Analysis of Enzymatic Degradation of Cellulose Microfibrils by Quantitative Surface Plasmon Resonance Imaging

by

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Cellulose is the most plentiful biopolymer on the planet, and as such, is a large potential energy source. Converting cellulose into ethanol first requires the disruption of the crystallinity of cellulose fibers and subsequent hydrolysis into glucose. The glucose can then be fermented, producing ethanol. The conversion of cellulose fibers to glucose is an energy intensive and costly step, which is a barrier to industrial production of cellulosic ethanol. The use of enzymes to facilitate this conversion is a promising approach. In the present study, the action of individual enzymes and combinations of enzymes from the Hypocrea jecorina secretome on bacterial cellulose fibers has been studied, to better understand their individual and synergistic action.

I have used a custom Surface Plasmon Resonance imaging (SPRi) device to measure changes in the thickness of cellulose fiber coverage of a thioglucose-functionalized
gold substrate upon exposure to enzymes. The cellulose fibers were deposited using a Langmuir-Blodgett technique, resulting in non-uniform cellulose coverage of the substrate. By defining local Regions of Interest (ROIs) of the cellulose-covered gold film, and by measuring the SPR curves at elevated temperature for the ROIs as a function of time, we are able to determine the rate and extent of degradation of the cellulose fibers within individual ROIs. We have fit the change in SPR angle over time after exposure to enzyme to an exponential decay function that allows us to determine the average time constant of action of these enzymes on the deposited cellulose fibers.

We have used the above procedure to measure the average time constants of action and the average degradation fraction (the change in average thickness divided by the initial average thickness) of cellulose fibers exposed to CBH-1, CBH-2, and EG-1, as well as combinations of these enzymes. We have measured an increase in the average degradation fraction and a decrease in the average time constants of action for cellulose fibers exposed to 23 µg/mL CBH-2 compared to fibers exposed to the same concentration of CBH-1. Additionally, for concurrent exposure of CBH-1 and EG-1 (with individual concentrations of 23 µg/mL), as well as concurrent introduction of CBH-1, CBH-2 and EG-1, we observed increases in the average degradation fraction and decreases in average time constants relative to the values measured for the individual enzymes. These measurements allow us to determine the relative activity of these enzymes and they demonstrate cooperativity and complementarity of action of the different enzymes.
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Chapter 1

Introduction and Background

1.1 An Overview of Cellulosic Ethanol Production

As the world’s supply of readily accessible fossil fuels is depleted, new sources of energy must be found and harnessed. Since cellulose represents a large portion of the earth’s biomass and is the most plentiful biopolymer, it provides a largely untapped reservoir of energy [1]. Efficient access to this energy requires conversion of cellulose fibers into ethanol. Some common sources of cellulose are corn stover, switchgrass, and wheat straw. Wheat straw presents an excellent candidate for use as a cellulose source for biofuel as it is produced as a by-product of a wheat harvest and is indigestible by humans.

1.1.1 The Composition and Structure of Lignocellulosic Biomass

The conversion process is challenging because of the structure of cellulose, which is hierarchical, as shown schematically in Figure 1.1. The base repeating monomer
is β-glucose. A single chain of cellulose consists of thousands of β-glucose units. β-glucose itself is a cyclical six carbon sugar with a five carbon ring, where the hydroxyl group bonded to the C-1 carbon is in a cis configuration with the CH₂OH group bonded to the C-5 carbon [2]. The structure is shown schematically at the bottom of Figure 1.2. Each strand of cellulose is additionally linked to neighbouring chains by hydrogen bonds in the crystalline regions of cellulose. These crystalline regions are resistant to hydrolysis, so disrupting them is an important step in the degradation of cellulose [3]. Bundles of the cross-linked cellulose chains form cellulose microfibrils and bundles of the microfibrils in turn form cellulose fibers.

Although cellulose forms a large portion of straw biomass, there are several other important components, such as lignin and hemicellulose. The presence of these extra components prevents the exposure of cellulose fibers to enzymes, and can serve to sequester enzymes during enzymatic catalysed hydrolysis [4]. Lignin is a heteropolymer produced from the enzyme mediated dehydrogenated polymerization of the three core lignin monomers, paracoumaryl alcohol, coniferyl alcohol, and sinapyl alcohol [5]. Additional aromatic carboxylic acids are present in some lignins, varying in composition between different species of plant [5]. Lignin is anchored to hemicellulose by covalent bonds in the secondary cell wall of woody plants [6]. Hemicellulose describes a number of different polysaccharides found in the plant cell wall matrix [7]. A schematic diagram of the secondary and primary plant cell walls is shown in Figure 1.3.

In addition to its presence in plant biomass, cellulose can also be produced by bacteria, such as *Gluconacetobacter xylinus*. Cellulose microfibrils are produced by
Figure 1.1: A schematic representation of the hierarchical structure of cellulose.

AFM image courtesy of Adam Raegen.
these microbes through the polymerization of glucose [8]. Bacterial cellulose is 
pure cellulose and does not contain additional complicating components such as 
lignin and hemicellulose. This property makes it ideal for studying the effects of 
different enzymatic treatments on pure cellulose.

1.1.2 Pretreatment of Lignocellulosic Biomass

As mentioned above, the presence of lignin and hemicellulose reduces the effi-
ciency of hydrolysis of cellulose. Because of this, it is desirable to remove as much 
of these other biopolymers as possible. To accomplish this in an industrial setting, 
the biomass is pretreated by either mechanical or chemical processing [4].

The most common method of mechanical separation is a steam explosion pre-
treatment, in which the biomass is exposed to high pressure, high temperature 
steam, promoting hemicellulose hydrolysis. The pressure is then reduced rapidly, 
resulting in explosive decompression of the biomass, which helps to expose the 
cellulose microfibrils [4, 9].

Chemical pretreatment can be accomplished through the use of acids. Con-
centrated acid processing has historically been used to digest hemicellulose, but 
proper containment and reuse of concentrated acid is costly and has prompted a 
shift to the use of more dilute acids. The addition of dilute acid solublizes the 
hemicellulose, which exposes cellulose microfibrils for subsequent hydrolysis [4].

After pretreatment of the biomass, the cellulose microfibrils are hydrolyzed into 
glucose. This is accomplished either by further acid treatment or enzymatic pro-
cessing. As enzymatic processing is the focus of this thesis, we will discuss the
Figure 1.2: An overview of the action of cellobiohydrolases 1 and 2, and \( \beta \)-glucosidases on cellulose. A cellulose polymer chain is broken into cellobiose units by the cellobiohydrolases, before being broken into \( \beta \)-glucose by \( \beta \)-glucosidases.
Figure 1.3: A schematic representation of the structure of a plant cell wall. A. Plasma membrane, B. Cellulose microfibril, C. Hemicellulose, D. Protein, E. Lignin, F. Primary wall, G. Middle lamella [7]
mechanism of enzymatic hydrolysis in detail.

1.2 The *Hypocrea jecorina* Cellulase System

One very promising approach to the industrial implementation of enzymatic hydrolysis of cellulose is to mimic a process that occurs efficiently in nature by fungi. There are a number of different classes of cellulases secreted by fungi which degrade cellulose microfibrils in different ways. Here we will focus on the action of the main classes of cellulases secreted by the *Hypocrea jecorina* fungus, commonly known as *Trichoderma reesei* in its anamorph (asexual reproduction) stage. There are three main classes of cellulases secreted by *Hypocrea jecorina*: the cellobiohydrolases, the endoglucanases, and the β-glucosidases [10].

The cellobiohydrolases act processively from the end of a cellulose chain, threading the chain through its active site, and cleaving the β-1,4 bond between cellobiose units [11]. In the present study, we will use cellobiohydrolase-1 (CBH-1) and cellobiohydrolase-2 (CBH-2). CBH-1 works from the reducing end of the cellulose chain, while CBH-2 works from the non-reducing end of the cellulose chain. Multiple isomorphous replacement x-ray crystallography has shown that both CBH-1 and CBH-2 active sites are enclosed by β strands and some connecting loops [12, 13].

The length and composition of the active sites does, however, differ in some ways. The CBH-1 active site tunnel length has been measured to be 40 Å, which is double the length of the CBH-2 active site tunnel. Additionally, the structure of these tunnels is different in some aspects. The CBH-2 tunnel consists of a single
β barrel and connecting loops, whereas the CBH-1 tunnel consists of two curved β sheets and connecting loops. Residue composition is also different between the two active sites, most notably in the number of tryptophans, which are known to interact with carbohydrates [12, 13]. The CBH-1 tunnel has four tryptophans, compared to three for CBH-2. It has been observed that CBH-2 also exhibits endoglucanase activity, which is not evident in CBH-1. The longer tunnel observed in CBH-1 is thought to play a role in this difference in activity since the longer tunnel makes it difficult for CBH-1 to disengage from the cellulose chain. This is thought to produce a more processive action of CBH-1, which has been observed [14, 15].

The endoglucanases do not work processively, but rather can bind to any point along the cellulose chain, cleaving the β-1,4 bonds at the binding location. This disrupts the crystallinity of the microfibril, and opens up new binding sites for the cellobiohydrolases. This is shown schematically in Figure 1.4. The ability of endoglucanases to bind at any point along the cellulose microfibril is enabled by the shape of their active site, which is a groove that is not enclosed by the loops that characterize the CBH-1 active site [14].

The β-glucosidases act on the water-soluble cellobiose produced by the other cellulases, cleaving the β-1,4 bond to form glucose. As β-glucosidases act on soluble cellobiose, their action is not inhibited by the structure, i.e. crystallinity of the cellulose microfibrils as in the case of the cellobiohydrolases and endoglucanases [16].
Figure 1.4: A schematic diagram showing the action of EG-1 in disrupting the crystallinity of cellulose microfibrils. The disruption in crystallinity produced by the breaking of $\beta$-1,4 bonds in the cellulose microfibrils by EG-1 increases cellulose binding site availability for cellbiohydrolases.
1.3 Surface Plasmon Resonance

To measure the action of cellulases on cellulose, we use a technique called Surface Plasmon Resonance imaging (SPRi). This is an optical imaging technique based on the physical phenomenon of Surface Plasmon Resonance (SPR).

1.3.1 The Theory of Surface Plasmon Resonance

Surface plasmons are collective electron density fluctuations on a metal surface that can be excited by an incident electromagnetic (EM) wave. At any interface between different materials 1 and 2, the boundary condition on the normal components of the electric field can be written as:

\[(\epsilon_2 E_2 - \epsilon_1 E_1) \cdot \hat{z} = 4\pi \sigma \quad (1.1)\]

where \(E_i, \ i = 1, 2\), are the electric fields, \(z\) is the unit vector perpendicular to the interface, \(\epsilon_i, \ i = 1, 2\), are the dielectric constants of the materials, and \(\sigma\) is the induced surface charge. Since the left hand side of Equation 1.1 is only nonzero for components of \(E\) that lie in the plane of incidence, defined by the interface and the normal to the interface, only \(p\)-polarized light can change the charge distribution at an interface. As surface plasmons are electron density fluctuations at a metal surface, only \(p\)-polarized light will be able to excite surface plasmons. We will now derive the dispersion relation for the excited surface plasmons at the interface between a dielectric and metal by applying Maxwell’s equations [17].

Consider an EM wave travelling through a dielectric, and incident on the interface between the dielectric and a metal at an angle \(\theta_i\) (See Figure 1.5). Snell’s law
Figure 1.5: A schematic diagram of an interface between two media showing refraction of the incident wave. The incident, transmitted, and reflected wavevectors are indicated as $k_i$, $k_t$, and $k_r$ respectively.
dictates that at the interface, the trajectory of the incident EM wave will be altered by refraction. Expressing Snell’s law in terms of wavevectors, we can write:

$$k_{xi} = k_{xt} \quad (1.2)$$

The equations describing the electric and magnetic fields of the EM waves are:

$$E_i = E_{i0} \exp[i(k_xx + k_zi z - wt)] \quad (1.3)$$

$$H_i = H_{i0} \exp[i(k_xx + k_zi z - wt)] \hat{y} \quad (1.4)$$

$$E_t = E_{t0} \exp[i(k_xx + k_zt z - wt)] \quad (1.5)$$

$$H_t = H_{t0} \exp[i(k_xx + k_zt z - wt)] \hat{y} \quad (1.6)$$

Two of Maxwell’s equations can be written as:

$$\nabla \times H - \epsilon \frac{\partial E}{\partial t} = 0 \quad (1.7)$$

$$\nabla \times E + \frac{\partial H}{\partial t} = 0 \quad (1.8)$$

By substituting $H_i$ and $H_t$ from Equations 1.4 and 1.6 into Equation 1.7, we obtain for the x components:

$$k_zi H_{yi} = -\omega \epsilon_d E_{xi} \quad (1.9)$$

$$k_zt H_{yt} = -\omega \epsilon_m E_{xt} \quad (1.10)$$

Since the tangential components of the electrical and magnetic fields must be continuous across the interface:
The x and z components of the wavevectors are related to the magnitude of the wavevector in the two media by:

\[ k_{zi}^2 + k_{x}^2 = k^2 \]  
\[ (1.12) \]

\[ k_{zt}^2 + k_{x}^2 = k^2 \]  
\[ (1.13) \]

Squaring Equation 1.11, and substituting Equation 1.12 and Equation 1.13, yields:

\[ \epsilon_d^2 (k_{zi}^2 - k_{x}^2) = \epsilon_m^2 (k_{zi}^2 - k_{x}^2) \]  
\[ (1.14) \]

Rearranging Equation 1.14 to solve for \( k_x^2 \) yields:

\[ k_x^2 = \frac{\epsilon_m^2 k_{zi}^2 - \epsilon_d^2 k_{zt}^2}{\epsilon_m^2 - \epsilon_d^2} \]  
\[ (1.15) \]

This can be simplified by relating the wavenumber of the EM wave in its medium to the wavenumber in a vacuum \( k \):

\[ k_x^2 = \frac{\epsilon_m^2 (\epsilon_d k_x^2) - \epsilon_d^2 (\epsilon_m k_x^2)}{\epsilon_m^2 - \epsilon_d^2} \]  
\[ (1.16) \]

Taking the square root of both sides of Equation 1.16 and simplifying, we can write:

\[ k_x = k \sqrt{\frac{\epsilon_d \epsilon_m}{\epsilon_m + \epsilon_d}} \]  
\[ (1.17) \]
This is the dispersion relation for the surface plasmons. The value of $k_x$ defined by Equation 1.17 and the value of the surface component of the wavevector of incident light, $k_{xi} = k \sqrt{\varepsilon_d}$, cannot be satisfied simultaneously. To achieve this, modifications must be made to the experimental geometry.

1.3.2 SPR Experimental Geometries

Two different experimental geometries are used to achieve coupling of the incident light to the surface plasmons. The first is the Otto configuration [18], in which the sample is placed between the metal and a dielectric (a high index glass prism) as seen in Figure 1.6A. The evanescent wave produced by total internal reflection from the lower surface of the prism satisfies the surface plasmon dispersion relation. The disadvantage to this configuration is that the exponential decay of the intensity associated with the evanescent wave requires a spacing between the high-index prism and metal film of approximately 200 nm. This is experimentally challenging, and is disrupted by the presence of contamination, such as dust particles.

The second configuration is the Kretschmann-Raether geometry [19], in which the metal is deposited as a thin layer, and the sample dielectric volume is located on the side of the metal film opposite to the high-index prism (see Figure 1.6B). This geometry is much more commonly used experimentally, as the prism can be pressed directly onto the metal film, or the metal film can be deposited directly onto the prism. This geometry allows the surface plasmon dispersion relation, Equation 1.17, and the dispersion relation for the in-plane component of the inci-
dent light to be matched at a specific angle of incidence.

1.4 Surface Plasmon Polaritons in the Kretschmann-Raether Geometry

As described in Section 1.3.1, sandwiching the metal film between two dielectrics allows the resonance condition to be satisfied. This can be shown mathematically by using a matrix formulation of the light scattering problem. Each layer present in the Kretschmann-Raether configuration can be represented by a $2 \times 2$ matrix. Scattering matrices have been used to describe the interaction of travelling waves with generic multilayer films, and to describe surface plasmon resonance [20, 21].

In the multilayer system shown in Figure 1.7, every interface between media with different indices of refraction gives rise to both transmitted and reflected EM waves. As a result, in any layer of the multilayer stack, there will be EM waves travelling in both the $+z$ and $-z$ directions. The electric fields of the EM waves travelling in either direction can be described by the vector:

$$E(z) = \begin{bmatrix} E^+(z) \\ E^-(z) \end{bmatrix}$$

(1.18)

The changes in $E^-(z)$ and $E^+(z)$ in going from $z = z_0$ to $z = z_1$ can be described by:

$$E(z_0) = SE(z_1)$$

(1.19)

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Figure 1.6: A. Schematic diagram of the Otto geometry of excitation for surface plasmons. Note that the sample dielectric width is necessarily quite small (approximately 200 nm). B. Schematic diagram of the Kretschmann-Raether surface plasmon geometry. The metal film is directly in contact with the prism, and has a thickness that is typically tens of nanometers.
Figure 1.7: Schematic diagram of the layers of the Kretschmann-Raether configuration used in the scattering matrix derivation. The layers are, from top to bottom: prism, metal layer, adsorbed layer, and buffer.
where $S$ is the $2 \times 2$ scattering matrix that transforms the electric fields as they travel from $z = z_0$ to $z = z_1$. It has the form:

$$S = \begin{bmatrix} S_{11} & S_{12} \\ S_{21} & S_{22} \end{bmatrix} \quad (1.20)$$

If we take $z = z_0$ and $z = z_1$ to be directly above and below a given interface within the multilayer structure, the scattering matrix takes the form:

$$S = I = \frac{1}{t_{01}} \begin{bmatrix} 1 & r_{01} \\ r_{01} & 1 \end{bmatrix} \quad (1.21)$$

where $r_{01}$ and $t_{01}$ are the Fresnel coefficients for reflection and transmission at the interface, which are defined as [22]:

$$r_{01} = \frac{n_1 \cos(\theta_0) - n_0 \cos(\theta_1)}{n_1 \cos(\theta_0) + n_0 \cos(\theta_1)} \quad (1.22)$$

$$t_{01} = \frac{2n_0 \cos(\theta_0)}{n_1 \cos(\theta_0) + n_0 \cos(\theta_1)} \quad (1.23)$$

where $\theta_0$ is the incident angle of the EM wave on the interface, and $\theta_1$ is the refracted angle. A schematic diagram of the geometry is shown in Figure 1.7. If we take $z = z_0$ and $z = z_1$ to coincide with the top and bottom surfaces of a given layer of uniform index of refraction separated by a uniform real index medium, the electric fields are changed by a phase shift $\beta = \frac{2\pi n_0}{\lambda} \cos(\theta)$, where $\theta$ is the angle between the $z$-plane normal and the angle of propagation of the transmitted EM wave in the layer. The scattering matrix has the form:
The scattering matrix for the entire multilayer system can be constructed by multiplying the matrices corresponding to each of the component layers successively. For the multilayer system of shown in Figure 1.7, the total scattering matrix can be written as:

$$S = L = \begin{bmatrix} e^{i\beta} & 0 \\ 0 & e^{-i\beta} \end{bmatrix}$$ (1.24)

where $I_{pm}$, $I_{mc}$, and $I_{cb}$ are the scattering matrices for the interfaces between prism and metal layer, metal layer and adsorbed layer, and adsorbed layer and buffer. $L_m$ and $L_a$ are the scattering matrices for the metal layer and adsorbed layer. By considering the incident electric field $E_i$ and the total reflected and transmitted electric fields $E_r$ and $E_t$, the $+z$ and $-z$ electric field of the EM waves at either side of the multilayer stack can be used to determine the reflection and transmission coefficients for the multilayer system. They are given by:

$$r = \frac{E^-(z_0)}{E^+(z_0)} = S_{21} \quad \frac{S_{11}}{S_{11}} \quad (1.26)$$

$$t = \frac{E^+(z_1)}{E^+(z_0)} = \frac{1}{S_{11}} \quad (1.27)$$

The reflectivity of the system is represented by $R = |r|^2$. By measuring the change in the reflectivity as a function of incident angle $\theta_i$ the characteristic SPR dip in reflectivity can be observed at an angle corresponding to the resonance condition. SPR curves calculated in this manner, representative of ROIs with and with-
out an adsorbed layer are shown in Figure 1.8. For angles of incidence close to that corresponding to the SPR condition, the light, instead of being reflected, is coupled into the metal film, exciting the surface plasmons, resulting in the dip in reflectivity. The shift in the resonance angle of the SPR curve to larger angles for the case of an adsorbed layer demonstrates the relationship between adsorbed film thickness, and resonance angle of the SPR curve. Additionally, an increase in the index of refraction of the adsorbed layer or buffer will also result in an increase in the SPR angle.

### 1.5 Traditional SPR Experiments

Surface Plasmon Resonance (SPR) has been used to measure adsorption and desorption rates of analyte-ligand pairs of molecules at surfaces [23]. For this experiment, the angle of incidence is scanned repeatedly in a range of angles centred about the resonance angle. This allows the tracking of the angle corresponding to the minimum intensity with time. The metal film used in the experiment is typically functionalized with a specific ligand that is designed to bind to the analyte introduced into the sample cell. As the analyte is introduced to the sample cell, a shift in the angle corresponding to the minimum intensity is observed. This angle can be referred to as the SPR angle. The desorption of the analyte can then be observed by changing the input flow from the analyte solution to buffer. The experiment can then be repeated by breaking the analyte-ligand bond, commonly known as the regeneration stage. This allows for the use of a single sample to collect hundreds of adsorption and desorption curves [23]. A schematic diagram of
Figure 1.8: Calculated SPR curves generated using the scattering matrix formalism. The black SPR curve was calculated with no adsorbed layer, while the red curve was calculated for an adsorbed film thickness of 10 nm. The indices of refraction were chosen to be \( n_p = 1.71129 \) for the high index glass prism, \( n_m = 0.181 + 5.3i \) for the metal film, \( n_a = 1.45 \) for the adsorbed film, \( n_b = 1.333 \) for the buffer. The thickness of the metal film was chosen to be 55 nm. The code for generating the curves was provided by Adam Raegen.
data obtained in one of these cycles is shown in Figure 1.9.

SPR experiments can also be conducted at a fixed angle of incidence $\theta_i$. Instead of monitoring the change in SPR angle versus time, as shown in Figure 1.9, the reflectivity of the sample is measured at a fixed $\theta_i$, assuming that the intensity-thickness relationship is roughly linear in the thickness of the adsorbed film. As a result, the value of $\theta_i$ must be judiciously chosen, and the range of changes in the thickness of the adsorbed layer that can be measured is limited. This allows for measurement of faster phenomena, as the speed of measurement is limited only by the sample rate of the photodetector.

### 1.5.1 Surface Plasmon Resonance Imaging (SPRi)

Surface Plasmon Resonance Imaging (SPRi) devices have also been commercially developed and are used in various applications. SPRi experiments allow the collection of adsorption and desorption curves for an analyte on multiple ligand binding areas in parallel. Imaging artefacts introduced by the collection of images at different angles of incidence $\theta_i$ limit most experiments to a measure of reflectivity at a fixed angle $\theta_i$ with time. As mentioned in Section 1.5, this limits the thickness of adsorbed layer able to be accurately measured.

In addition to adsorption kinetics measurements, SPR and SPRi are used extensively in biosensing applications. In a biosensor experiment, it is not the rate of adsorption or desorption that is measured, but rather the affinity of an analyte for a variety of ligands. The analyte is introduced to a number of different ligands corresponding to different regions of the sample, and the presence of specific binding
Figure 1.9: A SPR sensorgram depicting the stages of an SPR kinetics experiment. The baseline signal is the SPR angle of the sample in the presence of only buffer. Once the analyte is introduced into the cell, the adsorption signal is observed. Switching back to flowing buffer allows the observation of desorption of the analyte. Regeneration of the pure ligand surface is accomplished by flowing a compound that causes disassociation of the ligand and analyte. After all of the analyte is removed, buffer is re-introduced and the baseline is recovered.
for an analyte-ligand pair is determined by the observation of an increased shift in the reflectivity measured for the region corresponding to the pairing, compared to the shift measured for non-specific absorption of analyte. Experiments like these are extensively used in pharmacological studies for screening for new drugs. SPRi is often used for this application because of the increased throughput speeds for the evaluation of analyte-ligand combinations [24].

1.6 Scope of Thesis

The purpose of this thesis is to characterize the interactions of some of the main cellulases of the *Hypocrea jecorina* cellulase system with bacterial cellulose substrates, and to evaluate the synergy between enzymes. The action of the individual enzymes and combinations of these enzymes are examined by measuring changes in the average thickness of bacterial cellulose fiber films with time. This is accomplished by using a custom surface plasmon resonance imaging instrument previously developed by Scott Allen [21].

This study provides insight into the extent and manner in which different cellulases modify the cellulose substrate, with a view towards increasing the efficiency of enzymatic degradation of cellulose. Specifically, the role of the crystallinity of the cellulose fibers in preventing efficient cellulase action and the role of different enzymes in disrupting this crystallinity is explored.

In Chapter Two, I describe the method of preparing our cellulose fiber-coated gold films, as well as the method of measuring the degradation of the cellulose fibers by the cellulases using SPRi. In Chapter Three, I describe the data analysis
methods that I have developed to determine the action of cellulases from data obtained from the SPRi experiments. The parameters that I have used to describe the enzyme activity are outlined, and the statistical analysis used to determine the average action of the cellulases is described. In Chapter Four, I outline the results of the SPRi experiments and the corresponding analysis of the action of cellulases on bacterial cellulose fibers, and I discuss the implications regarding the method of action of the different cellulases and their synergies. In Chapter Five, I provide a summary of the results and suggest promising future experiments and analysis.
Chapter 2

Materials and Methods

2.1 Sample Preparation

Cellulose fibers were deposited onto gold-coated, high-index SF-10 glass slides (GWC Technologies Inc.). The substrates were cleaned and reused for several experiments. To remove the cellulose from the surface, the slides were wiped with ethanol-soaked lens paper. This process removes the cellulose fibers from the gold film. The slides were then immersed in aqua regia (3:1 by volume of concentrated hydrochloric acid to nitric acid) at room temperature for an hour to remove the gold layer. To ensure the removal of all contaminants, the slides were then immersed in a heated chloroform and ethanol (1:1 by volume) mixture and sonicated for five minutes. They were then heated and sonicated in 5% Hellmanex (Hellma) for five minutes. In between these treatments, the slides were sonicated in ethanol for five minutes. Finally, the slides were sonicated in three successive baths of Milli-Q water (resistivity of 18.2 MΩ·cm) for five minutes each. The slides were
stored in ethanol before gold and titanium layers were deposited.

To prepare the substrates for the SPRi experiments, a high index SF-10 glass slide (GWC Technologies, Inc.) was coated with a thin (∼1 nm thick) titanium adhesion layer and a ∼50 nm thick gold layer using thermal evaporation (base pressure ∼10⁻⁷ Torr, deposition rate of 0.1 nm/s). To improve the adhesion of cellulose microfibrils, the gold surface was functionalized by a thioglucose monolayer. This monolayer self-assembled by placing the gold-coated slide in a methanol-thioglucose solution for at least 24 h.

The cellulose fibers used in the degradation experiments were bacterial cellulose fibers secreted by *Gluconacetobacter xylinus* bacteria that were obtained from the laboratory of Professor Anthony Clarke. To prepare the cellulose fibers for deposition on the thioglucose-functionalized gold-coated glass slide, they were ball-milled in HPLC grade methanol. To separate the cellulose from any contaminants, the cellulose suspension was centrifuged at 870 × g, 1540 × g, and 1950 × g for 3 minutes at each rotation speed. After each centrifugation, excess methanol was removed, more methanol was added and the cellulose was resuspended by vortexing the mixture for one minute. Chloroform was subsequently added to the suspension to increase the rate of evaporation, which improved the quality of the deposition of the cellulose fibers. The composition of the final mixture was 7:3 chloroform:methanol by volume.

The bead-beaten cellulose fibers were then deposited onto the thioglucose functionalized gold-coated glass slide using a Langmuir-Blodgett (LB) transfer procedure. For these transfers, a KSV 5000 LB system was used. In this procedure, 1 mL of the methanol-chloroform cellulose suspension was carefully dropped by syringe
onto the surface of Milli-Q water in a polytetrafluoroethylene (PTFE) trough filled with Milli-Q water. A piece of clean filter paper (Whatman) was used as a Wilhelmy plate to measure surface tension. PTFE barriers on either end of the trough were then moved towards each other at a rate of 10 mm/min until a surface tension value of 5 mN/m was attained. The functionalized gold-coated glass slide was then dipped 1 cm into the water and removed at a rate of 25 mm/min. This same transfer technique has been used in atomic force microscopy (AFM) studies of enzymatic degradation of cellulose fibers in our collaboration [25].

The cellulose fiber coated slides were stored in a humid container with a reservoir of water to maintain the hydration of the cellulose fibers, as it has been demonstrated that that vacuum dehydration of cellulose affects enzymatic activity [26]. Although the cellulose will be partially dehydrated in suspension in the chloroform-methanol solution, cellulose has a demonstrated high affinity for water in desiccated environments. Several studies have investigated the use of cellulose to dehydrate ethanol, indicating that the stored cellulose should maintain some hydration [27, 28]. Water adsorption to cellulose-based materials (primarily wood chips) was measured for ethanol concentrations up to 97% [28].

2.2 Surface Plasmon Resonance Imaging System

A custom Surface Plasmon Resonance imaging (SPRi) device based on a commercial instrument (SPRImager, GWC Technologies, Inc.) was used to collect all of the cellulose degradation data [21, 29]. The main components of the system are shown in Figure 2.1, consisting of a light source, sample cell, and a CCD camera.
Figure 2.1: Schematic diagram of the custom SPR imaging apparatus.
A miniature quartz-halogen bulb was used as a white light source. The light from the bulb was focused onto a pinhole and collimated by a camera lens and then passed through a linear polarizer. The collimated light beam was then directed onto the sample, passing through a SF-10 (index of refraction \( n = 1.712 \) at \( \lambda = 794.7 \text{ nm} \)) prism and gold-coated SF-10 glass slide. Light reflected from the sample then passed through a narrow bandpass filter (center wavelength of 794.7 nm, FWHM of 1.5 nm, F1.5-794.7-4-25.0M, Melles Griot) and focused onto a CCD camera (WAT 902H2, Watec, 752 x 480 pixels). The angle of incidence \( \theta \), is defined by the angle between the incident light on the prism and the normal of the prism surface in contact with the glass slide. The prism and camera are mounted on two RS-232 controllable rotation stages (RT-3-10, Newmark Systems) to obtain a \( \theta - 2\theta \) rotation geometry for the sample and camera as shown in Figure 2.1. The rotation of the stages is controlled to a precision of 0.004°.

Details of the geometry of the sample cells are shown in Figure 2.2. Three separate 600 µL volume sample cells are formed by a viton gasket which rests between an inset in the Kel-F block and the gold-coated glass slide. Buffer and cellulase can be introduced into each of the sample cells using a syringe or micropipette through holes that are machined in the Kel-F holder. To prevent refraction at the interface between the prism and glass slide, a thin layer of index-matching fluid (\( n = 1.720 \)) (R.P. Cargille Laboratories) was placed on the slide and the prism was clamped to the glass slide within the flow cell, as can be see in Figure 2.2. The temperature of the cell was maintained by controlling the voltage applied to a resistive heating pad using a proportional-integrative-derivative (PID) temperature controller (Eurotherm 808).
Figure 2.2: Left: Photograph of the sample cells with prism. The holes in the optical table, which are on a 1” square lattice, provide a scale reference. Right: Schematic diagram of prism and sample cells. A: Clamp to press prism against gasket (D), forming the seal for the sample cells. B: SF-10 triangular prism. C: Gold-coated SF-10 slide. D: Viton gasket. E: Recesses in the Kel-F block that defines the volume of the sample cells. Openings are for the injection of buffer and cellulase.
2.3 Experimental Procedures

2.3.1 Buffer and Cellulase Preparation

For all experiments, a 50 mM pH 5.0 citrate buffer was used that was prepared by combining 0.3522 g of citric acid and 0.9321 g of sodium citrate in 100 mL of Milli-Q water. All cellulase solutions were provided by Iogen Corporation in pH 5.0, 50 mM citrate buffer. Individual cellulases were separated from a commercial Hypocrea jecorina cellulase secretome by Iogen Corporation. Individual cellulase components were tested for the presence of other major Hypocrea jecorina cellulases (CBH-1, CBH-2, EG-1, EG-2) by performing a Western Blot analysis. Cellulase solutions were stored in the frozen state, and thawed when needed for experiments.

At the beginning of each experiment, buffer was injected into the fluid cells using a 1 mL disposable tuberculin syringe (Fisher). The temperature of the fluid cells was then increased to 50°C. 50 µL cellulase solutions were prepared to the desired concentration and then heated in an incubator (Grant-bio PCH-2) to 50°C. After both buffer and cellulase reached a temperature of 50°C, the cellulase solution was injected into two of the fluid cells. Buffer was maintained in the third fluid cell to serve as a control. The quantity of each individual cellulase injected into the fluid cells was adjusted such that a final concentration of each cellulase in the fluid cell of 23 µg/mL was attained for most experiments. Experiments were also performed using lower concentrations of 1.8 µg/mL and 1.9 µg/mL.
2.3.2 SPR Imaging Data Collection

In the experiments, buffer was introduced into the fluid cells and SPR imaging data collection was initiated. The angle of incidence of the collimated light beam on the gold film was varied from $48^\circ$ to $53^\circ$, and images of the cellulose-coated gold film were collected at $0.1^\circ$ intervals. This was accomplished by rotating the sample cell and camera in a $\theta$-$2\theta$ configuration as shown schematically in Figure 2.1. The average intensity of regions of interest (ROIs) within the images was tracked as a function of angle of incidence $\theta_i$. Examples of ROIs within SPR images are shown in Figure 2.3, together with their corresponding SPR curves.

Tracking changes in SPR images as the angle of incidence $\theta_i$ is varied is complicated by two phenomena: beam walking and beam compression. Beam walking refers to the displacement of the light beam on the sample as $\theta_i$ is changed due to refraction of the light at the surface of the triangular prism [30, 21]. The second phenomenon is image compression or foreshortening, which refers to the increase in the illumination area of the sample as $\theta_i$ is increased, such that surface features occupy different fractions of the image as $\theta_i$ is varied [30, 21, 29]. Schematic representations of the effects of beam walking and image compression are shown in Figure 2.4. Both effects have been compensated by the image collection software as detailed by Allen [21]. The image collection software shifts the location and size of the ROI to compensate for these two effects so that one is measuring the average intensity of the same region of the cellulose-coated gold film at each value of the angle of incidence.

In each experiment, the specific angle range was repeatedly scanned. At each
Figure 2.3: A) SPR image collected at $\theta_i = 50^\circ$ in buffer, before introduction of the cellulase. B) SPR image collected at $\theta_i = 50^\circ$ 24 h after the injection of a solution containing 2.1 $\mu$g/mL CBH1 to the sample cell. C) SPR curves for the same highlighted ROIs before and 24 h after the introduction of the cellulase.
Figure 2.4: Schematic representations of the optical artefacts associated with scanning angle SPR imaging. 

A) The effect of beam walking, due to refraction of the light, in which the shift in the position of the light beam on the base of the prism is indicated by the distance $d$. B) The effect of image compression, or foreshortening in which the relative size of the ROI is smaller at oblique incidence ($\theta_i = 45^\circ$) (right) than for a smaller angle of incidence ($\theta_i = 15^\circ$) (left) due to the increased area of illumination for a uniform diameter beam at more oblique angles. $d_s$ is the size of a ROI on the gold film, and $d_c$ is the size of the image of that ROI on the camera. As the angle of incidence $\theta_i$ is increased, the size of the sample illuminated also increases. This results in the ROI on the gold film covering a reduced fraction of the illuminated sample. Consequentially, the length of the ROI on the camera in plane with the light beam, $d_{cr}$ is decreased as the angle of incidence $\theta_i$ is increased.
angle of incidence, the average intensity for each ROI and the time at which the image was collected was stored. This allowed us to track the time evolution of the SPR curves for each ROI. In each experiment, several ROIs that were representative of both cellulose-coated and bare regions were defined and measured.
Chapter 3

Data Analysis

In this Chapter, I describe the method that we have developed to extract the characteristic parameters of action of individual cellulases and combinations of cellulases on cellulose fibers.

3.1 Fitting and Extracting Kinetic Information From SPR Curves

A SPR curve measured for a region of interest (ROI) within an SPR image can be used to characterize the thickness and index of refraction of adsorbed material on a thin gold film. Typically, SPR curves are interpreted in terms of a film of uniform thickness on a gold film. By assuming index of refraction values for the adsorbed film and the surrounding buffer, the thickness of the film that is assumed to be of uniform thickness can be determined by applying the analysis described in Section 1.4. However, for the cellulose degradation experiments conducted in the present
study, the cellulose coverage, even within a ROI is very heterogeneous. For this case of a nonuniform coating of cellulose, the previously outlined approach is not sufficient.

The SPR curve measured for a substrate that is heterogeneously coated with cellulose fibers is typically broadened and much more shallow than the SPR curve measured for films of uniform thickness. This broadened, shallow SPR curve can be thought of as a weighted superposition of SPR curves for uniform films of different thicknesses. The reduction in the quality of the SPR resonance can be seen in the experimental data shown in Figure 2.3. Determining the best fit distribution of uniform film SPR curves to accurately describe the SPR curve measured for a ROI on a laterally inhomogeneous sample is a challenging problem that is being pursued independently in our laboratory by Dr Adam Raegen. I will instead examine the time dependence of the angle corresponding to the minimum intensity of the SPR curve following the introduction of cellulases, as well as the reflected intensity at the SPR angle (the angle corresponding to the minimum in reflected intensity of light). This angle provides information about the time dependence of the average thickness of the cellulose within a given ROI [23].

The SPR curve attained for a heterogeneously-coated ROI can be thought of as a superposition of a number of SPR curves for adsorbed films of uniform thickness, resulting in a less deep SPR curve. Following from this, the change in minimum reflected intensity gives an indication of the change in the heterogeneity of coverage of the ROI. Although this approach does not provide a complete description of the change in the distribution of cellulose coverage within each ROI with time, it does allow the determination of important information on the rate of action of
cellulases on the cellulose fibers coating the ROI.

## 3.2 Cellulase Kinetics Analysis

It is possible to measure the kinetics of cellulase action by finding the angle of incidence corresponding to the minimum reflected light intensity measured as a function of time after exposure to the cellulase. However, each measured intensity value has a certain level of noise due to fluctuations in the intensity in the light source and variability in cellulose coverage and composition. It is more precise to determine the angle and intensity of the minimum of the SPR curve by fitting a quadratic function to the reflected intensity versus angle of incidence data within a small range of angles (0.5°) centred on the minimum in reflected intensity. A representative data set and corresponding best fit are shown in Figure 3.1, and it can be seen that the quadratic function provides a very good fit to the data over this small range of angles.

We fit a quadratic function to the SPR curve minimum for every SPR curve obtained from each ROI over time. From this we extracted the angle values corresponding to the minimum intensity for each ROI as a function of time. We refer to this angle as the SPR angle. This procedure allows us to determine changes to the average thickness of the cellulose fibers over time. A representative data set of the raw SPR angle data (in red) versus time is shown in Figure 3.2, together with the best fit SPR angle corresponding to the minimum intensity (in black). This comparison demonstrates that the best fit angle is a better parameter for tracking changes in cellulose fiber coverage over time.
Figure 3.1: Average intensity of the ROI indicated in the SPR image shown in Figure 2.3 as a function of the angle of incidence $\theta_i$. The red line corresponds to the best fit of the data points to a quadratic function.
Figure 3.2: SPR angle versus time for a single ROI in Figure 2.3 after exposure to 2.1 µg/mL CBH-1. A comparison is shown between the data points obtained from fitting the minimum in the reflected intensity with a quadratic function (black) and those obtained by selecting the angle corresponding to the minimum intensity datapoint from the raw data (blue). The best fit of the black data points to Equation 3.1 (red) has a time constant of $\tau_a = 154 \pm 3$ min.
The time constant of action of the cellulases on the cellulose fibers within a given ROI can be determined by fitting the best fit SPR angle versus time to an exponential decay function of the form:

\[ \theta(t) = \theta_\infty + \Delta \theta e^{-t/\tau_a} \]  

where \( \theta_\infty \) is the asymptotic value of the SPR angle in the limit of long times, \( \Delta \theta \) is the difference between the initial measured SPR angle and \( \theta_\infty \), \( \tau_a \) is the corresponding time constant of action of the cellulases, and \( t \) is the time. A similar function is fit to the minimum reflected intensity versus time data:

\[ I(t) = I_\infty + \Delta I e^{-t/\tau_i} \]  

where \( I_\infty \) is the asymptotic minimum reflected intensity in the limit of long times, \( \Delta I \) is the difference between the initial measured minimum reflected intensity and \( I_\infty \), \( \tau_i \) is the corresponding time constant of action of the cellulases, and \( t \) is the time.

Many enzyme-catalyzed processes can be understood in terms of Michaelis-Menten kinetics. The Michaelis-Menten model of enzyme action on a substrate assumes a reversible binding of the enzyme to the substrate, forming an enzyme-substrate complex. The substrate-enzyme complex is broken when the substrate is irreversibly converted to product. This process can be described by:

\[ E + S \xrightleftharpoons[k_+]{k_-} ES \xrightarrow{r} E + P \]
where $E$ refers to the enzyme, $S$ refers to the substrate, and $P$ refers to the product, and $k_+, k_-$ and $r$ are the appropriate rate constants. Assuming that the intermediate ES is in a steady state \( \left( \frac{d[E S]}{dt} = 0 \right) \) yields a relation between the rate of action of the enzymes on the substrate, and the substrate concentration and rate constants:

\[
\frac{d[P]}{dt} = V_{\text{max}} \frac{[S]}{K_m} \frac{1}{1 + ([S]/K_m)}
\]

(3.3)

where $V_{\text{max}}$ is the maximum rate of reaction ($V_{\text{max}} = r[E_{\text{tot}}]$) and $K_m$ is the Michaelis constant \( K_m = \frac{k_+ + r}{k_-} \).

However, for the case of the action of the cellulolytic enzymes on cellulose fibers, this model has been found to be deficient [31]. The insolubility of the cellulose fibers, and the difference in reaction rate of the cellulases on crystalline and amorphous regions of cellulose fibers invalidates the use of this model to study the action of cellulases on cellulose fibers. While the rate of action of the cellulases is complicated by these factors, we have found that Equation 3.1 and Equation 3.2 provide a phenomenological model of the action of the cellulases studied on cellulose fiber-coated ROIs.

The change in the average thickness of the cellulose fiber coverage within a given ROI after the introduction of the cellulases can be determined by tracking the shift in the best fit value of the SPR angle. The fractional change in the average film thickness can be written as:

\[
\frac{\Delta h}{h_0} = \frac{\theta_0 - \theta}{\theta_0 - \theta_u}
\]

(3.4)
where $h$ is the average thickness of the cellulose film, $h_0$ is the original average thickness of the cellulose film, $\theta_u$ is the average SPR angle measured on three or four uncoated ROIs, and $\theta_0$ is the SPR angle for the cellulose coated ROI upon initial introduction of cellulase to the sample cell. Equation 3.4 provides another parameter, $\Delta h/h_0$, to describe the action of the cellulases on the cellulose fibers. This calculation assumes that the index of refraction of the cellulose fibers is constant for the duration of the experiment. This is not necessarily true, as in the process of degradation of a cellulose fiber, the crystallinity of regions of the fiber is disrupted. This will result in a change in the index of refraction of those regions. As a result, Equation 3.4 provides only an approximation of the extent of degradation of cellulose fibers coating an ROI.

### 3.2.1 Baseline Adjustment

In some of our datasets we have observed an upwards drift in SPR angle. This has been observed for ROIs coated with cellulose fibers and bare ROIs, as well as for ROIs in cells with and without cellulase introduced. An example of this drift can be seen in Figure 3.3. It is likely that the long-term drift is a result of evaporation, resulting in a higher molarity buffer containing a high concentration of cellulase. This produces an increase in the index of refraction of the buffer, which shifts the SPR angle to a slightly larger value. The initial increase in SPR angle observed in both the bare and coated ROIs is possibly due to an additional increase in index of the buffer from the production of cellobiose. A final cellobiose concentration of $\sim 0.4$ mM was previously measured for similarly prepared cellulose covered gold-
coated glass slides exposed to a *Hypocrea jecorina* cellulase mixture [29].

The drift produces a distortion of the cellulose degradation signal that is described by Equation 3.1. To correct for this, the baseline SPR angle data from uncoated ROIs are subtracted from the raw SPR angle data for a cellulose coated ROI. An example of the SPR angle data before and after the correction was applied is shown in Figure 3.3. Data corrected in this manner will be marked with an asterisk (*) in any results. The magnitude of the shift in the SPR angle seen in the uncoated ROI seen in Figure 3.3 corresponds to a shift in index of refraction of the buffer and enzyme solution of only $2 \times 10^{-3}$. As the fluid cells were originally covered by a piece of viton, there was not an adequate seal to prevent this evaporation. Later experiments used a parafilm covering on the fluid cells to minimize the impact of evaporation on the SPR signal.

### 3.3 Statistics

Since the degradation of cellulose will vary between different ROIs, it is necessary to average the time constants and fractional change in cellulose coverage measured for several ROIs to properly characterize the action of the cellulases. These variations are likely due to local variations in the geometry of the cellulose fiber distribution and in the ratio of crystalline and amorphous cellulose, resulting in different binding sites available to the cellulases.

An example of the distribution of best fit time constants of action after the introduction of CBH-1 to cellulose fibers is shown in Figure 3.4. The distributions are approximately symmetrical, which allows us to use the standard deviation of
Figure 3.3: SPR angle data versus time for A) coated ROI (black) and uncoated ROI (red) from the same sample cell, each exposed to 23 µg/mL CBH-2, B) baseline-corrected data for the same coated ROI. The red line corresponds to the best fit of the data in plot B to Equation 3.1 with a best fit value of $\tau_a = 36.3 \pm 0.7$ min. The Corrected Angle seen in plot B is the difference between the SPR angle measured for a cellulose-coated ROI, and a uncoated, baseline ROI.
Figure 3.4: Histograms of time constants of action obtained from best fits of A. SPR angle to Equation 3.1 and B. minimum reflected intensity to Equation 3.2. C. Histogram of fractional degradation of cellulose coated ROIs.
the dataset to represent the spread in the time constants of action. The error in the mean value is given by the standard deviation of the mean, which is given by:

\[ \sigma_\tau = \frac{\sigma_x}{\sqrt{N}} \]  

(3.5)

In Equation 3.5, \( \sigma_\tau \) is the standard deviation of the mean, \( \sigma_x \) is the standard deviation of the dataset, and \( N \) is the number of data points within the dataset. This parameter is used to characterize the error in the mean time constants of action reported in Chapter 4.

We note that not all ROIs yield SPR angle and minimum reflected intensity data that fit well to the exponential decays specified by Equation 3.1 and Equation 3.2. These anomalies may be due to the presence of bubbles, or fluctuations in light source intensity. These fits are discarded by eye and not included in the datasets that were used in the calculation of the average time constants of action or average fractional change in cellulose coverage. For each cellulase or cellulase combination, over 30 ROIs were used to calculate the average time constants of action and average degradation fraction. For the representative CBH-1 data set shown in Figure 3.4, the average time constant of action is \( 45 \pm 5 \) min for the minimum reflected intensity and \( 69 \pm 6 \) min for the SPR angle. The average fractional change in cellulose coverage is \( 0.55 \pm 0.03 \). The distribution of fractional changes in cellulose coverage that can be seen in Figure 3.4 has a significant spread, with a standard deviation of 0.16. Significant differences in between datasets likely indicate variability in the cellulose crystallinity and coverage.
Chapter 4

Results and Discussion

In this chapter, I describe the results of SPRi experiments performed by injecting cellulases into the SPR fluid cell. These experiments are distinct from those performed by flowing the cellulases through the fluid cell. There are two main reasons for preferring injection experiments over flow experiments. In flow experiments, air bubbles are very often introduced to the system. Air bubbles in the vicinity of an ROI drastically change the SPR signal due to the change in index, invalidating our SPR angle and minimum reflecting intensity tracking for that ROI. Additionally, industrial cellulosic ethanol production is conducted with an introduction of a fixed mass of cellulase, rather than a continuous flow. A summary of the results obtained in fluid flow experiments is given in Appendix A.
4.1 Injection Experiment Results

4.1.1 Single Cellulase Experiments

Enzymatic degradation experiments were performed using concentrations for individual cellulases of 23 µg/mL. A summary of the average time constants associated with the cellulose degradation, determined according to the method in Section 3.2 is given in Table 4.1. The error in the time constant values is the standard deviation of the mean obtained for a number of ROIs in the same SPR image. The degradation fraction, representing the average fraction of cellulose removed from an ROI during an experiment after exposure to a cellulase is also listed in Table 4.1, which is provided at the end of this chapter. These values are calculated using Equation 3.4. Values obtained from datasets involving baseline corrections, as described in Chapter 3, are marked with an asterisk.

As mentioned in Section 3.2, $\tau_i$ is calculated from a best fit of Equation 3.2 to the minimum reflected intensity of the measured SPR curve for the ROI over time. The measured SPR curve can be viewed as a weighted superposition of SPR curves corresponding to different uniform thicknesses of cellulose. Thus, the minimum intensity of the SPR curve is representative of the heterogeneity of coverage of cellulose of an ROI, and $\tau_i$ is a measure of the rate at which the heterogeneity of cellulose coverage of the ROI changes over time. A smaller time constant represents a faster decrease in the heterogeneity of cellulose coverage. $\tau_i$ and $\tau_a$ values measured for the action of cellulase on cellulose fibers may differ, due to the geometry of the exposed surface of the cellulose fibers. More elevated and exposed
regions of the cellulose fibers may be degraded first, producing smaller $\tau_i$ values.

The average values of $\tau_a$ calculated for exposure to CBH-1 and CBH-2 were $69 \pm 6$ min and $35 \pm 5$ min respectively. Similarly, a larger $\tau_i$ value was calculated for ROIs exposed to CBH-1 ($\tau_i = 45 \pm 5$ min), compared to those exposed to CBH-2 ($\tau_i = 14 \pm 1$ min). This indicates an increased rate of reduction of the heterogeneity of cellulose coverage for ROIs exposed to CBH-2. The distributions of time constants of action for cellulases acting on cellulose fibers, as well as examples of minimum reflected intensity data, SPR angle and best fits to these data are given in Figure 4.1 for CBH-1 and Figure 4.2 for CBH-2.

The average degradation fraction calculated for cellulose fibers exposed to CBH-1 is $0.55 \pm 0.03$, and for CBH-2 is $0.75 \pm 0.02$. The larger degradation fraction observed for exposure to CBH-2 indicates that there are more binding sites available for CBH-2 than for CBH-1. An increased number of available binding sites results in an increased number of glycosidic bonds accessible to cleaving by cellulase. A disruption in crystallinity of the cellulose fiber will result in more binding sites available to the cellulases. An increase in the number of binding sites would also contribute to the increased rate of action for CBH-2. Similar increases in enzymatic hydrolysis of cellulose have been previously measured for increased binding site availability [32].

### 4.1.1.1 Low Cellulase Concentration Experiments

We performed experiments in which a low concentration of EG-1 and CBH-2, 1.8 µg/mL and 1.9 µg/mL respectively, was exposed to cellulose fibers. The resulting average $\tau_a$ and $\tau_i$ values obtained from the best fits are shown in Table 4.1. The
Figure 4.1: A) SPR angle data versus time, for a single ROI after exposure to 23 µg/mL CBH-1. The red line corresponds to the best fit of the data to Equation 3.1 with a best fit value of $\tau_a = 38.4 \pm 0.3$ min. B) Minimum reflected intensity data versus time for a single ROI after exposure to 23 µg/mL CBH-1. The red line corresponds to the best fit of the data to Equation 3.2 with a best fit value of $\tau_i = 54.0 \pm 0.2$ min.
Figure 4.2: A) SPR angle data versus time, for a single ROI after exposure to 23 µg/mL CBH-2. The red line corresponds to the best fit of the data to Equation 3.1 with a best fit value of $\tau_a = 36.8 \pm 0.7$ min. B) Minimum reflected intensity data versus time for a single ROI after exposure to 23 µg/mL CBH-2. The red line corresponds to the best fit of the data to Equation 3.2 with a best fit value of $\tau_i = 19.1 \pm 0.6$ min.
CBH-2 results demonstrate an increase in both average time constants of action compared with the results obtained from the higher concentration of 23 µg/mL. A \( \tau_i \) value of \( 289 \pm 23 \) min and a \( \tau_a \) value of \( 232 \pm 11 \) for the 1.9 µg/mL CBH-2 experiments were obtained. In addition, there was a reduction in the average degradation fraction for the low concentration CBH-2 experiments, measured to be \( 0.171 \pm 0.008 \) for the 1.9 µg/mL CBH-2 experiments, compared to a value of \( 0.75 \pm 0.02 \) for the 23 µg/mL experiments. The decrease in degradation fraction for exposure to a lower concentration of CBH-2 may be a result of a decrease in cellulase activity over time. In other studies, a decrease in cellulase activity with time has been measured after exposure to cellulase [33]. In an AFM study by Igarashi et al., clusters of cellulase would immobilize on the cellulose fiber over time, reducing overall activity [34]. As the lower concentration of CBH-2 has a longer time constant of action, this may result in less degradation of cellulose due to the decrease in cellulase activity over time.

For the 1.8 µg/mL EG-1 experiments, \( \tau_a \) best fit values of \( 282 \pm 2 \) min and \( \tau_i = 170 \pm 10 \) min were obtained. An average degradation fraction of \( 0.19 \pm 0.02 \) was obtained for the 1.8 µg/mL EG-1 experiments. In some EG-1 SPR angle datasets, an initial increase in SPR angle is observed, similar to that observed in Figure 4.5. This feature can be attributed to adsorption of the enzyme to the cellulose fibers upon the introduction of cellulase to the system, as subtracting the SPR angle data for an uncoated ROI eliminates this feature as shown in Figure 4.3.
Figure 4.3: A) SPR angle data versus time, for a single ROI after exposure to 1.8 µg/mL EG-1. B) The same ROI seen in plot A after the background SPR signal for a bare ROI has been subtracted, as outlined in Section 3.2.1. The red line corresponds to the best fit of the data in plot B to Equation 3.1 with a best fit value of $\tau_a = 350 \pm 3.5$ min.
Figure 4.4: A) SPR angle data versus time, for a single ROI after exposure to 1.9 µg/mL CBH-2. The red line corresponds to the best fit of the data to Equation 3.1 with a best fit value of $\tau_a = 194 \pm 2$ min. B) Minimum reflected intensity data versus time for a single ROI after exposure to 1.9 µg/mL CBH-2. The red line corresponds to the best fit of the data to Equation 3.2 with a best fit value of $\tau_i = 260 \pm 2$ min.
Figure 4.5: A) SPR angle data versus time, for a single ROI after exposure to 1.8 µg/mL EG-1. The red line corresponds to the best fit of the data to Equation 3.1 with a best fit value of $\tau_a = 253 \pm 3$ min. B) Minimum reflected intensity data versus time for a single ROI after exposure to 1.8 µg/mL EG-1. The red line corresponds to the best fit of the data to Equation 3.2 with a best fit value of $\tau_i = 237 \pm 3$ min.
4.1.1.2 Comparisons With Results of Atomic Force Microscopy Experiments

In our collaborative research project, atomic force microscopy (AFM) was used to measure the effect of exposure of bacterial cellulose fibers to CBH-1 and EG-1 at room temperature [35]. In these experiments, EG-1 and CBH-1 were introduced at concentrations of 4-7 µg/mL and 23 µg/mL respectively [35]. Cellulase activity was tracked with time of exposure to the enzymes by performing an analysis of the volume of the cellulose fibers from the topography AFM images. Typically, the volume change of different regions of a cellulose fiber was measured. Representative plots and images from the AFM study are shown in Figure 4.6. The end regions refer to kinks and the ends of cellulose fibers, where many more cellulose chain ends are exposed to action by the cellulases.

CBH-1 was observed to degrade the cellulose fiber over the course of an hour before the activity of the cellulase was greatly diminished. The reduction in volume of the end and crystalline regions after exposure to CBH-1 was observed to be different. The volume of the end regions of the cellulose fiber decreased by approximately 30%, and that of the crystalline regions decreased by approximately 20%, indicating that the structure of the cellulose fibers affects the amount of cellulose that can be degraded. The one hour time of action measured in the AFM volume analysis experiments is similar to the average time constant of action \( \tau_a = 69 \pm 6 \) min measured in the present SPR imaging experiments. The average degradation fraction measured in our SPR experiments with 23 µg/mL CBH-1 was 0.55 ± 0.03. This is a decrease in cellulose volume that is greater by a factor of 2 than that obtained for the end region and by a factor of 3 than that obtained for the crystalline
Figure 4.6: Volume analysis of cellulose fibers after exposure to 23 µg/mL CBH-1 (top) and 4–7 µg/mL EG-1 (bottom), with accompanying AFM topography images. Plots and images were provided courtesy of Amanda Quirk [35].
region, measured in the AFM experiments. This discrepancy may be due to the different experimental conditions. For example, the AFM experiment was conducted at room temperature, while the SPR experiment was conducted at 50 °C.

Upon introduction of EG-1 to the cellulose fibers, both swelling and a reduction in the volume of the cellulose fibers was observed using AFM. For end regions of the cellulose fibers, an initial degradation which occurred for 0.5 hours was followed by a long-term swelling of the end regions. For crystalline regions of the cellulose fibers, a small amount of initial degradation was overwhelmed by swelling in those regions during the first 10 minutes of action. The initial swelling was followed by long term degradation of the crystalline regions of the cellulose fibers. Whereas the AFM experiments allow for analysis of individual regions of a cellulose fiber, the present SPR imaging experiments instead allow the tracking of the average behaviour of a large number of cellulose fibers. From these measurements, fits to the minimum reflected intensity and SPR angle data yielded average time constants of action of $\tau_i = 170 \pm 10$ min and $\tau_a = 282 \pm 2$ min.

4.1.2 Multiple Cellulase Results

In addition to the single cellulase experiments, a series of experiments were performed using combinations of cellulases. For each experiment, the individual cellulase concentration was 23 µg/mL. The resulting best fit $\tau_i$ and $\tau_a$ time constant values are given in Table 4.1. CBH-1 and EG-1 acting together on cellulose fibers results in average $\tau_i$ and $\tau_a$ values of $14 \pm 2$ min and $15 \pm 1$ min respectively. These $\tau_i$ and $\tau_a$ values are much smaller than those measured for CBH-1 acting alone on
cellulose fibers: $\tau_i = 45 \pm 5$ min and $\tau_a = 69 \pm 6$ min. Also, an increased average degradation fraction of $0.60 \pm 0.02$ for the action of the combination of CBH-1 and EG-1 was measured, which was slightly greater than the value of $0.55 \pm 0.03$ measured for CBH-1 acting alone. Examples of minimum reflected intensity data and SPR angle data as well as the best fits to these data are shown in Fig. 4.7 for the combined action of CBH-1 and EG-1.

The reduction in the average time constants of action for the combination of CBH-1 and EG-1, compared to that measured for CBH-1 acting alone, indicates an increased rate of action, whereas the slight increase in the degradation fraction indicates that significant new regions of the cellulose fibers are not available for degradation by the addition of EG-1. As EG-1 is an endoglucanase, it will serve to increase the number of binding sites available to CBH-1 by disruption of $\beta$-1,4 linkages located randomly along the cellulose chains. This increase in the available binding sites for CBH-1 increases the number of CBH-1 cellulases acting on the cellulose fiber at any given moment, resulting in a shorter time constant of action. Over the course of the experiment, no net additional binding sites are made available, as there is not a significant increase in degradation fraction.

CBH-1, CBH-2 and EG-1 acting together on cellulose fibers resulted in very short time constants of action. An average $\tau_a$ value of $5.7 \pm 0.7$ min was obtained. We were not able to obtain a reliable value of $\tau_i$ due to the fast action of the cellulases. Examples of minimum reflected intensity data and SPR angle data as well as the best fits to the data, are shown in Figure 4.8 for the combined action of CBH-1, CBH-2, and EG-1. A degradation fraction of $0.79 \pm 0.01$ was obtained for cellulose fibers exposed to the combination of CBH-1, CBH-2, and EG-1. Both the
Figure 4.7: A) SPR angle data versus time, for a single ROI after exposure to 23 µg/mL CBH-1 and EG-1, individually. The red line corresponds to the best fit of the data to Equation 3.1 with a best fit value of $\tau_a = 12.1 \pm 0.3$ min. B) Minimum reflected intensity data versus time for a single ROI after exposure to 23 µg/mL CBH-1 and EG-1, individually. The red line corresponds to the best fit of the data to Equation 3.2 with a best fit value of $\tau_i = 7.9 \pm 0.2$ min.
Figure 4.8: A) SPR angle data versus time, for a single ROI after exposure to 
23 µg/mL CBH-1, CBH-2, and EG-1, individually. The red line corresponds to 
the best fit of the data to Equation 3.1 with a best fit value of $\tau_a = 4.7 \pm 0.2$ min. 
B) Minimum reflected intensity data versus time for a single ROI after exposure to 
23 µg/mL CBH-1, CBH-2, and EG-1, individually.
reduced average time of action and increased degradation fraction obtained for the combination of these cellulases indicate greater activity, compared with experiments performed using single cellulases and experiments performed with combinations of two cellulases. This indicates that additional binding sites are available for cellulase action, and that additional regions of the cellulose fibers are degraded. As CBH-2 binds to the opposite end of the cellulose chain from CBH-1 (the non-reducing end of the cellulose chain) additional binding sites will be available, likely resulting in an increased average degradation rate. The increased average degradation fraction relative to that measured for the combination of CBH-1 and EG-1 indicates that new areas of cellulose fibers are accessible to the cellulases.

The increase in average degradation fraction for cellulase combination experiments, compared with individual cellulase experiments indicates that an increased number of binding sites are available with the additional cellulases present. Together with the overall decrease in both time constants of action, $\tau_i$ and $\tau_a$, for the cellulase combination experiments, there is some evidence for cooperativity of action of the different cellulases studied. However, further experiments are required to quantitatively determine the extent of synergy obtained for combinations of cellulases. In addition to results detailed in this chapter, evidence of synergy of action of CBH-1 and CBH-2 is described in Appendix A.
<table>
<thead>
<tr>
<th>Cellulase</th>
<th>Individual Cellulase Concentration (µg/mL)</th>
<th>Average Time Constant of Action (min)</th>
<th>Average Degradation Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\tau_i$</td>
<td>$\tau_a$</td>
</tr>
<tr>
<td>CBH-1</td>
<td>23</td>
<td>45 ± 5</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>CBH-2</td>
<td>23</td>
<td>14 ± 1</td>
<td>35 ± 5*</td>
</tr>
<tr>
<td>EG-1</td>
<td>1.8</td>
<td>170 ± 10</td>
<td>282 ± 2</td>
</tr>
<tr>
<td>CBH-2</td>
<td>1.9</td>
<td>289 ± 23</td>
<td>232 ± 11</td>
</tr>
<tr>
<td>EG-1 + CBH-1</td>
<td>23</td>
<td>14 ± 2</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>EG-1 + CBH-1 + CBH-2</td>
<td>23</td>
<td>&lt;1</td>
<td>5.7 ± 0.7*</td>
</tr>
</tbody>
</table>

Table 4.1: Average time constants of action of cellulase types on ROIs. $\tau_i$ and $\tau_a$ represent the average time constants of action of the cellulase or cellulase mixture corresponding to changes in the minimum reflected intensity and angle, respectively, measured for different ROIs in an SPR image, and fit to Equation 3.1 and Equation 3.2. The degradation fraction is calculated using Equation 3.4, which represents the fraction of cellulose removed from the cellulose fibers in an ROI.
Chapter 5

Summary and Conclusions

5.1 Summary of Results

The results of the present study can be split into two categories: improvements to experimental procedures and cellulose degradation results.

5.1.1 Experimental Improvements

In the present study, a number of improvements were made over the original experimental design detailed by Scott Allen [21]. Instead of collecting reflected intensity measurements at a few points along the SPR curve to determine the inflection point, and using the change in inflection point to determine the rate of action of cellulases on cellulose fibers, entire SPR curves are now collected. From these curves, the minimum reflected intensity and SPR angle are used as parameters measuring the heterogeneity of coverage and average thickness of coverage of cellulose fibers. For accurate measurement of the SPR angle, a quadratic function was fit to
the data points in a small range of angles around the minimum in reflected intensity to obtain the best fit angle corresponding to the minimum in reflected intensity. This reduces the effect of noise that is present in individual measurements, and increases the precision of the measured SPR angle. The data analysis was automated by MATLAB code which allowed quicker analysis of large numbers of ROIs. Instead of manually analyzing dozens of ROIs per experiment in OriginLab, with each ROI taking several minutes to analyze, the MATLAB code can parse an entire experiment, and analyze all of the ROIs in minutes.

5.1.2 Experimental Results

The action of the individual cellulases of the *Hypocrea jecorina* cellulase complex was measured on bacterial cellulose fibers produced by *Gluconacetobacter xylinus*. The activity of CBH-1 and CBH-2 acting alone was measured using a concentration of 23 µg/mL. The average time constants of action, $\tau_a$ and $\tau_i$, were smaller for CBH-2 compared to those measured for CBH-1. Additionally, an increased degradation fraction was measured for cellulose fibers exposed to CBH-2. This suggests that there are a greater number of binding sites available to CBH-2, compared to CBH-1. Single enzyme experiments using CBH-2 and EG-1 were performed using a smaller concentration of 1.9 µg/mL and 1.8 µg/mL.

In addition to the single enzyme experiments, a series of cellulase synergy experiments was conducted. Experiments were performed by introducing CBH-1 and EG-1 concurrently to cellulose fibers, as well as introducing all three of the cellulases together concurrently, each with a concentration of 23 µg/mL. For ex-
periments involving the concurrent introduction of CBH-1 and EG-1, and the concurrent introduction of all three enzymes, decreases in the average time constants of action were measured, together with increases in the average degradation fraction, relative to those observed in the single cellulase experiments. These results suggest synergy between CBH-1, CBH-2 and EG-1 in degrading cellulose fibers.

5.2 Future Work

There are several changes to the experimental apparatus and procedure that could improve the quality of the data collected. For the sample cell it would be very useful to have more accessible chambers and an easier method of sealing the chambers from the atmosphere. Currently, an aluminum bar blocks easy access to the center sample cell. For long experiments, evaporation of the buffer occurs as the current method of sealing the sample cells (parafilm) can fail (peel off), resulting in considerable evaporation of buffer in the sample cells. A better method of sealing the sample cells would improve the long term stability of the apparatus.

Although we have achieved insight into the action of the *Hypocrea jecorina* cellulase system of cellulose, additional experiments could be conducted to improve our understanding. Measuring the effect of cellulase concentration on the action of individual cellulases and cellulase combinations would help to understand the synergy between cellulases. Specifically, an experiment measuring the degradation of cellulose fibers upon introduction of $23 \mu\text{g/mL}$ EG-1 would improve our understanding of any cooperative action between EG-1 and the cellobiohydrolases. In addition to this, performing AFM measurements of the topography of
ROIs before and after exposure to cellulases would allow the determination of the rate of removal of cellulose. We could then correlate these changes in cellulose coverage to changes in the SPR curves. Additionally, experiments involving other components of lignocellulosic biomass such as lignin would help in interpreting the action of cellulases on industrial substrates. In particular, these experiments would help determine whether lignin serves to sequester the cellulases, retarding degradation of the cellulose fibers.

In addition, there are improvements to the analysis procedure that would increase the amount of useful information extracted from these experiments. For example, fitting a convolution of SPR curves corresponding to different uniform thicknesses of cellulose films to experimental data would provide information on changes to distribution of cellulose coverage with time of exposure to cellulases within an ROI.
Appendix A

Flow Experiments

In addition to the cellulase injection experiments outlined in Chapter 4, a series of experiments in which buffer and cellulase were flowed continuously over the cellulose-coated gold substrates were conducted. The differences in procedure and apparatus, as well as the results of these experiments are outlined below.

A.1 Methods and Materials

The cellulose and substrate preparation steps outlined in Section 2.1 for the injection experiments are identical to those used for the flow experiments. However, the buffer and cellulase preparation does differ for the flow experiments. The buffer used in the flow experiments is a 50 mM pH 5.4 citrate buffer instead of the 50 mM pH 5.0 citrate buffer used in the injection experiments. The flow experiments were conducted at pH 5.4 as previous experiments conducted by Allen [21]. The pH of the buffer was changed for the injection experiments to more closely re-
flect conditions for industrial cellulosic ethanol production. In addition, different cellulase concentrations were used for the flow experiments, as listed in Table A.1.

The optical layout shown in Figure 2.1 for the injection experiments was also used in our flow experiments, with modifications to the prism and sample cell configuration. The configuration used for the flow experiments is shown in Figure A.1. The chambers for the flow experiments are smaller (~50 µL) than those used in the injection experiments (~600 µL). Additionally, the Viton gasket defines only two sample cells. To flow the buffer and enzyme into the sample cells, a 12-roller peristaltic pump (Ismatec) with Tygon tubing (Cole-Parmer) is used in line after the sample cells. Before the sample cells a degasser (4-channel Micro-Elite, Alltech) is used to reduce the number of air bubbles. All components of the system are connected by PEEK™ tubing (UpChurch Scientific). The Tygon tubing is only used in the peristaltic pump, and is connected to PEEK™ tubing by an adaptor.

Before data collection begins, buffer is flowed into both sample cells, which are heated to 50 °C by a resistive heating pad. After a temperature of 50 °C is reached, the same data collection protocols outlined in Section 2.3.2 are used to collect SPR curves for a selection of ROIs for the course of the experiment. The inlet to one of the sample cells is then changed from flowing buffer to cellulase in buffer, with buffer continuously flowed through the other sample cell as a control.

A.2 Results and Discussion

The data analysis techniques outlined for the injection experiments in Chapter 3 have also been used to analyze the results of the flow experiments. For exposure
Figure A.1: Left: Schematic diagram of prism and sample cells. A: Clamp to press prism against gasket (D), forming the seal for the sample cells. B: SF-10 triangular prism. C: Gold-coated SF-10 slide. D: Viton gasket. E: Channels through which the buffer and cellulase solutions are flowed. Right: Top-down view of Viton gasket. F: Viton Gasket. G: Sample cells formed by the gasket. H: Channel through which the buffer and cellulase solutions are flowed.
to 23 µg/mL CBH-1 and 22 µg/mL CBH-2, the average values of $\tau_a$ were 89 ± 5 min and 79 ± 5 min, respectively. The average $\tau_i$ values calculated for exposure to the same concentrations of CBH-1 and CBH-2 were 53 ± 3 min and 39 ± 3 min, respectively. Both of the average $\tau_a$ and $\tau_i$ values measured for exposure to CBH-2 were smaller than those measured for exposure to CBH-1. The corresponding average degradation fractions were 0.52 ± 0.03 and 0.48 ± 0.03 for exposure to CBH-1 and CBH-2, respectively.

For exposure to 8.8 µg/mL EG-1, an average value of $\tau_a = 140 ± 30$ min and an average value of $\tau_i = 93 ± 6$ were measured. The corresponding value of the average degradation fraction was measured to be 0.15 ± 0.03. Both of the average time constants of action measured for EG-1 exposure are longer than those measured for CBH-1 and CBH-2. Additionally, the degradation fraction measured for EG-1 is significantly lower (by a factor of ~3) than that measured for CBH-1 and CBH-2. Although the concentration of EG-1 was lower than that of CBH-1 and CBH-2, the dramatic difference in activity that was measured indicates a much higher activity for CBH-1 and CBH-2 than for EG-1 exposed to cellulose fibers.

For the simultaneous introduction of 23 µg/mL CBH-1 and 22 µg/mL CBH-2, average values of $\tau_a = 27 ± 2$ min, and $\tau_i = 19 ± 1$ min were obtained. An average degradation fraction of 0.45 ± 0.06 was also measured. Although the time constants of action measured for the simultaneous introduction of CBH-1 and CBH-2 were smaller than for the individual cellulases, the degradation fraction values obtained for exposure to CBH-1 and CBH-2 individually and in combination were very similar. This result indicates that although the rate of action of the cellobiohydralases may be improved by simultaneous introduction of the enzymes, the fraction of
cellulose digested is comparable.

Both the change in heterogeneity and change in thickness of cellulose coverage of an ROI can be described by exponential decay functions. Generally, two independent decay processes acting on one substrate can be described by the product of two exponentials:

\[ A = A_0 e^{-t/\tau_1} e^{-t/\tau_2} \]  \hspace{1cm} (A.1)

This can be simplified to a single exponential decay function with an effective time constant of action:

\[ \tau_{eff} = \frac{\tau_1 \tau_2}{\tau_1 + \tau_2} \]  \hspace{1cm} (A.2)

Using Equation A.2 to determine the predicted time constants of action for independent and simultaneous action of CBH-1 and CBH-2 on the same cellulose substrate yields values of \( \tau_i = 22.5 \pm 0.1 \) min and \( \tau_a = 41.9 \pm 0.1 \) min. The measured average time constants of action for simultaneous action of CBH-1 and CBH-2 on the same cellulose substrates were \( \tau_i = 19 \pm 1 \) min and \( \tau_a = 27 \pm 2 \) min. This indicates a decrease in the measured time constants of action of 16% for \( \tau_i \), and 36% for \( \tau_a \) compared to the calculated values. The decrease in time constants of action indicates cooperative action of CBH-1 and CBH-2 on cellulose.

While the differences in experimental conditions between the injection and flow experiments makes direct comparison difficult, similar trends are evident in both types of experiments. Average time constants of action, and degradation fractions measured for exposure to CBH-1 and CBH-2 are similar, though smaller time con-
stants of action for CBH-2 have been measured for both injection and flow experiments. Average $\tau_i$ values measured tend to be smaller than their corresponding average $\tau_a$ values for the action of all cellulases and cellulase combinations. As mentioned in Section 4.1.1, the smaller time constants of action for changes in heterogeneity may be a result of regions of increased sample thickness having increased accessibility to cellulases, allowing for them to be degraded faster. In addition, the average time constants of action for CBH-2 have been measured to be smaller for both flow and injection experiments. Similarly, average degradation fractions for exposure to CBH-2 were shown to be larger than for exposure to EG-1 for both flow and injection experiments.
Table A.1: Average time constants of action of cellulase types on ROIs. $\tau_i$ and $\tau_a$ represent the average time constants of action of the cellulase or cellulase mixture corresponding to changes in the minimum reflected intensity and angle, respectively, measured for different ROIs in an SPR image, obtained by fitting the SPRI data to Equations 3.1 and 3.2. The degradation fraction is calculated using Equation 3.4, which represents the fraction of cellulose removed within a ROI. The EG-1 results were obtained from 5 ROIs. For each of the other results, at least 15 ROIs were used.
Bibliography


