Towards hydrophobin-mediated surface modification of lignocellulosic fractions: characterization of Class I hydrophobin interactions with cellulose and lignin and subsequent impact on *T. reesei* cellulase CBHI binding and activity

By

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A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
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Abstract

Class I hydrophobins have the ability to form amyloid-like rodlets upon exposure to an air-water interface, but the role of assembly in surface modification is unclear. Their binding to lignocellulose, a potential feedstock for chemicals, materials and energy, was tested, using aeration to induce hydrophobin assembly. Five class I hydrophobins were tested, both with and without aeration, for their ability to bind to cellulose and lignin, and significant differences in film mass and viscoelasticity could be observed. Films were further characterised by testing the binding of the Trichoderma reesei enzyme cellobiohydrolase I (CBHI) to lignin and cellulose after hydrophobin binding. Again, aeration was observed to alter the interactions hydrophobins had with both the surface and enzyme. This study displays the importance of hydrophobin assembly on observed properties of the protein, and highlights the possibility of tuning hydrophobin interactions with surfaces and proteins by aeration.
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List of Abbreviations Used

QCM-D   Quartz-Crystal Microbalance with Dissipation monitoring
CBHI    Cellobiohydrolase I
DNS     3,5-dinitrosalicylic acid
ThT     Thioflavin T
TFA     Trifluoroacetic acid
PCA     Principal Component Analysis
HOPG    Highly Oriented Pyrolytic Graphite
SWCN    Single-Wall Carbon Nanotube
BSA     Bovine Serum Albumin
CAZyme  Carbohydrate Active Enzyme
Cel7A   Endo-β-1,4-glucanase I
AFM     Atomic Force Microscopy
ESEM    Environmental Scanning Electron Microscopy
DMAc    Dimethylacetamide
TMSC    Trimethylsilyl Cellulose
SDS     Sodium Dodecyl Sulfate
AAA     Amino Acid Analysis
CBM     Carbohydrate Binding Module
WCA     Water Contact Angle
1. Introduction
1.1 Lignocellulosic Biomass as a Feedstock

If one were to imagine a renewable, environmentally-friendly feedstock of complex chemical structure, plant biomass is certainly one of the first images to come to mind. The majority of plant biomass is made of lignocellulose, an intricate network of polymers that makes up the primary and secondary cell walls, and is responsible for protecting the cells from enzymatic attack by pathogens.\textsuperscript{1} The three main components of lignocellulose are cellulose, lignin and hemicellulose, the ratio of which varies widely between different plants. As a major component of any plant, lignocellulose is the most abundant biopolymer on the planet, and bio-renewable. Further, large amounts of lignocellulose are present in the waste streams of the forestry and agricultural industries, as well as in municipal solid waste.\textsuperscript{2} Agriculture and forestry are globally ubiquitous industries, and this means the lignocellulose from their waste streams is theoretically available anywhere in the world, all year round.

The major applications of lignocellulosic biomass thus far focus heavily on the cellulose fraction, while under-utilizing the lignin and hemicellulose. Cellulose is well established as a source of fibers for textiles, paper, and thermal insulation.\textsuperscript{3,4} Recently, there has also been large interest in the degradation of cellulose to glucose, which would then be fermented to produce ethanol for biofuels.\textsuperscript{5} First generation biofuels utilized food crops such as corn, sugar cane and soybeans and, despite rising yields, the entire premise was plagued by the ethical dilemma of diverting water, land and resources from food crops to fuel while hunger and famine are ever-present on the planet. Second generation biofuels, which are made from lignocellulose, would not compete with food crops, as the lignocellulose is abundant in the non-edible waste stream of agricultural and forestry industries.
With 280 million tons of plastic being produced globally in 2013, primarily for packaging, and less than half that amount entering landfills, there is a large push to find an alternative feedstock for plastics from renewable sources.\textsuperscript{6} A bioplastic is defined as plastic that is either based on a renewable source, or a plastic that is itself biodegradable, but more attention has been focused on the use of renewable sources.\textsuperscript{7} Petroleum based plastic is a difficult thing to replace, as it is easy to process, resists water and bacteria, is lightweight and has excellent mechanical properties. There are several bioplastic candidates that are slowly replacing these plastics where possible but they are typically lacking in mechanical strength or shelf life.\textsuperscript{8–10}

### 1.2 Hydrophobins in Enzymatic Cocktails

As lignocellulose separation and modification is improved, studies have focused on organisms that have done this for millennia. In particular, enzymatic conversion of cellulose and hemicellulose components to sugars is preferred when considering subsequent fermentation of the sugars to fuels and chemicals. Such enzymatic processes, however, are often incomplete; certain enzymes may also be prone to non-productive association with lignin-enriched residues.\textsuperscript{11} In an effort to overcome these challenges and identify more stable enzymes, several groups have investigated key enzymes that are secreted by fungi during their growth on lignocellulosic substrates.\textsuperscript{12} While focus was mainly on finding enzymes with ideal substrate selectivity, low product inhibition and stable activities, there is a growing focus on altering the lignocellulose in a controlled, non-destructive manner to decrease recalcitrance.\textsuperscript{13,14} One example is swollenins, proteins that have been shown to increase rates of activity and final yields by disrupting the enzyme-resistant structure of the lignocellulose.\textsuperscript{15}

Hydrophobins are a class of non-catalytic proteins found in filamentous fungi. They are typically 7–20 kDa, and are characterised by their ability to assemble at interfaces.\textsuperscript{16} Due to their
high surface activity, they play a number of roles in fungal development related to interface modification, from waterproofing the fungi itself, to improving adhesion onto a substrate, to lowering the water tension so that the fungi can grow into the air.\textsuperscript{16,17}

Hydrophobins are typically separated into class I and class II. Class I, which is further divided into class IA and class IB, is characterised by the relatively low sequence similarities between members, as well as the ability to form insoluble, functional amyloids when exposed to an interface.\textsuperscript{16,18,19} Class II hydrophobins, conversely, have sequences of high similarity, and instead assemble into less robust films at an interface. Class I hydrophobins will naturally adsorb at an interface, and this adsorption induces a conformational shift into a $\beta$-sheet rich structure that allows for the formation of amyloid fibrils, also known as rodlets.\textsuperscript{20,21} Importantly, the rodlets of some hydrophobins have shown the ability to increase the binding and stability of enzymes to different surfaces, and may play a role in fungal degradation of substrates.\textsuperscript{20,22} The non-assembled form of several hydrophobins do not display the same effects, highlighting the importance of sample preparation, and the testing of hydrophobins in both states to fully understand possible functions.

The recalcitrance of lignocellulosic biomass is a large hurdle hindering the utilisation of an otherwise ideal feedstock. While there has been extensive work in the discovery and optimisation of the enzymes required to release or modify the different fractions of lignocellulose, non-catalytic proteins are an under-explored avenue of mitigating the difficulty of modifying the feedstock. This study aims to characterise the binding of several class I hydrophobins to cellulose and lignin in both their assembled and monomeric states, and to determine the effect of these hydrophobin films on cellulase binding. Understanding these interactions may not only improve enzymatic modification of lignocellulose, but provide insight
into how the assembly state of hydrophobins can be exploited to tailor interactions with specific substrates.

1.3 Research Hypotheses

A. Hydrophobins across the spectrum of Class IB will adsorb differentially to cellulose and lignin

While hydrophobins across the spectrum of class IB have relatively high sequence homology, this does not preclude the possibility of differences in their binding profiles to lignin and cellulose. Each hydrophobin must be tested on each surface to determine which ones bind, and to what extent binding occurs.

B. Adsorption of class IB hydrophobins to cellulose and lignin will be altered by overnight aeration

Aeration is typically used to induce rodlet formation of class I hydrophobins by introduction of an air-water interface into solution. The five hydrophobins will be tested before and after overnight aeration to determine their binding to both cellulose and lignin. It is expected that the assembly induced by overnight aeration will alter the binding of these proteins to the different substrates.

C. Adsorbed class IB hydrophobins will differentially affect subsequent binding and activity of cellulases on cellulose and lignin

Hydrophobins have shown the ability to alter the activity and binding of enzymes at an interface, and this ability will be determined for the five tested hydrophobins in regards to a cellbiohydrolase, and endo-1,4-β-D-glucanase. Just as overnight aeration is expected to affect
binding to the substrate, it is also expected to alter the effect each hydrophobin has on cellulase binding and activity.

1.4 Research Objectives

1. Characterise the binding of four hydrophobins across the spectrum of class IB, and one hydrophobin from the mixed region of class I to both cellulose and lignin.

2. Characterise the binding of the hydrophobins to cellulose and lignin after overnight aeration to induce rodlet assembly.

3. Determine the effect of each hydrophobin coating on the subsequent binding of cellobiohydrolase I to lignin and cellulose.

4. Determine the effect of a hydrophobin coating on cellulose on the activity of cellulases.

Using a Quartz Crystal Microbalance with Dissipation monitoring (QCM-D), it is possible to monitor, in real-time, the binding of different hydrophobins to cellulose and lignin, as well as the subsequent binding and washing of an enzyme of interest.

1.5 Outline of Thesis Document

The research herein is presented in two chapters. In the first chapter, the process of producing, purifying and verifying the purity of the hydrophobins is outlined. Further, the binding of five hydrophobins was tested with and without aeration on lignin and cellulose using a quartz crystal microbalance (QCM-D). The resultant frequency and dissipation data were plotted on ΔD vs ΔF plots, and fit to mathematical models to determine the extent and rate of mass binding to the surface.

The second chapter outlines the testing of cellobiohydrolase I (CBHI) binding to lignin and cellulose in the presence of each of the hydrophobin films formed. Again, the QCM-D is
used to determine the extent of mass binding to the surface, and to compare this to cellulose and lignin surfaces with no hydrophobins present as controls. Further work was then done to study the effect of SC16 to CBHI activity on cellulose through the use of a 3,5-dinitrosalicylic acid (DNS) assay. The chapter outlines the work done to determine the linear range of the assay, the ideal substrate, as well as the concentration of enzymes in solution, and then determines the effect of increasing SC16 concentrations on said activity.
2. Literature Review
2.1 Hydrophobins

In 1984, Wessels et al. discovered a series of homologous genes that were expressed during fruiting body initiation in the filamentous fungi *Schizophyllum commune*. The genes all encoded small proteins with a characteristic eight-cysteine pattern and N-terminal secretion signal. Due to the moderately hydrophobic nature of these proteins, they were given the name “hydrophobins”, and these proteins have since been identified in a number of roles throughout the fungal life cycle.

Hydrophobins are typically between seven and twenty kilodaltons in size, non-catalytic, and found in Ascomycota and Basidiomycota fungi. While sequence similarity is low among hydrophobins, their structures are all centered on four disulphide bridges and a β-barrel core. In between the β-strands that comprise the β-barrel core are several loop regions, and these vary widely between hydrophobins. These loop regions differ in length, hydrophobicity and structure, and as the core of the protein is relatively conserved, it is thought that these regions are responsible for the differences found in the assemblies and adherence to different surfaces.

Their ability to assemble at interfaces is apparently used by the fungi to alter the physiochemical properties of a surface, and many fungi contain genes for multiple hydrophobins that are differentially expressed along the fungal life cycle, and in response to various stimuli. *Neurospora crassa* mutants with the gene for the hydrophobin EAS knocked out were found to lack the rodlet layer on their surface that both repelled water, and aided in the dispersion of spores. Further, hydrophobins are used by fungi when growing out of the water and into the air, as the proteins lower the surface tension of the water when bound to the air-water interface.
Hydrophobins have also been implicated in symbiotic exchanges with plant roots and pathogenicity.\textsuperscript{27,29}

This same surface activity has been linked to “gushing” in beer, the name given to a phenomenon in which beer spontaneously foams and rushes out of a container.\textsuperscript{30,31} Sarlin et al. have found that even 1 μg of a hydrophobin isolated from \textit{Trichoderma reesei} in 330mL beer is enough to induce this effect. It is hypothesized that the hydrophobins coat and stabilize carbon dioxide bubbles, preventing their diffusion into solution, and that these bubbles rapidly expand when the container is opened, leading to foam and, tragically, wasted beer.

As more hydrophobins were discovered, sequence patterns emerged that allowed them to be classified as either class I or class II. Class I hydrophobins have low sequence similarity among them, but they all assemble into highly stable, insoluble films at interfaces.\textsuperscript{16,21,24} In contrast, class II hydrophobins show relatively high sequence similarities, and form films that can be dispersed with detergents or alcohol. Class II hydrophobins typically have a compact β-barrel core and 3 loop regions, with loop 2 containing an alpha helix while loops 1 and 3 are short and disordered (Figure 1).\textsuperscript{21} The loop regions in class I hydrophobins have much more variability, although in general these regions tend to be longer and less ordered. Another noteworthy difference in structure is the presence of an amyloidogenic sequence in some class I hydrophobins, which seem to promote the formation of amyloid fibrils.\textsuperscript{20,21} Amyloid fibrils are characterized by stacking of proteins that are high in β-sheet structures, and it has been shown that there is a conformational shift in the structure of class I hydrophobins, once they reach a critical concentration, into a β-sheet rich structure.\textsuperscript{24}
Figure 1: Schematic Representation of Secondary Structural Elements in Class I Hydrophobins SC16 (Schizophyllum commune), EAS (Neurospora crassa), DewA (Aspergillus nidulans), and MPGI (Magnaporthe oryzae) and Class II Hydrophobins HFBI (Trichoderma reesei), HFBII (Trichoderma reesei), and NC2 (Neurospora crassa). β-Sheets are shown in red, and α-Helices in purple. \( L_1, L_2, \) and \( L_3 \) refer to the loop regions in the protein.

In a 2016 study by Pham et al, the assembly of the class I hydrophobin MPGI, from \( M. \) oryzae, was monitored in real time using thioflavin T (ThT), a dye that fluoresces when bound to the extended β-sheet structure of amyloid fibrils.\(^{20}\) Solutions of MPGI were monitored over time in a 96-well plate in the presence of ThT while being shaken on an orbital platform. It was found that increasing concentrations of MPGI did not alter the rate of assembly, and removal of the air-water interface by adding buffer up to the lid inhibited assembly completely. They found that the kinetics of assembly of MPGI could be modelled by a “dock-lock” process, in which they suggest that monomers must first bind to the interface, before a conformational shift exposes the amyloidogenic sequence of the protein, forming an assembly-competent form of the monomer.

Samples of class I hydrophobins are typically exposed to pure trifluoroacetic acid (TFA) to resolubilize the protein and remove any assemblies in solution; assembly can then be induced at a later time through aeration.\(^{32}\) Whether done through vigorous methods such as vortexing, or gentle methods such as head-over-tail shaking, the key factor seems to be the introduction of an air-water interface, with the rate of assembly being proportional to the interfacial area introduced, as well as the hydrophobin concentration in solution.\(^{20,33}\) It is this interfacial binding that is thought to induce conformational changes in the loop regions of hydrophobins, forming beta
sheets that can stack into amyloid rodlets possibly due to the selective partitioning of the hydrophobic residues to the hydrophobic side of the interface, and vice-versa.\textsuperscript{21,34} In this way, interfaces stabilize conformations of the protein that would otherwise not be seen, and these may be the forms necessary for rodlet assembly.

The presence of an interface is also important in the assembly of other, disease-related, amyloid-forming proteins such as α-synuclein and islet amyloid polypeptide.\textsuperscript{35,36} When exposed to amyloid seeds, these proteins typically have a lag phase before fibril formation as monomers shift into an assembly-competent conformation, but the presence of an interface has been shown to reduce or eliminate this lag period, as well as increasing the total amount of aggregates formed over time. This interfacial effect is thought to be driven by the surface-active nature of many amyloid-forming proteins, as adsorbing to an interface increases the local concentration, promoting conformational shifts.\textsuperscript{35,36} However there are some significant contrasts to hydrophobins, as hydrophobins do not show this initial lag phase in assembly, they cannot assemble in the absence of an interface, and seeding a solution of monomeric hydrophobins with pre-assembled rodlets does not increase the rate of assembly.\textsuperscript{20} Until recently, amyloids were only known for their association with degenerative diseases such as Alzheimer’s, Parkinson’s and Huntington’s.\textsuperscript{19} Hydrophobins are members of a growing list of functional amyloids, in which the formation of β-sheet rich structure is not a pathogenic occurrence, but the functional conformation of the protein. Alternatively, this also raises the question of whether or not the proteins are capable of binding and modifying surfaces without the formation of amyloid fibers.

A study by Gandier et al. went on to propose a subdivision of class I into class IA and IB.\textsuperscript{21} A total of 1046 predicted hydrophobins were analyzed using a principal component analysis of the sequence alignment matrix, and a clear separation of class I hydrophobins from
Ascomycota and Basidiomycota emerged. A principal component analysis (PCA) is a statistical procedure that seeks to reduce the dimensions of a data set while maintaining as much diversity as possible. With each hydrophobin representing a point in a multi-dimensional dataset, the PCA seeks to find the vectors in the set that capture the most variance among the points. Principal component 1 (PC1), as such, is the vector across which the data points spans the furthest, and PC2 would be second, etc. The PCA showed, as expected, that class II hydrophobins clustered away from the rest quite readily, due to the high sequence similarity among members, while class I hydrophobins were much less concentrated (Figure 2). However, it also showed that the Ascomycota and Basidiomycota class I hydrophobins were separated, with a mixed region in between. Class IA consists of the Ascomycota hydrophobins, while class IB was made up of Basidiomycota hydrophobins. All class I hydrophobins with previously determined structures (EAS, DewA and MPGI) were found in the mixed region of the PCA, suggesting they are not representative of other class I hydrophobins, but SC16, from *Trichoderma reesei*, may be representative of class IB hydrophobins.
Applications involving hydrophobins have utilized their ability to assemble into amphipathic films in a variety of fields, from food applications to biosensors, pharmaceuticals and more.\textsuperscript{24} One such application revolves around their ability to stabilize dispersions of solids, liquids or gases in hydrophilic solvents such as water. Highly oriented pyrolytic graphite (HOPG) and carbon nanotubes are two materials whose potential is limited by their tendency to aggregate in polar solvents.\textsuperscript{37} Both class I and class II hydrophobins have been used to stabilize dispersions in a simple, one-step process. Yang et al. used the class I hydrophobins EAS and HYD3 to stabilize single-wall carbon nanotubes (SWCNs), graphene, HOPG and mica in aqueous solutions. They found that the hydrophobins could bind both to the hydrophobic HOPG, graphene and SWCNs, as well as the hydrophobic mica with no chemical treatments or additional steps. Further, suspensions of hydrophobic drugs can be stabilized in water with hydrophobins, increasing the viability of oral or intravenous delivery.\textsuperscript{25} Valo et al. found that
they could form hydrophobin-coated drug nanoparticles by adding the class II hydrophobin HFBII to an aqueous solution before precipitating the hydrophobic drug. A GFP tag was also added to the hydrophillic side of the protein allowing them to visualize the particles under a fluorescence microscope, and showing the possibility of adding tags to the outside of the particle for targeting or other purposes. In another study, the class I hydrophobin SC3 was added to the drugs cyclosporine A and nifedipine to increase their availability for oral administration. Not only did they see an increase in availability, but cyclosporine A stabilized with SC3 also resulted in a slower uptake, leading to a more stable, longer lasting drug effect.

The chemical and physical stability of the amyloid assemblies formed by class I hydrophobins have also led to successes in their application for surface modification. Fokina et al. have used the class I hydrophobins DewA and DewB, from Aspergillus nidulans, to functionalize a surface by fusing them to a laccase protein. Isolation of the protein was not needed, as the cells were sonicated after production, and incubating the polystyrene with the cell lysate overnight was sufficient to coat the surface in the protein. This facile coating method has also been used to confer antimicrobial properties onto the surface of a medical device, such as the fusion of HGFI with the bacteriocidal peptide Pediocin PA-1, and the creation of biliary plastic stents that resisted fouling. Further, it has been shown that a film of the hydrophobin Vmh2 or Pac3 can reduce Staphylococcus epidermidis attachment and biofilm formation on polystyrene as well as other multiple medically relevant materials with no need for added tags or modifications.

The hydrophobin Vmh2, from Pleurotus ostreatus, is noted for the ability of the assembled form of the protein to strongly bind proteins to the surface, including enzymes with retained activity. It was shown that bovine serum albumin (BSA) and laccase enzymes
deposited on the surface of a Vmh2-coated steel sample plate resisted washing over time, and that the laccase enzyme retained activity for at least 50 hours afterwards. Further, a layer of Vmh2 was deposited on a steel sample loading plate for matrix assisted laser desorption/ionization (MALDI) analysis and allowed for the washing and desalting of the protein sample prior to analysis while mitigating sample loss, without the use of high-performance liquid chromatography (HPLC) or other techniques. This same Vmh2 coating was also used to non-covalently immobilize enzymes used in protein characterization, such as trypsin and alkaline phosphatase, to steel sample plates. Samples could be loaded on the plate, incubated in the presence of several immobilized enzymes, and analyzed by MALDI MS/MS in a relatively short time, with very low sample volumes.46

Several class I hydrophobins have also been used in the design of materials for regenerative medicine. HGFI, from Grifola frondosa, was fused to a vascular endothelial growth factor (VEGF) to aid in the regulation of migration and survival of endothelial cells on poly(e-caprolactone).47 DewA has also been fused to either a RGD or LG3 tag to increase the adhesion of human cells on a polystyrene and teflon without increasing the risk of bacterial infection.40,48,49 It was found that both proteins increased the binding of mesenchymal stem cells (MSCs), osteoblasts and fibroblasts to a polystyrene or titanium surface, while showing no effect on the growth of these cells, or the binding of Staphylococcus aureus.

The main bottleneck in hydrophobin applications thus far is the lack of industry-scale production methods.16,19 BASF is thus-far the sole example of production in the gram per litre scale necessary for large-scale applications of the proteins.50 Their recombinant proteins, H*Protein A and H*Protein B, were created by fusing the class I hydrophobin DewA with either the full Bacillus subtilis protein yaaD or a truncated yaaD, respectively, and produced in E. coli
with kilogram yields. Other sources report the use of an overproducing *T. reesei* strain, or the use of *Saccharomyces cerevisiae* to produce class II hydrophobins, with yields of approximately 250-600 mg/mL.\textsuperscript{51,52} Most class I hydrophobins however have not reached the same success, with yields of 10 mg/L being reported.\textsuperscript{53} This may be due to the difficulty the formation of amyloid fibers adds to the purification, or simply due to the fact that class I hydrophobins are less studied in general. Novel methods of increasing these yields include the use of foam fractionation, to take advantage of foam stabilization effects of hydrophobins, and biofilm reactors, which allow for higher concentration of crude product and the possibility of continuous production.\textsuperscript{54}

Despite the multitude of applications exploiting the ability of hydrophobins to create chemically and physically robust films on surfaces, these have primarily focused on the functionalization of inorganic substrates such as polystyrene, SiO\textsubscript{2}, Teflon, glass, titanium, and graphite.\textsuperscript{16,19,24} The main advantage of hydrophobins in surface modification is the fact that they can quickly form ordered films on surfaces at mild pH and temperature without the need for any further chemical modifications. However, hydrophobins have also been shown to interact with carbohydrate-active enzymes (CAZymes), altering activity on lignocellulose. The hydrophobins RolA and MPG1, from *Aspergillus oryzae* and *Magnaporthe oryzae*, respectively, were found to increase the concentration of enzymes on the surface of a substrate, and stabilize their activity over time.\textsuperscript{20,22} MPG1 increased the binding of the cutinase Cut2 to a HOPG surface, increasing its activity over time and resisting repeated washes. RolA similarly undergoes a conformational shift upon binding to polyester polybutylene succinate-co-adipate, and subsequently concentrates the polyesterase CutL1 on the surface.\textsuperscript{55} Assembly state of hydrophobins seems to have an
impact on binding and modification of surfaces, and understanding this relationship may also provide further means to control and tailor their effects on lignocellulosic fractions.

### 2.2 Lignocellulosic Biomass

Lignocellulose is the crosslinked, robust network of polysaccharides, proteins and lignin that confer physical stability and enzymatic resistance to cell walls in plant cells.\(^1,5^6\) The waste streams of agriculture (in the form of corn stover, sugarcane bagasse, and other non-edible portions of plants), municipal waste (yard trimmings and food waste), and the forestry industry (pulp and paper waste, wood residue) produce a steady stream lignocellulose that is not utilised fully. Energy crops such as switch grass, poplar and willow also represent potential energy crops for the production of lignocellulose that can grow in land that is otherwise unsuited for food production. As a renewable, abundant source of a diverse, biodegradable polymers that is easily accessible and not limited by geography, lignocellulose is being focused on as a viable alternative to fossil fuel.\(^1^4,5^7,5^8\) It is estimated that the US and Canada together have the potential to produce a billion dry tons of lignocellulosic biomass annually, with the US producing mostly agricultural residues and energy crops while Canada produces more wood residues.\(^2\) 150 million dry tons of forestry waste is estimated to be available per year from sawdust and woodchips from sawmills, and energy crops in the US have exceeded a production of three billion dry tons per annum.

Lignocellulose is primarily composed of cellulose (45-55%), lignin (20-30%) and hemicellulose (25-30%), as well as a small fraction of lipids, protein and other trace components, although these proportions can vary in different plants.\(^4,5^\) Woody biomass, for example, has more cellulose and lignin present, while grassy biomass contains a higher percentage of hemicellulose. Cellulose (Figure 3A) consists of a chain of \(\beta(1 \rightarrow 4)\)-linked glucose monomers hundreds to tens
of thousands of monomers long. The monomer unit of cellulose is cellobiose, a disaccharide made of two glucose molecules linked by a $\beta(1\rightarrow4)$-glycosidic bond. Cellulose is produced by cellulose synthases, which come together in bundles (“rosettes”) of 36, in the plasma membrane.\textsuperscript{59} These 36 synthases produce a linear chain of $\beta(1\rightarrow4)$-linked glucose monomers, and these all come together to form an elementary cellulose fibril, or microfibril, that is between 500 and 15,000 monomers long.\textsuperscript{1,60} These microfibrils then pack further into macrofibrils, which confer the mechanical stability needed in plant cell walls. The majority of cellulose is in crystalline form, with chains being tightly packed together by an extended network of hydrogen bonds and hydrophobic stacking, while a small portion is amorphous, and more susceptible to enzyme attack.

Hemicelluloses (Figure 3B) have a less tightly packed structure due to the mixed linkages and branching found between the monomers, and the fact that there are approximately 20 different saccharide monomers found in it.\textsuperscript{1} Hemicelluloses have lower molecular weights relative to cellulose, with a degree of polymerization of 70-200 compared to cellulose’s 500-15,000. Their composition also varies based on source, such as the xylan-rich hemicellulose of agricultural biomass and hardwood (deciduous) trees, or the (galacto)glucomannan rich hemicellulose of softwood (coniferous) trees.

Lignin, the third most abundant component, is composed of the phenolic monomers coniferyl alcohol, coumaryl alcohol, and sinapyl alcohol cross-linked in a large, polyaromatic structure (Figure 3C).\textsuperscript{61} Softwood lignin is composed mainly of coniferyl alcohol, with small amounts of sinapyl alcohol, while hardwoods are often a mix of coniferyl and sinapyl alcohols, with small amounts of coumaryl alcohol. The complexity and variability of lignin structures precludes the creation of a definitive model, but there are patterns present, such as relative
occurrence of covalent linkages between monolignol subunits, and the covalent binding of lignin polymers to polysaccharides in the cell wall to form a lignin-carbohydrate complex. The molecular weight and exact structures of lignin have been difficult to analyze or isolate, as isolation methods tend to break down the native form during the process. In plant cells, lignin is the rigid, impermeable shield responsible of resisting chemical and enzymatic attacks. Lignin is known to be a major factor in increasing the difficulty of lignocellulose modification due to its tendency to non-specifically bind enzymes, and cover the cellulose fibrils.

Among the fractions of lignocellulose, cellulose has found the most commercial success, mainly in the production of textiles and pulp and paper products such as paper and cardboard. Many industries seek to utilize cellulose as a feedstock for hexose sugars for a variety of platform chemicals and, with the advent of second generation biofuels, for subsequent fermentation to ethanol. Rather than degrading cellulose to its base sugars, other industries have taken advantage of nature’s ability to reliably produce a complex polymer, and isolated the cellulose intact. Cellulose acetate is the most commercially successful of these industries, with
applications in filters, textiles, LCD screens and x-ray films. There are also a growing number of bioplastics and textile fibers being created, either using cellulose as the main component, or incorporating crystalline cellulose fragments (cellulose nanofibers) into the structure to impart mechanical strength.

From an industrial standpoint, lignin has historically been thought of as nothing more than a barrier between us and the cellulose we seek to extract. As per its function in a plant cell, lignin forms a physical barrier to chemical and enzymatic attack on the cell wall by non-specifically binding enzymes and releasing inhibitory byproducts when degraded. Once removed from the lignocellulose, the lignin would be burned to provide heat and electricity to other processes. Typical routes to remove lignin include the use sulfite pulping or alkaline treatment to dissolve the lignin, but these methods also lead to degradation of the polymer. As new technologies seek to take advantage of the diverse chemical structures in lignin, specifically the aromatic compounds, pretreatments are focusing more and more on the removal of lignin with minimal degradation. The lignin may then undergo specific degradation reactions to produce a variety of bulk and fine chemicals, including monomers for polymers such as polyethylene terephthalate and styrene. Again, there is a lot to be gained from the production of these polymers from larger lignin structures, rather than degrading the polymer and immediately building it back up.

Despite the multitude of potential uses for lignocellulosic biomass, its adoption is hindered by the high recalcitrance of the material. The high cost of the enzymes is already a major factor, but this problem is compounded by the properties of lignocellulose that hinder enzyme action. Lignin is known to lower enzyme efficacy by unproductively binding enzymes that cannot degrade it, cellulose crystallinity lowers the available surface area for enzymatic
treatment, and even when enzymes do manage to degrade cellulose, the products of this reaction act as inhibitors, stifling further degradation. Pretreatment methods have been focused on the removal of lignin and disruption of cellulose crystallinity, and there is a large effort in the discovery of enzymes with low product inhibition and high stability over time.

2.3 Pretreatment of Lignocellulosic Biomass

As established industries that utilize lignocellulose sought mainly to degrade it to sugars, many available pretreatment methods were designed to remove lignin and disrupt the crystallinity of cellulose in the most economical way possible, rather than focusing on preserving the complexity of the starting materials. Acid, alkaline and liquid hot water treatments are the main pretreatment methods used, and all of them aim to degrade and remove the lignin and hemicellulose. Conversely, newer pretreatments have emerged that can separate the fractions in a milder manner, such as organosolv or the use of ionic liquids. Organosolv treatment is used to isolate lignin in its natural form, using organic solvents such as methanol, ethanol and acetone at temperatures ranging from 150-220°C.

Biological pretreatments seem like an attractive option due to the specificity of enzymes, low energy requirements, high yields and lack of harmful chemicals. However, the disadvantages are also easy to see, as the organisms used, mainly fungi, require long incubation times, and by extension large reactors, as well as careful growing conditions. Research has been aimed at determining the transcriptomic and secretomic responses of fungal monocultures, or combinations of fungal species, in response to different substrates and environmental conditions, to discover new carbohydrate-active enzymes (CAZymes) with ideal activities for industrial use. The soft-rot fungi *Trichoderma reesei* is one of the most studied sources of CAZymes, and has a long history in industrial production of enzymes, despite the fact that its genome has
fewer genes for CAZymes than many other fungi. The fact that *T. reesei* can degrade lignocellulose as efficiently as other fungi suggests that there are other, non-enzyme factors dictating its efficiency, and research has expanded to search for these possible factors.

Some of these factors include non-enzymatic proteins which increase the activity and stability of cellulytic enzymes by altering the structure of the lignocellulosic substrate. Expansins are one such class of proteins, which disrupt the hydrogen bonding in lignocellulose to reduce cellulose crystallinity, a process termed amorphogenesis. This activity is necessary in plant cells to make room for growth, but has been shown to also increase cellulase activity. Swollenins, an expansin-like protein found in *T. reesei*, have been shown to increase the activity of cellulases on cellulose, but only on select substrates. The protein was found to have no effect on pure cellulose, suggesting interactions with other fractions as well.

Hydrophobins present a novel method of specific surface modification that can modulate enzyme binding and activity without degrading the substrate unnecessarily. MPGI and HFBII have shown the ability to recruit enzymes to a surface, and other hydrophobins may show other, exploitable interactions with enzymes once bound to lignocellulose. Further, the robust, chemically-resistant nature of class I hydrophobin assemblies is well documented, allowing for their use in a wider variety of temperatures and pH than other proteins.

2.4 Quartz Crystal Microbalance

A Quartz Crystal Microbalance uses the piezoelectric nature of a thin quartz crystal to measure changes in mass over time. As an alternating current is passed through the material, vibrations occur at a specific frequency, and constructive interference will occur at resonant frequencies. Mass added to or taken from the crystal will lower and raise this resonant frequency, respectively. By having one side of this crystal attached to a surface in a flow chamber, it is
possible to monitor in real time the resonant frequency of the crystal while it is linked to the surface changes occurring on the other side of the surface it is attached to. Further, multiples of the resonant frequency, typically the 3rd, 5th, 9th, and 11th, can be monitored for extra information, and are referred to as “overtones”. The mass of analyte bound to the surface was related to the change in frequency by the equation $\Delta m = -C \Delta f / n$, where $\Delta m$ is mass bound to the surface, $\Delta f$ is the change in frequency, $n$ is the overtone used, and $C = 17.7 \text{ ng} \cdot \text{Hz} \cdot \text{cm}^{-2}$. This is known as the Sauerbrey equation, and it holds true as long as the analyte on the surface is rigid and thin enough to follow the vibrations of the sensor.

Early QCM experiments were primarily used as mass balances in vacuums, testing corrosion and deposition on surfaces when exposed to gases. However, as experiments in liquid environments became more common in the 1980’s, the Sauerbrey equation was no longer sufficient to properly model the data. The Sauerbrey equation assumes that the addition of mass to the crystal would simply mimic the effects of a thicker crystal. If the film on the surface becomes significantly hydrated, soft or thick, the film will not follow the vibrations of the sensor, as the wave created by the motion of the crystal cannot propagate properly through the medium, and the resultant energy loss will cause the Sauerbrey equation to underestimate the mass bound. The viscoelastic nature of these films required more information to properly model, and this led to the development of a Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D). The dissipation factor, $D$, is a dimensionless measure of the rate at which energy is lost in the sensor once the current is turned off, or $D = \frac{E_{\text{Dissipated}}}{2\pi E_{\text{Stored}}}$, where $E_{\text{Dissipated}}$ is the energy lost per vibration of the sensor, and $E_{\text{Stored}}$ is the total energy in the sensor. The dissipation is a function of the viscosity and the elasticity of the film, and significant dissipation changes (greater than $2 \times 10^{-6}$) indicate a viscoelastic layer is being deposited or formed.
Measuring dissipation and frequency changes at multiple overtones is critical for modelling viscoelastic films, as the changes may be dependant on the frequency, leading to variations between different overtones (Figure 4). The different overtones also decay over various lengths, with the fundamental frequency (1\textsuperscript{st} overtone) decaying 250nm from the sensor surface, and all others decaying sooner. Monitoring the frequency and dissipation changes in multiple overtones simultaneously allows for the determination of the viscoelastic properties of the film, as well as accurate determination of mass and thickness, using the Voigt model. The Voigt model, pictured as a dashpot and a spring in parallel, represents the adsorbed film using the complex shear modulus, according to the equation $G = G' + iG'' = \mu_f * \frac{i2\pi f \eta_f}{\omega}$, where $G'$ is the storage modulus (elasticity) and $G''$ is the loss modulus (viscosity). $\mu_f$ is the storage (elastic) modulus of the film, $f$ is the resonant frequency, and $\eta_f$ is the viscosity of the film. As seen in the equation, the viscoelasticity ($G$), which will alter the frequency change observed, is dependent on the frequency being monitored (overtone). The model assumes that the film on the sensor surface is evenly distributed, and sticks to the surface well enough to prevent any slipping, and that there is enough bulk fluid above the film to consider it infinite.
It is possible to compare frequency changes within similar experiments as long as the viscoelasticity is not altered significantly, for example two proteins binding to the same surface, both with small dissipation changes. However comparing a viscoelastic surface to a rigid one, or binding of a large protein compared to a small one does require viscoelasticity to be taken into
account, and the Voigt model can output parameters such as film thickness and mass to directly compare these data.

The capabilities of the QCM-D led to an increase in its potential applications, specifically in its use as a biosensor. It allowed researchers to study the interaction between DNA, protein, lipids, and even cells with different surfaces in real time, and showed how the density, hydration, or elasticity of a film could be altered over time. Lee et al. studied the effects of buffer and pH on a chitosan brush layer, watching the film swell and collapse when exposed to different conditions. Other groups have also characterised the swelling and collapse of varying polymer films, as well as the binding of cells, proteins or drugs to different materials to design anti-fouling surfaces.

The use of cellulose films in QCM-D is nothing new, and interactions between enzymes and different forms of cellulose have been studied. However, the interactions between enzymes and their substrate depends on more than just the chemical composition of the substrate, but the macroscopic organization as well. Nanocrystalline cellulose, also known as cellulose nanowhiskers, consists of highly crystalline portions of cellulose in portions that are 100-1000nm long. Nanofibrillar cellulose is similar, but much longer, and consisting of crystalline and amorphous regions, while regenerated cellulose involves dissolution of the cellulose in an appropriate solvent (with or without chemical modification of the cellulose), followed by spin coating or film preparation, and regeneration. One method of cellulose dissolution involves chemical modification of the cellulose to trimethylsilyl cellulose, and regeneration of the cellulose after spinning or film formation through the use of gaseous HCl (Figure 5). Once regenerated, the cellulose is shown, by AFM and ATR-IR, to be mostly in an amorphous state.
This amorphousness, while less representative of the natural state of cellulose, increases enzyme activity on the cellulose, and confers reproducibility between samples.

Figure 5: Chemical Modification of Cellulose into TMSC by dissolution into LiCl/Dimethylacetamide (DMAc), followed by addition of Hexamethyldisilazane. Cellulose was regenerated after spin-coating by exposure to HCl gas for 2 min.
3. Chapter 1: Binding of Class IB Hydrophobins to Lignin and Cellulose

3.1 Background

Hydrophobins are a class of secreted proteins found in filamentous fungi, typically 100 residues long. They form a β-barrel core, and several disordered loop regions. They are identified by their characteristic eight cysteine pattern, and ability to form films at interfaces. Their high surface activity is utilised by the fungi to alter their environment, aiding in adhesion, waterproofing of aerial structures, and coating of spores to avoid immunogenic detection. Industrially, hydrophobins have been used to disperse hydrophobic materials, such as drugs, in water, to stabilize foam in food products, and to non-covalently immobilize proteins to surfaces.

Hydrophobins are separated into class I and class II based on sequence homology and the physio-chemical properties of their assemblies, with class I being further subdivided into IA and IB based on sequence. Class II hydrophobins are readily recognizable due to their high sequence homology, and the films they form at interfaces are known to be soluble in ethanol or sodium dodecyl sulfate (SDS). Class I hydrophobins form much more resistant assemblies, requiring strong acids such as trifluoroacetic acid (TFA) to disrupt, due to the fact that the monomeric form restructures into a β-sheet-rich structure upon assembly, allowing the formation of amyloid-like rodlets. Unlike other amyloids, the formation of these rodlets only occurs once the protein is bound to an interface.

Typically, amyloid formation has been associated with diseases such as Huntington’s and Alzheimer’s, but there is a growing list of proteins for which the amyloid form of the protein is the active form. MPGI, a class I hydrophobin found in Magnaporthe oryzae, has been shown to increase the adhesion and stability of cutinases to the surface of rice plants, but only once it is assembled. This may suggest that the requiring the presence of an interface is a control
mechanism for the assembly of these proteins, and that the function of a hydrophobin may
necessitate the monomeric or assembled form of the protein.

Lignocellulose is a combination of cellulose, lignin, and hemicellulose into a chemically
and enzymatically resistant material. Formed in the cell wall of plant cells, lignocellulose confers
the structural stability to the cell, as well as forming a physical shield to protect from physical,
chemical and enzymatic degradation. However, once the fractions are separated, they represent
an abundant, bio-renewable feedstock for materials, platform chemicals, and biofuels. Typically,
lignocellulose is subjected to acid, alkaline, or hot water pre-treatment to degrade and remove the
lignin and hemicellulose and make the cellulose more amenable to enzyme activity. However
recent technologies have increased the value of lignin, recognizing it as the most abundant
biological source for aromatic compounds. Now, there is more of an emphasis on targeted,
specific modification of lignocellulose, to separate fractions with minimal damage to the
polymers. Enzymes with high substrate specificity are typically used to degrade and modify the
lignocellulose in predictable ways, but they are hindered by the high rates of non-specific
binding to lignin, and the recalcitrant crystallinity found in cellulose.

Hydrophobins may provide a means to specifically alter the surface of different fractions
of lignocellulose, and optimize the interactions of enzymes with the appropriate substrate, and
assembly state of the protein may play a critical role in the control of this modification. To test
this, five hydrophobins were recombinantly produced in *E. coli*, and tested for binding to
regenerated cellulose and softwood lignin using a quartz crystal microbalance with dissipation
monitoring (QCM-D). The hydrophobins were then tested again, after the samples were aerated
overnight to induce rodlet assembly. The data obtained from the QCM-D measurements was
modelled to provide values for the mass of protein bound to the surface. Determining which
hydrophobins bind specifically to different fractions of lignocellulose, and how assembly of the hydrophobin alters this binding, may allow for the targeted modification of lignocellulosic fractions.

Four hydrophobins (Table 1) from across the spectrum of Class IB, and one hydrophobin from the mixed region of class I (PcaHyd3) were tested for binding to cellulose and softwood lignin using a QCM-D, and the resultant films were characterized by modelling. The effect of overnight aeration, and by extension protein assembly, was then tested as well on the same surfaces.

Table 1: Summary of Five Hydrophobins Tested

<table>
<thead>
<tr>
<th></th>
<th>MW (kDa)</th>
<th>Class</th>
<th>Organism of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PcaHyd2</td>
<td>10.4</td>
<td>IB</td>
<td><em>Phaenerochaete carnosa</em></td>
</tr>
<tr>
<td>PcaHyd1</td>
<td>11.5</td>
<td>IB</td>
<td><em>Phaenerochaete carnosa</em></td>
</tr>
<tr>
<td>PcaHyd3</td>
<td>8.9</td>
<td>IM</td>
<td><em>Phaenerochaete carnosa</em></td>
</tr>
<tr>
<td>SC16</td>
<td>9.9</td>
<td>IB</td>
<td><em>Schizophyllum commune</em></td>
</tr>
<tr>
<td>SerpLa1</td>
<td>8.3</td>
<td>IB</td>
<td><em>Serpula lacrymans</em></td>
</tr>
</tbody>
</table>

3.2 Materials and Methods
3.2.1 Production and Purification of Hydrophobins from *E. coli*

The five hydrophobins studied in this project were produced in *E. coli* Origami B(DE3) cells obtained from Millipore Sigma (cat. No. 70837). The hydrophobin genes were fused to an N-terminal His tag and TRX solubilisation tag, with a thrombin cleavage site separating them, in a pET-32a(+) vector obtained from Novagen (cat. No. 69015-3) (Supplementary 1). Cells were plated onto LB Agar containing 100μg/mL carbenicillin, 15 μg/mL kanamycin and 12.5 μg/mL tetracycline overnight. A single colony was then chosen to seed 50mL of LB broth with the same antibiotics, and this was incubated at 37°C overnight on a shaking platform at 200rpm. 22mL of this culture was then used to seed a 1L culture, which was incubated at 37°C and 200rpm. Once
the OD of the culture was between 0.6 and 0.8 (Measured with a Thermo Scientific Genesys 20 Spectrophotometer), the hydrophobin expression was induced by the addition of Isopropyl β-D-\(\text{1-thiogalactopyranoside (IPTG)}\) to a final concentration of 300\(\mu\)M. The cultures were then incubated overnight at 21°C and 200rpm.

Following 16-18 hours of induction, the culture was spun down in a centrifuge at 8900g for 20 minutes in a JLA.1000 rotor from Beckman Coulter. The pellet was resuspended in 20mL binding buffer (20mM Sodium Phosphate at pH7.4, with 0.5M NaCl and 20mM Imidazole.) and 200\(\mu\)L of 100mM PMSF was added along with 200\(\mu\)L of 10mg/mL lysozyme. The suspension was left shaking on ice for 60min, before being sonicated for 10 minutes (3s on, 4s off). The sonicated samples were then spun down in a JA-12 rotor (Beckman Coulter) at 23,000g for 90min, and the supernatant was kept for purification.

Purification involved the use of a 5mL pre-packed His-Trap FF Crude column obtained from GE Healthcare (cat. No. 04007041EF). The samples were loaded into the column with a syringe before an FPLC was used to elute the protein of interest using a gradient elution from 20mM to 500mM Imidazole; fractions containing protein were detected by UV adsorption. Eluted protein was then pooled in dialysis tubing (Thermo Scientific, 3,500 MW cut-off, cat. No. 68035) and dialized in dialysis buffer (25mM Tris-HCl buffer at pH 7.5 with 100mM NaCl and 2mM CaCl\(_2\)). 60-100 units of a His-tagged enterokinase obtained from GenScript (cat. No. 203004-500) were also added to the sample to remove the upstream His-tag and solubilisation tag from the hydrophobin (Figure 6). The next day, the sample was run over the pre-packed His-Trap column again, and this time the flowthrough and wash fractions were kept, as the hydrophobins no longer possessed a His-tag to bind to the column. The purity of this sample was assessed using a 15% SDS gel that was silver-stained for visualization, as the selected
hydrophobins do not bind coomassie. The sample was concentrated using a Corning Spin-X UF 6 spin concentrator (cat. No. 431482) and a buffer exchange was performed using a Bio-Rad Econo-pac 10DG Desalting column (cat. No. 732-2010) from elution buffer into ammonium acetate. Some of this solution was sent for Amino Acid Analysis (AAA) at Sick Kids Hospital (see below), while the rest was split up into 200μL aliquots, which were then flash frozen in liquid nitrogen and freeze-dried. The aliquots were kept in a room temperature desiccator until needed.

3.2.2 Silver Stain of SDS-PAGE Gel

Gels intended for silver staining were first incubated in fixing solution (50mL MeOH, 50mL H_2O, 12g Trichloroacetic acid) for at least 30min on a shaking platform at room temperature. The gel was then rinsed with milliQ water three times, each time for a duration of 20min. Next, the gel was incubated in 5μg/mL Dithiothreitol for 30min, followed by a 0.2% silver nitrate solution for another 30min. At this point on, the gel was kept in the dark as much as possible. The gel was then rinsed briefly with milliQ water before being incubated in 3% Na_2CO_3 for 30min. To develop, the gel was incubated in 3% Na_2CO_3 with 50μL/100mL of 37%
formaldehyde. Developing was done with the gel in the dark, until bands had developed sufficiently, and then stopped with a 15% acetic acid solution.

3.2.3 Amino Acid Analysis of Hydrophobin Samples

Samples of each batch of hydrophobins were sent to the SPARC BioCentre at Sick Kids Hospital in Toronto for Amino Acid Analysis (AAA). AAA involves the hydrolysis of a protein with 6N HCl, followed by derivatization of the individual residues. The sample is then analysed by HPLC to quantify the moles of each residue, allowing for quantification of the original sample and, given the protein’s sequence, determination of sample purity.

3.2.4 Chemical Modification of Cellulose to Trimethylsilyl cellulose (TMSC)

Two grams of microcrystalline cellulose were suspended in 80 mL of N,N-Dimethyl Acetamide (DMA) and stirred at 120°C for three hours. The slurry was allowed to cool to 100°C, and 6g of anhydrous LiCl were added. Constant stirring was continued until the solution cooled to room temperature. The solution was then heated to 80°C and 20mL of hexamethyldisilazane (HMDS) was added 1mL at a time, over the course of an hour. The mixture was cooled to room temperature, and the TMSC was precipitated by pouring into 400mL of methanol. The TMSC was then collected by vacuum filtration and dried in a vacuum desiccator overnight.

3.2.5 Preparation of Cellulose and Softwood Lignin QCM-D Sensors

Softwood Organosolv lignin was dissolved in dioxane at 0.5% w/v. Approximately 65μL of this solution was then coated onto a SiO₂ sensor obtained from Biolin Scientific using a Laurell Tech WS-400BZ-6nPP Lite spin coater. The spin coating program had four steps: 800rpm for 25s, 1200rpm for 25s, 2500rpm for 30s, and 6000rpm for 25s. The sensor was then placed in an oven at 60-70°C overnight.

TMSC was dissolved at 0.5% w/v in chloroform, and 65μL was coated onto a sensor using the same spin coater protocol as the lignin sensors. These sensors were then left in the oven
overnight at 60-70°C before being placed in a vacuum desiccator along with a beaker of 10% HCl. A vacuum pump was used to bring the HCl to a rolling boil for 8sec, and then the desiccator was sealed and left for 2min. Once the vacuum was released, the now-regenerated cellulose sensors were taken and placed in the QCM-D.

3.2.6 Preparation of Hydrophobin Sample
The freeze-dried sample of hydrophobin was taken from the desiccator, and 250μL of TFA was added. The tube was sonicated for 30min, and then dried in a fume hood. Once all the TFA had evaporated, 1mL of buffer was added to the tube and it was sonicated for a further 30min.

Overnight aeration was done with 50μg of protein in 2mL of buffer, inside of a 15mL falcon tube. The sample was put on a rotisserie shaker, and left head-over-tail shaking for 20-24 hours. All samples were run on the QCM-D at 5μg/mL.

3.2.7 QCM-D Experiment Setup
All QCM-D experiments were done using a QSense Analyzer obtained from Biolin Scientific. Once the sensors were placed in the modules and secured, the 1st fundamental frequency was found for each module to ensure the sensor was placed correctly. If the dissipation was between 30-50×10⁻⁶, it was considered to be placed correctly, and the buffer was flown through the QCM-D at 50μL/min. Once the buffer had been running for approximately an hour, the 3rd to 11th overtones were found. The QCM-D was run until a stable baseline had been achieved for at least 15min, and then the inlet tubes were placed into the hydrophobin sample. This was run until the observed frequency shift was less than 1Hz every 15min, and the tube was placed back into the buffer to wash. Once the frequency change was again less than 1Hz per 15 min, the experiment was stopped. The QCM-D was washed with 50mL of 2% SDS followed by 50mL of Milli-Q water.
3.2.8 Analysis of QCM-D Data

The Dfind program (version 0.50) from Biolin Scientific was used to model the data obtained and determine the mass of protein bound to each surface. To properly model the mass of protein binding to the sensor, the Voigt model requires the density ($\rho_L$) and viscosity ($\eta_L$) of the fluid, as well as the density ($\rho_f$), viscosity ($\eta_f$), thickness ($d_f$) and elasticity ($\mu_f$) of the film on the sensor. Further, the model assumes a laterally homogenous film on the surface that is tightly adsorbed to the sensor surface, and a Newtonian fluid being flown through the chamber, that is considered infinite. The density and viscosity of water at 23°C were used for the fluid, as 1mM buffer is very similar to water. Modelling was attempted by considering the cellulose or lignin to be one layer, and the hydrophobins to be another layer formed on top, each with their own set of parameters, but this was unsuccessful as the hydrophobins do not form a discrete, separate layer on top of the cellulose and lignin. As such, it was modelled as one layer, with the density of both the cellulose/lignin and the hydrophobin films being averaged together.

The density of the cellulose/lignin before hydrophobin addition was estimated by finding the resonant frequencies for the same sensor before and after it had been coated in cellulose or lignin (Supplementary 2). This gives a measure of the frequency and dissipation changes on the sensor incurred by coating, and this could be used to estimate mass and density of the cellulose or lignin film. Afterwards, the density of the cellulose or lignin was adjusted based on assumptions of how hydrophobin binding would change them. The density of cellulose was not expected to change drastically, as it is already hydrated and close to the density of water, but lignin, which is more hydrophobic, was expected to have a lower density after protein binding. Protein films are typically 85% or above water, so the density is typically very close to 1000 kg/m$^3$. By fixing $\rho_L$, $\eta_L$ and $\rho_f$, the Voigt model is used by the program to estimate the remaining parameters (fitted parameters), produce expected frequency and dissipation values for
this set of parameters, and compare that to the experimental values by a chi-square test. The fitted parameters are then adjusted, within a limit set by the user, to minimise the chi-square value.

Determining the rate of mass binding to each surface was done by plotting the initial, linear rate of mass adsorption over time for each experiment, up until the rate of binding slowed (Supplementary 5). This plot was then fit to a linear curve, where the slope represented the rate of mass binding to the surface in ng/min.

3.3 Results
3.3.1 Production and Purification of Hydrophobins
PcaHyd2, PcaHyd1 and Batch 5A and 5B of SC16 were produced in-house for the experiments of this thesis, while PcaHyd3 and SerpLa1 were produced by a collaborator (Prof. Steve Smith, Queen’s University). All protein batches were quantified by Amino Acid Analysis at the Sparc Biocenter in Sick Kids Hospital, Toronto, Ontario. 5mg of protein was produced in batch 5 of SC16 from 4L of culture, before being split into batch 5A and 5B. 1mg of PcaHyd2 and 3.4mg of PcaHyd1 were produced, in 4L of culture each. Purity of the protein samples was confirmed by running on a 10% SDS gel and silver staining (Supplementary 3).

Purity of the hydrophobin batches was further assessed by amino acid analysis, by comparing the expected amount of each amino acid to the actual amount present, relative to the other amino acids (Figure 7). Mostly, the averaged ratios of individual amino acids hover around 1.0, indicating a pure sample.
3.3.2 Binding of Class IB Hydrophobins to Cellulose

SC16 and PcaHyd2 are the only hydrophobins tested to show binding to cellulose, with frequency changes between 5-15Hz, and dissipation changes below $2 \times 10^{-6}$ (Figure 8). Further, the spread of the different overtones for each hydrophobin is small, although PcaHyd2 shows a larger variation in frequency changes between overtones. Interestingly, addition of SC16 to the cellulose shows a negative dissipation change. Although it is not a large change, it implies increasing density and rigidity of the film, and this was not seen with any other experiment.
SerpLa1, PcaHyd1 and PcaHyd3 did not show any binding to cellulose in 1mM NaOAc at pH 4.5.

Binding of the same five hydrophobins to cellulose was drastically altered when samples were subjected to overnight aeration. PcaHyd1, PcaHyd3 and SerpLa1 still showed essentially no binding to the surface after aeration. However, PcaHyd2 binding decreased, almost to nothing, after samples were aerated overnight. The largest difference is in the binding of SC16, which after aeration shows a much higher frequency and dissipation change, coupled with a large spread of the overtones.

Figure 8: Summary of Frequency Changes (Hz) and Dissipation Changes (1x10^-6) seen upon Binding of Hydrophobins to Cellulose. Hydrophobins samples were all made at 5μg/mL, and experiments were run at 23°C in 1mM NaOAc buffer at pH4.5, at 50μL/min. Hydrophobin samples were aerated by gently shaking 50μg in 2mL head-over-tail in a 15mL Falcon tube at room temperature for 20-24 hours. (n=2)
3.3.3 Binding of Class IB Hydrophobins to Lignin

Binding to softwood lignin was seen with all five hydrophobins, with very small dissipation changes, and with the overtones showing very little spread (Figure 9).

After aeration, binding to softwood lignin was not seen for any hydrophobin other than SC16. Just as in the cellulose binding experiments, SC16 now bound with a larger frequency and dissipation change, as well as spreading of the overtones.

![Figure 9: Summary of Frequency Changes (Hz) and Dissipation Changes (1x10^-6) seen upon Binding of Hydrophobins to Softwood Lignin. Hydrophobin samples were all made at 5μg/mL, and experiments were run at 23°C in 1mM NaOAc buffer at pH4.5. Hydrophobin samples were aerated by gently shaking 50μg in 2mL head-over-tail in a 15mL Falcon tube at room temperature for 20-24 hours. (n=2)](image)

3.3.4 Modelling of QCM-D Data

3.3.4.1 Determining Density of Cellulose, Softwood Lignin, and Hydrophobin Films

To model the frequency and dissipation data collected, the program used (Dfind 0.50) required an estimate of the average density of everything on the sensor. In this case, the film was a mixture of the hydrophobins and the surface they were being tested on. Hydrophobin films were assumed to be mostly water, with a density ranging from 1020-1040 kg/m³. The density of
the cellulose and softwood lignin films were obtained by comparing baseline data for an uncoated sensor and the same sensor after coating. The cellulose film on the sensor was found to have a density of around 1050 kg/m$^3$, as the film was hygroscopic and swelled when in the QCM-D (Supplementary 4), while the density of the softwood lignin film was estimated at 1200 kg/m$^3$.

With these data, a range of densities were input into the modelling software to determine which value allowed for the best fit, while having reasonable values for thickness and mass bound. Softwood lignin alone had a density of 1200 kg/m$^3$, but 1150 kg/m$^3$ gave the best fit once non-aerated hydrophobin samples had been bound to the surface, and 1100 kg/m$^3$ for aerated hydrophobin samples. For the cellulose, the film was already highly hydrated and viscoelastic before the addition of hydrophobins, so any protein binding did not alter the density significantly from 1050kg/m$^3$.

### 3.3.4.2 Modelling Protein Mass over Time on Cellulose

The program displays the two options that fit the best, mathematically, and it is up to the user’s discretion to choose which one is more realistic based on the sample being tested. In this case, testing non-aerated proteins on lignin gave a possible film thickness of either 2-3nm or 7-8nm, and the model that gave an estimate of 2-3nm was chosen, as there is literature citing the thickness of a hydrophobin monolayer as approximately 2-3nm.$^{16,21,27}$ Typically, one of these results assumes a viscoelastic layer, and one assumes a rigid layer, although this is not always the case. It is important to note, however, the mass given by the QCM-D model is a “wet” mass, as it includes any water trapped in the film or bound to the proteins as well.

PcaHyd2, PcaHyd3 and SC16 were the three hydrophobins that showed binding to cellulose (Figure 10). Without aeration, PcaHyd2 showed the largest variation between
replicates, with mass on the sensor reaching 200-350 ng/cm$^2$. After aeration, PcaHyd2 consistently bound a maximum of approximately 100 ng/cm$^2$. SC16 is the protein with the largest change, with 160 ng/cm$^2$ binding without aeration, and approximately 3500ng bound to the same area after aeration.

![Figure 10: Summary of Resultant Mass per Area for Each Hydrophobin on Cellulose. QCM-D experiments were run at 23°C and 50μL/min in 1mM NaOAc buffer at pH 4.5, with 5μg/mL solutions of hydrophobins. Hydrophobins were aerated for 20-24h by gentle head-over-tail shaking at room temperature on a rotisserie shaker, with 50μg of hydrophobins in 2mL of buffer. (n=2)](image)

When determining the rate of mass binding to a cellulose surface, aerated SC16 was clearly faster than other hydrophobins at a rate of ~36nm/min (Figure 11). All other samples bound at a rate of ~10ng/min or less.
3.3.4.3 Modelling Protein Mass over Time on Softwood Lignin

With the exception of SerpLa1 and aerated SC16, each hydrophobin formed a film that was 200-300 ng/cm² on the surface of the softwood lignin (Figure 12). SerpLa1 differs from the rest in that maximal binding was at around 40-80 ng/cm², regardless of whether the sample was aerated or not. SC16 and SerpLa1 were also the only two hydrophobins to bind to lignin at all after aeration. SC16, just as on cellulose, showed a much higher dissipation and frequency change after aeration, while SerpLa1 remained mostly the same.
Figure 12: Summary of Resultant Mass per Area for Each Hydrophobin Film on Softwood Lignin. QCM-D experiments were run at 23°C and 50μL/min in 1mM NaOAc buffer at pH 4.5, with 5μg/mL solutions of hydrophobins. Hydrophobins were aerated for 20-24h by gentle head-over-tail shaking at room temperature on a rotisserie shaker, with 50μg of hydrophobins in 2mL of buffer. \( (n=2) \)

While aerated SC16 bound multiple times faster than other samples to cellulose, the fastest binding to lignin was seen in the non-aerated PcaHyd2 and SC16 samples (Figure 13). Non-aerated PcaHyd1 and PcaHyd3, despite binding to a similar amount, showed significantly lower rates of binding, suggesting that different interactions may be mediating lignin binding in each case.
Figure 13: Summary of the rate of binding of each hydrophobin to lignin, with and without aeration. QCM-D experiments were run at 23°C and 50μL/min in 1mM NaOAc buffer at pH 4.5, with 5μg/mL solutions of hydrophobins. Hydrophobins were aerated for 20-24h by gentle head-over-tail shaking at room temperature on a rotisserie shaker, with 50μg of hydrophobins in 2mL of buffer. (n=2)

3.4 Discussion

Differences in viscoelasticity of a film are known to cause errors in the Sauerbrey equation when correlating mass to a frequency shift in QCM-D experiments. This means that it is not accurate to directly compare frequency shifts between experiments, as the relationship between frequency and mass change. As such, it is necessary to fit the data to an appropriate model that accounts for differences in viscoelasticity, and to compare experiments with outputs of said model. The viscoelasticity of cellulose can be seen when setting up QCM-D experiments, as the frequency and dissipation changes over time reflect the swelling of the cellulose as buffer is flown over a dry sensor. Thickness is one viable method of comparing the resultant hydrophobin films, but the film thickness given by the Dfind program is a weighted average of
the film across the entire sensor, and if the film only covers the sensor partially the uncovered sections bring down the average. As such, mass per area was chosen as the measure with which to compare and contrast the different data sets obtained. This is also subject to the same averaging over the sensor surface, but the value given is less misleading.

Once coated onto a sensor, the cellulose film was estimated to have a density of 1050kg/m³, consistent with the assumption of a hydrated, viscoelastic film. The density of the lignin was estimated to be approximately 1200kg/m³. As expected, this is significantly higher than the cellulose film, and the relatively hydrophobic layer does not swell and take in water when placed in the QCM-D.

A negative dissipation change corresponds to an increase in rigidity, or an increased density. The only case in which this occurred was the binding of non-aerated SC16 to cellulose. The cellulose film is known to be swollen with buffer before the hydrophobin is introduced, so it may be possible that the hydrophobin is penetrating the cellulose film and displacing water, collapsing the cellulose film to some degree.80,81 Conversely, the large, positive dissipation change seen when aerated SC16 samples bind to lignin and cellulose support the theory that the aeration induces assembly of oligomeric rodlets, as rodlets are larger and exposed to much more solvent, leading to a viscoelastic film. The large difference in mass of SC16 bound to the surface of lignin and cellulose goes to support the theory that aeration, or the assembly state of the protein, can be used to control hydrophobin interactions with different surfaces. This also highlights the necessity of using a model to compare results from viscoelastic films to those of rigid films, as SC16 binding after aeration resulted in a frequency shift that was 4-5 times larger than its non-aerated counterpart, but a film mass that was approximately 15 times heavier.
As a relatively hydrophobic fraction of lignocellulose, lignin is known to non-specifically bind proteins due to hydrophobic interactions.\textsuperscript{1} Adding an inexpensive protein to lignocellulose to bind to the lignin before adding enzymes, a practice known as “lignin blocking”, is one method of mitigating enzyme loss through non-specific binding.\textsuperscript{88} Bovine serum albumin (BSA) is one popular protein for this purpose. All hydrophobins, with the exception of SerpLa1, bound similarly to lignin before aeration, resulting in a 200-300ng/cm\textsuperscript{2} film. PcaHyd3 is from class IM, while the other hydrophobins are from class IB, but there doesn’t seem to be a unifying theme among the four class IB proteins, or a significant difference in PcaHyd3.

As expected, aerated SC16 showed the highest rate of binding over time to cellulose, as it showed the most bound to the surface at equilibrium. The large dissipation change seen upon binding also suggests a larger amount of water is coupled to the film, adding to the observed mass and inflating the binding rate. The binding of aerated SC16 to lignin, however, was not the fastest of the tested samples. PcaHyd2 and SC16 showed higher rates of binding despite much lower mass bound at equilibrium. Further, the rates of PcaHyd2 and SC16 were significantly higher than PcaHyd3 and PcaHyd1, despite all four samples showing similar amounts of mass bound to the surface at equilibrium. While a numerical value for the affinity constant for each sample cannot be elucidated simply though the QCM-D, the rates of binding can be correlated to it, and compared qualitatively amongst the different samples as long as it is assumed that each sample forms a film with similar amounts of coupled water. Luckily, all samples other than aerated SC16 show a small dissipation change when binding to lignin, and while this does not give information on the extent of hydration of each sample, it does imply that they are all similarly hydrated. As such, the higher rate of binding of SC16 and PcaHyd2 to lignin compared
to PcaHyd1 and PcaHyd3 may suggest a different, stronger interaction between these proteins and the lignin surface.

It has been shown that the rate of hydrophobin assembly is dependent on the protein concentration as well as the extent of aeration, and that assembly can be monitored over time by an increase in ThT fluorescence.\textsuperscript{20,21} However, there is recent, unpublished work completed by Gandier et al. suggesting a hydrophobin may intrinsically assemble at a different rate, given the same concentration and aeration. When aerating the five hydrophobins tested here at 80μg/mL for 20-24h, only SC16 showed significant ThT binding, suggesting it was the only one properly assembled into the expected amyloid-like rodlets. These data suggest that the aerated samples of PcaHyd2, PcaHyd1, PcaHyd3 and SerpLa1 might not be equivalent to the amyloid-like assemblies of SC16, which may explain the large difference in binding.

However, the aerated samples of PcaHyd2, PcaHyd1, PcaHyd3 and SerpLa1 did not resemble their non-aerated counterparts either when binding to lignin and cellulose. For example, non-aerated PcaHyd3 will bind to lignin but not cellulose, while an aerated sample will bind to cellulose and not to lignin. If hydrophobic interactions are the driving force of hydrophobin binding to lignin, and aerated samples do not bind, conformational shifts that hide hydrophobic regions may be a plausible explanation for the loss of binding. This suggests there is a structural difference in the protein, such as assembly state or conformation, that is altering the binding profile, even if that change is not the expected rodlet formation. The presence of another, transition form of the protein that is different from both the assembled, amyloid-like form and monomeric form of the protein may explain the differences aeration induces in binding, despite the lack of ThT binding. One way to support this theory would be to take a sample of hydrophobins other than SC16 and aerate it until it has been confirmed by ThT binding that
amyloid-like assemblies are present, and then test the binding of this sample to cellulose and lignin. If the assumptions are correct, it will behave like neither of the samples presented here. Measuring the zeta potential of the proteins and the water contact angle of the sensor surface before hydrophobin binding may also give further insight into the surfaces charges present on the hydrophobins and on the cellulose and lignin, as well as any changes incurred in the proteins by overnight aeration. With this data, the key factors mediating hydrophobin binding may be determined for each protein on each surface, and may explain differences seen upon aeration, or between hydrophobins.

3.5 Conclusion
The goal of this study was to determine how each of the five hydrophobins bound to cellulose and softwood lignin, and how this binding was altered by overnight aeration. Overnight aeration had a drastic impact on the binding profile of SC16 to lignin and cellulose, giving insight into the importance of handling of samples for any potential applications. New data suggest that the concentration of hydrophobins and time used for overnight aeration are insufficient to induce proper rodlet assembly in PcaHyd2, PcaHyd1, PcaHyd3 and SerpLa1, so it is likely that the rodlet form of the protein was not being tested, but some other assembled form. While not the original goal of the study, these data may yet prove useful in understanding assembly of class I hydrophobins, and the possibilities in manipulating assembly state to alter binding.

4. Effect of Hydrophobin Binding on Cellulase Binding and Activity
4.1 Background
Lignocellulose, composed of lignin, cellulose and hemicellulose, is an abundant, renewable source of platform chemicals, materials and energy that is currently underutilized due
to the difficulty of separating and modifying the fractions. Enzymatic modification of lignocellulose is an attractive option due to the benign reaction conditions and high specificity of enzymes. However, enzyme efficacy is significantly hindered, as enzymes irreversibly bind to the lignin fraction of lignocellulose, cellulose is packed tightly into a crystalline form that is inaccessible, and the formation of reaction products quickly inhibits the enzymes in solution. Hydrophobins have shown a multitude of biological functions involving the physio-chemical alteration of interfaces, including the recruitment and stabilization of enzymes to a substrate surface.

Previously, the binding of several class I hydrophobins, in both their monomeric and assembles states, has been tested on cellulose and lignin using a QCM-D. Further research was done to determine the subsequent effect of these hydrophobin films on binding and activity of cellulases. Cellulases are typically organized into three groups that synergistically degrade cellulose to sugars: cellobiohydrolases, endocellulases, and β-glucosidases. Endocellulases cleave the β(1→4)-linkages within the cellulose fibers, cellobiohydrolases produce cellobiose by acting from the ends of cellulose fibers, and β-glucosidases convert cellobiose to glucose monomers.

Cellobiohydrolase I (CBHI) is a cellobiohydrolase found in *T. reesei*, and endo-β-1,4-glucanase I (Cel7A) is an endocellulase found in *Trichoderma longibrachiatum*. They contain a catalytic domain and a carbohydrate binding module (CBM) from family 1. CBMs are non-catalytic domains that aid in the binding of carbohydrate-active enzymes (CAZymes) to their substrate. The binding of class I CBMs to cellulose is due in part to three solvent-exposed aromatic residues on the flat face of the protein that are spaced in a way that allows for van der Waals interactions with consecutive glucose monomers in cellulose. There are also several polar
residues on the same face thought to play a part in hydrogen bonding with the cellulose, aiding in binding.

However, there are also data to suggest that CBMs can promote unproductive binding to lignin, and that it is driven by hydrophobic interactions. Studies have found that by altering the hydrophobic residues on the surface of the CBM, or acetylating the surface to impart hydrophilicity, they could mitigate the binding of the enzyme to lignin.\textsuperscript{91,92} Further, they found that while the catalytic domain of CBHI alone has lower activity on cellulose than the full protein, activity of the catalytic domain alone was not slowed by the presence of lignin, while the whole protein was.\textsuperscript{11}

Herein, binding of cellobiohydrolase I was tested on lignin and cellulose using a QCM-D. Enzyme binding to cellulose and softwood lignin was tested at 23\degree C, at which it was confirmed by DNS assay that there is no cellulase activity. These surfaces were either left uncoated, or coated in a sample of hydrophobins, with or without overnight aeration to induce assembly.

The impact of hydrophobin association to cellulase on cellulase activity was also tested, this time using cellulose in suspensions and the 3,5-dinitrosalycilic acid (DNS) assay (also known as the Filter Paper Assay by IUPAC) to detect release of glucose, cellobiose, and cello-oligosaccharides. Activity assays were performed using CBHI and Cel7A together, given their well described synergistic action.

4.2 Materials and Methods
4.2.1 Cellulase Enzymes

Cellobiohydrolase I (CBHI) (Accession number: CAH10320.1), from \textit{Trichoderma reesei}, was a kind gift from Professor M. Sandgren of the Swedish University of Agricultural Sciences in Uppsala, Sweden.
Endo-1,4-β-D-glucanase I (Cel7A) (Accession Number: CAA43059.1), from
Trichoderma longibrachiatum, was purchased from Megazyme (cat. No. E-CELTR).

4.2.2 Cellulase QCM-D Experiments
To test the effect of hydrophobin films on CBHI binding to lignin and cellulose, coated sensors were first placed in the QCM-D and exposed to the hydrophobin being tested, as outlined in Chapter 3. Once the wash step was completed, and a stable signal had been achieved for 15-20 minutes, the solution was changed to a solution of cellobiohydrolase I at 15μg/mL in the same 1mM Sodium Acetate buffer, at pH 4.5. The experiment was run until the signal stabilized, and then the sensor was washed again. All experiments were run at 23°C and 50μL/min.

4.3.3 Cellulase Dinitrosalicylic Acid (DNS) Experiments
Cellulose Filter Paper (Whatman, grade 1, cat. No. 1001-110) or Avicel PH-101 (Fluka Analytical, 50μm, cat. No. 11365) (25mg) was mixed with varying amounts of a 1:1 ratio of CBHI and Cel7A, and the sample was filled to 1mL with buffer and incubated in an Eppendorf thermomixer R at 40°C and 1300rpm. If SC16 was added to the sample, it was either pre-incubated with the cellulose, pre-incubated with the cellulases, or added into the cellulase/cellulose immediately before incubation at 40°C. Pre-incubation involved the addition of the SC16 to either the cellulose or cellulase, followed by gentle head-over-tail shaking for 30min at room temperature.

To make the DNS solution, 30g of sodium-potassium tartrate was dissolved in 50mL of water, while 1g of 3,5-dinitrosalicylic acid was dissolved in 20mL of 2M NaOH. The two solutions were then mixed and filled to 100mL with water, and stirring was continued until a homogenous solution was obtained.

Once the cellulose/cellulase samples had been incubated for the appropriate time, the samples were spun down, and 400uL of the supernatant was transferred to another tube. 400uL
of DNS solution was added to the tube, and the sample was incubated at 95°C for 5min. The samples were then spun down, and 150μL were aliquoted into 3 wells each of a clear 96-well plate. Absorbance was measured at 560nm in a Tecan Infinite M200 plate reader, and compared to a glucose standard to determine concentration.

4.3 Results
4.3.1 QCM-D Results

Without aeration, all tested hydrophobins showed ability to block cellulase binding to lignin, albeit to different extents (Figure 14). Notably, the lowest mass deposited onto lignin surfaces was observed using SerpLa1 (Table 2), which was correlated to lowest impact of CBHI binding to lignin. Despite similar amounts of binding of PcaHyd2 to both cellulose and lignin, PcaHyd2 shows to have no significant effect on CBHI binding to cellulose, while blocking nearly all binding of the enzyme to lignin. As a result, SC16 was the only hydrophobin to show a significant effect on binding of CBHI to cellulose, and blocked nearly 40% of that binding.
Table 2: Mass of each hydrophobin bound to cellulose and lignin, and maximal subsequent binding of CBHI (ng/cm²) without aeration (A) and with aeration (B). Boxes marked with "ND" (not detected) show experiments where the mass of protein bound is below the detection limit of the QCM-D, and "N/A" marks experiments that were not done, as CBHI binding was not assessed on surfaces where no hydrophobin binding was seen. (n=2)

<table>
<thead>
<tr>
<th>Hydrophobin</th>
<th>Cellulose Mass (ng/cm²)</th>
<th>Lignin Mass (ng/cm²)</th>
<th>CBHI Mass (ng/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PcaHyd2</td>
<td>111.4</td>
<td>7.4</td>
<td>ND</td>
</tr>
<tr>
<td>PcaHyd3</td>
<td>225.3</td>
<td>28.7</td>
<td>ND</td>
</tr>
<tr>
<td>SC16</td>
<td>3460</td>
<td>183.8</td>
<td>ND</td>
</tr>
<tr>
<td>SerpLa1</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>PcaHyd2</td>
<td>111.4</td>
<td>7.4</td>
<td>ND</td>
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<td>PcaHyd3</td>
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<td>SerpLa1</td>
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<td>0</td>
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<td>ND</td>
</tr>
</tbody>
</table>

Legend:
- A: Mass of CBHI bound to cellulose and lignin, and maximal subsequent binding of CBHI (ng/cm²) without aeration.
- B: Mass of each hydrophobin bound to cellulose and lignin, and maximal subsequent binding of CBHI (ng/cm²) with aeration.
- ND: Not detected.
- N/A: Not assessed.

(n=2)
After aeration, PcaHyd2, PcaHyd1 and PcaHyd3 did not bind to lignin, and as such did not affect enzyme binding. The final mass of PcaHyd2 absorbed to cellulose was halved after aeration, yet the aerated sample was able to reduce CBHI binding, whereas before aeration it had no effect (Figure 15). It was determined above that aeration increases the mass of the SC16 film formed on cellulose and lignin by 21 and 8 times, respectively. Consequently, the resultant layer of aerated SC16 nearly doubled the amount of CBHI bound to the surfaces of both lignin and cellulose. SerpLa1 shows a small change after aeration, as slightly less protein was bound to the surface of lignin, and its ability to block CBHI binding was halved.
Initial attempts to test CBHI activity on cellulose using the QCM-D by running the experiment at 40°C were inconclusive. Although CBHI was functional at this temperature, changes in frequency and dissipation values seen could be a result of enzyme activity, cellulose degradation, hydrophobin release from the surface, and the binding/unbinding of the CBHI enzyme all combined. As such, impact of hydrophobin association to cellulose on cellulase action was evaluated using the DNS assay to quantify reducing sugars.

4.3.2 DNS Assays

To determine the linear range of the assay, a calibration curve was performed using glucose as a standard. One molecule of glucose has one reducing end, and so the concentration of reducing ends will be known exactly. Figure 16 shows the results of this experiment, and shows that the linear relationship between absorbance and concentration of reducing ends remains
stable past 10mM. However, it also shows that concentrations below 500μM are beyond the limit of detection of the assay, and as such set the range of the assay for further experiments.

Avicel PH-101 (50μm) and Whatman cellulose filter paper (grade 1) were both used as substrates for DNS assays run with varying amounts of a 1:1 mixture of CBHI and Cel7A for 2, 6 and 24h (Figure 17). The synergistic effect between the endocellulase Cel7A and the exocellulase CBHI is visible in the increase of the mixed cellulase samples over either respective enzyme alone. Further, these data show that the enzymes were able to degrade Avicel faster than filter paper at the time points tested. As such, subsequent assays were run with Avicel.
Figure 17: DNS Assay Results for 2, 6 and 24 Hours on Cellulose Filter Paper (Top) and Avicel (Bottom). 25mg of each substrate was mixed with 50μg of enzyme and incubated at 40°C for the appropriate time.

To determine the ideal cellulase concentration for DNS assays, 25mg of Avicel was incubated with a 1:1 ratio of different amounts of CBHI and Cel7A, and reducing ends were
quantified after 2h and 22h (Figure 18). The results suggest that samples with 20μg or 50μg of each cellulase would result in values that are above the limit of detection of the assay, and not too high. 50μg of each enzyme were used in subsequent experiments, as 20μg of each enzyme may fall below the 500μM threshold if enzyme activity was hindered by a hydrophobin.

SC16 samples were pre-incubated with either the Avicel or the enzyme cocktail, for 30min while on a rotisserie shaker at room temperature, and then incubated at 40°C 2h or 20h (Figure 19). Neither time point showed any significant differences in enzyme activity, regardless of the amount of hydrophobin added.
Next, the previous DNS assays were repeated using aerated SC16 samples. Again, pre-incubation (either SC16 with enzyme or SC16 with substrate) was 30min, and then samples were
incubated at 40°C for 2 or 20h (Figure 20). Again, there is little to no difference in the resultant yield of reducing ends at 2h or 20h.

Figure 20: Summary of DNS Assay with Aerated SC16 at 2 Hours (Top) and 20 Hours (Bottom). Varying amounts of Aerated SC16 were incubated with either 25mg of Avicel or 50μg of each Cel7A and CBHI, before all components were mixed and incubated at 40°C. (n=3)
4.4 Discussion

All hydrophobins tested, without aeration, showed the ability to block CBHI binding to lignin. Hydrophobic interactions are the main factor in CBM binding to lignin, so blocking that binding suggests hydrophobins also bind to lignin through interaction of hydrophobic regions on the surface. Further, the extent to which the hydrophobin blocks enzyme binding to lignin seems correlated with the mass of hydrophobin bound, as SerpLa1 has the lowest binding to lignin, and the weakest effect on CBHI binding.

Non-aerated PcaHyd2 bound to both cellulose and lignin to a similar extent, yet PcaHyd2 did not affect binding of CBHI to cellulose and blocked essentially all binding of the enzyme to lignin. This suggests PcaHyd2 may be differently oriented on lignin versus cellulose surfaces, leading to exposure of different amino acids available to interaction with CBHI, or that the two proteins interact with different features of the cellulose surface.

After aeration, PcaHyd2 is able to block nearly 20% of enzyme binding to cellulose, despite the fact that there is less than half the mass bound to cellulose in the aerated sample compared to the non-aerated sample. CBMs drive CBHI binding to both cellulose and lignin, but by different mechanisms.11 The binding of PcaHyd2 to cellulose and lignin may be altered by the overnight aeration, as hydrophobic residues on the surface are hidden, and polar residues exposed, inhibiting lignin binding and altering its interaction with cellulose. This further supports the idea that, although not assembled into an amyloid fibril as expected, the overnight aeration was enough to induce conformational changes that alter the binding of the protein.

After aeration, SC16 nearly doubled the amount of CBHI bound to the surface of both cellulose and lignin, whereas without aeration, SC16 blocked 30% of CBHI binding to cellulose, and nearly all binding to lignin. The ability of aeration to reverse the impact of hydrophobin
coatings is consistent with recognized impacts on hydrophobin assembly, and highlights the potential to control hydrophobin coating properties through pre-treatment. This interaction may be due to the thicker, more hydrated layer formed by the aerated SC16 trapping more CBHI within it rather than a specific recruitment of the enzyme to the surface, as this effect was not shown to alter the activity of the enzymes in the DNS assay. The SC16 may yet have an impact on enzyme activity, perhaps conferring stability at different pH or temperature conditions, or substrate selectivity when exposed to more complex substrates such as lignocellulose, but these will have to be tested independently in further DNS assays.

In the case of non-aerated hydrophobins to lignin, it is likely that the effect on CBHI binding after film formation is due to the coverage of the hydrophobic lignin by the hydrophobin film, and the surface of the film being unsuitable for CBHI binding. However, the mechanism of the altered binding is unclear for other hydrophobin samples on other surfaces, as it may be as a result of partial coverage of the surface, CBHI binding spots on the hydrophobin film itself, or CBHI in solution trapped in the hydrated film. Aerated SC16, for example, saw twice the mass of CBHI bound to the surface, but it is unclear if the SC16 is laid flat on the surface or extended outwards. Typical imaging methods such as atomic force microscopy (AFM) and scanning electron microscopy (SEM) are insufficient to determine this, as they require the sample to be dried down before imagining, collapsing the cellulose film. Environmental scanning electron microscopy, however, would allow for imaging of the cellulose surface under hydrated conditions, and may allow for determination of how the SC16 rodlets are bound to the surface.

4.5 Conclusion

While new data suggests that only SC16 was in the assembled rodlet form that was expected after overnight aeration, the differences in impact on CBHI binding between non-
aerated and aerated samples are still significant in other hydrophobins. The effect of aeration suggests a method of controlling the impact hydrophobins have on lignocellulosic fractions by altering the handling of the protein prior to coating.

While binding of CBHI was modified by SC16, and this modification was dependant on whether the SC16 samples were aerated or not, no significant difference in enzyme activity could be seen by the DNS assay. Regardless of aeration, SC16 concentration or incubation time, the effect of SC16 was not discernable. However, the DNS assay only tests the concentration of reducing ends, and thus is only an indication of enzyme activity. The SC16 film may play a role in stability of the cellulases under different pH or temperature conditions, or it may aid in the mitigation of non-specific binding to lignin. The DNS assay may be used to test the impact of SC16 on cellulase activity at different pH’s and temperatures, or a more complex substrate, containing cellulose and lignin, may be used in place of the Avicel.
5. Conclusions

In this study, the effect of aeration was tested on several class I hydrophobins’ ability to bind to cellulose and lignin, as well as their effect on subsequent enzyme binding and activity. It was hypothesized that differential binding would be seen amongst the hydrophobins tested, as well as within each hydrophobin, based on whether or not the sample was aerated. This hypothesis was confirmed by QCM-D data, in which the differential binding of each hydrophobin to cellulose and lignin was tested and quantified, and in which the effect of aeration was highlighted. The thick, viscoelastic film formed by SC16 only after aeration is indicative of the large, oligomeric rodlet expected after overnight aeration, and, while not as drastic as SC16, PcaHyd1, PcaHyd2 and PcaHyd3 all showed differences in their binding profiles after aeration. These data support the hypothesis that aeration induces conformational shifts in the proteins, and can be used as a tool to tune the interactions of the protein with a surface.

In chapter 2, the subsequent effect each hydrophobin film had on cellulase binding and activity was tested. Again, the difference induced by overnight aeration was clear, and indicative of a conformational shift in the protein. A DNS assay was used to test the effect SC16 had on the activity of a mixture of cellobiohydrolase I and Endo-1,4-β-D-glucanase I on Avicel, but was unable to find a significant effect.

From an engineering perspective, understanding and exploiting hydrophobin surface modification may present a novel method of increasing enzyme yield, stability and efficacy on lignocellulosic substrates in an environmentally friendly, non-destructive manner. In addition, the effect of aeration on hydrophobin binding has been demonstrated, and understanding precisely how aeration affects the structure of hydrophobins may present a method for further tailoring surfaces in a controlled, facile manner. The results presented here may aid in the
rational discovery and application of hydrophobins for surface modification by developing the techniques to study protein interactions (QCM-D), and the methodology for properly handling samples.

6. Future Work

The DNS assay has shown that it can be used to quantify enzyme activity on insoluble substrates, but the effect of SC16 was not seen in these experiments. By utilizing a more complex substrate than Avicel, one that contains lignin as well as cellulose, there may be visible differences in enzyme activity upon hydrophobin addition. PcaHyd2 is a promising candidate for these experiments, as it has shown the ability to block CBHI binding to lignin, but not cellulose. Further, the DNS assay can be performed under different pH or temperature conditions, to determine if the presence of hydrophobins confers and stability to enzyme activity.

Several complimentary methods may also present means to further characterise the hydrophobin films formed, such as environmental scanning electron microscopy (ESEM), water contact angle measurements (WCA), and zeta potential measurements. Typically, surface imagining techniques such as scanning electron microscopy and atomic force microscopy require dry samples. Imagining hydrophobin films on cellulose by this method would lead to collapse of the film or the cellulose, and would fail to give further understanding of film features, such as the orientation of SC16 rodlets on the surface. ESEM allows for surface imaging under hydrated conditions, and this would give valuable insight into the how the hydrophobin films are formed, such as information on whether the SC16 rodlets are laid flat on the surface or extending out into solution. WCA measurements on cellulose and lignin, before and after film formation, would also give insight into how the hydrophobicity of each surface changes, and the type of surface presented by the hydrophobin film. Finally, zeta potential allows for the determination of the
potential observed on the surface of the hydrophobin assemblies. These methods may provide more specific data on the interactions seen between hydrophobin samples, lignin and cellulose surfaces, and CBHI, to explain the differences seen in binding and film formation before and after aeration.

The QCM-D has proven to be a useful tool in monitoring the binding or proteins to a surface, as it provides insight on rates, viscoelastic properties, mass and thickness in real time. Further characterisation of hydrophobin films, by testing the effects of different surfaces, aeration conditions, pHs or hydrophobin mutations on viscoelastic properties, may provide invaluable insight into the key residues and structural features needed that dictate observed properties. This in turn may eventually allow for the rational design of a hydrophobin for specific applications, and the understanding of how aeration can be used to control the function of this protein. The work performed in this study has suggested that surface binding and enzyme interactions are drastically altered by aeration of hydrophobins, but understanding the key residues involved in this change may allow for the prediction of other protein interactions of interest, and rational design of a hydrophobin.
7. Works Cited


85–88.


(70) Härkkinen, M., Valkonen, M. J., Westerholm-Parvinen, A., Aro, N., Arvas, M., Vitikainen,


8. Supplementary

Supplementary 1: Vector Map of pET-32a(+) vector used for Hydrophobin Production in *E.coli*
Supplementary 2: Frequency and Dissipation Changes Incurred by Coating SiO$_2$ Sensors with Cellulose. Baseline data before coating (left of black line) was stitched to baseline data after coating (right of black line), and resultant changes were used to predict mass and thickness of the cellulose film.
Supplementary 3: Sample Silver Stained Gels of Purified SC16 and PcHyd1. PcHyd1 (11.5kDa) seems to be above the 15kDa ladder marker, but identity and purity was confirmed by Amino Acid Analysis (Figure 7B)
Supplementary 4: Sample QCM-D Data showing the Frequency and Dissipation Change of a Dry Cellulose Sensor as Buffer is Flown Over. Falling Frequency and Rising Dissipation Indicate a Swelling, Viscoelastic Layer.

Supplementary 5: Graph of mass over time of hydrophobin binding with the linear range highlighted (red arrow). The slope of the linear region was used as the rate of mass binding in ng/min.