *K. chersonesos* mutant. With SYPRO staining no bands are more distinct in treatment conditions for *K. chersonesos*.

In the experimental conditions with 60:40 PBAT in 2 % malt extract medium (Figure 18) the band at about 40 kDa is more distinct in treatment conditions for *K. chersonesos* wild type and mutant and the band at about 50 kDa is only more distinct in the wild type strain. In 0.2 % malt extract medium without glucose (Figure 20) the band at about 30 kDa is more distinct in treatment conditions for *K. chersonesos* wild type and mutant and the band at about 40 kDa is only more distinct in the wild type strain.

In the experimental conditions with 90:10 PBAT in 2 % malt extract medium (Figure 22) the band at about 40 kDa is more distinct in treatment conditions for *K. chersonesos* wild type and mutant. In 0.2 % malt extract medium without glucose (Figure 24) the band at about 40 kDa is more distinct in treatment conditions for *K. chersonesos* wild type and mutant and the band at about 30 kDa is only more distinct in the wild type strain.

Concluding these results, the intensity of the protein band at about 40 kDa can be observed to be affected positively by the presence of PBAT in every chemical composition, compared to the according control conditions. The intensity of the bands at about 58, 28, and 27 kDa is only increased by the presence of 50:50 PBAT. The abundance of the proteins at about 50 kDa is increased by the presence of 50:50 and 60:40 PBAT, while the protein amount at about 30 kDa is increased by the presence of 60:40 and 90:10 PBAT.

While the hydrolase activity of the secretome as a whole, determined by the hydrolase activity assay, is not significantly influenced by the presence of PBAT, the secretion of certain types of proteins is increased in treatment conditions. This can be seen in the differential banding patterns of the secretomes at the mentioned protein sizes.

A shotgun-proteomics approach is carried out to give more information about the identity and differential secretion of proteins by *K. chersonesos* growing in control conditions and presence of poly(1,4-butylene adipate-co-terephthalate).

7.5.2 Interference of melanin with protein visualisation

As it can be seen in all examined conditions with optimal media and *K. chersonesos* wild type (Figure 14, Figure 18, and Figure 22), the fungal melanin, released into the culture medium alongside the secretome as sole melanin molecules or melanin granules[3,122], is stained by silver staining as well. Furthermore, melanin is also directly attached to the extracellular proteins.[173] Due to the interference of melanin with SYPRO staining, it is speculated that the protein-bound melanin takes away the binding space for the SYPRO dye, which causes the interference with this method of protein visualisation. The interference of melanin with both protein visualisation methods makes analysis of the differential protein secretion cumbersome.

7.6 Possible future applications

The black fungus *Knufia chersonesos* is able to degrade milled poly(1,4-butylene adipate-*co*-terephthalate) of varying chemical compositions in liquid cultures with different content of nutrients.

The degradation process of poly(1,4-butylene adipate-*co*-terephthalate) is hypothesised to be achieved by the primary cleaving of the polyester into oligomers and monomers through hydrolytic enzymes secreted into the medium by the *K. chersonesos*. The secondary degradation is done by absorption of the monomers adipic acid, butandiol, and terephthalic acid into the fungal biomass, usage as nutrient source for the metabolism, and finally mineralisation. It was hypothesised by Kasuya *et al.* that the degradation of PBAT and the uptake of the aliphatic monomers adipic acid and butandiol is a relatively fast process and the uptake or metabolisation of the aromatic monomer terephthalic acid is the limiting kinetic step in fungal PBAT degradation.[109] Therefore terephthalic acid is accumulated in the medium upon PBAT degradation.

7.6.1 PBAT waste management and recycling

Due to the ability of *K. chersonesos* to degrade PBAT, management including recycling of waste PBAT, stemming from 50:50 PBAT commercially distributed by BASF[174], would be

possible. In addition to that, *K. chersonesos* could be tested for the ability to degrade other polyester materials not known for biodegradability.

K. chersonesos and the secreted exoenzymes, utilised for PBAT degradation, could be applicable by the means of recycling the partly fossil-based polyester and surface functionalisation of PBAT.[96,174] When *K. chersonesos* is used in culture to degrade waste PBAT, terephthalic acid is accumulated in the medium and could be recycled for polymers based on terephthalic acid, such as poly (ethylene terephthalate) (PET).[175]

With the exoenzymes secreted by *K. chersonesos* the degradation of waste PBAT could be performed without having to sacrifice the aliphatic components of the polyester in the process, as these are uptaken and used as nutrients by the fungus. To realise this second option, supernatant of an actively growing and an upscaled culture of *K. chersonesos* would have to be added to the waste PBAT in order to induce degradation. When the transfer of culture supernatant is not feasible, due to possible contaminations with the black fungus or insufficient production of enzymes, another possibility would be the identification and production of hydrolytic fungal exoenzymes by heterologous expression, as *K. chersonesos* is not suitable for high protein production attributable to its slow growth rate. This however, has not yet been experimentally investigated, but is already in planning phase.

7.6.2 PBAT surface functionalisation

As briefly mentioned before, hydrolytic enzymes from culture supernatant or heterologous expression could be used as highly specific molecular tools for the biochemical surface functionalisation of polyester materials, while retaining the advantageous bulk polymer properties.[96]

7.6.3 Future research on black fungi

Extremotolerant or extremophilic organism have long been neglected by the scientific community as rare and exotic colonisers of biological niches.[45] Whilst, precisely this trait renders these organisms as interesting objects of scientific research and treasure boxes of protein designs capable of functioning in unfavourable conditions. As all biological functions mediated by proteins have to be processed in organisms tolerating extreme environments as well, the proteins were adapted to these environmental conditions.[176] Therefore, researching proteins used by extremotolerant or extremophilic organisms, could enable researchers to broaden the range of conditions a specific protein function can be used in.

Especially fungi should be prioritised in this research, as their proteins used for the digestion of nutrients are secreted directly into their surroundings and therefore are fully exposed to the environmental conditions.[41] This would increase the diversity of the conditions a specific enzyme activity can be used in, which is generally advantageous for industrial application. In biotechnological process design less attention has to be focused on altering the process conditions to be compatible with the enzymes used.

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9 Supplementary information

9.1 Visuals of PBAT degradation experiments with *K. chersonesos*

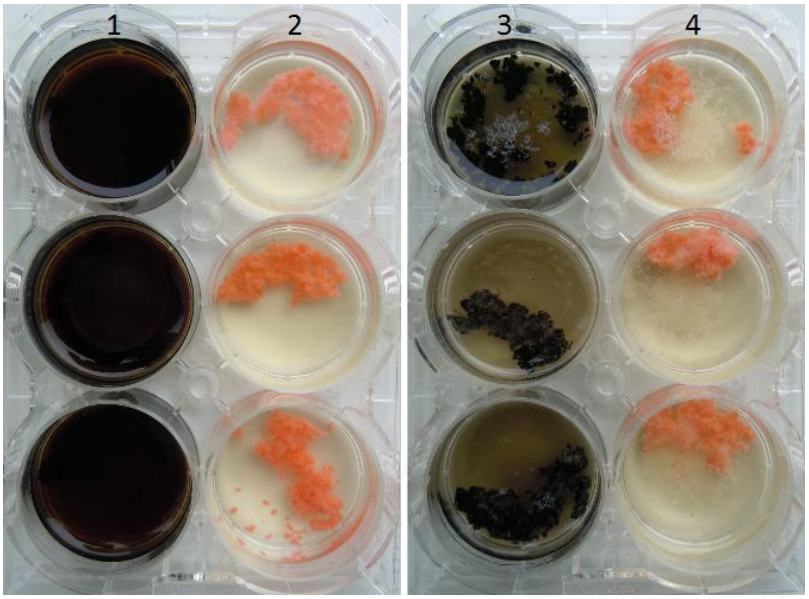

Figure 28: Control condition of *K. chersonesos* wild type (1) and mutant (2) in 2 % malt extract medium for 15 days and treatment condition of *K. chersonesos* wild type (3) and mutant (4) in 2 % malt extract medium treated with 50:50 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT, EcoFlex Blend C1200, 68818836WO, 500 µm maximum particle size) for 15 days.

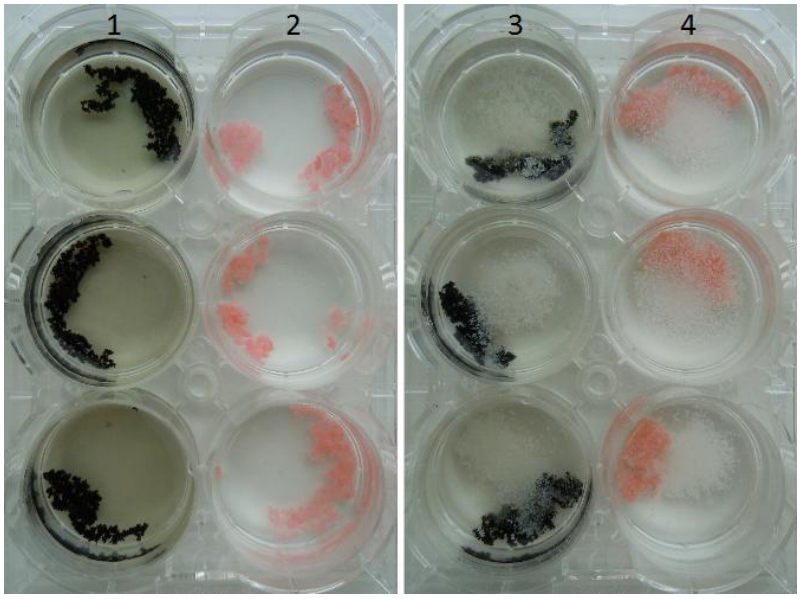

Figure 29: Control condition of *K. chersonesos* wild type (1) and mutant (2) in 0.2 % malt extract medium without glucose for 15 days and treatment condition of *K. chersonesos* wild type (3) and mutant (4) in 0.2 % malt extract medium without glucose treated with 50:50 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT, EcoFlex Blend C1200, 68818836WO, 500 µm maximum particle size) for 15 days.

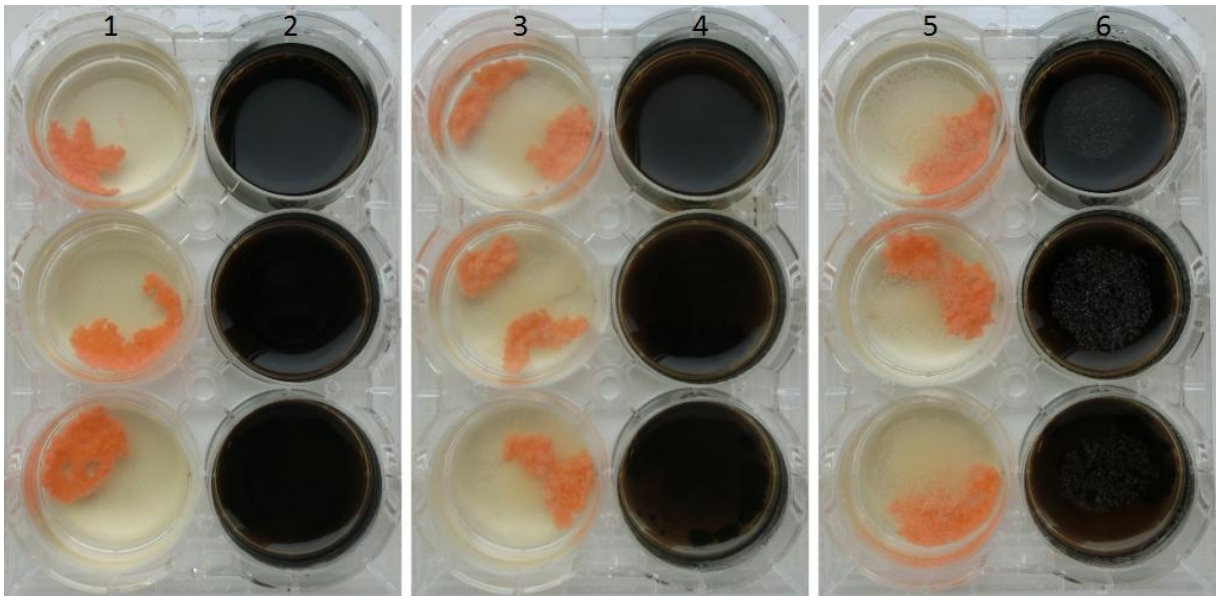

Figure 30: Control condition of *K. chersonesos* wild type (2) and mutant (1) in 2 % malt extract medium for 15 days, treatment condition of *K. chersonesos* wild type (4) and mutant (3) in 2 % malt extract medium treated with 60:40 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) for 15 days, and treatment condition of *K. chersonesos* wild type (6) and mutant (5) in 2 % malt extract medium treated with 90:10 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) for 15 days.

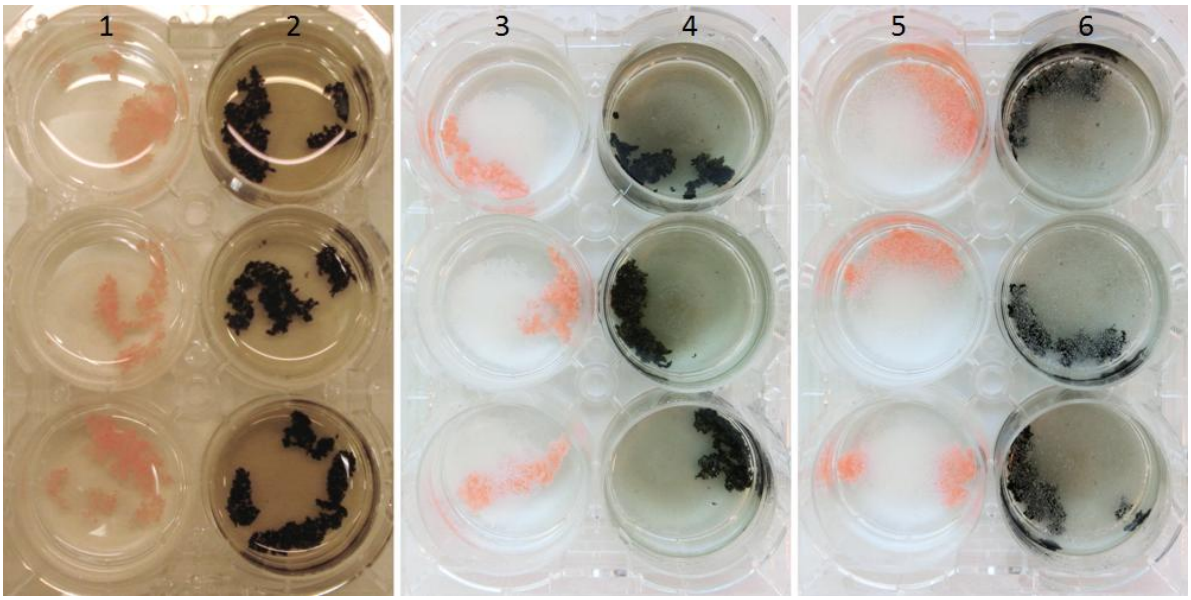

Figure 31: Control condition of *K. chersonesos* wild type (2) and mutant (1) in 0.2 % malt extract medium without glucose for 15 days, treatment condition of *K. chersonesos* wild type (4) and mutant (3) in 0.2 % malt extract medium without glucose treated with 60:40 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) for 15 days, and treatment condition of *K. chersonesos* wild type (6) and mutant (5) in 0.2 % malt extract medium without glucose treated with 90:10 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) for 15 days.

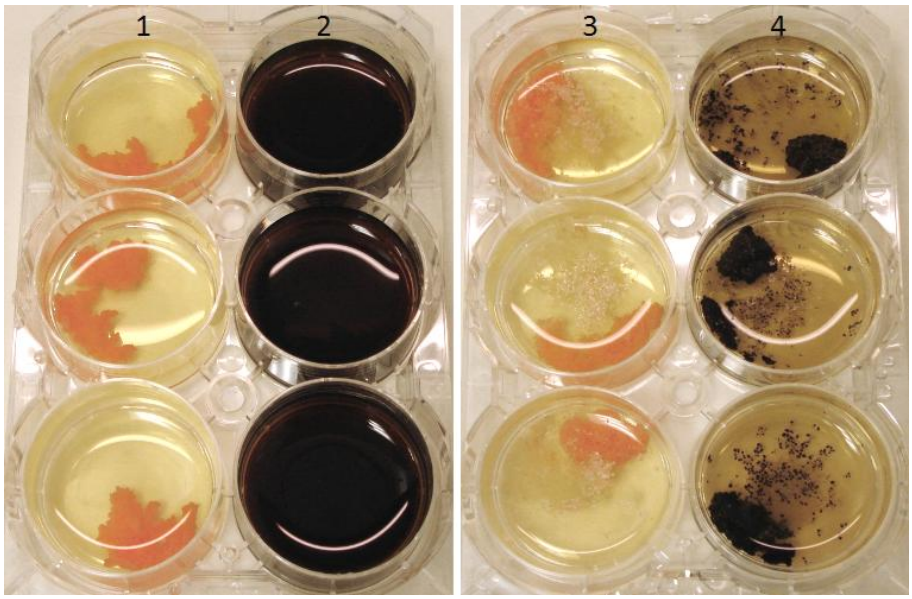

Figure 32: Control condition of *K. chersonesos* wild type (2) and mutant (1) in 2 % malt extract medium for 15 days and treatment condition of *K. chersonesos* wild type (4) and mutant (3) in 2 % malt extract medium treated with 50:50 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) for 15 days.

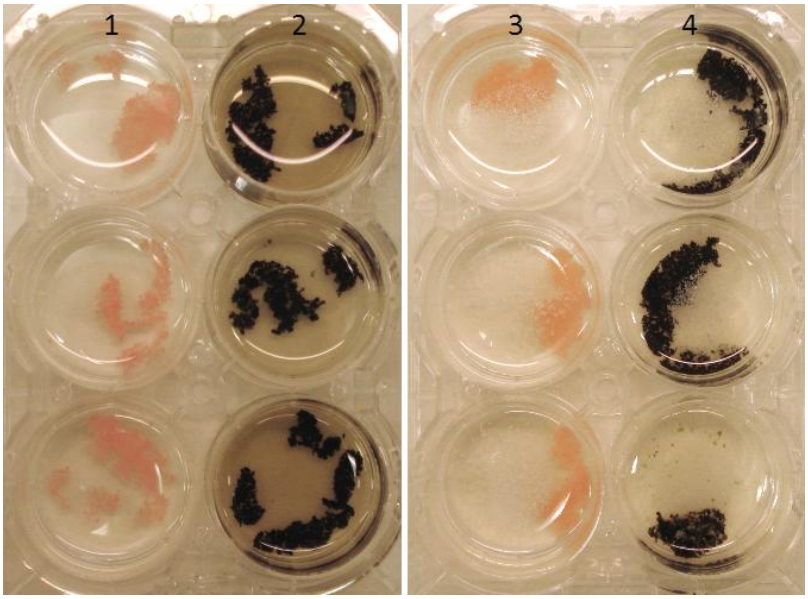

Figure 33: Control condition of *K. chersonesos* wild type (2) and mutant (1) in 0.2 % malt extract medium without glucose for 15 days and treatment condition of *K. chersonesos* wild type (4) and mutant (3) in 0.2 % malt extract medium without glucose treated with 50:50 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) for 15 days.

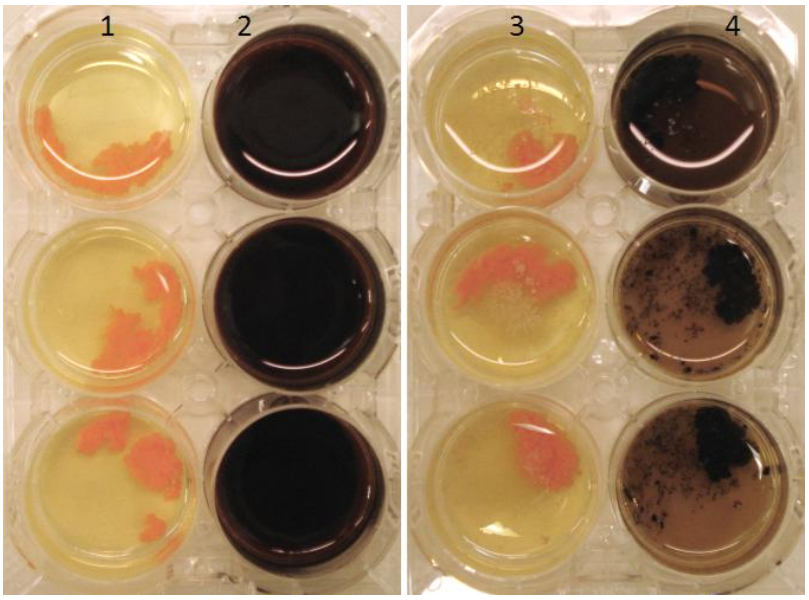

Figure 34: Control condition of *K. chersonesos* wild type (2) and mutant (1) in 2 % malt extract without glucose medium for 15 days and treatment condition of *K. chersonesos* wild type (4) and mutant (3) in 2 % malt extract medium without glucose treated with 50:50 poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) for 15 days.

9.2 Data

Table 12: Data of the protein concentration measurements. The concentrated samples marked in dark gray were diluted 1/10 before measurement and concentrated samples marked in light gray were diluted 1/5 before measurement.

Condition, Medium, Fungus, PBAT	Protein concentration						
	Concentrated sample / [µg/µl]				β(Protein)	Mean	Std
	Read 1	Read 2	Read 3	Mean	[µg/µl]	[µg/µl]	[µg/µl]
Control, 2 % ME-Glu, K. chersonesos mutant	0.858	0.856	0.859	**0.858**	0.0858	**0.0767**	**0.0086**
	0.690	0.683	0.688	**0.687**	0.0687		
	0.755	0.756	0.760	**0.757**	0.0757		
Control, 2 % ME-Glu, K. chersonesos wild type	0.082	0.080	0.079	**0.802**	0.0802	**0.0808**	**0.0025**
	0.080	0.079	0.078	**0.787**	0.0787		
	0.085	0.083	0.083	**0.835**	0.0835		
Treatment, 2 % ME-Glu, K. chersonesos mutant, PBAT 50:50	1.100	1.090	1.090	**1.093**	0.1093	**0.1028**	**0.0057**
	0.995	0.987	0.988	**0.990**	0.0990		
	1.010	1.000	0.993	**1.001**	0.1001		
Treatment, 2 % ME-Glu, K. chersonesos wild type, PBAT 50:50	0.130	0.130	0.129	**1.297**	0.1297	**0.1284**	**0.0033**
	0.127	0.123	0.124	**1.247**	0.1247		
	0.132	0.131	0.130	**1.310**	0.1310		
Control, 0.2 % ME-Glu, K. chersonesos mutant	0.238	0.239	0.242	**0.240**	0.0240	**0.0238**	**0.0007**
	0.243	0.244	0.245	**0.244**	0.0244		
	0.232	0.230	0.231	**0.231**	0.0231		
Control, 0.2 % ME-Glu, K. chersonesos wild type	0.120	0.120	0.120	**0.120**	0.0120	**0.0113**	**0.0006**
	0.110	0.110	0.110	**0.110**	0.0110		
	0.110	0.110	0.110	**0.110**	0.0110		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 50:50	0.152	0.153	0.152	**0.152**	0.0152	**0.0144**	**0.0010**
	0.135	0.134	0.131	**0.133**	0.0133		
	0.146	0.146	0.146	**0.146**	0.0146		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 50:50	0.022	0.022	0.022	**0.110**	0.0110	**0.0114**	**0.0005**
	0.024	0.022	0.022	**0.113**	0.0113		
	0.024	0.024	0.024	**0.120**	0.0120		
Control, 2 % ME, K. chersonesos mutant	0.684	0.689	0.692	**0.688**	0.0688	**0.0734**	**0.0051**
	0.784	0.790	0.792	**0.789**	0.0789		
	0.721	0.728	0.728	**0.726**	0.0726		
Control, 2 % ME, K. chersonesos wild type	0.082	0.082	0.081	**0.815**	0.0815	**0.0833**	**0.0065**
	0.079	0.078	0.077	**0.778**	0.0778		
	0.092	0.091	0.090	**0.905**	0.0905		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 50:50	0.930	0.934	0.936	**0.933**	0.0933	**0.0904**	**0.0027**
	0.884	0.882	0.874	**0.880**	0.0880		
	0.897	0.898	0.900	**0.898**	0.0898		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 50:50	0.142	0.142	0.140	**1.413**	0.1413	**0.1439**	**0.0041**
	0.149	0.149	0.148	**1.487**	0.1487		
	0.142	0.142	0.141	**1.417**	0.1417		
Control, 2 % ME, K. chersonesos mutant	0.741	0.747	0.751	**0.746**	0.0746	**0.0812**	**0.0057**
	0.822	0.852	0.854	**0.843**	0.0843		
	0.841	0.848	0.851	**0.847**	0.0847		
Control, 2 % ME, K. chersonesos wild type	0.063	0.060	0.060	**0.608**	0.0608	**0.0613**	**0.0016**
	0.060	0.060	0.060	**0.600**	0.0600		
	0.064	0.063	0.063	**0.632**	0.0632		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 60:40	0.853	0.857	0.851	**0.854**	0.0854	**0.0951**	**0.0090**
	1.030	1.030	1.030	**1.030**	0.1030		
	0.966	0.972	0.973	**0.970**	0.0970		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 60:40	0.070	0.069	0.069	**0.692**	0.0692	**0.0757**	**0.0057**
	0.079	0.078	0.078	**0.782**	0.0782		
	0.080	0.080	0.080	**0.797**	0.0797		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 90:10	0.807	0.812	0.812	**0.810**	0.0810	**0.0849**	**0.0104**
	0.960	0.967	0.972	**0.966**	0.0966		
	0.766	0.770	0.771	**0.769**	0.0769		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 90:10	0.068	0.068	0.067	**0.673**	0.0673	**0.0636**	**0.0032**
	0.063	0.063	0.060	**0.618**	0.0618		
	0.063	0.063	0.060	**0.617**	0.0617		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 60:40	0.245	0.247	0.251	**0.248**	0.0248	**0.0303**	**0.0052**
	0.348	0.350	0.351	**0.350**	0.0350		
	0.311	0.311	0.312	**0.311**	0.0311		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 60:40	0.198	0.199	0.202	**0.200**	0.0200	**0.0187**	**0.0012**
	0.183	0.184	0.183	**0.183**	0.0183		
	0.175	0.176	0.179	**0.177**	0.0177		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 90:10	0.212	0.214	0.216	**0.214**	0.0214	**0.0240**	**0.0025**
	0.256	0.260	0.274	**0.263**	0.0263		

	0.243	0.243	0.241	**0.242**	0.0242		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 90:10	0.163	0.165	0.170	**0.166**	0.0166	**0.0167**	**0.0013**
	0.154	0.155	0.155	**0.155**	0.0155		
	0.179	0.181	0.184	**0.181**	0.0181		
Control, 2 % ME, K. chersonesos mutant	0.600	0.661	0.645	**0.635**	0.0635	**0.0732**	**0.0133**
	0.673	0.677	0.680	**0.677**	0.0677		
	0.834	0.912	0.906	**0.884**	0.0884		
Control, 2 % ME, K. chersonesos wild type	0.143	0.143	0.142	**1.427**	0.1427	**0.1431**	**0.0047**
	0.150	0.149	0.145	**1.480**	0.1480		
	0.139	0.139	0.138	**1.387**	0.1387		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 50:50 EcoFlex	0.760	0.766	0.770	**0.765**	0.0765	**0.0800**	**0.0075**
	0.745	0.746	0.756	**0.749**	0.0749		
	0.891	0.886	0.881	**0.886**	0.0886		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 50:50 EcoFlex	0.161	0.161	0.159	**1.603**	0.1603	**0.1593**	**0.0009**
	0.161	0.159	0.157	**1.590**	0.1590		
	0.159	0.159	0.158	**1.587**	0.1587		
Control, 0.2 % ME-Glu, K. chersonesos mutant	0.461	0.467	0.469	**0.466**	0.0466	**0.0407**	**0.0069**
	0.325	0.331	0.338	**0.331**	0.0331		
	0.421	0.425	0.424	**0.423**	0.0423		
Control, 0.2 % ME-Glu, K. chersonesos wild type	0.184	0.186	0.187	**0.186**	0.0186	**0.0245**	**0.0054**
	0.292	0.294	0.289	**0.292**	0.0292		
	0.253	0.261	0.259	**0.258**	0.0258		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 50:50 EcoFlex	0.389	0.391	0.393	**0.391**	0.0391	**0.0351**	**0.0081**
	0.404	0.403	0.404	**0.404**	0.0404		
	0.256	0.259	0.257	**0.257**	0.0257		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 50:50 EcoFlex	0.296	0.299	0.299	**0.298**	0.0298	**0.0271**	**0.0031**
	0.236	0.239	0.237	**0.237**	0.0237		
	0.274	0.280	0.278	**0.277**	0.0277		

Table 13: Data of the hydrolase activity measurements

Condition, Medium, Fungus, PBAT	**Hydrolase activity**					
	Technical replicates			**Measurements**	**Mean**	**Std**
	Read 1	**Read 2**	**Read 3**	**[U/ml]**	**[U/ml]**	**[U/ml]**
Control, 2 % ME-Glu, K. chersonesos mutant	0.02090	0.02430	0.02433	0.02318	**0.02242**	**0.00071**
	0.02098	0.02205	0.02228	0.02177		
	0.02233	0.02194	0.02267	0.02231		
Control, 2 % ME-Glu, K. chersonesos wild type	0.01114	0.01296	0.01160	0.01190	**0.01215**	**0.00167**
	0.01487	0.01358	0.01334	0.01393		
	0.01138	0.01116	0.00932	0.01062		
Treatment, 2 % ME-Glu, K. chersonesos mutant, PBAT 50:50	0.01929	0.01971	0.02113	0.02004	**0.02091**	**0.00133**
	0.02318	0.02217	0.02196	0.02244		
	0.02003	0.01970	0.02104	0.02025		
Treatment, 2 % ME-Glu, K. chersonesos wild type, PBAT 50:50	0.00721	0.00595	0.00735	0.00684	**0.00489**	**0.00177**
	0.00315	0.00359	0.00339	0.00337		
	0.00450	0.00455	0.00429	0.00445		
Control, 0.2 % ME-Glu, K. chersonesos mutant	0.00881	0.00739	0.00814	0.00811	**0.00818**	**0.00074**
	0.00742	0.00737	0.00763	0.00747		
	0.00879	0.00899	0.00909	0.00895		
Control, 0.2 % ME-Glu, K. chersonesos wild type	0.00427	0.00434	0.00404	0.00422	**0.00390**	**0.00067**
	0.00290	0.00316	0.00334	0.00314		
	0.00455	0.00440	0.00411	0.00435		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 50:50	0.00494	0.00513	0.00537	0.00515	**0.00554**	**0.00035**
	0.00617	0.00612	0.00516	0.00582		
	0.00597	0.00577	0.00522	0.00565		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 50:50	0.00261	0.00238	0.00246	0.00248	**0.00205**	**0.00040**
	0.00219	0.00184	0.00185	0.00196		
	0.00197	0.00158	0.00156	0.00171		
Control, 2 % ME, K. chersonesos mutant	0.01990	0.01861	0.01715	0.01855	**0.02016**	**0.00170**
	0.02345	0.02006	0.02229	0.02193		
	0.01974	0.02023	0.02001	0.01999		
Control, 2 % ME, K. chersonesos wild type	0.01073	0.01157	0.01130	0.01120	**0.01030**	**0.00079**
	0.00896	0.01134	0.00888	0.00973		
	0.00946	0.00963	0.01085	0.00998		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 50:50	0.01857	0.01603	0.02160	0.01873	**0.01607**	**0.00307**
	0.01758	0.01779	0.01495	0.01677		
	0.01296	0.01392	0.01125	0.01271		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 50:50	0.00250	0.00496	0.00388	0.00378	**0.00391**	**0.00093**
	0.00521	0.00512	0.00434	0.00489		
	0.00157	0.00356	0.00403	0.00305		
Control, 2 % ME, K. chersonesos mutant	-	-	-	-	**-**	**-**
	-	-	-	-		

	-	-	-	-		
Control, 2 % ME, K. chersonesos wild type	-	-	-	-	-	-
	-	-	-	-		
	-	-	-	-		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 60:40	-	-	-	-	-	-
	-	-	-	-		
	-	-	-	-		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 60:40	-	-	-	-	-	-
	-	-	-	-		
	-	-	-	-		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 90:10	-	-	-	-	-	-
	-	-	-	-		
	-	-	-	-		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 90:10	-	-	-	-	-	-
	-	-	-	-		
	0.00385	0.00362	0.00350	0.00366		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 60:40	0.00389	0.00362	0.00355	0.00369	**0.00366**	**0.00003**
	0.00385	0.00357	0.00350	0.00364		
	0.00343	0.00324	0.00305	0.00324		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 60:40	0.00321	0.00279	0.00273	0.00291	**0.00296**	**0.00026**
	0.00274	0.00282	0.00261	0.00272		
	0.00389	0.00409	0.00429	0.00409		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 90:10	0.00385	0.00399	0.00414	0.00399	**0.00385**	**0.00033**
	0.00329	0.00343	0.00373	0.00348		
	0.00401	0.00409	0.00404	0.00404		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 90:10	0.00262	0.00277	0.00296	0.00278	**0.00376**	**0.00087**
	0.00428	0.00454	0.00453	0.00445		
	0.01351	0.01345	0.01312	0.01336		
Control, 2 % ME, K. chersonesos mutant	0.01301	0.01218	0.01220	0.01247	**0.01425**	**0.00236**
	0.01772	0.01679	0.01629	0.01693		
	0.00684	0.00609	0.00590	0.00628		
Control, 2 % ME, K. chersonesos wild type	0.00573	0.00553	0.00512	0.00546	**0.00668**	**0.00147**
	0.00925	0.00819	0.00752	0.00832		
	0.01276	0.01331	0.01331	0.01313		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 50:50 EcoFlex	0.01063	0.01056	0.01116	0.01078	**0.01221**	**0.00125**
	0.01242	0.01275	0.01299	0.01272		
	0.00355	0.00389	0.00395	0.00380		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 50:50 EcoFlex	0.00295	0.00338	0.00337	0.00323	**0.00383**	**0.00061**
	0.00451	0.00468	0.00418	0.00446		
	0.00733	0.00719	0.00707	0.00720		
Control, 0.2 % ME-Glu, K. chersonesos mutant	0.00617	0.00610	0.00612	0.00613	**0.00677**	**0.00056**
	0.00698	0.00706	0.00688	0.00697		
	0.00303	0.00293	0.00293	0.00296		
Control, 0.2 % ME-Glu, K. chersonesos wild type	0.00273	0.00268	0.00273	0.00271	**0.00312**	**0.00050**
	0.00354	0.00372	0.00374	0.00367		
	0.00272	0.00277	0.00311	0.00287		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 50:50 EcoFlex	0.00326	0.00331	0.00345	0.00334	**0.00322**	**0.00031**
	0.00318	0.00346	0.00372	0.00345		
	0.00137	0.00143	0.00142	0.00141		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 50:50 EcoFlex	0.00071	0.00079	0.00088	0.00079	**0.00123**	**0.00038**
	0.00146	0.00149	0.00149	0.00148		

Table 14: Data of the terephthalic acid concentration measurements with HPLC

Condition, Medium, Fungus, PBAT	**Terephthalic acid concentration**			
	c(Ta)	**β(Ta)**	**Mean**	**Std**
	[mmol/l]	**[mg/ml]**	**[mg/ml]**	**[mg/ml]**
	0.0000	0.0000		
Control, 2 % ME-Glu, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME-Glu, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.1159	0.0193		
Treatment, 2 % ME-Glu, K. chersonesos mutant, PBAT 50:50	0.1040	0.0173	**0.0184**	**0.0010**
	0.1118	0.0186		
	0.1148	0.0191		
Treatment, 2 % ME-Glu, K. chersonesos wild type, PBAT 50:50	0.0984	0.0163	**0.0175**	**0.0014**
	0.1021	0.0170		
	0.0000	0.0000		
Control, 0.2 % ME-Glu, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		

Control, 0.2 % ME-Glu, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 50:50	0.1456	0.0242	**0.0257**	**0.0018**
	0.1667	0.0277		
	0.1516	0.0252		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 50:50	0.1211	0.0201	**0.0206**	**0.0015**
	0.1344	0.0223		
	0.1170	0.0194		
Control, 2 % ME, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 50:50	0.1020	0.0169	**0.0171**	**0.0009**
	0.1091	0.0181		
	0.0985	0.0164		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 50:50	0.0905	0.0150	**0.0155**	**0.0007**
	0.0909	0.0151		
	0.0985	0.0164		
Control, 2 % ME, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 60:40	0.2311	0.0384	**0.0340**	**0.0038**
	0.1947	0.0323		
	0.1889	0.0314		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 60:40	0.1707	0.0284	**0.0318**	**0.0030**
	0.2013	0.0334		
	0.2028	0.0337		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 90:10	0.0384	0.0064	**0.0067**	**0.0004**
	0.0427	0.0071		
	0.0391	0.0065		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 90:10	0.0378	0.0063	**0.0063**	**0.0005**
	0.0349	0.0058		
	0.0409	0.0068		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 60:40	0.1839	0.0306	**0.0295**	**0.0010**
	0.1774	0.0295		
	0.1722	0.0286		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 60:40	0.0901	0.0150	**0.0265**	**0.0103**
	0.2088	0.0347		
	0.1793	0.0298		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 90:10	0.0495	0.0082	**0.0093**	**0.0009**
	0.0582	0.0097		
	0.0600	0.0100		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 90:10	0.0458	0.0076	**0.0069**	**0.0011**
	0.0441	0.0073		
	0.0340	0.0056		
Negative Control, 2 % ME, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 0.2% ME-Glu PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 2 % ME, PBAT 60:40	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 0.2% ME-Glu PBAT 60:40	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 2 % ME, PBAT 90:10	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 0.2% ME-Glu PBAT 90:10	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos wild type	0.0008	0.0001	**0.0001**	**0.0001**
	0.0000	0.0000		

	0.0007	0.0001		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 50:50 EcoFlex	0.0869	0.0144	**0.0157**	**0.0018**
	0.0900	0.0150		
	0.1071	0.0178		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 50:50 EcoFlex	0.0921	0.0153	**0.0152**	**0.0012**
	0.0842	0.0140		
	0.0989	0.0164		
Control, 0.2 % ME-Glu, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 0.2 % ME-Glu, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 50:50 EcoFlex	0.1061	0.0176	**0.0191**	**0.0023**
	0.1075	0.0179		
	0.1306	0.0217		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 50:50 EcoFlex	0.0968	0.0161	**0.0167**	**0.0007**
	0.1048	0.0174		
	0.1000	0.0166		

Table 15: Data of the mono(4-hydroxybutyl) terephthalate concentration measurements with HPLC

Condition, Medium, Fungus, PBAT	**Mono(4-hydroxybutyl) terephthalate concentration**			
	c(BTa)	**β(BTa)**	**Mean**	**Std**
	[mmol/l]	**[mg/ml]**	**[mg/ml]**	**[mg/ml]**
Control, 2 % ME-Glu, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME-Glu, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME-Glu, K. chersonesos mutant, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME-Glu, K. chersonesos wild type, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 0.2 % ME-Glu, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 0.2 % ME-Glu, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 60:40	0.0324	0.0077	**0.0052**	**0.0022**
	0.0166	0.0039		
	0.0169	0.0040		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 60:40	0.0441	0.0105	**0.0107**	**0.0002**
	0.0446	0.0106		

	0.0456	0.0109		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 90:10	0.0004	0.0001	**0.0003**	**0.0002**
	0.0024	0.0006		
	0.0013	0.0003		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 90:10	0.0041	0.0010	**0.0010**	**0.0000**
	0.0044	0.0010		
	0.0041	0.0010		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 60:40	0.0740	0.0176	**0.0211**	**0.0037**
	0.0871	0.0208		
	0.1049	0.0250		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 60:40	0.0312	0.0074	**0.0148**	**0.0070**
	0.0654	0.0156		
	0.0897	0.0214		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 90:10	0.0197	0.0047	**0.0055**	**0.0007**
	0.0238	0.0057		
	0.0255	0.0061		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 90:10	0.0119	0.0028	**0.0030**	**0.0001**
	0.0130	0.0031		
	0.0127	0.0030		
Negative Control, 2 % ME, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 0.2% ME-Glu PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 2 % ME, PBAT 60:40	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 0.2% ME-Glu PBAT 60:40	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 2 % ME, PBAT 90:10	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 0.2% ME-Glu PBAT 90:10	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos mutant	0.0029	0.0007	**0.0005**	**0.0001**
	0.0018	0.0004		
	0.0021	0.0005		
Control, 2 % ME, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 50:50 EcoFlex	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 50:50 EcoFlex	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 0.2 % ME-Glu, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 0.2 % ME-Glu, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 50:50 EcoFlex	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 50:50 EcoFlex	0.0067	0.0016	**0.0015**	**0.0003**
	0.0049	0.0012		
	0.0073	0.0017		

Table 16: Data of the bis(4-hydroxybutyl) terephthalate concentration measurements with HPLC

Condition, Medium, Fungus, PBAT	**Bis(4-hydroxybutyl) terephthalate concentration**			
	c(BTaB)	**β(BTaB)**	**Mean**	**Std**
	[mmol/l]	**[mg/ml]**	**[mg/ml]**	**[mg/ml]**
Control, 2 % ME-Glu, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME-Glu, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		

Treatment, 2 % ME-Glu, K. chersonesos mutant, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME-Glu, K. chersonesos wild type, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 0.2 % ME-Glu, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 0.2 % ME-Glu, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 60:40	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 60:40	0.0009	0.0003	**0.0001**	**0.0001**
	0.0000	0.0000		
	0.0001	0.0000		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 90:10	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 90:10	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 60:40	0.0016	0.0005	**0.0004**	**0.0002**
	0.0014	0.0004		
	0.0006	0.0002		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 60:40	0.0000	0.0000	**0.0007**	**0.0008**
	0.0014	0.0004		
	0.0050	0.0015		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 90:10	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 90:10	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 2 % ME, PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 0.2% ME-Glu PBAT 50:50	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Negative Control, 2 % ME, PBAT 60:40	0.0000	0.0000	**0.0014**	**0.0012**
	0.0065	0.0020		
	0.0073	0.0023		
Negative Control, 0.2% ME-Glu PBAT 60:40	0.0069	0.0021	**0.0014**	**0.0012**
	0.0069	0.0022		
	0.0000	0.0000		
Negative Control, 2 % ME, PBAT 90:10	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		

	0.0000	0.0000		
Negative Control, 0.2% ME-Glu PBAT 90:10	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 2 % ME, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos mutant, PBAT 50:50 EcoFlex	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 2 % ME, K. chersonesos wild type, PBAT 50:50 EcoFlex	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 0.2 % ME-Glu, K. chersonesos mutant	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Control, 0.2 % ME-Glu, K. chersonesos wild type	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos mutant, PBAT 50:50 EcoFlex	0.0000	0.0000	**0.0000**	**0.0000**
	0.0000	0.0000		
	0.0000	0.0000		
Treatment, 0.2 % ME-Glu, K. chersonesos wild type, PBAT 50:50 EcoFlex	0.0018	0.0005	**0.0006**	**0.0002**
	0.0017	0.0005		
	0.0028	0.0009		

9.3 List of illustrations

9.4 List of tables

9.5 List of Formulas

10 Appendix

10.1 Abstract

Knufia chersonesos is an oligotroph and extremotolerant model organisms for non-pathogenic black fungi, available as melanised wild type and natural non-melanised mutant. *K. chersonesos* is not only able to tolerate and grow on aromatic hydrocarbons, but also to degrade aliphatic-aromatic copolyesters, which makes it a biotechnological interesting candidate for biodegradation of synthetic polyesters. In experiments *K. chersonesos* was cultivated in the presence of the aliphatic-aromatic copolyester poly(1,4-butylene adipate-*co*-terephthalate) (PBAT) in liquid culture. The culture supernatant was analysed with p-nitrophenyl butyrate assay for hydrolase activity, HPLC for PBAT degradation products, and SDS-PAGE for the secretome. PBAT was degraded by *K. chersonesos*, with the degradation rates descending from 60:40 over 50:50 to 90:10 PBAT. The degradation rates of 50:50 and 90:10 PBAT were increased by limitation of nutrients to the *K. chersonesos*. The hydrolase activity in the culture supernatant is not influenced by the presence of PBAT, which suggest constitutive secretion of hydrolases. The melanisation of the fungal cell wall decreases the hydrolase activity measured in the culture supernatant. Unlike the hydrolase activity, the secretion of proteins in the sizes 58, 50, 40, 28, and 27 kDa is increased in the presence of 50:50 PBAT in SDS-PAGE analyses. The protein secretion in the sizes 50, 40, 30 kDa and 40, 30 kDa are increased for 60:40 PBAT and 90:10 PBAT, respectively. Potential applications of *K. chersonesos* and its secreted hydrolases lie in recycling and surface functionalisation of PBAT.

10.2 Zusammenfassung

Knufia chersonesos ist ein oligotropher und extremotoleranter Modellorganismus für nicht pathogene Schwarze Pilze, welcher als melanisierter Wildtyp und natürliche nicht melanisierte Mutante vorkommt. *K. chersonesos* toleriert nicht nur aromatische Kohlenwasserstoffe im Wachstumsmedium und nutzt diese als Nährstoffquelle, sondern ist zudem in der Lage aliphatisch-aromatische Copolyester abzubauen, was diesen Organismus interessant für den biotechnologischen Abbau von synthetischen Polyestern macht. Im Experiment wurde *K. chersonesos* in Gegenwart des aliphatisch-aromatischen Copolyesters Poly(1,4-butylenadipat-*co*-terephthalat) (PBAT) in Flüssigkultur kultiviert. Der Kulturüberstand wurde mit dem p-Nitrophenylbutyrat Assay auf Hydrolase-Aktivität, HPLC auf PBAT-Abbauprodukte und SDS-PAGE auf das Secretom des Schwarzen Pilzes analysiert. PBAT wird von *K. chersonesos* abgebaut, wobei die Rate des Abbaus von 60:40 über 50:50 zu 90:10 PBAT abfällt. Im Falle von 50:50 und 90:10 PBAT wird die Abbaurate durch ein nährstoffarmes Kulturmedium erhöht. Die Hydrolase-Aktivität im Kulturüberstand wird nicht durch die Anwesenheit von PBAT beeinflusst, was auf eine konstitutive Sekretion von Hydrolasen schließen lässt. Die Melanisierung der Pilzzellwand reduziert die gemessene Hydrolase-Aktivität im Kulturüberstand. Im Gegensatz zur Hydrolase-Aktivität wird die Sekretion von Proteinen mit 58, 50, 40, 28, und 27 kDa durch die Gegenwart von 50:50 PBAT erhöht. Die Sekretion von Proteinen in den Größen 50, 40, 30 kDa wird durch 60:40 PBAT und 40, 30 kDa durch 90:10 PBAT erhöht. Potentielle Anwendungen von *K. chersonesos* und seiner sezernierten Hydrolasen liegen im Bereich des Recyclings und der Oberflächenfunktionalisierung von PBAT.