THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Microbial strategies for deconstruction of bark components

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Cover: Fungi thriving in a woodland environment. Artist: Diane Sandall
Back: *Pseudomonas abieticivorans*. Artist: Diane Sandall

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“Det är bara lite *otur* i arbetet.”

- *Farfar*

[on failure]

Preface

This dissertation serves as partial fulfilment of the requirements for obtaining the degree of Doctor of Philosophy at the Department of Life Sciences at Chalmers University of Technology. The work was funded by the Swedish Energy Agency. The PhD studies were carried out between April 2019 and September 2023 under the supervision of Assoc. Prof. Johan Larsbrink and co-supervision of Assoc. Prof. Merima Hasani. The thesis was examined by Prof. Pernilla Wittung-Stafshede.

The majority of the work in this thesis was carried out at the Division of Industrial Biotechnology (IndBio) and Forest Products and Chemical engineering at Chalmers University of Technology. Proteomics experiments were conducted during a research visit at the Norwegian University of Life Sciences funded by a grant from Nils Philblads foundation. Elemental analysis experiments were conducted at Chalmers Materials Analysis Laboratory (CMAL). Nuclear magnetic resonance experiments were conducted at the Department of Chemistry and Chemical Engineering by Dr. Alexander Idström. Mass spectrometry experiments were executed at the Separation Science Laboratory at AstraZeneca by Annika Langborg Weinmann. Methanolysis experiments were conducted at by Ekaterina Korotkova at Johan Gadolin Process Chemistry Centre, Åbo Akademi University. Crystallization experiments were performed at Gothenburg University and X-ray diffraction data were collected at the MAX IV Laboratory by Dr. Tom Coleman. Transmission electron microscopy experiments were performed at Gothenburg University by Sebastian Valenzuela.

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Abstract

Bark is the outermost part of a tree, and it is a protective layer against external threats such as microorganisms and environmental stressors. Bark consists of various polymers including lignin, cellulose, hemicellulose, and it also contains a large fraction of compounds known as extractives. The polymers and extractives are assembled into a heterogenous complex matrix, forming a highly recalcitrant material. Despite its protective role, bark is degraded in nature by microorganisms, yet little is known about the specific microorganisms involved and how they affect bark composition.

In this thesis, I have investigated different strategies that individual species and microbial communities employ to degrade bark and how enzymes hydrolyze pure polysaccharides and extractive compounds, focusing on spruce bark degradation. I analyzed a microbial community growing on spruce bark over six months and observed significant effects on the extractives, especially resin acids at the start of the cultivation. The community was dominated by bacteria, and guided by metagenomics, a new *Pseudomonas* species was isolated, sequenced, and shown to degrade the major resin acids present in spruce bark. The role of filamentous fungi in the microbial community was unclear, despite their reputation as exceptional lignocellulose degraders. Therefore, I studied fungi from the Basidiomycota and Ascomycota phyla known to employ different lignocellulose degradation strategies. I showed that the Basidiomycetes can degrade/modify resin acids, while the Ascomycetes instead appeared to tolerate resin acids. All fungi investigated were able to degrade the bark polysaccharides, with significant differences in pectin and xylan degradation. To understand xylan degrading mechanisms in more detail, I studied the growth of taxonomically different yeasts and biochemically characterized their xylanases. One of the yeasts, *Wickerhamomyces canadensis*, grew poorly on xylan but its growth was boosted when co-cultured with another yeast, *Blastobotrys mokoenaii*. This suggests that *W. canadensis* is a secondary degrader of xylan. For in-depth studies of extractive-degrading enzymes, I biochemically characterized three tannases from the bacterium *Clostridium butyricum* and demonstrated their ability to cleave oak bark tannins. My work contributes to our understanding of the microbial degradation of bark and the strategies employed by microbial communities, individual species, and enzymes to degrade bark.

Keywords: Bark, microbial communities, fungi, yeast, bacteria, polysaccharides, extractives, enzymes, proteomics.

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Amanda Sörensen Ristinmaa, Albert Tafur Rangel, Alexander Idström, Sebastian Valenzuela, Eduard J. Kerkhoven, Phillip B. Pope, Merima Hasani, and Johan Larsbrink
Resin acids play key roles in shaping microbial communities during degradation of spruce bark. *Submitted for publication. Preprint available online.*
DOI: 10.1101/2023.04.19.537524

II. Amanda Sörensen Ristinmaa, Ekaterina Korotkova, Magnus Ø. Arntzen, Vincent G. H. Eijsink, Chunlin Xu, Anna Sundberg, Merima Hasani, and Johan Larsbrink
Exploring fungal strategies for degradation of spruce bark and its extractives and polysaccharides. *Manuscript.*

III. Jonas Laukkonen Ravn, Amanda Sörensen Ristinmaa, Tom Coleman, Johan Larsbrink, and Cecilia Geijer (2023)
Yeasts have evolved divergent enzyme strategies to deconstruct and metabolize xylan, *Microbiology Spectrum*, *11*(3), e00245-23.
DOI:10.1128/spectrum.00245-23

IV. Amanda Sörensen Ristinmaa, Tom Coleman, Leona Cesar, Annika Langborg Weinmann, Scott Mazurkewich, Gisela Brändén, Merima Hasani, and Johan Larsbrink (2022)
Structural diversity and substrate preferences of three tannase enzymes encoded by the anaerobic bacterium *Clostridium butyricum*, *Journal of Biological Chemistry*, *298*(4), 1-14.
DOI:10.1016/j.jbc.2022.101758

Contribution summary

I. First author. I planned the majority of the experiments. I performed the experimental work together with my co-authors. I analyzed the data, interpreted the results and wrote the manuscript.

II. First author. I planned the majority of the experiments. I performed the experimental work together with my co-authors. I analyzed the data, interpreted the results and wrote the manuscript.

III. Second author. I planned and performed the recombinant protein production and purification and characterized enzymes using *p*NP-assays and ran the HPAEC-PAD together with co-workers, interpreted the data and contributed to writing the manuscript.

IV. First author. I conceived the idea. I planned the majority of the experiments. I performed the experimental work together with my co-authors. I analyzed the data, interpreted the results and wrote the manuscript.

Oral and poster presentations of this thesis work

- Poster presentation "*Clostridium butyricum* encodes three functionally and structurally diverse tannase enzymes active on water-soluble oak bark extractives" at the European Molecular Biology Organization in Denmark 2021: Enzymes of the future meeting (Lyngby, Denmark, Dec 2021)
- Pecha Kucha presentation at Ekmandagarna (Stockholm, Sweden, Feb 2022)
- Oral and poster presentation "Characterization of three tannases from *Clostridium butyricum* active on oak bark extract" at the Symposium on Biomaterials, Fuels and Chemicals (New Orleans, USA, Apr 2022)
- Poster presentation "Deciphering key stages, compounds, and microorganisms during microbial degradation of spruce bark" at the Carbohydrate Bioengineering Meeting 14 (Ås, Norway, Oct 2022)
- Oral and poster presentation "Filamentous fungi display different behavior during spruce bark degradation" at the Carbohydrate-Active Enzymes for Glycan Conversions Gordon Research Seminar (GRS), and poster presentation at the associated Gordon Research Conference (GRC; Andover, USA, Jun 2023)

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Abbreviations

16S ribosomal RNA	16S rRNA
Acid insoluble residue	AIR
Acid-soluble lignin	ASL
Arabinose	Ara
Arabinoxylan	AX
Auxiliary activities	AA
Average nucleotide identity	ANI
Carbohydrate binding module	CBM
Carbohydrate esterase	CE
Carbohydrate-active enzyme	CAZyme
Carbohydrate-Active enZYmes Database	CAZy
Collision cross section	CSS
Degree of polymerization	DP
Domain of unknown function	DUF
Galactoglucomannan	GGM
Galactose	Gal
Galacturonic acid	GalA
Gas chromatography	GC
β–Glucogallin	GG
Glucose	Glc
Glucuronoarabinoxylan	GAX
Glucuronoxylan	GX
Glycoside hydrolase	GH
Hexyl gallate	HX
High-performance anion exchange chromatography with pulsed amperometric detection	HPAEC-PAD
High-performance liquid chromatography coupled to photodiode array	HPLC-PDA
Homogalacturonan	HG
Internal transcribed spacer	ITS
Lytic polysaccharide monooxygenase	LPMO
Mannose	Man
Mass spectrometry	MS
Metagenome-assembled genome	MAG
Methyl gallate	MG
p-Nitrophenyl acetate	*p*NP-Ac
Operational taxonomic unit	OTU
Propyl gallate	PG
Polysaccharide lyase	PL

Polysaccharide Utilization Locus	PUL
Pyrolysis gas chromatography high-resolution mass spectrometry	py–GC–HR-MS
Rhamnogalacturonan I and II	RG-I and RG-II
Rhamnose	Rha
Sigma 70 factor subunit of the DNA polymerase	*rpoD*
National Center for Biotechnology Information	NCBI
Nuclear magnetic resonance spectroscopy	NMR
Two-dimensional nuclear magnetic resonance spectroscopy	2D-NMR
Ultra-high performance liquid chromatography tandem mass spectrometry	UHPLC-MS/MS
Xylooligosaccharide	XO
Xylogalacturonan	XG
Xyloglucan	XyG
Xylose	Xyl

1. Introduction

Bark functions as a protective skin for trees, safeguarding them against external threats. Trees have evolved a diverse arsenal of defensive compounds aimed at counteracting decay; these are found in higher concentrations in the bark. Nevertheless, despite the accumulation of these compounds, microorganisms are still capable of colonizing and growing on trees. The main objective of this thesis was to identify microorganisms and enzymes responsible for breaking down the components found in bark, and to subsequently employ these enzymes for the degradation of bark constituents.

1.1 Bark is an abundant and underutilized resource

While only present on the outside, bark still comprises a significant (10-15%) portion of a tree's total volume at harvest (1). According to the United Nations Food and Agriculture Organization report from 2020, global consumption of round wood reached 3.91 billion m^3, resulting in an estimated 191.5 million tons of bark generated annually by the forest industry (2). Despite these high production amounts, bark is a side stream and its primary use is in heat generation through combustion (direct burning) within the timber and paper industry, along with some usage in horticulture (3). Traditionally, the bark of trees has been considered an unusable waste product with little value due to its high moisture content and heterogeneous composition.

1.2 The importance of bark

Some trees can live for thousands of years, and need protection against changes in temperature or humidity, mechanical damage, and microorganisms. However, because trees cannot physically flee from danger, they must rely on other means of protection including the shielding mechanisms provided by bark. Certain tree species, such as eucalyptus even shed their bark, as a means of removing moss and other epiphytes or parasites that may adhere to it (4). Disruption of bark by bark beetles or animals can have dire consequences for the tree, as bark covers the phloem, which is the primary source of nutrient transport in plants (5).

Bark is composed of a wide range of molecules, making it highly structurally diverse. In addition to cellulose, hemicelluloses, and lignin, which are the main components of wood, bark typically contains an abundance of extractives, which act as antioxidants, antimicrobials, insecticides, and water-repelling waxes (1). They combine to form a highly recalcitrant hierarchical structure, which is resistant to biological degradation. The extractives in bark are defined as compounds that can be extracted through treatments with non-polar and polar solvents, hence the name. The total content of extractives varies across tree species, but may amount to 20%–40% of the bark dry weight (1).

1.3 Antimicrobial bark components

Bark is a source of many natural products, such as tannins, resin acids, and other polyphenolic compounds, which are found among extractives. These compounds possess a variety of beneficial properties, including antioxidant, anti-inflammatory, and antimicrobial activities (3). Bark has been used in medicine for centuries: salicylic acid or aspirin comes from the willow tree bark, and Taxol, a compound that has been used to treat breast and ovarian cancer is obtained from Pacific yew tree bark (6).

Tannins extracted from bark have been used for centuries in the leather industry in the tanning process, whereby they bind to the proteins in the hide to make them more durable and water-resistant (7). As a result of binding to and precipitating microbial proteins, tannins protect plants from microbial pathogens. Compounds from bark can also be toxic. Resin acids give rise to allergic reactions following skin contact which may occur if they are used as printing ink on textiles (8). They are considered highly toxic to aquatic organisms, bioaccumulates and results in long-term toxicity, with lethal concentrations of around a few milligrams per liter (8, 9).

Despite its primary function in protecting trees against microbial attack and the presence of extractive compounds, bark nevertheless undergoes degradation in nature. To fully utilize the potential of bark and its components through biochemical means, it is essential to understand the overall biological degradation process.

1.4 Microorganisms associated with bark

Bark houses a diverse community of bacteria, fungi, and other organisms on its surface, and these microorganisms play important ecological roles, such as breaking down organic matter, facilitating nutrient cycling, and contributing to the decomposition of dead wood. Traditionally the study of microorganisms has been limited to those that can be grown as isolated species, even though in nature no microorganism grows in isolation (10).

Despite the antimicrobial properties of bark, it is gradually broken down by microorganisms in environmental communities, or by microbiota residing in the gut of insects such as the bark beetle (11). Curiously, the genome of the European spruce bark beetle has revealed many genes associated with polysaccharide degradation, but no genes associated with xenobiotic metabolism, which would enable degradation of extractives (11). This may seem paradoxical but could be due to symbiosis with the insect's gut microbiome and their ability to detoxify the bark's chemical defenses. Alternatively, the beetle could simply avoid the bark and only bore through it to focus on wood degradation. As evidenced by the bark beetle, many of the microorganisms found in microbial communities often have specific metabolic requirements or depend on the presence of other microbial species.

Isolation of individual species or enrichments of co-cultures of two or more species are an important and widely applied method for characterizing novel bacteria and fungi found in association with trees (12). These utilize a plethora of enzymes to degrade and/or transform the complex structures of the many different polymers and extractive compounds present in bark. To break down polymers common to both wood and bark microorganisms have been found to possess specialized enzymes termed carbohydrate-active enzymes (CAZymes) (13). Instead, there is limited knowledge regarding enzymes targeting of bark-enriched components, with certain lipases, tannases, and cutinases having been shown to degrade individual extractive components (14).

1.5 Aim of the thesis

The overall aim of the thesis was to study which microorganisms deconstruct bark, and the enzymes they can employ. This approach aimed to provide a holistic understanding of how bark was metabolized in nature (Fig. 1.1). As the composition of bark varies vastly between different tree species, in my thesis I have focused on industrially important spruce bark, tannins extracted from oak bark, as well as pure polysaccharides and model substrates for enzymatic investigations. To this end, the following overarching research question was formulated:

How is bark degraded by microorganisms?

To answer this rather broad question, I have used a combination of different techniques and divided the overall research question into more specific sub-questions:

Which species can grow on bark?

Given the limited understanding of bark degradation, my first step was to identify which microorganisms grew on bark either as isolated species or within a microbial community. I studied the development of the microbial community over time and isolated different species on resin acids, with the primary goal of finding microorganisms that could grow on them as a sole carbon source. I have also studied how individual fungal species degraded bark, focusing on five taxonomically different species. Filamentous fungi can be frequently found growing on the outside of fallen trees, suggesting that they play a part in the bark decomposition process.

At present, it is not known how microbial communities or individual species degrade bark, i.e., whether they simply ignore the presence of extractives or degrade/modify them, and how they break drown polysaccharides in the presence of extractives. Therefore, the following research question was formulated:

What are the compositional changes during microbial bark degradation?

As the antimicrobial properties of bark are attributed to extractives, we sought to identify the effect of microorganisms on these compounds, in addition to the wood polymers. Microorganisms involved in bark degradation at different stages were identified by linking changes in bark composition and microbial community abundance.

From the previous research on microbial communities, the role of fungi during bark degradation was unclear since the community was dominated by bacteria. In our next study we monitored the degradation process for individual fungal species to compare their substrate preferences on bark, focusing primarily on polysaccharides and extractives.

During growth of microorganism, bark undergoes changes driven by enzymatic activity, including polysaccharide and extractive degradation. Therefore, the following research question was formulated:

How can individual enzymes be used to degrade bark components?

To gain a better understanding of how microorganisms degrade bark components, I focused on enzymes that had received limited attention thus far. Specifically, I worked with yeast enzymes belonging to uncharacterized xylanase subfamilies, as well as tannases. The enzymes were purified, and their activity was characterized on pure substrates.

Although yeasts are ubiquitous across various habitats, their role in the breakdown and metabolism of xylan, as well as their potential contributions to its natural turnover, remain poorly understood. The CAZymes of these organisms have remained unexplored in comparison to those of bacteria and filamentous fungi. Therefore, three taxonomically different yeast species were grown on xylan, and their putative xylanases were expressed, purified, and characterized. This effort aimed to enhance our understanding of the enzymatic strategies employed by these yeasts to break down xylan.

Whereas xylanases act on polymers which are shared by both xylem and bark, extractives occur at significantly higher concentrations in bark. As a result, my next research effort focused on identifying enzymes capable of targeting tannins. One such class of enzyme is represented by tannases, which act specifically on ester-linked galloyl units found in hydrolysable tannins. Although only a limited number of tannases have been characterized so far, *Clostridium butyricum*, encodes multiple tannases, prompting an investigation into the reasons behind this multiplicity.

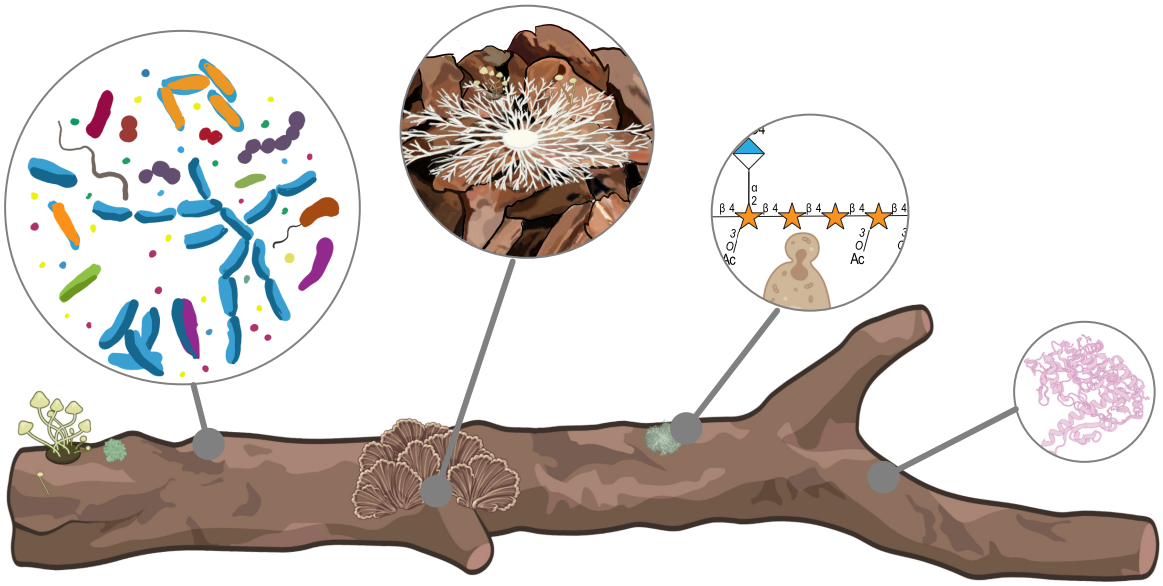


Figure 1.1. Overview of the four different research topics investigated in this thesis. The four different aspects of bark degradation investigated in this thesis, from the left: mixed microbial communities, single fungal species growing on bark, single yeast species growing on polysaccharides, and biochemical characterization of pure enzymes. Created in Biorender.com.

Main points

- Bark should not be regarded as a waste resource.
- Extractives are a major part of bark.
- Microorganisms act together to degrade lignocellulose.
- Enzymes catalyze the degradation of compounds found in bark.

2. Bark composition

To defend the tree from microbial and environmental threats, bark has evolved into a complex structure. It comprises an inner living layer of secondary phloem, known as the inner bark (1), whose cells enable the transport of photosynthesis products throughout the tree (15). In addition, the inner bark plays a crucial role in wound healing and serves as a storage site for carbohydrates, water, and secondary metabolites (16). The outer part of the tree is the outer bark, which consists primarily of dead or non-active tissue and provides stability to the stem and protection to the tree (17).

Bark has a complex hierarchical structure with a different composition compared to wood (xylem). Many of the polymers present in the xylem, such as cellulose, hemicelluloses, and lignin (18) are also present in bark but their proportions differ: bark contains less cellulose and hemicelluloses, but more lignin (Fig. 2.1) (19). The main difference between bark and xylem, however, is the significantly higher proportion of extractives in bark (20), which as previously mentioned contribute to its defensive properties.

The composition of bark also depends upon the tree species; certain extractive compounds tend to dominate in certain types of bark. For instance, birch bark is rich in betulin, while oak bark is rich in tannins, and spruce bark contains significant amounts of resin acids (21). These compounds contribute to the unique properties and functions of bark in each species. Even the structure of bark has different morphologies depending on its source; cork oak bark has a thick, spongy, and insulating layer of cork that covers the tree's trunk. Instead, in birch or spruce, bark remains more intact, forming a cohesive outer layer (4).

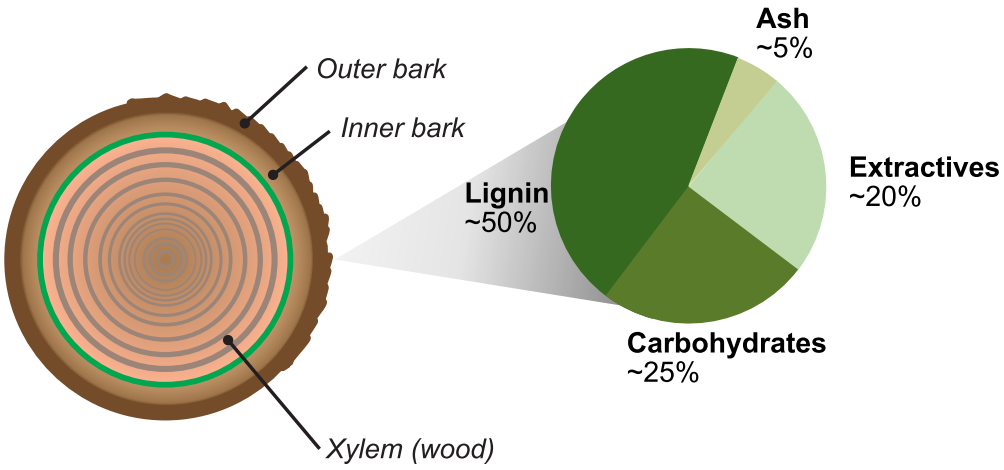


Figure 2.1. Overview of the structure of the tree stem. The tree encompasses inner and outer bark, while the rest of the stem is categorized as wood (xylem). The pie chart on the right illustrates the composition of bark. Modified from Paper I. Credit to Dr Johan Larsbrink for the illustration of the stem.

2.1 Extractives act as decay-delaying agents

Extractives from softwood and hardwood differ greatly from each other and are enriched with different types of compounds. The choice of solvent determines the type of molecule that can be extracted from bark. Broadly speaking, extractives are categorized into hydrophilic or hydrophobic, depending on whether they are extracted with hexane or water, respectively (Fig. 2.2) (22).

Extractives play a very important role in the resilience of wood to degradation. Their removal makes wood more susceptible to decay, whereas adding extractives from a durable species enhances the durability of more degradation-susceptible wood (23-25). Bioassays have demonstrated that the antifungal properties of extractives play a pivotal role in the resistance of hardwood to decay (26-28). Notably, resin acids have been found to inhibit cellulose hydrolysis (29). In my work, I have focused on spruce bark extractives, as well as tannins from oak bark.

2.2 Hydrophilic extractives: tannins

Hydrophilic extractive compounds include various components, among them polyphenols. Polyphenols, encompass flavonoids, phenolic acids, and tannins. Tannins are a major component of bark in many species and encompass a large group of compounds (Fig. 2.2). Oak bark, which has long been recognized as a rich source of tannins, has extensively been used in leather tanning processes (30). In plants, the decay delaying properties of tannins have been attributed to binding and precipitation of proteins (31). Based on their structure tannins are classified into four subgroups; gallotannins, ellagitannins, condensed tannins, and complex tannins (Fig. 2.2) (32). Hydrolysable tannins, which can be degraded by tannases, include gallotannins and ellagitannins, and are composed of a galloyl and/or ellagoyl moiety linked to a glucose unit. Condensed tannins have carbon-carbon bonds between catechin units; whereas complex tannins have galloyl moieties ester linked to a catechin unit.

2.3 Hydrophobic extractives: resin acids, fats, and suberin

Hydrophobic spruce bark extractives comprise mainly resin acids, fats, waxes, and suberin; each of them plays a distinct role and exhibits specific chemical properties. Resin acids are diterpenoid compounds found in conifer resin and at elevated concentrations in bark (1). They are released from the wood during the pulping process and have historically been found in high concentrations in pulp and paper mill effluents (33). Due to their poor water solubility, resin acids tend to accumulate in sediments (8), posing a significant threat to fish and crustaceans, for whom they are highly toxic (9). The long-lasting toxicity of resin acids derives from their bioaccumulation (34). Moreover, their hydrophobic nature allows them to cross cell membranes, further contributing to their toxic effects and antimicrobial activity (9). The most abundant resin acid in spruce bark is dehydroabietic acid (Fig. 2.2) (35). Other extractives found in spruce bark extractives include terpenes known as sterols, of

which β-sitosterol is the primary sterol in softwoods. When sterols are ester linked with a fatty acid, they are called steryl esters.

Fats and waxes are nontoxic metabolites found in bark: fats are esters of glycerol and fatty acids of various lengths, whereas waxes consist of long fatty acids ester linked to alcohols (1). These compounds, including triglycerides, free fatty acids, diacylglycerides, and monoacylglycerides, are extracted from bark using organic solvents. The carbon chain lengths of fatty acids in bark range from 16 to 22, although longer chains have also been reported (17).

Suberin is a constituent of the outer bark and is particularly abundant in cork. It is composed of waxes and phenols, forming a polyester structure consisting of fatty acids and hydroxy fatty acids (36). The composition of suberin varies depending on tree species (17).

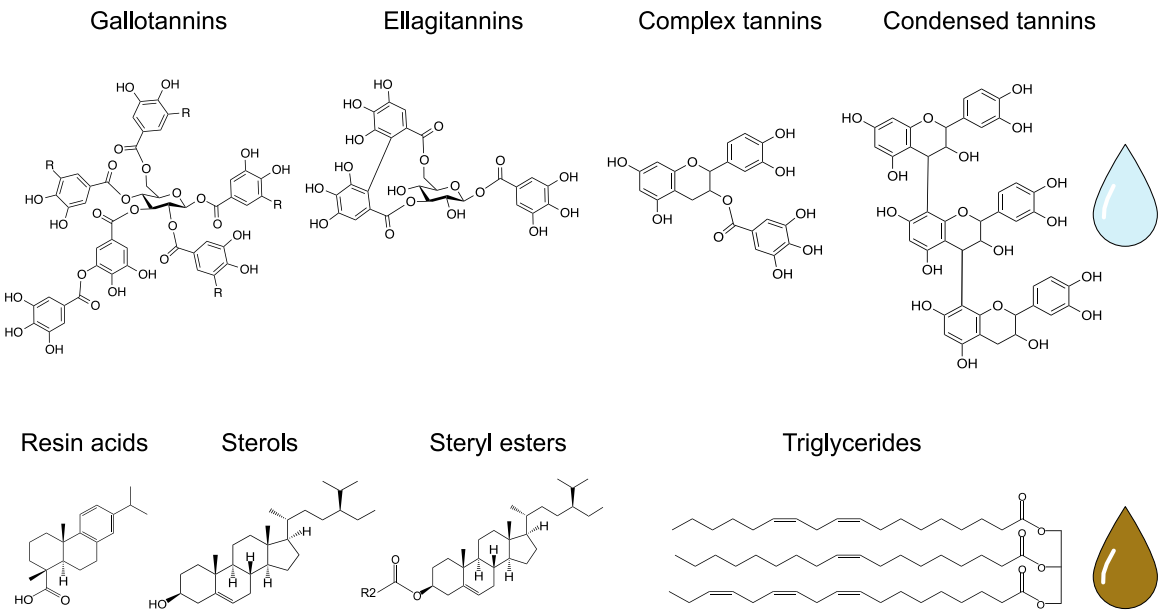


Figure 2.2. Extractive subgroups and representative structures. Hydrophilic (blue drop) to hydrophobic (brown drop) compounds found in bark. Oak bark extractives are composed of all the represented hydrophilic compounds, but predominantly gallotannins (tannic acid if R = gallate) and ellagitannins. Spruce bark extractives are mainly composed of hydrophobic compounds (in steryl ester, R2 = fatty acid). Modified from Papers I & IV.

2.4 Extraction and analysis of bark extractives

The first step in the analysis of extractives involves separating these molecules from bark. This is done using a Soxhlet extractor, an apparatus designed for the continuous extraction of analytes from a solid into a solvent (Table 2.1) (37). The process starts by heating a distillation flask containing the solvent of choice, holding a chamber with the solid material to be extracted (bark) at the bottom of the distillation arm. The heat causes vapors to rise, which then condense back into the flask through a water-cooled condenser dripping back into the chamber with bark. When the liquid in the extraction chamber reaches the top of a bent tube (siphon), the extract-enriched solvent is siphoned back into the flask. High extraction yields (often more than 15) are achieved through multiple extraction cycles (38). In my work, I used acetone as the extraction solvent because of its ability to extract a wide range of molecules, as outlined in **Papers I** & **II**. It is also possible to perform sequential extractions by varying the solvent's hydrophobicity to target specific types of compounds. As this is laborious to do via Soxhlet extraction, accelerated solvent extraction offers a highly automated extraction technique, whereby solvents are applied at high temperatures under pressure. The elevated temperature accelerates extraction kinetics, while the elevated pressure prevents boiling at temperatures above the normal boiling point of the solvent. In contrast to Soxhlet extraction, this approach offers a quicker and more effective extraction process but requires access to specialized instrumentation.

In my investigation of tannin hydrolysis, I made a surprising observation: certain extractives from bark could be obtained without using either high temperatures or the laborious Soxhlet extraction. Initial trials on tannase hydrolysis using milled oak bark as a substrate led to gallic acid release. This was in stark contrast to later experiments with water-washed oak bark, which did not result in gallic acid release after tannase treatment. Taken together, these findings indicated that water-soluble tannins were removed during the washing process, prompting me to use mild water extraction to generate tannin substrates in **Paper IV**.

Given the diverse nature of extractives, a wide range of techniques is required for their identification and quantification. In my research, I combined gas chromatography (GC) and nuclear magnetic resonance (NMR) to quantify and identify lipophilic extractives from spruce bark, as well as high-performance liquid chromatography (HPLC) to identify tannins from oak bark extract (Table 2.1). GC enables detailed characterization, particularly for volatile and low-molecular-weight compounds. The specific method used for identification and quantification varies depending on the type of extractive.

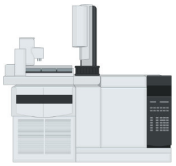
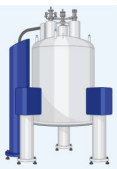
Quantification and identification of lipophilic extractives using GC can be performed with short or long columns. Short GC capillary columns have allowed for the quantification of high-molecular-weight triglycerides, although this method does not offer the highest resolution (39). Originally developed in the 1990s for monitoring wood extractives in papermaking processes, this method involves the rapid determination of extractive classes;

fatty acids, resin acids, sterols, steryl esters, and triglycerides (37). The quantification of these classes of extractives is achieved by adding four internal standards (heneicosanoic acid, betulinol, cholesteryl heptadecanoate, and dipalmitoyl oleyl glycerol) to the sample. This approach provides a general analysis, which was particularly valuable in **Paper I**, where the aim was to assess the overall impact of microbial treatment on bark extractives without any specific target compounds in mind. For the identification and quantification of individual components, longer columns with thicker films are employed. GC can be coupled to mass spectrometry (MS) or a flame ionization detector (FID); GC-MS is valuable for qualitative characterization; whereas FID is more suitable for compound quantification. Nevertheless, both identification and quantification can be achieved via GC-MS analysis. In such cases, validation is typically performed using the NIST MS search program and the NIST/EPA/NIH Mass spectral database 2011 (NIST 11), as well as available standards. In **Paper I**, GC-MS was used for compound identification, while GC-FID was used for quantification. In **Paper II**, both identification and quantification were achieved by GC-MS with standards.

NMR spectroscopy is the gold standard for the identification of molecules (40). However, NMR requires a relatively large sample size, and in the case of solution-state NMR, the sample must be well-dissolved. In **Paper I**, we used 2D-NMR to identify and analyze the spectra of dehydroabietic acid, the main extractive found in spruce bark. Furthermore, we used it to identify a ferulic ester compound, possibly originating from suberin.

Quantification and identification of gallic acid is facilitated by the presence of the aromatic ring, which enables detection by ultraviolet-visible spectroscopy. Hence, I employed HPLC coupled to a photodiode array (HPLC-PDA) instead of GC to monitor tannin degradation by tannases. Identification of compounds in complex matrices such as tannins from oak bark, is greatly facilitated by using ultra high-performance liquid chromatography tandem mass spectrometry UHPLC-(MS/MS) and collision cross section (CSS) (41). To aid in compound identification, in addition to using available standards, I created a custom library based on previously reported tannin molecules from oak bark extractives. There is no consensus on what constitutes a valid metabolite identification, but this allowed me to putatively identify tannin molecules by comparing their spectral similarity with the library even in the absence of available standards (40).

Table 2.1. Overview of the methods used in this thesis for quantification and identification of bark extractives. Created using BioRender.

Extractive type		Analytical method	Structural information	Paper
Total extractives		Soxhlet	• Gravimetric analysis • Hydrophobicity	Paper I & II
Hydrophobic extractives		GC-MS/FID	• Identification and quantification of hydrophobic extractives • Quantification of extractive groups using a short column method	Paper I & II
Hydrophobic extractives		2D-NMR	• Structural elucidation of target lipophilic extractives	Paper I
Tannins		HPLC-PDA	• Quantification of gallic acid	Paper IV
Tannins		UPLC-MS/MS -CSS	• Identification of tannins	Paper IV

2.5 Bark polysaccharides

Polysaccharides are complex polymers consisting of monosaccharides linked by covalent glycosidic bonds, which connect the anomeric carbon in carbohydrates to other compounds. These linkages give rise to a diverse array of structures, ranging from simple oligosaccharides to highly complex and branched chains, extending to millions of monosaccharide units. Matrices composed of different polysaccharides (and lignin) linkages and building blocks make up the cell wall, which provides both a mechanical and chemical protection against degradation and impedes the breakdown of lignocellulose.

The diverse array of plant polysaccharides provide essential functions in plant growth, energy storage, as well as structural integrity of plant cells and tissues (42). On the one hand, structural polysaccharides such as cellulose are incorporated into the cell walls, which represent the main load-bearing structures in plants. On the other hand, storage polysaccharides serve as reserves of energy and building blocks within plant cells (42). These polysaccharides can be stored inside the cell (e.g., starch) or in the cell wall (43). In fungi, glycogen serves as a storage form of glucose; whereas chitin is an essential constituent of fungal cell walls (44).

2.6 Cellulose: the primary polysaccharide in spruce bark

Cellulose, the dominant polysaccharide in bark (constituting approximately 30% of the dry weight), is the most abundant polymer on Earth (45). It is composed solely of β-(1 → 4)-linked glucose (Glc) units that form long straight chains (Fig. 2.3). The number of glucose units in a chain, known as the degree of polymerization (DP), varies depending on the plant species and the extraction method employed. Generally, the DP is lower in bark compared to the xylem (17).

Although cellulose has a relatively simple structure consisting of straight glucan chains, adjacent glucose units from neighboring cellulose polymers interact through hydrogen bonding between their hydroxyl groups and via hydrophobic interactions below and above the pyranose ring planes, resulting in the formation of microfibrils (45). These microfibrils exhibit regions of varying order, accounting for the presence of both crystalline and less ordered regions (46). The crystalline regions pose a challenge for enzymatic degradation. So far, only lytic polysaccharide monooxygenase have been reported capable to degrade these crystalline regions (47).

2.7 Hemicelluloses are composed of a wide range of polysaccharides

Hemicelluloses refer to a diverse group of polysaccharides closely associated with cellulose in plant cell walls. They are usually extracted using alkaline solvents (48). Unlike cellulose, hemicelluloses are composed of many different monosaccharides and exhibit lower chemical and thermal stability (49). These polysaccharides contribute to the overall strength, cross-linking, and hydration of the cell wall (5). While hemicelluloses are primarily heteropolysaccharides, they include some homopolysaccharides (49). An important characteristic of hemicelluloses, such as xylans and mannans, is their ability to form covalent bonds with lignin, also known as lignin-carbohydrate complexes (50, 51). This interaction contributes to the recalcitrance of lignocellulosic biomass, which makes it more resistant to enzymatic degradation. Hemicelluloses can be classified into different subclasses based on the chemical composition of their backbone.

Mannans are the most abundant type of hemicellulose in both spruce wood and bark, constituting approximately 10% of the dry weight in the latter (17). There are two types of mannan in spruce bark: glucomannan (GM) and galactoglucomannan (GGM). In GGM, which is the most abundant of the two in softwood, the backbone consists of stretches of β-(1 → 4)-D-mannose (Man) and β-(1 → 4)-D-glucose (Glc) units, which can be substituted with α-(1 → 6)-linked-galactose (Gal) residues, and acetylated (Fig. 2.3) (52). In contrast, GM does not contain any Gal residues. The solubility of GGM in water and its properties in solution, such as viscosity, are influenced by the level of substitution on the backbone (49). In other plant species, mannans can exist as linear backbones (e.g., ivory nut mannan seeds), or free of galactose decorations (e.g., in certain hardwoods) (49, 53).

Xylans are the second most abundant type of hemicelluloses found in spruce bark, comprising approximately 5% of the dry weight (17). They consist of a backbone composed of β-(1 → 4)-linked-D-xylose (Xyl) residues, which can be *O*-acylated and substituted by α-(1 → 2)-linked (methyl)-glucuronic acid, α-(1 → 2)-and/or α-(1 → 3)-linked arabinosyl (Ara) units, and phenolic compounds (Fig. 2.3) (49). These various decorations give rise to different types of xylans, commonly classified as arabinoxylan (AX), glucuronoxylan (GX), and glucuronoarabinoxylan (GAX). In spruce bark, the predominant type of xylan present is GAX. While most studies of microbial xylan degradation have focused on bacteria and filamentous fungi; in **Paper III**, I characterized xylanases from yeasts using both AX and GX substrates, thereby improving our understanding of their xylan-degrading mechanisms.

Although not commonly associated with spruce bark, xyloglucan (XyG) has been detected in bark through glycome profiling, which is not unexpected given that it is found in the primary cell wall of all terrestrial plants (54). XyG consists of a linear backbone of β-(1 → 4)-linked glucan units, which is decorated with xylosyl units. These xylosyl units can be further substituted with galactosyl, fucosyl, and/or arabionofuranosyl units (52). The

specific type and degree of substitution varies depending on the plant species. In the case of spruce bark, fucosylated XyG has been reported (54).

2.8 Starch is a storage polysaccharide in bark

In most plants, carbohydrates are stored as starch; whereas fungi store energy in the form of glycogen. Both starch and fungal glycogen are composed of glucose, with varying branching and supramolecular organization of the glucan chain being predominately α-(1 → 4)-linked. Such order results in the formation of helices and a relatively soluble polysaccharide dictated by its DP (55). Starch consists of two main components: amylose and amylopectin which both have a backbone of α-(1 → 4)-linked Glc units, but the latter contains approximately 5% α-(1 → 6)-linked Glc moieties, resulting in branching (Fig. 2.3) (56, 57). Due to their similarities, distinguishing between starch and fungal glycogen can be challenging, as I will discuss in section 2.10.

2.9 Pectins are charged polysaccharides

Pectins encompass a diverse group of large and complex charged polysaccharides that contain a significant amount of galacturonic acid (GalA) and are typically negatively charged (58). They can be classified into several types, including homogalacturonan (HG), xylogalacturonan (XG), and rhamnogalacturonan (RG-I and RG-II) (Fig. 2.3). HG is the simplest and is characterized by a backbone of α-(1 → 4)-linked GalA units that may be acetylated or methylated (59). These linear chains can bind calcium ions, resulting in the formation of a gel-like structure that contributes to cell-cell interactions (49). XG is moderately more complex, featuring β-(1 → 3)-xylosyl substitutions. In contrast, RG-II is highly intricate, consisting of multiple side chains composed of at least ten different sugars on the backbone of α-(1 → 4)-linked GalA. RG-I is also complex and has a variable backbone composed of α-(1 → 4)-linked GalA and α-(1 → 2)-linked rhamnose (Rha) residues, with side chains mainly constituted of Gal and Ara (59). In spruce, the estimated pectin content is approximately 9.0% in the outer bark and 12.6% in the inner bark (17).

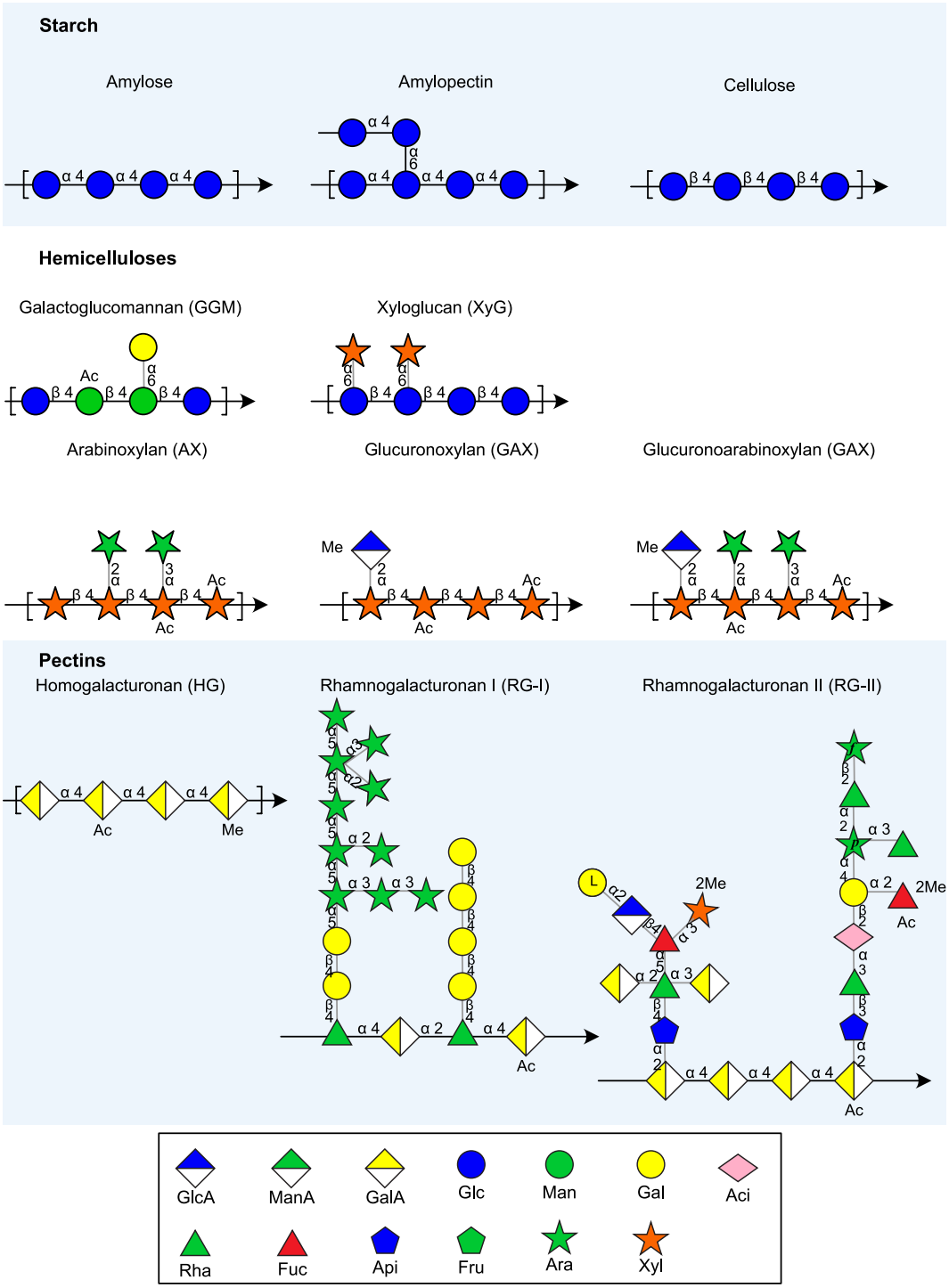


Figure 2.3. Representative structures of common polysaccharides found in spruce bark, with linkages indicated. Monosaccharide symbols follow the Symbol Nomenclature for Glycans (60). Methyl groups and acetyl groups are denoted by “Me” and “Ac”, respectively.

2.10 Polysaccharide analysis

Polysaccharides are analyzed by broad spectrum of techniques, from basic assessments of total sugar composition to more sophisticated approaches that investigate glycosidic linkages, substitution patterns, and other structural characteristics (61, 62). For a general understanding of polysaccharide composition, the relative amounts of different monosaccharides are often determined (Table 2.2) (63). Total or partial hydrolysis followed by chromatographic quantification of monosaccharides is one of the most common methods. Enzymes specific for certain types of glycosidic linkages can degrade selected polysaccharides, and the resulting changes/products provide insights into the structural features and properties of the polysaccharide. Ultimately, the choice of analysis method depends on the specific research objectives and the level of detail required to address the scientific questions related to polysaccharide composition, structure, and function. In my research, I studied microbial and enzymatic degradation of individual polysaccharides, more specifically xylan, and the degradation of polysaccharides in more complex bark samples, including those treated with microorganisms. The following techniques allowed me to assess the effect of microbial growth on the bark polysaccharides.

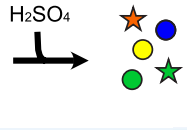
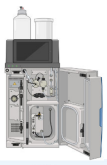
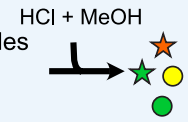
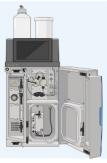
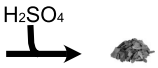
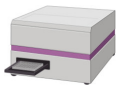
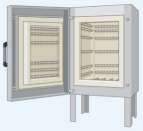
Total sugar composition of bark is most commonly performed using sulfuric acid, which allows the complete hydrolysis of polysaccharides into monosaccharides (64). A disadvantage of using sulfuric acid is the loss of structural information about side group locations or linkages. Samples are completely hydrolyzed using 72% sulfuric acid, and after filtering off any remaining solid residue, the hydrolysate is used to determine total carbohydrate composition. The different monosaccharides in the hydrolysate are quantified using high performance anion exchange chromatography pulsed amperometric detection (HPEAC-PAD). In **Papers I** & **II**, I used sulfuric acid hydrolysis to assess how microbial degradation influenced the composition of bark. However, one notable limitation of hydrolyzing the entire sample is the inability to determine the specific polymer sources of the resulting monosaccharides. This was particularly evident in **Paper II**, where it would have been valuable to identify the origins of Ara and Gal, but for which we could only assume to derive from pectin and/or xylan and mannan.

Besides monosaccharides, HPAEC-PAD is a crucial method for the detection and quantification of oligosaccharides. In **Paper III**, I employed this technique to quantify and characterize the various hydrolysis products generated when treating GX and AX with xylanases from yeast. This technique proved instrumental in comparing the substrate preferences of different glycoside hydrolases, allowing me to identify and quantify the resulting xylooligosaccharides. One drawback is the need for standards to identify and quantify the peaks observed in the obtained chromatogram.

To simultaneously analyze neutral and acidic sugars, an alternative hydrolysis method called acid methanolysis was employed in **Paper II**. Unlike sulfuric acid hydrolysis, acid methanolysis uses hydrochloric acid and methanol. One advantage of this technique is that it does not hydrolyze cellulose, allowing selective quantification of glucose originating from hemicellulose and pectin (65, 66). Combining acid methanolysis with sulfuric acid hydrolysis enables the quantification of glucose from cellulose. Akin to sulfuric acid hydrolysis, methanolysis does not provide information about the origin of the monosaccharides from either pectin or hemicellulose. Here, samples are hydrolyzed with 2 M hydrochloric acid in anhydrous methanol, degrading the polymers into methyl glycosides. The resulting products are then derivatized using hexamethyldisilazane and trimethylchlorosilane, which results in silylated derivatives. Identification and quantification of monosaccharides are achieved using GC-FID. Due to the silylation process, multiple peaks are observed for each monosaccharide in the obtained chromatogram (65).

Enzymes can be used to selectively degrade polysaccharides and enable their quantification. Owing to their specificity, enzymes such as amylases and amyloglucosidase are capable of degrading starch, allowing for quantification of total starch content (67). In **Paper II**, I used these enzymes to identify and quantify starch, which is relatively abundant in bark, and distinguish it from cellulose and hemicelluloses. However, discriminating between starch and fungal glycogen is less straightforward, as they share similar overall linkages (see section 2.8) (56). Consequently, when fungi grow on bark, it becomes difficult to differentiate between these two components. This limitation emphasizes the need for further investigation and exploration to differentiate starch and fungal glycogen.

Table 2.2. Methods used in this thesis to analyze spruce bark polysaccharides, lignin, and ash. Monosaccharide symbols follow the Symbol Nomenclature for Glycans (60). Created using BioRender.

Polymer	Analytical method	Structural information	Paper
Polysaccharides 	 HPAEC-PAD	• Depolymerisation of cellulose, hemicelluloses and pectin	Paper I & II
Polysaccharides 	 GC-FID	• Depolymerisation of hemicelluloses and pectins, not cellulose	Paper II
Starch/Glycogen 	 HPAEC-PAD	• Depolymerisation of starch/glycogen	Paper II
Xylan 	 HPAEC-PAD	• Oligosaccharide quantification	Paper III
Lignin 	 Gravimetric + UV	• Low selectivity • No structural information	Paper I & II
Ash 	 Gravimetric	• No structural information	Paper I & II

2.11 Lignin and ash in bark

Lignin, an aromatic macromolecule found in plant cell walls, is composed of monolignols linked by radical coupling mechanisms that lead to both ether and C-C bonds (68). The three most abundant monolignols are *p*-coumaroyl alcohol, coniferyl alcohol, and sinapyl alcohol, and additional monolignols can be present in lower concentrations (5). In softwood lignin, coniferyl alcohol is the dominant species, while *p*-coumaroyl alcohol is present in lower amounts (5). While wood and bark lignin are generally considered to have a similar overall structure, phenolic compounds such as hydroxy stilbene glucosides, are incorporated into the lignin structure of spruce bark, acting as true lignin monomers (69). Lignin analysis is highly complicated, and even more so in bark owing to the presence of tannins, suberin, and stilbene glucosides. These compounds tend to precipitate under acidic conditions (employed during total acid hydrolysis), contributing to the acid-insoluble material interpreted as lignin in bark (54).

When compared to wood, bark contains more minerals, which are referred to as ash. The main mineral found in bark is calcium, which makes up approximately 82%-95% of the mineral content. It is present in the form of calcium oxalate crystals, along with potassium and magnesium (70). The total ash content in bark, can vary between 5%-10% based on dry mass, which is much higher than the corresponding value (1%) in wood (17).

2.12 Analysis of lignin and ash in bark

Accurately quantifying lignin in a complex sample poses inherent difficulties as it relies on total (acid) hydrolysis of the material. This leads to interference from other components when both soluble and insoluble lignin are quantified following hydrolysis. Insoluble lignin, or the acid-insoluble residue (AIR), is gravimetrically quantified after hydrolysis and filtration of the residual solid sample (Table 2.2). Consequently, non-lignin acid-insoluble materials, such as ash, tannins, suberin, or chitin (e.g., from fungi), are also quantified as part of the AIR (54, 70-72). Acid-soluble lignin is measured by a spectrophotometric method using the acid-solubilized liquid fraction. While these techniques offer valuable means of quantifying lignin content and were both used in **Papers I** & **II**, they do not provide further insights into the specific structural characteristics of lignin. Instead, the structure of lignin can be derived using two-dimensional NMR (2D-NMR), although in-depth description of such analysis is outside the scope of this thesis. Moreover, lignin can be quantified by pyrolysis gas chromatography high-resolution mass spectrometry (py–GC-HR-MS), which has been used successfully to measure lignin during fungal growth, without relying on gravimetric analysis (73, 74).

Similar to the quantification of lignin, the determination of ash content in bark relies on gravimetric analysis and was used in **Papers I** & **II.** This method involves subjecting the bark sample to oxidation at a temperature of 575°C, resulting in the combustion of organic material and leaving behind the inorganic ash residue (Table 2.2) (75). The accuracy of this method depends on the accurate measurement of the initial sample weight and the resulting ash.

Main points

- Bark is the outer tissue of the tree and has a protective role.
- The composition of bark differs from that of xylem.
- Extractives and lignin are abundant in bark.
- Polysaccharides can be quantified using hydrolysis.
- Quantification of bark lignin is difficult due to extractives.

3. Microbial growth on bark

Microorganisms account for roughly half of all biomass on earth and are found everywhere: deep within the earth, in the air, in the sea, on the surface/inside of plants and animals, and in the soil (76). Microbial communities residing in soil are incredibly diverse, comprising an astonishing array of bacteria, fungi, and archaea. Indeed, estimates suggest more than 10^6 individual species-level operational taxonomic units (OTUs) in a single soil type (77). Filamentous fungi, bacteria, and yeasts are key microorganisms of many microbial communities, with distinct substrate preferences, and key features, such as lignocellulose and extractive degradation/detoxification, that enable them to grow on organic matter.

There are two main approaches to studying microorganisms and microbial communities: culture-dependent and culture-independent (Fig. 3.1). The latter, rely on techniques such as amplicon sequencing, which typically targets specific gene regions in the 16S ribosomal RNA (16S rRNA) for bacteria or the ITS (Internal Transcribed Spacer) region for fungi (78, 79). As these two genetic markers are conserved and found in most species, they are very useful for identification and can be employed for comparing the relative abundance of different genera. Alternatively, the entire DNA from the sample can be extracted and sequenced, allowing for the assembly (binning) of reads into larger contigs or even complete genomes, in a process referred to as metagenomics. One of the key advantages of metagenomic analysis is the ability to sequence unculturable organisms, which are estimated to make up a large proportion (95%-99%) of species (78). The study of individual microorganisms or culture-based methods involves the isolation and enrichment of microorganisms or ordering them from microbial culture collections. By examining both isolated microorganisms and microbial communities, it is possible to investigate their phenotypic traits, such as morphology and growth. This is achieved through the evaluation of growth, the degradation of target compounds, and/or the analysis of the proteins produced, all of which reveal the functional potential of these species.

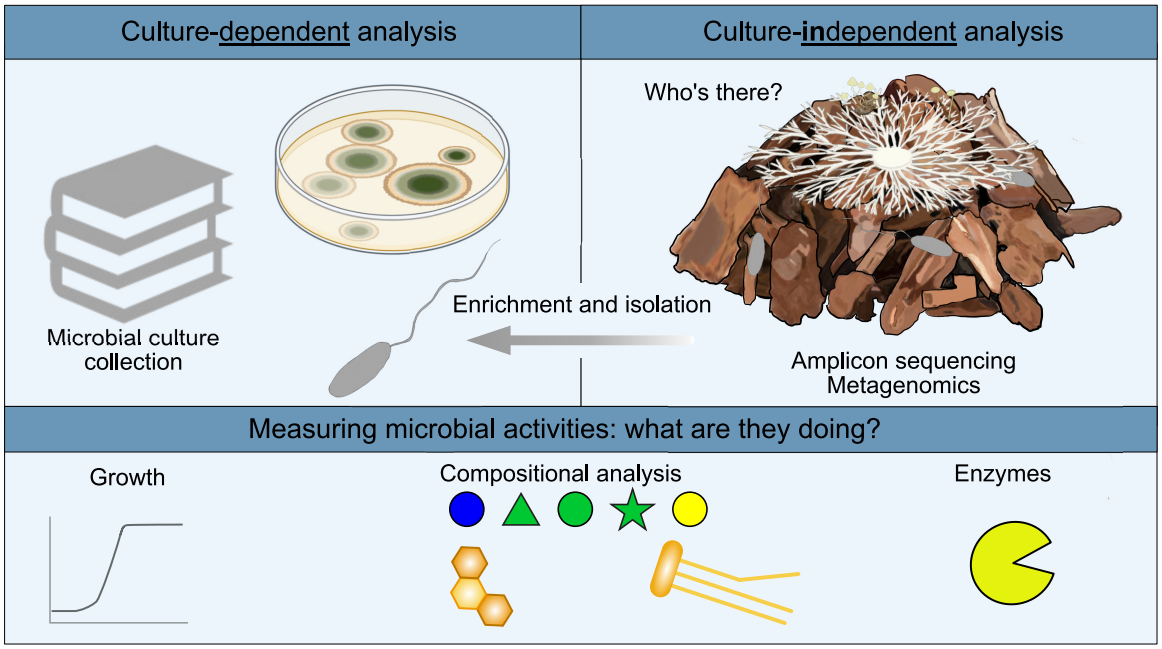


Figure 3.1. Overview of the methods used to study microorganisms in this thesis. Studies can either be culture-independent, such as in metagenomics where all DNA is sequenced (right), or culture-dependent, where individual species are isolated and studied (left). Both methods can also be combined to gain a more comprehensive understanding of the microbial community. The overarching goal is to monitor what the microbial community is doing during growth, either through compositional analysis of the carbon source or screening the activity of the enzymes produced.

3.1 The microbial community of bark

A wide variety of microorganisms colonize plants; some of them can be beneficial (mutualistic) while others can be detrimental (pathogenic) or neutral (commensal) (80). Microbial communities on the outside of the tree are composed of lichens, fungi that grow on the tree surface although they can also penetrate the outer bark, and bacteria. Fungi exhibit an extraordinary ability to break down complex polymers (81). Bacteria are ubiquitous and abundant, encompass a vast array of species, and display remarkable versatile metabolic activities (76). Indeed, they were suggested to represent the primary degraders of bark, due to the suitability of this substrate as a nutrient source (82).

Knowledge on bark-residing microorganism is scarce, but microbial community composition is known to differ between bark and wood during degradation (83). Moreover, the specific sampling point on the tree is a major factor (84), and the presence of bark has been found to slow down the degradation of the underlying wood (84). Recently, bark-residing methanotrophic bacteria have gained increasing attention, because they reduce methane emissions from trees, thereby contributing to the mitigation of global warming (85). While fungal communities on bark have received even less attention, they undergo significant changes during degradation (86). Nevertheless, the composition and development of microbial communities during the active decomposition of bark cannot be directly compared to similar studies focusing on wood degradation. This disparity arises

from the abundance of toxic extractive compounds in bark (83, 87), which despite their antimicrobial properties, have been frequently overlooked.

3.2 Rapid resin acid-degradation by a microbial community

Considering the limited understanding of bark microbial degradation and the impact of microbial growth on bark composition, the purpose of **Paper I** was to gain an understanding of this fundamental process. First, we created an inoculum from industrial spruce bark, which was used to inoculate gamma-irradiated sterile bark. Several bark samples were used throughout the study—one sample per replicate and time point—and, hence, an entire plate was required for both sequencing and chemical compositional analyses (and many plates were made).

We concentrated on extractives, as these are the compounds enriched in bark, but also investigated the effects on polysaccharides and lignin using the methods outlined in chapter 2. In **Paper I**, we observed a significant drop in resin acid concentration, especially during the initial growth phase, with minimal differences in other bark components (polymers) (Fig. 3.2). Among resin acids only 7-oxodehydroabietic acid remained relatively stable throughout the cultivation period, suggesting ongoing resin acid degradation as this compound is a known degradation intermediate (88). The slight increase in dehydroabietic acid on week 2 might indicate its release from the bark matrix, suggesting that some resin acids are weakly linked or that abietic acid undergoes microbial transformation into dehydroabietic acid during cultivation (89).

A combination of ITS (see **Paper I**) and 16S rRNA sequencing was used to investigate the bacterial and fungal communities (in this thesis, only the bacterial community data will be presented). The main species of rapid bark colonizers were from Pseudomonadota (formally known as Proteobacteria) and Bacteroidota (Bacteroidetes). While the relative abundance of Pseudomonadota species decreased after the initial colonization, they remained the dominant group. Interestingly, after 8-12 weeks, the relative abundances of species belonging to Bacteroidota and Verrucomicrobiota increased significantly. This shift suggested a potential transition towards carbohydrate utilization, as these phyla include specialists in carbohydrate metabolism.

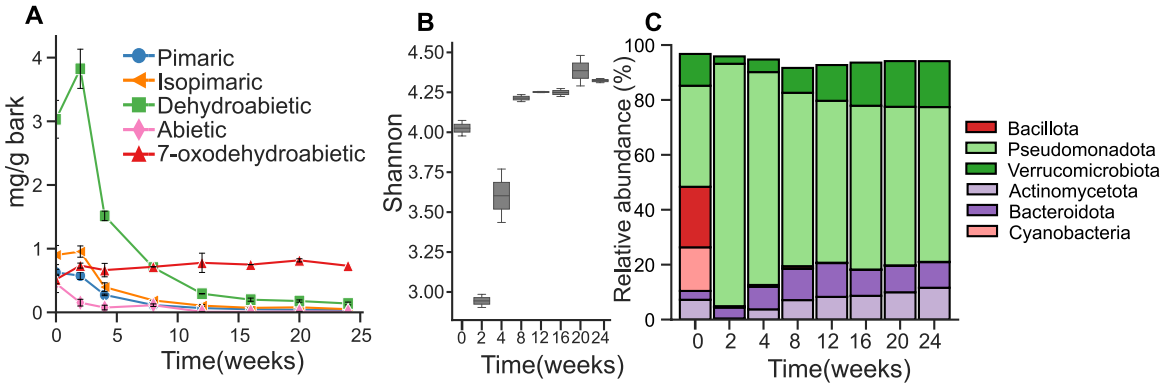


Figure 3.2. Effect of inoculating bark with a culture containing a mixture of both bacteria and fungi. A) Content of individual resin acids over time; only 7-oxodehydroabietic acid was unaffected by degradation. B) Alpha diversity of the bacterial community. C) Relative abundance of the phyla in the bacterial community over time. Mean values and standard deviations are based upon duplicate biological experiments. Adapted from Paper I.

During growth on bark, the diversity of the bacterial community decreased in the earliest (two week) sample, suggesting enrichment of microorganisms capable of degrading or tolerating resin acids (Fig. 3.2). To gain a more comprehensive understanding of the underlying biological processes, metagenome sequencing was performed on the two-week sample, allowing for the reconstruction of metagenome-assembled genomes (MAGs) (Fig. 3.3). Taxonomic analysis of the metagenomic data confirmed that bacteria were the dominant organisms, accounting for 76% of the contigs, followed by eukaryotes (8%) and unclassified organisms (18%). MAGs were analyzed using BLAST to search for a *dit* gene cluster previously shown to be essential for resin acid degradation, and a varying degree of completeness was seen. Only Pseudomonadota species were found to have *ditA1* homologs, encoding an essential resin acid-degrading protein (Fig. 3.3) (90). MAG 15, which contained all essential genes appeared to be the most prevalent MAG in the culture with a relative abundance of 19.2%. To gain further insight into the resin acid-degrading population present at two weeks, bacterial colonies able to grow on abietic acid were enriched and isolated, as described in section 3.4.

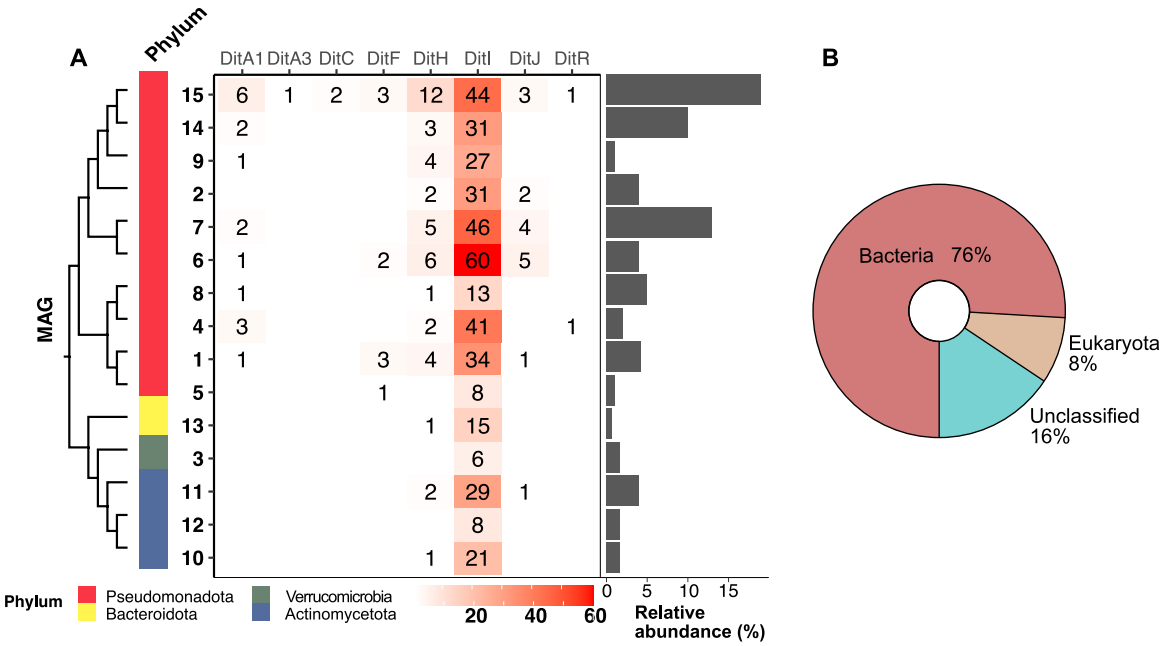


Figure 3.3. Metagenome Assembled Genomes (MAGs) from the two-week spruce bark degradation sample. A) Taxonomic classification is based on the Genome Taxonomy Database, and phyla are indicated by color (91). MAGs are clustered according to proteome-based Genome Blast Distance Phylogeny distance using the TYGS database (92). This type of phylogenetic clustering was used as no 16S rRNA sequence could be extracted from MAG12. The relative abundance of MAGs (determined via CoverM v. 0.6.1.) within the metagenome is shown on the right. Only genes within the *dit* cluster shown to be essential for resin acids degradation are displayed. B) Comparison of fungal, bacterial, and unclassified contig abundance in the sample. Modified from Paper I.

3.3 Isolation and characterization of a new bacterial species

The isolation and characterization of novel bacterial species is fundamental in microbiology. These studies expand our knowledge on microbial diversity, ecological roles, and potential biotechnological applications of newly discovered microbial species. Generally, the initial step involves the enrichment and isolation of a specific bacterial species of interest (Fig. 3.4). To identify the bacterial species, genotypic identification methods, such as sequencing of the 16S rRNA genome region are employed. To this end, it is crucial to obtain a full-length, high-quality sequence and manually inspect the chromatograms (Sanger ab1 files). While the 16S rRNA region is widely used for bacterial identification; it may not always discriminate between different species within a genus because of its relatively slow rate of evolution. Thus, in the case of *Pseudomonas*, multiple housekeeping genes (e.g., *gyrA* or *rpoD*) can confirm the identity of the isolate as a new species (93). In the past, species identification relied mainly on biochemical and morphological studies, but nowadays it is based on whole-genome sequencing. A comparison is conducted between the sequenced genome and known type strains, and a cut-off value of 94%~96% average nucleotide identity (ANI) is typically used to determine species differentiation (94).

Once a genome with annotated features is acquired, the next step is to determine the biological functions or activities associated with, for example, the protein-coding regions. This is where errors can occur as many genes are assigned based on homology rather than experimentally validated data. An example of database used to assign functional annotation is Pfam (95), which portrays proteins as hidden Markov models and encompasses a wide array of sequences from diverse microorganisms. Additionally, TIGRFAM offers curated multiple sequence alignments for the classification of protein sequences (96). InterPro along with eggNOG-mapper make up a compilation of protein sequence databases, performing searches and linking matches to hits with other information (e.g., Gene Onthology and EC-numbers) (97, 98). Furthermore, there are also specialized enzyme databases, such as dbCAN, which can be used to annotate CAZymes (99).

Characterization of a newly discovered bacterial species involves a comprehensive analysis of its morphological and phenotypic attributes, which can vary depending on the taxonomic group, and is used to differentiate the new species from closely related ones. Bergey's Manual serves as a valuable resource for obtaining phenotypic data of related species (100). Finally, both sequence data and the new species must be deposited in public databases, such as The National Center for Biotechnology Information (NCBI) and culture collections such as DSMZ, ATCC or GUCC, to ensure accessibility of the new strain. At the same time, a record of novel microbial taxa should be submitted to the International Journal of Systematic and Evolutionary Microbiology.

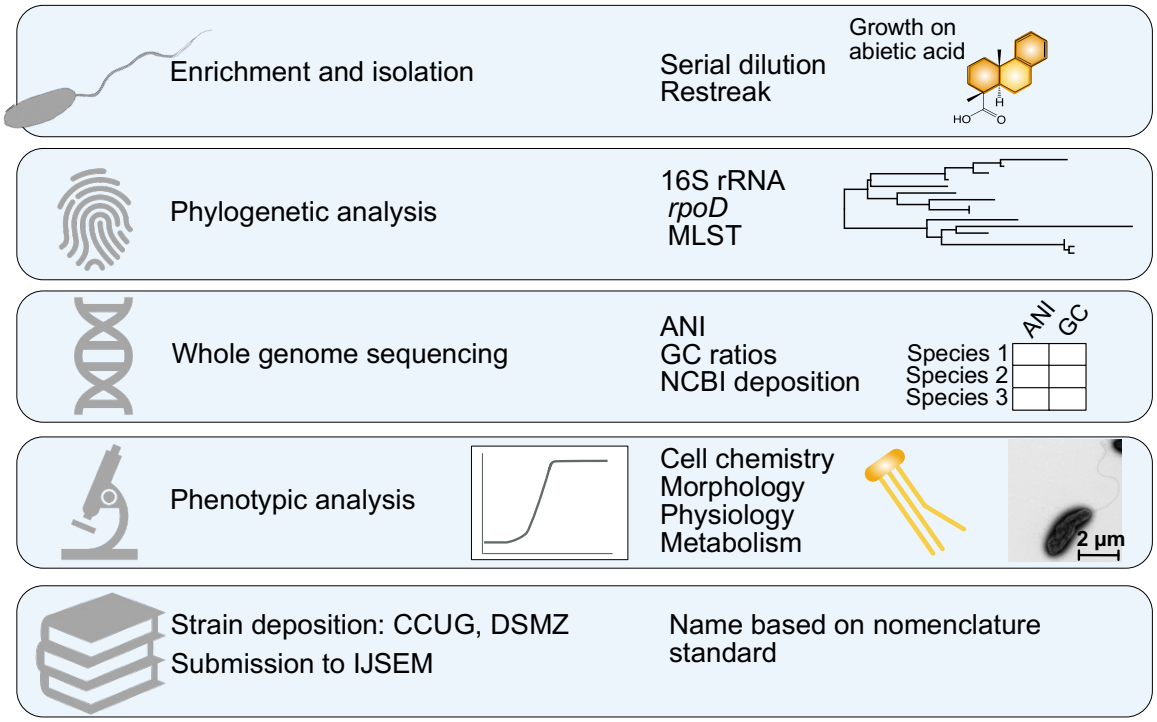


Figure 3.4. Overview of isolation and characterization of a bacterial species from a microbial community growing on spruce bark. The steps used for the isolation of *Pseudomonas abieticivorans* (described below) are shown on the right. First, the species of interest must be isolated as a pure culture, and the genus is determined by sequencing either the 16S rRNA or housekeeping genes such as *rpoD*. Multiple housekeeping genes can be used to discriminate between different bacterial species using multilocus sequence typing (MLST). If the isolate is suspected to be a new bacterial species, its whole genome is sequenced and compared to previously sequenced isolates using the ANI and GC ratio. A subset of traits is used to discriminate the isolate from known organisms and, finally, the strain is deposited in a culture collection, named according to nomenclature standards, and published in the International Journal of Systematic and Evolutionary Microbiology (IJSEM).

3.4 *Pseudomonas abieticivorans* sp. nov — a resin acid-degrading bacterium isolated from spruce bark

Given that resin acid degradation is a major event during the early stages of spruce bark breakdown (see section 3.2), I set to study resin acid-degrading microorganisms in more detail. Based on the compositional analysis of the two-week microbial community sample, I successfully enriched for bacteria capable of growing on abietic acid as a sole carbon source and isolated a strain initially named Barky PIA16 (**Paper I**) (Fig. 3.5). Based on metagenomic data, I suspected the bacterium to be either a member of *Pseudomonas*, *Burkholderia*, or *Paraburkholderia* as these were abundant at two weeks. To identify the strain, I first amplified the 16S rRNA gene by PCR and conducted a BLAST search of the resulting sequence in the NCBI database, confirming the isolate's classification as a *Pseudomonas* species. To overcome the poor resolution of the 16S rRNA gene in *Pseudomonas*, I instead analyzed the sequence of another housekeeping gene, the sigma 70 factor subunit of DNA polymerase (*rpoD*) (93). The closest match to the *rpoD* sequence

displayed a similarity of 91.96%, which is well below the recommended threshold of 97% for defining new *Pseudomonas* species (93).

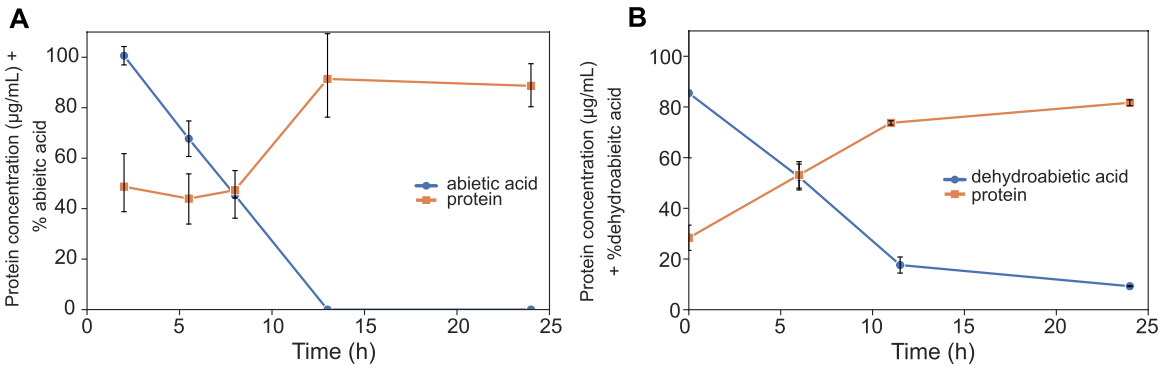


Figure 3.5. Growth of *Pseudomonas abieticivorans* PIA16 on resin acids. A) Growth on abietic acid. B) Growth on dehydroabietic acid. The intracellular protein concentration was used as a proxy for growth because optical density monitoring was complicated by the insoluble nature of resin acids. Means and standard deviations are based on duplicate biological experiments. Adapted from Paper I.

Based on the low similarity of the *rpoD* sequence (<97%), the whole genome of Barky PIA16 was sequenced and run through the DSMZ Type (Strain) Genome Server (92) to check for similarities with already isolated type strains within the *Pseudomonas* genus. All ANI percentages were below 95% (78), indicating that Barky PIA16 was a new species. Additional comparisons of the 16S rRNA sequences with all NCBI data were conducted. ANI values for the ten most related strains were all much lower than the recommended 95% cut-off, and thus a new species name was proposed: *Pseudomonas abieticivorans* (a.bi.e.ti.ci.vo'rans. N.L. neut. n. acidum abieticum, abietic acid; L. pres. part. vorans, eating; N.L. part. adj. abieticivorans, eating abietic acid).

To check if the isolated *Pseudomonas abieticivorans* PIA16 was indeed present in the bark consortium, MAG genomes (section 3.2) were compared to the *P. abieticivorans* PIA16 genome. The ANI match (>99%) between the isolate and the highly abundant MAG15 (relative abundance of 19.2%) supported it being the same species. This highlights the importance of *P. abieticivorans* in the early bark microbiome, yet how it degrades resin acids remains to be determined.

3.5 Bacterial degradation of resin acids

As mentioned in section 2.3**,** resin acids are abundant in spruce bark, with the two main types being dehydroabietic acid and abietic acid (35). Microorganisms with the ability to mineralize resin acids have been isolated from various sources, from enriched bioreactors to papermill effluents (34, 101). The majority have been identified as Gram-negative, and only a few as Gram-positive (89, 102). Most of these bacteria can metabolize resin acids, but only a minor amount can transform them (103). Typically, bacteria that have been isolated on abietane-type resin acids cannot grow on pimarane resin acids; whereas those isolated on pimarane resin acids can degrade abietane-type resin acids (89).

The initial steps of dehydroabietic acid and abietic acid metabolism have been proposed based on intermediates from culture media and gene deletions (Fig. 3.6) (89, 103). The first studies employed a bacterium called *Pseudomonas abietaniphila*, which grew on abietic acid, dehydroabietic acid, palustric acid, and 7-oxodehydroabietic acid. Genome analysis revealed the presence of a core and an extended *dit* gene cluster (as briefly touched upon in section 3.3) shown to harbor genes involved in the degradation of abietane diterpenoids (104). Within this cluster, a ring hydroxylating dioxygenase called *dit*A1 was identified, and found responsible for catalyzing the formation of a catecholic intermediate. To investigate its role, *dit*A1 knockout mutants were generated in both *Paraburkholderia xenovorans* (previously *Burkholderia xenovorans*) and *P. abietaniphila*, and the strains were unable to grow on resin acids, instead accumulating 7-oxodehydroabietic acid (104, 105). It was also demonstrated that *dit*A1 required a ketone at position C7, as dehydroabietic acid could not serve as a substrate for the enzyme (88, 104).

The initial steps in resin acid degradation rely on P450 monooxygenases, with two proposed initiation pathways: oxidation of the A ring at position C3 or of the B ring at position C7 (89). Two P450 monooxygenases were identified within the extended *dit* cluster. In the case of *P. abietaniphila*, a *dit*Q knockout (KO) mutant strain grew on all resin acids mentioned above, suggesting the presence of an alternative degradation pathway, possibly involving a second P450 enzyme, which was later identified (106). Notably, the crude lysate enriched in *dit*Q bound to dehydroabietic acid, but not abietic acid, palustric acid, 7-oxodehydroabietic acid or isopimaric acid (106). *P. xenovorans* on the other hand, encodes for two P450 enzymes, *dit*Q and *dit*U, and knockout of *dit*Q resulted in impaired growth on dehydroabietic acid and palustric acid, but had no effect in cells grown on abietic acid or 7-oxodehydroabietic acid (105). The second P450 enzyme, *dit*U, was unable to support growth on abietic acid but could facilitate growth on 7-oxodehydroabietic acid and dehydroabietic acid (105).

As mentioned in section 3.2, the genome sequence of PIA16 (and MAG15) revealed an intact *dit* cluster (and extended *dit* cluster) similar to the one found in *P. abietaniphila* (Fig. 3.6), which likely explains its ability to degrade abietane-type resin acids (104). A novel

and interesting phenotype of this organism, however, is its ability to degrade pimarane-type resin acids (see **Paper I**), which warrants further investigation.

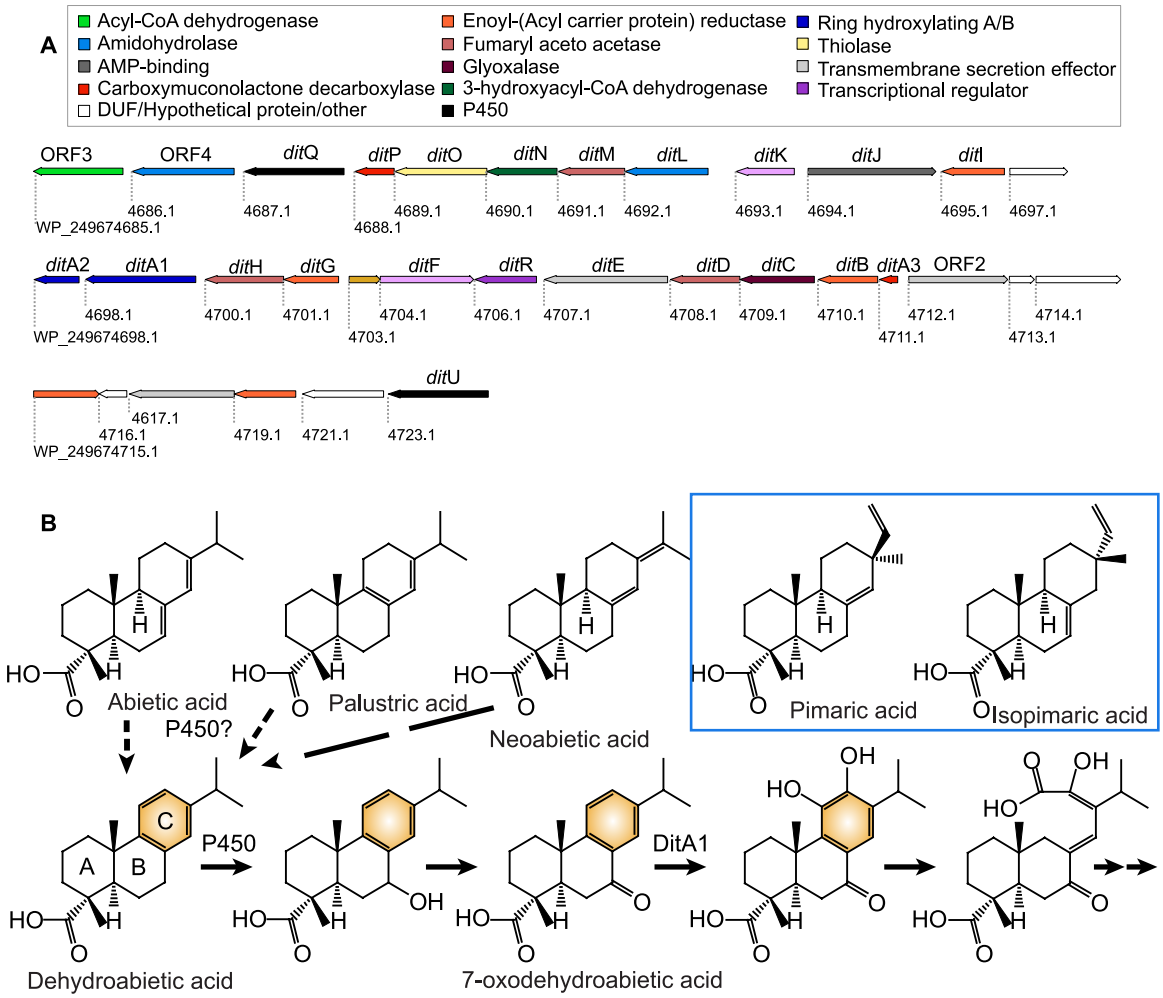


Figure 3.6. The identified *dit* cluster in *Pseudomonas abieticivorans* which spans 34.6 kbp and proposed degradation pathway. A) The *dit* cluster in *P. abieticivorans*. GenBank accession numbers are shown below each gene, gene names are reported above, and their functional annotations according to Pfam are color coded. B) Summary of proposed biochemical pathways for aerobic abietane degradation. Dashed lines denote multiple reactions. Credit to Dr Johan Larsbrink for Figure B. Modified from Paper I.

3.6 Fungal degradation of lignocellulose and extractives

Fungi, which encompass both filamentous fungi and yeasts, are among the main biomass degraders in nature. As solid substrates cannot be taken up directly by fungi, they secrete enzymes into the extracellular environment to degrade polymers and then take up smaller oligosaccharides or monosaccharides (44). Traditionally, fungi have been classified based on which mechanisms they use to degrade lignocellulose and the type of rot they exhibit (Fig. 3.7). White-rot fungi from the Basidiomycota phylum, efficiently degrade lignin, either in combination with cellulose or cellulose and hemicellulose (107). Brown-rot fungi, also belonging to the Basidiomycota phylum, selectively depolymerize cellulose and

hemicellulose while leaving brown lignin relatively intact (107). Soft-rot fungi, found in both the Ascomycota and Basidiomycota phyla, cause a softening and disintegration of the wood structure (12). They target primarily the cellulose and hemicellulose components of lignocellulose, resulting in only limited degradation of lignin (81). Yeasts are unicellular microorganisms found both in the Ascomycota and Basidiomycota phyla (108). Ecological studies of filamentous fungi and yeasts during natural decay of wood show elevated yeast diversity in delignified wood (109). Fungi act as the main lignocellulose-degrading organisms, providing monomeric or oligomeric saccharides that support yeast growth (109).

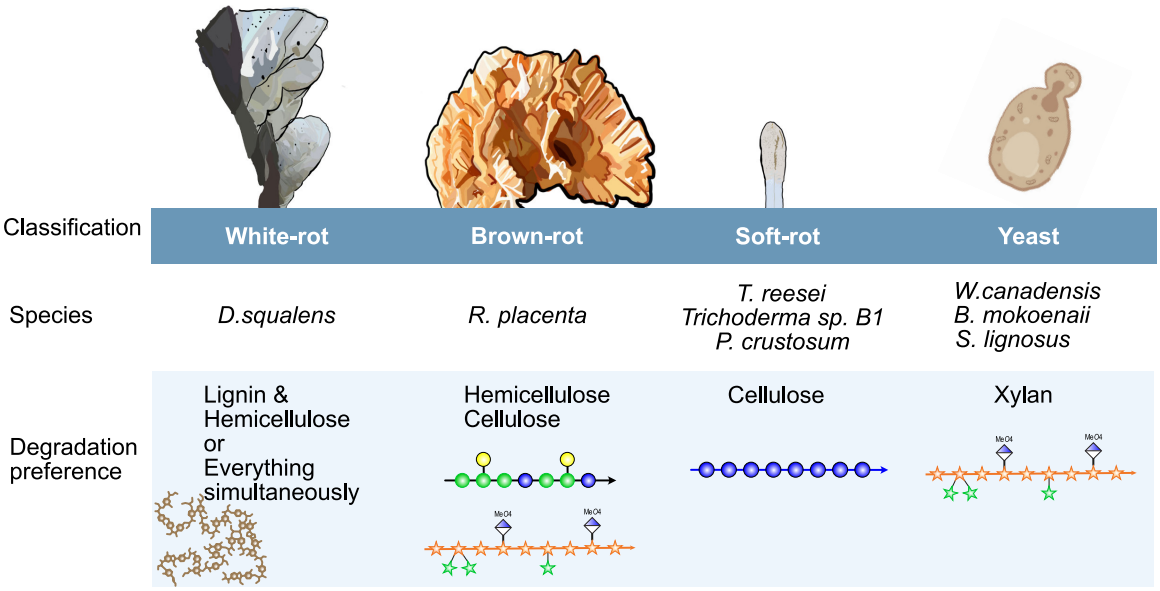


Figure 3.7. Overview of fungi capable of degrading bark or its components, along with their substrate specificity. The top row shows the general classification of fungi, based either on the type of decay or the type of fungus; the middle row shows the fungal species used in this thesis; and the bottom row lists the proposed substrate preference for each or these species: *Dichomitus squalens, Rhodonia placenta*, *Trichoderma reesei*, *Trichoderma sp. B1*, *Penicillium crustosum*, *Blastobotrys mokoenaii*, *Scheffersomyces lignosus*, and *Wickerhamomyces canadensis*.

While fungi are known to degrade wood polymers present in bark, their ability to break down extractives is less clear. Extractive degradation has been studied mainly in the context of pitch control, as these compounds cause pitch deposits during pulp and paper manufacturing (35, 110). This has led to the discovery of the sapstain fungus *Ophiostoma piliferum*, which breaks down the triglycerides in wood and leaves the polysaccharides relatively intact (110). *Phlebiopsis gigantea*, which metabolizes non-toxic triglycerides using lipases (111, 112) is more understood. In many cases, degradation is not limited to extractives but also includes polysaccharides and lignin (113, 114).

Studies on the degradation of more toxic components such as resin acids have been carried out in Scots pine, revealing that the extracellular culture fluid from the brown rot fungus

Rhodonia placenta (formally: *Postia placenta*) degraded lignans and resin acids (115). The response of *Phanerochaete chrysosporium* towards tannin-containing oak acetonic extract revealed upregulation of CytP450 and a glutathione transferase, suggesting their requirement for detoxification (116). Very few studies have focused on the degradation of both extractives and polymers in bark. A proteomic study using 2D gel electrophoresis examined the filamentous fungus *Aspergillus nidulans* growing on cork oak bark (117). Two white-rot fungi, *Phanerochaete velutina* and *Stropharia rugosoannulata*, grown on Scots pine bark were found to degrade hemicelluloses, cellulose, and certain acetone extractives, although the specific compounds were not examined (118).

3.7 The role of fungi in bark degradation

My findings on microbial communities growing on spruce bark (see section 3.2) could not point to a specific role of fungi in the degradation process due to their low abundance within the overall population. Nevertheless, initial experiments in **Paper II** indicated that filamentous fungi grew well on bark. Consequently, as presented in section 3.6, I selected five taxonomically diverse fungi, which exhibited distinct lignocellulose degradation modes. The three fungi from the Ascomycota phylum comprised a fungus isolated from a tree stump in Hälsingland County, Sweden (*Trichoderma* sp. B1), a rapidly growing green contaminant (*Penicillium crustosum*), and *Trichoderma reesei*, a cellulolytic filamentous fungus. White-rot and brown-rot Basidiomycetes were represented by *Dichomitus squalens* and *R. placenta*, respectively. The fungi were cultivated on bark for six months and their growth was evaluated.

Measuring the growth of filamentous fungi presents some challenges owing to their morphology, and it is further complicated by the use of insoluble substance such as bark as a carbon source. Proxy strategies include measuring extracellular protein concentration, ergosterol content, fungal biomass weight, or quantifying mass loss in the material (119). In my case, I opted for the latter (Fig. 3.8), although this approach entailed measuring the combined mass of bark and fungi, and not only fungal growth. The fungi displayed different rates of growth, with *P. crustosum* rapidly colonizing bark without any measurable lag-phase, and *D. squalens* showing the highest total mass loss (Fig. 3.8). The fungi exhibited different growth patterns, suggesting different substrate preferences. This prompted me to perform a compositional analysis to determine the substrate preferences of each fungus.

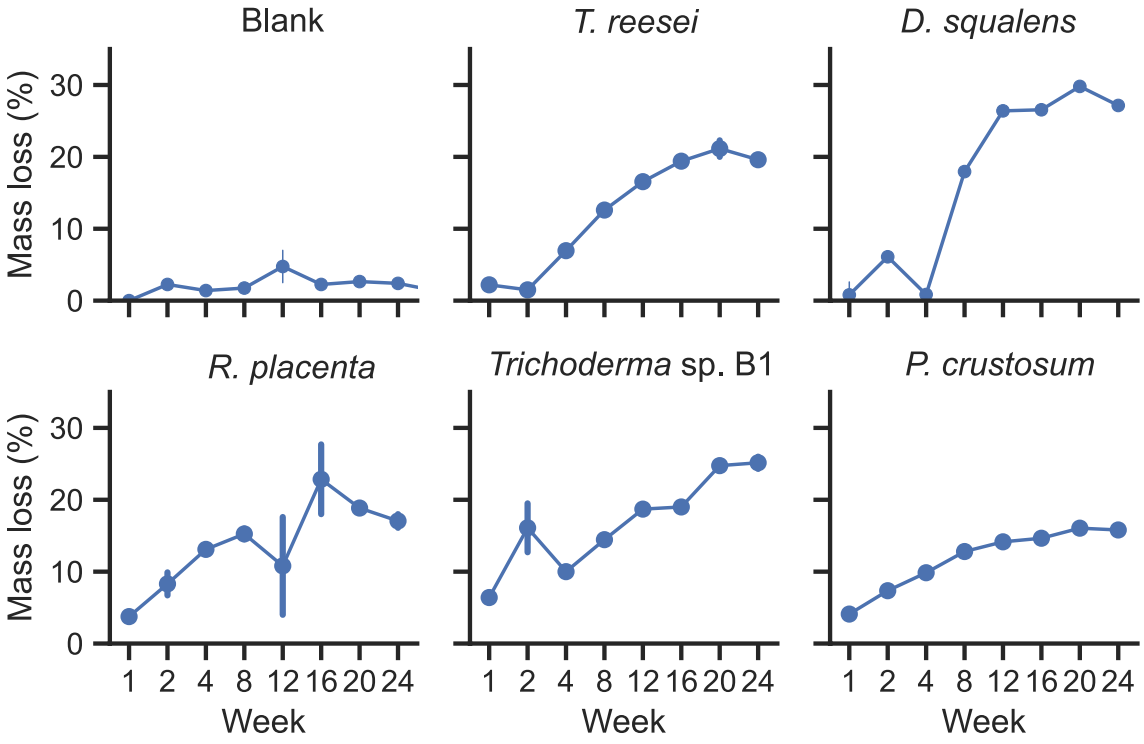


Figure 3.8. Growth of filamentous fungi during six months (24 weeks) using spruce bark as the sole carbon source. Measurements were based on overall mass loss. No fungus was added to the blank (control) sample. The plates were incubated at 20°C. Values correspond to the mean of duplicate biological experiments. Adapted from Paper II.

Extractives have been associated with the decay-delaying properties of bark. Specifically, I studied the effect of fungal growth on resin acids, sterols, and fatty acids (Fig. 3.9). Unsurprisingly, all fungi could degrade fatty acids (e.g., hexadecenoic acid), but their ability to break down dehydroabietic acid and β-sitosterol varied substantially. In particular, stark difference was found for Ascomycota and Basidiomycota species, with the former having difficulty degrading dehydroabietic acid, whereas Basidiomycetes were able to degrade or modify it. Similarly, β-sitosterol accumulated in cultures of *P. crustosum, D. squalens*, and *Trichoderma* sp. B1, suggesting that they could utilize steryl esters as a source of energy for growth, but had limited ability to further metabolize β-sitosterol. Instead, *T. reesei* exhibited no degradation. Finally, *R. placenta* could degrade/modify β-sitosterol, indicating it could metabolize both sterols and steryl esters. As filamentous fungi are particularly well known for their ability to degrade polysaccharides, this aspect was examined further.

Distinguishing the origin of monosaccharides, whether derived from bark or fungal biomass, and identifying the specific polysaccharides after complete hydrolysis of samples presented significant challenges during polysaccharide analysis. Even though certain monosaccharides, such as Xyl and Rha, are almost exclusively associated with GAX and pectin, respectively, others, such as Ara and Gal, may originate from both hemicellulose and pectin side chains. Moreover, Glc and Man are found in cellulose, starch, glycan, mixed-linked-β-glucan, mannan in the fungal cell wall, and/or GGM. To determine exactly

which polymer components can or cannot be degraded by fungi, it is necessary to ascertain substrate specificities through extraction of the polymers from bark or antibodies. Total hydrolysis of bark, which was employed here, serves as a good initial indicator of how individual monosaccharide abundance changes during fungal growth. Importantly, it can (in some cases) be linked to specific polysaccharides and serve as a basis for future studies.

The largest differences in polysaccharide degradation were found for pectin and GAX (Fig. 3.9). After 12 weeks, the white-rot fungus *D. squalens* achieved significant removal of Xyl (64%) and Rha (66%), indicating efficient degradation of both GAX and pectin. In contrast, the other Basidiomycetes *R. placenta* displayed a clear preference for pectin, as indicated by removal of 47% Rha at week 12, along with simultaneous break down of Xyl, albeit to a lesser extent. A similar trend was observed with *P. crustosum*, which exhibited even greater preference for early-stage pectin degradation, having removed 66% of Rha at week 12, and degrading Xyl continuously until week 24. In comparison, the two *Trichoderma* species showed limited degradation of Rha (~27%; week 12) and a preference for Xyl instead (~60%; week 12). These differences in pectin and GAX, show that the fungi have clear polysaccharide preferences when grown on bark.

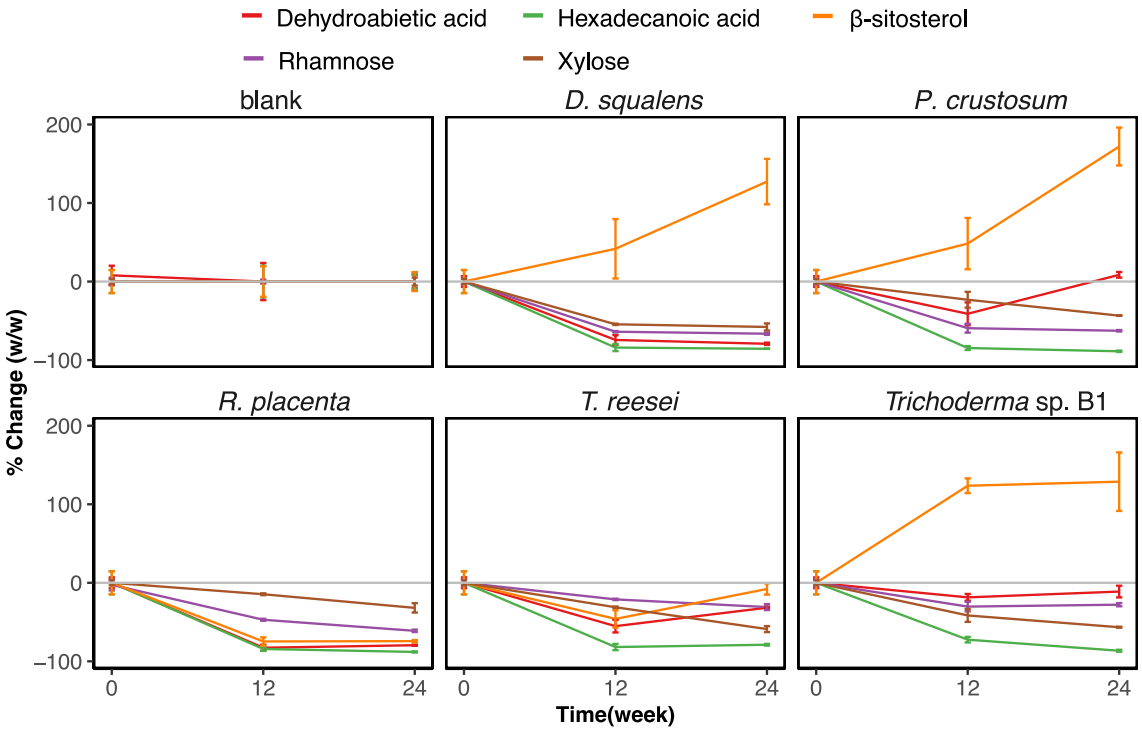


Figure 3.9. Impact of the growth of five filamentous fungi on selected extractive compounds in spruce bark. No fungus was added to the blank sample. An increase in the amount of compound is indicated by a positive value, and a decrease by a negative value. Data represent the means from triplicate biological experiments and error bars show the standard error. Modified from Paper II.

Notably, little to no lignin degradation was observed (see **Paper II**), which could be due to many different reasons, the simplest being that the fungi did not degrade lignin. However, the methodology used for measuring Klason lignin is easily disturbed by any other acid-insoluble material. Samples from fungal cultures contain substantial amounts of mycelium, which is largely constituted of acid-resistant chitin (and glucan). To bypass these disturbances, one could remove the fungal biomass before hydrolysis or quantify chitin in the AIR by boiling the sample in concentrated hydrochloric acid for several hours. In both cases, however, quantification would still rely on gravimetric analysis, at which point one may opt to use py-GC-HR-MS (73, 74).

Rather than degrading resin acids and sterols, the Ascomycota species investigated here tend to tolerate these compounds and, akin to *P. gigantea*, they may rely on ABC transporters to maintain their tolerance (111, 112). Successful growth of *D. squalens* on bark seems to be due to its ability to degrade/modify almost all bark components, ranging from extractives to pectin and GAX. Therefore, the proteins produced during growth on bark were investigated, as outlined in section 4.3.

3.8 Scavenging xylan oligosaccharides enables growth of *Wickerhamomyces canadensis*

Next, I investigated the microbial strategies of yeasts for metabolizing pure xylan. Yeasts have pivotal roles in various biological processes such as fermentation, as well as in biotechnological applications (120, 121). Most studies have focused on *Saccharomyces cerevisiae* or lipid-producing species, but recent research has demonstrated that yeasts could also break down complex biomass (122) including xylan (123). Yeasts produce xylanolytic enzymes such as xylanases (122), which can be either secreted, remain in the cell or become attached to the cell surface, thereby providing the yeast with a range of strategies for xylan degradation.

The production and secretion of enzymes is an energetically expensive process for microorganisms, and extracellular enzymes become ‘public goods’ in the microbiome, thus benefitting organisms other than the producer. Therefore, many microorganisms have evolved sophisticated strategies to limit wasting energy on enzyme secretion, by keeping enzymes within close proximity to the cell. This is the case of cellulosomes, which were first discovered in bacteria (124), and have later also been found in some anaerobic fungi (125). Tethering of enzymes to the cell surface reduces the chances of enzyme loss in the environment and ensures that the products remain close to the cell, thereby minimizing the risk of nutrient theft by opportunistic microorganisms seeking to scavenge released oligosaccharides.

Paper III focuses on three xylan-degrading yeasts: *Blastobotrys mokoenaii*, *Scheffersomyces lignosus*, and *W. canadensis.* Their genomes were mined to

reveal their strategies for xylan degradation. These three yeasts were selected based on a previous publication that screened 40 out of 332 genome-sequenced yeast for growth on xylan (122). The three candidates grew on xylan, were from different parts of the taxonomic tree, and displayed different xylanase CAZyme profiles, as well as predicted subcellular localization.

To determine the subcellular localization of potential xylanases, we measured the xylanase activities in the secretome, intact yeast cells, and lysed cells using the dinitrosalicylic acid (DNS) assay and a chromogenic substrate to assay for β-xylosidase activity (Fig. 3.10). Extracellular xylanase activity was observed only for *B. mokoenaii*; whereas xylanolytic activity was associated with the cell surface in *S. lignosus*. *W. canadensis* cells that had been grown on xylan alone exhibited no xylanase activity, while those incubated with xylan and xylooligosaccharides (XOs) displayed extracellular xylanase activity. All species exhibited β-xylosidase activity.

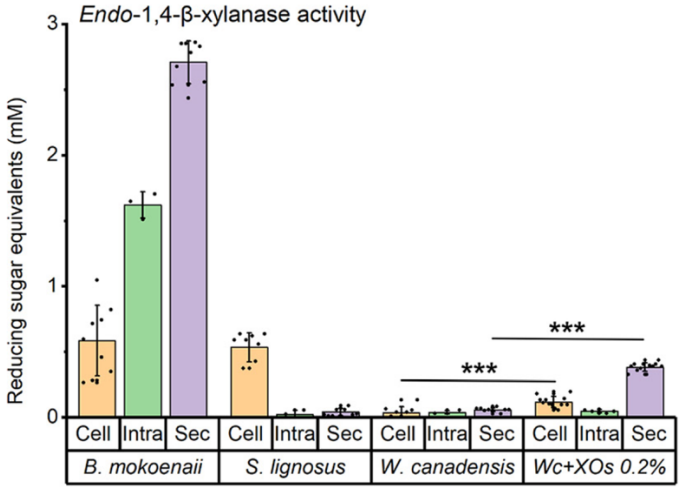


Figure 3.10. Localization of xylanolytic activity in three yeast species. Yeast subcellular xylanase activity originating from the secretome (Sec), cells (Cell), and intracellular material (Intra) from lysed cell fractions was compared using dinitrosalicylic acid reducing sugar assays in at least triplicate experiments. Values represent the means and error bars denote standard deviations. Adapted from Paper III.

Compared to the other two yeasts, *W. canadensis* grew poorly on xylan, but demonstrated xylanase activity following addition of XOs, indicating that the latter triggered its xylanolytic system (Fig. 3.11). Furthermore, addition of XOs to xylan significantly enhanced the growth rate of *W. canadensis*, surpassing growth observed when using XOs alone or xylan as the sole carbon source. Moreover, the same effect was observed when the two heterologously expressed xylanases, *Bm*Xyn11A and *Wc*Xyn5_49A (further discussed in section 4.4), were added. To test whether the strategy employed by *W. canadensis* to grow on xylan involved scavenging of released XOs, we grew the strain in a co-culture with *B. mokoenaii* and *S. lignosus*. *B. mokoenaii* secretes xylanases while in *S. lignosus*, the activity is cell-associated (Fig. 3.10). In both liquid (see **Paper III**) and agar plates, co-culture with *B. mokoenaii* boosted growth of *W. canadensis.* On plates, a clearing zone

derived from xylan hydrolysis by *B. mokoenaii* was observed, followed by growth of *W. canadensis* (Fig. 3.11).

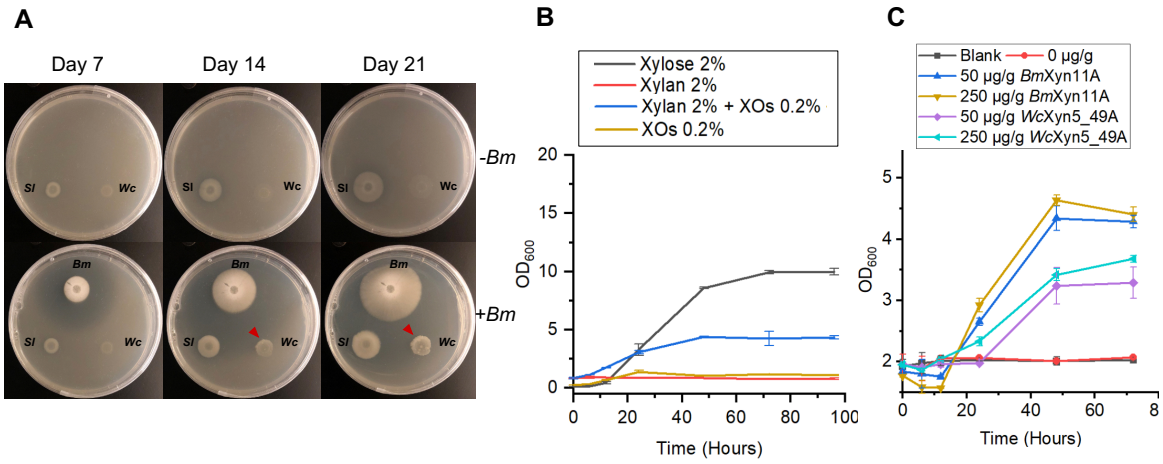


Figure 3.11. *W. canadensis* growth on xylan is boosted by the addition of xylooligosaccharides. A) Growth of the three yeasts on 0.4% beechwood glucuronoxylan agar plates with (+Bm) or without (–Bm) the addition of *B. mokoenaii*. The clearing zones correlate with xylanase-mediated xylooligosaccharide release and enable *W. canadensis* growth (red arrows). B) Yeasts grown in 10 mL Delft minimal medium with 2% beechwood glucuronoxylan or 2% xylose as the sole carbon source in biological triplicates. XOs, xylooligosaccharides C) Growth of *W. canadensis* in 2% beechwood glucuronoxylan supplemented with *Bm*Xyn11A and *Wc*Xyn5_49A at lower (50 mg/g xylan) or higher (250 mg/g xylan) concentrations in biological triplicates. *Bm*, *Blastobotrys mokoenaii*; *Sl*, *Scheffersomyces lignosus*; *Wc*, *Wickerhamomyces canadensis.* Adapted from Paper III.

This finding prompts the exploration of co-culturing species such as *B. mokoenaii*-*W. canadensis* to obtain valuable insights into whether opportunistic species exhibit a similar behavior when acting upon different types of polysaccharides. This comparative analysis could unveil the broader scope of polysaccharide degradation among yeasts but also other microorganisms.

Main points

- Microorganisms can act together to degrade bark.
- Microorganisms with complex metabolism can be difficult to isolate.
- Bacteria seem to the dominate initial stages of bark degradation.
- Bacteria can degrade resin acids, which has been ascribed to the presence of the *dit*-cluster.
- Fungi can grow on bark and influence both polysaccharides and extractives.
- Xylan-degrading yeasts employ different strategies, including secretion of xylanases or tethering them close to the cell.

4. Biochemical degradation of bark

Advancements in genomics have radically transformed the field of enzyme discovery. With the entire DNA of an organism being easily analyzed, it is now possible to locate the genes expected to encode for proteins of interest. This can be achieved either by searching for genes that are similar to known proteins or by employing computational tools to predict the genes' function. Proteomics reveals a microorganism's complete or partial set of proteins produced under specific conditions and can be used to detect putative new proteins. Once potential proteins have been detected or identified in the genome, they can be expressed in a production host and purified for further characterization. This process may entail screening the enzyme's activity against different substrates, ascertaining its optimal pH and temperature, investigating its mechanism of action, and solving its structure (Fig. 4.1).

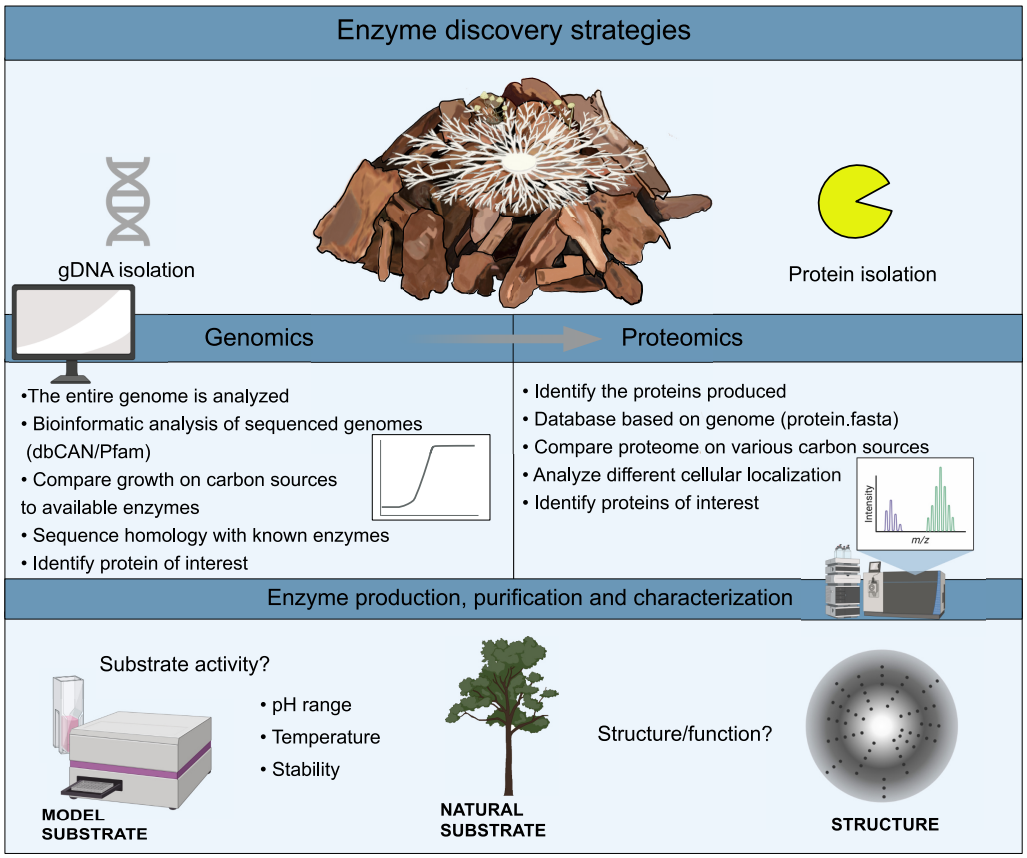


Figure 4.1. Overview of methods used to identify enzymes in this thesis. Discovery of enzymes can start with sequencing of a genome or mining an already sequenced organism's genome for interesting features by, for example, sequence comparison to known enzymes, or relating types of CAZymes to the physiology of the organism. Proteomics analysis requires knowledge of an organism's genome sequence, so that peptides can be mapped to an appropriate template. Because the proteome varies depending on the environment, it can yield information about growth on different carbon sources. Once proteins of interest are identified, they can be heterologously expressed and characterized on both model and natural substrates. Created in Biorender.com.

4.1 Bark-degrading enzymes are highly diverse

As has been discussed in chapter 2, bark is a highly complex matrix, containing not only the polymers found in the wood—lignin, hemicellulose, and cellulose—but also a highly diverse range of extractives (1). The heterogenous composition of bark requires an arsenal of different enzymes, capable of targeting wood polymers as well as extractives, to hydrolyze it. Degradation and modification of polysaccharides can be accomplished by CAZymes, which are categorized according to their functions and families based on amino acid sequence similarity in the Carbohydrate-Active enZYmes Database (CAZy) (13). In contrast, extractive-acting enzymes are not found in any consolidated database, although some, such as cutinases (esterases active on suberin) found in carbohydrate esterase family 5, appear in CAZy (36). It is possible to find some extractive-acting enzymes in BRaunschweig ENzyme Database (126), as well as the ESTerases and alpha/beta-Hydrolase Enzymes and Relatives database (127). At present, the best characterized extractive-degrading enzymes are lipases (EC 3.1.1.3) and tannases (EC 3.1.1.20), but probably the complete set of such enzymes encompasses a multitude of functions and families, many hitherto undiscovered (14).

4.2 Carbohydrate-active enzymes can be co-localized in the genome

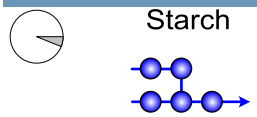
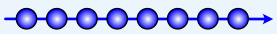
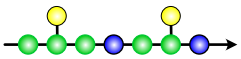
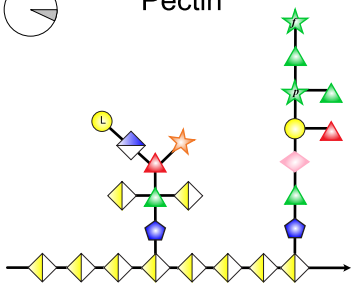
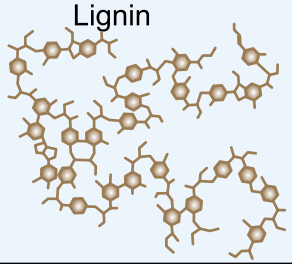
CAZymes are responsible for assembling, modifying, and degrading carbohydrates and glycoconjugates. Moreover, some CAZy families are involved in the deconstruction of lignin (13, 128). In my work, I have focused on the degradative capacity of CAZymes. As previously mentioned, CAZymes are organized in the CAZy database in the following five enzyme classes and associated modules (13):

- Glycoside Hydrolases (GHs) – Break glycosidic bonds by hydrolysis. This thesis will further discuss GHs below.
- GlycosylTransferases – Form glycosidic linkages by synthesizing di- and oligo-polysaccharides.
- Polysaccharide Lyases – Break glycosidic bonds through elimination reactions in uronic acid-containing polysaccharides, such as pectins.
- Carbohydrate Esterases (CEs) – Break ester-linked side groups decorating the polysaccharide backbone and/or side chains.
- Auxiliary Activities (AAs) – Redox enzymes, such as Lytic Polysaccharide Mono Oxygenases (LPMOs).
- Carbohydrate Binding Modules – Proteins with the ability to bind to carbohydrates, typically linked to other CAZymes.

There are essentially endless structural variations when coupling sugars together, and this is mirrored in the diversity of enzymes cleaving glycosidic bonds (129). The GH class is the most numerous in CAZy, with new families being discovered continuously; just recently GH family 184 was created (13, 130). GHs can cleave both the glycan backbones and side chains, depending on the type. The presence and/or absence of certain GHs in the genome can indicate, which carbon sources the microorganism can or cannot metabolize.

Nonetheless, prediction of substrate preference can be complicated by the polyspecificity of some CAZy families such as GH5, whose members have different or even multiple functions and can cleave a wide range of glycosidic bonds. Substrate predictions for some GH families have been aided by the further division into subfamilies, thereby improving accuracy (13). There are also a few monospecific CAZy families with only one known activity. I have taken advantage of substrate predictions to correlate known CAZymes with glycans during *D. squalens* degradation of bark (**Paper II**), as well as for the identification of putative xylanases in yeast (**Paper III**), and MAGs (**Paper I**) (section 3.3 and Table 4.1). In spite of the division into CAZy subfamilies, certain structural elements are common to multiple types of polysaccharides. For example, arabinosyl side groups are present in both pectin and arabinoxylan, thereby preventing exact substrate prediction.

Table 4.1. Structure of the main polymers found in spruce bark and associated enzymes. Major linkages found in these polymers and the corresponding CAZy families based on **Papers I & II**. GH, glycoside hydrolase; CBM, carbohydrate-binding module; AA, auxiliary activity; CE, carbohydrate esterase; PL, polysaccharide lyase. Symbol nomenclatures were assigned according to the Symbol Nomenclature for Glycans. Polymer abundance in bark is from Fengel *et al.* (1984) (17).

	Major linkages	CAZy families
Starch 	α-(1→4)-D-Glc α-(1→6)-D-Glc	**GH**: 13, 31 **CBM**21
Cellulose 	β-(1→4)-D-Glc	**AA**: 3*, 9, **GH**: 5*,6, 7, 12*, 44* **CMB**1
(Galacto)glucomannan 	β-(1→4)-D-Man β-(1→4)-D-Glc α-(1→4)-D-Gal *O*-Acetyl	**GH**: 26, 27, 36 125
Arabinoglucuronoxylan 	β-(1→4)-D-Xyl 4-*O*-Me-α-(1→2)-D-GlcA α-(1→2)-L-Ara	**GH**:10, 11, 30, 51,115 **CE**: 1, 2, 4, 6, 15 **CBM:** 13
Pectin 	α-(1→4)-D-Gal*p*A α-(1→2)-L-Rha	**PL:** 1, 4, 9, 10, 26 **GH**: 28, 78, 105, 138 **CE:** 8,19,12
Lignin 	β-aryl-ether phenylcoumaran diphydroxy biphenyl	**AA**: 1, 2

Annotation of a new genome with CAZy families is usually facilitated by the developers of CAZy, who perform semi-automatic curation of the genome. However, since 2012 there is also a web-based server called dbCAN (now updated to dbCAN3) which allows users to analyze their own protein sequences with automated CAZyme annotation (99, 131). In my thesis work, I used dbCAN2 to analyze the putative CAZymes derived from my metagenome-assembled genomes (MAGs), and *Pseudomonas* isolate. Although many candidates displayed a low potential for carbohydrate utilization, MAG13 stood out. MAG13 was taxonomically classified within the Bacteroidota phylum, a phylum known to excel in biopolymer metabolization (132). In several Bacteroidetes, the genes necessary for the capture and degradation of a polysaccharide co-localize in so-called polysaccharide utilization loci (PULs) (133, 134). Consequently, domains of unknown functions (DUF) found within a putative PUL could represent new hitherto unknown CAZymes acting on the PUL's target glycan. While I did not find any new putative CAZymes in my MAGs, MAG13 contained a putative PUL (#3) targeting starch (Fig. 4.2). This PUL contained common PUL features, such as homologs to SusC and SusD from the archetypal starch utilization system (Sus) of *Bacteroides thetaiotaomicron*. These form a protein complex where SusD (or SusD-like proteins) binds to carbohydrate fragments, and shuttles them into the SusC-like protein pore, for import into the periplasm (134). Also, a SusE-like protein was identified, putatively providing additional binding capacity on the cell surface. Catalytic domains, which target the glycan of interest are another key part of PULs and in the case of PUL 3 from MAG13 they encompass a putative GH13 α-amylase/pullulanase and GH31 α-glucosidase, for starch hydrolysis.

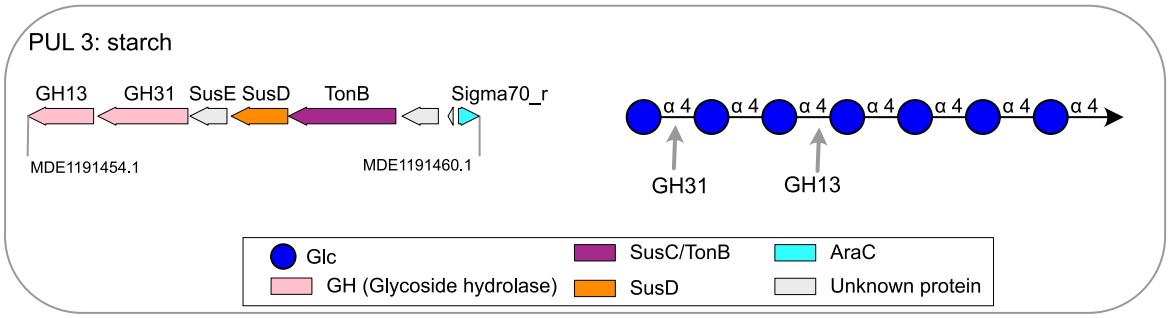


Figure 4.2. Overview of PUL3, a putative starch degrading PUL identified in MAG13 (Phylum: Bacteroidetes). Symbol nomenclature were assigned according to the Symbol Nomenclature for Glycans. NCBI GenBank accession numbers are annotated at the start and end of the PUL. Adapted from Paper I

4.3 Discovery of bark-enzymes using proteomics

Proteomics enables the identification and study of total proteins in a sample, and it is a valuable tool for enzyme discovery as well as their subcellular localization. Advancements in proteomics have been closely linked to the development of MS tools suitable for analyzing proteins and peptides (135-137), along with effective search databases compiled from a growing number of sequenced genomes. In contrast to the genome, which can be considered as "fixed", the proteome of a microorganism is highly dynamic and can change depending on environmental factors such as nutrient availability, stress, and temperature (138, 139).

Different cultivation set-ups, such as liquid cultures or solid-state cultures, lead different proteins being produced (140). Opposed to liquid cultures, solid-state cultures closely resemble the environment in which microorganisms grow in nature. A problem that can arise from such a set-up is separation of the proteins from the sample, as lignin and possibly extractives may bind to proteins, or alternatively be extracted together with them. Cell lysis can also cause problems when focusing on secreted proteins. I encountered both problems in **Paper II**, whereby the initial intent was to extract proteins while simultaneously characterizing the bark. However, when precipitating the proteins using trichloroacetic acid and acetone, I ended up with an insoluble pellet that I struggled to re-suspend. It appears that the extended period before attempting resuspension might have caused the pellet to become challenging to dissolve. Without a doubt, the presence of extractives also played a role in the difficulty of dissolving the pellet. An alternative proteomics workflow was devised; it was based on using lower amounts of bark and growing the fungi on agarose plates with a filter to separate them from the secreted proteins (141-143). As fungi rely on protein secretion (section 3.6), we wanted to study the extracellular environment or secretome, without interference from non-secreted proteins. Any contaminating proteins derived from cell lysis could confound the secretome results.

Specifically, I was interested in determining, which proteins were produced by *D. squalens* when growing on bark (discussed in section 3.7). To achieve this goal, I grew the fungus on acetone-extracted bark (washed to remove extractives), untreated bark, glucose, and galactomannan to discriminate changes in the proteome between extractives and carbohydrates. Using *in silico* prediction methods, it is possible to anticipate which proteins may be present in the extracellular matrix. For example, I used a combination of three prediction algorithms to determine the sub-cellular location of *D. squalens* proteins: SignalP, Phobius, and WolfPSort (144-146). Each of these algorithms uses unique methodologies. In Eukaryotes, SignalP identifies the "standard" secretory signal peptides that are transported via the Sec translocon and cleaved by signal peptidase I (145). By combining these tools, one can predict, which proteins are likely to be extracellular. Based on prediction of secretion signals, I filtered these entries to determine a profile closer to the "true" secretome. I encountered significant problems due to cell lysis and only found 17% of annotated extracellular proteins in the exoproteome (*in silico* prediction was 7.5%). I

concluded that there was significant cell lysis during growth on bark. Owing to the high number of intracellular proteins in the secretome, I only analyzed those, which were predicted to be extracellular and did not rely on protein quantification. As I will show below, despite significant sample lysis, one can still learn something from the observed proteome.

Focusing on known CAZymes, I found very little difference between the *D. squalens* proteome in acetone-extracted bark and untreated bark. Most CAZymes were found in the bark sample, indicating that extraction removed some polysaccharides, as previously observed (19). Overall, the CAZyme repertoire in these two samples was similar, and it targeted all known spruce bark polysaccharides. One of the most interesting findings concerned enzymes identified as putatively active on XyG, a polysaccharide not typically associated with spruce bark. In the past, antibodies have been used to identify XyG in spruce bark harvested both in winter and summer (54), further supporting the presence of XyG in this substrate. To gain insight into potential new CAZymes and/or extractive-degrading enzymes, I focused on the proteins exclusive to the two bark samples (Fig. 4.3).

Of particular note was the high number of auxiliary activity domains, especially AA9 proteins (A0A4Q9PQC0, A0A4Q9Q8Z1, A0A4Q9QAL2, A0A4Q9QAL2) (Fig. 4.3). These enzymes are commonly thought to act only cellulose, but some have recently been shown to be active on xylan if cellulose is present (147-150). This indicates that AA9 enzymes may be either directly or indirectly dependent on extractives. One such example is gallic acid, a compound found in tannins, which has been shown to act as a reductant for some LPMOs (151).

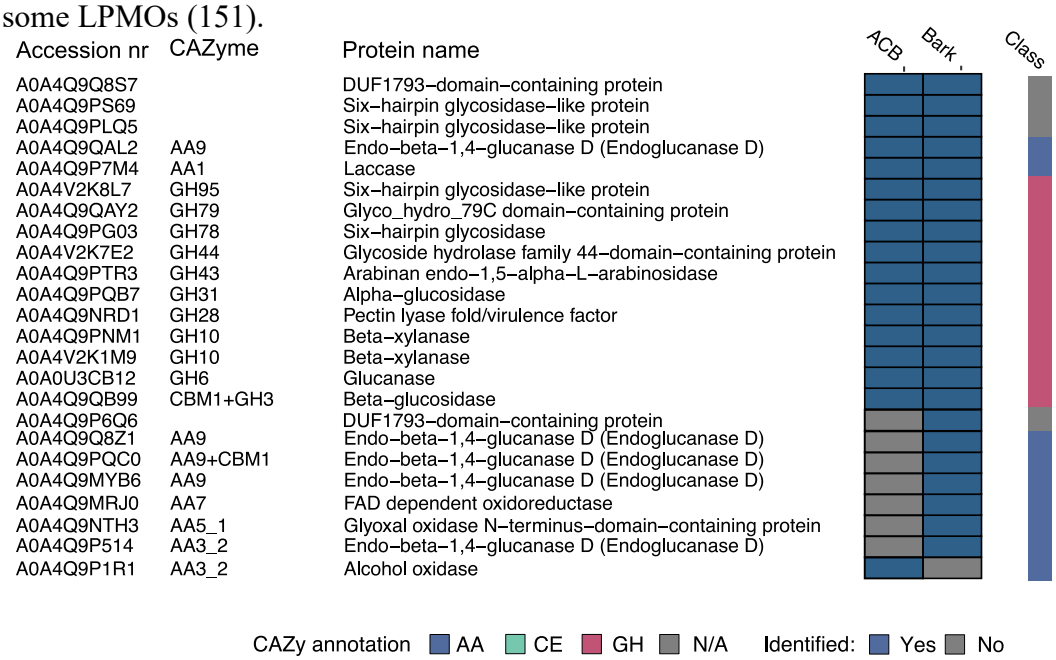


Figure 4.3. Map displaying the extracellular proteins detected exclusively in the two bark samples, after filtering of intracellular entries. The results are clustered based on identified carbon source, colored blue if found and grey if not identified. On the right, the bar is colored according to CAZy class. AA, Auxiliary activity; GH, Glycoside Hydrolase; CE, Carbohydrate esterase; N/A, No CAZy classification; ACB, Acetone-extracted bark. Modified from Paper II.

When I examined proteins that were not annotated as CAZymes, two domains of unknown function (A0A4Q9Q8S7, A0A4Q9P6Q6) were identified (Fig. 4.4). Gene Ontology revealed that they both belonged to carbohydrate metabolic process proteins and harbored three domains of unknown function (DUF5127, DUF4965, DUF1793) (Pfam: PF17168, PF16335, PF08760). Specifically, the two last domains were part of the superfamily of six-hairpin glycosidases. Comparing the AlphaFold2-generated structure for A0A4Q9Q8S7 against all entries in the Protein Database using Dali, yielded β-xylosidase and glucosyleramidase as the closest entries (152). Further evidence suggests that these two proteins are putative CAZymes. Schmerling et al. (2022) were able to generate a soluble protein with an almost identical domain architecture from *Streptomyces misionensis* (containing an extra DUF4965 domain) (153). Despite its classification as a glutaminase, it displayed no activity on glutamine substrates. Instead, it exhibited low β-galactosidase activity (153). These two domains would be of great interest to produce and characterize against both model and natural substrates, to determine if they are indeed active against polysaccharides in bark or, possibly, fungal glycan.

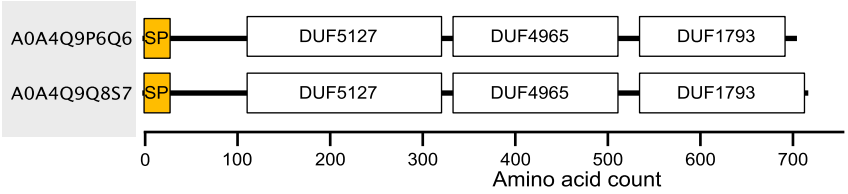


Figure 4.4. Domain architecture of the two proteins containing domains of unknown function. Adapted from Paper II.

4.4 Linking growth to genome supports xylanase identification

Xylanases, which are numerous and very well-described in bacteria and filamentous fungi, are found in GH families 5, 7, 8, 10, 11, 12, 30, 43, 98, and 141 in CAZy (154, 155). Xylanases can either be endo- or exo-acting; while the former bind along the xylan backbone releasing longer xylooligosaccharides (XOs); the latter cleaves the xylan chain from the reducing or non-reducing end, releasing shorter XOs (154). Yeasts can be found in all environments (156); yet, their xylan-degrading capability remains less studied than in the case of filamentous fungi or bacteria.

In our investigation of the xylanolytic strategies employed by yeasts (see section 3.8 and **Paper III**), we studied purified individual putative xylanases produced by *B. mokoenaii*, *S. lignosus*, and *W. canadensis* to explore their different functions. *S. lignosus* encoded a likely xylanase from the monospecific GH family 10 (GH10: *Sl*Xyn10A). Instead, *B. mokoenaii* encoded an enzyme from GH11 (*Bm*Xyn11A), which includes both endo-xylanase and β-xylosidase activities (155). Microorganisms capable of thriving on xylan, despite not encoding conventional GH10 or GH11 enzymes, are of strong interest to researchers, because they might employ alternative mechanisms for xylan degradation. *W. canadensis* lacked any apparent xylanases and, instead, encoded four different GH5 enzymes, a family which possesses a variety of different endo-acting activities on β-linked

glycans. The only putative xylanase, based on previously characterized enzymes, was from subfamily GH5_22 (157). Thus, I worked with *Wc*Xyn5_22A, but also *Wc*Xyn5_49A which is from a presently uncharacterized subfamily. To identify the likely candidates responsible for xylan depolymerization, putative xylanases were cloned, expressed, purified, and analyzed biochemically.

The above four enzymes were biochemically characterized on beech glucuronoxylan (GX) and wheat arabinoxylan (WAX). After 24 h on GX (for WAX; see **Paper III**), all enzymes reached maximum hydrolysis (Fig. 4.5). The enzymatic treatments resulted in different XOs profiles, with *Bm*Xyn11A producing mainly smaller oligosaccharides (xylose, xylobiose, xylotriose), while *Sl*Xyn10A yielded longer ones (xylobiose, xylotriose, xylotetraose, xylopentose, xylohexose). The XO distribution between these two enzymes corresponds well with previous studies where GH11 xylanases need three unsubstituted xylose units, while GH10 can accommodate more branched xylans (158, 159). As typical endo-acting enzymes they cleave linkages within the polysaccharide backbone, producing oligosaccharides differing in length (Fig. 4.5). During hydrolysis of GX, *Wc*Xyn5_22A generated minor amounts of XOs; whereas and *Wc*Xyn5_49A generated xylobiose, xylotriose, and xylotetraose. No activity was detected on *p*NP-xylopyranoside, indicating it was an endo-xylanase. This work demonstrates for the first-time xylanase activity in GH family 5_49 and represents, therefore, a unique discovery.

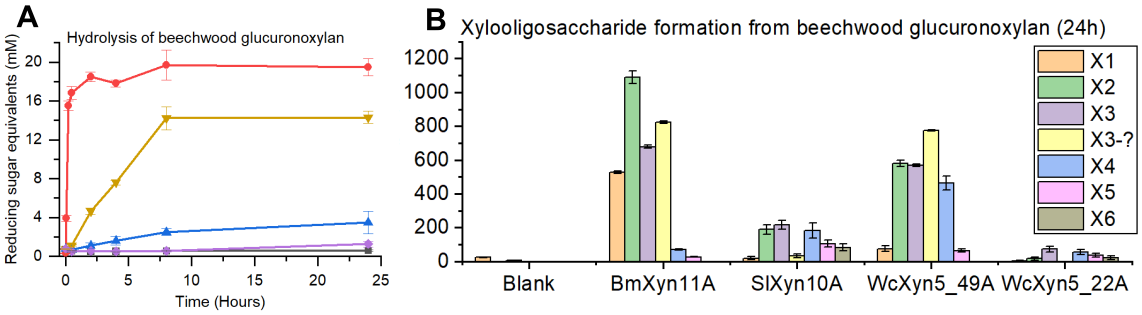


Figure 4.5. Effect of xylanase treatment on beechwood glucuronoxylan. A) Hydrolysis of beechwood glucuronoxylan based on reducing sugar equivalents released over time and B) Xylooligosaccharide formation after 24 h. Xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentose (X5), xylohexose (X6) and an unidentified peak between X3 and X4, named (X3-?). Data are presented as the average of two replicates with error bars denoting the standard error of the mean. Modified from Paper III.

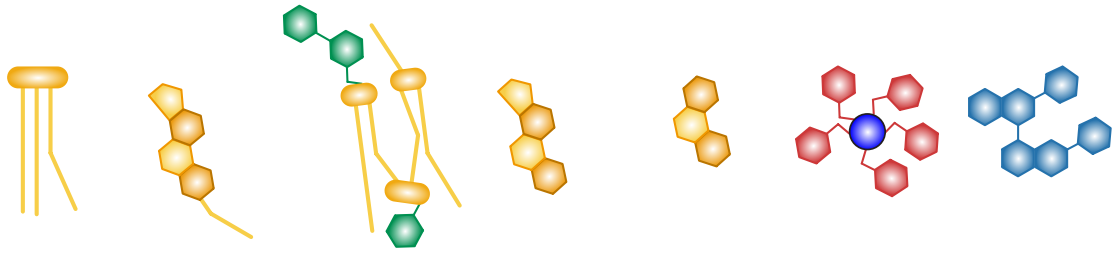
Notably, in CAZy, the GH5_49 subfamily is only present in Saccharomyceta (13), and has been identified in 319 out of the 332 yeast genomes that were the basis for this study (160). This prompts questions regarding the role of GH5_49 enzymes, in combination with other enzymes (e.g., from GH5_22), in xylan degradation by yeast. To study this, yeast GH5_49 enzymes should be studied in greater detail.

4.5 Extractive-degrading enzymes are highly diverse

As described in chapter 2, extractives are a highly diverse class of compounds possessing a variety of linkages, including esters and carbon-carbon bonds. Hence, microorganisms need to deploy an extensive number of enzymes to metabolize them (Fig. 4.6). Furthermore, some extractives are small hydrophobic molecules, and some steps of the degradation process may take place inside the cell wall. Finally, poor solubility in water and/or a large size of certain extractives requires the use of extracellular enzymes (14).

To degrade triglycerides, the white-rot fungus *P. gigantea* produces an extracellular lipase (EC 3.1.1.3), enabling growth on microcrystalline cellulose supplemented with sapwood extract of *Pinus taeda* (111). Steryl esters, which are quite abundant in certain extracts, are hydrolyzed by steryl esterases (EC 3.1.1.13). These enzymes have broad substrate specificity and are capable of hydrolyzing triacylglycerols, *p*-nitrophenyl esters, and steryl esters (161). Suberin is degraded by small serine esterases (20–25 kDa) called cutinase (EC 3.1.1.74). Cutinases are thought to be secreted by plant pathogens to either disrupt the protective layer and allow access to the underlying cells, or to enable the use of released fatty acids as carbon sources (36, 162).

At present, little is known about the degradation of condensed tannins (section 2.2). One theory is that it depends on a cascade of mono- or di-oxygenase enzymes to perform initial oxidation steps followed by hydrolysis (163). The identification of enzymes responsible for degrading condensed tannins requires further research. Hydrolyzable tannins, instead, are cleaved by enzymes called tannin acyl hydrolases, or tannases (164), which I studied in **Paper IV** (Fig. 4.6).



Triglycerides	Steryl ester	Suberin	Sterols	Resin acids	Hydrolyzable tannins	Condensed tannins
non-toxic	non-toxic	physical barrier	membrane interacting	membrane interacting	binds, precipitates proteins	binds, precipitates proteins
lipases	steryl esterases lipases	cutinases	unknown	P450 laccases?	tannases	unknown

Figure 4.6. Major extractive types. The list ranges from the more hydrophobic triglycerides, steryl esters, suberin, sterols, and resin acids, to hydrophilic extractives, such as hydrolysable and condensed tannins. For each extractive, their protective role in bark and the corresponding enzymes responsible for their hydrolysis are mentioned.

4.6 Tannases target ester linkages in hydrolysable tannins

Tannases (EC 3.1.1.20) target ester and depside linkages in hydrolysable tannins (165). Even though tannases have been identified in numerous organisms, such as bacteria, fungi, yeast, and plants (166-168), only a few fungal tannases have been subjected to biochemical analysis (167). There is somewhat more information available on bacterial tannases, at present, only two microorganisms, namely *Lactiplantibacillus plantarum* (formerly: *Lactobacillus plantarum*) and *Streptococcus gallolyticus*, have been reported to have more than one tannase (169-172). The reason behind this multiplicity in encoded tannases remains unclear. Much like extractives in general, hydrolysable tannins are also composed of structurally diverse compounds, so it is likely that different substrate specificities are found within the tannase enzyme family. To determine substrate specificity and screen enzyme activities we employed model substrates that mimicked features of the natural substrate. This approach made it possible to compare enzyme activity with respect to various substitutions (Fig. 4.7) (173).

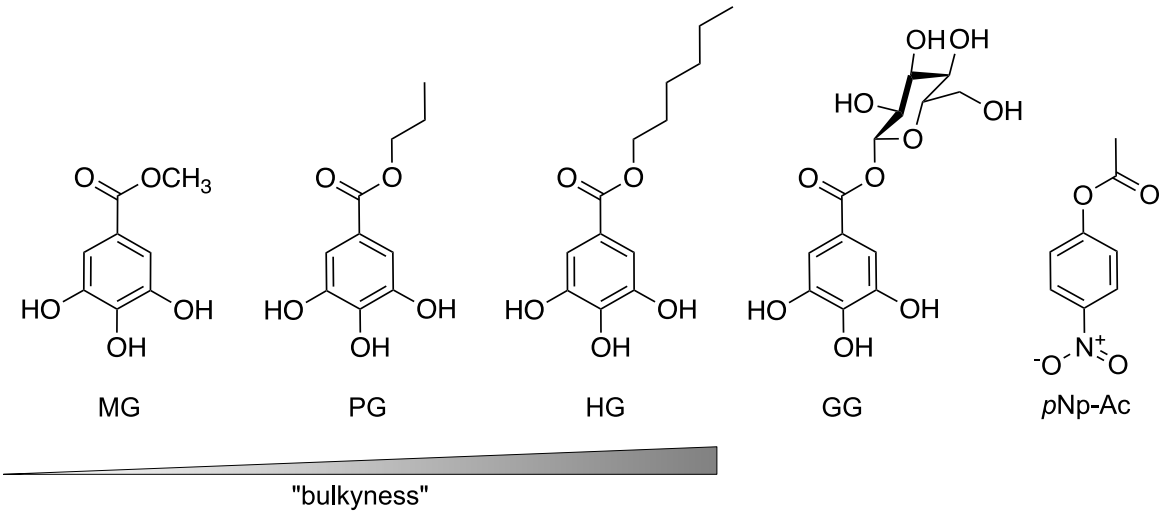


Figure 4.7. Model substrates used to gain insight into tannase substrate preference. MG, Methyl gallate; PG, Propyl gallate; HG, Hexyl gallate; GG, β-Glucogallin; *p*NP-Ac, *p*-Nitrophenyl acetate. Adapted from Paper IV.

In **Paper IV**, model substrates were used to compare substrate preferences of three tannases from *C. butyricum*. The three enzymes showed marked differences, with *Cb*Tan1 and *Cb*Tan3 displaying a clear preference towards β-glucogallin, while *Cb*Tan2 was much more promiscuous (Table 4.2). The K_m was about 10-fold higher compared to other enzymes, which indicates discrepancy between the tested model substrates and the linkage targeted by the enzyme (172). Still, even if activity is observed on model substrates, it does not necessarily mean that the enzymes can perform the same activity on more complex, natural substrates. As oak bark is known to contain abundant hydrolysable tannins, I tested the ability of *Cb*Tan1, 2, and 3 to degrade this natural mixture of tannins and, therefore, determine if they displayed the same specificity on this more complex substrate.

Table 4.2. Kinetic parameters of *C. butyricum* tannases on model substrates. Tannase activity was measured using the rhodanine assay, while acetyl esterase activity was measured using *p*NP-Ac. The enzymes showed no activity on methyl ferulate (data not shown). The standard deviation was determined from triplicate experiments and calculated using OriginPro software v. 9.6.0.172. Adapted from Paper IV.

Enzyme	Substrate	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)
*Cb*Tan1	MG	10.8 ± 1.18	2.40 ± 0.33
	PG	12.0 ± 1.60	2.60 ± 0.43
	GG	3.50 ± 0.70	72.8 ± 22.2
	HG	Cannot be saturated up to 10 mM	3.60 ± 0.22
	*p*NP-Ac	Cannot be saturated up to 10 mM	0.07 ± 0.004
*Cb*Tan2	MG	23.4 ± 2.68	7.99 ± 1.13
	PG	22.2 ± 2.26	6.17 ± 0.76
	GG	23.0 ± 3.70	10.2 ± 1.80
	HG	Cannot be saturated up to 10 mM	6.40 ± 0.22
	*p*NP-Ac	Cannot be saturated up to 10 mM	0.03 ± 0.002
*Cb*Tan3*	MG	24.3 ± 3.52	1.64 ± 0.09
	PG	7.20 ± 0.90	2.24 ± 0.30
	GG	8.99 ± 1.70	52.9 ± 12.5
	HG	Cannot be saturated up to 10 mM	1.28 ± 0.004
	*p*NP-Ac	No detectable activity at 10 mM	

*As *Cb*Tan3 was not fully pure, the values represent k_{cat}^{obs} and K_m^{obs}

The release of gallic acid can be monitored with several methods. The simplest is a colorimetric assay, in which rhodanine binds to gallate, causing the formation of a pink compound that can be quantified using a spectrophotometer. Alternatively, HPLC-PDA can be used to measure the peak area of gallate. To measure gallate in a complex substrate such as oak bark extract, I used HPLC-PDA, which improved the detection range and minimized interference from any unwanted side reactions involving rhodanine. As observed with the model substrates, all three enzymes released gallate from the oak bark extract, albeit at different rates (Fig. 4.8). To determine if the three enzymes displayed different substrate preferences during oak bark extract hydrolysis, I mixed the enzymes in the same concentration as when used individually (0.12 nM total enzyme). The addition of the three tannases together gave rise to slightly improved hydrolysis, indicating that the three enzymes complement each other and target different types of tannins in the oak bark extract or different moieties present in tannins.

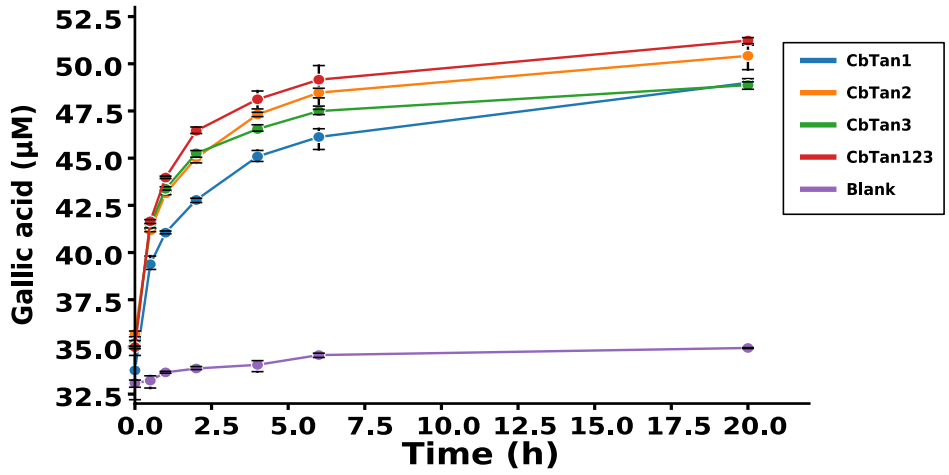
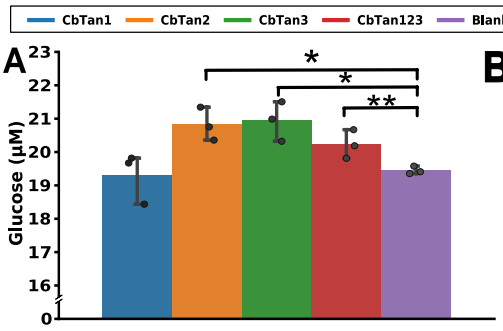


Figure 4.8. Effect of tannase treatment on oak bark water extract. Shown is the released gallic acid in the enzymatically treated oak bark extract over time for each enzyme, compared to a sample containing no enzyme (blank). All reactions contained 0.12 nM total enzyme, for both individual enzymes and when all three were combined. Adapted from Paper IV.

Tannins extracted from bark are highly diverse. Hydrolyzable tannins, more specifically, gallotannins, contain glucose at their center and are attached by ester linkages to gallate (36). The ability of the three enzymes to hydrolyze gallotannins was monitored by measuring the glucose released by the samples after 20 h of enzyme treatment (Fig. 4.9). Reactions with *Cb*Tan2, *Cb*Tan3, and *Cb*Tan1–3 (all three enzymes combined) were able to release glucose from the extract (albeit a very small amount) however, the release was not improved when using *Cb*Tan1.

To identify which compounds could be hydrolyzed by tannases, I employed reversed-phased liquid chromatography and UHPLC-(MS/MS)-CSS. A compound was putatively identified via a match in a custom library of tannin molecules found in oak bark from the literature (174-178). It must be emphasized that when working with MS/MS, the curation of a detailed and accurate compound library is critical and may be a limiting factor in compound identification. Using this library, we were indeed able to identify differences in the substrate preferences for the *Cb*Tan enzymes, with all enzymes able to degrade di- and tri- galloyl glucose (Fig. 4.9). The only observed difference was in the degradation of compounds with identical structure to two of the model substrates, methyl gallate, and galloyl glucose. According to our compound identification, only *Cb*Tan2 was able to degrade these two substrates, in contrast to our biochemical data (see above). As such, I could not confirm that *Cb*Tan1 and *Cb*Tan3 were able to degrade these compounds in the extract. Thus, the increase in glucose from *Cb*Tan2 might be derived from galloyl glucose. In contrast, I could not determine the compound from which glucose was released during *Cb*Tan3 incubation. One possible reason for this discrepancy between model and natural substrates could be enzyme inhibition by ellagic acid (or other compounds). Alternatively, the galloyl unit might be positioned on the wrong carbon on the gallate-glucose molecule, preventing both *Cb*Tan1 and *Cb*Tan3 from degrading it.



B

Putative identification	Blank	*Cb*Tan1	*Cb*Tan2	*Cb*Tan3
Methyl gallate	+	+	ND	+
Gallic acid*	+	+	+	+
Ellagic acid*	+	+	+	+
Galloyl glucose	+	+	ND	+
Digalloyl glucose	+	ND	ND	ND
Trigalloyl glucose	+	ND	ND	ND

Figure 4.9. Effect of tannase treatment on tannin containing oak bark water-extract (20 h incubation). A) Released glucose. The stars indicate p-values: *≤0.01, **≤0.05; as determined by a Student t-test in Python. B) Putative identification of compounds in the oak bark extract using UPLC-MS/MS-CSS. Compounds are annotated as detected (+) or not detected (ND) in triplicate experiments based on molecular mass, CCS, fragmentation, and standards (*). Modified from Paper IV.

Data gathered here suggest that the three *C. butyricum* tannases exhibit unique substrate preferences, both on model and natural substrates. The divergent preferences could potentially result from diverse biological functions within the microorganism, and they might enable *C. butyricum* to thrive in environments rich in a wide range of tannins, such as those found in both soil and the gut.

Main points

- The genome of an organism can be used to discover new enzymes.
- Bacteroidetes metabolize (and sense) complex polysaccharides using polysaccharide utilization loci.
- Proteomics is a powerful tool for enzyme discovery, allowing to see changes in response to environmental perturbations.
- Enzymes acting on bark are highly diverse and probably unknown.
- Tannases are active on hydrolyzable tannins from oak bark and release gallate and glucose during hydrolysis.

5. Conclusions

The work presented in this thesis focuses on bark degradation by microbial communities and individual species, as well as the characterization of pure enzymes that are active on bark components. This chapter discusses how the research questions stated in **chapter 1** have been accomplished.

Which species can grow on bark?

To address this question, growth of a mixed consortium was monitored for up to six months and evaluated using amplicon sequencing, as outlined in **Paper I**. Resin acid-degrading bacteria were enriched throughout this period and after a while the population seemed to shift towards more carbohydrate focused. The dominant bacterium at two weeks, *P. abieticivorans* PIA16, was successfully isolated, sequenced, and phenotypically characterized.

In my opinion, this is a major step forward in the understanding of bark degradation because it investigates the long-term degradation of bark and shows enrichment of resin acid-degrading bacteria during growth. This sets the basis to explore whether the degradation of other types of bark also occurs through the breakdown of extractive molecules that restrict the growth of nearly all organisms except a few, but it also raises new questions. Would another batch of spruce bark enrich for a similar set of genera or microorganisms with comparable phenotypes? I believe that it is highly likely that we would find microorganisms, which perform the same metabolic activity (i.e., degradation of resin acids) as resin acids are an abundant extractive in spruce bark; however, the dominant bacterial species might not necessarily be the same. Hence, in the future, one may isolate more novel species of resin acid bacteria from the yet-little-studied tissue spruce bark.

Notably, the fungal population within the community discovered in **Paper I** only accounted for only a small proportion (1-8%) of the obtained reads/contigs, making it challenging to ascertain the impact of fungi on bark composition. Consequently, we turned our attention to single fungal isolates in **Paper II**. All fungal taxa, from Ascomycota to Basidiomycota, grew on bark albeit at different rates, suggesting diverse strategies for growth which was successfully evaluated in the next sub-question.

What are the compositional changes during microbial bark degradation?

The degradation of bark by a mixed consortium was evaluated using compositional analysis in **Paper I**. Our findings indicated that resin acids underwent early degradation during microbial breakdown of bark, coinciding with a significant loss in diversity of the microbial community. In contrast, during the same time frame there was only minimal impact on polysaccharides. This observation is in stark contrast to the results obtained in **Paper II**, which explored single fungal species growing on bark. Carbohydrate analyses showed

significant differences among fungi, particularly with respect to xylan and pectin degradation. Furthermore, fungi displayed different approaches towards extractive compounds, ranging from tolerance to detoxification and/or complete metabolism.

Paper II is a novel and informative study because involves a variety of fungal species cultivated over prolonged periods. Even after six months, the degradation of polysaccharides and/or extractives continues, underscoring the slow nature of bark breakdown. I think this study shows important differences in bark degradation in fungi and together with **Paper I** highlights differences between bacteria and fungi. In my opinion, even when selecting fungi from different taxa, there is an inherent bias towards species that exhibit rapid growth. Consequently, this could result in preference for less specialized microorganisms with more general degradation patterns, compared to more niche yet slow-growing ones. The latter, although challenging to cultivate due to their slow growth, could employ alternative strategies and possess enzymes, which are more relevant for molecules such as extractives.

How can enzymes be used to degrade bark components?

Different enzymes target specific types of molecules. For instance, xylanases target xylan, a type of hemicelluloses in bark; whereas tannases target tannins. Both these types of enzymes have been investigated in **Paper III** and **Paper IV**, respectively.

In **Paper III** we characterized three xylanases from three different yeast strains: *B. mokoenaii*, *S. lignosus*, *W. canadensis*. The most interesting outcome of the study was the inability of *W. canadensis* to utilize xylan as the sole carbon source unless supplemented with xylooligosaccharides or exogenous xylanases (i.e., GH5_22), or co-culturing with *B. mokoenaii*. Apparently, *W. canadensis* depends on neighboring cells for the initial breakdown of xylan to trigger its own xylan-degrading machinery. Notably, we also characterized the first xylanase activity in GH5_49

I think this study raises the question of how widespread and different the strategies of polysaccharide degradations are. Typically, studies are done on single organisms; however, to observe reliance on other producers for initiating xylan degradation, one needs to carry out additional experiments such as co-cultures and/or supplementation with small amounts of oligosaccharides. These results also prompt a re-evaluation of the yeasts that were initially observed to not grow on xylan during the screening experiment. They could be subjected to another round of screening on plates containing both xylan and xylooligosaccharides (122), of particular interest are yeasts that encode GH5_22 and GH5_49 enzymes.

In **Paper IV** we examined the tannases and *C. butyricum*. The underlying reason for multiple tannase-encoding genes being present in *C. butyricum* remains unclear. One possibility is that these genes might help the microorganism in either metabolizing or detoxifying tannins of different types, which we were not able to distinguish in our

experiments. The three tannase enzymes were phylogenetically dissimilar and had somewhat different activity on model substrates. Notably, these enzymes were found to be active in degrading oak bark tannins, which had not been previously demonstrated for tannases.

The question of why microorganisms encode multiple versions of the same type of enzyme remains perplexing. Why not produce one enzyme with a broad substrate specificity? A reasonable assumption is to prepare for different environmental conditions, metabolic pathways, or different substrates. This enzyme redundancy could also provide a degree of flexibility, enabling microorganisms to respond efficiently to different changes in the environment. It also serves as a form of back-up, ensuring that if one form is less effective under certain conditions, another form might still perform the required function. While the above study investigated the specificity of these enzymes, we cannot establish whether they are actually expressed and, if so, whether they are produced under the same conditions. The more fundamental question regarding whether *C. butyricum* can utilize tannins as a carbon source remains unanswered.

How is bark degraded by microorganisms?

In my work I have used different techniques to answer this overarching research question. I have achieved my initial objective of finding microorganisms capable of growing on bark by working with both single species and microbial communities. This also led to the isolation of a new *Pseudomonas* species, capable of degrading resin acids. Additionally, I characterized both xylanases and tannases, which are responsible for degrading the xylan and tannins, respectively. Based on my research, it is evident that there are still numerous avenues left to explore and tasks to undertake (see future perspectives). Overall, this thesis emphasizes how bark degradation is a gradual process and how bark is a valuable resource containing many compounds of interest (e.g., extractives) for both research and practical applications.

6. Future perspectives and outlook

In order to augment how the biological capabilities of bark can be utilized, it is imperative to understand its degradation by microorganisms, both by microbial communities and isolated species. This could enable the emergence of novel methods that facilitate alternative uses of this side-stream. This thesis provides, at least in part, some initial understanding of microbial degradation of bark using microbial communities, pure cultures, and purified enzymes focusing on both extractives and polysaccharides.

6.1 Are we barking up the wrong tree?

Although assessing the most suitable utilization of bark, may fall outside the scope of my thesis, I will share my own ideas on this.

Is burning bark preferable to its further utilization? I would argue that it is not. First and foremost, bark is a rich source of various compounds with potential medicinal and antimicrobial properties. Some of these bioactive compounds, such as antioxidants and tannins, have demonstrated health benefits (179, 180), and can be used to develop new medications or used as antimicrobial coatings. Microorganisms living on bark could also speed up agricultural processes, aiding in the breakdown of organic material.

I have focused on the bark and its degradation, but an alternative avenue might be to harness the fungi that grow on it. In my research on fungal growth on bark, I noted the rapid colonization of the bark's surface by *D. squalens*, resulting in the formation of a dense mycelium layer. This mycelium holds promise as a biomaterial, because of its tough durable properties. For example, bark could be inoculated with fungal mycelium, which would grow on the surface and produce an encasing with downstream use as packaging material.

However, it is important to note that the utilization of bark is not without challenges. Excessive harvesting of bark from trees can negatively impact ecosystems and threaten biodiversity. In conclusion, bark can be much more than just fuel. Its diverse components offer a range of applications, spanning from antimicrobial to new biomaterials.

6.2 Branching out into future research

During my research, I discovered that microbial communities cultivated on spruce bark exhibit an early degradation of resin acids (**Paper I**). It would also be valuable to investigate if similar effects to other extractive molecules can be observed during degradation of bark from other tree species. The same inoculum can be used to examine the succession of microbial communities growing on different types of bark. This would provide valuable insights on their respective degradation processes. One question is whether betulin or other molecules seem to have the same kind of role as 'gate-keeper' molecules in other bark types. One possibility is that some types of bark do not have such a dominant molecule, and their transformation is dictated by an interaction between several compounds (Fig. 6.1).

Instead of relying on an inoculum of unknown microorganisms, an alternative approach is to use a synthetic community of well-defined microorganisms. Such a strategy would make it easier to replicate experiments and to envision industrial applications. In **Paper III**, the degradation of xylan by *W. canadensis* was investigated, revealing the dependence on other microorganisms to degrade xylan. This finding opens up the possibility of using co-cultures with two or three species to improve the breakdown of such complex material as bark. The use of bark as a growth medium has two limitations: the significant presence of extractives, which impedes microbial growth, and the high lignin content, which few organisms can use as a carbon source. One potential approach to overcome these challenges involves the use of a synthetic consortium (Fig. 6.1). This consortium would consist of the resin acid-degrading bacterium *P. abieticivorans* working in synergy with a lignin-degrading fungus. By leveraging this cooperative arrangement, it becomes possible to produce a high-polymer material suitable for application as a substrate in bioreactors.

For the degradation of abietane-type diterpenoids, the *dit* cluster is a key mechanism used by resin acid degrading bacteria. There is currently little understanding of how pimarane-type resin acids are degraded or how microorganisms cope with such insoluble substrates. Notably, the isolate *P. abieticivorans* PIA16 degraded degrade both pimarane and abietane type resin acids. Accordingly, RNA-seq could be used to compare the expression profiles of the said cluster, as well as other relevant proteins (e.g., P450s) across these different types of resin acids (Fig. 6.1). The resulting analysis could aid in identifying an additional cluster responsible for pimarane degradation, and its cellular localization. Resin acids are insoluble in aqueous media, so it would be worthwhile to investigate whether extracellular enzymes are involved in their degradation, because the *dit* cluster is predicted to be expressed intracellularly. Alternatively, it is possible that certain enzymes from the *dit* cluster are secreted into the extracellular environment through outer membrane vesicles, as observed during growth of *Pseudomonas putida* on lignin (181). Exploring these possibilities would provide valuable insights into the mechanisms involved in resin acid degradation, shedding light on both intracellular and extracellular degradation. By heterologoously expressing and purifying enzymes from this cluster, one could further

investigate the specificities of the encoded enzymes and possibly enhance the chemical modification of resins. Thus, resin acids could be tailored for use as films or biomaterials.

As with enzymes in general, it is difficult to predict the specific substrates of tannases based solely on their amino acid sequence. Enzymes active on tannins from *C. butyricum* were studied in **Paper IV** and, in this study, I constructed a phylogenetic tree using already characterized tannases to try to find correlations with substrate preferences. However, no correlation was found. An emerging strategy for preforming substrate to enzyme domain prediction entails network analysis, which has been shown within the CAZy database to enable better substrate preference prediction (155). In the future, when characterizing new tannases, and trying to predict their function from amino acid sequences, this could prove a useful tool (Fig. 6.1).

How do we identify new bark-acting enzymes in the future? The identification of CAZymes benefits significantly from the CAZy database. I often found myself envious of researchers working with CAZymes due to the relative ease of their annotation and, to some extent, substrate prediction. Consequently, I propose development of a comparable database that consolidates knowledge regarding extractive-degrading enzymes (Fig. 6.1). The initial step would involve the compilation of data on enzymes known to interact with both hydrophobic and hydrophilic extractives. While such a database is a long-term project, the studies presented within this thesis are an excellent starting point. Information similar to that found in CAZy will enable bark valorization through the development of new biochemical, biological, and biotechnological methods.

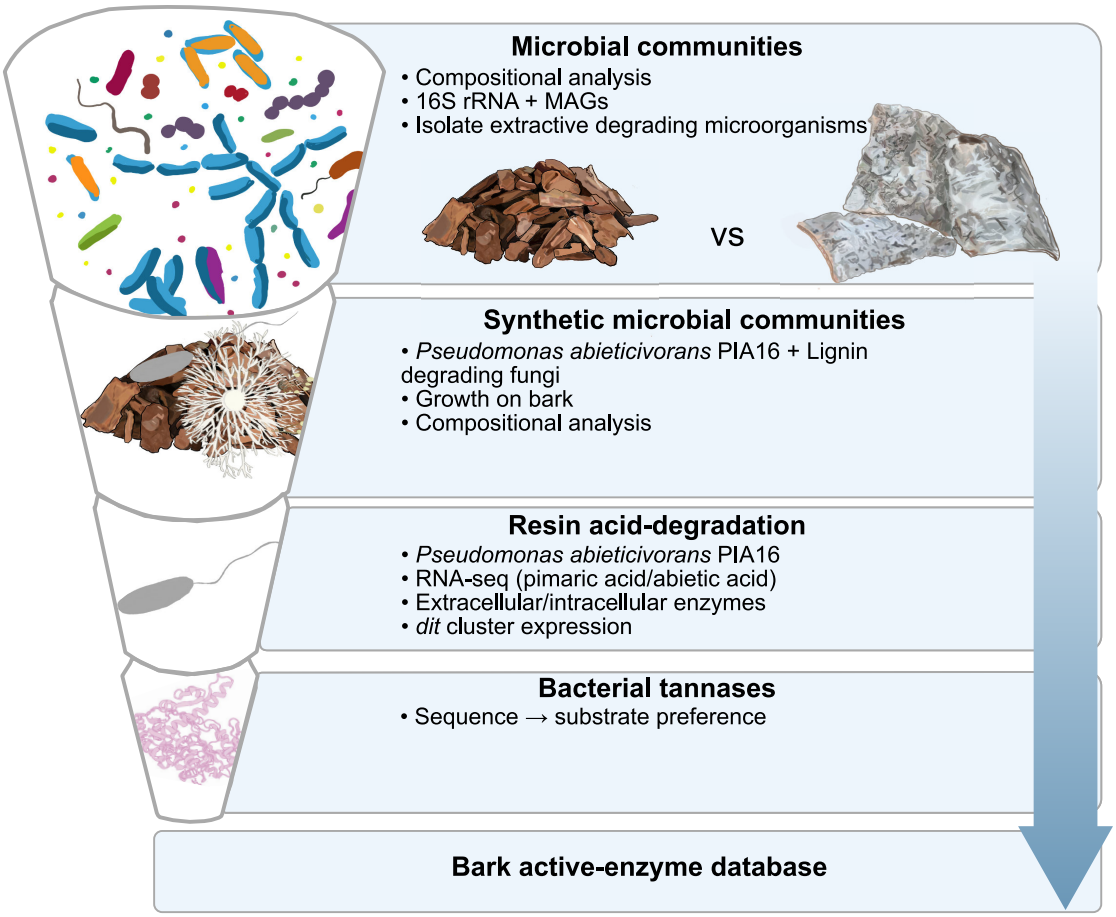


Figure 6.1. Summary of future perspectives on bark research.

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