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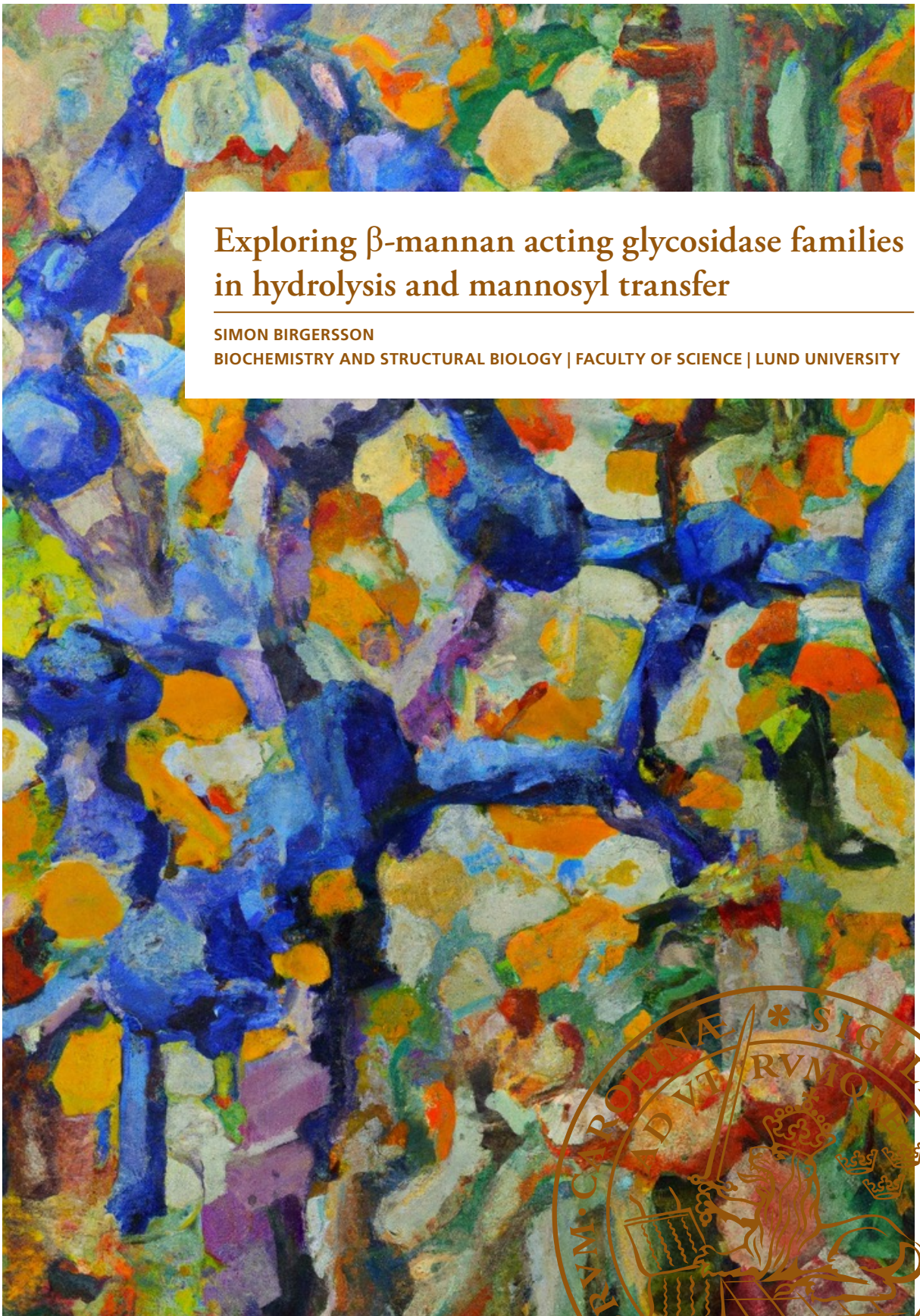

Exploring β-mannan acting glycosidase families in hydrolysis and mannosyl transfer

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by Simon Birgersson

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Abstract
The thesis revolves around the study of glycoside hydrolases (GH), specifically GH5 β-mannanases and GH36 α-galactosidase (**I-III**), as well as a GH130 mannoside phosphorylase (**I**, **IV**). These enzymes are investigated for their actions on and conversion of hemicellulosic β-mannan, including food-grade galactomannan and softwood galactoglucomannan, along with manno-oligosaccharides (MOS) derived from such polymers. The focus lies on exploring the transferase activity, specifically transglycosylation, of retaining GHs, elucidating the molecular and structural mechanisms governing these reactions, and assessing their potential applications in synthesizing novel glycosides like MOS and allyl glycosides from renewable glycans (**II**, **III**).

Paper I explores the utilization of β-mannan-oligosaccharides (MOS/GMOSs) by *Roseburia hominis*, and its interactions with *Bifidobacterium adolescentis*, revealing differential utilization patterns, cross-feeding of acetate, and potential synergistic roles of specific enzymes shown to be upregulated in the presence of MOS/GMOS by *R. hominis*. In **Paper II**, A novel double mutant (R171K/E205D) of the catalytic module (CM) of the family GH5 Trichoderma reesei β-mannanase (*Tr*Man5A) showed enhanced transglycosylation capacity, particularly with mannotetraose and allyl alcohol, surpassing wild-type *Tr*Man5A and offering potential applications in the enzymatic synthesis of novel biomaterials and glycopolymers through allyl glycoside production. The +2 subsite of *Me*Man5A, a GH5 β-mannanase from the blue mussel, was studied by substituting two tryptophans with alanines in **Paper III**. These substitutions impaired *Me*Man5A's hydrolytic activity, affecting transglycosylation with saccharides but not alcoholysis. In **Paper IV**, the mannan oligosaccharide phosphorylase *Rh*MOP130A, first investigated in **Paper I** was investigated in terms of its structure and studied for its synthetic potential in the reverse phosphorlysis reaction direction, allowing for elongation of mannan oligosaccharide using activated mannose phosphate. *Rh*MOP130A showed the ability to elongate allyl mannose, such as produced in **Paper II**, making it an interesting tool for modification of novel glycosides.

The findings of this thesis enhance our comprehension of the molecular factors and reaction parameters that impact the effective conversion of β-mannans into novel glycosides. This showcases the viability of utilizing β-mannanases in the enzymatic synthesis of novel bio-materials.

Key words
glycoside hydrolases, β-mannanase, transglycosylation, enzymatic synthesis, enzyme synergy, enzyme engineering, MALDI-ToF, HPLC, NMR

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Contents

List of publications

This thesis is based on the following publications, referred to by their Roman numerals:

I Abhishek Bhattacharya, Lovisa Majtorp, **Simon Birgersson**, Mathias Wiemann, Krishnan Sreenivas, Phebe Verbrugghe, Olivier Van Aken, Ed W. J. Van Niel, and Henrik Stålbrand
Cross-Feeding and Enzymatic Catabolism for Mannan-Oligosaccharide Utilization by the Butyrate-Producing Gut Bacterium Roseburia hominis A2-183
Microorganisms, **2022**, 10, 2496

II Samuel J. Butler, **Simon Birgersson**, Mathias Wiemann, Monica Arcos-Hernandez, Henrik Stålbrand
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III **Simon Birgersson**, Johan Morrill, Olof Stenström, Mathias Wiemann, Ulrich Weininger, Pär Söderhjelm , Mikael Akke, and Henrik Stålbrand
Flexibility and Function of Distal Substrate-Binding Tryptophans in the Blue Mussel β-Mannanase *Me*Man5A and Their Role in Hydrolysis and Transglycosylation
Catalysts, **2023**, 13, 1281

IV **Simon Birgersson** and Lovisa Majtorp, Mathias Wiemann, Derek Logan, and Henrik Stålbrand
Structure of β-mannan oligosaccharide phosphorylase *Rh*MOP130A and synthesis of elongated glycosides
Manuscript

Author contributions

Paper I: I planned, executed, and analysed a part of the experiments. These experiments were focused on one of three in detail characterised enzymes, the GH130 β-mannan oligosaccharide phosphorylase *Rh*MOP130A for which I characterized its enzymatic properties. This included studying phosphorolysis reactions for different mannooligosaccharides, using MALDI-ToF, HPAEC-PAD, and a phosphate colorimetric assay for identification, quantification, and activity. I also conducted bioinformatics analysis on *Rh*MOP130A and *Rh*MnBP1, comparing them to known proteins. Additionally, I contributed to discussing all data in the paper and played a significant role in developing and editing the manuscript.

Paper II: I led the conceptualization, method development, planning, and execution of part of the experiments with a focus on the enzyme synergy as well as NMR-analysis. I took part in drafting manuscript content for these part. The contribution involved characterizing the GH27 α-galactosidase Aga27A, establishing an HPLC method for product separation, and analyzing results. He planned, performed, and analysed all NMR experiments for the paper. He also played a key role in scientific discussion, data analysis, and manuscript revision after peer review.

Paper III: I contributed experimentally to the transglycosylation screening and evaluation of *Me*Man5A in the paper, as well as the bioinformatical analysis. I also contributed to the presentation, interpretation and conclusions surrounding the NMR experiment, as well as for the MD simulations. I was the main contributor to writing the original draft of the manuscript with input from the co-authors, determining the direction of the paper, as well as editing the manuscript after review.

Paper IV: I led the conceptualization, method development, planning, and execution of biochemical catalysis experiments. I also played a key role in scientific discussion, data analysis, and manuscript writing, drafting the text and manuscript content of the biochemical catalysis experiments. I gave significant input to the discussion and writing of the full manuscript, including interpretation and presentation of all data.

Acknowledgements

Here it is, the result of my PhD, in one book. It has been hard work, sometimes stressful but overall a very positive experience which I owe to the great support, assistance, encouragement, and sometimes distraction that I've got from so many people close to me. A great thank you to all of you, I owe you a lot. A few special mentions are below but I'd thank you all!

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underneath the cartoon persona I found a gentle, funny, and very interested person and I consider you one of my dear friends from my time here.

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Popular science summary

Life thrives through ubiquitous chemical reactions, with plants generating sugars via photosynthesis, serving as vital energy-rich molecules for diverse organisms. Utilizing these sugars, plants construct distinct anatomical features such as leaves, stalks, and stems, each tailored for specific functions. Wood, categorized as *lignocellulosic biomass*, comprises cellulose, hemicellulose, and lignin. While wood naturally degrades over centuries, enzymatic processes can significantly expedite this degradation. Enzymes, acting as biological catalysts, are found everywhere in nature and can transform, degrade, or synthesize molecules, catalyzing reactions at rates over a million times faster than spontaneous occurrences.

This thesis delves into enzymes involved in lignocellulosic biomass degradation, focusing on hemicellulose hydrolysis and *transglycosylation* reactions. Transglycosylation connects saccharides to other molecules, forming new molecules, called *glycoconjugates*, which can include materials like plastics (polymers) and compounds like surfactants (detergents). several types of enzyme are studied, such as β-mannanases and α-galactosidases for their potential in this regard.

Studying a sugar digesting bacteria found in the human gut in **Paper I**, certain genes for enzymes in a specific usage area (*Rh*MosUL) in the DNA were more active in *R. hominis* in the presence of non-digestible sugar. Studying these enzymes helps us understand how beneficial bacteria in the human gut use non-digestible sugars and this study emphasizes the importance of cooperation and interactions between different bacteria that break down mannans in the same gut environment.

Using β-mannanases in **Paper II**, this thesis demonstrates the transglycosylation of mannan-saccharides to an alchol molecule called allyl alchol, creating new reactive molecules. Alterations to the enzyme's active-site cleft enhance transglycosylation efficiency. Enzyme synergy, observed when combining a β-mannanase and an α-galactosidase reaction with galactomannan, significantly increases synthesis. The versatility of the studied α-galactosidase is showcased by its ability to use various acceptors, expanding the range of potential glycoconjugates.

Another mannanase, like the one found in blue mussels (called *Me*Man5A) can also create new bonds in addition to breaking them using transglycosylation. In **Paper III**, the focus was on a specific part of *Me*Man5A that has two important components (tryptophans W240 and W281). Changing these components in the enzyme affected how well it worked. The changes made the enzyme less effective in breaking down galactomannan, a type of mannan. The study used different techniques to understand how the enzyme and mannans interact. The results showed that both tryptophans are important for how the enzyme binds to mannans. One of them (W240) is rigid and

specific, while the other (W281) is flexible and helps with binding. The changes in the enzyme also affected its ability to create new bonds with certain substances, like alcohols. Overall, the results of this thesis helps us understand how these enzymes work and how they can be changed to do different things.

in **Paper IV** This research looks into how *Rh*MOP130A, a specific enzyme from the gut bacteria *Roseburia hominis*, works in reversing a process called phosphorolysis which is similar to hydrolysis but uses phosphate to break the sugar bond. It belongs to a group of enzymes called GH130. The study found that this enzyme can use certain sugars as building blocks, especially mannose, and has a specific structure that allows it to work on longer sugar chains. Interestingly, it can also use different substances like those created via transglycosylation to make their sugar parts longer, showing its potential for making different kinds of products.

In summary, this thesis enhances the understanding of factors influencing efficient enzymatic conversion of β-mannans, demonstrating the potential to produce reactive glycoconjugates and materials from renewable resources, with implications for future studies and the utilization of enzymes in biochemical production processes.

Populärvetenskaplig sammanfattning på svenska

Livet frodas genom kemiska reaktioner, där växter genererar socker genom fotosyntes som i sin tur fungerar som livsviktiga energirika molekyler för olika organismer. Genom att använda dessa socker bygger växter distinkta delar som blad, stjälkar och stammar, var och en anpassad för specifika funktioner. Trä består av*lignocellulosisk biomassa*, som i sin tur är uppbyggt av cellulosa, hemicellulosa och lignin. Även om trä naturligt bryts ned över århundraden kan enzymatiska processer avsevärt påskynda detta. Enzymer, som agerar biologiska katalysatorer, är mångfaldiga och kan omvandla, bryta ned eller syntetisera molekyler, katalysera reaktioner med hastigheter över en miljon gånger snabbare än spontana händelser.

Denna avhandling granskar enzymer som är involverade i nedbrytning av lignocellulosisk biomassa och fokuserar på hydrolys- och transglykosyleringsreaktioner för hemicellulosa. *Transglykosylering* kopplar samman sackarider, antingen med socker eller med andra molekyler, och bildar nya produkter som kan användas till, t.ex., material som plaster (polymerer) eller föreningar som tensider (tvättmedel). Flera typer av enzymer studeras, såsom β-mannanaser och α-galaktosidaser för deras transglykosyleringspotential, samt ett mannofosforylas, ett relativt understuderat typ av enzym med spännande egenskaper.

I **Artikel I** studeras en bakterie (*Roseburia hominis*) som bryter ned socker i människans

tarm. Vissa gener för enzymer i ett specifikt användningsområde (*Rh*MosUL) i DNA var mer aktiva i *R. hominis* i närvaro av socker. Studier av dessa enzymer hjälper oss förstå hur gynnsamma bakterier i människans tarm använder sockerarter som ej är nedbrytbara av människan, och betonar vikten av samarbete och interaktioner mellan olika bakterier som bryter ned mannan i samma tarmmiljö.

I **Artikel II** undersöks användningen av β-mannanaser för transglykosylering, för att sätta ihop socker (mannan) med funktionella molekyler, vilket skapar nya intressanta produkter. Ändringar i enzymets aktiva säte ökar effektiviteten av transglykosylering. Enzymsynergi, observerad vid kombination av ett β-mannanas och ett α-galaktosidas i en transglykosyleringsreaktion med galaktomannan ökade signifikant syntesen av dessa produkter.

Ett annat mannanas finns i blåmusslor (kallad *Me*Man5A), kan också skapa nya bindningar utöver att bryta ned dem med hjälp av transglykosylering. I **Artikel III** så var fokus på en specifik del av *Me*Man5A:s aktiva säte som har två viktiga aminosyror (tryptofanerna W240 och W281). När dessa byttes ut så påverkades enzymets prestanda. Ändringarna gjorde enzymet mindre effektivt i nedbrytningen av galaktomannan, en typ av mannan. Studien använde olika tekniker för att förstå hur enzymet interagerar med mannan. Resultaten visade att båda tryptofanerna är viktiga för hur enzymet binder socker. En av dem (W240) är styv och specifik, medan den andra (W281) är flexibel och hjälper till med bindningen. Ändringarna i enzymet påverkade också dess förmåga att skapa nya bindningar med socker men inte med alkoholer.

i **Paper IV** undersöks enzymet textitRhMOP130A från GH familj 130, som kommer ifrån tarmbakterien *Roseburia hominis*, och hur det fungerar. *Rh*MOP310A kan använda en process som kallas omvänd fosforolys som kan förlänga sockergrupper. Studien fann att detta enzym kan använda vissa sockerarter som byggstenar, särskilt mannos, och har en specifik struktur som gör att det kan arbeta på längre sockerkedjor. Intressant nog kan den också använda olika ämnen, så som de som skapas via transglykosylering för att göra deras sockergrupper längre, vilket visar dess potential för att göra olika typer av produkter.

Sammantaget hjälper resultaten av denna avhandling oss att förstå hur dessa enzymer fungerar och hur de kan förändras för att utföra olika uppgifter. Hoppet med denna avhandling är att bidra till ökad förståelse för faktorer som påverkar effektiv enzymatisk omvandling av β-mannaner och att visa potentialen att producera reaktiva glykokonjugat från förnybara källor med hjälp av enzymer.

Exploring β-mannan acting glycosidase families in hydrolysis and mannosyl transfer

Chapter 1

Introduction

There is an increasing interest in supplementing fossil fuels as the main carbon source for the production of important resources in society such as fuels and plastics with renewable sources[1]. This shift, together with adapting a more circular economy can help to reduce carbon emissions[2,3]. Renewable carbon sources, derived from organic matter and sustainable feed-stocks, offer a promising alternative to traditional petrochemical feed-stocks. Biomass, algae, and waste utilization technologies play key roles in this transition[4,5]. The technological development, mostly for the production of biofuels has been driven by the utilization of feed-stocks rich in sugar and starch, such as corn, sugar cane, sugar beet, and barley, known as *first generation feed-stocks*[6]. These have been in focus due their low cost of production and agricultural history, but the future viability of these first generation feed-stocks have come into question as they compete with food and feed as well as requiring fertile soil and irrigation[7].

The move from first-generation to *second-generation feed-stocks*, i.e. from non-food biomass sources signifies a major leap in sustainable resource utilization. While first-generation feed-stocks, like food crops, face scalability and competition issues[6],

Second-generation feed-stocks like agricultural residues and non-food biomass such as lignocellulose are more abundant and have significant potential to be more sustainable[6]. One such second generation feed-stock is *softwood hemicellulose galactoglucomannan*, which is highly prevalent in Sweden, found in pine[8,9] and spruce[10,11]. These tree species are well developed resources in the forestry industry, for generation of paper and pulp, and heating power from forestry waste. However, some of these waste streams can contain galactoglucomannans that could be further valorised into high value products[12].

The degradation of plant biomass such as hemicellulosic galactoglucomannan in na-

ture is facilitated by a variety of enzymes, such as such as retaining GH5 β-mannanases[1], and proteins. *Retaining* glycoside hydrolases refers to their catalytic mechanism where the stereochemical orientation of the cleaved product is conserved. These types of enzymes can also catalyze the formation of glycoside bonds via *transglycosylation*, which can be used to synthesize novel glycosides, which is explored in this thesis (**Papers II-III**). These glycosides show a lot of potential for applications, such as bio-surfactants, [13, 14] or in novel biomaterials [15], to name a few.

The aim of the work has been to explore the molecular aspects of enzymatic β-mannan conversions, including such as catalyzed by different glycoside hydrolases and related phosphorylases, thereby contributing to the knowledge of these reactions. The primary objective is to investigate the transferase function, particularly transglycosylation, of retaining glycoside hydrolases (GHs). In principle, retaining GHs may catalyze transglycosylation reactions applicable for enzymatic synthesis, but this may vary even within the same GH family. The thesis work aims to contribute to understanding the molecular and structural mechanisms governing these reactions and evaluating their potential for synthesizing innovative glycosides, such as MOS and allyl glycosides, utilizing sustainable glycans. The inverting β-mannan oligosaccharide phosphorylase was included as an novel alternative route for enzymatic synthesis of the above mentioned glycosides, via reverse phosphorolysis.

By studying glycoside hydrolases with various specificities, the results herein contribute to furthering the application of β-mannanases for synthesis of novel renewable glycosides. In **Paper I**, three enzymes with from different enzyme families that are utilized by the β-mannan degrading bacteria *Roseburia hominis* are characterized. In **Paper II**, the β-mannanase *Tr*Man5A was studied in terms of transglycosylation, and the study showed how different glycoside hydrolases can be combined to increase the yield of β-mannan conversion synergistically. In **Paper III**, another β-mannan degrading enzyme derived from blue mussel, *Me*Man5A was studied, and the effect amino acid residues in the active site have on facilitating substrate binding in GH5 retaining β-mannanases.

Taken together, this work explores the relationship between β-mannan acting enzymes and their substrates, and the interactions between them. The utilization of hemicelluloses and polymeric mannans as substrates for enzymatic conversion is underexplored. This is also true for the application of enzyme synergy to improve substrate utilization in transglycosylation reactions. Significant effort has been placed in this project on the study enzymatic function, transglycosylation capacity, and evaluation of the reaction products. This endeavor has contributed to the broader investigation of enzymes as catalytic entities for the production of specialized chemicals derived

[1]Explained in section 2.2.4

from renewable hemicellulosic resources.

Chapter 2

Background

2.1 Lignocellulose

Lignocellulose is a complex and abundant biopolymer composed of cellulose, hemicellulose, and lignin interconnected in a matrix[16], making it recalcitrant to enzymatic degradation[17,18]. It is the main structural component of the secondary cell wall of plants, provides mechanical strength to cell walls, and aids in the formation of stable plant structures. Lignocellulose can be utilized as a renewable source of energy, produce biofuels, chemicals, and materials, and as an important source of energy and biomass for humans and animals[19]. The relative amounts of cellulose, hemicellulose, and lignin vary depending on the host organism. The structure of lignocellulose consists of cellulose that is wrapped by hemicellulose (dry matter accounting for 20–35 %) and lignin (dry matter accounting for 5–30 %[20]), see Figure 2.1.

In Sweden, a large source of lignocellulose is from forestry, commonly from softwoods such as spruce and pine. These softwoods are rich in hemicellulose (25%[21]) with the most abundant hemicellulose being *O-acetyl galactoglucomannan*[22]. This hemicellulose source tends to be under-utilized in paper and pulp production[23], and is often burned for energy production[16], but in contrast to cellulose hemicellulose can be water soluble and very suitable for biocatalysis.

These hemicellulose-rich waste stream could potentially be valorized into high value products such as biofuels or platform chemicals[24]. There is an increasing interest for utilization of lignocellulose as a sustainable second generation carbohydrate feedstock, as compared to first generation feedstocks that compete with food production[7].

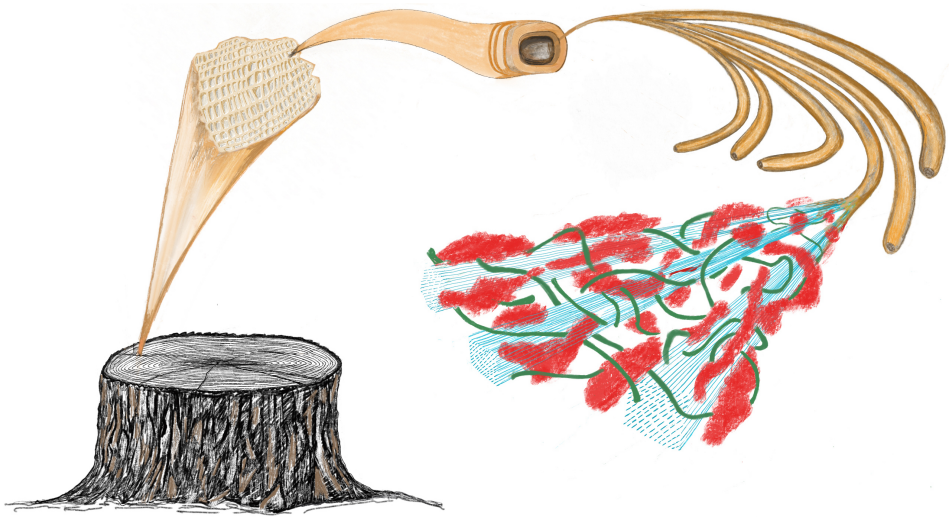

Figure 2.1: Structure of lignocellulose in wood biomass, featuring cells and the cell wall in woody tissue. Macrofibrils, consisting of the primary wood components, form an interwoven mesh composed of cellulose microfibrils (blue stripes), hemicellulose (green lines), and lignin (red blobs). *Figure used with permission from Samuel Butler.*

2.1.1 Cellulose

Cellulose is widely recognized to be the most abundant renewable organic resource on Earth and is widespread in higher plants, bacteria, marine algae, and other biomass[25]. Cellulose is at its most basic a linear homopolymer composed of glucose units linked β-1,4 glycosidic bonds[26]. The cellulose polysacharides then form a superstructure through interpolymer interactions, such as hydrogen bonds which makes up the cellulose found in nature[27].

The chemical formula of cellulose is $(C_6H_{10}O_5)_n$, excluding the terminal units where n, called the degree of polymerization (DP), represents the number of glucose groups. The polysaccharide chain is linear, and the linearity leads to a strong tendency for cellulose molecules to interact together by intra- and intermolecular hydrogen bonding, whereby the molecules bundle together to form microfibrils consisting of both highly ordered crystalline- and less ordered amorphous regions[28].

A cellulose fibril is a small, stretching unit which aggregate and then constitute the structure of natural and synthetic fiber materials, e.g. textile fibers. They also make long molecular chains gathered into bundles in one direction[29]. Natural cellulose can have up to 10.000 to 15.000 glucose units[21], and the fibril contains approximately 60–80 cellulose molecules. Hydrogen bonds are formed between adjacent molecules. Microfilament is composed of fibrils and is fixed in size. Macrofilament is has more

than one microfilament, and its size varies with the sources or processing conditions of raw materials.

Cellulose synthesis occurs through multi-enzyme complexes in the plasma membrane, termed cellulose synthase complexes, which synthesize the glucan polymer while positioning the glucans to achieve the regular secondary structure[28,30,31]. In forming the secondary structure of cellulose, both crystalline and amorphous regions are created, coexisting within individual fibrils[29]. The crystalline regions, tightly packed, present poor accessibility for hydrolytic enzymes, contributing significantly to the recalcitrance of lignocellulose[32,33]. The cellulose microfibrils constitute a robust and rigid structure, acting as the skeletal backbone in plant cell walls. In the secondary cell wall, these microfibrils interweave with polymeric hemicelluloses and lignin, forming macrofibrils that enhance strength, rigidity, and act as a chemical and physical barrier inhibiting cellulose degradation[34].

In nature, cellulose facilitates the growth of organisms. Beyond its ecological role, cellulose, along with other lignocellulose components, serves as a long-term carbon storage, mitigating increasing CO_2 concentrations in the atmosphere due to its stable structure and abundance[30]. The glucan structure stores energy, providing a sustained energy source for microbes[26,30]. From a human perspective, cellulose has been utilized as a key component of lignocellulose for millennia. It has found applications in linen and cotton fabrics and as the primary component in paper. The pulping and paper industry generates substantial quantities of fractionated cellulose annually[35]. Mechanical and chemical treatments alter both the cell wall structure and cellulose within it. Consequently, the DP of refined cellulose extracts varies based on source and treatment method, influencing material and chemical properties[36]. The applications originating from cellulose continue to diversify, with wood cellulose being employed in established applications and emerging candidates for commercialization, including advanced composite materials and various food and health applications[37,38,39]. With a heightened interest in sustainable fuels, cellulose has become a focal point of extensive research for efficient saccharification and subsequent ethanol fermentation[40,41,42]. This increased cellulose demand will lead to more hemicellulose waste as byproduct, meaning that efficient utilization of hemicellulose is of utmost interest.

2.1.2 Hemicellulose

Hemicellulose, distinct from cellulose, is a group of branched polysaccharide whose composition varies based on plant species, environmental factors, and specific plant parts. Hemicellulose is found throughout plant life and is one of the most abundant resources for organic material[43]. Besides its prevalence in softwoods, it is also found in algae, mosses, and other plants[44,45]. Its main chain may comprise different sugar

monomer units, with varying bonds between them. Hemicellulose is categorized by the type of glycan moeities it contains, such as xylan, arabinan, galactan, and mannan[43].

Hemicelluloses undergo synthesis within the Golgi apparatus, involving a plethora of enzymes and synthase complexes due to their varied structure[43,46,47]. These molecules exhibit smaller sizes compared to cellulose, with an average degree of polymerization (DP) ranging from 100-200, although isolation methods may lead to partial degradation, resulting in reported average DPs of up to 400[21,48]. Within the plant cell wall, hemicelluloses serve as structural glycans, closely associating with cellulose and lignin. They intertwine with cellulose microfibrils, forming amorphous structures that contribute significantly to both structural integrity and the cell wall's recalcitrance[32,43]. Covalent lignin-carbohydrate complexes (LCCs) further contribute to the recalcitrance of lignocellulosic biomass[49]. In addition to their roles in the cell wall, hemicelluloses function as storage carbohydrates in seeds[43].

As a principal component of lignocellulose, hemicelluloses have garnered increased attention as a feedstock for bio-based chemicals and fuels[37]. Hardwoods tend to exhibit of more xylan-based hemicellulose whilst mannan is more commonly found in softwoods such as pine or spruce[19]. Softwood hemicelluloses, primarily composed of hexose-sugars, are already employed in ethanol fermentation[50] and have been utilized for the production of various fermentation products[51]. β-Mannan hemicellulose have varied structures, such as linear β-mannans, glucomannans, galactomannans, and galactoglucomannans (GGM), discussed further below. Controlled partial hydrolysis could render these polysaccharides suitable for applications such as prebiotic supplements, including some arabinoxylan and glucomannan oligosaccharides[52].

2.1.3 Lignin

Lignin is a complex aromatic polymer composed of phenylpropane units. The exact molecular composition of lignin is difficult to determine due to its random linkages[53]. Lignin is referred to as the second most abundant organic resource on earth[54]. In contrast to cell wall polysaccharides, lignin is distinct as it is made up of substituted phenyl propylene units instead of monosaccharide units[55]. The synthesis of lignin is intricate, involving numerous enzymes in the phenylpropanoid pathway[56]. It commences with the conversion of L-phenylalanine into primary monolignols, which serve as the monomers in the lignin polymer[55]. Softwood lignin mainly comprises guaiacyl units, while hardwood lignin includes both syringyl and guaiacyl units, with hydroxyphenyl units present in small quantities in both hardwood and herbaceous lignin[57,58].

Lignin encloses the lignocellulosic biomass. From a chemistry point of view, phenyl-

propanoid derivatives are the basic units of the lignin; they combine into high molecular substances by ether bonds or carbon-carbon bonds. According to the physical characteristics, lignin is hard, which increases the robustness of the cell wall[25]. Commonly, the cell wall of plants with a supporting function and mechanical action always contains a high lignin content. The lignin content is about 27–32 % in woody plants and about 14–25 % in herbaceous plants[59]. Lignin is the name of a group of substances; their inhomogeneity is manifested in different species of plants, length of growing season, and different parts of the plants. Even in the different morphologies of cells the structures of lignin are not the same[20].

The amorphous and highly variable structure of lignin interacts with hemicellulose and cellulose in the cell wall, contributing additional strength and resistance to the structure[49]. Lignin partially envelops polysaccharides in the cell wall, forming a physical barrier that restricts access for hydrolytic enzymes[33]. Additionally, lignin has been observed to engage in non-productive binding events with cellulases, partly due to carbohydrate-binding modules (CBM) attached to the enzymes[60].

2.1.4 β-Mannan saccharides

This thesis focuses on enzymes involved in mannans conversion, with interest in using primarily hemicellulosic β-mannans as saccharide substrates. Significant quantities of mannans are presently part of many industrial waste streams, e.g. from paper and pulp industries, rendering them an untapped resource[12 12,61,62]. β-Mannans predominantly consist of easily fermentable hexoses, prompting interest in their application for fermentative bio-fuel and bio-based chemical production[43]. Additionally, beyond their existing uses outlined below, β-mannans have been employed in the enzymatic synthesis of innovative glycoconjugates, holding potential applications as surfactants and bio-polymers[13] (**Paper II-III**). The molecular structure of mannan hemicellulose can vary, but primarily falls into one of four categories: linear mannan, glucomannan, galactomannan, or galactoglucomannan. These vary in which sacharides form the β-1,4 backbone and which degree of α-1,6 galactosyls substituents are present. Linear mannan and galactomannan have polymannan backbones, with the difference that galactomannan have α-1,6 galactosyl substituents decorating it[63]. Glucomannan and galactogluomannan in contrast have glucosyl units dispersed through the mannan backbone chain[44,64], alongside acetylations at the C2 and C3 positions of its backbone hexosyls[64,65,66] (Fig. 2.2). galactoglucomannan also possesses similar galactosyl substitutions to galactomannan[63] (Fig. 2.2).

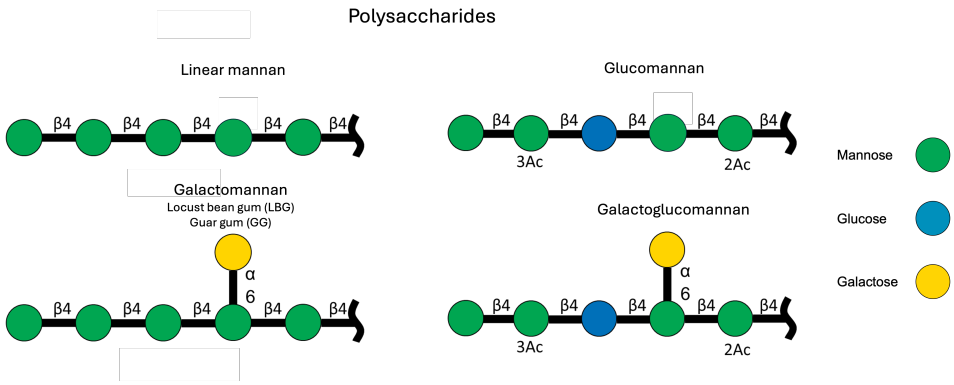

Figure 2.2: Schematic representation of the structure of mannan, glucomannan, galactomannan and galactoglucomannan using the symbol nomenclature for glycans (SNFG) scheme [67,68]. Mannosyl units are colored green, glucosyls are colored blue, and galatosyls are yellow. The polysaccharide backbone is made up of β-1,4-linked saccharides whilst the galactosyl substituents are connected through α-1,6 linkages. The acetylated mannosyls are decorated at either the C2 or C3 oxygens. End right end of the polysaccharide chain indicates a continuation or the reducing end.

Linear β-mannans are classified as homopolymers, featuring backbones exclusively comprised of β-1,4-mannosyl units, albeit with a minor galactose content[43,69]. These mannans serve as predominant structural elements in specific algae and are commonly present in the endosperms of palm nuts[69]. Mannans extracted from the "Ivory nut" of the tagua palm (*Phytelephas sp.*) exhibit two fractions: one crystalline and one microfibular, with approximate degrees of polymerization (DP) of 10-20 and 40-80, respectively. Both fractions demonstrate essential insolubility in aqueous solutions, prompting the hypothesis that linear mannans may initially undergo synthesis as more soluble galactomannan precursors, subsequently subjected to in situ de-galactosylation by galactosidases[43,69].

Glucomannans, in contrast, feature backbones predominantly composed of mannose, interspersed with glucose units in a non-repeating pattern. The relative proportion of mannose to glucose varies depending on the source, potentially influenced by substrate availability (GDP-mannose and GDP-glucose) for a synthase capable of utilizing both substrates[43,70]. Analogous to linear mannans, glucomannan backbones exhibit a low degree of glycosyl substitution but are partially acetylated, enhancing solubility[70]. Konjac glucomannan, derived from the bulbo-tuber of the konjac plant (*Amorphophallus konjac*), maintains a mannan to glucan ratio of approximately 1.6:1, with a 5-10% degree of acetylation and a reported DP around 6000[44,70]. Despite its water solubility, it demonstrates thickening and gelling properties, extensively employed in diverse food applications[70].

Galactomannans, characterized by β-1,4-linked mannosyl units with pendent α-1,6-linked galactosyl units exhibit considerable variability in the galactose to mannose

ratio, ranging from 55% to 85% mannan[71]. Some galactomannans also display low degrees of acetylation[71]. While these polysaccharides are found in diverse sources, such as coffee and coconuts, they are particularly abundant in legumes[43,72]. Locust bean gum (LBG) and guar gum, derived from *Ceratonia siliqua* and *Cyamopsis tetragonoloba*, respectively, represent economically significant galactomannans with applications in the food and feed industry[45,73].

The solution characteristics of galactomannans are contingent on their degree of polymerization (DP) and the mannose to galactose ratio. LBG, with an approximate mannan:galactan composition of 3:1 to 4:1 and a DP around 1500, contrasts with guar gum, which exhibits corresponding values of 2:1 and reported DPs ranging from 4000 to 8000[45,73]. Challenges in determining molecular weights arise from their thickening properties, leading to widely varied reported molecular masses. In a study by Pitkanen et al., it was postulated that overestimation of higher DP values is likely due to aggregation of galactomannan molecules[71]. The water solubility of galactomannans, attributed to galactose substituents hindering intermolecular interactions, varies between guar gum, which readily dissolves in water at room temperature, and LBG, requiring heating for optimal solubility[45]. The removal of galactosyl units reduces the solubility of the intact polysaccharide[71].

Galactosyl units on the guar gum backbone occur in blocks, creating sections with heavy galactosylation interspersed with sections largely composed of linear mannan. LBG exhibits a more evenly distributed structure, with shorter block-wise, random, and ordered regions, contributing to variations in in-solution characteristics[45,71]. These distinctive features influence the specific applications of galactomannans in commercial settings[70]. Enzymatic modification, particularly through partial hydrolysis, emerges as a potential strategy to enhance the utilization of galactomannans, providing a means for tailored polysaccharides and oligomers, thereby avoiding the constraints associated with sourcing from various natural origins[71].

GGM (galactoglucomannan), constituting up to 25% of the dry weight of softwood, stands out as the predominant hemicellulose in this context[74]. The well-characterized GGM from softwoods exhibits a non-repeating backbone comprised of β-1,4-linked mannosyl and glucosyl units, featuring pendent α-1,6-linked galactosyl units and partial O-acetylation at the C2 or C3 position of the mannosyl units[35,48,75]. The DP of GGM typically ranges from 100-150, and the degree of acetyl substitution varies between 0.2-0.4, contingent upon the source[35,75]. Notably, the method of extraction plays a pivotal role in influencing these characteristics, as alkaline extraction may lead to de-acetylation of GGM, and severe conditions may result in the partial hydrolysis of the heteropolymer[48]. GGM can be categorized into two fractions based on galactose content. The approximate composition of the low-galactose GGM is mannan:glucan:galactan at 3-4:1:0.1, while the higher galactose fraction comprises 3-4:1:1.

The high-galactose fraction also exhibits higher solubility compared to the other[21,35].

Serving a similar function other hemicelluloses in lignified plant tissues, GGM contributes to the overall strength of the intricate matrix comprising cellulose, hemicellulose, and lignin. Extensive studies have been conducted on GGM isolates from softwood[75,76,77].

Being a major component of softwood, GGM has garnered increasing interest as a feedstock for the production of value-added chemicals[74]. The potential applications of hemicellulosic mannans encompass saccharification and subsequent or simultaneous fermentation. In this context, GGM, alongside hydrolyzed cellulosic glucan, can be converted into bio-ethanol or other bio-based chemicals[41,78]. The paper and pulping industry has already implemented fermentation of residuals for bio-ethanol production, with room for development to enhance the process economy[50,79]. GGM has attracted significant attention as a potential component or precursor in various high-value chemicals. For instance, it has been employed in the production of hydrogels, demonstrating improved wound healing properties and enhanced material characteristics[80,81]. Additionally, GGM-derived oligosaccharides has exhibited potential as an oxygen-barrier material, integrating into polymeric materials as a substitute for synthetic polymer components[38,82]. In mouse models, GGM has displayed potential prebiotic properties both in vitro and in vivo[83,84], aligning with the attributes of numerous cellulosic and hemicellulosic oligosaccharides[52]. As more potential applications emerge and processing methods evolve, the viability of GGM as a feedstock in future commercial applications is promising. Consequently, this renewable and abundant bio-resource is likely to emerge as an increasingly significant feedstock in the near future.

2.2 Glycoside Hydrolases

Glycoside hydrolases (GHs) are a group of enzymes that catalyze the hydrolysis of glycosidic bonds, and are found in all domains of life, from bacteria to humans. These enzymes are important for many biological processes, including the breakdown of dietary carbohydrates in the gut microbiome[85], degrading plant matter to be used as sustenance for soil bacteria[86], and restructuring of plant cell walls[87]. GHs are generally described based on their specificity, e.g. β-1,4-linkages between mannosyls as is the focus of this thesis (see Table 2.1), but there are more broadly acting GHs as well. Besides catalytic domains, GHs may contain additional domains, such as carbohydrate-binding modules (CBMs), which are important for substrate proximity and binding[74].

Table 2.1: Shows the enzymes studied in this thesis.

Enzyme	Family	Specificity	Clan	Fold	Mechanism	Paper
*Tr*Man5A	GH5	β-1,4-mannanase	A	$(\beta/\alpha)_8$ barrel	retaining	II
*Me*Man5A	GH5	β-1,4-mannanase	A	$(\beta/\alpha)_8$ barrel	retaining	III
Aga27A	GH27	α-1,6-galactosidase	D	$(\beta/\alpha)_8$ barrel	retaining	II
*Rb*Gal36A	GH36	α-1,6-galactosidase	D	$(\beta/\alpha)_8$ barrel	retaining	I
*Rb*Man113A	GH113	β-1,4-mannosidase	A	$(\beta/\alpha)_8$ barrel	retaining	I
*Rb*MOP130A	GH130	β-1,4-mannophosphorylase	-	5-fold β-propeller	inverting	I, IV

2.2.1 Classifications of Glycoside Hydrolases

GHs are classified in a few different ways, such as by their EC number (General, 3.2.1.X) which is based on the chemical reactions for which GHs act as catalysts[88]. This does not account for host species, origin, sequence identity between members of the same number, and requires characterization of the enzyme to facilitate classification.

GHs are also classified into GH *families*[89]. This classification is maintained by the carbohydrate active enzyme database (CAZy, found at `www.cazy.org`)[90]. This classification is instead based on amino acid sequence similarity. Since important residues for catalysis are highly conserved, this grants the possibility of predicting the specificity of the enzyme[91]. CAZy also incorporate many other types of glycoside interacting proteins, such as carbohydrate esterases, polysacharide lyases, and glycosyl transferases, as well as the CBM domains which can help facilitate substrate binding in GHs. As more and more enzymes are discovered and studied, both new families[92], and new family divisions occur[93]. This divisions split the GH family into "subfamilies" with more closely related enzymes, potentially aiding specificity prediction.

As more and more structures of GHs were resolved, the possibility to compare tertiary structures of GHs became possible. This led to the classification of GH families into "clans" based on on the three-dimensional fold similarity as well as their mechanism[94].

2.2.2 Structure and Reaction Mechanisms

GHs generally catalyse the cleavage of glycosidic bonds, but do so in a few different modes. The can be *endo-* or *exo*-acting, i.e they cleave internal or terminal glycosidic bonds, either from the reducing or non-reducing end[89]. The predominant exo- or endo-acting nature of a glycoside hydrolase is often dictated by the topology of its active site. Active sites of glycoside hydrolases typically fall into one of three main topologies: *pocket*, *cleft*, or *tunnel*[89]. A pocket topology, common in exo-acting GHs, features a pocket-formed active site where saccharides bind. This pocket can be created by a single enzyme or through interactions in oligomeric complexes. Examples

of which are α-1,6-galactosidases such as *Rh*Gal36A in **Paper I** and Aga27A in **Paper II** Conversely, the cleft or groove topology, found in many endo-acting glycoside hydrolases, is more open, allowing for diverse saccharide binding such as *Tr*Man5A[95] in **Paper II** or *Me*Man5A[96] in **Paper III**. The tunnel topology is an extension of the cleft, featuring more restricted access due to extended loops around the active site[97]. Cellobiohydrolases such Cel7A from *T. reesei* have tunnel shaped active sites and acts to depolymerize insoluble cellulose via a processive mechanism[98].

GHs can also be *retaining* or *inverting*, i.e. they retain or invert the anomeric configuration at the cleaved bond. Generally the catalysis is performed by two catalytic residues, possessing carboxylate groups on their side chains, acting as nucleophile and acid/base[99], but other mechanisms also occur. Some retaining glycosidases use a mechanism known as *substrate-assisted catalysis*. These enzymes lack the catalytic nucleophile and instead utilize the N-acetyl group of the substrate as an internal nucleophile, forming an oxazoline/oxazolinium ion intermediate[88]. A water molecule then attacks the intermediate, resulting in retention of the anomeric configuration. This mechanism is observed in various enzyme families, such as GH18 chitinases[100], GH20 hexosaminidases, and GH84 endo-β-N-galactosaminidases[101]. Despite a conserved general acid/base residue (typically carboxylic acid residues like glutamic acid or aspartic acid), differences exist among GH families in terms of the hydrogen bonding network and the presence of a third residue influencing the assisting residue[88].

Inverting GHs operate via a single-step, single-displacement mechanism. This process involves transition states resembling oxocarbenium ions. Two amino acid side chains, commonly glutamic or aspartic acids, usually positioned 6-11 Å apart, provide general acid and general base assistance during the reaction[102].

The retaining double displacement mechanism instead involves a two steps, in the *glycosylation* step, one residue acts as a nucleophile, forming a glycosyl-enzyme covalently bound intermediate, while the other serves as an acid catalyst. In the *deglycosylation* step, a glycoside acceptor hydrolyses the covalent intermediate, with the second residue acting as a base catalyst. The acid/base group's pKa value changes for optimal function throughout the reaction process (Fig: 2.3)[103].

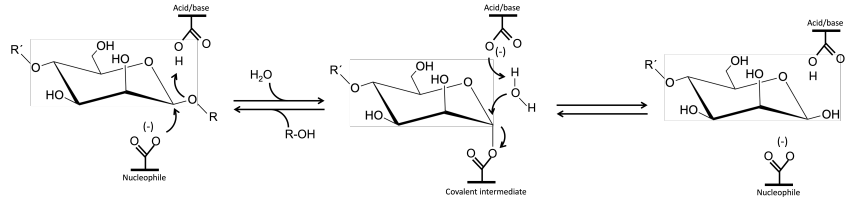

Figure 2.3: Reaction scheme for retaining β-glycosidases. Initial attack on the anomeric carbon by the nucleophile, followed by protonation by the acid/base residue to form the covalent glycoside enzyme intermediate step. This state is broken by a second nucleophilic attack by the glycosyl acceptor (here water) leading to deglycosylation of the enzyme.

2.2.3 Subsite nomenclature

In order to bind its substrate, glycoside hydrolases possess glycan monomer binding subsites within its active site[104]. These subsites are designated using a numbering system, where integers are used to measure the amount of sites away from the point of cleavage, which occurs between subsites -1 and +1. The side of the positive integers denote subsites in the reducing end direction of the substrate, whilst the negative subsites are towards the nonreducing end (Fig 2.4). Negative subsites are commonly termed *glycone subsites*, and the positive are herein termed *aglycone subsites*. The amino acid residues present in these subsites heavily influence the substrate binding mode, specificity, and tranglycosylation propensity of the enzyme.

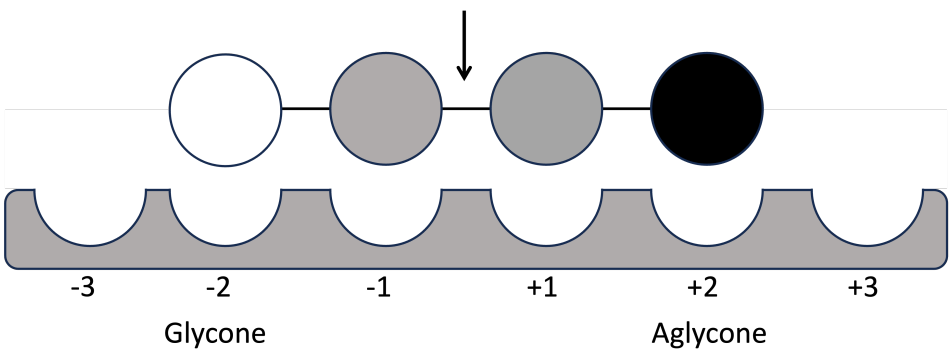

Figure 2.4: Schematic representation of a glycoside hydrolase binding site, with six glycan monomer binding subsites, bound to a saccharide of degree of polymerization (DP) 4. The white circle is the nonreducing end sugar monomer, grey circles are internal monomers, and the black circle is the reducing end monomer. The point of cleavage is indicated by an arrow between subsite -1 and +1 and subsites are designated by integers based on their distance from the point of cleavage. Negative and positive subsites are herein referred to as glycone and aglycone subsites respectively. Different glycoside hydrolases have more or less of these subsites depending on the enzyme.

2.2.4 β-Mannanases

β-Mannanase (EC:3.2.1.78) are GHs that act to depolymerize with attack on internal β-1,4-linkages in mannan-containing saccharides[105]. There are four families that exhibit β-mannanase activity, GH5, GH26, GH113 and GH134[106] (Table 2.2). Except for GH134, GH5, GH26, and GH113 belong to the same structural clan GH-A and exhibit distant homology, evident in their shared $(\beta/\alpha)_8$ barrel three-dimensional structure, identical catalytic amino acids (Glu/Glu, serving as the catalytic nucleophile and proton donor), and the same catalytic mechanism resulting in the retention of the anomeric configuration (Table 2.2). While GH5 and GH26 are well-established families with numerous characterized members, GH113 is a relatively recent and less-explored family.

Table 2.2: Shows the GH families that exhibit β-mannanase activity.

Family	Clan	Fold	Mechanism
GH5	A	$(\beta/\alpha)_8$ barrel	retaining
GH26	A	$(\beta/\alpha)_8$ barrel	retaining
GH113	A	$(\beta/\alpha)_8$ barrel	retaining
GH134	-	lysozyme fold	inverting

Most β-mannanases cleave their substrate at random internal β-1,4-mannoside linkages leading to difficulty in predicting the product profile[107]. This is not always the case as there are examples of β-mannanases that are processive[108], or leave defined hydrolysis products[109]. β-Mannanases are found in many different environments, from the human gut microbiome (**Paper I**), to fungi (**Paper II**), plants[110], extremophiles[111], and even expressed by some *Animalia* (**Paper III**). β-mannanases tend to display an open cleft shaped active site that allow for binding of several sugar subunits, as examplified in the crystal structure of *Tr*Man5A (pdb: 1QNR[95]), studied in **Paper II** and shown in Figure 2.5. β-mannanase families GH5 and GH113 have been shown to posses transglycosylation capacity[112] but interestingly not for GH26 even though the catalytic mechanism is the conserved[85,113]. investigations into mutations in GH26 enzymes to facilitate transglycosylation have been done[114] but the cause of this discrepancy is not entirely known. A theory is that it has to due with the generally less strict substrate specificity in GH5/GH113 as compared to GH26, allowing for more different binding configurations covering both glycone and aglycone subsites[115,116].

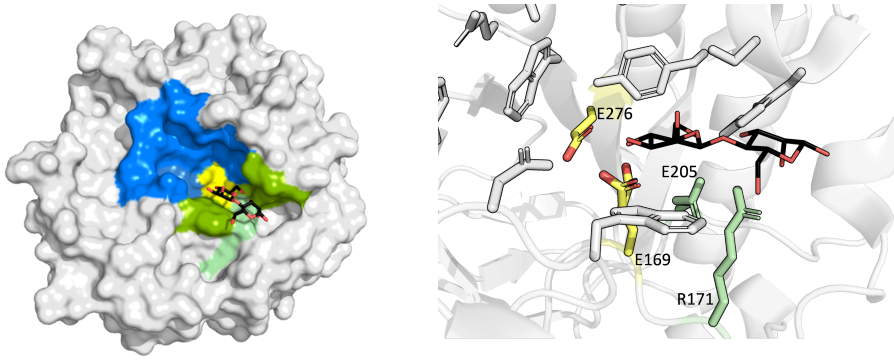

Figure 2.5: Left: Surface view of GH5 endo β-mannanase *Tr*Man5A from *Trichoderma reesei* (pdb: 1QNR [95]) with glycone (negative) subsites highlighted in blue and aglycone (positive) subsites highlighted in green, catalytic residues in yellow. a mannobiose molecule, shown in black is bound in the +1 and +2 subsites. **Right:** close up on the +1 and +2 subsites of *Tr*Man5A with a bound mannobiose molecule in black. Yellow residues are catalytic glutamates, E169 acting as acid/base and E276 acting as nucleophile[117]. Green residues are R171 and E205, which were mutated to further accomodate transglycosylation acceptor binding **(Paper II)**.

2.2.5 α-Galactosidases

α-Galactosidases (EC:3.2.1.22) catalyzes cleavage of terminal, non-reducing α-1,6-galactosyls residues in α-galactosylated saccharides[105]. Specifically, the enzyme facilitates the transfer of a water molecule to the glycosidic bond, resulting in the separation of the α-galactose unit from the rest of the bound glycoside. α-Galactosidases are found across 6 GH families, GH4, GH27, GH36, GH57, GH97, and GH110[106,118] (table 2.3), where GH27 and GH36 belong to clan GH-D which shares the same $(\beta/\alpha)_8$ barrel structural motif as in GH-A (Table 2.3). The products of these enzymes maintain the anomeric configuration of their substrates after cleavage, using a similar mechanism as GH-A with the exception of the catalytic residues being aspartates instead of glutamates. The reason why α-galactosidases generally don't cleave internal α-1,6-bonds like an endo-acting enzyme is explained by its pocket shaped active site[118].

Table 2.3: Shows the GH families that exhibit α-galactosidase activity

Family	Clan	Fold	Mechanism
GH4	-	Rossmann fold	NAD+ assisted
GH27	D	$(\beta/\alpha)_8$ barrel	retaining
GH36	D	$(\beta/\alpha)_8$ barrel	retaining
GH57	-	$(\beta/\alpha)_7$ barrel	retaining
GH97	-	$(\beta/\alpha)_8$ barrel	retaining, inverting
GH110	-	parallel β-helix	inverting

The majority of GH-D sequences noted as α-galactosidases belongs to either the

GH27 or GH36 families (studied in **Paper I** and **Paper II** respectively), which have been shown to possess transglycosylation capacity utilizing different donor substrates, including 4-nitrophenyl-α-D-galactopyranoside (pNP-Gal) [119], oligosaccharides [120].

2.2.6 Manno-phosphorylases

Glycoside phosphorolases (GPs) (EC: 2.4.1.x) [121], found in CAZy families GT4, GT35, GH13, GH65, GH94, and GH130, utilize phosphate instead of water to enforce catalysis [106,122] (Table 2.4). Inverting GHs show a similar mechanism to inverting GPs as they both utilize a direct displacement reaction mechanism. However, they differ in catalytic machinery, where inverting GHs involve two amino acid residues (proton donor and catalytic base), while inverting GPs require only a single catalytic residue, instead using inorganic phosphate as a replacement for the catalytic base [123]. Anomeric configuration inversion in GPs occurs in a single step: the target sugar monomer at the non-reducing end is attacked by the catalytic base at the C1 position. A proton from the acid/base residue is then captured by the inter-glycosidic oxygen, forming an oxocarbenium ion-like transition state. Subsequent bond cleavage yields a glycosyl-phosphate with inverted configuration and a shorter oligosaccharide [124]. Certain bacteria, such as *Roseburia*, utilize a β-1,4-mannooligosaccharide phosphorylase (MOP) to degrade β-1,4-mannobiose into α-d-mannose-1-phosphate and mannose (**Paper I**). Both mannosyl glucose phosphorlyases (MGP) and MOP are found in glycoside hydrolase family 130 (GH130), which also includes related enzymes targeting β-1,2 mannosidic linkages [125].

Table 2.4: Shows the Cazy families that exhibit phosphorylase activity.

Family	Clan	Fold	Mechanism
GT4	GT-B	-	retaining
GT35	GT-B	-	retaining
GH13	-	$\beta/\alpha)_8$ barrel	retaining
GH65	-	-	inverting
GH94	-	$(\alpha/\alpha)_6$	inverting
GH130	-	5-fold β-propeller	inverting

GH130 is further divided into three subfamilies (GH130_1, GH130_2, and GH130_NC) based on phylogenetic analysis [126]. GH130_1 encompasses MGPs , GH130_2 includes MOP, 1,4-β-mannosyl-N-acetylglucosamine phosphorylase, and β-1,4-mannosyl-[N-glycan] phosphorylase [126]. The specificity for substrates is determined by the recognition of mannosyl units within the - 1 and +1 subsites. While the recognition in the -1 subsite remains largely similar to that in mannosylglucose phosphorylases (including the attack of glycosidic oxygen by mannose OH-3 in the -1 subsite), there

are differences in the +1 subsite, leading to the exclusion of glucose recognition[125]. Instead, mannose and, in some cases, GlcNAc may be accommodated in this site. Additionally, enzymes from the GH130_2 group, unlike GH130_1 members, incorporate other positive subsites, explaining their capacity to act on larger oligosaccharides[125,126]. GH130_NC involves phosphorylases and hydrolases targeting β-1,2 mannosidic linkages[125]. GH130 contains as of March 14th 2024, 4994 sequences with only 20 functionally characterized and 12 available pdb structures (`cazy.org`). Recently an expansion of the GH130 subfamilies was suggested, dividing the family into 15 distinct cluster based on sequence similarity[127].

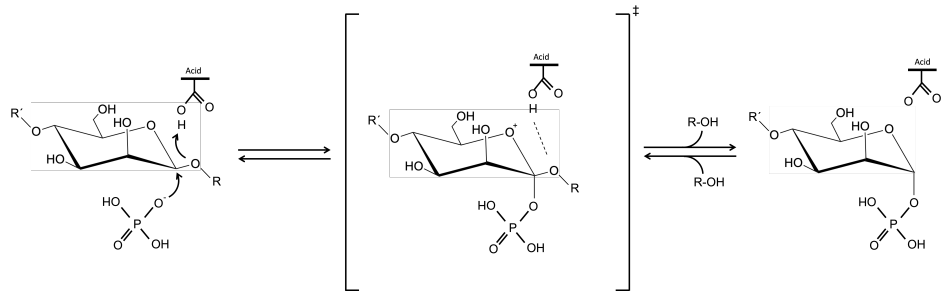

Figure 2.6: schematic representation of mechanism of action for inverting glycoside phosphorolases such as those in family GH130. The reaction left to right is referred to as phosphorolysis, and right to left as reverse phosphorolysis, yielding an elongated saccharide.

Reverse hydrolyis can be hard to achieve for glycoside hydrolases due to the relative abundance of water compared to sugars in solution. For glycoside phosphorylases however, the free energy associated with breaking the ester-linkage in the sugar-1-P product of phosphorylases is similar to that of the glycosidic linkage of the glycan substrate[121]. This enables the equilibrium position to be shifted favorably towards synthesis, i.e reverse phosphorylysis through manipulation of reaction conditions[128]. Phosphorylases can thus be utilized in both reaction directions, exploiting their ability to degrade carbohydrates for the production of commercially valuable phosphorylated sugars, such as D-mannose-1-phosphate (M1P). A combination of phosphorolysis and reverse phosphorolysis (synthetic) reactions offers the possibility synthesis of diverse and intricate carbohydrate molecules[123].

2.2.7 Carbohydrate binding modules

Hydrolytic enzymes, can consist of several domains besides the one responsible for catalysis. Non-catalytic carbohydrate-binding modules (CBM), play a crucial role in the degradation of insoluble polysaccharides[129]. These auxiliary domains primarily function to bind polysaccharides, ensuring close proximity and prolonged interac-

tion with the substrate for effective carbohydrate hydrolysis[130]. Notable examples of insoluble polysaccharides targeted by these enzymes encompass cellulose, chitin, β-glucans, starch, glycogen, inulin, pullulan, and xylan . As of Febuary 2024, CBMs are classified into 101 families according to the Cazy database based on amino acid similarity. By facilitating interaction between the catalytic domain and its substrate, CBMs may enable and enhance catalysis. A recent study showed that removal of the CBM of *Es*GH5_8 and *Xc*GH5_8, two GH5_8 mannanases decreased their activity towards insoluble substrate and shifting substrate preference towards mannooligosaccharides[131]. CBMs exhibit significant diversity in substrate specificity and other properties, making them a valuable resource for study to increase substrate utilization in both hydrolysis and transglycosylation[132]. The *Trichoderma reesei* GH5_7 β-mannanase *Tr*Man5A carries a cellulose binding CBM of CBM family 1 and enhances the enzyme activity towards complex substrates including both cellulose and β-mannan hemicellulose[133].

2.2.8 Synergistic interaction between enzymes

In nature, the enzymatic degradation of complex polysaccharide structures is facilitated by multiple enzymes operating in concert to break down the complex structures of polysaccharides into its component monosaccharides[134].

This allows for enzymes to circumvent potential substrate limitations, such as steric hindrance obstructing the enzyme substrate complex from successfully forming[135], thus increasing the productivity of other cooperating enzymes. This is referred to as *enzyme synergy*, an approach which can be utilized for finding the required combination of enzymes for efficient conversion of a given substrate[136]. An example of enzyme synergy *in vivo* are from expressed proteins in *Polysaccharide Utilization Loci* (PULs) encoded in different types of polysaccharide degrading bacteria[137,138].

In vitro, this type of approach is also applied in degradation of more complex poly- and oligo- saccharides, using a cocktail of different complimenting enzymes to further increase product yields[23,139,140]. To measure the combinatory effect of enzymes, the degree of synergy (DS) can be determined. DS is defined as the ratio of the product yield for the co-incubation divided by the sum of the single incubations[135] (eq 2.1). If DS is >1, this indicates synergistic effect, i.e the co-incubation yield is higher than the sum of the single yields indicating a positive effect. A DS≈1 indicates little to no synergistic effect, indicating that the enzymes act independently. if DS<1, the combinatory effect of co-incubation is negative, i.e the enzymes operate worse together than apart by some factor, e.g. steric hindrances or product inhibition.

$$DS = \frac{yield_{co\ incubation}}{\sum_{i=1}^{n} yield_{single\ incubation,\ i}} \tag{2.1}$$

Enzyme synergy can be divided into *homosynergy*, where enzymes of similar specificity cooperate beneficially, or *heterosynergy* where the enzyme cooperate via different specificities[141]. In **Paper II**, to circumvent the limited substrate utilization when incubating GH5_7 β-mannanase *Tr*Man5A with *locust bean gum* galactomannan (LBG) and allyl alcohol for translgycosylation reactions, a GH27 α-galactosidase Aga27A was employed to debranch the β-mannan backbone, exposing more cleavage sites for *Tr*Man5A. The results showed that not only did the contributions to transglycosylation increase for both individual enzymes, the contribution of allyl-galactose from Aga27A increased the total transglycosylation yield as well.

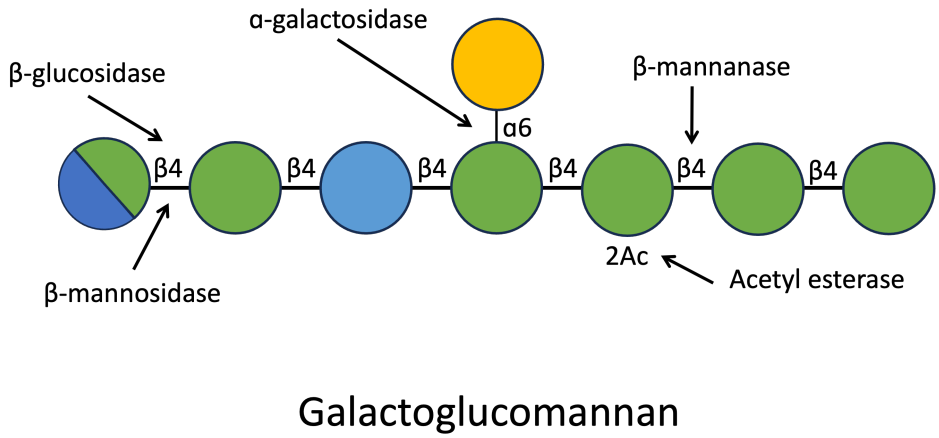

Figure 2.7: Schematic representation of a fragment of galactoglucomannan with mannosyls in green, glucosyls in blue, galactosyls in yellow, and "Ac" indicates acetyl units. The reducing end is shown towards the right. The various glycoside hydrolase specificities required for efficient conversion of galactoglucomannan are shown. The enzymes that have been examined in this thesis are β-mannanases, β-mannosidases, and α-galactosidases.

2.3 Transglycosylation

2.3.1 Mechanism behind transglycosylation

Some glycoside hydrolases (GHs) are capable of forming new glycosidic bonds through an alternative reaction path to hydrolysis, but for it to be possible, some factors are required. In order to break the covalent intermediate of the retaining mechanism (as seen in Figure 2.8), a glycosyl acceptor molecule is required. In hydrolysis this role is performed by a water molecule which acts a nucleophile to break the covalent intermediate[103], but this role can be filled by other molecules as well. This results in a transfer of the glycosyl to another molecule, a process which is referred to as

transglycosylation.

Enzymatic transglycosylation is of interest for scaled up biotechnological applications, due to their relatively "softer" conditions acting at $\leq$ 60°C, pHs around 5-8 and at atmospheric pressures, in comparison to other chemical modifications of glycosides, which often also requires cumbersome protection/deprotection steps to ensure reaction specificity[142,143] that are generally very good at.

GHs vary in their propensity towards transglycosylation vs. hydrolysis, some almost entirely catalysing this type of reaction, referred to as transglycosylases (TGs)[144], although they are not necessarily able to transfer onto other molecules than saccharides, such as alcohols[145]. An example is the GH5 β-mannanase from tomato LeMan4A, with different isoforms displaying varying transglycosylation preferences[144].

Enzymes with native transglycosylation capabilities play roles in plant cell wall modification and oligo- or polysaccharide synthesis. TGs share evolutionary connections with hydrolysing GHs, often found in the same families. Despite high transglycosylation with saccharides, this doesn't guarantee the ability to use other acceptors, such as alcohols. There are also another type of carbohydrate active enzymes that are able to form glycosidic bonds, referred to as glycoside transferases (GTs). In contrast to other GHs, GTs utilize nucleotide sugar substrates and this type of enzyme is responsible for synthesis of many common polysaccharides *in vivo*[146,147].

In vivo, transglycosylation reactions catalysed by GHs are involved in the biosynthesis of polysaccharides and modification of plant cell walls[146]. *In vitro*, transglycosylation reactions can be used to synthesize novel glycosides and, or to modify the structure of existing molecules.

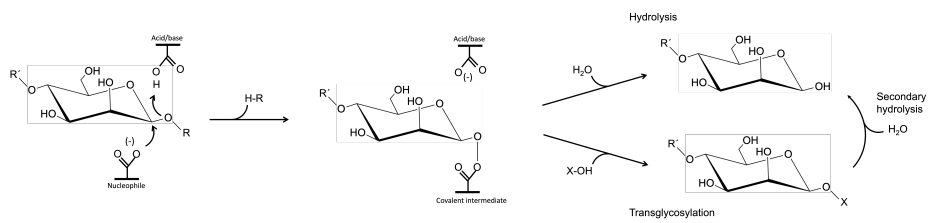

Figure 2.8: Transglycosylation reaction scheme. The covalent enzyme glycoside intermediate formed during cleavage for retaining double displacement acting enzymes can be broken by nucleophilic attack of either a water molecule, or another hydroxyl containing glycoside acceptor (X-OH), leading to hydrolysis and transglycosylation respectively. Further downstream hydrolysis reactions of transglycosylation products can occur if the enzyme has activity towards it, leading to *secondary hydrolysis*.

Retaining glycoside hydrolases primarily involved in hydrolysis may also exhibit transglycosylation[145,148,149]. This research is of interest for enzymatic synthesis, however their often significant primary hydrolysis compete with transglycosylation and possi-

ble degradation of transglycosylation products via hydrolysis (referred to as *secondary hydrolysis*[148]) have prompted extensive enzyme engineering efforts to enhance transglycosylation performance (**Papers II-III**)[146,150].

To assess the transglycosylation capacity of enzymes, the *rS / rH* ratio, denoting the rate of synthesis (rS) over the rate of hydrolysis (rH), can be calculated as described by Van Rantwijk et al[148]. The ratio reflects the balance between catalytic events leading to synthesis (transglycosylation) and hydrolysis in a given reaction. In the context of retaining glycosidases, it signifies the disruption propensity of the covalent glycosyl-enzyme intermediate by water (hydrolysis) or another glycosyl acceptor, such as allyl alcohol **Paper II** or alkane alcohols (**Paper III**). Inverting GHs may also be applicable in enzymatic synthesis, via *reverse hydrolysis*. In contrast to generally kinetically controlled retaining GHs described above, synthesis by reverse hydrolysisis is thermodynamically controlled[148,151]. The reveresibility of these cleavages was studied for the β-mannan oligosaccharide phosporylase *Rh*MOP130A in **Paper IV**.

$$\frac{r_S}{r_H} = \frac{[translgycosylation\ products]}{[hydrolysis\ products]} \tag{2.2}$$

2.3.2 Engineering strategies for increased transglycosylation

The glycosynthase method stands out as a successful strategy for enhancing the synthetic capacity of retaining glycoside hydrolases. By mutating the nucleophile of a retaining β-glycosidase to, e.g. an alanine and utilizing activated donors, yields of synthetic products can be increased. This approach minimizes product hydrolysis, a common limitation in transglycosylation catalyzed by retaining GHs with hydrolytic activity. While effective, the glycosynthase method has previously required fluorinated glycosyls and may involve extended reaction times or high enzyme loading[152,153].

Rational engineering approaches leverage previous structural and mechanistic insights to modify transglycosylation capacity by targeting specific amino acids. This strategy has successfully altered transglycosylation in various retaining glycoside hydrolases by focusing on substrate interactions, water interactions, or catalytic residues, e.g. as performed in **Papers II-III**[154].

Random mutagenesis provides an alternative for improving transglycosylation capacity, allowing the discovery of variants with improved properties without prior structural or mechanistic knowledge. However, it necessitates extensive screening, which can be challenging for transglycosylation reactions compared to hydrolytic activities[100].

While the presented methods have strengths and weaknesses, they are not mutually exclusive, and studies demonstrate the potential for combining approaches for comprehensive enzyme engineering. In a study performed by Teze et al., an approach for semi rational mutagenesis is presented where *in silico* analysis of related protein sequences identifies 6–12 single-mutant candidates that enhance transglycosylation yields[155].

Chapter 3

Methods

The following section provides a condensed overview and does not encompass all methodologies employed in the papers forming the foundation of this thesis. For a comprehensive understanding of the methods employed, refer to the respective individual papers. Instead, this section will concentrate on the general principles underlying the key methods in the project.

3.1 Activity determination of glycoside hydrolases

3.1.1 Para-nitrophenol assay

Several different methods can be used for assessing the activity and substrate specificity of glycoside hydrolases. Commonly used are colorimetric assays which attempts to relate cleavage events to the increase of absorbance in the sample at a specific wavelength. For *exo*-acting glycoside hydrolases such as α-galactosidases, a common colorimetric assay makes use of *4-Nitrophenyl* α*-D-galactopyranoside* (pNP-gal) [156,157,158] (**Paper I-II**) or equivalent pNP-glycoside. The hydrolytic cleavage of the *paranitrophenyl* (pNP) group from the galactose moiety leads to the release of para-nitrophenol, which under basic conditions is deprotonated to its phenolate state, which can be detected using a spectrophotometer measuring the absorbance at 405 nm. By incubating an enzyme with pNP-gal under known conditions, then stopping the reaction by excess addition of sodium carbonate, which ensures that the pH is high enough for pNP to predominately be in the phenolate state [156], followed by absorbance measurement, one can relate the absorbance via a standard curve of known pNP concentrations to the enzyme activity. In **Paper I**, the activity of GH36 α-galactosidase *Rh*Gal36A was

determined using the pNP-gal assay. In **Paper II**, the activity and stability of the GH27 α-galactosidase Aga27A was determined for the co-incubation with GH5_7 β-mannanase *Tr*Man5A.

3.1.2 DNS reducing end assay

For evaluating *endo*-acting GHs such as β-mannanases, another assay can be used to determine their acitivty against polysaccharide substate, e.g. galactomannan. The assay makes use of *3, 5-dinitrosalicylic acid* (DNS)[159,160,161] (**Paper I-III**). DNS reacts with and oxidizes reducing sugars, leading to the conversion of DNS to *3-amino-5-nitrosalicylic acid*, which has an absorbance maximum for light at 540 nm[162]. The increase in absorbance at 540 nm can thus be related to the increase in concentration of reducing end sugars via a standard curve of the expected saccharide product, which in turn corresponds to the amount of cleavage events[163]. The fact that the signal is proportional to the amount of reducing sugars in the sample means that a high background absorbance becomes an issue for reactions with oligomeric substrates, leading to a shorter dynamic range of measurable acitivities. In **Paper II**, the DNS assay was used to quantify the specific activity of GH5_7 β-mannanase *Tr*Man5A and its mutants when incubated with locust bean gum galactomannan (LBG), as well as determining the release of reducing end saccharides from a co-incubation with GH27 α-galactosidase Aga27A. In **Paper III**, the DNS assay was used to determine the activity of GH5_10 β-mannanase *Me*Man5A alongside its mutant variants against LBG.

3.1.3 Inorganic phosphate quantification assay

Another colorimetric assay used for enzyme activity determination involves quantification of free inorganic phosphate using a colorimetric malachite green phosphate assay kit (MAK308, Sigma-Aldrich, St. Louis, MO, USA)[164,165]. Quantifying phosphate is used in many different applications, but was primarily used within the scope of this project for activity determination of the GH130_2 β-mannan oligosaccharide phosphorylase *Rh*MOP130A in **Papers I** and **IV**. The enzyme was incubated in the reverse phosphorolysis reaction direction[123,126], i.e. with α-D-mannose 1-phosphate (10 mM) and a suitable mannosyl acceptor. The incubation is terminated after a set period of time, and then incubated with Malachite green, which reacts with free phosphate to form a blue-colored complex. This color formation is rapid and can be easily quantified using a spectrophotometer measuring absorbance at 620 nm. This assay is quite sensitive, and many precautions need to be considered when to acquire reliable results, such as avoiding phosphate contamination either from buffer or reaction

components.

3.1.4 HPAEC-PAD for reducing end saccharide quantification

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is a commonly applied method for acquiring quantitative data of the saccharide product profile from reactions with glycoside hydrolases[166]. A benefit of the method is the possibility to compare the release of sacharides from both oligomeric and polymeric substrates. The method of separation is based on interaction with a positively charged stationary phase in the column, and thus a strong base is utilized as eluent since sugars are weak anions at higher pH[23,52]. Detection is made possible by using an electrode that oxidizes the sugars and then the conductivity of the solution is recorded[166]. This can then be used to identify the type of sugar using suitable controls, and determine the concentration of sugars via a standard curve. HPAEC-PAD is utilized in **Papers I, III** and **IV**.

3.2 Screening and evaluation of transglycosylation activty for glycoside hydrolases

3.2.1 Mass spectrometry for product identification

For initial assessment of product profile, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF MS) was utilized (**Papers II-IV**). The sample is deposited onto a stainless steel target plate along with a matrix solution (10 mg/ml of 2,5-dihydroxybenzoic acid or DHB), used to facilitate ionization. The sample is then allowed to dry, resulting in the crystallization of the matrix with the sample[167]. The deposited sample is then subjected to analysis using a MALDI-ToF mass spectrometer, where the sample is ionized by absorbing light from the matrix molecule. The ionized analytes are subsequently separated within the mass spectrometer based on their weight and charge[168]. By calculating the theoretical monoisotopic mass of anticipated transglycosylation products and comparing it to the mass-to-charge ratio (m/z) values observed in the spectrum from the mass spectrometric analysis, it is possible to determine whether the molecules appear to be present in the sample or not[169,170]. While MALDI-ToF MS enables swift examination of a large number of samples to identify the presence of transglycosylation products, obtaining quantitative data from this method can be challenging[168].

3.2.2 HPLC for non-reducing end glycoside quantification

To achieve quantitative analysis of transglycosylation products, high-performance liquid chromatography (HPLC) was utilized. HPLC is a widely employed analytical technique capable of separating a diverse range of molecules by selecting appropriate mobile and stationary phases[171,172]. The choice of detector depends on the properties of the analytes. For analytes with functional groups exhibiting light absorbance, a UV-Vis detector set at an appropriate wavelength can be employed[172], as demonstrated in **Paper II** and **IV** where allyl glycosides were detected by measuring UV absorbance at 195 nm (the absorbance maximum of allyl groups). In cases where the analyte lacks absorbance, a more general detector like a charged aerosol detector (CAD) or an in-line mass spectrometer can be used instead[172].

Hydrophilic interaction chromatography (HILIC) was implemented to differentiate saccharides and transglycosylation products in the form of glycosides (**Paper II** and **IV**). In these studies, samples were injected into a mobile phase mixture of *acetonitrile* (ACN) and water, were water acts as the strong eluent and allowed to pass though a NH_2 column (LUNA amino, 250 × 4.6 mm, 3 µm, Phenomenex, Torrance, CA, USA). This separates the analytes based on their hydrophilicity, where more hydrophilic compounds retain for longer. in **Paper II** and **IV**, an injection program developed in-house was utilized, where an initial isocratic flow of 15:85 H_2O:ACN ran for 5 min, after which the water concentration was increased gradually to 30 % over 10 min to "push out" more retaining higher DP saccharides. This allowed for an efficient analysis of enzyme reactions yielding allyl glycosides, where different DP analytes could be quantified, even allowing for the separation of allyl-β-mannoside and allyl-α-galactoside in synergy reactions (**Paper II**). By employing standards with known concentrations, it became possible to identify and measure the analytes. In situations where no standards were available, a combination of fraction collection and analysis of the fractions using MALDI-ToF MS was employed to verify the identity of the peaks in the chromatogram resulting from the HPLC separation.

3.2.3 Evaluation of transglycosylation reactions

In **Paper II**, the transglycosylation capacity of the examined glycoside hydrolases was assessed through various methods. One parameter used to gauge transglycosylation capacity was yield, which quantifies how much of the loaded donor substrate had been converted into transglycosylation or hydrolysis products. While yield calculations provide information about how much of the donor substrate has been transformed into transglycosylation products, they offer limited insight into the underlying reactions, such as the extent of secondary hydrolysis of transglycosylation products (as in

Figure 2.8) in the reaction.

To address secondary hydrolysis, the rate of synthesis over the rate of hydrolysis (r_S/r_H) [148, 173] was computed in **Paper II** using a generic formula depicted as equation 2.2. The r_S/r_H-value can also be interpreted as a metric describing the preference of the retaining GH to catalyze transglycosylation reactions rather than hydrolysis reactions [148], especially when measured early in the reaction before secondary reactions, like secondary hydrolysis (Figure 2.8), have had time to occur. A small r_S/r_H ratio indicates a preference for catalyzing hydrolysis, while a larger value suggests a preference for transglycosylation.

3.3 Structural analysis of glycoside hydrolases and their reactions component

3.3.1 Structural analysis of glycoside hydrolases

Due to the potential challenges and time demands associated with acquiring the 3D structure of a protein via X-ray crystallography, Cryo-EM or NMR, alternative methods that do not depend directly on experimental methods may be more attractive. Alternative approaches for investigating glycoside hydrolase-substrate interactions involve structure modeling or prediction using new methods such as as Alphafold [174,175], coupled with overlays with crystal structures that have bound substrates (as demonstrated in **Papers II - IV**) [176,177], or by docking potential substrates to the generated models [178]. Both of these approaches can be used for determining putative catalytic residues or substrate interactions.

3.3.2 Determination of substrate binding mode preference for glycoside hydrolases

A glycoside hydrolase can bind a substrate in multiple positions, yielding various hydrolytic products (see Fig 2.4) [114,179]. While the product profile can indicate the preference for generating specific products, it cannot reveal the precise positioning of the substrate in the active site. One approach to discern the preferred binding mode is by using a sugar alcohol as a substrate, which generates both a shortened sugar alcohol and a regular sugar upon hydrolysis [96]. The reaction can then be analyzed via, e.g. HPLC to determine relative amounts of these species. However, concerns may arise regarding the interaction of the reduced sugar with the glycoside hydrolase in comparison to a natural non-reduced oligosaccharide.

In **Paper III**, an alternative method was employed to analyze the preferred substrate binding mode. This involved using reducing end oligosaccharides as substrates in combination with HPAEC-PAD and MALDI-ToF MS[114]. By incubating the glycoside hydrolase and the oligomeric substrate in ^{18}O-labeled water ($H_2^{18}O$), the newly formed reducing end incorporates ^{18}O. MALDI-ToF MS analysis enables the determination of the ratio of labeled (^{18}O-incorporated) and unlabeled product, providing insight into the original productive binding preference of the substrate for that specific hydrolysis product[114,117]. This method allows for a comprehensive estimation of the relative preferred binding mode for a specific oligosaccharide (see Fig 4 in **Paper III**). Care must be taken to account for factors such as the presence of any ^{16}O-water in the reaction and the natural occurrence of mannooligosaccharide isotopes.

3.3.3 NMR methods

Nuclear magnetic resonance (NMR), alongside X-ray crystallography, stands as one of the most prevalent techniques for obtaining 3-dimensional conformational data on high molecular weight substances with high resolution[180]. NMR relies on nuclear spin, an inherent property of atoms that allows nuclei to act similar to magnets. Only nuclei with nonzero spin numbers, such as ^{1}H, ^{2}H, ^{13}C, and ^{15}N, can be directly observed in NMR experiments. During an NMR experiment, the sample is placed in a homogeneous magnetic field within a small tube. Nuclei with nonzero spin numbers precess around the applied field due to the torque generated by the interaction between the angular momentum and the field. The NMR signal is acquired by disrupting the *equilibrium state* (where the bulk magnetization is aligned with the applied field), such as applying a radio pulse orthogonally to the static magnetic field. The pulse rotates the bulk magnetization, generating transverse magnetization. This transverse magnetization , induces a current in the coil, serving as the Free Induction Decay (FID) signal for NMR experiments[180,181]. Fourier transformation converts this time-based signal into a frequency-based spectrum (Hz or Rad), providing information on both chemical and conformational structures[180].

Chapter 4

Results and discussion

4.1 Summary of papers

4.1.1 Paper I: Exploring an mannan degrading enzymes in a mannooligosaccharide utilization locus

The human gut microbiota plays a crucial role in processing complex nutrients, impacting both health and disease. Short-chain fatty acids (SCFAs) produced during glycans fermentation by the human gut microbiota (HGM), especially butyrate, are known to have implications for human health[182,183].

β-Mannans, complex dietary fibers such as galactomannans that carry α-galactosyl side groups, are used widely in the food and feed industry besides being found as a component in softwood hemicellulose. While β-mannan metabolism is recognized as a key glycan utilization pathway in the HGM, research on the utilization of (galacto)-β-mannan-oligosaccharides (MOS/GMOS) remains limited. Notably, while studies on inulin and xylan utilization in these bacteria have been performed, research on β-mannan and MOS/GMOS utilization loci in *Roseburia spp.* and *Bifidobacterium spp.* has been limited[137,161]. The mechanisms behind the utilization of β-mannans, specifically MOS/GMOS are largely underexplored and of great interest for further study. Although β-mannans are recognized as vital dietary fibers and thickeners, the mechanisms underpinning their breakdown and utilization by prevalent bacteria in the HGM, especially regarding MOS/GMOS, lack comprehensive investigation.

The metabolic pathways, involved genes, and carbohydrate-active enzymes (CAZymes) facilitating the degradation of these complex glycans remain poorly understood. Additionally, the dynamics of cross-feeding interactions between different gut bacterial

species have been studied in the context of other carbohydrates, e.g. arabinoxylans, little attention has been given to MOS/GMOS utilization, specifically focusing on secondary mannan degraders such as *Bifidobacterium adolescentis* and *Roseburia hominis*[184].

Understanding whether these interactions display cooperative or competitive behaviors within shared nutrient niches in the human gut is crucial for understanding their relationship within the HGM. Furthermore, identification and characterization of the enzymes responsible for substrate degradation would provide insights into efficient degradation of these complex saccharides, allowing for application in production of novel glycosides. Therefore, **Paper I** concerns the bacteria *Roseburia hominis* A2-183 DSMZ 16839 (referred to as *R. hominis* from here on), found in the HGM, in terms of cross-feeding and metabolic interactions between *Bifidobacterium adolescentis* ATCC 15703 (referred to as *B. adolescentis* from here on) an acetate producer, and *R. hominis* (butyrate producer) during MOS/GMOS utilization.

The aim of **Paper I** was thus to investigate potential cross-feeding and metabolic interactions between *B. adolescentis* and *R. hominis* during MOS/GMOS fermentation, with a focus on their cooperative or competitive behavior. Additionally, **Paper I** aims to further elucidate the mechanistic role of a putative *R. hominis* MOS/GMOS utilization locus (*Rh*MosUL) by conducting transcript analysis of specific *Rh*MosUL genes and characterizing selected proteins encoded by this locus, such as *Rh*MOP130A, *Rh*Gal36A, and *Rh*GH113A, which are shown to be upregulated during *R. hominis* growth on MOS/GMOS (Figure 3 in **Paper I**).

Paper I shows how *B. adolescentis* and *R. hominis* process carbohydrates like glucose and MOS/GMOS individually and when cocultured. It uncovers their growth dynamics, revealing glucose competition and differential responses to MOS/GMOS. *B. adolescentis* produces acetate from both glucose and MOS/GMOS, while *R. hominis* consumes acetate and generates butyrate mainly from MOS/GMOS (Figure 1C/D in **Paper I**). Notably, enzyme analyses demonstrate *B. adolescentis*'s extracellular α-galactosidase activity, absent in *R. hominis*. The findings in **Paper I** show distinct substrate preferences, differential enzyme activities, and cross-feeding behavior for the bacterium *R. hominis* within the gut microbiota.

4.1.1.1 Difference in carbohydrate utilization for mono- and cocultures

The growth of *B. adolescentis* and *R. hominis* was studied in monocultures using glucose as the substrate, with *R. hominis* exhibiting a dependency on acetate. When cocultured, both strains exhibited lower cell concentrations compared to monocultures, suggesting competition for glucose (Figure 1A in **Paper I**). When MOS/GMOSs were

used as the substrate, the growth curves of *B. adolescentis* in mono- and cocultures were comparable, while *R. hominis* showed slightly higher cell concentrations after 24 hours in coculture compared to monoculture fermentations (Figure 1A in **Paper I**). Additionally, the growth of *R. hominis* was slower in the first 6 hours of coculture on both glucose and MOS/GMOS (Figure 1 in **Paper I**). Tuncil et al.[185] did a study comparing the relative expression of *Bacteroides ovatus* and *Bacteroides thetaiotaomicron* cazymes in cocultivation. There they showed the hierarchical utilization of glycans by *B. ovatus* and *B. thetaiotaomicron* by species-specific preferences and transcriptional responses to substrate depletion, with polymer length influencing glycan preference[185]. To evaluate this further, the short-chain fatty acid (SCFA) production of *R. hominis* and *B. adolescentis* was studied as well.

In terms of SCFA, *B. adolescentis* in monoculture on glucose primarily produced acetate, followed by lactate and formate. In *R. hominis* monocultures, acetate was consumed while butyrate, lactate, and formate were produced (Figure 1C in **Paper I**). When *B. adolescentis* was cultivated on MOS/GMOS, acetate and formate concentrations increased up to 16 hours, with a decrease in lactate. For *R. hominis* on MOS/GMOS, butyrate was the major organic acid produced (Figure 1D in **Paper I**). Similarily, in a study by Kim et al.[186], cross-feeding interactions between *Faecalibacterium prausnitzii* and *Bifidobacterium* strains, particularly *Bifidobacterium catenulatum*, in coculture with fructooligosaccharides as an energy source, demonstrated increased viable cell count, enhanced butyrate production, and anti-inflammatory effects both *in vitro* and *in vivo*, suggesting potential implications for treating inflammatory bowel disease[186].

During cocultivation, a substantial decrease in acetate was observed, potentially due to initial dominance of *B. adolescentis* resulting in acetate production, which is then utilized by *R. hominis* (Figure 1E in **Paper I**). This was supported by the significant growth of *R. hominis* observed after 3 to 6 hours of cultivation on glucose or MOS/GMOS, respectively. The significant production of butyrate in cocultivation was also observed after 12 hours, compared to 6 hours in monocultures (Figure 1 in **Paper I**).

To investigate the impact of cocultivation on carbohydrate utilization, MOS/GMOS were studied in mono- and coculture fermentations. As described above, both strains efficiently utilized glucose in monocultures, indicating potential competition for this substrate during cocultivation. The utilization patterns of MOS/GMOS differed in coculture compared to monocultures. While *R. hominis* showed a preference for certain types of MOS and GMOS, *B. adolescentis* exhibited uptake of MOS with a degree of polymerization (DP) of 2–3 and galactose. Interestingly, the utilization profile of MOS/GMOS was distinct in coculture, with rapid disappearance of most detectable oligosaccharides. This pattern of behaviour for cocultures have been seen before, such as in a study by Zeybek et al.[187], where xylan was made possible as a carbon source

by *Bifidobacterium*, by cocultivation with *Bifidobacterium animalis* subsp. *lactis*, with *B. animalis* subsp. *lactis* benefiting from coculture with xylan-hydrolyzing *Bacteroides* species[187].

The presence of galactose in the fermentation medium during the growth of *B. adolescentis* on MOS/GMOS was examined in both monoculture and coculture. Significant α-galactosidase activity was detected in the extracellular fractions of *B. adolescentis*, indicating hydrolysis of substrates. In contrast, no α-galactosidase activity was observed in *R. hominis* extracellular fractions. Furthermore, incubation of extracellular fractions with specific substrates confirmed that *B. adolescentis* possesses an extracellular α-galactosidase. No β-mannosidase activity was detected in any of the extracellular fractions.

4.1.1.2 Upregulation of MOS utilization locus indicates involved enzymes

The study investigated the role of a putative MOS utilization locus in *R. hominis* (*Rh*MosUL) and other genes in MOS/GMOS utilization through expression analysis using RT-qPCR. Six reference genes were selected for normalization, and the three most stable ones (rho, DnaJ, and rpoB) were used for target gene expression analysis. Ten target genes were evaluated in *R. hominis* during growth on different substrates. Genes within *Rh*MosUL and others involved in acid production showed significant differential expression on MOS/GMOS compared to monosaccharides (Figure 3 in **Paper I**). These genes are implicated in MOS/GMOS utilization. In a similar study of another butyrate producer, *Roseburia intestinalis* by La Rosa et al.[137], revealed that conserved a gene locus expressing several similar carbohydrate acting proteins that allow *R. intestinalis* to depolymerize β-mannan plant polysaccharides[137], including a GH26 β-mannananase, ABC-transport mannan protein, GH36 α-galactosidase, GH130 mannophosporylases, among others[137]. 4 sequences were initially of interest, and analysed for potential expression.

Bioinformatic analysis of *Rh*MOP130A, *Rh*Man113A, *Rh*Gal36A, and *Rh*MosBP supported their putative functions. Important catalytic residues were conserved in these enzymes. *Rh*MosBP likely has a MOS-binding function, as indicated by its homology with *Bl*MnBP1[161], and thus excluded from expression. Recombinant *Rh*MOP130A, *Rh*Man113A, and *Rh*Gal36A proteins were expressed, purified, and characterized. *Rh*MOP130A and *Rh*Gal36A were found to be hexameric and tetrameric respectively, using Native PAGE, consistent with previous reports on GH130[188] and GH36 enzymes[156].

4.1.1.3 Characterization pf RhMosUL enzymes

*Rh*MOP130A, a predicted GH130 β-1,4 mannan-oligosaccharide phosphorylase, demonstrated stability and specific activity at pH 5.5 and 37°C, it retained >95% activity for 72 hours and remained stable for 5 hours at pH 6. Its optimal activity was at pH 5.5, exhibiting a specific activity of 1.16 ± 0.11 kat/mol at 37°C using M_2 as the acceptor saccharide. This enzyme showed no activity towards mannose or glucose but displayed considerable activity with M_3, M_4, and M_5 as acceptor (Table 1 in **Paper I**). In phosphorolysis tests, *Rh*MOP130A exhibited activity against M_3, preferring substrates with three mannosyl units (Table 1 in **Paper I**). Its phosphorolysis activities resembled those of gut bacterial GH130 enzymes but differed from GH130_1 mannoside hydrolases[125], suggesting a preference for longer substrates like MOS over shorter ones. Moreover, during analysis, the enzyme demonstrated synthesis activity, creating products with higher polymerization levels than the initial substrate. This has been observed previously in GH130_2 phsophorylases, such as for *Ri*GH130_2 in La Rosa et al[137], or in the thermoactive glycoside phosphorylase from hyperthermophile *Thermotoga maritima* (TM1225) in a study by Grimaud et al[189]. However, *Rh*MOP130A showed no activity against galactosylated mannooligosaccharides like GM_3 or G_2M_5, unlike its activity on undecorated saccharides (Figure 4A in **Paper I**).

Based on similarities to GH113 endo-β-mannanases[190,191], *Rh*Man113A was tested with polymeric β-mannans but displayed low activity (≈0.02 katal/mol for locust bean gum galactomannan and ≈0.01 for konjac glucomannan at pH 6). In contrast, known GH113 enzymes, *Aa*ManA[190] and *Ax*Man113A[191], exhibited high activity with these substrates. When *Rh*Man113A interacted with M_4, increased activity was observed. Analysis revealed the formation of hydrolytic products M_1, M_2, and M_3 from M_4, with M_1 as the primary product and M_2 accumulating without being consumed (**Paper I**, figure 5). *Rh*Man113A's optimal activity was observed at pH 6. Among oligosaccharides M_2–M_6, it showed no detectable activity towards M_2, favoring M_4 and M_5 as primary substrates, followed by M_3 and M_6 (Table 2 in **Paper I**). Notably, it exhibited no activity towards galactosylated mannotriose (GM_3) (Table 2 in **Paper I**), indicating the importance of a non-galactosylated end mannosyl unit for its hydrolytic function.

*Rh*Gal36A exhibited activity (17.1 ± 0.7 katal/mol at 30°C, pH 5.5) against GM_3, resembling that of *Bo*Gal36A, a GH36 α-galactosidase found in *B. ovatus*[192]. *Rh*Gal36A effectively removed α-galactose substituents from GM_3 and G_2M_5 within 24 hours, leaving behind galactose, undigested M_3, and M_5 as end-products. Digestion of raffinose by *Rh*Gal36A produced galactose and sucrose after 24 hours, suggesting a preference for galactomannooligosaccharides (GMOSs) rather than raffinose. Notably, *Rh*Gal36A displayed the ability to remove internal galactose units from di-

galactosylated mannopentaose (Table 3 in **Paper I**), a characteristic commonly seen in GH27 α-galactosidases[157] but only observed in GH36 α-galactosidases in some cases, such as in *Bo*Gal36A[137,156,192]. *Rh*Gal36A's capacity to remove galactose units from galactosylated mannotriose suggests it could render the remaining saccharide accessible for other enzymes such as *Rh*MOP130A and *Rh*Man113A (Figure 4.1). These enzymes might be hindered by galactose substituents in the substrate, as indicated in the analysis.

The characteriztion of the *Rh*MosUL encoded enzymes *Rh*Gal36A, *Rh*MOP130A, and the distally encoded *Rh*Man113A indicates a synergistic mode of conversion for internal hydrolysis of MOSs/GMOSs, which is substantiated by their simultaneous upregulation during cultivation on MOS/GMOSs. Utilizing a concert of enzymes in this way allows bacteria such as *R. hominis* to effectively convert more complicated sugars with many different enzymatic targets for its metabolism[137].

4.1.1.4 Proposed substrate degradation pathway

R. hominis utilizes MOS with a DP up to 6, reflecting *Rh*Man113A and *Rh*MOP130A's specificities. *Rh*MOP130A, a mannan-oligosaccharide phosphorylase, operates on DP 3–6, slightly favoring M_3 (Table 1 in **Paper I**), while *Rh*Man113A, an exo-oligomannosidase, targets M_3-M_6, favoring M_4 (Table 2 in **Paper I**). Both are hindered by galactose side groups on mannan-oligosaccharides. However, *Rh*Gal36A can de-galactosylate GM_3 and G_2M_5 (Table 3 in **Paper I**), likely working sequentially with *Rh*Man113A and/or *Rh*MOP130A, as observed in other PULs, such as in *Bacteriodes ovatus*[193], *R. intestinalis*[137], in *Faecalibacterium prausnitzii*[138] (proposed mode of action in **Paper I**, figure 6, also in Figure 4.1). This study provided information concerning MOS/GMOS utilization in *R. hominis*, distinct from primary polysaccharide degraders like *B. ovatus*[193] and emphasizing the potential roles of *R. intestinalis*[137] and *R. hominis* in mannan degradation in the gut microbiota. Taken together, the findings in **Paper I** contribute to a better understanding of glycan-dependent metabolic interactions in the gut microbiota and may lead to the discovery of novel carbohydrate-active enzymes as well as underscore the importance of complimentary specificities in degradation of complex saccharides by cazymes.

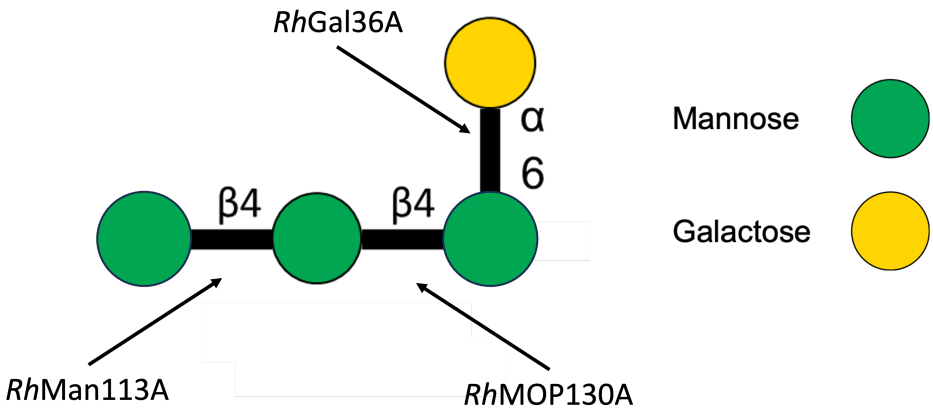

Figure 4.1: Proposed degradation pathway of MOS/GMOS for characterized enzymes in **Paper I**. *Rh*Gal36A acts initially to cleave off potential galactosyls substituents followed by the cleavage of the mannosyl backbone by *Rh*Man113A and *Rh*MOP130A, where *Rh*Man113A acts on terminal residues and *Rh*MOP130A cleaves internal and terminal mannosyl bonds via phosphorolysis.

4.1.2 Paper II: Improving transglycosylation through enzyme synergy

The study focused on investigating the transglycosylation capacity of a fungal GH5_7 endo-β-mannanase from *Trichoderma reesei* (*Tr*Man5A). The full-length native enzyme, which includes a carbohydrate binding module, was truncated leaving just catalytic module of *Tr*Man5A (referred to as WTcm) and two variants of WTcm, R171K (previously studied in the group[117]) and R171K/E205D (Figure 4.3), were examined.

4.1.2.1 Comparison of aglycone modified TrMan5A mutants

The initial assessment of transglycosylation capacity involved the use of mannotetraose (M_4) as the substrate, along with two acceptors: methanol and allyl alcohol. The initial screening of product formation using MALDI-TOF MS and HPLC revealed that all four enzymes produced transglycosylation products with 1 to 3 mannosyl units (Figure 2 in **Paper II**), with the R171K/E205D variant demonstrating the highest yield (Figure 3 in **Paper II**). Interestingly, the primary product for *Tr*Man5A and WTcm was found to be allyl mannotrioside (AM_3), indicating that the primary productive binding mode was from subsite -3 to +1 for M_4, whereas a previous study suggested that WTcm primarily preferred to bind M_4 in the -2 to +2 subsites when using methanol or butanol as acceptor[117]. However, another study reported similar observations of hexyl mannotrioside being a major product for *Tr*Man5A when using M_4 as the donor substrate and hexanol as the acceptor[13].

It was previously established that transglycosylation with the saccharide acting as the acceptor occurred with WTcm using lower M_4 concentrations[13]. Therefore, it was hypothesized that both *Tr*Man5A and WTcm initially preferred using M_4 as the acceptor, leading to the generation of elongated saccharides. These elongated saccharides were then proposed to serve as the glycosyl donor in subsequent reactions, resulting in the production of substantial amounts of AM_3.

A further comparison of the transglycosylation capacity between *Tr*Man5A and R171K/-E205D over extended incubation times was conducted. R171K/E205D displayed the highest apparent transglycosylation capacity among the variants (Table 1 in **Paper II**, Table 4.1). When M_4 was used as the substrate, both *Tr*Man5A and R171K/E205D exhibited continuous production of allyl mannosides during the initial time period. However, as M_4 was depleted, both enzymes displayed secondary hydrolysis, which refers to the hydrolysis of the allyl mannosides[148]. This phenomenon was more pronounced for R171K/E205D compared to *Tr*Man5A (Figure 4 in **Paper II**). Secondary hydrolysis of transglycosylation products is observed in many different types of GHs, and can reduce apparent translgycosylation yields. in a study on the GH20 lacto-N-biosidase (LnbB) by Castejón-Vilatersana et al[194], the aim was to reduce hydrolytic activity and confer transglycosylation activity for the synthesis of lacto-N-tetraose (LNT) from p-nitrophenyl β-lacto-N-bioside and lactose, resulting in mutants with residual hydrolase activity yielding transglycosylating enzymes producing LNT at 10-30%[194]. In addition, several attempts at modelling secondary hydrolysis have been carried out in order to provide improved transglycosylation yields[195,196].

The rate of synthesis over rate of hydrolysis (r_S/r_H) was calculated based on the formation of allyl mannosides and hydrolysis products, showing that R171K/E205D is superior to the wild type in terms of allyl transfer using oligomeric glycosyls donor substrate[148,173]. Further analysis indicated significant secondary hydrolysis with R171K/-E205D, as the experimental yield of allyl mannosides was substantially lower than the theoretical yield based on the initial r_S/r_H-value. For *Tr*Man5A, on the other hand, the experimental yield exceeded the theoretical yield. This was attributed to the occurrence of transglycosylation with saccharides as glycosyl acceptors, a reaction not considered in the initial r_S/r_H calculation.

Table 4.1: Comparative overview of GH5 β-mannanases that have been mutated and their propensity for transglycosylation. The wild type enzyme is highlighted in **bold**, with the mutations in rows below. The used glycosyl donor, acceptor, observed products, product yields if available, preferred binding mode are also shown. "M_X": using saccharides, e.g. either original substrate or hydrolysis products as glycosyl acceptor. "N.D": *Not Determined*.

Enzyme	Donor	Acceptor	Products	Yields	Binding mode	Reference
WT MeMan5A	M_5	M_X, alcohols	M6/M7/M8/alcohol mannosides	N.D	from -3 to +2	**Paper III**
W240A	M_5	M_X, alcohols	alcohol mannosides	N.D	from -3 to +2	**Paper III**
W281A	M_5	M_X, alcohols	alcohol mannosides	N.D	from -3 to +2	**Paper III**
W240A/W281A	M_5	alcohols	alcohol mannosides	N.D	No preference	**Paper III**
WT TrMan5A	M_4	allyl alcohol	allyl-β-mannosides	7.7%	from -2 to +2	**Paper II**
R171K/E205D	M_4	allyl alcohol	allyl-β-mannosides	8.97%	from -3 to +1	**Paper II**
WT TrMan5A	LBG	HEMA	ethyl-metacrylate mannosides	7.42%	from -2 to +2	[197]
AnMan5A	M_4	M_X	M5/M6	2%	from -2 to +2	[176]
ManAS289W	M_4	M_X	M5/M6	5%	from -2 to +2	[176]
AnMan5B	M_4	M_X	M5/M6/M7	18.7%	N.A	[145]
AnMan5C	M_4	M_X	M5/M6	16%	from -2 to +2	[176]
ManCW283S	M_4	M_X	M5/M6	9%	from -2 to +2	[176]

When locust bean gum galactomannan was used as the donor substrate, the yield of allyl mannosides decreased for both wt *Tr*Man5A and R171K/E205D, although *Tr*Man5A demonstrated higher production of allyl mannosides compared to R171K/-E205D (Table 2 in **Paper II**).

Analysis of the reaction mixture of *Tr*Man5A with locust bean gum and allyl alcohol using MALDI-TOF MS revealed the presence of a heptasaccharide, indicating the accumulation of inaccessible saccharide products (Figure 8 in **Paper II**). *Tr*Man5A would be able to degrade these longer saccharides if they only consisted of mannosyl units, but since the substrate was galactomanan, it is likely that the observed peaks were from shorter saccharides that instead possessed galactosyl substitutions. It was thus proposed that higher degrees of galactosyl substitutions may hinder *Tr*Man5A, resulting in reduced locust bean gum conversion[113]. Steric hindrance caused by backbone decorating substituents have been shown to reduce acitivity in polymeric substrate acting mannanases before, such as in a study by Arnling Bååth et al.[77], where increased acetylation of the mannan backbone showed reduced activity in *Cellvibrio japonicus* enzymes GH5 CjMan5A and GH26 CjMan26A[77].

4.1.2.2 coincubation for increased transglycosylation yields

*Tr*Man5A had been theorized to be worse at galactomannan conversion due to steric hindrances caused by themsubstrates galactosyl substitution, leading to worse utilization of the substrate. To address this, an α-galactosidase (Aga27A) was introduced to the reaction mixture and coincubated with *Tr*Man5A (Figure 4.2). This addition aimed to remove galactosyl substitutions that may hinder *Tr*Man5A, shown

to be required for efficient conversion of complex β-mannans (**Paper I**)[198]. The results showed a significant reduction in the presence of heptasaccharides, supporting the idea that without the galactosidase, there was an accumulation of galactosylated oligosaccharides (Figure 8 in **Paper II**). The presence of Aga27A also resulted in a nearly five-fold increase in the yield of allyl glycosides (Table 3 in **Paper II**). This increase was partly attributed to the transglycosylation activity of Aga27A, a catalysis not previously described for this enzyme. Between the publication of **Paper II** and this thesis , further studies have been carried out examining the transglycosylation behaviour of Aga27A, comparing it to the GH36 α-galactosidase, *Bo*Gal36A[192], where Aga27A was shown to be slightly worse at generating translgycosylation products with a variety of glycosyl donors and acceptor when compared to *Bo*Gal36A, but Aga27A in turn showed much less pronounced secondary hydrolysis[192].

Both enzymes exhibited a higher yield of transglycosylation products when coincubated compared to when incubated alone with locust bean gum galactomannan, indicating a synergistic effect similar to previous studies concerning locust bean gum hydrolysis[157,199]. Enzyme synergy is a recognized phenomenon in the hydrolysis of polysaccharides, evident in both exo- and endo-acting cellulases[200]. This cooperative action has also been documented in xylanases and arabinofuranosidases[184], as well as in β-mannanases and α-galactosidases[198].

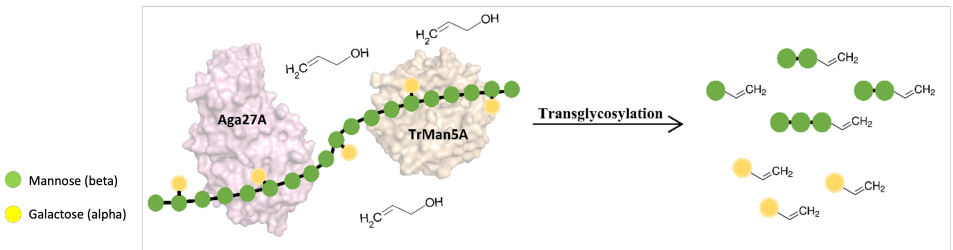

Figure 4.2: Schematic representation of enzyme synergistic approach for transglycosylation reactions using locust bean gum galactomannan as glycoside donor and allyl alcohol as acceptor.

To summarize, the effect of alterations in positive numbered aglycone binding subsites, specifically the +2 subsite in *Tr*Man5A, was studied for the potential application of transglycosylation using the alcoholic glycosyl acceptor molecule allyl alcohol. Reducing steric hindrance in the +2 subsite proved successful when utilizing oligomeric glycosyl donor substrate, but not for polysacccharides (Table 2 in **Paper II**). *Tr*Man5A showed lower transglycosylation yields than R171K/E205D with M_4 as the glycosyl donor, but higher when using locust bean gum instead. This difference was attributed in part to the prevalence of transglycosylation reactions with *Tr*Man5A and M_4, where saccharides served as acceptors, a feature observed in many GH5 β-mannanases[107,154] (**Paper III**). Taken together, the results in**Paper II** show the impor-

tance of considering the substrate used when determining the most effective enzyme for conversion. This limitation was successfully addressed by the addition of Aga27A in the enzyme synergy experiment, providing the contribution of allyl-α-galactose (Figure 9 in **Paper II**) reaction products as well as providing a synergistic contribution to overall yields (Table 3 in **Paper II**), resulting greatly increased production of allyl-glycosides. Enzyme synergy for hydrolysis of complex polysaccharides have been studied in previously [23,141,201], but the application of transglycosylation reactions with alcoholic acceptors provides an interesting new one pot reaction for functionalization of renewably derived mannan into novel glycosides.

Enzyme synergy has been utilized for hydrolysis of polysaccharides in studies before, both for xylan [198], and for mannan [141] conversion, but **Paper II** is in our understanding potentially the first example of the utilization of enzyme synergy for transglycosylation of polysaccharides using an alcohol as glycosyl acceptors. This approach, where screening enzymes or mutants of interest on oligosaccharide substrates for transglycosylation ability then building a library of complimentary specificities may be valid for other substrates as well, such as xylan or glucan based polysaccharides providing a powerful method for efficient production of functionalized glycosides from renewable feed-stocks.

4.1.3 Paper III: Studying the role of aglycone binding tryptophans in GH5_10 MeMan5A

Paper III concerns the GH5_10 β-mannanase *Me*Man5A from blue mussel (*Mytilus edulis*), and its interactions with glycosides in its aglycone binding subsites. *Me*Man5A features two semi-conserved tryptophans (W240 and W281) in the distal subsite +2 of its active site cleft (Figure 4.3). These tryptophans are positioned to interact with glycosides in the +2 subsites, where the loop hosting W281 was proposed to be flexible based on the higher B-factor as compared to other active site loops in the crystal structure of *Me*Man5A (pdb:2C0H) [96]. Flexible loops have shown to play an important role in substrate interaction in some GHs, such as in the GH3 barley β- glucohydrolase, were movements of a tryptophan carrying loop provides the enzyme with processivity with its movements, shown to be eliminited when mutated out [202].

In order to study the effect of these tryptophans on glycosyl interactions in the +2 aglycone subsite of *Me*Man5A, three mutant versions of the enzyme was expressed. These three mutants consisted of alanine substitutions for either W240 (W240A), W281 (W281A) or both (W281A/W240A, or double mutant). The impact of these mutations was assessed in terms of enzyme kinetics, product patterns, subsite binding, and transglycosylation using saccharides and alcohols as acceptors.

4.1.3.1 Aglycone tryptophan deletion in MeMan5A affects hexosyl subsite binding affinity

The substitutions mentioned above were shown to lead to reductions in catalytic efficiency, specifically three-fold (W281A), five-fold (W240A), or 20-fold (W240A/W281A) when using galactomannan as substrate and determined via DNS assay (Table 2 in **Paper III**). This trend was similar for incubations with either mannotetraose (M_4) and mannopentaose (M_5) as substrate as well (Table 2 in **Paper III**), indicating that lessened interaction in the +2 aglycone subsite caused by the alanine substitution has a negative effect on binding the substrate.

The effect of single sugar monomer binding subsites on overall productive substrate binding capability have been studied in different types of GHs, such as in xylan acting GH10 enzymes previously, such as in a xylanase from *Pseudomonas fluorescens* subsp. *cellulosa* (XYLA) and from the *Cellulomonas fimi* exoglucanase (Cex) in a study by Charnock et al[203]. The results revealed differences in the topology of their active sites, with Cex having three glycone and two aglycone binding sites, while XYLA had three glycone and four aglycone binding sites. Site-directed mutagenesis on XYLA indicated the crucial roles of aromatic residues, in the binding subsites, reducing activity on xylo-oligosaccharides when mutated out due to reduced possible binding across the catalytic site[203]. To investigate this further, we determined the preferred binding mode of *Me*Man5A to M_5 using isotope labelled water ($H_2^{18}O$) to determine ratio of binding positions.

The enzyme variants were incubated with M_5, and samples were collected at regular intervals for analysis. The hydrolysis of oligosaccharides can result in products with the same degree of polymerization through various binding modes. To differentiate between these modes and determine *Me*Man5A's preference, experiments were conducted using ($H_2^{18}O$) and analyzed with MALDI-ToF MS. This allowed for the calculation of ratios between labeled and unlabeled products with the same degree of polymerization. The ratios of manno-oligosaccharide products obtained from these experiments was combined with product concentrations determined through HPAEC-PAD to assess the relative preference of each *Me*Man5A variant for different M_5 binding modes[114], presented in Figure 4 in **Paper III**. The results showed that wild type *Me*Man5A as well as the single mutated variants W240A and W281A showed a preference for binding M_5 from subsite -3 to +2 (Figure 4 in **Paper III**), indicating significant mannose-unit binding in the +2 subsite remained intact even after substituting either W240 or W281. The W281A substitution had no effect on binding modes, whereas a slight shift was observed with W240A, which accounted for the increased production of mannose (M_1) and M_4 by this variant. In the case of W240A/W281A, the preference for binding M_5 from subsite -3 to +2 was lost, and

M_5 binding from subsite -4 to +1 became equally prevalent (Table 4.1). This shift may be attributed to a reduction in favorable mannosyl interactions in the +2 subsite.

4.1.3.2 Flexibility of W281 involved in binding interactions

To examine the nature of the interactions between W240, W281, and glycans in the +2 aglycone subsite, NMR studies of WT *Me*Man5A and its variants were studied in incubations with different glycans (Figure 5 in **Paper III**). The presence of mannobiose (M_2) and mannotriose (M_3) had no discernible impact on the chemical shift of W281, indicating no significant interaction or change in orientation. However, the introduction of di-galactosylated mannopentaose (G_2M_5) led to a noticeable change in the chemical shift of W281, suggesting a direct binding or sensing event (Figure 5 in **Paper III**. Notably, six of the tryptophan peaks (including W281) displayed changes in chemical shift, corresponding to the number of tryptophans present in *Me*Man5A's active site (Figure 5 in **Paper III**)[96]. Transversal ^{15}N relaxation rates (R_2) for these residues were also studied, and showed that W281 exhibited similar rates in both the unbound and M_2-bound states (Table 3 in **Paper III**), which were lower compared to other tryptophans. This indicates a higher level of flexibility on a short time scale for W281. However, the interaction with G_2M_5 resulted in an increase in R_2, implying a reduction in W281 flexibility via a direct binding event (Table 3 in **Paper III**).

Molecular dynamics (MD) simulations were also carried out to further study the flexibility of W281 and W240. *Me*Man5A's apo structure revealed that the loop containing W281 exhibited limited flexibility (Figure 1B, S4, S5 in **Paper III**). However, the side chain of W281 displayed significant flexibility, deviating by up to 11.2 Å from its position in the crystal structure. When a ligand (M_6) from a homolog structure of the GH5_8 β-mannanase StMandC (pdb: 4Y7E)[204] was positioned in subsites -3 to +3, the MD simulation showed a reduction in W281 flexibility and movement of the loop backbone. In the presence of the ligand, W281 primarily maintained one position, engaging in stacking interactions with the mannosyl unit in subsite +2, where the conformational adjustment involved both loop backbone and side-chain movements. surface loops with hydrophobic residues have shown dynamics relevant for glycan binding previously in GHs, such as for StMandC[204] and others[19,205]. On the other hand, the side chain of W240 remained rigid throughout the simulation, forming a hydrogen bond with the subsite +2 mannosyl C2-OH. This would provide *Me*Man5A with mannosyl specificity in the +2 binding subsite. Taken together, the flexibility of W281 in the apo-enzyme and its exposure near the binding cleft's edge indicates its potential role in substrate capture and its contribution to positioning of the glycan in the active site cleft, where the rigid W240 is poised for binding at the cleft's bottom.

4.1.3.3 Effect of MeMan5A aglycone modification on transglycosylation specificity

The transglycosylation experiments conducted in this study demonstrate the involvement of +2 subsite tryptophans in glycan acceptor interactions during transglycosylation. Substantial decrease in transglycosylation capacity with saccharide acceptors was observed upon W281 substitution. Similarly, substituting W240 leads to a notable reduction in transglycosylation with saccharide acceptors, and complete elimination of saccharide transglycosylation capacity is observed in the W240A/W281A variant (Table 1 in **Paper III**). This indicates that both W240 and W281 contribute to glycan acceptor-binding. While hydrophobic residues like tryptophans are known to be crucial in glycan interactions[206], the substitutions W240A and W281A did not affect transglycosylation with alcohol acceptors. This is evidenced by the production of alkyl mannosides using methanol and hexanol as acceptors(Figure 3 in **Paper III**).

Similar interactions in the +2 subsite of other GHs[207], including GH5_7 β-mannanase *Tr*Man5A are observed in other studies **Paper II**[145], e.g. subsititution of R171 in the +2 subsite to lysine abolishes transglycosylation with saccharides while maintaining activity with alcohols (**Paper II**)[13]. This suggests that both polar and non-polar residues in the +2 subsite of GH5 mannanases of different subfamilies play significant roles in glycan acceptor interactions. Given the partial conservation of R171 in *Tr*Man5A[95] and W240/W281 in *Me*Man5A (Fig 1A in **Paper III**) within subfamilies GH5_7 and GH5_10, respectively, similar functions may potentially apply to numerous other GH5 mannanases. Thus, **Paper III** establishes potential approaches for targeted mutations to enhance the transglycosylation yields of these enzymes when utilizing alcohols as acceptors for the production of novel glycosides.

4.1.4 Paper IV: Exploring reverse phosphorolysis in GH130_2 β-mannan oligosaccharide phosphorylase RhMOP130A

The gut microbiota utilizes carbohydrate-active enzymes (CAZymes), including glycoside phosphorylases (GPs), to break down complex carbohydrates like β-mannan. While glycoside hydrolases are considered the main contributors, other carbohydrate active enzymes such as GPs, and more specifically GH130 enzymes, contribute in glycan breakdown[123]. This study focused on the GH130_2 β-mannan oligosaccharide phosphorylase *Rh*MOP130A, initially expressed in **Paper I**, exploring its molecular basis, potential for synthesizing novel glycosides through reverse phosphorolysis[125], with the intention to create elongated glycosides. The investigation delved into specificity, comparing acceptors for product formation and kinetics, and analyzes the enzyme's structure/function relationship through a new crystal structure.

4.1.4.1 Acceptor screening

Reverse phosphorolysis product formation in *Rh*MOP130A reactions was analysed using MALDI-TOF MS. Various acceptors were tested, revealing successful elongation with mannose, glucose, cellobiose, and specific mannosides (Table 1 in **Paper IV**). Both mannosyls and glucosyls acted as acceptors, suggesting a lack of binding specificity at the C2 hydroxyl. Galactose was ineffective as a glycoside acceptor, instead indicating the importance of the C4 hydroxyl orientation for successful elongation.

Different glycoside donors were tested, with no elongation using α-D-glucose 1phosphate as glycoside donor. Successful synthesis up to DP 6 with M_2, allyl α-D-mannopyranoside (AM_1), and M1P was detected (Figure 1 in **Paper IV**).

A thermoactive glycoside phosphorylase from *Thermotoga maritima* (TM1225) was investigated by Grimaud et al[189]. TM1225 also exhbited reverse phosphorolysis acitivty on β-mannan oligosaccharides displaying synthesis of mannan chains with a degree of polymerization up to 16 at 60 °C, resulting crystal formation of pure linear mannans like those found in the ivory nut[189]. TM1225 is the closest homolog to *Rh*MOP130A, and its products were determined to be connected through β-1,4-mannosidic bonds via NMR, meaning that it operates via an inverting mechanism[189].

4.1.4.2 Quantification

To explore the catalytic behavior of *Rh*MOP130A in the reverse phosphorolytic direction, reactions with M1P as the glycosyl donor were conducted. Notably, activity towards AM_1 as an acceptor was higher compared to mannose (M_1) (Table 2 in **Paper IV**), suggesting a potential interaction of the allyl group in the enzyme's active site. The specific activity of *Rh*MOP130A was determined in the reverese phosphorolysis direction using M_1, M_2, and AM_1, where no activity towards M_1could be detected (Table 2 in **Paper IV**). The activity of M_2 was approximately 12 x higher than for AM_1, indicating a more favorable interaction in the acceptor binding subsites.

For a time-resolved study, incubations with 10 mM M1P and 10 mM M_2 were performed for up to 24 hours, with product quantification using HPAEC-PAD (Figure 2 in **Paper IV**). The initial decrease in M_2 indicated the dominant formation of M_3, suggesting the incorporation of M1P into M_2 via reverse phosphorolysis. After 1 hour, a varied product profile with quantifiable peaks corresponding to M_3-M_6 was observed. Peaks with higher retention times, likely originating from higher degrees of polymerization (DP) mannooligosaccharides, were also detected up to a maximum elongation of around DP10 (Figure 2 in **Paper IV**). Similar peaks were observed for TM1225 when operating in the reverse phosporolysis direction, albeit M_6 was used as

the initial glycosyl acceptor instead[189].

Peaks were quantified and the product profile of each time point was analyzed (Figure 3 in **Paper IV**). After 24 hours, a peak corresponding to M_1 appeared, potentially resulting from breakdown of M1P or phosphorolysis of M_2 using liberated inorganic phosphate from a previous reaction. Controls showed no M1P degradation with or without *Rh*MOP130A in the timeframe of the reaction. Reverse synthesis reactions with M_1 and M1P showed no activity when analyzed via the phsophate quantification assay (Table 2 in **Paper IV**), but when analyzed by the more sensitive MALDI-ToF, revealed peaks corresponding to elongated products (Figure S1A in **Paper IV**), indicating limited but productive binding of a DP2 saccharide.

Additionally, incubations with 10 mM AM_1 and 10 mM M1P for up to 4 hours were analyzed by hydrophilic interaction liquid chromatography (HILIC) high-performance liquid chromatography (HPLC) (Figure 4 in **Paper IV**). Peaks indicating the formation of allyl α-D-mannobiose (AM_2) was confirmed via MALDI-ToF of collected fractions. Comparing productivity with AM_1 as an acceptor to M_2, AM_2 formation after 4 hours (0.48 ± 0.19 mM) was lower than the corresponding amount of M_3 produced (2.2 ± 0.83 mM), supporting that *Rh*MOP130A performs with reduced activity when using allylated mannoside substrates.

4.1.4.3 Structural analysis

The BLASTp analysis of *Rh*MOP130A in the PDB database confirmed its classification and function, with the closest match being identified as the Thermotoga maritima GH130 mannoside-phosphorylase (TM1225), sharing 61.5% identity and known for its activity on mannan-oligosaccharides[189]. MSA with homologs revealed the conservation of catalytic and phosphate-binding residues (Figure S6 in **Paper IV**).

The crystal structure of *Rh*MOP130A from was resolved at 2.3Å, revealing a five-fold β-propeller domain structure (Figure 6A in **Paper IV**), a characteristic feature of GH130 enzymes[188]. Additionally, the crystallographic refinement statistics and X-ray diffraction data collection for *Rh*MOP130A were presented (table 3 in **Paper IV**), providing comprehensive details on the structural determination process. Conserved residues forming the active site were located in a central cavity. *Rh*MOP130A formed a hexameric quaternary structure, in line with results from **Paper I**, (Figure 6B/C in **Paper IV**) with two sets of trimers stacked on top of each other (Figure 7B in **Paper IV**), creating a hollow passage for substrate entry and product exit. Potential loop interactions around the active site could contribute to substrate specificity.

Conserved residues N50, R65, Y109, D110, R156, N157, R174, K215, H234, Y245, and

D307 were identified through sequence alignment (Figure S6 in **Paper IV**) and superimposition with a 4-O-β-D-mannosyl-D-glucose phosphorylase from *Bacteroides fragilis* NCTC 9343 (*Bf*MGP), found in the human gut (pdb:3WAS, Figure 6A in **Paper IV**)[188] . The altered residue at position 71 (G71) contributed to less steric hindrance at subsite +1, potentially influencing *Rh*MOP130A's improved activity towards AM1 compared to M_1 or G_1 alone although no structure of the complex was obtained. W210 from a neighboring subunit formed a putative +2 subsite, which could show the potential importance of oligomerization for substrate interaction.

*Rh*MOP130A exhibited unique loop compositions (Figure 6D in **Paper IV**), including a lid loop (Loop 1) with glycine and histidine residues facilitating substrate interactions. Loop 2, containing a glycine instead of arginine, contributed to increased promiscuity at subsite +1 compared to *Bf*MGP. Loop 3 in *Rh*MOP130A was shorter, allowing accessibility to longer oligomeric substrates. Unlike other GH130 enzymes such as *Bf*MGP[188], *Rh*MOP130A lacked protruding N- and C-tab helices and N-tab loop, relying on extensive surface interactions for oligomerization.

The active site accessibility in *Rh*MOP130A, visualized through a +2 putative subsite (Figure 7A in **Paper IV**), demonstrated the potential for favorable binding of longer MOSs. The residues R156, N157, R174, K215, H234, and Y245 were conserved between *Rh*MOP130A and 3WAS, showing favorable positioning for phosphate binding (Figure 6B in **Paper IV**).

Taken together, the findings suggest that *Rh*MOP130A exhibits promising enzymatic potential for generating allyl glycosides with an elongated glycoside moiety, offering a valuable avenue for synthesizing allyl glycosides with controlled chain lengths. This enzymatic approach can facilitate the production of allyl glycosides with DP $\geq$ 2, addressing limitations observed in previous studies (**Paper II**)[85]. The ability to control glycoside chain length holds significance for diverse applications such as surfactant and glycopolymer production[208], showcasing the potential application of this enzymatic process in various fields.

4.2 Synthesis of novel glycosides from renewable mannan as an environmentally friendly product stream for biochemical production

Utilizing enzymes for the conversion of hemicellulosic β-mannan to either oligosaccharides (partial hydrolysis[201]) or monosugars (total hydrolysis or "saccharification"[23]) have been demonstrated previously[209,210], where either single enzymes[211] or cocktails consisting of several enzymes acting in concert[23,209] have been employed to effectively

degrade polymeric mannan[199,210,212]. The partial hydrolysis products of these feedstocks have seen applications as prebiotics[23,84], and the resulting monosugar from total hydrolysis are commonly of interest for bioethanol fermentation[213]. But the application of transglycosylation reactions using polymeric mannan is still understudied and underutilized. In **Paper II**, locust bean gum galactomannan was converted into allyl-glycosides utilizing a combination of the endo-β-mannanase *Tr*Man5A and the α-galactosidase Aga27A. these allyl glycosides could potentially be modified in a downstream reaction using relatively simple click chemistry reactions for further functionalization[214].

Transglycosylation reactions for the purpose of enzymatic synthesis (as in **Papers II-III**) have been studied previously, and have displayed potential application for production of medicine and food products[215] as well as for the production of biomaterials[15] and surfactants[13,14]. But the required reactants, such as defined oligosaccharides are expensive to acquire commercially and require industrial operations to produce, either via synthesis or purification followed by partial hydrolysis[216].

As noted earlier, hemicellulosic mannan-rich feedstocks are currently utilized for hydrolysis. However, enzymatic conversion of these glycans through reactions such as transglycosylation (such as in **Paper II** and reverse phosphorolysis (such as in **Paper IV**) can be employed to produce other less explored, novel, and potentially high-value compounds further valorizing this underutilized waste stream[79,109].

*Tr*Man5A and *An*Man5B have been utilized for transglycosylation of mannosides onto 2-hydroxyethyl methacrylate (HEMA), yielding HEMA-saccharide conjugation products[197], which were sucessfully copolymerized with N-isopropylacrylamide (NIPAm) to form glyco-decorated polymer chains which displayed thermoresponsive properties when characterized[15].

Complex hemicellulosic mannans provide challenges for effective conversion, requiring building knowledge about the molecular determinants of transglycosylation as well as the substrate specificity of various GHs. Building a library of GHs with and without transglycosylation capacity of different substrate specificities can in the future allow for the possibility to target specific polysaccharide substrates and directing the reaction towards an intended transglycosylation product.

4.3 Applying Enzyme synergy for transglycosylation increases yields

In vivo, the degradation of hemicellulosic β-mannan polysaccharides are facilitated by a conjunction of enzymes[134]. in **Paper I**, the utilization mechanism of MOS/GMOS

by human gut bacterium *R. hominis* via the utilization locus *Rh*MosUL was proposed. The GH36 α-galactosidase *Rh*Gal36A, supported by biochemical data, eliminates α-galactosyl substitutions, enabling the depolymerization of linear mannan-oligosaccharides by the exo-oligomannosidase *Rh*Man113A and the β-mannan oligosaccharide phosphorylase *Rh*MOP130A. Malgas et al. offer a comprehensive review of the structures of xylan and mannan in nature, emphasizing the cooperative actions of xylanolytic[217] and mannanolytic enzymes[198] (Table 4.2).

Table 4.2: Examples of enzyme synergy incubation for the conversion of hemicellulose. Targeted substrate shown in the leftmost column, followed by coincubated enzymes, then intended products, and Degree of Synergy (DS), i.e the relative production of the intended product upon coincubation, lastly the source of the information.

Substrate	Enzymes	Products	DS	Reference
Locust bean gum	*Tr*Man5A, Aga27A	allyl glycosides	4.4	**Paper II**
		allyl-β-mannosides	2.1	
		allyl-α-galactosides	1.3	
	*Tr*Man5A and *An*Man2A		0.9	218
	Aga27A, Man26A	reducing end sugars	1.1	198
	Aga27A, GH5 ManA		1.6	
Guar Gum galactomannan	Aga27A and Man5A		1.1	
Beechwood glucoronxylan Wheat arabinoxylan	Xyna2A, XT6	xylo-oligosaccharides	1.14 1.1	141
Sugarcane hemicellulose	CflXyn11A, TrCel7B	reducing end sugars	6.3	219
Corn stover hemicellulose	CEL, XYL, and *Ta*Man5	reducing end sugars	1.7 1.6	220
Softwood paper pulp	*P. fluorescens* GH26 ManA, Aga27A	reducing end sugars galactose	4.5 3.7	221

To achieve the efficient hydrolysis of a similar complex polymeric substrate, e.g. locust bean gum galactomannan for the purpose of transglycosylation, a similar approach can be applied[23]. In **Paper II**, the GH27 α-galactosidase Aga27A was coincubated with *Tr*Man5A to address the proposed steric hindrance posed by galactosyl substitutions hindering *Tr*Man5A from efficiently cleaving the β-mannan backbone. The results demonstrated a significant reduction in the peak corresponding to galactosylated heptasaccharidedetectide in the coincubation, indicating the effective removal of galactosyl substitutions, allowing for attack by *Tr*Man5A. Furthermore, the coincubation led to a 4.4-fold increase in the yield of allyl glycosides (Figure 10 in **Paper II**. The synergistic effect of the two enzymes, enhancing access to the substrate, resulted in higher yields of transglycosylation products compared to individual incubations.

Leveraging a synergistic combination of glycoside hydrolases can enhance the efficient utilization of renewable natural substrates in biotechnological conversion processes. In addition to employing enzyme synergy strategies, the identification of glycoside hydrolases (GHs) with transglycosylation capacity, particularly those unaffected by

various substitutions and the presence of diverse sugar units in the main chain, is important for working with complex polymeric donor substrates[150]. This not only applies to β-mannans but extends to other renewable heteroglycans like xylans[198]. Exploring the impact of the choice of donor substrate on transglycosylation capacity is also of interest, as a deeper understanding of this aspect can inform reaction designs aimed at maximizing transglycosylation yields.

In this thesis, factors influencing the suitability of retaining glycoside hydrolases for transglycosylation have been discussed. **Papers II** revealed that secondary product hydrolysis adversely impacted transglycosylation product yields. Notably, R171K/E205D experienced significant degradation of AM_3 over prolonged incubation (Figure 4 in **Paper II**). **Paper II** also highlighted the importance of understanding the complex reaction pathways possible for β-mannanases with transglycosylation capacity, such as transglycosylation where saccharides act as glycosyl acceptors in *Tr*Man5A, impacting the apparent initial allyl transglycosylation capacity, as apparent in rS/rH estimation (Table 1 in **Paper I**), where secondary hydrolysis could be detected, proved limited in fully describing the reaction course.

4.4 Alterations in active site affects specificity and product profile

The -1 subsite of glycoside hydrolases is important for catalysis, hosting the glycan moiety that contorts during the transition state of hydrolysis in glycoside hydrolases[222], and hosting the catalytic residues that facilitate cleavage. Substituting conserved residues in the -1 subsite of retaining GHs can generate variants with improved transglycosylation compared to the wild-type[146,155]. In a study be Teze et al., they outline a sequence independent semi-rational approach for enhancing transglycosylation yields through rapid *in silico* analysis of protein sequences, identifying semi-conserved residues, usually around the -1 subsite, for mutation to increase transglycosylation, and was shown to work even for distally related GHs[155].

In several studies of retaining GHs, positive-numbered subsites have been described to influence transglycosylation by interacting with or restricting potential acceptor molecules[117,176,223] (**Paper II** and **III**). In **Papers II** and **III**, alterations in the aglycone subsites of the GH5_7 β-mannanase *Tr*Man5A and GH5_10 *Me*Man5A were examined respectively. *Tr*Man5A variants with more open space in the +2 sugar monomer binding subsite (R171K and R171K/E205D) showed increased transglycosylation capacity using allyl alcohol as an acceptor and mannotetraose as a donor compared to wild-type (Figure 4.3, left). The modifications eliminated competing transglycosylation reactions involving saccharide acceptors due to reduced mannosyl binding

affinity, resulting in enhanced allyl mannoside production. For *Me*Man5A, mutated variants (W240A, W281A, W240A/W281A) showed greatly reduced transglycosylation capacity using saccharides as acceptors whilst retaining its propensity for alcohol transglycosylation(Figure 4.3, right). This shows that modifications in aglycone subsites can affect GH5 mannanases from different subfamilies.

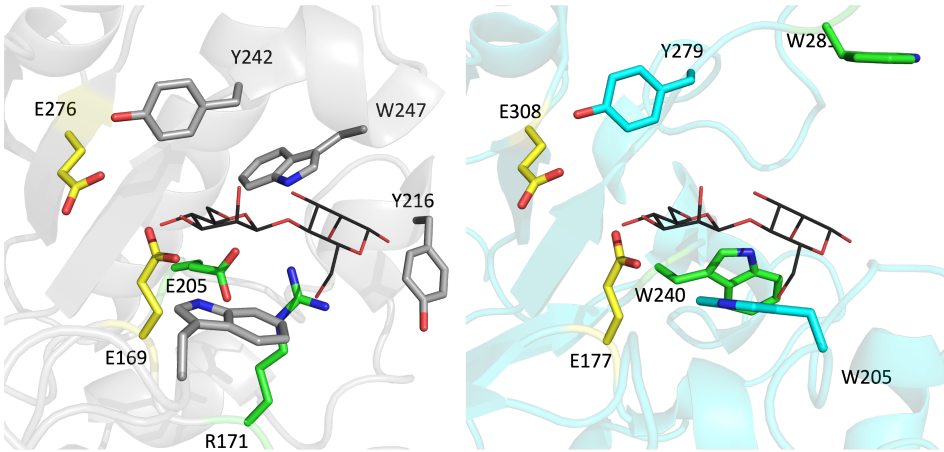

Figure 4.3: Structural superimposition of GH5 β-mannanases *Tr*Man5A (**Paper** II) and *Me*Man5A (**Paper** III), zoomed in on +1 and +2 aglycone binding subsites. **Left:** in **Paper** II the residues R171 and E205 (green) were mutated to lysine and aspartic acid respectively. **Right:** in **Paper** III, two tryptophans, W240 and W281 (green) were mutated into alanines. Both mutations negatively affected transglycosylation with saccharides whilst retaining it with alcohols.

Existing literature extensively establishes the importance of aglycone subsites in modifying transglycosylation behavior in various retaining GHs, demonstrating diverse aglycone modifications and their effects on transglycosylation capacity[117,146,203]. Similarly to *Me*Man5A, acceptor interactions through tryptophan residues in the +1 sugar monomer binding subsite in homologous GH5 β-mannanases AnMan5A and AnMan5C has been studied[13,176].

Another approach to modifying the aglycone subsites involves altering the active site to increase its openness, enabling the accommodation of specific acceptors. For instance, a study on a GH13 glucansucrase demonstrated improved transglycosylation with the bulky flavonoid luteolin through screening of a mutant library[223]. This enhancement was attributed to amino acid substitutions that facilitated better accommodation of luteolin in the aglycone subsites, thereby increasing transglycosylation. A similar mechanism may explain the higher initial transglycosylation observed in the R171K/E205D variant compared to the R171K variant in **Paper II**. Here, the improved transglycosylation of R171K, compared to the wild-type *Tr*Man5A, was attributed to suppressed saccharide transglycosylation likely due to reduced binding in the +2 sub-

site[117]. The additional enhancement in transglycosylation for R171K/E205D was suggested to result from better accommodation of the allyl alcohol in the active site.

Another strategy to boost the transglycosylation capacity of glycoside hydrolases involves reducing the accessibility of the enzyme's catalytic site to water molecules. For example, Jamek and colleagues demonstrated that inserting a loop near the active site of a GH20 β-N-acetylhexosaminidase increased transglycosylation by shielding the catalytic site, thereby reducing hydrolysis[224]. in table 4.3, the transglycosylation capacity of mannan acting GHs studied in this thesis is presented. Rational engineering of aglycone subsites, guided by knowledge of the glycoside hydrolase's structure, proves to be a potent strategy for enhancing transglycosylation efficiency by reducing saccharide transglycosylation reactions.

Table 4.3: Presents a comparison of the transglycosylation capacity between studied mannan acting enzymes. The assessment is based on the observation of peaks with m/z corresponding to transglycosylation products identified through MALDI-ToF MS analysis. A plus sign (+) indicates the presence of observed transglycosylation products, while a minus sign (-) signifies the absence of such products. Glycosyl donors used are specified in column "M_N", with the propensity of saccharide transfer noted along the length of the saccharide listed in parentheses, along with the corresponding mannooligosaccharide substrate.

Enzyme	Acceptor				
	M_N	Methanol	Hexanol	Propargyl alcohol	allyl alcohol
WT *Me*Man5A	+ (M_5), **III**	+	+	+	
W240A *Me*Man5A	+ (M_5), **III**	+	+		
W281A *Me*Man5A	+ (M_5), **III**	+	+		
W240A/W281A *Me*Man5A	- (M_5), **III**	+	+		
*Tr*Man5A	+ (M_4)[117]	+			+
R171K *Tr*Man5A	- (M_4)[117]	+			+
R171K/E205D *Tr*Man5A	- (M_4), **II**	+			+
Aga27A	-[192]	+[192]		-[192]	+[192]

4.4.1 Substrate binding behaviour influences product profile

The architecture and topology of the active site plays a crucial role in determining the substrate interactions and product outcomes of GHs. The binding at the initial stage significantly impacts both hydrolysis and transglycosylation processes, influencing whether the GH is endo- or exo-acting, substrate specificity, and product profiles.

For exo acting enzymes, the active site is often closed off, often referred to as pocket shaped such as for the GH27 α-galactosidase Aga27A (**Paper II**, Figure 4.4, bottom left), superimposed with meilbiose from its homolog, a GH27 α-galactosidase MEL1 from *Saccharomyces cerevisiae* (PDB: 3LRL,[225]). This limits Aga27A to cleaving of single α-1,6-galactosyl bonds such as those found in galacto(gluco)mannan, and generating single galactosyls as products. In **Paper IV**, the mannan oligosaccharide phsophorylase *Rh*MOP130A was structurally determined via x-ray crystallography.

*Rh*MOP130A, features an interesting active site (Figure 4.4, bottom right, including mannose glucose and phosphate from superimposed homolog structure, a *Bacteroides fragilis* GH130 mannosyl-D-glucose phosphorylase, pdb: 3WAS[188]). *Rh*MOP130A is capable of acting on MOs of various chain length (**Paper I**), potentially due to its rather unique tryptophan residue forming a putative +2 subsite for hexosyl binding (Figure 6 in **Paper IV**).

The structure of the active sites in endo-β-mannanases, exemplified by *Tr*Man5A (Figure 4.4, **Paper II**), affects their ability to handle different polymeric substrates[89]. The more open active site clefts of these enzymes allow accommodation of galactosyl-substituted mannose-based polysaccharides[106,226]. Furthermore, the interaction with galactosyl substituents proves vital for catalytic efficiency towards galactomannans. The glycone and aglycone subsites influence the preferred binding mode of oligosaccharide substrates and the length of the glycosyl-enzyme intermediate, subsequently impacting hydrolysis or transglycosylation product formation. In **Paper III**, modifications in the active site effected hexosyl binding affinity in the aglycone subsites of *Me*Man5A, which in turn resulted in a shift in binding mode and altered the resulting product profile. Understanding the limitations imposed by the enzyme's active site is essential for achieving specific characteristics in the end products, such as saccharide chain length.

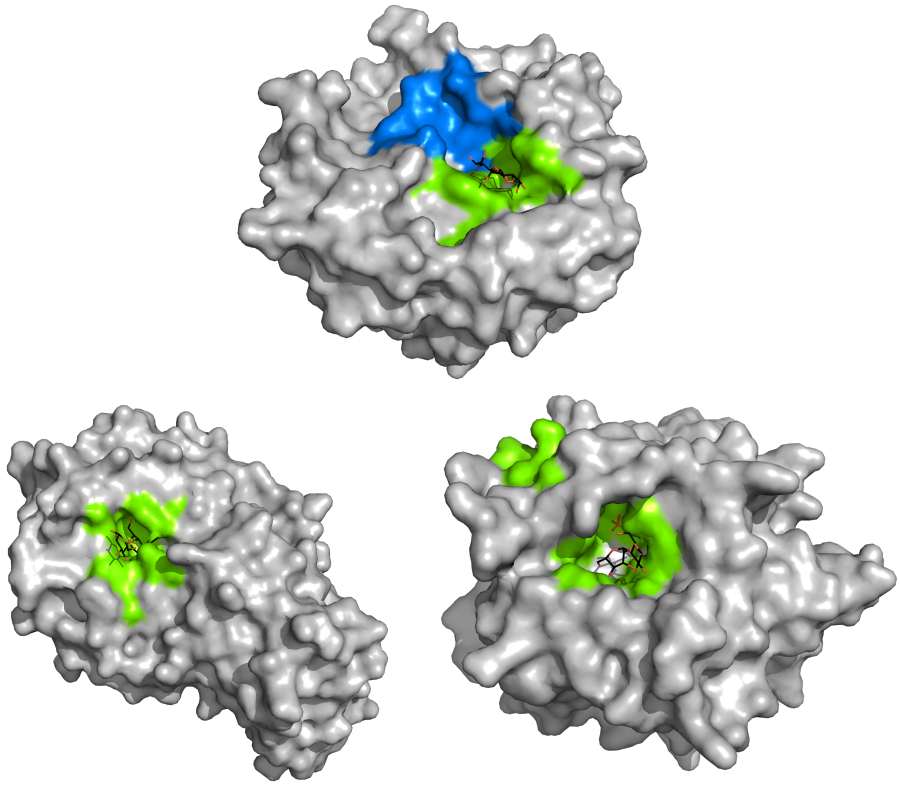

Figure 4.4: Surface view demonstrating the active site topology of different enzyme studied in this thesis. **top:** crystal structure of GH5 endo-mannanase *Tr*Man5A (pdb:1QNR) studied in **Paper II**, with its cleft shaped active site (- subsites in blue, + subsites in green), with mannobiose bound across +1 $\rightarrow$ +2 from the structure. **Bottom left:** alphafold predicted structure of GH27 α-galactosidase Aga27A (**Paper II**) with its pocket shaped active site, with melibiose ligand from structural superimposition with GH27 α-galactosidase MEL1 from Saccharomyces cerevisiae (pdb: 3LRL). **Bottom right:** Crystal structure of single subunit of *Rh*MOP130A (**Paper IV**, not yet deposited to pdb), with its putative active site, with mannoglucose and phosphate from structural superimposition with homolog mannosyl-D-glucose phosphorylase (*Bf*MGP) from *Bacteroides fragilis* (pdb: 3WAS).

4.4.2 Effect on aglycone subsites for substrate and acceptor interactions

Several studies have demonstrated the role of positive-numbered (aglycone) subsites in transglycosylation for retaining GHs[117,207,223]. Aglycone subsites may interact with or restrict molecules acting as acceptors, such as water for hydrolysis or saccharides/alcohols for transglycosylation. **Paper II** focused on altering aglycone subsites in GH5 β-mannanase *Tr*Man5A, revealing increased transglycosylation capacity in variants (R171K and R171K/E205D) with allyl alcohol as an acceptor and M_4 as a donor substrate. These modifications eliminated competing transglycosylation reactions involving M_4 as an acceptor. This observation has been made in the R171K variant of *Tr*Man5A before[117], where the reduced binding affinity in +2 was shown to displace

the preferred binding mode of *Tr*Man5A and M_4 from -2 to +2 in wt *Tr*Man5A to -3 to +1, as well as eliminating transglycosylation using sugar acceptors[117].

Mutations in the aglycone subsites of GH5_10 β-mannanase *Me*Man5A performed in **Paper III** showed interesting differences in transglycosylation behaviour. Product analysis revealed that *Me*Man5A can utilize alcohols as acceptors, expanding its potential for synthetic applications. WT *Me*Man5A displayed transglycosylation products when incubated with M_5, as well as when using methanol, hexanol, and propargyl alcohol as acceptors with M_5 as donor substrate. Similar reactions were conducted with W240A and W281A mutants, interestingly, there were no apparent differences in transglycosylation using alcohol acceptors among wild type *Me*Man5A, W240A, W281A, or W240A/W281A, whilst limiting saccharide transglycosylation for the single mutants and eliminating it entirely for the double mutant. This demonstrates that substitutions in the +2 subsite of *Me*Man5A did not significantly impact the enzyme's ability to utilize alcohols as acceptors whilst reducing hexosyl interactions therein.

A similar observation has been made in two homologous GH5 β-mannanases from *Aspergillus nidulans*, where the disparity in transglycosylation with saccharides between the two was attributed to the presence or absence of a tryptophan in the +1 subsite of each respective enzyme[176]. Variants where the tryptophan was swapped between the two, also resulted in swapped transglycosylation propensity compared to their respective wild types[176] (Table 4.1).

Taken together, removal of hydrophobic residues like tryptophans in aglycone binding subsites have shown to reduce sugar binding affinity, directing the reaction towards using non sugar glycosyl acceptors instead, providing a helpful tool for modifying the transglycosylation behaviour of β-mannanases.

4.4.3 Improving transglycosylation yields

Previous studies have highlighted the significance of saccharide interactions in positively numbered subsites in saccharide transglycosylation by GHs[145], including GH5 enzymes[117,176]. For instance, a single arginine substitution in the +2 subsite of *Tr*Man5A significantly reduced transglycosylation activity with saccharide acceptors[117]. Further mutations in the +2 subsite of *Tr*Man5A were carried out in **Paper II**, which showed to increase initial transglycosylation yields when incubation the enzyme with M_4 (Table 4.1), potentially due to a combination of reduced steric hindrance in the binding sites for alcohol acceptors and reduced affinity for sugar acceptors. A similar observation was made for *Me*Man5A (**Paper III**), where removing to sugar interacting tryptophans in the +2 subsites were shown to displace the preferred binding mode of M_5, and eliminating transglycosylation with sugar acceptors without altering transg-

lycosylation propensity for various alcohol acceptors (Table 4.3). Performing rational mutagenesis of residues in aglycone subsites can thus alter binding affinity, providing altered product profiles, shifting acceptor preferences and allowing for more directed transglycosylation reactions, providing biochemists with tools for determining the product outcomes for glycan conversion according to the desired application.

Furthermore, *Tr*Man5A variants was incubated with locust bean gum galactomannan and allyl alcohol, showed wild type *Tr*Man5A to be most effective, likely due to favorable saccharide interaction in the +2 subsite of the wild type compared to the double mutant. However in these experiments, limited transglycosylation yields when compared to available mannosyl units in the galactomannan substrate were observed. Thus, enzymatic reactions were conducted using the enzymes *Tr*Man5A and Aga27A individually, as well as in combination, with the same conditions applied as in previous experiments involving only *Tr*Man5A were performed (Figure 9-10 in **Paper II**).

Importantly, Aga27A exhibited a considerable capacity for transglycosylation, producing allyl-galactosides with nearly 16% of the available galactosyl substituents, marking the first report of Aga27A's transglycosylation ability. The transglycosylation reactions catalyzed by both enzymes resulted in a substantial increase in substrate utilization, with almost 10% of saccharide units in the LBG substrate incorporated into transglycosylation products with allyl alcohol, marking a 4.4-fold increase in total produced allyl glycosides (table 4.2). This synergistic relationship between *Tr*Man5A and Aga27A in enhancing transglycosylation yields represents a novel aspect in enzymatic synthesis, suggesting increased feasibility for the production of novel biochemicals using natural hemicellulosic donors.

4.5 Applications of functionalized glycosides, e.g. novel biomaterial, surfactants provide downstream flexibility

Functionalization of glycosides presents diverse applications, ranging from surfactants[14] to biopolymers and novel biomaterials[15], and enzymatic production of these glycosides presents an environmentally friendly means of achieving these promising reactants. There has been a notable surge in interest surrounding the utilization of glycans in the production of various chemicals and materials, marking a shift from fossil-derived feedstocks to more sustainable, renewable alternatives[227].

Paper II highlight the successful enzymatic synthesis of allyl glycosides. A significant hurdle in scaled-up glycoconjugate production, namely the high cost of activated substrates or defined oligosaccharides often used in their enzymatic synthesis[228], was

effectively addressed by demonstrating the use of the more cost-effective polymeric donor substrate locust bean gum galactomannan. Furthermore, the paper showcased increased yields through enzymatic synergy, yielding both allyl-β-mannosides and allyl-α-galactosides. These findings underscore the heightened feasibility of employing enzymatic synthesis in novel glycoside production, paving the way for the development of biobased chemical products with enhanced sustainability and potential cost-effectiveness.

These allyl-functionalized glycoconjugates can be utilized in subsequent chemical reactions, where the allyl group acts as a handle in processes such as "click-chemistry" thiol-ene reactions[214,229,230]. Such chemical modifications have proven successful for allylated glycosides, forming chemical bonds with thiolated compounds[230,231,232]. The thiol-ene reactions, known for their versatility and specificity, have found applications in various polymer and materials synthesis, notably under mild aqueous conditions without the need for toxic catalysts, and ensuring high yields[233]. Thus enyzmatically produced allyl glycosides have the potential to be a flexible reactant for novel applications in the future.

*Me*Man5A, highlighted in **Paper III**, demonstrated the synthesis of alkane-mannosides like hexyl-mannotriose, presenting potential applications as biosurfactants (Figure 3 in **Paper III**). This direct addition of a hydrophobic tail could bypass the additional step of thiol-ene click chemistry, could streamline the production of biobased surfactants.

The presented findings in **Paper IV** demonstrate the feasibility of employing allyl-α-mannose as an acceptor in reverse phosphorolysis reactions, yielding elongated allyl mannosides. This offers a potential avenue for enzymatically synthesizing allyl glycosides with an elongated glycosyl moiety, a capability previously limited when utilizing transglycosylation for addition of allyl alcohol to mannan oligosaccharides as seen in **Paper II** or other alcohol glycoside acceptors[197].

Given a readily available supply of phosphorylated sugars to act as glycosyl donors, the reverse phosphorolysis reaction catalyzed by GH130 mannan oligosaccharide phosphorylases could be developed to a straightforward second-step reaction within a potential process stream. A future possibility to circumvent the need for phosphorylated mannosides could potentially be the *in situ* generation through initial phosphorolysis reaction, allowing elongation of a desired glycoside instead, perhaps via coincubation of enzymes that are only capable of the reaction in one direction each, or with different specificities but more research on this subject is required.

Chapter 5

Conclusions and future perspectives

This study delves into the function of enzymes acting on β-mannans and the interplay with their corresponding substrates, emphasizing a focus on transglycosylation with the intended target of underutilized β mannan hemicellulose as substrate for enzymatic conversion to generate interesting novel glycosides.

This includes the study of glycoside hydrolases (GH) (GH5 β-mannanases and GH36 α-galactosidase) (**Papers I**-**III**) and a GH130 mannoside phosphorylase (**Papers I** and **IV**) acting on and catalyzing the conversion of hemicellulosic β-mannan (food grade galactomannan, softwood galactoglucomannan) and manno-oligosaccharides (MOS) derived from such polymers. Of particular interest is the transferase activity of retaining GHs (transglycosylation), the molecular and structural governance of such reactions and their potential applicability in synthesis of novel glycosides (such as MOS and allyl glycosides) from renewable glycans (**Papers II** and **III**). The focus of the project has thus on the examination of enzymatic function, transglycosylation capacity, and assessment of reaction products in several GH families.

The research presented in **Paper I** focuses on the metabolism of β-mannans and their oligosaccharides (MOS/GMOS) by the human gut bacteria *Roseburia hominis*. the investigation of a putative MOs utilization locus in *R. hominis* sheds light on the upregulation of specific genes and a few key enzymes were selected for characterization (the phosphorylase *Rh*MOP130A, and the hydrolases *Rh*Man113A and *Rh*Gal36A). *Rh*MOP130A and *Rh*Man113A only showed activity towards non-galactosylated MOs. Notably, *Rh*MOP130A exhibited synthesis activity, generating products with higher polymerization levels than the initial substrate, futher studied in **Paper IV**. *Rh*Gal36A effectively removed α-galactose substituents from GMO substrates (GM_3 and G_2M_5), suggesting that it acts initially before secondary cleavage by *Rh*Man113A and *Rh*MOP-

130A. The proposed substrate degradation pathway was thus suggested as a sequential action of *Rh*Gal36A, followed *Rh*Man113A, and *Rh*MOP130A, highlighting the importance of enzymes with complementary specificities in the conversion of relatively simple substrates like GMOs/MOs.

Utilizing enzyme synergy allows for conversion of complex substrates, such as GH36 α-galactosidase *Rh*Gal36A removing galactosyl substituents for *Rh*Man113A and *Rh*MOP-130A to cleave the mannan oligosaccharide backbone in **Paper I**. To allow utilization of the more abundant and complex renewable hemicellulose galactoglucomannan as a donor substrate, further exploration of both protein engineering and enzyme synergy can be explored to enhance conversion yields. These strategies align with ongoing investigations into galactoglucomannanM refining as part of a biorefinery concept[23,234].

The transglycosylation capacity of the GH5_7 β-mannanase *Tr*Man5A and its variants were explored in**Paper II**. Following initial assessment, both wild-type *Tr*Man5A and its R171K/E205D variant were selected for further investigation. The double mutant exhibited higher transglycosylation capacity than the wild-type with M_4 as donor, but it also showed an increase in secondary hydrolysis. Conversely, when utilizing the larger and bulkier galactomannan as the donor substrate, the wild-type *Tr*Man5A outperformed R171K/E205D in transglycosylation. The discrepancy was hypothesized to be linked to the R171K substitution eliminating saccharide transglycosylation, while the E205D substitution potentially facilitated allyl alcohol accommodation. Addition of GH27 α-galactosidase Aga27A greatly increase the yield of allyl glycosides, showing synergistic behaviour. **Paper II** showed the significance of aglycone subsites in transglycosylation by retaining GHs and the novel application of enzyme synergy for transglycosylation using allyl alcohol as an acceptor.

These synthesized allyl glycosides hold promise for integration into innovative biomaterials using traditional radical polymerization and click-chemistry methods, as discussed briefly in **Paper II**[214,229]. In the future, these active compounds can be further modified for a variety of possible applications, such as biomaterials or surfactants. Previously, transglycosylation products using *Tr*Man5A, locust bean gum galactomannan as glycosyl donor substrate and 2-hydroxyethyl methacrylate (HEMA) as a glycosyl acceptor[197] have been successfully co-polymerized with N-isopropylacrylamide to form a novel thermoresponsive polymer[15], showing further potential for application of alcohol transglycosylation products.

Paper III, concerned *Me*Man5A, a GH5_10 β-mannanase from blue mussel, and its two tryptophans W240 and W281 positioned in the +2 aglycone binding subsite of *Me*Man5A. These residues were mutated out and replaced with alanines and studied. Binding mode analysis revealed altered preferences in W240A/W281A, suggesting diminished mannosyl interactions in the +2 subsite. NMR and MD analysis inves-

tigated the flexibility of W281 and showed that it is positioned more statically for binding in presence of a ligand. Transglycosylation with sugar acceptors was reduced in the single mutants and eliminated in the double mutant, leaving translgycosylation with alcohols unaffected. This study investigates substrate interactions in *Me*Man5A, providing insight into the function of residues in aglycone binding subsites of retaining GHs. The findings provide strategy for targeted mutations in GH5 mannanases to improve their transglycosylation capabilities, particularly when utilizing alcohols as acceptors for novel glycoside production.

The mutational work performed on *Tr*Man5A has yielded and increased transglycosylation yield with manno-oligosaccharides (**Paper II**), but these mutations were limited to the aglycone binding subsites assuming that the altered binding acceptor binding affinity would affect transglycosylation behaviour. But, in a study by Teze et al.[155], they designed a semi rational sequence based approach for suggesting mutations that would increase transglycosylation. One of the families this method was applied to as proof of concept was GH2, a fellow clan GH-A member using a β-mannosidase from *Cellulomonas fimi*, *Cf* Man2A as a model enzyme. Several glycone binding subsite residues were suggested for mutation that lead to significantly increased saccharide transglycosylation (e.g., W362H, R360K, W612H, and W6223H). As many of these are structurally conserved in *Tr*Man5A, these would be interesting targets for future mutation (corresponding mutations in *Tr*Man5A would be W56H, R54K, W206H, and Y323H, respectively) and could perhaps further direct reactions catalysed by *Tr*Man5A towards translgycosylation rather than hydrolysis.

Mutations in aglycone binding subsites performed herein have all shown to result in decreased transglycosylation using sugar acceptors, but using a rational mutational approach, enhancement of acceptor binding could be possible albeit challenging in the future. Providing the aglycone binding sites of GHs with higher affinity towards a certain saccharide could lead to higher saccharide transfer yields, making it an interesting approach for synthesis of specifically defined saccharides.

In **Paper IV**, the investigation centers on the β-mannan oligosaccharide phosphorylase GH130_2 enzyme *Rh*MOP130A, a glycoside phosphorylase found in the gut bacterium *Roseburia hominis*, first expressed and characterized in **Paper I**. The study explores the molecular basis of *Rh*MOP130A, and explores its potential for synthesizing novel glycosides through reverse phosphorolysis as well as providing a crystal structure of the enzyme.

In summary, the work contributes to understanding transglycosylation by retaining GHs, offering insights into substrate choices, secondary hydrolysis, and aglycone subsite engineering. The potential applications extend beyond β-mannans, showcasing principles applicable to diverse GHs. In addition, the thesis presents an alternative

approach for enzymatic synthesis of β-mannosides via revere phosphorolysis as well as providing structural insights in the acitve site of the phosphorylase. The thesis examplifies the importance of detailed knowledge of enzyme structures and future complex structures would aid in revealing the enyzms mechanism and provide basis for design of potentially improved variants for synthesis. The collective findings pave the way for further enzyme engineering, aiming to advance transglycosylation and reverse phosphorolysis from proof-of-concept to large-scale applications in hemicellulose valorization.

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