EXPLORING PROTEIN STRUCTURE-FUNCTION RELATIONSHIPS IN XANTHANASES FROM GLYCOSIDE HYDROLASE FAMILY 9

by

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

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Abstract

The hydrolysis of xanthan is a commercially relevant reaction. This is due to the widespread use of this polysaccharide as a rheology modifier in many industries. Therefore, investigations into the structure and function of enzymes capable of altering xanthan properties, including endo-xanthanases, is warranted.

This thesis describes investigations into the structure-function relationship of PspXan9, a bacterial xanthanase from glycoside hydrolase family 9 (GH9). To do this, enzyme kinetic assays were performed to determine preferred biochemical conditions, substrate specificity and Michaelis-Menten kinetics. These revealed that PspXan9 was highly specific for xanthan following a pretreatment with xanthan lyase, and notably, required calcium for increased activity and stability. The appearance of only two products over the course of a reaction, as monitored by high-performance size exclusion chromatography coupled to a UV-detector (HPSEC-UV), demonstrated that hydrolysis of lyase-treated xanthan with PspXan9 occurred in a processive fashion. However, the identity of these products as lyase-treated xanthan tetrasaccharides and octasaccharides was only realized following analysis with liquid chromatography coupled to mass spectrometry (LC-MS), tandem mass spectrometry (MS/MS) and 1D- and 2D-Nuclear Magnetic Resonance (NMR). Analysis of the PspXan9 x-ray crystal structure was used to reveal structural insights that guided targeted mutations to perform subsite mapping. Changes resulting from each mutation were tested for activity, and degree of processivity through viscometric and HPSEC-UV analysis. Lastly, protein similarity networks were utilized to find a putative GH9 xanthanase subgroup as well as a possible progenitor GH9 whose protein expression was attempted.
Lay Summary

Xanthan is a carbohydrate that is produced by a type of pathogenic bacteria to allow it to infect and kill many different food crops including cauliflower, broccoli and kale. However, xanthan has also been found to have numerous applications and is currently being used in household products as stabilizers and thickeners. These products include salad dressings, toothpaste and certain medications. As the prevalence of xanthan in industry increases, the desire to discover ways of altering its properties also increases. One useful way to do this is through enzyme modification. This work serves as an investigation toward identifying and understanding enzymes capable of digesting xanthan in hopes of realizing their potential for future applications.
Preface

Chapter 2: Structure and Biochemical Analysis of PspXan9: An Endo-Processive Multi-Modular Xanthanase from Paenibacillus nanensis, was written by me, Sean McDonald, and reviewed and revised by my supervisor Prof. Dr. Harry Brumer. I performed enzyme biochemical analysis including determination of optimal conditions, and carbohydrate analytics including HPSEC-UV, LC-MS, MS/MS and NMR experiments. Nicholas McGregor, a PhD student formerly in the Brumer Lab, assisted with mass spectrometric acquisition and analysis. The cloning, expression and purification of PspXan9 was carried out by the biotechnology company Novozymes A/S. A version of this chapter including work from our collaborators is found in the publication:


Chapter 3: Structural Determinants Conferring the Processivity of PspXan9 Endo-Xanthanase was written by me, Sean McDonald, and reviewed and revised by my supervisor, Prof. Dr. Harry Brumer. It contains original work with experiments performed, designed and analyzed by Sean McDonald under the guidance of Harry Brumer. The exception is the cloning, expression and purification of PspXan9 variants which was completed by the biotechnology company Novozymes A/S.
Chapter 4: Conclusions and Future Outlook was written by me, Sean McDonald and reviewed and revised by my supervisor Prof. Dr. Harry Brumer. The bioinformatics analysis of the sequence similarity network, and phylogenetic tree constructed was completed with the aid and guidance of Alexander Viborg, a post-doctoral fellow formerly in the Brumer Lab.
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List of Abbreviations

2-D NMR: Two-Dimensional Nuclear Magnetic Resonance
AA: Auxiliary Activity
Ac: Acetate
BCA: Bicinchoninic Acid
bMLG: Barley Mixed-Linkage Glucan
BSA: Bovine Serum Albumin
bX: Beechwood Xylan
CAZy: Carbohydrate Active Enzyme Database
CAZymes: Carbohydrate-Active Enzymes
CBM##: Carbohydrate-Binding Module Family ##
CBM: Carbohydrate-Binding Module
CE: Carbohydrate Esterase
CID: Collision-Induced Dissociation
CHES: N-cyclohexyl-2-aminoethanesulfonic acid
CMC: Carboxymethylcellulose
COSY: Correlation Spectroscopy
EIC: Extracted Ion Chromatogram
ESI: Electrospray Ionisation
GH##: Glycoside Hydrolase Family ##
GH: Glycoside Hydrolase
Glc: Glucose
GlcA: Glucuronic acid
HEC: Hydroxyethylcellulose

HEPES: 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid

HMBC: Heteronuclear Multiple Bond Correlation

HPAEC-PAD: High Performance Anion Exchange Chromatography Coupled to Pulsed Amperometric Detection

HPLC: High-Performance Liquid Chromatography

HPSEC-UV: High Performance Size-Exclusion Chromatography coupled to UV-detector

HSQC: Heteronuclear Single Quantum Coherence

IMAC: Immobilized Metal Affinity Chromatography

kDa: Kilodalton

LC-MS: Liquid Chromatography Coupled to Mass Spectrometry

m/z: Mass-to-Charge Ratio

Man: Mannose

MES: 2-(N-Morpholino)Ethanesulfonic Acid

MLG: Mixed-Linkage Glucan

MS/MS: Tandem Mass Spectrometry

MS: Mass Spectrometry

NMR: Nuclear Magnetic Resonance

NOESY: Nuclear Overhauser Effect Spectroscopy

PDB ID: Alphanumeric Protein Structure Identifier within the Protein Data Bank

PDB: Protein Data Bank

PL##: Polysaccharide Lyase ##

PL: Polysaccharide Lyase
QTof: Quadrupole Time-of-Flight

RI: Refractive Index

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SEC: Size-Exclusion Chromatography

SSN: Sequence Similarity Network

TIC: Total Ion Current

TOCSY: Total Correlation Spectroscopy

tXyG: Tamarind Xyloglucan

UPLC: Ultra-Performance Liquid Chromatography

UV: Ultraviolet

wAX: Wheat Arabinoxylan
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First and foremost, I would like to thank my supervisor Prof. Dr. Harry Brumer for giving me the opportunity to work as a graduate student in his lab. The guidance and mentorship that he has provided me with over the years has been and will continue to be invaluable academically, professionally and extracurricularly. Without his assistance I would not have a clear path towards my future career as a scientist. Appreciation is also due to my committee members Prof. Dr. Nobuhiko Tokuriki and Prof. Dr. Lawrence for meeting with me to ensure my progress was on track and also for critically reading this thesis. I would also like to extend a special thanks to Prof. Dr. David Bird for giving me undergraduate research opportunities as well as giving me the courage to pursue graduate studies.

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I am very appreciative of ECOSCOPE for giving me a truly unique opportunity in which to complete my graduate studies. This of course includes the additional training and education, the internship as well as the support system fostered within the community of ECOSCOPE trainees.

Finally, I would like to thank my family and friends for their endless love and support. This journey would have been unimaginable without the many phone calls, movies and visits over the last four years. I will do my best to make you all proud.
Dedication

I dedicate this thesis to my Mom and Dad, Kyle and Janet. Thank you for always being there in my times of need.

“The best way to predict the future is to invent it.”

— Alan Fay, Chief Scientist for Atari
Chapter 1: Introduction

1.1 Xanthan

In the late 1950s, scientists from the Northern Regional Research Laboratory (NRRL) of the United States Department of Agriculture were tasked with finding a cheaper, domestic alternative to the commonly used imported plant gums, such as tragacanth, that are often used as binders and thickeners\(^1\). Previous work on dextran led researcher Allene Jeanes et al to realize that microbial polysaccharides may serve as potential plant gum-substitutes considering their unique properties. After an extensive survey, they found that the bacterial plant pathogen *Xanthomonas campestris* secreted a large, water-soluble exopolysaccharide, which demonstrated high viscosity in low concentrations\(^2,3\). This polysaccharide was named xanthan after its *Xanthomonas* source and is now considered the main virulence factor of members of the genus\(^4\). During infection, *X. campestris* pathovar *campestris* attacks the plant vasculature system where it takes up residence by producing xanthan and preventing the flow of nutrients throughout the plant.

The unique structure of the polysaccharide provides it with equally unique rheological properties that numerous industries have harnessed today\(^3,5-7\). In general, these applications utilize the properties of xanthan as a thickener, stabilizer, emulsifier and lubricant. Xanthan natively exists as a double helix with each molecule consisting of a $\beta(1 \rightarrow 4)$-glucan backbone with alternative glucose substitutions at the C3 position\(^8,9\) (Figure 1.1). These substitutions consist of a trisaccharide sidechain which is comprised of an $\alpha(1 \rightarrow 3)$-mannosyl residue linked to a $\beta(1 \rightarrow 2)$-glucuronic acid and terminated with a $\beta(1 \rightarrow 4)$-mannosyl residue. Both mannoses can be substituted with 6-$O$-acetylation, primarily the inner $\alpha(1 \rightarrow 3)$-mannose, although the terminal mannose is instead more often pyruvylated, to form a 4,6-$O$-[(S)-1-carboxyethylidene] moiety. The acetylation and pyruvylation are highly dependent on production conditions such as bioreactor...
used, growth conditions and strain selection\textsuperscript{7}. Recent experiments using atomic force microscopy have shown that the presence of these functional groups, as well as temperature and ionic strength all highly affect the conformation of xanthan which can exist as an ordered anti-parallel helix, a disordered random coil, or in a state between these states as a partially renatured confirmation with as many as 8 different forms (Figure 1.2)\textsuperscript{8, 10}.

**Figure 1.1 – Structure of xanthan.** (A) A single pentasaccharide repeating unit consisting of a $\beta(1 \rightarrow 4)$-glucan backbone with a trisaccharide sidechain appended to every other glucose residue. The sidechain is comprised of an $\alpha(1 \rightarrow 3)$-mannosyl residue linked to a $\beta(1 \rightarrow 2)$-glucuronic acid and terminated with a $\beta(1 \rightarrow 4)$-mannosyl residue. The inner mannose is often 6-\textit{O}-acetylation (see R-group), whereas the terminal mannose is often pyruvylated, to form a 4,6-\textit{O}-\{(S)-1-carboxyethylidene\} moiety. (B) The predicted 3D structure of a single xanthan unpyruvylated pentasaccharide produced by GLYCAM 3D Carbohydrate Builder (https://dev.glycam.org/). Carbon atoms and bonds are shown in teal; oxygen atoms are shown in red or purple if they can be substituted (see A).
Figure 1.2 – Xanthan confrontational behaviour. Models proposed for the conformational behavior of xanthan in solution. a) native ordered double stranded double helix; b) disordered xanthan strains; c) proposed conformations for renatured xanthan; d) partially dissociated native strains; e) network formation by the association of disordered xanthan segments; f) partially dissociated renatured strains; g) network formation by stacking of ordered xanthan segments; h) network formation by stacking of completely ordered xanthan molecules. Reprinted (adapted) from Kool Doctoral Thesis. Copyright 2014 Wageningen University.
The xanthan production process involves the stirred fermentation of *X. campestris* on either glucose or sucrose media. To achieve sufficient amounts of xanthan, fermentation is allowed to continue for at least 100 h\(^7\). After appropriate xanthan production, the *X. campestris* cells are typically pasteurized and removed via centrifugation or filtration. Finally, the polysaccharide is precipitated following the addition isopropanol, dried and milled. Because of its numerous applications, over 80 kilotonnes is produced annually generating over US$400M\(^7\).

### 1.1.1 Degradation of Xanthan by Carbohydrate-Active Enzymes

Enzyme modification of xanthan has become of recent interest due to the large number of industrial applications utilizing this polysaccharide\(^11\). However, the complex structure of xanthan requires an assortment of different classes of carbohydrate-active enzymes (CAZymes)\(^12\) to hydrolyze all of the monosaccharide linkages\(^13\). Information regarding CAZymes can be found in the CAZy database, which serves as the main repository. Instead of organizing CAZymes based on the International Union of Biochemistry and Molecular Biology Enzyme Commission (EC) number, the CAZy database has sorted CAZyme families based on amino acid sequence\(^14\). This allows predictions to be made regarding a specific enzyme family’s structural fold, and even its catalytic mechanism. CAZymes fall into five distinct classes: glycoside hydrolases (GH), polysaccharide lyases (PL), glycosyl transferases (GT) carbohydrate esterases (CE) and auxiliary active enzymes (AA)\(^12,14\). In the context of this thesis, only the PL and GH classes of enzymes are relevant.
1.1.1.1 **Polysaccharide Lyases**

Polysaccharide lyases (PL; EC 4.2.2.-) are a class of enzymes containing 37 families, all of which break glycosidic linkages through eliminative cleavage\(^{15}\). To do this, PLs bind hexuronic acids through the presence of uronate-recognition sites that hold its substrate in place through hydrogen bonding\(^{15,16}\). Once in place, the C5 proton is abstracted, and donated to the oxygen in the glycosidic bond, leading to cleavage and the formation of a Δ4,5-ene-hexuronic acid. This unsaturation leads to the formation of a UV-detectable moiety at 235 nm that is often used in both PL activity and product analysis.

1.1.1.2 **Glycoside Hydrolases**

Glycoside hydrolases (GHs; EC 3.2.1.-) make up the largest class of CAZymes with currently 165 families\(^{17}\). While these families differ in sequence, fold and specificity,\(^{14,18}\) they generally perform catalysis through either an inverting (anomeric conformation changed) or retaining (anomeric conformation maintained) mechanism\(^{19}\). Inverting GHs facilitate the hydrolysis of glycosidic bonds by providing a catalytic base to deprotonate water and a catalytic acid to donate a proton to the oxygen of the glycosidic bond. As a result, the water experiences an increase in nucleophilicity and attacks the anomeric centre resulting in the formation of a new reducing-end.

Similar to inverting GHs, the catalytic acid/base of retaining GHs donates a proton to the oxygen of the glycosidic bond; however, retaining GHs instead provide their own nucleophile, typically in the form of an amino acid with a carboxyl-containing sidechain to initiate hydrolysis. The nucleophile attacks the anomeric carbon leading to the formation of a glycosyl-enzyme
intermediate. The acid/base residue is then deprotonated by a nearby water, which subsequently attacks the anomeric carbon, effectively deglycosylating the enzyme and completing cleavage.

Despite generally having similar amino acid sequence, fold and catalytic mechanism, single a GH family can have three very different modes of action: \textit{exo}-acting, \textit{endo}-dissociative acting and \textit{endo}-processive acting\textsuperscript{20}. Hydrolysis by \textit{exo}-acting GHs with either a pocket or crater active site is restricted to hydrolysis of the ends of the carbohydrates. Similarly, \textit{exo}-acting GHs with tunnel-shaped active sites also act from the ends of carbohydrates; however, these enzymes typically are able to engage in multiple hydrolytic events without dissociating from the substrate\textsuperscript{18}. This generally results in the production of oligosaccharides with consistent molecular masses\textsuperscript{20}. Conversely, \textit{endo}-dissociative-acting GHs have active sites that are described as clefts that facilitate binding at any point on the carbohydrate\textsuperscript{18}. Following hydrolysis, these enzymes will dissociate from the substrate before subsequent hydrolytic events. This stochastic cleavage pattern results in the release oligosaccharides with a diverse range of molecular masses (Figure 1.3). \textit{Endo}-processive GHs also have active site clefts and therefore are capable of binding in the middle of a carbohydrate; however, after binding \textit{endo}-processive GHs engage in multiple hydrolytic events without dissociating. It is generally thought that the ability to prevent dissociation by \textit{exo}- (with active site tunnels) and \textit{endo}-processive GHs is either a result of an appended noncatalytic carbohydrate-binding modules (CBMs)\textsuperscript{21}, or from an extensive sugar-binding platform with subsites that are mainly comprised of aromatic residues such as tryptophan and tyrosine\textsuperscript{20}. 
Figure 1.3 – Representative GPC chromatograms from endo-dissociative and endo-processive exo-processive acting xyloglucanases. (A) Endo-hydrolytic activity of a Paenibacillus pabuli GH5 xyloglucanase shifts the molar mass of native tamarind polysaccharide toward lower values, without the appearance of Glc4-based XGOs (dotted line) until the end of the depolymerization. (The peak at 18.5–19 min is due to buffer. Data from Gloster et al., 2007.) (B) The exo-processive/endo-dissociative hydrolytic activity of a GH74 xyloglucanase from Chrysosporium lucknowense (Grishutin et al., 2004) produces XGO_{Glc4} (dotted line) at early stages of the reaction without a dramatic shift in the molar mass of the polysaccharide (Eklöf and Brumer, unpublished data; C. lucknowense GH74 xyloglucanase purchased from Dyadic NL). Reprinted (adapted) with permission from Methods in Enzymology (2012) 510: 97-120. https://doi.org/10.1016/B978-0-12-415931-0.00006-9. Copyright 2012 Elsevier.
1.1.1.3 Predicted Enzymes for Xanthan Saccharification

Presently, the xanthan lyase (4.2.2.12) from the polysaccharide family 8 (PL8), is the most thoroughly characterized type of xanthan degrading enzyme\textsuperscript{15, 16, 23-30}. The xanthan lyase acts by cleaving the terminal mannose through $\beta$-elimination, resulting in the formation of an unsaturation between C4 and C5 of the glucuronic acid ($\Delta$4,5-ene-GlcA). Studies have shown that the eliminative cleavage allows for $\beta$-(1 $\rightarrow$ 4)-glucanases to efficiently depolymerize the lyase-treated xanthan backbone\textsuperscript{31, 32}. Prior research on xanthan degradation has also isolated several xanthan depolymerizing enzymes (endo-xanthanases). These enzymes generally require $\beta$-eliminative cleavage of the d-Man-[4,6-O- (S)-1-carboxyethylidene]p-$\beta$(1 $\rightarrow$ 4)-d-GlcAp glycosidic bond, provided by the xanthan lyase, before hydrolysis of the xanthan backbone can occur. However, Li, et al reported one notable exception to this with an endo-xanthanase from Microbacterium sp. XT11 that is capable of acting without any xanthan lyase pretreatment\textsuperscript{33}. Unfortunately, these studies have not identified the CAZy family that these endo-xanthanases belong to, which hinders the structural understanding of determinants for their substrate specificity. Fortunately, whole-genome studies of the xanthan-degrading bacterium Microbacterium sp. XT11, have pointed to a gene encoding a PL8 found with genes of several GH families and necessary sugar transporters all located on a single transcriptionally coregulated gene cluster\textsuperscript{13}. These include extracellular GHs with known $\beta$-endo-glucanase activity (GH9) and intracellular GHs with $\beta$-exo-unsaturated glucuronidase activity (GH88), $\beta$-exo-glucosidase activity (GH3) and $\alpha$-exo-mannosidase (GH38) activity. Sequential degradation by Bacillus sp. GL1 suggests that the PL8 xanthan lyase must initiate the degradation by cleaving the pyruvlyated terminal $\beta$(1 $\rightarrow$ 4)-mannose (or pyruvylated mannose)$^{32}$. Lyase-treated xanthan may then be depolymerized by a $\beta$(1 $\rightarrow$ 4)-glucanase, such as a GH9 enzyme. The remaining trisaccharide is then dismantled subsequently by the removal of
the unbranched glucose by a $\beta(1 \rightarrow 4)$-glucosidase (GH3), unsaturated glucuronic acid by a $\beta(1 \rightarrow 2)$-glucuronoyl hydrolase (GH88) and lastly the appended $\alpha(1 \rightarrow 3)$-mannose by a $\alpha(1 \rightarrow 3)$-mannosidase (GH38)\textsuperscript{12,32}.

1.2 Techniques Used for the Characterization of an *Endo*-Xanthanase

Kinetic and product analyses must be completed to fully characterize a glycoside hydrolase. Biochemical analysis allows for an understanding of substrate specificity, changes in activity due to different conditions and kinetic parameters. Product analysis can be used to determine the enzyme mode of action, the products that are released as well as the structure of these products. This section is by no means meant to provide an exhaustive description of the wide breadth of methods available; however, it is instead meant to describe the assays used within this thesis.

1.2.1 Enzyme Kinetics

1.2.1.1 Bicinchoninic Acid Reducing Sugar Assay

The quantification of reducing-ends can be used as a proxy for determining the rate of hydrolysis performed by glycoside hydrolases. One method to do this is utilizing the bicinchoninic acid reducing sugar (BCA) assay. This assay exploits the reducing power of the aldehyde form reducing sugars, to reduce cupric (Cu$^{2+}$) ions to their cuprous (Cu$^{+}$) form\textsuperscript{34}. Disodium 2,2’-bicinchoninic acid, in a high pH buffer, typically carbonate buffer, can then bis-chelate the reduced copper ions to form a BCA-Cu$^{+}$ complex which is readily detectable at 562 nm (Figure 1.4). Recent optimizations have outlined a means of complex activity characterization of glycoside hydrolases in a low-volume format allowing for numerous parallel reactions to be performed in PCR tubes\textsuperscript{35}. 
Figure 1.4 – A schematic outline of the BCA assay. The disodium 2,2′-bicinchoninic acid and Cu$^{2+}$ ions exist separately until the presence of a reducing sugar in its aldehyde form reduces the copper ions which can then be readily bis-chelated by bicinchoninic acid.

1.2.2 Product Analysis

1.2.2.1 Analysis of Endo-Xanthanase Products by HPSEC-UV

There are many types of chromatography columns that can be employed for the separation of carbohydrates; however, the use of certain columns may not be practical depending on the nature of the carbohydrate of study. For instance, High Performance Anion Exchange Chromatography (HPAEC) is limited to being performed at a high pH not only for increasing analyte retention through deprotonation of the sugar hydroxyl groups, but also because it is necessary for electrochemical detection with a gold electrode\textsuperscript{36}. For this reason, anionic polysaccharides often will require use of higher “pusher” concentrations typically in the form of sodium acetate to release retained analytes. Xanthan provides an extreme example due to its high molecular weight which is stoichiometrically proportional to the number of negatively charged sidechains\textsuperscript{7}. Unfortunately, this combination makes elution of the polysaccharide under standard HPAEC conditions unfeasible, therefore, other means of separation must be considered.
In contrast, High Performance Size Exclusion Chromatography (HPSEC) is capable of separating carbohydrates simply based on size, typically without the additional complexities of other separation techniques such as dynamic gradients and multiple eluents\(^3\). Instead, isocratic gradients of distilled water or at most solutions containing salts or buffers to control for ionic strength or pH, can be employed. In the case of anionic polysaccharides, such as xanthan, it may be necessary to add salts to prevent interactions with the stationary phase.

Refractive index (RI) detection is often coupled with HPSEC as it is a robust detection method that detects indiscriminately\(^3\). However, if the analyte of interest has a UV-active moiety, then UV detectors can be a highly convenient means of discriminating analytes. In the case of detecting carbohydrates, UV detectors are typically reserved for synthesized conjugates with UV detectable aglycones. However, the resultant product formation following $\beta$-elimination by polysaccharide lyases on glycosyl linkages of uronic acids leads to the formation of a conjugated double bond system that can be readily detected at 235 nm\(^1\). This is the case when xanthan lyase acts on the sidechains of xanthan to form a terminal $\Delta 4,5$-ene-GlcA residue, as previously described in Section 1.1.1.\(^{26, 27}$.

### 1.2.2.2 Identification of Endo-Xanthanase Products with LC-MS and MS/MS

Mass spectrometry is a widely applied analytical technique in carbohydrate analysis as it aids in the identification of analytes based on mass-charge ratio ($m/z$)\(^3\). This unit is effectively the molecular mass divided by the charge that the molecule carries. These charges are applied to carbohydrates through several different ionization techniques including electrospray ionization (ESI), which is used heavily in this thesis. ESI is beneficial for two reasons, the first is that it is able to apply multiple charges to a single ion which, because mass is divided by charge, allows
molecules with large molecular weights to be observed\textsuperscript{39}. The second reason that ESI is beneficial is its ability to perform “soft ionization” or in other words, provide ionization with limited in-source fragmentation\textsuperscript{40}. Following the creation of ions, they can be readily detected through mass spectrometry, but this will reveal very little information about the structures detected compounds apart from their mass-charge ratio.

To elucidate further information, tandem mass spectrometry or MS/MS is required. To do this, mass spectrometers can use ion-filters to separate ions with mass-to-charge ratios of interest. These filtered ions are then fragmented by being subjected to a collision gas that is typically composed of nitrogen, argon or helium. The resultant MS/MS spectra reveals fragments of the parent ion that are known as product ions.

Fragmentation of carbohydrates can happen on either side of the oxygen on the glycosidic linkage, or intra-ring fragmentation may occur. These fragmentations have a standard nomenclature that is outlined in great detail by Domon & Costello\textsuperscript{41}. This nomenclature follows the following pattern: a detected fragment can either belong to the side of the carbohydrate corresponding to the reducing (X, Y and Z ions) or non-reducing (A, B or C ions) end. Subscripts following the ion-type identify the bond position within the carbohydrate structure. X, Y and Z ions start from 0 (e.g. X\textsubscript{0}) at the reducing-end, while A, B and C ions start at 1 (e.g. A\textsubscript{1}) at the non-reducing-end. X and A ions are intra-ring fragments with the X ion containing the reducing-end side of the fragment and the A ion contains the non-reducing-end side. These ions are preceded by a superscript that denotes the ring bonds being fragmented with starting at 0 with the bond between the ring oxygen and the reducing-end, and continuing clockwise to 5. For example, intra-ring fragmentation of the second hexose in a trisaccharide that occurs at the bond between the C2 and C3, as well as the bond between C4 and C5 is created that contains the reducing-end side of the
ion, would be identified as \(2,4\text{X}_{1}\). The remaining four fragment ion types occur at the glycosidic linkage with Y and C ions fragmenting with the glycosidic oxygen and Z and B ions fragmenting without it. Identifying these ions can often be difficult due to the nature of different cleavage patterns varying from simple cleavage to the involvement of up to three proton transfers, therefore, it is strongly recommended to use the aforementioned review by Domon & Costello as a guide\(^{41}\).

If it is necessary to use mass spectrometry on a mixture of compounds, then it is often advisable to perform liquid chromatography for compound separation to aid in their analysis. In the case of carbohydrates, retention with typical alkyl-bonded silica reverse phase columns can often be difficult as they are highly polar analytes as a result of their high abundance of hydroxyl groups. To overcome this, porous graphitized carbon (PGC) columns can be utilized as the flat graphite surface stationary phase allows for excellent adsorption of more planar molecules, such as carbohydrates\(^{42}\). Indeed, separation of stereoisomers, such as anomers, and closely related compounds differing by a single functional group is often possible.

1.2.2.3 Nuclear Magnetic Resonance Spectroscopy of Carbohydrates

The application of nuclear magnetic resonance (NMR) serves as a very powerful tool in the characterization of carbohydrate structures and conformations\(^{43, 44}\). Key benefits include the low quantity of sample required to achieve adequate signal as well as the ability to reclaim precious material once analysis has been finished.

Depending on the purpose and extent of characterization, numerous NMR methods may be used in conjunction with each other\(^{43, 44}\). \(^1\text{H} \ 1\text{D}-\text{NMR} \) can enable the identification of peaks associated anomeric protons found in the sugar. This is based on associated peaks being located between \(\delta 4.4 - \delta 5.5 \text{ ppm} \) in an established “anomeric range” of the spectra. Additionally, \(J\)-
coupling constants of approximately 1-2 Hz are typically associated with \( \alpha \)-anomeric protons, whereas coupling constants of \( \sim 8 \) Hz are more typical with \( \beta \)-anomeric protons. Unfortunately, majority of the data associated with H2-H6 peaks all appear approximately \( \delta \) 3.0 - \( \delta \) 4.0 ppm and therefore can be buried by each other. This issue is exacerbated by the presence of additional sugar residues such as the analysis larger molecules like oligosaccharides and polysaccharides. To overcome this, other methods can be utilized to gain better separation of individual peaks. One such method \( ^1 \text{H} \) 1D-TOCSY (total correlation spectroscopy) where the irradiation of protons within the aforementioned anomeric range can facilitate the identification of chemical shift found in each sugar spin system. While this provides fairly clean spectra that can be used for assigning coupling constants and splitting patterns, little information is provided about which peak corresponds to which proton. Instead, overlaying spectra from 2D-TOCSY and 2D-COSY (correlation spectroscopy) allows the assignment of \( ^3J \)-coupled protons (COSY) while ensuring that the proton is within a desired spin system (TOCSY).

Once protons are assigned, their \( ^1J_{C-H} \)-coupled carbons can be readily assigned through HSQC (heteronuclear single quantum coherence) data. Similarly to \( ^1 \text{H} \) proton NMR data, there are expected ranges for anomeric carbon signals with \( \alpha \)-anomers falling between \( \delta \) 97 - \( \delta \) 101 ppm and \( \beta \)-anomers between \( \delta \) 103 - \( \delta \) 105\textsuperscript{44}. The oxygen found in the glycosidic bond makes it impossible for many NMR techniques to identify neighbouring sugar residues\textsuperscript{43, 44}. Therefore, HMBC (heteronuclear multiple bond correlation) experiments enable \( ^2,^3J_{C,H} \)-coupling through glycosidic linkages. Lastly, NOESY and ROESY experiments can be completed to gain through-space information garnering information about carbohydrate conformations.
1.2.2.4 Viscometric Analysis

Tracking enzymatic hydrolysis of polysaccharides through viscometric analysis can offer information about how the depolymerization occurs\textsuperscript{20}. For instance, when studying the glycoside hydrolases, viscometry can provide details about the mode of action that a particular GH acts on the substrate. This stems from the idea that endo-dissociative and exo-/endo-processive enzymes hydrolyze polysaccharides differently leading to hydrolysis causing the viscosity to reduce at different rates. For instance, endo-dissociative GHs are capable of decreasing the viscosity of solutions rapidly based their ability to stochastically cleave polysaccharides which increases the likelihood of the immediate drop in the degree of polymerization of products during reactions\textsuperscript{20}. In comparison, a gradual decrease in viscosity is observed with exo-acting and endo-processive GHs, as these maintain association with the substrate between hydrolytic events and therefore are slow to reduce the overall degree of polymerization of a polysaccharide (Figure 1.5). The major disadvantage of the assay is that it only provides information about GHs of interest in relation to other enzymes capable of acting on the same substrate. This can include enzyme variants or GHs from different families.
Figure 1.5 – An example of a viscometric assay to determine the degree of processivity of PoGH74 and its variants compared to the endo-dissociative BoGH5 on xyloglucan. The BoGH5 (black squares) is used as an endo-dissociative control to compare the degree of processivity of the endo-processive wildtype PoGH74 (red circles) and its subsite variants: PoGH74cat(W406A) (+2 subsite) (green diamonds), PoGH74cat(W347A) (+3 subsite) (blue triangles), PoGH74cat(W348A) (+5 subsite) (purple triangles), PoGH74cat(Y372A) (+6 subsite) (cyan hexagons). Reprinted (adapted) with permission from *Biochem J* (2018) 475 (24): 3963-3978. https://doi.org/10.1042/BCJ20180763. Copyright 2018 Portland Press.
1.3 Aims of Thesis

Since the food industry was granted permission to use xanthan by the FDA in 1969, its applications have expanded to cosmetics, pharmaceutical, and petroleum industries. Indeed, this has led to a forecasted annual increase of xanthan production by 5-10% worldwide\(^{46}\). This is due to xanthan’s chemical structure lending it favourable properties as a viscosity modifier capable of handling extreme salt concentrations, temperatures and pH. This widespread use has garnered intense interest from the biotechnology industry who intend to find enzymes capable of depolymerizing xanthan and modifying them to be tailored for specific applications\(^{47,48}\). Using molecular biology and microbiology techniques, bacterial strains producing enzymes capable of acting on xanthan were discovered by Novozymes A/S. Subsequent steps were taken to identify, express and purify a \(\beta\)-glucanase from \textit{Paenibacillus nanensis} belonging to glycoside hydrolase family 9 (GH9) capable of depolymerizing xanthan coined “\(PspXan9\)”. However, since \(PspXan9\) is the first identified GH9, to hydrolyze xanthan, little is understood regarding how its structure affects function.

To gain more insights into the structure-function relationship of \(PspXan9\), the first objective of this thesis is to provide a comprehensive understanding of the kinetic parameters, mode of action and product analysis of \(PspXan9\). Once this has been established, it is hypothesized that single point mutations to predicted amino acid residues of significance (catalytic and subsite residues, et cetera) will be sufficient to confer superior characteristics to \(PspXan9\) for desired applications. Therefore, the second aim is to use a structure-guided approach to rationally engineer \(PspXan9\) by selecting these amino acid residues of significance and comparing them to the previously established data from the first objective. In theory, it will be possible to alter catalytic
activity, mode of action and even products produced. This can be useful in industrial applications if the desired applications are not met by wildtype \textit{PspXan9}.
Chapter 2: Biochemical Analysis of PspXan9: An Endo-Processive Multi-Modular Xanthanase from Paenibacillus nanensis

2.1 Introduction

With increasing uses of xanthan in household products, interest has grown in developing a xanthan depolymerase (*endo*-xanthanase) that can serve as laundry detergent additive. To find novel xanthanases, the biotechnology company, Novozymes A/S, collected forest soil samples from China and attempted to grow bacteria in growth media using xanthan as the sole carbon source. Following the amplification of 16S rRNA, it was revealed that *Paenibacillus nanensis* (98.1% sequence identity to *P. nanensis* in GenBank: AB265206) was one of the bacteria capable of growing solely on xanthan47.

Although glycoside hydrolase family 9 is historically considered to be comprised of cellulases49, a glycoside hydrolase family 9 (GH9) enzyme from *P. nanensis* (herein PspXan9; GenBank MG661269), was suspected as being able to depolymerize xanthan. This is because recent studies have provided evidence that GH9 enzymes can have high specificities towards non-cellulose substrates, notably Cel9X from *Clostridium cellulolyticum* which is highly specific for hydrolyzing xyloglucan50 as well as the *endo*-xanthanase, MiXen, from *Microbacterium* sp. XT1133,51. Despite all of these noncellulase GH9 enzymes being active on a cellulosic backbone, these recent examples indicate a wider substrate specificity within this family than previously suspected.

PspXan9 was able to demonstrate a benefit in laundry detergent application assays47; however, the mechanism and structure of the enzyme was poorly understood. Therefore, x-ray crystallography completed by our collaborators Olga V. Moroz, Elena Blagova, Gideon J. Davies and Keith S. Wilson at the University of York, United Kingdom revealed an impressive hexa-
modular structure consisting of an N-terminal Ig-like (termed X229) domain, a GH9 catalytic module, three β domains and a C-terminal carbohydrate binding module (CBM) family 84 (Figure 2.1).

Figure 2.1 – X-ray crystal structure of PspXan9 highlighting its unique modularity. Top: Crystal structure of GH9 xanthanase from Paenibacillus nanensis (PspXan9) solved at 2.04 Å. The overall fold is coloured by domain from the N-terminus: X229 domain, Ig-like, (1–90 aa) (violet), GH9 catalytic module (91–559 aa) (blue), β1 (560–657 aa) (green), β2 (658–814 aa) (yellow), β3 (815–918 aa) (orange), and CBM84 domain (919–1055 aa) (red). Bottom: A schematic diagram showing the unique organization of the domains of PspXan9 structural. Calcium ions are shown as teal spheres.
As GH9 enzymes can function with as little as the GH9 catalytic domain, the presence of the five additional modules raises questions about how they impact the function of PspXan9. This is especially true for the β2 domain which hangs directly over the GH9 catalytic module which could indicate a role in binding or activity. Additionally, calcium ions may play an important role considering there are five calcium ions found throughout the enzyme. Of these, there are three bound by the GH9 catalytic module, one bound by both the GH9 catalytic module and the β1 domain, and finally, one bound by the CBM84 domain. Dependence on calcium ions for stability has been observed in other GH9 enzymes; however, it is currently unclear what role these ions serve in the PspXan9.

To gain a better understanding of PspXan9, kinetics were completed to confirm the enzyme’s substrate specificity, and to determine kinetic parameters on xanthan and its derivatives. Thorough analytical studies were performed on enzymatic products to determine the mode of action by monitoring the cleavage pattern over time, as well as a thorough analysis of the limit digest products to determine the site of hydrolysis. This study aims to lay the foundation for future studies on PspXan9 as well as to provide insights into the potential for industrial endo-xanthanases.

2.2 Materials and Methods

2.2.1 Cloning, Expression and Purification

Purified PspXan9 protein was supplied by Novozymes A/S who performed the cloning, expression and purification, as described in Moroz, et al. Briefly, the PspXan9 gene was cloned into a Bacillus subtilis expression vector containing an Bacillus clausii secretion signal, BcSP followed by an N-terminal polyhistidine affinity tag (6x-HisTag; HHHHHHHPR-) for downstream purification as described previously. The recombinant B. subtilis clone was cultivated in LB
media for 5 days at 30 °C, before the enzyme was collected from the supernatant. *Psp*Xan9 was then purified by three rounds of chromatography starting with hydrophobic interaction chromatography (HIC), followed by immobilized metal ion affinity chromatography (IMAC) and completed with size-exclusion chromatography (SEC) to desalt the enzyme sample. Fractions deemed to be > 95% pure, based on results from SDS-PAGE, were pooled and buffer exchanged in 20 mM Tris-HCl, pH 8.5.

Upon receiving the enzyme from Novozymes A/S, intact protein mass spectrometry was conducted using a nanoACQUITY UPLC coupled to a Xevo G2-S QTof (Waters Co., Milford, MA, USA) system, essentially according to methods previously described\textsuperscript{54}. *Psp*Xan9 was determined to be 115972 Da (Figure 2.2), which is 3 Da above the calculated molecular weight from the mature amino acid sequence. Protein concentration was calculated from \(A_{280}\) values using the theoretical extinction coefficient of 199860 M\(^{-1}\)cm\(^{-1}\) based on the deduced mature amino acid sequence.
Figure 2.2 – Purity and molecular mass analysis of recombinant PspXan9. Intact mass spectrum of GH9 PspXan9. Inset: SDS-PAGE analysis of PspXan9 (lane ‘GH9’) versus a molecular weight ladder (lane ‘L’).


2.2.2 Carbohydrate Sources

Xanthan gum, “xanthan”, (item no. XA105) was purchased from Spectrum Chemical, New Brunswick, NJ, USA. Barley mixed-linkage glucan (bMLG), tamarind seed xyloglucan (tXyG), beechwood xylan (bGX), and wheat-flour arabinoxylan (wAX) were all acquired from Megazyme (Bray, Ireland). The cellulose analogues carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC) were purchased from Acros Organics (Morris Plains, NJ, USA) and Amresco (Solon, OH, USA), respectively.

Lyase-treated xanthan gum (polytetrameric xanthan) was prepared by incubating 0.5 U mL⁻¹ of xanthan lyase from Bacillus sp. (EC 4.2.2.12, CAZy family PL8, Megazyme cat. no. E-XANLB) in 2.5 g L⁻¹ xanthan and 50 mM HEPES-NaOH, at pH 7.0. The Δ4,5-unsaturation of the
glucuronic acid was monitored in a quartz cuvette with 1-cm pathlength at 235 nm with a Cary 60 UV-Vis spectrophotometer, as previously stated\textsuperscript{27}. Once the absorbance plateaued, another 0.5 U mL\textsuperscript{-1} xanthan lyase was added and monitored to ensure completion. The resultant polysaccharide was extracted by precipitation as previously described\textsuperscript{27,32}; however, chilled 2-propanol was used instead of ethanol. Following precipitation, 2-propanol was decanted and finally centrifuged at 4500 $\times$ $g$ for 20 min to aid in removing the remaining supernatant. Residual 2-propanol was removed by placing the precipitated polysaccharide in a vacuum desiccator and left for 72 h.

### 2.2.3 Enzyme Kinetic Analysis

$PspXan9$ activity on polysaccharides was determined by quantifying the production of reducing-ends using a low-volume copper/2,2$'\text{-}$bicinchoninic acid (BCA) reducing sugar assay, as previously described\textsuperscript{35}. D-glucose standard curves were prepared at glucose concentrations between 0-125 μM in 50 mM HEPES-NaOH, pH 7 and contained 2 mM CaCl\textsubscript{2} when appropriate. For all kinetic assays, enzyme substock solutions of appropriate concentrations were prepared in 50 mM HEPES-NaOH, pH 7.0 containing 0.1 g L\textsuperscript{-1} bovine serum albumin (BSA) to avoid nonspecific adsorption to vessel surfaces. Due to limited availability of $PspXan9$, 0.1 g L\textsuperscript{-1} BSA served as an analogue of boiled enzyme in negative controls, unless otherwise stated. Reactions were terminated after 10 min by the 1:1 v/v addition of BCA reagent and the formation of the Cu$^{+}$-BCA complex was stimulated by heating samples to 80 °C for 20 min. Finally, the Cu$^{+}$-BCA complex absorbance was measured at 562 nm.

The activity of $PspXan9$ was optimized for CaCl\textsubscript{2} concentration, temperature and pH on 0.5 g L\textsuperscript{-1} lyase-treated xanthan in 50 mM HEPES-NaOH, pH 7.0, unless otherwise stated. The optimum calcium concentration was determined by incubating 1.59 nM $PspXan9$ with calcium
concentrations between 0-10 mM CaCl₂ at 37 °C. The effect of temperature on activity was determined by incubating 0.8 nM of \textit{Psp}Xan9 at temperatures ranging from 30-79 °C using a temperature gradient in a Bio-Rad Thermal Cycler S1000. The pH-profile was found by incubating 0.8 nM of \textit{Psp}Xan9 at 55 °C in 50 mM buffers ranging from pH 3.6-10.6. The buffers were as follows: Na-acetate (pH 3.6-5.5), MES-NaOH (pH 5.38-7.0), HEPES-NaOH (pH 7.0-8.5) and CHES-NaOH (pH 8.5-10.6).

The effect of calcium on the thermostability of \textit{Psp}Xan9 was determined by comparing residual activity of enzymes incubated at various temperatures over 48 hours in the presence or absence of CaCl₂. Reaction mixtures containing either 0 or 20 mM CaCl₂, were incubated with 16 nM of \textit{Psp}Xan9 at temperatures between 45-69 °C, maintained by a Bio-Rad Thermal Cycler S1000. Residual activity was assayed by adding the incubated enzyme to reaction mixtures containing 0.5 g L⁻¹ lyase-treated xanthan in 50 mM HEPES-NaOH, pH 7.0 at 37 °C for 10 min before terminating the reaction with ice-cold BCA reagent. The final CaCl₂ concentration for all residual activity reactions was 2 mM.

Enzyme specificity and specific activity was determined on selected substrates by adding appropriate concentrations of \textit{Psp}Xan9 to 2 g L⁻¹ of either xanthan, lyase-treated xanthan, bMLG, tXyG, bGX, wAX, CMC or HEC. Final \textit{Psp}Xan9 concentrations were 0.4 nM for lyase-treated xanthan, 191 nM for xanthan and CMC, and 383 nM for bMLG, tXyG, bGX, wAX, and HEC. Reactions were carried out in 50 mM HEPES, pH 7.0 with 2 mM CaCl₂ at 55 °C, over 10 min. One unit of activity (U) is defined as the amount of enzyme needed to release 1 μmol of glucose-equivalent reducing-ends in 1 min.

The kinetic parameters $k_{\text{cat}}$ and $K_m$ were determined for \textit{Psp}Xan9 on lyase-treated xanthan. 0.4 nM of \textit{Psp}Xan9 was assayed with lyase-treated xanthan concentrations between 0.01-2 g L⁻¹
in 50 mM HEPES-NaOH, pH 7.0 with 2 mM CaCl₂ at 55 °C, over 10 min. OriginPro software (OriginLab) was used to fit the data to the Michaelis-Menten equation.

2.2.4 Enzyme Product Analysis

2.2.4.1 HPSEC-UV

High-performance size exclusion chromatography coupled to a UV-detector (HPSEC-UV) was used to visualize the breakdown products of lyase-treated xanthan following digestion with \( PspXan9 \). 16 nM of \( PspXan9 \) was incubated with 2 g L\(^{-1} \) lyase-treated xanthan in 50 mM HEPES-NaOH, pH 7 with 2 mM CaCl₂ at 55 °C. Samples were taken periodically and the reaction was immediately inactivated upon addition to 80 °C H₂O in a 1:1 v/v ratio. The elution of lyase-treated xanthan partial digest products was monitored at 235 nm and separation was achieved using Ultrahydrogel\(^{TM} \) 6 x 40 mm Guard Column in series with a Ultrahydrogel\(^{TM} \) Linear 7.8 x 300 mm column with an isocratic gradient of 0.5 M NaCl with a flowrate of 0.6 mL min\(^{-1} \) over 45 min. The column and compartment temperature were set to 60 °C and 40 °C, respectively. These experiments were carried out on a ICS-5000 DC HPLC using Chromeleon software version 7 (ThermoFisher).

2.2.4.2 LC-MS & MS/MS

Exact mass determination of partial digestion products after 2 h as well as limit digestion products by were performed in negative-ion mode using a nanoACQUITY UPLC coupled to a Xevo G2-S QTof (Waters Co., Milford, MA, USA). Separation was achieved using a Hypercarb Kappa 3 μm porous graphitized carbon column (150 x 0.32 mm). Eluent A was comprised of 95% 25 mM ammonium formate, pH 5, and 5% acetonitrile; eluent B was comprised of 5% 0.5 M
ammonium formate, pH 5, and 95% acetonitrile. Samples were eluted at a flow rate of 8 μL min\(^{-1}\) with the following gradient: 0-5 min: 95% eluent A and 5% eluent B; 5-25 min: linear gradient to 70% eluent A and 30% eluent B; 25-25.1 min: linear gradient to 95% eluent A and 5% eluent B; 25.1-45 min (25.1-30 min for reduced samples): 95% eluent A and 5% eluent B. When appropriate, the reduction of 2 g L\(^{-1}\) limit digestion products was achieved by the addition of 1 M NaBH\(_4\) (S678-25, Fisher Scientific) after the products were diluted in 50 mM sodium carbonate buffer, pH 10. After the reduction had proceeded for 12 h, it was quenched by lowering the pH to 5-6 with 1 M acetic acid. The final concentration of the products was adjusted to 1 g L\(^{-1}\). The target masses for MS-MS were 661 \(m/z\), 703 \(m/z\) and 663 \(m/z\) with collision energies of 15 eV and 25 eV for the latter.

2.2.4.3 NMR

Sample preparation for NMR analysis began by incubating 38 nM PspXan9 with 12.5 g L\(^{-1}\) of lyase-treated xanthan in 5 mM HEPES, pH 7.0 with 2 mM CaCl\(_2\). Complete degradation was confirmed using HPSEC-UV as described above (data not shown). Removal of any acetyl groups on the α\((1 \rightarrow 3)\)-mannosyl residue was completed by raising the pH to 10 using 28-30% NH\(_4\)OH (1336-21-6, Fisher Scientific). The sample was then desalted in ultra-pure H\(_2\)O using a 100 x 2.6 cm Bio-Gel P-2 column with a 0.5 mL min\(^{-1}\) flowrate. Fractions absorbing at 235 nm were pooled and lyophilized. Prior to NMR experiments, the sample was dissolved in 99.9% D\(_2\)O before being lyophilized. This was repeated twice to ensure adequate proton removal. Finally, the oligosaccharides were resuspended in D\(_2\)O at a concentration of 7 g L\(^{-1}\).

All NMR experiments were carried out on a Bruker Avance 600 equipped with a z-gradient TCI cryoprobe, with a \(^1\)H resonance at 600.15 MHz and a \(^13\)C resonance of 150.92 MHz. These
included the collection of 1D-1H spectrum, and 1D-1H TOCSY NMR with irradiations at predicted anomeric protons including δ 4.29, 4.44, 4.64, 5.20 and 5.36 ppm. On top of this, several 2D experiments were conducted including homonuclear 1H 2D-TOCSY and 1H 2D-COSY and heteronuclear 1H,13C 2D-HSQC and 1H13C 2D-HMBC with the latter being optimized at 5 and 10 Hz. The HOD peak (δ 4.70 ppm) served as a reference for all 1H experiments, whereas 13C experiments were referenced to external methanol in D2O (δ 49.00 ppm).

2.3 Results and Discussion

2.3.1 Catalytic Activity and Specificity of Recombinant PspXan9

2.3.1.1 Activity Optimization and Thermostability of PspXan9

To gain insights into optimal conditions of PspXan9 activity, calcium concentration, temperature and pH were screened. Compared to its absence, PspXan9 demonstrated a 4.75-fold increase in activity on lyase-treated xanthan in the presence 2 mM CaCl2 (Figure 2.3A). Interestingly, the addition of CaCl2 concentrations higher than this led to a gradual monotonic decline in activity. Before temperature optimization and thermostability trials were completed, PspXan9 activity was measured in different pH buffers ranging from 3.6-10.6. The pH-rate profile demonstrated a bell-shaped curve with activity peaking between 7.0-7.5, thus pH 7.0 was selected for future kinetic studies (Figure 2.3C).

Using ideal pH and calcium concentrations, PspXan9 temperature optimization trials showed the highest activity could be achieved at 63 °C (Figure 2.3B). However, immediately following this peak activity, a rapid 8.6-fold drop was observed from 63-72 °C at a decline of 4.1 \times 10^3 \text{ min}^{-1} \text{ per °C}. To test both the importance of calcium on PspXan9 stability, as well as to determine the appropriate thermal conditions to establish kinetic parameters, a thermostability
assay was performed. Remarkably, in the absence of calcium (Figure 2.4A), a minimum of 40% decline in activity was observed at all assayed temperatures (45-69 °C) in the first hour. Indeed, activity at all temperatures was completely abrogated after just 10 h (Figure 2.4B). Conversely, in the presence of calcium, full activity was maintained at all assayed temperatures below 60 °C during the first hour (Figure 2.4C). Incubations at 60 °C were the highest temperature to maintain activity over the 48 h assay but demonstrated a t1/2 of approximately 22 h (Figure 2.4D). Incubations at temperatures as high as 54 °C were capable of sustaining 75% residual activity over 48 h. For these reasons, kinetic parameters were measured at 55 °C subsequently.

Figure 2.3 – Optimization of PspXan9 activity. (A) The effect of calcium concentration on the hydrolytic activity of PspXan9 at 37 °C in 50 mM HEPES-NaOH buffer, pH 7.0 at 37 °C. (B) Temperature-rate profile in the presence of 2 mM CaCl₂ in 50 mM HEPES-NaOH buffer, pH 7.0. (C) pH-rate profile at 55 °C in the presence of 2 mM CaCl₂ in 50 mM HEPES-NaOH buffer, pH 7.0. For each, the rate of reducing-end formation with 0.5 g L⁻¹ of lyase-treated xanthan as the substrate was determined after a 10 min incubation. Error bars represent the standard deviation over three replicates. Reprinted (adapted) with permission from ACS Catal. 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
Figure 2.4 – The effect of Ca2+ ions on thermostability of PspXan9. (A) Remaining activity of 16 nM PspXan9 following incubation at the indicated temperatures; (B) shows an expansion of the data in panel A to cover a longer time period. (C) residual activity of 16 nM PspXan9 in the presence of 20 mM CaCl2 following incubation at the indicated temperatures; (D) an expansion of the data in panel C to cover a longer time period. After dilution, enzyme activity assays contained a final concentration of 2 mM CaCl2 (cf. Figure 2.3C). Error bars represent the standard deviation over three replicates. Reprinted (adapted) with permission from ACS Catal. 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.


2.3.1.2 Substrate Specificity of *PspXan9*

![Diagram of xanthan and products](image)

**Figure 2.5** – The structure of xanthan and products following incubation with PL8 xanthan lyase and *PspXan9*. Xanthan lyase cleaves the side chain GlcA-β(1 → 4)-pyruvylated mannose glycosidic linkage from native polypentameric xanthan (A), resulting in the formation of a Δ4,5-ene-GlcA residue and release of pyruvylated mannose. The resulting polytetrameric xanthan (B) is the preferred substrate for the GH9 endoxanthanase, which yields tetrasaccharides (C) as limit-digest products. Acetylation is variable. Reprinted (adapted) with permission from *ACS Catal.* 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.

In comparison with other tested polysaccharides, *PspXan9* demonstrated highest specificity for lyase-treated xanthan (Table 2.1; Figure 2.5). Indeed, activity on untreated xanthan was 635-times lower, which was similar to unbranched polysaccharides including artificial β(1 → 4)-glucan derivatives carboxymethyl cellulose (CMC) and hydroxyethyl cellulose (HEC), as well as barley mixed linkage β(1 → 3)/β(1 → 4)-glucan (bMLG). *PspXan9* was even less active on the highly branched β(1 → 4)-glucan, xyloglucan,55 and β(1 → 4)-xylans.56 Initial-rate kinetics were performed on lyase-treated xanthan and fit using the Michaelis-Menten equation to determine $k_{cat}$ and $K_m$ (1.69 × 10^4 min⁻¹ and 0.0962 g L⁻¹, respectively; Figure 2.6).

**Table 2.1** – Kinetic analysis of the activity of *PspXan9* on polysaccharide substrates.
<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Specific Activity (U mg⁻¹)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>polypentameric xanthan (Figure 2.5A)</td>
<td>0.285 ± 0.010</td>
</tr>
<tr>
<td>lyase-treated xanthan (Figure 2.5B)</td>
<td>181 ± 9</td>
</tr>
<tr>
<td>CMC</td>
<td>0.492 ± 0.022</td>
</tr>
<tr>
<td>HEC</td>
<td>0.180 ± 0.099</td>
</tr>
<tr>
<td>bMLG</td>
<td>0.213 ± 0.029</td>
</tr>
<tr>
<td>tXyG</td>
<td>0.0679 ± 0.0005</td>
</tr>
<tr>
<td>bGX</td>
<td>0.0614 ± 0.0014</td>
</tr>
<tr>
<td>wAX</td>
<td>0.0449 ± 0.0132</td>
</tr>
</tbody>
</table>

*One enzyme unit (U) is defined as the amount of enzyme required to release 1 μmol of glucose reducing-end equivalents per minute.

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Figure 2.6 – Michaelis-Menten kinetics of *PspXan9* activity on lyase-treated xanthan. *PspXan9* kinetics were determined by quantifying activity after a 10 min incubation with lyase-treated xanthan concentrations between 0.01-2 g L⁻¹. Error bars represent the standard deviation over three replicates. Reprinted (adapted) with permission from *ACS Catal.* 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
2.3.2 Structural Analysis of \textit{Psp}Xan9 Products

2.3.2.1 HPSEC-UV and LC-MS Analysis of \textit{Psp}Xan9 Degradation Products

Analysis of product formation over time using HPSEC-UV was used to gain insight into the mode of action employed by \textit{Psp}Xan9 (Figure 2.7). After just 5 min of hydrolysis, a reduction of a large $M_w$ peak (lyase-treated xanthan) eluting at 19.1 min, accompanied by the immediate formation of two partially resolved low $M_w$ peaks (eluting at 32.4 min and 33.2 min) was observed (Figure 2.7F). The lack of intermediary product peaks suggests that \textit{Psp}Xan9 hydrolyzes lyase-treated xanthan in a processive manner. Interestingly, after approximately 1 h of hydrolysis, the larger of the two product peaks (eluting at 32.4 min) can be seen decreasing (Figure 2.7D). By 9.5 h of hydrolysis, \textit{Psp}Xan9 achieves complete conversion of lyase-treated xanthan to a single product eluting at 33.2 min (Figure 2.7F). To better understand the relationship between the two observed low-$M_w$ peaks, the samples taken after the 2 h and 9.5 h time points were also subjected to LC-ESI-MS for analysis (Figure 2.8).

LC-MS revealed the presence of two products found in the 9.5 h sample despite it appearing to be a single peak in the HPSEC-UV (Figure 2.8B). These had detected masses of 703 $m/z$ and 661 $m/z$ which correspond to the lyase-treated tetrasaccharide repeating unit in both the acetylated and nonacetylated forms, respectively (Figure 2.5B). Extracted ion chromatograms of the sample taken after 2 h of hydrolysis were used to visualize individual products from the somewhat complex total ion current (TIC) chromatogram (Figure 2.9). In addition to the aforementioned tetrasaccharide products (Figure 2.8B and Figure 2.9B-C), products with masses matching varying degrees of acetylation of lyase-treated xanthan octasaccharides (1305 $m/z$, 1347 $m/z$ and 1389 $m/z$; Figure 2.8B and Figure 2.9 F-H) as well as nonpyruvylated xanthan pentasaccharides (883 $m/z$ and 925 $m/z$; Figure 2.9D-E) were also present. The existence of nonpyruvylated pentasaccharides may
be a result of the high specificity of the *Bacillus* sp. xanthan lyase to the pyruvylated form of the β-D-mannosyl terminal residue\(^{26}\). Although the pentasaccharide will not show up in the HPSEC-UV analysis, the two low \(M_w\) product peaks correspond to lyase-treated xanthan oligosaccharides with backbones of either four or two glucose residues.
Figure 2.7 – HPSEC-UV analysis of xanthan oligosaccharide products from a timecourse digestion with \textit{PspXan9}. 2 g L\textsuperscript{-1} of lyase-treated xanthan (A) was incubated with 16 nM \textit{PspXan9} at 55°C and sampled at various time points (B-F) for analysis by HPSEC-UV. Reprinted (adapted) with permission from \textit{ACS Catal.} 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
Figure 2.8 – Timecourse degradation products and subsequent analysis through LC-MS. (A) Limit digest products are observed following 9.5 h of incubation (see Figure 2.7 for a full timecourse). (B) Mass spectrum of the unsaturated octasaccharide product (turquoise) found at 32.4 min elution time after a 2 h incubation, and the mass spectrum of acetylated (green) and nonacetylated (magenta) unsaturated tetrasaccharides found in the partial digest (2 h) and the limit digest (9.5 h) and elution at 33.2 min (see Figure 2.9 for extracted ion chromatograms). Reprinted (adapted) with permission from ACS Catal. 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
Figure 2.9 – LC-MS chromatograms of partial digest products from *PspXan9*. Products from 2 h of degradation by 16 nM *PspXan9* were separated over 45 min by liquid chromatography and analyzed using mass spectrometry with a scan range of 100-2000 m/z in negative-ion mode. The resulting total ion current chromatogram (A) and extracted ion chromatograms (B-H) of expected products are displayed above, with corresponding structures and m/z values indicated on the right. Resolution of reducing-end anomers and acetylated variants is observed for some products. Non-carbohydrate peaks in the TIC chromatogram are denoted with an asterisk symbol (*). Reprinted (adapted) with permission from *ACS Catal.* 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
2.3.2.2 Identification of \textit{PspXan9} Products Using LC-MS/MS

When comparing extracted ion chromatograms of 661 \textit{m/z} and 703 \textit{m/z}, it was observed that there was an increased complexity upon the addition of the acetyl group, namely a doubling in peak formation (Figure 2.9B-C and Figure 2.10A). Following the NaBH\textsubscript{4} reduction, the chromatogram of the 663 \textit{m/z} tetrasaccharide alditol is simplified with only a single peak (Figure 2.10A). This suggests that the disappearance of peak complexity corresponds with the reduction of the sugars and therefore the loss of $\alpha/\beta$ anomeric peaks (not assigned). However, because both 661 \textit{m/z} and 703 \textit{m/z} chromatograms should only have two anomeric peaks, the cause of the additional two peaks in the 703 \textit{m/z} chromatogram remains uncertain. It was supposed that the four peaks in the 703 \textit{m/z} EIC may indicate an alternative position of the 6-\textit{O}-acetyl-group. Prior studies have demonstrated that under specific conditions, migrations of acetyl groups on partially acetylated sugars can take place\textsuperscript{57}. Therefore, to find the position of the acetyl group on xanthan tetrasaccharides, MS/MS was conducted on 703 \textit{m/z} (Figure 2.11). Unfortunately, the absence of mannosyl intra-ring $^{1,4}\text{X}_{1\alpha}$, $^{0,3}\text{A}_{2\alpha}$, $^{0,2}\text{A}_{2\alpha}$ and $^{2,5}\text{A}_{2\alpha}$, product ions lead to the inability to assign the acetyl position.

To identify whether the site of \textit{PspXan9} hydrolysis occurred on the sidechain-branched glucose or the unbranched glucose (Figure 2.5C), tandem mass spectrometry (MS/MS) was conducted on the nonacetylated tetrasaccharide (661 \textit{m/z}) as well as the alditol derivative of the limit digest (663 \textit{m/z}). An additional byproduct of the reduction was the deacetylation of all tetrasaccharides due to the extended incubation in carbonate buffer (pH 10) necessary to prevent premature NaBH\textsubscript{4} decomposition\textsuperscript{58}. When comparing the MS/MS spectra of the precursor ion with its reduced derivative (661 \textit{m/z} and 663 \textit{m/z}), diagnostic product ions of sidechain position were identified by a $\Delta \textit{m/z}$ of 2 increase signifying the presence of the glucitol rather than the glucose.
reducing-end (Figure 2.10C, Figure 2.12 and Figure 2.13). These product ions included the increase of the $Z_1$ ($481 \text{ m/z}$ to $483 \text{ m/z}$) and $Y_1$ ($499 \text{ m/z}$ to $501 \text{ m/z}$) and conservation in mass of the $B_1$ ($161 \text{ m/z}$) and $C_1$ ($179 \text{ m/z}$) nonreducing-end product ions. The increase in mass of sidechain containing product ions ($Z_1$ and $Y_1$) indicates the presence of the glucitol and therefore the site of cleavage on the sidechain-branched glucose.

Figure 2.10 – Extracted ion chromatograms of lyase-treated xanthan tetrasaccharides, their derivatives and subsequent MS/MS analysis. (A) Extracted ion chromatograms showing the acetylated (green) and nonacetylated (magenta) unsaturated tetrasaccharides limit digest products and their subsequent reduced and deacetylated form (blue) after treatment with NaBH$_4$. Two chromatographic peaks were observed for each species due to the resolution of reducing-end anomers. (B) MS/MS results comparing the nonacetylated unsaturated tetrasaccharide (magenta) and the NaBH$_4$-reduced derivative (blue) with key ion fragments identified above their corresponding masses (see Figure 2.11, Figure 2.12 and Figure 2.13 for full MS/MS spectra). Reprinted (adapted) with permission from *ACS Catal.* 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
Figure 2.11 – MS/MS of the 703 m/z precursor ion, corresponding to the acetylated, lyase-treated xanthan tetrasaccharide, from lyase-treated xanthan degradation by PspXan9. (A) The fragmentation pattern of the 703 m/z precursor ion; (B) The MS/MS spectrum of the product ions from the fragmentation of 703 m/z. Selected regions are magnified and diagnostic peaks labelled in bold. Standard carbohydrate fragment nomenclature is used\textsuperscript{41}. The presence of a forward slash in a peak label indicates the presence of alternative assignments. Regions within the bounds of arrows found below the x-axis, indicate the amount of magnification (if any) the region is being displayed. Reprinted (adapted) with permission from ACS Catal. 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
Figure 2.12 – MS/MS of the 663 m/z precursor ion, corresponding to the reduced, lyase-treated xanthan tetrasaccharide, from lyase-treated xanthan degradation by PspXan9. (A) The fragmentation pattern of the 663 m/z precursor ion; (B) The MS/MS spectrum of the product ions from the fragmentation of 663 m/z. Selected regions are magnified and diagnostic peaks labelled in bold. Standard carbohydrate fragment nomenclature is used\textsuperscript{41}. The presence of a forward slash in a peak label indicates the presence of alternative assignments. Regions within the bounds of arrows found below the x-axis, indicate the amount of magnification (if any) the region is being displayed. Reprinted (adapted) with permission from ACS Catal. 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
Figure 2.13 – MS/MS of the 661 m/z precursor ion, corresponding to the nonacetylated, lyase-treated xanthan tetrasaccharide, from lyase-treated xanthan degradation by \textit{PspXan9}. (A) The fragmentation pattern of the 661 m/z precursor ion; (B) The MS/MS spectrum of the product ions from the fragmentation of 661 m/z. Selected regions are magnified and diagnostic peaks labelled in bold. Standard carbohydrate fragment nomenclature is used\textsuperscript{41}. The presence of a forward slash in a peak label indicates the presence of alternative assignments. Regions within the bounds of arrows found below the x-axis, indicate the amount of magnification (if any) the region is being displayed. Reprinted (adapted) with permission from \textit{ACS Catal.} 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
2.3.2.3 Identification of *PspXan9* Products Using NMR

To further support this proposal, NMR experiments were performed. Complete proton assignment was achieved through a combination of selective $^1$H 1D-total correlation spectroscopy (TOCSY) (Figure 2.14), 2D-homonuclear total correlation spectroscopy (TOCSY; Figure 2.15) and 2D-homonuclear correlation spectroscopy (COSY; Figure 2.16) experiments. Anomeric assignments were accomplished irradiating proton peaks between δ 4.29 and 5.36 ppm and comparing the resulting spectra to literature values$^{43,44,59}$ and authentic standards (data not shown).

Interestingly, full magnetization transfer of the α-Man (δ 5.36 ppm) spin system was not successful leading to only the formation of a small single peak (dd) at δ 4.29 ppm (Figure 2.14E). Subsequent irradiation of the δ 4.29 ppm (Figure 2.14F) peak lead to the complete magnetization transfer of the spin system, suggesting that the transfer was impeded due to weak equatorial coupling through the mannosyl H2. Additionally, irradiation of δ 4.29 ppm generated the formation of two apparent singlet anomeric peaks ($^3J_{H1,H2}$; Table 2.2) at δ 5.35 and 5.38 ppm, instead of the expected single peak. This, in addition to complexity of the H3-H5 peaks, suggests the possibility of conformational flexibility of the α-Man residue.

Irradiation of δ 4.34 ppm uncovered the Δ4,5-ene-GlcA spin system, which was readily identified by the presence of the H4-vinyl proton at δ 5.74 ppm, as well as the presence of only four protons (Figure 2.14G and Figure 2.15). The 2D-COSY spectrum later revealed that, despite being within the anomeric range, the proton at δ 4.34 ppm was, in fact, H2 of the Δ4,5-ene-GlcA (Figure 2.16) residue. Instead, H1 of the Δ4,5-ene-GlcA residue was buried beneath the HOD peak at δ 4.82 and 4.83 ppm. As with α-Man, the presence of two apparent doublets instead of a single H1 peak suggests conformation flexibility either of the Δ4,5-ene-GlcA residue consequence of the conformation flexibility seen in α-Man.
Figure 2.14 – Selective $^1$H 1D-TOCSY experiment of deacetylated lyase-treated xanthan tetrasaccharides generated from lyase-treated xanthan degradation by \textit{PspXan9}. (A) Anomeric protons were targeted based on the full 1D-NMR spectrum, using the established anomeric proton region from between 4.3-5.5 ppm. Spectra corresponding to individual spin systems are displayed in various colors with: (B) the reducing-end (r.) β-glucose in red. (C) the reducing-end (r.) α-glucose in magenta. (D) the nonreducing-end (n.r.) β-glucose in light blue. (E) α-mannose in green with irradiation on H1 at δ 5.36 ppm. (F) α-mannose in green with irradiation on H2 at δ 4.29 ppm. (G) β-Δ4,5-ene-gluconic acid in orange. Proton integrations are presented on the full $^1$H 1D-NMR spectrum above their corresponding peak. Irradiations from $^1$H 1D-TOCSY experiments are displayed by black stars above corresponding peaks. Reprinted (adapted) with permission from \textit{ACS Catal.} 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
Figure 2.15 – $^1$H 2D-TOCSY experiment of deacetylated lyase-treated xanthan tetrasaccharides generated from lyase-treated xanthan degradation by PspXan9. Each crosspeak indicates coupling between protons through magnetization transfer. Crosspeaks originating from the same spin system are shown in matching colours specific to each monosaccharide with the $\alpha/\beta$ reducing-end glucose shown in magenta and red, respectively; the nonreducing-end $\beta$-glucose shown in light blue; nonreducing $\alpha$-mannose in green and $\beta$-$\Delta 4,5$-ene-glucuronic acid in orange. The remaining positive peaks are shown in dark blue and negative peaks are shown in grey. The crosspeaks that couple with the H2 diagonal peak of mannose are also shown in green due to the poor magnetization transfer from the anomeric proton. Reprinted (adapted) with permission from ACS Catal. 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
Figure 2.16 – $^1$H 2D-TOCSY experiment overlaid with a $^1$H 2D-COSY experiment of deacetylated lyase-treated xanthan tetrasaccharides generated from lyase-treated xanthan degradation by PspXan9. The $^1$H 2D-TOCSY spectrum (see Figure 2.15 for more details) is overlaid with data from $^1$H 2D-COSY allowing visualization of the crosspeaks that are $^3J$-coupled to neighboring vicinal protons. Peaks from $^1$H 2D-TOCSY are presented in dark blue, whereas $^1$H 2D-COSY peaks are shown in red. Reprinted (adapted) with permission from ACS Catal. 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.

$^1$H$^{13}$C HSQC experiments allowed for the majority of $^{13}$C assignments to be completed by correlating the assigned protons to their neighbouring carbons ($^1J_{C,H}$; Figure 2.17, Table 2.2). However, $^1$H$^{13}$C HMBC, $^2^3J_{C,H}$-coupling experiments were required to assign the C5 and C6 of
the Δ4,5-ene-GlcA residue which have no $^1J_{C-H}$ correlation (Figure 2.18) as well as to discern the carbohydrate sequence of the tetrasaccharide (Figure 2.17 and Figure 2.19. The assignment of Δ4,5-ene-GlcAC$_5$ was made possible through correlations with either the H3 ($^3J_{C5,H3}$) and the vinyl proton ($^2J_{C5,H4}$), whereas Δ4,5-ene-GlcAC$_6$ could only be assigned with $^3J$-coupling with the H4-vinyl proton. $^3J$-couplings were also necessary to sequence the tetrasaccharide as well as identify the sidechain position on the tetrasaccharide. Evidence including the diagnostic crosspeaks $^3J_{\text{Man-C1,}\alpha/\beta-\text{Glc(r.)-H3}}, \quad ^3J_{\alpha/\beta-\text{Glc(r.)-C3,Man-H1}},$ as well as the lack of intra-ring correlations from the C3/H3 of Glc(n.r.) suggest that the sidechain on the tetrasaccharide is on the reducing-end glucose (Figure 2.17 and highlighted in Figure 2.19).
Figure 2.17 – Complete assignment of proton and carbon nuclei from deacetylated lyase-treated xanthan tetrasaccharides generated from lyase-treated xanthan degradation by PspXan9 using $^1$H,$^{13}$C HSQC and $^1$H,$^{13}$C HMBC experiments. Each red crosspeak corresponds to a $^1$J_{C,H}-coupling interaction from the HSQC experiment. $^2$J_{C,H}- or $^3$J_{C,H}-couplings were generated with HMBC experiments optimized for couplings at 5 Hz or 10 Hz, and are represented by blue and purple crosspeaks, respectively. The dotted arrows aid in tracing the path between the red HSQC crosspeaks of both of the C3-H3 peaks from the $\alpha/\beta$-glucose reducing-end (Glc(r.)) glucose through blue/purple HMBC crosspeaks to the red HSQC crosspeak of the C1-H1 of mannose. The notation states the sugar residue and its corresponding atom(s) below it. The sugar residue containing the carbon atom is always listed first, followed by the residue containing the proton atom. However, if both carbon and proton atoms exist within the same residue, the residue is only listed once. Abbreviations used: r., reducing-end; n.r., nonreducing-end; Glc, glucose; Man, mannose; Δ4,5-GlcA; Δ4,5-ene-glucuronic acid. Reprinted (adapted) with permission from ACS Catal. 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
Figure 2.18 – Assignment of Δ4,5-ene-GlcA and a schematic illustrating the correlations for determination of C5 and C6 nuclei from deacetylated lyase-treated xanthan tetrasaccharides generated from lyase-treated xanthan degradation by PspXan9 using $^1$H,$^13$C HSQC and $^1$H,$^13$C HMBC experiments. Each red crosspeak corresponds to a $^1$$J_{C,H}$-coupling interaction from the HSQC experiment. $^2$$J_{C,H}$- or $^3$$J_{C,H}$-couplings were generated with HMBC experiments optimized for couplings at 5 Hz or 10 Hz, and are represented by blue and purple crosspeaks, respectively. The notation states the sugar residue and its corresponding atom(s) below it. The sugar residue containing the carbon atom is always listed first, followed by the residue containing the proton atom. However, if both carbon and proton atoms exist within the same residue, the residue is only listed once. Abbreviations used: r., reducing-end; n.r., nonreducing-end; Glc, glucose; Man, mannose; Δ4,5-GlcA; Δ4,5-ene-glucuronic acid. Reprinted (adapted) with permission from ACS Catal. 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
Figure 2.19 – Summary of $^1$H$^{13}$C spectra of the diagnostic region. (A) The diagnostic region of the $^1$H,$^{13}$C HSQC, and HMBC 2D-NMR spectra for the identification of the PspXan9 products (see Figure 2.17 for full 2D-heteronuclear NMR spectrum). Each red crosspeak corresponds to a $^1$J$_{C,H}$-coupling interaction from the HSQC experiment. $^2$J$_{C,H}$- or $^3$J$_{C,H}$-couplings were generated with HMBC experiments optimized for couplings at 5 or 10 Hz and are represented by blue and purple crosspeaks, respectively. The dotted arrows aid in tracing the path between the red HSQC crosspeaks of both of the C3–H3 peaks from the $\alpha/\beta$-glucose reducing-end (Glc(r.)) glucose through blue/purple HMBC crosspeaks to the red HSQC crosspeak of the C1–H1 of mannose. (B) Structure of the unsaturated xanthan tetrasaccharide and key 2D-heteronuclear interactions illustrating those as seen in (E). Note that for panels (A) and (B), roman numerals (i–iv) show important correlations and their position within the unsaturated xanthan tetrasaccharide. Abbreviations used: r., reducing-end; n.r., nonreducing-end; Glc, glucose; Man, mannose; $\Delta 4,5$-GlcA; $\Delta 4,5$-ene- GlcA. Reprinted (adapted) with permission from ACS Catal. 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.
**Table 2.2 – $^1$H and $^{13}$C assignments of the lyase-treated xanthan tetrasaccharide.**

<table>
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<th>Monosaccharide</th>
<th>$\delta$ (ppm)</th>
<th>Multiplicity</th>
<th>Coupling constant (J) (Hz)</th>
<th>Monosaccharide</th>
<th>$\delta$ (ppm)</th>
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<td>$Glc(r)$ -</td>
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<td>m</td>
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<td>$\beta C2$</td>
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<tr>
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<td>n.a.</td>
<td>$C6$</td>
<td>n.a.</td>
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<td>$C6$</td>
<td>168.65</td>
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</table>

n.a. (not assigned) indicates that an unambiguous assignment was not possible due to overlapping peaks.

2.4 Conclusions

To improve the use of xanthan in diverse applications, controlled modification of its physical/chemical properties is presently desired. Therefore, discovery and characterization of specific xanthanases is a topic of current interest.

*Psp*<sup>Xan</sup>9 is one of these xanthanases, which we have demonstrated to have higher specificity for xanthan following a pretreatment with a PL8 xanthan lyase than it has for any other polysaccharide, including untreated xanthan which *Psp*<sup>Xan</sup>9 had 635-times lower activity on. This specificity is unique among GH9 members which is an enzyme family more often associated with the hydrolysis of cellulose and its derivatives. The absence of calcium ions was determined to have a detrimental effect on both the activity and thermostability of *Psp*<sup>Xan</sup>9.

A combination of HPSEC-UV and LC-MS showed that the enzyme behaves in a processive manner by releasing only products with backbones consisting of either four (octasaccharides) or two (tetrasaccharides) glucose residues, but eventually will hydrolyze all products to the latter. Data from MS/MS and NMR both support that *Psp*<sup>Xan</sup>9 cleaves lyase-treated xanthan at the branched glucose residue. Although not as thoroughly characterized, ESI-MS analysis by Nankai *et al* suggested that tetrasaccharide structures with the same sidechain position were formed during the depolymerization of xanthan by *Bacillus* sp. GL1<sup>32</sup>. This site of hydrolysis is unique as prior MS/MS<sup>60</sup> and NMR<sup>59</sup> experiments have shown that the hydrolysis of untreated xanthan by fungal cellulases occurs at the unbranched glucose.

The methods developed and data collected in this chapter will allow comparisons to be made with results collected following future site-directed mutagenesis conducted on desired amino residues. Therefore, future studies on *Psp*<sup>Xan</sup>9 will attempt to modulate its mode of action from processive to dissociative. This should allow the enzyme to be more useful in applications where
rapid viscosity reduction is required. To do this, the crystal structure of \textit{PspXan9} will be used as a guide to predict subsite residues, which can play important roles in mode of action.
Chapter 3: Structural Determinants Conferring the Processivity of PspXan9 Endo-Xanthanase

3.1 Introduction

The limitations of chemical reactions coupled with the complex structure of xanthan make modification exceedingly difficult. However, the exploitation of nature’s powerful arsenal of industrially relevant carbohydrate-active enzymes, is largely considered to be the future of efficient alterations to xanthan. One such example is an extracellular glycoside hydrolase family 9 (3.2.1.-) \( \beta-(1 \rightarrow 4) \)-xanthanase produced by *Paenibacillus nanensis* known as *PspXan9*. It was discovered during a survey for xanthan degrading enzymes that could be applied in laundry detergents. The enzyme exhibits a unique hexamodular structure consisting of an N-terminal immunoglobulin (Ig)-like domain, a GH9 catalytic domain, three all \( \beta \)-sheet domains (\( \beta_1 \), \( \beta_2 \) and \( \beta_3 \)) and a C-terminal CBM84 domain.

*PspXan9* acts by hydrolytically cleaving the xanthan \( \beta-(1 \rightarrow 4) \) bond at the sidechain-substituted glucose releasing tetrasaccharides as the ultimate limit digest products (Figure 3.1). HPLC data collected during partial digests showed that *PspXan9* releases only products with backbones consisting of only two (tetrasaccharide) or four (octasaccharide) glucose units. Once all of the polysaccharide is degraded, *PspXan9* then cleaves all the remaining octasaccharides into tetrasaccharides. Its large active site cleft in combination with the lack of intermediary products suggests that the enzyme operates in an endo-processive manner. This mode of action allows for multiple hydrolytic events to occur while the polysaccharide processes through the enzyme. This differs from endo-dissociative GHs which dissociate after every hydrolytic event. In industrial applications, one disadvantage of GHs with the endo-processive mode of action, when compared
to endo-dissociative GHs, is the gradual decrease in viscosity (Figure 1.5). While this may be beneficial during some applications, others may require a more rapid rheological adjustment.

**Figure 3.1 – The structure of xanthan and products following treatment with PL8 xanthan lyase and PspXan9.** Xanthan lyase cleaves the side chain GlcA-β(1 → 4)-pyruvylated mannose glycosidic linkage from native polypentameric xanthan (A), resulting in the formation of a Δ4,5-ene-GlcA residue and release of pyruvylated mannose. The resulting polytetrameric xanthan (B) is the preferred substrate for the GH9 endo-xanthanase, which yields tetrasaccharides (C) as limit-digest products. Acetylation is variable. Reprinted (adapted) with permission from *ACS Catal.* 2018, 8, 7, 6021-6034. https://doi.org/10.1021/acscatal.8b00666. Copyright 2018 American Chemical Society.

In this study, we aim to design PspXan9 variants that act in an endo-dissociative manner. To do this, variant selection was guided by structural alignments with previously subsite-annotated endo-processive enzymes from the glycoside hydrolase family 9. After determining the activity of each variant, the reduction of relative viscosity was determined for each enzyme. Moreover, the degradation of lyase-treated xanthan was monitored for each mutant in order to measure the degree of processivity of each. This research aims to provide a basis for the structure-functional relationship that confers the endo-processive nature of PspXan9 as well as expand the industrial applications of the enzyme by enabling rapid alterations to viscosity.
3.2 Materials and Methods

3.2.1 Identification of Key Residues Providing \textit{PspXan9} with Processivity

\textit{PspXan9} subsite residues were predicted based on comparison with the archetypal \textit{endo}-
processive GH9, Cel9 (formerly E4) from \textit{Thermobifida fusca} (previously \textit{Thermomonospora fusca})\textsuperscript{62,63}. Superimposition of the x-ray crystal structures of the two GH9 enzymes, \textit{PspXan9} (PDB: 6FHJ)\textsuperscript{31} and the ligand-bound structure of \textit{TfCel9} (PDB: 4TF4), was accomplished using the \textit{cealign} function from PyMOL (Schrödinger, Inc)\textsuperscript{64}. Potential \textit{PspXan9} subsite residues were identified by finding complementary \textit{TfCel9} residues that were previously annotated as subsites. In the absence of comparable residues, bulky amino acids known for their C-H/π stacking interactions with carbohydrates (tryptophan, tyrosine and phenylalanine)\textsuperscript{65} were selected based on their proximity to the cellulose-derivate ligand in the 4TF4 crystal structure. The annotation of \textit{PspXan9} subsites followed the established CAZy nomenclature\textsuperscript{66} and followed the same orientation as \textit{TfCel9}\textsuperscript{62}.

3.2.2 Cloning, Site Directed Mutagenesis, Expression and Purification

The cloning, mutagenesis, and subsequent expression and purification of 17 selected \textit{PspXan9} variants (listed in Table 3.1 in Section 3.3.2) were performed and supplied for this study by Novozyme A/S in a similar fashion in Moroz, \textit{et al}\textsuperscript{31} and described in brief in Section 2.2.1. The purity of these proteins assessed by SDS-PAGE. For each variant, 2 μg of protein was loaded into each well.
3.2.3 Carbohydrate Sources

Lyase-treated xanthan was prepared as previously described by Moroz, et al.\(^3\) and in Section 2.2.2. In brief, 0.5 U mL\(^{-1}\) xanthan lyase from Bacillus sp. was incubated with 2.5 g L\(^{-1}\) of xanthan for several days. The reaction was determined to be completed when there was no increase in UV absorbance observed at 235 nm. The lyase-treated xanthan polysaccharide was precipitated with 2-propanol and purified by discarding the supernatant. After repeated washes with 2-propanol, the precipitate was dried in a vacuum desiccator.

3.2.4 Specific Activity of PspXan9 Mutants

The specific activities of PspXan9 wildtype and its variants were measured using the optimized reaction conditions of 2 g L\(^{-1}\) lyase-treated xanthan at 55 °C in 50 mM HEPES, pH 7.0 with 2 mM CaCl\(_2\) using the bicinchoninic acid (BCA) reducing sugar assay, as previously described (Section 2.2.3).\(^3\) One unit of activity (U) is defined as the amount of enzyme needed to release 1 μmol of glucose-equivalent reducing-ends in 1 min.

3.2.5 Carbohydrate Analytics

3.2.5.1 Viscometric Analysis

The degree of processivity was evaluated by monitoring the reduction of flow time of the sample in relation to the degree of hydrolysis using an Ubbelohde viscometer (Size 1B, K = 0.05 c, Q Glass Company, Inc., U.S.A.) submerged in a water bath to hold a static temperature of 30 °C. Initial concentrations of PspXan9 and its variants were adjusted in 50 mM HEPES-NaOH, pH 7.0 and 0.1 g L\(^{-1}\) of BSA so that a final concentration of 0.02 U of each variant was present following a 1:100 dilution upon its addition into the reaction mixture. It is worth noting that the
final concentration of BSA at 0.001 g L⁻¹ did not impact viscosity (data not shown). For each time point, flow time was normalized using the equation \((t_{\text{sample}} - t_{\text{buffer}}) t_{\text{buffer}}^{-1}\) where \(t_{\text{sample}}\) is the flow time at a given time point and \(t_{\text{buffer}}\) is 50 mM HEPES, pH 7.0 and 2 mM CaCl₂. \(t_{\text{buffer}}\) was determined to be 0.34 min.

The viscometric reaction mixture contained 2.5 g L⁻¹ lyase-treated xanthan, 50 mM HEPES-NaOH, pH 7.0 and 2 mM CaCl₂. The mixture was centrifuged for 30 min at 10000 \(\times\) \(g\) to ensure the removal of any insoluble particles present. The soluble fraction was then decanted, and aliquots were collected by withdrawing the solution while agitated by a magnetic stir bar. The aliquots were stored at room temperature to prevent any aggregation from occurring.

Degree of hydrolysis was determined by performing the BCA reaction and comparing the number of reducing-ends at a given time point to the total number of possible reducing-ends in a limit digest. Limit digests were created by incubating the viscometric reaction mixture with 1 \(\mu\)g mL⁻¹ of the Y362A variant over 72 h. Complete digestion was confirmed by diluting the sample to 1 g L⁻¹ and analyzed using the HPSEC-UV method as described in Section 2.2.4.1 (data not shown).

### 3.2.5.2 HPSEC-UV

An ICS-5000 DC HPLC system, operated by Chromeleon software version 7 (ThermoFisher), was used to perform high-performance size-exclusion chromatography coupled with a UV detector (HPSEC-UV) to both qualitatively and quantitatively analyze lyase-treated xanthan partial hydrolysis product formation over time by \(\text{PspXan9}\) and its variants. Due to initially incoherent oligosaccharide quantification data, lyase-treated xanthan was assessed by comparing chromatography timecourse data using final concentrations of xanthan between 0.125
and 1 g L\(^{-1}\) (data not shown). Finally, the HPSEC-UV method was optimized for final lyase-treated xanthan concentrations of 0.25 g L\(^{-1}\).

For each sample, 0.005 U of each \textit{Psp}Xan9 variant was incubated with 0.5 g L\(^{-1}\) of lyase-treated xanthan at 55 °C in 5 mM HEPES-NaOH, pH 7.0 and 2 mM CaCl\(_2\). Time points were taken over a 24 h period by terminating the reaction through the addition of the sample to 95 °C H\(_2\)O in a 1:1 ratio. 10 μL from each sample was then eluted through an Ultrahydrogel 6 x 40 mm Guard Column in series with a Ultrahydrogel Linear 7.8 x 300 mm column at a flowrate of 0.6 mL min\(^{-1}\) using a 0.5 M isocratic gradient of NaCl for 45 min. Elution was monitored at an absorbance of 235 nm. Quantification of oligosaccharides over time was determined chromatographically by using the area under the curve (AUC) of both the polysaccharide (17.0-30.0 min) and oligosaccharide peaks (30.1-35 min) using the following equation:

\[
\text{oligosaccharide production (\%)} = \frac{x}{x + y}
\]

Equation 3.1 operates under the assumption that the total %AUC (polysaccharide (\(y\)) plus oligosaccharide (\(y\)) peaks) account for the total presence of the \(\Delta 4,5\)-ene glucuronic acid conjugated system; thus \(x + y\) must equal 100.

### 3.3 Results and Discussion

#### 3.3.1 Residue Selection for Mutagenesis

The prediction of sugar-binding subsites found in the \textit{Psp}Xan9 (PDB: 6FHJ) active site was guided through structural alignments with of the crystal structure of \textit{Tj}Cel9A (PDB: 4TF4)
which contained cellotetraose (–4 to –1) and cellobiose (+1 to +2) ligands (Figure 3.2). Using this strategy, the –2 (F420), –1 (Y165), +1 (W532) and +2 (Y487) subsites of *PspXan9* were readily assigned. In many cases, large differences of the position or rotation in key residues required assumptions to be made. These differences may have been due to the absence of ligand in the xanthanase structure, or the large differences between the substrates of the two enzymes (lyase-treated xanthan and cellulose). For instance, the –4 (W369) and –3 (Y368A) subsites of *PspXan9* which did not align to either cellotetraose or the corresponding subsites of *TfCel9A*. Assignment of these subsites, were then made by selecting aromatic residues that were found within the approximate position of a predicted subsite (the distance between oxygen atoms in neighbouring glycosidic bonds is ~5.5 Å; data not shown). Lastly, despite the absence of direction from both *TfCel9A* and liganded cellotetraose, the existence of a –5 subsite was predicted and Y362, Y401 and Y641 were targeted for study accordingly. Chemically inert alanine mutants were subsequently designed for all identified subsites and previously suggested catalytic residues to identify the role each subsite residue plays in both activity and processivity.

The *PspXan9* active site is structurally unique because of the presence of the β2-module that hangs directly over the active site (Figure 2.1 & Figure 3.2). The W719 and W680 residues, found on this module seem to provide addition sugar-binding at the +2 and a potentially novel +3 subsite (Figure 3.2). Therefore, alanine mutants of each were devised. We also observed that the β2-module is anchored to the GH9 catalytic module through a < 3.5 Å, D528-K720 interdomain salt-bridge (Figure 3.2B). D528N were K720Q mutants were designed to remove the ionic interactions, while conserving residue size.
Figure 3.2 – Summary of selected residues for mutagenesis. (A) Structural overview of individual subsite residues selected in the *Psp*Xan9 active site. (B) i) Selected catalytic residues (magenta) and interdomain salt-bridge residues (red/blue) spanning the GH9 catalytic module and the β2 domain; ii) a 180 °C rotated view of the salt-bridge. Cellotetraose and cellobiose ligands (black) were aligned from PDB: 4TF4.
3.3.2 Variant Production and Purity Assessment

A total of 15 of the 17 selected mutations were successfully produced recombinantly (Figure 3.2 & Table 3.1). Fortunately, this included at least one variant for each subsite, the catalytic acid mutant, E536A, and one of the putative supporting base variants, D158A. The two variants that were not able to be produced were the putative −5 subsite residue Y641A and the catalytic nucleophile D161A.

The purity of the variants and molecular weight was assessed by SDS-PAGE which indicated that all samples contained a band consistent with calculated molecular weight of ~116 kDa (Table 3.1; Figure 3.3)\textsuperscript{31}. In majority of the samples there was also the presence of a single contaminating band that appears to have a molecular weight of just below ~63 kDa. Since these protein samples were purified utilizing an N-terminal 6x-HisTag, there is a strong possibility is that this contaminating band also contains the tag. The theoretical mass of the N-terminal Ig-like domain and GH9 domain was calculated to be 61135 Da which seems to fit the contaminating band (Figure 3.4). Since this band could be the result of pre-immobilized metal affinity chromatography (IMAC) proteolysis, confirmation of this would be achievable through the presence of this peak following further attempts of IMAC purification. If the band persists, in resultant SDS-PAGE gels, then the presence of the 6x-HisTag can be presumed.
Table 3.1 – Summary of *PspXan9* variant production.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Site</th>
<th>Production</th>
<th>Calculated Molecular Weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K720Q</td>
<td>+ salt-bridge</td>
<td>✓</td>
<td>115968</td>
</tr>
<tr>
<td>D528N</td>
<td>– salt-bridge</td>
<td>✓</td>
<td>115967</td>
</tr>
<tr>
<td>Y362A</td>
<td>–5 subsite</td>
<td>✓</td>
<td>115876</td>
</tr>
<tr>
<td>Y401A</td>
<td>–5 subsite</td>
<td>✓</td>
<td>115876</td>
</tr>
<tr>
<td>Y641A</td>
<td>–5 subsite</td>
<td>✗</td>
<td>115876</td>
</tr>
<tr>
<td>W369A</td>
<td>–4 subsite</td>
<td>✓</td>
<td>115853</td>
</tr>
<tr>
<td>Y368A</td>
<td>–3 subsite</td>
<td>✓</td>
<td>115876</td>
</tr>
<tr>
<td>F420A</td>
<td>–2 subsite</td>
<td>✓</td>
<td>115892</td>
</tr>
<tr>
<td>Y165A</td>
<td>–1 subsite</td>
<td>✓</td>
<td>115876</td>
</tr>
<tr>
<td>E536A</td>
<td>Cat. acid</td>
<td>✓</td>
<td>115910</td>
</tr>
<tr>
<td>D161A</td>
<td>Nuc./Base</td>
<td>✗</td>
<td>115924</td>
</tr>
<tr>
<td>D158A</td>
<td>Nuc. Helper</td>
<td>✓</td>
<td>115924</td>
</tr>
<tr>
<td>W532A</td>
<td>+1 subsite</td>
<td>✓</td>
<td>115853</td>
</tr>
<tr>
<td>Y487A</td>
<td>+2 subsite</td>
<td>✓</td>
<td>115876</td>
</tr>
<tr>
<td>W719A</td>
<td>+2 subsite</td>
<td>✓</td>
<td>115853</td>
</tr>
<tr>
<td>F231A</td>
<td>+2 subsite</td>
<td>✓</td>
<td>115892</td>
</tr>
<tr>
<td>W680A</td>
<td>+3 subsite</td>
<td>✓</td>
<td>115853</td>
</tr>
</tbody>
</table>

Figure 3.3 – Molecular mass approximation and assessment of purity of *PspXan9* variants. Molecular weight from the ladder (*M*<sub>w</sub>) is compared with the bands from each variant sample labelled above its lane. Molecular weight of each band of the *Mw* ladder is indicated to the side of the ladder.
3.3.3 Activity of PspXan9 Variants

Appropriate enzyme concentrations for specific activity measurements were determined by finding the amount of enzyme necessary to produce a spectrophotometric signal equal to that of 100 μM – 125 μM of glucose reducing-ends over the 10 min assay time (Table 3.2). The activity of all variants was lower than wildtype PspXan9 on lyase-treated xanthan (Table 3.3). At least one variant from the –5 and +2 subsites maintained a residual activity above 45%. The residual activities in the catalytic acid knockout mutant was similar to those reported in other studies at 0.1%63, 69. While also demonstrating unsurprisingly feeble activity, the supporting general base mutant, D158A, was not as poor as the +1 subsite mutant W532A. However, the catalytic acid mutant (E536A) predictably had the lowest residual activity with only 0.125 U mg⁻¹.

Alternatively, D528N, which was anticipated to have similar activity to K720Q, instead has 5-fold higher activity (Table 3.3). This suggests there to be other contributing factors leading to this significant difference, such as the nearby +2 subsite, W719. The proximity of subsite mutations to the catalytic residues seems to dictate the extent to which PspXan9 activity was lost. This is especially true when comparing the external –5 subsites variants with residual activity between 58-74% (Table 3.3). However, this trend does not apply to the positive subsites with W680A (putative +3 subsite) having lower activity than all +2 subsites. Considering that GH9 members commonly only have two positive subsites62, 70, the extreme drop in activity in the putative +3 subsite variant suggests that it may be uniquely important for activity on lyase-treated xanthan.
Table 3.2 – The required concentration of each variant to produce a desired spectrophotometric signal using the BCA reducing sugar assay.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Enzyme (μg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>0.05</td>
</tr>
<tr>
<td>Y362A</td>
<td>0.05</td>
</tr>
<tr>
<td>W369A</td>
<td>0.05</td>
</tr>
<tr>
<td>Y401A</td>
<td>0.05</td>
</tr>
<tr>
<td>Y487A</td>
<td>0.05</td>
</tr>
<tr>
<td>D528N</td>
<td>0.05</td>
</tr>
<tr>
<td>F231A</td>
<td>0.1</td>
</tr>
<tr>
<td>Y368A</td>
<td>0.1</td>
</tr>
<tr>
<td>W719A</td>
<td>0.1</td>
</tr>
<tr>
<td>K720Q</td>
<td>0.1</td>
</tr>
<tr>
<td>D158A</td>
<td>0.5</td>
</tr>
<tr>
<td>F420A</td>
<td>0.75</td>
</tr>
<tr>
<td>W680A</td>
<td>1</td>
</tr>
<tr>
<td>Y165A</td>
<td>1.25</td>
</tr>
<tr>
<td>W532A</td>
<td>1.25</td>
</tr>
<tr>
<td>E536A</td>
<td>2</td>
</tr>
</tbody>
</table>
### Table 3.3 – Activity comparison between *PspXan9* subsite and catalytic residue variants.

<table>
<thead>
<tr>
<th>Site of Mutation</th>
<th>Specific Activity (U mg(^{-1}))</th>
<th>Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype</td>
<td>236 ± 4</td>
<td>100</td>
</tr>
<tr>
<td>Y362A –5 subsite</td>
<td>175 ± 9</td>
<td>74</td>
</tr>
<tr>
<td>Y401A –5 subsite</td>
<td>137 ± 7</td>
<td>58</td>
</tr>
<tr>
<td>D528N Interdomain salt-bridge</td>
<td>128 ± 8</td>
<td>54</td>
</tr>
<tr>
<td>Y487A +2 subsite</td>
<td>109 ± 6</td>
<td>46</td>
</tr>
<tr>
<td>F231A +2 subsite</td>
<td>57.3 ± 4.4</td>
<td>24</td>
</tr>
<tr>
<td>W369A –3/–4 subsite</td>
<td>43.9 ± 2.3</td>
<td>19</td>
</tr>
<tr>
<td>W719A +2 subsite</td>
<td>43.0 ± 0.8</td>
<td>18</td>
</tr>
<tr>
<td>Y368A –3 subsite</td>
<td>28.1 ± 2.3</td>
<td>12</td>
</tr>
<tr>
<td>K720Q Interdomain salt-bridge</td>
<td>26.5 ± 3.0</td>
<td>11</td>
</tr>
<tr>
<td>Y165A –1 subsite</td>
<td>5.08 ± 0.44</td>
<td>2.2</td>
</tr>
<tr>
<td>W680A +3 subsite</td>
<td>4.07 ± 0.23</td>
<td>1.7</td>
</tr>
<tr>
<td>F420A –2 subsite</td>
<td>3.73 ± 0.18</td>
<td>1.6</td>
</tr>
<tr>
<td>D158A Supporting General Base</td>
<td>2.36 ± 0.32</td>
<td>1.0</td>
</tr>
<tr>
<td>W532A +1 subsite</td>
<td>0.614 ± 0.532</td>
<td>0.3</td>
</tr>
<tr>
<td>E536A General Acid</td>
<td>0.125 ± 0.080</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*One enzyme unit (U) is defined as the amount of enzyme required to release 1 μmol of glucose reducing-end equivalents per minute.

### 3.3.4 Altering the Mode of Action of *PspXan9*

Two methods were used to determine the mode of action of each of wildtype *PspXan9* and its variants. The first performed was a viscometric assay where an *endo*-xanthanase is determined to be processive if more hydrolysis is required to reduce the specific flow time when compared to a more dissociative variant. The second method utilizes HPSEC-UV to compare the rate at which of low molecular weight products (octa-/tetra-saccharides) with rapid rates indicating the higher degrees of processivity.
3.3.4.1 Viscometric Analysis of Reaction Progress

Viscometric assays tracking the degradation of lyase-treated xanthan by PspXan9 and its variants with higher than 10% residual activity were performed to assess the effect of each mutation on the degree of processivity (Figure 3.4). This 10% residual activity threshold means that Y165A (−1), W680A (+3), F420A (−2), W532A (+1) and the catalytic residues were not included in this viscometric analysis. Before any enzyme degradation took place, the average specific flow time \([t_n-t_0]/t_0\) of lyase-treated xanthan alone was determined to be 108; however, following PspXan9 activity, the specific flow time reached as low as 9 (Figure 3.4A). While D528N was able to achieve this after ~17% of hydrolysis, Y368A was remarkably able to achieve a specific flow time of 14 after just 2% hydrolysis prior to its premature termination. This rapid drop in specific flowtime after a relatively low amount of hydrolysis indicates that Y368A is the most dissociative of all PspXan9 variants. Conversely, the gradual drops in the specific flow time of lyase-treated xanthan observed by D528N, Y362A, Y487A and the wildtype enzyme suggest that these are all is the most processive variants.
Figure 3.4 – Viscometric analysis monitoring the breakdown of lyase-treated xanthan by *PspXan9* and its variants. Normalized flow time of lyase-treated xanthan in 50 mM HEPES-NaOH, pH 7.0 with 2 mM CaCl₂ was compared to the degree of hydrolysis experienced over time following the addition of 0.02 U of enzyme. Each variant is listed in the legend by their degree of processivity based on these results. A) Full data set conducted over 24 h of incubation. B) Magnified region over the first 4% of hydrolysis. The horizontal magenta line indicates an arbitrary reference point with a specific flow time of 40. Vertical lines drawn from each variant label are used to aid in data visualization.
Apart from these two extremes, it was necessary focus at a smaller scale to gain a more precise comparison of the other variants. Three groups of variants became apparent when observing the degree of hydrolysis necessary for each enzyme to drop the specific flow time of lyase-treated xanthan to 40 (Figure 3.4B). These groups included is the most processive group (D528N > Y362A > Wildtype > Y487A), an intermediate group (W369A and Y401A), and lastly the most dissociative group (Y368A > F231A > W719A > K720Q). According to this, the −3 subsite, followed by the +2 subsite are highly important in maintaining the processivity in wildtype PspXan9. The lack of change in processivity of Y487A (+2 subsite) may indicate either a limited contribution to binding, or an incorrect assignment as a subsite residue. This would instead imply that F231A and W719A are providing all of the binding at this position.

Interestingly, the interdomain salt-bridge variants K720Q and D528N were indicated as having completely opposite modes of action. In the case of the D528N mutation, processivity may have been maintained as a result of its ability to form a hydrogen bond with K720, if the modules are able to stay within a suitable distance. Despite, the K720Q amino acid variant also still having the potential to form a hydrogen bond with the D528 residue, it is possible that the K720Q variant loses processivity as a result of destabilizing the neighbouring +2 subsite residue, W719. Evidence of this is the similarity between K720Q and W719A mutants when observing the degree of hydrolysis necessary to reduce lyase-treated xanthan to a specific flow time of 40 (Figure 3.4).

Several studies have monitored the reduction of viscosity of xanthan reducing as a result of enzymatic hydrolysis from promiscuous cellulases on xanthan without lyase treatment\textsuperscript{71, 72}. However, to our knowledge, this is the first study that not only attempted to compare hydrolysis of enzyme variants, but also the first time that attempts have been made to compare xanthanase variants in hopes of ascertaining changes to the enzyme mode of action. In theory, it is expected
that if dissociative variants are assayed, there will be a rapid reduction in viscosity compared to processive variants (Figure 1.5). This should be readily apparent as it was in previous studies that analyzed the changed in viscosity reduction on other polysaccharides including CMC\textsuperscript{73}, chitosan\textsuperscript{74} and xyloglucan\textsuperscript{75, 76}. While the aforementioned reports on CMC and chitosan compared the degree of processivity between different enzymes, the two studies on xyloglucan examined changes in viscosity reduction between GH74 subsite variants in a similar fashion to this thesis.

Arnal \textit{et al}, reported showed that the processive wildtype enzyme required 8\% hydrolysis to reach its shortest flow time recorded, whereas the most dissociative variants were able to reach this same flow time much sooner after only 1\% hydrolysis\textsuperscript{75}. Matsuzawa \textit{et al}, similarly found that the most dissociative variants tested were able to reach the lowest specific flow time measurement of the processive wildtype, with 3-times less hydrolytic events\textsuperscript{76}. Interestingly, unlike Matsuzawa \textit{et al}, mutations made by Arnal \textit{et al}, displayed intermediate degrees of processivity, which took twice the number of hydrolytic events as the most dissociative variants, but still 4-times less than the processive wildtype GH74\textsuperscript{75}.

If the degree of processivity was decreased, we expected that the \textit{PspXan9} subsite variants would show a significant change (as seen in Figure 1.5) in the amount of hydrolysis required for viscosity reduction. Instead, the most processive mutants (wildtype, Y487A, Y362A D528N) only required 2\% more hydrolysis than most dissociative \textit{PspXan9} variants (Y362A, F231A, W719A and K720Q) to arrive at a specific flow time of 40 (Figure 3.4B). This change in degree of processivity seems more akin to the differences between the most dissociative GH74 variants and the variants with an intermediate degree of processivity as observed by Arnal \textit{et al}\textsuperscript{75}. This begets the question whether these mutations are not alone capable of diminishing processivity or whether
the viscometric assay itself is able to distinguish the degree of processivity of \textit{endo}-xanthanases. Therefore, HPSEC-UV data was collected to provide further support of these findings.

### 3.3.4.2 Tracking \textit{PspXan9} Reaction Products Over Time Using HPSEC-UV

The depolymerization of lyase-treated xanthan by \textit{PspXan9} and its variants was compared both qualitatively and quantitatively using HPSEC-UV (Figure 3.5, Figure 3.6, Figure 3.7, Figure 3.8, and Figure 3.9). Wildtype \textit{PspXan9} degrades lyase-treated xanthan substrate leading to the formation of two relatively resolved peaks eluting at 32.4 min and 33.2 min (Figure 3.5), that through LC-ESI-MS, have previously been determined to be comprised of octasaccharides (1390 Da, 1348 Da and 1306 Da) and tetrasaccharides (704 Da and 662 Da) with various degrees of acetylation\textsuperscript{31}. Once majority of the substrate was consumed, \textit{PspXan9} begins to cleave the octasaccharide into the final tetrasaccharide products, as shown in Section 2.3.2.

Similar to the chromatogram of wildtype (Figure 3.5), the chromatograms of –5 subsite variants Y362A and Y401A (Figure 3.6A-B, respectively) indicated that the tetrasaccharide (\(\beta-D\)-\textit{GlcP}-(1→4)[\(\alpha-D\)-\textit{GlcAP}-\(\beta-(1→2)\)-\textit{ManP}-\(\alpha-(1→3)\)]-\(\beta-D\)-\textit{GlcP}-OH) is the dominant initial product. While other subsite variants, W369A (–4; Figure 3.6C), Y368A (–3; Figure 3.6D) and all +2 subsite mutants (F231A, Y487A and W719; Figure 3.7B-D), initially produce the two oligosaccharide peaks in roughly equal amounts, followed by the eventual decrease of the larger \(M_w\), 32.4 min peak and growth of smaller \(M_w\) peak eluting at 33.2 min. These minor differences between the wildtype chromatographic profile and the +2 subsite mutants suggests that these feebly contribute to substrate binding.
Figure 3.5 – Timecourse analysis of wildtype *PspXan9* using HPSEC-UV. 0.5 g L⁻¹ of lyase-treated xanthan was incubated with 0.005 U of wildtype *PspXan9* at 55°C and sampled at various time points for analysis by HPSEC-UV at 235 nm. Coloured boxes are indicative of the time used for quantifying both polysaccharide (red) and oligosaccharides (blue). Experiment is similar to the timecourse digest in Section 2.3.2.1, only with lower enzyme concentration to match variants.
Figure 3.6 – Timecourse analysis of PspXan9 – subsites variants using HPSEC-UV. 0.5 g L⁻¹ of lyase-treated xanthan was incubated with 0.005 U of each PspXan9 – subsite variant at 55°C and sampled at various time points for analysis by HPSEC-UV at 235 nm. Coloured boxes are indicative of the time used for quantifying both polysaccharide (red) and oligosaccharides (blue).
Conversely, the –2, –1, +1 and +3 subsites all resulted in HPSEC-UV traces that were notably different from that of wildtype PspXan9. For example, the F420A (–2) mutant seems to have lost the ability to generate tetrasaccharide products altogether, but instead shows two dominant peaks formed (31.25 min and 32.4 min) after 5 min of hydrolysis and eventual disappearance of the 31.25 min peak after 30 min of incubation (Figure 3.6E). Following this, the 32.4 min octasaccharide peak dominates and there is no evidence of the formation of any tetrasaccharides peak eluting at 33.2 min. The same larger molecular weight peak eluting at 31.25 min can also be observed in HPSEC-UV traces of –1 (Y165A; Figure 3.6F), and to a greater extent in the +1 and +3 subsites (W532A and W680A; Figure 3.7A & E, respectively). Additionally, an oligosaccharide eluting after ~30.0 min is also being produced by the W532A mutant which persists for up to 1 h of hydrolysis. The presence of the larger molecular weight products, while not necessarily equating to a decrease of the degree of processivity, does clearly show the importance of these subsites in facilitating the regulation of consistent processing along the lyase-treated xanthan backbone.

The chromatographic profiles of the salt-bridge variants D528N and K720Q are qualitatively similar to each other with the formation of two product peaks at 32.4 min and 33.2 min (Figure 3.8). These both seem to mimic the HPSEC-UV chromatogram of wildtype PspXan9 with the 33.2 min (tetrasaccharide) peak being the dominant product peak at early time points. However, D528N is more similar to wildtype PspXan9 as both are able to completely hydrolyze the substrate peak after just 120 min of hydrolysis, whereas traces of this peak are still apparent in the K720Q timecourse after 240 min of hydrolysis. This suggests that K720Q could be more dissociative than D528N which not only supports results from the viscometric data (Figure 3.5) but also demonstrates its likeness to the neighbouring W719A (+2; Figure 3.7D) variant.
Figure 3.7 – Timecourse analysis of *PspXan9* + subsites variants using HPSEC-UV. 0.5 g L\(^{-1}\) of lyase-treated xanthan was incubated with 0.005 U of each *PspXan9* + subsite variant at 55°C and sampled at various time points for analysis by HPSEC-UV at 235 nm. Coloured boxes are indicative of the time used for quantifying both polysaccharide (red) and oligosaccharides (blue).
Figure 3.8 – Timecourse analysis of *PspXan9* interdomain salt-bridge variants using HPSEC-UV. 0.5 g L\(^{-1}\) of lyase-treated xanthan was incubated with 0.005 U of each *PspXan9* salt-bridge variant at 55°C and sampled at various time points for analysis by HPSEC-UV at 235 nm. Coloured boxes are indicative of the time used for quantifying both polysaccharide (red) and oligosaccharides (blue).

Quantitative analysis of the HPSEC-UV traces was completed to gain more insights into the degree of processivity exhibited by each variant (Figure 3.9). At each time point measured, comparisons were made between the presence of polysaccharide (16.0-30.0 min) to the presence of oligosaccharide (30.0-35.0 min) in Figures 3.5-3.8 and were divided by the total absorbance at 235 nm in the chromatogram from between 16.0-35.0 min to express the production of oligosaccharides as a percent using Equation 3.1. The slower generation of oligosaccharide products is indicative of a variant with a lower degree of processivity. The –4 subsite mutant, W369A, achieved ~100% oligosaccharide production after just 30-60 min of incubation demonstrating a high degree of processivity, superior to even wildtype *PspXan9* (Figure 3.9).
Majority of the other mutants seem to cluster in a relatively tight group with similar degrees of processivity. Of these, the F420A (−2; Figure 3.6E) and W680A (+3; Figure 3.7E) subsites, which were observed producing larger molecular weight products, also exhibited a higher degree of processivity than wildtype *PspXan9*. In addition to producing larger molecular weight products, W532A (+1; Figure 3.7A), was one of the most dissociative variants. Lastly, the −1 subsite mutant, Y165A, appears to have significantly lower processivity than all other variants achieving only ~60% oligosaccharide production during the time provided (Figure 3.9). However, for this same reason, it opens questions to whether or not its enzyme activity is diminishing over time. Unfortunately, reducing sugar assays were not conducted in parallel to the HPSEC-UV experiments, otherwise linearity over the full 1440 min timecourse would indicate that the enzyme is still completely functional. Upon closer inspection of the Y165A chromatogram (Figure 3.6F), it also seems odd that there is an absence of noteworthy oligosaccharide formation until between the 30 min and 60 min time points. In fact, there is also a lack of significant changes in the polysaccharide window, coloured in red (Figure 3.6F). This is troubling considering the same amount of enzyme activity units were used for all variants. If there is enzyme activity during the first 30-60 min of the assay, then we expect to see changes to the chromatogram at some point. However, since it appears as though Y165A could be behaving in a more *endo*-dissociative manner, it is also possible that the larger molecular weight oligosaccharides are simply co-eluting with the main polysaccharide peak (Figure 3.6F). However, the complete lack of change in substrate peak appearance could suggest that there may be some unexpected interactions occurring that are preventing larger oligosaccharides from being visualized.

These unexpected results may be due to xanthan’s complex anionic polymeric structure adding complexity to its analysis. For example, Matsuda *et al* found that at low enough xanthan
concentrations (≤ 1 g L⁻¹), the xanthan double helix can completely dissociate at temperatures higher than 80 °C due to xanthan itself not providing the solution with a high enough ionic strength to maintain intramolecular interactions. Upon cooling, each molecule of xanthan can self-associate into aggregates if it is in a solution of adequate ionic strength (≥ 0.1 M NaCl). Studies have shown that aggregation causes shifts in the observed molecular weight making it impossible to accurately determine. Considering that to deactivate the enzyme before HPSEC-UV analysis, the samples were heated to 95 °C and then injected into a mobile phase of 0.5 M NaCl, it is very possible that aggregation may be occurring. This may explain the absence of any large oligosaccharide peak formation on until 30-60 min following incubation with Y165A (Figure 3.9F).
Figure 3.9 – Quantification of lyase-treated xanthan oligosaccharide generation by *PspXan9* and its variants over time. The HPSEC-UV data (Figure 3.6, Figure 3.7 & Figure 3.9) from each variant was collected and used to quantify the oligosaccharide production. The percentage was determined at each time point by comparing the amount of oligosaccharide area to the total amount of peak area (see Equation 3.1). Each variant is listed in the legend by its degree of processivity based on these results.
Another possibility is that the 2 mM CaCl₂ added to PspXan9 reactions may be playing a role with complicating the chromatographic analysis. This is because divalent ions, such as calcium, have been shown through NMR experiments to form crosslinkages between the anionic xanthan sidechains. In fact, it has been shown that xanthan will achieve its maximum gel-like character in the presence of CaCl₂ when a ratio of 1:2 Ca²⁺: COO⁻ is achieved. Once this ratio reaches 1:1, and there is a calcium ion for both the pyruvylated mannose and the glucuronic acid, the gel-like characteristics are completely lost. However, effects at lower concentrations of CaCl₂, more similar to those used in this study, have also been shown to maintain (if added before) or return (if added after) xanthan to its native conformational state following denaturation through heating. It is worth noting that the referenced experiments were not performed on lyase-treated xanthan, so knowing exactly how the addition of 2 mM CaCl₂ impacted the conformation of the polysaccharide and whether this is played a role in the unexpected results in HPSEC-UV chromatograms.

3.4 Conclusions

In certain applications, the rapid reduction of viscosity of industrial carbohydrates is sometimes desired. These situations may range from the removal of textiles soiled with xanthan during a laundry cycle or the reduction of xanthan-containing high viscosity fractioning fluid enabling the outflow of petroleum products during drilling. Glycoside hydrolases have to ability to either degrade their substrates gradually, in exo- or endo-processive modes of action, or quickly through endo-dissociative action. The widespread use of the microbial polysaccharide xanthan throughout numerous industries, has led to recent interest in its modifications by enzymes.
PspXan9 has been identified as an endo-processive xanthanase, capable of depolymerizing the lyase-treated xanthan backbone. Here, we attempted to rationally engineer PspXan9 to act in an endo-dissociative mode of action so that it can rapidly reduce the viscosity of xanthan-containing solutions. To do this we used a structurally guided approach to identify key aromatic residues found in the sugar-binding subsites of PspXan9. In total, 15 variants were recombinantly produced including representatives including an interdomain salt-bridge, all negative (−5 to −1) and positive subsites (+1 to +3), and the catalytic acid and a supporting catalytic base. These enzymes were then subjected to viscometric and chromatographic analyses to determine their degree of processivity in relation to wildtype. With this approach, our results suggest that the removal −1 subsite is associated with the loss in both activity and processivity, whereas removal of the −4 subsite enhances processivity, while maintaining moderate activity. However, considerations for xanthan’s unique secondary structure may limit the legitimacy of these conclusions and warrant refining present means of analysis or development of alternative methods. For instance, the results from the viscometric assay indicated that the Y368A (−3 subsite) variant is strongly dissociative, reducing the viscosity more rapidly than the other variants. However, the HPSEC-UV results imply the Y368A (−3 subsite) variant may actually be intermediately dissociative. This ambiguity between the experiments suggests that factors within the experimental design are not accounting for the complexity of the xanthan and its interactions with assay components such as salt concentrations and temperature. Mutations to the −2, −1, +1 and +3 subsites allow the generation of larger unidentified molecular weight oligosaccharides. Therefore, further attempts to isolate and characterize these should be made to better understand the effect of the subsite as well as to serve as future ligands in crystal structures.
Chapter 4: Conclusions and Future Outlook

The growing need for highly pure and stable hydrocolloids with unique properties has led to increased interest into microbial polysaccharides that can be not only used in new products but also be used as replacements for their plant gum predecessors\(^1\). However, due to expensive production costs and low yield for many, xanthan serves as the main example of this notion being a reality\(^3\). Indeed, due to its diverse uses spanning multiple industries, in 2011 xanthan accounted for 6% of global hydrocolloid market. Because of its widespread use, there have been considerable efforts given to finding xanthan depolymerizing enzymes. These enzymes have the potential of being used in industrial processes \textit{in situ}\(^4\), or to generate xanthan oligosaccharides for specific applications\(^4\). Therefore, the work in this thesis sought to characterize the novel xanthan depolymerizing enzyme \textit{PspXan9}.

4.1 Summary of Findings

Chapter 2 contains a biochemical investigation into \textit{PspXan9} as well as a thorough analysis of its products. Utilizing reducing sugar assays to quantify enzyme kinetics and determine substrate specificity, we found that \textit{PspXan9} is highly specific to lyase-treated xanthan over all other substrates. This is also true for native xanthan which is hydrolyzed significantly slower indicating that a prior xanthan lyase treatment is necessary. A combination of HPSEC, LC-MS, MS/MS and 1D/2D NMR spectroscopy were used to determine not only the mode of action of \textit{PspXan9}, but also its products and site of cleavage. \textit{PspXan9} cleaves the backbone of the lyase-treated xanthan at the branched glucose releasing either octasaccharide or tetrasaccharide products. The absence of all other intermediately sized products indicated that \textit{PspXan9} operates in an \textit{endo}-processive mode of action.
In Chapter 3, we investigated the structure-function relationship that enables PspXan9 to act in an endo-processive manner and aimed to abrogate its ability to process along the substrate. This was done by rationally engineering PspXan9 using structurally guided techniques. Based on prior structural knowledge of processive GH9 cellulases and our recently solved PspXan9 structure, we focused on selecting aromatic residues for site directed mutagenesis based on the assumption that these often serve as sugar-binding subsites of carbohydrates. Using viscometric and chromatographic analyses, we probed the effects of mutations of the PspXan9 subsites. Our data suggests that the –1 subsite is crucial for the processive nature of PspXan9. However, when considering the data from variants analyzed by both viscometric and chromatographic means, there are inconsistencies. This may be the result of secondary structure lyase-treated xanthan behaving differently in certain conditions in one assay and not the other.

4.2 Future Directions

To further understand the structure-function relationship between GH9 endo-xanthanases and lyase-treated xanthan, more research needs to be performed. This includes ligand bound structural studies utilizing either the octasaccharide limit digest products from the –2 subsite (F420A) variant or even purification of the larger molecular weight products using partial digests of –3 (Y368A), –1 (Y165A), +1 (W532A) or +3 (W680A) subsites using similar techniques as described in Section 2.2.4.

Additional efforts should be taken to discover and characterize new GH9 xanthanases with the hope of finding enzymes that are stable in the absence of calcium and can act in an endo-dissociative manner. Initial efforts to find novel xanthanases were made by scouring the CAZy database looking for GH9 members in the bacterial genomes that contained PL8 enzymes. This
method identified GH9 members from *Microbacterium testaceum* StLB037 (BAJ75824.1; Section 2.3.1), *Paenibacillus borealis* DSM 13188 (AIQ58732.1) and *Paenibacillus* sp. FSL P4-0081 (AIQ30003.1) as potential xanthanases. To provide further support, subsequent efforts were made utilizing sequence similarity networks (SSN) with the assistance of the bioinformatician Dr. Alex Viborg, who was a Post-Doctoral Fellow in the Brumer Group.

All GH9 sequences deposited in the CAZy database, including the putative GH9 xanthanases, were combined with the identified GH9 xanthanases from patents, along with an GH9 of interest from *Acetivibrio cellulolyticus* (*Ac*GH9; WP_083878892.1). This enzyme stood out as it was the most similar non-Bacilli, non-Actinobacteria GH9 sequence to *Psp*Xan9 with a percent identity of 39%. An SSN was created using all-versus-all pairwise local sequence alignments at a threshold of 1e-60. At this cut off, a single cluster formed containing all aforementioned GH9 members (Figure 4.1A). Ten random representative GH9 sequences were then selected from the other SSN clusters, in addition to with all of the members of the putative xanthanase cluster. These sequences were aligned using MAFFT (multiple alignment using fast Fourier transform). Using this multiple sequence alignment, RAxML (Randomized Axelerated Maximum Likelihood) was performed to compute a phylogenetic tree. Again, all putative GH9 xanthanases forming a monophyletic group along with *Ac*GH9 (Figure 4.1B). This not only suggests that there is a GH9 xanthanase subgroup, but it also provides strong evidence that *Ac*GH9 may be a xanthanase coming from a novel host.
Figure 4.1 – Bioinformatics identification of xanthanases from glycoside hydrolase family 9. (A) A sequence similarity network (SSN) of 1800 full length GH9 protein sequences, filtered with an E value of $10^{-60}$, including: CAZy GH9 sequences, patented xanthanases\textsuperscript{47}, and AcGH9. (B) Phylogenetic tree constructed from the 10 randomly selected sequences from G1-4 and G6-11 in addition to the entire G5 cluster. Description of side indicates the known mode of action or activity, if possible.

Several attempts were made to recombinantly produce this enzyme in an \textit{E. coli} host, including the use of maltose-binding protein and glutathione S-transferases solubility tags. However, these failed to yield soluble protein. Future attempts at AcGH9 production are strongly encouraged as it will expand the diversity of the GH9 family.

Together, this research aims to serve as a guideline for modifying the mode of action of not only \textit{endo}-xanthanases but also glycoside hydrolases from family 9. Additionally, the variants
found in this study could offer future options to industry for the rapid depolymerization and reduction in viscosity of lyase-treated xanthan.
References


