Roskilde Universitet / Roskilde University

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Real-time Calorimetric Investigation of the slow-down effect in enzymatic hydrolysis of cellulose.

Figure 1. *Picture taken from our experiment. Indicating Regenerated amorphous cellulose (on the left), Avicel (on the right) and TAMIII calorimeter in the background.*

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2.Abstract

One of the current challenges facing the scientific community is optimising the enzymatic hydrolysis of cellulose, as this represents a bottleneck in the production of second generation bioethanol. This project focuses on exploring possible reasons as to why the rate of the enzymatic hydrolysis consistently shows an exponential decreases over time (known as the slow-down effect). More specifically, our investigation centers on how the structure of cellulose may be related to this effect.

To do so, our primary method was isothermal calorimetry using a TAMIII calorimeter in conjunction with the 'restart' method: after an initial standard hydrolysis where the conditions were the same for all three samples in each experiment, two of the three directly received either an additional dose of enzyme or additional fresh substrate - which replaced some of the original enzyme-substrate mix - in varying quantities. This was done over a series of 5 experiments, with either different standard conditions or changes made during the restarts. The TAMIII measured the heat produced by the hydrolyses in Watts, providing real-time insight into the reactions. We performed these experiments using Cellic CTec2 from Novozymes and two types of cellulose substrates, which differ in their structure: Avicel and regenerated amorphous cellulose (RAC).

Our findings showed comparable increases in reaction rate when either CTec2 or fresh Avicel was added. However, the increases when Avicel was added showed direct proportionality to the amount added and increased the average specific activities of the enzymes, whereas the addition of extra CTec2 resulted in reaction rate increases that were not proportional to the amount of enzymes present because of a decrease in overall specific activities. This has implications for the biofuel industry, where enzyme efficiency is highly important. Analysis of lines of best fit matching with our data yielded the conclusion that there may be two factors affecting the decrease in reaction rate: one with a major contribution and the other comparatively minor.

Based on our results and literature findings, we suggest that the major contributor to the slow-down effect was a decreasing amount of amorphous cellulose, which is degraded at a much higher rate than the crystalline form, and the minor part was slight denaturation of the cellulases. Furthermore, we confirmed that product inhibition is an unlikely cause of the slow-down within our range of Avicel conversion (13.49% - 22.15%). We could not make any conclusions relating to RAC as we experienced problems using this substrate in our experimental setup.

3. Investigation

3.1 Introduction

In the recent years industrial interest has shifted from conventional fossils fuels to alternative sources. This can be related to socioeconomic factors, which have played out, ranging from economic crises to environmental concerns, making its price fluctuate and its use environmentally unsustainable. One such alternative fuel source is the utilization of lignocellulosic biomass to produce ethanol (Raghuwanshi, 2014), which is known as second-generation biofuel.

Biomass can be obtained from most agricultural crop residue and is therefore a readily available source of potential energy (Kumar, 2008). One of the most recent methods of biomass conversion utilizes hydrolytic enzymes, which degrade it to fermentable sugars. However, cellulose, which is the most abundant constituent present in biomass, is not easily degradable and hence presents an obstacle to the practicality of this alternative source.

The enzymes used are cellulases, which facilitate degradation of biomass to sugars such as glucose, cellobiose and cello-oligosaccharides (Jutru, 2014). The focus of the researchers' efforts has been optimizing this conversion as to make it economically viable. To degrade biomass, a mixture of different cellulases, referred to as 'enzyme cocktail' is used, as to enhance the efficiency thereof. Other factors also come into play, such as stability of the mixture, product inhibition, specificity, synergism between different enzymes, productive binding to the cellulose, physical characteristics, and the composition of the lignocellulosic biomass (Heinelman, 2009).

It is difficult to achieve optimal results due to the lack of available knowledge regarding the mechanisms underlying enzyme activity in the hydrolysis of cellulose (Mielenz, 2001). One of those mechanical mysteries which is put into focus of this paper is the rate of cellulose hydrolysis, which appears to exponentially decrease over time, regardless of the excess amount of the substrate.

To examine this problem, we chose isothermal calorimetry as a unique method, which allows for real-time observation of the reaction. It seems to be an interesting approach to investigating how adding extra substrate or enzyme changes the course of the reaction and the extent of its effect on the slow-down of the rate of reaction.

3.2 Research question

This has paved way for our research question which is the center of this investigation:

"How is the 'slow-down' effect in enzymatic cellulose hydrolysis related to enzymatic issues and the structure of the substrate?"

3.3 Theory

3.3.1 Cellulose

Cellulose is an abundant organic compound, which consists of several hundred to several thousand β (1-4) linked glucose monomers, as shown in *figure 2*. It has a repeating unit of two glucose molecules connected via ketal linkage with one β glucose linkage flipped by 180 degrees (Horn *et al.*, 2012). Moreover, the unbranched cellulose chains are densely packed through hydrogen bonds between chains. It is a key component of primary cell walls of the plants, which gives them, among other things, rigidity and enables them to stand upright (Jin, 2015).

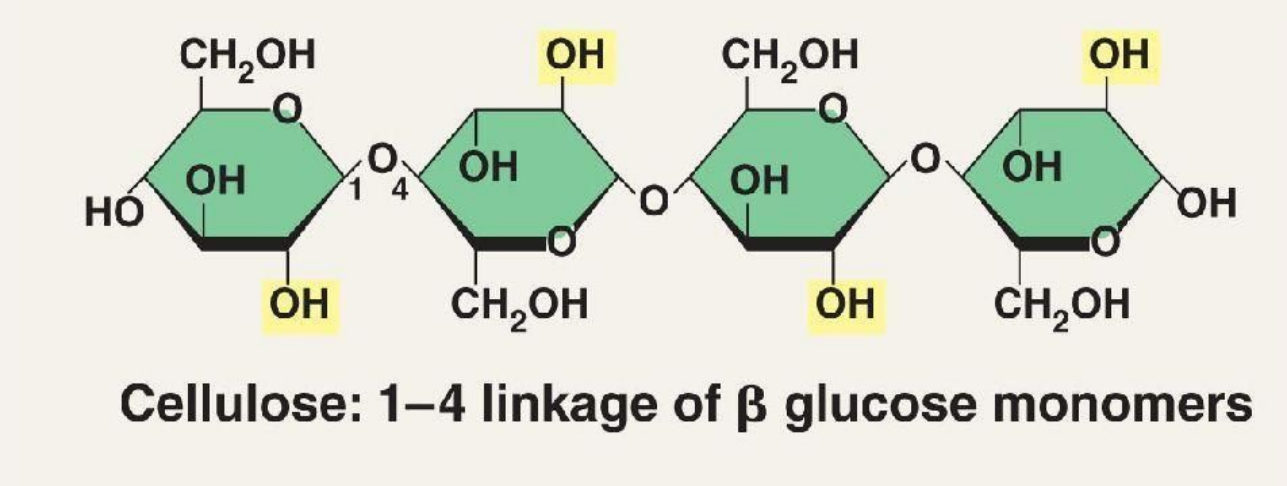

Figure 2. The basic structure of cellulose (Holtzclaw, 2010)

There are two types of hydrogen bonds in cellulose, the one between C_3 OH group and the oxygen of the pyranose ring within the same molecule and the other between C_6 OH group of the same molecule and oxygen of the glycosidic bond of another molecule as shown in *Figure* 2. Generally, β (1-4) glycosidic bonds are difficult to break. Additionally, these hydrogen bonds can make a very tight, ordered crystalline structure, which make the bonds difficult to access for water and cellulases. Amorphous cellulose, on the other hand, which has a more disordered, less tightly packed structure, allows for more penetration of cellulases (Wang, 2018). This is explained further in the following section (*Section 3.3.2),* which deals with these enzymes.

3.3.2 Cellulases

There are generally two ways to hydrolyse the cellulose; chemically and enzymatically (Jin, 2015). In the chemical method, highly concentrated acid is used to hydrolyse the cellulose under high temperature and pressure, but this method is not preferable because of the heterogeneous toxic by-products produced during the reaction (Jin, 2015). The other method involves enzymes:

Cellulases are a group of enzymes that hydrolyse the β(1-4) glycosidic bonds in cellulose chains. In nature, they are produced primarily by wood-degrading organisms such as fungi and bacteria (Zhang and Zhang, 2013). In general, there are three types of enzymes secreted for this purpose as can be seen in the figure below.

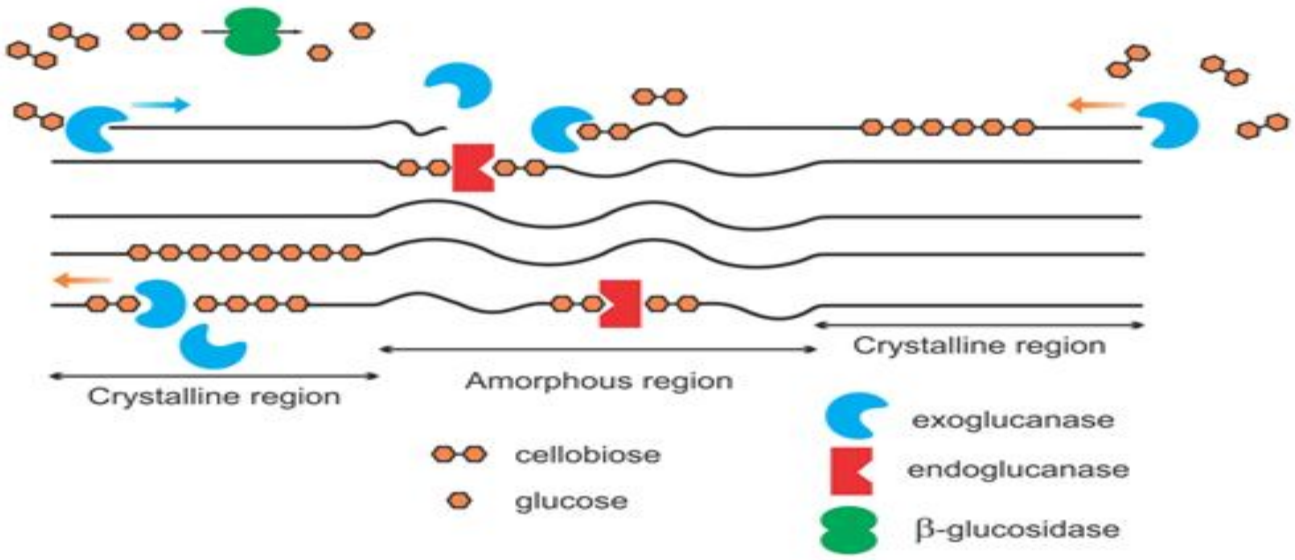

Figure 2. Shows the synergic action of different cellulases. Exoglucanase, Endoglucanase and β-glucosidase. (Akhtar et al., 2016)

Endoglucanases are one type of cellulase which cuts internal bonds in the cellulose chain and introduces several non-consecutive chain-breaks. Cellulases of the second type are called exoglucanases (also called cellobiohydrolases), which begin at the ends of the cellulose chains and hydrolyse them processively (without detaching in between) to cellobiose. β-glucosidases, the third type (which is not technically a cellulase but plays an important role in cellulose degradation), then convert the cellobiose into glucose (Horn *et al.*, 2012), as shown in *figure* 2.
Since each enzyme type described above has different capabilities, which function in cooperation with each other, enzyme cocktails (mixtures) have been investigated as a more effective alternative to pure enzymes. The breaks introduced by endoglucanases provide more chain ends for the cellobiohydrolases to attach to, and the cellobiose produced by the cellobiohydrolases provides the substrate required for β-glucosidases to produce glucose (the generally desired product) by further hydrolysis.

Cellulases have different affinities for crystalline and amorphous regions of the cellulose. A study done on cellobiohydrolases and endoglucanases showed that cellobiohydrolases have substrate binding subsites and product binding subsites and are able to hydrolyse crystalline substrate to produce mainly cellobiose, whereas endoglucanases have more open substrate binding groove, which show poor activity towards crystalline substrate and are presumed to act mainly on amorphous or distorted substrate and produce significant amount of glucose (Gruno et al., 2004). This is shown by the locations of the different enzymes in *Figure 2.*

All three of the aforementioned enzymes function in the pH range 5 - 5.5 (Novozymes, 2010), but the optimal temperature range for β-glucosidase activity, which is 45°C - 55°C, has a slightly higher lower limit than the optimal temperature range for the cellulases exoglucanase and endoglucanase (40°C - 50°C)(Gautam *et al.*, 2018).

3.3.3 Slow-Down Background

The decrease of the enzymatic activity during the course of interaction between the cellulases and the cellulose is what generally is referred to as the slow-down effect in literature. The reaction starts off rapidly, then over the course of time, it exponentially slows down. The reason(s) for this occurrence are not concretely known, although several possibilities have been suggested. Current research suggests that the decrease in the rate of the reaction is mainly substrate dependent, rather than enzyme dependent. This means that the traits of the substrate play a more significant role in the slow-down and overall rate of the reaction than the traits of the enzyme.

Factors that come into play are the structural features of the specific substrate, such as crystallinity, particle size, pore volume, lignin or hemicellulose content and surface area accessible to the enzyme (Lee, 1997; Fan *et al.,* 1980; Dusterhoft *et al.*, 1993).

In any sample of cellulose, there is great variability when it comes to any of the before mentioned parameters, which makes studying the structural features affecting the hydrolysis difficult (Lynd *et al.,* 2002). E.g. one sample may have longer chains of cellulose, whereas another may have shorter ones, and experimental results would hence vary depending on such differences.

Crystallinity is known to play a significant role in the hydrolysis of cellulose. Crystalline structures of cellulose are hydrolysed with more difficulty than its amorphous counterparts. Research has been conducted with pure cellulose, suggesting higher hydrolysis rate of amorphous cellulose compared to that of crystalline cellulose (Lynd *et al.,* 2002). If cellulases preferred to attack amorphous regions first, it would be expected that the crystallinity increases during the course of the reaction (Fan *et al.,* 1980). However, studies show conflicting results regarding this issue (Park *et al.,*2006; Lynd *et al.,* 2002).

Another factor is the surface area, which provides enzymes with a space to bind on. A clear correlation can be observed: the more surface area there is, the more space for enzymes to attack and break bonds. A study by Olsen *et al.*, 2016, suggests that before enzyme addition, Avicel exists like an aggregate of particles and enzyme addition breaks this aggregate apart into smaller particles. These small particles are responsive to enzymes and as long as the reaction proceeds, the number of these readily degradable particles decreases, leaving behind recalcitrant ones, which are equivalent to the non-accessible areas of the substrate.

Lignin and hemicellulose content can also be important variables affecting the enzyme activity in biomass. It has been discovered that the presence of either polymer decreases the hydrolysis rate (Yoshida *et al.,*2008; Jeoh et al., 2007). The study by Yoshida *et al.* found that cellulases bind to lignin irreversibly, decreasing the overall active enzyme concentration. However, the slow-down in cellulose hydrolysis has also been found to occur in the absence of lignin or hemicellulose (Olsen *et al.,* 2016). Thus our research will centre on the slow-down using pure cellulose and will not factor in the effects of other polymers, which may be present in biomass.

With regards to the enzyme kinetics, the heterogeneous degradation of cellulose is not as simply described as enzyme catalysis occurring in solution. This is because, for endoglucanases and cellobiohydrolases, adsorption and desorption from the surface of the substrate are essential steps in the process (lateral diffusion across the

surface of the substrate also plays a role for cellobiohydrolases)(Sørensen *et al.*, 2015). The processive action of cellobiohydrolases is essential to the breakdown of crystalline cellulose, but these have been found to be intrinsically slow in practice, compared to their computed rate of catalysis (Horn et al., 2012). The association and dissociation have been suggested as the cause for this slow reaction rate, with dissociation acting as the rate-limiting factor at lower temperatures, especially so for cellobiohydrolases because of the high affinity they have for cellulose (Sørensen et al., 2015).

This suggests a further enzymatic reason for the slow-down, which seems to occur more and more as the reaction progresses. If amorphous regions are degraded preferentially because of the higher affinity of endoglucanases for these regions, crystallinity increases, and the hydrolysis of cellulose relies on more on cellobiohydrolases, thus the reaction rate slows. However, as mentioned earlier in this section, research has been conflicted regarding the crystallinity increase (Park *et al.,*2006; Lynd *et al.,* 2002).

Another enzyme-related issue arises when it comes to the products released by the hydrolysis, since product inhibition is a common cause of decreasing reaction rate for many enzyme-catalysed processes. A study done on bacterial cellulose found that cellobiose is responsible for the inhibition of the early rate of hydrolysis. However, in case of natural cellulosic substrate, product inhibition is not usually not responsible for limiting the reaction rate (Gruno *et al.*, 2004). Using a cocktail of different enzymes may further limit such product inhibition, because as cellobiohydrolases break the cellulose polymer into cellobiose, □-glucosidases convert the cellobiose to glucose, which is far less inhibitory. In this way the cocktail acts so as to minimise product inhibition.

Thermal stability of the enzymes involved in hydrolysing cellulose has also been proposed as a reason for the loss of reaction rate. For example, a study done by Rodrigues, Felby, & Gama found that certain cellulases also contained in the Cellic CTec2 enzyme cocktail (used in this project) deactivate to an extent over 24 hours at 50°C (Rodrigues, Felby, & Gama, 2014). However, we do not expect this to be a key factor in the slow-down as this has also been found as the temperature where the enzyme cocktail was most effective (Novozymes, 2010).

We've therefore explored a hypothesis that the slow-down is affected by the substrate structure, or more specifically, it is affected by how amorphous/crystalline it is. From the preliminary literature research, it is clear that many factors may contribute to the slow-down, some of which are more prevalent than others. The key to finding the main reason for the slow-down, will be exploring which of these factors have greater effect.

3.4 Methodology

3.4.1 Materials & Calorimetry

Our approach makes use of a TAMIII isothermal calorimeter, as shown in the figure below:

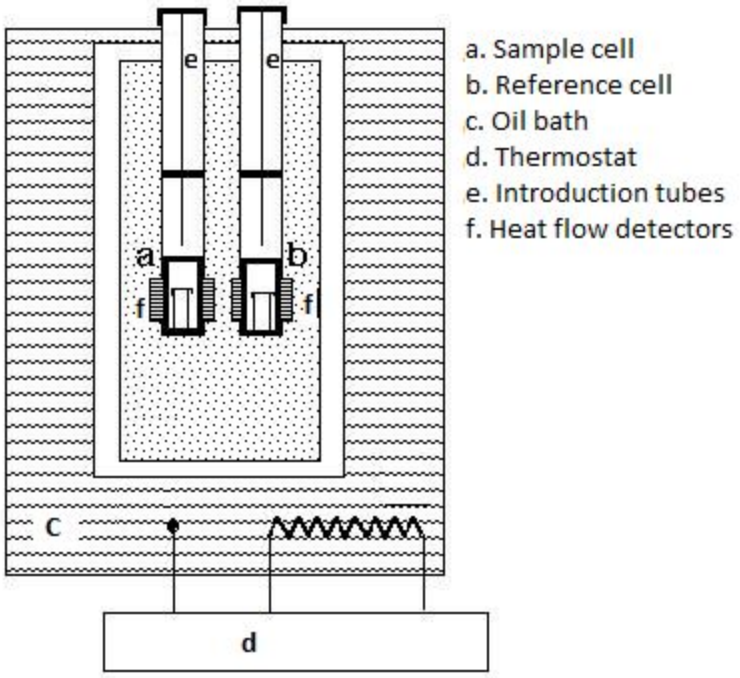

Figure 3: Simple diagram of a TAMIII isothermal calorimeter (Murasawa & Koseki, 2015). The above represents one 'channel' in each experiment.

The TAMIII calorimeter functions by measuring the heat flow (in µWatts = µJoules/second) required to maintain isothermal conditions between a sample (a.) and its respective reference cell (b.). This measurement is done using detectors (f.) attached to these cells. In order to be able to measure heat flow on the nanowatt scale, the TAMIII must maintain a stable internal temperature using an oil bath (c.), which thermally isolates the internal components from external temperature changes and is kept at the desired temperature by a thermostat (d.). By filling the reference cell with a nonreacting solution (such as buffer), one can track miniscule changes in temperature which should correspond only to changes in the sample. Since the detection of these sample temperature changes and correction of heat flow to the reference cell are nearly immediate, data can be measured in real-time (immediately as the changes occur) and with very small time-steps, allowing for a much more complete visualization of the hydrolysis than would be possible using slower methods such as quenched enzyme assays.

Aside from providing real-time data, calorimetry does not rely on properties such as colour or opacity, nor does it require a modified substrate. This makes it ideal in analysing reactions involving complex substrates such as cellulose or lignocellulosic biomass.

Figure 3 corresponds to one calorimeter. The TAMIII we worked with had 4 such calorimeters contained within it, which allowed for simultaneous data collection from three samples (since calorimeter 1 was not functional).

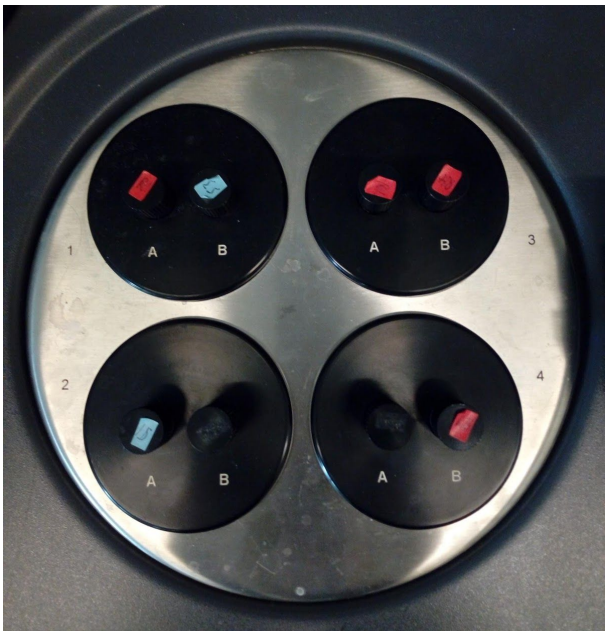

Figure 4: Top view of the four calorimeters within the TAMIII. The knobs (some with coloured labels) are the caps of introduction tubes (see Fig. 3 label 'e'). The A tubes contain sample cells and the B tubes the reference cells.

In our experiment we opted for the use of a cellulase cocktail over a single enzyme, as the different cellulases acting synergistically degrades cellulose far more quickly than any one component alone and therefore gives a stronger signal to be measured. The enzyme cocktail was Cellic CTec2 from Novozymes, which is an aggressive mix, meaning that it is rather effective in degrading cellulose. A quantitative activity value can be found in the study 'Celluclast and Cellic® CTec2: Saccharification/fermentation of wheat straw, solid-liquid partition and potential of enzyme recycling by alkaline washing' (Rodrigues *et al.*, 2014).

For substrate, we make use of two different types of cellulose: Regenerated amorphous cellulose (RAC, 5.3 g/L) and Avicel (100.33 g/L), which is also known as microcrystalline cellulose. These two cellulose types were selected based on their structure, since Avicel is more crystalline and RAC more amorphous.

An operational temperature of 45°C was selected, which was on the low end of the recommended temperature range (45°C - 50°C) given on the CTec2 application sheet provided by Novozymes (Novozymes, 2010) to reduce the likelihood of thermal denaturation of the enzymes but still ensure the hydrolysis occurred fast enough to obtain measurable results. The solutions containing the cellulose substrates had pHs within the recommended range of 5 - 5.5.

3.4.2 Experimental Procedure -Restart

The approach we used in this project has been called the 'restart' method (Kansou, 2017). The concept is to run a reaction under certain conditions, then change one of those conditions halfway and measure the resulting response. Our general restart procedure with the enzymatic hydrolysis of cellulose was as follows:

1. Establishing a baseline:

This was necessary for determining the heat flow of the substrate alone so as to later determine the heat produced by the reaction.

Firstly, the reference cells for each of the 3 calorimeters were filled with a standard acetic acid buffer. These were then hung halfway into their respective introduction tubes and held in place by a magnet, so that the cell was in contact with the calorimeter but not fully within the detector - hence hanging halfway. They were then left there for approximately 45 - 60 min to bring their temperatures up to that of the calorimeter (45^0C). Then the sample cells were filled with 3 ml of cellulose substrate and similarly hung halfway. This was sometimes done simultaneously with the hanging of the reference cells. After hanging halfway, the reference cells, then the samples were further lowered (slowly so as to minimize noise due to friction) until fully submerged in their calorimeters. The samples were left in and the heat flow values for the substrate measured for approximately one hour for the sake of convenience. This did not allow enough time for the sample temperature to fully equilibrate, and the final baseline was determined via extrapolation of the resulting signal using software on the TAMIII.

2. Standard hydrolysis

The initial hydrolysis was the same across all three calorimeters for each experiment. This was done to allow the slow-down to occur fully before the restart and to establish a standard reaction for each sample to which the restarted hydrolysis could be compared. It also allowed us to compare the three samples, since any differences not caused by the restarts would then show up in the standard hydrolyses as well.

The sample cells containing only the substrate were then removed from the calorimeters and precisely 30 µL of undiluted CTec2 stock added to each. They were then resealed, shaken, and left hanging halfway in the introduction tubes for 45 - 60 min before being lowered fully into the calorimeter. The reactions were then left to proceed for approximately 24 hours.

3. Restart

Here, differences between the three calorimeters were introduced in order to judge the effects of different variables on the reaction rates. As a rule, one calorimeter was always left unaltered to show how the reaction would proceed under the same initial conditions and over the same time period as the others, but with no changes made after the initial hydrolysis. Furthermore, whatever change was made on the second sample was doubled in amount for the third (e.g.: 0 µL of CTec2 reinjected into one sample, 30 µL in another, and 60 µL in the last).

The sample cells where a change was to be made were raised and either given new aliquots of CTec2 in varying doses, or fresh substrate. The sample cells were then, as for the initial hydrolysis, resealed, hung halfway for temperature equilibration, then lowered fully and allowed to react for another approximately 24 hours.

The unique conditions of each experiment are the following:

- Experiment 1: The cellulosic substrate used was Avicel and the restart involved injection of new CTec2 stock (0 µL, 30 µL and 60 µL).
- Experiment 2: The substrate was Avicel and the restart comprised of removing a certain amount of sample mixture (0 ml, 0.5 ml, and 1 ml) and replacing it with the same amount of fresh Avicel.
- Experiment 3: The substrate was RAC, and the restart was re-addition of CTec2 (0 µL, 30 µL and 60 µL), as in Experiment 1.
- Experiment 4: Substrate was RAC and restart was removal of sample mixture (0 ml, 0.5 ml and 1 ml) followed by replacement with the same amount of fresh RAC (same procedure as Experiment 2).
- Experiment 5: Substrate for initial hydrolysis was Avicel. Same procedure for substrate exchange as that for Experiments 2 and 4 was used, but the fresh substrate that replaced the removed mixture was RAC.

Through the enzyme re-addition restart experiments (1 and 3), the dependence of the reaction rate on enzyme concentration could be clarified. Furthermore, this indicated whether enzyme deactivation was a cause of the slow-down or not. Through the substrate exchange experiments, we could similarly determine the importance of fresh cellulose to the reaction rate, which might point to changes in the substrate structure as causing the slow-down. The aim of repeating the experiments with RAC as well as Avicel (and both in Experiment 5) was to gauge what effects the different structures of the two celluloses might have, further elucidating the role of the substrate here.

3.5 Results and Data Analysis

3.5.1 Raw data

The raw data for each experiment were collected in terms of heat flow (µWatts = µJ/sec) vs time, as described in *Section 3.4.1.* A representative example is shown below:

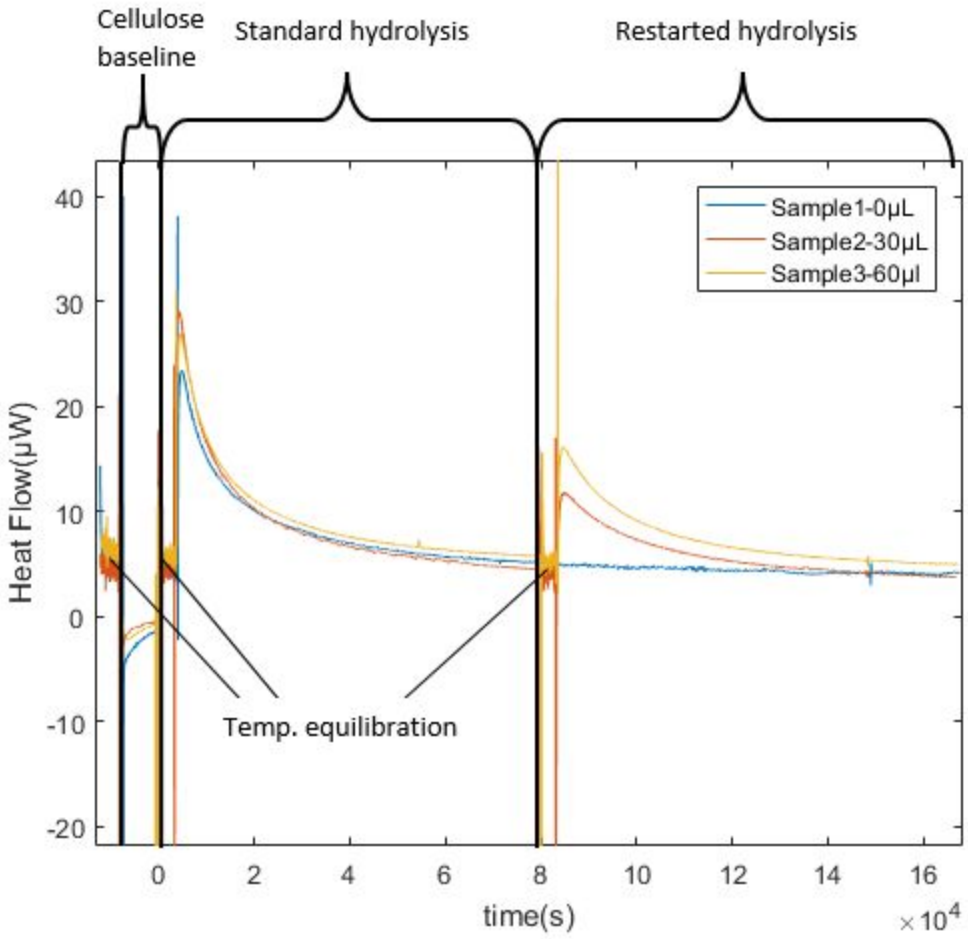

Figure 5: Raw data for Experiment 1 (repeated enzyme addition in Avicel). The volumes next to the sample names represents how much CTec2 was added to the samples for the restart.

The rest of the raw data graphs can be seen in *Section 5.1*. The heat flow values here are relative to that of the substrate alone, without any CTec2. This was achieved by subtracting the final value of the extrapolated cellulose baseline (see *Section 3.4.2, Establishing a baseline*) from all the other heat flow measurements. The starting time (t=0s) is taken as the time when the sample cells were placed into the introduction tubes (see *Figure 3*) in the TAMIII after the reaction was started.

3.5.2 Reaction rates

The raw data was then split into the first and second enzyme additions to remove the noise associated with temperature equilibration (shown in *Figure 5*) and time was reset for the restarted hydrolysis so that it also has t=0s as the starting time. Heat flow in µW was then converted to reaction rate ($\mu mol * s^{-1}$) using the enthalpy value for β(1-4) glycosidic bond hydrolysis, since the heat produced in the sample was due to the energy released by breaking these bonds. 'A calorimetric assay for enzymatic saccharification of biomass' (Murphy *et al.*, 2010), found ΔH to be approximately $-2.5kJ * mol^{-1}$ by hydrolyzing cellobiose alone. However, the same study found that other effects, which are not described here, contribute to a different

apparent enthalpy value for the hydrolysis of insoluble cellulose. Thus, the value must be found experimentally for each substrate. That determined by Murphy *et al*. for Avicel was $-4.32\ kJ*mol^{-1}$, corresponding to $-4.32*10^{9}\ \mu J*mol^{-1}$, which was the value we used. This led us to the following formula, where R is equal to the number of □(1-4) glycosidic bonds broken per second:

$$Heat\ Flow(\mu J/s) = \Delta H(\mu J/mol) * Rate(mol*s^{-1}) \quad \text{(eq.1)}$$

This equation was then changed to solve for Rate and multiplied by 10^6 to make the unit $\mu mol/s$.

$$R(\mu mol*s^{-1}) = \frac{HF(\mu Js^{-1})}{\Delta H(\mu Jmol^{-1})} * 10^6 = \frac{HF(\mu Js^{-1})}{(4.32*10^9)(\mu Jmol^{-1})} * 10^6 \quad \text{(eq.2)}$$

Lines of best fit for the data from both the standard and restarted hydrolyses were obtained using the Curve Fitting app in MATLAB and extrapolated backwards to the start point of the hydrolyses (when the samples were placed in the introduction tubes). This was done to give an approximate idea of the reaction rates during the temperature equilibration periods of the sample cells, which could not be accurately recorded since the temperature differences between the sample and reference cell can only be assigned to the energy released by hydrolysis once the base temperatures of the sample and the reference are equal. The equations for the lines of best fit can be seen in the legends of the graphs.

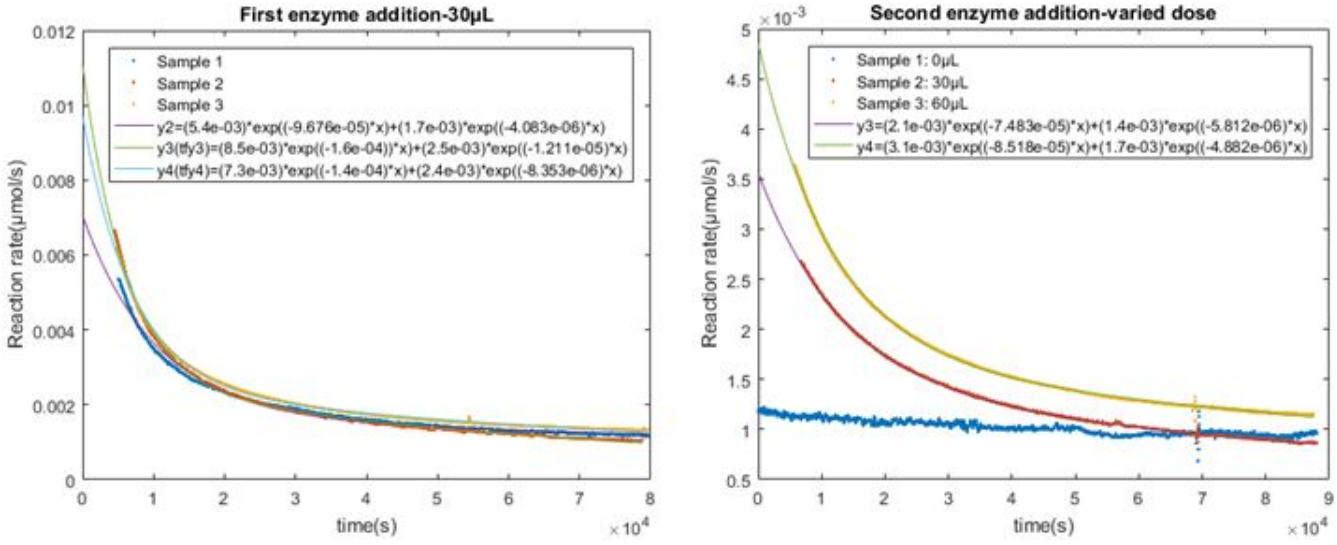

Figure 6: Experiment 1 (repeated CTec2 addition in Avicel) results with fits. The data from each calorimeter within the TAMIII can be seen to form two decay curves, or one in the case of sample 1. This decay represents the slow-down effect under investigation here. Sample 1's signal is noisy due to an empty reference cell.

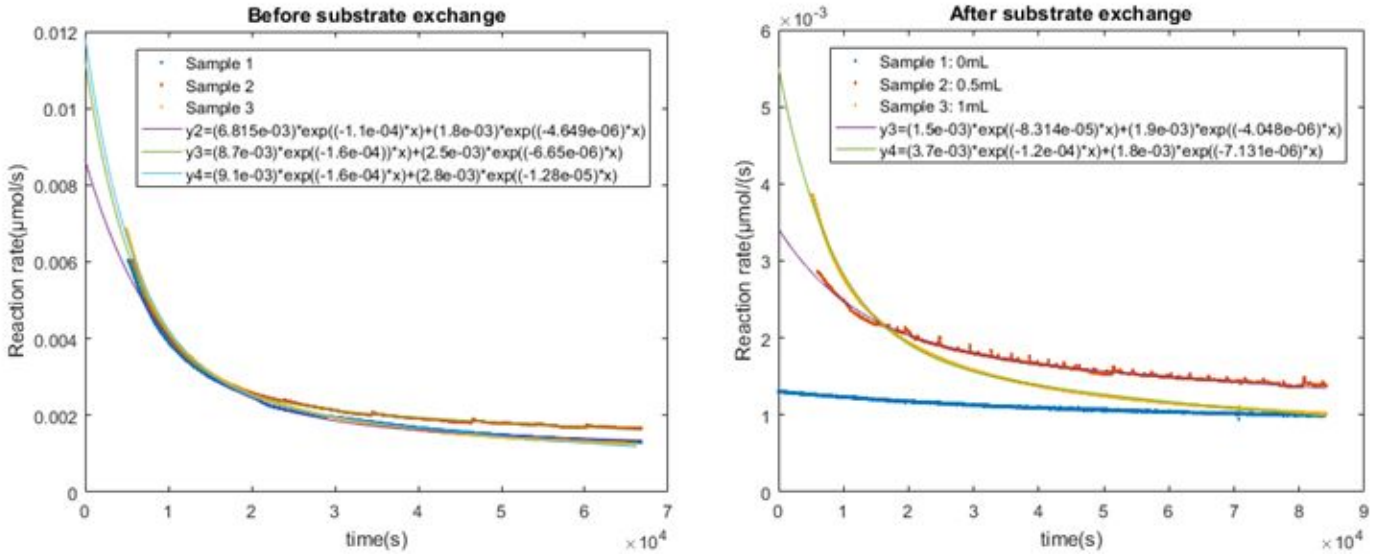

Figure 7: Experiment 2 (fresh Avicel exchange) results with fits. Here, the increased reaction rate after the restart is due to the addition of fresh substrate. Some noise is visible in sample 2's signal, especially in the second hydrolysis.

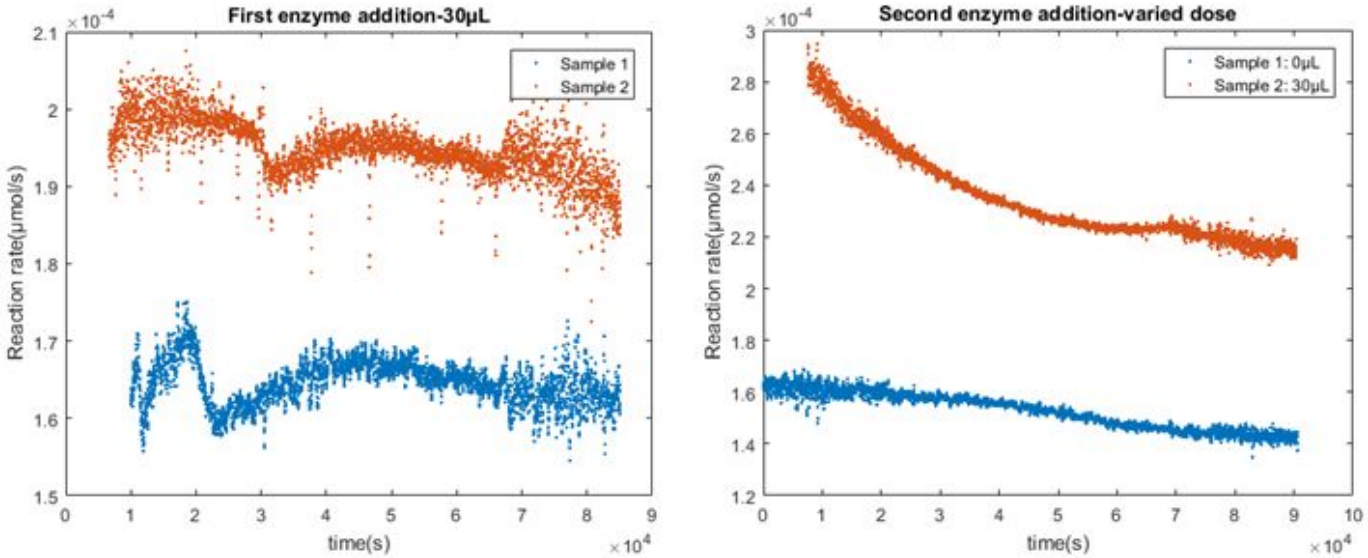

Figure 8: Experiment 3 (repeated addition of CTec2 in RAC) results. Satisfactory fits were not possible here. The results from one sample were excluded due to bad data.

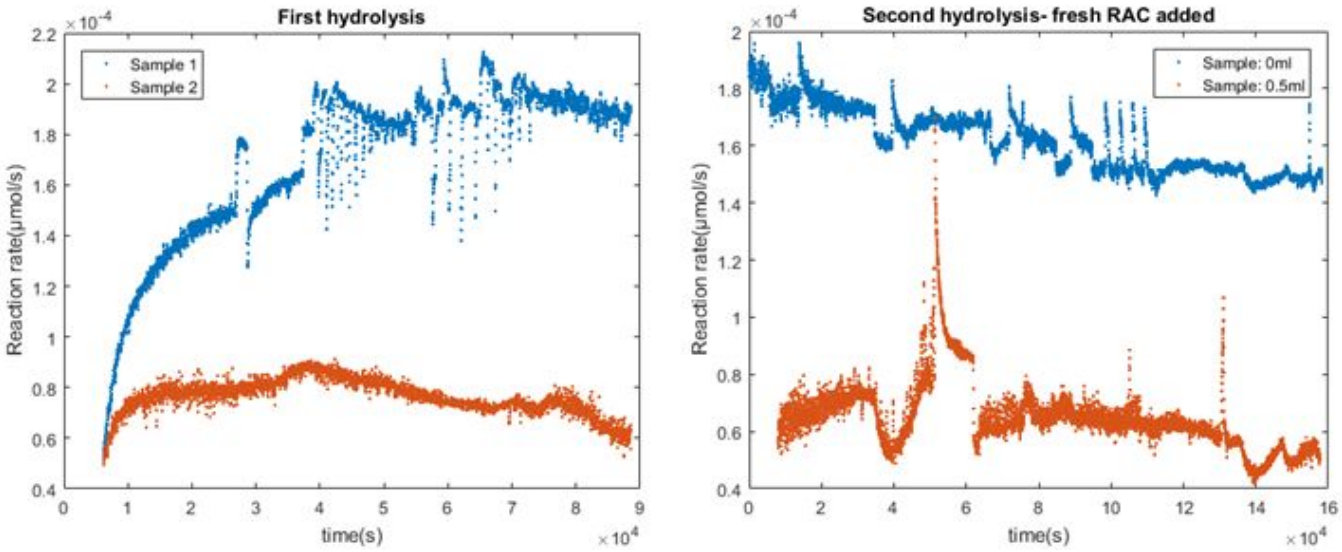

Figure 9: Experiment 4 (fresh RAC exchange) results. Fits were not possible and one sample's results were omitted due to bad data.

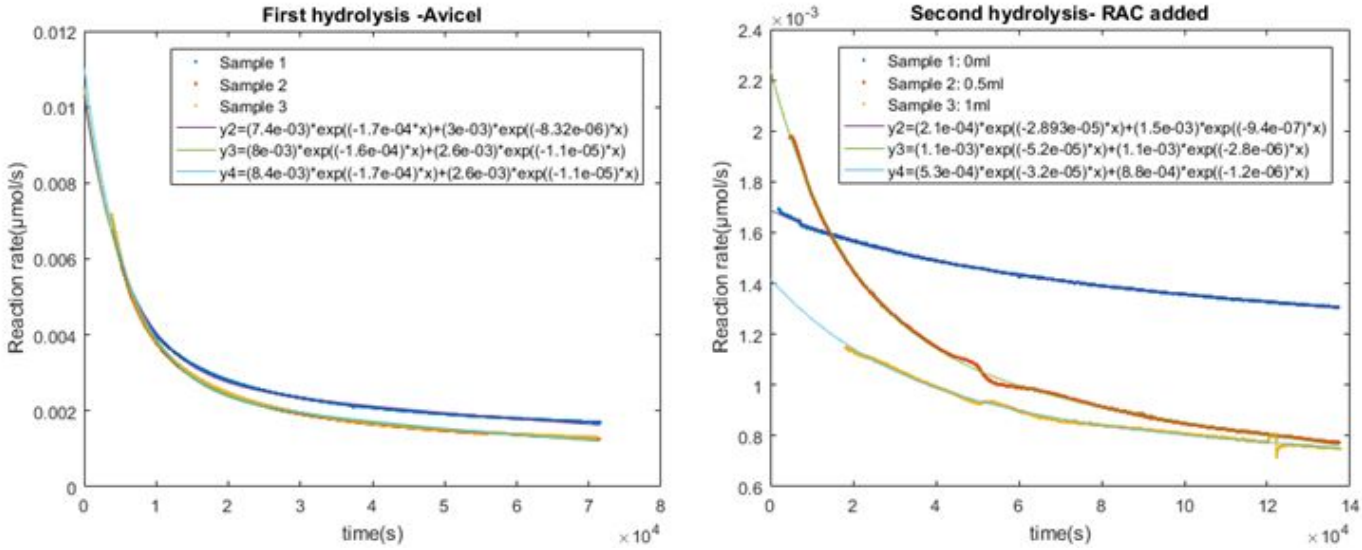

Figure 10: Experiment 5 (Avicel-RAC exchange) results.

3.5.3 Substrate conversion

The next step performed was integration of the reaction rate data, to find out how much of the cellulose had been converted over the course of the overall reaction (combining how much was degraded in the standard and restarted hydrolyses where necessary).

Table 1: Amount of cellulose degraded in each restart experiment. These values are understatements as they do not include the amount degradation which occurred during temperature equilibration periods.

	No restart	30 µL CTec2 added/ 0.5ml fresh substrate	30 µL CTec2 added/ 1 ml fresh substrate
Experiment	□(1-4) glycosidic bonds broken (µmoles)	□(1-4) glycosidic bonds broken (µmoles)	□(1-4) glycosidic bonds broken (µmoles)
1. CTec2 added (Avicel)	230.3	260.8	306.4
2. Fresh substrate added (Avicel)	225.3	292.4	272.3
3. CTec2 added (RAC)	26.20	36.58	-
4. Fresh substrate added (RAC)	39.59	16.91	-
5. Fresh RAC added (into Avicel)	370.1	302.3	285.2

The values in the above table were calculated using trapezoidal numerical integration of the reaction rate vs time data shown in *Figures 6-10*. The fraction of cellulose converted released from the insoluble polymer could then be calculated using the mass concentration of RAC and Avicel as well as the molecular weight (Mw) of glucose ($180.156 * 10^6 g/\mu mol$), using the assumption that every ß(1-4) glycosidic bond broken ultimately corresponds to the removal of one glucose molecule:

$$\%converted(\mu mol) = \frac{no.bonds\ broken(\mu mol) * Mw(glucose)(g/\mu mol)}{[cellulose](g/L) * sample\ volume(L)} * 100 \qquad \text{(eq.3)}$$

The most relevant resulting values are shown and discussed in *Section 3.6.5.*

3.5.4 Enzyme concentrations

For the purpose of discussion in *Section 3.6.2* it was necessary to determine the concentration of CTec2 during the different hydrolysis stages for each sample. Since the non-enzyme volume of each sample was the same initially, the concentration of CTec2 is taken as equal to the volume of enzyme present in the sample.

$$C_E = V_E \qquad \text{(eq.4)}$$

Concentrations are all the same in the standard hydrolysis ($C_{standard}$) in each experiment and remain the same for the samples where no restart was done. In the restart experiments involving a second enzyme addition, the

new concentrations ($C_{restart}$) are equal to $V_E + amount\ of\ enzyme\ added$. These values can be seen in *Table 2*.

In the case of the substrate exchange experiments, an amount of substrate-enzyme mix (x mL) was removed from the samples where fresh substrate was to be added, prior to its addition, giving new volumes (V2) while maintaining $C_{standard}$.

$$V_{standard} = 3 * 10^{-3}L + 30 * 10^{-6}L \quad \text{(eq.5)}$$

$$V2 = V_{standard} - x\ mL \quad \text{(eq.6)}$$

$$= (30 * 10^{-6}L + 2.5 * 10^{-3})L\ (where\ 0.5\ ml\ was\ exchanged) = (30 * 10^{-6}L + 2 * 10^{-3})L\ \ (where\ 1\ ml\ was\ exchange$$

The fresh substrate was then added, returning the mixture's volume to $V_{standard}$ and giving a new enzyme concentration ($C_{restart}$).

$$C_{standard} * V2 = C_{restart} * V_{standard} \quad \text{(eq.7)}$$

$$C_{restart} = (C_{standard} * V2)/V_{standard}$$

The enzyme concentrations after the restarts are shown below in *Table 2*:

Table 2: Enzyme concentration (or volume of enzyme present) in samples after restart ($C_{restart}$) .

	Enzyme addition restart		Substrate exchange restart	
Amount added (CTec2) or exchanged (cellulose) (L)	$30 * 10^{-6}$	$60 * 10^{-6}$	$0.5 * 10^{-3}$	$1 * 10^{-3}$
$C_{restart}$ (L)	$60 * 10^{-6}$	$90 * 10^{-6}$	$25.05 * 10^{-6}$	$20.10 * 10^{-6}$

3.6 Discussion

3.6.1 Restart Impacts on Reaction Rates

The most central aspect of the research done in this project is ultimately the assessment of the changes in reaction rates over time before and after the restart. To this end, the most relevant information to take from the results displayed in *Figures 6-10* is simply which variable has the greatest effect on restarting the hydrolysis reaction (enzyme or substrate) and what the relationship between the change in reaction rates following the restarts and the degree of change made is. To better understand this, *Tables 3 & 4* provide numerical descriptions of the effects that the restarts had on the reaction rates in the different samples.

Table 3: Change in reaction rate when CTec2 was added. Values are relative to the last data point of the standard hydrolysis for each sample. For the samples with no restart (OμL added/exchanged), this point is around when the

standard hydrolyses for the other samples were terminated to provide a comparison. The values assigned under 'Peak' represent the increase up to the first data point taken for the second restarted hydrolysis. Measured points were used rather than the extrapolations, since the fitted lines are not considered as reliable as the real data. The values assigned under 'End' represent the difference between the last points of the standard and restarted hydrolyses. Where there was no restart there is no need for a 'Peak value.

Experiment	Increase in reaction rate upon re-addition of cellulase cocktail (µmol/s)					
Volume of enzyme added (µL)	0		30		60	
	Peak	End	Peak	End	Peak	End
1. Substrate = Avicel	-	-2.2e-04	1.6e-03	-1.8e-04	1.2759	-1.8e-04
3. Substrate = RAC	-	-2.6e-05	1.0e-04	3.2e-05	-	-

Table 4: Change in reaction rate when fresh substrate was added. Same conditions apply as in Table 3.

Experiment	Change in reaction rate upon addition of fresh substrate (µmol/s)					
Amount of substrate exchanged (ml)	0		0.5		1	
	Peak	End	Peak	End	Peak	End
2. Substrate = Avicel	-	-3.1e-04	1.2e-03	-2.8-04	2.6e-03	-2.4e-04
4. Substrate = RAC	-	-4.1e-05	1.4e-06	-5.1e-06	-	-
5. Substrate = Avicel (1st hydrolysis), & RAC (2nd hydrolysis)	-	-3.9e-04	7.1e-04	-4.9e-04	-1.5e-04	-5.5e-04

As can be seen from the above tables, the increases of the peaks (where only Avicel was used) when enzyme or substrate was added are quite comparable in size. However, the proportionality of these increases to the amount added is interesting: the increase in the sample where 60 µL of CTec2 was added is only approximately 30% higher than when 30 µL was added in. Contrastingly, the peak increase in the sample where 1 ml of fresh Avicel was exchanged was more than double that of the one where 0.5 ml was exchanged, even though the

CTec2 concentration in the former was lower due to more enzyme being removed as part of the exchange. This shows that the reaction rate is proportional to the amount of fresh cellulose added.
Since literature indicates that amorphous cellulose is degraded preferentially (Pellegrini *et al.*, 2014), this suggests that the increase in reaction rate may be proportional to the amount of amorphous cellulose present. Supporting this is the fact that by the end of the restarted hydrolyses in Experiment 2 (Avicel exchange), the reaction rates had dropped by a very similar amount relative to the end of the standard hydrolyses as the sample where with no restart had over the same time period. This is because the reaction rate becomes almost steady after the peak region, which fits with the idea that once the amorphous regions have been quickly hydrolysed, giving rise to the increased reaction rates, the degradation of the crystalline regions proceeds a more or less steady rate (or only decreases very slowly). This could be due to the reliance on cellobiohydrolases at this stage, which are essential for degrading crystalline cellulose (see *Section 3.4.3*) and are inherently slow, whereas the more amorphous regions are degraded much faster by endoglucanases, thus giving rise to the peaks observed.

3.6.2 Enzyme Activity

As mentioned in the section above, *Figures 6-10* and *Tables 3-4* show that adding either substrate or fresh enzyme result in similar increases in the rate of cellulose hydrolysis. However, since cellulases are one of the most expensive components of second generation biofuel production (Hong *et al.,* 2013), it would be ideal to get the highest reaction rate using the lowest amount of these enzymes possible, to make the process the most economically beneficial. For this purpose, we divided the rates of reaction in restarted hydrolysis of each experiment by the concentration of CTec2 at that time, which can be seen in *Table 2,* to determine the reaction rate *per unit of enzyme*. This is known as the enzyme's specific activity, although in our case it is the average specific activity of all the cellulases in the CTec2 cocktail. Doing this also helps further clarify potential causes of the slow-down.

The resulting specific activity graphs can be seen below. The specific activities during the standard hydrolyses add little value since all the samples had the same CTec2 concentration during this period.

Enzyme addition restarts:

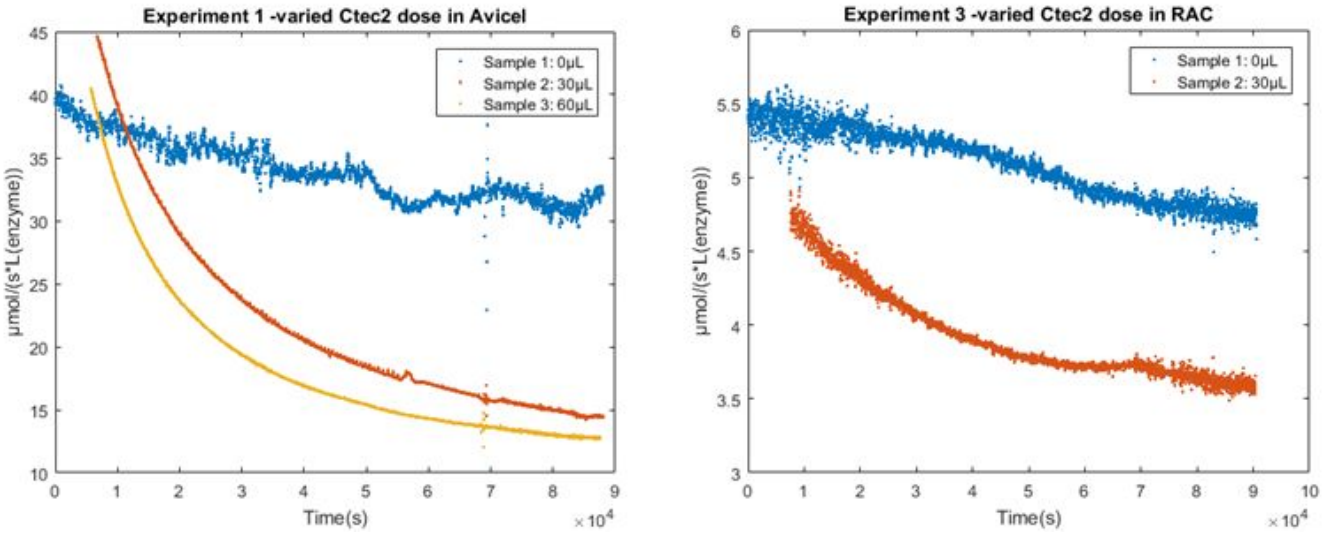

Figure 11: Enzyme activities in during the second hydrolysis period of each experiment, after an additional dose of CTec2 was added.

As can be seen from *Figures 6* and *8*, the addition of extra CTec2 does result in a boost in reaction rates, but this increase is small compared to the proportional increase in enzyme concentration, as stated in *Section 3.6.1.* This is reflected in *Figure 11*, where one can see that the sample where no restart was performed has the highest overall specific activity and the one where the most CTec2 was added has the lowest (although again this pattern does not seem proportional to the amount of CTec2 added). Furthermore, this occurs when extra CTec2 is added to either cellulose type. These specific activities help explain the conclusion that the increase in reaction rate after the restart is not proportional to the extra dose of CTec2: if the activity per unit enzyme decreases the more CTec2 is added, then there may be an overall increase in hydrolysis rate, but it will not be nearly as high as if each enzyme unit did as much work is it had done before. Therefore, the rate does not increase proportionally.

However, the specific activities for the restarted samples in *Figure 11* were briefly higher than the non-restarted one, which hints that there is some process affecting reaction rates where the addition of more cellulases is beneficial, but this effect does not last long.

When substrate is exchanged:

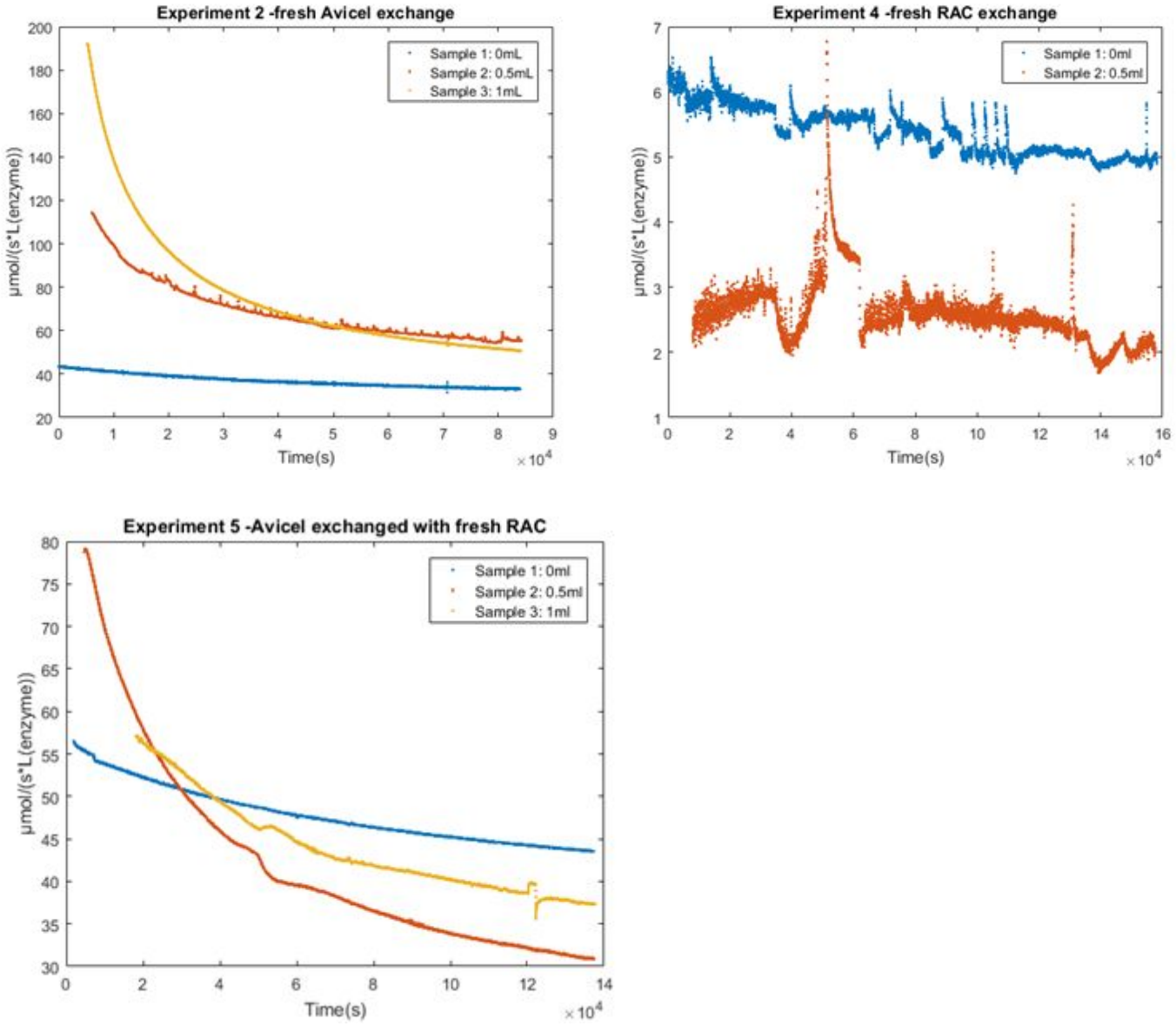

Figure 12: Enzyme activities in during the second hydrolysis period of each experiment, after part of the mixture was exchanged with fresh substrate.

The specific activities shown in the figure above behave quite differently to those after the enzyme addition restarts. The most notable part of *Figure 12* is the relatively high activities of the samples where a substrate restart was done compared to the one where it was not. Additionally, the activities remain higher long after the hydrolysis curve has levelled off. This indicates that adding fresh substrate does enhance the specific enzyme activity, even over a longer time span, which contrasts the opposite pattern that occurred when enzyme was added (see *Figure 11*). There, the more enzyme, the lower the average efficiency. Furthermore, the curves of the enzyme activities of the restarted samples soon dropped far below that of the unchanged

sample, indicating that in the long term, addition of more cellulases is not an efficient solution, whereas the addition of more Avicel is.

These results seem to indicate that the cellulases were in abundance, and that the amount of usable substrate was the limiting factor to the enzyme activity, which fits with the finding in *Section 3.6.1* that the increase in peak reaction rate was proportional to substrate added.

The results in *Figure 12*, Experiment 5 show mixed specific activities that do not seem to correlate with the amount of substrate added. This is likely due to the issues we had with RAC (see *Section 3.6.4*).

3.6.3 Interpreting Line of Best Fit Equations

Another interesting, yet not so obvious aspect of our results can be explored if we look at the curve fitting process. The curves we were able to fit (see *Section 3.5.2*) to the data signals for the samples in each experiment (except those using RAC only) all best followed a two-term exponential decay curve fit, which can be described by the following form (where *R* represents reaction rate in µmol/s and *t* is time(s)):

$$R = a * exp\,(b * t)\; + c * exp(d * t) \qquad \text{(eq.8)}$$

The data for each sample where no restart was done was simply fitted once as a whole to obtain a more accurate fit, also because there was no temperature equilibration period in the second half to compensate for. Experiment 5 is an exception, since some disturbance occurred during the handling of the other samples. Where a restart was done, the data was fitted separately for the first and second additions, as each restart represents a new hydrolysis curve. Coefficients for each fit (*a, b, c* and *d)* can be seen in the legends of the respective graphs (see *Figures 6-10)*.

Eq.8 simply shows that there are two factors affecting the overall decay of *R* (the reaction rate), each corresponding to one exponential decay term: $a * exp\,(b * t)$, and $c * exp(d * t)$. By treating the terms separately, the half-time of each term can be calculated. That is, how much time is needed for each process, to decay to half of its original rate.

For example, we can take the data from fitted equation from the sample in Experiment 1 where no restart was done:

$$R = 0.005364 * exp((-9.676e-05) * t)\; +\; (0.001687) * exp((-4.083e-06) * t) \qquad \text{(eq.9)}$$

Taking only the first decay term and its corresponding contribution (R_f) to the overall reaction rate (*R*):

$$R_f = 0.005364 * exp((-9.676e-05) * t) \qquad \text{(eq.10)}$$

Treatment with natural logarithms shows that the change in reaction rate is analogous to the change in concentration in a first order reaction:

$$ln(R_f) = ln(0.005364 * exp((-9.676e-05) * t))$$

$$ln(R_f) = -(9.676e-05 * t) + ln(0.005364) \quad \text{(eq.11)}$$

The general integrated equation for a first order reaction has the form $ln[A] = -k * t + ln[A_0]$, where A_0 is the initial concentration. Thus, one can see that *eq.6* has the same form and in this case the initial reaction rate is 0.005364, which matches our data. We can then use the first order formula for calculating half-time ($t_{1/2}$) to see how long each term takes for its contribution to *R* (overall reaction rate) to decrease by half:

$$t_{1/2} = 0.693/k \quad \text{(eq.12)}$$

k can then be substituted with its analogue from *eq.11*; $(9.676e-05)$, giving the half-time for R_f.

$$t_{1/2} = 0.693/k = 0.693/(9.676e-05) = 7162s$$

Similar treatment of the second exponential decay term in *eq.9* gives $t_{1/2} = 169700s$, thus showing that the second term gives a much slower decay. Performing this analysis on the other curves across all experiments (where this was possible) yielded similar results, ie: one exponential term always decayed dramatically faster than the other.

If a mathematical description can be derived from empirical data when describing natural phenomena, it is often so that it reflects underlying processes of the phenomena. Many examples of this practice can be found throughout the history of science, and it is a generally accepted idea amongst the scientific community.

For our experimental setup, this would imply that there are two ongoing real processes contribution to the total reaction rate; one that causes a fast decay and another which causes slow decay. Realising what these two processes represent in reality could help reveal the cause(s) of the slow-down. Possible explanations could include the following: the fast decay represents the decrease in accessible/preferentially-degradable substrate, and the slow decay might represent minor denaturing of the enzymes due to the heat of operation. The latter has been found by some studies to occur to a significant extent (see *Section 3.3.3*), but since the operational temperature was intentionally kept at the low end (45°C) of CTec2's optimal range in our experiments, it is unlikely that this was the major cause of the slow-down. However, there may still have been some denaturation, which could partially explain why the reaction rate and specific activities also increased initially when more CTec2 was added. More detailed analysis of the half-times in relation to the perturbations involved in the restarts could be done, but we have decided not to because of possible error arising from imperfect fits, which would necessitate further verification before any conclusions could be drawn.

3.6.4 RAC

An important point to mention regarding the discussion of our results is the behaviour of RAC during the respective experiments, where we observed great disturbances and unexpected behaviour from this substrate in the measured data, as can be seen from *Figures 8 and 9*. While the literature is in favour of RAC being a better substrate in terms of enzyme activity because of its high surface area (Pellegrini *et al.,* 2014), we could not confirm this with the obtained data. The signal was weak, peaks after additions were barely visible and the data was not very informative. This trend was observed in every single experiment involving RAC alone as a

substrate, suggesting that there was a problem with either the substrate, our method not being well-suited to it, or a combination of the two.

RAC has a gel-like structure because of its branching and therefore the ability to adhere and contain water molecules within its structure (Warren *et al*., 2017). Knowing this property might be able to explain as to why we observed such strange behaviour from the experiments. Since it is gel-like, its viscosity may have made it difficult for the enzymes to reach all areas of the substrate, even though the samples were shaken prior to the lowering of the ampules. We propose that a possible explanation for the observed phenomenon was that very little of the enzyme was able to diffuse onto attack sites on the substrate and react, thus lowering the signal that we might have seen if the enzyme could have reached the water-occupied areas. The samples could have been stirred throughout the course of the reaction in order to obtain more informative results and overcome the structure problem by letting enzyme continuously flow through the gel, allowing for easier access. However, this would have been with our experimental setup using the TAMIII since, for example, any break in the seals of the sample cells could lead to evaporation, which would affect the data measurements.

Another possible explanation for the weak signal could be the low concentration of RAC (5.3g/L) compared to Avicel (100.33g/L), but this is unlikely as the high surface area of RAC due to its amorphousness would mean that it effectively has a higher concentration of 'available substrate' per mass unit than Avicel and we would expect this to compensate for the difference in mass concentration in the samples. Furthermore, lower concentration does not explain the inconsistencies and messiness of the curves, which goes beyond the amplitude of the noise seen in some of the Avicel curves.

Lastly, it is possible that RAC was so degradable that it was hydrolysed to a great extent during the hour or so that it took to equilibrate temperatures between the sample and reference cells at the start of the experiments, and what we saw in the signal was only the tail-end of the hydrolysis. This is supported by the substrate conversion data in *Table 1*, which indicated shows the maximum degradation of RAC during the period measured was 39.59 µmols of β(1-4) glycosidic bonds broken, corresponding to 41.45% of RAC degraded by mass according to the method used in *eq.3*. This value, however, may not be reliable because of the unknown factors and noise associated with the signal. Since we know that for Avicel the highest reaction rates occur in the peak near the beginning, it follows that a significant portion of the total substrate conversion occurs during that time. If the same pattern were to be true for RAC, then we could simply be seeing very low reaction rates because there was significantly less substrate left to digest by the time accurate measurements began, and likely almost none at the end of the measured period since a further 41.45% was degraded during that time.

Because of these generally bad results for RAC, very few solid conclusions can be drawn from the respective data. However, it was still included for comparison where relevant.

3.6.5 Product inhibition

One final topic to cover in this discussion is the possibility of the slow-down having some relation to product inhibition. Although, as mentioned in *Section 3.3.3,* literature has indicated that product inhibition is not likely to be the main cause of this effect, it was nonetheless necessary for us to determine how much cellulose was degraded in each case in order to confirm this and contextualize our results (show what range of substrate

conversion we are working within). As per *Table 1,* the highest fraction of Avicel degraded that could be calculated simply (without accounting for substrate removed during the restart) is for the sample in Experiment 5 where no RAC was added (no restart): 370 µmoles of bonds were hydrolysed, which corresponds to 22.15% degradation of cellulose by weight. Similarly, for RAC the maximum conversion would be 41.45%, as mentioned in *Section 3.6.4*. Although the lines of best fit would theoretically allow us to account for the amount of cellulose degraded during the temperature equilibration of the sample cells, we decided to neglect this and use only the real data points, since the fits are not perfect and may diverge from the data most dramatically near the peak of each hydrolysis, where they would be most relevant. Therefore, the real conversion of cellulose would be higher. Furthermore, the values may be crude approximations of the actual amount of cellulose degraded during the measurable periods because it assumes that for every one ß(1-4) glycosidic bond broken, one glucose molecule is removed from the cellulose polymer. However, the last bond broken when a cellulase molecule reaches the end of a chain would release two glucose molecules. The assumption is that this discrepancy is relatively small since the number of chain ends is very small compared to the number of bonds within the chains. However, these values still confirm that product inhibition is an unlikely cause of the slow-down, at least for Avicel, because the substrate was only partially degraded (by just over 20%), hence releasing relatively little product. Furthermore, the slow-down occurs most radically very near the beginning of the reaction, when very little glucose would have accumulated.

3.7 Conclusion

Our aim for this project, as outlined in our research question, was to investigate potential causes of the slow-down effect and elucidate the mechanism behind it if possible. Because of the nature of our results and experimental setup, we could not go into the molecular level of detail in our interpretation of results without becoming speculative. Thus, the precise mechanism could not be uncovered.

The most important conclusion we have reached is that the maximum increases in reaction rates during the restarts are proportional to the amount of fresh substrate, not enzymes, added (although this could only be determined with Avicel). This, in combination with the decrease in enzyme efficiency when extra CTec2 was added, indicates that fresh substrate was the limiting factor and not the number of functional enzymes. However, slow-down still occurred after both restarts and the end reaction rates were approximately the same as when no change to the standard hydrolyses had been made. At this point, enzyme concentration was not a pivotal factor to reaction rate, since each sample, whether substrate or enzyme had been added, had a different CTec2 concentration from the non-restarted sample, which further emphasizes how this was not the limiting factor. Comparison of the specific activities of the CTec2 cocktail throughout the reactions showed that while adding fresh cellulose yields similar initial rate increases and end final rate values, the specific activities of the cellulases were much higher, meaning that it was a more efficient solution in terms of the amount of cellulase cocktail added compared to the reaction rates achieved. This has economic implications for the biofuel industry, which would of course benefit from achieving the highest enzyme efficiency possible, especially since the cost of enzymes is a large prohibitor of the mass commercial production of bioethanol.

It was also confirmed that product inhibition is an unlikely cause of the slow-down within our range of conversion for Avicel (13.49% - 22.15%), since the inhibitory effects of glucose (the final product of the hydrolysis) would be minimal at this level of degradation.

These findings agree with the general hypothesis we formed from reviewing relevant literature; that the reaction rates (and hence the slow-down) correlate primarily to changes in the structure of cellulose throughout the reaction. Namely, when there is more amorphous cellulose the reaction rate is higher because of the greater surface area for enzymes to attack and the faster action of endoglucanases, which prefer these regions. Thus, the peak increase in reaction rates is proportional to the amount of fresh substrate because new substrate contains undegraded amorphous regions of cellulose. As these amorphous regions become increasingly degraded, leaving a higher proportion of crystalline substrate, the available surface area decreases, and the reaction rate becomes more reliant on the action of the slower cellobiohydrolases, which are more suited to the degradation of crystalline regions. This may provide a reason for why the reaction rate becomes so low towards the later stages of the hydrolyses. Additionally, this can be interpreted using our analysis of the two-term exponential-decay lines fitted to the reaction rate data, which suggest one term has a major contribution to the slow-down while the other has only a minor one. Continuing with our hypothesis, the major, fast-decaying term may bear a relation to the amount of 'available' cellulose or surface area, implying that it is proportional to the reaction rate, which again explains why amount of fresh substrate is proportional to the initial reaction rate increase during restarts. The minor term is possibly due to slight denaturation of the enzymes in CTec2.

Additional results with an impact on this hypothesis would have been obtained if we had not experienced issues using RAC as a substrate in our experiments, as this would have allowed further assessment of the importance of the structure of cellulose by providing a more amorphous counterpart to Avicel.

As much as the above explanation fits our results, it is clearly not complete, and further investigation (see *Section 3.8)* is necessary to confirm or reject our hypothesised reasons and provide a comprehensive quantitative theory describing the slow-down. Despite this, our findings provide useful insight into the topic and support many of the findings in other studies done on enzymatic hydrolysis of cellulose.

3.8 Perspective

To conclude this project, we have come up with several suggestions as to how the course of future research could proceed so it would further strengthen, or reject our hypotheses and interpretation of the results.

Firstly, it was noted that RAC did not behave according to our expectations. To solve this issue, for which we speculate is related to the hydrogel structure of the substrate, it would be possible to continuously stir the samples containing RAC. This would allow a flow of the enzyme through the substrate, overcoming the structural problem. However, stirring in the calorimeter may bring additional problems and make the experiment more complex, as stirring would generate friction and hence increase heat flow from that cell. It would also require additional tools inserted into the cell for stirring, making the cells less sealed and increasing the possibility of evaporation of the sample. Further exploring the RAC issue, it could have been replaced with

either a more amorphous or a more crystalline substrate than Avicel. This would still allow comparison of the effect of the structural differences between the substrates, but without the hydrogel-related problems.

If data without interferences had been necessary, it would be possible to add enzyme via titration. This would allow to keep track of all heat flow changes, without noisy signal during the temperature equilibration times.

Furthermore, experiments could have been performed with several repetitions - this would allow for more reliable results and lessen the effect of possible errors that might have occurred randomly, during the experiment executions. Another possibility would be increasing the range of enzyme and substrate concentrations used - with differing those, it would be possible to explore what ratio of enzyme and substrate is optimal for the enzymatic activity.

Lastly, while a lot of data manipulation has been performed, it would be possible to further extend it. One of the ways to do so could be, for example, calculating half-times of the two slow-down factors found in each two-term exponential decay curve (as explained in *Section 3.6.3*). Then, it would be possible to compare the half-times before and after disturbing the initial hydrolysis reaction. This could maybe give one an insight into whether or not there is any difference in the decay speed before and after substrate/enzyme addition.

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6. Appendix

6.1 Raw Data Graphs

Heat flow in µW. Baselines were subtracted, and the time set so that t=0s at the point where the sample cell containing the enzyme/substrate mix was first lowered into the calorimeter (the spike at 0s). Figures were enlarged to show appropriate data regions.

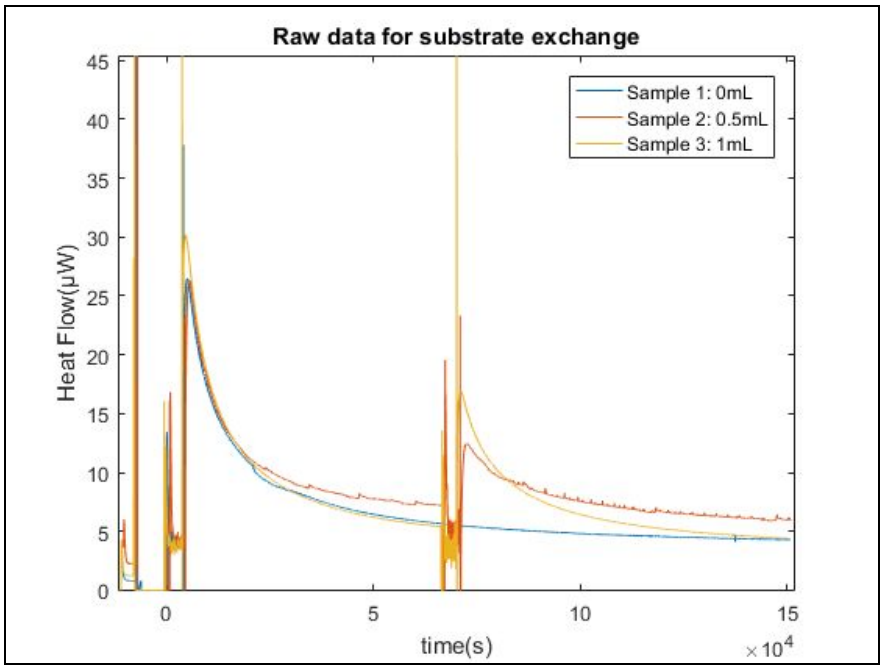

Figure 11: Raw data for Experiment 2- fresh substrate addition (Avicel)

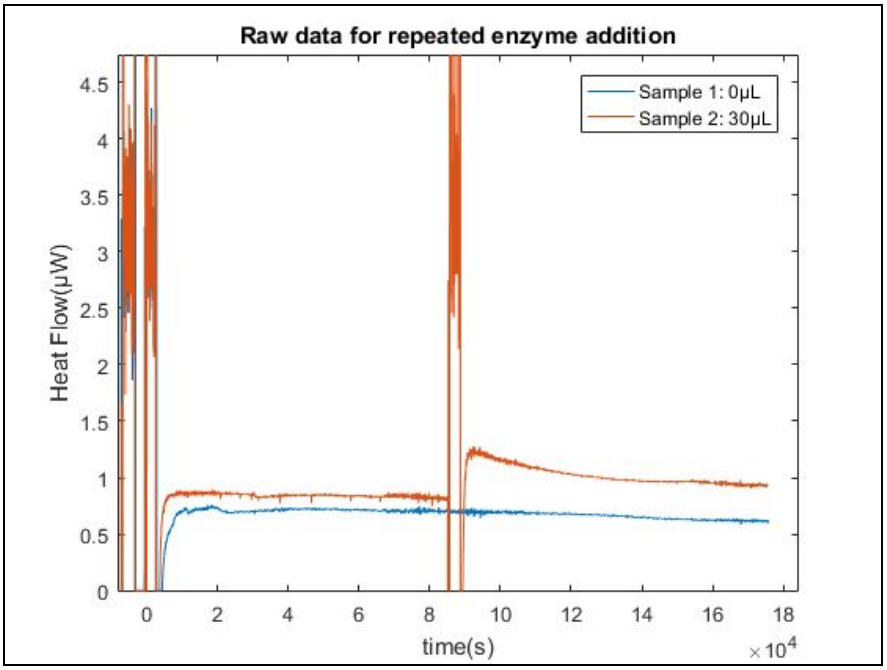

Figure 12: raw data for Experiment 3- repeated enzyme addition in RAC. Results for channel 4 omitted due to bad data.

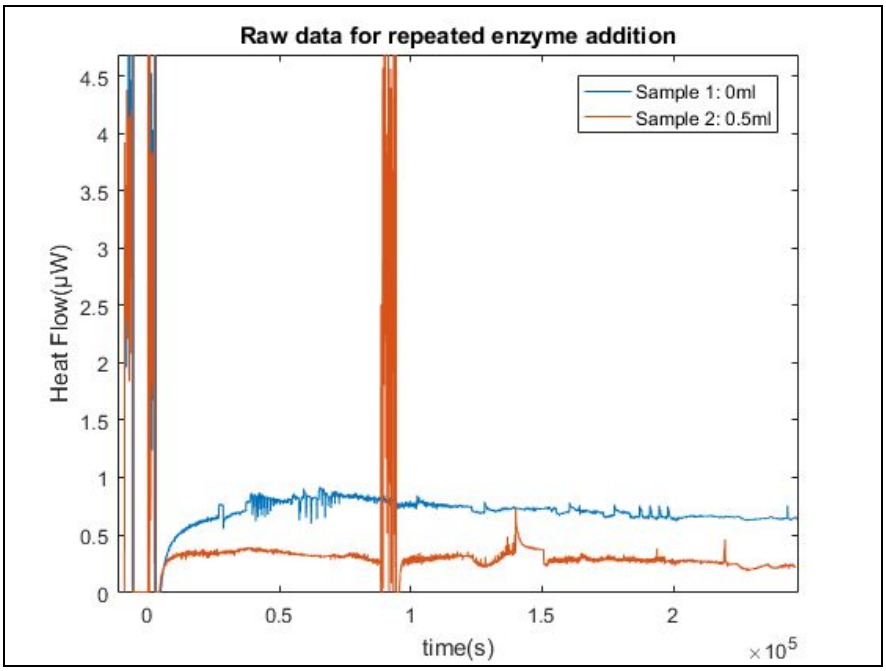

Figure 13: raw data for Experiment 4- fresh substrate addition (RAC). Results for channel 4 omitted due to bad data.

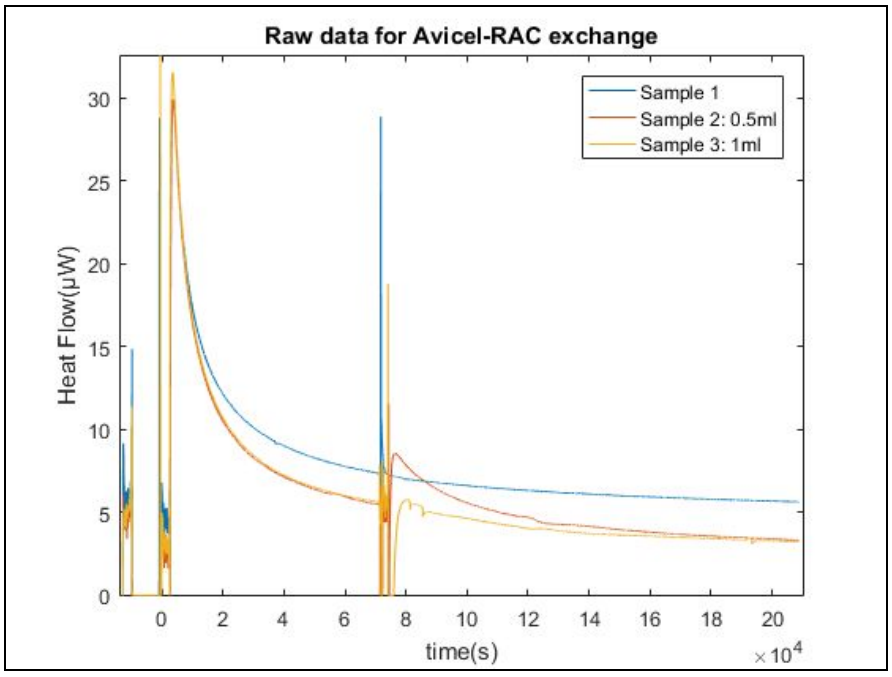

Figure 14: Raw data for Experiment 5- initial substrate Avicel, then with addition of fresh RAC.

6.2 Representative MATLAB code

C:\Users\Torben\Documents\MATLAB\Cellula...\Exp1enzadd.m Page 1

```
close all
clear all
[t2,w2]=importfile('0.txt',1,6003);
[t3,w3]=importfile('30.txt',1, 6003);
[t4,w4]=importfile('60.txt',1, 6003);

%converting to uW
w2=(w2*10^6);
w3=(w3*10^6);
w4=(w4*10^6);

%subtracting baseline
w2=w2-713.3;
w3=w3-555.6;
w4=w4-384.6;
for k=1:length(w2);
    if w2(k)<0;
        w2(k)=0;
    end
end
for p=1:length(w3);
    if w3(p)<0;
        w3(p)=0;
    end
end
for y=1:length(w4);
    if w4(y)<0;
        w4(y)=0;
    end
end
%subtracting time so that t=0s when enzyme is first lowered halfway
t2=t2-12250;
t3=t3-12200;
t4=t4-11870;
%splitting experiment into first and second enzyme additions
%(also restarting numbering from t=(2nd enzyme lowered)=0 for second addition)
tf4=t4(t4>(4787)&t4<(79600));
wf4=w4(t4>(4787)&t4<(79600));
tl4=t4(t4>(85350))-(79600);
wl4=w4(t4>(85350));
tf3=t3(t3>(4450)&t3<(78790));
wf3=w3(t3>(4450)&t3<(78790));
tl3=t3(t3>(85540))-(78790);
wl3=w3(t3>(85540));
tf2=t2(t2>(5055)&t2<(79600));
wf2=w2(t2>(5055)&t2<(79600));
tl2=t2(t2>(79600))-(79600);
wl2=w2(t2>(79600));
%overall ch2 for curve fitting
th2=t2(t2>(5055));
wh2=w2(t2>(5055));
```

```
%raw data (only baseline subtracted)
plot(t2, w2)
hold on
plot(t3, w3)
hold on
plot(t4, w4)
hold off
xlabel('time(s)')
ylabel('Heat Flow(µW)')
legend('Sample1-0µL','Sample2-30µL','Sample3-60µl')
%changing HF(w) into umol/s (assuming dH=-4.32*10^9uJ/mol for B(1-4)glycosidic bond)
dH=4.32*10^9;
rh2=(wh2/(dH))*10^6;
rf2=(wf2/(dH))*10^6;
rf3=(wf3/(dH))*10^6;
rf4=(wf4/(dH))*10^6;
rl2=(wl2/(dH))*10^6;
rl3=(wl3/(dH))*10^6;
rl4=(wl4/(dH))*10^6;
%enzyme concentrations and normalizing
v1=(3*10^(-3))+(30*10^(-6));
vf3=(3*10^(-3))+(60*10^(-6));
vf4=(3*10^(-3))+(90*10^(-6));
c1=(30*10^(-6));
c3=(60*10^(-6));
c4=(90*10^(-6));
nf2=rf2/c1;
nf3=rf3/c1;
nf4=rf4/c1;
nl2=rl2/c1;
nl3=rl3/c3;
nl4=rl4/c4;
figure
plot(tf2, nf2,'.')
hold on
plot(tf3, nf3,'.')
hold on
plot(tf4, nf4,'.')
hold off
xlabel('Time(s)')
ylabel('µmol/(s*L(enzyme))')
legend('Sample 1','Sample 2','sample 3')
title('Enzyme activity 1st hydrolysis')
figure
plot(tl2, nl2,'.')
hold on
plot(tl3, nl3,'.')
hold on
plot(tl4, nl4,'.')
hold off
xlabel('Time(s)')
```

```
ylabel('µmol/(s*L(enzyme))')
legend('Sample 1: 0µL','Sample 2: 30µL','Sample 3: 60µL')
title('Experiment 1 -varied Ctec2 dose in Avicel)
%extrapolated fit ch2 as a whole
for ty2=1:t2(6003);
    y2(ty2) = 0.005364*exp((-9.676e-05)*ty2) + (0.001687)*exp((-4.083e-06)*ty2);
end
tfy2=1:20:tf2(2483);
yf2=y2(1:20:tf2(2483));
halftime2f=0.693/(9.676e-05);
halftime2s=0.693/(4.083e-06);
%extrapolated fits for 1st addition
for tfy3=1:tf3(2487);
    yf3(tfy3)=0.008547*exp((-0.0001637)*tfy3) + 0.002536*exp((-1.211e-05)*tfy3);
end
tfy3=1:20:tf3(2487);
yf3=yf3(1:20:length(yf3));
halftime3ff=0.693/(0.0001637);
halftime3fs=0.693/(1.211e-05);
for tfy4=1:tf4(2509);
    yf4(tfy4) = 0.007254*exp((-0.0001414)*tfy4) + (0.002447)*exp((-8.353e-06)*tfy4);
end
tfy4=1:20:tf4(2509);
yf4=yf4(1:20:length(yf4));
halftime4ff=0.693/(0.0001414);
halftime4fs=0.693/(8.353e-06);
%extrapolated fits for 2nd addition
for tly3=1:tl3(2728)
    yl3(tly3)=0.002144*exp((-7.483e-05)*tly3) + 0.001416*exp((-5.812e-06)*tly3);
end
tly3=1:20:tl3(2728);
yl3=yl3(1:20:length(yl3));
halftime3lf=0.693/(7.483e-05);
halftime3ls=0.693/(5.812e-06);
for tly4=1:tl4(2737)
    yl4(tly4) = 0.003135*exp((-8.518e-05)*tly4) + (0.001718)*exp((-4.882e-06)*tly4);
end
tly4=1:20:tl4(2737);
yl4=yl4(1:20:length(yl4));
halftime4lf=0.693/(8.518e-05);
halftime4ls=0.693/(4.882e-06);

halftime2=vertcat(halftime2f,halftime2s);
halftime3f=vertcat(halftime3ff,halftime3fs);
halftime4f=vertcat(halftime4ff,halftime4fs);
halftime4l=vertcat(halftime4lf,halftime4ls);
halftime3l=vertcat(halftime3lf,halftime3ls);
T=table(halftime2,halftime3f,halftime3l,halftime4f,halftime4l);
figure
plot(tf2, rf2,'.')
hold on
```

```
plot(tf3, rf3,'.')
hold on
plot(tf4, rf4,'.')
hold on
plot(tfy2, yf2)
hold on
plot(tfy3, yf3)
hold on
plot(tfy4, yf4)
hold off
title('First enzyme addition-30µL')
xlabel('time(s)')
ylabel('Reaction rate(µmol/s)')
legend('Sample 1','Sample 2','Sample 3','y2=(5.4e-03)*exp((-9.676e-05)*x)+(1.7e-03)*exp
((-4.083e-06)*x)','y3(tfy3)=(8.5e-03)*exp((-1.6e-04))*x)+(2.5e-03)*exp((-1.211e-05
*x)','y4(tfy4)=(7.3e-03)*exp((-1.4e-04)*x)+(2.4e-03)*exp((-8.353e-06)*x))')
figure
plot(tl2, rl2,'.')
hold on
plot(tl3, rl3,'.')
hold on
plot(tl4, rl4,'.')
hold on
plot(tly3, yl3)
hold on
plot(tly4, yl4)
hold off
title('Second enzyme addition-varied dose')
xlabel('time(s)')
ylabel('Reaction rate(µmol/s)')
legend('Sample 1: 0µL','Sample 2: 30µL','Sample 3: 60µL','y3=(2.1e-03)*exp((-7.483e-05)
*x)+(1.4e-03)*exp((-5.812e-06)*x)','y4=(3.1e-03)*exp((-8.518e-05)*x)+(1.7e-03)*exp
((-4.882e-06)*x)')
% Integrating data for glucose conversion
avicel=100.33;
tot=avicel*(3*10^(-3));
gluc=(180.156*10^(-6));
cgluc2=cumtrapz(th2,rh2);
for h1=1:length(cgluc2)
    fracgluc2(h1)=(cgluc2(h1)*gluc*100)/tot;
end
tlo3=tl3+78790;
th3=vertcat(tf3,tlo3);
rh3=vertcat(rf3,rl3);
cgluc3=cumtrapz(th3,rh3);
for h2=1:length(cgluc3)
       fracgluc3(h2)=(cgluc3(h2)*gluc*100)/tot;
end
tlo4=tl4+79600;
th4=vertcat(tf4,tlo4);
rh4=vertcat(rf4,rl4);
```

```
cgluc4=cumtrapz(th4,rh4);
for h3=1:length(cgluc4)
       fracgluc4(h3)=(cgluc4(h3)*gluc*100)/tot;
end
figure
plot(th2, fracgluc2)
hold on
plot(th3, fracgluc3)
hold on
plot(th4, fracgluc4)
ylabel('% cellulose converted')
xlabel('time(s)')
legend('Channel2','Channel3','Channel4','location','northwest')
converted2=trapz(th2,rh2);
converted3=trapz(th3,rh3);
converted4=trapz(th4,rh4);
G=table(converted2,converted3,converted4);
%peak and end changes in restart from end of standard hydrolysis
dif2=rl2(length(rl2))-rf2(length(rf2));
peak3=rl3(1)-rf3(length(rf3));
dif3=rl3(length(rl3))-rf3(length(rf3));
peak4=rl4(1)-rf4(length(rf4));
dif4=rl4(length(rl4))-rf4(length(rf4));
D=table(dif2,peak3,dif3,peak4,dif4);
```