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BIOCHEMICAL AND MOLECULAR ANALYSES OF PLANT CELL WALL DEGRADING
ENZYMES FROM THE RUMEN BACTERIUM *RUMINOCOCCUS ALBUS* 8

BY

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THESIS

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Abstract

Ruminants rely on microorganisms inhabiting the rumen to digest plant matter for subsequent fermentation to provide the host with the majority of its energy demands. *Ruminococcus albus* 8 is a rumen bacterium that efficiently degrades and ferments plant cell wall polysaccharides, such as cellulose and hemicelluloses. Xylan, a hemicellulosic polymer of β -1,4 linked xylose monomers may be appended with arabinose, glucuronic acid, and acetyl residues. Several classes of enzymes attack the polysaccharide to release the constitutive monosaccharides. Lichenin is a linear polymer of glucose linked together by β -1,3 and β -1,4 glycosidic bonds. Lichenin can be found in lichens and common feeds such as barley, sorghum, and wheat.

A bioinformatic analysis of the genome of *R. albus* 8 revealed five genes encoding glycoside hydrolases predicted to hydrolyze the xylose backbone and several genes encoding putative accessory enzymes that are expected to remove side chains from xylans. The genes were cloned, heterologously expressed in *E. coli*, and biochemically characterized to determine how the enzymes function synergistically to release soluble sugars that can be fermented by *R. albus* 8. In an experiment designed to identify cellulase encoding genes in the genome of *R. albus* 8, four genes encoding lichenin degrading enzymes were identified. An enzyme mixture was created, and the components were shown to act synergistically to release, from the polysaccharide, cellobiose and cellotriose which are preferentially utilized by *R. albus*.

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TABLE OF CONTENTS

LIST OF FIGURES.....	vi
LIST OF TABLES.....	viii
CHAPTER 1. LITERATURE REVIEW.....	1
INTRODUCTION.....	1
PLANT CELL WALL POLYSACCHARIDES.....	4
MAJOR FIBROLYTIC MICROORGANISMS.....	6
POLYSACCHARIDE DEGRADATION.....	10
<i>Ruminococcus albus</i> GROWTH AND METABOLISM.....	18
CHARACTERIZED ENZYMES FROM <i>Ruminococcus albus</i>	21
OBJECTIVES.....	25
CHAPTER 2. XYLAN DEGRADING ENZYMES FROM <i>Ruminococcus albus</i> 8.....	26
ABSTRACT.....	26
INTRODUCTION.....	27
MATERIALS AND METHODS.....	29
RESULTS.....	36
DISCUSSION.....	62

CHAPTER 3. LICHENIN DEGRADING ENZYMES FROM <i>Ruminococcus albus</i> 8	66
ABSTRACT	66
INTRODUCTION	67
EXPERIMENTAL PROCEDURES.....	69
RESULTS	76
DISCUSSION	94
SUMMARY OF RESEARCH	98
FUTURE RESEARCH.....	102
Appendix A. Annotation of the glycoside hydrolases within the draft genome sequence of <i>Ruminococcus albus</i> 8	104
REFERENCES.....	110

LIST OF FIGURES

		Page
CHAPTER 1		
Fig. 1.1	Diagram of the reaction mechanisms of glycoside hydrolases.....	11
Fig. 1.2	Diagram of subsite recognition of a cellotetraose molecule bound to a cellobiohydrolase.....	13
Fig. 1.3	Diagram of the reaction mechanism of Asp-His-Ser acetyl esterases.....	17
CHAPTER 2		
Fig. 2.1	Hemicellulose degrading enzymes of <i>R. albus</i> 8.....	38
Fig. 2.2	Hydrolysis of xylan substrates by five putative endoxylanases from <i>R. albus</i> 8.....	42
Fig. 2.3	Carbohydrate esterase activities of the five endoxylanases from <i>R. albus</i> 8.....	44
Fig. 2.4	Qualitative binding studies of the five endoxylanases from <i>R. albus</i> 8.....	45
Fig. 2.5	Functional analyses of the gene products of the <i>R. albus</i> 8 <i>xyl3A</i> and <i>ara51A</i>	47
Fig. 2.6	Functional analyses of the <i>R. albus</i> 8 <i>Agu67A</i>	48
Fig. 2.7	Hydrolysis of increasing amounts of xylan substrates by <i>R. albus</i> 8 endoxylanases in the presence of two accessory enzymes (β -xylosidase and α -L-arabinofuranosidase)	52
Fig. 2.8	Optimization of xylan (soluble WAX) hydrolysis with the five endoxylanases and their accessory enzymes from <i>R. albus</i> 8.....	53
Fig. 2.9	HPAEC analysis of xylan (soluble WAX) hydrolysis with the five endoxylanases and their accessory enzymes from <i>R. albus</i> 8.....	54
Fig. 2.10	Optimization of xylan (oat spelt xylan) hydrolysis with the five endoxylanases and their accessory enzymes from <i>R. albus</i> 8.....	55
Fig. 2.11	Optimization of xylan (birchwood xylan) hydrolysis with the five endoxylanases and their accessory enzymes from <i>R. albus</i> 8.....	56

Fig. 2.12	Enzymatic treatments of undegraded products with enzyme mixtures.....	58
Fig. 2.13	Enzymatic treatments of undegraded products from soluble wheat arabinoxylan.....	60
Fig. 2.14	Enzymatic treatments of undegraded products from birchwood xylan.....	61
CHAPTER 3		
Fig. 3.1	Cloning of 4 glycoside hydrolases from <i>Ruminococcus albus</i> 8.....	78
Fig. 3.2	Characterization of the substrate specificities of 4 glycoside hydrolases from <i>Ruminococcus albus</i> 8.....	80
Fig. 3.3	Comparison of products of hydrolysis from polysaccharides incubated with Ra0453, Ra0505 or Ra2830.....	82
Fig. 3.4	Activities of GH5 enzymes with cello-oligosaccharides and cellulosic substrates.....	84
Fig. 3.5	Products of hydrolysis of mixed linkage β -1,3-1,4-gluco-oligosaccharides by Ra0453, Ra2830, Ra0505 and Ra1595.....	86
Fig. 3.6	Degradation pattern of mixed linkage β -1,3-1,4-gluco-oligosaccharides by Ra0453, Ra2830, Ra0505 and Ra1595.....	87
Fig. 3.7	Hydrolysis of lichenin by Ra0453, Ra2830 and Ra0505 with time.....	90
Fig. 3.8	Determination of synergistic activities of Ra0453, Ra2830, Ra0505, and Ra1595 during hydrolysis of lichenin.....	91
Fig. 3.9	Total degradation of lichenin by mixtures of Ra0453, Ra2830, Ra0505, and Ra1595.....	93

LIST OF TABLES

	Page
CHAPTER 1	
Table 1.1	Previously cloned and sequenced enzymes from <i>R. albus</i>22
CHAPTER 2	
Table 2.1	Primers used for cloning genes predicted to degrade xylan 40
Table 2.2	Synergistic effects of multiple endoxylanases with Xyl3A and Ara51A..... 50
CHAPTER 3	
Table 3.1	Primers used for cloning genes encoding lichenin degrading enzymes... 70
Appendix A	
Table A.1	Glycoside hydrolases identified within the draft genome sequence of <i>Ruminococcus albus</i> 8..... 106

CHAPTER 1. LITERATURE REVIEW

INTRODUCTION

Coevolution of higher organisms and microorganisms into mutually beneficial symbiotic relationships has been studied for many years. Many mammals have bacterial communities incorporated into the digestive tract to provide a mutually beneficial relationship. Hindgut fermenters, including swine and horses, allow bacteria to ferment the components of the feed that the host is unable to digest or absorb. In these animals, the cecum or colon is the main site of microbial fermentation that leads to end products that can be further metabolized by the host [1]. In contrast, ruminants, including cows, goats, giraffes, and reindeer, house the majority of their microbial community in the fore-stomach or rumen. A large consortium of microbes from all domains of life inhabits the rumen, including eubacteria, methanogenic archaea, protists, fungi and viruses [1]. The ruminant animal relies on the organisms inside the rumen to convert the recalcitrant plant matter, composed of various polysaccharides that would normally be unavailable to the host, into a bio-available source of energy and nutrients. The symbiotic microbes ferment the plant matter before the host can directly obtain nutrients from the ingested material. The host, in turn, utilizes the products of fermentation as well as the microbial biomass that accumulates to satisfy their nutritional requirements [1]. The symbiosis of plant degrading microbes and their herbivorous host has provided scientists with a relatively constant system in which to explore native gut populations and their role in degradation of plant matter.

Microorganisms metabolize the recalcitrant plant matter into energy and nutrients that they subsequently transfer to the host in the form of fermentation products. Short-chain fatty acids, also known as volatile fatty acids (VFAs), are the main products of microbial fermentation in the rumen utilized by the host. The epithelial lining of the rumen absorbs the VFAs and then the host can metabolize of these compounds for energy. The fermentation products satisfy as much as 70% or more of the host's energy requirements. The main short chain fatty acids produced from fermentation include acetate, propionate and butyrate, and they are normally found in a ratio of 65:20:15, respectively. The ratio can vary based on the type of diet consumed. As the VFAs are absorbed, their fates differ based on their biochemical properties. The peripheral tissues use acetate for energy, as it is converted to acetyl-CoA and subsequently enters the TCA cycle. Acetate is also efficiently used by the adipose tissue for lipogenesis from acetyl-CoA [2]. Propionate, a three carbon fatty acid, is transported to the liver along with valerate (a five carbon fatty acid) to enter gluconeogenesis, providing up to 90% of the host's blood glucose demands [3]. Ruminants strongly rely on gluconeogenesis for blood glucose because the microbes in the rumen rapidly ferment any soluble sugars before they reach the small intestine. Butyrate, a four carbon fatty acid, is utilized for energy by the rumen epithelial cells during absorption and very little butyrate is released into the blood [2]. Butyrate is also produced via fermentation in the human colon, where it is used for energy by colonocytes or transported to the liver for ATP production [4].

Research to understand the mechanisms of bacterial fermentation in the rumen is not only of scientific interest, but also of industrial importance. The enzymes found in ruminal microbes can be used as supplements in animal feed to improve feed efficiency

and livestock production [5]. Some rumen bacteria, including *Ruminococcus albus*, can produce ethanol, by the mixed acid fermentation pathway, which holds significance to the biofuel industry for the production of bioethanol from lignocellulosic biomass.

Techniques to cultivate and isolate the microbes responsible for the fermentation of plant matter in the rumen were developed in the late 1940's by Hungate [1]. Predominant ruminal bacteria were isolated on anaerobic media that contained rumen fluid to simulate their natural habitat. The isolated bacteria were characterized based on their ability to utilize polysaccharides as well as other factors, including cell morphology, fermentation products and nutrient requirements. The primary cellulose degrading microbes include *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. Bacteria involved in the fermentation of hemicellulosic polysaccharides include *Prevotella bryantii*, *Ruminobacter amylophilus*, *Butyrivibrio fibrisolvens*, as well as *R. albus*, and *R. flavefaciens*. These bacteria are involved in the conversion of the recalcitrant polysaccharides into fermentation products that the host animal is able to metabolize.

There are also highly specialized bacteria that seem to be extremely limited in terms of energy sources, utilizing the fermentation products from other bacteria. For example, *Oxalobacter formigenes* decarboxylates oxalate into formate and the coupled transport creates a proton gradient which is its only source of energy conservation [6]. *Megasphaera elsdenii* and some *Selenomonas ruminantium* strains are also highly specialized bacteria that convert lactate into other volatile fatty acids [7]. Scientific research on the metabolism of these bacteria has been ongoing since their isolation,

and of particular interest to our group, are the fibrolytic bacteria that degrade and ferment the plant cell wall polysaccharides, cellulose and hemicellulose.

PLANT CELL WALL POLYSACCHARIDES

The plant cell wall is complex in both structure and composition. There are three main structural components: cellulose, hemicellulose and lignin. Research on ryegrass shows that the plant cell wall is composed of decreasing concentrations of the following polysaccharides: cellulose, glucurono-arabinoxylan, xyloglucan, rhamnogalacturonan (pectin), mixed linkage glucans, and galactans [8]. Lignin, the major structural non-polysaccharide polymer, is composed of monomers of phenolic coumaric acid derivatives: *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Lignin is found in all vascular plants and provides strength and rigidity to terminally differentiated cells [9]. This randomly bonded structure is extremely difficult to degrade (requiring radical intermediates) and surrounds the structural polysaccharides, preventing access to the polysaccharides and increasing the recalcitrance of plant cell walls to degradation. The lignin present in ruminant feeds decreases the nutritional quality of the feed [9].

Cellulose. The main structural component of the cell wall is cellulose, the largest store of glucose. As a structural polysaccharide found in plant cell walls, cellulose is an unavailable energy source for most animals. The recalcitrant nature of cellulose is attributed to the high degree of crystallinity, formed by an extensive hydrogen bonding network, as well as the stability of the β -1,4 glycosidic linkage holding together the glucose monomers within the repeating cellobiose units. The recalcitrance of cellulose is also due to the stability of the glycosidic bonds, shown to have a half-life of approximately 22 million years [10]. During synthesis, the hydrogen-bonding network

can be disrupted, forming regions that are amorphous or partially soluble. These amorphous regions are more accessible to enzymatic attack. A study of *Ruminococcus albus* 7 grown on insoluble cellulose has shown that the limiting factor for this bacterium's growth is the depolymerization of cellulose [11]. Cellulose is generally complexed with other cross-linking polysaccharides, such as β -glucans and xyloglucans, to hold together multiple cellulose microfibrils [12].

Hemicellulose. Hemicellulose refers to polymers of sugars, primarily pentose sugars, found surrounding cellulose microfibrils, including xylan, mannan, and arabinan. Substituted xylan, the dominant component of hemicellulose, is composed of a backbone of xylose monomers held together by a β -1,4 glycosidic bond. The xylose residues may be appended with other substituents including arabinose, glucuronic acid, acetate, and feruloyl esters. Different sources of xylan are composed of different proportions of substituents. Wheat arabinoxylan is composed of 33% arabinose and 66% xylose, while birchwood xylan is composed of 1% arabinose, 89% xylose and 8% glucuronic acids [13, 14]. The ferulic acid branches have been implicated in crosslinking to lignin. Other hemicellulosic polysaccharides can be composed of multiple sugars in the backbone, such as glucomannan (glucose and mannose) and xyloglucan (xylose and glucose) [8].

Mixed-linkage glucans. Glucans, other than cellulose, are present in a variety of cell walls. Mixed linkage glucans, composed of glucose linked together by β -1,3 and β -1,4 glycosidic bonds, are found in the Poales order of grasses, which include common ruminant feeds such as barley, sorghum and wheat. The β -glucans are commonly found as crosslinking glycans, which hold together cellulose microfibrils [12]. Another source

of β -glucans are lichens, a mutualistic symbiosis between algae and fungi [15]. Lichenin, a reserve polysaccharide found in lichens, is a mixed linkage glucan containing 30% β -1,3 linkages and 70% β -1,4 linkages [16]. Reindeer and caribou consume lichens during the winter season when forage is low. Approximately 1.3 to 4.9 kilograms of lichens are consumed during this season [17, 18].

MAJOR FIBROLYTIC MICROORGANISMS

The bacteria responsible for the degradation of these polysaccharides are of particular interest to both nutritionists and microbiologists. Individual bacterial contribution to the rumen microbiome is a major research endeavor. To determine the bacterial population, researchers use a wide range of techniques including cultivation methods, PCR-based 16S RNA quantification, quantitative fluorescence in situ hybridization (qFISH), and meta-genomic analysis.

As DNA sequencing technologies have progressed, our knowledge of microbial populations has improved. Although we have many isolated bacteria from several genera and species, the phylogenetic diversity in the rumen microbiome is not well represented in the bacterial isolates, despite having been studied for over 60 years [19, 20]. Quantification of predominant cellulolytic bacteria, including *Fibrobacter succinogenes*, *Ruminococcus albus* and *R. flavefaciens*, shows that *Ruminococcus* sp. account for about 8% of the 16S RNA, while the individual species mentioned account for less than 1%. *F. succinogenes* also account for approximately 1% [21]. Meta-genomic and 16S RNA encoding sequence analysis of the rumen phyla revealed that Firmicutes were the predominant bacteria in the fiber adherent fraction as well as the liquid fraction, followed by Bacteroidetes, while Fibrobacteres was not a dominant

phylum [22]. *Prevotella ruminicola* and *P. bryantii* are major hemicellulolytic species isolated from the rumen that fall into the Bacteroidetes phylum.

Fibrobacter succinogenes. *Fibrobacter succinogenes* is a gram-negative staining rod-shaped bacterium originally isolated as *Bacteroides succinogenes* in 1947 by Hungate [23]. In terms of cellulose degradation in the rumen, *F. succinogenes* is considered one of the dominant cellulolytic microbes [24]. Although this microbe is able to degrade hemicellulosic polysaccharides, it is referred to as a specialist because it utilizes only cellulose and its hydrolytic products as its energy source. Degradation of hemicellulose is hypothesized to serve as a method to gain access to the cellulose microfibrils buried in the hemicellulose. It has been shown that adhesion to cellulose is important for degradation and microbial growth [25, 26]. *F. succinogenes* has been proposed to degrade cellulose by a mechanism in which glucan chains are channeled into the periplasm where hydrolytic cleavage occurs [27]. In 2011, the complete genome sequence of *F. succinogenes* S85 became public. Genes predicted to be involved in polysaccharide degradation were found through a bioinformatic search of the genome and several have been previously cloned and characterized [28]. Characterized glycoside hydrolases from this organism are able to work together synergistically to release reducing sugars from cellulose [29, 30]. Hemicellulose degrading enzymes from *F. succinogenes* have also been characterized including endo-xylanases [31-36], arabinofuranosidases [37], acetylxylan esterases [38-40]. During periods of exponential growth, the bacterium stores an intracellular polysaccharide, which can comprise up to 30% of the dry cell weight [41]. Based on a recent report using qFISH, *F. succinogenes* makes up approximately 0.3% of the total cell number in the rumen [42].

Prevotella bryantii. *Prevotella bryantii*, isolated in 1957 [43], is another gram-negative staining rod-shaped hemicellulose degrader. This bacterium degrades and metabolizes hemicellulosic substrates, such as xylan [44]. The *Prevotella* genus belongs to the order Bacteroidales, and based on qFISH data, this order constitutes over 12% of the microbes in the rumen [42]. The *Bacteroides* and *Prevotella* genera share a unique system to sense and regulate digestion of polysaccharides termed Sus-like systems [45, 46]. A hybrid two component regulatory protein senses the presence of periplasmic polysaccharide fragments and transmits the signal to a cytoplasmic DNA binding domain [47]. Subsequently, the expression of genes encoding enzymes involved in substrate degradation are up-regulated. This phenomenon is shown to function in the degradation of xylan in *Bacteroides ovatus* [48] as well as *Prevotella bryantii* [49]. *P. bryantii* has at least 109 genes putatively involved in polysaccharide degradation and has been shown to up-regulate genes encoding xylan degrading enzymes when grown in the presence of wheat arabinoxylan [49]. *P. ruminicola* and *P. bryantii* are both hemicellulolytic isolates from the rumen and their genomes share many fibrolytic enzymes involved in hemicellulose degradation, in contrast, the synteny of the genes is low and some families of fibrolytic enzymes are distinctly abundant in *P. ruminicola* and poorly represented in *P. bryantii* [50]. Cytoplasmic carbohydrate stores have been shown to compose up to 60% of the dry cell weight of *P. bryantii* [41]. Discrepancies between ruminal counts can be attributed to the method of detection, and the microbiome can change based on the species, diet, geographic origin, and even animal to animal variation [42].

Ruminococcus species. *Ruminococcus albus* and *Ruminococcus flavefaciens* are gram-positive staining cocci that are able to degrade and ferment both cellulose and hemicellulose. Upon isolation in 1951, *R. albus* was characteristically white, and *R. flavefaciens* was separately categorized because of its yellow pigment. Later, *R. albus* strain 7 was isolated which produces a yellow pigment; and shown to degrade cellulose more efficiently than the white pigmented strains [51]. *R. albus* strains 7 and 8 produce a bacteriocin, albusin, which is able to depress the growth of *R. flavefaciens* FD-1 [52, 53]. It has been shown that *R. albus* was dominant in cell number over *R. flavefaciens* and *F. succinogenes* when grown on cellulose during competition experiments [54]. Similar to *F. succinogenes*, adhesion of *R. albus* cells to cellulose is important for degradation and utilization of the substrate, and adhesion defective mutants cannot utilize cellulose effectively [55, 56]. Some reports show that *R. albus* is a dominant cellulolytic microbe in the rumen [57], yet the more recent qFISH data estimates that *R. albus* contribute only 0.41% of the bacterial population and *R. flavefaciens* comprises 0.56% [42]. The same report also shows that when family specific primers are used, Ruminococcaceae contribute over 10% of the bacterial cells in the rumen. It has been shown that *R. flavefaciens* contains genes for scaffoldins and dockerin linked enzymes reminiscent of a cellulosomal model for cellulose degradation, with a crystal structure for the cohesin domain later reported [58-60].

Rumen fungi. Fungi isolated from the rumen have been shown to play an important role in polysaccharide degradation, and therefore the GH genes they possess have been cloned and characterized. *Orpinomyces joyonii* contains three cloned endoglucanases from GH family 5, CelA, CelB29 and CelB2 [61-63]. *Neocallimastix frontalis*

possesses CelA, an endo-glucanase belonging to GH family 5 [64]. *Neocallimastix patriciarum* cDNA clones show four cellulases, CelA [65, 66], CelB [65, 67], CelC [65], and CelD [68]. Finally, two *Piromyces rhizinflata* cellulases have been cloned and characterized [69]. Xylan degrading enzymes have also been cloned from ruminal fungi including a β -xylosidase from *Neocallimastix frontalis* [70], two endo-xylanases from *Neocallimastix frontalis* [71], and three xylan esterases from *Neocallimastix patriciarum* [72].

POLYSACCHARIDE DEGRADATION

Ruminal microbes have evolved to efficiently degrade plant cell wall polysaccharides. Genomes from the major fibrolytic ruminal bacteria, *F. succinogenes* S85, *P. bryantii* B₁₄, *P. ruminicola* 23 and *R. albus* (strains 7 and 8) have been sequenced, and all possess a myriad of putative carbohydrate active enzymes implicated in the degradation of cellulose and hemicelluloses. Glycoside hydrolases (GH) are categorized by amino acid sequence similarity, secondary and tertiary structure, and catalytic mechanism. They are sorted in the Carbohydrate Active enZYme (CAZy) Database (<http://www.cazy.org/>) into GH families [73]. Most of the GH families show a high degree of substrate specificity (i.e. GH family 10 and 11 xylanases), while others may be somewhat promiscuous (GH family 5 enzymes).

Catalysis. Most of the GH families in the database function via one of two general acid-base catalytic mechanisms of glycosidic bond hydrolysis include the inverting mechanism (Fig. 1.1A), where the orientation of hydroxyl group on the anomeric carbon is changed, and the retaining mechanism (Fig1.1B), during which the orientation of the hydroxyl group remains the same [74]. Work to elucidate the

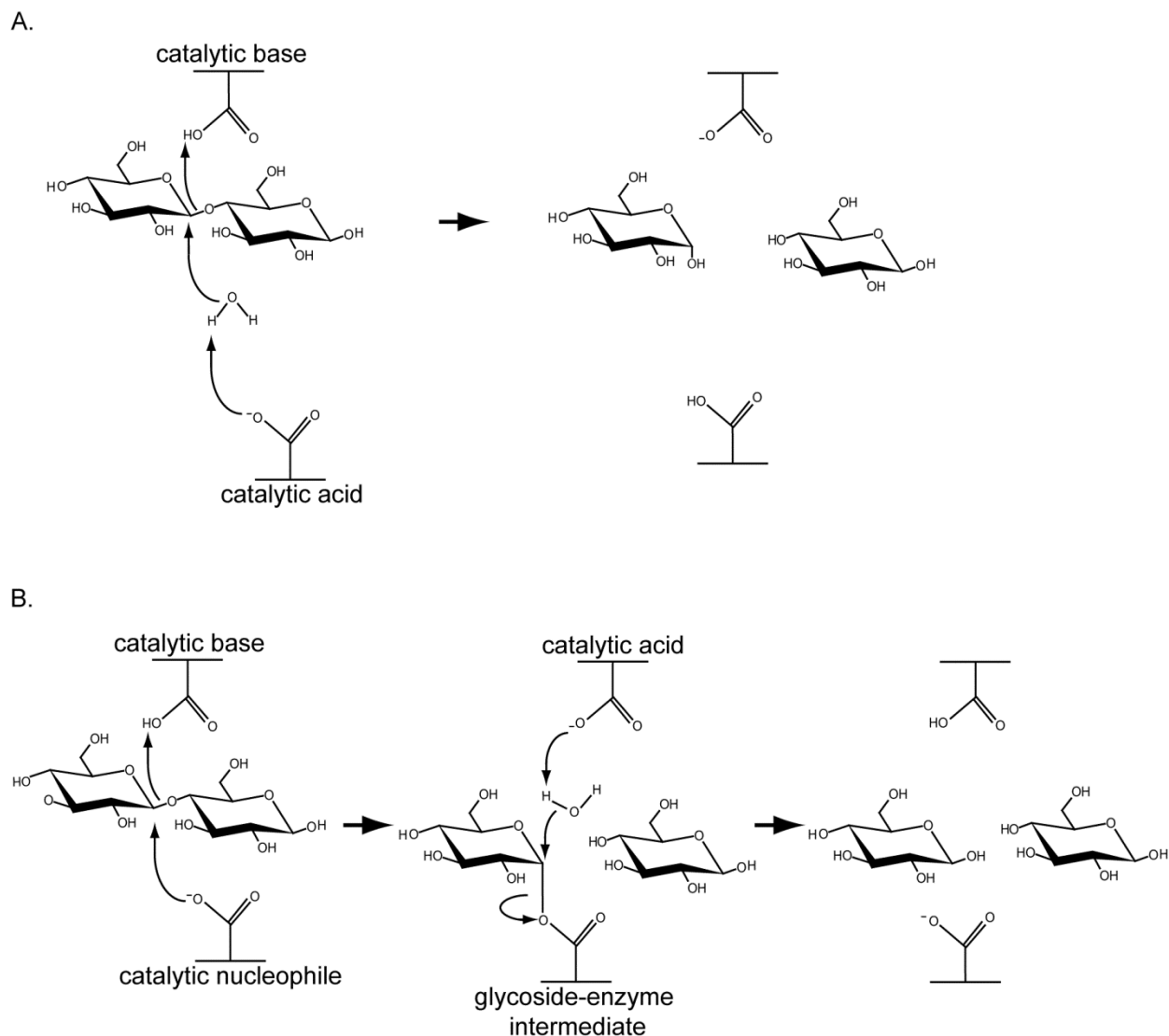


Figure 1.1. Reaction mechanisms of glycoside hydrolases. (A) The one-step inverting mechanism utilizes a catalytic acid that deprotonates a water molecule which attacks the anomeric carbon, consequently changing configurations. (B) The two-step retaining mechanism involves a nucleophilic amino acid directly attacking the anomeric carbon creating a covalent bond between the sugar residue and the enzyme, forming a glycoside-enzyme intermediate with an inverted orientation at the anomeric carbon. Subsequently, a catalytic acid deprotonates a water molecule which attacks the anomeric carbon, restoring the original configuration and the sugar is released from the enzyme.

mechanism of glycosidic bond cleavage has been carried out by creating catalytic mutants and studying the rescue of catalysis or creating covalently linked intermediates of the substrate-protein transition state and obtaining crystal structure of the enzymes [75]. The nomenclature for how an individual GH domain recognizes individual sugar monomers within a polysaccharide chain has changed over the years, but has settled upon labeling them as numerical subsites (Fig. 1.2). The subsites that bind sugars to the non-reducing end of the cleavage site are counted as negative integers, decreasing in number leading away from the cleavage site. The subsites to the reducing end of the cleavage site are label with positive integers, increasing in number leading away from the cleavage site [76].

Modular domain structure. Many GH enzymes exist in a single polypeptide chain along with other accessory domains. Protein domains referred to as carbohydrate binding modules (CBM) are used in conjunction with hydrolytic domains to improve the enzyme's ability to hydrolyze its substrate. The function of a CBM is accomplished by increasing the enzyme's affinity for and proximity to the substrate [77]. Carbohydrate binding modules can be specific or non-specific for the substrates that they bind [77, 78]. Due to their unique characteristics, CBMs have been adopted for use in other research areas, including affinity tags and analyzing carbohydrate content [79]. Some proteins may also be composed of two separate catalytic domains that act on the same or different polysaccharides [80-82].

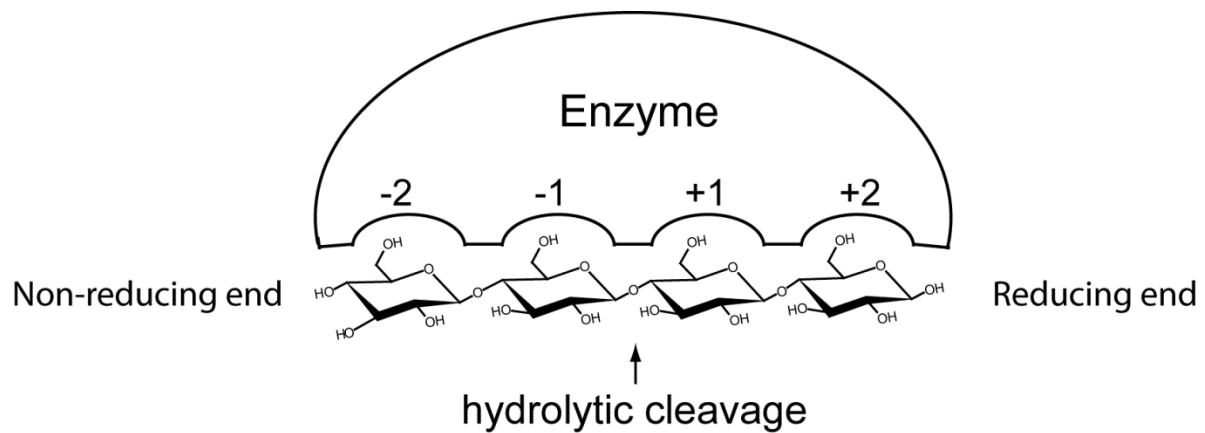


Figure 1.2. Subsite recognition of a cellotetraose molecule bound to a cellobiohydrolase. Sugars leading away from the cleavage site to the non-reducing end are numbered with negative integers. Sugars leading away from the cleavage site to the reducing end are labeled with positive integers.

Cellulose degradation. Cellulose degradation, in the classical model, requires the concerted action of three classes of enzymes. Endo-glucanases (EC 3.2.1.4) randomly cleave β -1,4 linkages in amorphous regions of cellulose, producing two new ends to the glucan chains. Exo-glucanases (EC 3.2.1.91) bind to glucan chain ends and processively cleave off repeating units of cellobiose. Endo- and exo- glucanases, when incubated together on cellulose have been shown to synergistically release hydrolysis products [83]. Exo-glucanases can be reducing end specific or non-reducing end specific and the different types of exo-glucanases show synergy with each other as well [84]. The final step in cellulose degradation is converting cellobiose or cello-oligosaccharides into glucose monomers, which is achieved by β -glucosidases (EC 3.2.1.21) or cellodextrinases (EC 3.2.1.74). β -glucosidases are able to bind and recognize their substrate in the -1 sugar binding subsite and the +1 region may vary depending on the protein and the specific substrate it acts upon [85]. The cooperative activity between β -glucosidases and mixtures of endo- and exo- glucanases in producing glucose has been previously reported [83].

Though the classical model states that a minimum of three enzymes is required for the complete depolymerization of cellulose, most cellulolytic organisms have multiple enzymes within each category, including *R. albus*. Variations on this classical model of cellulose degradation have been discovered. Processive endo-glucanases are able to cleave random linkages in glucan chains, like endo-glucanases, and continue to processively release cellobiose, like exo-glucanases [86, 87]. These enzymes can display synergy with endo-glucanases and exo-glucanases as well as β -glucosidases [86]. Cellobiose phosphorylases (EC 2.4.1.20) or cellodextrin phosphorylases (EC

2.4.1.49) are glycoside hydrolases that cleave cellobiose or cello-oligosaccharides (DP=n) using phosphate instead of a water molecule to produce glucose-1-phosphate and glucose or cello-oligosaccharides (DP=n-1), respectively [88]. Using an intracellular phosphorylase enzyme allows the bacterium to save energy during the uptake and degradation of disaccharides. There has also been a report of an enzyme from GH family 10 that has hydrolytic activity on glucan chains as well as xylan chains in one catalytic domain [89].

Cellulosomes are multi-enzyme complexes that are composed of scaffoldin, a large protein whose purpose is to scaffold other proteins in close proximity, and other enzymes that possess activity on polysaccharides, either hydrolytic or binding. The proteins are held together by protein domains with affinity for each other, cohesins found on scaffoldins and dockerins found on catalytic and binding domains. Cellulosomes have been found in gram-positive clostridial bacteria from the soil, such as *Clostridium thermocellum* [90], and cellulosomal protein encoding genes can be found in the rumen bacterium such as *Ruminococcus flavefaciens* [60]. The increased proximity of enzymes that work cooperatively is thought to increase synergy and enhance degradation. The cellulosomes are able to attach to polysaccharides via CBMs linked to dockerins, and then also bind to the bacterium by cell wall bound cohesins. The cell is brought close to the substrate, and the soluble oligosaccharides produced by hydrolytic action of the cellulosome are subsequently internalized and fermented [91, 92].

Hemicellulose degradation. Hemicelluloses require many different enzymes for degradation. In substituted xylan, the backbone of β -1,4 linked xylose units is cleaved

by endo-xylanases which break the long polymer into shorter fragments during successive catalytic events. Endo-xylanases from GH family 10 and GH family 11 both degrade long polymers of xylan into shorter fragments, but their subsites allow for the incorporation of substituents on different sugars in relation to the site of hydrolysis [93]. From xylo-oligosaccharides, xylose is released by β -xylosidases in a similar manner to β -glucosidases. GH family 3 contains both of these activities and their mode of action is the same. Some bacteria harbor multiple GH family 3 enzymes, and this may be due to a need to degrade oligosaccharides that vary in the location of substitution [94]. The substituents on xylan are removed by a variety of accessory enzymes. Arabinose units, bound to the backbone of arabinoxylans, are hydrolyzed by α -L-arabinofuranosidases. Acetyl groups are removed by acetylxylan esterases using a mechanism similar to those of serine proteases (Fig. 1.3). Glucuronic acid residues are removed by α -glucuronidases. Lastly, ferulic acid esterases cleave off feruloyl ester from the backbone. All of these enzymes work synergistically to release monomeric sugars for the bacterium to ferment. Arabinases, mannanases, and β -mannosidases have also been shown to degrade hemicelluloses of arabinan, mannan, and manno-oligosaccharides, respectively. Carbohydrate binding modules have been found linked to hemicellulose degrading enzymes and increase substrate specificity and degradation.

Mixed linkage glucan degradation. Mixed linkage glucans, such as lichenin, can be degraded by many cellulases and laminarinases (cleave β -1,3 linkages) to some extent, but GH family 16 lichenases are more effective at lichenin deconstruction [95]. Cellulases have affinity for the β -1,4 linkages, laminarinases can cleave the internal β -1,3 linkages. The action of endo-glucanases are hindered by the presence of β -1,3

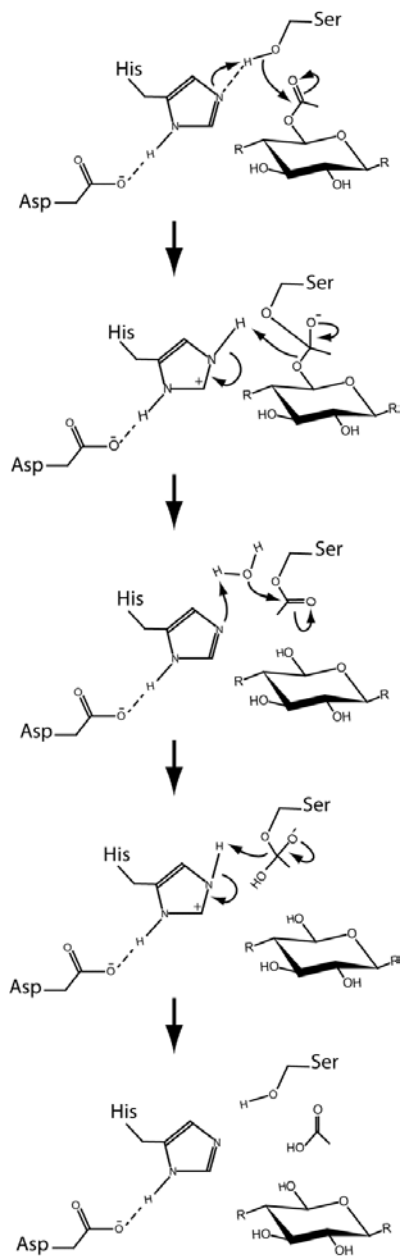


Figure 1.3. The reaction mechanism of Asp-His-Ser acetyl esterases.

The mechanism employs an aspartate, histidine and serine for catalytic cleavage of the acetyl residue, forming an acetylated serine as an intermediate product before hydrolytic cleavage and release of acetate. This mechanism is similar to those of serine proteases.

linkages and the action of lamininases are impeded by the presence of the β -1,4 linkage. Licheninases have higher affinities for the mixed linkages present in lichenin or barley glucan, and allow for β -1,3 and β -1,4 linkages in the sugar binding subsites. Oligosaccharides released by hydrolysis need to be further degraded by β -glucosidases with affinity for β -1,3 linkages created at the non-reducing end [96].

***Ruminococcus albus* GROWTH AND METABOLISM**

Several strains of *R. albus* have been isolated and each may have different growth requirements [97, 98], so it is important to not generalize the results from one study to all of the strains of *R. albus*. Many studies have been conducted on strains 7 and 8. *R. albus* 7 is a cellulolytic coccus, and is capable of degrading over 80% of xylan including flax hemicelluloses and oat hull hemicelluloses. Interestingly, the same strain is not able to grow on xylose monomers [97]. A strong requirement for branched chain fatty acids including iso-butyric, and 2-methylbutyric acids has been shown for strain 7 [99, 100]. The branched chain amino acids are subsequently incorporated into lipids that are found in the membrane fraction [100]. The organism is also sensitive to pH. In a chemostat, cells grow optimally at a pH of 6.8 and are washed out below 5.9 [101].

Hydrolysis of cellulose by spent culture media, from *R. albus* RAM grown on cellulose, was studied and the cellulolytic enzymes lose activity after exposure to oxygen [102]. This phenomenon is not unexpected from a strict anaerobe. Media without rumen fluid was unable to support growth of *R. albus* 8 on cellulose, and it was proposed that certain growth factors in rumen fluid promote degradation of cellulose. Phenylpropionic acid (PPA) was shown to be required, at 3 μ M, for *R. albus* 8 to grow

on cellulose [103]. Cultures containing PPA showed that the cellulase activity was maintained in the cell pellet, while the PPA deprived culture had a majority of the cellulase activity remaining in the supernatant [104]. Cells grown on PPA containing media, when visualized using transmission electron microscopy, showed a more extensive and lobed capsule. This finding may signify a cellulosomal multi-protein complex, as there are dockerin domains in some previously characterized proteins. In opposition to this theory, there are no scaffoldin-like proteins identified within the partially sequenced genome. Later phenylacetic acid [105] was also found to stimulate digestion of cellulose at 5 μ M concentration [106]. While insoluble cellulose degradation is improved with PPA and PAA addition, these molecules are not required for utilization of xylan [107].

The mode of cellulose degradation by *R. albus* has yet to be elucidated, although many pieces of the puzzle have been presented. Continuous culture studies determined that the end products of fermentation are acetate, ethanol, formate, hydrogen and carbon dioxide [11]. The ultrastructure of *R. albus* shows a thick cell wall and fibers that may play a role in adherence of cells to the plant cell wall [108]. Adhesion plays an important role in cellulose degradation, with several adhesion defective mutants unable to degrade cellulose [55, 56, 109]. Research done on two different strains of *R. albus*, strain 8 and strain 20, have shown that adhesion is mediated by type IV pilin proteins, CbpC [110] and GP25 [111], respectively, which were previously thought to be only found in bacteria with two cell walls. The adhesion deficient mutant *R. albus* 20 also has decreased cellulase activity. Other researchers have isolated an adhesion defective mutant of *R. albus* 8 that lacks two proteins, Cel9B and Cel48A [56]. These proteins are

putative glycoside hydrolases predicted to be involved in cellulose hydrolysis. Interestingly, this adhesion defective mutant has no difference in expression of pili proteins. The two glycoside hydrolases were later shown to contain a novel CBM that is only found in *Ruminococcus albus* and classified within its own family, CBM family 37, which is able to bind to polysaccharides as well as the bacterial cell wall [112]. This module is proposed to function as a shuttle between the bacterial cell wall and the polysaccharide substrate for the attached catalytic domain.

Growth of *R. albus* B199 on cello-oligosaccharides, the products of cellulose hydrolysis, has been studied. This strain has a faster growth rate on cello-oligosaccharides with a degree of polymerization (DP) greater than 2 as compared to cellobiose or glucose. Also the growth yields were greater on the cellodextrins. Cellobiose phosphorylase and cellodextrin phosphorylase activities were located in the cytoplasm and their activities are four fold higher in cells grown on cellodextrins and cellobiose versus cells grown on glucose [113]. This suggests that *R. albus* B199 imports cello-oligosaccharides into the cytoplasm where they are degraded and utilized for energy. It has been previously shown in *Clostridium thermocellum* that cellodextrins with a DP of 5 or lower are internalized before degradation and this is an ATP dependent event [114].

R. albus is postulated to produce 9 high energy phosphate bonds from 1 cellobiose molecule [115]. This is an interesting observation in that *R. albus* is predicted to metabolize sugars to acetate, ethanol, formate, carbon dioxide and hydrogen. Cellobiose catabolized into pyruvate yields 5 ATP using the energy conserving cellobiose phosphorylase. Conversion of pyruvate to acetate and ethanol through mixed

acid fermentation yield another 2 ATP after the redox balancing steps [116]. A total of 7 ATP can be produced through substrate level phosphorylation. This requires *R. albus* to be able to utilize other energy conserving steps to save or create ATP. These observations are of interest for future study.

CHARACTERIZED ENZYMES FROM *Ruminococcus albus*

Ruminal bacteria and their glycoside hydrolases have been studied for decades and many enzymes have been biochemically characterized, including cellulolytic and hemicellulolytic enzymes from *R. albus*, *R. flavefaciens*, *F. succinogenes*, and *P. bryantii*. Recent interest in biofuel production has revitalized research in enzymes capable of hydrolyzing lignocellulosic biomass, in order to identify more active enzyme to bypass that high cost of depolymerization. Due to *R. albus* 8's ability to hydrolyze both cellulose and hemicellulose, the enzymes from this organism are of interest in formulating a mixture of enzymes able to cooperatively release more soluble sugars from plant cell wall polysaccharides.

Research on *R. albus* enzymes has led to the cloning and characterization of a variety of enzymes. Little work has been done to characterize cellulases specifically from strain 8. Howard and White constructed genomic libraries from *R. albus* 8 and found 5 distinct clones harboring cellulases [117]. No subsequent work has been done to identify the specific genes present in these clones. Other strains of *R. albus*, including F-40, SY3, and AR67 have been used for more detailed characterization and accession numbers for proteins with available sequence data are available in Table 1.1.

Table 1.1. Previously cloned and sequenced enzymes from *R. albus*

Accession #	<i>R. albus</i> strain	Secreted (predicted) ^a	GH family ^b	CBM Family ^b	CE family ^b
Cellulases					
EgI (CelA)	AAA26469	F-40	Yes	GH 5	
EgIV (CelB)	BAA32286	F-40	No	GH 5	
EgV (CelC)	BAA92146	F-40	Yes	GH 5	
Cel5D	BAA92430	F-40	Yes	GH 5	2xCBM4_9
Cel9A ^c	BAB64431	F-40	Yes	GH 9	CBM3
EgA	AAA26467	SY3	No	GH 5	
EgB	CAA38693	SY3	Yes	GH 5	
Xylanases					
XynA	AAA85198	7	Yes	GH 11	CBM 4_9 CE 4
XynB ^c	BAB39493	7	Yes	GH 11	CBM 4_9 CE 4
XynC ^c	AB057589	7	Yes	GH 30	CBM 4_9 CBM 37

^a Signal peptides were predicted using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>)

^b Domain identity was predicted using Pfam (<http://pfam.sanger.ac.uk/>)

^c Proteins were not biochemically characterized

Seven endo-glucanases have been cloned from *R. albus* strain F-40, six of which have been biochemically characterized. EG1 (CelA) has been cloned, sequenced, and the purified protein activity was tested on cellulosic substrates showing optimal activity at 37°C and pH 6.5 [118, 119]. The products of short term hydrolysis include cellotetraose, cellopentaose and cellohexaose, while long term hydrolysis produces glucose, cellobiose and cellotriose. The transcriptional start site of this gene was also mapped, and this information may be used in future research to determine possible promoter elements [119]. EgII [120] and EgIII [121] were cloned and characterized, but do not have published sequences. EgIV (CelB) was also sequenced, shown to contain a signal peptide and GH5 domain, and its hydrolytic activity tested on soluble carboxymethyl cellulose [122]. When CBMs from *C. stercorarium* were fused to this enzyme, the protein was able to hydrolyze cellulose faster and to a greater extent due to the newly conferred binding ability [123]. EgV (CelC) contains a dockerin domain in addition to a catalytic module. After purification, the enzyme was shown to degrade CMC and lichenin and, to a lesser extent, xylan in optimal conditions (40 °C at pH 7) [124]. The main products of hydrolysis from cello-oligosaccharides include cellobiose and cellotriose. Cel5D also contains a N-terminal GH family 5, dockerin domain, and two CBM family 4 domains at the C-terminus. Activity was tested against CMC and cello-oligosaccharides, and the primary hydrolysis products include cellobiose and cellotriose similarly to CelC [125]. Cel9A was cloned with Cel5D but was not expressed. This enzyme contains a GH family 9 domain at the N-terminus followed by CBM family 3 and a dockerin at the C-terminus.

R. albus SY3 has had two endo-glucanases cloned and characterized, EgA having CMCase and xylanase activity, and EgB having only CMCase activity [126]. Both EgA and EgB were unable to bind specifically to cellulose and lack CBMs. The β -glucosidase from *R. albus* AR67 has hydrolytic activity on β -1,3 linkages as well as β -1,4 linkages [127]. The β -glucosidase of *R. albus* F-40 has activity on β -1,4 linkages, but was not tested on β -1,3 linkages [128]. The enzyme was able to rapidly hydrolyze artificial substrates such as *para*-nitrophenol glucoside, which is not linkage specific. Kinetic analysis reveals a much higher K_m for cellobiose as compared to *p*NP-glucoside, which may be due to a preference for a different disaccharide as the natural substrate. An exo-glucanase from *R. albus* F-40 was cloned and characterized, but the gene sequence was not determined [129]. Viscosity changes of enzyme incubated with CMC were not representative of an endo-acting enzyme. Also, the low activity on insoluble cellulose and high activity on *p*NP-cellobioside led the authors to characterize this enzyme as a cellobiosidase (exo-glucanase).

Although many genes in *R. albus* 8 contain dockerin domains, this organism is not thought to employ a cellulosome for polysaccharide degradation due to a lack of scaffoldin genes. In contrast to this theory, a cellulosomal like protein complex was purified from *R. albus* F-40 and shown to contain at least 15 different enzymes, most of which show hydrolytic activity on xylan or CMC [130]. A similar cellulosome-like complex has not been shown for *R. albus* 8 or any other *R. albus* strains, and there is currently no publicly available genome sequence for *R. albus* F-40. It is possible that strain F-40 may use a cellulosomal system while the other strains only contain dockerin domains as remnant of a lost cellulosomal system.

Although several endo-glucanases from *R. albus* show cellulolytic and xylanolytic activities, few xylanase genes have been cloned from this organism. Three xylanases from *R. albus* 7 were cloned and sequences were deposited: XynA, XynB, and XynC, accession number AB057589. Of these three GH family 11 encoding genes, only XynA was characterized. The *R. albus* 7 XynA has hydrolytic activity on oat spelt xylan and birchwood xylan and no hydrolysis of cellulosic substrates was detected [131].

OBJECTIVES

Although many polysaccharide-degrading enzymes have been characterized from *R. albus*, there is still much to be determined about their cooperative ability to hydrolyze cellulose and hemicellulose. Only one endo-xylanase has been characterized from *R. albus* 7 and this enzyme alone is not sufficient to deconstruct xylan into fermentable monosaccharides. In an effort to understand the molecular mechanisms of plant cell wall degradation in *R. albus* 8, we generated a partial genome sequence of the bacterium to allow us to test the following hypotheses:

- 1. The *Ruminococcus albus* 8 genome has evolved the gene encoding enzymes that allow the bacterium to degrade plant cell wall polysaccharides synergistically to fermentable sugars.**
- 2. A unique set of enzymes has been evolved to target different polysaccharides present in plant cell wall polysaccharides.**

CHAPTER 2. XYLAN DEGRADING ENZYMES FROM *Ruminococcus albus* 8¹

ABSTRACT

Ruminococcus albus 8 is a ruminal bacterium capable of metabolizing hemicellulose and cellulose, the major components of plant cell wall. The enzymes that allow this bacterium to capture energy from the two polysaccharides, therefore, have potential application in plant cell wall depolymerization, a process critical to biofuel production. For this purpose, a partial genome sequence of *R. albus* 8 was generated. The genomic data depicted a bacterium endowed with multiple forms of plant cell wall degrading enzymes. The endoxylanases of *R. albus* 8 exhibited diverse modular architectures, including incorporation of a catalytic module, a carbohydrate binding module and a carbohydrate esterase module in a single polypeptide. The accessory enzymes of xylan degradation were a β -xylosidase, an α -L-arabinofuranosidase, and an α -glucuronidase. We hypothesized that due to the chemical complexity of the hemicellulose encountered in the rumen, the bacterium uses multiple endoxylanases, with subtle differences in substrate specificities, to attack the substrate, while the accessory enzymes hydrolyze the products to simple sugars for metabolism. To test this hypothesis, the genes encoding the predicted endoxylanases were expressed, and the proteins were biochemically characterized either alone or in combination with accessory enzymes. The different endoxylanase families exhibited different patterns of

¹ Moon Y.H., Iakiviak M., Bauer S., Mackie R.I., Cann I.K. 2011. Biochemical analyses of multiple endoxylanases from the rumen bacterium *Ruminococcus albus* 8 and their synergistic activities with accessory hemicellulose-degrading enzymes. *Appl. Environ. Microbiol.* 77(15):5157-69

product release, with the family 11 endoxylanases releasing more products in synergy with the accessory enzymes from the more complex substrates. Aside from the insights gained in hemicellulose degradation by *R. albus* 8, this report should enhance our knowledge on designing effective enzyme cocktails for release of fermentable sugars in the biofuel industry.

INTRODUCTION

Production of biofuels from plant biomass is under intensive research in regard to their potential as alternative fuels, and also as a means to reduce greenhouse gas emission. The world produced approximately 87 giga-liters of liquid biofuels in 2008, and higher production levels are anticipated due to current and future innovative technologies [132]. Second generation biofuels are focused on the conversion of feedstock such as lignocellulose from corn residues, sugarcane bagasse, and switchgrass into competitive alternative fuels [13, 133]. Of the many bioenergy feedstock, Switchgrass and Miscanthus are expected to become significant substrates in the future for bioconversion to bioethanol [132].

Xylan, the most abundant hemicellulose, is a heteropolymeric substrate consisting of a repeating β -1,4-linked xylose backbone decorated with acetyl, arabinofuranosyl, and 4-O-methyl glucuronyl groups. Additionally, xylan may be cross-linked to lignin by aromatic esters. Thus, to depolymerize xylan efficiently to its constituent monosaccharides, a xylan-fermenting organism must possess a set of specialized enzymes. These enzymes include endo-1,4- β -xylanases (EC 3.2.1.8), β -D-xylosidases (EC 3.2.1.37), α -L-arabinofuranosidases (EC 3.2.1.55), α -glucuronidases

(EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72), and ferulic/coumaric acid esterases (EC 3.1.1.73) [81].

Ruminococcus albus 8 is widely known as one of the most actively fibrolytic ruminal bacteria. This bacterium degrades cellulose and hemicellulose in forages such as alfalfa and grass hays [51, 99, 134, 135]. It has been shown that *R. albus* 8 produces a wide range of proteins with glycoside hydrolase activities [103, 136, 137]. However, the glycoside hydrolases present in *R. albus* 8 have received limited investigation. Previous studies on *R. albus* 8 have focused on cellulose degrading enzymes (Cel5G, Cel9B, Cel9C, and Cel48A) and rarely on its hemicellulases. The limited work on hemicellulases include the work of Greve *et al.* [137] who purified an α -L-arabinofuranosidase from the extracellular broth of cultures of this bacterium and determined its biochemical activities. Furthermore, Xu *et al.* [78] cloned a xylanase gene (*xyn11C*) from *R. albus* 8; however, gene expression resulted in an insoluble form of the gene product. In other *R. albus* strains, specifically *R. albus* 7, only one xylanase gene (*xynA*) has been cloned and the gene product biochemically characterized [138].

Recently, we produced a draft genome sequence of *R. albus* 8, and bioinformatic analysis revealed a bacterium with multiplicity of glycoside hydrolase (GH) and carbohydrate esterase (CE) encoding genes. Furthermore, in a report on transcriptomic analysis of the ruminal hemicellulolytic bacterium *Prevotella bryantii* B₁₄, it was observed that this bacterium releases multiple forms of enzymes, such as endoxylanases, during depolymerization of soluble wheat arabinoxylan [49]. This observation suggested that highly hemicellulolytic ruminal bacteria employ diverse endoxylanases to enhance release of nutrients from complex polysaccharides.

To investigate the potential application of *R. albus* 8 enzymes in the hydrolysis of complex hemicellulosic substrates, 11 genes predicted to encode hemicellulose-targeting enzymes were cloned and expressed to study their synergistic activities. Using biochemical approaches, five of the proteins were assigned functions of endoxylanases, and one each was assigned β -xylosidase, α -L-arabinofuranosidase, and α -glucuronidase activities, respectively. Further characterization of the enzymes demonstrated that individual endoxylanases functioned synergistically with accessory enzymes to yield monosaccharides from model xylan substrates. Surprisingly, in the study of synergism between the endoxylanases and the accessory enzymes, five endoxylanases added at a total concentration similar to the individual endoxylanases yielded about the same levels of endproducts. In addition, larger hydrolysis products that accumulated in the reaction mixture, when extracted from TLC plates, were converted to monosaccharides by enzymes that were present in the original reaction mixture, suggesting endproduct inhibition. Since enzymatic hydrolysis of feedstock represents one of the critical bottlenecks in the biofuel industry, it is our anticipation that insights gained from this study, which focused on the interplay of several hemicellulose-targeting enzymes, will aid in the design of efficient enzyme cocktails for depolymerization of feedstock in the biofuel industry.

MATERIALS AND METHODS

Bacterial strains- *R. albus* 8 was obtained from the Department of Animal Science, University of Illinois at Urbana-Champaign. *E. coli* JM109 and BL21-CodonPlusTM (DE3) RIL competent cells and the PicoMaxx high fidelity PCR system were purchased from Stratagene (La Jolla, CA). The pET-46b EK/LIC cloning kit were

obtained from Novagen (San Diego, CA). The DNeasy Blood and Tissue kit and the QIAprep Spin Miniprep Kit were obtained from QIAGEN, Inc. (Valencia, CA). The Talon Metal Affinity resin was purchased from Clontech Laboratories, Inc. (Mountain View, CA). Amicon Ultra-15 centrifugal filter units with 10 kDa molecular mass cut-off (MMCO) and 50 kDa MMCO were obtained from Millipore (Billerica, MA).

Genome sequencing and annotation- A single colony of *R. albus* 8 was cultured at 37°C in DSMZ Medium 436 (<http://www.dsmz.de/>). The cells were harvested by centrifugation at 20,000 x g at 4°C for 15 minutes. DNA was isolated from saturated cultures and purified using the Qiagen DNeasy Blood and Tissue kit with an integrated RNase treatment step. The genome of *R. albus* 8 was partially sequenced by the W. M. Keck Center for Comparative and Functional Genomics at University of Illinois. The genomic sequence data were generated with ½ plate of FLX PE 454 data and ½ plate of 8 kb paired-end GS FLX Titanium data using a Genome Sequencer instrument from 454 Life Sciences (Branford, CT). The partial genome sequence was assembled into 2 scaffolds using 172 contigs. The Rapid Annotation using Subsystem Technology (RAST) server [105] was used for the auto-annotation of the *R. albus* 8 draft genome sequence. Prediction of signal peptides was performed by using LipoP 1.0 server (<http://www.cbs.dtu.dk/services/LipoP>)

Gene cloning, expression and protein purification- The genes in this study were amplified by the PicoMaxx high fidelity PCR kit using genomic DNA as template. Each amplicon was then digested with the exonuclease activity of T4 DNA polymerase and annealed to a similarly digested pET-46b vector and transferred into *E. coli* JM109 by electroporation. After plasmid extraction, the insert in the plasmid construct was

sequenced to confirm the integrity of the cloned gene (W. M. Keck Center for Comparative and Functional Genomics at University of Illinois). The correct plasmid constructs were introduced into *E. coli* BL-21 CodonPlus (DE3) RIL competent cells by heat shock transformation, and grown overnight on lysogeny broth (LB) agar plates supplemented with ampicillin (100 µg/mL) and chloramphenicol (50 µg/mL) at 37 °C. After 12 hr, a single colony was used to inoculate fresh LB broth (10 mL) supplemented with the same antibiotics (ampicillin and chloramphenicol) and cultured with aeration for 8 h at 37 °C. The pre-cultures were then used to inoculate fresh LB broth (1 L) supplemented with ampicillin and chloramphenicol and the cultures were incubated at 37 °C with vigorous shaking (225 rpm/min). At an optical density at 600 nm of 0.3, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and the temperature was shifted to 16 °C and culturing continued. After 15 h of growth, the cells were harvested by centrifugation (4000 ×g, 15 min, 4°C). The cell pellets were then re-suspended in 30 mL of lysis buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.0) and ruptured by two passages through an EmulsiFlex C-3 cell homogenizer from Avestin (Ottawa, Canada). Each cell lysate was clarified by centrifugation at 20,000 × g for 30 min at 4 °C to remove the cell debris. The recombinant proteins were then purified using Talon Metal Affinity resin according to the supplier's protocol with the exception that Tris-based binding (50 mM Tris-HCl, 300 mM NaCl, pH 7.5) and elution (50 mM Tris-HCl, 300 mM NaCl, 250 mM Imidazole, pH 7.5) buffers were employed. Aliquots of eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according Laemmli's method [139] and protein bands were visualized by staining with Coomassie brilliant blue G-250. Elution fractions were

pooled and the proteins were exchanged into protein storage buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) by three successive concentration and dilution cycles with Amicon Ultra-15 centrifugal filter units. The protein concentrations were calculated by absorbance spectroscopy at 280 nm using a NanoDrop 1000 from Thermo Scientific (Waltham, MA) with the following extinction coefficients: 79,300 M⁻¹cm⁻¹, 165,480 M⁻¹cm⁻¹, 166,050 M⁻¹cm⁻¹, 124,680 M⁻¹cm⁻¹, 141,180 M⁻¹cm⁻¹, 74,720 M⁻¹cm⁻¹, 85,260 M⁻¹cm⁻¹, and 126,740 M⁻¹cm⁻¹ for Xyn10A, Xyn10B, Xyn11D, Xyn11E, Xyn11F, Xyl3A, Ara51A, and Agu67A, respectively.

Hydrolysis of soluble wheat arabinoxylan, oat spelt xylan, and birchwood xylan- The five endoxylanases (0.5 μM) were incubated with each substrates (1-8% w/v, final concentration) in citrate buffer (50 mM sodium phosphate, 150 mM NaCl, pH 6.5) at 37 °C for 15 hours. The concentration of reducing ends were estimated using the *para*-hydroxybenzoic acid hydrazide (PAHBAH) assay as described previously [140] with glucose as a standard. For qualitative identification of the hydrolysis products, the reactions were resolved by thin layer chromatography (TLC). The mobile phase was composed of *n*-butanol:acetic acid:H₂O, 10:5:1 (v/v/v) and 10cm x 20cm TLC plates were used. For quantitative analysis of the products of hydrolysis, the samples were analyzed by high performance anion-exchange chromatography (HPAEC). For HPAEC, 100 μL of each diluted sample was analyzed on a System Gold HPLC instrument from Beckman Coulter (Fullerton, CA) equipped with CarboPac™ PA1 guard (4 x 50 mm) and analytical (4 x 250 mm) columns from Dionex Corporation (Sunnyvale, CA) and a Coulochem III electrochemical detector from ESA Biosciences (Chelmsford, MA). Xylose, arabinose, and xylo-oligosaccharides (X2-X4) were injected as standards. For

quantification of arabinose and xylose concentrations in the samples, calibration curves were generated with known concentrations of xylose, arabinose, and xylo-oligosaccharides (X2-X4). Where synergistic activities of xylanases and accessory enzymes were investigated, each enzyme was added at 0.5 μ M, unless otherwise stated as in experiments where different enzyme concentrations were varied to optimize enzyme mix.

Esterase activity- Acetyl xylan esterase activity was assayed using acetylated oat spelt xylan and *para*-nitrophenyl (*p*NP)-acetate (SIGMA). Acetylated oat spelt xylan was prepared in accordance with the method described by Mitchell et al. [141]. Ten microliters of the enzyme under investigation (final concentration of 0.5 μ M) was incubated with 2% (w/v) acetylated oat spelt xylan in a buffer (50 mM Na-phosphate with 150 mM NaCl, pH 6.5), and the released acetic acid was measured using an acetic acid detection kit (Megazyme, Bray, Ireland) described in our previous report [40]. For *para*-nitrophenyl (*p*NP) linked substrates, 100 μ L of each enzyme (final 0.5 μ M) was incubated with 1 mM *p*NP-acetate in a buffer (50 mM Na-phosphate, 150 mM NaCl, 10% dimethyl sulfoxide, pH6.5) and the rate of *p*NP release was determined by monitoring the absorbance at 400 nm continuously.

Binding of insoluble polysaccharides- Oat spelt xylan (OSX) and birchwood (BWX) as ligands were purchased from Sigma-Aldrich (St. Louis, MO). Since OSX and BWX contain some soluble components, the soluble fractions were removed as described by Yoshida *et al.* [37, 40]. Briefly, one gram of substrate was stirred in 100 mL of distilled water for 12 h. After centrifugation (4,000 \times g, 10 min, room temperature), the precipitate was further washed with 100 mL of distilled water and centrifuged (4,000

× g, 10 min, room temperature). The insoluble fractions were lyophilized and then ground into small particles in a mortar, producing insoluble OSX (is-OSX) and insoluble BWX (is-BWX). The binding of proteins to insoluble substrates was carried out as follow: one mL of 2 μM proteins in 50 mM sodium phosphate buffer (pH 6.5), containing 150 mM NaCl was mixed with 20 mg of insoluble polysaccharides. The reaction mixture was gently mixed at 4 °C for 1 h. The insoluble polysaccharide was precipitated by centrifugation (13,000 rpm, 4 °C, 1 min). For qualitative binding assessment, the unbound protein (supernatant) and bound protein (precipitate) fractions was resolved on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Blanks were prepared by incubating the protein without insoluble polysaccharide in the reaction buffer. For the quantitative binding capacity of the proteins to insoluble polysaccharides, the intensities of the resolved protein bands were calculated using the Gene Tools software program (SYNGENE, Frederick, MD).

Determination of specific activities of Xyl3A and Ara51A with pNP-linked sugars- The specific activities of Xyl3A and Ara51A with *para*-nitrophenyl (pNP) linked substrates were assayed using a thermostated Cary 300 UV-Vis spectrophotometer from Varian Inc. (Palo Alto, CA). Fifteen different substrates from Sigma-Aldrich (St Louis, MO) were screened including: pNP-α-L-arabinopyranoside, pNP-α-L-arabinofuranoside, pNP-β-D-fucopyranoside, pNP-α-L-fucopyranoside, pNP-α-D-galactopyranoside, pNP-β-D-galactopyranoside, pNP-α-D-glucopyranoside, pNP-β-D-glucopyranoside, pNP-β-D-maltopyranoside, pNP-α-D-maltopyranoside, pNP-α-D-mannopyranoside, pNP-β-D-mannopyranoside, pNP-α-L-rhamnopyranoside, pNP-β-D-xylopyranoside, and pNP-β-D-cellobioside. The different pNP substrates (1.0 mM, final

concentration) were incubated at 37 °C in the presence or absence of Xyl3A or Ara51A (50 nM, final concentration) in a buffer composed of 50 mM Na-phosphate buffer and 150 mM NaCl (pH 6.5) for 30 min, and the rate of *p*NP release was determined by monitoring the absorbance at 400 nm continuously.

Hydrolysis of aldouronic acid by Agu67A- The enzyme, Agu67A or Xyl3A (final 0.5 μ M, 50 mM Na-phosphate with 150 NaCl), was incubated with the aldouronic acid mixture (final 1.2 mg/mL) at 37 °C individually or together to determine their potential synergistic activity. After 1 hour, the reactions were terminated by boiling for 10 minutes. The hydrolysis products were analyzed by high performance anion-exchange chromatography (HPAEC) as described above. Xylose, and xylo-oligosaccharides (X2-X4) were used as standards, and the concentrations in the samples were calculated as described above.

Specific activities of multiple endoxylanases from *R. albus* 8 on xylan substrates-The specific activities of the five endoxylanases were determined at 37°C in 50 mM sodium phosphate (pH 6.5) and 150 mM NaCl. Each protein (50 nM or 100 nM) was incubated with 10 mg/mL of the following polysaccharides: soluble wheat arabinoxylan with medium viscosity (Megazyme, Bray, Ireland), oat spelt xylan, and birchwood xylan (Sigma-Aldrich, St. Louis, MO) and the reducing ends were quantified with a *para*-hydroxybenzoic acid hydrazide (*p*HBAH) assay [140] with glucose as a standard.

Purification of incompletely degraded products for further hydrolysis - The intermediate products accumulating from hydrolysis of soluble wheat arabinoxylan and

birchwood xylan were separated by thin layer chromatography (TLC) as described above. After drying the TLC plates, each product was scratched and then extracted with sterile distilled water. The purified products of soluble wheat arabinoxylan (final 0.17%) were reacted with β -xylosidase and α -L-arabinofuranosidase (each final 5 μ M) separately or in combination. Likewise, the purified products of birchwood xylan were reacted with β -xylosidase and α -glucuronidase either individually or in combination (each final 5 μ M).

RESULTS

Sequencing and auto-annotation of the genome of *R. albus 8*- The draft genome sequence of *R. albus 8* was composed of 3,873,351 base pairs aligned in 2 scaffolds containing 172 contigs with an average G+C content of 45.4%. The partial genome, which was from data representing 120X coverage, was auto-annotated using Rapid Annotation using Subsystem Technology (RAST), which predicted 3550 gene-coding sequences. Further bioinformatic analysis of glycoside hydrolase (GH) encoding genes was performed to determine the complexity of the enzymes employed by *R. albus 8* to degrade complex polysaccharides. The presence of 61 putative GH genes from 22 different families points to an organism evolved to degrade different polysaccharides. Of the 37 genes putatively involved in degradation of hemicellulose, 12 appeared to be involved in the degradation of substituted xylan, including endoxylanases, β -xylosidases, α -glucuronidases, α -L-arabinofuranosidases, and acetyl xylan esterases.

Domain architecture of multiple endoxylanases and their accessory enzymes from *R. albus 8*- Five putative endoxylanases were identified in the genome

of *R. albus* 8. The domain architecture of the proteins (ORF2725, ORF2882, ORF997, ORF1984, ORF2008) are shown in Figure 2.1A. The genes have been deposited at the Genbank database with accession numbers, JF314316, JF314317, JF314318, JF314319, and JF314320, respectively. Based on the amino acid sequences, two of the proteins belonged to GH family 10 (GH10), and they were designated *R. albus* 8 Xyn10A and Xyn10B. As shown in Fig. 2.1A, Xyn10A was the simplest among the putative endoxylanases. The analyses suggested that Xyn10A, made up of 377 amino acids, is composed of only a GH10 catalytic module. In contrast, Xyn10B, made up of 830 amino acids, exhibited a centrally located GH10 catalytic module flanked by a putative CBM family 4_9 (CBM4_9) at the N-terminus and two putative CBMs of families 22 and 37 (CBM22 and CBM37) tandemly located at the C-terminus. The remaining three putative endoxylanases belong to GH family 11 (GH11) and were named *R. albus* 8 Xyn11D, Xyn11E, and Xyn11F, since three different GH11 xylanases have already been cloned from this bacterium [78, 138]. The polypeptides of Xyn11D, Xyn11E, and Xyn11F are composed of 757, 682, and 540 amino acids, respectively. Each putative GH11 endoxylanase is characterized by a N-terminally located GH11 catalytic module flanked at the C-terminus by a CBM22 module. Similar to Xyn10B, the CBM22 of Xyn11D is followed by a CBM37 module and C-terminal to the second CBM is a putative carbohydrate esterase family 4 (CE4) module. Both Xyn11E and Xyn11F are similar to Xyn11D; however Xyn11E is missing the CBM37 module and Xyn11F lacks the CE4 module. Except for Xyn10A, each of the putative endoxylanases has an N-terminal signal peptide, suggesting that these enzymes are extracellularly located.

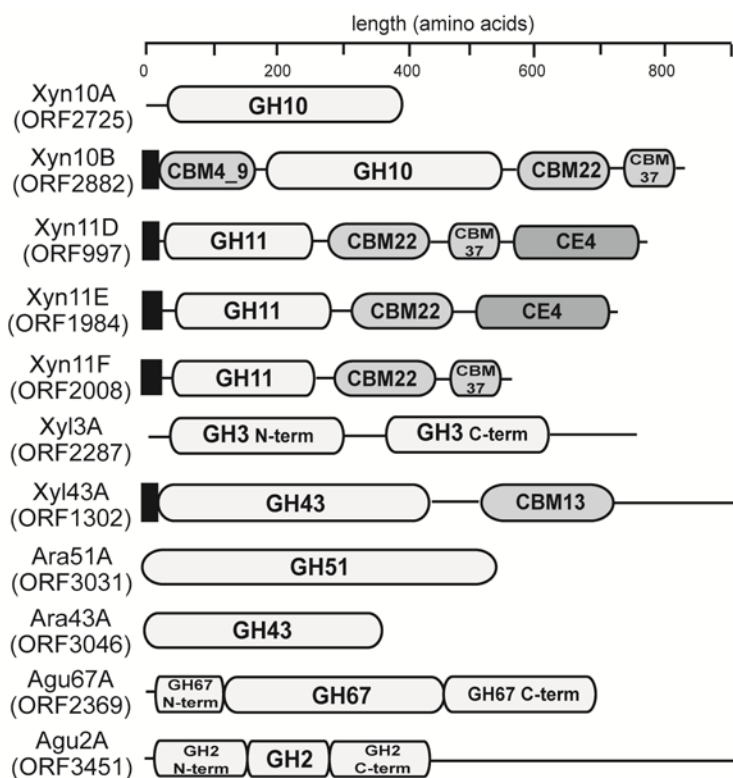
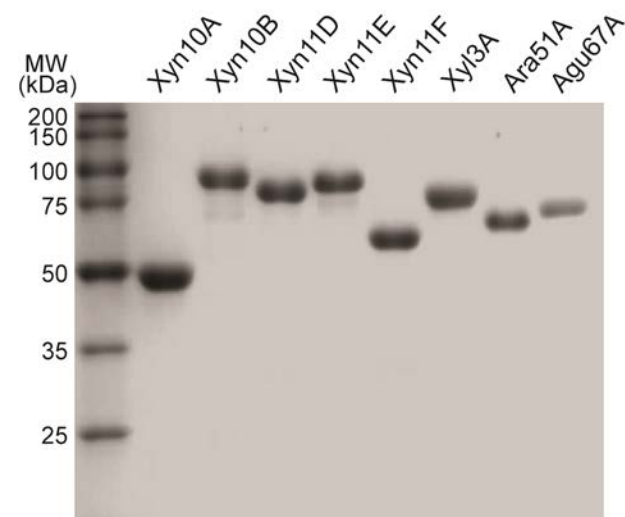
A.**B.**

Figure 2.1. Hemicellulose degrading enzymes of *R. albus* 8. (A) Domain architecture of five putative endoxylanases (Xyn10A – Xyn11F) and six putative accessory enzymes identified in the partial genome sequence of *R. albus* 8. The functional domains were assigned by using the Pfam online server (<http://www.sanger.ac.uk/Software/Pfam/>). The numbers in the names refer to the predicted GH family of the protein. (B) Purification of the predicted GH family proteins described in (A). Only the proteins for which functions were assigned in the study are presented. The elution fractions from cobalt affinity chromatography were pooled for each protein and analyzed by 12% SDS-PAGE.

Unlike the complexity seen in the endoxylanases, the six polypeptides identified as accessory enzymes that aid the endoxylanases to release monosaccharides from xylans were simpler in domain architecture. Based on biochemical analysis, only three of the polypeptides (Xyl3A, Ara51A, and Agu67A) exhibited such activities (Fig.2.1A), and their respective genes have been assigned the Genbank accession numbers JF314321, JF314323, and JF314325, respectively. The *R. albus* 8 β -xylosidase (Xyl3A), α -L-arabinofuranosidase (Ara51A), and α -glucuronidase (Agu67A) were composed of 691, 489, and 650 amino acids, respectively, and they were composed of only their respective catalytic modules, i.e., GH3, GH51, and GH67 family modules. Therefore, from Fig. 2.1A, our biochemical analysis did not lead to functional assignments for Xyl43A, Ara43A, and Agu2A, which were assigned Genbank accession numbers of JF314322, JF314324, and JF314326, respectively.

Cloning, expression, and purification of multiple endoxylanases and their accessory enzymes from *R. albus* 8- The product of each gene was expressed as a polypeptide with a N-terminal hexa-histidine tag to facilitate protein purification. The PCR primers (Table 2.1) were designed to remove the signal peptides, where present, to ensure accumulation of recombinant proteins intracellularly during heterologous gene expression in *E. coli* cells. The predicted molecular masses of Xyn10A, Xyn10B, Xyn11D, Xyn11E, and Xyn11F were 45.0, 90.5, 81.3, 73.7, and 57.5 kDa, respectively. As shown in Fig. 2.1B, the molecular masses estimated by SDS-PAGE analysis agreed with the predicted values. The predicted molecular masses of Xyl3A, Ara51A, and Agu67A were 77.3, 57.1, and 75.4 kDa, respectively, and therefore agreed with the estimates from SDS-PAGE analysis.

Table 2.1. Primers used for cloning genes predicted to degrade xylan.

Primer names	Primers	
	Orientation	Sequence (5' → 3') ^a
Xyn10A ^b (ORF2725)	Forward	5'- <u>GACGACGACAAGATG</u> CTGACAGGCTGCGGCTCTTC-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> TATTTTTCTGCAAGCGATGGGTCTTTTGC-3'
Xyn10B (ORF2882)	Forward	5'- <u>GACGACGACAAGATGG</u> CACCTGTAAGCGCACAGGAGGTC-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> TACATAACAGTTACGGTAACTGCGTTTTTGATAGC-3'
Xyn11D (ORF997)	Forward	5'- <u>GACGACGACAAGATGG</u> ACCAGACTTATTACGACAATAAGACAGGTACC-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> TCAGGATACTTTGGTGTATATCTGACCGC -3'
Xyn11E (ORF1984)	Forward	5'- <u>GACGACGACAAGATGG</u> ACGATGACAGCAGATCGGTTTCTGC-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> TATCCGATCTTCCTGTAGATAGTCCCGC-3'
Xyn11F (ORF2008)	Forward	5'- <u>GACGACGACAAGATGG</u> GAGAAGGTGCTTTACACATCGCCGC-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> TACCTTACAGTAACAACATATAGCATTCTTGATGGC-3'
Xyl3A (ORF2287)	Forward	5'- <u>GACGACGACAAGATG</u> AAAAAATATTTAGACGAGTCGCTTTCCGGCTGAG-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> TCACTTGTATCTCGGTGCTTACGC-3'
Xyl43A (ORF1302)	Forward	5'- <u>GACGACGACAAGATGG</u> CCGCAAACAAAGCCCGTGATC-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> TAAAGCTAGTGCCATTTTTCTCAGCAGTAC-3'
Ara51A (ORF3031)	Forward	5'- <u>GACGACGACAAGATG</u> AAAAAACTCAAAGCAGTCGTAACACATTAGACA-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> TCAAAAAAGTCTTACGCTTACTACCGACAG-3'
Ara43A (ORF3046)	Forward	5'- <u>GACGACGACAAGATGG</u> ACGAAAAACTGATCTTCAACAAACCCTGG-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> TTAGTTGTGCGTTACATTATCAAAGCTGAACAC-3'
Agu67A (ORF2369)	Forward	5'- <u>GACGACGACAAGATGG</u> TATTTCAAACCTCTGGCTGAACTACAAAAAACTCG-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> TCAGTTGTATATCTTTGCCCCCTGC-3'
Agu2A (ORF3451)	Forward	5'- <u>GACGACGACAAGATG</u> ATAAATAAAATCGACTTGTGCGGGGCTTG-3'
	Reverse	5'- <u>GAGGAGAAGCCCGG</u> CTATCTGCCTGCCGCATAATCGGTCAGCG-3'

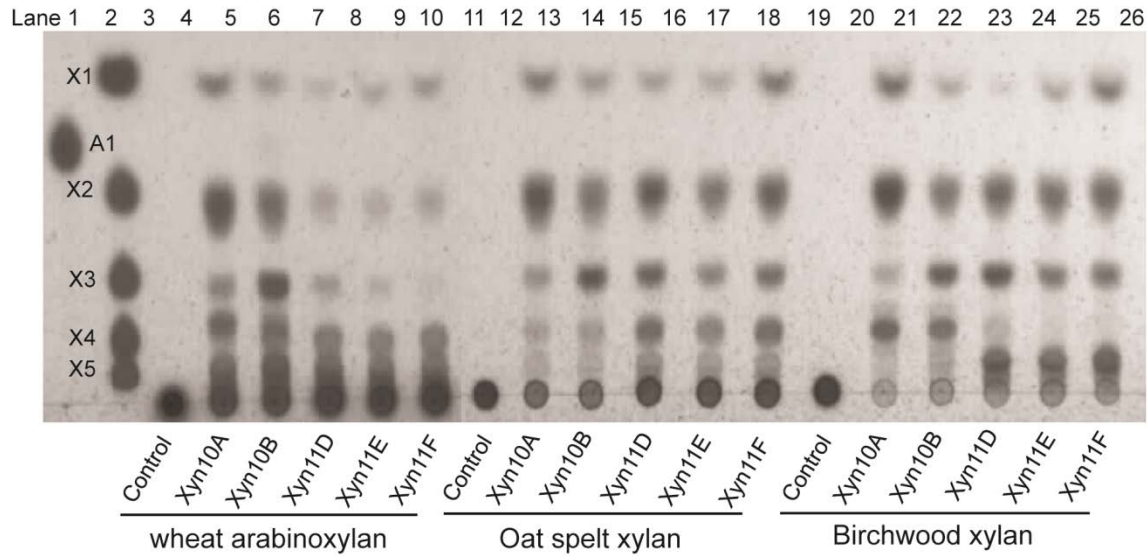
^a Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

^b Primers were designed to clone the respective gene in frame with an N-terminal hexahistidine fusion tag encoded by the plasmid vector, pET-46b using ligation independent cloning according to the Ek/LIC protocol from Novagen (San Diego, CA). Underlined sequence indicates sequence complementary to the plasmid vector.

Enzymatic activities of the five putative endoxylanases from *R. albus* 8- To determine whether the putative endoxylanases possess their predicted activities, each enzyme was examined for release of products from three different xylan sources. The substrates were soluble wheat arabinoxylan (WAX), oat spelt xylan (OSX), and birchwood xylan (BWX). As shown in Fig. 2.2A, each enzyme released products ranging from xylose to xylo-oligosaccharides of varying lengths from all three substrates. The patterns of the products released by the GH10 proteins, in general, were different from those of the GH11 proteins. Thus from WAX, the GH10 enzymes were more efficient in releasing xylobiose and xylotriose compared with the GH11 enzymes. Likewise, with birchwood xylan, the GH10 enzymes released a product that showed similar migration to xylo-tetraose on the TLC. In contrast, on birchwood xylan, the GH11 enzymes released a product that migrated similarly to xylo-pentaose, and this product was missing in the hydrolyzates of the GH10 proteins. Similar product release patterns were, however, observed for all five enzymes with oat spelt xylan, although release of the xylo-tetraose-like product was less with the GH10 enzymes. None of the enzymes released arabinose from the three substrates. Therefore, the biochemical data confirmed the five proteins as endoxylanases.

As shown in Fig. 2.2B, each of the endoxylanases showed the highest specific activity on the less complex substrate WAX. In general, the GH11 endoxylanases also showed higher activities on WAX than the GH10 enzymes. For Xyn10A, Xyn10B, and Xyn11D, the specific activities were higher on OSX than BWX. This observation was more pronounced for Xyn10B and Xyn11D compared with Xyn10A. The Xyn11E and Xyn11F of *R. albus* 8, by contrast, exhibited similar specific activities for OSX and BWX.

A.



B.

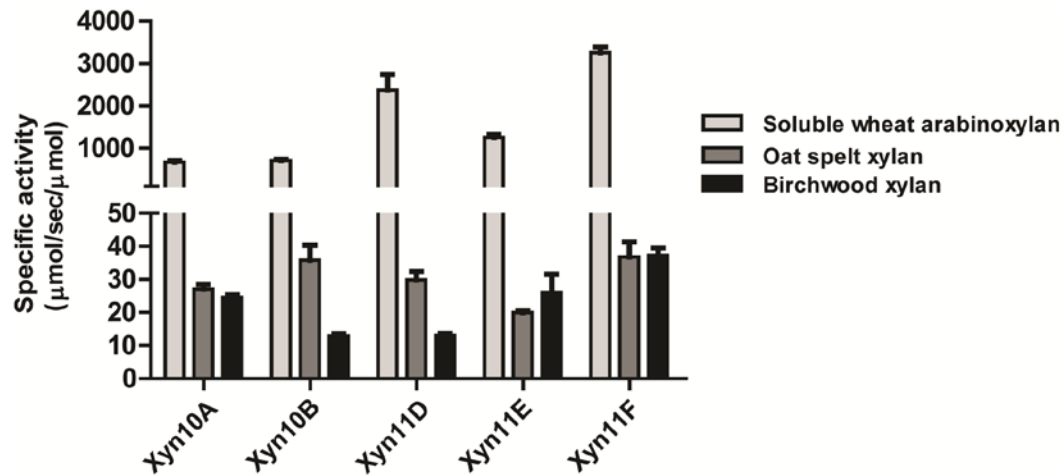


Figure 2.2. Hydrolysis of xylan substrates by five putative endoxylanases from *R. albus* 8. (A) Thin layer chromatography (TLC) analysis of end products of hydrolysis. The *R. albus* 8 putative endoxylanases Xyn10A, Xyn10B, Xyn11D, Xyn11E, and Xyn11F (final 0.5 μM each) were incubated with wheat arabinoxylan (WAX) or oat spelt xylan (OSX) or birchwood xylan (BWX) at 37 $^{\circ}\text{C}$ for 15 h. Each substrate was at a final concentration of 1% (w/v). The products were resolved by TLC followed by staining with methanolic orcinol. Arabinose (A1) and xylo-oligosaccharide standards (X1-X5) were spotted on the plate in lanes 1 and 2, respectively, to serve as markers for the identification of the released products. (B) The specific activities of the five endoxylanases. Each xylan substrate (final 1% (w/v)) in 50 mM Na-phosphate buffer with 150 mM NaCl (pH 6.5) was incubated with each endoxylanase for determination of specific activities. The final concentrations of the enzymes were 50 nM for reactions with soluble wheat arabinoxylan and 100 nM for oat spelt xylan and birchwood xylan as substrate. Bars are shown with standard errors for three independent experiments.

Carbohydrate Esterase (CE) activities of the *R. albus* 8 endoxylanases-

Domain architecture suggested that two of the *R. albus* 8 endoxylanases harbor carbohydrate esterase activities (Fig. 2.1A). The esterase modules belong to CE4 family, which has been reported to encode activities such as acetyl xylan esterase, chitin deacetylase, and peptidoglycan N-acetylmuramic acid deacetylase (<http://www.cazy.org/>). Therefore, all five enzymes were tested for their capacity to release products from the artificial substrate *p*NP-acetate and acetylated oat spelt xylan, a natural substrate. Only Xyn11D showed detectable activity on the two substrates, and the activity on the natural substrate was about five times higher than on *p*NP-acetate (Fig. 2.3). There was no activity detected for Xyn11E, although its CE4 module shared 57% identity with that of Xyn11D (Fig. 2.3).

Carbohydrate binding activities of the *R. albus* 8 endoxylanases- Other than Xyn10A, each of the *R. albus* 8 endoxylanases harbored either one or more CBMs. The proteins were, therefore, analyzed for binding to two potential substrates: insoluble oat spelt xylan (is-OSX) and insoluble birchwood xylan (is-BWX). Little to no binding activity was detected for Xyn10A with each of the substrates, as expected, and negligible binding activity was detected with Xyn11E. A larger proportion of Xyn10B, Xyn11D, and Xyn11F, however, bound to is-OSX, suggesting that at least one of the multiple CBMs in each of these polypeptides recognizes linkages in is-OSX (Fig. 2.4). The three endoxylanases also bound to is-BWX, although the amount of proteins that bound to the same amount of substrate as is-OSX was about half in each case, suggesting that the proteins have higher binding affinity for is-OSX.

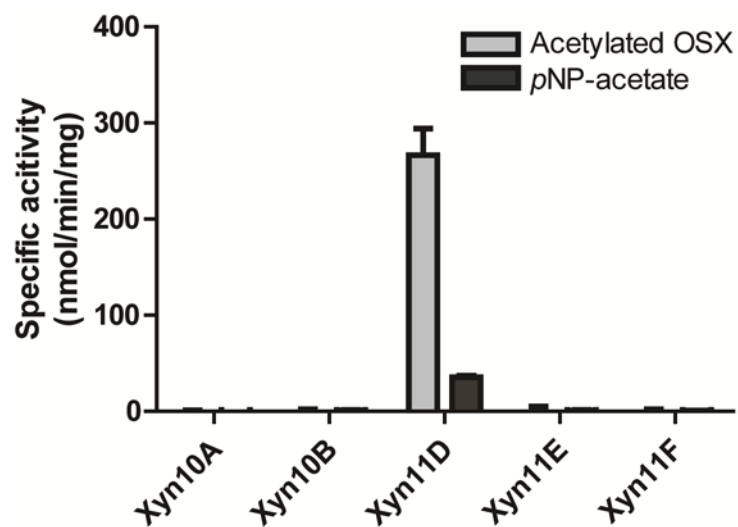


Figure 2.3. Carbohydrate esterase activities of the five endoxylanases from *R. albus* 8. Each endoxylanase (final 0.5 μ M each) was incubated with either acetylated OSX (final 2% (w/v)) or pNP-acetate (final 1 mM). The specific activities were then estimated by determining the release of acetic acid using an acetic acid detection kit or the release of pNP at an absorbance of 400 nm.

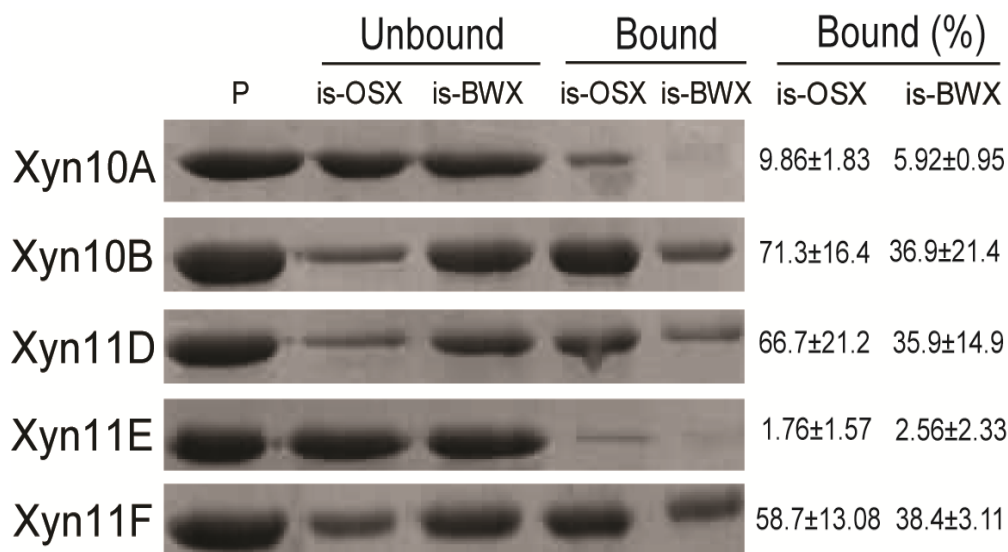


Figure 2.4. Qualitative binding studies of the five endoxylanases from *R. albus* 8. The insoluble OSX (is-OSX) or the insoluble BWX (is-BWX) was incubated with enzyme (2 μ M) at 4°C. Each substrate was present at 2% (w/v). Lane P represents the same amount of protein (2 μ M) incubated in the same buffer, but without substrate. After centrifugation, the supernatants after incubation with is-OSX or is-BWX were loaded on SDS-PAGE as the unbound proteins. The precipitated (pelleted) fractions were washed three times with buffer and eluted with the same volume of loading buffer and loaded on SDS-PAGE gels as the bound proteins. The percentages of bound proteins were calculated by analyzing the intensities of the protein bands using Gene Tools software program.

Enzymatic activities of accessory enzymes for xylan hydrolysis- Six different proteins were predicted to participate with endoxylanases to release fermentable sugars from xylans. These included two putative xylosidases (Xyl3A and Xyl43A), two putative arabinofuranosidases (Ara51A and Ara43A), and two putative glucuronidases (Agu67A and Agu2A) (Fig. 2.1A). In each case, the predicted activity was detected in only one of the two proteins. Thus, Xyl3A, Ara51A, and Agu67A exhibited β -xylosidase, α -L-arabinofuranosidase, and α -glucuronidase activities, respectively. The activity of the β -xylosidase was initially demonstrated with *p*NP- β -D-xylopyranoside, and although the enzyme also released some products from *p*NP- α -L-arabinofuranoside, this activity was very low (Fig. 2.5A). The results thus suggested that Xyl3A possesses both β -xylosidase and α -L-arabinofuranosidase activities. Screening for activity with different *p*NP-linked sugars also suggested that Ara51A possesses α -L-arabinofuranosidase activity. The enzyme was further tested with natural substrates such as wheat arabinoxylan, sugar beet arabinan, larch arabinan, and oat spelt xylan, and the arabinose released was quantified as the amount of reducing ends resulting from the enzymatic hydrolysis (Fig. 2.5B). The product from incubation with wheat arabinoxylan was confirmed as arabinose through TLC (results not shown). The α -glucuronidase activity was determined by use of aldouronic acids (2 parts aldotetrauronic acid: 2 parts aldotriuronic acids: 1 part aldobiuronic acid) as substrates. As shown in Fig. 2.6, incubation of aldouronic acids with Agu67A led to release of undecorated xylo-oligosaccharides, such as xylobiose, xylotriose, and xylotetraose. Incubating Xyl3A with the aldouronic acids led to an increased amount of xylose (97.3 mM) compared to the control or substrate without enzyme (15.6 mM), suggesting that

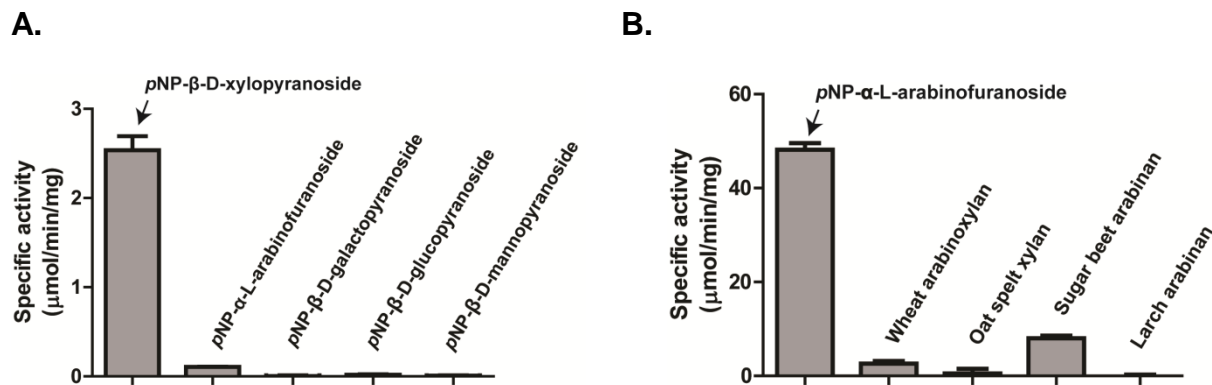


Figure 2.5. Functional analyses of the gene products of the *R. albus* 8 *xyl3A* and *ara51A*. (A) Hydrolysis of *pNP*-linked sugars by *R. albus* 8 Xyl3A. The Xyl3A protein was incubated with different sugars linked to *pNP* and the cleavage of the linkage estimated as the amount of *pNP* released into the reaction mixture. (B) Hydrolysis of arabinose containing substrates by *R. albus* 8 Ara51A. The catalytic activity of Ara51A was estimated by use of *pNP*-linked substrates and also with natural substrates (wheat arabinoxylan, oat spelt xylan, sugar beet arabinan, and larch arabinan). The catalytic activity on *pNP*-linked substrates were determined as described in (A), and the activities on the natural substrates were determined by quantifying the amounts of reducing ends released during incubation with Ara51A.

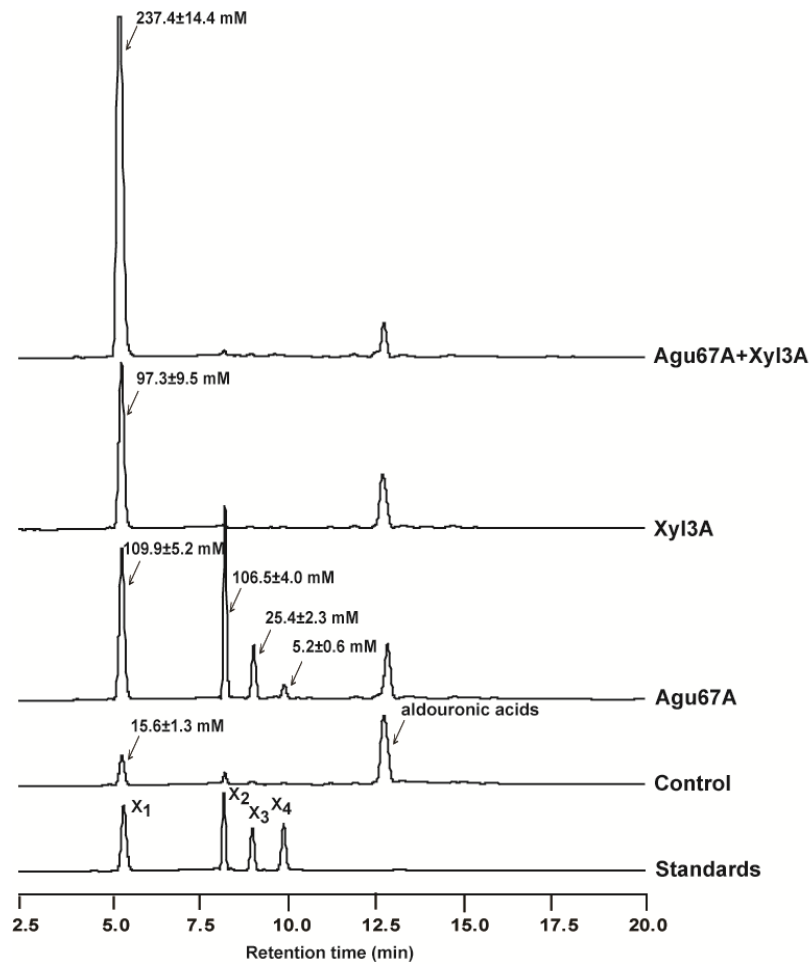


Figure 2.6. Functional analyses of the *R. albus* 8 Agu67A. The catalytic activity of the *R. albus* Agu67A was determined by its capacity to release xylo-oligosaccharides from aldouronic acids and also to function synergistically with a β -xylosidase (*R. albus* 8 Xyl3A) to release xylose from aldouronic acids. The *R. albus* 8 Agu67A (final 0.5 μ M) was incubated with aldouronic acids (final 0.12% (w/v)) for 1 hour at 37 °C in the absence or presence of Xyl3A (final 0.5 μ M). The hydrolytic products were analyzed by HPAEC-PAD and identified by comparison of peaks with retention times of standard xylo-oligosaccharides.

Xyl3A is able to cleave xylose from the aldouronic acid mixture or that there were xylo-oligosaccharides lacking the glucuronic acid side chain in the substrate. As expected, the two enzymes acted synergistically to release large amounts of xylose (237.4 mM) from the aldouronic acids. Thus, the α -glucuronidase, Agu67A, was able to cleave the methyl glucuronic acid side chains, making the xylo-oligosaccharides available for hydrolysis to xylose by Xyl3A.

Synergistic activities of individual endoxylanases with accessory enzymes in xylan hydrolysis- The capacities of Xyl3A and Ara51A to function synergistically with the various endoxylanases from *R. albus* 8 were investigated. On WAX, OSX and BWX, Xyl3A released xylose and no arabinose (Table 2.2). This observation suggests that some degraded substrates were already present in the substrate, allowing cleavage from the ends by Xyl3A. Ara51A, on the other hand, released arabinose from WAX and OSX (Table 2.2) as was expected (Fig. 2.5B). Irrespective of the enzymes used to hydrolyze birchwood xylan, arabinose was not released, suggesting that there is little to no arabinose in this substrate or the linkages are not susceptible to the enzymes. The Xyl3A and Ara51A of *R. albus* 8 functioned synergistically to release products, at least from WAX and OSX (Table 2.2). In the presence of Xyl3A and Ara51A, each endoxylanase from *R. albus* 8 released amounts of xylose and arabinose from wheat arabinoxylan and oat spelt xylan that suggested synergistic activities among the three enzymes (Table 2.2). The family 10 endoxylanases (Xyn10A and Xyn10B) released more products (both xylose and arabinose) than the family 11 enzymes when incubated with the accessory enzymes with WAX as the substrate. In contrast, on the more complex substrates (OSX and BWX), the family 11 endoxylanases released more

Table 2.2. Synergistic effects of multiple endoxylanases with Xyl3A and Ara51A

Endo-xylanase	Xyl3A	Ara51A	Wheat arabinoxylan ^a		Oat spelt xylan ^a		Birchwood xylan ^a	
			Xylose (mM)	Arabinose (mM)	Xylose (mM)	Arabinose (mM)	Xylose (mM)	Arabinose (mM)
-	+	-	3.42±0.71	N.D. ^b	8.83±1.70	N.D.	8.08±0.25	N.D.
-	-	+	N.D.	33.5±7.42	N.D.	4.90±0.39	N.D.	N.D.
-	+	+	40.3±7.50	41.5±5.94	24.6±2.23	6.14±0.08	10.3±1.22	N.D.
Xyn10A	+	+	152.6±1.31	76.8±2.21	94.9±3.05	13.6±1.13	89.6±5.11	N.D.
Xyn10B	+	+	137.9±3.00	65.6±2.39	65.1±0.98	10.6±0.54	71.1±2.05	N.D.
Xyn11D	+	+	128.4±11.56	56.2±3.74	165.7±7.26	21.2±1.01	117.5±3.96	N.D.
Xyn11E	+	+	114.2±22.07	53.2±2.32	119.7±1.96	17.2±0.30	83.3±8.67	N.D.
Xyn11F	+	+	109.7±17.11	53.4±1.72	150.5±5.11	19.8±2.53	99.3±14.4	N.D.

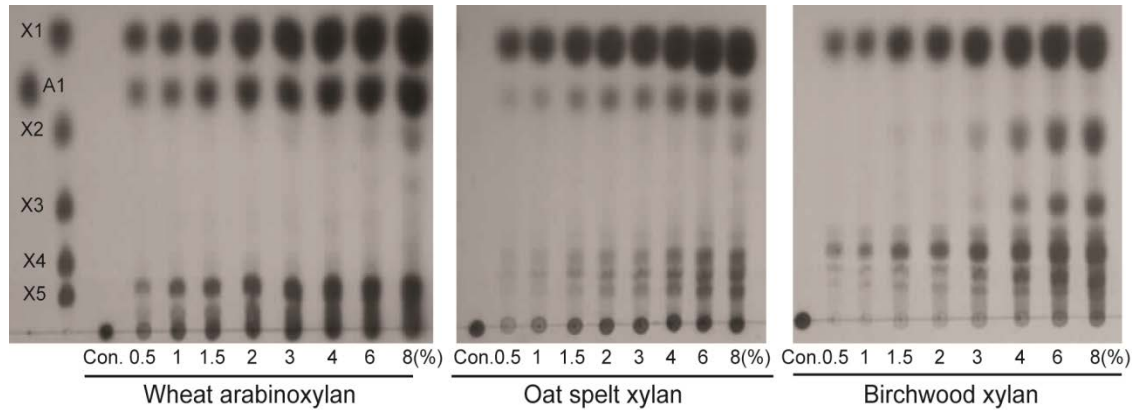
^aThe experiments were performed in triplicates, and data are reported as means ± standard deviation from the mean. Each enzyme, either endoxylanase or accessory protein (Xyl3A or Ara51A) was present at a concentration of 0.5 μM. The respective enzyme or a combination of enzymes was incubated with the xylan substrates (8% (w/v)) in 50 mM Na-phosphate buffer with 150 mM NaCl (pH 6.5) at 37 °C for 15 h. The products were determined by an HPLC method as described in the Materials and Methods. ^bN.D. indicates that the concentrations for xylose and arabinose were below the detection limit.

products in synergy with Xyl3A and Ara51A when compared with the GH10 endoxylanases functioning together with the accessory enzymes (Table 2.2).

Synergistic activities of the five endoxylanases with accessory enzymes in xylan hydrolysis- Our recent analysis of four GH3 enzymes from the rumen xylanolytic bacterium *Prevotella bryantii* suggested that multiple β -xylosidases in the same organism do not have redundant activities [94]. We, therefore, analyzed the effect of incubating the five endoxylanases together with the accessory enzymes in the presence of increasing amounts of WAX, OSX, and BWX. As shown in both Fig. 2.7A and 2.7B, at the same enzyme concentration, increasing any of the three substrates led to release of more products. Whereas on wheat arabinoxylan and oat spelt xylan both xylose and arabinose were detected (Fig. 2.7A or TLC data), arabinose was absent in the hydrolysis products of birchwood xylan. There were more products released from the less complex WAX compared with the more complex OSX and BWX (Fig. 2.7B). In fact, at higher substrate concentrations (>2%) almost twice the reducing ends from oat spelt xylan and birchwood xylan were detected for soluble wheat arabinoxylan.

Optimization of enzymes to yield larger amounts of monosaccharides- At a substrate concentration of 8%, accumulation of larger products were observed for each substrate (Fig. 2.7A). Thus, the enzyme concentrations were varied to determine whether the larger products can be converted to monomeric sugars. In lane 2 of Fig. 2.8, 2.9, 2.10, and 2.11, the enzyme concentrations were the same as in the experiment for 8% substrate concentration in Fig. 2.7. The five endoxylanases are present at 0.1 μ M each (Fig. 2.8, 2.9, 2.10, 2.11). Increasing the concentration of the endoxylanases rather decreased product yield in the hydrolysis of WAX (Fig. 2.9 lane 3). Lane 4 shows

A.



B.

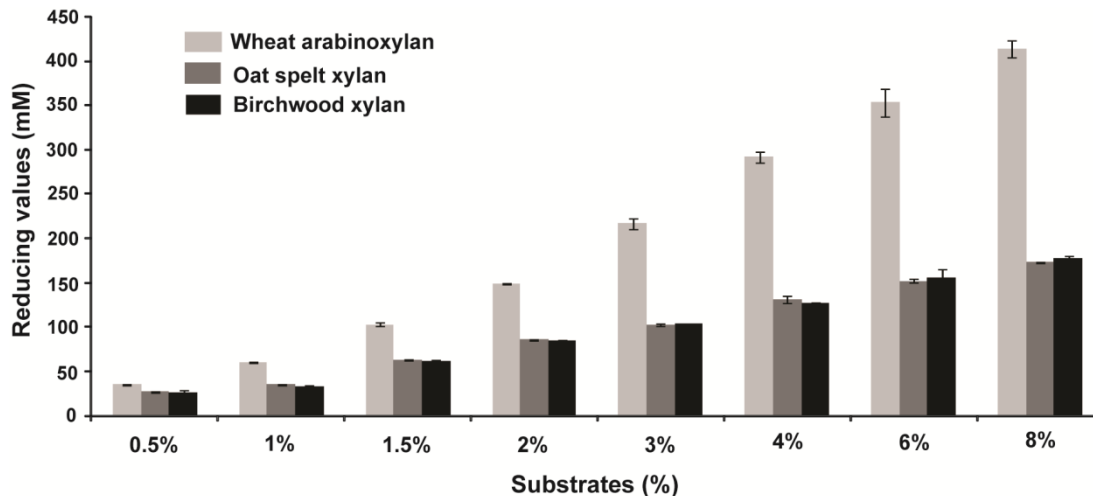


Figure 2.7. Hydrolysis of increasing amounts of xylan substrates by *R. albus* 8 endoxylanases in the presence of two accessory enzymes (β -xylosidase and α -L-arabinofuranosidase). (A) Thin layer chromatography of products of hydrolysis by *R. albus* 8 xylan hydrolyzing enzymes. In each lane, other than the control where enzymes were not added, the enzymes were added as a mixture of endoxylanases (Xyn10A, Xyn10B, Xyn11D, Xyn11E, and Xyn11F at 0.1 μ M each), β -xylosidase (Xyl3A at 0.5 μ M), and α -L-arabinofuranosidase (Ara51A at 0.5 μ M). The enzyme mixtures were incubated with xylan substrates (wheat arabinoxylan or oat spelt xylan or birchwood xylan at 0.5 to 8% (w/v)) for 15 h at 37 $^{\circ}$ C. The hydrolysis products (0.5 μ L) were resolved by TLC and staining with methanolic orcinol. Xylo-oligosaccharide standards (X1-X5) and arabinose (A1) were also spotted on the TLC plates as markers. (B) Quantification of products in the experiment shown in (A) with a reducing sugar assay. The reducing sugar concentrations were calculated from the absorbance at 410 nm with comparison to a standard curve generated with known concentrations of glucose.

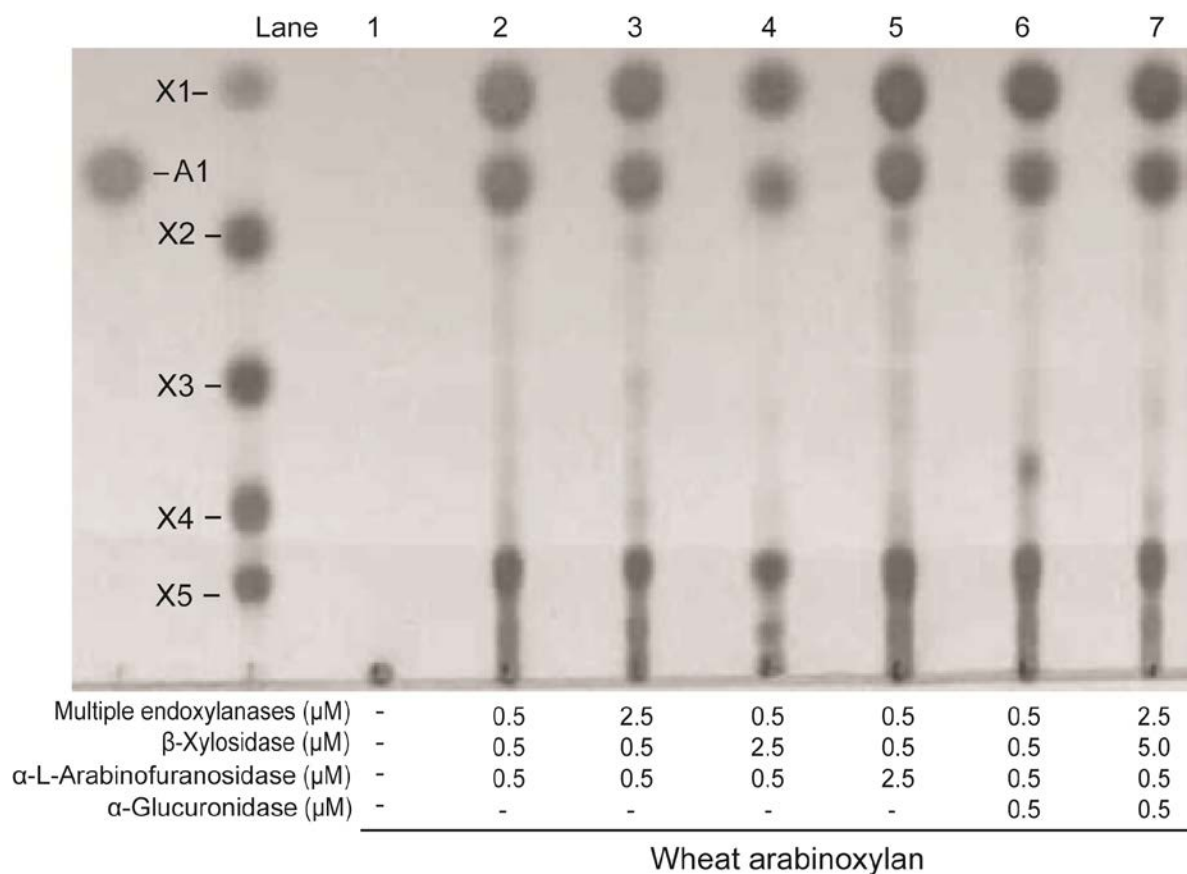


Figure 2.8. Optimization of xylan (soluble WAX) hydrolysis with the five endoxylanases and their accessory enzymes from *R. albus* 8. In lane 1 is substrate without enzymes to serve as a control experiment. The experiment in figure 2.7 with 8% of the appropriate substrate was repeated in lane 2, and the concentration of the multiple xylanases were increased (2.5 μM) in lane 3, β -xylosidase concentration was increased (2.5 μM) in lane 4, α -L-arabinofuranosidase concentration was increased (2.5 μM) in lane 5, α -glucuronidase was added (0.5 μM) in lane 6, and both the multiple endoxylanases and β -xylosidase were increased in lane 7. The products of hydrolysis were resolved by TLC and stained with methanolic orcinol. Arabinose (A1) and xylo-oligosaccharides (X1-X5) were used as standards.

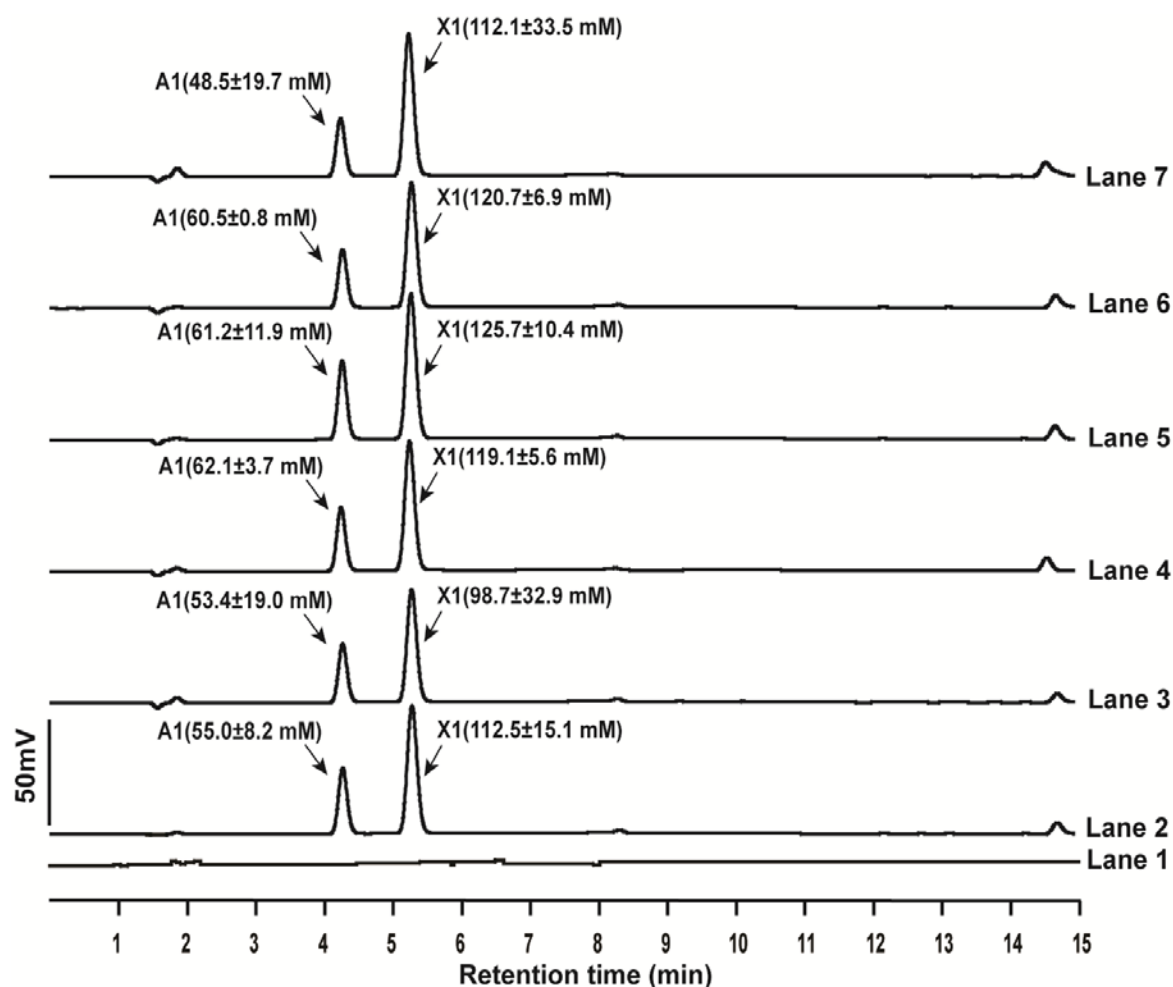
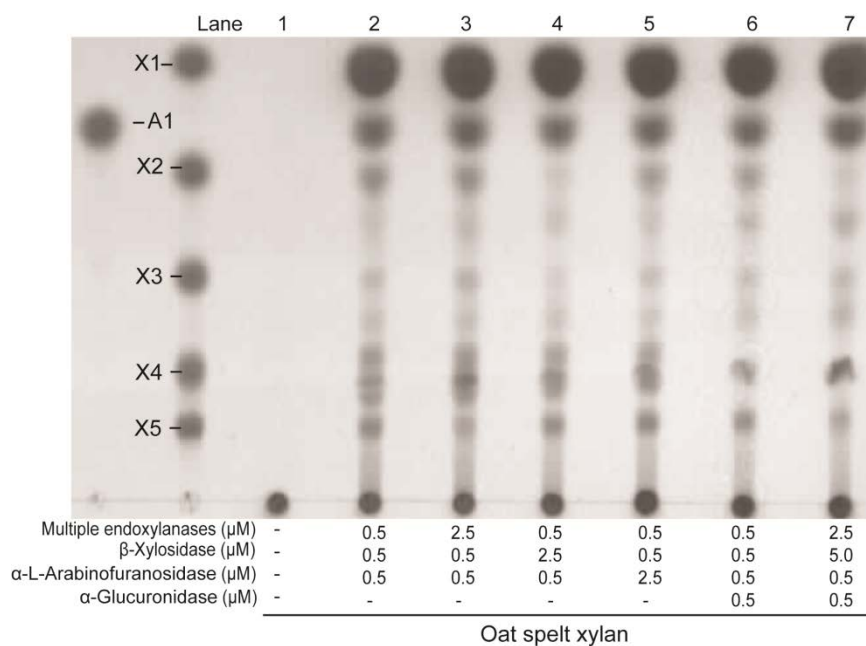


Figure 2.9. HPAEC analysis of xylan (soluble WAX) hydrolysis with the five endoxylanases and their accessory enzymes from *R. albus* 8. Quantification of monosaccharides released during optimization of soluble wheat arabinoxylan hydrolysis by *R. albus* 8 enzymes. The products of hydrolysis presented in figure 2.8 were analyzed by high performance anion exchange chromatography (HPAEC) followed by detection with a pulsed amperometric detector (PAD). The products of hydrolysis were identified by comparison of peaks with retention times of standards; arabinose (A1) and xylo-oligosaccharides (X1-X3). Standard curves were derived to estimate the amounts of monosaccharides released in each experiment. All experiments were performed three times and representative curves were shown. The values of monosaccharides released represent the mean of three experiments \pm standard error.

A.



B.

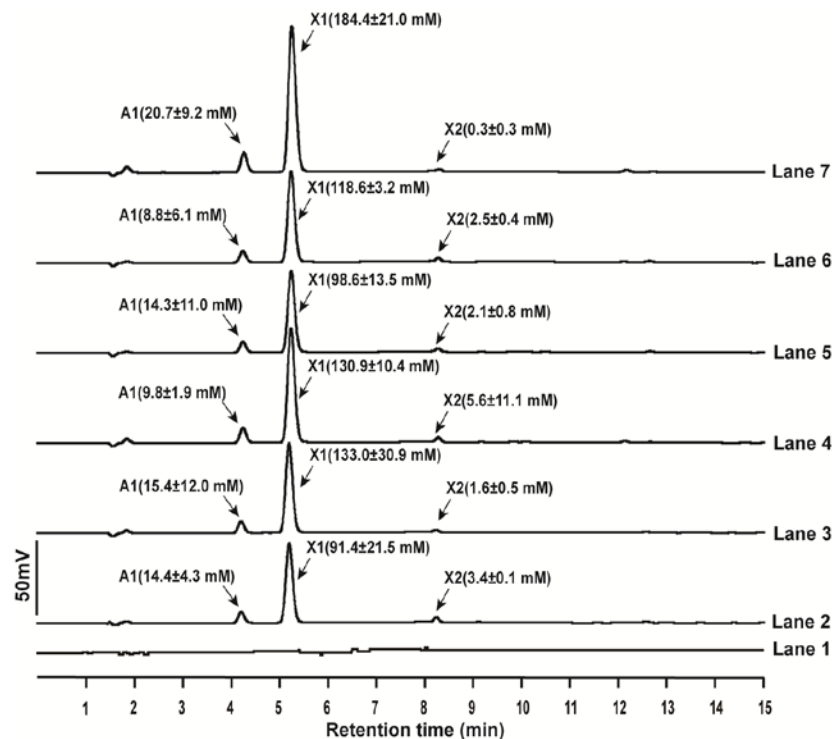
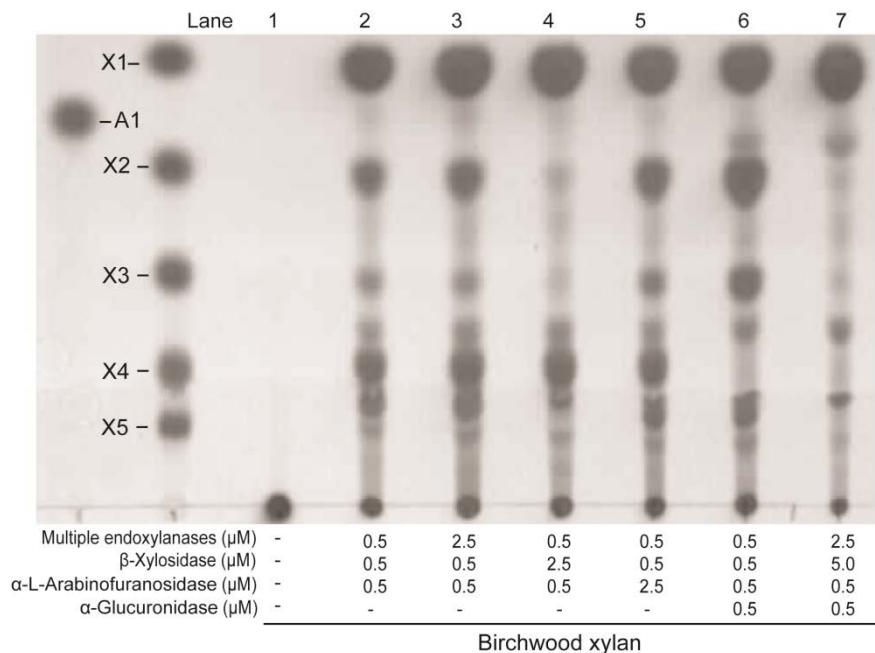


Figure 2.10. Optimization of xylan (oat spelt xylan) hydrolysis with the five endoxylanases and their accessory enzymes from *R. albus* 8. (A) The experiments are the same as described in Fig. 2.8 except for oat spelt xylan as the substrate. (B) The experiments are the same as described in Fig. 2.9 except for oat spelt xylan as the substrate.

A.



B.

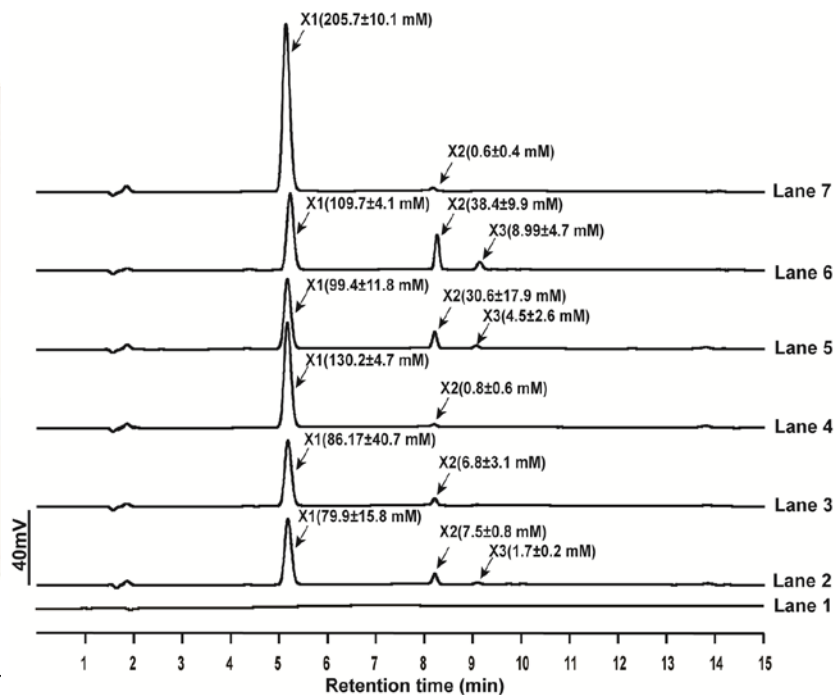


Figure 2.11. Optimization of xylan (birchwood xylan) hydrolysis with the five endoxylanases and their accessory enzymes from *R. albus* 8. (A) The experiments are the same as described in Fig. 2.8 except for birchwood xylan as the substrate. (B) The experiments are the same as described in Fig. 2.9 except for birchwood xylan as the substrate.

that increasing the β -xylosidase slightly increased the yields of arabinose (55 mM versus 62 mM) and xylose (112.5 mM versus 119.1 mM). A similar observation was made for lane 5, where the arabinofuranosidase concentration was increased. Addition of the α -glucuronidase at the same concentration as the other enzymes (0.5 μ M) also led to increased product release (lane 6). Since a product migrating to the same position as xylopentaose was observed (Fig. 2.8), the concentrations of the endoxylanases and the β -xylosidase were increased by 5 and 10 times, respectively, and also α -glucuronidase was added. However, this did not result in either removal of this product or an increase in release of monosaccharides (lane 7). Note, however, that a product that migrated between X3 and X4 in the presence of α -glucuronidase was absent in lane 7 (Fig. 2.8, 2.9). Similar experiments, as described for WAX, were carried out for the more complex xylans, and all experiments led to increases in monosaccharide yield. Interestingly, the enzyme concentration in lane 7 that did not yield any effect on soluble WAX led to twice the amount of xylose released in OSX (lane 2 versus lane 7 of Fig. 2.10B), and similar results were also obtained for birchwood xylan (Fig. 2.11A and 2.11B). With birchwood xylan, adding the α -glucuronidase led to an increase in products including the levels of xylobiose and xylotriose (lane 6). By adding more endoxylanases and β -xylosidase to the reaction mixture about 2.5 fold of xylose was released compared to the initial level of products (Fig. 2.11B, lane 2 versus lane 7).

Hydrolysis of accumulating large size products- The incompletely degraded products of WAX (SWAX-P1 and SWAX-P2 in lane 2 of Fig. 2.12A and 2.12B) were initially digested with a β -xylosidase and an arabinofuranosidase, since this substrate is

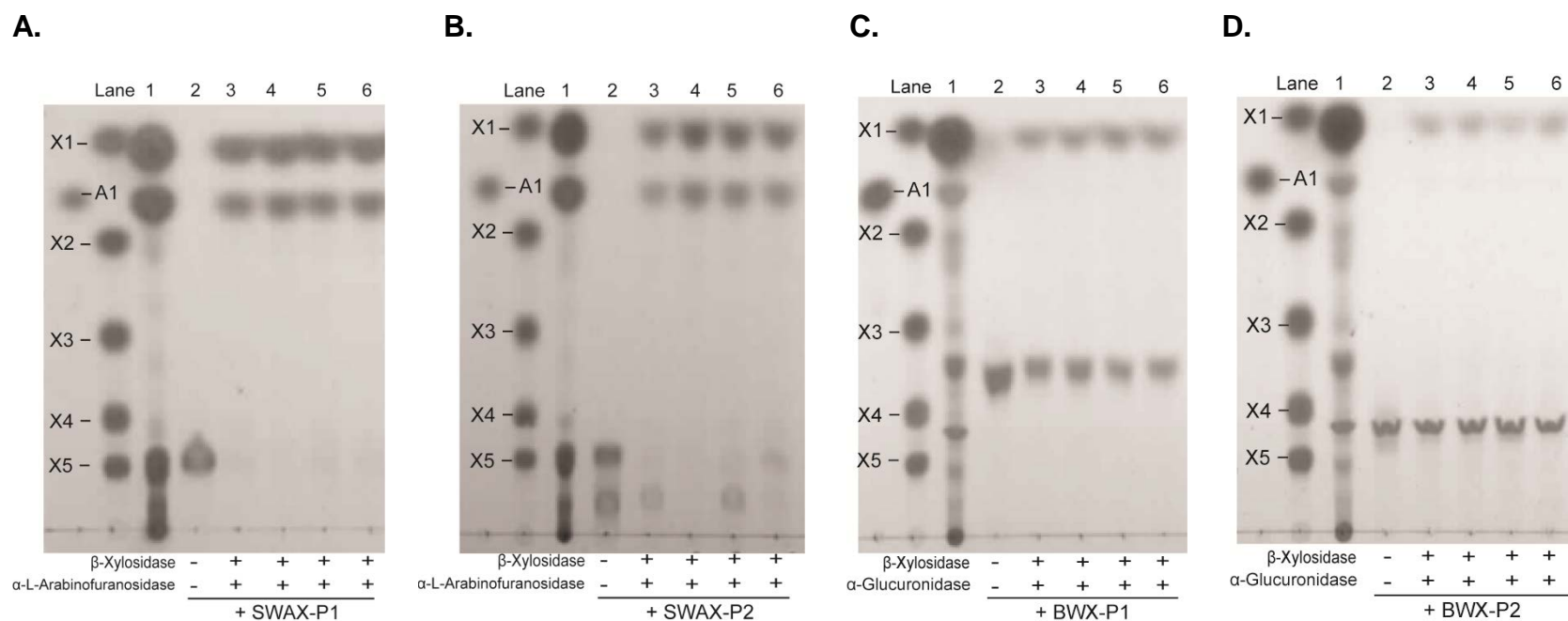


Figure 2.12. Enzymatic treatments of undegraded products with enzyme mixtures. β -Xylosidase and α -L-arabinofuranosidase (final 5 μ M each) were reacted with each product (final 0.17% for SWAX-P1 or SWAX-P2 and final 0.31% for BWX-P1 or BWX-P2) in a 50 mM Na-phosphate buffer containing 150 mM NaCl (pH 6.5) at 37 $^{\circ}$ C for 15 hours. (A) Enzymatic treatment of SWAX-P1. Lane 1, soluble WAX hydrolysis products (Lane 7 sample in figure 2.8); lane 2, SWAX-P1 before enzymatic reaction; lane 3, SWAX-P1 after reaction with β -xylosidase (Xyl3A) and α -L-arabinofuranosidase (Ara51A) from *R. albus* 8; lane 4, SWAX-P1 after reaction with β -xylosidase (Xyl3A) from *Prevotella bryantii* B₁₄. and α -L-arabinofuranosidase (Ara51A) from *R. albus* 8; lane 5, SWAX-P1 after reaction with β -xylosidase (Xyl3B) from *Prevotella bryantii* B₁₄. and α -L-arabinofuranosidase (Ara51A) from *R. albus* 8; lane 6, SWAX-P1 after reaction with β -xylosidase (Xyl3C) from *Prevotella bryantii* B₁₄. and α -L-arabinofuranosidase (Ara51A) from *R. albus* 8. (B) Enzymatic treatment of SWAX-P2. The experiments in the lanes are the same as described in Fig. 2.12A, except for the substrate. (C) Enzymatic treatment of BWX-P1. The experiments in the lanes are the same as described in Fig. 2.12A except for the use of α -glucuronidase (Agu67A) instead of α -L-arabinofuranosidase (Ara51A). (D) Enzymatic treatment of BWX-P2. The experiments in the lanes are the same as described in Fig. 2.12C, except for the substrate.

mostly β -1,4 linked xylose chains with arabinose side chains. The Xyl3A of *R. albus* 8 and several β -xylosidases from the rumen bacterium *P. bryantii* B₁4 (Xyl3A, Xyl3B, and Xyl3C) were tested. It was observed that the arabinofuranosidase released arabinose and products similar to xylo-oligosaccharides from SWAX-P1 (Fig. 2.13B). Thus, various combinations of β -xylosidases with the arabinofuranosidase were investigated for the capacity to degrade SWAX-P1 and SWAX-P2. As shown in Fig. 2.12A and 2.12B, these incompletely degraded products were converted to xylose and arabinose, suggesting that the undegraded WAX products were short xylose chains decorated with arabinose. A similar experiment was carried out for the incompletely degraded products from birchwood xylan hydrolysis. In this case, we used the different β -xylosidases and α -glucuronidase, since glucuronic acid side chains are common in this substrate. Although no hydrolysis of the substrate (BWX-P1) was observed with the β -xylosidases (Fig. 2.14A), a product that migrated above the substrate was seen on reacting with α -glucuronidase (Fig. 2.14B). The diverse β -xylosidases and the *R. albus* α -glucuronidase were, therefore, used in combination to digest BWX-P1 (Fig. 2.12C) and BWX-P2 (Fig. 2.12D). In each case xylose was released, although there also remained undegraded product, suggesting that there are linkages that are not susceptible to the two enzymes. Addition of an acetyl xylan esterase and a ferulic acid esterase to the two enzymes did not enhance hydrolysis of either BWX-P1 or BWX-P2 by the β -xylosidases and α -glucuronidase (results not shown).

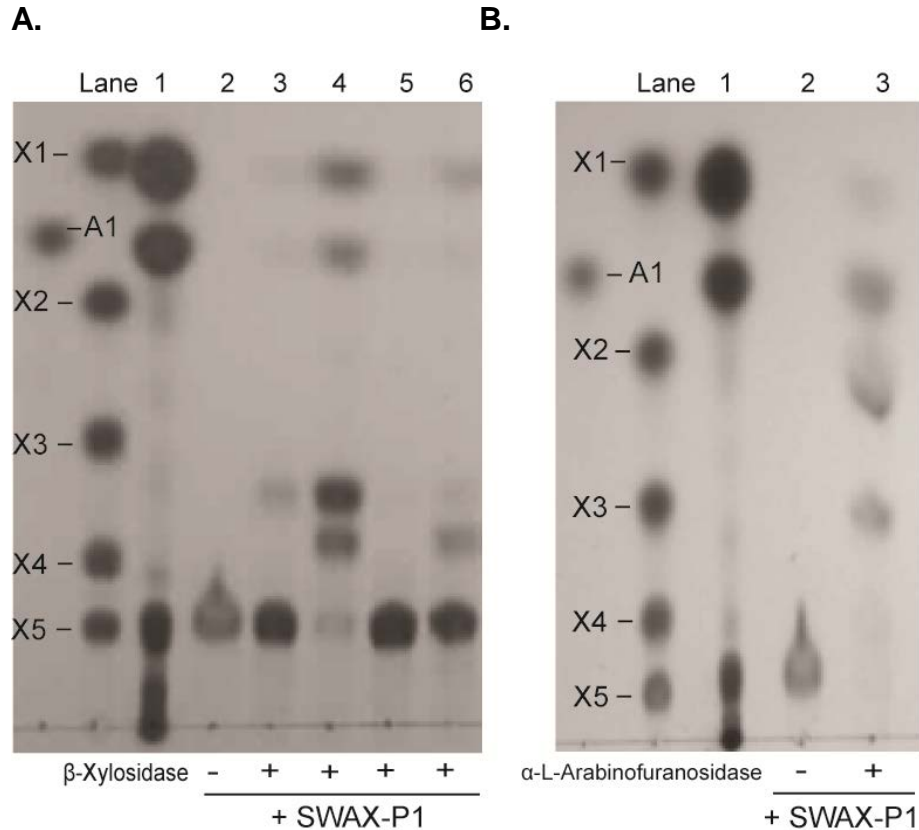


Figure 2.13. Enzymatic treatments of undegraded products of soluble wheat arabinoxylan. Each enzyme indicated was used at a final concentration of 5 μ M. The individual enzymes were reacted with SWAX-P1 (final 0.17%) and BWX-P1 (final 0.31%) in 50 mM Na-phosphate buffer with 150 mM NaCl (pH 6.5) at 37 °C for 15 hours. (A) Enzymatic treatment of SWAX-P1 with β -xylosidases. Lane 1, soluble WAX hydrolysis products (Sample from lane 7 in figure 2.8); lane 2, SWAX-P1 before enzymatic reaction; lane 3, SWAX-P1 after reaction with β -xylosidase (Xyl3A) from *R. albus* 8; lane 4, SWAX-P1 after reaction with β -xylosidase (Xyl3A) from *Prevotella bryantii* B₁₄; lane 5, SWAX-P1 after reaction with β -xylosidase (Xyl3B) from *Prevotella bryantii* B₁₄; lane 6, SWAX-P1 after reaction with β -xylosidase (Xyl3C) from *Prevotella bryantii* B₁₄. (B) Enzymatic treatment of SWAX-P1 with α -L-arabinofuranosidase (Ara51A). Lane 1, soluble WAX hydrolysis products (Sample from lane 7 in figure 2.8); lane 2, SWAX-P1 before enzymatic reaction; lane 3, SWAX-P1 after reaction with α -L-arabinofuranosidase (Ara51A) from *R. albus* 8.

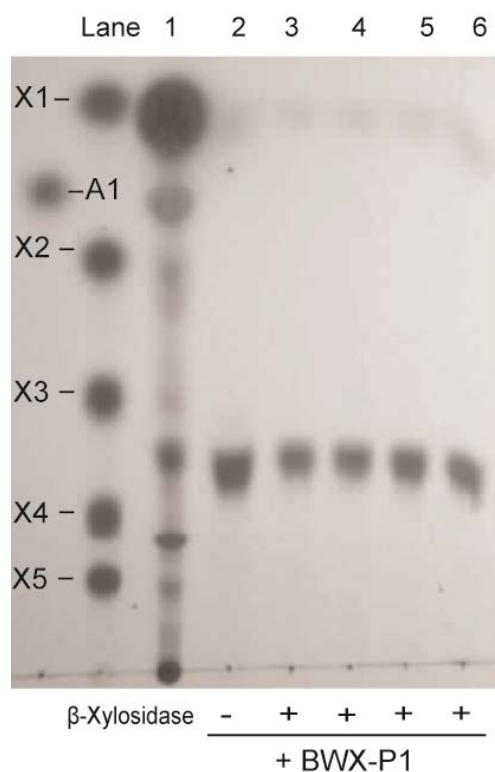
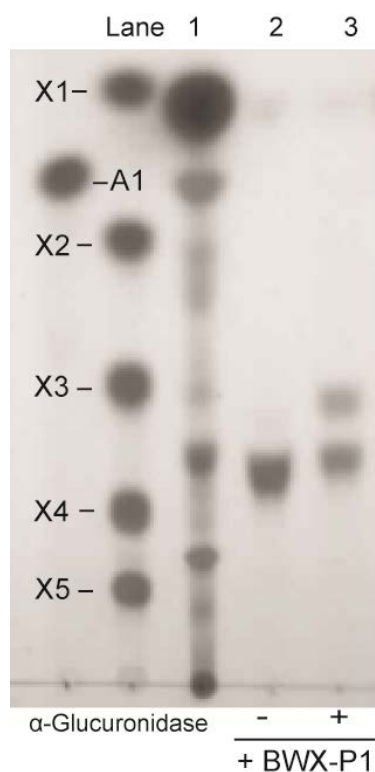
A.**B.**

Figure 2.14. Enzymatic treatments of undegraded products from birchwood xylan. (A) Enzymatic treatment of BWX-P1 with β -xylosidases. The experiments in the lanes are the same as described in Fig. 2.13A except for BWX-P1 replacing SWAX-P1. The BWX-P1 is the undegraded product present in lane 7 of Fig. 2.11A. (B) Enzymatic treatment of BWX-P2 with α -glucuronidase (Agu67A). The experiments in the lanes are the same as described in Fig. 2.13B except for the use of α -glucuronidase (Agu67A) instead of α -L-arabinofuranosidase (Ara51A). The products of hydrolysis were resolved by TLC and stained with methanolic orcinol. Arabinose (A1) and xylo-oligosaccharides (X1-X5) were used as standards.

DISCUSSION

The cow rumen harbors diverse microbes that harness glycoside hydrolases and carbohydrate esterases to depolymerize plant cell wall in order to capture nutrients. The highly cellulolytic ruminal bacterium *Fibrobacter succinogenes* contains hemicellulolytic genes, however it metabolizes cellulose and its degradation products, cellobiose and glucose [142, 143]. In contrast, *Prevotella bryantii*, an organism found in the same ecosystem, only utilizes hemicellulose [44]. Thus *R. albus* 8, the bacterium studied in this report, has an advantage in being able to grow on a variety of plant cell wall polysaccharides [97, 134, 144].

The partial genome sequence of *R. albus* 8 provided a view of the degradative potential of this bacterium on diverse plant cell wall polysaccharides. The majority of the GH genes were predicted to encode hemicellulose-degrading enzymes, and for xylan hydrolysis, the bacterium employs enzymes from GH10, GH11, GH3, GH43, and GH51 families. It is important to note that *R. albus* 8 exhibits preference for cellobiose utilization [145]. However, xylose, the main sugar component of most xylans, is also rapidly metabolized for energy [146]. The activities of the *R. albus* 8 endoxylanases showed interesting differences in terms of products released from the different substrates. Differences between the GH10 and GH11 xylanases were expected based on previous reports that GH10 xylanases can more efficiently degrade highly substituted arabinoxylans [147]. These trends were visible in Fig. 2.2A, which may be due to an active site that can accommodate arabinose decorations on the β -1,4-linked xylose chains [147, 148].

Among the GH11 proteins, although only little variation in the endproducts was detected, interesting differences in the domain architectures were observed (Fig. 2.1A). This diversity of endoxylanases may aid *R. albus* 8 to effectively utilize xylans that exhibit different substitutions in the context of the plant cell wall. This hypothesis was supported by the synergy of individual xylanases with two accessory proteins (β -xylosidase, and the α -L-arabinofuranosidase) (Table 2.2). For WAX, it was observed that Xyn10A and Xyn10B were better able to release xylose and arabinose in the presence of the two accessory enzymes compared to the Xyn11 proteins. In contrast, the GH11 endoxylanases, in general, were superior to the GH10 enzymes in releasing products from the more complex xylans (OSX and BWX) in the presence of the accessory enzymes (Table 2.2). Thus, the endproducts and functions of the various endoxylanases in this study varied. This observation further supports our previous report that rather than being redundant, the multiple forms of a particular enzyme in a plant cell wall degrading organism exhibit subtle differences to optimize utilization of the complex substrate [94].

Members of CBM37 bind to different substrates [78]. The differential binding activities observed with the CBM containing endoxylanases may be due to differences in the combinations of CBM families found in the different polypeptides. Interestingly, CBM22 and CBM37 are the most common CBM's in the genome of *R. albus* 8, and these were also the CBM families present in the endoxylanases in this study (data not shown). When both types of CBM's were present in a single polypeptide, higher amounts of protein bound to substrate, suggesting synergistic binding of the two CBM's

to substrate. It is also possible that the CBM22 found in the xylanases does not bind to the xylan substrates, as observed with Xyn11E.

Microorganisms used in industrial fermentation for production of biofuels lack the enzymes necessary to release fermentable sugars from cellulosic (hemicellulose and cellulose) substrates. Their successful application in production of biofuels from lignocellulose, therefore, will require initial treatment, such as enzymatic hydrolysis, to release fermentable sugars. The multiple endoxylanases showed synergistic activities with two accessory proteins (β -xylosidase and α -L-arabinofuranosidase) during hydrolysis of the xylan substrates. However, some larger products still remained in the reaction mixture (Fig. 2.7A), indicating that additional enzymes may be needed to further hydrolyze the substrates. Oat spelt xylan and birchwood xylan are reported to contain uronic acids at 1.8% and 10.3%, respectively [149], while soluble wheat arabinoxylan contains almost no uronic acid. The hydrolysis patterns of our enzymes reflected these reported estimates. Generally, the most abundant hemicellulose constituent of hardwoods, including birchwood, is *O*-acetyl-(4-*O*-methylglucurono)xylan which contains one α -(1 \rightarrow 2)-linked 4-*O*-methylglucuronic acid substituted every 10-20 xylose residues [150, 151]. Two-fold and 2.5-fold increases in endproducts (xylose) were released from oat spelt xylan and birchwood xylan, respectively, by varying the concentrations of the endoxylanases, β -xylosidase, and α -L-arabinofuranosidase, and also by including an α -glucuronidase (Fig. 2.10B and 2.11B). In contrast, an increase in endproducts was not seen with WAX as substrate in the presence of α -glucuronidase (Fig. 2.9). This observation was expected, since 4-*O*-methylglucuronic acid substitutions are likely absent in the WAX used in this study.

It was anticipated that using multiple endoxylanases instead of a single endoxylanase, in combination with the accessory enzymes will yield more endproducts, especially with OSX and BWX as substrate. However, the results for the multiple endoxylanases and single endoxylanase were similar or rather better in some cases with a single endoxylanase. The molecular basis of this observation is unclear. However, it is our hypothesis that on more complex plant matter, rather than the model substrates used in this study, the benefits associated with multiple endoxylanases will be observed.

Aside from the interest in the application of these enzymes in plant cell wall depolymerization in the biofuel industry, the current report provides insights into the lifestyle of a major ruminal bacterium *R. albus* 8. Ruminal bacteria and other microorganisms that derive nutrients from plant cell wall encounter diverse chemical linkages, and a response to this challenge is to evolve a large number of enzymes with subtle differences in enzymatic activities, as reported for *P. bryantii* B₁₄ [94], to optimize substrate hydrolysis. In this report, we provide biochemical evidence on how *R. albus* 8 might harness its arsenal of glycoside hydrolases to degrade different xylans to monomeric sugars that can then be metabolized. The synergistic effects of different xylanolytic enzymes have been previously demonstrated [152-154]. However, to our knowledge, this is the first report in which, at least, all of the enzymes minimally required for degradation of a hemicellulose were assembled from the same organism.

CHAPTER 3. LICHENIN DEGRADING ENZYMES FROM *Ruminococcus albus* 8²

ABSTRACT

Ruminococcus albus 8 is a fibrolytic ruminal bacterium capable of utilization of various plant cell wall polysaccharides. A bioinformatic analysis of a partial genome sequence of *R. albus* revealed several putative enzymes likely to hydrolyze glucans including lichenin, a mixed linkage polysaccharide of glucose linked together in β -1,3 and β -1,4 glycosidic bonds. In the present study, we demonstrate the capacity of four glycoside hydrolases (GH), derived from *R. albus*, to hydrolyze lichenin. Two of the genes encoded GH family 5 enzymes (Ra0453 and Ra2830), one encoded a GH family 16 enzyme (Ra0505), and the last gene encoded a GH family 3 enzyme (Ra1595). Each gene was expressed in *E. coli*, and the recombinant protein was purified to near homogeneity. Upon screening on a wide range of substrates, Ra0453, Ra2830, and Ra0505 displayed different hydrolytic properties as they released unique product profiles. The Ra1595 protein, predicted to function as a β -glucosidase, preferred cleavage of a nonreducing end glucose when linked by a β -1,3 glycosidic bond to the next glucose residue. The major product of Ra0505 hydrolysis of lichenin was predicted to be a glucotriose that was only degraded by Ra0453 to glucose and cellobiose. Most importantly, the four enzymes functioned synergistically to hydrolyze lichenin to glucose,

² Iakiviak M, Mackie RI, Cann IK. 2011. Functional analyses of multiple lichenin-degrading enzymes from the rumen bacterium *Ruminococcus albus* 8. *Appl. Environ Microbiol.* 77(21):7541-7550.

cellobiose, and cellotriose. This lichenin degrading enzyme mix should be of utility as an additive to feeds administered to monogastric animals, especially those high in fiber.

INTRODUCTION

The rumen microbiome has evolved efficient mechanisms to degrade plant cell wall polysaccharides into metabolizable energy. *Ruminococcus albus* is a fibrolytic bacterium found in the rumen of many herbivores [1, 42, 155, 156]. This bacterium is capable of efficient fermentation of cellulose as well as hemicellulose for growth. Degradation of cellulose, consisting of glucose monomers polymerized by β -1,4 glycosidic bonds, requires the concerted action of 3 classes of enzymes; endo-glucanases (EC 3.2.1.4), exo-glucanases (EC 3.2.1.91), and β -glucosidases (EC 3.2.1.21), which can be in non complexed or complexed (cellulosomal) state [91, 92, 157-159]. The hemicellulose xylan is a complex heteropolymer consisting of a β -1,4 linked xylose backbone that may be appended with a variety of substituents and requires a wide array of enzymes for full depolymerization [13, 46, 49]. Mixed linkage β -glucans are composed of glucose monomers that are linked together by β -1,3 and β -1,4 glycosidic bonds. These polymers are involved in maintaining the plant cell wall structure and providing stored energy in the plant order Poales, with members such as barley, wheat, and ryegrass [8, 12]. Lichenin, another mixed linkage β -glucan, is found as a storage polysaccharides in lichens, which are forage for reindeer and caribou during the winter season [15, 17, 18].

Bacterial enzymes specific for lichenin degradation are classified within the glycoside hydrolase (GH) family 16 lichenases (EC 3.2.1.73) and selectively hydrolyze β -1,4 glycosidic bonds of 3-O substituted glucosyl residues [160]. Lichenases have

been studied through biochemical assays as well as crystal structures and binding studies, and the residues involved in substrate recognition and hydrolysis, in some enzymes, have been identified [161-163].

The enzymes involved in cellulose deconstruction, including endo- and exo- glucanases from GH families 5 and 9, interact with and hydrolyze lichenin at non specific β -1,4 linkages [95]. Laminarin, a β -1,3 linked glucose polymer with β -1,6 linked glucose substituents, is hydrolyzed by GH16 laminarinases (EC 3.2.1.39), which also have the ability to degrade internal β -1,3 linkages within lichenin [96].

Several biochemical studies have been conducted on lichenin degrading enzymes of ruminal origin; however only two enzymes of the ruminal cellulolytic bacterium *Fibrobacter succinogenes* have been extensively characterized at the biochemical level [69, 82, 164, 165]. A bioinformatic search of a partial genome of *Ruminococcus albus* 8 revealed many genes encoding putative glycoside hydrolases. Three cloned endo-glucanases, two from *R. albus* F-40 and one from *R. albus* SY3, were capable of hydrolyzing lichenin, but the mechanisms of their activities were not characterized in detail [124, 166, 167]. A clearer understanding of the *R. albus* enzymes used to degrade complex polysaccharides into fermentable sugars will provide mechanistic insights into the role of strains of this bacterium in their natural habitat. In this study, three genes encoding putative lichenin degrading enzymes and one gene encoding a putative β -glucosidase in *R. albus* 8 were expressed, and the hydrolytic activities on polysaccharides and oligosaccharides were biochemically characterized. Insights into the biochemical function of each enzyme and how they contribute in synergy to

effectively degrade lichenin into glucose, cellobiose and cellotriose for subsequent utilization by the organism are discussed.

EXPERIMENTAL PROCEDURES

Materials. *R. albus* 8 genomic DNA was obtained from the Department of Animal Sciences, University of Illinois at Urbana-Champaign [103]. *Escherichia coli* JM109 and *E. coli* BL21-CodonPlus (DE3) RIPL competent cells and PicoMaxx high-fidelity DNA polymerase were purchased from Stratagene (La Jolla, CA). The pET-46 Ek/LIC vector kit was obtained from Novagen (San Diego, CA). QIAprep Spin Miniprep kit was obtained from Qiagen (Valencia, CA). The 1,3:1,4- β -gluco-oligosaccharides, cello-oligosaccharides, laminaribiose, lichenin, laminarin, glucomannan, and wheat arabinoxylan were purchased from Megazyme (Bray, Ireland). The glucose oxidase reaction reagents were obtained from Pointe Scientific Inc. (Canton, MI). All other reagents were of the highest possible purity and were purchased from Sigma-Aldrich (St. Louis, MO).

Gene cloning, expression, and protein purification. A search of the partial genome sequence of *R. albus* 8 yielded more than 10 genes predicted to encode enzymes capable of cleaving β -1,4 or β -1,3 glycosidic bonds. All of these genes have been cloned, expressed, and partially characterized (Iakiviak et al. unpublished data). Two genes, designated Ra0453 and Ra2830, were annotated as putative exoglucanases, Ra1595 was predicted to encode a β -glucosidase and Ra0505 was predicted to be a lichenase. The genomic DNA of *R. albus* 8 was extracted using the DNeasy Blood and Tissue kit (Qiagen). The primer pairs, listed in Table 3.1, were used to amplify their target genes by using the PicoMaxx PCR Kit (Agilent Technologies) and

TABLE 3.1. Primers used for cloning genes encoding lichenin degrading enzymes.

Gene	Location	Sequence ^a
<i>ra0453</i>	Forward	5'- <u>GACGACGACAAGATGAAGGTATTTGATGGTTTTTCAGAAGG</u> -3'
	Reverse	5'- <u>GAGGAGAAGCCCGGT</u> TACAGGTATTTGACC -3'
<i>ra2830</i>	Forward	5'- <u>GACGACGACAAGATGGCATCTGCACCCGAC</u> -3'
	Reverse	5'- <u>GAGGAGAAGCCCGGT</u> TATTTTTGTAGCTTGCTG -3'
<i>ra0505</i>	Forward	5'- <u>GACGACGACAAGATGGATAATGCCGAAAAGAATGACAGCAAAGATGAGG</u> -3'
	Reverse	5'- <u>GAGGAGAAGCCCGGT</u> TTATTCAGCGGCGGTGAAGCG -3'
<i>ra1595</i>	Forward	5'- <u>GACGACGACAAGATGGCAATACTCGACTGGAACAAATATCTCG</u> -3'
	Reverse	5'- <u>GAGGAGAAGCCCGGT</u> TTATGAATTAAGCCCCGTGTTTTCCGGC -3'

^a Nucleotides added to facilitate ligation independent cloning

R. albus 8 genomic DNA as the template. The forward primers were designed to delete the putative signal peptides present in two of the genes. Thus from Ra0505, the PCR amplification deleted the N-terminally located sequence MKKITALTLAFMTVFSLSACG, and from Ra2830, MLKKIISGVTAASACTIVLSTASGVIVHDAPAASTVSA was deleted. The signal peptides were predicted using the SignalP v3.0 online server [168]. Each PCR product was cloned into the pET-46b vector (Ek/LIC, Novagen), and the ligation products were transformed into *Escherichia coli* JM109 using electroporation. After selection on Lysogeny Broth (LB) plates supplemented with ampicillin at 100 µg/ml, five colonies were picked and cultured in LB medium containing the same antibiotic at the same concentration. Plasmids were purified from each culture using the QIAprep Spin Miniprep Kit (Qiagen). To confirm that the plasmids contain the desired genes, DNA sequencing was performed on the DNA inserts (W. M. Keck Center for Comparative and Functional Genomics).

For gene expression, each plasmid containing the correct DNA insert was transformed into *E. coli* BL21-CodonPlus (DE3) RIPL competent cells using the heat shock method, and grown on LB agar plates, supplemented with ampicillin (100µg/ml) and chloramphenicol (50µg/ml), for 16 hours. For each gene, five colonies were picked and inoculated into LB medium (10ml), with ampicillin (100µg/ml) and chloramphenicol (50µg/ml), and grown for 6-8 hours at 37°C with vigorous shaking. After the pre-cultures reached saturation, each culture was transferred to fresh LB medium (1L) containing ampicillin and chloramphenicol, at the same concentrations stated above, and grown at 37°C with shaking until the optical density at 600 nm (OD₆₀₀) reached 0.5. Gene expression was induced by adding isopropyl-β-D-thio-galactopyronoside (IPTG) to a

final concentration of 0.1mM, and the cultures were incubated at 16°C for 16 hours. Cells were collected by centrifugation (5000 x g, 15 min, 4 °C) and re-suspended in 30ml of lysis buffer (50mM Tris, 300mM NaCl, pH7.5). The cell suspensions were lysed using an EmulsiFlex C-3 cell homogenizer from Avestin (Ottawa, Canada). The cell debris were separated by centrifugation (20,000 x g, 30 min, 4 °C), and the clarified lysate was incubated at 4°C for 1 hour with Talon Metal Affinity Resin (Clontech). The bound proteins were eluted from the resin using an elution buffer composed of the lysis buffer supplemented with 150mM imidazole. Subsequently, the fractions containing the Ra0453, Ra0505, and Ra2830 proteins were subjected to gel filtration (HiLoad 16/60 Superdex 200 prep grade column, GE Healthcare) on an AKTExpress fast protein liquid chromatograph (FPLC, GE Healthcare). The proteins were in a buffer composed of 50mM Tris, 150mM NaCl, pH 7.5, and the same buffer was used to develop the chromatography. The highly purified protein fractions (based on SDS-PAGE) were dialyzed against a storage buffer (50mM Tris, 150mM NaCl, pH 7.5) and stored at 4°C. The *R. albus* 8 Ra1595 protein was subjected to gel filtration chromatography with a buffer containing 50mM sodium phosphate, pH7.5. The eluted proteins were applied to an anion exchange column (5 mL HiTrap Q HP column, GE Healthcare) with the buffer for the gel filtration chromatography serving as the equilibration buffer. To elute the bound proteins, a gradient was developed with the equilibration buffer containing NaCl at 1 M concentration. The highly purified protein fractions were dialyzed against the protein storage buffer, described above, and stored at 4°C until used. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described elsewhere [139], and the proteins were visualized by staining with Coomassie brilliant

blue G-250. The concentration of each purified protein was determined using absorbance spectroscopy by the method described by Gill and von Hippel [169]. Briefly, the NanoDrop 1000 from Thermo Fisher Scientific Inc. (Waltham, MA) was used to measure protein concentration based on the molecular mass and extinction coefficients of Ra0453 (41.2 kDa), Ra0505 (29.1 kDa), Ra1595 (101.4 kDa), and Ra2830 (63.4 kDa), which were estimated as $84020 \text{ M}^{-1} \text{ cm}^{-1}$, $56510 \text{ M}^{-1} \text{ cm}^{-1}$, $108600 \text{ M}^{-1} \text{ cm}^{-1}$, and $171200 \text{ M}^{-1} \text{ cm}^{-1}$, respectively.

Hydrolysis of *para*-nitrophenyl linked sugars. Recombinant enzymes were screened on a library of *para*-nitrophenol (*p*NP) linked glycosides including: *p*NP- α -L-arabinopyranoside, *p*NP- α -L-arabinofuranoside, *p*NP- β -D-fucopyranoside, *p*NP- α -L-fucopyranoside, *p*NP- α -D-galactopyranoside, *p*NP- β -D-galactopyranoside, *p*NP- α -D-glucopyranoside, *p*NP- β -D-glucopyranoside, *p*NP- β -D-maltopyranoside, *p*NP- α -D-maltopyranoside, *p*NP- α -D-mannopyranoside, *p*NP- β -D-mannopyranoside, *p*NP- α -L-rhamnopyranoside, *p*NP- β -D-xylopyranoside, and *p*NP- β -D-cellobioside. All *p*NP-linked substrates were purchase from Sigma Aldrich (St. Louis, MO). The *R. albus* 8 Ra0453, Ra0505, Ra1595, and Ra2830 at $0.5 \mu\text{M}$, in each case, were incubated with substrate (1 mM) for 30 minutes, in a buffer containing 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0, at 37°C in a thermostated Synergy II multi-mode microplate reader from BioTek Instruments Inc. (Winooski, VT). The release of *p*NP was measured at 400 nm every 31 seconds using the path-length correction feature to convert the absorbance values recorded to correspond to a 1 cm path-length. Absorbance values calculated were converted to concentrations using a predetermined extinction coefficient of $10.601 \text{ mM}^{-1} \text{ cm}^{-1}$ for *p*NP at pH 7.0 and a wavelength of 400 nm . To

determine the optimal pH, *p*NP-cellobioside was dissolved to a final concentration of 1mM in a series of buffers including: citrate buffer (50mM sodium citrate, 150mM sodium chloride, pH 5.0, 5.5, 6.0), phosphate buffer (50mM sodium phosphate, 150mM sodium chloride, pH 6.0, 6.5, 7.0, 7.5, 8.0), and bicine buffer (50mM BICINE, 150mM sodium chloride, pH 8.0, 8.5, 9.0). The specific activities of Ra0453 (6 nM) and Ra2830 (100 nM) were calculated at each pH using an extinction coefficient of *p*NP determined for the corresponding buffer. The enzymes were in a concentration that provided linear release of *p*NP. By comparing relative activity at each pH, buffer A (50mM sodium phosphate, 150mM sodium chloride, pH6.5) was determined to be the optimal buffer. Therefore, all subsequent reactions were carried out in buffer A. The specific activities of Ra1595 were determined for *p*NP- β -D-glucopyranoside (1mM) and *p*NP- β -D-cellobioside (1mM), using an enzyme concentration of 40nM and 100nM, respectively. Concentration of *p*NP released was calculated using a predetermined extinction coefficient of 4.0033 mM⁻¹cm⁻¹ for *p*NP at pH 6.5.

Hydrolysis of polysaccharides. Hydrolysis of natural substrates, including glucomannan, lichenin, laminarin, and carboxymethyl cellulose, present at 0.5%(w/v) by three of the proteins (Ra0453, Ra0505, Ra2830) were performed at 37°C at concentrations that resulted in a linear release of products. Avicel, phosphoric acid swollen cellulose (PASC), and Whatman's filter paper (WFP) at concentrations of 2% (w/v) were incubated with Ra0453 and Ra2830 (1 μ M) at 37°C for 16 hours. The phosphoric acid swollen cellulose was prepared as described elsewhere [170]. The release of reducing sugars was detected using the *para*-hydroxy benzoic acid hydrazide (*p*HBAH) assay as described by Lever [140]. The specific activities of the enzymes with

each polysaccharide as substrate were determined based on the amount of reducing ends release by each enzyme per time. Cellobiose was used to derive a standard curve for the *p*HBAH assay. To examine the products of hydrolysis, polysaccharides incubated with enzymes were separated using thin layer chromatography (TLC) with a mobile phase composed of *n*-butanol, acetic acid, and water (10:5:1) and visualized by spraying with methanolic orcinol and heating at 75°C for 15 minutes. The products of hydrolysis were also separated using high performance anion exchange chromatography (HPAEC) with a System Gold® HPLC instrument from Beckman Coulter (Fullerton, CA) fitted with a CarboPac™ PA-1 guard column (4 x 50 mm) and a CarboPac PA1 analytical column (4 x 250 mm) from Dionex Corporation (Sunnyvale, CA). The eluted saccharides were then detected with a pulsed Model 5040 Amperometric Analytical Cell and a Coulochem® III electrochemical detector (PAD) from ESA Biosciences (Chelmsford, MA) as described elsewhere [94]. To identify and quantify the mono- and oligo- saccharides produced from enzymatically degraded polysaccharides, peak retention times and peak areas from the chromatographs of samples were compared to those of commercially available saccharides analyzed as standards. For time course hydrolysis and determination of synergistic interactions of enzymatic activity on lichenin, the substrate under investigation at 0.5% (w/v) was digested with individual enzymes or different mixtures of Ra0453, Ra0505, Ra1595, and Ra2830 present in solution at 0.5 μM, 0.05 μM, 0.1 μM and 0.5 μM, respectively.

Hydrolysis of gluco-oligosaccharides. Enzymatic hydrolysis of cello- and β-1,3-β-1,4-gluco- oligosaccharides was carried out with 10mM substrate concentration at 37°C. To determine specific activities of Ra1595 on cellobiose and laminaribiose, the

enzyme was added at concentrations that provided a linear release of product. Glucose production was measured with the glucose oxidase reagent set purchased from a commercial vendor (Pointe Scientific) and used according to the instructions of the manufacturer. The hydrolyzed sample and standards with varying concentrations of glucose (5 μ l) were added to the preheated reagent (400 μ l) and incubated at 37°C for 5 minutes in the thermostated Synergy II multi-mode microplate reader. The absorbance was measured at 510 nm and the products released in the reaction mixture were estimated using a standard curve generated with known glucose concentrations. Hydrolysis products of cello- and β -1,3- β -1,4-gluco- oligosaccharides were separated and visualized with thin layer chromatography as described above. The time course of cellohexaose hydrolysis by Ra0453 and Ra2830 was performed to gain insight to their mode of hydrolysis of cellulosic substrates. The substrate (10 mM) was incubated with Ra0453 or Ra2830 at concentrations of 2 μ M and 75 nM, respectively. The reaction products were then separated and detected using HPEAC-PAD as described above. Since initial screening showed that Ra0505 and Ra1595 lack cellulolytic activity, their hydrolysis of cellulosic substrates was not further investigated.

RESULTS

Domain analysis of four lichenin degrading enzymes from *R. albus* 8. A bioinformatics approach was used to identify glycoside hydrolase (GH) genes predicted to encode polysaccharides degrading enzymes in a partial genome sequence of *Ruminococcus albus* 8 (Genbank Accession No. NZ_ADKM000000000: K. E. Nelson et al. unpublished data). A myriad of genes coding for putative glycoside hydrolases that target cellulose were identified and expressed. Two genes encoding GH5 enzymes

(Ra0453 and R2830) were selected for further biochemical characterization based on their annotation as putative exo-glucanases, an activity critical to cellulose degradation. Each gene was expressed with an N-terminal Hexa-Histidine tag (6-His tag) in *E. coli* BL-21 CodonPlus RIPL cells to facilitate purification of the gene product. The two proteins degraded lichenin during initial screenings of multiple substrates. Therefore, other putative GH genes (Ra0505 and Ra1595) encoding proteins likely to target lichenin were identified, cloned, and expressed for protein purification and functional characterization in combination with Ra0453 and Ra2830. The objective here was to determine whether these enzymes functioned synergistically to degrade lichenin. The Genbank accession numbers of Ra0453, Ra0505, Ra1595, and Ra2830 are EGC02962, EGC02853, EGC01435, and EGC04285, respectively.

In Figure 3.1A, the domain architectures, as determined by Pfam (<http://pfam.sanger.ac.uk/>), of the four cloned enzymes are presented, with arrows designating the location of primers. Two of the proteins (Ra2830 and Ra0505) were predicted to possess signal peptides. Therefore, the forward PCR primers were designed to remove the signal peptides to ensure accumulation of the recombinant gene products in the *E. coli* cells. Whereas Ra0453 is composed of only a GH5 domain, the GH5 module of Ra2830 was flanked by an N-terminal signal peptide and a C-terminal dockerin-like domain. The two enzymes are classified within the same family, however, their GH5 catalytic domains only shared 14% identity. Ra0505, which also has an N-terminal signal peptide, contained a GH16 catalytic domain. The GH16 module is known to occur in glycoside hydrolases with lichenase (EC 3.2.1.73), laminarinase (EC 3.2.1.6), agarase (EC 3.2.1.81), or xyloglucan:xyloglucosyl transferase (EC 2.4.1.207)

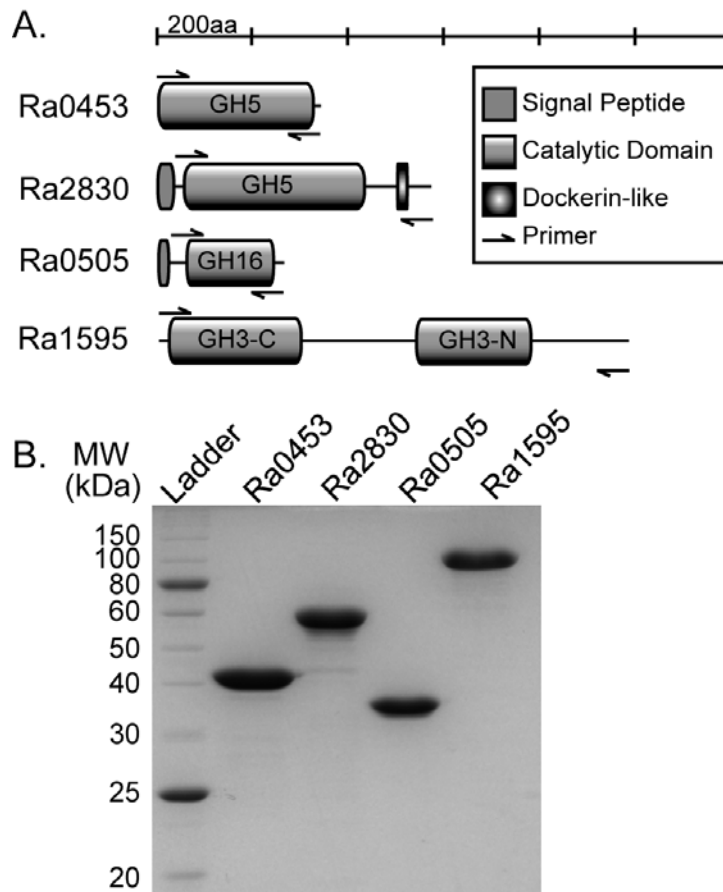


Figure 3.1. Cloning of 4 glycoside hydrolases from *Ruminococcus albus* 8. (A) Domain architecture of the cloned glycoside hydrolases from *R. albus* 8. Arrows designate the location of the primers within the gene sequence. Annotation of the domains was performed using Pfam (<http://pfam.sanger.ac.uk/>). Abbreviations: GH, Glycoside Hydrolase; C, Carboxy terminus; N, amino terminus. (B) Purification of Ra0453, Ra2830, Ra0505, and Ra1595 was performed by Cobalt affinity chromatography followed by gel filtration. Ra1595 was further purified by anion exchange chromatography. The highly purified proteins were analyzed by 12% SDS-PAGE.

activities. The *R. albus* 8 Ra1595 possessed the two domains known to occur in GH family 3 proteins, which are usually β -glucosidases (EC 3.2.1.21) or β -xylosidases (EC 3.2.1.37). An interesting feature of Ra1595 is that its C-terminal and N-terminal domains are usually located at the N-termini and C-termini, respectively, of members of this GH family. The linker region between the two domains (GH3-C and GH3-N) also appears longer than that of other GH3 proteins. Whereas the linker regions of other GH3 proteins examined were approximately 65-100 amino acid residues long [94], Ra1595 has a linker of 225 amino acid residues.

Analyses of purified lichenin degrading enzymes. The *R. albus* Ra0453, Ra2830, Ra0505, and Ra1595 were purified to near homogeneity (Fig 3.1B) and stored at 4°C until used. The predicted molecular masses of the four proteins (Ra0453, 41.2 kDa), (Ra2830, 63.4 kDa), (Ra0505, 29.1 kDa), and (Ra1595, 101.4 kDa) were similar to their values estimated by SDS-PAGE. The purified enzymes were screened for activity on a wide range of polymeric substrates by incubating each enzyme (0.5 μ M) with individual substrates (0.5% w/v) for 16 hours (data not shown). Specific activities were determined with a reducing sugar assay for substrates that were degraded by the enzymes (Fig. 3.2). Despite their low amino acid sequence identity, Ra0453 and Ra2830 hydrolyzed the same polysaccharides, although with different specific activities. The highest specific activity of Ra0453 was with lichenin as a substrate, followed by glucomannan, WAX, and CMC. As shown in the results for Ra0453, the activity on the soluble mixed linkage β -glucan (lichenin) was approximately 1000 times higher than CMC. The *R. albus* Ra2830 also had the highest specific activity on lichenin, and the activities on CMC and glucomannan, which were similar, were about 10 times lower

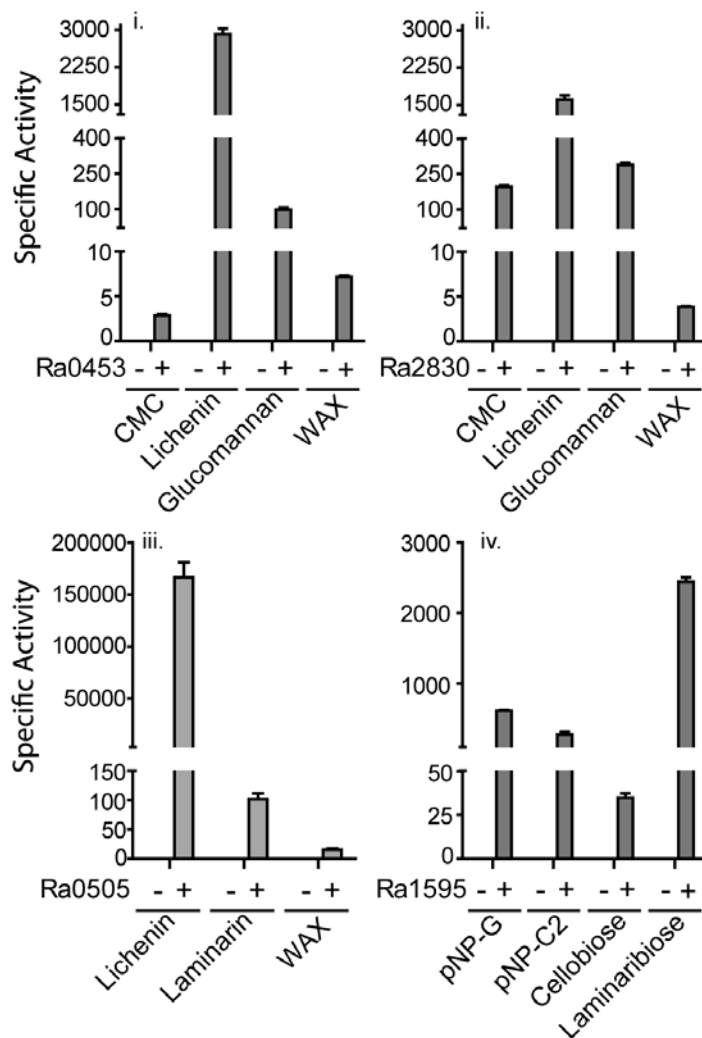


Figure 3.2. Characterization of the substrate specificities of 4 glycoside hydrolases from *Ruminococcus albus* 8. Specific activities (nmol products released per nmol enzyme per minute) with soluble substrates, including carboxymethyl cellulose (CMC), lichenin, glucomannan, wheat arabinosyl (WAX), and laminarin present at 0.5% (w/v), were reported for (i) Ra0453, [167] Ra2830, and (iii) Ra0505. Reducing ends in the reaction products were analyzed by the *para*-hydroxybenzohydride (PAHBAH) reducing sugar assay using cellobiose as the reducing end standard. Specific activities for the hydrolysis of *para*-nitrophenyl (*p*NP) linked glycosides, including *p*NP-glucose (*p*NP-G) and *p*NP-cellobiose (*p*NP-C2), and also cellobiose and laminaribiose by (iv) Ra1595 were determined. Hydrolysis of *p*NP linked glycosides was quantified by measuring absorbance of *p*NP release at 410 nm. Cellobiose and laminaribiose degradation was quantified by measuring glucose production using the Pointe Scientific Glucose Oxidase kit. Reactions were performed in triplicates, and the bars correspond to the standard error of the mean. The (-) and (+) signs mean absence and presence, respectively, of an enzyme in a particular row.

than the lichenase activity. For this enzyme, the activity on WAX was very low, as observed for Ra0453. Very large amounts of reducing ends were released from lichenin by Ra0505 at a very low enzyme concentration. The specific activity of this enzyme, on lichenin, was 50 to 100 fold higher than those of Ra0453 and Ra2830, respectively. Whereas Ra0505 did not have hydrolytic activity on β -1,4 linked glucans (based on CMC as a substrate), it hydrolyzed laminarin, a β -1,3 linked glucan, at a low rate. There was also release of a small amount of reducing ends from WAX, at a comparable rate to those of Ra0453 and Ra2830. The GH3 enzyme, Ra1595, was able to degrade *p*NP-glucoside and *p*NP-cellobioside, but the hydrolysis of cellobiose was much lower, i.e., approximately 18-fold lower than hydrolysis of *p*NP-glucoside. By using a glucose oxidase-coupled assay, it was determined that laminaribiose, composed of two glucose monomers linked by a β -1,3 glycosidic bond, was degraded 70 times faster than cellobiose, indicating that this enzyme prefers to cleave β -1,3 glycosidic bonds.

HPLC analysis of end products from polysaccharide degradation. To determine whether differences in end products were displayed during hydrolysis of polysaccharide substrates by Ra0453, Ra2830, and Ra0505, the individual enzymes were incubated with the different polysaccharides overnight, and the soluble products were analyzed by HPAEC-PAD (Fig 3.3). Comparison of Ra0453 and Ra2830 activity on cellulosic substrates (Fig. 3.3A and 3.3B) demonstrated that Ra0453 produces a small amount of cellobiose, whereas Ra2830 released much higher amounts of cellobiose, as well as producing some cellotriose and glucose. This finding shows that although each of the two enzymes contains a GH5 module, their substrate recognition and catalytic properties are different. The hydrolysis of konjac glucomannan by both

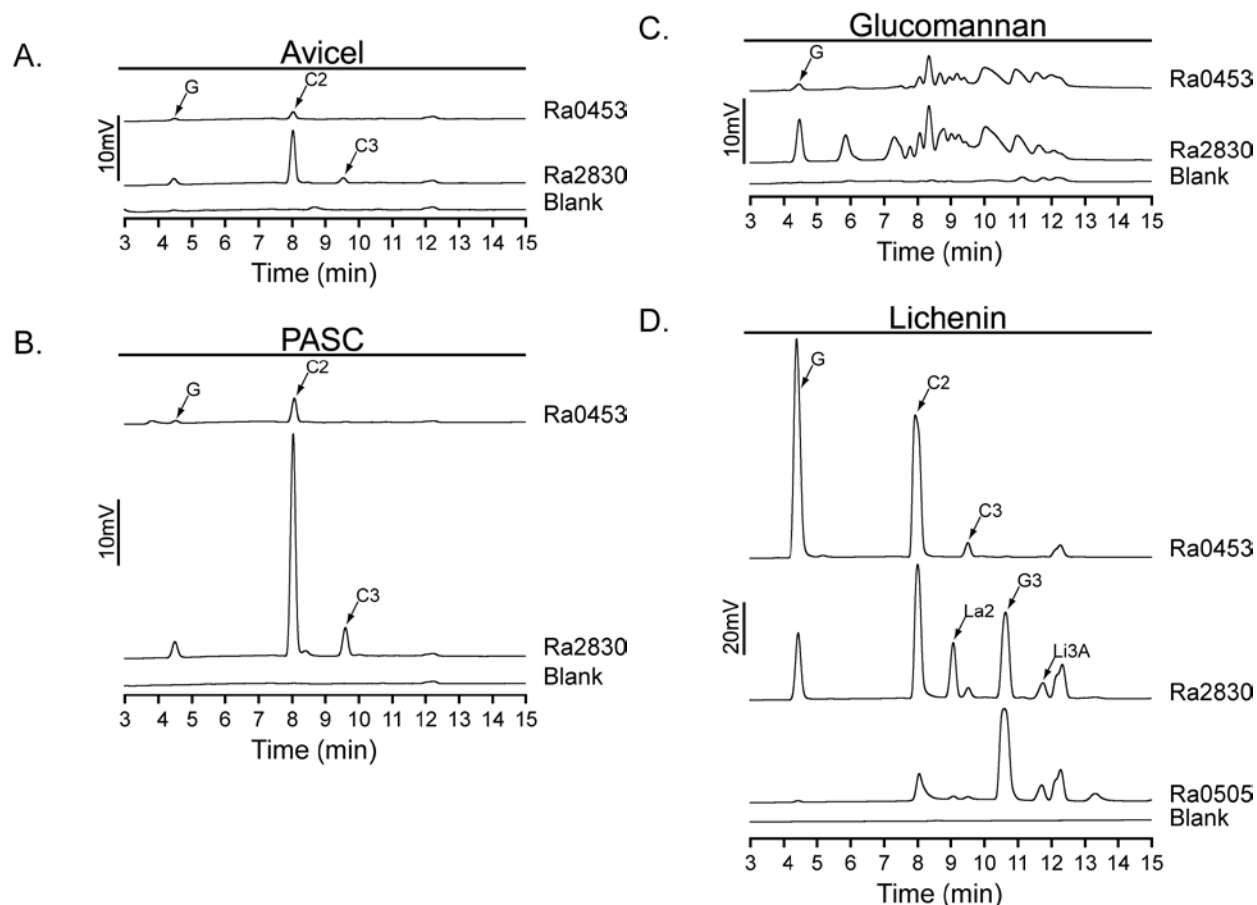


Figure 3.3. Comparison of products of hydrolysis from polysaccharides incubated with Ra0453, Ra0505 or Ra2830. Chromatographs of products of hydrolysis from (A) Avicel, (B) phosphoric acid swollen cellulose (PASC), (C) glucomannan, and (D) lichenin. Enzymatic degradation of substrates (0.5% w/v) was performed for 16 h with 0.5 μ M enzyme concentration of Ra0453, Ra0505, or Ra2830. Reaction products were analyzed by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD). Sample peaks were identified by comparison of sample retention times to those of commercially available oligosaccharides. Abbreviations: G, glucose; C2, cellobiose; C3, cellotriose; La2, laminaribiose; Li3A, β -1,3-1,4-glucotriose; G3, unidentified oligosaccharide.

GH5 enzymes resulted in glucose and many oligosaccharides with identities that are not easily resolved (Fig. 3.3C).

The products of lichenin degradation by Ra0453, Ra2830, and Ra0505 showed interesting differences (Fig. 3.3D). The *R. albus* Ra0453 enzyme seems to produce mainly glucose, cellobiose, cellotriose, and a fourth peak unresolved because it represents multiple peaks. This pattern is different from the end products profile of Ra2830, which contained a variety of products including glucose, cellobiose, laminaribiose, cellotriose, a β -1,3-1,4-glucotriose (Li3A), and an unidentified product, which we labeled unidentified oligosaccharide. The GH16 enzyme, Ra0505, mainly produced the latter unidentified oligosaccharide (G3 as in Ra2830 under Fig. 3.3D) in addition to a smaller proportion of cellobiose and Li3A.

Hydrolysis of cello-oligosaccharides. The GH5 proteins Ra0453 and Ra2830 are both able to hydrolyze cellulosic substrates, although they differ in the rate and extent of degradation. An experiment using cello-oligosaccharides of different lengths was carried out to determine the capacity to release smaller products. Both enzymes were able to hydrolyzed cello-oligosaccharides of degree of polymerization greater than 3. In addition, Ra0453 degraded cellotriose (Fig. 3.4Ai, lane 5), whereas no products were observed with Ra2830 incubated with this substrate (Fig. 3.4Aii, lane 5). Based on both HPAEC-PAD and TLC analyses, the major product of oligosaccharide degradation, from both enzymes, is cellobiose, which has been shown as a preferred substrate of *R. albus* 7 [145], a relative of the bacterium under study.

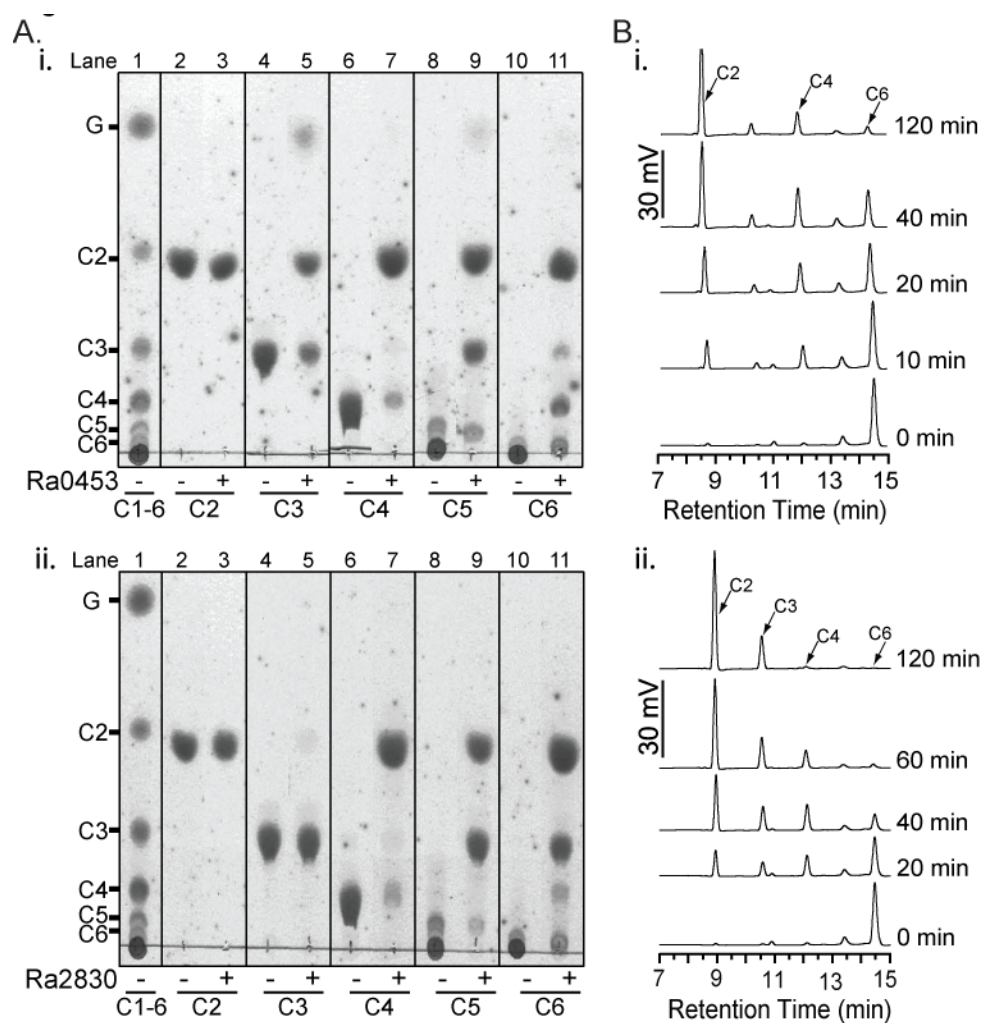


Figure 3.4. Activities of GH5 enzymes with cello-oligosaccharides and cellulosic substrates. (A) Products released from cello-oligosaccharides, with a degree of polymerization (DP) from 2-6. Substrates were incubated with and without (i) Ra0453 or (ii) Ra2830. Oligosaccharides (10 mM) were incubated with enzyme (0.5 μ M) for 16 h at 37°C and reaction products were resolved using thin layer chromatography. (B) Time course for hydrolysis of cellohexaose with (i) Ra0453 (2 μ M) or (ii) Ra2830 (0.075 μ M). Products were separated and detected by HPAEC-PAD. Abbreviations: G, glucose; C2, cellobiose; C3, cellotriose; C4, cellotetraose; C5, cellopentaose; C6, cellohexaose; C1-6, mixture of glucose and cello-oligosaccharides (DP 2-6). The (-) and (+) signs mean absence and presence, respectively, of an enzyme in a particular row.

To further analyze the action by which the enzymes hydrolyze longer cello-oligosaccharides, they were incubated with celohexaose for a time course analysis. The products were analyzed by HPAEC-PAD (Fig. 3.4B). The *R. albus* 8 Ra0453 mostly cleaved cellobiose from celohexaose to produce cellotetraose, which was then hydrolyzed into two cellobiose units (Fig. 3.4Bi). This pattern is suggestive of an exocellulolytic mode of action, as cellobiose constitutes the repeating unit in cellulose. Conversely, Ra2830 displayed an endo-cellulolytic mode of action, producing relatively equal amounts of cellobiose, cellotriose and cellotetraose from celohexaose initially, with further degradation to shorter products, mostly cellobiose, with time (Fig. 3.4Bii).

Hydrolysis of β -1,3- β 1,4 gluco-oligosaccharides. All of the cloned enzymes had high activity on lichenin or soluble oligosaccharides that can be produced from lichenin hydrolysis. To obtain further insight into the types of bonds cleaved and the subsite recognition by these enzymes, Ra0453, Ra2830, Ra0505, and Ra1595 (0.5 μ M) were incubated with gluco-oligosaccharides that contain various configurations of the β -1,3 and β -1,4 glycosidic bonds (Figure 3.5).

The *R. albus* Ra0453 hydrolyzed Li3B into glucose and cellobiose (Fig. 3.5i, lanes 6 and 7), Li4A into cellobiose (2 glucose units joined by a β -1,4 glycosidic linkage) and laminaribiose (2 glucose units joined by β -1,3 glycosidic linkage), Li4B into cellobiose and glucose (Fig. 3.5i lanes 10 and 11), Li4C into cellobiose (Fig. 3.5i, lanes 12 and 13), and Li5A into cellobiose and Li3A (Fig. 3.5i lanes 14 and 15). Upon analyzing the substrates that were extensively degraded (Li3B, Li4B and Li4C), a β -1,3 linkage is cleaved when a β -1,4 linkage is present between the -1 and -2 subsites (Fig. 3.6Bi). The production of cellobiose and glucose from Li4B can be explained by the slow hydrolysis

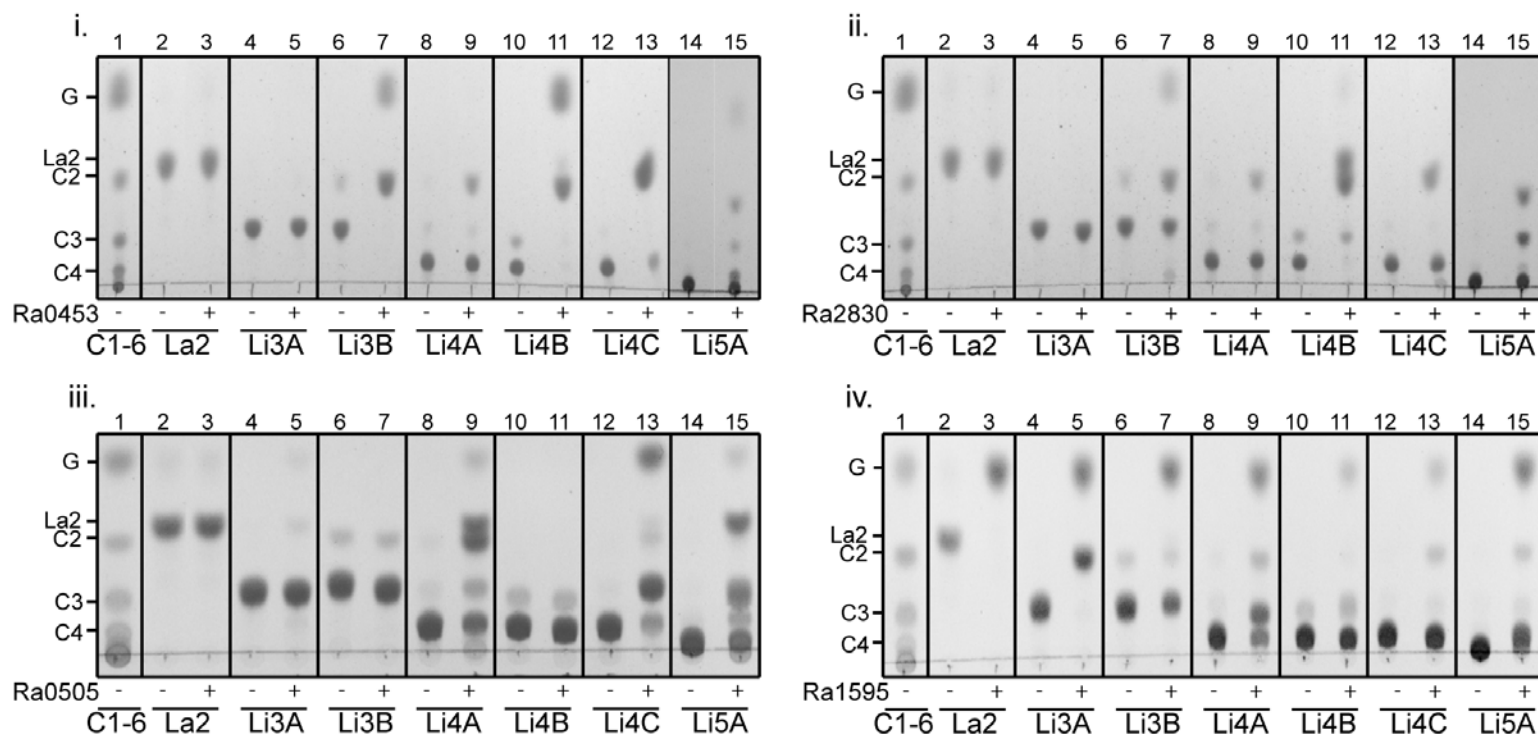


Figure 3.5. Products of hydrolysis of mixed linkage β -1,3-1,4-gluco-oligosaccharides by Ra0453, Ra2830, Ra0505 and Ra1595. Various β -1,3-1,4-glucooligosaccharides (10 mM), with a degree of polymerization of 2-5, were incubated in the presence or absence of 0.5 μ M (i)Ra0453, [167]Ra2830, (iii)Ra0505, or (iv)Ra1595 for 16 h at 37°C. Reaction products and cello-oligosaccharide standards (G, C2-4) were resolved using thin layer chromatography. Abbreviations: G, glucose; C2, cellobiose; C3, cellotriose; C4, cellotetraose; La2, laminaribiose; Li3A, β -1,3-1,4-gluco-oligosaccharide; Li3B, β -1,4-1,3-gluco-oligosaccharide; Li4A, β -1,3-1,4-1,4-gluco-oligosaccharide; Li4B, β -1,4-1,4-1,3-gluco-oligosaccharide; Li4C, β -1,4-1,3-1,4-gluco-oligosaccharide; Li5A, β -1,3-1,4-1,4-1,4-gluco-oligosaccharide. The (-) and (+) signs mean absence and presence, respectively, of an enzyme in a particular row.

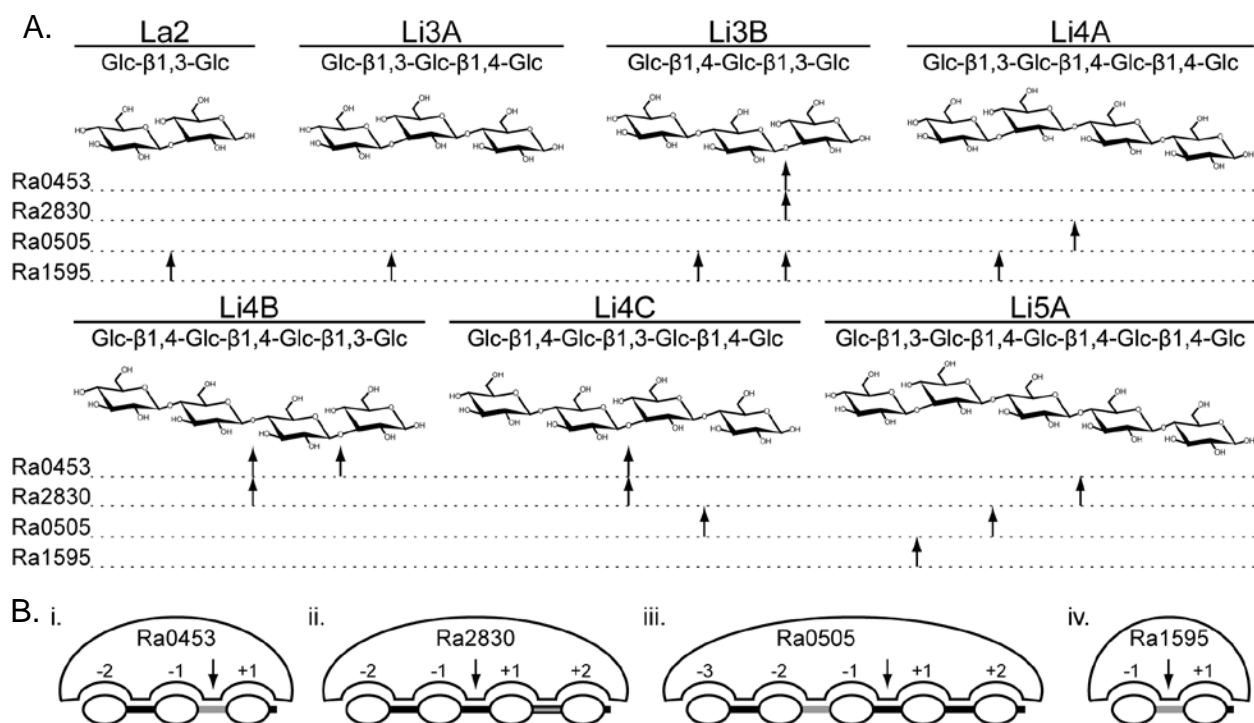


Figure 3.6. Degradation pattern of mixed linkage β -1,3-1,4-gluco-oligosaccharides by Ra0453, Ra2830, Ra0505 and Ra1595. (A) Chair structures of various oligosaccharides used in this study. The cleavage sites of the *R. albus* 8 enzymes (Ra0453, Ra2830, Ra0505 or Ra1595) are depicted as arrows pointing to the glycosidic bonds. Cleavage patterns were confirmed using HPAEC-PAD; sample peaks were identified by comparison of sample retention times to those of commercially available oligosaccharides. (B) Predicted sugar binding subsites in Ra0453, Ra2830, Ra0505, and Ra1595 based on the pattern of hydrolysis of oligosaccharides. Black lines represent β -1,4 glycosidic linkages, gray lines designate β -1,3 glycosidic linkages, and gray and black lines represents the ability of Ra2830 to bind to β -1,3 or β -1,4 linked glucose units.

of cellotriose (further explained later), which is produced from the initial cleavage of the β -1,3 linkage present in the tetra-saccharide (Fig. 3.6A).

The *R. albus* Ra2830 was also able to degrade Li4B, and unlike Ra0453, the products included cellobiose and laminaribiose (Fig. 3.5ii lanes 10 and 11). Other oligosaccharides degraded by Ra2830 include Li3B and Li4C (Fig. 3.5ii), which were cleaved at the β -1,3 glycosidic bond. Hydrolysis of this linkage was incomplete after 16 hours, indicating that these are not preferred substrates.

The *R. albus* Ra0505 was able to degrade Li4A, Li4C, and Li5A by hydrolyzing a β -1,4 linkage when a β -1,3 linkage is present between the -1 and -2 subsites (Fig. 3.6Bii). This activity was evident in oligosaccharides with a degree of polymerization greater than 3 (Fig. 3.5iii).

The *R. albus* Ra1595 degraded laminaribiose rapidly. In contrast, cellobiose was degraded at a much slower rate (Fig. 3.2). These findings indicated a preference for hydrolysis of the β -1,3 glycosidic bond. The experiment with the mixed linkage cello-oligosaccharides as substrates (Fig. 3.5iv) suggested that Ra1595 preferentially releases the glucose molecule in a β -1,3 glycosidic bond at the nonreducing end. Hydrolysis of Li3A produced glucose and cellobiose, and hydrolysis of Li4A produced glucose and cellotriose with a small amount of cellobiose (Fig. 3.5iv). When the nonreducing end β -1,3 linked glucose molecule is cleaved, the cello-oligosaccharide intermediate accumulates in the solution. However, Li3B was partially degraded into glucose with no accumulation of a disaccharide. This hydrolysis pattern can be attributed to the slow release of the β -1,4 linked glucose present at the nonreducing end

of the chain, then rapid hydrolysis of the laminaribiose intermediate that is linked by a β -1,3 glycosidic bond.

Hydrolysis of lichenin by Ra0453, Ra2830, and Ra0505. The *R. albus* 8 Ra0453, Ra2830, and Ra0505 were shown to hydrolyze several polysaccharide substrates (Fig. 3.2 and Fig. 3.3) with the highest activities displayed on lichenin. To examine the products released by each of the enzymes during hydrolysis of lichenin, a time course for hydrolysis of this substrate was performed. In Figure 3.7A, representative chromatograms show a timed release of products from lichenin hydrolysis as analyzed by HPAEC-PAD. The concentrations of the products that matched the elution of standards were calculated and plotted in Figure 3.7B. The *R. albus* Ra0453 (50 nM) hydrolyzed lichenin within 2 hours into cellotriose, cellobiose and glucose (Fig. 3.7A, Ra0453). Upon continued incubation, the cellotriose was converted to glucose and cellobiose. At the same enzyme concentration as Ra0453, Ra2830 degraded lichenin at a slower rate and released a mixture of products simultaneously (Fig. 3.7A and Fig. 3.7B, Ra2830), and the endproducts profile remain the same over a period of 12 hours, indicating that there are no intermediate oligosaccharides that are further degraded with time. The Ra0505 of *R. albus* 8 was incubated with lichenin at 5 nM, which is ten times lower than the concentrations of the other two enzymes; however, the degradation of lichenin was complete within 2 hours. The major product was the unidentified oligosaccharide and there were no detectable intermediate sugars.

Synergistic hydrolysis of lichenin by Ra0453, Ra2830, Ra0505, and Ra1595. The *R. albus* 8 Ra1595 yielded almost no detectable products from lichenin, based on HPLC analysis (Fig. 3.8A). However, since it degraded oligosaccharides derived from

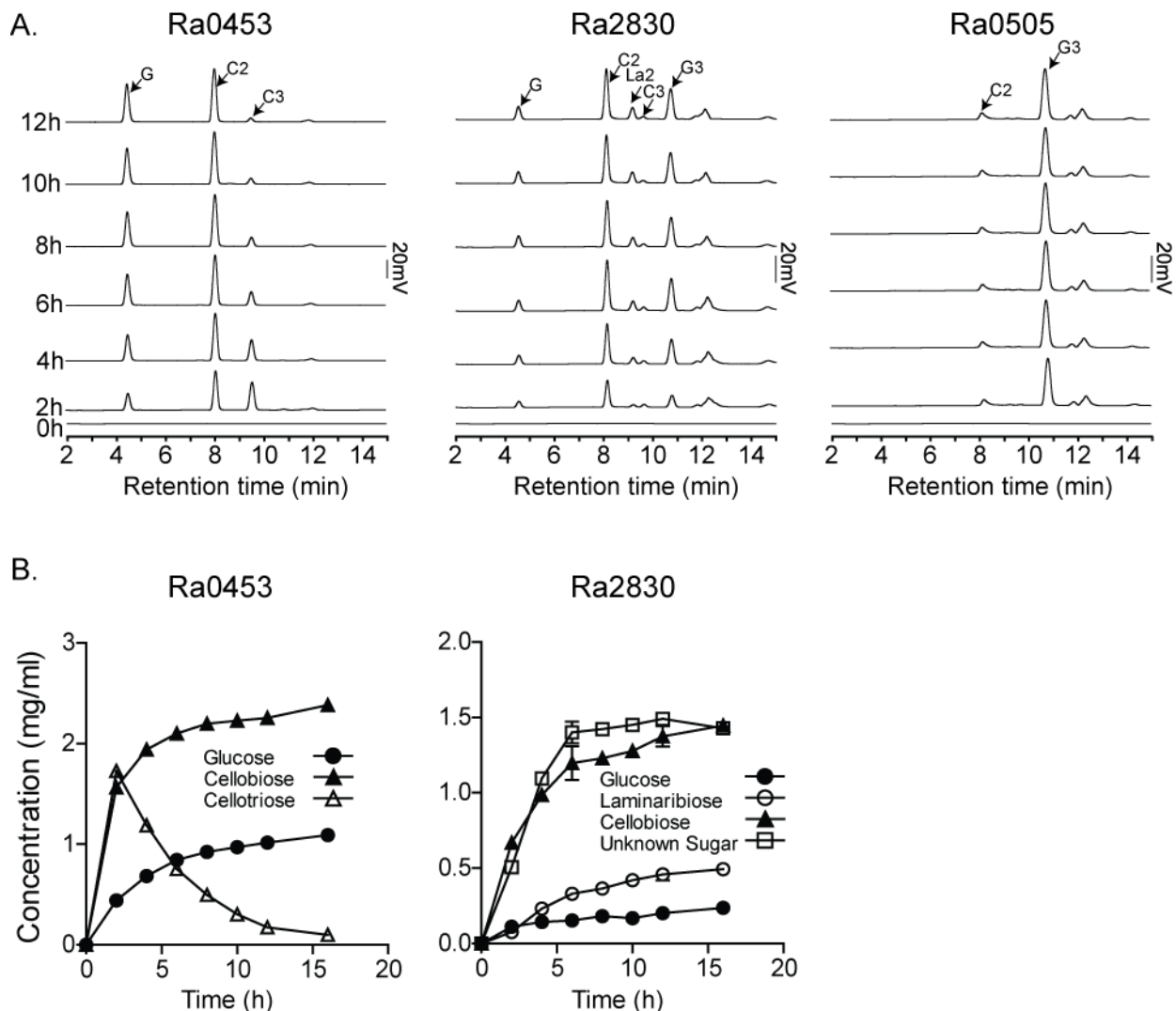


Figure 3.7. Hydrolysis of lichenin by Ra0453, Ra2830 and Ra0505 with time. Lichenin at a final concentration of 0.5% (w/v) was incubated for various lengths of time with Ra0453, Ra2830 or Ra0505 at final concentrations of 0.5 μ M, 0.5 μ M and 0.05 μ M respectively. (A) Identification of products of soluble oligosaccharide hydrolysis of the three enzymes by HPAEC-PAD. Sample peaks were identified by comparison of sample retention times with those of commercially available oligosaccharides. (B) Concentrations of sugars released during hydrolysis of lichenin. Concentrations were determined by analyzing peak areas of mono-, di-, and tri-saccharides and comparing to standard curves generated with known concentrations of the corresponding sugars. Reactions were performed in triplicates, and error bars correspond to standard error of the mean. Abbreviations: G, glucose; C2, cellobiose; C3, cellotriose; La2, laminaribiose; G3, an unidentified oligosaccharide.

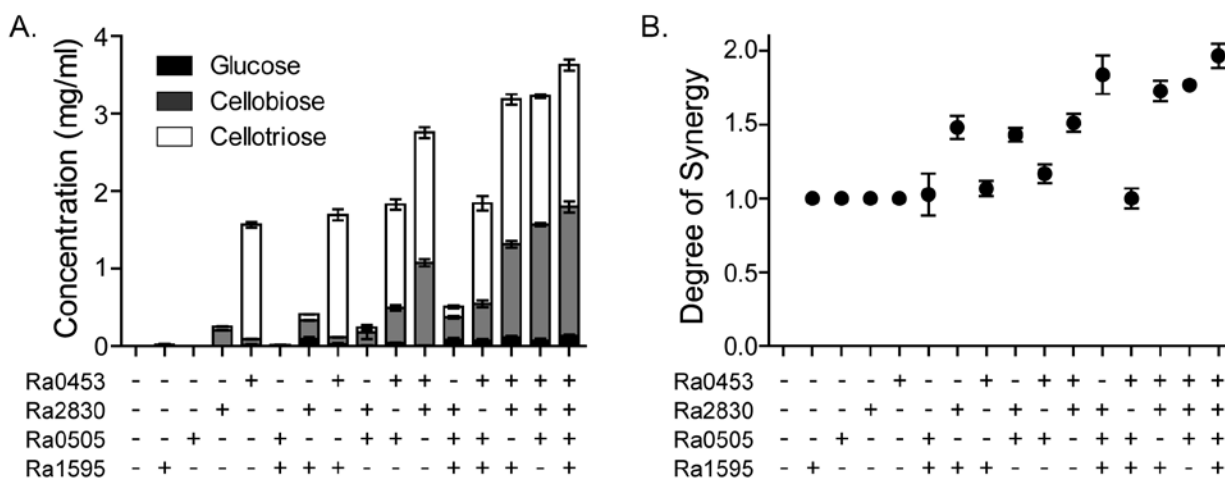


Figure 3.8. Determination of synergistic activities of Ra0453, Ra2830, Ra0505, and Ra1595 during hydrolysis of lichenin. All possible combinations of the four enzymes Ra0453, Ra2830, Ra0505, and Ra1595 present at final concentrations of 0.5 μ M, 0.5 μ M, 0.05 μ M, and 0.1 μ M, respectively, were incubated with lichenin (0.5% w/v) for 1 minute. (A) Concentrations of glucose [67], cellobiose (gray) and cellotriose (white) present in the sample after hydrolysis. Concentrations were determined by comparison of the peak areas of the sample to those of standard curves generated with known concentrations of the corresponding sugars. (B) Degree of synergy of enzyme mixtures calculated by using the following formula: $DOS = \frac{\text{Total sugars released from enzyme mixture}}{\sum(\text{Total sugars released from incubation with the individual enzymes in the mixture})}$. Reactions were performed in triplicates, and the bars correspond to standard error of the mean. The (-) and (+) signs mean absence and presence, respectively, of an enzyme in a particular row.

lichenin, it was analyzed together with the polysaccharide degrading enzymes to determine if combinations of the four enzymes will synergistically release products. All possible combinations of enzyme mixtures were investigated, and the soluble sugars released were analyzed by HPAEC-PAD. Short term hydrolysis (Fig. 3.8), performed for only 1 minute, showed pronounced release of cellobiose and cellotriose in mixtures containing Ra0453. In order to quantify the degree of synergy (DOS), the following formula was used; $DOS = \frac{[\text{Total sugars released from enzyme mixture}]}{\sum([\text{Total sugars released from incubation with individual enzymes}])}$. A majority of the mixtures showed some amount of synergistic activity. Combinations of two or three enzymes lacking Ra2830 showed a lower DOS compared to mixtures containing Ra2830. When Ra2830 is co-incubated with Ra1595 on lichenin, there is a 4-fold increase in the concentration of glucose released, leading to a DOS of approximately 1.5. Mixtures containing Ra0453 were able to release larger amounts of glucose and cello-oligosaccharides and the unidentified oligosaccharide was not detected. The mixture containing all of the enzymes released the most glucose, cellobiose and cellotriose, which corresponded to approximately 85% conversion of the substrate in the reaction mixture into soluble sugars. After 12 hours of hydrolysis (Fig. 3.9), mixtures containing Ra0453 were able to release only glucose, cellobiose and cellotriose, while mixtures lacking Ra0453 contained large amounts of the unidentified oligosaccharide (G3 in Fig 3.9). The unidentified oligosaccharide was purified from thin layer chromatography plates and subjected to hydrolysis by each of the enzymes investigated in the present study, and only Ra0453 hydrolyzed this oligosaccharide to cellobiose and glucose (Fig. 3.9B, lane

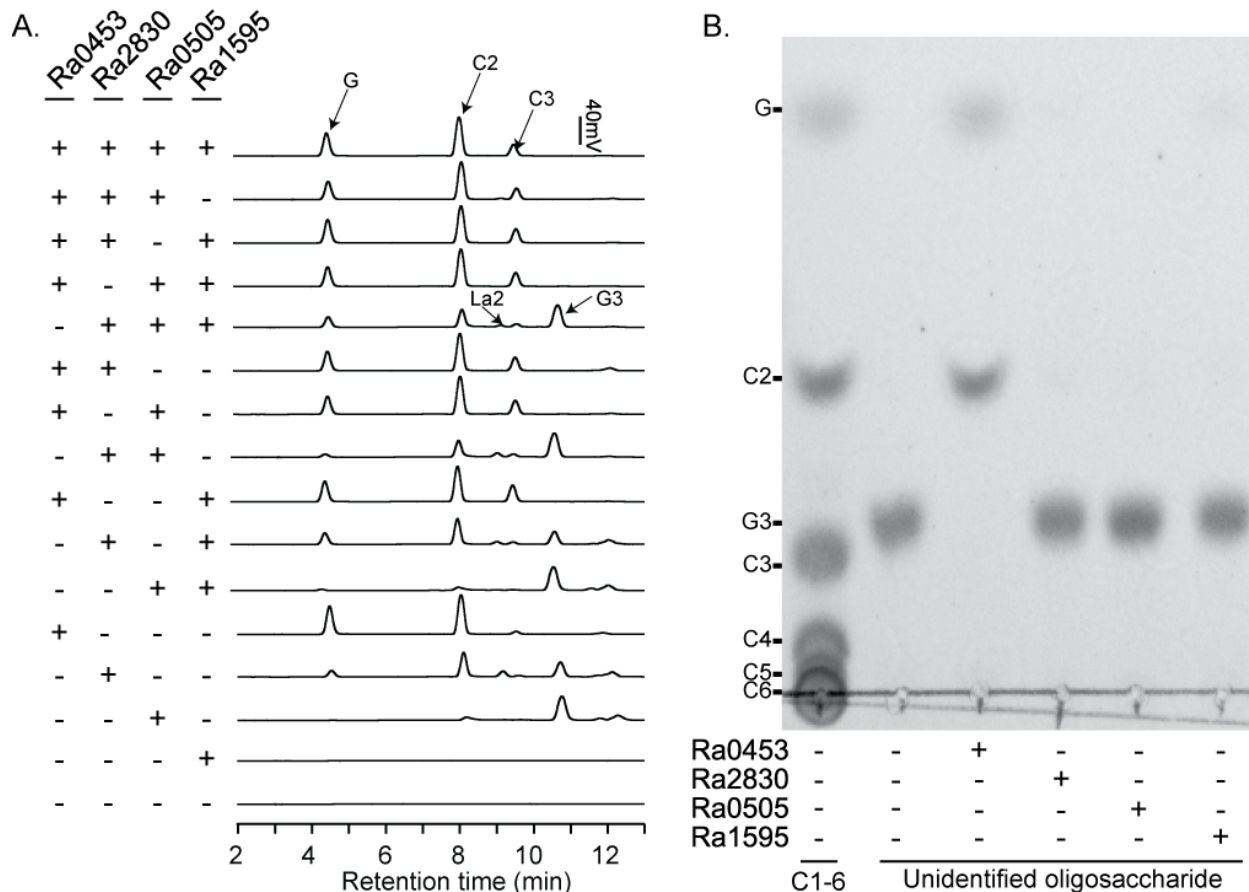


Figure 3.9. Total degradation of lichenin by mixtures of Ra0453, Ra2830, Ra0505, and Ra1595. All possible combinations of the enzymes Ra0453, Ra2830, Ra0505, and Ra1595 present at a final concentration of 0.5 μ M, 0.5 μ M, 0.05 μ M, and 0.1 μ M, respectively, were incubated with 0.5% (w/v) lichenin for 12 h. (A) HPAEC-PAD chromatographs of representative samples are shown displaying the oligosaccharides produced following degradation. Sample peaks were identified by comparison of sample retention times to those of commercially available oligosaccharides. (B) Visualization of products released from the unidentified oligosaccharide (G3 in Fig. 3.9A), incubated with or without Ra0453, Ra2830, Ra0505 and Ra1595. The unidentified oligosaccharide was incubated with 0.5 μ M enzyme concentration for 16 h at 37°C and reaction products were resolved using thin layer chromatography. Abbreviations: G, glucose; C2, cellobiose; C3, cellotriose; C4, cellotetraose; C5, cellopentaose; C6, cellohexaose; C1-6, mixture of glucose and cello-oligosaccharides (DP 2-6). G3, unidentified oligosaccharide.

3). Thus, this hydrolytic activity of Ra0453 leads to the absence of the unidentified oligosaccharide in the end products of enzyme mixtures containing Ra0453.

DISCUSSION

In the plant cell wall of several commonly used livestock feeds, β -glucans serve as cross-linking glycans, holding cellulose microfibrils together and potentially shielding them from microbial degradation. However, lichens possess mixed linkage β -glucans as a storage polysaccharide, which is consumed by reindeer and caribou as forage during winter [17, 18]. The mixed linkages found in the feed of ruminants have led to evolution or acquisition of diverse glycoside hydrolases to hydrolyze complex polysaccharides, and these enzymes include cellulases from GH families 5 and 9 [95], as well as GH16 lichenin specific glucanases [160]. In a recent metagenomic study, GH family 16 enzymes were found to comprise 1.74% of the total glycoside hydrolase genes in fiber adherent bacteria of the bovine rumen, providing support for the importance of mixed linkage glucan hydrolysis [171].

The *R. albus* 8 Ra0505 effectively hydrolyzes lichenin, and homologs of this enzyme are found in the sequenced genomes of several important ruminal fibrolytic bacteria including *Butyrivibrio proteoclasticus* B316, *Prevotella ruminicola* 23, *Ruminococcus flavefaciens* FD-1, two homologs in *Fibrobacter succinogenes* S85, and two homologs in *R. albus* 7. Interestingly, the genome sequence of *P. ruminicola* 23 shows a region that may be involved in lichenin utilization. Within this region are genes coding for a TonB dependent transporter, a GH16 enzyme, a GH5 enzyme, and a GH3 enzyme in an operon-like organization. The glycoside hydrolases in the putative operon exhibited homology to the enzymes characterized in this study including Ra0505,

Ra0453 and Ra1595. The data presented here may, therefore, provide a model for the mechanism by which *P. ruminicola* 23 degrades and metabolize mixed linkage glucans. A lichenase (XynD) from *R. flavefaciens* 17 has been cloned and biochemically characterized. The polypeptide of the *R. flavefaciens* 17 XynD is composed of two catalytic domains: a GH11 xylanase and a GH16 lichenase [82]. The GH16 domain of XynD and that of Ra0505 share 55% amino acid sequence identity. In contrast to *R. flavefaciens* strain 17, the sequenced genome of *R. flavefaciens* strain FD-1 contains a gene coding for a single polypeptide that harbors three tandem repeats of the GH16 module.

The *R. albus* 8 Ra0453 is able to efficiently degrade the mixed linkage glucan into glucose, cellobiose and celotriose, mostly products that lack β -1,3 linkages. Several characterized GH5 enzymes possess hydrolytic activity on lichenin. These enzymes have high activity on cellulosic substrates and are likely to hydrolyze the β -1,4 linkages within mixed linkage glucans and not the β -1,3 linkages [164, 172, 173]. The results presented in this study show that Ra0453 is able to hydrolyze the β -1,3 linkages present in β -1,3-1,4 glucans and have no activity on β -1,3 glucans such as laminarin (data not shown). It appears that a β -1,4 linkage present between the -1 and -2 subsites is required by Ra0453 for the hydrolysis of the β -1,3 glycosidic bond (Fig 3.6i). Although Ra0453 is capable of hydrolyzing the mixed linkage polysaccharide, this enzyme is predicted to be intra-cellularly located due to the lack of a signal peptide, and therefore it may degrade the products of lichenin breakdown after transport into the cell. This hypothesis is supported by the release of the unidentified oligosaccharide product of

lichenin hydrolysis by Ra2830 and Ra0505 (enzymes with signal peptide), which is converted to glucose and cellobiose by Ra0453.

There is potential for Ra0453 to be used as feed additive for diets made with barley grain. Previous studies have shown that inclusion of a β -1,3-1,4 glucanase improved the nutritive value of barley based broiler diets [5]. In contrast to other licheninases, the hydrolysis of mixed linkage glucans by Ra0453 releases a fair amount of glucose with prolonged incubation, and inclusion in feeds may provide additional nutritional benefits, especially to monogastric animals.

There have been two previous studies conducted on β -glucosidases from *R. albus* strains F-40 and strain AR67 [127, 128]. In the study conducted by Ware et al., the GH3 protein had greater hydrolytic activity on β -1,3 linked laminaribiose and laminarin than the β -1,4 linked cellobiose and cello-oligomers [127]. The study performed by Ohmiya et al. characterized a β -glucosidase [128] that shares 79% amino acid sequence identity with Ra1595. This enzyme was able to degrade pNP- β -D-glucoside within 2 hours, however, hydrolysis of cellobiose was incomplete after 15 hours. In our study, Ra1595 exhibited a similar activity since it was able to hydrolyze pNP- β -D-glucoside faster than cellobiose (Fig. 3.2iv). We have shown that Ra1595 preferentially degrades laminaribiose, and therefore it can be classified as a β -1,3 glucosidase. The products from Ra2830 catalyzed hydrolysis of lichenin include laminaribiose, and Li3A, which are suitable substrate for Ra1595. As expected, these two enzymes acted synergistically to release more glucose.

Based on the results from the present study, a model for utilization of lichenin by *R. albus* 8 likely involves secretion of Ra2830 and Ra0505 into the extracellular space to degrade this complex plant cell wall polysaccharide. The two enzymes hydrolyze the β -glucans into a variety of oligosaccharides that are transported into the cell as substrates for further degradation by the other enzymes (Ra0453 and Ra1595) predicted to be intra-cellularly located. The β -glucosidase Ra1595 degrades laminaribiose and Li3A (produced by Ra2830) into glucose and cellobiose. The *R. albus* 8 Ra0453 degrades the unidentified oligosaccharide (produced by Ra0505 and Ra2830) into glucose and cellobiose, and these products correspond to the final products seen in the mixture of all four enzymes (Fig. 3.9). The genome of *R. albus* 8 codes for a putative cellobiose phosphorylase that likely catalyzes the phosphorolysis of cellobiose into glucose and glucose-1-phosphate which can then enter glycolysis [88, 113]. Future studies on enzyme localization, especially for Ra0453, and gene expression profiles will be required to validate this model.

SUMMARY OF RESEARCH

In this work, a bioinformatics analysis of the genome sequence of *R. albus* 8 was performed to identify genes involved in the degradation of polysaccharides. Initially, a focus was placed on genes coding for predicted glycoside hydrolases capable of degrading xylan and hydrolysis of the substituents commonly found on the xylan backbone. Two endo-xylanase encoding genes containing GH family 10 catalytic domains and three endo-xylanase genes coding for GH family 11 catalytic domains were cloned and heterologously expressed in *E. coli* and purified to near homogeneity. All but one of the polypeptides contained carbohydrate-binding modules. In addition to CBMs, Xyn11D and Xyn11E contain carbohydrate esterase domains, which function to remove acetyl residues found on xylan chains. These five enzymes were able to hydrolyze hetero-xylans to produce unique product profiles showing that the GH10 and GH11 enzymes can cleave the xylan in distinct locations respective of the substituents. As expected, hydrolysis of the soluble wheat arabinoxylan was more rapid than insoluble xylans most likely due to greater accessibility of the xylan chains to the enzymes.

After further analysis of complementary enzymes on the genome, a GH3 β -xylosidase (Xyl3A) was cloned and purified from this organism. This enzyme was shown to preferentially hydrolyze xylose linked substrates. In conjunction with a GH67 α -glucuronidase (Aug67A), an enzyme predicted to remove glucuronic acid residues appended to xylose units, the Xyl3A was able to synergistically release more xylose units from aldouronic acids. Finally, a GH 51 α -arabinofuranosidase (Ara51A), was

shown to release arabinose units from substituted xylan. A mixture created including all of the endo-xylanases, Xyl3A, and Ara51A was capable of synergistic release of xylose and arabinose from hetero-xylans.

Upon higher substrate loading, a larger proportion of intermediate products accumulated, including xylobiose and an oligosaccharide that migrated near xylohexaose. The xylobiose accumulation was alleviated when excess Xyl3A was added to the mixture, and the other oligosaccharide was degraded when Ara51A was included in the mixture. Inclusion of all of these enzymes allowed the release of more xylose and arabinose.

Most *Ruminococcus* species are not able to utilize the monomers of substituted xylan for growth which may be a reflection of the organism's ability to transport these sugars into the cell. As a result, oligosaccharide must be degraded in the cytoplasm which can be related to the absence of a signal peptide within the accessory enzymes. Most of the endo-xylanases contain signal peptides because their respective substrates are too large to be transported into the cell. A single GH 10 did not possess a signal peptide. While it is possible for this polypeptide to be exported using a separate pathway, it may also be likely that this gene product remains inside the cell, hydrolyzing small oligosaccharides into smaller chains for more rapid metabolism. The accessory enzyme, Xyl3A, Ara51A, and Agu67A, all lack signal peptides and are likely retained in the cytoplasm. When small oligosaccharides produced from the action of the endo-xylanases enter the cell, the accessory enzymes may cleave the substituents and release sugar monomers for fermentation.

R. albus 8 was isolated as a fibrolytic bacterium capable of hydrolysis of cellulose. While analyzing the genome for enzymes capable of cellulose hydrolysis, two genes (Ra0453 and Ra2830) were of initial interest due to their predicted functions as exo-glucanases. Upon cloning and expression of these two genes, they were shown to have higher rate of hydrolysis on lichenin, a soluble mixed linkage glucan. Another gene (Ra0505) predicted to have lichenin hydrolyzing capabilities was then cloned and showed to have a high rate of lichenin hydrolysis. These three enzymes showed unique product profiles. While Ra0453 only produced glucose cellobiose and cellotriose, the other two enzymes produced an oligosaccharide of unknown identity. While searching for β -glucosidases that can degrade cellobiose, a GH 3 enzyme (Ra1595) was found to rapidly hydrolyze the β -1,3 linked oligosaccharides produced from lichenin hydrolysis by Ra2830 and Ra0505. The β -glucosidase synergistically released more glucose and cellobiose when added to lichenin with Ra2830 and Ra0505. Interestingly, only Ra0453 was able to degrade the unknown oligosaccharide into glucose and cellobiose.

Cellular localization of these gene products would be very helpful in identification of the pathway of lichenin hydrolysis. The signal peptide containing Ra2830 is likely extracellularly located, and due to its higher cellulose degrading ability also functions as a cellulase with the ability to hydrolyze lichenin whenever the substrate is present. Ra0505 is likely an extracellular licheninase and produces the unidentified oligosaccharide which is subsequently transported into the cell. Upon entering the cell Ra0453, lacking a signal peptide, is able to hydrolyze the substrate into glucose and cellobiose, which are then fermented by the bacterium. Products of lichenin hydrolysis

by Ra2830 enter the cell where Ra1595 can further degrade the β -1,3 linkage to produce fermentable sugars.

FUTURE RESEARCH

This work provides a foundation of knowledge in understanding the enzymes employed by *R. albus* 8 to degrade xylan and lichenin into readily fermentable sugars. The cellulolytic potential of *R. albus* 8 enzymes has yet to be explored in detail. Analysis of the draft genome sequence reveals nine potential endo-glucanases (GH family 5) and three exo-glucanases (GH family 9) which have been cloned in our lab. Preliminary analysis of the gene products show that seven GH family 5 enzymes and one GH family 9 enzyme possess hydrolytic activity on amorphous cellulose. Further characterization of each enzyme is required to fully elucidate the mechanisms of cellulose degradation.

The genes presented in this work were identified through a bioinformatic search by annotating predicted gene functions, which has limitations. As seen with Ra0453 and Ra2830, the predicted exo-glucanase function was mis-annotated and this may apply to the predicted function of other genes. As seen with other rumen bacteria, many glycoside hydrolase containing genes are highly up-regulated for fermentation of a specific polysaccharide [49]. Future studies would benefit from using a transcriptomic approach to identify genes differentially regulated during growth on different carbon sources, such as cellulose and xylan. This would provide insight into the genes regulated by *R. albus* 8, highlighting those that are preferentially expressed for the degradation of cellulose or xylan.

Genes identified for polysaccharide depolymerization may contain a signal peptide implying extracellular localization of gene products and the lack of a signal peptide implies cytoplasmic localization. This can be tested by preparing cellular

fractions and performing quantitative proteomics or 2D gel electrophoresis and N-terminal sequencing. Verification of enzyme localization can provide insight into the pathway of polysaccharide degradation, especially in regards to transport of intermediate products for cytoplasmic deconstruction or complete degradation of oligosaccharides in the extracellular space.

Ruminococcus albus is unable to grow on the monosaccharides, glucose and xylose, while capable of rapid fermentation of cellulose and xylan. This presents the possibility of transport of oligosaccharide into the cell and the inability to transport monosaccharides. ABC transporters are encoded on the genome of this organism, but due to the lack of gene organization into operons, it is difficult to predict the solute that is transported. Identification of the substrate transported is a crucial step that can be tested by cloning the transporter into another organism and determining if this organism would be able to uptake the molecule. If the gene involved in oligosaccharide uptake is transcriptionally regulated in *R. albus* 8, this would be identified using RNA seq.

Rumen bacteria generally lack genetic systems for the manipulation of the genome. Ideally, individual enzyme contribution to the deconstruction of a polysaccharide would be tested by creating deletion mutants and testing growth rate on the polysaccharide of interest. Therefore, it is of interest to pursue techniques to create a genetic system in *R. albus* 8.

To fully understand how rumen bacterium can ferment complex polysaccharides, further research is essential to elucidate the mechanism of hydrolysis as well as the mechanism of gene regulation. As this work provides answers to key steps in this topic, it also offers many questions that are yet to be answered.

Appendix A. Annotation of the glycoside hydrolases within the draft genome sequence of *Ruminococcus albus* 8

The genome of *Ruminococcus albus* 8 was sequenced at the W.M. Keck Center for Comparative and Functional Genomics using the GS FLX Titanium General Library and 8 kb span paired end library methods. Sequences obtained from the general library were aligned onto the paired end library. The resulting draft genome sequence contained 2 scaffolds comprised of 172 contigs with an estimated genome coverage of 120x. The estimated genome size was 3.9 MB with a G+C content of 45.4%.

An in depth bioinformatic analysis of the genome was performed to identify all putative glycoside hydrolase (GH) encoding genes present. Several representative members of each glycoside hydrolase family in the Carbohydrate Active enzyme (CAZy) database were aligned onto the genome using the Basic Local Alignment Search Tool (BLAST) to identify potential genes of interest. The genes identified were then used to search the NCBI genomic database using BLAST to identify the most closely related, characterized enzymes. Domain architectures were identified using the Conserved Domain Database (CDD) and Pfam (pfam.sanger.ac.uk). The putative GH encoding genes from *R. albus* 8 were used to search the draft genome to identify any other potential genes of interest.

Using this approach, a total of 61 CAZy genes were identified from 22 families. The genes identified reveal the versatility of *R. albus* 8 for polysaccharide degradation. A total of 4 families are involved in cellulose degradation and 8 families are involved in hemicellulose degradation. Genes encoding hydrolytic enzymes involved in mannan,

arabinan, and pectin deconstruction are also coded within the genome of *R. albus* 8. Almost 10% of the glycoside hydrolases possess dockerin sequences within the polypeptide, yet there were no scaffoldin encoding genes identified. In lieu of a cellulosomal system, *R. albus* 8 shows variation in the domain architectures of the glycoside hydrolases and accessory domains. Many catalytic modules are coded on the same polypeptide as carbohydrate binding modules or even other catalytic domains with complementary function (i.e. xylanase and acetyl xylan esterase).

The *R. albus* 8 draft genome revealed the presence of four GH families with the predicted function of cellulose degradation: β -glucosidases from GH family 3, endoglucanases from GH family 5, and exo-glucanases from GH families 9 and 48. A total of 20 genes were annotated with the predicted function of complete cellulose degradation. A total of 30 genes were predicted to be involved in hemicellulose degradation, including 15 genes specific for xylan degradation. The GH families involved in xylan degradation include β -xylosidases from GH 3, endo-xylanases from GH 8, 10, and 11, arabinofuranosidases from GH 43 and 51, and α -glucuronidases from GH 2 and 67.

The annotation of these genes provides a glimpse into the fibrolytic versatility of *Ruminococcus albus* 8 and provided initial targets for gene cloning and characterization.

Table A.1. Glycoside hydrolases identified within the draft genome sequence of *Ruminococcus albus* 8.

GH/CE Family	PEG #	Domain Architecture				Putative Activity	Percent Identity	Enzyme(Accession #)/ Organism		
		200aa								
GH 2	1321							β -galactosidase	33%	BbgIII(ABE27152)/ <i>Bifidobacterium bifidum</i>
GH 2	1636							β -galactosidase	40%	Lactase(P70753)/ <i>Actinobacillus pleuropneumoniae</i>
GH 2	3451							β -glucuronidase/ β -galactosidase	20%	β -galactosidase(P00722)/ <i>Escherichia coli</i>
GH 2	3468							β -mannosidase	33%	β -mannosidase(2VJX_A)/ <i>Bacteroides Thetaiotaomicron</i>
GH 3	1595							β -glucosidase	78%	β -glucosidase(P15885)/ <i>R. albus</i>
GH 3	2003							β -glucosidase	36%	Glucohydrolase(AAB36385)/ <i>Thermobispora bispora</i>
GH 3	2287							β -glucosidase/ β -xylosidase	52%	β -xylosidase B(CAD48309)/ <i>Clostridium stercorarium</i>
GH 3	3307							β -glucosidase	54%	β -glucosidase(CAB08072)/ <i>Clostridium stercorarium</i>
GH 4	2355							α -galactosidase/ α -glucosidase	23%	α -glucosidase(1OBB_B)/ <i>Thermotoga maritima</i>
GH 5	185							β -1,4-endoglucanase	83%	Endo-1,4- β -Glucanase VII(BAA92430)/ <i>R. albus</i>
GH 5	259							β -1,4-endoglucanase	73%	Endoglucanase 4(Q07940)/ <i>R. albus</i>
GH 5	325							β -1,4-endoglucanase	78%	Endoglucanase B(CAA38693)/ <i>R. albus</i>
GH 5	453							β -1,4-endoglucanase	44%	Cellodextrinase A(P16169)/ <i>R. flavafaciens</i>
GH 5	711							β -1,4-endoglucanase	28%	Endo-1,4-beta-Glucanase V(BAA92146)/ <i>R. albus</i>
GH 5	903							β -1,4-endoglucanase	50%	Endo- β -1,4-glucanase G(Q05332)/ <i>Clostridium thermocellum</i>
GH 5	1553							β -1,4-endoglucanase	31%	Endo-1,4-beta-Glucanase(P23661)/ <i>R. albus</i>










Table A.1. (cont.)

GH 5	1793		β -1,4-endomannanase	60%	Mannanase(BAA25878)/ <i>Bacillus circulans</i>
GH 5	1831		β -1,4-endoglucanase	43%	Endo-1,4- β -Glucanase V(BAA92146)/ <i>R. albus</i>
GH 5	2461		β -1,4-endoglucanase	59%	CelB(AAD30364)/ <i>Caldicellulosiruptor sp.</i>
GH 5	2535/ 2536		β -1,4-endoglucanase	55%	Endo-1,4- β -Glucanase V(BAA92146)/ <i>R. albus</i>
GH 5	2830		β -1,4-endoglucanase	52%	1,4- β -Endo/Exo-glucanase B(P10474)/ <i>Caldicellulosiruptor saccharalyticus</i>
GH 5	2979		β -1,4-endoglucanase	43%	Xyloglucanase(2JEP_A)/ <i>Paeanibacillus pabuli</i>
GH 8	1474		β -1,4-endoxylanase	57%	Xylanase(1WU4_A)/ <i>Bacillus Halodurans</i>
GH 9	2259		Cellobiohydrolase	37%	1,4-Cellobiohydrolase(P0C2S1)/ <i>Clostridium thermocellum</i>
GH 9	2875		Cellobiohydrolase	34%	1,4-Cellobiohydrolase(P0C2S1)/ <i>Clostridium thermocellum</i>
GH 9	2876		Cellobiohydrolase	37%	Endo-1,4- β -glucanase A(P22534)/ <i>Caldicellulosiruptor saccharolyticus</i>
GH 9	3241		Cellobiohydrolase	33%	1,4-Cellobiohydrolase(P0C2S1)/ <i>Clostridium thermocellum</i>
GH 10	2725		β -1,4-endoxylanase	40%	Endo-1,4-beta-xylanase B(P26223)/ <i>Butyrivibrio fibrisolvens</i>
GH 10	2882		β -1,4-endoxylanase	49%	Endo-1,4- β -xylanase Y(P51584)/ <i>Clostridium thermocellum</i>
GH 11 CE 4	997		β -1,4-endoxylanase & deacetylase	49%	Xylanase A(AAA85198)/ <i>R. albus</i>
GH 11 CE4	1984		β -1,4-endoxylanase & deacetylase	70%	Xylanase A(AAA85198)/ <i>R. albus</i>
GH 11	2008		β -1,4-endoxylanase	60%	Xylanase B(BAB39493)/ <i>R. albus</i>
GH 16	505		β -1,3-1,4-endoglucanase	57%	β -1,3-1,4-endoglucanase(P27051)/ <i>Bacillus licheniformis</i>

Table A.1. (cont.)

GH 25	645		β -1,4-N-acetylmuramic acid	31%	Lysozyme(1JFX_A)/ <i>Streptomyces coelicolor</i>
GH 25	1133		β -1,4-N-acetylmuramic acid	31%	Lysozyme(1JFX_A)/ <i>Streptomyces coelicolor</i>
GH 25	1300		β -1,4-N-acetylmuramic acid	30%	Lysozyme(1JFX_A)/ <i>Streptomyces coelicolor</i>
GH 25	3198		β -1,4-N-acetylmuramic acid	30%	Lysozyme(1JFX_A)/ <i>Streptomyces coelicolor</i>
GH 26	793		β -1,4-mannanase	32%	β -1,4-mannanase(AAC44232)/ <i>Caldicellulosiruptor saccharolyticus</i>
GH 26	1372		β -1,4-mannanase	35%	β -1,4-mannanase(AAC44232)/ <i>Caldicellulosiruptor saccharolyticus</i>
GH 26	1906		β -1,4-mannanase	29%	β -1,4-mannanase(AAC44232)/ <i>Caldicellulosiruptor saccharolyticus</i>
GH 26	2739		β -1,4-mannanase	40%	Mannanase 26B(BAB19050)/ <i>Clostridium thermocellum</i>
GH 26 CE3	3353		β -1,4-mannanase/ acetyl xylan esterase	36%	Mannanase 26B(BAB19050)/ <i>Clostridium thermocellum</i>
GH 28	2353		Pectinase	34%	Pectate lyase(AAW84064)/ Uncultured bacterium
GH 31	3069		α -galactosidase	26%	α -galactosidase A(Q9ALJ4)/ <i>Geobacillus stearothermophilus</i>
GH 31	3092		α -xylosidase	49%	α -glycosidase(1XSI_A)/ <i>Escherichia coli</i>
GH 31	2050		α -galactosidase	51%	α -galactosidase A(Q9ALJ4)/ <i>Geobacillus stearothermophilus</i>
GH 43	1302		Endoarabinase/ β -xylosidase	32%	β -xylosidase(P07129)/ <i>Bacillus pumilus</i>
GH 43	1555		α -N-arabinofuranosidase xylosidase	37%	Xylosidase/Arabinosidase(P48790)/ <i>Clostridium stercoararium</i>
GH 43/ GH 54	3033		Arabinosidase/ Xylosidase	33%	α -L-arabinofuranosidase(ACP50519)/ <i>Penicillium purpurogenum</i>
GH 43	3034		Endoarabinase/ Arabinosidase/ xylosidase	36%	Arabinanase(ACE73680)/ <i>Geobacillus stearothermophilus</i>

Table A.1. (cont.)

GH 43	3046		α -N-arabinofuranosidase	51%	α -N-arabinofuranosidase(P82594)/ <i>Streptomyces chartreusis</i>
GH 43	3372		Endoarabinase	39%	Arabinanase(ACE73680)/ <i>Geobacillus stearothermophilus</i>
GH 48	2561		β -1,4-endoglucanase/ β -1,4-exoglucanase	40%	Cellobiohydrolase S(1L1Y_A)/ <i>Clostridium thermocellum</i>
GH 51	3031		α -L-arabinofuranosidase	57%	α -L-arabinofuranosidase(AAC28125)/ <i>Clostridium stercorarium</i>
GH 53	322		β -1,4-endogalactanase	50%	β -1,4-endogalactanase(Q65CX5)/ <i>Bacillus licheniformis</i>
GH 67	2369		α -glucuronidase	50%	α -D-glucuronidase(1K9D_A)/ <i>Geobacillus stearothermophilus</i>
GH 77	2141		4- α -gluconotransferase	48%	4- α -gluconotransferase(Q59266)/ <i>Clostridium butyricum</i>
GH 88	564		D-4,5 unsaturated β -glucuronyl hydrolase	26%	unsaturated rhamnogalacturonyl hydrolase(2GH4_A)/ <i>Bacillus subtilis</i>
GH 88	2354		D-4,5 unsaturated β -glucuronyl hydrolase	23%	unsaturated rhamnogalacturonyl hydrolase(O31521)/ <i>Bacillus subtilis</i>

REFERENCES

1. Bryant, M.P., *Bacterial species of the rumen*. Bacteriol. Rev., 1959. **23**(3): p. 125-53.
2. Bergman, E.N., *Energy contributions of volatile fatty acids from the gastrointestinal tract in various species*. Physiol. Rev., 1990. **70**(2): p. 567-90.
3. Nafikov, R.A. and D.C. Beitz, *Carbohydrate and lipid metabolism in farm animals*. J. Nutr., 2007. **137**(3): p. 702-5.
4. Guilloteau, P., et al., *From the gut to the peripheral tissues: the multiple effects of butyrate*. Nutr. Res. Rev., 2010. **23**(2): p. 366-84.
5. Von Wettstein, D., J. Warner, and C.G. Kannangara, *Supplements of transgenic malt or grain containing (1,3-1,4)- β -glucanase increase the nutritive value of barley-based broiler diets to that of maize*. Br. Poult. Sci., 2003. **44**(3): p. 438-49.
6. Stewart, C.S., S.H. Duncan, and D.R. Cave, *Oxalobacter formigenes and its role in oxalate metabolism in the human gut*. FEMS Microbiol. Lett., 2004. **230**(1): p. 1-7.
7. Hobson, P.N. and R.J. Wallace, *Microbial ecology and activities in the rumen: Part 1*. Crit. Rev. Microbiol., 1982. **9**(3): p. 165-225.
8. Chesson, A., A.H. Gordon, and J.A. Lomax, *Methylation analysis of mesophyll, epidermis, and fiber cell-walls isolated from the leaves of perennial and italian ryegrass*. Carbohydr. Res., 1985. **141**(1): p. 137-147.
9. Bonawitz, N.D. and C. Chapple, *The genetics of lignin biosynthesis: connecting genotype to phenotype*. Annu. Rev. Genet., 2010. **44**: p. 337-63.

10. Wolfenden, R., X.D. Lu, and G. Young, *Spontaneous hydrolysis of glycosides*. J. Am. Chem. Soc., 1998. **120**(27): p. 6814-6815.
11. Pavlostathis, S.G., T.L. Miller, and M.J. Wolin, *Fermentation of insoluble cellulose by continuous cultures of Ruminococcus albus*. Appl. Environ. Microbiol., 1988. **54**(11): p. 2655-9.
12. Burton, R.A. and G.B. Fincher, *(1,3;1,4)- β -D-glucans in cell walls of the poaceae, lower plants, and fungi: a tale of two linkages*. Mol. Plant, 2009. **2**(5): p. 873-82.
13. Dodd, D. and I.K. Cann, *Enzymatic deconstruction of xylan for biofuel production*. Glob. Change. Biol. Bioenergy, 2009. **1**(1): p. 2-17.
14. Kormelink, F.J.M. and A.G.J. Voragen, *Degradation of different [(glucurono)arabino]xylans by a combination of purified xylan-degrading enzymes*. Appl. Microbiol. Biotechnol., 1993. **38**(5): p. 688-695.
15. Ahmadjian, V., *Lichens*. Annu. Rev. Microbiol., 1965. **19**: p. 1-20.
16. Chanda, N.B., E.L. Hirst, and D.J. Manners, *A comparison of isolichenin and lichenin from Iceland Moss (Cetraria Islandica)*. J. Chem. Society, 1957(May): p. 1951-1958.
17. Holleman, D.F., J.R. Luick, and R.G. White, *Lichen intake estimates for reindeer and caribou during winter*. J. Wildl. Manage., 1979. **43**(1): p. 192-201.
18. Kumpula, J., *Winter grazing of reindeer in woodland lichen pasture. Effect of lichen availability on the condition of reindeer*. Small Rumin. Res., 2001. **39**(2): p. 121-130.
19. Kim, M., M. Morrison, and Z. Yu, *Status of the phylogenetic diversity census of ruminal microbiomes*. FEMS Microbiol. Ecol., 2011. **76**(1): p. 49-63.

20. Hungate, R.E., *The anaerobic mesophilic cellulolytic bacteria*. Bacteriol. Rev., 1950. **14**(1): p. 1-49.
21. Russell, J.B., R.E. Muck, and P.J. Weimer, *Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen*. FEMS Microbiol. Ecol., 2009. **67**(2): p. 183-97.
22. Brulc, J.M., et al., *Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases*. Proc. Natl. Acad. Sci. U. S. A., 2009. **106**(6): p. 1948-53.
23. Hungate, R.E., *Studies on cellulose fermentation: III. The culture and isolation for cellulose-decomposing bacteria from the rumen of cattle*. J. Bacteriol., 1947. **53**(5): p. 631-45.
24. Kobayashi, Y., T. Shinkai, and S. Koike, *Ecological and physiological characterization shows that Fibrobacter succinogenes is important in rumen fiber digestion - review*. Folia Microbiol. (Praha), 2008. **53**(3): p. 195-200.
25. Jun, H.S., et al., *Outer membrane proteins of Fibrobacter succinogenes with potential roles in adhesion to cellulose and in cellulose digestion*. J. Bacteriol., 2007. **189**(19): p. 6806-15.
26. Gong, J. and C.W. Forsberg, *Factors affecting adhesion of Fibrobacter succinogenes subsp. succinogenes S85 and adherence-defective mutants to cellulose*. Appl. Environ. Microbiol., 1989. **55**(12): p. 3039-44.
27. Ransom-Jones, E., et al., *The Fibrobacteres: an important phylum of cellulose-degrading bacteria*. Microb. Ecol., 2012. **63**(2): p. 267-81.

28. Suen, G., et al., *The complete genome sequence of Fibrobacter succinogenes S85 reveals a cellulolytic and metabolic specialist*. PLoS One, 2011. **6**(4): p. e18814.
29. Qi, M., H.S. Jun, and C.W. Forsberg, *Cel9D, an atypical 1,4- β -D-glucan glucohydrolase from Fibrobacter succinogenes: characteristics, catalytic residues, and synergistic interactions with other cellulases*. J. Bacteriol., 2008. **190**(6): p. 1976-84.
30. Qi, M., H.S. Jun, and C.W. Forsberg, *Characterization and synergistic interactions of Fibrobacter succinogenes glycoside hydrolases*. Appl. Environ. Microbiol., 2007. **73**(19): p. 6098-105.
31. Cho, K.K., et al., *Molecular cloning and expression of a novel family A endoglucanase gene from Fibrobacter succinogenes S85 in Escherichia coli*. Enzyme Microb. Technol., 2000. **27**(7): p. 475-481.
32. Hu, Y.J., et al., *Cloning of a xylanase gene from Fibrobacter succinogenes 135 and its expression in Escherichia coli*. Can. J. Microbiol., 1991. **37**(7): p. 554-61.
33. Jun, H.S., et al., *Characteristics of a cluster of xylanase genes in Fibrobacter succinogenes S85*. Can. J. Microbiol., 2003. **49**(3): p. 171-80.
34. Marrone, L., K.A. McAllister, and A.J. Clarke, *Characterization of function and activity of domains A, B and C of xylanase C from Fibrobacter succinogenes S85*. Protein Eng., 2000. **13**(8): p. 593-601.
35. Matte, A. and C.W. Forsberg, *Purification, characterization, and mode of action of endoxylanases 1 and 2 from Fibrobacter succinogenes S85*. Appl. Environ. Microbiol., 1992. **58**(1): p. 157-68.

36. Paradis, F.W., et al., *The xynC gene from Fibrobacter succinogenes S85 codes for a xylanase with two similar catalytic domains*. J. Bacteriol., 1993. **175**(23): p. 7666-72.
37. Yoshida, S., et al., *Domain analysis of a modular α -L-Arabinofuranosidase with a unique carbohydrate binding strategy from the fiber-degrading bacterium Fibrobacter succinogenes S85*. J. Bacteriol., 2010. **192**(20): p. 5424-36.
38. Kam, D.K., et al., *Characteristics of adjacent family 6 acetylxylan esterases from Fibrobacter succinogenes and the interaction with the Xyn10E xylanase in hydrolysis of acetylated xylan*. Can. J. Microbiol., 2005. **51**(10): p. 821-32.
39. McDermid, K.P., C.W. Forsberg, and C.R. MacKenzie, *Purification and properties of an acetylxylan esterase from Fibrobacter succinogenes S85*. Appl. Environ. Microbiol., 1990. **56**(12): p. 3805-10.
40. Yoshida, S., R.I. Mackie, and I.K. Cann, *Biochemical and domain analyses of FSUAxe6B, a modular acetyl xylan esterase, identify a unique carbohydrate binding module in Fibrobacter succinogenes S85*. J. Bacteriol., 2010. **192**(2): p. 483-93.
41. Russell, J.B., *Strategies that ruminal bacteria use to handle excess carbohydrate*. J. Anim. Sci., 1998. **76**(7): p. 1955-63.
42. Kong, Y., et al., *Quantitative fluorescence in situ hybridization of microbial communities in the rumens of cattle fed different diets*. Appl. Environ. Microbiol., 2010. **76**(20): p. 6933-8.

43. Bryant, M.P., et al., *Bacteroides ruminicola* n. sp. and *Succinimonas amylolytica*; the new genus and species; species of succinic acid-producing anaerobic bacteria of the bovine rumen. J. Bacteriol., 1958. **76**(1): p. 15-23.
44. Miyazaki, K., et al., *Degradation and utilization of xylans by the rumen anaerobe Prevotella bryantii* (formerly *P. ruminicola* subsp. *brevis*) B₁₄. Anaerobe, 1997. **3**(6): p. 373-81.
45. Martens, E.C., et al., *Complex glycan catabolism by the human gut microbiota: the Bacteroidetes Sus-like paradigm*. J. Biol. Chem., 2009. **284**(37): p. 24673-7.
46. Dodd, D., R.I. Mackie, and I.K. Cann, *Xylan degradation, a metabolic property shared by rumen and human colonic Bacteroidetes*. Mol. Microbiol., 2011. **79**(2): p. 292-304.
47. Miyazaki, K., et al., *Involvement of the multidomain regulatory protein XynR in positive control of xylanase gene expression in the ruminal anaerobe Prevotella bryantii* B₁₄. J. Bacteriol., 2003. **185**(7): p. 2219-26.
48. Martens, E.C., et al., *Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts*. PLoS Biol., 2011. **9**(12): p. e1001221.
49. Dodd, D., et al., *Transcriptomic analyses of xylan degradation by Prevotella bryantii and insights into energy acquisition by xylanolytic bacteroidetes*. J. Biol. Chem., 2010. **285**(39): p. 30261-73.
50. Purushe, J., et al., *Comparative genome analysis of Prevotella ruminicola and Prevotella bryantii: insights into their environmental niche*. Microb. Ecol., 2010. **60**(4): p. 721-9.

51. Hungate, R.E., *Microorganisms in the rumen of cattle fed a constant ration*. Can. J. Microbiol., 1957. **3**(2): p. 289-311.
52. Chen, J., D.M. Stevenson, and P.J. Weimer, *Albusin B, a bacteriocin from the ruminal bacterium Ruminococcus albus 7 that inhibits growth of Ruminococcus flavefaciens*. Appl. Environ. Microbiol., 2004. **70**(5): p. 3167-70.
53. Odenyo, A.A., et al., *The use of 16S rRNA-targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria: development of probes for Ruminococcus species and evidence for bacteriocin production*. Appl. Environ. Microbiol., 1994. **60**(10): p. 3688-96.
54. Chen, J. and P. Weimer, *Competition among three predominant ruminal cellulolytic bacteria in the absence or presence of non-cellulolytic bacteria*. Microbiology, 2001. **147**(Pt 1): p. 21-30.
55. Miron, J., et al., *An adhesion-defective mutant of Ruminococcus albus SY3 is impaired in its capability to degrade cellulose*. J. Appl. Microbiol., 1998. **84**(2): p. 249-254.
56. Devillard, E., et al., *Ruminococcus albus 8 mutants defective in cellulose degradation are deficient in two processive endocellulases, Cel48A and Cel9B, both of which possess a novel modular architecture*. J. Bacteriol., 2004. **186**(1): p. 136-45.
57. Weimer, P.J., et al., *Effect of diet on populations of three species of ruminal cellulolytic bacteria in lactating dairy cows*. J. Dairy Sci., 1999. **82**(1): p. 122-34.

58. Alber, O., et al., *Cohesin diversity revealed by the crystal structure of the anchoring cohesin from Ruminococcus flavefaciens*. *Proteins*, 2009. **77**(3): p. 699-709.
59. Rincon, M.T., et al., *Unconventional mode of attachment of the Ruminococcus flavefaciens cellulosome to the cell surface*. *J. Bacteriol.*, 2005. **187**(22): p. 7569-78.
60. Ding, S.Y., et al., *Cellulosomal scaffoldin-like proteins from Ruminococcus flavefaciens*. *J. Bacteriol.*, 2001. **183**(6): p. 1945-53.
61. Liu, J.H., et al., *An endoglucanase from the anaerobic fungus Orpinomyces jayonii: characterization of the gene and its product*. *Can. J. Microbiol.*, 1997. **43**(5): p. 477-85.
62. Qiu, X., et al., *Isolation and analysis of two cellulase cDNAs from Orpinomyces jayonii*. *Gene*, 2000. **245**(1): p. 119-26.
63. Ye, X.Y., T.B. Ng, and K.J. Cheng, *Purification and characterization of a cellulase from the ruminal fungus Orpinomyces jayonii cloned in Escherichia coli*. *Int. J. Biochem. Cell Biol.*, 2001. **33**(1): p. 87-94.
64. Fujino, Y., et al., *Cloning, sequencing, and expression of an endoglucanase gene from the rumen anaerobic fungus Neocallimastix frontalis MCH3*. *Biosci. Biotechnol. Biochem.*, 1998. **62**(9): p. 1795-8.
65. Xue, G.P., et al., *Cloning and expression of multiple cellulase cDNAs from the anaerobic rumen fungus Neocallimastix patriciarum in Escherichia coli*. *J. Gen. Microbiol.*, 1992. **138**(7): p. 1413-20.

66. Denman, S., G.P. Xue, and B. Patel, *Characterization of a Neocallimastix patriciarum cellulase cDNA (celA) homologous to Trichoderma reesei cellobiohydrolase II*. Appl. Environ. Microbiol., 1996. **62**(6): p. 1889-96.
67. Zhou, L., et al., *Intronless celB from the anaerobic fungus Neocallimastix patriciarum encodes a modular family A endoglucanase*. Biochem. J., 1994. **297 (Pt 2)**: p. 359-64.
68. Xue, G.P., K.S. Gobius, and C.G. Orpin, *A novel polysaccharide hydrolase cDNA (celD) from Neocallimastix patriciarum encoding three multi-functional catalytic domains with high endoglucanase, cellobiohydrolase and xylanase activities*. J. Gen. Microbiol., 1992. **138**(11): p. 2397-403.
69. Tsai, C.F., X. Qiu, and J.H. Liu, *A comparative analysis of two cDNA clones of the cellulase gene family from anaerobic fungus Piromyces rhizinflata*. Anaerobe, 2003. **9**(3): p. 131-40.
70. Garcia-Campayo, V. and T.M. Wood, *Purification and characterisation of a β -D-xylosidase from the anaerobic rumen fungus Neocallimastix frontalis*. Carbohydr. Res., 1993. **242**: p. 229-45.
71. Gomez de Segura, B. and M. Fevre, *Purification and characterization of two 1,4- β -xylan endohydrolases from the rumen fungus Neocallimastix frontalis*. Appl. Environ. Microbiol., 1993. **59**(11): p. 3654-60.
72. Dalrymple, B.P., et al., *Three Neocallimastix patriciarum esterases associated with the degradation of complex polysaccharides are members of a new family of hydrolases*. Microbiology, 1997. **143 (Pt 8)**: p. 2605-14.

73. Henrissat, B. and G. Davies, *Structural and sequence-based classification of glycoside hydrolases*. *Curr. Opin. Struct. Biol.*, 1997. **7**(5): p. 637-44.
74. Zechel, D.L. and S.G. Withers, *Glycosidase mechanisms: anatomy of a finely tuned catalyst*. *Acc. Chem. Res.*, 2000. **33**(1): p. 11-8.
75. Zechel, D.L. and S.G. Withers, *Dissection of nucleophilic and acid-base catalysis in glycosidases*. *Curr. Opin. Chem. Biol.*, 2001. **5**(6): p. 643-9.
76. Davies, G.J., K.S. Wilson, and B. Henrissat, *Nomenclature for sugar-binding subsites in glycosyl hydrolases*. *Biochem. J.*, 1997. **321 (Pt 2)**: p. 557-9.
77. Herve, C., et al., *Carbohydrate-binding modules promote the enzymatic deconstruction of intact plant cell walls by targeting and proximity effects*. *Proc. Natl. Acad. Sci. U. S. A.*, 2010. **107**(34): p. 15293-8.
78. Xu, Q., et al., *A novel family of carbohydrate-binding modules identified with *Ruminococcus albus* proteins*. *FEBS Lett.*, 2004. **566**(1-3): p. 11-6.
79. Shoseyov, O., Z. Shani, and I. Levy, *Carbohydrate binding modules: biochemical properties and novel applications*. *Microbiol. Mol. Biol. Rev.*, 2006. **70**(2): p. 283-95.
80. Su, X., R.I. Mackie, and I.K. Cann, *Biochemical and mutational analyses of a multidomain cellulase/mannanase from *Caldicellulosiruptor bescii**. *Appl. Environ. Microbiol.*, 2012. **78**(7): p. 2230-40.
81. Dodd, D., et al., *Biochemical analysis of a β -D-xylosidase and a bifunctional xylanase-ferulic acid esterase from a xylanolytic gene cluster in *Prevotella ruminicola* 23*. *J. Bacteriol.*, 2009. **191**(10): p. 3328-38.

82. Flint, H.J., et al., *A bifunctional enzyme, with separate xylanase and $\beta(1,3-1,4)$ -glucanase domains, encoded by the xynD gene of Ruminococcus flavefaciens*. J. Bacteriol., 1993. **175**(10): p. 2943-51.
83. Irwin, D.C., et al., *Activity studies of eight purified cellulases: Specificity, synergism, and binding domain effects*. Biotechnol. Bioeng., 1993. **42**(8): p. 1002-13.
84. Barr, B.K., et al., *Identification of two functionally different classes of exocellulases*. Biochemistry, 1996. **35**(2): p. 586-92.
85. Nam, K.H., M.W. Sung, and K.Y. Hwang, *Structural insights into the substrate recognition properties of β -glucosidase*. Biochem. Biophys. Res. Commun., 2010. **391**(1): p. 1131-5.
86. Reverbel-Leroy, C., et al., *The processive endocellulase CelF, a major component of the Clostridium cellulolyticum cellulosome: purification and characterization of the recombinant form*. J. Bacteriol., 1997. **179**(1): p. 46-52.
87. Gilad, R., et al., *CelI, a noncellulosomal family 9 enzyme from Clostridium thermocellum, is a processive endoglucanase that degrades crystalline cellulose*. J. Bacteriol., 2003. **185**(2): p. 391-8.
88. Hidaka, M., et al., *Structural dissection of the reaction mechanism of cellobiose phosphorylase*. Biochem. J., 2006. **398**(1): p. 37-43.
89. Shi, P., et al., *Paenibacillus sp. strain E18 bifunctional xylanase-glucanase with a single catalytic domain*. Appl. Environ. Microbiol., 2010. **76**(11): p. 3620-4.

90. Lamed, R., E. Setter, and E.A. Bayer, *Characterization of a cellulose-binding, cellulase-containing complex in Clostridium thermocellum*. J. Bacteriol., 1983. **156**(2): p. 828-36.
91. Fontes, C.M. and H.J. Gilbert, *Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates*. Annu. Rev. Biochem., 2010. **79**: p. 655-81.
92. Bayer, E.A., et al., *The cellulosomes: multienzyme machines for degradation of plant cell wall polysaccharides*. Annu. Rev. Microbiol., 2004. **58**: p. 521-54.
93. Faulds, C.B., et al., *Synergy between xylanases from glycoside hydrolase family 10 and family 11 and a feruloyl esterase in the release of phenolic acids from cereal arabinoxylan*. Appl. Microbiol. Biotechnol., 2006. **71**(5): p. 622-9.
94. Dodd, D., et al., *Functional diversity of four glycoside hydrolase family 3 enzymes from the rumen bacterium Prevotella bryantii B14*. J. Bacteriol., 2010. **192**(9): p. 2335-45.
95. Kanda, T., et al., *Substrate specificities of exo- and endo-type cellulases in the hydrolysis of β -(1----3)- and β -(1----4)-mixed D-glucans*. J. Biochem., 1989. **105**(1): p. 127-32.
96. Driskill, L.E., M.W. Bauer, and R.M. Kelly, *Synergistic interactions among beta-laminarinase, β -1,4-glucanase, and β -glucosidase from the hyperthermophilic archaeon Pyrococcus furiosus during hydrolysis of β -1,4-, β -1,3-, and mixed-linked polysaccharides*. Biotechnol. Bioeng., 1999. **66**(1): p. 51-60.
97. Dehority, B.A., *Degradation and utilization of isolated hemicellulose by pure cultures of cellulolytic rumen bacteria*. J. Bacteriol., 1965. **89**: p. 1515-20.

98. Bryant, M.P. and I.M. Robinson, *Some nutritional requirements of the genus Ruminococcus*. Appl. Microbiol., 1961. **9**(2): p. 91-5.
99. Dehority, B.A., H.W. Scott, and P. Kowaluk, *Volatile fatty acid requirements of cellulolytic rumen bacteria*. J. Bacteriol., 1967. **94**(3): p. 537-43.
100. Allison, M.J., et al., *Metabolic function of branched-chain volatile fatty acids, growth factors for ruminococci. II. Biosynthesis of higher branched-chain fatty acids and aldehydes*. J. Bacteriol., 1962. **83**: p. 1084-93.
101. Russell, J.B. and D.B. Dombrowski, *Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture*. Appl. Environ. Microbiol., 1980. **39**(3): p. 604-10.
102. Smith, W.R., I. Yu, and R.E. Hungate, *Factors affecting cellulolysis by Ruminococcus albus*. J. Bacteriol., 1973. **114**(2): p. 729-37.
103. Hungate, R.E. and R.J. Stack, *Phenylpropanoic acid: Growth factor for Ruminococcus albus*. Appl. Environ. Microbiol., 1982. **44**(1): p. 79-83.
104. Stack, R.J. and R.E. Hungate, *Effect of 3-phenylpropanoic acid on capsule and cellulases of Ruminococcus albus 8*. Appl. Environ. Microbiol., 1984. **48**(1): p. 218-23.
105. Aziz, R.K., et al., *The RAST Server: rapid annotations using subsystems technology*. BMC Genomics, 2008. **9**: p. 75.
106. Stack, R.J., R.E. Hungate, and W.P. Opsahl, *Phenylacetic acid stimulation of cellulose digestion by Ruminococcus albus 8*. Appl. Environ. Microbiol., 1983. **46**(3): p. 539-44.

107. Reveneau, C., et al., *Phenylacetic and phenylpropionic acids do not affect xylan degradation by Ruminococcus albus*. Appl. Environ. Microbiol., 2003. **69**(11): p. 6954-8.
108. Patterson, H., et al., *Ultrastructure and adhesion properties of Ruminococcus albus*. J. Bacteriol., 1975. **122**(1): p. 278-87.
109. Rakotoarivonina, H., et al., *Proteomic identification of CBM37-containing cellulases produced by the rumen cellulolytic bacterium Ruminococcus albus 20 and their putative involvement in bacterial adhesion to cellulose*. Arch. Microbiol., 2009. **191**(4): p. 379-88.
110. Pegden, R.S., et al., *Adherence of the gram-positive bacterium Ruminococcus albus to cellulose and identification of a novel form of cellulose-binding protein which belongs to the Pil family of proteins*. J. Bacteriol., 1998. **180**(22): p. 5921-7.
111. Rakotoarivonina, H., et al., *Adhesion to cellulose of the Gram-positive bacterium Ruminococcus albus involves type IV pili*. Microbiology, 2002. **148**(Pt 6): p. 1871-80.
112. Ezer, A., et al., *Cell surface enzyme attachment is mediated by family 37 carbohydrate-binding modules, unique to Ruminococcus albus*. J. Bacteriol., 2008. **190**(24): p. 8220-2.
113. Lou, J., K.A. Dawson, and H.J. Strobel, *Cellobiose and cellodextrin metabolism by the ruminal bacterium Ruminococcus albus*. Curr. Microbiol., 1997. **35**(4): p. 221-7.

114. Strobel, H.J., F.C. Caldwell, and K.A. Dawson, *Carbohydrate transport by the anaerobic thermophile Clostridium thermocellum* LQRI. *Appl. Environ. Microbiol.*, 1995. **61**(11): p. 4012-5.
115. Hungate, R.E., *Polysaccharide storage and growth efficiency in Ruminococcus albus*. *J. Bacteriol.*, 1963. **86**: p. 848-54.
116. Desvaux, M., *Unravelling carbon metabolism in anaerobic cellulolytic bacteria*. *Biotechnol. Prog.*, 2006. **22**(5): p. 1229-38.
117. Howard, G.T. and B.A. White, *Molecular cloning and expression of cellulase genes from Ruminococcus albus 8 in Escherichia coli bacteriophage lambda*. *Appl. Environ. Microbiol.*, 1988. **54**(7): p. 1752-5.
118. Deguchi, H., et al., *Purification and properties of the endo-1,4- β -glucanase from Ruminococcus albus and its gene product in Escherichia coli*. *J. Ferment. Bioeng.*, 1991. **71**(4): p. 221-225.
119. Ohmiya, K., et al., *Structure of a Ruminococcus albus endo-1,4- β -glucanase gene*. *J. Bacteriol.*, 1989. **171**(12): p. 6771-5.
120. Ohmiya, K., K. Maeda, and S. Shimizu, *Purification and properties of endo-(1-4)- β -D-glucanase from Ruminococcus albus*. *Carbohydr. Res.*, 1987. **166**(1): p. 145-155.
121. Watanabe, Y., et al., *Purification and properties of the endo-1,4- β -glucanase-III from Ruminococcus albus*. *J. Ferment. Bioeng.*, 1992. **73**(1): p. 54-57.
122. Karita, S., et al., *Cloning and sequencing of a novel endo-1,4- β -glucanase gene from Ruminococcus albus*. *J. Ferment. Bioeng.*, 1993. **76**(6): p. 439-444.

123. Karita, S., K. Sakka, and K. Ohmiya, *Cellulose-binding domains confer an enhanced activity against insoluble cellulose to Ruminococcus albus endoglucanase IV*. J. Ferment. Bioeng., 1996. **81**(6): p. 553-556.
124. Ohara, H., et al., *Sequence of egV and properties of EgV, a Ruminococcus albus endoglucanase containing a dockerin domain*. Biosci. Biotechnol. Biochem., 2000. **64**(1): p. 80-8.
125. Taguchi, H., et al., *Cloning of the Ruminococcus albus cel5D and cel9A genes encoding dockerin module-containing endoglucanases and expression of cel5D in Escherichia coli*. Biosci. Biotechnol. Biochem., 2004. **68**(7): p. 1557-64.
126. Poole, D.M., et al., *Nucleotide sequence of the Ruminococcus albus SY3 endoglucanase genes celA and celB*. Mol. Gen. Genet., 1990. **223**(2): p. 217-23.
127. Ware, C.E., A.H. Lachke, and K. Gregg, *Mode of action and substrate specificity of a purified exo-1,4- β -D-glucosidase cloned from the cellulolytic bacterium Ruminococcus albus AR67*. Biochem. Biophys. Res. Commun., 1990. **171**(2): p. 777-86.
128. Ohmiya, K., et al., *Isolation and properties of β -glucosidase from Ruminococcus albus*. J. Bacteriol., 1985. **161**(1): p. 432-4.
129. Ohmiya, K., et al., *Purification and properties of cellobiosidase from Ruminococcus albus*. J. Bacteriol., 1982. **150**(1): p. 407-9.
130. Ohara, H., et al., *Characterization of the cellulolytic complex (cellulosome) from Ruminococcus albus*. Biosci. Biotechnol. Biochem., 2000. **64**(2): p. 254-60.

131. Nakamura, M., et al., *Molecular cloning, nucleotide sequence and characteristics of a xylanase gene (xynA) from Ruminococcus albus 7*. Anim. Sci. J., 2002. **73**(5): p. 347-352.
132. Somerville, C., et al., *Feedstocks for lignocellulosic biofuels*. Science. **329**(5993): p. 790-2.
133. Somerville, C., et al., *Toward a systems approach to understanding plant cell walls*. Science, 2004. **306**(5705): p. 2206-11.
134. Bryant, M.P., et al., *Characteristics of ruminal anaerobic cellulolytic cocci and Cillobacterium cellulosolvens n. sp.* J. Bacteriol., 1958. **76**(5): p. 529-37.
135. Dehority, B.A., *Rate of isolated hemicellulose degradation and utilization by pure cultures of rumen bacteria*. Appl. Microbiol., 1967. **15**(5): p. 987-93.
136. Greve, L.C., et al., *Muralytic Activities of Ruminococcus albus 8*. Appl. Environ. Microbiol., 1984. **47**(5): p. 1141-5.
137. Greve, L.C., J.M. Labavitch, and R.E. Hungate, *α -L-arabinofuranosidase from Ruminococcus albus 8: purification and possible role in hydrolysis of alfalfa cell wall*. Appl. Environ. Microbiol., 1984. **47**(5): p. 1135-40.
138. Nakamura M., N.T., Takenaka A., Aminov R. I., Ogata K., Tajima K., Matsui H., Benno Y., and Itabashi H., *Molecular cloning, nucleotide sequence and characteristics of a xylanase gen (xynA) from Ruminococcus albus 7*. Animal Science Journal, 2002. **73**: p. 347-352.
139. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. Nature, 1970. **227**(5259): p. 680-5.

140. Lever, M., *A new reaction for colorimetric determination of carbohydrates*. Anal. Biochem., 1972. **47**(1): p. 273-9.
141. Mitchell, D.J., Grohmann, K., Himmel, M. E., Dale, B. E. and Schroeder, H. A., *Effect of the degree of acetylation on the enzymatic digestion of acetylated xylans*. J. Wood. Chem. Technol., 1990. **10**: p. 111-121.
142. Matte, A., C.W. Forsberg, and A.M. Verrinder Gibbins, *Enzymes associated with metabolism of xylose and other pentoses by Prevotella (Bacteroides) ruminicola strains, Selenomonas ruminantium D, and Fibrobacter succinogenes S85*. Can. J. Microbiol., 1992. **38**(5): p. 370-6.
143. Miron, J. and D. Ben-Ghedalia, *Digestion of cell-wall monosaccharides of ryegrass and alfalfa hays by the ruminal bacteria Fibrobacter succinogenes and Butyrivibrio fibrisolvens*. Can. J. Microbiol., 1993. **39**(8): p. 780-6.
144. Gradel, C.M. and B.A. Dehority, *Fermentation of isolated pectin and pectin from intact forages by pure cultures of rumen bacteria*. Appl. Microbiol., 1972. **23**(2): p. 332-40.
145. Thurston, B., K.A. Dawson, and H.J. Strobel, *Cellobiose versus glucose utilization by the ruminal bacterium Ruminococcus albus*. Appl. Environ. Microbiol., 1993. **59**(8): p. 2631-7.
146. Thurston, B., K.A. Dawson, and H.J. Strobel, *Pentose utilization by the ruminal bacterium Ruminococcus albus*. Appl. Environ. Microbiol., 1994. **60**(4): p. 1087-92.
147. Biely, P., et al., *Endo- β -1,4-xylanase families: differences in catalytic properties*. J. Biotechnol., 1997. **57**(1-3): p. 151-66.

148. Fujimoto, Z., et al., *Crystal structures of decorated xylooligosaccharides bound to a family 10 xylanase from Streptomyces olivaceoviridis E-86*. J. Biol. Chem., 2004. **279**(10): p. 9606-14.
149. Sun, H.J., et al., *Preparation of (1-->4)- β -D-xylooligosaccharides from an acid hydrolysate of cotton-seed xylan: suitability of cotton-seed xylan as a starting material for the preparation of (1-->4)- β -D-xylooligosaccharides*. Carbohydr. Res., 2002. **337**(7): p. 657-61.
150. Timell, T.E., *Recent progress in the chemistry of wood hemicelluloses*. Wood Sci. Technol., 1967. **1**: p. 45-70.
151. Teleman, A., et al., *Characterization of O-acetyl-(4-O-methylglucurono)xylan isolated from birch and beech*. Carbohydr. Res., 2002. **337**(4): p. 373-7.
152. Koukiekolo, R., et al., *Degradation of corn fiber by Clostridium cellulovorans cellulases and hemicellulases and contribution of scaffolding protein CbpA*. Appl. Environ. Microbiol., 2005. **71**(7): p. 3504-11.
153. de Vries, R.P., Kester, H. C. M., Poulsen, C. H., Benen, J. A. E., Visser, J., *Synergy between enzymes from Aspergillus involved in the degradation of plant cell wall polysaccharides*. Carbohydr. Res., 2000. **327**: p. 401-410.
154. Raweesri, P., P. Riangrunrojana, and P. Pinphanichakarn, *α -L-Arabinofuranosidase from Streptomyces sp. PC22: purification, characterization and its synergistic action with xylanolytic enzymes in the degradation of xylan and agricultural residues*. Bioresour. Technol., 2008. **99**(18): p. 8981-6.

155. Orpin, C.G., et al., *Seasonal changes in the ruminal microflora of the high-arctic Svalbard reindeer (*Rangifer tarandus platyrhynchus*)*. Appl. Environ. Microbiol., 1985. **50**(1): p. 144-51.
156. Yang, S.L., et al., *Bacterial diversity in the rumen of Gayals (*Bos frontalis*), Swamp buffaloes (*Bubalus bubalis*) and Holstein cow as revealed by cloned 16S rRNA gene sequences*. Mol. Biol. Rep., 2010. **37**(4): p. 2063-2073.
157. Doi, R.H., *Cellulases of mesophilic microorganisms: cellulosome and noncellulosome producers*. Ann. N. Y. Acad. Sci., 2008. **1125**: p. 267-79.
158. Kumar, R., S. Singh, and O.V. Singh, *Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives*. J. Ind. Microbiol. Biotechnol., 2008. **35**(5): p. 377-91.
159. Lynd, L.R., et al., *Microbial cellulose utilization: fundamentals and biotechnology*. Microbiol. Mol. Biol. Rev., 2002. **66**(3): p. 506-77, table of contents.
160. Planas, A., *Bacterial 1,3-1,4- β -glucanases: structure, function and protein engineering*. Biochim. Biophys. Acta., 2000. **1543**(2): p. 361-382.
161. Gaiser, O.J., et al., *Structural basis for the substrate specificity of a *Bacillus* 1,3-1,4- β -glucanase*. J. Mol. Biol., 2006. **357**(4): p. 1211-25.
162. Piotukh, K., et al., *Protein-carbohydrate interactions defining substrate specificity in *Bacillus* 1,3-1,4- β -D-glucan 4-glucanohydrolases as dissected by mutational analysis*. Biochemistry, 1999. **38**(49): p. 16092-104.
163. Tsai, L.C., et al., *Mutational and structural studies of the active-site residues in truncated *Fibrobacter succinogenes* 1,3-1,4- β -D-glucanase*. Acta. Crystallogr. D Biol. Crystallogr., 2008. **64**(Pt 12): p. 1259-66.

164. Palackal, N., et al., *A multifunctional hybrid glycosyl hydrolase discovered in an uncultured microbial consortium from ruminant gut*. Appl. Microbiol. Biotechnol., 2007. **74**(1): p. 113-24.
165. Taylor, E.J., et al., *How family 26 glycoside hydrolases orchestrate catalysis on different polysaccharides: structure and activity of a Clostridium thermocellum lichenase, CtLic26A*. J. Biol. Chem., 2005. **280**(38): p. 32761-7.
166. Romaniec, M.P.M., et al., *Cloning of Ruminococcus albus endo- β -1,4-glucanase and xylanase genes*. Lett. Appl. Microbiol., 1989. **9**(3): p. 101-104.
167. Kawai, S., et al., *Molecular cloning of Ruminococcus albus cellulase gene*. Agric. Biol. Chem., 1987. **51**(1): p. 59-63.
168. Emanuelsson, O., et al., *Locating proteins in the cell using TargetP, SignalP and related tools*. Nat. Protoc., 2007. **2**(4): p. 953-71.
169. Gill, S.C. and P.H. von Hippel, *Calculation of protein extinction coefficients from amino acid sequence data*. Anal. Biochem., 1989. **182**(2): p. 319-26.
170. Wood, T.M., *Preparation of Crystalline, Amorphous, and Dyed Cellulase Substrates*. Method. Enzymol., 1988. **160**: p. 19-25.
171. Hess, M., et al., *Metagenomic discovery of biomass-degrading genes and genomes from cow rumen*. Science, 2011. **331**(6016): p. 463-467.
172. Foong, F., et al., *Nucleotide-sequence and characteristics of endoglucanase gene engB from Clostridium cellulovorans*. J. Gen. Microbiol., 1991. **137**: p. 1729-1736.
173. Voget, S., H.L. Steele, and W.R. Streit, *Characterization of a metagenome-derived halotolerant cellulase*. J. Biotechnol., 2006. **126**(1): p. 26-36.