KINETIC INVESTIGATION AND MODELING OF CELLULASE ENZYME

USING NON-CRSYTALLINE CELLULOSE AND

CELLO-OLIGOSACCHARIDES

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Suma Peri

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Suma Peri

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Signature of Author

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Date of Graduation

THESIS ABSTRACT

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Enzymatic hydrolysis of Cellulose depends on many factors; physical properties of substrate (composition, crystallinity, degree of polymerization etc.), enzyme synergy (origin, composition etc.), mass transfer (substrate adsorption, bulk and pore diffusion etc.) and intrinsic kinetics. Most of these effects occur concurrently, therefore cannot be distinguished from each other. Accurate assessment of intrinsic kinetics requires pure form of cellulosic substrates unhindered by mass transfer resistances, or physical factors of substrate. Non-crystalline cellulose (NCC) and Cello-oligosaccharides (COS) are the products of our laboratory which were used as substrates to study the enzymes. The most notable difference seen in this study is that the activity measured by initial rates against NCC is two orders of magnitude higher than that against crystalline cellulose. Since removal of physical barrier primarily increases the hydrolysis by Endo-glucanase, a

significant amount of cello-oligosaccharides and cellobiose was seen to accumulate in hydrolysis of NCC. Cellobiose gradually disappeared whereas cello-oligosaccharides remained constant throughout the enzymatic hydrolysis. The actions of Endo-Glucanases and Exo-Glucanases during the synergism were much more easily distinguished when NCC was used as the substrate. From the experiments conducted on COS, it became apparent that Exo-glucanases cannot act on the soluble substrates. On the other hand, β-glucosidase acts on the cellobiose as well as the cello-oligosaccharides. To find the inhibitory effects, hydrolysis intermediates and products (NCC, cello-oligosaccharides, cellobiose and glucose) are externally supplied at the initial stages of hydrolysis. The time course data on cellulose, COS, cellobiose, and glucose were taken and incorporated into a comprehensive kinetic model that accounts for inhibitory effects of reaction intermediates and products (cello-oligosaccharides, cellobiose and glucose) to determine the kinetic parameters. The model has shown a good agreement with experimental data.

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Style manual or journal used Bioresource Technology

Computer software used Microsoft Office XP (Professional)

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I. INTRODUCTION

More than 93% of all energy consumed in US is for fuel purposes. It was reported that in 2005, 63% of oil consumed in US were imported from middle-east. This energy consumption is increasing by approximately 10% every year (Annual Energy Outlook with Projections to 2025, DOE). Bioethanol is a clean, renewable energy source that has been identified as an important alternative to petroleum for a variety of environmental, economical, and strategic reasons (Wyman, 1996). Bioethanol is important to US energy security because it is produced from domestically grown lignocellulosic biomass feedstocks such as cornstover, wheat straw, and sugar cane bagasse. Since the Clean Air Act Amendments of 1990, there has been a strong demand for ethanol as an oxygenate blended with gasoline. Several ethanol blends have been tested. E10 (10% ethanol, 90% gasoline) is the most commonly distributed, with nearly 4 billion gallons being distributed in 2004. E20 (20% ethanol, 80% gasoline) is used in some other countries and is being considered for promotion in the United States. E85 and E95 (85% and 95% ethanol, respectively) have been successfully tested in North America in government fleet vehicles, flexible-fuel vehicles (FFVs), and urban transit buses. Ethanol is also an effective tool for reducing air toxics that come from the transportation sector.

Today, U.S. produces approximately 4 billion galloons of fuel ethanol per year. At about 3.1% of U.S. gasoline consumption, ethanol represents a small percentage of

our total transportation fuel supply. But demand is growing for this alternative to petroleum and for ethanol production to keep up with increasing demand; feedstock supplies for the fuel must also keep pace. Plentiful, domestic, cellulosic biomass feedstocks such as herbaceous and woody plants, agricultural and forestry residues, and a large portion of many municipal solid waste and industrial waste streams, can be converted to ethanol.

Despite the increasing popularity for bioethanol, there are many technical issues yet to be addressed in the process of converting biomass to ethanol. Cellulosic biomass is a complex mixture of plant cell wall carbohydrate polymers known as cellulose and hemi cellulose, plus lignin and a smaller amount of other compounds known as extractives. To produce ethanol from biomass feedstocks, two key processes must occur. First, the hemi cellulose and cellulose portions of the biomass must be broken down into simple sugars through a process called saccharification. Second, the sugars must be fermented to make ethyl alcohol or ethanol. A variety of thermal, chemical, and biological processes can be used to produce ethanol from biomass. For example saccharification can be carried by acid or enzymatic hydrolysis. Due to the stringent environmental regulations acid hydrolysis even though well established, lost its popularity. Enzymatic hydrolysis is currently the primary route for ethanol production. Among the many aspects of enzymatic hydrolysis, understanding of mechanism and enzymatic kinetics is an important research element. This information will help us in designing more economical saccharification processes and subsequent fermentation to produce bio-ethanol.

The substrate properties and the interactions with the enzyme are of vital importance in determining the effectiveness of cellulose hydrolysis. The physical

structure (Crystallinity, particle size, pore size, accessible area) of cellulose are among the major factors that hinder the enzymatic reaction by cellulase. Under the influence of the above characteristics, it is difficult to obtain the intrinsic kinetic information. Hence there is a need for a substrate which can reveal the true characteristics of the enzyme. One such substrate is the Non-Crystalline cellulose, a product of our laboratory. Because of the concentrated acid treatment, the hydrogen bonds in the cellulose are broken and the substrate was made available for the enzyme.

The objective of this study is to investigate the intrinsic enzymatic kinetics of hydrolysis of cellulosic biomass. This investigation was carried out in three different phases.

Chapter 1 covers the enzymatic hydrolysis experiments performed on Non-crystalline cellulose prepared in our lab. As this cellulosic substrate is amorphous and its hydrolysis is free of mass transfer and chemical resistances, it provides good insights into enzymatic kinetic mechanism.

Chapter 2 explains a dilute acid partial hydrolysis method devised for the production of cello-oligosaccharides. Strategy to obtain a uniform distribution of cello-oligosaccharides production is discussed. Hydrolysis of cello-oligosaccharides in presence of β-glucosidases and cellulases is also investigated independently.

In Chapter 3 a multi-step experimental approach is discussed to investigate the mechanism and intrinsic kinetics of enzymatic hydrolysis of non-crystalline cellulose. Also discussed is a long range mechanistic model to explain the experimental observations and the validation of the model predictions with experimental data.

II. LITERATURE REVIEW

1. Enzyme-Cellulose Interactions:

The enzyme-substrate interaction varies from one enzyme-substrate complex to another. The formation of enzyme substrate complex is usually by the weak Vander Waals forces and hydrogen bonding. The substrate binds to a specific site of the enzyme called as the active site .As the size of the substrate is very small when compared to the size of the enzyme, it easily fits into any region of the enzyme to form the complex. (Shuler and Kargi, 2nd Edition).

2. Cellulose:

Cellulose contains simple repeating units of glucose, but has a complex structure because of the long chains of glucose subunits joined together by β–1, 4–linkages (Lynd et al., 2002). Cellulose occurs in several crystalline phases, as well as in allomorphs resulting from the disruption of the non-covalent forces that normally bind the individual carbohydrate strands together within the crystal lattice. A region of cellulose that is highly disrupted, and thus low in crystallinity, is referred to as amorphous cellulose (Atalla, 1993; Tomme et al., 1995). The stabilizing factors in cellulose are weak individually but collectively form strong bonds. The chains are in layers held jointly by Vander Waals forces and hydrogen–bonds (intramolecular and intermolecular) (Gan et al., 2003). About thirty to forty individual cellulose molecules are arranged into units called

protofibrils, which are further arranged into larger units called microfibrils. These in turn assemble into cellulose fibers. (Mosier et al., 1999).The polymer is not entirely crystalline in nature even though the micro fibrils are tightly packed to form a crystalline structure. The tightly packed and well ordered sites are spaced by loosely arranged ones called amorphous regions (Levy et al., 2002).

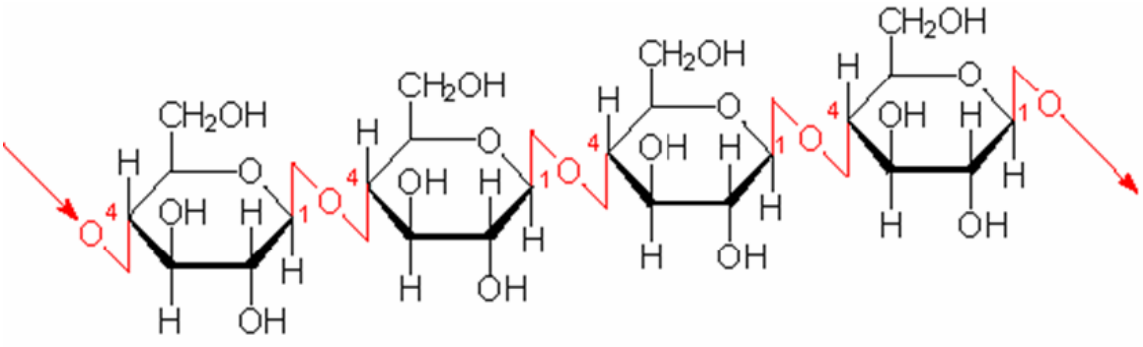

FIGURE II-1: Diagram showing the β –1, 4–linkages in a cellulose chain. (Samejima et al., 1998). The red dotted lines are the intermolecular H–bonds. Such formed cellulose chains are held by intramolecular H-bonds.

3. Pure Cellulosic Substrates:

Pure cellulosic materials can be divided into wood based fibers and non-wood derived fibers. Cotton is a natural polymer of cellulose consisting of the soft fibrous structure which comes under the non-wood derived fibers. Chemically Cotton is cellulose polymer made up glucose molecules. Structurally cotton fiber has fibril and microfibrils and cellulose that give it various physical properties. Cotton cellulose is more crystalline than the wood cellulose. The degree of polymerization of cotton ranges from 3000-4000. Avicel and Alpha cellulose are the fibers processed from the wood cellulose. Avicel is a microcrystalline substrate produced by limited acid hydrolysis of native cellulose to yield highly-ordered forms of cellulose by removing most of the amorphous regions. However,

microcrystalline substrates such as Avicel have complex ultra structures that interfere with enzymatic attack on the microcrystals (Saddler and Penner, 1995). Alpha cellulose is a high grade wood pulp that is acid and lignin free. It contains long chains and durable plant fibers. It is an amorphous alkali-resistant cellulose. It may be derived from wood, linen, cotton, and other plant materials. Typical composition of Alpha cellulose is: 92.2% Glucan, 3.4% Xylan, and 3.2% Mannan on dry basis with negligible ash content. The degree of polymerization of alpha cellulose and Avicel ranges from 200-300.

4. Cellulases and Their Modes of Action:

Cellulases include three main types of enzymes, Endoglucanases, Cellobiohydrolases or Exo glucanases and β-glucosidases.These enzymes can either be free (mostly in aerobic microbes) or grouped in a multicomponent enzyme complex (cellulosome) found in anaerobic cellulolytic bacteria (Bayer et al., 1998). Cellulases from different sources have also been reported to show similar modes of action (Mosier et al., 1999).The enzymatic hydrolysis of the glycosidic bonds takes place through general acid catalysis involving two carboxylic acids (Mosier et al., 1999).

i ***Endoglucanases:*** Endoglucanases forms intermediate compounds with cellulose chains and hydrolyses them at random, giving rise to less polymerized chains and soluble reducing sugars(Bravo et al.,2001).The indiscriminate action of Endoglucanases progressively increases the accessibility of cellulose chain ends, in this manner increasing the specific surface area of the substrate for Exocellulase activity. Endoglucanase attacks the β-1, 4 glycosidic bonds within the amorphous regions of cellulose chains

(Mosier et al., 1999). The products of this attack are oligosaccharides of various lengths and subsequently new chain reducing ends (Lynd et al., 2002).

ii ***Exoglucanases:*** Exoglucanases degrade crystalline cellulose most efficiently and act in a processive mode and bind to the reducing or non–reducing ends of cellulose polysaccharide chains, releasing either glucose (glucohydrolases) or cellobiose (cellobiohydrolases) as major products (Lynd et al., 2002).

iii ***β-glucosidases:*** These complete the hydrolysis of cellulose. They hydrolyze cellobiose, a potential inhibitor of cellobiohydrolases (Lemos et al., 2003). The catalytic activity of β-glucosidase is inversely proportional to the degree of substrate polymerization.

5. Enzymatic Hydrolysis of Cellulose:

As a result of the insolubility and heterogeneity of native cellulose, it is recalcitrant to enzymatic hydrolysis. The degradation of crystalline cellulose is a complex process requiring the participation of many enzymes (Schwarz, 2001). As cellulose can be regarded as the most abundant and biologically renewable resource for bioconversion, its exploitation can be maximized on hydrolysis to glucose and other soluble sugars which can be further fermented into ethanol for use as liquid fuel (Eriksson et al., 2002). Cellulases are the enzymes responsible for the cleavage of the β–1, 4–glycosidic linkages in cellulose. They are members of the glycoside hydrolase families of enzymes that hydrolyze oligosaccharides and / or polysaccharides (Schülein, 2000).

The rate of conversion of cellulose fibers to individual, easily hydrolysable

shorter chains depends on some factors. For enzymatic hydrolysis of natural cellulose, as cited by (Lynd et al., 2002), a number of determining factors of hydrolysis rate have been postulated, including:

i ***Crystallinity:*** This is generally regarded as a key factor influencing cellulose hydrolysis at both enzymatic and microbial levels. The highly crystalline regions of cellulose chains are recalcitrant to hydrolysis, as a result of their tightly packed nature which prevents accessibility of the enzymes. The rate of hydrolysis slows down in presence of more crystalline regions.

ii ***Degree of polymerization:*** The rate of hydrolysis is low if the chain length is longer.

iii ***Particle size:*** Within any given cellulose sample, there is a great measure of unevenness of the size and shape of individual particles, which thus affects the rate of hydrolysis.

iv ***Pore volume:*** The pore structure of cellulosic materials must be able to accommodate particles of the size of a cellulolytic enzyme. The greater the availability, the more the enzymes that are adsorbed (Mosier *et al*., 1999).

v ***Accessible surface area:*** Most cellulose chains are hidden within the microfibrils, which prevents exposure to enzymes and thus limiting the rate of hydrolysis.

6. Models for Hydrolytic Action of the Enzyme:

Non-mechanistic models are used for data correlation under some conditions without an explicit calculation of the adsorbed cellulase concentrations. They do not enhance in understanding the enzyme reaction.

The models which are based on concentration as the only variable that describe the state of the substrate and/or are based on a single cellulose hydrolyzing activity are termed as ‘‘semi-mechanistic.’’ In particular, models featuring the concentration as the only substrate state variable are referred to as ‘‘semi-mechanistic with respect to substrate,’’ and the models with a single cellulose hydrolyzing activity are referred to as “semi-mechanistic with respect to enzyme.” Most of the hydrolysis models proposed to date for design of industrial systems fall into the category of semi-mechanistic models. Limitations of this model lie in understanding the level of substrate and enzyme features.

Models including adsorption model, substrate state variables with concentration, and multiple enzyme activities are identified as ‘‘functionally based models.’’ These are useful for developing and testing understanding at the level of substrate features and multiple enzyme activities, including identification of rate-limiting factors. The limitations for designing the models are in the molecular levels, the state of model development and data availability (Zhang and Lynd, 2004).

Finally, models based on structural features of cellulase components and their interaction with their substrates are termed ‘‘structurally based models.’’ To a greater extent, structurally based models are useful for molecular design as well as testing and developing understanding of the relationship between cellulase structure and function. Derivation of meaningful kinetic models based on structural models requires major advances in protein functions.

III. ENZYMATIC HYDROLYSIS OF NON-CRYSTALLINE CELLULOSE

ABSTRACT

Hydrolysis of cellulose by cellulase enzymes is a solid-liquid heterogeneous reaction. As such the reaction is strongly affected by the non-reaction resistances caused most notably by the crystalline structure. Other non-reaction factors include surface area, diffusion of enzyme, substrate and product, and adsorption of enzyme onto non-cellulosic components. These non-reaction factors mimic the true nature of the hydrolytic enzymatic reaction. For this study, non-crystalline cellulose produced in our laboratory is used as the substrate. This material is drastically different from natural cellulose in that the crystalline structure is disrupted. This chapter focuses on the hydrolytic reactivity of cellulase enzyme against this novel substance. The most notable difference seen in this study is that the activity measured by initial rates against NCC is an order-of-magnitude higher than that against the crystalline cellulose. Since removal of physical barrier primarily increases the hydrolysis by Endo-glucanase and consequently by Exo-glucanase, a significant amount of cello-oligosaccharides and cellobiose were seen to accumulate in the early phase of hydrolysis.

1. INTRODUCTION

Cellulose hydrolysis by cellulases can be explained in terms of the synergistic action of Endo-glucanase, Exo-glucanase and B-Glucosidase (Bravo et al., 2001). The mechanism of cellulose hydrolysis involves the disruption of physical structure, creating more number of reacting ends, thus making the substrate easily available for the enzyme to be acted upon. This step is followed by the primary hydrolysis involving the release of soluble intermediates and the secondary hydrolysis where the low molecular weight intermediates are converted into glucose (Zhang and Lynd, 2004). The physical structure (Crystallinity, particle size, pore size, accessible area) of cellulose are among the major factors that hinder the enzymatic reaction by cellulase. Under the influence of the above characteristics, it is difficult to obtain the intrinsic kinetic information. Among the fundamentally defined questions with applied implications, the role of substrate properties and the interactions within the enzyme are of vital importance in determining the effectiveness of cellulose hydrolysis. Most of the publications included the study of the properties of different cellulosic substrates, factors influencing the hydrolysis and proposals to minimize or eliminate those hindering factors. The basic study regarding the disruption or modification of the physical structure of the cellulose can throw some light in understanding the behavior of the cellulases.

There are various solvents which can dissolve and alter the structure of cellulose. Ionic liquid solvents which are expensive can be used as nonderivatizing solvents for cellulose. They solubilize cellulose through hydrogen bonding form hydroxyl functions to the anions of the solvent (Rogers et.al., 2002). In the method of producing derivatized

cellulose, acetylation, nitration, sulfation are the most common reactions known in the literature. The degree of substitution on cellulose depends on the extent of the reaction and concentration of the reaction medium (Schweiger, 1978). Acid treatments to dissolve cellulose for a less time and precipitating the swollen cellulose in DI water or organic solvents produce a substrate entirely different from cellulose. Regenerated cellulose after treatment with hydrochloric acid (Penner and Chin Hsu, 1991), degraded cellulose produced by action of sulfuric acid, phosphoric acid swollen cellulose (PASC) are some of the substrates which show significant differences from the cellulose. PASC also known as experimentally generated amorphous cellulose is used as substrate for studying the enzyme components (Stalbrand et.al., 1998).

For fundamental studies on the cellulase, novel Non-crystalline Cellulose invented in our laboratory is used as the substrate (Pending US Patent, Lee and Harraz, Application No. 60/762439, 2006)**.** Non-crystalline cellulose (NCC) is produced from alpha cellulose by treatment with concentrated sulfuric acid. This treatment followed by re-precipitation of the above mixture in water leads to the formation of NCC. During this re-precipitation process, the cellulose particles were seen to be agglomerating. Since the hydrogen bonds which hold the crystalline structure are broken, the Crystallinity of NCC is essentially removed.

This substrate when sonicated forms well dispersed homogeneous slurry with water. The main objective of this work is to investigate the kinetic behavior of enzymatic hydrolysis against NCC and seek additional information that may not be available from crystalline cellulose.

2. MATERIALS AND METHODS

2.1 Materials:

Cotton (supplied by Buckeye Tech) and Alpha cellulose (Sigma Aldrich; Cat. No.C8002; Lot No.114K0244) which are pure forms of cellulose are used as raw materials for producing Non Crystalline Cellulose (NCC). Avicel PH-101 (Fluka; Cat. No.11365; Lot No. 1094627), microcrystalline cellulose was used as a standard reference substrate. Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257), was obtained from NREL and has an average activity of 31.2 FPU/mL. Novozyme 188 β-glucosidase (Novo Inc., Lot no.11K1088) has an activity of 750 CBU/mL. The NCC was stored in wet state (approximately 80% MC) under refrigeration to perform the compositional and digestibility analysis.

2.2 Experimental Procedure:

2.2.1 Compositional Analysis:

Small portions of the NCC samples were freeze-dried overnight, crushed to smaller particle size, and analyzed for composition in duplicate using NREL LAP standard method No.002. The NCC was observed to form a very rigid structure upon heating which could not be completely hydrolyzed even when grinded to a fine particle size. Hence the samples were freeze-dried to overcome the inaccuracy due to incomplete glucan hydrolysis when dried at 45^{o} C. The compositions analyzed are tabulated (Table III-1).

TABLE III-1: Composition Analysis of Avicel, Alpha cellulose and NCC.

	Avicel	Alpha cellulose	NCC
MC %	3.78	4.56	4.86
Glucan	97.26	76.58	87.27
Xylan		21.87	10.51
Ash			1.91
Total	97.26	100.18	100.18

2.2.2 Enzymatic Hydrolysis:

The hydrolysis of cellulose was performed in 250 mL shake flasks with a working volume of 100 mL. 1 g glucan (1% w/v dry basis) was taken as basis for each falsk.0.4 mL of tetracycline (10 mg/mL in 70% ethanol) and 0.3 mL of cyclohexamide (10 mg/mL in distilled water) were added as antibiotics to prevent any type of growth. Sodium citrate buffer (0.05 M) was used to bring the final working volume to 100 mL. The pH of 4.5 can be assumed to be maintained throughout the reaction because of the buffer addition. All the components were assumed to have a density of 1g/mL in the flask. Substrate blanks and Enzyme blanks were run to account for any glucose contribution from the samples and any protein from enzymes.

The flasks were heated for 1 hour at 50^{o}C before the addition of the 1 mL of cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257). The enzyme solutions were diluted to 1,3,15 FPU/mL by adding appropriate amounts of buffer solutions just prior to their addition into the flasks. The experiment was conducted in a New Brunswick Scientific (Edison, NJ) Series 25 incubator shaker at 50°C and 150 rpm. Samples were taken at 0, 0.25, 0.5, 0.75,1, 2, 3, 4, 5, 6, 12, 24, 36, 48, 72, and 96 Hours and boiled for 5 min to kill the enzyme, thus confirming the ceasing of the reaction. Then

the samples were centrifuged, and analyzed for glucose, cellobiose and higher cello-oligosaccharides using HPLC.

2.2.3 Analysis:

The samples after the carbohydrate analysis and the enzyme hydrolysis were analyzed for sugars using HPLC equipped with RI detector and Bio-Rad's Aminex HPX-87P column maintained at 85^{o}C with DI water as the mobile phase. Glucose and soluble cello-oligosaccharides formed during hydrolysis were quantified using HPLC. The cellulose digestibility was calculated by the following equation,

Cellulose Digestibility = (Cellulose digested (g)/ Initial Cellulose (g)) x100%

Where the cellulose digested was calculated by the sum of glucose and equivalent glucose from cellobiose and cello oligosaccharides measured by HPLC.

3. RESULTS AND DISCUSSIONS

The enzymatic hydrolysis experiments were conducted to assess the kinetic behavior of cellulase enzyme when the physical structure of cellulose is disrupted. It was observed that the reaction shifts nearly towards homogenous reaction after 20 hours of the reaction time. As shown in the Figure III-1, because of the disruption of the hydrogen bonds in the cellulose structure, the crystalline region disappears and hence the components of cellulase enzyme directly start their mode of activity on the substrate. The Endo-glucanase component increases the concentration of the reducing ends by acting on the interior parts and thus decreasing the degree of polymerization of the cellulose substrate. Consequently fragmented insoluble cellulose with high reducing ends and soluble cello-oligomers are formed. Having the reducing ends readily available for the

Exo-glucanase, it rapidly solubilizes the insoluble cellulose to soluble cellobiose. Hence, it was seen that the initial rates of the enzymatic hydrolysis were high.

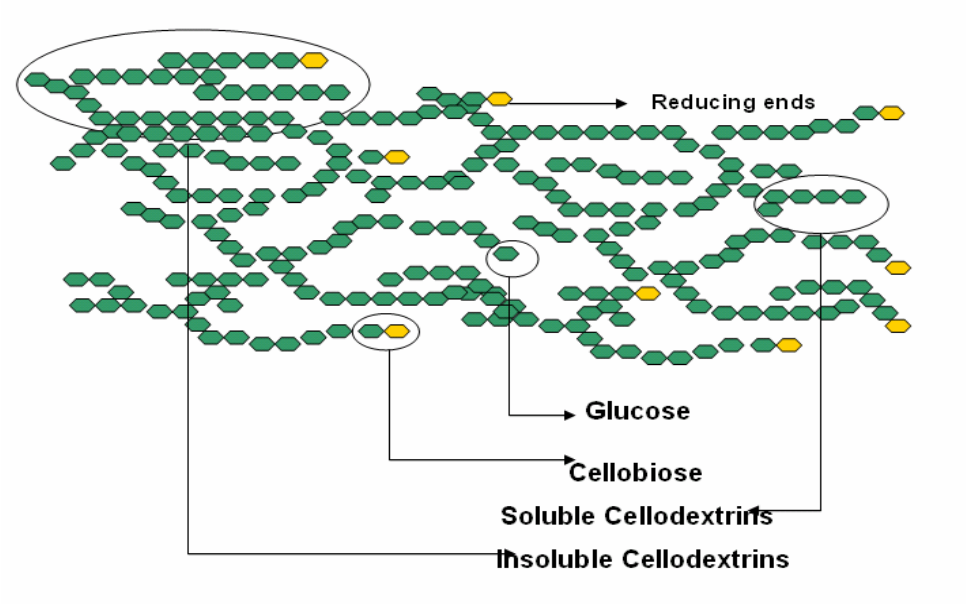

FIGURE III-1: Schematic Diagram of NCC Structure

The extent of hydrolysis of NCC was higher when compared to the reference substrates Avicel and untreated cellulose as shown in Figure III-2. With NCC as substrate upon cellulase hydrolysis, oligomers and cellobiose were seen to accumulate during the early stages of the reaction, evidenced by HPLC chromatographs of Figure III-3. This shows that soluble cello-oligomers were significant fraction of the intermediate products of Endo-enzyme. It is apparent from the Figure III-4 that as the time increases cellobiose gradually disappear but oligomers were not digested till the end. This indicates that the Exo-glucanase does not act on the soluble cello-oligomers to produce cellobiose. In the conventional definition of digestibility, only glucose and cellobiose were considered for the calculation of enzyme hydrolysis. The total hydrolysis as given by the enzyme should also involve the cello-oligomers.

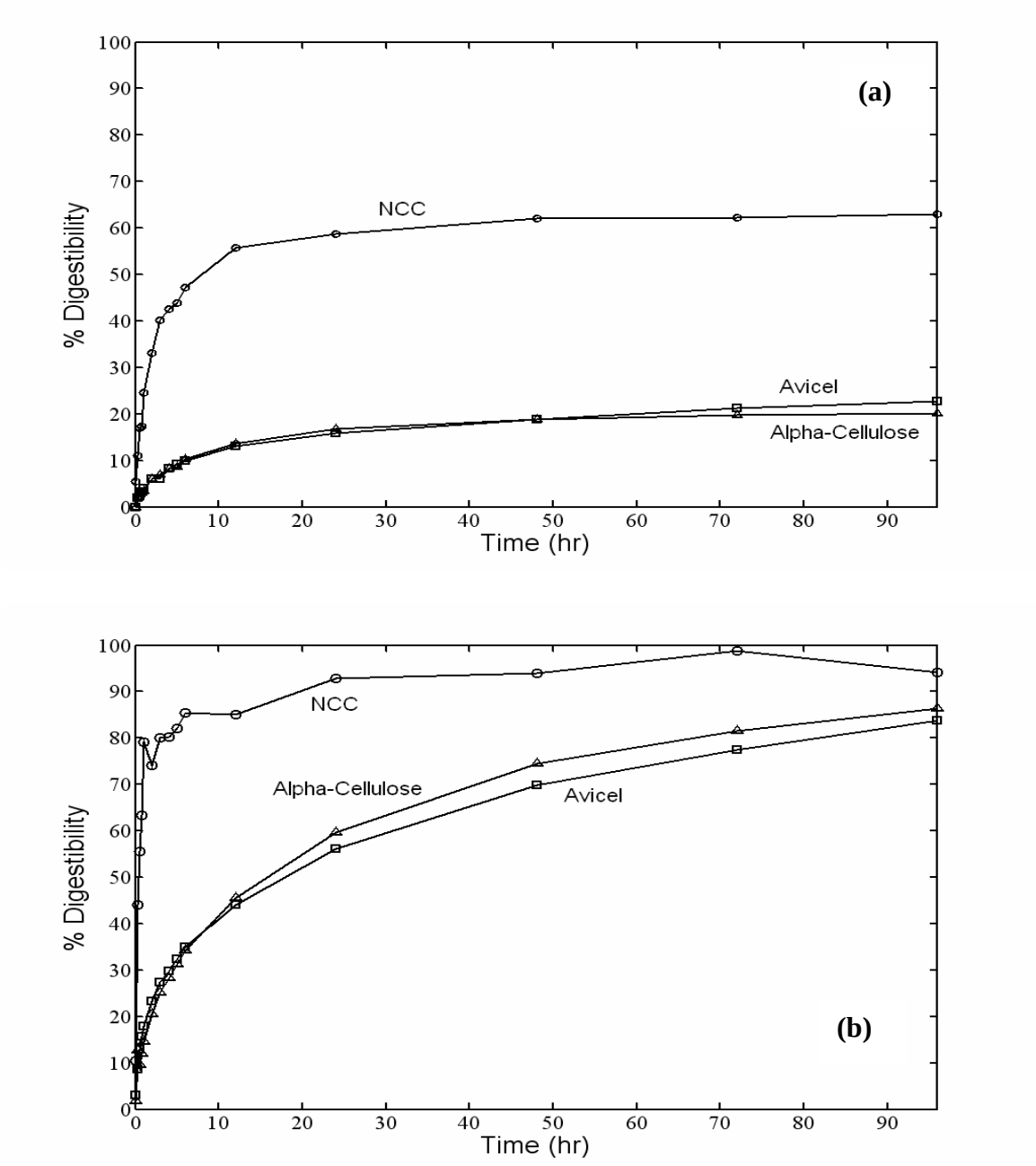

FIGURE III-2: Comparison of hydrolysis of NCC with Alpha cellulose and standard reference Avicel for an enzyme loading of a**)** 1 FPU/g-Glucan (1% glucan loading) **b)** 15 FPU/g-Glucan (1% Glucan loading).

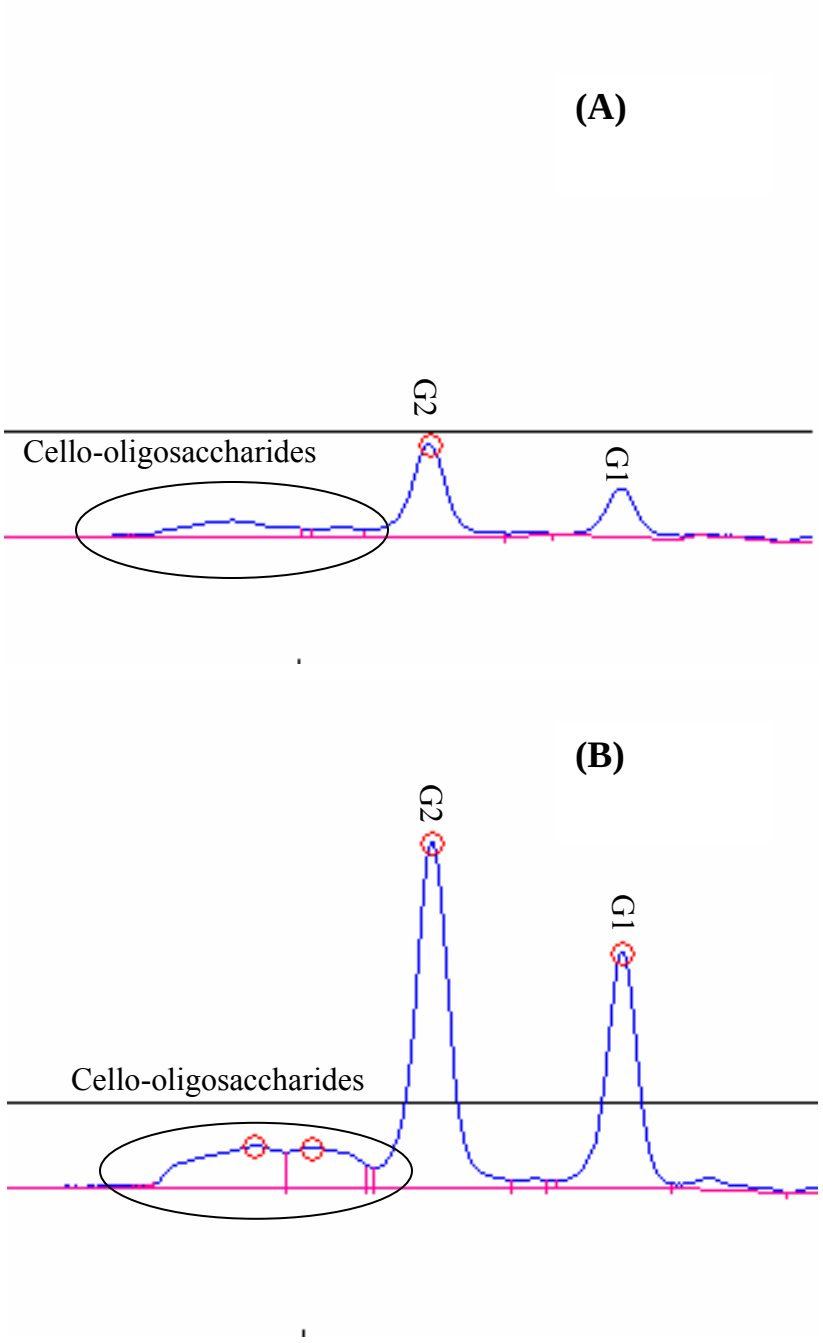

FIGURE III-3: HPLC chromatographs showing the formation of Cello-oligosaccharides during the enzymatic hydrolysis of NCC with an enzyme loading of 1FPU/g-Glucan. (1% glucan loading) **(A)** Time=0hour **(B)** Time=1hour

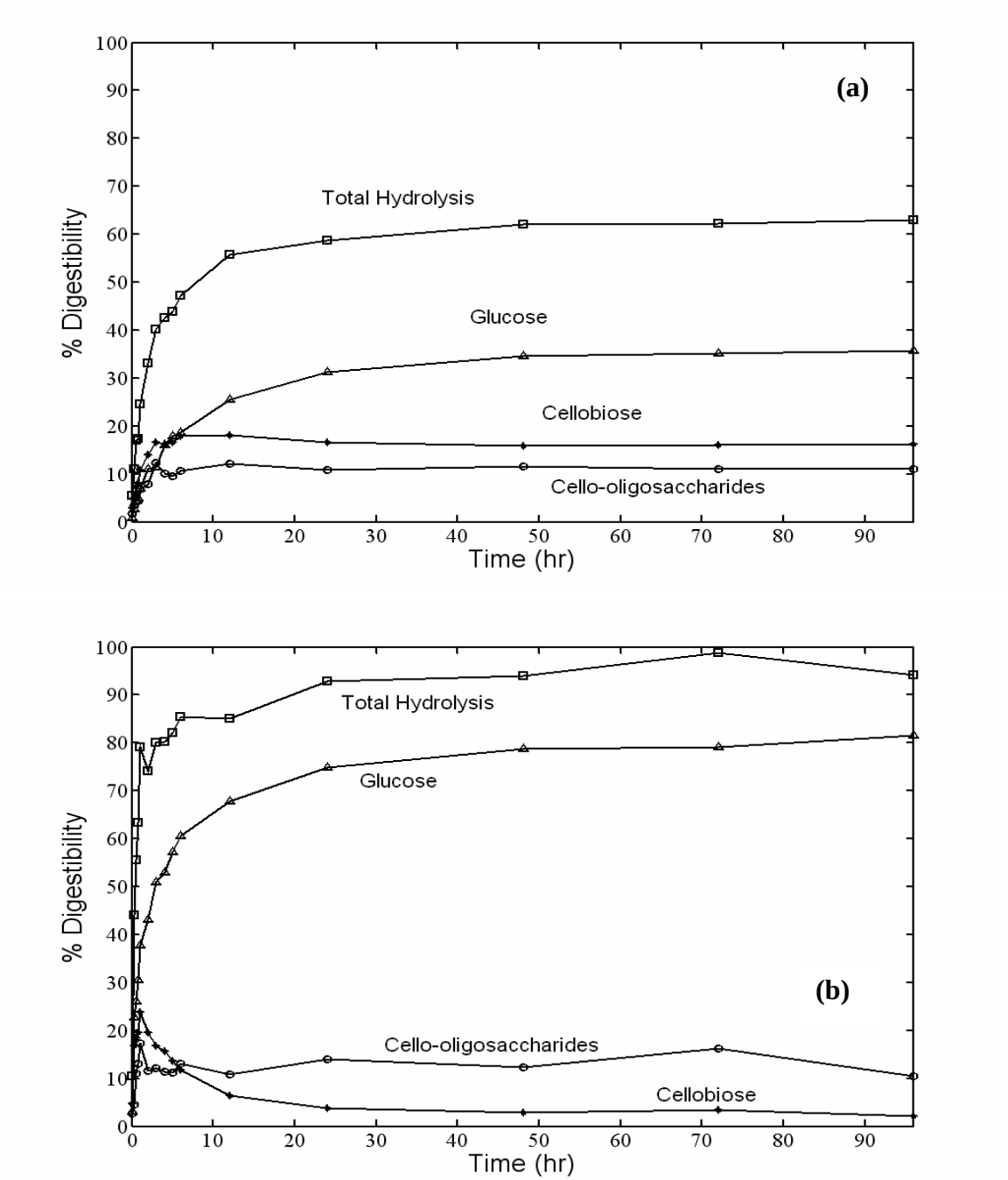

FIGURE III-4: NCC digestibility with an Enzyme loading of **a)** 1 FPU/g-Glucan (1% Glucan loading) **b)** 15 FPU/g-Glucan (1% Glucan loading)

When oligomers were considered the total hydrolysis of NCC had increased by 10%. This shows a significant amount of cello-oligomer formation during the reaction which was not completely hydrolyzed to glucose due to lack of enzyme or some components of enzyme not contributing towards the conversion.

The results of hydrolysis of sonicated NCC (Figure III-5a) indicated very high initial hydrolysis rates when compared to the original NCC (Figure III-4a). The digestibility for 96 hours was reported to be 20% higher than that of unsonicated NCC. This indicates that breaking the particle size and making a homogenous reaction mixture would affect the enzyme-substrate reaction positively. The data for the hydrolysis of sonicated NCC when Tween20 is added are also presented in Figure III-5b. Surfactants also showed a 10% increase in the formation of Glucose. The formation of cello-oligosaccharides in sonicated NCC was higher than that of the cello-oligosaccharides seen during the hydrolysis of sonicated NCC when Tween20 was added. A comparison of sonicated NCC, and sonicated NCC with Tween20 hydrolyzed to COS, cellobiose and glucose is seen in Figure III-5.

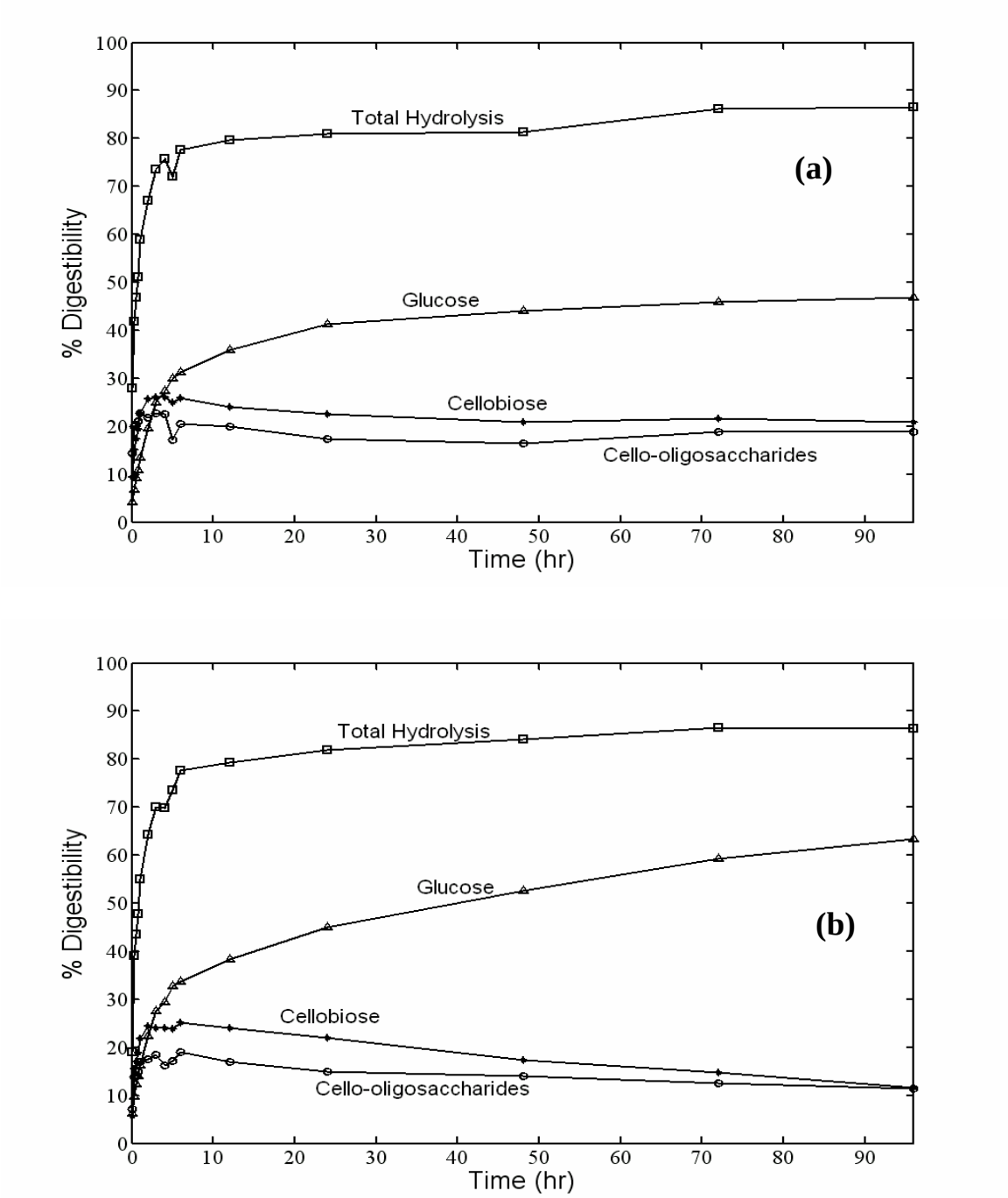

FIGURE III-5: Comparison of Hydrolysis of **a)** sonicated NCC and **b)** sonicated NCC+1%Tween20 with cellulase loading of 1FPU/g-Glucan.

4. CONCLUSIONS

Hydrolysis of NCC exhibited extremely high initial rates. The reaction essentially ceased after 24 hours. In the enzymatic hydrolysis of NCC, a significant amount of cellobiose and cello-oligomers were formed as reaction intermediates. Cellobiose gradually disappeared whereas cello-oligomers remained constant throughout the enzymatic hydrolysis. The actions of Endo-Glucanases and Exo-Glucanases were easily distinguished when NCC was used as the substrate.

IV. SYNTHESIS AND HYDROLYSIS OF CELLO-OLIGOSACCHARIDES

ABSTRACT

Cello-oligosaccharides (COS) are prepared from pure cellulosic material (cotton) by a relatively simple process: dissolution into concentrated sulfuric acid followed by hydrolysis and purification. Concentrated solution of COS thus prepared is lyophilized to get the soluble sugars ranging from G_2-G_6. A fairly uniform distribution of all the sugars in the oligosaccharides is obtained in this process. COS is then used in understanding the behavior of the cellulase enzymes. COS are easily hydrolyzed by acid to glucose but they are not significantly digested by the cellulase enzyme. Hydrolysis of COS with b-glucosidase results in higher digestion than the cellulase enzyme. This explains the inhibition caused by COS on Exo and Endo-glucanases. The behavior of the homogeneous reaction of COS and the cellulase enzymes are discussed in this chapter.

1. INTRODUCTION

Cello-oligosaccharides play an important role in exploring the intrinsic kinetics of enzymatic hydrolysis and also in enzyme characterization. They have been used to study the aspects of microbial cellulose utilization including regulation of cellulase synthesis, cell growth, and bioenergetics. They are proved to have lessening effects on cholesterol levels when consumed as food (Wakabayashi et al., 1995; Cummings and Macfarlane, 1997).

General methodology of cello-oligosaccharides preparation involves partial hydrolysis of cellulose to produce cello-oligosaccharides followed by some separation processes to remove acid and/or salts resulted from acid hydrolysis procedures.

HCl fuming method reported by Miller et al. (1960, 1963) is widely used in cello-oligosaccharides preparation. It involves hydrolysis of microcrystalline cellulose by HCl fuming followed by usage of reduced pressure to remove HCl, ion exchange to remove residual salts and solvents to precipitate cello-oligosaccharides to facilitate acid removal. Many variations were proposed in the acid and salt removal techniques in the literature.

Using 80% sulfuric acid for primary hydrolysis, followed by a secondary hydrolysis at dilute acid concentration is an alternative for Miller's method (Voloch et al., 1984). Another alternative method for acid hydrolysis method involves cello-oligosaccharides preparation via acetylation of cellulose (Dickey and Wolform, 1949; Wolfram and Dacon, 1952) which found to be more expensive and time consuming. Zhang and Lynd (2003) recently proposed a concentrated acid (80% by volume of 37% hydrochloric acid and 20% by volume of 98% sulfuric acid) hydrolysis of Avicel followed by acetone precipitation, washing by ion exchange, and neutralization with

barium hydroxide. Further separation of constituent cello-oligosaccharides was carried out by series of Bio-Rad AG50W-X4 and Bio-Gel P4 columns. They reported a cello-oligosaccharide yield of 20% with a non-uniform distribution. Apparently this method is cost effective and obviated many limitations posed from a charcoal-cellite column used for chromatographic separations of constituent cello-oligosaccharides.

All the above mentioned acid hydrolysis methods uses high concentrations of hydrochloric acid and/or sulfuric acid in the hydrolysis step. On considering safety and environmental concerns posed by usage of concentrated acids there comes an explicit need to explore the possibilities of using relatively diluted acids for hydrolysis of micro-crystalline cellulose to produce more evenly-distributed cello-oligosaccharides.

Objective of the current study is to study the cellulase enzyme behavior using soluble cello-oligosaccharides as substrates.

2. MATERIALS AND METHODS

2.1 Materials:

Cotton (supplied by Buckeye Tech) is used as a raw material for synthesizing Cello-oligosaccharides (COS). Acetone (Fisher Sci. Histological grade; Cat No. Lot No. 060206-36) was used to precipitate the sugars. Sulfuric acid (Technical grade) from Fisher Scientific was used as a reaction reagent. Avicel PH-101 (Fluka; Cat. No.11365; Lot No. 1094627), microcrystalline cellulose was used as a standard reference substrate. Cello-oligosaccharides obtained from Sigma (Cat No.C8071) was used as a standard substrate in comparing the produced cello-oligosaccharides. Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257), was obtained from NREL and had an average

activity of 31.2 FPU/mL. Novozyme 188 β-glucosidase (Novo Inc., Lot no.11K1088) had an activity of 750 CBU/mL.

2.2 Experimental Procedures:

2.2.1 Compositional Analysis:

The Cello-oligosaccharides produced using sulfuric acid was analyzed for composition in duplicate using NREL LAP standard method No.015.

2.2.2 Acid Hydrolysis:

0.3 ± 0.001 g of Cello-oligosaccharides was weighed in a 100ml pressure tube. 3 ml of 72% sulfuric acid was added to the COS. This mixture was shaken for the complete dissolution of the COS in sulfuric acid. Immediately 84 ml of DI water was added to make it a 4% acidic solution. Then the pressure tubes were subjected to a high temperature at 121^{o}C for one hour in the auto clave. Because of the low acid concentration and high temperature, the COS is assumed to be hydrolyzed to glucose.

2.2.3 Enzymatic Hydrolysis:

The hydrolysis of cellulose was performed in 250 mL shake flasks with a working volume of 100 mL. 1 g glucan (1% w/v dry basis) was taken as basis for each flask. 0.4 mL of tetracycline (10 mg/mL in 70% ethanol) and 0.3 mL of cyclohexamide (10 mg/mL in distilled water) were added as antibiotics to prevent any type of growth. Sodium citrate buffer (0.05 M) was used to bring the final working volume to 100 mL. The pH of 4.5 can be assumed to be maintained throughout the reaction because of the buffer addition. All the components were assumed to have a density of 1g/mL in the flask. Substrate blanks and Enzyme blanks were run to account for any glucose contribution from the samples and any protein from enzymes.

The flasks were heated for 1 hour at 50^{o}C before the addition of the 1 mL of cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257). The enzyme solutions were diluted to 1, 3, 15 FPU/mL by adding appropriate amounts of buffer solutions just prior to their addition into the flasks. The experiment was conducted in an New Brunswick Scientific (Edison, NJ) Series 25 incubator shaker at 50°C and 150 rpm. Samples were taken at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 12, 24, 36, 48, 72, and 96 Hours and boiled for 5 min to kill the enzyme, thus confirming the ceasing of the reaction. Then the samples were centrifuged, and analyzed for glucose, cellobiose using HPLC.

2.2.4 Analysis:

The samples after the carbohydrate analysis and the enzyme hydrolysis were analyzed for sugars using HPLC equipped with RI detector and Bio-Rad's Aminex HPX-87P column maintained at 85^{o}C with DI water as the mobile phase. The cellulose digestibility was calculated by the following equation,

Cellulose Digestibility = (Cellulose digested (g)/ Initial Cellulose (g)) x100%

Where the cellulose digested was calculated by the sum of glucose and equivalent glucose from cellobiose and cello oligosaccharides measured by HPLC.

3. RESULTS AND DISCUSSIONS

Cellulose hydrolysis by sulfuric acid for production of cello-oligosaccharides is a well-known procedure. Cellulose dissolution occurs at an appreciable rate when the concentration of sulfuric acid is above 65%. The concentration, solid to liquid ratio, time and temperature for the production of Cello-oligosaccharides differ form each substrate. We have found that appreciable yield of cello-oligosaccharides can be obtained using

72% sulfuric acid. Use of acetone helps in the removal of free sulfuric acid present after the reaction. Retention time and the quantity of acetone addition, control the precipitation of cello-oligosaccharides. Hence desired degree of polymerization (DP) can be obtained by varying the above controlling factors. Anion exchange resin was used to remove the residual free acid and the cellulose sulfate present in the solution of cello-oligosaccharides. This helped in reducing the ash content in the final product. When calcium or barium hydroxides were used to remove the acid traces, the final product showed an appreciable increase in the amount of ash. Thus obtained cello-oligosaccharides were analyzed and the composition is reported as 96.31% pure Glucan. The ash content was minimal (0.79%). The distribution of all the sugars in the final product was fairly uniform. Because of the uniform distribution, the hydration factor for calculating the glucan equivalent of the cello-oligosaccharides can be taken as 0.9282. This value is the average of all the hydration factors of the individual cello-oligomers ranging from cellobiose to cellohexose. The HPLC peaks for certain concentrations were compared with the standard cello-oligosaccharides purchased from Sigma and were reported (Figure IV-1).

Acid Hydrolysis of cello-oligosaccharides resulted in 93% conversion of the oligomers to the monomer (glucose) in 20 min when the reaction was carried in an autoclave at 121^{o}C. When these soluble substrates were subjected to hydrolysis by cellulase enzyme, they were not completely hydrolyzed to glucose inspite of the reaction being homogeneous which is evident from the HPLC peaks before and after the exposure to the hydrolyzing enzymes shown in Figure IV-2.

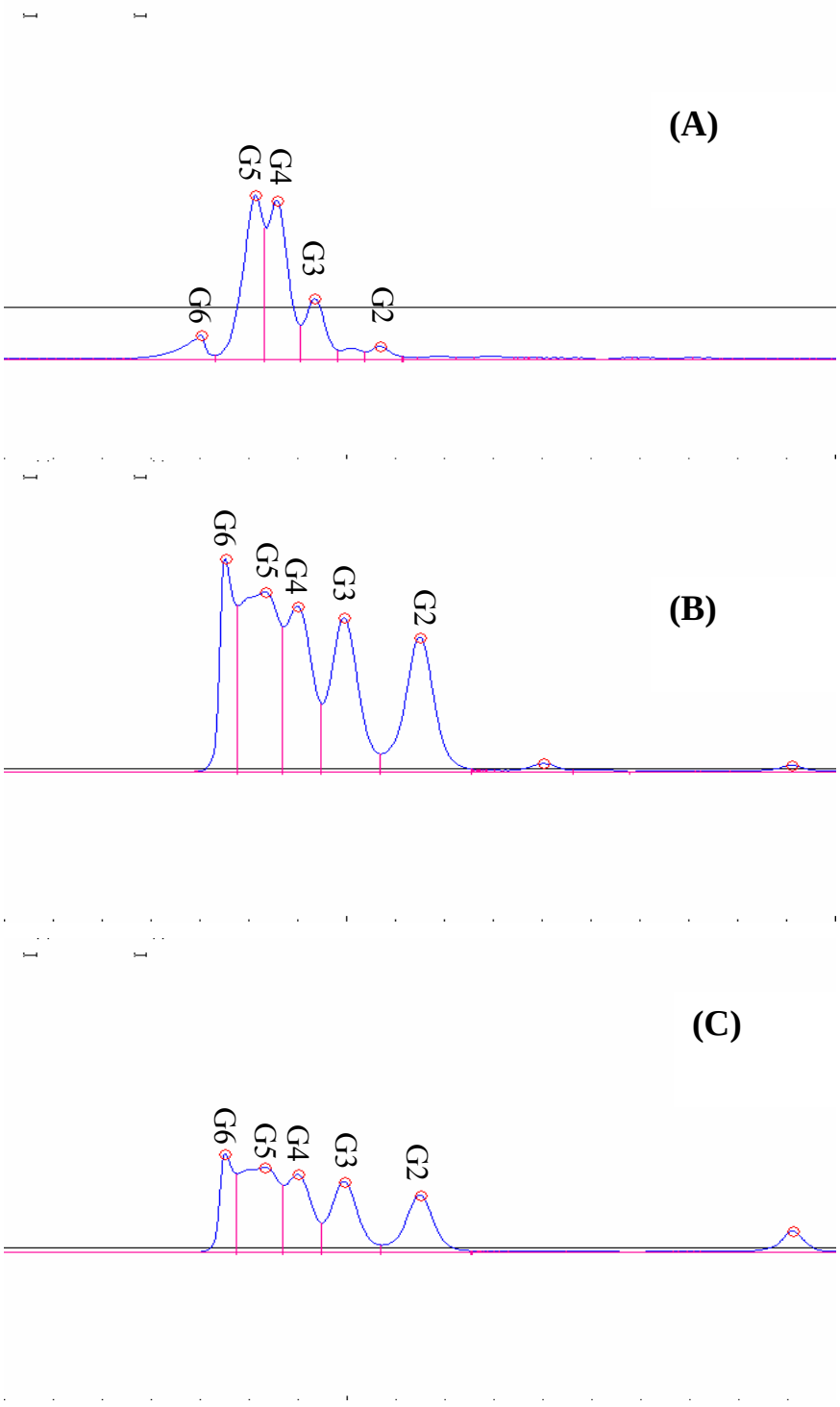

FIGURE IV-1: HPLC chromatographs of **(A)** Sigma Standards Cello-Oligosaccharides (Cat No C8071): Concentration: 5 g/L **(B)** Cello-Oligosaccharides: Concentration: 10 g/L, **(C)** Concentration: 2 g/L.

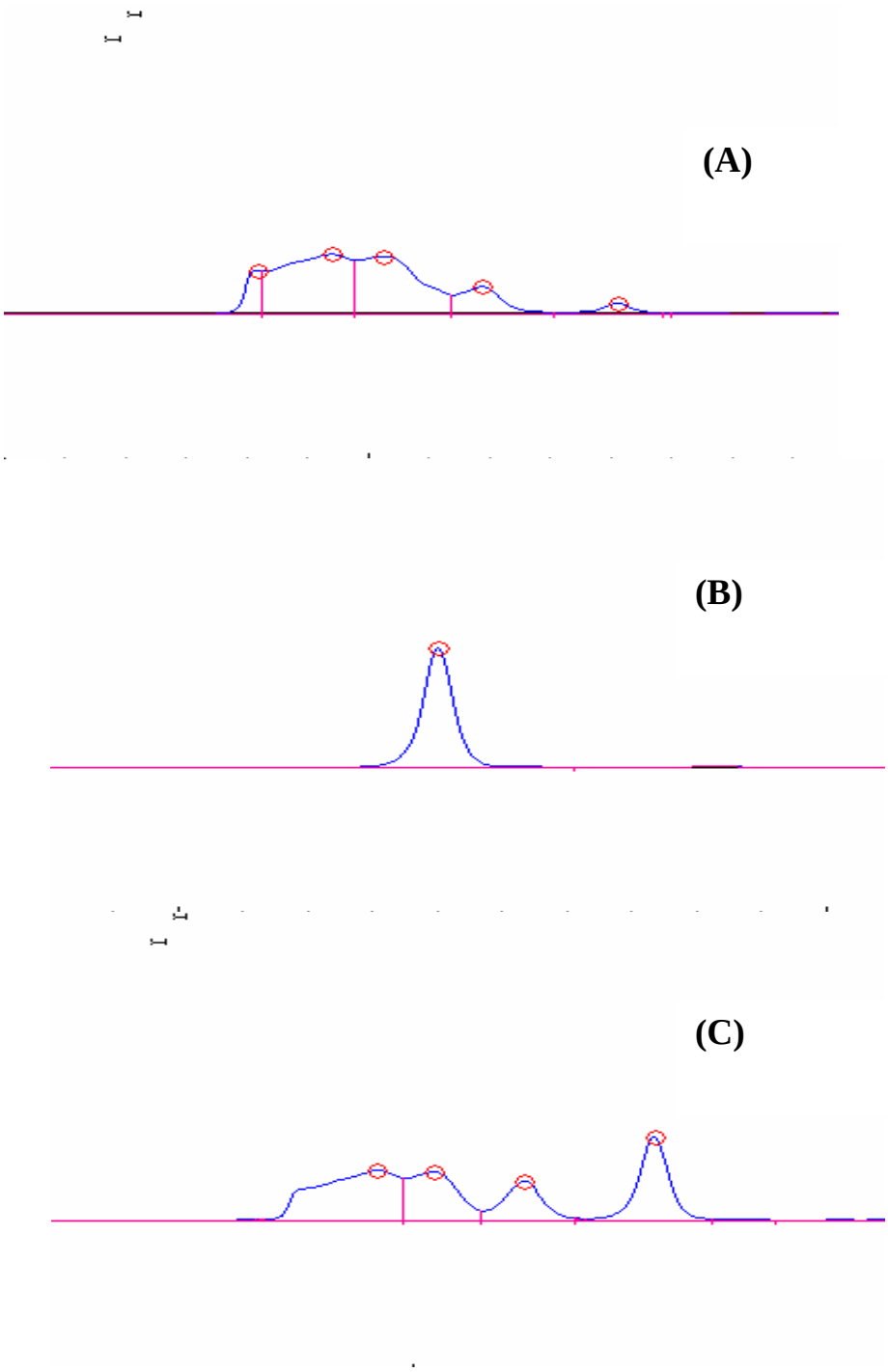

FIGURE IV-2: HPLC chromatographs picturing the hydrolysis of Cello-Oligosaccharides. **(A)** Cello-oligosaccharides before hydrolysis, **(B)** Cello-oligosaccharides after acid hydrolysis, **(C)** Cello-oligosaccharides after enzyme hydrolysis (96 Hours)

It is apparent from the data shown in Table IV-1, IV-2, IV-3 and Figure IV-3 that the Cello-oligosaccharides exhibit poor hydrolysis rates with cellulase enzyme. The reason could be explained in terms of the chain length and finding of the proper active binding site for the enzyme on the substrate. As the Endo-cellulase molecule slides on the cellulose chain, it makes a random cleavage so that the Exo enzyme can easily work on the reducing ends to produce cellobiose. Due to the reduction in the chain length, the Exo enzyme lacks in locking with the active site on the substrate to form the enzyme substrate complex. The dimers and trimers of glucose molecules may occupy the sites other than the active sites and thus inactivating the enzyme. These cello-oligosaccharides act as inhibitors to the Endo and Exo enzymes.

TABLE IV-1: Enzymatic hydrolysis of COS (Enzyme loading -1 FPU/g Glucan)

Time (hours)	%Glucose	% Cellobiose	Total Hydrolysis
0	0.00%	3.97%	3.97%
6	3.06%	4.52%	7.58%
12	3.63%	4.52%	8.14%
24	5.38%	4.66%	10.04%
48	5.94%	4.73%	10.67%
72	6.52%	4.71%	11.23%
96	7.31%	4.88%	12.19%

TABLE IV-2: Enzymatic hydrolysis of COS (Enzyme loading - 3 FPU/g Glucan)

Time (hours)	%Glucose	%Cellobiose	Total Hydrolysis
0	0.00%	4.02%	4.02%
6	4.32%	4.63%	8.95%
12	5.21%	4.66%	9.87%
24	6.39%	4.87%	11.26%
48	7.18%	4.94%	12.11%
72	7.17%	5.04%	12.21%
96	7.80%	5.18%	12.98%

TABLE IV- 3: Enzymatic hydrolysis of COS (Enzyme loading - 15 FPU/g Glucan)

Time (hours)	%Glucose	%Cellobiose	Total Hydrolysis
0	1.37%	4.08%	5.45%
6	7.10%	5.18%	12.28%
12	7.79%	5.20%	12.99%
24	9.27%	5.71%	14.98%
48	10.44%	5.87%	16.31%
72	10.85%	5.90%	16.74%
96	11.67%	6.08%	17.75%

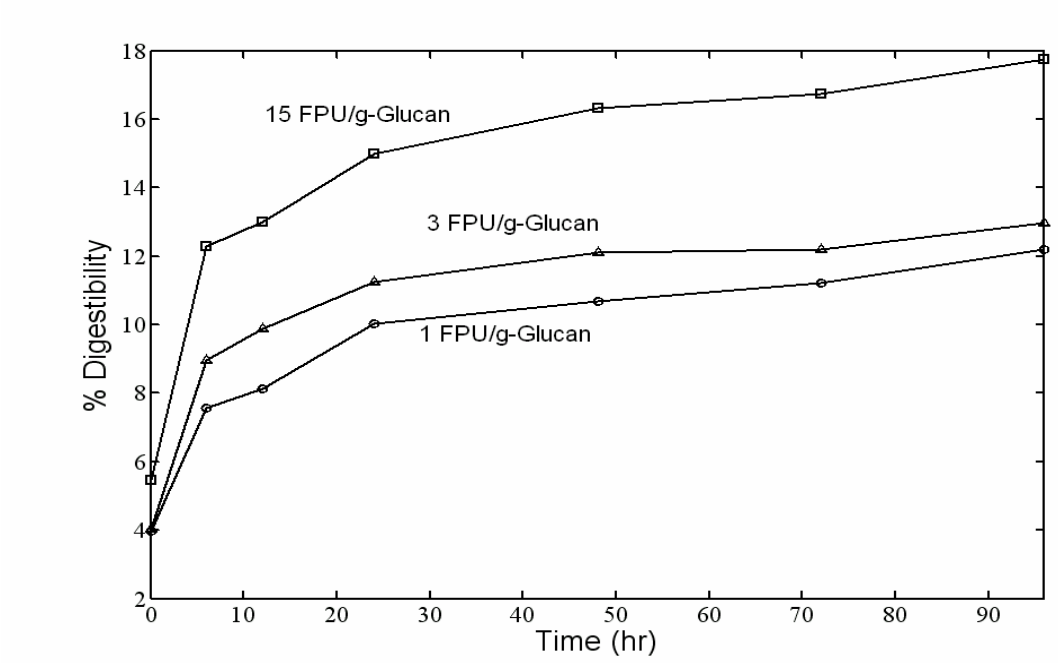

FIGURE IV-3: Hydrolysis of cello-oligosaccharides with different cellulase enzyme loadings.

When this cellulase enzyme was supplemented with β-glucosidase, the hydrolysis rate increased and the oligosaccharides were digested to the monomeric sugars. When the hydrolysis was carried on Cello-oligosaccharides with β-glucosidase alone, the extent of hydrolysis was higher than with only cellulases. Figure IV-4 shows the extent of hydrolysis of cello-oligosaccharides in presence of cellulases and β-glucosidases independently. This explains that β- glucosidase acts on the cellobiose as well

as the oligosaccharides and produces glucose. Cellulase enzymes cannot act on the soluble substrates. Comparison of cellulase hydrolysis of cello-oligosaccharides with that of insoluble substrate Avicel is shown in Figure IV-5. In presence of cellulase enzyme Avicel was hydrolyzed to 85% whereas, COS was subjected to hydrolysis to an extent of 18%.

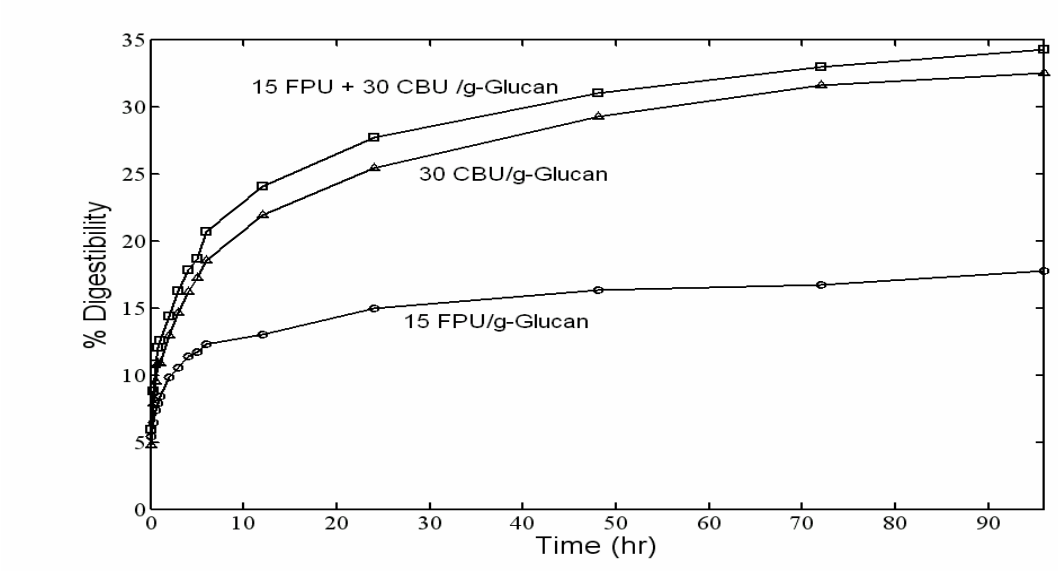

FIGURE IV-4: Hydrolysis of COS with cellulase and cellulase supplemented with β-glucosidase.

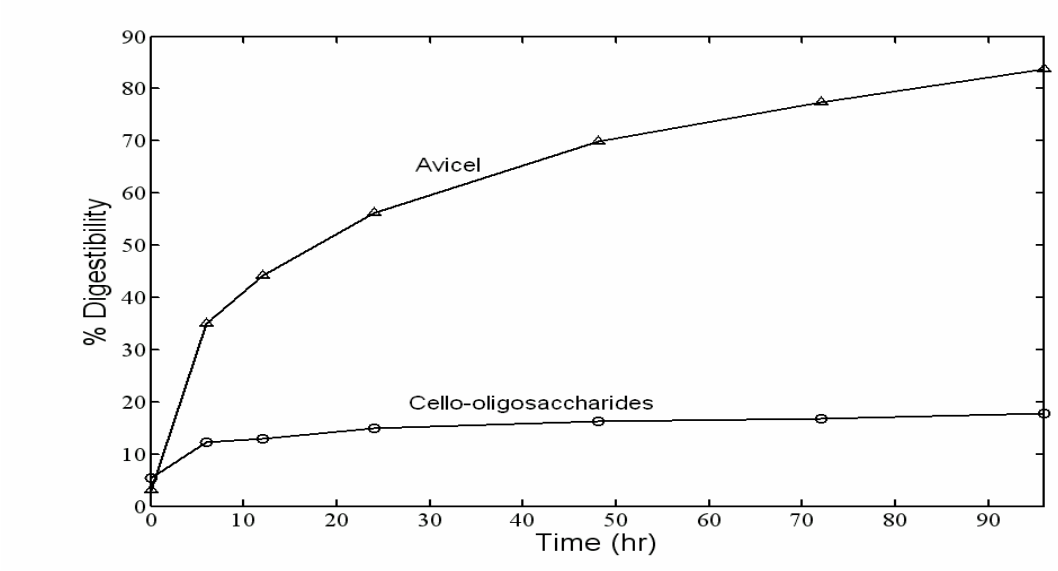

FIGURE IV-5: Hydrolysis of COS and Avicel with a cellulase loading of 15 FPU/g-Glucan (1% Glucan loading)

4. CONCLUSIONS

COS were easily hydrolyzed by sulfuric acid, but were not significantly hydrolyzed by cellulase. Since the cellulase enzymes were designed to work on solid substrates, the COS (DP 2 to 10) do not fit into the active cites of the enzymes. Hydrolysis of COS by Spezyme CP was slower than that of Avicel. COS were inhibitory to Endo and Exoglucanases. It was found that COS can be hydrolyzed by β-glucosidases.

V. INTRINSIC KINETICS OF CELLULASE ENZYME AS OBSERVED WITH HYDROLYSIS OF NON-CRYSTALLINE CELLULOSE

ABSTRACT

A multi-step approach was taken to investigate the intrinsic kinetics of the cellulase enzyme complex as observed with hydrolysis of NCC. In the first stage, initial rate mechanistic models were built using the enzymatic hydrolysis experiments performed on various substrates; Non-crystalline cellulose, Alpha cellulose and Avicel. It was found that most of these kinetic models gave good predictions in the initial stages of the enzymatic hydrolysis with acceptable precision, but failed to explain the reducing reaction rates in the later stages. In the second stage, assessment of effect of reaction intermediates and products on intrinsic kinetics of enzymatic hydrolysis was performed using the data obtained from NCC, in the isolation of external factors like mass transfer effects, physical properties of substrate etc. Various experiments were performed in series to identify the individual inhibitory effects from hydrolysis intermediates (soluble cello-oligosaccharides, cellobiose) and product (glucose). These studies revealed that, these inhibitions are simultaneous in nature and can not be quantified independently. In the final stage, based on some assumptions a comprehensive intrinsic kinetics mechanism was proposed. These assumptions were made from the experimental observations on the concentration profiles of NCC, soluble cello-oligosaccharides, cellobiose and glucose as

the time course of enzymatic hydrolysis. Four reactions were identified as important steps in the mechanism of enzymatic hydrolysis. The reactions were: NCC to Cellobiose, Cellobiose to Glucose, NCC to Soluble cello-oligosaccharides (COS), and COS to Glucose. From batch experiments using NCC, the time-course data on cellulose, COS, Cellobiose, and Glucose was taken. This data was used to estimate the parameters in the kinetic model that accounts for inhibitory effects of reaction intermediates and products (COS, Cellobiose and Glucose). The model predictions of NCC, COS, Cellobiose and Glucose profiles, has shown a good agreement with experimental data generated from hydrolysis of different initial compositions of substrate (NCC supplemented with COS, Cellobiose and Glucose).

1. INTRODUCTION

Kinetics and further modeling studies of Hydrolysis are useful in different stages of processing of biomass to fermentable sugars. They span the entire domain of operations, namely; enzyme characterization, substrate preparation, reactor design, and optimization of feeding profiles of substrate in a fed-batch operation. There can be two kinds of modeling approaches, empirical and mechanistic modeling. Empirical models relate the factors using a pure mathematical correlation, without any insight into the underlying mechanism. These are easy to develop and are useful in enzyme characterization and substrate preparation. Whereas, mechanistic models are developed from the reaction mechanisms, mass transfer considerations and other physical parameters which affect the extent of hydrolysis. As these models address the underlying dynamics of the process, they can be extensively used in every stage. Mechanistic models vary in their complexity based on the utility they were intended to use for. These models are quite useful in describing the reaction mechanism between ligninocellulosic biomass and enzyme. We need to consider many factors which determine the rate and extent of hydrolysis of biomass for developing mechanistic models. There will be many parameters which bear direct or indirect effects on the degradation of cellulose to fermentable sugars in presence of enzyme, as reported in the literature. Broadly they can be classified as follows (Brown , 2004);

- ***Enzyme Characteristics***: Adsorption of enzyme onto ligninocellulosic biomass prior to reaction; Intermediate and end-product inhibition which is either competitive or noncompetitive; Synergy and thermodynamic considerations of the various enzyme compounds; Mass transfer limitations affecting the transport of

the enzyme to substrate

- ***Substrate Characteristics***: Lignin distribution; Presence of other components such as hemicellulose, proteins and fats; Particle size; Crystallinity; Degree of polymerization

A comprehensive model has to incorporate all these factors; nevertheless there is no constraint on the amount of experimental data and computation power. As discussed earlier the complexity of the mechanistic models varies based on the underlying assumptions made in model development. To quantify the enzymatic hydrolysis using simplistic models, it can be divided into two stages; initial stage where the rate of hydrolysis is almost linear, and later stage where rate continuously decreases and saturates (Brown, 2004). The factors affecting the reaction rates in two stages are distinct in each case;

- ***Initial Stage:*** Product inhibition is not important, least affected by mass transfer resistances, chemical pretreatment plays an important role in initial rates, and pseudo-steady state can be assumed.
- ***Later Stage:*** Rate is higher initially but reduces later due to product inhibition, pseudo-steady state assumptions do not apply as there will be accumulation of intermediates, and substrate characteristics changes (crystallinity, degree of polymerization etc). (Klyosov, 1990; Valjamae et al., 1998; Zhang et al., 1999)

In summary, enzymatic hydrolysis of lignocellulosic biomass depends on many factors; physical properties of substrate (composition, crystallinity, degree of polymerization etc.), enzyme synergy (origin, composition etc.), mass transfer (substrate adsorption, bulk and pore diffusion etc.) and intrinsic kinetics. In past 50 years many

researchers contributed to the understanding of these factors and their effects on rate and extent of cellulose hydrolysis (Zhang and Lynd, 2004). Most of these are competing effects and they can not be distinguished from each other. Accurate estimation of intrinsic kinetics requires pure form of cellulosic biomass to surpass the mass transfer resistances and effects of physical properties of substrate and enzyme complex.

In enzyme catalyzed reaction networks inhibitory effects of the reaction intermediates and products play an important role. These inhibitors bind to enzyme active site and reduce their activity. The substrate may act as the inhibitor in some cases. Hence, the intrinsic reaction kinetics of enzymatic cellulose hydrolysis is also subjected to mediation by a host of factors like inhibitory effects from reaction intermediates and products, enzyme adsorption etc. Furthermore, the influence of each factor is difficult to be quantified in isolation as many factors are interrelated during the hydrolytic reaction. In this work, a three step approach was adapted to investigate the intrinsic cellulase kinetics on hydrolysis of NCC.

- Critical evaluation of Initial Rate Mechanistic Models for Enzymatic Hydrolysis
- Independent inhibition studies with reaction intermediates and products
- Comprehensive intrinsic kinetic studies of cellulase Hydrolysis of NCC

To carry out this sequential study, various enzymatic hydrolysis experiments were performed on NCC, alpha cellulose and avicel.

2. MATERIALS AND METHODS

2.1 Materials:

2.1.1 Enzyme:

Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257), was obtained from NREL and has an average activity of 31.2 FPU/mL. Novozyme 188 β-glucosidase (Novo Inc., Lot no.11K1088) has an activity of 750 CBU/mL. The enzyme solutions were pre-diluted to 1, 3, and 15 FPU/mL by adding appropriate amounts of buffer solutions.

2.1.2 Substrate:

Alpha cellulose, Avicel and NCC were used as substrates in multiple enzymatic hydrolysis experiments to investigate the intrinsic enzymatic kinetics.

- ***Alpha cellulose*:** Alpha cellulose (Sigma Aldrich catalog number: C8002, Lot No. 114K0244) is a pure form of cellulose. It was analyzed for sugars, moisture and ash content according to NREL procedures. Ash content was negligible.
- ***Avicel*:** Avicel PH-101 (Fluka; Cat. No.11365; Lot No. 1094627), is also called microcrystalline cellulose was used as a standard reference substrate. It is prepared from cellulosic fibers (wood pulp) by partial acid hydrolysis and then spray drying of the washed pulp slurry, but it still contains a substantial amount (about 30 to 50%) of amorphous cellulose (Krassig,1993).
- ***NCC*:** Cotton (supplied by Buckeye Tech) and Alpha cellulose which are pure forms of cellulose are used as raw materials for producing Non Crystalline Cellulose (NCC).

The composition of these substrates is given in Table V-1.

TABLE V-1: Composition (% weight) of various substrates used for enzymatic hydrolysis.

Constituents	Avicel	Alpha Cellulose	NCC (Freeze-Dried)
Glucan	97.26	76.58	87.27
Xylan	0.00	21.81	10.51
Galactan	0.00	0	0
Arabinan	0.00	0.74	0
Mannan	0.00	1.05	0.49
Ash	0.00	0	1.91
Total	97.26	100.18	100.18

2.2 Experimental Procedure:

2.2.1 Enzymatic Hydrolysis:

The hydrolysis of cellulose was performed in 250 mL shake flasks with a working volume of 100 mL. 1 g glucan (1% w/v dry basis) was taken as basis for each falsk.0.4 mL of tetracycline (10 mg/mL in 70% ethanol) and 0.3 mL of cyclohexamide (10 mg/mL in distilled water) were added as antibiotics to prevent any type of growth. Sodium citrate buffer (0.05 M) was used to bring the final working volume to 100 mL. The pH of 4.5 can be assumed to be maintained throughout the reaction because of the buffer addition. All the components were assumed to have a density of 1g/mL in the flask. Substrate blanks and Enzyme blanks were run to account for any glucose contribution from the samples and any protein from enzymes.

The flasks were heated for 1 hour at 50^{o}C before the addition of the 1 mL of cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257). The enzyme solutions were diluted to 1,3,15 FPU/mL by adding appropriate amounts of buffer solutions just prior to their addition into the flasks. The experiment was conducted in a New Brunswick Scientific (Edison, NJ) Series 25 incubator shaker at 50°C and 150 rpm. Samples were taken at 0, 0.25, 0.5, 0.75,1, 2, 3, 4, 5, 6, 12, 24, 36, 48, 72, and 96

Hours and boiled for 5 min to kill the enzyme, thus confirming the ceasing of the reaction. Then the samples were centrifuged, and analyzed for glucose, cellobiose and higher cello-oligosaccharides using HPLC.

2.2.2 Analysis:

Then the samples were centrifuged, and analyzed for glucose, cellobiose and higher cello-dextrin's using HPLC. The samples after the carbohydrate analysis and the enzyme hydrolysis were analyzed for sugars using HPLC equipped with RI detector and Bio-Rad's Aminex HPX-87P column maintained at 85^{o}C with DI water as the mobile phase. The cellulose digestibility was calculated by the following equation

Cellulose Digestibility = (Cellulose digested (g)/ Initial Cellulose (g)) x100%

where, the cellulose digested was calculated by the sum of glucose and equivalent glucose from cellobiose and cello oligosaccharides measured by HPLC.

2.3 Critical evaluation of Initial Rate Mechanistic Models for Enzymatic Hydrolysis:

In this work, a comparative study is made among the mechanistic models available in the literature to capture the initial stages of hydrolysis using three different substrates; NCC, Alpha-cellulose and Avicel. Later long range prediction capabilities of these models were explored. Following is the broad class of initial rate mechanistic models tested as described by (Brown, 2004)

a) ***MM kinetics with competitive/noncompetitive inhibition, with/without quasi-steady state approach:*** Early workers (Ghose and Das, 1971; Dwivedi and Ghose,1979) showed that hydrolysis of Solka Floc and hydrolysis of alkali treated bagasse by *Trichoderma reesei cellulase* followed M-M kinetics.

Later one was described by competitive inhibition. In an independent study Howell and Stuck (1975) found that M-M model with noncompetitive inhibition applied to Solka Floc hydrolyzed with *Trichoderma viride* cellulase. Gan *et al.* (2002) adopted quasi-steady state approach and yet arrived to a mechanistic model similar to M-M kinetics.

$$\frac{dS}{dt} = \frac{-kSE}{K_m + S} \qquad \ldots (1)$$

Where, S is the concentration of the substrate, E is the enzyme concentration, K_m is saturation constant and k is the rate constant.

b) ***Shrinking site hydrolysis model with langmuir-type adsorption isotherm:*** Considering the changing characteristics of the cellulose structure, Humphrey (1979) used the shrinking-site hydrolysis model with a Langmuir-type adsorption isotherm. Recently Movagharnejad *et al.* (2003) have extended the shrinking-site model to rice pollards, sawdust, wood particles, and used paper.

$$\frac{dS}{dt} = \frac{-kS^{4/3}E}{\alpha + E} \qquad \ldots (2)$$

c) ***Two phases of cellulose: amorphous and crystalline:*** Wald *et al.* (1984) proposed a two-parameter model that considered the two phases of cellulose (amorphous and crystalline) including an adsorption step. Rice straw substrate was modeled as a shrinking sphere with the amorphous region surrounding the crystalline core. The model has a similar mathematical form as M-M, except an enzyme term appears in the denominator, rather than a substrate term.

$$\frac{dS}{dt} = \frac{-kSE}{\alpha + E} \qquad \ldots (3)$$

Here, α is the desorption constant for the enzyme and substrate surface.

d) ***Hydrolysis of both cellulose and cellobiose:*** Fan and Lee (1983) developed a mechanistic model that describes the hydrolysis of cellulose and cellobiose, but did not include an adsorption step.

$$\frac{dS}{dt} = -k - \frac{\mathrm{K}SE}{\alpha + S} \qquad \ldots (4)$$

e) ***MM kinetics with competitive inhibition and Langmuir adsorption:*** Huang (1975) modeled the hydrolysis of cellulose by *T. viride* cellulase using the M-M mechanism with competitive inhibition, but he also included a Langmuir adsorption step.

$$\frac{dS}{dt} = \frac{-kSE}{\alpha + S + \varepsilon E} \qquad \ldots (5)$$

f) ***MM kinetics with Noncompetitive inhibition and Langmuir adsorption:*** Holtzapple *et al*. (1984) proposed the HCH-1 model, which is essentially the MM mechanism with an adsorption step and noncompetitive inhibition. Brown and Holtzapple *et al.* (1990) developed the pseudo-steady state approximation for the HCH- 1 model.

$$\frac{dS}{dt} = \frac{-kSE}{\alpha + \phi S + \varepsilon E}$$

$$where,\ \phi = \frac{\left(S - E - \alpha\right) + \sqrt{\left(\alpha + E - S\right)^2 + 4\alpha S}}{2S} \qquad \ldots (6)$$

These six categories of the models cover the gamut of mechanistic models to model cellulose hydrolysis. In some cases, the constants are interpreted differently. In other cases, the models are applied multiple times to each enzyme and substrate component

(Brown, 2004). A critical evaluation of these models was done to test their efficacy of explaining long range kinetics on the hydrolysis of NCC, avicel and alpha-cellulose. The data was fit to these available models so they can be compared on an equal basis.

2.4 Independent inhibition studies with reaction intermediates and products:

Enzymatic hydrolysis of lignocellulosic biomass depends on many factors; physical properties of substrate (composition, crystallinity, degree of polymerization etc.), enzyme synergy (origin, composition etc.), mass transfer (substrate adsorption, bulk and pore diffusion etc.) and intrinsic kinetics. In past 50 years many researchers contributed to the understanding of these factors and their effects on rate and extent of cellulose hydrolysis (Zhang and Lynd, 2004). Most of these are competing effects and they can not be distinguished from each other. Accurate estimation of intrinsic kinetics requires pure form of cellulosic biomass to surpass the mass transfer resistances and effects of physical properties of substrate and enzyme complex. For this purpose, enzymatic hydrolysis is performed on Non-crystalline cellulose produced in our laboratory.

In this part of the study, inhibitory effects of soluble cello-oligosaccharides, cellobiose and glucose on enzymatic hydrolysis of NCC are considered. These reaction intermediates and products were externally added to the substrate (NCC) initially. Later the cellulose concentration profiles were studied after introducing the enzyme complex to the substrate solution. Table V-2 shows the list of enzymatic hydrolysis experiments performed.

TABLE V-2: Experimental planning for carrying out the independent inhibition studies of hydrolysis intermediates (COS and Cellobiose) and product (Glucose).

	Experiments	
Study Performed	**With 1 FPU/g-Glucan enzyme**	**With 3 FPU/g-Glucan enzyme**
	Pure NCC substrate	Pure NCC substrate
COS inhibition	(NCC + 5% COS) substrate	(NCC + 5% COS) substrate
	(NCC + 10% COS) substrate	(NCC + 10% COS) substrate
	Pure NCC substrate	Pure NCC substrate
Cellobiose inhibition	(NCC + 5% Cellobiose) substrate	(NCC + 5% Cellobiose) substrate
	(NCC + 10% Cellobiose) substrate	(NCC + 10% Cellobiose) substrate
	Pure NCC substrate	Pure NCC substrate
Glucose inhibition	(NCC + 5% Glucose) substrate	(NCC + 5% Glucose) substrate
	(NCC + 10% Glucose) substrate	(NCC + 10% Glucose) substrate

The series of experiments were designed in such a way that, the initial concentration of one of the components (cello-oligosaccharides, cellobiose or glucose) supplemented with NCC was varied on keeping others constant. Idea behind this strategy was that, the resultant variations in the hydrolysis rates can be solely attributed to the constituent whose composition is varied.

2.5 Comprehensive intrinsic kinetic studies of cellulase Hydrolysis of NCC:

The focus of this study was not to propose altogether a new phenomenological reaction mechanism, but to qualitatively and quantitatively analyze the underlying steps in enzymatic hydrolysis and to come up with a well rounded understanding of controlling factors of intrinsic kinetics. The analysis presented in this work highlights the dynamically changing reaction rates, inhibitory effects of reaction intermediates and products (cello-oligosaccharides, cellobiose and glucose), variability in available active enzyme. The saturating kinetics in a finite batch time was also considered. Overall

simplified mechanism of the hydrolysis of cellulose (NCC) can be given by the schematic shown in Figure V-1. Cellulose is break down to smaller chain length cello-oligosaccharides (insoluble: DP>15, soluble: DP<15) by the action of Endo-glucanases. Further breakdown of insoluble cello-dextrins to glucose-dimer (cellobiose) is catalyzed by Exo-glucanases. β-glucosidases act on both soluble oligosaccharides and cellobiose and converts them to fermentable sugar (glucose). (Beldman et al., 1985; Henriksson, 1997; Valijamae et al., 1998)

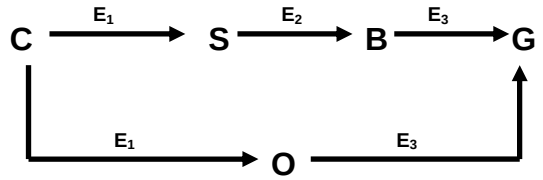

FIGURE V-1: Schematic showing the simplified mechanism of the enzymatic hydrolysis of NCC. C: Non Crystalline Cellulose, S: Insoluble Cello-dextrins, O: Soluble Cello-oligosaccharides, B: cellobiose, G: glucose, E_1: Endo-glucanases, E_2: Exo-glucanases, E_3: β-glucosidases.

The following assumptions were made to simplify the mechanism and derive the pertinent mathematical model:

- The cellulase system (E) of Endo-glucanases (E_1), Exo-glucanases (E_2) and β-glucosidase (E_3) is considered as having a constant composition for the given complex. They may be independently inhibited by the reaction intermediates and products in distinct manner.
- The reducing sugars inhibit the enzyme in a reversible and competitive/non-competitive manner (Gusakov and Sinitsyn, 1992; Holtzapple et al., 1990).
- Cellulase adsorption to the substrate surface is reversible and is governed by simple Langmuir type adsorption isotherm (Huang, 1975; Lee and Fan, 1982;

Mandels et al., 1971; Moloney and Coughlan, 1983).

- Cellulose and insoluble cello-oligosaccharides possess similar inhibitory effects on enzymes and also their hydrolysis kinetics are assumed to be similar.
- Resistances offered by the crystallinity and varying composition with respect to the degree of hydrolysis were neglected as these studies were carried out on non-crystalline cellulose.
- As the time scale of hydrolysis is much larger than the time scale of bulk diffusion of enzyme, mass transfer resistances were considered to be negligible (Fan et al., 1981; Fan and Lee, 1983; Lynd et al., 2002).

Along with the simplifying assumptions that were stated earlier, it was also assumed from the intuition that the enzymes catalyzing each reaction step are inhibited by different reaction intermediates and products differently as they are distinct in their action and behavior, even though they were considered to be a single complex quantitatively. The following were the detailed inhibitory mechanisms deduced:

- Enzymes E_1 and E_2 were subjected to non-competitive inhibition by soluble cello-oligosaccharides, cellobiose and glucose. It was observed that as the glucose concentration increases, the inhibition rate of E_1 and E_2 increases exponentially. From the numerical experiments it was found that the probability of glucose (inhibitor) binding to the enzyme is three times higher than the probability of substrate binding.
- Enzymes E_3 were solely inhibited by glucose in competitive inhibition.

At this juncture the mechanism can be re-drawn as shown in Figure V-2. As the properties of insoluble cello-oligosaccharides and cellulose are assumed to be the same,

conversion of cellulose to cellobiose was lumped into a single step.

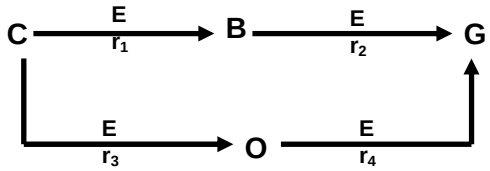

FIGURE V-2: Schematic of NCC hydrolysis mechanism after further simplification

From these insights, rate of each reaction can be written as follows:

$$r_1 = \frac{k_1 CE}{\left(K_c + C\right)\left(1 + \frac{B}{K_{BI}} + \frac{O}{K_{OI}} + \frac{G^3}{K_{GIn}^3}\right)\left(E + K_E\right)}$$

$$r_2 = \frac{k_2 BE}{\left(K_B\left(1 + \frac{G}{K_{GI}}\right) + B\right)\left(E + K_E\right)} \qquad \ldots (7)$$

$$r_3 = \frac{k_3 CE}{\left(K_c + C\right)\left(1 + \frac{B}{K_{BI}} + \frac{O}{K_{OI}} + \frac{G^3}{K_{GIn}^3}\right)\left(E + K_E\right)}$$

$$r_4 = \frac{k_4 OE}{\left(K_O\left(1 + \frac{G}{K_{GI}}\right) + O\right)\left(E + K_E\right)}$$

In these rate equations,

$k_i\ (i = 1,2,3,4)$ are the primary rate constants,

$K_C, K_B, and\ K_O$ are cellulose saturation constant, cellobiose saturation constant and soluble cello-oligosaccharides saturation constant respectively,

$K_{BI}, K_{OI}, and\ K_{GI}$ are inhibition constant of cellobiose, soluble cello-oligosaccharides and glucose respectively for cellulases,

K_E is the desorption equilibrium constant for cellulases onto the NCC surface

$C, O, B, and\ G$ are concentrations of NCC, soluble cello-oligosaccharides, cellobiose and glucose respectively.

Further, the accumulation rates of cellulose, soluble cello-oligosaccharides, cellobiose and glucose were written as,

$$\frac{dC}{dt} = -r_1 - r_3;\ \ \frac{dO}{dt} = r_3 - r_4;\ \ \frac{dB}{dt} = r_1 - r_2;\ \ and\ \ \frac{dG}{dt} = r_2 + r_4 \qquad \ldots (8)$$

Model parameters were estimated using a nonlinear trajectory optimization as explained in the following section.

2.6 Model Parameter Estimation using Nonlinear Trajectory Optimization:

The models represented by equations (1 to 8) can be represented as

$$\frac{dx}{dt} = f(x,\theta) \qquad \ldots (9)$$

Here, x is the concentration vector which encompasses cellulose, soluble cello-oligosaccharides, cellobiose and glucose; θ represents the vector of model parameters. Integrating this differential equation(s) yield time course data of concentrations. Hence, the predicted concentration vector of the enzymatic hydrolysis reaction components can be represented as,

$$\hat{x}(t) = \int_0^t f(x,\theta) \qquad \ldots (10)$$

where, $\hat{x}(t)$ is the predicted concentration vector of cellulose, soluble cello-oligosaccharides, cellobiose and glucose. The objective function which is to be minimized to solve for the model parameters is given as,

$$\underset{\theta}{Min} \sum_{t=0}^{t_{batch}} \left[\hat{x}(t) - x(t)\right]^2 \qquad \ldots (11)$$

Subjected to,

$$\theta^L \leq \theta \leq \theta^H$$

In this work, integration of differential equations representing the model equations is performed using ***ode45*** routine in MATLAB®. On the outer frame work, the nonlinear constrained optimization is performed using ***fmincon*** routine. After this algorithm was converged for each data set, optimal set of parameters which yield the predictions closer to the experimental values were obtained.

3. RESULTS AND DISCUSSIONS

Enzymatic Hydrolysis data of NCC, Alpha cellulose and Avicel were shown in Figure V-3. This data was used to estimate the model parameters of the initial rate kinetic models. On performing trajectory optimization using nonlinear constrained optimization on the hydrolysis data of different substrates at three enzyme loadings, model parameters were obtained, which were tabulated in Table V-3. Except MM model all the other five models represented the initial hydrolysis rates efficiently, for all three substrates for three different enzyme loadings, which is evident from Figures V- 4, V- 5 and V-6. MM kinetics was giving acceptable performance with high enzyme loading.

Even though these models can explain the initial progression of the enzymatic hydrolysis, they failed to predict the later stages of the hydrolysis. This was shown in the Figure V-7. Initial rate mechanistic models assume 100% hydrolysis as they do not consider the decelerating reaction rate due to the increasing enzymatic inhibition with the increase in hydrolysis time. Another cause for failure may be creeping up due to ignoring the effects of hydrolysis intermediates like cello-oligosaccharides and cellobiose. Hence, there was an explicit need of accommodating the increasing inhibition rates with batch

time, while modeling the kinetics of enzymatic hydrolysis.

TABLE V-3: Model parameters of different initial rate mechanistic models for different substrates.

	Model Parameter	NCC	Alpha Cellulose	Avicel
Model – a	*k (g/(g.hr))*	0.1226	0.0136	0.0135
	K_m *(g/L)*	39.8700	43.1200	43.867
Model – b	$K\ (L/g)^{(1/3)}.hr^{-1}$	0.0917	0.0356	0.0343
	α *(g/L)*	10.5465	39.7600	41.7800
Model – c	*k (g/L)*	0.1751	0.0760	0.0734
	α *(g/(g.hr))*	8.9627	39.6700	38.89
Model – d	*k (g/(L.hr))*	0.0001	0.0948	0.0607
	K (g/(g.hr))	0.1751	0.0778	0.0839
	α *(g/L)*	8.9632	83.1290	90.0000
Model – e	*K (g/(g.hr))*	0.9599	0.0786	0.0636
	α *(g/L)*	39.765	41.2500	40.898
	ε *(g/g)*	5.5029	0.9531	0.7307
Model – f	*K (g/(g.hr))*	0.0599	0.0726	0.0584
	α *(g/L)*	1.8915	37.9700	41.7800
	ε *(g/g)*	0.3500	0.8986	0.6861

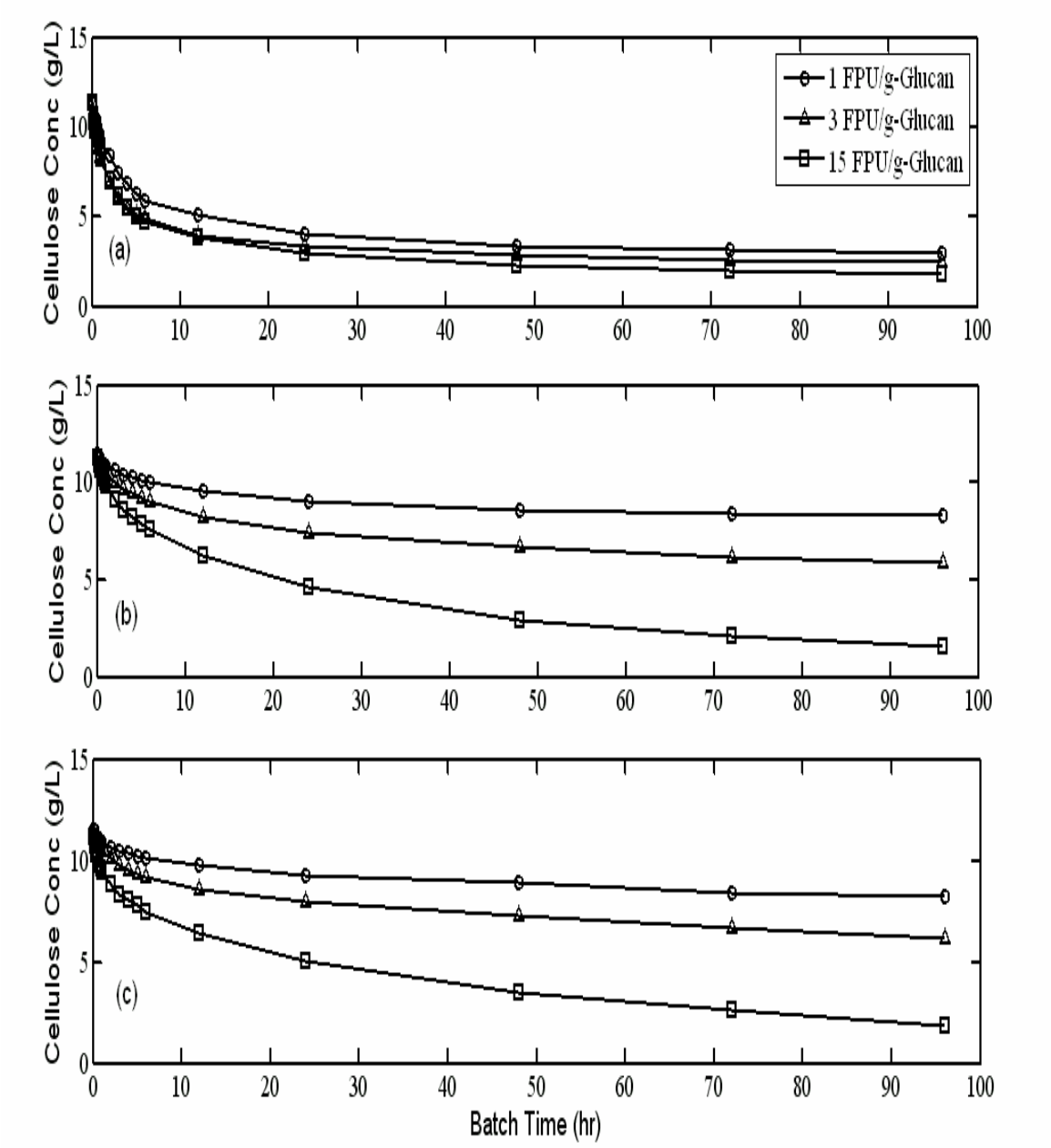

FIGURE V-3: Experimental hydrolysis data of various cellulosic substrates with three different enzyme loadings (1, 3 and 15 FPU/g-Glucan); (a) NCC, (b) Alpha Cellulose, and (c) Avicel.

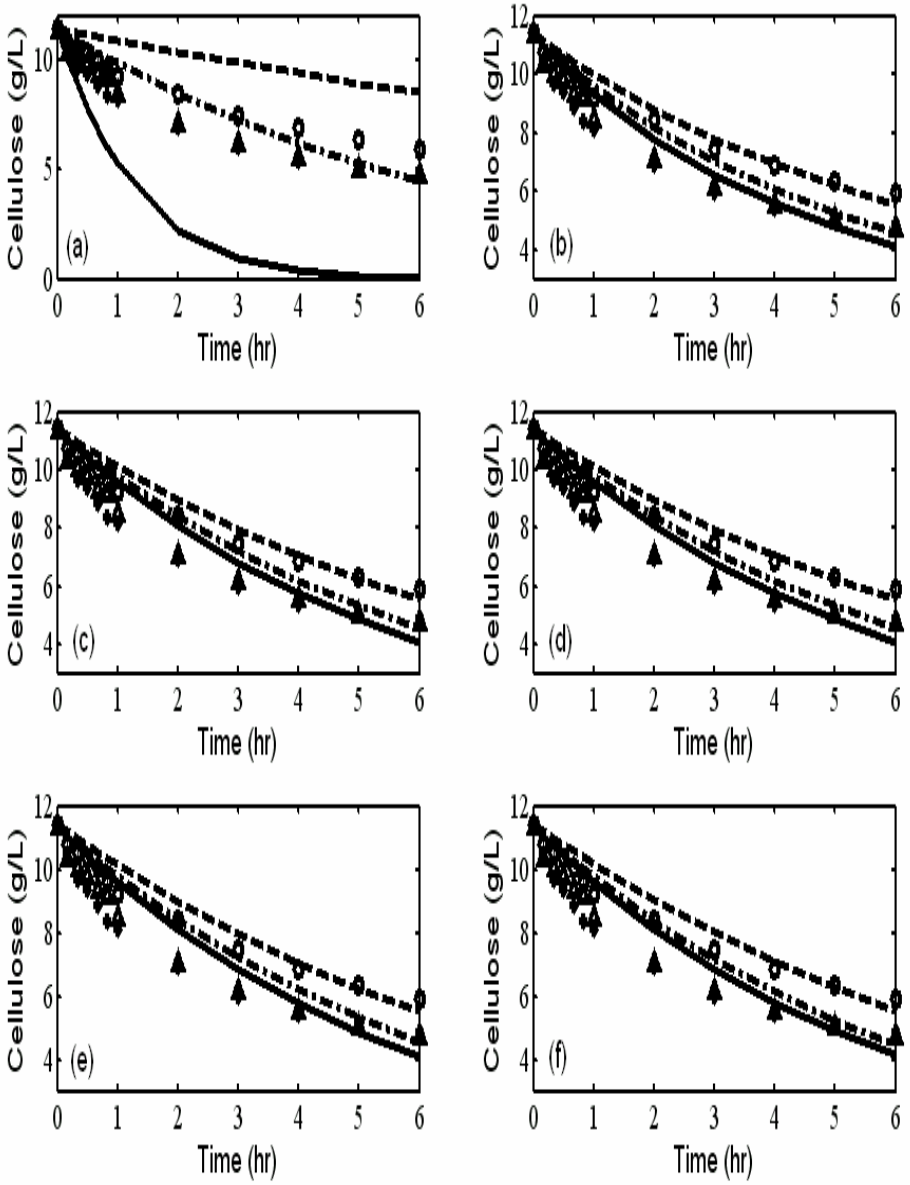

FIGURE V-4: Comparison of experimental data for the initial stage of hydrolysis of NCC with model (a-f) predictions at three enzyme loadings (1, 3 and 15 FPU/g-Glucan). Experimental data is shown by markers as discrete points: (o) with 1 FPU enzyme loading, (+) with 3 FPU enzyme loading, (Δ) with 15 FPU enzyme loading. Model predictions are plotted by lines: (---) with 1 FPU enzyme loading, (-.-.) with 3 FPU enzyme loading, (___) with 15 FPU enzyme loading.

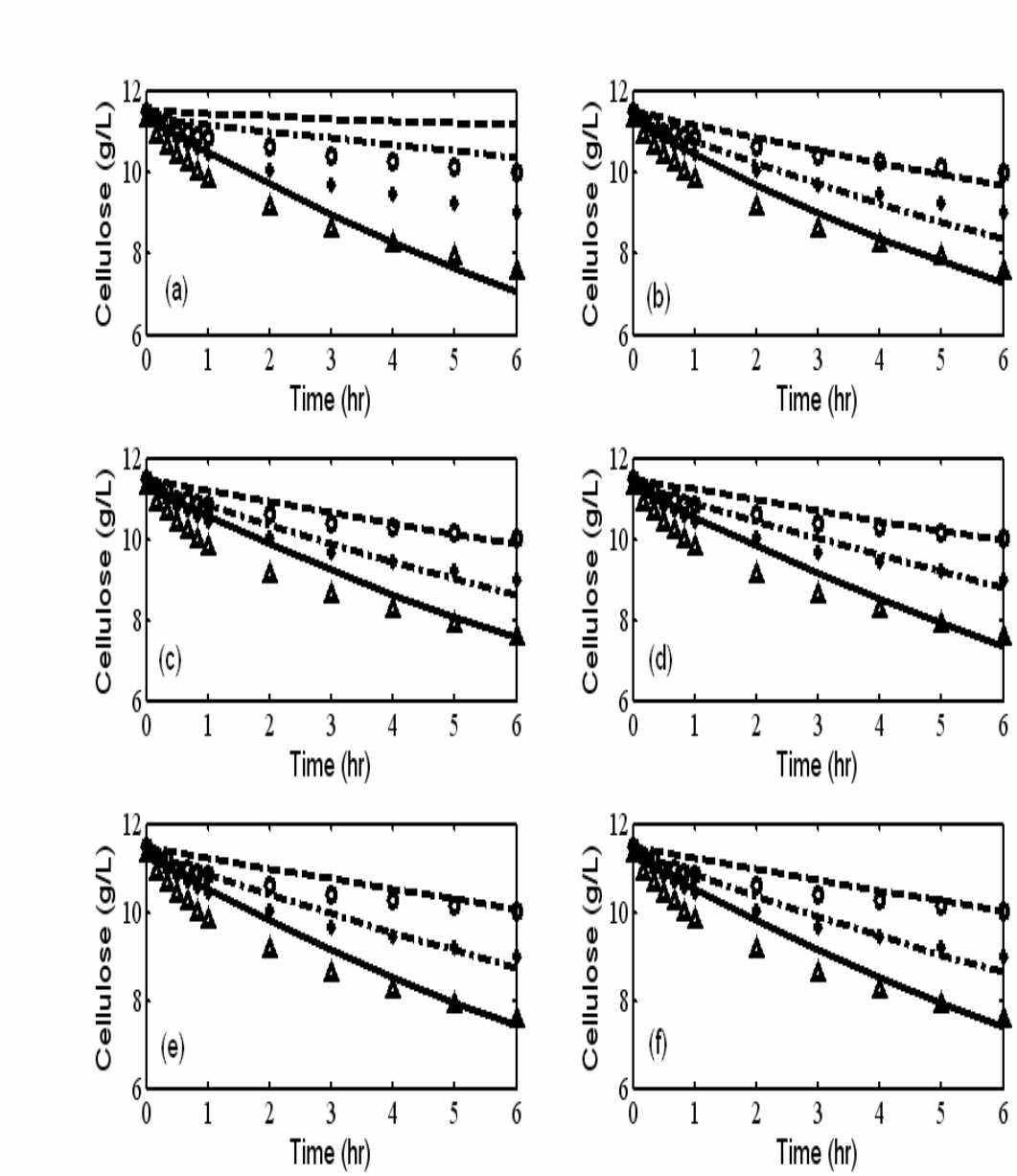

FIGURE V-5: Comparison of experimental data for the initial stage of hydrolysis of Alpha Cellulose with model (a-f) predictions at three enzyme loadings (1, 3 and 15 FPU/g-Glucan). Experimental data is shown by markers as discrete points: (o) with 1 FPU enzyme loading, (+) with 3 FPU enzyme loading, (Δ) with 15 FPU enzyme loading. Model predictions are plotted by lines: (---) with 1 FPU enzyme loading, (-.-.) with 3 FPU enzyme loading, (___) with 15 FPU enzyme loading.

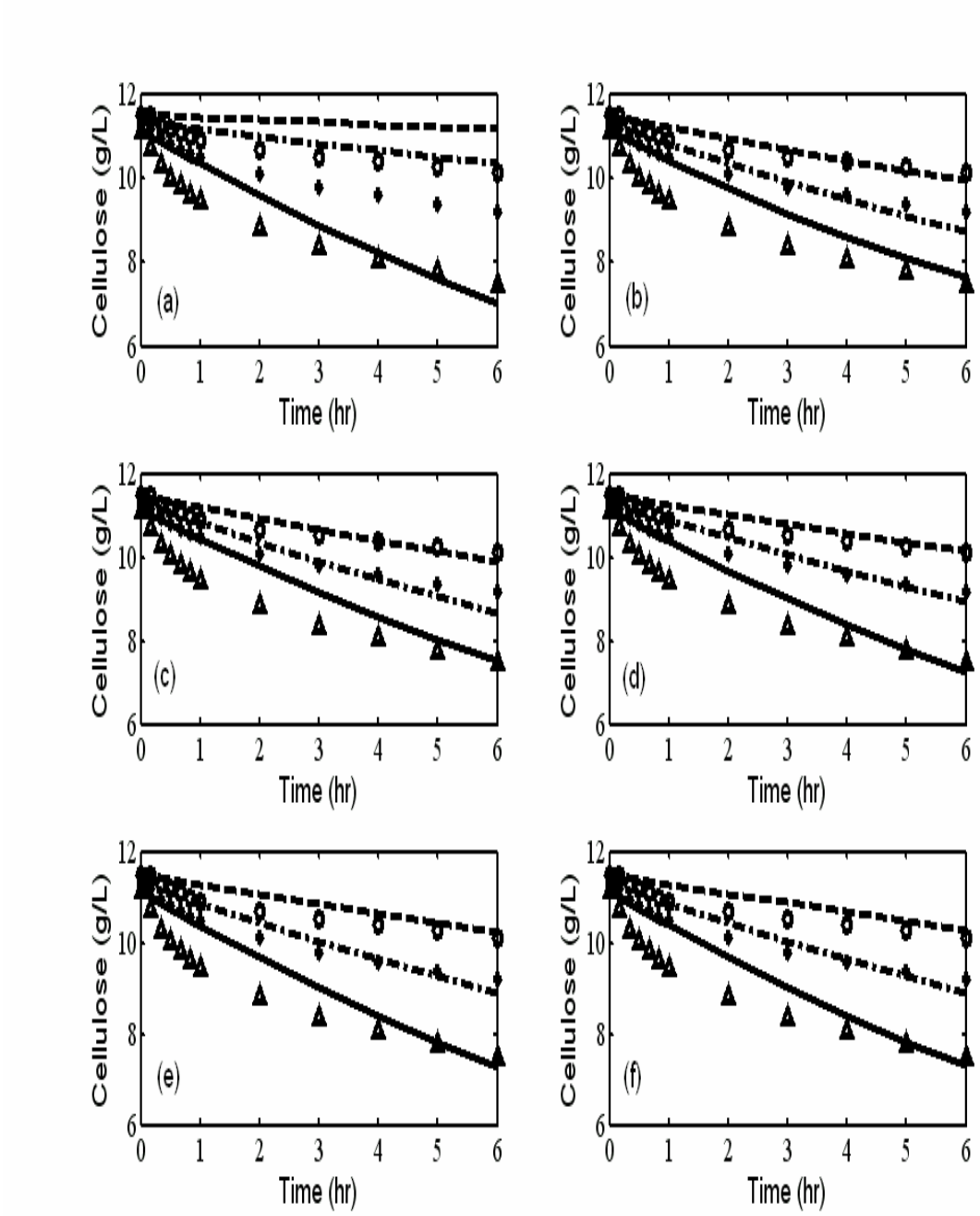

FIGURE V-6: Comparison of experimental data for the initial stage of hydrolysis of Avicel with model (a-f) predictions at three enzyme loadings (1, 3 and 15 FPU/g-Glucan). Experimental data is shown by markers as discrete points: (o) with 1 FPU enzyme loading, (+) with 3 FPU enzyme loading, (Δ) with 15 FPU enzyme loading. Model predictions are plotted by lines: (---) with 1 FPU enzyme loading, (-.-.) with 3 FPU enzyme loading, (___) with 15 FPU enzyme loading.

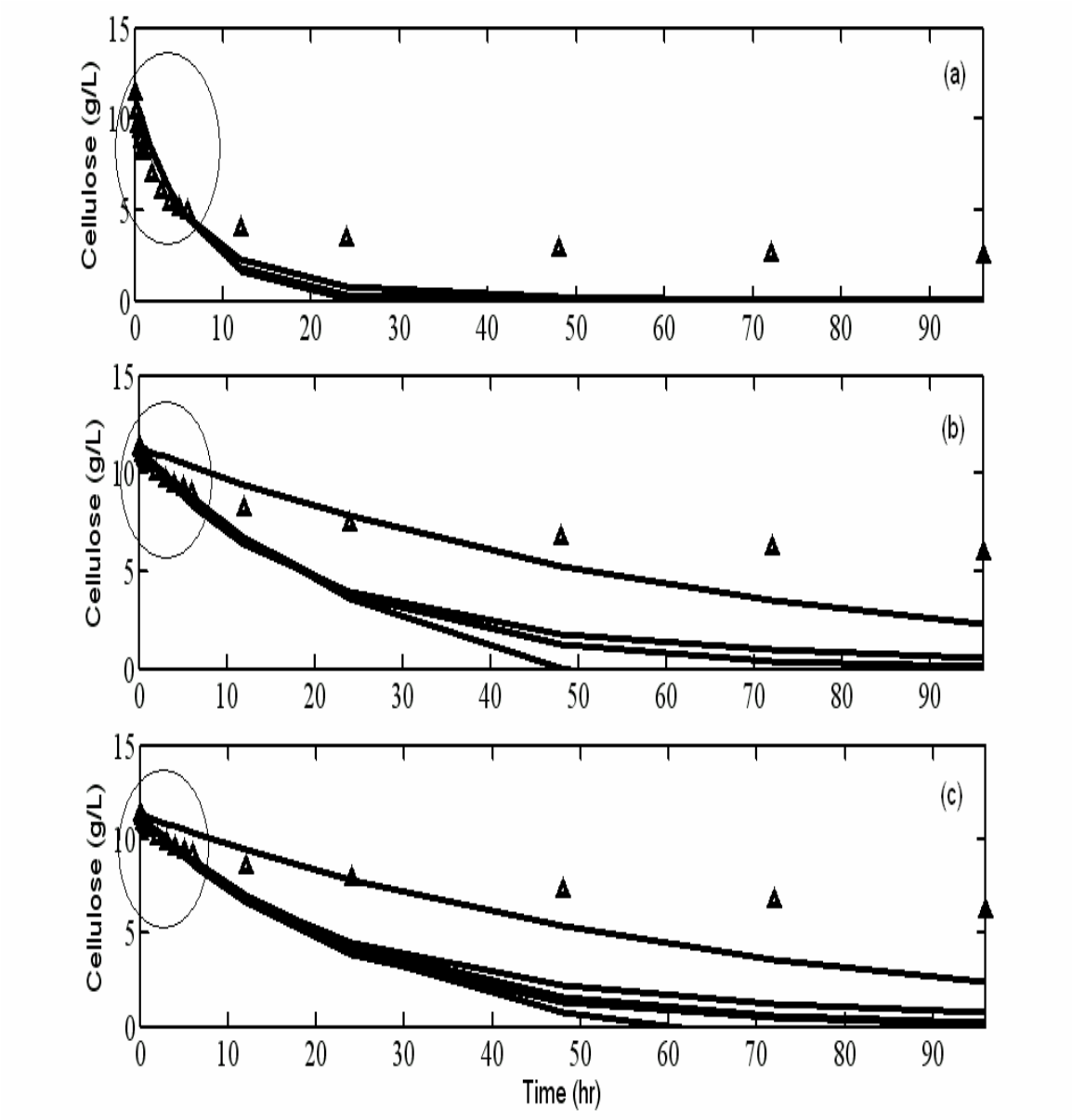

FIGURE V-7: Performance of mechanistic models under study for long range enzymatic hydrolysis of different substrates; (a) NCC, (b) alpha cellulose, and (c) Avicel. Experimental data is represented by triangles and various model predictions are represented by continuous lines. Circled portion of the plots is the initial stage of the hydrolysis (0 to 6hr).

To study the individual contribution from cello-oligosaccharides, cellobiose and glucose on the inhibition rates of cellulase, a fixed set of enzymatic hydrolysis experiments were performed on NCC supplemented with cello-oligosaccharides, cellobiose or glucose. Figure V-8 shows the Cellulose hydrolysis in three different cases, with pure NCC, with an addition of 5% Cello-oligosaccharides and with 10% Cello-oligosaccharides. These three hydrolysis experiments were performed at both 1FPU and 3 FPU/g-Glucan enzymes loading. It is evident from the Figure V-8 that at higher initial oligomer concentrations, the initial reaction hydrolysis rate lasted for relatively smaller time and also resulted in reduced extent of hydrolysis. It can be inferred from these observations that oligomers strongly inhibit the hydrolysis rates.

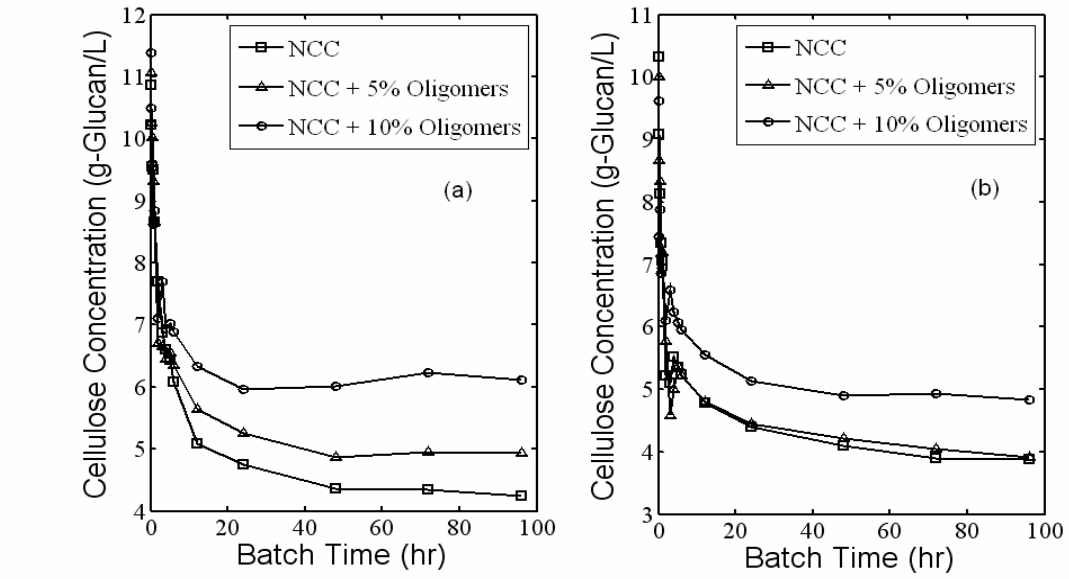

FIGURE V-8: Enzymatic hydrolysis of NCC substrate with initial addition of Cello-oligosaccharides in different proportions a) enzyme loading of 1 FPU/g-Glucan b) enzyme loading of 3 FPU/g-Glucan.

Figure V-9 and V-10 were plotted for different initial compositions of substrate (NCC) with Cellobiose and Glucose respectively with two enzyme loadings (1 FPU and 3 FPU/g-Glucan) in each case.

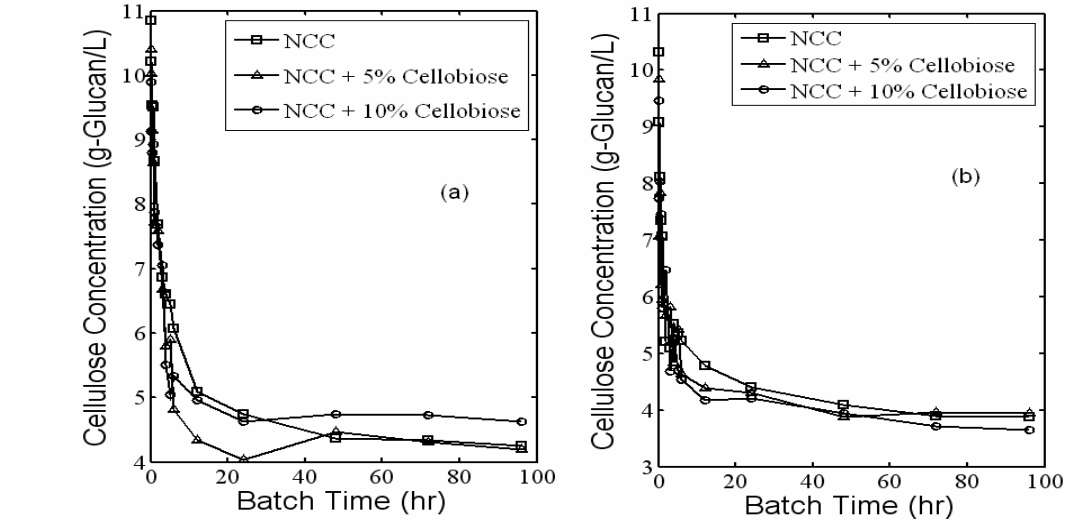

FIGURE V-9: Enzymatic hydrolysis of NCC substrate with initial addition of Cellobiose in different proportions a) enzyme loading of 1 FPU/g-Glucan b) enzyme loading of 3 FPU/g-Glucan.

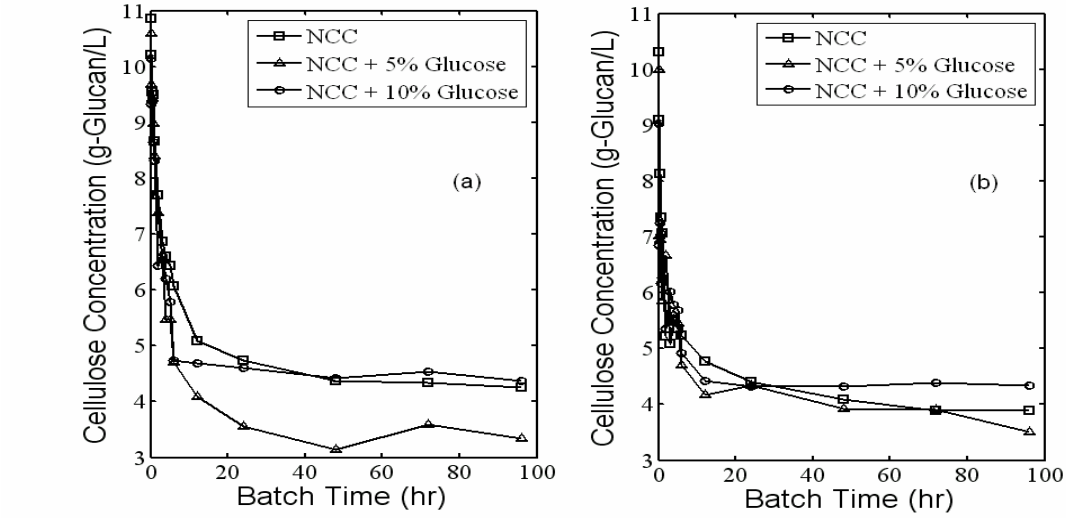

FIGURE V-10: Enzymatic hydrolysis of NCC substrate with initial addition of Glucose in different proportions a) enzyme loading of 1 FPU/g-Glucan b) enzyme loading of 3 FPU/g-Glucan.

In both the cases, the effect of external additions was indistinct as the rate and extent of hydrolysis with increase in amount of addition (either Cellobiose or Glucose) found to follow a random order. At 5% of addition, the higher initial rate prolonged for longer time and extent of hydrolysis was also higher. Conversely, further increase in external Cellobiose / Glucose addition to 10% resulted in reduction in the final hydrolysis extent. This may be resulted due to the following reasons;

- Along with cellulose decomposition, these additions might be triggering some intermediate steps in the cellulose hydrolysis to form glucose
- Due the accumulation of cello-oligomers, cellobiose and glucose the effect of initial addition of either cellobiose or glucose was not distinct.
- Initial high concentrations of glucose / cellobiose may inhibit the cellulose flux towards soluble cello-oligomers and hence may reduce the inhibition on cellulases, resulting in higher extent of hydrolysis.

This brief study on external additions of cello-oligosaccharides, cellobiose and glucose exemplified the need to come up with a comprehensive method to investigate the inhibitory effects of all these compounds together as these interactions are inter-dependant in nature.

As shown above, the reaction kinetics of enzymatic cellulose hydrolysis is subjected to mediation by a host of factors. Furthermore, the influence of each factor is difficult to be quantified in isolation as many factors are interrelated during the hydrolytic reaction. As discussed in the section 2.5, in synthesizing a mathematical representation of the hydrolytic reaction kinetics, a strategy was adopted to incorporate vital information with respect to the reaction mechanism, but without unnecessary over-complication by

attempting to incorporate all the interwoven events in the complex heterogeneous reaction. Initially, the parameters for the proposed model were identified on the data obtained from cellulase hydrolysis of pure NCC at two enzyme loadings (1 FPU and 3 FPU) using nonlinear constrained trajectory optimization. These model parameters are listed in Table V-4. Later the proposed methodology was validated against different sets of experimental data. The experimental design is presented in Table V-5.

TABLE V-4: Parameter values of proposed comprehensive kinetic model for cellulase hydrolysis of NCC.

S.No.	Parameter	Description	Numerical Value
1	k_1 *(g/g.min)*	Rate constant	38.29625
2	k_2 *(g/g.min)*	Rate constant	32.92130
3	k_3 *(g/g.min)*	Rate constant	20.62100
4	k_4 *(g/g.min)*	Rate constant	14.83944
5	K_C *(g/L)*	Saturation constant for NCC	9.348311
6	K_B *(g/L)*	Saturation constant for Cellobiose	13.400910
7	K_O *(g/L)*	Saturation constant for COS	14.277510
8	K_{OI} *(g/L)*	Inhibition constant for COS	8.686783
9	K_{BI} *(g/L)*	Inhibition constant for Cellobiose	5.200752
10	K_{GI} *(g/L)*	Inhibition (competitive) constant for Glucose	0.080118
11	K_E *(g/L)*	Enzyme desorption constant	0.038113
12	K_{GIn} *(g/L)*	Inhibition (non-competitive) constant for Glucose	0.431098

TABLE V-5: Experimental planning for carrying out the comprehensive intrinsic kinetic studies of cellulase hydrolysis of NCC.

	Experiments	
Set	**With 1 FPU/g-Glucan enzyme**	**With 3 FPU/g-Glucan enzyme**
1	Pure NCC substrate	Pure NCC substrate
2	(NCC + 5% COS) substrate	(NCC + 5% COS) substrate
3	(NCC + 10% COS) substrate	(NCC + 10% COS) substrate
4	(NCC + 5% Cellobiose) substrate	(NCC + 5% Cellobiose) substrate
5	(NCC + 5% Glucose) substrate	(NCC + 5% Glucose) substrate

From Figures V-11 to V-15, it is evident that in any case proposed methodology predicted the concentration profiles of cellulose, cello-oligosaccharides, cellobiose and glucose which were in close agreement with experimental data. Hence, we believe that this model can comfortably explain the entire enzymatic hydrolysis batch with high precision.

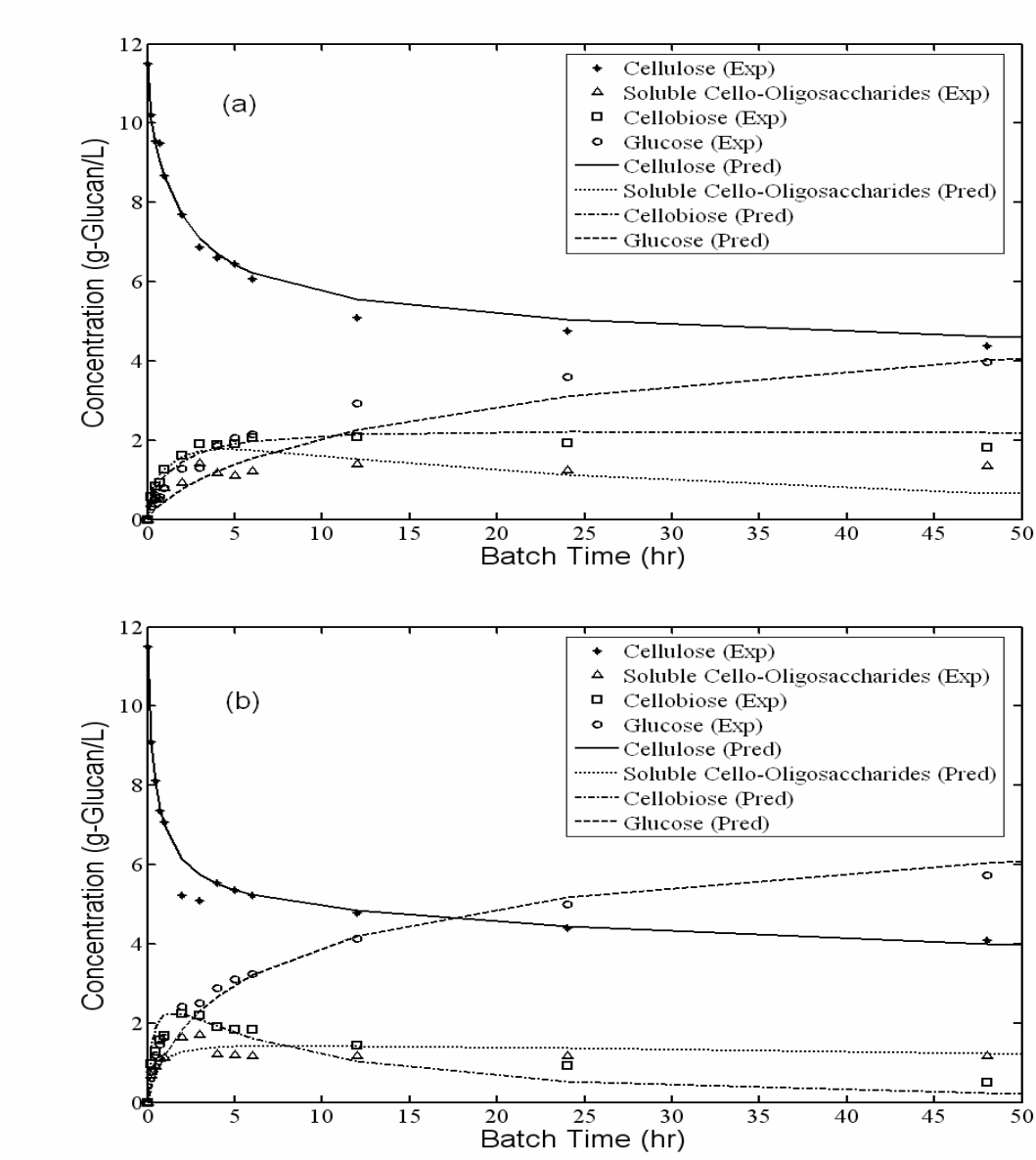

FIGURE V-11: Experimental and predicted concentration profiles of cellulose, soluble cello-oligosaccharides, cellobiose and glucose in the enzymatic hydrolysis of pure non-crystalline cellulose a) with 1 FPU/g-Glucan enzyme loading, b) with 3 FPU/g-Glucan enzyme loading.

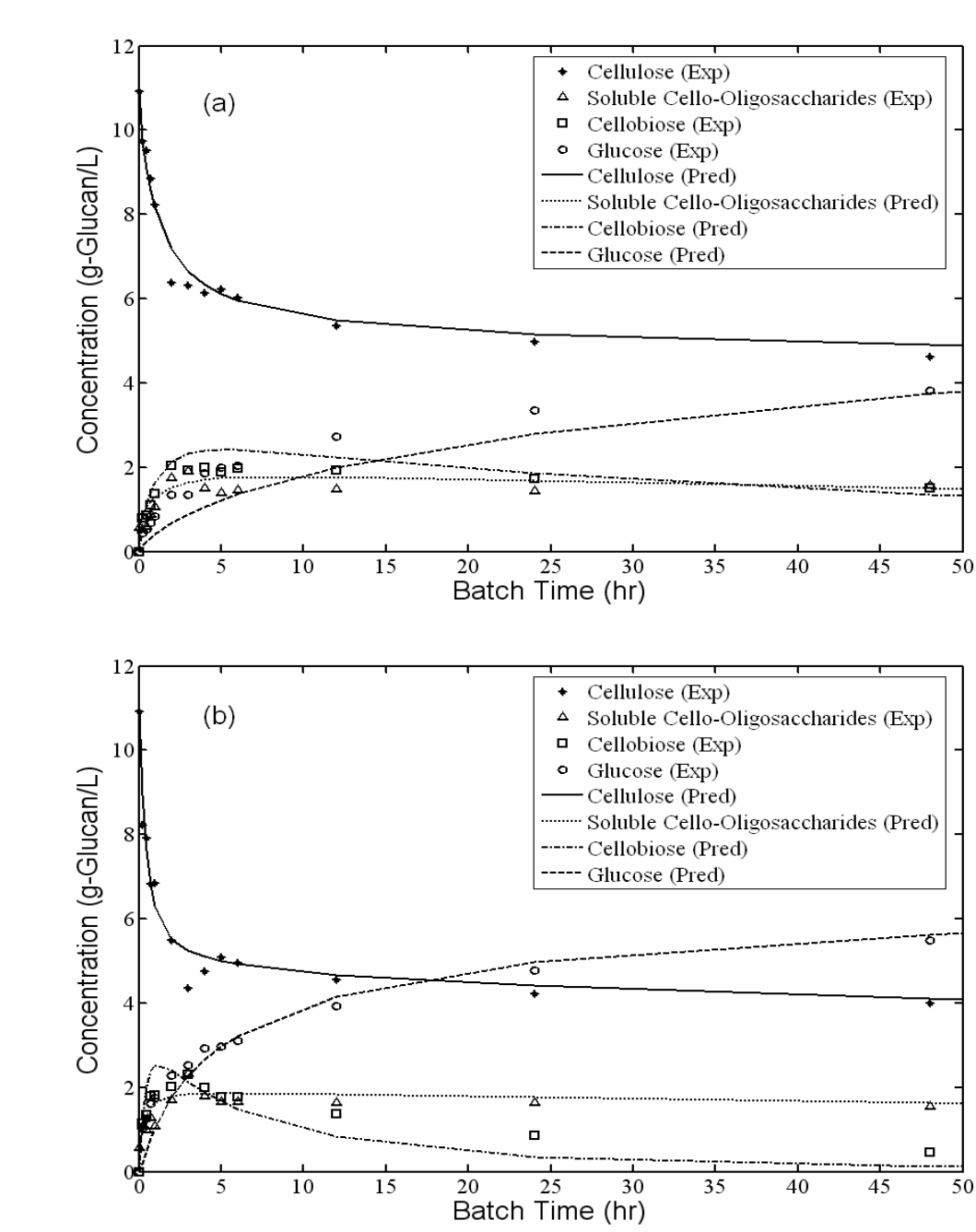

FIGURE V-12: Experimental and predicted concentration profiles of cellulose, soluble cello-oligosaccharides, cellobiose and glucose in the enzymatic hydrolysis of non-crystalline cellulose with 5% cello-oligosaccharides a) with 1 FPU/g-Glucan enzyme loading, b) with 3 FPU/g-Glucan enzyme loading.

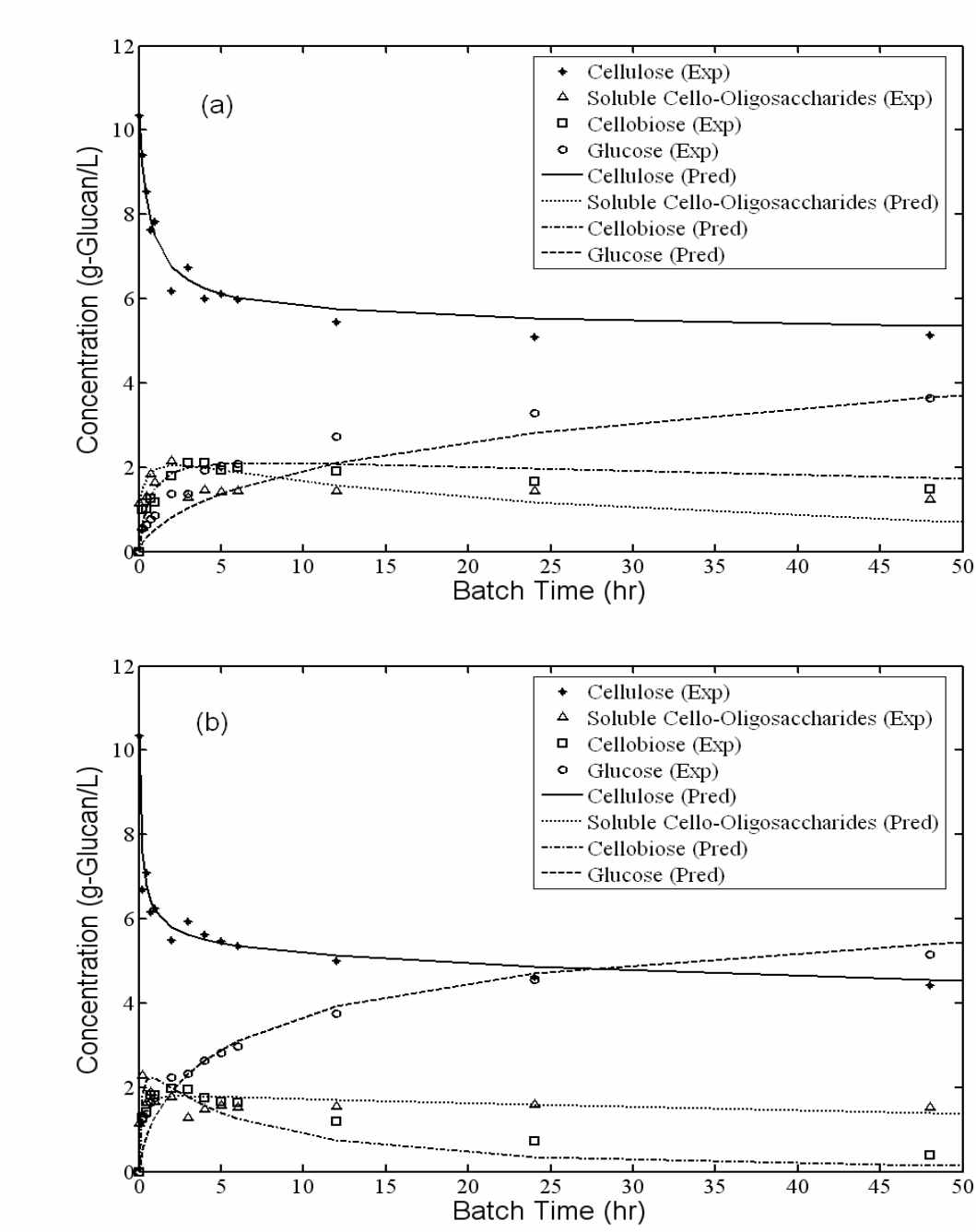

FIGURE V-13: Experimental and predicted concentration profiles of cellulose, soluble cello-oligosaccharides, cellobiose and glucose in the enzymatic hydrolysis of non-crystalline cellulose with 10% cello-oligosaccharides a) with 1 FPU/g-Glucan enzyme loading, b) with 3 FPU/g-Glucan enzyme loading.

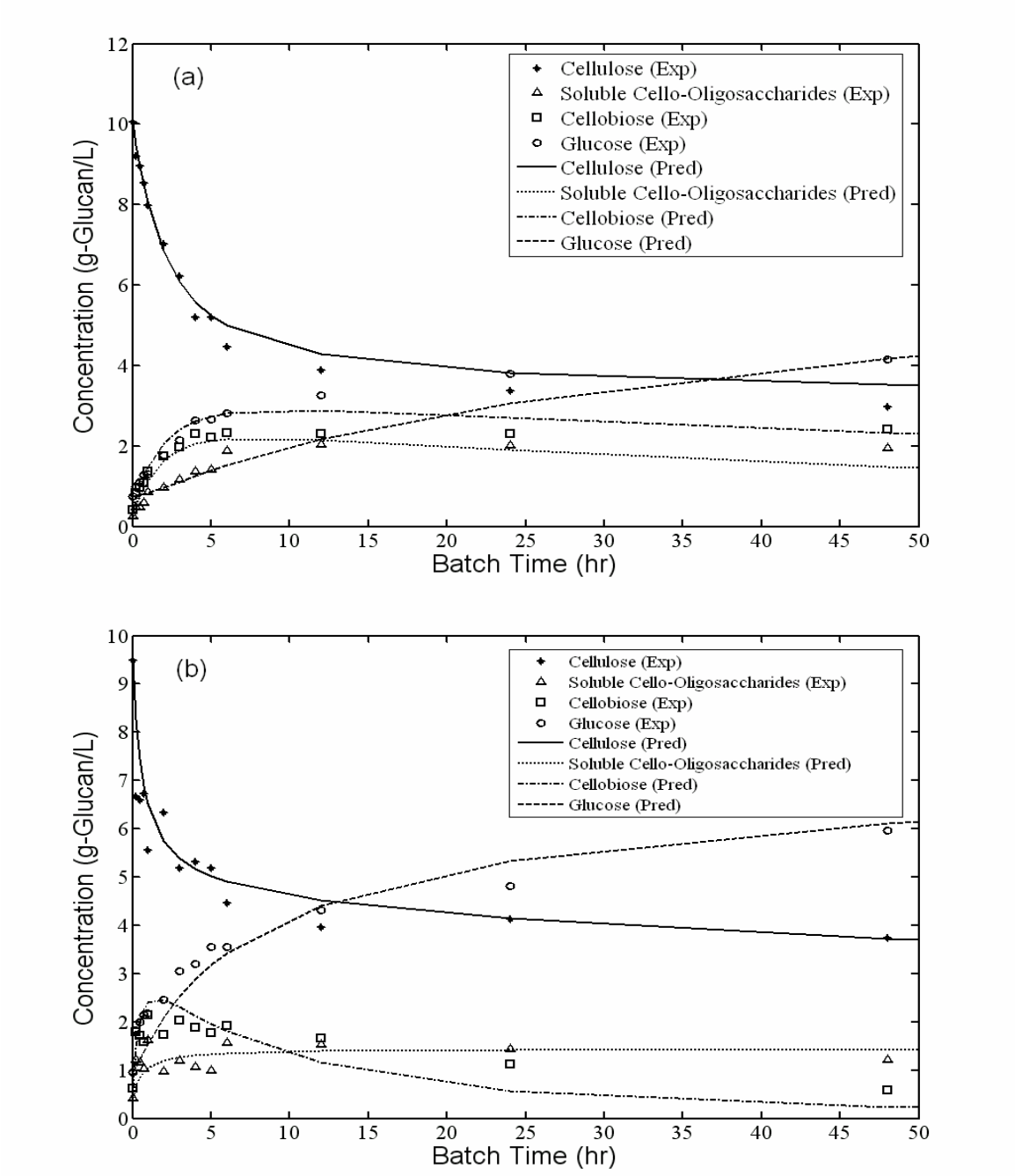

FIGURE V-14: Experimental and predicted concentration profiles of cellulose, soluble cello-oligosaccharides, cellobiose and glucose in the enzymatic hydrolysis of non-crystalline cellulose with 5% Glucose a) with 1 FPU/g-Glucan enzyme loading, b) with 3 FPU/g-Glucan enzyme loading.

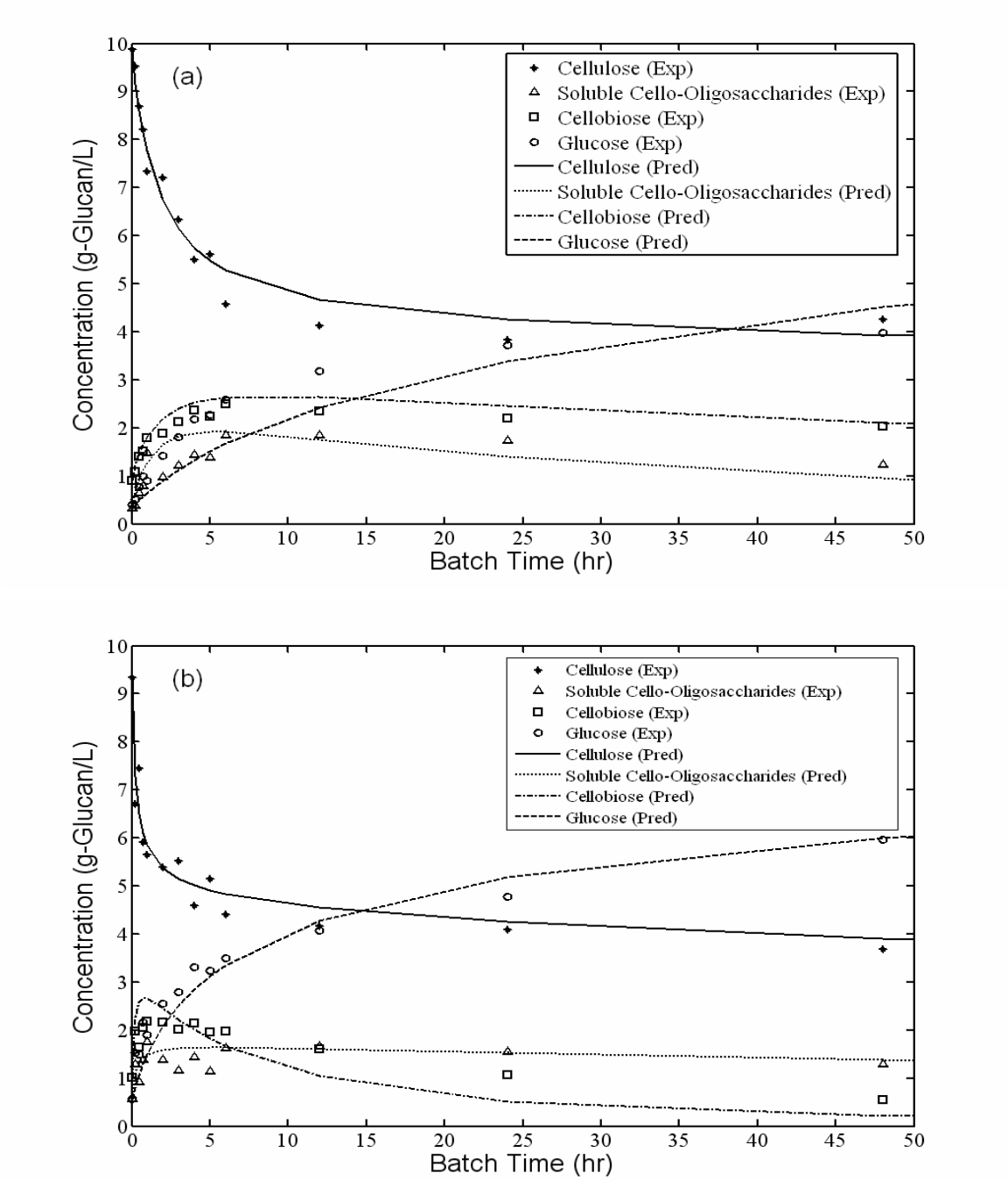

FIGURE V-15: Experimental and predicted concentration profiles of cellulose, soluble cello-oligosaccharides, cellobiose and glucose in the enzymatic hydrolysis of non-crystalline cellulose with 5% Cellobiose a) with 1 FPU/g-Glucan enzyme loading, b) with 3 FPU/g-Glucan enzyme loading.

4. CONCLUSIONS

Existing mechanistic models are capable to explain the progression of enzymatic hydrolysis of cellulose efficiently in the initial stages of hydrolysis, but failed to model later stages. From a series of experiments it was found that independently quantifying the inhibitory effect of hydrolysis intermediates (soluble cello-oligosaccharides and cellobiose) and product (glucose) on cellulases, is not possible as these effects are simultaneous in nature and can not be delinked from each other.

This experimental investigation coupled with mathematical modeling and simulation analysis highlights the changing reaction dynamics of batch cellulose hydrolysis which is influenced by substrate binding of enzyme and competitive / non-competitive product inhibition. As a result of revisiting the extensively studied subject with new analysis and experiments, some new and additional understandings of the enzymatic reaction kinetics are offered:

- Cellulose is break down to smaller chain length cello-oligosaccharides (insoluble: DP>15, soluble: DP<15) by the action of endo-glucanases. Further breakdown of insoluble cello-oligomers to glucose-dimer (cellobiose) is catalyzed by Exo-glucanases. β-glucosidase acts on both soluble cello-oligosaccharides and cellobiose and converts them to fermentable sugar (glucose).
- Cellulase adsorption to the substrate surface is reversible and is governed by simple Langmuir type adsorption isotherm.
- The reducing sugars inhibit the enzyme in a reversible and competitive/non-competitive manner
- Enzymes endo-glucanases and exo-glucanases were subjected to non-competitive

inhibition by soluble cello-oligosaccharides, cellobiose and glucose.

- As the glucose concentration increases, the inhibition rate of endo-glucanases and exo-glucanases increases. The probability of glucose (inhibitor) binding to the enzyme is three times higher than the probability of substrate binding.
- Enzymes β-glucosidases were solely inhibited by glucose in competitive inhibition.
- With NCC as substrate accumulation of significant amounts of cellobiose and soluble cello-oligosaccharides was observed.

The proposed model is considered not fully comprehensive especially in respect to lacking consideration of the synergistic actions of different cellulase enzyme components which are difficult to ascertain from a biochemistry point of view. Nonetheless, this work offers an approach to the analysis of the enzymatic hydrolysis kinetics, especially the effect of enzyme-substrate and enzyme-product interactions.

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APPENDICES

APPENDIX – I

MATLAB CODES TO BUILD INITIAL RATE MECHANISTIC MODELS

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%  "Parameter.m" : Main function for building the initial rate mechanistic
%%  models for cellulase hydrolysis of treated unrefined cotton, alpha
%%  cellulose avicel and cello-oligomers
%%  requires "Objectivefn.m" and "KineticModel.m" as supporting functions
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

clc; clear all;
warning off;

global dat Data Type;

%% Experimental Hydrolysis Data used
%% for building Initial rate mechanistic models

%treated unrefined : time(hr) 1FPU 3FPU 15FPU
dat1 = [0       0.698   0.871   1.398
0.167   6.419   9.482   10.225
0.333   8.312   16.039  12.766
0.500   9.914   18.446  14.729
0.667   12.893  23.196  18.811
0.833   16.390  27.310  20.319
1       19.834  29.325  26.343
2       26.676  39.877  38.570
3       35.206  48.063  46.421
4       40.267  53.174  51.895
5       45.045  55.620  56.177
6       48.448  57.618  58.757];

% alpha cellulose : time(hr) 1FPU 3FPU 15FPU
dat2 = [0       0.000   1.350   1.733
0.167   1.773   2.509   5.183
0.333   2.256   4.883   7.446
0.500   4.329   6.037   9.521
0.667   4.515   6.982   11.053
0.833   5.231   7.937   13.025
1       5.559   9.105   14.624
2       7.698   12.709  20.566
3       9.474   15.914  25.149
4       10.533  17.838  28.291
5       11.797  19.928  31.290
6       12.966  21.759  34.153];
```

```
%avicel : time(hr) 1FPU 3FPU 15FPU
dat3 = [0          0.000    1.587    3.154
0.167    0.000    2.776    6.679
0.333    2.122    4.890    10.490
0.500    2.824    5.971    12.941
0.667    3.538    7.169    14.727
0.833    4.395    8.108    16.456
1        5.144    8.658    17.841
2        7.082    12.215   23.211
3        8.560    14.956   27.388
4        9.495    16.753   29.809
5        10.775   18.571   32.432
6        11.945   20.228   35.026];

%cello oligomers : time(hr) 1FPU 3FPU 15FPU
dat4 = [0          3.974    4.021    5.449
0.167    4.514    3.850    6.058
0.333    4.427    4.090    6.865
0.500    4.682    4.166    7.386
0.667    4.700    5.381    7.701
0.833    4.912    5.560    8.110
1        5.121    5.843    8.440
2        5.577    6.777    9.854
3        6.023    7.451    10.527
4        6.595    8.066    11.398
5        7.072    8.487    11.689
6        7.582    8.950    12.283];


Type =2;
dat = dat2;

% Bounds for the model parameters to be estimated for ensuring
% feasibility
LB = [1e-4 1e-4 1e-4]';
UB = [9 40 50]';

% Initial assumption for model parameters
X = [0.1 30 20]';

% Optimization routine
options = optimset('Display','iter','TolFun',1e-8, 'MaxFunEvals', 1000);
X=fmincon('Objectivefn',X,[],[],[],[],LB,UB,[],options)


% Plotting predicted and experimental extent of hydrolysis (1 FPU case)
S = (100-dat(:,2))*11.494/100;
n = length(S);
figure(1)
plot(dat(:,1), Data(1:n,2));
hold;
plot(dat(:,1), S,'*');

% Plotting predicted and experimental extent of hydrolysis (3 FPU case)
S = (100-dat(:,3))*11.494/100;
plot(dat(:,1), Data(n+1:2*n,2),'r');
```

```
plot(dat(:,1), S,'r*');

% Plotting predicted and experimental extent of hydrolysis (15 FPU case)
S = (100-dat(:,4))*11.494/100;
plot(dat(:,1), Data(2*n+1:end,2),'k');
plot(dat(:,1), S,'k*');

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
```

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%  "Objectivefn.m" : Function which calculates the objective function to be minimized
%%  in the optimization of model parameter estimates for initial rate
%%  mechanistic models
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

function f = Objectivefn(X)

global dat Data Xp Type E;

T= dat(:,1);
err = 0;
E0 = [1 3 15]';
Data = [];

for ii=1:3
   S = (100-dat(:,ii+1))*11.494/100;  % Substrate concentration in g/L
   E = 20*E0(ii);                     % Enzyme concentration in g/L
   Xp = X;
   Data = [Data; T(1) S(1)];
   Sp = S(1);
   S0 = Sp;
   % Prediction of the time course Data of cellulose
   for i=1:length(T)-1
      Tstep = T(i+1);
      [t,y]=ode45('KineticModel',[0 Tstep],S0);
      Sp = y(end);
      Data = [Data; T(i+1) Sp];
      err = err + (Sp-S(i+1))^2;  % Square of prediction error
   end
end

f = err/length(T); %  Objective function value

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
```

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%  "KineticModel.m" : Function in which different initial rate mechanistic models were  simulated
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

function f = KineticModel(t,C)

global Xp Type E;

%% MM kinetics with competitive/noncompetitive inhibition, with/without quasi-steady state approach
if (Type==1)
   k = Xp(1);
   Km = Xp(2);
   S = C(1);               % component concentrations
   r = k*S*E/(Km+S);        % reaction rate
   dCSdt = -r;              % component accumulation rates
end

%% Shrinking site hydrolysis model with langmuir-type adsorption isotherm
if (Type==2)
   k = Xp(1);
   alfa = Xp(2);
   S = C(1);                % component concentrations
   r = k*E/(E+alfa)*S^(4/3); %reaction rate
   dCSdt = -r;               % component accumulation rates
end

%% Two phases of cellulose: amorphous and crystalline
if (Type==3)
   k = Xp(1);
   alfa = Xp(2);
   S = C(1);                  % component concentrations
   r = k*E/(E+alfa)*S;         % reaction rate
   dCSdt = -r;                 % component accumulation rates
end

%% Hydrolysis of both cellulose and cellobiose
if (Type==4)
   k = Xp(1);
   K = Xp(2);
   alfa = Xp(3);
   S = C(1);                  % component concentrations
   r = k+K*E/(E+alfa)*S;       % reaction rate
   dCSdt = -r;                 % component accumulation rates
end

%% MM kinetics with competitve inhibition and langmuir adsorption
if (Type==5)
   K = Xp(1);
   alfa = Xp(2);
   eps = Xp(3);
   S = C(1);                  % component concentrations
   r = K*E*S/(eps*E+alfa+S);   % reaction rate
   dCSdt = -r;                 % component accumulation rates
end

%% MM kinetics with competitive inhibition and langmuir adsorption
```

```
if (Type==6)
   K = Xp(1);
   alfa = Xp(2);
   eps = Xp(3);
   S = C(1);                    % component concentrations
   phi = ((S-E-alfa)+((alfa+E-S)^2+4*alfa*S)^0.5)/2/S;
   r = K*E*S/(eps*E+alfa+phi*S);% reaction rate
   dCSdt = -r;                  % component accumulation rates
end

f = dCSdt;

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
```

APPENDIX – II

MATLAB CODES TO BUILD COMPREHENSIVE KINETIC MODEL

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%% "ParameterEst.m" : Program to estimate the optimal model parameters
%% for the proposed comprehensive kinetic model
%% required function are: "Objectivefn.m" and "KineticModel2.m"
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

clc; clear all;
warning off;

global T C1 O1 B1 G1 H1 E11 C2 O2 B2 G2 H2 E12 Data;

%% figure properties
set(0,'DefaultLineLineWidth',2);
set(0,'DefaultaxesLineWidth',2);
set(0,'DefaultaxesFontSize',14);
set(0,'DefaultTextFontSize',14);
set(0,'DefaultAxesFontName','Times');

NCCdata;  % Experimental Data

% 1 - NCC hydrolysis data with 1 FPU enzyme
% 2 - NCC hydrolysis data with 3 FPU enzyme
% 3 - NCC+5%o hydrolysis data with 1 FPU enzyme
% 4 - NCC+5%o hydrolysis data with 3 FPU enzyme
% 5 - NCC+10%o hydrolysis data with 1 FPU enzyme
% 6 - NCC+10%o hydrolysis data with 3 FPU enzyme
% 7 - NCC+5%G hydrolysis data with 1 FPU enzyme
% 8 - NCC+5%G hydrolysis data with 3 FPU enzyme
% 9 - NCC+10%G hydrolysis data with 1 FPU enzyme
% 10 - NCC+10%G hydrolysis data with 3 FPU enzyme
% 11 - NCC+5%B hydrolysis data with 1 FPU enzyme
% 12 - NCC+5%B hydrolysis data with 3 FPU enzyme
% 13 - NCC+10%B hydrolysis data with 1 FPU enzyme
% 14 - NCC+10%B hydrolysis data with 3 FPU enzyme

% converting data in % glucose to g/L for an initial substrate conc of 1% glucon
dat = dat9;
E11 = E9*10.965/30;            % Enzyme concentration (g/L)
T = dat(:,1);                  % Batch time
H1 = dat(:,2)+dat(:,3)+dat(:,4);  % Percent Hydrolysis
C1 = (100-H1)*11.494/100;       % Substrate concentration cellulose (g/L)
O1 = dat(:,2)*11.494/100;       % Soluble Cello-Oligomers concentration (g/L)
B1 = dat(:,3)*11.494/100;       % Cellobiose concentration (g/L)
G1 = dat(:,4)*11.494/100;       % Cellulose concentration (g/L)
```

```
dat = dat9;
E12 = E9*10.965/30;              % Enzyme concentration (g/L)
H2 = dat(:,2)+dat(:,3)+dat(:,4);  % Percent Hydrolysis
C2 = (100-H2)*11.494/100;         % Substrate concentration cellulose (g/L)
O2 = dat(:,2)*11.494/100;         % Soluble Cello-Oligomers concentration (g/L)
B2 = dat(:,3)*11.494/100;         % Cellobiose concentration (g/L)
G2 = dat(:,4)*11.494/100;         % Cellulose concentration (g/L)

%% Initial approximation for model parameters
% rate constants
k1 = 0.016;
k2 = 0.016;
k3 = 0.016;
k4 = 0.036;

% saturation constants
Kc = 40.12;
Kb = 29.12;
Ko = 13.12;

% inhibition constants
Koi = 12.12;
Kbi = 8.12;
Kgi = 6.12;

% constant in langmuir isotherm
Ke = 10.12;

%% Lower and upper constraints for model parameters
LB = [1e-4, 1e-4, 1e-4, 1e-4, 0, 0, 0, 0.01, 0.01, 0.01, 0.001];
UB = [60, 60, 40, 40, 100, 100, 100, 1000, 1000, 1000, 1000 ];
Param = [k1 k2 k3 k4 Kc Kb Ko Koi Kbi Kgi Ke]';

%% Nonlinear Trajectory Optimization to estimate the model parameters
options = optimset('Display','iter','TolFun',1e-6, 'MaxFunEvals', 10000);
X = fmincon('Objectivefn',Param,[],[],[],[],LB,UB,[],options);

n = length(T);

%% Plotting the experimental values and predicted time course data (1FPU)
figure(1)
plot(T, [C1 O1 B1 G1],'k*');
hold;
plot(T,Data(1:n,2:end),'k');

%% Plotting the experimental values and predicted time course data (3FPU)
figure(2)
plot(T, [C2 O2 B2 G2],'k*');
hold;
plot(T,Data(n+1:end,2:end),'k');

%% Saving model parameters
save ParametersModified X;
```

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%  "Objectivefn.m" : Objective function in nonlinear trajectory optimization for
%%  model parameter estimation for comprehensive kinetic model representing
%%  cellulase hydrolysis of NCC
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

function f = Objectivefn(X)

global T C1 O1 B1 G1 H1 E11 C2 O2 B2 G2 H2 E12 Data E Xp;

err = 0;
Xp = X;

Cp = C1(1);  % Cellulose concentration
Op = O1(1);  % Oligomer concentration
Bp = B1(1);  % Cellobiose concentration
Gp = G1(1);  % Glucose concentration
E = E11;     % Enzyme concentration

%% data: batch time, oligomers, cellobiose, glucose and cellulose
Data = [T(1) Cp Op Bp Gp];
C0 = [Cp Op Bp Gp]';

% Prediction of the time course Data
for i=1:length(T)-1

   Tstep = T(i+1)-T(1);
   [t,y]=ode45('KineticModel2',[0 Tstep],C0);

   Cp = y(end,1);
   Op = y(end,2);
   Bp = y(end,3);
   Gp = y(end,4);

   mid = length(T)/2;

   Data = [Data; T(i+1) Cp Op Bp Gp ];
   mid = length(T);
   %if(i<length(T)-2)
      err = err + abs(i^2)*(2*(Cp-C1(i+1))^2 + (Op-O1(i+1))^2 + (Bp-B1(i+1))^2 + 2*(Gp-G1(i+1))^2) ;
   %end

end

Cp = C2(1);
Op = O2(1);
Bp = B2(1);
Gp = G2(1);
E = E12;

% data: batch time, oligomers, cellobiose, glucose and cellulose
Data = [Data; T(1) Cp Op Bp Gp];
C0 = [Cp Op Bp Gp]';

% Prediction of the time course Data
```

```
for i=1:length(T)-1

   Tstep = T(i+1)-T(1);
   [t,y]=ode45('KineticModel2',[0 Tstep],C0);

   Cp = y(end,1);
   Op = y(end,2);
   Bp = y(end,3);
   Gp = y(end,4);

   mid = length(T)/2;

   Data = [Data; T(i+1) Cp Op Bp Gp ];
   %if(i<length(T))
      err = err + abs(i^2)*(2*(Cp-C2(i+1))^2 + (Op-O2(i+1))^2 + (Bp-B2(i+1))^2 + 2*(Gp-G2(i+1))^2) ;
   %end

end

f = err/2/length(T);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
```

```
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%  "KineticModel2.m" : Function to simulate the comprehensive kinetic model
%%  proposed for cellulase hydrolysis of NCC
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

function f = KineticModel2(t,Conc)

global Xp E;

% kinetic parameters
X = Xp;

k1 = X(1);
k2 = X(2);
k3 = X(3);
k4 = X(4);
Kc = X(5);
Kb = X(6);
Ko = X(7);
Koi = X(8);
Kbi = X(9);
Kgi = X(10);
Ke = X(11);

% component concentrations
C = Conc(1);   % Cellulose
O = Conc(2);   % COS
B = Conc(3);   % Cellobiose
G = Conc(4);   % Glucose

% individual reaction rates (Scheme -1)  : very good for O and B
r1 = k1*C*E/(Kc+C)/(E+Ke)/(1+B/Kbi+G^3/Kgi+O/Koi);
r2 = k2*B*E/(Kb*(1+G/Kgi)+B)/(E+Ke);
r3 = k3*C*E/(Kc+C)/(E+Ke)/(1+B/Kbi+G^3/Kgi+O/Koi);
r4 = k4*O*E/(Ko*(1+G/Kgi)+O)/(E+Ke);

% component accumulation rates
dCdt = -r1-r3;  % Cellulose accumulation
dOdt = r3-r4;   % COS accumulation
dBdt = r1-r2;   % Cellobiose accumulation
dGdt = r2+r4;   % Glucose accumulation


f = [dCdt dOdt dBdt dGdt]';

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
```