# ENZYMATIC HYDROLYSIS OF CELLULOSIC FIBER

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# ENZYMATIC HYDROLYSIS OF CELLULOSIC FIBER

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# SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| BET | Brunauer-Emmett-Teller |
| CAC | Cellulose accessibility to cellulase |
| CBD | Carbohydrate Binding Domain |
| CD | Catalytic Domain |
| c-PAM | Cationic Polyacrylamide |
| CI (FTIR) | Crystallinity Index (Fourier Transform Infrared Spectroscopy) |
| EC | Endoglucanase |
| FQA | Fiber Quality Analyzer |
| FPU | Filter Paper Units |
| HW | Hardwood |
| L | Contour Length |
| RPS | Recycled Paper Sludge |
| SSA | Specific Surface Area |
| SSF | Simultaneous Saccharification and Fermentation |
| SW | Softwood |
| TOC | Total Organic Carbon |

# SUMMARY

Low cost cellulosic wastes like paper sludge, municipal wastes, solid wastes from food, packing etc. contain a high amount of cellulose which can be converted to bioethanol by two steps: (1) solubilization of cellulosic fibers to monosaccharides (2) conversion of monosachharides to bioethanol via fermentation. At present the implementation of this technology has been deterred by high cost for enzymes. Enzymatic hydrolysis of cellulosic fibers shows a biphasic behavior with an initial fast step followed by a slow step leading to low cellulose conversion rates. Low hydrolytic conversion rates necessitate the use of a high enzyme dosage to obtain meaningful cellulose conversion rates which make the implementation of this entire technology economically infeasible. The objective of this study is to get a better understanding of the mechanism of enzymatic hydrolysis of fibers to glucose and to investigate the effect of cationic polymers on enzymatic hydrolysis rates. To achieve the first objective, we performed experiments so as to study changes in morphological and physiochemical properties like fiber length, percentage of fines, crystallinity index, kink angle, kink index, mean curl, total organic carbon and glucose production with time. We used bleached kraft softwood, hardwood, and unbleached softwood fiber as cellulosic substrate and pergalase as cellulase enzyme. All of the experiments were carried out at experimental conditions of a temperature of 50 ˚C and a pH of 5.0 which maximize enzymatic activity. We studied the impact of recycling and refining on hydrolysis rates by measuring total organic carbon and glucose production. We found that refining increases enzymatic conversion rates by about as much as 20 %, however refining being energy intensive makes its

implementation economically unfavorable. We found a novel way of enhancing hydrolysis rates by the use of cationic polyacrylamides. The effect of cationic polacrylamides was studied on both hardwood and softwood fibers at similar experimental conditions. Cationic polyacrylamides produced a maximum rate increase of 20 % in hydrolytic conversion rates for hardwood fibers. Even though, the increase in hydrolysis rates for softwood fibers was smaller than hardwood fibers, it was still significant. We further studied the effect of parameters like polymer concentration, cationicity and molecular weight to find a relation between properties of polymers and the increase in enzymatic hydrolysis.

# CHAPTER 1

# INTRODUCTION

Biological conversion of low cost cellulosic residues like paper sludge, agricultural and forestry residues, municipal solid waste, and food waste to fermentable sugars reduces the waste disposal costs and concomitantly meets the growing demand for energy (Walker and Wilson, 1991; Lynd et al., 2002; Gan et al., 2003; Zhang and Lynd, 2004; Kumar et al., 2008). These sugars can further be converted to fuels and chemicals like ethanol, organic acids or biodegradable plastics etc (Walker and Wilson 1991; Zhang and Lynd, 2004, 2006; Marques 2008). The production of ethanol as a fuel from cellulosic fraction was first proposed in 1960s (Cheung and Anderson, 1997). The conversion of waste cellulosic residues to bio-ethanol involves delignification of cellulose, depolymerization of carbohydrate polymers to free sugars via enzymes and fermentation of these sugars to produce ethanol (Cheung and Anderson, 1997; Lee, 1997). The major limitations in the technology of conversion of waste biomass to bioethanol are the low hydrolytic conversion rates and the high cost of enzymes which make the implementation of overall process uneconomical (Fan et al., 1980; Lynd et al., 2002; Gan et al., 2003; Zhang and Lynd, 2004; Kumar et al., 2008). The mechanism of enzymatic hydrolysis of cellulose is not clearly understood because of the complexity of heterogeneous nature of substrate and the multi-component cellulase system. The understanding of the mechanism of enzymatic hydrolysis is critical to the work towards increasing the hydrolytic conversion rates and developing a technologically feasible and economically viable process for conversion of waste lignocellulosics to bio-fuels. Expansion of fermentation technologies to the low-cost lignocellulosic biomass holds a great potential for in terms

of energy reserves for future. Although cellulose may be hydrolyzed by non enzymatic methods, the utility cost of enzymatic hydrolysis is much lower compared to the alternative methods of acidic hydrolysis because it is carried out at milder reaction conditions, higher product yields, fewer side reactions, less energy demand and less reactor resistance to pressure and corrosion (Lee, 1997; Zuhair, 2007; Marques et al. 2008). Enzymes are also environment friendly and non toxic or corrosive. However, commercial application of enzymatic hydrolysis of cellulose has been deterred by the high cost of enzymes, slow reaction rate and lack of an effective reactor system for the complex heterogeneous nature of the reaction in a solid-liquid system (Gan et al., 2003; Zhang and Lynd, 2004, 2006).

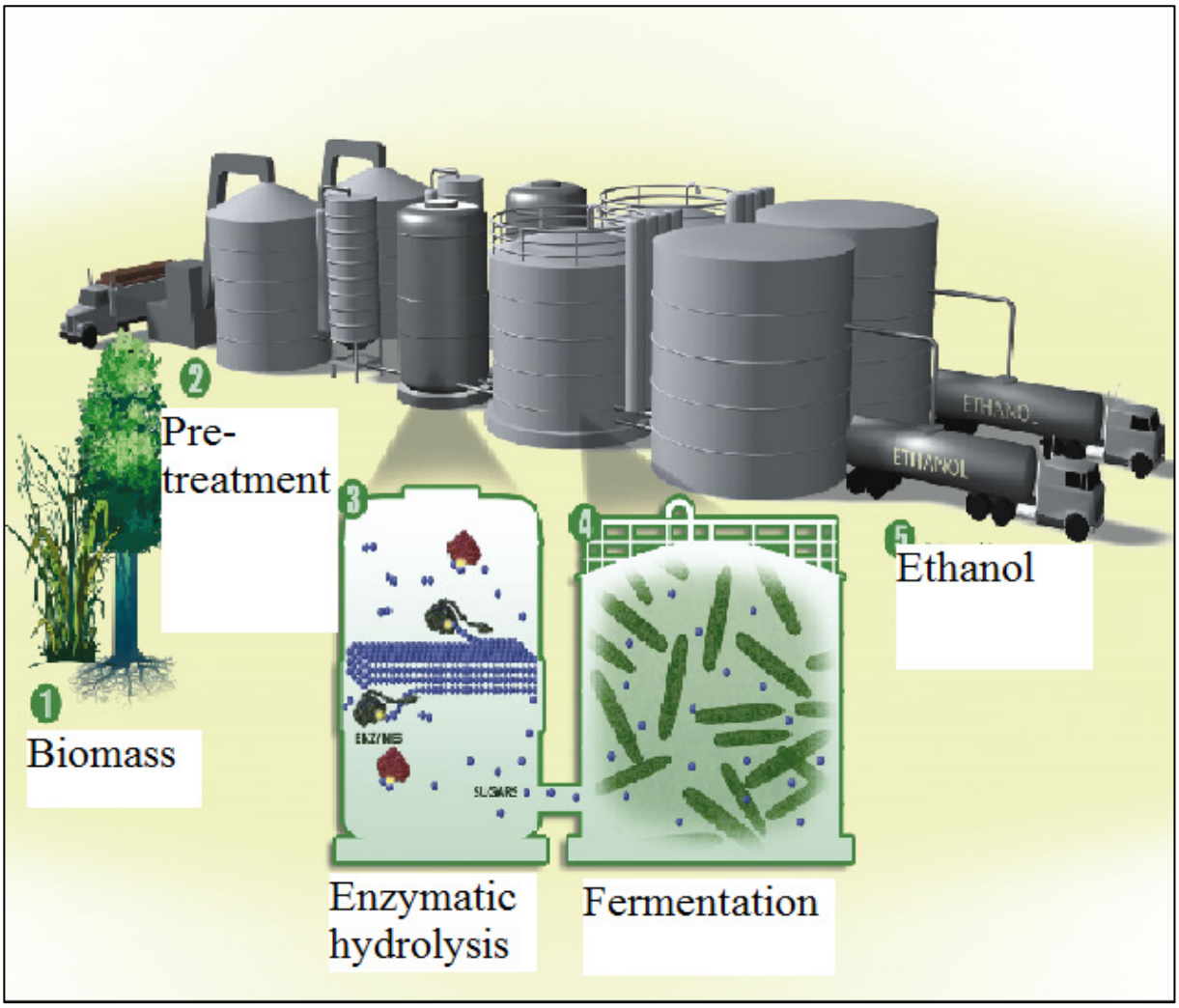



Figure 1.1: Overview of lignocellulosics to biofuels conversion

(NREL: http://www.nrel.gov/biomass/)

## 1.1 Bioconversion of paper sludge to glucose

The macroscopic view of this project is the bioconversion of waste paper sludge to fermentable sugars. There has been an increase in paper recycling endeavor due to environmental concerns, governmental regulations and economic considerations. During paper recycling, about 15-20% of the reused pulp fibers is damaged by shortening and ends up as waste sludge. The high water holding capacity of recycled paper sludge (RPS)

makes dewatering and disposal difficult (Lark et al., 1997). The various processes to dispose of RPS are land filling, land spreading, and incineration. Incineration facilities can be damaged by temperature fluctuations caused by high water content. Landfill space is limited and uncontrolled fermentation of organic wastes can cause emission of greenhouse gases such as methane and carbon dioxide (Schartman and Jeffries, 1999; Moon et al., 2009). The biological conversion of sludge to value added bio products like bioethanol, organic acids serves both the purpose of reducing solid waste disposal cost and generating energy. About 5 million metric tons of waste paper sludge (oven dry) is disposed of annually in the United States (Fan and Lynd, 2007). RPS approximately contains 50%-80% of cellulose depending on the source of the sludge (Schartman and Jeffries, 1999). Because of its high cellulose content it can be potentially converted to fermentable sugars which can further be either converted to bioethanol or other organic chemicals which would not only utilize the waste fibers but also reduce disposal costs (Fan and Lynd, 2007; Marques et al.,2008). The advantages of this solid feedstock are high enzymatic digestibility due to its low lignin content and low particle size and environmental benefit of reduction of waste volume (Lark et al., 1997; Fan and Lynd 2007; Romani et al., 2007). Industrial biosludge has been evaluated to have a good potential for ethanol production by simultaneous saccharification and fermentation (SSF). In a typical SSF process, the cost of cellulase enzyme for hydrolysis accounts for a large portion of the overall cost of conversion of biomass to ethanol (Schartman and Jeffries, 1999). So, the primary concern in the commercialization of this process is reduction in the enzyme dosage. When RPS is used, then one can use lower enzyme dosage rates as the repulped fibers have been treated extensively by mechanical and chemical procedures

such as refining, drying and so on and therefore do not require much size reduction procedure (Schartman and Jeffries, 1999).

## 1.2 Objective

The purpose of the present study is to understand the mechanism of enzymatic hydrolysis and investigate different methods for enhancing the enzymatic hydrolysis. A major portion of the study is concentrated on the effect of cationic polyacrylamides in accelerating the enzymatic hydrolysis rates. One of the unexpected findings of our lab is that the cationic polymers increase the enzymatic hydrolysis rates for corn starch (Banerjee et al., in press). My work is focused on studying the effect of cationic polyacrylamides on enzymatic hydrolysis of cellulosic fibers. Some of the differences between enzymatic hydrolysis of corn starch and cellulose fiber are 1) solubility of substrates 2) particle size of substrates and 3) interactions between polymer and enzyme. These differences may lead to a difference in behavior of cellulosic fibers towards enzymatic hydrolysis.

# CHAPTER 2

# BACKGROUND

## 2.1 Hardwood and Softwood fibers

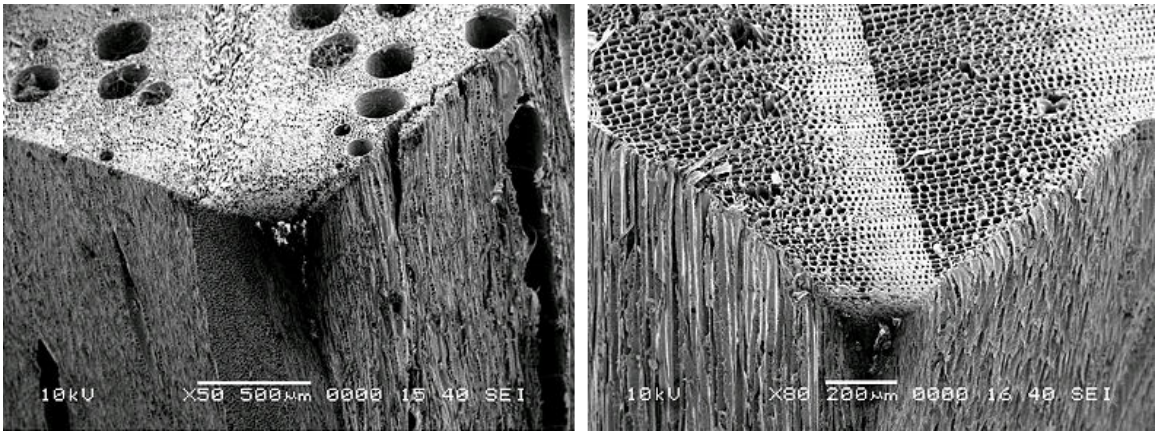



Figure 2.1: Hardwood and softwood fiber (Gullichsen and Fogelholm, 1999)

Softwoods are referred to as coniferous woods since they have seeds that are produced in cones and not covered, while hardwood trees produce covered seeds within flowers. However, these general names cannot be used exclusively as a measure of "hardness" because considerable overlap occurs in the range of average specific gravities of softwoods and hardwoods; some softwoods are quite hard, and some hardwoods are relatively soft (Gullichsen and Fogelholm, 1999). Another classification is based on the retention of needle- or scale-like leaves by most softwoods, as opposed to the annual leaf shedding by most hardwoods (Gullichsen and Fogelholm, 1999). Hardwood fibers are somewhat similar to softwood fibers with some differences; *e.g.* they are rounder and shorter. Both of them have same constituents i.e. cellulose, hemicellulose, lignin etc. but they differ in proportion of these constituents (Gullichsen and Fogelholm, 1999)

Table 2.1: Constituents of hardwood and softwood fibers

| | **Hardwood** | **Softwood** |
|---|---|---|
| **Length** | ~ 1 mm | 2~4 mm |
| **Cellulose** | 45% | 45% |
| **Hemicellulose** | 25% | 17% |
| **Lignin** | 20% | 28% |

## 2.2 Lignocellulose

Lignocellulosics are primarily made up of plant cell wall which is a composite of three biopolymers- cellulose, hemicellulose and lignin. It is composed of about 30-50% cellulose, 20-35% hemicellulose, 10-15 % lignin (Gullichsen and Fogelholm, 1999). Cellulose is the major constituent of the plant cell wall and is a linear biopolymer of d-glucose with a molecular weight of about half million. It is linear, unbranched and mostly crystalline in nature. Hemicelluloses are composed of short highly branched chains of various sugars: mainly xylose, arabinose (five-carbon), and further galactose, glucose and mannose (all six-carbon). It also contains smaller amounts of non-sugars such as acetyl groups. Hemicellulose, because of its branched, amorphous nature, is relatively easy to hydrolyze (Gullichsen and Fogelholm, 1999). Lignin is a large complex, variable, hydrophobic, cross-linked, three dimensional aromatic polymer of phenylpropane and methoxy groups, which gives rigidity to plant structure. It is extremely resistant to

chemical and enzymatic degradation and therefore is a residue in ethanol production. It is degraded by only few organisms mainly by fungi, into higher value products such as organic acids, phenols and vanillin (Lee, 1997).

## 2.3 Cellulose

Cellulose is the most abundantly naturally occurring biopolymer and has been used as a source of food and energy for years. It is a linear polymer of glucose units linked by β-(1-4) glycosidic bonds. It consists of both crystalline and amorphous components each of which show a different digestibility towards enzymatic hydrolysis and the percentage of regions is variable depending on the source of biomass. The cellulose chains are held in layers through vander waal's forces, intermolecular and intra molecular hydrogen bonding (Zhang and Lynd, 2004).

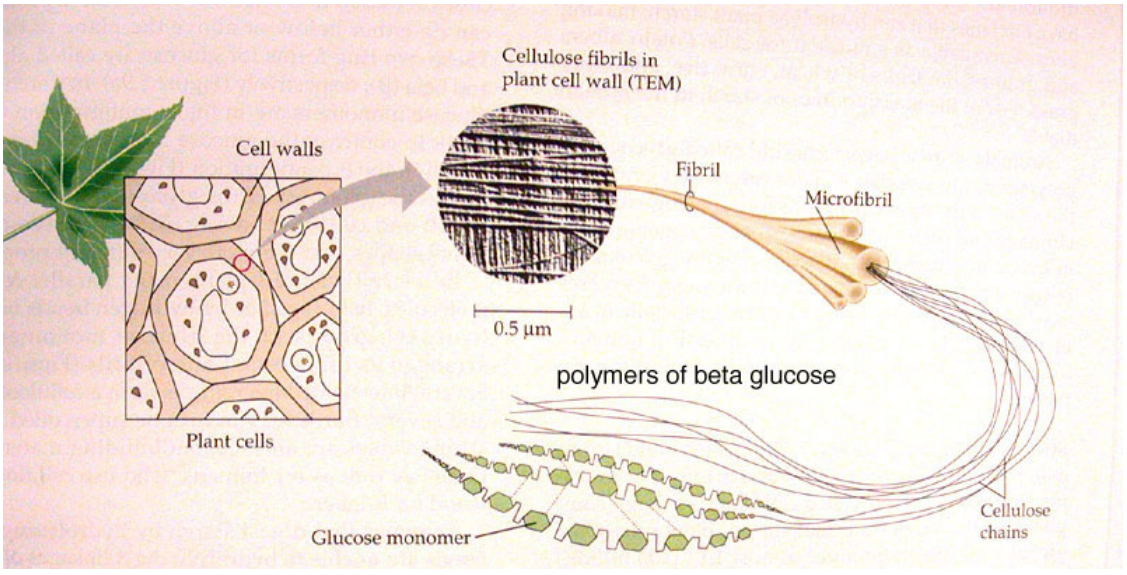



Figure 2.2: Microscopic view of plant cell walls down to cellulose

(NREL: http://www.nrel.gov/biomass/)

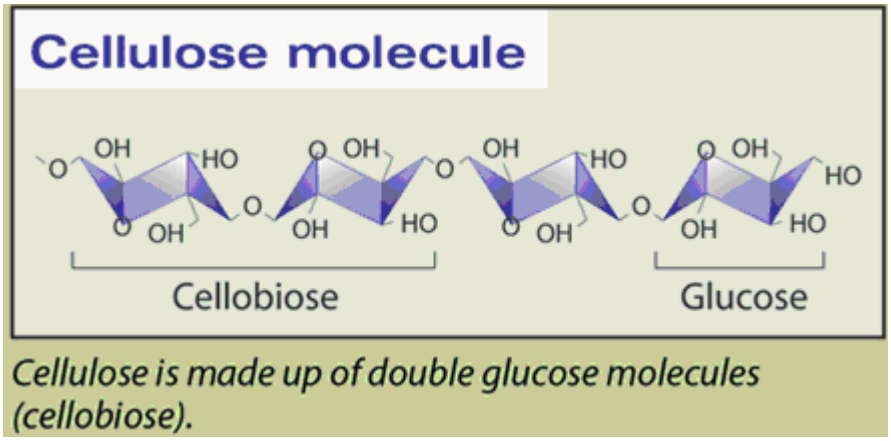



Figure 2.3: Structure of cellulose

(NREL: http://www.nrel.gov/biomass/)

Celluloses from different sources are same at the molecular level but they differ in their crystalline order structure and their association with other biopolymers like hemi cellulose, lignin. It is not difficult to hydrolyze β-(1-4) glycosidic bonds but the presence of intra and inter hydrogen bonds stiffens the chains and packs them tightly enough to prevent penetration not only by enzymes but also by small molecules like water which explains insolubility of cellulose in water.

## 2.4 Cellulases

Cellulases are enzymes that hydrolyze β-(1-4) glycosidic bonds in cellulose. They are produced both by bacteria and fungi. However, the enzymes produced by aerobic fungus, *Trichoderma reesei* are broadly studied for enzymatic hydrolysis of cellulose. These enzymes constitute non-complexed cellulase systems i.e. systems based on synergistic discrete action of individual components rather than that of a stable complex (Meinke et al., 1995).The general structure of most of the cellulases can be broken down into two

structural parts: the catalytic domain (CD) and the carbohydrate binding domain (CBD), both of which are connected via a flexible linker peptide. CBD promotes the adsorption of the cellulase to the crystalline region of the cellulosic substrate and facilitates the hydrolysis by bringing its catalytic domain in close proximity with cellulose chains (Abuja et al.,1988).The adsorption of cellulases onto cellulosic substrates is affected by the degree of polymerization, crystallinity, pH and temperature (Lynd et al., 2002; Zhang and Lynd, 2004)

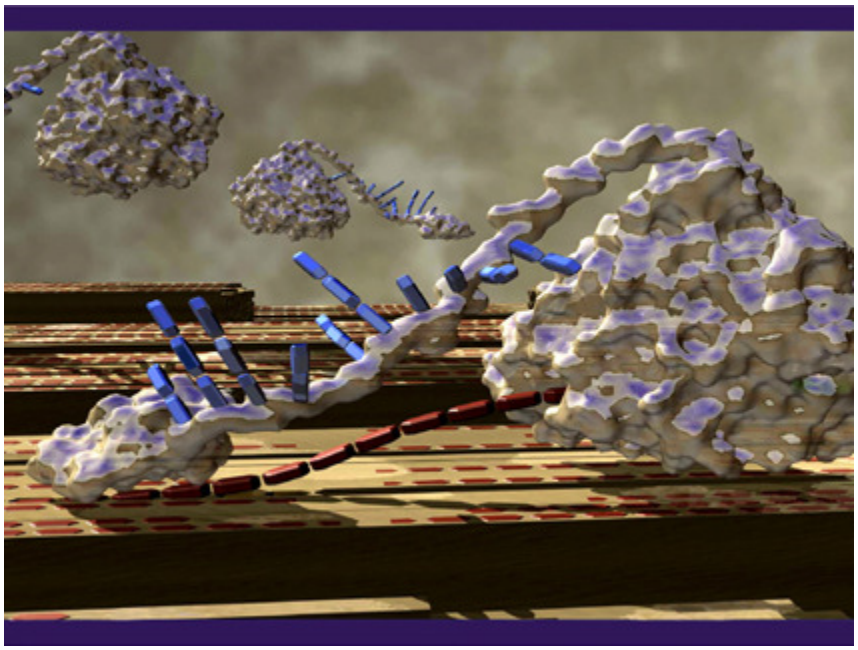


Figure 2.4: Cellulose breakdown (CBH 1)

(NREL: http://www.nrel.gov/biomass/)

Cellulases can be broadly divided into three classes based on their catalytic action as endoglucanases (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC3.2.1.21). Endoglucanases randomly attack the amorphous regions inside the cellulose chains on the surface of microfibrils and produce oligosachharides of varying

lengths and create new chain ends for exoglucanases. Exoglucanases hydrolyse the cellulose chain from ends producing cellobioses or two units of glucose. The hydrolysis of exoglucanases is restricted to the ends of cellulose chains as their access to substrate is hindered by their structure (Meinke et al., 1995). β-glucosidase hydrolyzes cellobiose units to form glucose units. The activity of cellulase systems is greater than the collective sum of individual activities, a phenomenon known as synergism. Synergism is a function of multiple forms of cellulases and the kind of cellulose i.e. amorphous or crystalline. There are different types of synergism: exo-endo , exo-exo , exo-gluco etc. Out of all these synergistic actions, synergism between exoglucanase and endoglucanase is the most important (Lynd et al., 2002; Kumar et al., 2008). As mentioned earlier, cellulose has both crystalline and amorphous regions and it is easier to hydrolyze amorphous regions in comparison to crystalline regions (Coughlan, 1992). Crystalline regions are resistant to attack by endoglucanases and the bonds cleaved are re-formed owing to stability of crystalline glucan chains. Therefore, synergism between endoglucanases and exoglucanases is vital to hydrolysis of cellulose. Exoglucanases chop off cellobiose units from newly created ends formed by endoglucanase, thereby preventing the reformation of bonds. Cellobiose is known to inhibit both exoglucanases and endoglucanases and makes this action rate limiting step in cellulose degradation (Lee, 1997). Therefore, the presence of β-glucosidase which converts cellobiose to glucose propels the reaction in the forward direction.

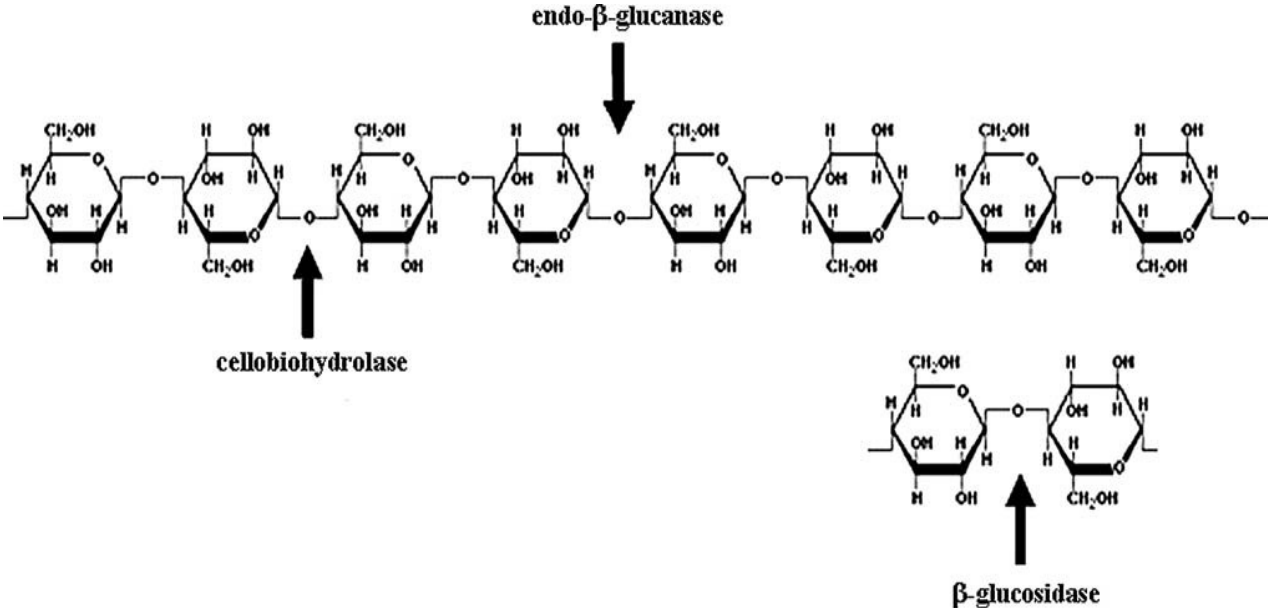



Figure 2.5: Synergistic action of cellulases (Kumar et al., 2008)

Trichoderma reesei is known to produce at least two exoglucanases, five endoglucanases and two beta-glucosidases (Vinzant et al., 2001). The two exoglucanases are explained by the two ends, i.e. reducing and non-reducing ends of crystalline cellulose and are also supported by synergy between exo-exo glucanases. However, the role of five endoglucanases is not clearly understood and is attributed to different nature of binding sites available on cellulose chains. Two β-glucosidases are explained by enzymes needed for breaking down cellobioses and short oligosaccharides to glucose. Although enzyme composition is an important factor which influences hydrolysis but accessibility of substrates has been found to be the overriding factor which affects hydrolysis (Ortega et al., 2001).

## 2.5 Effect of structural features on enzymatic hydrolysis

Although the composition of cellulase system influences enzymatic hydrolysis, the overriding factor is the dependency on structural features of cellulose such as crystallinity, lignin content, particle size, pore volume and accessible surface area which are specific to source and nature of substrate (Fan et al., 1980; Dusterhoft et al., 1993; Lee, 1997). The initial rate of enzymatic hydrolysis is relatively rapid and decreases over time. The biphasic behavior of enzymatic hydrolysis has been investigated by researchers for a long time now, however there is still no definite answer and the mechanism is only vaguely understood. It is a complex process which is a function of many variables and is further complicated by changing reaction dynamics. The fine structure variability of cellulose makes it difficult to obtain a discrete population of particles with identical structural features. Because of a great variability in the shape and size of the particles within any cellulose sample, measurements of structural features are only the average values of those features. Thus, experiments are limited to comparing measurements of hydrolysis among cellulose particles of average structural features and it is hard to study the effect of a particular structural feature on hydrolysis rates (Lynd et al., 2002). Because of interrelationships among various structural features, one can not identify a particular structural feature as the reason for slowing down of hydrolysis rates. For example, mechanical treatment of fibers to reduce crystallinity also increases the accessible surface area of cellulose. Therefore the increase in enzymatic hydrolysis can be due to either a decrease in crystallinity or an increase in surface area or a combination of both these factors. Similarly, structural discontinuities that contribute to an increase in

pore volume also lower the average crystallinity. It is impossible to alter one fine structural feature without altering others (Lynd et al., 2002).

### 2.5.1 Crystallinity

Common methods for the characterization of crystalline cellulose structure are based on X-ray (Kolpak and Blackwell, 1976; Krassig, 1993) or infrared (IR) absorption (Fink et al., 1985; Youn Oh et al., 2005). As mentioned earlier, crystalline regions are more difficult to hydrolyze than amorphous regions. Studies with pure cellulose have shown that the rate of hydrolysis of amorphous cellulose is five to thirty times higher than that of crystalline cellulose (Ghana et al., 1993; Lynd et al., 2002; Ortega et al. 2007; Zuhair, 2007). It has been reported that a lower starting crystallinity index produces higher saccharification rates (Fan et al., 1980). Studies have also been conducted by ball milling of crystalline cellulose, which decreases both particle size and crystallinity which conform an enhancement in enzymatic hydrolysis rate with a reduction in crystallinity (Chang and Holtzapple, 2007; Yoshida 2008). However, it is not clear if this increase in hydrolysis rate is a concerted effect of decrease in particle size and reduction in crystallinity. Cellulose is made up of both crystalline and amorphous fractions and if enzymes preferentially attack amorphous regions then one expects crystallinity to increase over the course of hydrolysis (Fan et al., 1980). There are conflicting results in support of this postulation. A few groups of investigators have reported an increase in crystallinity as hydrolysis proceeds (Saddler, 1986; Park et al., 2007). Several other studies did not find any appreciable increase in crystallinity during the course of enzymatic hydrolysis (Lynd et al., 2002). Sinitsyn et al., 1989 found that crystallinity initially increases with time, then decreases and finally levels off.

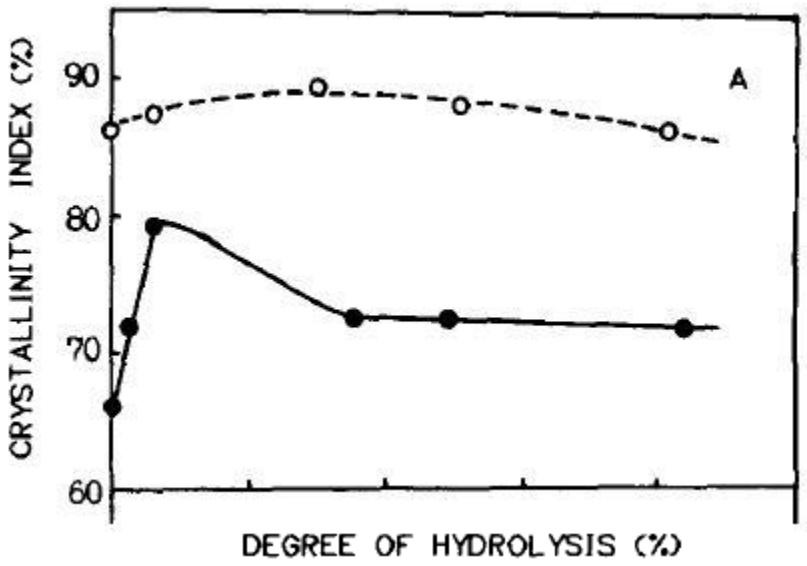



Figure 2.6: Crystallinity changes during hydrolysis (Sinitsyn et al., 1989)

Papers that have reported a direct correlation between hydrolysis rates and crystallinity have mostly used pure cellulosic substrates. The equivocal results and uncertainty of methodologies used to measure crystallinity make it difficult to conclude at this time if crystallinity index is a major determinant of the rate of enzymatic hydrolysis (Lynd et al., 2002; Mansfield et al., 1999; Yoshida et al., 2008).

### 2.5.2 Accessible surface area

Accessible surface area is other parameter which has been extensively studied with regard to its effect on hydrolysis rate. As cellulases need to adsorb on to the surface of cellulose prior to hydrolysis, it is intuitive that the initial rate of enzymatic hydrolysis is directly proportional to surface area accessible to enzymes. Cellulosic particles have inner and outer surface area and inner surface area is two-three orders higher than

external surface for most of the cellulosic samples. One of the popular methods to measure surface is Brunauer-Emmett-Teller (BET) method which measures the surface area available to a nitrogen molecule (Fan et al., 1980). The drawbacks of this method are that (1) nitrogen molecule is smaller than a cellulase molecule and (2) this technique is suited to measure surface areas of a dried cellulosic substrate. Because of the drawbacks of this technique, researchers using this method have found little evidence in support of the theory of surface area being a major determinant of hydrolysis (Mansfield et al., 1999). The internal surface area can be measured by solute exclusion, small-angle X-ray scattering , water vapor sorption etc. (Stone et al., 1969; Grethlein 1985) amongst which, solute exclusion technique is the most popular. It determines the area available in the form of pores and cavities in the fiber wall which are accessible to dextran molecules (Stone et al., 1969). The advantage of this method is that it can be used for measuring accessible surface areas of hydrated substrates. Stone et al, 1969 and Grethlein, 1985 using this method found linear correlations between the initial hydrolysis rates and the pore size accessible to a molecule of size 30-50 Å. But also to be noted is that dextran molecules cannot distinguish between the surface area at which enzymatic hydrolysis occurs from the area which does not have sites for enzymatic attack (Gilkes et al., 1992) which means that this technique can lead to an overestimation of internal surface area. Hong et al., 2007 proposed a new technique for measuring cellulose accessibility to cellulase (CAC) based on the langmuir adsorption of a nonhydrolytic fusion protein containing a cellulose-binding module and a green fluorescent protein resembling the cellulase molecule of Trichoderma Reesei. They also found a good correlation between substrate reactivity and the accessible surface area. It was reported by few authors that

specific surface area (SSA) decreases initially and then increases with time. At the start of the reaction enzymes rapidly hydrolyze amorphous regions leading to a rapid initial decrease in SSA but these enzymes also cause defragmentation of substrate which increases overall SSA with time (Klyosov, 1981). Hong et al., 2007 found out that CAC decreases over the course of enzymatic hydrolysis. Fan et al., 1980 have reported that SSA may not significantly affect the hydrolysis rates and concluded that sensitivity of hydrolysis rates to surface area depends on the specific substrate and varies widely for different substrates. Because of the limitations of current active cellulase adsorption methods for measuring surface area, there is an inconsistency in the results and no clear quantitative conclusion has been made between hydrolysis rate and cellulose accessibility.

### 2.5.3 Lignin content

It is reported in various papers that the presence of lignin decreases enzymatic hydrolysis rates (Mansfield et al., 1999; Zhang and Lynd, 2004; Yoshida et al., 2008). Lignin acts as a barrier and prevents the enzymes from binding to cellulose. It adsorbs irreversibly to cellulases molecules, thereby decreasing free enzyme available for hydrolysis of cellulose substrate (Yoshida et al., 2008). It blocks the progress of cellulase down the cellulose chain (Mansfield et al., 1999). For samples with low lignin and low crystallinity, a higher fraction of enzymes can adsorb onto cellulosic surface and are rapidly hydrolyzed while for samples with low lignin and high crystallinity, even though more enzymes adsorb onto the cellulosic surface, the hydrolysis rates are still slow.(Chang and Holtzapple, 2000).

# CHAPTER 3

# EXPERIMENTS

## 3.1 Experimental conditions

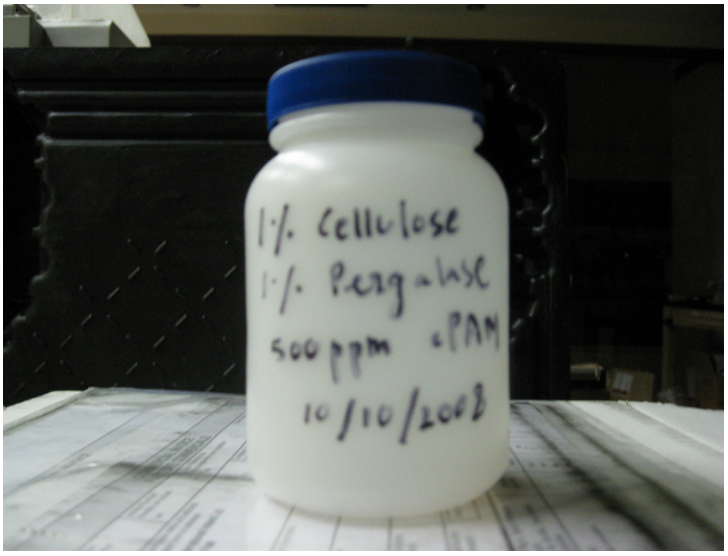



Figure 3.1: Bioreactor

The batch reactor system is made up of a 200 ml plastic bottle of 5 cm diameter used as bioreactor. This bioreactor is filled with sodium citrate buffer of concentration 0.05 M and pH 5 and the cellulosic fiber is suspended in this bioreactor. The substrate used is bleached kraft softwood, Weyerhaeuser, Canada, Lot-95623 GPOP and bleached hardwood, Alabama River. We also used unbleached softwood fiber with a kappa no. of 110.8, lobolloy pine, IP-64 Replicate, for few experiments. Pergalase 7457 from Genencor was the enzyme used for all of the experiments. Its activity was measured by the DNS method (Miller, 1959) and was calculated to be 15 filter paper units (FPU) / ml. Fiber consistency is defined as percentage of weight of fiber to the total weight of solvent and fiber. We used a fiber consistency of 1% for all the experiments unless specified. We used cationic polyacrylamides (c-PAMs) of different cationicity and molecular weights

from SNF polymers for investigating the effect of cationic polymers on enhancement of enzymatic hydrolysis.


Figure 3.2: Rotary water bath shaker

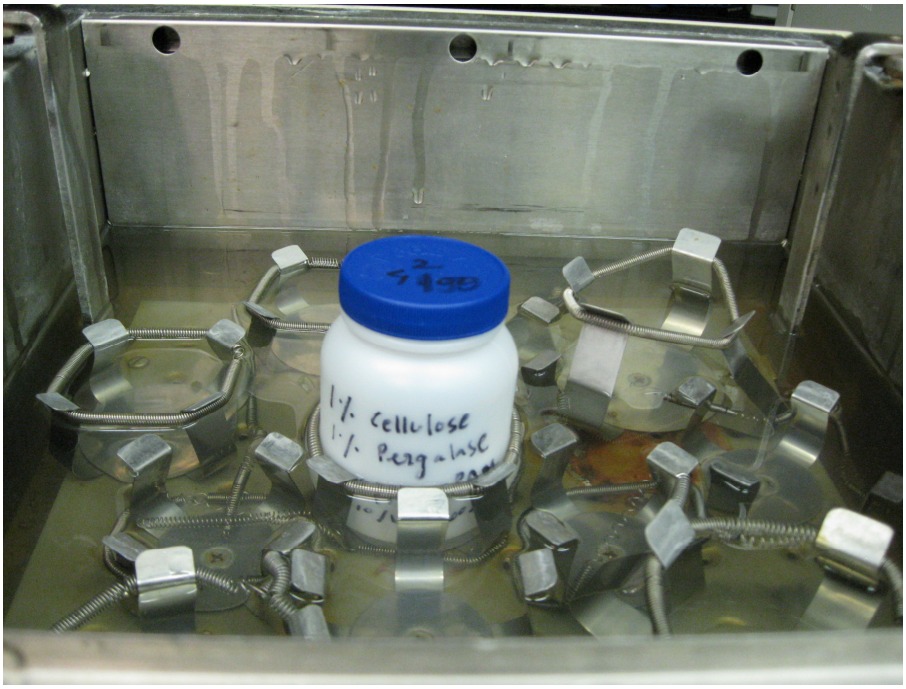



Figure 3.3: Bioreactor in a rotary water bath shaker

After addition of enzyme, the bioreactor is kept in a rotary water bath shaker maintained at 50 ˚C and agitated at 150 rpm. The temperature and pH have been optimized for maximum enzyme activity (Cheung and Anderson, 1996). The experiment is run for 48 hours and samples are collected throughout this period, which are analyzed for changes in fiber length, production of dissolved carbon and glucose. Fiber length is measured by using fiber quality analyzer (FQA), dissolved carbon by total organic analyzer (TOC) and glucose by megazyme glucose assays (Kunst et al., 1988). The results are reported after subtraction of the initial TOC contributed by the enzyme.

## 3.2 Measurement of cellulase activity

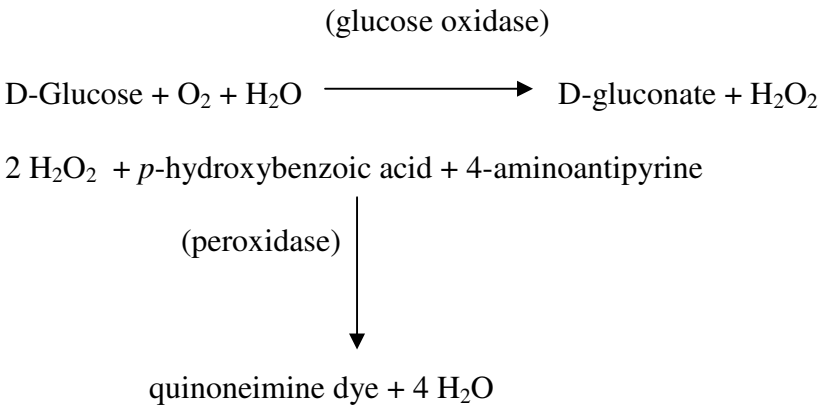
The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating filter paper cellulase units by IUPAC (Adney and Baker, 1996). The assay procedure therefore involves finding a dilution of the original enzyme stock such that a 0.5 mL aliquot of the dilution will catalyze 4% conversion in 60 minutes (or, in practical terms, finding two dilutions that bracket the 4%-conversion point so closely that the required dilution can be obtained, with reasonable accuracy, by interpolation) and then calculating the activity (in FPU/mL) of the original stock from the dilution required.

## 3.3 Measurement of Glucose

The megazyme d-glucose assay employs a high purity glucose oxidase and peroxidase and can be used with confidence for the specific measurement of d-glucose in extracts of plant materials or foods (Kunst et al., 1988).

**Principle:**

The reactions involved are:




The quinoneimine dye is pink in color whose absorbance can be measured by UV spectrophotometer which can be correlated to the glucose concentration by using the standard curves of absorbance vs. concentration of glucose.

## 3.4 Fiber quality analyzer


Figure 3.4: Fiber quality analyzer

The OpTest Laboratory Fiber quality analyzer is an instrument which rapidly, accurately and automatically measures the quality of cellulose fibers. The fiber qualities measured by the laboratory FQA are:

1) Fiber length: It measures the true contour length (L) against the projected length (l).

2) Fiber curl: Curl is the gradual and continuous curvature of a fiber. FQA reports it as curl index. The definition of curl index  is the ratio of the true contour length L of the fiber divided by the projected length (l) of the fiber minus 1. The curl index is calculated for each fiber.

Curl Index = (L/l) - 1

3) Percentage of fines: Fines are referred to fibers whose length is less than 1mm. It measures the percentage of fines at any point of time.

4) Kink index: It is the abrupt change in fiber curvature. It is calculated according to Kibblewhite's equation (FQA Manual, 2000).

5) Kink angle: The average kink angle is the average of all kink angles greater than 20 degrees, divided by the total number of detected kinks.

## 3.5 Total organic carbon analyzer


Figure 3.5: TOC analyzer

The TOC-V is an instrument that measures the amount of total carbon, inorganic carbon and total organic carbon in water." Oxidative combustion -infrared analysis" is widely used TOC measurement method.

# CHAPTER 4

# RESULTS

## 4.1 Images of fiber breakdown

During enzymatic hydrolysis, cellulosic fibers are broken down by endo and exo gluconases and converted to shorter oligosaccharides which are ultimately converted to glucose by the action of β-glucosidase. The images of fibers during the course of the experiment have been captured using a microscope of high resolution (50 X) so as to give a pictorial representation of changes in fiber length with time. This experiment was carried out at 1% consistency and 1% enzyme dosage for softwood fibers. The following images were taken at time 0, 1, 4, and 10 hours respectively.

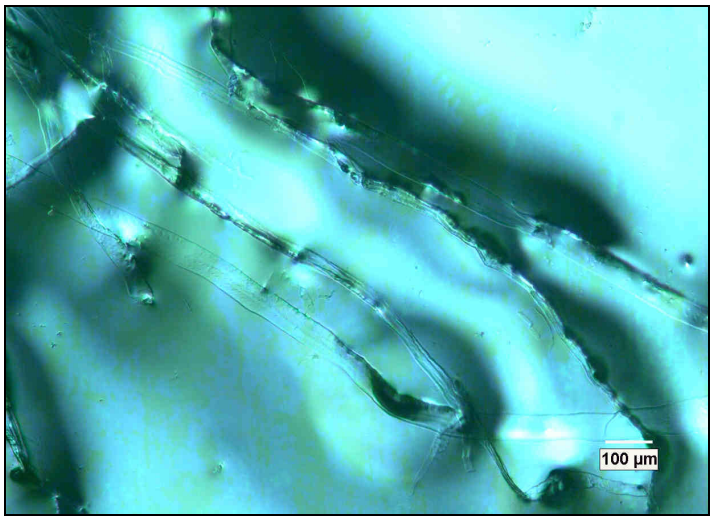



Figure 4.1: The image corresponds to fiber lengths at t= 0 hr

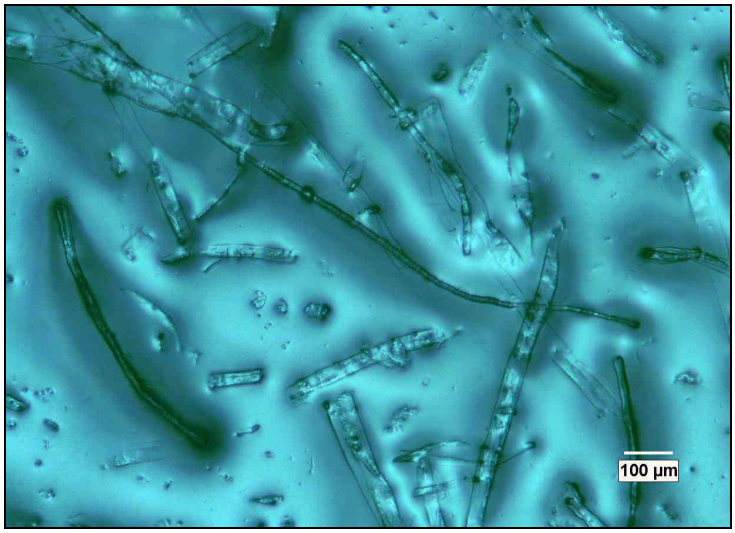



Figure 4.2: The image corresponds to fiber lengths at t= 1 hrs

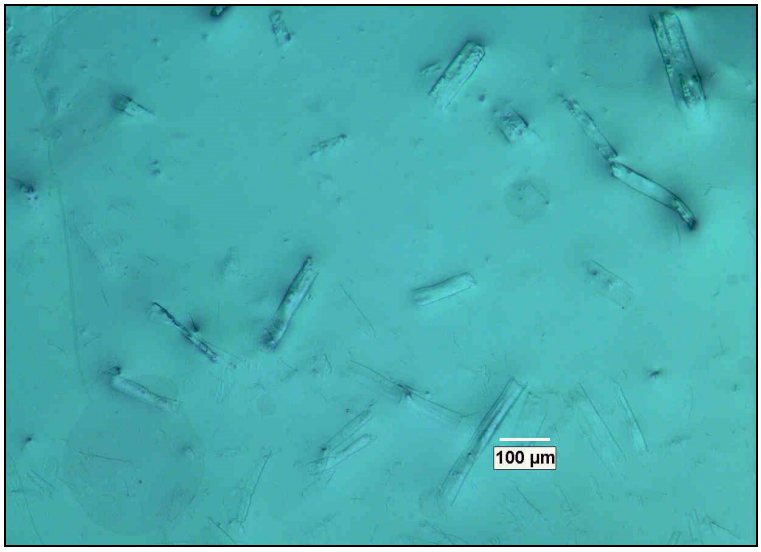



Figure 4.3: The image corresponds to fiber lengths at t= 4 hrs

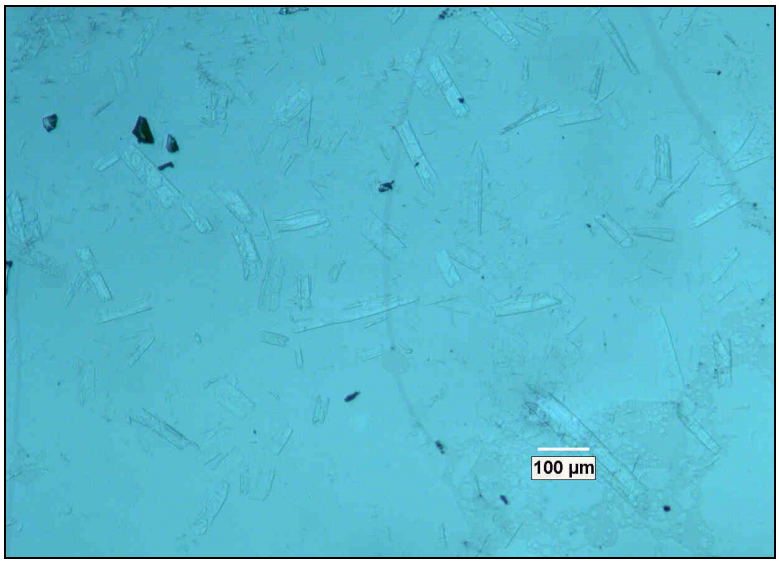



Figure 4.4: The image corresponds to fiber lengths at t=10 hrs

The images at times corresponding to 4 and 10 hours are not clear as the fibers are not three dimensional anymore because of the chewing action of enzymes, as a result of which, the microscope could not be focused to take clear images.

## 4.2 Enzymatic hydrolysis of softwood and hardwood fiber

We studied enzymatic hydrolysis behavior of hardwood and softwood fibers and they showed a slightly different behavior towards enzymatic hydrolysis. We compared parameters like fiber length, percentage of fines, mean curl, kink angle, kink index, total organic carbon and glucose production. These experiments were carried out at 1% consistency of hardwood and softwood fibers and 1% enzymatic dosage of pergalase (15 FPU/ml). Samples were collected at various points of time, for two days and then

analyzed for changes in fiber length, percentage of fines, total organic carbon and glucose.

### 4.2.1 Fiber length

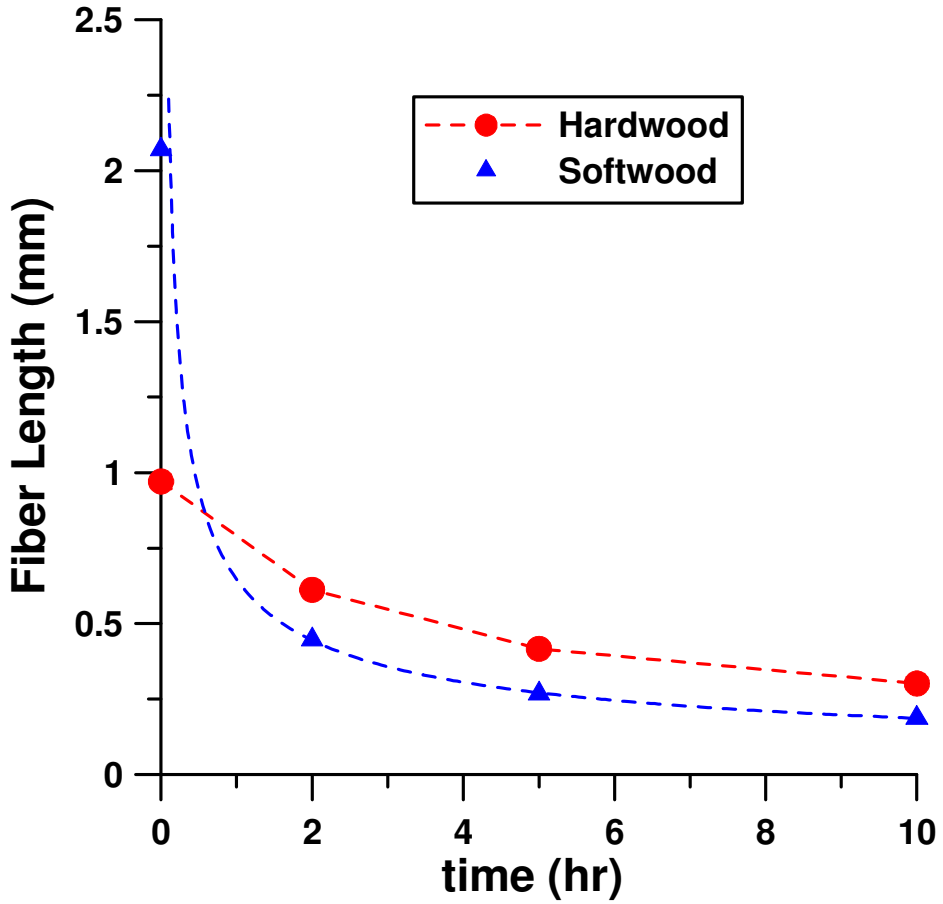



Figure 4.5: Plot of length vs. time for hardwood and softwood fiber(over 10 hrs)

Enzymatic hydrolysis exhibits biphasic behavior for change in length, production of TOC and production of glucose. As can be seen in the graphs for hardwood and softwood a fiber, length hydrolysis consists of two steps: (1) a fast initial step (2) followed by a slow step. We can also infer from the above figure that the enzymatic hydrolysis curve is steeper for softwood than it is for hardwood (for the particular chosen substrates). In the

first two hours, there is an 80% reduction in the length for softwood fibers as compared to 40% reduction in length for hardwood fibers. The conclusion of this experiment is that enzymatic hydrolysis for softwood fibers is faster than that of hardwood fibers, at least in the initial stages. This is probably because of higher accessible cellulosic area of and lower crystallinity of the particular softwood fibers chosen as substrate.

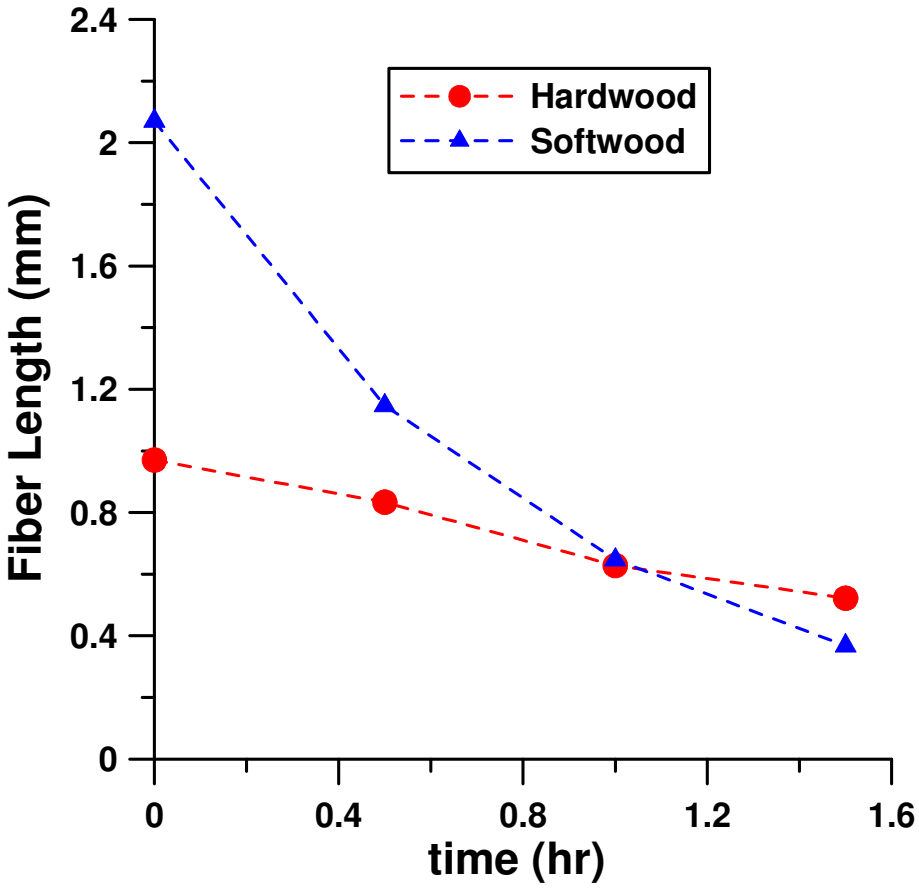



Figure 4.6: Plot of length vs. time for hardwood and softwood fibers (over 2 hours)

In order to understand the kinetics of the fast phase of hydrolysis, we carried out an experiment with similar conditions as mentioned above and an enzymatic activity of 13

FPU/ml and data points were collected after every thirty minutes for the first two hours. We can conclude from the above graph that the hydrolytic rates are close to linear during the initial phase of enzymatic hydrolysis for both hardwood and softwood fibers.

### 4.2.2 Percentage of fines

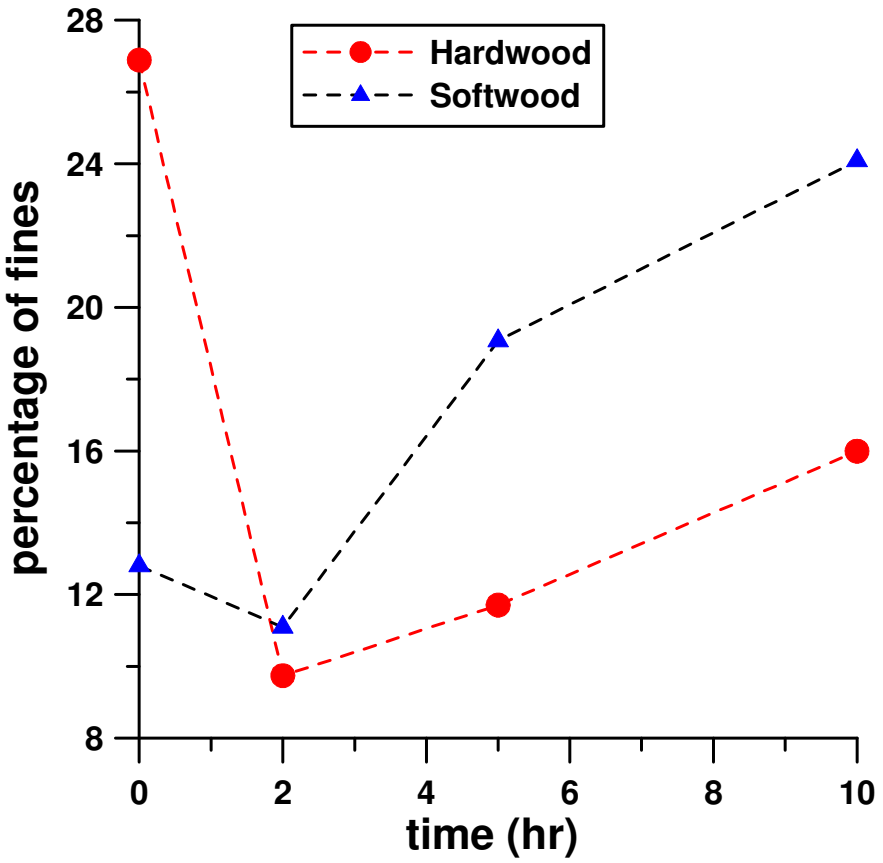



Figure 4.7: Plot of % fines vs. time for hardwood and softwood fiber

The hydrolysis curves for changes in fines % showed a totally different behavior. Hardwood fibers in our study had a higher % of fines (~ 30%) to begin with than did the softwood fibers (~ 10%). The hardwood fines were attacked first because of which we

see a sharp dip in the percentage of fines and then an increase in the percentage of fines when the longer fibers are being converted to fines. For softwood, the long fibers are attacked and converted to fines continually, which is why the percentage of fines keeps increasing. This also explains why the length hydrolysis curves are steeper for softwood as compared to hardwood.

### 4.2.3 Total organic carbon production

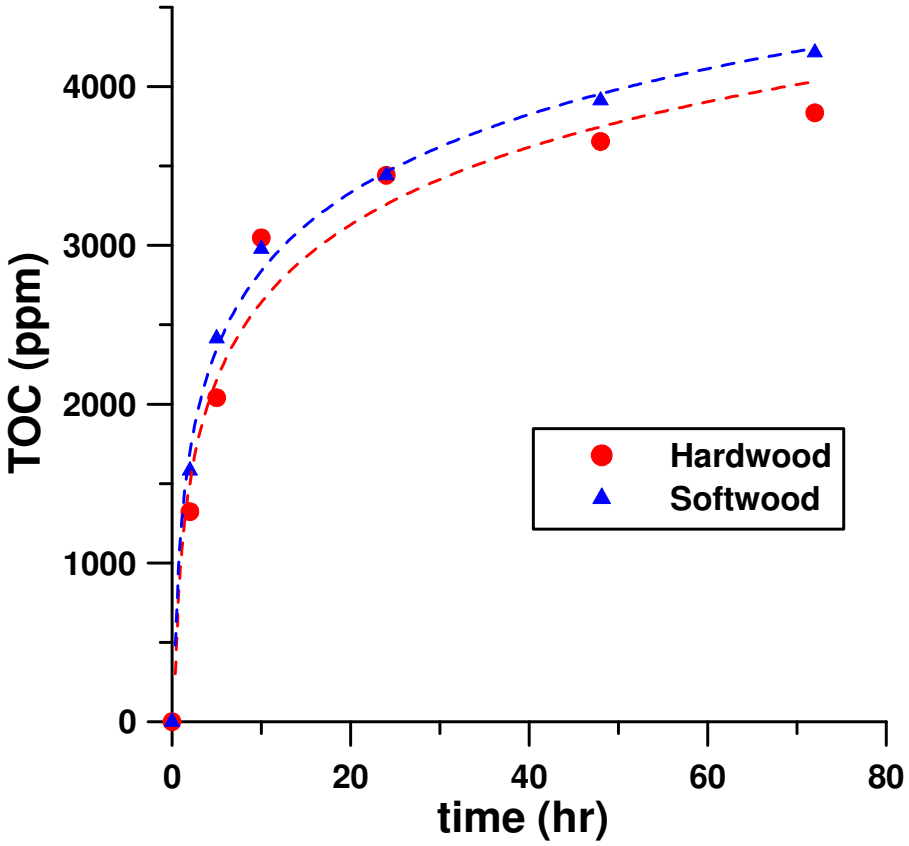



Figure 4.8: Plot of TOC vs. time for hardwood and softwood fiber

As can be seen from the above graph, total dissolved carbon production for softwood is higher than that of hardwood.

### 4.2.4 Glucose production

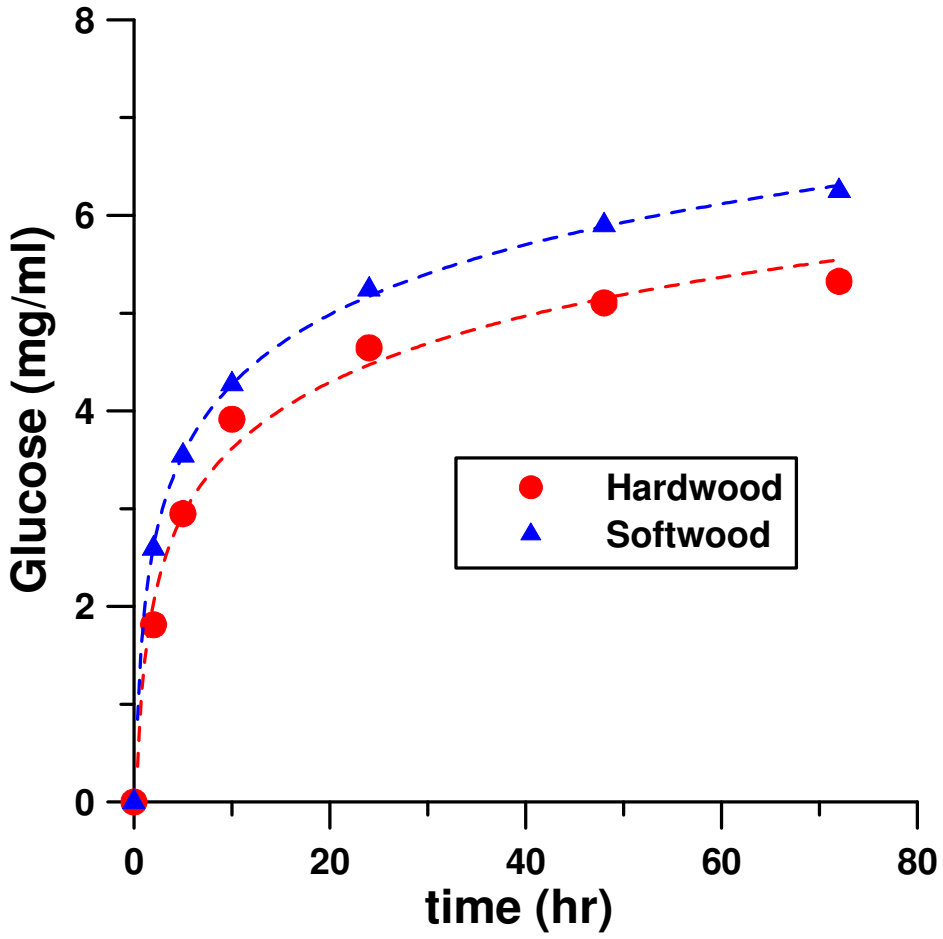



Figure 4.9: Plot of glucose vs. time for hardwood and softwood fiber

Glucose hydrolysis curves show a similar trend as the TOC curves. Glucose production is higher for softwood fibers as compared to hardwood fibers. We observed a linear relationship between TOC and glucose for hardwood and softwood fibers and therefore measurement of one quantity can be used for prediction of the other quantity provided that the experimental conditions are maintained the same.

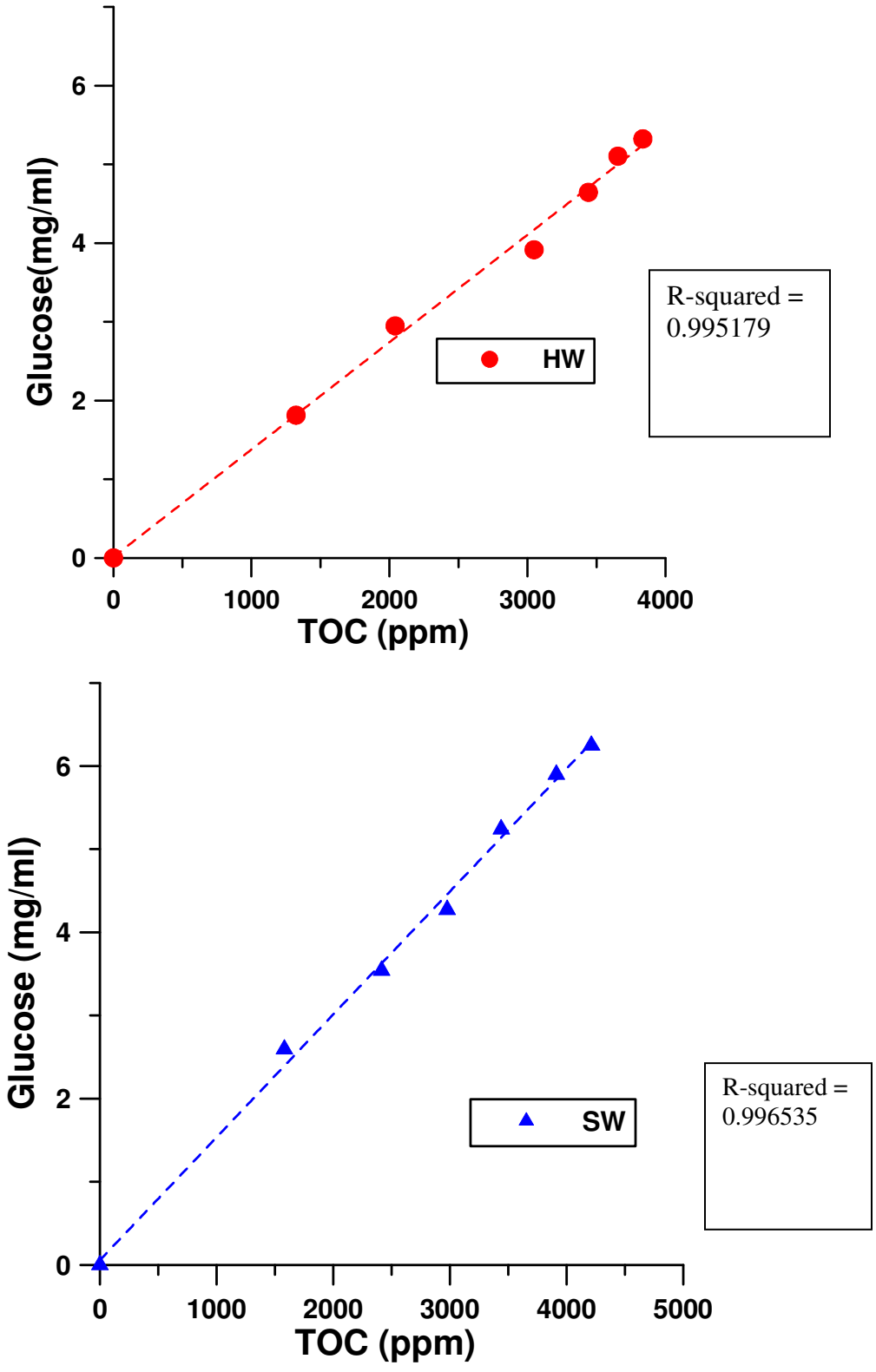



Figure 4.10: Plot of glucose vs. TOC for (a) hardwood and (b) softwood fiber

We measured kinetics of other parameters like mean curl, kink angle etc. which gave an insight into morphological changes of fibers with time during enzymatic hydrolysis.

### 4.2.5 Mean curl

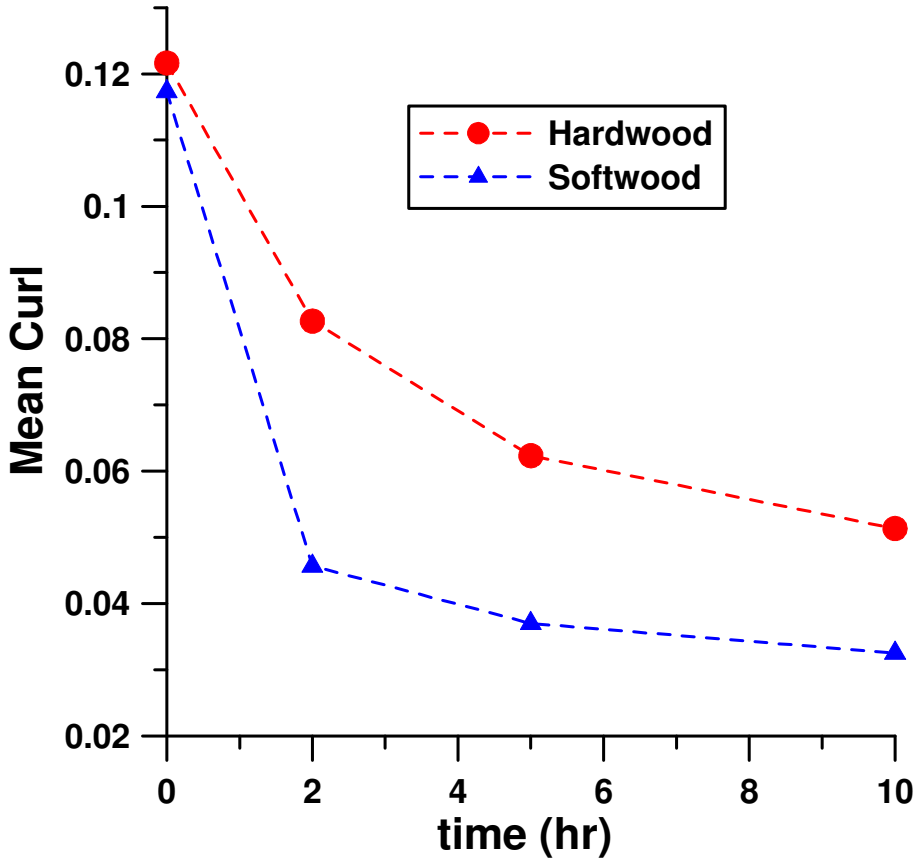



Figure 4.11: Plot of mean curl vs. time for hardwood and softwood fiber

The curl of hardwood and softwood fibers decreases over the course of enzymatic hydrolysis. As mentioned earlier, curl is defined as the ratio of difference between the measured length and projected length to the original length of fiber. A decrease in curl over time means that the fibers are becoming flatter with time which means shorter straighter fibers are being created by the chewing action of enzymes.

### 4.2.6 Kink angle

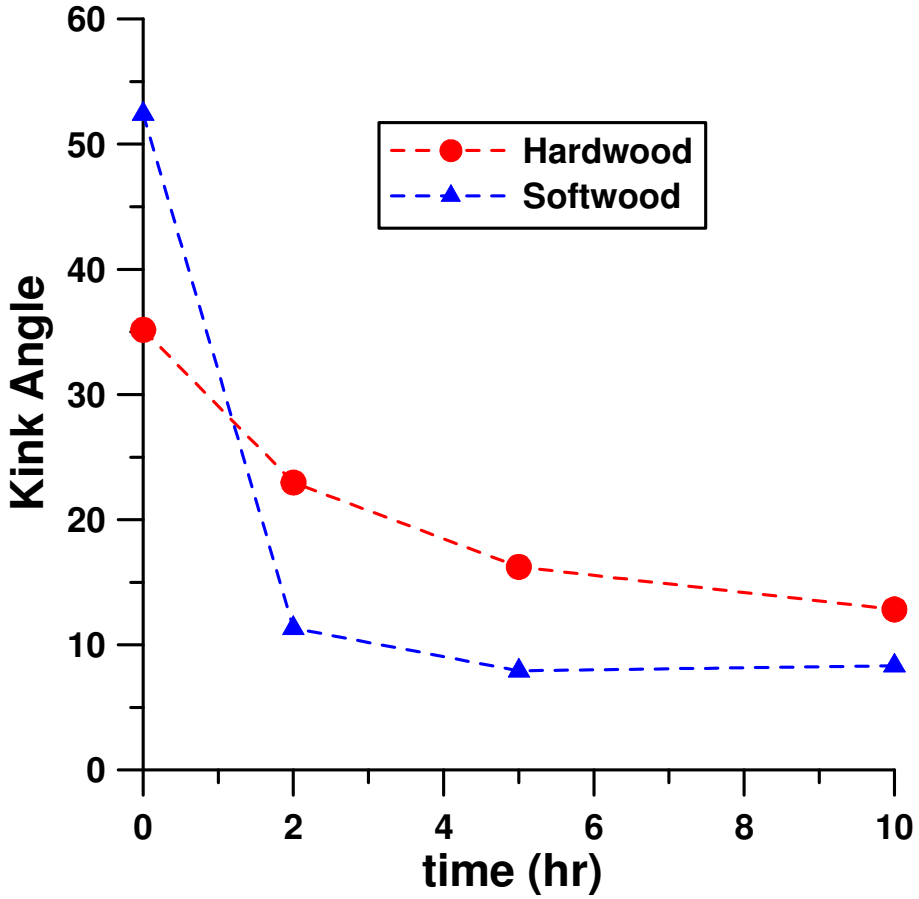



Figure 4.12: Plot of mean kink angle vs. time for hardwood and softwood fiber

As we can see from the graph, the average kink angle decreases during the course of enzymatic hydrolysis which brings us to the same conclusion that, shorter fibers are being created with time through enzymatic action. Creation of straighter shorter fibers leads to a decrease in average kink angle.

### 4.2.7 Kink index

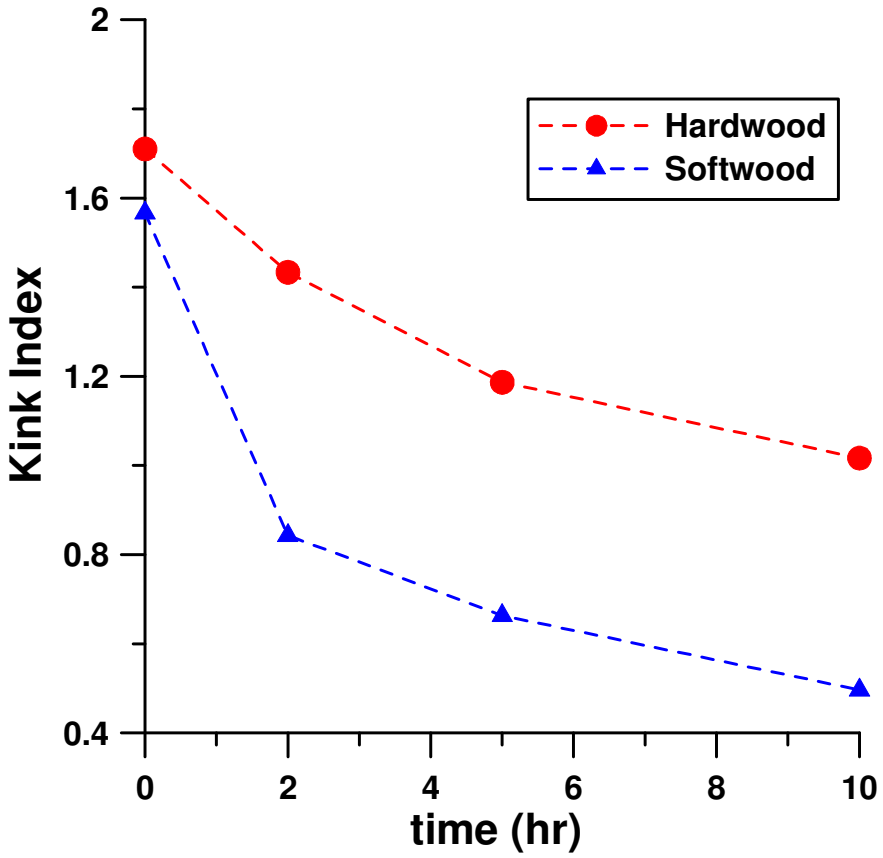



Figure 4.13: Plot of mean kink index vs. time for hardwood and softwood fiber

Dislocation is a structure containing slip planes and slip lines in cellulose fibers. These localized changes or distortions of cellulose microfibrils in growing trees due to wind action, increase in size during pulping and cooking etc. These dislocations are less ordered structure or more amorphous and thereby targeted by cellulases during enzymatic action (Ander et al., 2008). So, during the beginning of enzymatic hydrolysis, cellulases attack the amorphous regions leading to the formation of kinks in the cellulosic fibers. The graph of kink index is similar to the trend shown by graphs of mean curl and kink angle, which further confirms our speculation.

## 4.3 Crystallinity measurements

We measured crystallinity of softwood fiber (1% fiber consistency, 1% enzyme dosage) during the course of enzymatic hydrolysis by FT-IR spectroscopy (Akerholm et al., 2004; Youn et al., 2005). Pellets of 1.5 mg of cellulosic samples were prepared by mixing with 200 mg of spectroscopic grade KBr. FTIR spectra were recorded using a Nicolet 550P spectrometer with detector at 4 $cm^{-1}$ resolution and 64 scans per sample. CI obtained by FTIR, CI (IR), was evaluated from the ratios of the absorption bands such as $A_{1430}/A_{894}$. As the location of the characteristic peak maximum varied from sample to sample, the height was determined at slightly different wave numbers.

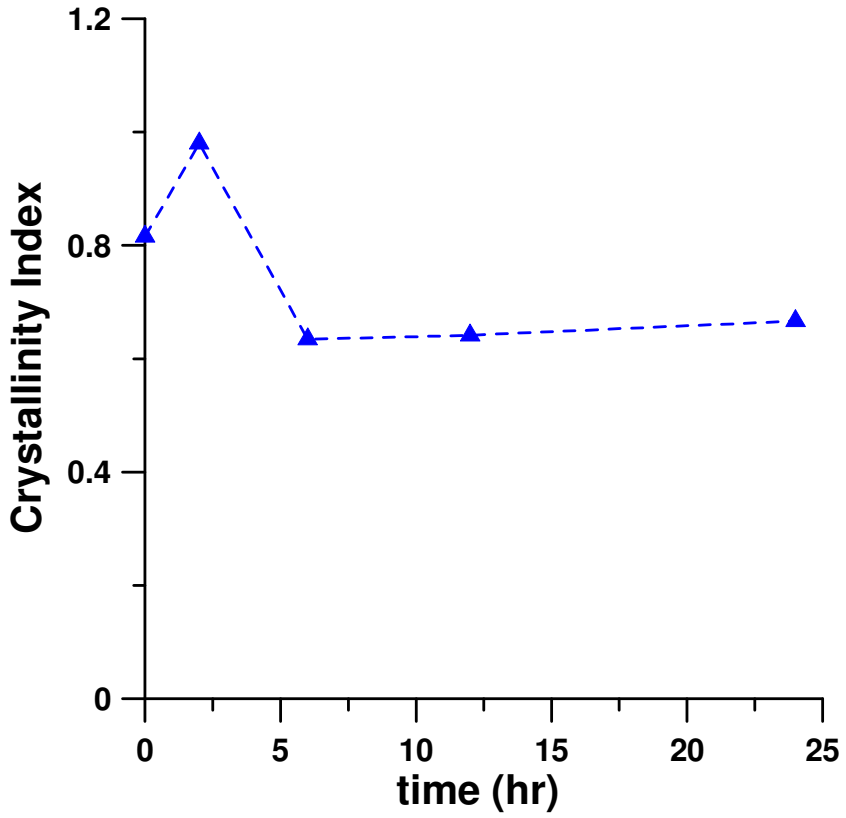



Figure 4.14: Relative changes in CI (FTIR) during the course of enzymatic hydrolysis for softwood fiber (similar to fig.

The crystallinity index increases initially which means that enzymes are preferentially attacking the amorphous regions and the depletion of amorphous regions is causing the crystallinity of the sample to increase. It increases up to a point, after which crystallinity starts falling, which indicates that crystalline region is being degraded by cellulases. And finally, crystallinity is almost constant with time which coincides with the second phase of enzymatic hydrolysis, when hydrolysis becomes slow.

## 4.4 Effect of enzyme loading

We carried out an experiment to determine the effect of enzyme loading on hydrolytic conversion rates. It was carried out at 1% consistency of softwood fibers and various enzyme loadings of 1%, 0.5%, 0.2 %, 0.1%, 0.05%, and 0.01% of pergalase of 15 FPU/ml. TOC and glucose readings were taken for this experiment. Because of a linear relationship between TOC and glucose, we will discuss only TOC results.

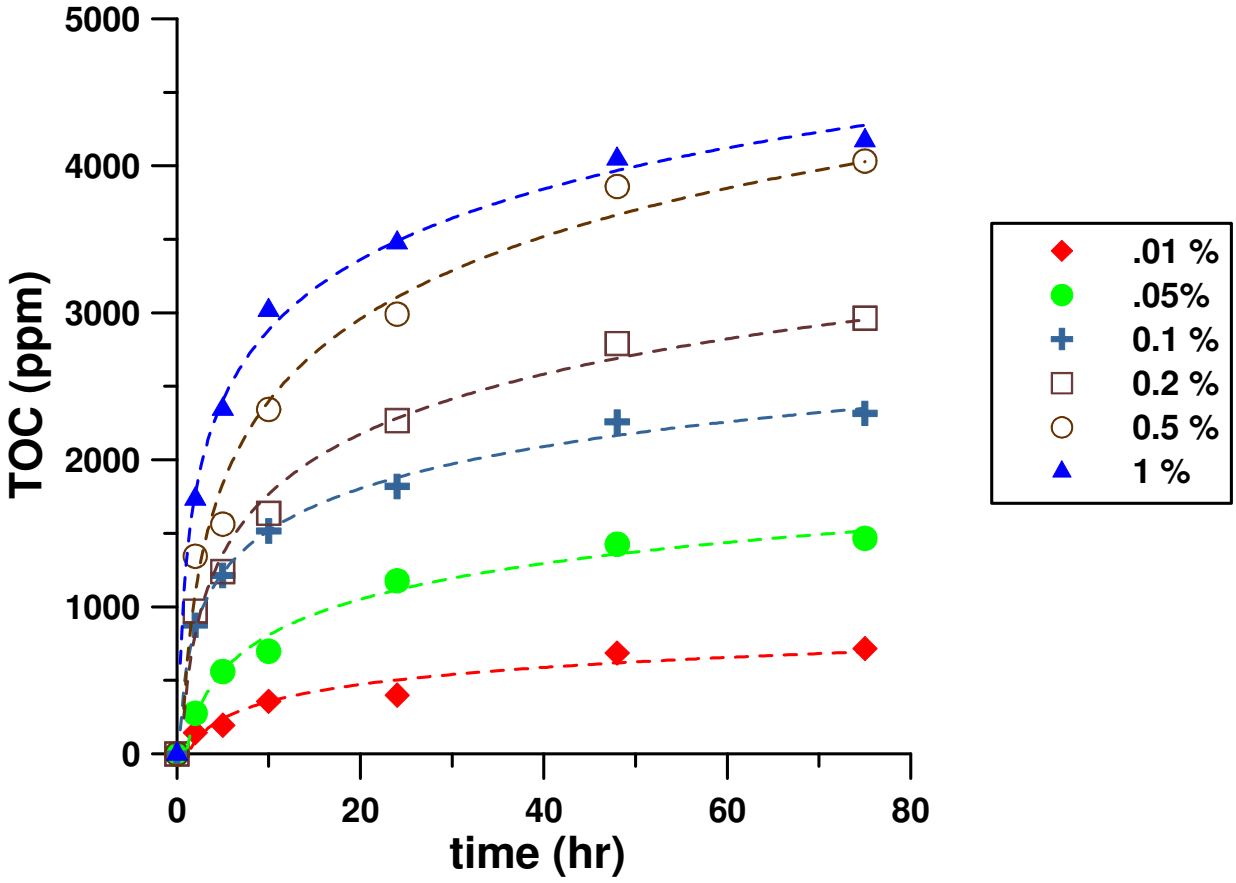



Figure 4.15: TOC production vs. time for different enzyme loadings

As we can see from the graph, TOC production increases with the enzymatic loading.

The following graph shows TOC conversion vs. enzymatic loading. TOC conversion rate is defined as $\left(\frac{TOC_{value}}{TOC_{\max imum}} \times 100\right)$.

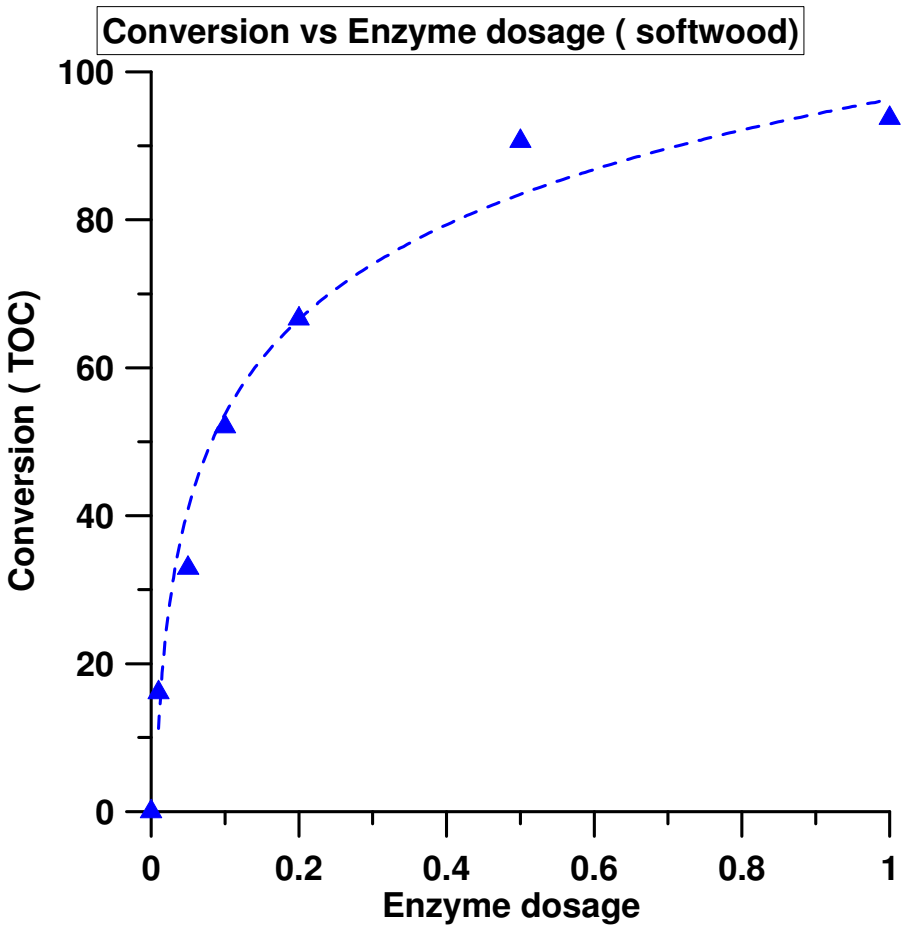



Figure 4.16: Conversion rates vs. enzymatic loading

The TOC conversion vs. enzymatic loading curve shows a logarithmic behavior and saturates with time. Therefore, one needs to optimize the enzyme loading with the hydrolytic conversion rates. At the current TOC hydrolytic conversion rates for enzymatic hydrolysis, one needs to use a higher enzymatic loading for getting conversion rate high enough to make the implementation of this technology economically feasible. There is a need to come up with alternative techniques so as to obtain higher conversion rates at lower enzymatic loadings.

## 4.5 Effect of lignin

We carried out a few experiments to determine the effect of lignin on hydrolysis rates. Lignin affects the hydrolysis rates by reducing the exposed surface area of cellulose and by binding with enzymes, thereby making fewer enzymes available for hydrolysis of cellulose. We compared brown softwood fiber of kappa# 113 and bleached softwood for changes in fiber length and production of dissolved carbon and glucose so as to study the effect of lignin on enzymatic hydrolysis.

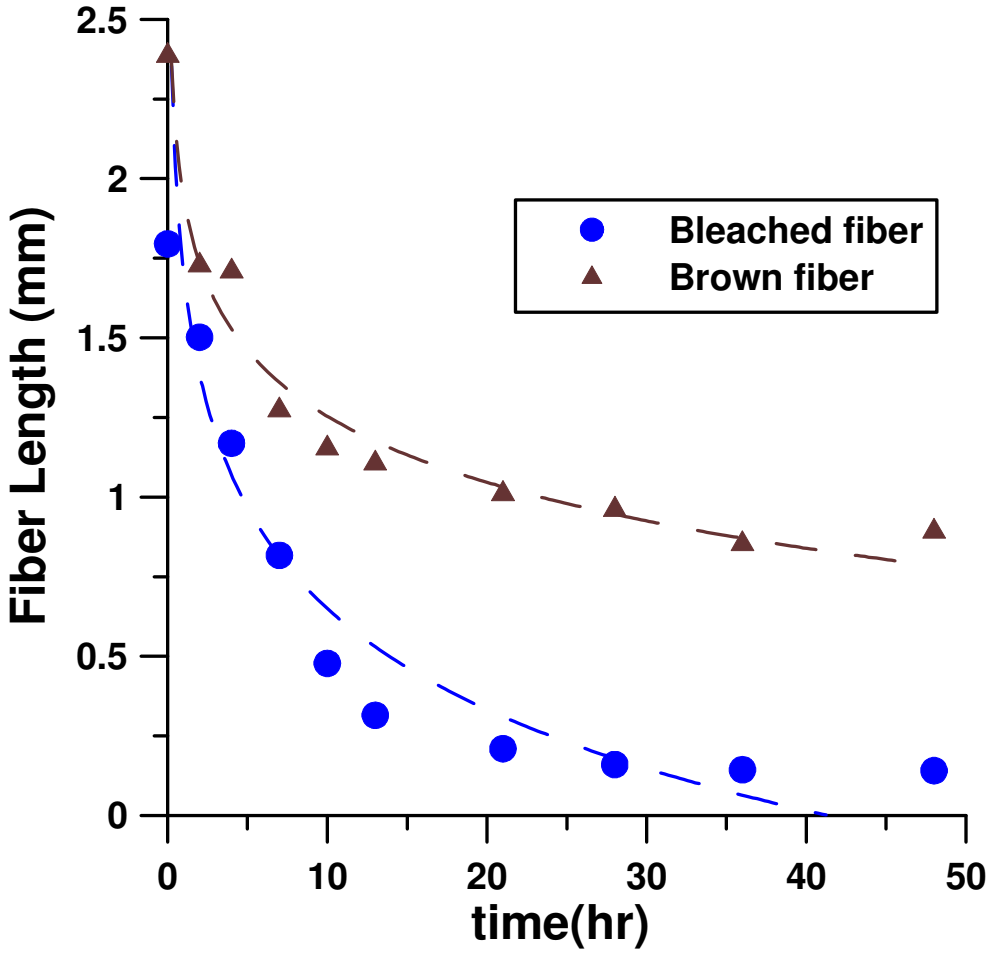



Figure 4.17: Length hydrolysis curves for bleached and brown fiber

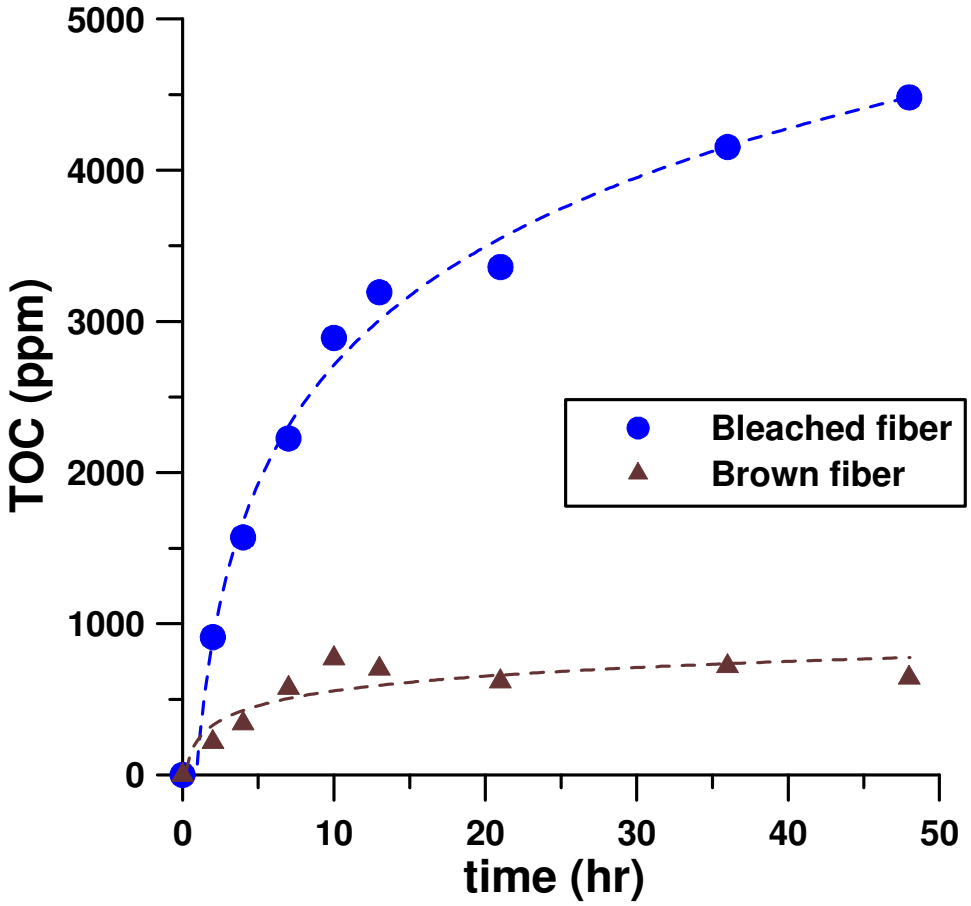



Figure 4.18: Production of total organic carbon for bleached and brown fiber

Therefore, as is clearly seen from the graphs, lignin inhibits fiber breakdown and total organic carbon production; thereby we can safely conclude that lignin inhibits enzymatic hydrolysis rates. The graph for glucose production is similar to the TOC production graph.

## 4.6 Effect of linear cationic polymers

One of the accidental discoveries in our lab was the finding that cationic polymers increase the enzymatic hydrolysis rates of corn. In this project, we investigated the effect of cationic polyacrylamides (c-PAMs) on enzymatic hydrolysis rates of cellulosic fibers. The effect of cPAMs was studied both on bleached and unbleached hardwood and softwood fibers.

### 4.6.1 Effect of linear cationic polymer: Bleached hardwood fiber

Let us look at the effect of polymers on fiber length breakdown and TOC production for hardwood fibers with experimental conditions being 1% consistency of fiber, 1% enzymatic dosage and 500 ppm of FO-4800 cPAM.

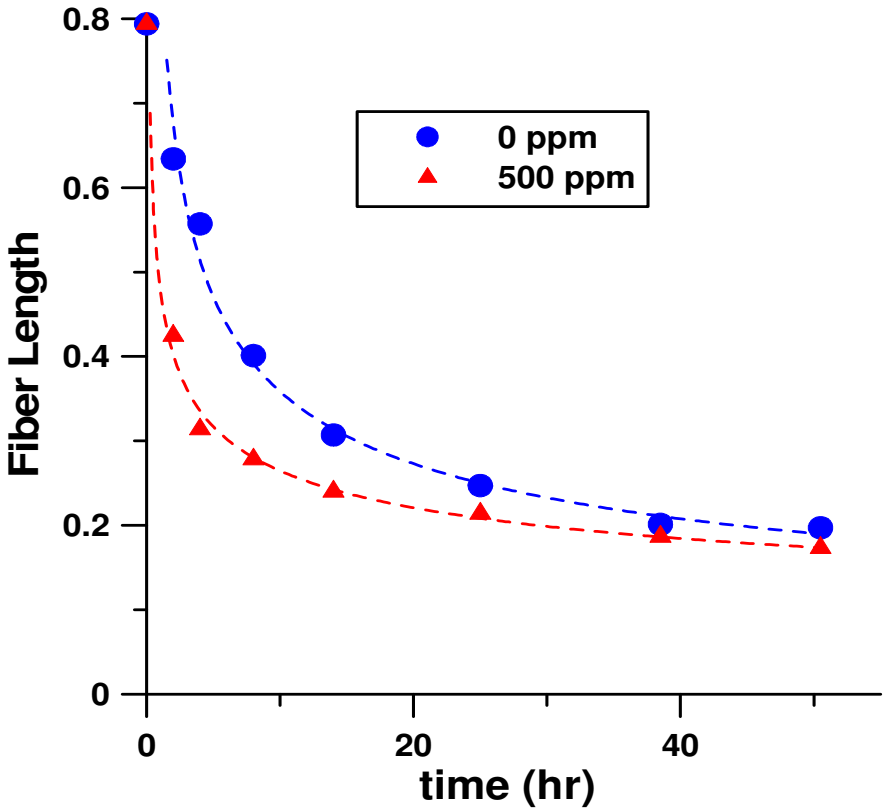



Figure 4.19: Effect of linear cationic polymer on fiber length of hardwood fiber

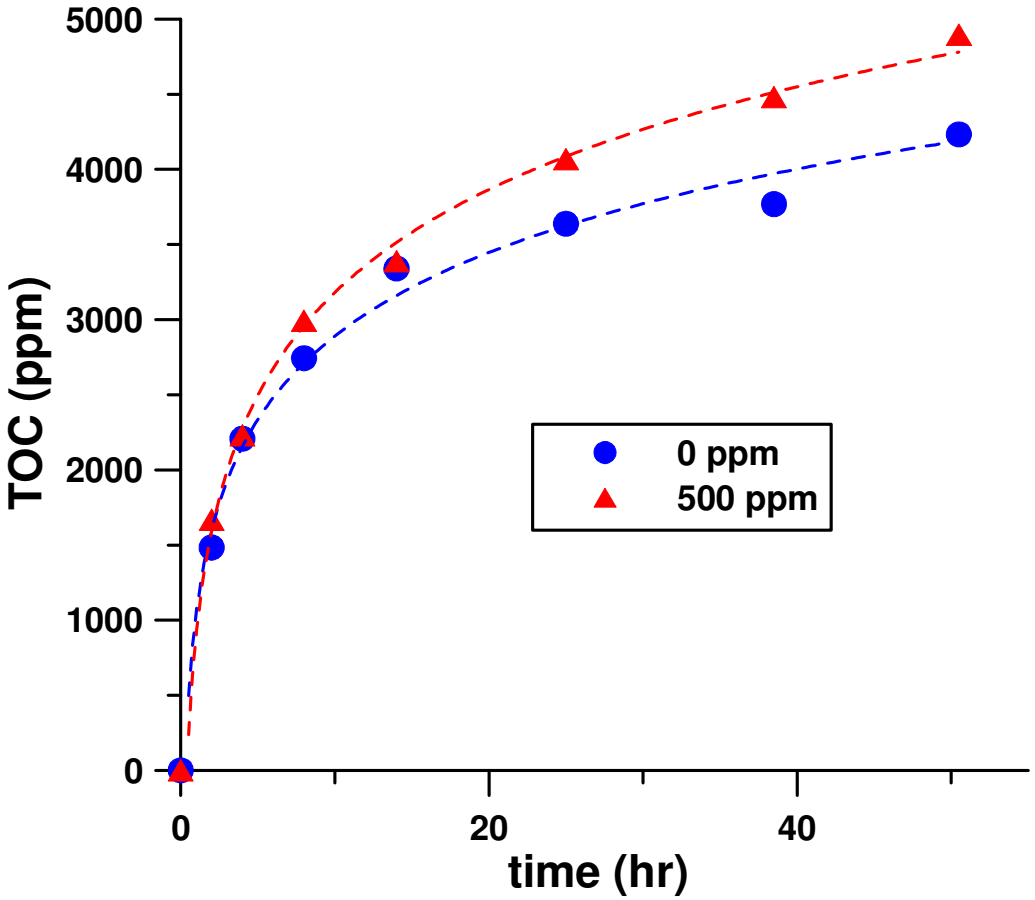



Figure 4.20: Effect of linear cationic polymer on TOC of hardwood fiber

As can be seen from the above graphs, cationic polymer not only speeds up the breakdown of hardwood cellulosic fibers but also increases total organic carbon production for hardwood fibers.

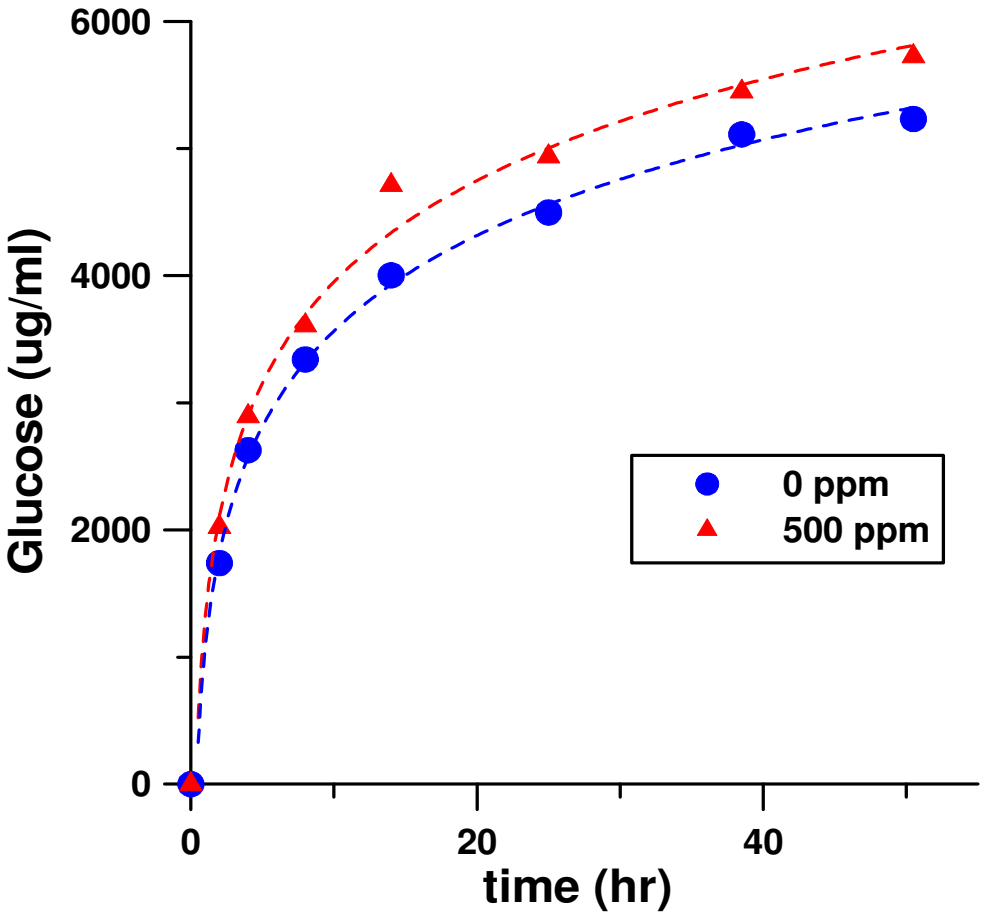



Figure 4.21: Effect of linear cationic polymer on glucose production of hardwood fiber

As is clearly seen from the above graph, cationic polymer increases production of glucose for hardwood fibers. In conclusion linear cationic polymers enhance enzymatic hydrolysis rates for hardwood fiber.

### 4.6.2 Effect of linear cationic polymer: Bleached softwood fiber

We carried out similar experiments with softwood fibers as the substrate and we found that cationic polymers do not have the same effect on softwood fibers as hardwood fibers. The increase in enzymatic hydrolysis rates for softwood fibers is less than that for hardwood fibers. This is because enzymatic hydrolysis rate for softwood fibers is inherently higher than that for hardwood; therefore, polymer does not play a role in further enhancing the hydrolysis rate as it does for hardwood fibers.

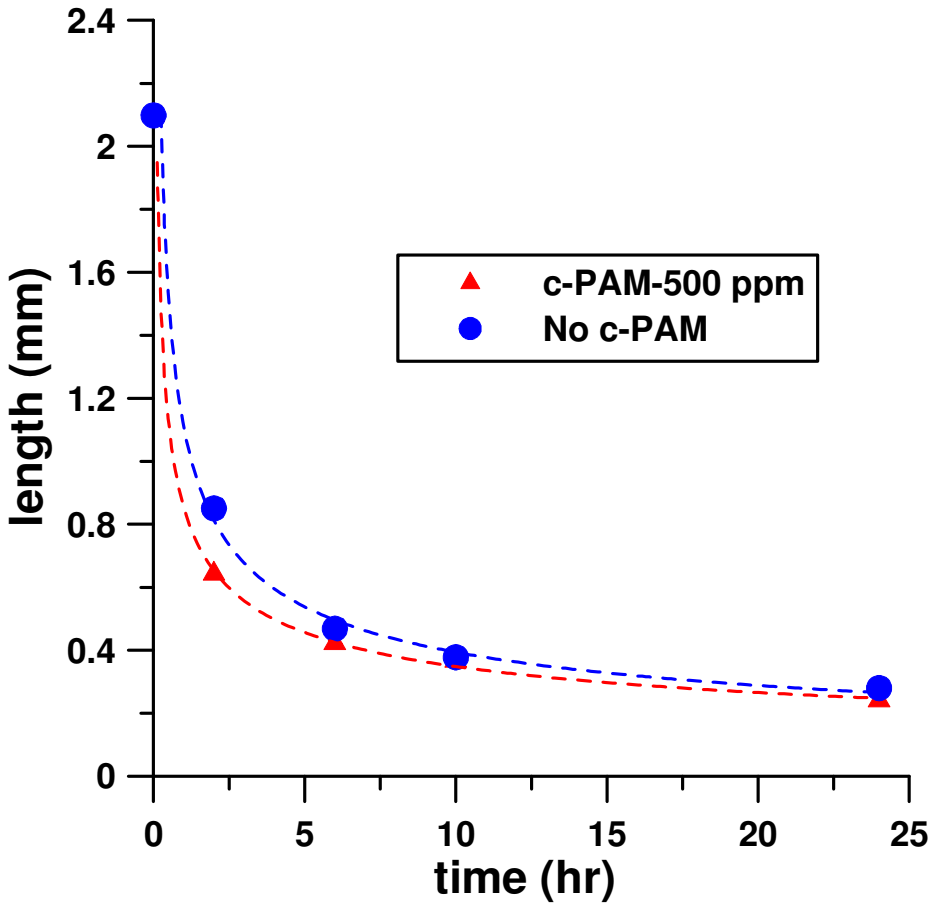



Figure 4.22: Effect of linear cationic polymer on fiber length of softwood fiber

As we can see from the above graph, the effect of cationic polymers on breakdown of softwood fibers is not as high as it is for hardwood fibers. However, TOC production for softwood fibers is enhanced by the use of cationic polymers which is shown in the graph below.

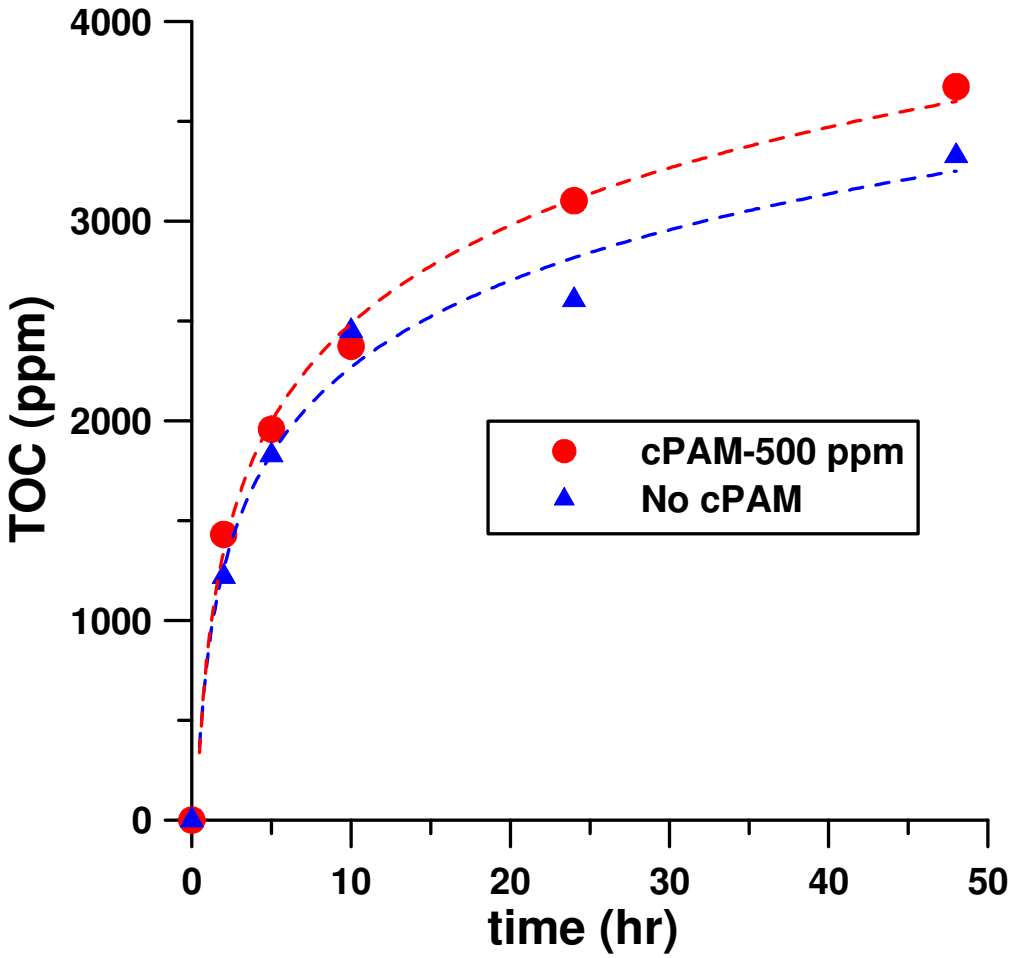



Figure 4.23: Effect of linear cationic polymer on TOC of softwood fiber

So, in conclusion, polymers don't enhance the fiber breakdown for softwood fiber however, they increase the production of total organic carbon and glucose production for softwood fiber.

### 4.6.3 Effect of linear cationic polymer: Unbleached softwood fiber

The effect of polymers on brown fiber's enzymatic hydrolysis was studied and we found contrasting results by using different polymers. We used cationic polymers FO 4350 (25 % cationcity) and 4800 (80% cationicity) which had different effect on enzymatic hydrolysis. FO4350 acts inhibitively on the enzymatic hydrolysis of brown fiber while FO 4800 acts positively and produces an increase in the enzymatic hydrolysis. The inhibition is attributed to the negative interactions of lignin with certain cationic polymers.

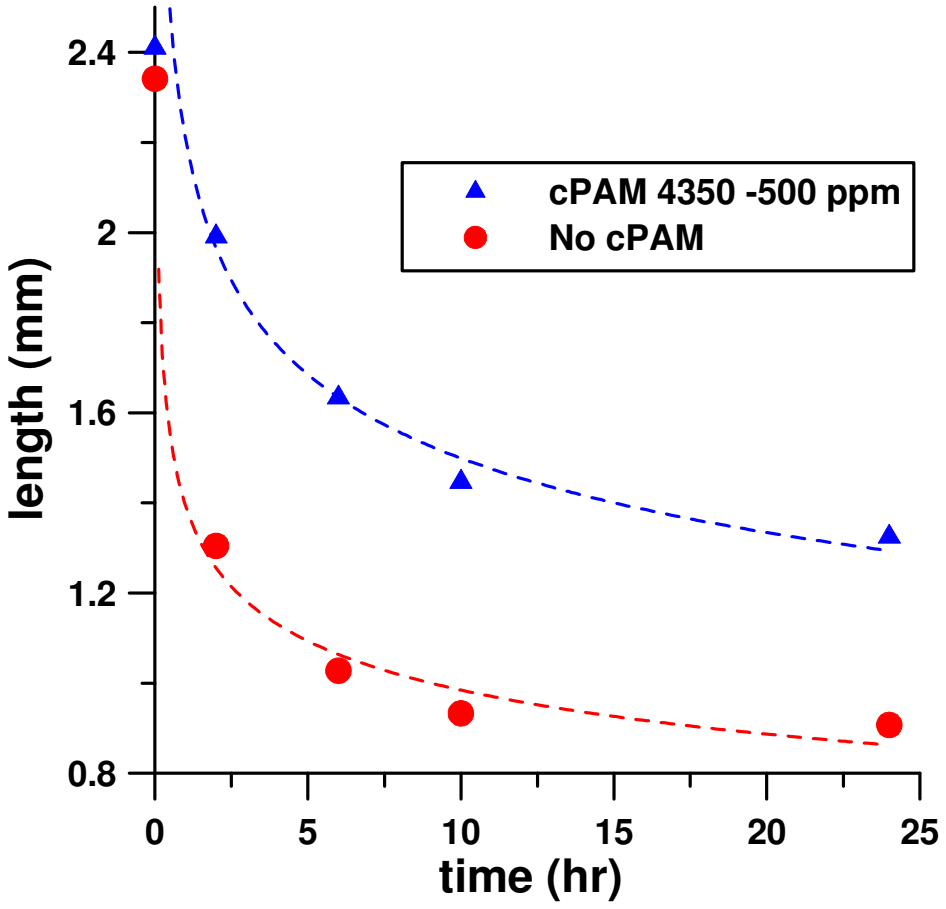



Figure 4.24: Effect of linear polymer (FO 4350) on length hydrolysis of brown fiber.

As we can clearly infer from the graph, cationic polymer FO 4350 inhibits the fiber breakdown of brown fibers. The following graph shows the effect of polymer FO 4800 on brown fiber enzymatic hydrolysis.

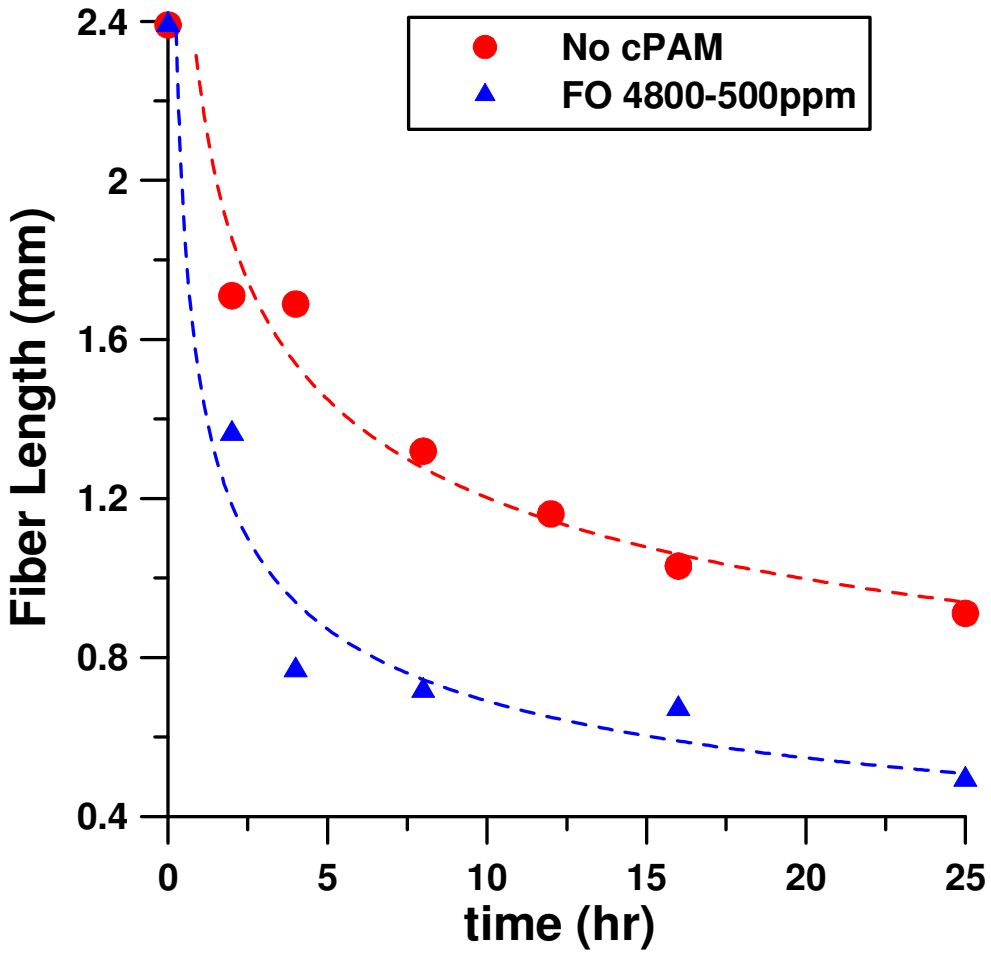



Figure 4.25: Effect of linear polymer (FO 4800) on length hydrolysis of brown fiber.

The above graph shows the positive effect of polymer FO-4800 on enzymatic hydrolysis of brown fibers. The reason behind certain polymers working negatively towards enzymatic hydrolysis of brown fiber hydrolysis is not clear.

### 4.6.4 Effect of nature of polymers

In order to study the effect of cationicity of polymers, we picked up polymers of different cationicity: FO 4190 (10% cationicity), F0 4350 (25 % cationicity), F0 4690 (60 % cationicity and FO 4800 (80 % cationicity). Softwood fibers at 1% consistency and an enzymatic dosage of 0.2 % pergalase was used for this experiment.

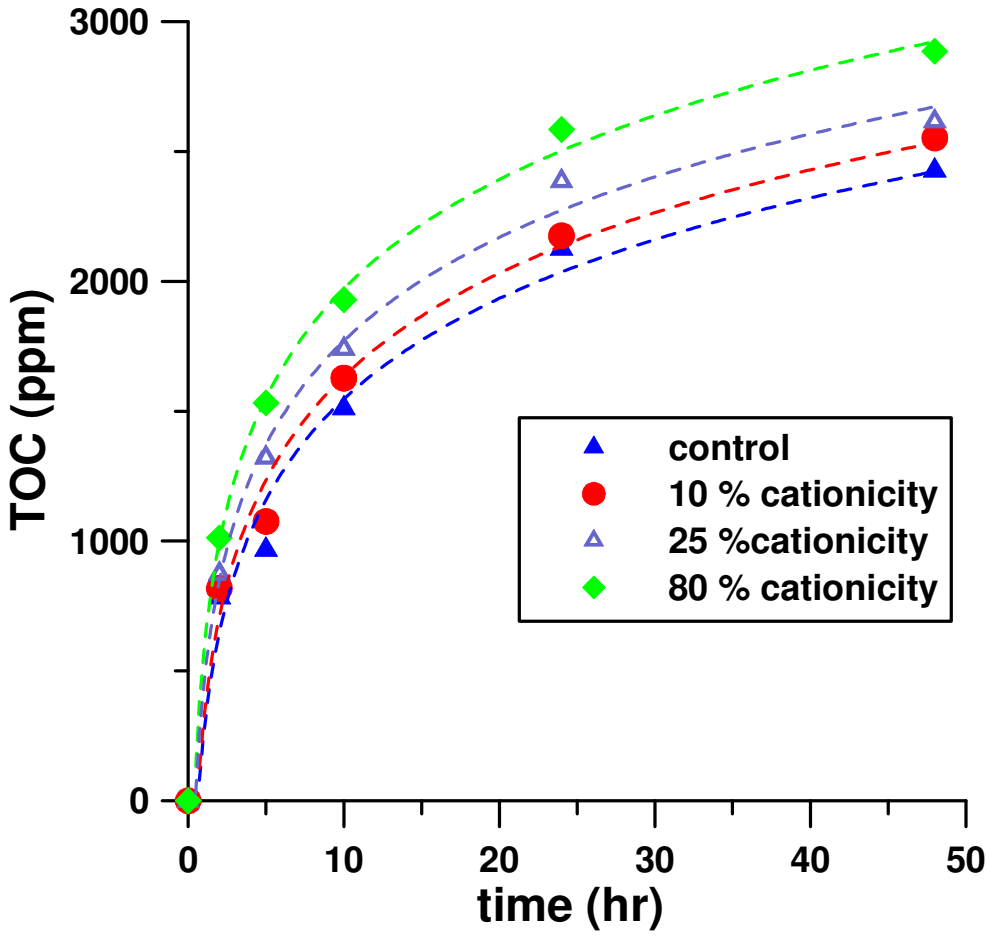



Figure 4.26: Effect of cationicity of polymers on TOC production

As can be seen in the graph 80% cationic polymer produces maximum TOC followed by 25% cationic polymer and then followed by 10% cationic polymer. From the results, it was concluded that an increase in enzymatic hydrolysis is directly proportional to

polymer cationicity which confirms the speculation that interactions between cationic polymers and cellulosic substrate are  of electrostatic nature.

### 4.6.5 Effect of polymer concentration

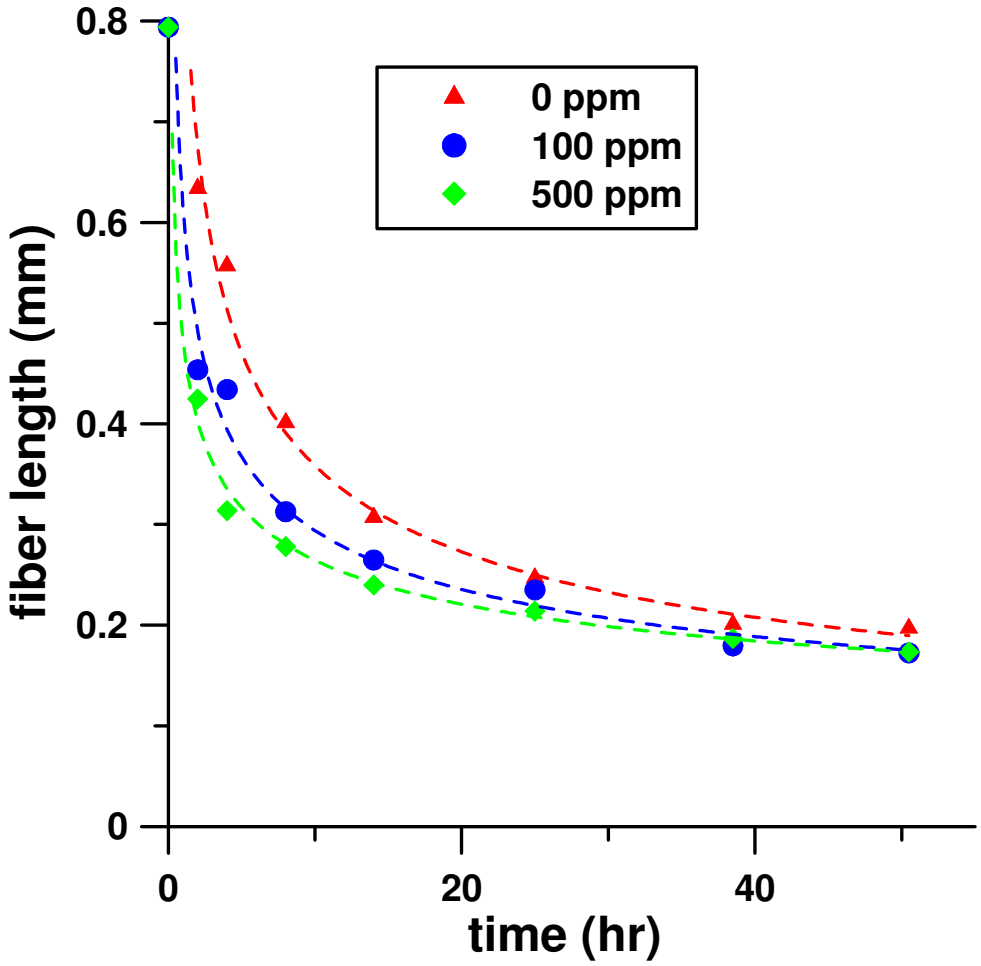



Figure 4.27: Effect of polymer concentration on breakdown of fibers.

We carried out experiments to study the effect of polymer concentration on enzymatic hydrolysis rates. We selected concentrations of 0 ppm, 100 ppm and 500 ppm and found out that the increase in enzymatic hydrolysis rates is directly proportional to the polymer concentration up to an upper limit beyond which hydrolysis rates are inhibited.

## 4.7 Effect of refining and recycling

In order to study the effect of refining and recycling on enzymatic hydrolysis, we carried out experiments with once refined and recycled and once recycled softwood fibers. The fibers were refined by using PFI laboratory pulp beater, mill #139 and freeness was measured by using canadian standard freeness tester, serial # 2729. The freeness of virgin fiber was measured to be 590 ml while that of refined fiber was 428 ml. The following graph compares the total organic carbon produced by different kind of fibers.

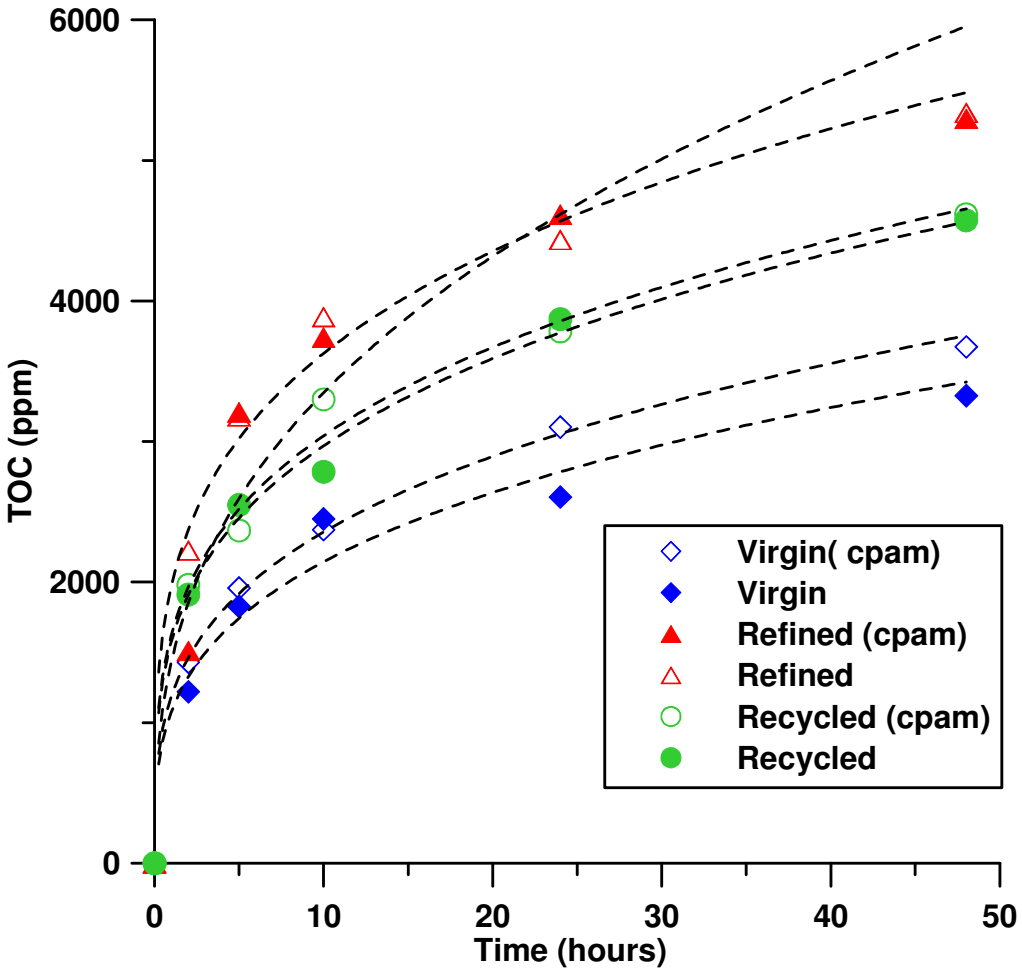



Figure 4.28: Effect of recycling and refining on enzymatic hydrolysis

As can be seen from above graph, refined fibers produce maximum dissolved carbon, followed by recycled fibers, and then the virgin fibers. This result was confirmed by glucose measurements as well. Cellulose conversion is defined as the ratio of amount of glucose produced to the weight of the initial cellulosic fiber (Moon et. al, 2008). Refined fibers showed a maximum cellulose conversion of about 66% followed by recycled fibers which showed a conversion of about 59% while the virgin fibers showed a conversion of 56%.

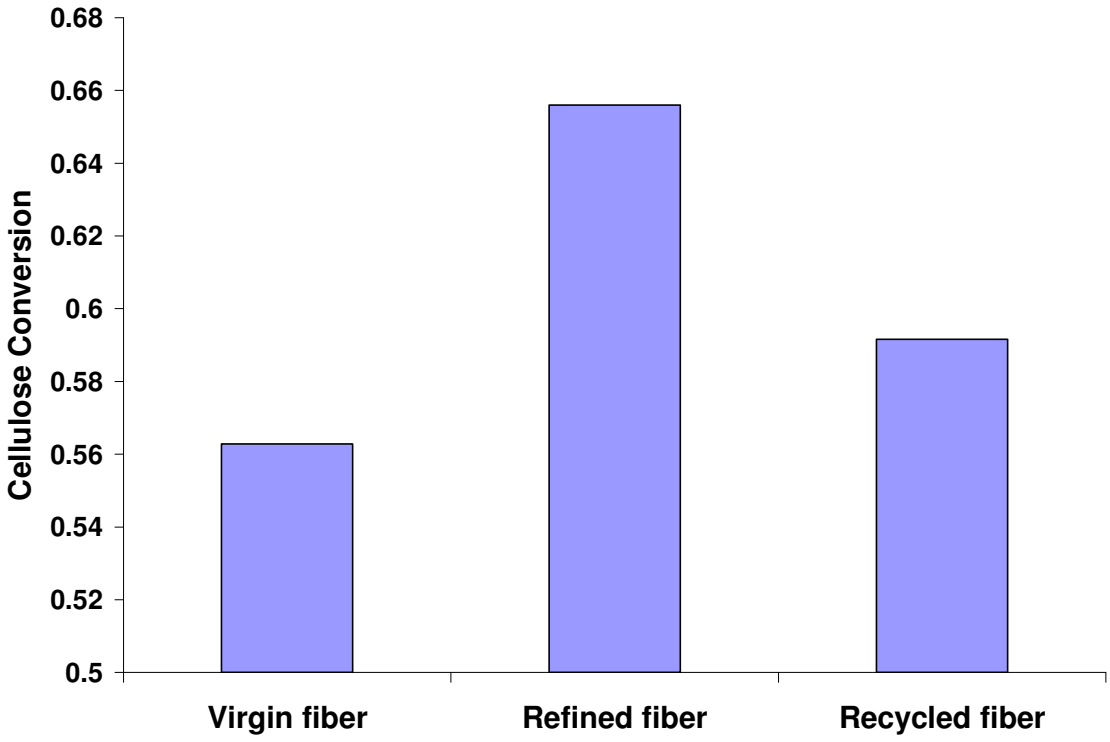



Figure 4.29: Cellulose conversion rates for different fibers

Refined fiber produces maximum increase in enzymatic hydrolysis and the speculated reason behind this observation is that beating of fibers increases surface area and decreases crystallinity, which might have increased enzymatic hydrolysis rate. However,

refining is energy intensive and it seems less likely that the overall economics will turn out favorable for the practical implementation. Recycled fiber as a substrate has higher enzymatic hydrolysis rates as compared to virgin fibers.

# CHAPTER 5

# CONCLUSIONS

Expansion of saccharification and fermentation technologies to low cost cellulosic residues like waste paper sludge holds a lot of potential in term of producing energy from waste, reducing green house gas emissions, boosting country's rural economics, reducing the dependence of US on foreign countries for oil and reducing the air, water and soil contamination associated with land disposal of organic wastes. The biological process of conversion of waste paper sludge to bioethanol requires: (1) depolymerization of the carbohydrate polymers to produce free sugars; and (2) fermentation of mixed hexose and pentose sugars to produce ethanol. The rate limiting step is the depolymerization of cellulose to produce free sugars because of the insolubility of cellulosic fibers. Enzymatic hydrolysis is superior to acid hydrolysis as it requires milder experimental conditions, consumes less energy and produces fewer side products. However, enzymatic hydrolysis has low conversion rates and enzymes being expensive, makes the implementation of this technology economically infeasible. We focused on understanding the mechanism of enzymatic hydrolysis of cellulose fibers to glucose and studying the effect of cationic polyacrylamides in enhancing the hydrolysis rates. From the experiments the following were concluded:

1) Enzymatic hydrolysis of softwood fibers is steeper and faster than hardwood fibers (specific to substrate samples). We noticed that the hydrolytic rates are close to linear in the first phase of enzymatic hydrolysis.

2) The percentage of fines for hardwood fibers decreases first and then increases while for softwood fibers, the percentage of fines continually increases with time. Therefore, during the beginning of enzymatic hydrolysis, fines are attacked for hardwood fibers while longer fibers are attacked for softwood fibers.
3) Lignin inhibits enzymatic hydrolysis as confirmed by length hydrolysis, total organic carbon and glucose production.
4) Crystallinity measurements for softwood fibers by FTIR spectroscopy showed that initially, crystallinity increases, then decreases and finally levels off. This observation is in conformation with the investigations made by researchers about crystallinity index changes during enzymatic hydrolysis.
5) The study of effect of refining and recycling on enzymatic hydrolysis showed that both refining and recycling enhance enzymatic hydrolysis. The increase is attributed to a probable increase in the accessible surface area, a decrease in crystallinity and an increase in the percentage of fines. Between refining and recycling, refining produces a higher increase in hydrolysis rates. However, refining is energy intensive which makes its implementation economically infeasible.
6) We found a novel way of increasing the enzymatic hydrolysis conversion for cellulosic fibers by using cationic polyacrylamides. Linear cationic polymers increase the effective binding of enzyme to the cellulosic substrate thereby increasing the enzymatic hydrolysis rates. Not only does do they speed up the break down of fibers, but they also increase the production of total organic carbon and glucose. The effect of cationic polymers in increasing the hydrolysis rates is higher for hardwood

fibers than for softwood fibers. Some of the other findings with regard to cationic polymers are as following:

(a) The increase in hydrolytic rates is independent of molecular weight of polymers.

(b) The increase in hydrolysis rates is proportional to the cationicity of polymers.

(c) The increase is proportional to concentration of polymers, however there is an upper limit to this concentration beyond which hydrolysis rates are inhibited.

(d) The effect of linear cationic polymers on brown fiber has shown conflicting results.

## 5.1 Future work

The effect of linear cationic polymers on enzymatic hydrolysis of corn starch is being investigated by my other lab members. It has been found that a few cationic polymers increase enzymatic hydrolysis rates of corn starch by as much as 50%. The increase in hydrolysis rates for cellulosic fibers is small yet significant. Therefore, the future work could be focused on trying to answer a few questions such as (1) Why is the increase in hydrolysis rates by cationic polymers different for corn starch and cellulosic fibers (2) What could be done to further increase the hydrolysis rates for cellulosic fibers (3) Why do not all polymers i.e. cross linked work favorably towards enzymatic hydrolysis or specifically what properties of polymers are important for enhancing enzymatic hydrolysis (4) Finding correlation between properties of polymers and an increase produced by polymers.

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