STUDIES ON PARTIALLY OXIDIZED CELLULOSE AND BAGASSE DERIVED CELLULOSE: CHEMICAL MODIFICATION, HYDROLYTIC BEHAVIOR AND APPLICATIONS

A thesis submitted to the

UNIVERISITY OF PUNE

For the degree of

DOCTOR OF PHILOSOPHY

In

(CHEMISTRY)

By

Mr. SHAIKH HAMID

Research guide

DR. A. J. VARMA

POLYMER SCIENCE AND ENGINEERING DIVISON

NATIONAL CHEMICAL LABORATORY

PUNE- 411 008, INDIA

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राष्ट्रीय रासायनिक प्रयोगशाला
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
डॉ. होमी भाभा मार्ग पुणे - 411 008. भारत
NATIONAL CHEMICAL LABORATORY
(Council of Scientific & Industrial Research)
Dr. Homi Bhabha Road, Pune - 411 008. India.

CERTIFICATE

Certified that the work incorporated in this thesis entitled "**Studies on partially oxidized cellulose and bagasse derived cellulose: Chemical modification, hydrolytic behavior and applications**" submitted by Mr. Shaikh Hamid was carried out by the candidate under my supervision/guidance. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

August, 2010

Dr. A. J. Varma
(Research Guide)

Communication Channels
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EPABX : +91-20-25893300
+91-20-25893400
FAX
Director's Office : +91-20-25902601
COA's Office : +91-20-25902660
COS&P's Office : +91-20-25902664
WEBSITE
www.ncl-india.org

Declaration by the Candidate

I declare that the thesis entitled **"Studies on partially oxidized cellulose and bagasse derived cellulose: Chemical modification, hydrolytic behavior and applications"** submitted for the degree of Doctor of Philosophy to the University of Pune, has been carried out by me at the National Chemical Laboratory, Pune, under the supervision of Dr. A. J. Varma. The work is original and has not been submitted as a part or full by me for any degree or diploma to this or any other university.

August, 2010 Shaikh Hamid

Dedicated to my beloved Amma

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August, 2010 *Shaikh Hamid*

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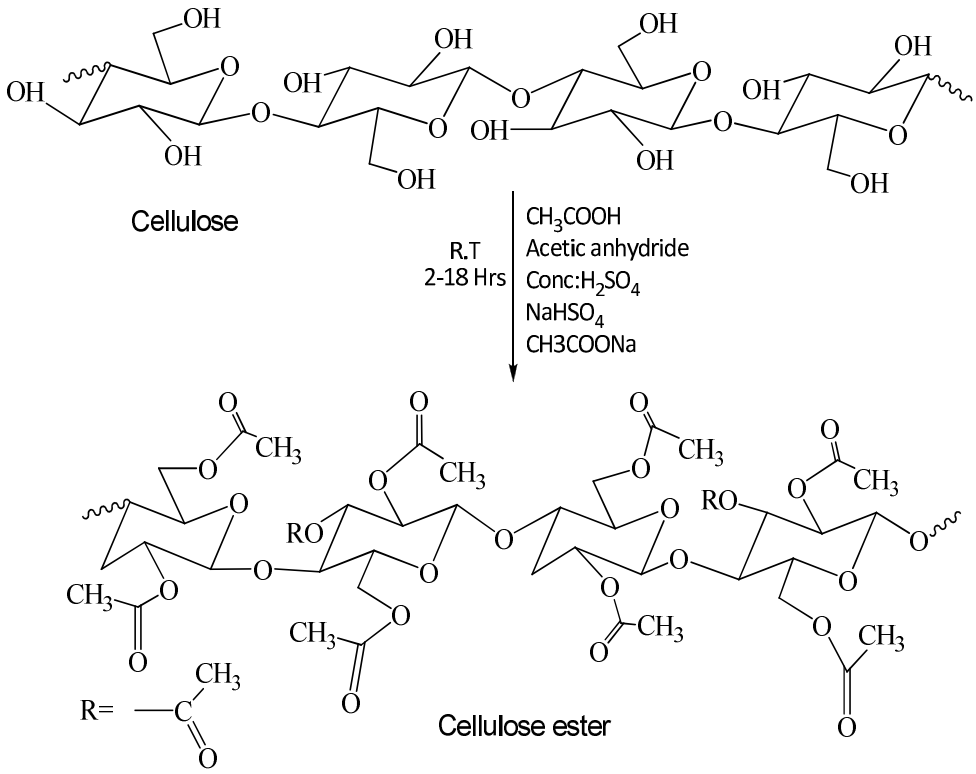

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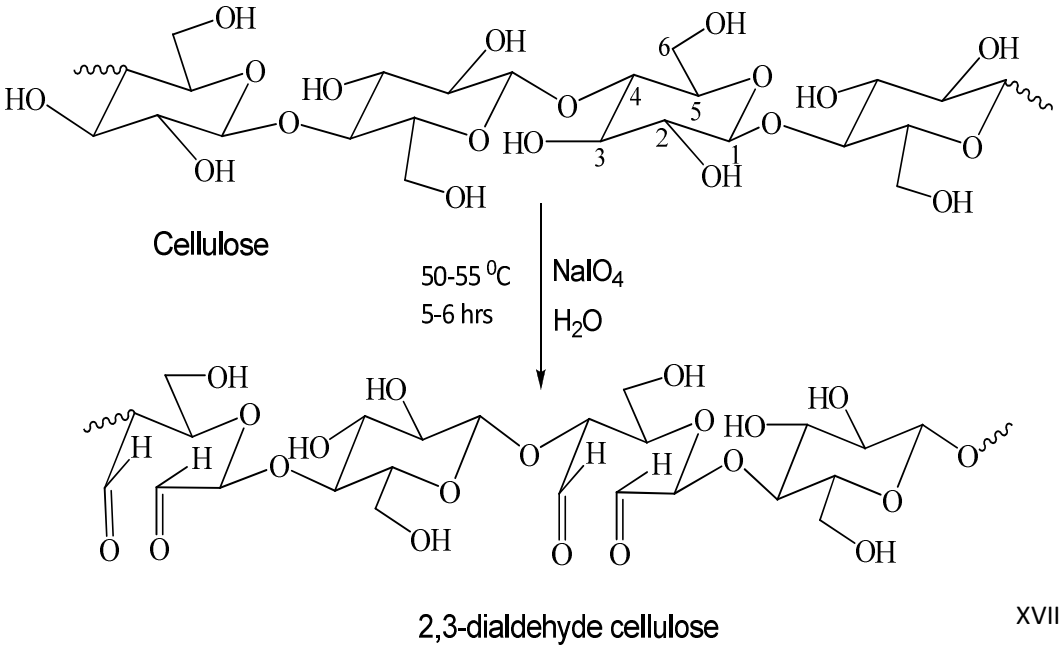

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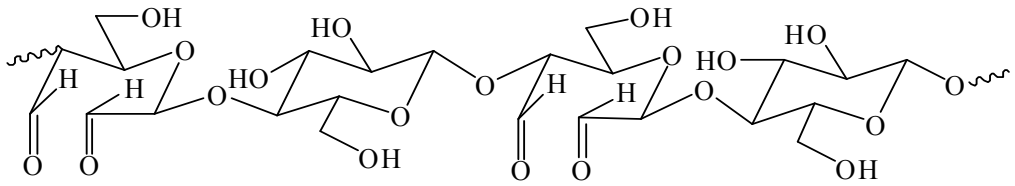
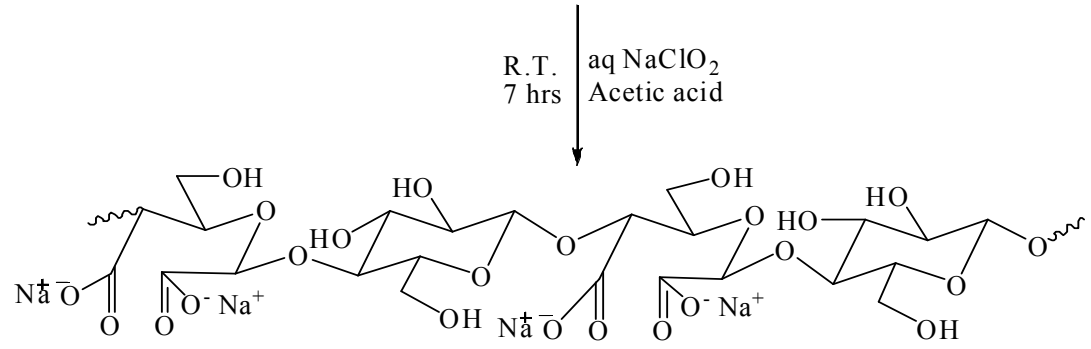


2,3-dialdehyde cellulose



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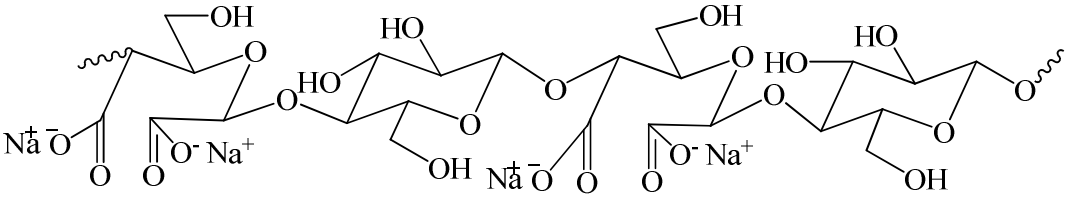

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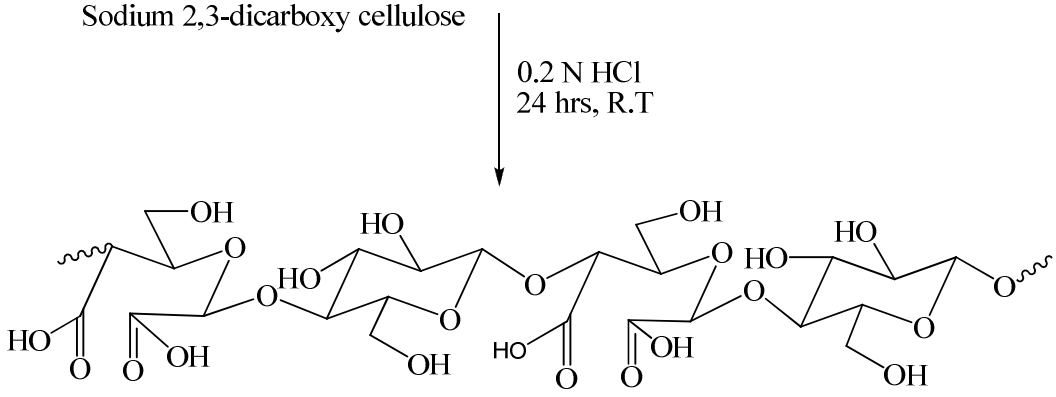

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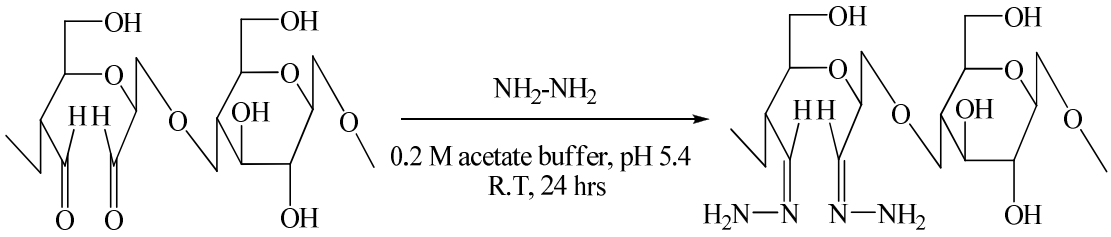

2, 3-dialdehyde cellulose

2, 3-dihydrazone cellulose

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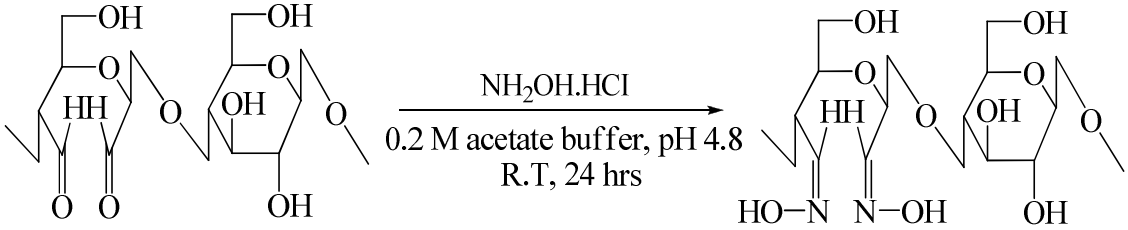

2, 3-dialdehyde cellulose

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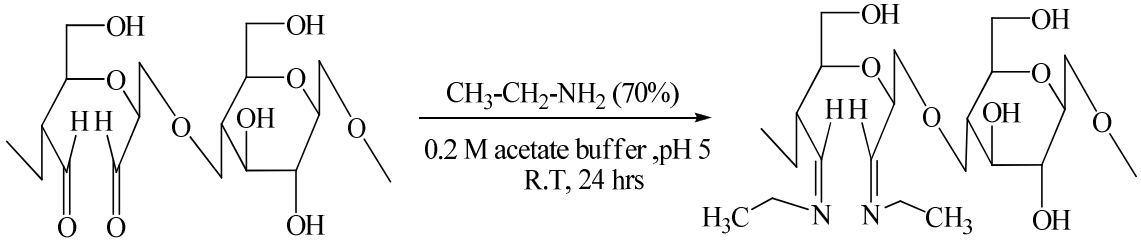

2, 3-dialdehyde cellulose

2, 3-diethylimine cellulose

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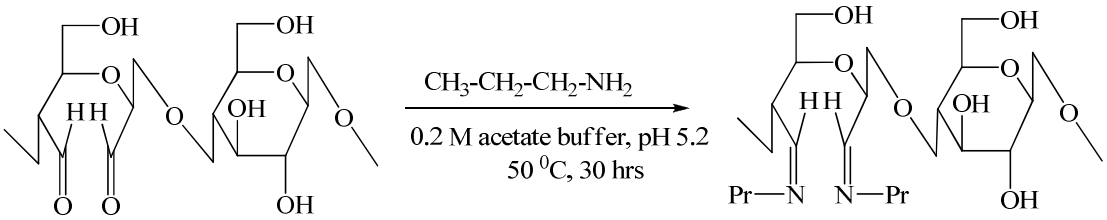

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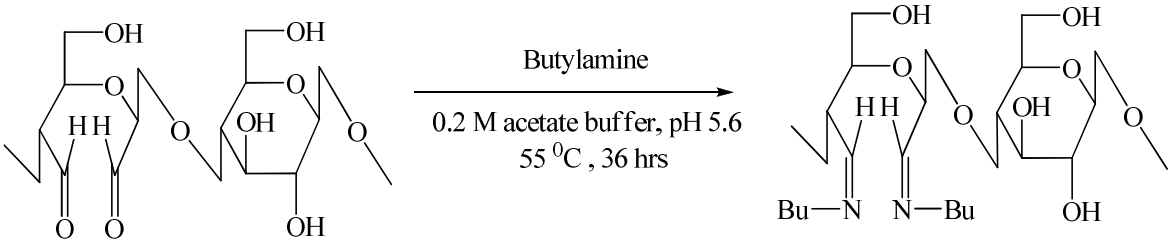

2, 3-dialdehyde cellulose — 2, 3-dibutylimine cellulose

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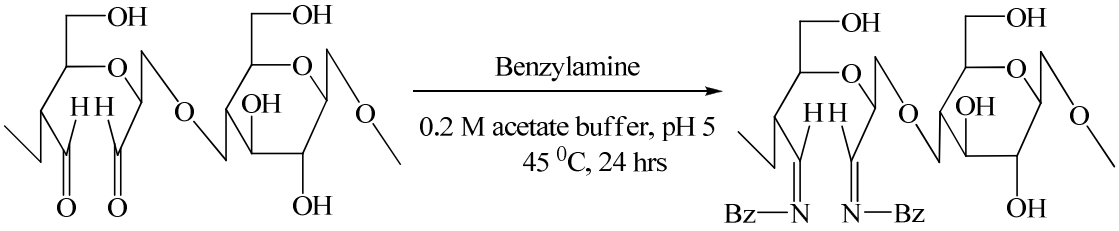


2, 3-dialdehyde cellulose — 2, 3-dibenzylimine cellulose

Abbreviations

NICM	National Collection of Industrial Microorganism
CP 123	Cellulose powder 123
K	Kappa Number
FPase	Filter paper cellulase
CMCase	Carboxymethyl cellulase
pNPG	p-Nitro phenyl β-D-glucopynoside
DNS	dinitrosalysilic acid
HPIC	High pressure ion chromatography
AGU	Anhydro glucose unit
DAC	2, 3-dialdehyde cellulose
NaDCC	Sodium 2, 3-dicarboxy cellulose
DCC	2, 3-dicarboxy cellulose
CP-MAS	Charged polarized magic angle spinning
EDAX	Energy dispersive X-ray spectroscopy
CA	Cellulose acetate
DS	Degree of substitution
GPC	Gel permeation chromatography
WAXRD	Wide-angle X-ray crystallography
ASTM	American Standard Testing Methods
DSC	Differential scanning chromatography
TGA	Thermogravimetric analysis
SEM	Scanning electron microscopy
CLSM	Confocal laser scanning microscopy
TEMPO	2,2,6,6-Tetramethylpiperidine-1-oxyl

Abstract of the thesis

Introduction

This research thesis is based on studying and developing the chemistry and technology of cellulosic polysaccharides. Cellulose, a complex polysaccharide consisting of glucose units, is the most abundant of all naturally occurring organic materials and has become one of the most important commercial raw materials in current years. Cellulose is present in plant cell walls, complex structures composed mostly of lignocellulosic materials which are a matrix of cross-linked polysaccharide networks, glycosylated proteins and lignin. This matrix has three main components: cellulose (polymer of hexose sugar), hemicellulose (polymer of pentose and hexose sugars), and lignin (phenylpropanoid polymer).

Cellulose is structural uniform polymer in which anhydroglucose unit (AGU) are linked by β-1,4-glycosidic bonds resulting in a homo-polymer with three hydroxyl groups per anhydroglucose units (AGU). Due to these glucosidic linkages, the most stable conformation for cellulose is that in which each glucose unit is rotated 180° relative to the preceding unit, which makes cellobiose, is the basic repeating unit of cellulose.

In a context of "second generation" biobased materials, utilizing agricultural waste by-products as sources for cellulose and lignocellulosic feed stocks are becoming increasingly necessary as it is not present in the human food chain as well as it can reduce dependency to some extent from materials derived from fossil resources. Lignocellulosic materials such as sugarcane bagasse mainly comprised of cellulose, hemicellulose, and lignin and it is a by-product of sugarcane industry. It is the most

abundant lignocellulosic waste feed stock and approximately 100 million tons of bagasse on dry basis is annually produced in India. It is generally burned to generate energy for sugar industries, and remaining thousands tons of bagasse are discarded, leading to a big environmental problem. And therefore within a concept of "biorefinery" the systematic utilization of bagasse has received much interest due to environmental concerns and huge quantity of bagasse produced.

Similarly due to availability of three reactive hydroxyl groups in each anhydroglucose units of cellulose, a great variety of structural modification and combinations are possible, and thus they can act as excellent materials for advanced and highly functional biopolymers. Traditionally, cellulose is employed for making some key commodity polymers and paper production. The chemistry of cellulose is undergoing renewed interest in current years mainly due to depleting of fossil resources, regulations on using synthetic materials, growing environmental awareness and economic considerations. Chemical modification of cellulose via oxidation is one of the reactions which lead to the product having several desirable properties and various interesting applications such as tissue engineering scaffold, antimicrobial cellulose, wound healing, antitutumor, inmunostimulant, adhesion-prevention and precursor for cellulosic derivatives. Thus extraction of high quality of cellulose from non-wood origin, functionalizations and chemical modification of cellulose further broadens the potential application of this biopolymer.

In the present study, sugarcane bagasse was chosen as the lignocellulosic biomass material, and from which the polysaccharide component, cellulose, hemicellulose were enriched by selective removal of lignin. This work is directed towards the extraction of cellulose from non-wood origin biomass, specifically sugar cane bagasse, followed by the evaluation of bagasse as a biobased materials under the

concept of 'biorefinery', for various applications such as synthesis of biodegradable cellulose acetate from cellulose, production of enzyme form bagasse polysaccharides, hydrolysis of chemically treated bagasse by the same enzymes for biofuel and biobased feedstock production and synthesis of a series of partially oxidized cellulose from pure cellulose having different extent of aldehyde groups on the same cellulose chain. This oxidized cellulose was further modified to carboxylate, carboxy cellulose and Schiff bases. These are the new series of functionalized cellulosic co-polymers that can be used for developing new applications. Further, the various oxidized cellulose derivatives synthesized were subjected to enzymatic hydrolysis and fluorescence visualization to study the effects of different functionalities on the cellulose.

The thesis has been divided into the following seven chapters

Chapter 1: Introduction (Literature review):

This chapter gives a detailed review of literature on various pretreatment techniques for fractionation of complex lignocellulosic biomass for extraction of cellulose and other polymeric components. It also give insight into sustainable value added materials can be made from lignocelluloses. It also details the various oxidation methods of cellulose, determination of oxidized functionality and application of oxidized cellulose in general. This chapter also includes the scope and objectives of the thesis.

Chapter 2: Studies on extraction of pure cellulose from bagasse and its application in producing cellulose acetates with novel application of residual hemicellulose as plasticizer: This chapter describes the study of utilization of

bagasse cellulose as a raw material to synthesis of cellulose acetate, which is a well known biodegradable polymer produced from cotton and wood cellulose. A detailed investigation on the effect of residual hemicellulose in extracted cellulose used in the synthesis of cellulose acetate was carried out. Cellulose acetate made from bagasse cellulose was extensively characterized using FTIR, TGA, DSC, GPC, HPIC, wide angle x-ray diffraction, viscometry and mechanical properties.

By this study it was demonstrated that the residual hemicellulose need not be considered as an impurity; rather it can be used in acetylated form as a plasticizer. It also acts as a biodegradable additive for cellulose acetates.

Chapter 3: Studies on production of hydrolytic enzymes from bagasse polysaccharides

This chapter describes the study of systematic delignification of bagasse by various chemical pretreatments to obtain delignified bagasse polysaccharides with various levels of purification. These polysaccharides were evaluated as a carbon source for the production of cellulase and xylanase enzymes. It was shown that these polysaccharides could be excellent carbon sources for cellulase production by *Penicillium janthinellum* NCIM 1171 and *Trichoderma viride* NCIM 1051. Chemically pretreated bagasse samples show highest β-glucosidase and xylanase activity, even when compared to pure cellulose powder (CP-100).

Chapter 4: Studies on enzymatic hydrolysis of delignified bagasse polysaccharides

In this chapter delignified bagasse polysaccharides were investigated as a substrate for hydrolysis to produce glucose, xylose, and arabinose sugars by using the same cellulases and xylanases produced earlier using the purified bagasse

polysaccharides as a carbon source. Effect of temperatures on hydrolysis was also studied in detail. It was found that under similar condition pure cellulose powder (CP-100) hydrolyzes up to 55-60% while delignified bagasse samples hydrolyzes up to 90-95 %. These sugars are valuable platform chemicals to produce a variety of fuels (ethanol, butanol) and other chemicals which produced only from petroleum sources.

Chapter 5: Synthesis and structural properties of dialdehyde cellulose and its derivatives

This chapter describes the synthesis of partially oxidized dialdehyde cellulose from highly pure cellulose to develop a series of dialdehyde cellulose having different extents of aldehyde groups (different levels of oxidation) on the same cellulose chain. This dialdehyde cellulose further modified to carboxy, carboxylate and Schiff's bases. These derivatives were exhaustively characterized by FTIR, ^{13}C CP-MAS spectroscopy, thermal analysis and wide angle X-ray diffraction and their structure-property relationships were established. Thus, we were successful in oxidized cellulose partially to obtain several dialdehyde celluloses and other biodegradable oxidized cellulosic co-polymers bearing various functionalities on the same cellulose chain.

Chapter 6: Study of morphology and hydrolytic behavior of dialdehyde cellulose and its derivatives

This chapter describes the hydrolysis behavior of all these derivatives by hydrolytic enzymes. It was observed that dialdehyde cellulose hydrolyzes at a slower rate than cellulose while dihydrozone cellulose resisted enzymatic hydrolysis and shows antimicrobial property. It also detailed the morphologies of oxidized cellulose and

other oxidized cellulosic derivatives. Scanning electron microscopy provided the complete morphological picture of dialdehyde cellulose and its derivatives. It was found that cellulose fibrils break down upon oxidation and subsequent clustering of fiber occurs by further derivatization.

It is also observed that the introduction of COOH, COONa and C=N functionalities on dialdehyde cellulose leads to fluorescence emitting cellulose derivatives. Modified cellulosic derivatives showing fluorescence at red, green and blue wave length, and thus this can open a new application of cellulose derivatives in biotechnology as biological imaging, and nanotechnology as fluorescence emitting cellulosic nano-particles, sensor technology and fluorescence emitting biodegradable materials for various applications.

Chapter 7: Summary and conclusions

This chapter summarizes the results and describes salient conclusions of the investigations reported in the thesis with the suggestion for future work.

Chapter 1

Literature review: cellulose from lignocellulosic biomass: Isolation, applications and oxidative modifications

1.1 Introduction

Plant residues generated from commodity crops are abundant, cheap and renewable and are suitable substrates for extraction of cellulose, a valuable industrial polymer. It has been estimated that about 2000 million tons of by-products are generated every year worldwide from major crops such as corn, wheat, rice, soybeans, sorghum and sugarcane (Huda et al., 2007). All of these by-products are composed of considerable amounts (35–45%) of cellulose that can be extracted in the form of solid fibers. This waste agricultural residue can be used to obtain about 390 million tons of cellulose, more than five times the current cellulose consumption in the world (Huda et al., 2007). Such large scale availability of cellulose will help the textile, biofuel, chemicals, industrial solvents and polymer industry to be sustainable.

It has been reported that many lignocellulosic by-products including cornstover, rice, wheat straw, sorghum stalks, pineapple and banana leaves can be used to obtain cellulose with properties suitable for textile, composite and other industrial application (Reddy and Yang, 2007, 2006, 2005; Sinha and Ghosh, 1977; Doraiswamy and Chellamani, 1993). In addition, problems associated with disposing these by-products after harvest will also be reduced if these by-products are used for industrial applications. Therefore, agricultural residues are being worked upon as key sustainable organic raw materials for further research and development.

Lignocellulosic biomass can be converted into cellulose and soluble sugar via a large number of approaches but this conversion is complicated due to its complex and recalcitrant nature (Yang and Wyman, 2008). Thus, there is need to find more suitable and practical applications for this lignocellulosic agricultural waste.

The present thesis describes the studies carried out on i) sugarcane bagasse derived cellulose ii) value added products from lignocellulose iii) various pretreatment techniques used for such lignocellulosic biomass to remove protective layers of lignin and thereby increase the accessibility to enzymes to carry out effective hydrolysis iv) results of actual enzymatic hydrolysis of lignocellulose materials and applications of these hydrolytic enzymes in various fields and v) oxidative modifications of cellulose, determination of oxidized cellulose structures and Schiff bases of oxidised cellulose. This thesis covers all these aspects of sugarcane based lignocellulose and cellulose. The detail literates of relevant topics are presented in this chapter.

1.2. Sugarcane bagasse as lignocellulosic feedstock

Sugarcane (*Saccharum officinarum*) is a grass which is harvested for its sucrose content. After extraction of juice from the sugarcane, the plant material that remains is termed as 'bagasse'. Bagasse is an interesting raw material for isolation of carbohydrate polysaccharides, such as cellulose (~45% by weight) and hemicellulose (~30% by weight). Chemically, bagasse has low ash content (~2.5%) compared to other crop residues such as rice straw and wheat straw, which has 17.5% and 11.0% ash respectively. Such low ash containing organic raw material can be used advantageously in industrial conversion processes using microbial cultures (Pandey et al., 2000).

1.3. Chemical composition of bagasse

Bagasse contains mainly cellulose, hemicellulose, lignin polymers and mineral. The detailed elemental analysis of bagasse is given in table 1.1.

Table 1.1: Analysis of bagasse (Jonathan Scurlock, 2008)

Chemical components in bagasse(dry basis)	%	Elemental analysis of bagasse (dry basis)	%
Cellulose	40-45	Carbon	45-47
Hemicellulose	25-30	Hydrogen	5-6
Lignin	18-20	Oxygen	45-50
Others*	10-15	Ash	2.5-3.5

* Sucrose, wax, protein, pectin, minerals, etc.

1.3.1. Cellulose

Cellulose is an isotactic (1→4)-β-D-polyacetal form of cellobiose which in turn consists of two glucose units as shown in figure 1.1. Due to (1→4)-β-D-glucosidic linkage, the most stable conformation for cellulose is one in which each glucose unit is rotated 180° relative to the preceding unit, so that 'cellobiose' is the actual repeating unit. This discovery won Staudinger the Nobel Prize in Chemistry in 1953. It has been found that adjacent cellulose chains are coupled via hydrogen bonds and Van der Waal's forces resulting in a parallel alignment and a crystalline structure. Several elementary fibrils are joined together forming microfibrils, which are further bundled into larger macrofibrils leading to the rigidity and strength of the cell walls of the plant materials (Chanliaud et al., 2002)

The number of glucose repeating units of cellulose depends on the plant from which it is derived, but normally it ranges from 500 to 15000 glucose units. The number of glucose units in the chain is called the degree of polymerization (DP) and is not a fixed value but rather a distribution over a range of DP (polydispersity).

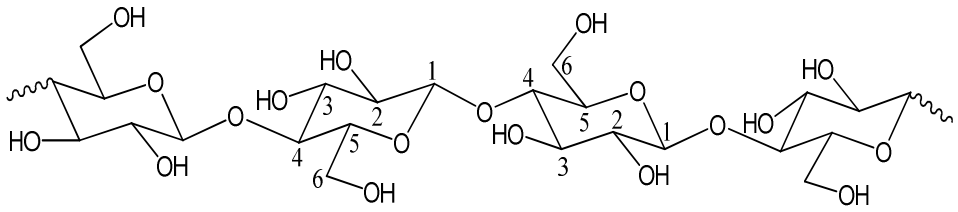


Figure 1.1: Chemical structure of cellulose: 1, 4-β-D-glucopyranose monomers and cellobiose repeating units

The DP and polydispersity of cellulose extracted from different sources is highly variable. Cellulose in exists mainly in highly ordered microcrystalline structures ("crystalline" or "supramolecular" regions) and lower order regions ("amorphous" regions). The crystalline structure can adopt several different crystal lattice structures and up to five different forms have been reported (Zugenmaier, 2001). Depending on the source of cellulose the crystallinity varies between 50 and 90% (Hone, 1994). Cellulose is insoluble in water even though it can swell to some extent; consequently native cellulose is not very reactive. The reactivity of cellulose can be greatly enhanced by various forms of treatment such as swelling under basic conditions, acid degradation or mechanical grinding which break down the fibrillar aggregations and increase surface area.

Although cellulose is insoluble in common organic solvent, some special solvents like cuprammonium, some ionic liquids and mixture of inorganic salt with high ionic strength solvents such as dimethylacetamide, dimethylsulphoxide can dissolve cellulose with relatively harsh condition (Zhu, 2006). In solution, the cellulose molecules exist as coils with a slight helical twist along the cellulose backbone. This twist is caused by interamolecular hydrogen bonds primarily between the hydroxyl group on C-3 and the pyranose ring oxygen in the adjacent glucose monomer and between the hydroxyl groups on C-6 and C-2 of the adjacent

glucose unit. The intra molecular bonds are responsible for the rigidity of cellulose (Krassig et al., 2001).

To extend the industrial applications of cellulose, its chemical structure has to be modified; modifications are most often performed by reacting the hydroxyl groups under heterogeneous conditions in such a way that the solubility of the cellulose in organic solvents is increased. The modification reaction takes place in a two-phase system where the first phase involves reaction on the amorphous regions and the second (slower) phase involves reactions on the crystalline regions.

1.3.2. Hemicellulose

After cellulose, the next major polysaccharide resource available to mankind is plant hemicelluloses. Unlike cellulose, hemicelluloses have lower degree of polymerization (DP) values, typically between 50–300. They are heterogeneous in their chemical constitution, and therefore mostly amorphous polymers. Most hemicelluloses are polymers of pentoses (mostly xylose and arabinose), hexoses (mostly mannose, glucose, and galactose), 4-*O* methyl glucronic acid and galacturonic acid residues. Trace amounts of rhamnose are also present in some hardwoods. In contrast to cellulose, the sugars present in hemicelluloses are easily hydrolyzable. The main component in hardwood hemicellulose is glucuronoxylan (Figure 1.2) while soft-wood hemicellulose consists of arabinoglucuronoxylan (Figure 1.3) and galactoglucomannans (Figure 1.4) as main components.

Hemicellulose is surrounded by a complex biopolymer called lignin which provides a protective sheath to the hemicellulose-cellulose framework; hemicellulose fraction can be isolated from the plant biomass even prior to delignification.

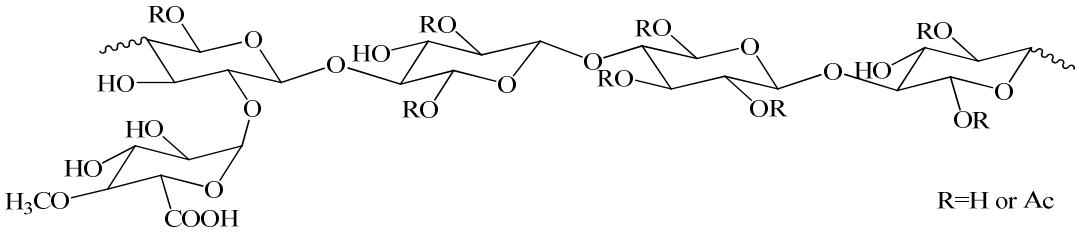


Figure 1.2: Structure of glucuronoxylan in hardwood

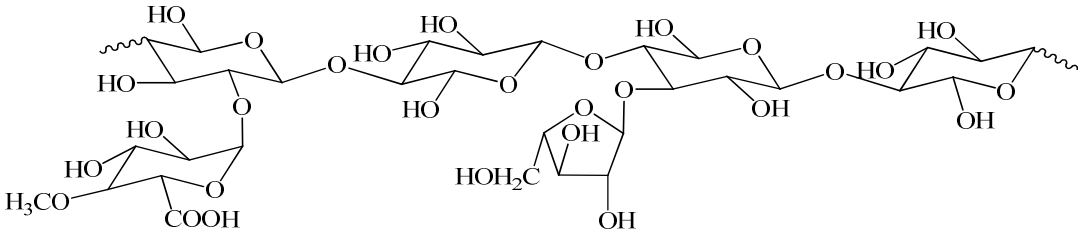


Figure 1.3: Structure of arabinoglucuronoxylan in softwood

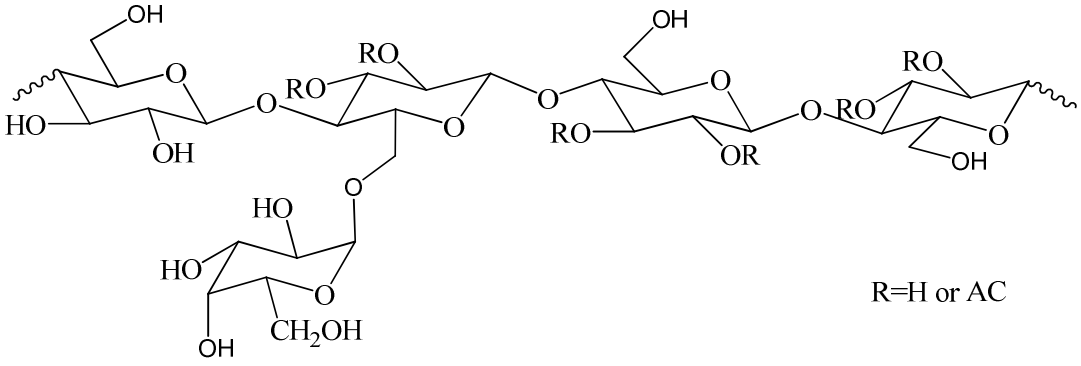
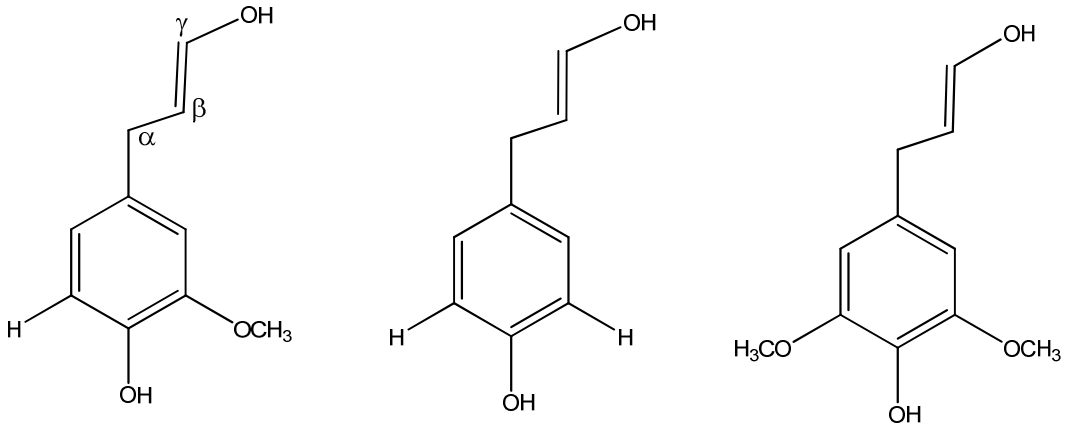


Figure 1.4: Structure of galactoglucomannans in softwood

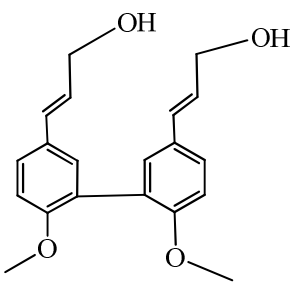
1.3.3. Lignin

Lignin is an amorphous, cross-linked and three dimensional biopolymers consisting coniferyl, sinapyl, and *p*-coumaryl alcohol as a repeating units (Zakzeski et al., 2010; Pu, Jiang and Ragauskas, 2007). Figure 1.5 shows primary building blocks of lignin structures. Soft wood lignin is mainly composed of coniferyl alcohol units while hardwood lignin composed of coniferyl and sinapyl alcohol units. The complex crosslinked structure of lignin, reinforced by cellulose fibers, gives strength to the lignocellulose biomass. Figure 1.6 shows some of the common linkages between phenylpropionic phenolic units of lignin (Chakar and Ragauskas, 2004).

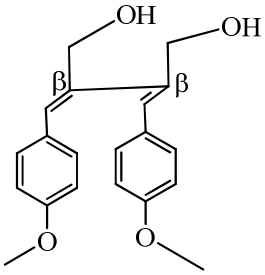


Guaiacyl/coniferyl alcohol p-Coumaryl alcohol Sinapyl/Syringyl alcohol

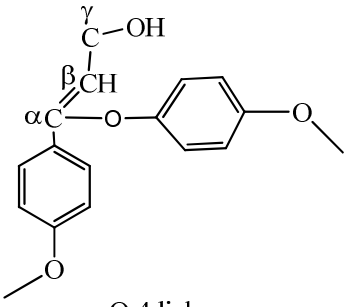
Figure 1.5: Building blocks of lignin



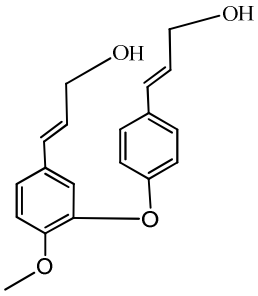
5-5- linkage



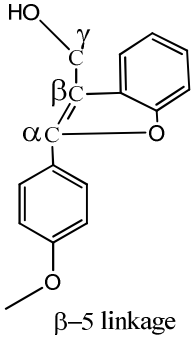
β-β- linkage



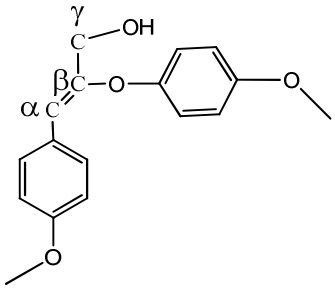
α-O-4 linkage



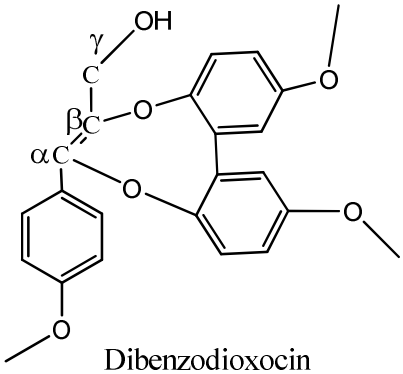
4–O–5 linkage



β–5 linkage



β–O–4 linkage



Dibenzodioxocin

Figure 1.6: Linkages between phenylpropane units in lignin

1.4. Value-added products from lignocellulosic biomass

Fermentable sugars such as glucose, xylose and arabinose, are the important products obtained from lignocellulosic biomass (Li et al., 2008; Kim and Hong, 2001). These reducing sugars can be obtained by either chemical or enzymatic hydrolysis of lignocellulose. The efficiency of hydrolysis depends on the enzyme characteristics, and upto 100% hydrolysis have been reported. For example, in the enzymatic hydrolysis using celluclast® along with novozym®, 100% of saccharification has been reported (Marques et al., 2008).

Production of cellulases from lignocellulose has been extensively studied (Adsul et al., 2004; Wen et al., 2005; Muthuvelayudham and Viruthagiri, 2006; Pothiraj et al., 2006; Daroit et al., 2007; Gao et al., 2008). Phytases, mannanases and amylases are also produced by microorganisms using lignocellulose as the main feedstock (Bhavsar et al., 2008). Similarly, other enzymes, such as xylanases, pectinases, pectin liase, protease, ligninases are produced from a wide range of lignocellulosic biomass (Rezende et al., 2002; Pandey and Pandey, 2002; Elisashvili et al., 2006; Dobrev et al., 2007; Mohana et al., 2008; Couto and Sanromana, 2005, 2006; Songulashvili et al., 2007; Elisashvili et al., 2008). Several efforts have been made to increase the production of enzymes through strain improvement by mutagenesis and recombinant DNA technology. Cloning and sequencing of the various genes of interest could economize the enzymes production processes (Kumar et al., 2008). The applications of the above enzymes in industries such as biofuel, food, brewery, textile laundry, chemical and pulp and paper is well established (Wen et al., 2005; Muthuvelayudham and Viruthagiri, 2006; Pothiraj et al., 2006; Daroit et al., 2007; Gao et al., 2008).

Conversion of lignocellulose to biofuels provides the best economically feasible and green second-generation renewable alternative to petroleum. Significant advances continued to be made towards bioconversion of lignocellulose biomass wastes into bioethanol, biodiesel, biohydrogen and biomethane.

Similarly, cellulose based derivative such as cellulose esters, cellulose ethers and mixed derivatives can be obtained from lignocellulose. Lignocellulosic polymer composites are among the emerging products made from lignocellulose. These biocomposites are very promising in producing sustainable current and future green materials to achieve durability without use of toxic chemicals. (Alemdar and Sain, 2008)

1.5. Fractionation of lignocellulosic biomass by various pretreatments

As mentioned earlier, lignocellulose is a complex material, leading to challenges in its application for further processing. Therefore, fractionation of lignocellulosic materials is essential for obtaining high purity α-cellulose for deriving various cellulose based chemicals and other polymeric constituents. Similarly pure hemicellulose and lignin, if isolated, could be used for various industrial applications as new feedstocks for new applications. The key characteristic of fractionation includes selective separations of each component of biomass and recovery of each component in high yield and high purity. Steam explosion and hydrothermal fractionation have been identified as able to meet many of these requirements. For clean fractionation of biomass, pretreatment steps are very essential. The purpose of pretreatment of lignocellulosics is to alter their physical structure of the constituent biopolymers in order to make the cellulose and hemicelluloses more accessible for down streaming processing. Effective pretreatment technologies should have some important parameters which

includes, minimization of degradation, limiting the formation of inhibitor by-products, prevention of lignin alterations and economic viability (Yang et al., 2004). Thus, without effective pretreatments, further technological progress in the conversion of lignocellulosic biomass to chemicals feedstock will not be feasible. Once an effective pretreatment of biomass is carried out, then it is relatively easy for downstream processing for e.g. for enzymatic hydrolysis for biofuel production or for fractionating polymeric constituents for various applications. The detailed study of various pretreatments gives a valuable insight to the understanding of the complexity of the lignocellulosic biomass, the limitation of each method, and how to innovatively combine two or more method to achieve a defined target. These pretreatments technique comprise, mechanical physiochemical, chemical, physical and biological pretreatments. Following are the pretreatments used to alter physical/chemical structure of recalcitrance lignocellulosic biomass.

1.5.1. Mechanical pretreatment

Mechanical pretreatment includes grinding, fiber cutting, milling (e.g. ball milling, two-roll milling, hammer milling, colloid milling, and vibro energy milling) to make the lignocellulosic materials easy to handle. These pretreatments increase the accessible surface area, size of pores and decrease the crystallinity as well as degrees of polymerization of cellulose. This is often the first step in the pretreatment of biomass (Hu et al., 2008; Sun and Cheng, 2002). It is shown that without any pretreatment corn stover with sizes of 53-75 μm was 1.5 times more productive than larger corn stover particles of 425-710 μm size (Zeng et al., 2007). Due to reduction in crystallinity by ball milling, more than 50% of saccharification of straw cellulose could be possible at mild hydrolytic condition (Sidiras and Koukios., 1989). Mais et al (2002) reported that by using ball mill reactor first and

then SO_2 impregnated steam-explosion for Douglas fir wood chips, total hydrolysis could be achieved at low enzyme loading (10 filter paper units/g of cellulose). Jin and Chen (2006) studied superfine grinding of steam-exploded rice straw; rice straw was cut to 5–8 cm and steam exploded at 180, 195, 210 and 220 °C for 4–5 minutes by saturated steam. The steam treated material was then pulverized using a vegetation disintegrator, and was put into superfine grinding using a fluidized-bed jet mill. The enzymatic hydrolysis of the superfine ground straw shows the highest hydrolytic rate and gives very high reducing sugar.

However, this type of pretreatment is not sufficient for isolation of all major polymeric constituent of biomass in pure form, and has to be combined with further chemical or biological treatment. Also, ball milling involves significant energy cost, is unable to remove lignin (which restricts the access of the enzymes for hydrolysis) and is therefore seldom used as the sole pretreatment methods.

1.5.2. Thermal pretreatment or pyrolysis

Thermal or pyrolysis has also been used for the pretreatment of lignocellulosic materials. Lignocellulose is rapidly decomposes to gaseous products and residual char when treated at temperatures greater than 300 °C (Kilzer and Broido, 1965; Shafizadeh and Bradbury, 1979). However, at lower temperatures, the decomposition is much slower, and the products formed are less volatile. The pyrolysis process is enhanced when carried out in the presence of oxygen (Shafizadeh and Bradbury, 1979). Pyrolysis pretreatment resulted in 80-85% conversion of carbohydrate component to sugars when mild acid hydrolysis (1 N H_2SO_4, 97 °C, 2.5 h) is carried out after pyrolysis pretreatment (Fan et al., 1987). Pyrolysis treatment resulted in conversion of biomass into syngas from which production of liquid fuels by Fischer-Tropsch (FT) process can be achieved (Zwart

et al., 2006). However, removal of char and ash which is formed at high temperatures is the biggest obstacle in these treatments.

1.5.3. Radiation exposure pretreatment

Irradiation by gamma ray, electron beam and microwaves can improve enzymatic hydrolysis of cellulosic materials (Hu et al., 2008). Kumakura (1983) studied the effect of irradiation for pretreatment of bagasse prior to its enzymatic hydrolysis; the pretreated bagasse resulted in double the yield of glucose by hydrolysis compared to the untreated one. They also reported that the cellulose component of the lignocellulose materials can be degraded by irradiation to fragile fibers and low molecular weight oligosaccharides and even cellobiose. High irradiation can also break the ring structure of cellulose. The enzymatic hydrolysis of filter paper with no lignin contents was not improved by irradiation pretreatment. Furthermore, enzymatic hydrolysis of newspapers with small amounts of lignin was slightly improved by irradiation. Therefore the effect of radiation should be correlated with the presence of lignin as well as the morphological structure such as crystallinity and density (Kumakura and Kaetsu, 1978, 1982, 1983).

However, not many workers have followed up this line of investigation, and there appear to be practical problem in commercial exploitation of these methods. Just like mechanical grinding to increase surface area not being stood alone pretreatment, so also for the irradiation methods. This pretreatment will have to combine with other methods and considering the capital costs and health issue is not likely to be of interest in the industrial application.

1.5.4. Physical pretreatments

Steam explosion of lignocellulosic biomass (Hu et al., 2008; Cantarella et al., 2004;

Sun and Cheng., 2002; Converti et al., 2000; Karr et al., 1998) and hot water treatment (Hu et al., 2008; Mosier et al., 2005) are two important methods of physical pretreatment with potential for industrial exploitation. Of the two, steam explosion method has attracted maximum attention, as evident from published literature. These methods are considered to be technologically and economically viable.

1.5.4.1. Steam explosion without catalyst

This method involves bringing into contact biomass particles with steam at elevated temperatures (~180–240 °C, 10-30 bar pressure) with residence times of ~2–5 min, followed by explosive decompression. This treatment promotes hemicellulose hydrolysis to a large extent and increases the pore volume of the biomass. Sudden explosive release of the steam pressure results in separating the cellulosic fiber further, and increases the surface area. This enables enzymes to penetrate the biomass more effectively for hydrolysis of the cellulose.

Extensive research has been conducted on steam explosion pretreatments (Hsu, 1996; McMillan, 1994; Saddler et. al., 1993). Hemicellulose is thought to be hydrolyzed by the acetic and other acids released during steam contact. Acetic acid is generated from hydrolysis of acetyl groups associated with the hemicellulose which further catalyzes hydrolysis and glucose or xylose degradation takes place. Water itself is believed to act as an acid at high temperatures (Weil et al., 1997; Baugh et al., 1988).

This steam explosion based autohydrolysis pretreatment has been shown to be effective with agricultural residues and hardwoods (Cantarella et al., 2004). Special care should be taken in selecting the steam explosion conditions in order to avoid excessive degradation of the physical and chemical properties of the biomass materials specially cellulose and to avoid formation of a more recalcitrant residue.

1.5.4.2. Hot water autocatalyzed pretreatments (Hydrothermal treatment)

In this pretreatment lignocellulosic heated at 180–230 ° C in the presence of hot water in closed vessel results in extensive hemicellulose hydrolysis. The temperature, time and weight/volume ratio of biomass determines the extent of hemicellulose, cellulose, and lignin removal, as well as their degradation products. These degraded products act as an inhibitor for enzymatic action. This method can be optimized for use with several type of agricultural biomass, especially where the equipment for steam explosion in not available.

Liquid hot water pretreatments results in the cleavage of *O*-acetyl and uronic acid substitutions from hemicellulose to generate acetic and other organic acids. The release of these acids promotes to catalyze formation and removal of oligosaccharides. However, the polysaccharides and especially hemicellulose may be further hydrolyzed to monomeric sugars which are subsequently partially degraded to aldehyde if mineral acid was added additionally. The enzyme inhibitors produced are principally furfural from pentoses and 5-hydroxymethyl furfural from hexose (Palmqvist and Hahn-Hagerdal, 2000).

1.6. Physico-chemical pretreatments

In this pretreatment chemical reaction in addition to physical defibration is involved. In this method steam explosion of biomass in the presence of acid, alkali, ammonia, carbon dioxide, and sulfur dioxide are carried out for enhanced hydrolysis of lignocellulosic materials.

1.6.1. Steam explosion in the presence of acids

The presence of an acid, particularly a mineral acid like sulphuric acid, is considered to be more effective in the removal of hemicellulose from plant biomass

with steam explosion process than use of only steam (Hu et al., 2008; Jorgensen et al., 2007; Sun and Cheng, 2002; Vlasenko et al., 1997). The use of an acid is of particular importance with soft wood (Jorgensen et al., 2007). Steam pretreatment with acid is considered to the method of choice for industrial application (Hu et al., 2008; Jorgensen et al., 2007). It is reported that this method also leads to a decrease in the amount of inhibitory chemical produced (Jorgensen et al., 2007, Sun and Cheng, 2002). Agricultural material like straws, corn stover and hardwood have also been extensively investigated and the promising results were found by this method (Jorgensen et al., 2000; Vlasenko et al., 1997).

1.6.2. Steam explosion in the presence of alkali

The presence of an alkali during the steam pretreatment followed by explosive decompression has been reported to yield significant success (Himmel et al., 2007). However, judging from literature, more efforts have been concentrated on acid steam explosion rather than alkali steam explosion of biomass. In the presence of alkali delignification occurs along with hemicellulose decomposition, thereby leading to more complex by-products.

1.6.3. SO_2 steam explosion pretreatment

Steam pretreatment can be performed with addition of sulfur dioxide (SO_2) to improve recovering both cellulose and hemicellulose fractions. This treatment can be carried out by adding 1-4% sulfur dioxide (w/w substrate) at elevated temperatures (160-230 °C) for few minutes (Eklund, Galbe, Zacchi., 1995). Eklund *et al* (1995) studied steam pretreatment of willow with the addition of SO_2 or H_2SO_4 in order to recover both cellulose and hemicellulose. The maximum glucose yield ~95% was obtained when the willow was treated with 1% SO_2 at 200 °C. However, the yield of xylose recovery by SO_2 was not as high as pretreatment with

dilute sulfuric acid. The sulfur dioxide rapidly diffuses into biomass pores before it is converted into H_2SO_4 providing superior performance compared to the direct use of an acid catalyst. However SO_2 is highly toxic and special care is needed in its handling which can increase the cost of method. Recent literature shows that there is very limited interest in this method.

1.6.4. Ammonia explosion pretreatment

The concept of Ammonia Fiber Explosion (AFEX) pretreatment is similar to steam explosion. In AFEX pretreatment, biomass is treated with liquid anhydrous ammonia at moderate temperatures (90–100°C and pressure 17–20 bar) for 5-10 minutes (Jorgensen et al., 2007) and then the pressure is rapidly released. In this process, the combined chemical and physical effects such as lignin solubilization, hydrolysis of hemicellulose, cellulose decrystallization and increased surface area takes place which enables nearly complete enzymatic conversion of cellulose and hemicellulose to fermentable sugars (Mosier et al., 2005). In this process, nearly all of the ammonia can be recovered and re-used whereas the remaining serves as nitrogen source for microbes in downstream processes (Dale et al., 1982). It is also characterized by high yield and there is no need of neutralization after pretreatment.

In Ammonia Recycled Percolation (ARP) process, ammonia solution passes through biomass at elevated temperatures (150–170^{0}C) and a fluid velocity of 1 cm/min with residence time of 14 minutes and then ammonia is separated and recycled. Under these conditions, aqueous ammonia causes depolymerization and cleavage of lignin-carbohydrate linkages. The ammonia freeze explosion pretreatment reduces lignin content and removes some hemicellulose with reduction in crystallinity of cellulose. Liquid ammonia causes cellulose swelling and

a phase change in the crystal structure from cellulose I to cellulose III. Alizadeh et al (2005) reported a six-fold increase in enzymatic hydrolysis yield and about 2.5-fold increase in ethanol yield of switch grass after this pretreatment. Thus this pretreatment has several advantages, such as near quantitative ammonia recovery, avoidance of any wash stream, long shelf life, low degradation of sugars, and low inhibitor formation. However, cost of ammonia and especially of ammonia recovery makes this pretreatment expensive.

1.6.5. CO_2 explosion treatment

Analogous to steam explosion and AFEX pretreatment, carbon-dioxide pretreatment has also been investigated with lignocellulosic biomass. For sugarcane bagasse, CO_2 explosion was found to be more economical than AFEX (Sun and Cheng 2002). When compared to steam explosion, it was found to produce less amount of inhibitory compounds. Kim et al (2001) have shown that in the presence of water, supercritical CO_2 can efficiently improve the enzymatic digestibility of hardwood as well as softwood. One step pretreatment by CO_2 explosion and enzymatic hydrolysis simultaneously have also been studied (Park, Ryu and Kim, 2001) and 100% glucose yield was claimed.

However, Zheng et al (1995) reported his experimental results which indicated that subcritical carbon dioxide is less effective than supercritical. A reason for such retardation in subcritical carbon dioxide is likely to be low diffusion in liquid carbon dioxide. In comparison with supercritical temperatures, carbon dioxide molecules at subcritical conditions find it relatively hard to penetrate the pores in the cellulosic structures. The higher pressure of carbon dioxide resulted in the higher glucose yield, which indicates that higher pressure is desirable for faster penetration of the carbon dioxide molecules into the cellulosic pores.

This method has several advantages such as availability at relatively low cost, non-toxic nature, nonflammability, easy recovery after extraction, and environmental acceptability (Zheng and Tsao, 1996). However, the effectiveness of this method is debatable; according to one report (Wyman, 1999) this method is not very encouraging, while another report claims supercritical CO_2 pretreatment to be effective in improving cellulose hydrolysis (Zeng et al., 1998). For industrial application this supercritical CO_2 process may prove to be too expensive and cost factors will dictate the future course of action.

1.6.6. Wet explosion pretreatment

In wet explosion, an oxidizing agent like hydrogen peroxide is introduced during pretreatment and it is subjected to sudden explosive decompression. The plant biomass thus obtained has lost some of the lignin and hemicellulose (Jorgensen et al., 2007). Part of the lignin is degraded, and part of the hemicellulose is oxidized. This method appears to have potential for further development into a viable pretreatment.

1.7. Chemical pretreatments

Chemical pretreatments are the most obvious methods of preparing the lignocellulose biomass substrate for hydrolytic study by enzymes, due to the large number of well studied reactions between individual components of the lignocellulosic structure and reactive chemicals like mineral acids, Lewis acids, strong alkali, divalent alkali like lime, weak base like ammonia, alkaline peroxides, strong oxidizing agent like ozone, organosolv or extractive solvent (Hu et al., 2008; Jorgensen et al., 2007; Himmel et al., 2007; Mosier et al., 2005; Galbe and Zacchi, 2002; Sun and Cheng, 2002; Zeng et al., 1998; Vlasenko,1997; Dien and Nguyen, 2005; Bjerre et al., 1996) are well investigated, and all these method have potential

for being adapted for commercialization. As always, the final choice will depends on the specification of products which are desired.

1.7.1. Pretreatment with mineral acids

Dilute mineral acid pretreatment have been found to be both effective and economical. These dilute acids hydrolyze the hemicellulose to their constituent pentose and hexose sugar, thereby disrupting the tight complex between lignin, hemicellulose and cellulose.

Dilute acid pretreatment has been extensively studied and typically employs low concentration range between 0.5–2% H_2SO_4 at the temperature of 160–220 ^oC to remove hemicelluloses and enhance cellulase digestion of cellulose (Willfoer et al., 2005). However, time, temperature and acid concentration have to be optimized for each type of plant biomass as the pentose sugar (like xylose) gets converted to furfural which acts as an inhibitor for enzymes. Concentrated acid, especially sulphuric and hydrochloric acid have also been investigated. These acids hydrolyze the cellulose component but cause degradation of pentose sugar of hemicellulose.

Acid pretreatment has some limitations including corrosion that requires expensive corrosion resistant reactors (Sun and Cheng, 2002). The acid needs to be recovered or neutralized before the sugars proceeds to fermentation. Disposal of neutralized salt are also problematic (Hinman et al., 1992; Wooley et al., 1999).

1.7.2. Pretreatment with alkali

Alkali pretreatment especially with NaOH takes advantage of its reaction with lignin leading to delignification. Alkali pretreatment methods are similar to the kraft paper pulping methods. The major effect of the alkaline pretreatment is the removal of lignin from the biomass, thus improving the reactivity of the remaining polysaccharides towards enzyme accessibility. In addition, alkali pretreatments

remove acetyl and the various uronic acid substitutions on hemicellulose, making it easier for enzymes to attack the hemicellulose polymer molecule (Chang et al., 2001). Alkali pretreatment processes utilize lower temperatures and pressures compared to other pretreatment methods. Other alkali reagents such as potassium, calcium, and ammonium hydroxide can also be used as reactants for alkali pretreatments.

Pretreatment of lime has also been investigated (Hu et al., 2008; Jorgensen et al., 2007). Lime pretreatment removes amorphous substances (e.g., lignin and hemicellulose) which increase the crystallinity index of cellulose. However, incorporation of some calcium into the lignocelluloses biomass cannot be avoided, and some calcium is converted to salt that is difficult to recover. However lime is an inexpensive, safe to handle and non corrosive reagent, so these factors may favour its use in particular situation.

An improved version of this pretreatment is the utilization of oxygen under alkaline conditions. An oxidative lime treatment (Chang et al., 2001) and other wet-oxidations (Lissens et al., 2004) have been shown to improve the effectiveness of this pretreatment technology especially for woody biomass.

1.7.3. Pretreatment using organosolv as extractive solvent

In organosolve pretreatment, lignocellulosic biomass are treated with organic solvents like methanol, ethanol, acetone, ethylene glycol etc. in the presence of dilute mineral or organic acids to penetrate the biomass structure and break the lignin bonds with the hemicellulose. This pretreatment is also carried out in combination with water i.e. ethanol/water system (Pan et al., 2006), water/acetone (Hasegawa et al., 2004), methyl isobutyl ketone/ethanol/water system (Bozell et al., 1995). Organosolv gives enhanced solubilizing properties due to the organic

component. For economic reasons, the use of low-molecular-weight alcohols such as ethanol and methanol has been favored over alcohols with higher boiling points such as ethylene glycol and tetrahydrofurfuryl alcohol. Since ethanol inhibits hydrolytic enzymes, it should be removed completely before enzymatic hydrolysis. The main advantage of the use of solvents over other chemical pretreatments is that relatively pure, low molecular weight lignin is recovered as a by-product (Sun and Cheng, 2002). However negative feature of this methodology is that residual solvents inhibit the action of enzymes.

1.7.4. Pretreatment with oxidant like ozone and hydrogen peroxide

Pretreatment of lignocellulosic materials with ozone, referred as "ozonolysis" pretreatment. This pretreatment can effectively solubilize lignin and part of hemicellulose while cellulose molecules remain predominantly unaffected. The main parameters to be controlled for improved efficiency of ozonolysis pretreatment are moisture content of the sample, particle size, and ozone concentration. Pretreatment of sugarcane bagasse with hydrogen peroxide greatly increases its accessibility towards enzymes. About 5% of lignin and major fraction of hemicellulose were solubilised by 2% hydrogen peroxide at 30 ^{0}C within 8 hours, leading to 95% yield of glucose from cellulose using appropriate cellulases (Sun and Cheng, 2002). Similarly, combination of peroxides with acetic acid removed almost 97% lignin from sugarcane bagasse, keeping major fraction (62%) of hemicellulose intact which results in 93.5% of hydrolysis (He Tan et al., 2010). However, ozonolysis is generally expensive since a large amount of ozone is required. Also health hazards due to exposure to ozone are another factor that diminishes its important for large scale application.

1.8. Microwave-chemical pretreatment

It has been suggested that microwave-chemical pretreatments is more effective than conventional heating with chemicals since the reaction is reported to accelerate during this process (Zhu et al., 2005). Microwave chemical treatments such as microwave/alkali, microwave/acid/alkali, and microwave/acid/alkali/H_2O_2 have been studied for rice straw and it has been observed that xylose could be recovered by microwave/acid/alkali and microwave/acid/alkali/H_2O_2 treatments and not alone by microwave/alkali treatments. Microwave/acid/alkali/H_2O_2 treatments give the highest rate of enzymatic hydrolysis for rice straw (Zhu et al., 2006). However, no technologies have been demonstrated with such system.

1.9. Biological pretreatments

Physical and thermo-chemical methods generally required expensive reactors or equipment requiring high energy inputs. Biological pretreatment, the other hand, use various types of fungi under ambient condition with low energy requirements and is a safe and environmentally friendly method. In biological pretreatment, microorganisms such as brown-, white-, and soft-rot fungi are used to degrade lignin and hemicellulose of plant biomass (Galbe and Zacchi, 2007). Brown rots mainly degrade cellulose, whereas white and soft rots fungi attack both cellulose and lignin. Cellululase-less mutant of white-rot fungi have also been developed and used for delignification reaction without losing cellulose (Sun and Cheng, 2002). Other enzymes like laccases, polyphenol oxidases, hydrogen peroxide producing enzymes and quinine-reducing enzymes also degrade lignin thereby enriched polysaccharides fraction in biomass. Some bacteria can also be used for biological pretreatment of lignocellulosic materials. Kurakake et al (2007) studied the

biological pretreatment of office paper with two bacterial strains, *Sphingomonas paucimobilis* and *Bacillus circulans* for enzymatic hydrolysis, and obtained up to 94% sugar by combining these strains. Biological pretreatment in combination with other pretreatment such as organic solvent treatments has also been studied to reduce the energy input required for the separation of wood components. The biological pretreatments saved up to 15% of the electricity needed for ethanolysis (Itoh et al., 2003). It has been shown that treating rice straw with white-rot fungi *Pleurotus ostreatus* followed by AFEX treatments gave significantly higher glucan and xylan conversions under mild AFEX conditions than required directly for AFEX pretreatment (Balan et al., 2008). The biological pretreatments are also useful for removal of specific components such as antimicrobial substances (Srilatha et al., 1995).

Low energy requirement, no chemical requirement and mild environmental conditions are the main advantages of biological pretreatment. However, the treatment rate is very low in most biological pretreatment processes (Sun and Cheng, 2002), and due to this drawback no large scale technologies have been feasible.

1.10. Pulsed-Electric-Field pretreatment (PEF)

Using high field strengths in the range of 5-20 kV/cm, plant cells can be significantly ruptured. By applying electric pulses with high field strengths, PEF pretreatment can create permanent pores in the cell membrane and hence facilitate the entry of acids or enzymes used to break down the cellulose into its constituent sugars. Pulsed-electric field (PEF) pretreatment involves the application of a short burst of high voltage to a sample placed between two electrodes. The advantages of PEF pretreatment is that it can be carried out at

ambient conditions and energy use is low because pulse times are very short (100 μs). Additionally, very simple equipment is needed (Kumar et al., 2009). However, this research is in a preliminary stage.

1.11. Inhibitor produced during pretreatments

The drastic condition of some pretreatments leads to the formation of several compounds which act as inhibitors for hydrolytic enzymes. These compounds are formic acid, acetic acid, furfural, 5-hydroxymethyfurfural, syringaldehyde, 4-hydroxy-benzaldehyde, levulinic acid and vanillin (Cantarella et al., 2004). Therefore, pretreatments conditions should be adjusted to minimize inhibitor formation and detoxification methods should be simple. Table 1.2 gives the effects of various pretreatments on biomass components. An effective pretreatment is characterized by several criteria which includes, i) avoidance of the need for reducing the size of biomass particles, ii) preserve the cellulose and hemicellulose fractions, iii) limit the formation of degradation products that may inhibit fermentative microorganisms, and iv) minimize the energy demand. Consequently, over the last few years, numerous pretreatment approaches have been investigated. The major conclusion is that almost all pretreatment systems can be gainfully utilized alone or in combination with other systems, if we understand thoroughly the structure of biomass so produced.

Table 1.2: Effect of various pretreatment on lignocellulosic components (Mosier et al., 2005)

Pretreatment	Cellulose	Hemicellulose	Lignin
Dilute acid	Some depolymerization	80–100% solubilization, primarily to monomers	Little or no solubilization, extensive redistribution
Steam explosion	Some depolymerization	80–100% solubilization to a mixture of monomers oligomers, and degradation products	Little or no solubilization, extensive redistribution
Hydrothermal processes	Some depolymerization	80–100% solubilization,	No solubilization
Organic solvents with water	Some depolymerization	Substantial solubilization (varies but can be nearly complete)	Substantial solubilization (varies but can be nearly complete)
AFEX	Some decrystallization	Solubilization from 0 to 60% depending on moisture; ~90%hydrolyzed to oligomers	Some solubilization (10–20%)
Sodium hydroxide pretreatment	Substantial swelling, type I 3 type II	Substantial solubilization (~50%)	Substantial solubilization (~50%)
Lime pretreatment	---	Significant solubilization (to 30%) under some but not all conditions	Partial solubilization (~40%)

1.12. Hydrolysis of lignocellulosic biomass

Following the pretreatments of lignocelluloses, hydrolysis is carried out to break down cellulose and hemicelluloses into fermentable sugars such as glucose and xylose. Strong acids such as sulfuric acid and halogen acids are capable of hydrolyzing a wide variety of lignocelluloses into simple fermentable sugars (Wyman, 1996). Enzymatic hydrolysis is an environmentally friendly alternative that involves using carbohydrate degrading enzymes (cellulases and hemicellulases) to hydrolyze lignocelluloses into fermentable sugars.

1.12.1. Enzymatic hydrolysis of celluloses

In hydrolysis of cellulose, the cellulose is converted into glucose sugars as,

$$(C_6H_{10}O_5)_n + nH_2O \longrightarrow n\,(C_6H_{12}O_6)$$

This reaction is catalyzed by dilute acid, concentrated acid, or enzymes (cellulases). Without any pretreatment the enzymatic hydrolysis are yields typically < 20 % of sugar whereas yields after pre-treatment often exceed 90 %.

Enzymatic hydrolysis of cellulose is typically carried out by cellulases. Unlike conventional hydrolysis using concentrated acid or alkaline reagents, enzymatic hydrolysis requires mild conditions. Cellulases are a group of hydrolytic enzymes capable of converting insoluble cellulose to glucose and produced by several bacterial and fungal species. These enzymes are produced as a cellulase system of several distinct enzymes by microorganisms, plants and animals.

The system has traditionally been assigned three types of enzymes including endoglucanases (endo-1, 4-β glucanases or 1, 4- β-D-glucan 4-glucanohydrolases), cellobiohydrolases (exo-1, 4 β-glucanases or 1, 4-β-D-glucan cellobiohydrolases) and cellobiases (β-glucosidases or β-D-glucoside glucohydrolases (Sarkka and

Suominen, 1996). Generally endoglucanases randomly hydrolyze the 1, 4- β-bonds within cellulose molecules, there by producing reducing and nonreducing ends. Cellobiohydrolases (exogluconases) cleave cellobiose units from the nonreducing ends of cellulose polymers and cellobiases hydrolyze cellobiose to the single monomer sugar, glucose. Commercially available preparations of cellulases are mixtures of the three types of enzymes described above. Celluloytic enzymes isolated from various sources differ in their molecular characteristics (molecular weight, amino acid composition and sequence, isoelectric point, carbohydrate content, adsorbability onto cellulose, catalytic activity and substrate specificity. Further, cellulase components from any organism occur in a number of forms, thereby creating an almost infinite number of potential cellulase systems. Each system is different in its composition and catalytic characteristics in terms of ability to hydrolyze cellulose. The individual components of a cellulase system combine to act synergistically toward insoluble cellulose. That is, the action of two or more individual cellulolytic components is greater than the sum of the action of each component alone. At present, mutant strains of *Aspergillus niger* and *Trichoderma viridae* are amongst the best available commercial sources of cellulases (Ashadi, 1996). Excellent commercial cellulases are also available from Genencor International like Accellerase®DUET, Accllerase®1500, Accellerase®XY Accellerase®XC (Genencor) and from Novozymes such as Cellic®, CTec2 and HTech2 (Novozymes).

1.12.2. Enzymatic hydrolysis of hemicelluloses

Similarly, hemicellulose (xylan) hydrolysis involves three main types of enzymes, endo-β-1-4-xylanase which primarily targets the internal β-1-4 glycosidic bonds between xylose units, exoxylanase that releases xylobiose units and β-xylosidase

that releases xylose from xylobiose and short chain xylooligosachharides. Also, there are several ancillary enzymes that are responsible for cleaving side-groups. These include α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, ferulic acid esterase, and p-coumaric acid esterase. Hemicellulose hydrolyzing systems also act as synergistically as was the case with celluloses. The number of enzymes required for hemicellulose hydrolysis is much greater than for cellulose hydrolysis, but accessibility towards the substrate is significantly easier since xylan does not forms tight crystalline structures like cellulose (Gilbert and Hazlewood, 1993).

1.13. Cellulase and xylanase production methods

Traditionally, these hydrolytic enzymes are produced using the submerged fermentation (SmF) method in which the cultivation of microorganisms occurs in an aqueous solution containing nutrients. An alternative to the traditional SmF method is the solid state cultivation (SSC) method which involves the growth of microorganisms on solid materials in the absence of free liquids (Cannel and Young, 1980). During the last few years, renewed interest in SSC has developed due to the recognition that many microorganisms including genetically modified organisms (GMO) may produce their products more effectively by SSC (Pandey et al., 1999). The enzymes produced by SmF must be concentrated and freeze-dried before usage because of liquid cultivation (it uses large volumes of water). However, the enzymes produced by SSC do not require concentration because of its solid cultivation and can be used on site. Thus, generally the SSC process is simpler and less expensive than the SmF process. Table 1.3 gives details of some cellulosic substrate used for cellulase productions. In these processes, the cost of the carbon source has been estimated to be the major contribution to cellulase

production cost. A successful commercial enzymatic hydrolysis process for cellulose must be cost efficient. Various purified celluloses, such as Avicel, Solka Floc, etc., have been used as carbon source for cellulase productions (Domingues et al., 2000., Esterbauer et al., 1991) but these are too expensive to be applied in large scale processes.

Table: 1.3: Some cellulosic substrates and organism used for enzyme production

Substrate	Organism(s)	references
Pure culture of cellulolytic microorganism (anaerobic)		
Avicel	*ClostridiumThermocellum , Ruminococcus albus, Methanobrevibacter Smithiti, Piromyces sp. Methanobacteriumformicu*	Lynd et al.,2001 1998,1997, Pavlostathis et al., 1988
Sigmacel	*Ruminococcus flavefaciens, Fibrobacter succinogenes (Selenomonas ruminantium or Streptococcus bovis*	Chen et al., 2001 Weimer.,1993 Shi and Weimer, 1992
Ball-milled filter paper	*Ruminococcus flavefaciens, Fibrobacter succinogenes, Ruminococcus albus, Clostridium polysaccharolyticum*	Kistner and Kornelius,1990 1983 Morrison, Mackien and Kistner, 1990
Bagasse	Aspergillus flavus	Ojumu Tunde

		Victor et al., 2003
Bagasse	*T. reesei*	Aguiar, 2001
corncob	Aspergillus flavus	Ojumu Tunde Victor et al., 2003
Sawdust	Aspergillus flavus	Ojumu Tunde Victor et al., 2003
sugar beet cellulose	*Trichoderma reesei* Rut C-30	Olsson et al., 2003
corn stover	Fusarium oxysporum	Panagiot et al.,2003
Pure culture of cellulolytic microorganism (Aerobic)		
Avicel	*Thermomonospora* sp. strain N-35	Meyer and Humphrey,1982
Solka Floc	*Trichoderma reesei*	Hendy et al.,1984
Purified powdered cotton	*Trichoderma reesei*	Ghose and Sahai, 1979
Ball-milled wood	*Trichoderma viride (reesei)*	Pietersen, 1977
Pure cultures of noncellulolytic microorganisms with added cellulase		
Delignified rice straw	*Pichia stipiti*	Hoshino et al.,1997
Pretreated wood	*Saccharomyces cerevisiae*	South and Lynd, 1993
Paper sludge	*Saccharomyces cerevisiae*	Fan and Lynd, 2007

1.14. Applications of cellulases and xylanases

1. Cellulases and hemicellulases are the most important groups of enzymes which are employed in the processing of lignocellulosic polysaccharides materials for the productions of biofuels and biobased chemical feedstocks.
2. Cellulase can improve the appearance of cotton fabric by removing small fibers created during washing and wearing.
3. Cellulase is one of the important components in enzyme blends used for formulating environmentally-friendly detergents by replacing surfactants and builders.
4. Cellulase is also applied for cost reduction in paper production processes by reducing refining energy (Sharyo and Masaki, 2009).
5. Xylanase in synergism with several other enzymes, such as mannanase, ligninase, xylosidase, glucanase, glucosidase, etc., can also be used for the generation of biofuels, and xylitol from lignocellulosic biomass (Dominguez, 1998; Olsson and Hahn-Hagerdal, 1996).
6. The most promising application of xylanases is in the prebleaching (biobleaching) of pulp. Enzyme application improves pulp fibrillation, water retention and selective removal of xylans from dissolving pulps (Bajpai, 1999).
7. The xylanases are also used in bakery for improving the quality of bread which increases in specific bread volume.
8. Incorporation of xylanase into a rye-based diet of broiler chickens results in reduced intestinal viscosity, thus improving both the weight gain of chicks and their feed conversion efficiency (Bedford and Classen, 1992).
9. α-L-Arabinofuranosidase and β-D-glucopyranosidase have been used in food processing for aromatizing musts, wines and fruit juices (Spagna et al., 1998).

1.15. Cellulose isolation

Cellulose can be extracted from lignocellulosic by-products using bacteria, fungi, mechanical and chemical methods. To extract cellulose from biomass, common chemical reagents used are alkali and mild acids. In recent years some ionic liquids have also been used for clean extraction of cellulose from biomass in which only cellulose fraction is dissolved in ionic liquids and can be separated by regeneration. Sodium hydroxide is the most commonly used alkali for cellulose extraction. Cellulose can be extracted before or after pretreatment, but before pretreatment other component e.g. hemicellulose and lignin may degrade and are difficult to isolate. Acid such as sulfuric acid and oxalic acid in combinations with detergent have also been used for cellulose extraction (Henriksson et al., 1997; Doraiswamy and Chellamani, 1993). For enzymatic extraction, a combination of enzymes such as hemicellulases, cellulases and pectinases are generally used with a pre- or post-chemical pretreatment of lignocellulose. Multienzyme complexes that can express 10–15 enzyme activities and provide better cellulose quality have also been developed (Hans Sejr, 2004). In general pure cellulose can be isolated from lignocellulosic biomass by various chemical and biochemical treatments. Traces of lignin in the cellulose are generally removed by bleaching processes to obtain cellulose with high α-cellulose content and > 90% brightness. Chemical concentrations, reaction time and temperature are the main factors which determine the quality of isolated cellulose. These processes are explained in detail in the Encyclopedia of Chemical Technology (Kirk-Othmer Encyclopedia of Chemical Technology, Kadla, J. F and Dai, Q., John Wiley & Sons, Vol. 21, 2006, pp-1-47).

1.16. Oxidative modification of cellulose

Chemical modification of cellulose via oxidation leads to products having several desirable properties and interesting applications. Oxidised celluloses have been used in tissue engineering scaffold ((Verma et al., 2008), antimicrobial cellulose (Hou et al., 2008), wound healing material (Finn, 1992), antitutumor material, inmunostimulant (Tokunaga, 1998) adhesion-prevention material (Jelinkova, 2002) and as a precursor for cellulosic derivatives (Chavan, V. B., 1995). Oxidized cellulose is also used in adsorption of Zwitterionic drugs from aqueous and organic solution (Dmitry et al., 2004).

1.16.1. Oxidation of cellulose

Due to availability of one primary and two secondary reactive hydroxyl groups on each anhydroglucose unit of cellulose, a number of chemical modifications are possible. These three hydroxyl groups can undergo chemical reactions typical for all primary and secondary alcoholic groups, such as esterification, nitration, etherification, and oxidation. The reactivity of these hydroxyl groups are in order, C-1 (primary) > C-2 > C-3 (secondary). During cellulose oxidation, aldehyde, ketone, and carboxyl groups may form on the cellulose chain, depending on the nature of the oxidizing agent, its specificity, and conditions of oxidation (Calvini et al., 2004). In oxidation of cellulose the major problem is the difficulty of producing oxidised cellulose which is homogeneous in chemical structure. Several of the oxidants employed are apparently not selective as to particular hydroxyl groups of the anhydroglucose unit in the cellulose molecules. When the oxidation is mild, the products usually consist of an oxidized portion and an unchanged residue of unreacted or only slightly modified cellulose. More drastic oxidation condition leads to degradation of cellulose. Therefore, it is necessary to control the whole oxidation process very carefully.

1.16.2. Oxidation of C-2—C-3 hydroxyl group

C-2-C-3 bond of anhydroglucose unit are selectively oxidized by sodium metaperiodate by Malaprade reaction to enrich carbonyl groups on cellulose chain (Malaprade, 1928). The product of the reaction is also known as dialdehyde cellulose (DAC) since two aldehyde groups are formed as shown in figure 1.7. This oxidation occurs through diol cleaving mechanism proposed by Buist and Bunton (1954) in which the hydroxyls reacts with periodate and formation of cyclic periodate ester takes place which subsequently undergoes rearrangements resulting in cleavage of C-C bond and formation of C=O bonds as shown in figure 1.8.

This dialdehyde cellulose can be used as starting material for various cellulosic derivatives (acids, imines, oximes) such as 2-3 dicarboxycelluose, 2-3-dihydrazone and 2-3 dioxime cellulose which may have many interesting applications. This oxidation causes significant changes in the properties of cellulose. The periodate oxidation is accompanied by decrease in crystallinity (Varma et al., 1997). Ung-Jin Kin (2000) also made similar observations when highly crystalline cellulose from *Cladophora sp* was treated with metaperiodate. Besides the decreases in crystallinity there are uneven distributions of aldehyde groups as shown by Calvini et al (2006). It has been proposed that the oxidation proceeds heterogeneously.

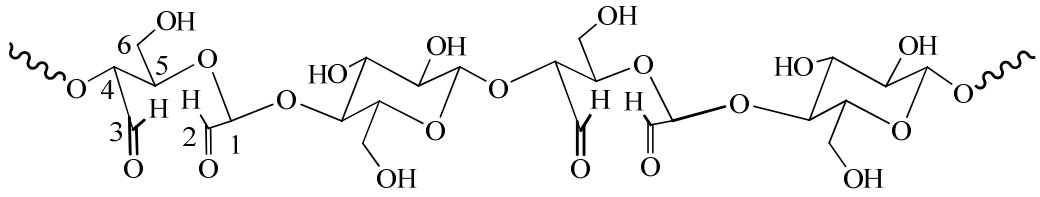


Figure 1.7: Structure of partially oxidized 2, 3-dialdehyde cellulose (DAC)

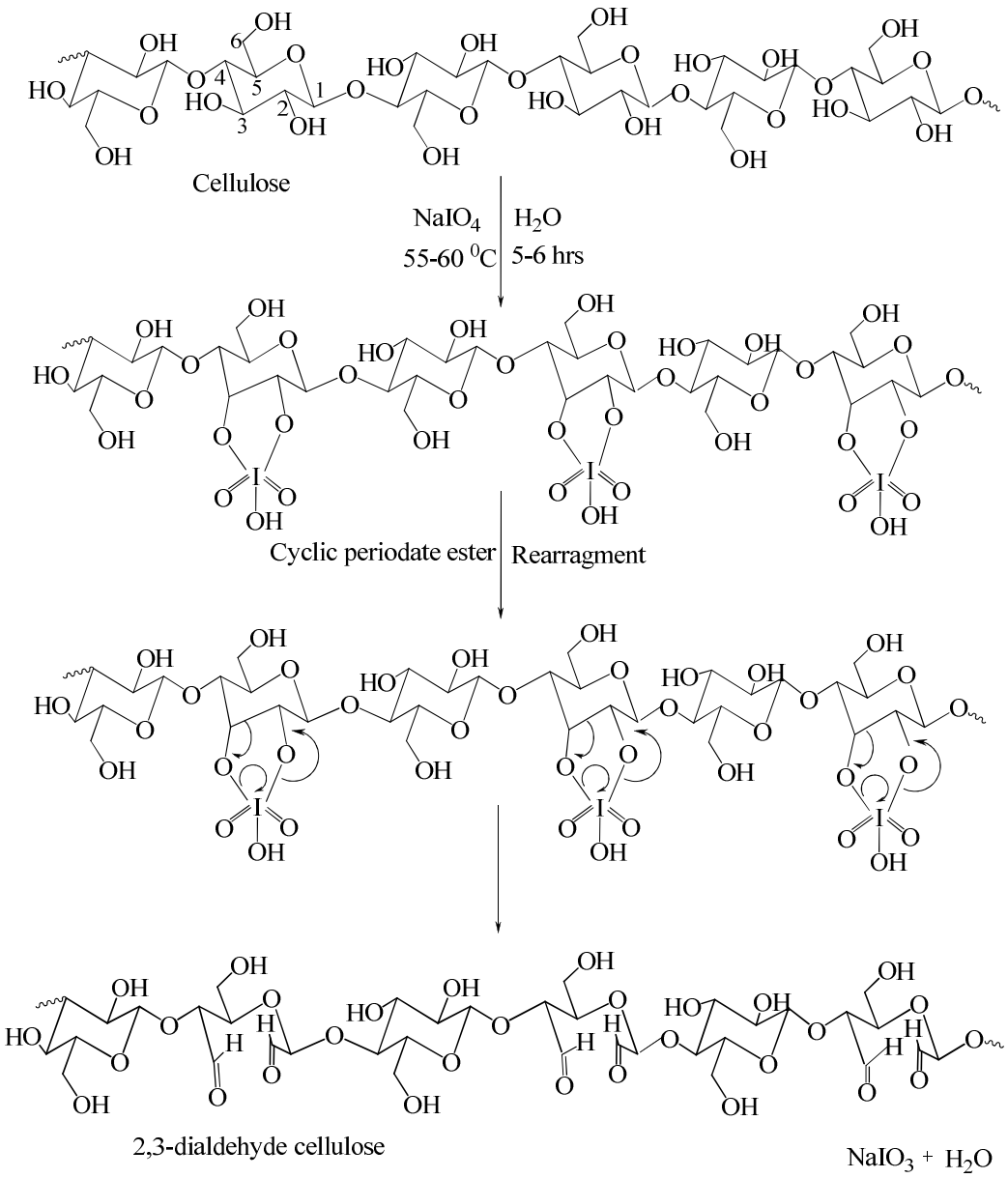


Figure 1.8: Mechanism of periodate oxidation of cellulose (partial oxidation)

1.16.3. Oxidation of primary hydroxyl group

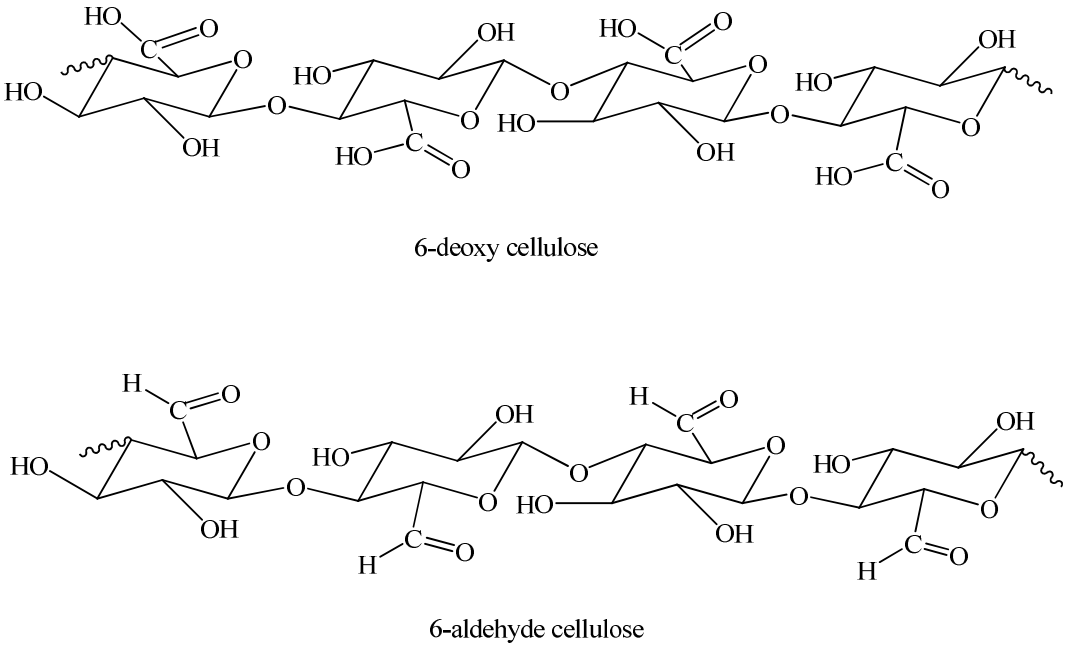


Figure 1.9: Structure of 6-deoxy and 6-aldehyde cellulose

Oxidation of primary hydroxyl of cellulose leads to 6-carboxy cellulose which is also known as cellouronic acid. Oxidized cellulose containing carboxyl group mainly at primary position represents an important class of biodegradable polymers. Primary hydroxyl oxidations of cellulose have been well studied and a number of methods to produce them are available (Maurer and Reiff 1943; Nieuwenhuizen et al., 1985; Davis et al., 1993). Catalytic oxidation of cellulose by 2, 2, 6, 6-tetramethyl-1-piperidinyloxy radical (TEMPO) has been reported to effectively convert C-6 primary hydroxyl group to carboxylate via a reactive aldehyde-intermediate (Saito et al., 2006). In this oxidation, nitrosonium ion (the oxidized form of TEMPO) is the oxidizing species which is generated in situ by the reaction between TEMPO with other oxidants such as hypo bromide ions. The

generation of hypobromide ions takes place from bromide salts and sodium hypochlorite. After oxidizing the nitrosonium ion, it converted into N-hyroxy-2, 2, 6, 6-tetramethyl piperidine, the reduced form of TEMPO. The detail catalytic oxidation pathway is shown in figure 1.10. Selective oxidation of primary alcohol of cellulose and other polysaccharides by using TEMPO-Lacasse system was patented by Jetten et al., 2000 and Viikari et al., 1999. TEMPO–NaBr–NaClO system was applied for the water soluble polysaccharides like amylodextrin, pullulan and potato starch and corresponding polyuronic acid obtained qualitatively (Jiang and Ragauskas, 2005; Bragd et al., 2004 and de Nooy et al., 1995).

Other oxidizing agents used to oxidized primary hydroxyl of cellulose are nitrogen tertraoxide (N_2O_4) or nitrogen dioxide (NO_2). NO_2 and N_2O_4 exist in equilibrium and can be used in gaseous or solution form in an appropriate organic solvent (Ashton.1968). Similarly $H_3PO_3/NaNO_2/NaNO_3$ (Besemer et al., 1998), H_3PO_4/NO_2 (Bertocchi., 1995) N_2O_4/dichromate (Walimbe et al., 1978), $H_3PO_4/NaNO_2$ (Painter., 1977), $HNO_3/H_2SO_4/NaNO_2$ (Wanleg H., 1956) was also applied. Oxidation of primary hydroxyl of cellulose to C-6-aldehyde can be obtained by photolysis of the 6-azido-6-deoxy cellulose (Clode and Horton, 1971).

The carboxyl groups on cellulose are of major interest in the field of medical application such as wound healing, inmunostimulant, antitumor and adhesion-prevention (Finn., 1992; Tokunaga and Naruse, 1998; Jelinkova et al., 2002; Wiseman, 2002). A commercially available hemostat Surgicell™ (Ethicon, Inc.: Somerville, NJ, USA) is used for various surgical procedures, and are made from oxidation of primary hydroxyl of cellulose into carboxylic acid cellulose (Krause, 2003). Surgicell™ are also explored for other medical application such as treatment

of skin graft donor sites and to supplement cartilage grafts (Uysal et al., 2006). Another commercial FDA approved oxidised cellulose, Interceed® (Ehticon, Inc.: Somerville, NJ, USA) are made from regenerated oxidised cellulose used in gynecological pelvic surgery (Waxman, 2000). Similarly, microdispersed oxidised cellulose hemostatic powder Seal-On™ (Alltracel Pharma Ltd, Dublin, Ireland) are used as a first aid product to treat cuts, abrasion, burns and lacerations. The presence of carboxyl group also helps in immobilizing drugs through ionic as well as covalent bonding (Yurkshtovich et al., 2002, Zhu et al., 2001).

This oxidized cellulose containing 16-24% carboxyl content is commercially available and used to stop bleeding during surgery. It is also used to prevent the formation and reformation of post-surgical adhesion (Johnson & Johnson Patient Care Inc., 1989). Oxidized cellulose having carbonyl content as low as 3% used as bioabsorbable materials (Ashton, 1968). It has been found that the hemostatic and absorption properties of oxidized cellulose increase with increasing carboxyl contents. Thus oxidiesd cellulose has huge potential as a medicinal material and further functionalizations and chemical modification of cellulose broadens the potential application of this biopolymer.

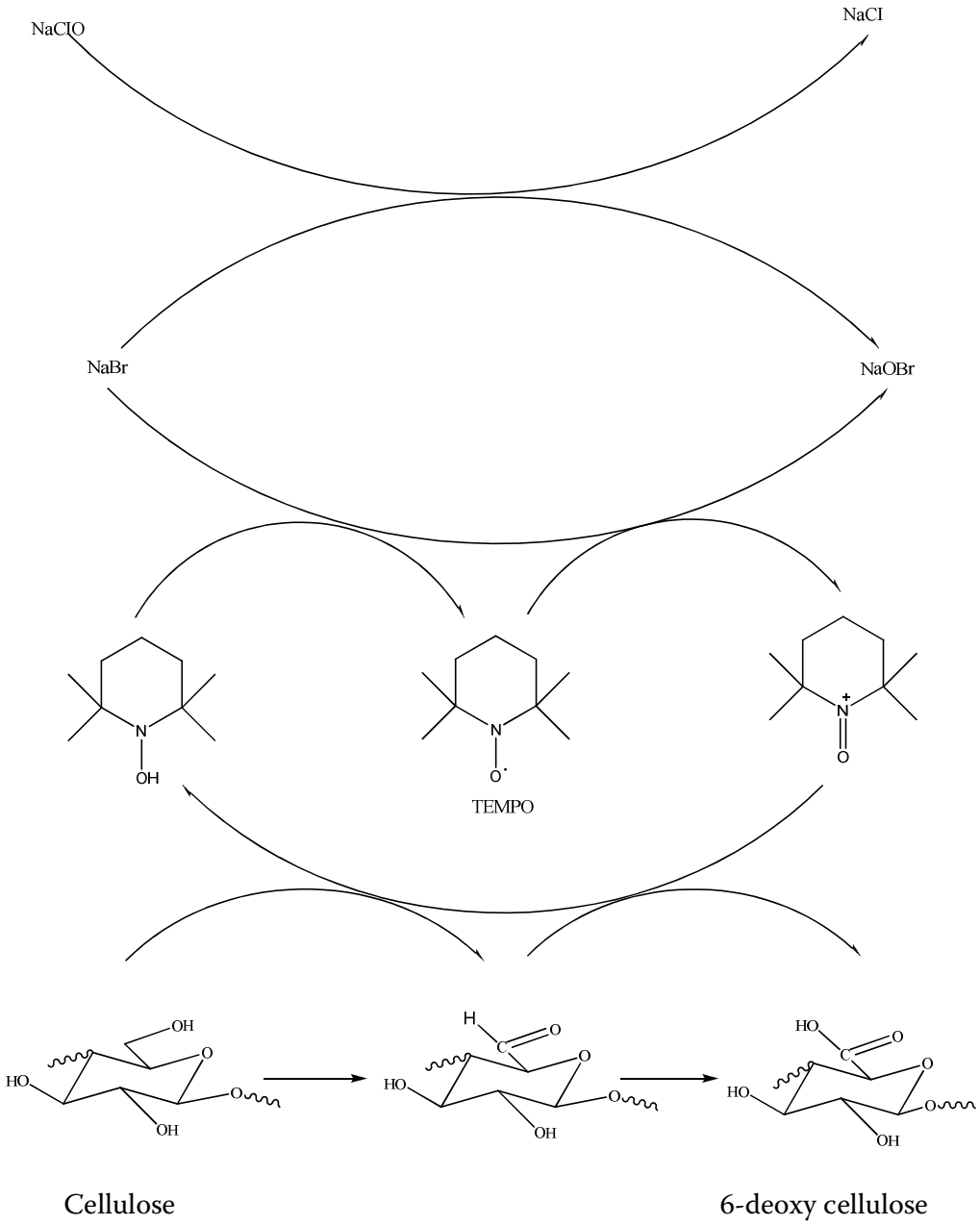


Figure 1.10: Catalytic pathway of TEMPO mediated oxidation of cellulose

1.16.4. Oxidation at C-2 /C-3 hydroxyl of cellulose

Hydroxyl groups at C-2 and/or C-3 can be oxidized to introduce keto groups on cellulose (Yalpani et al., 1984). Very few reported methods are available for oxidizing selectively the hydroxyl groups in a controlled manner either at C-2 or C-3 positions of the cellulose. Keto groups on this position may form diketo structure under alkaline condition due to keto-enol tautomerism and hence these carbonyl groups are unstable in alkaline condition as shown in figure 1.11

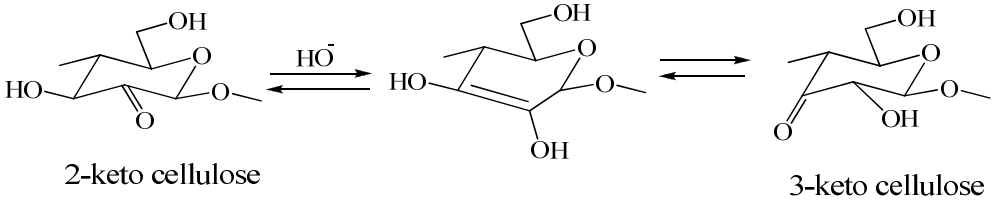


Figure 1.11: Keto-enol tautomerism in carbonyl group in keto cellulose under alkaline condition

Keto group at C-3 is considered to be less reactive than keto group at C-2 (Lewin and Mark, 1997). In alkaline condition keto groups at C-2 leads to the formation of new reducing end group while keto group at C-3 position forms non reducing by β-elimination as shown in figure 1.12. Thus depending on orientation of keto group in alkaline condition their reaction product may differ.

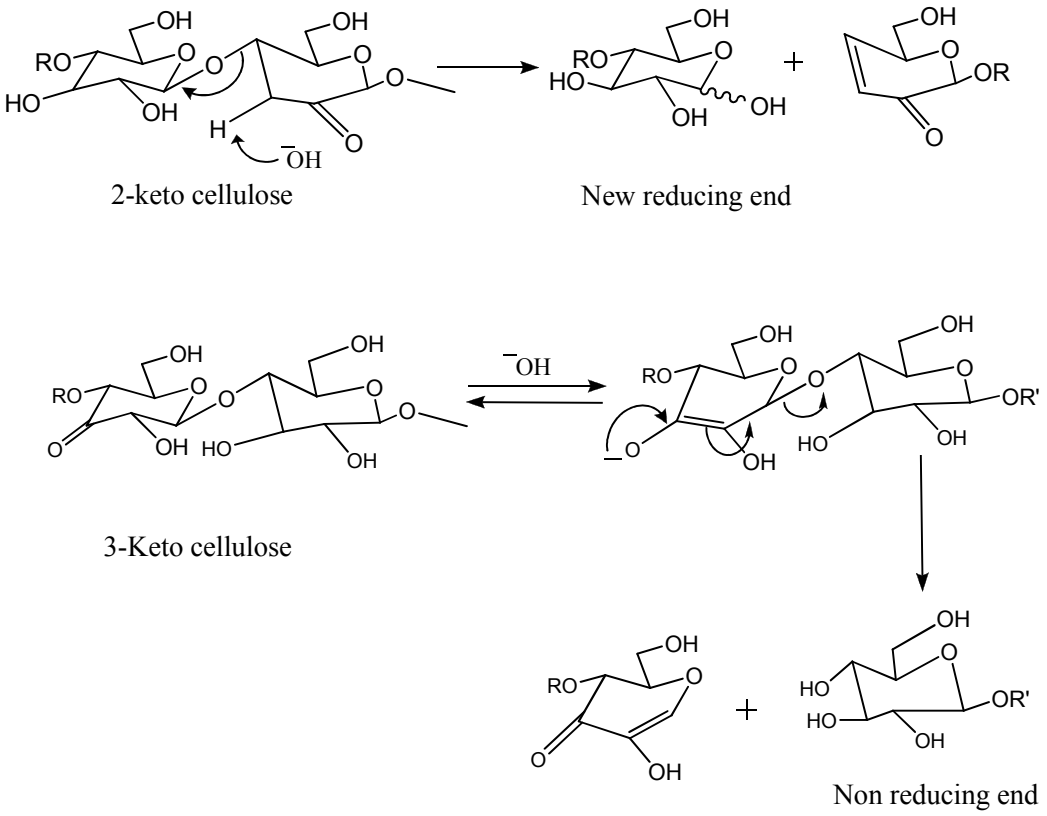
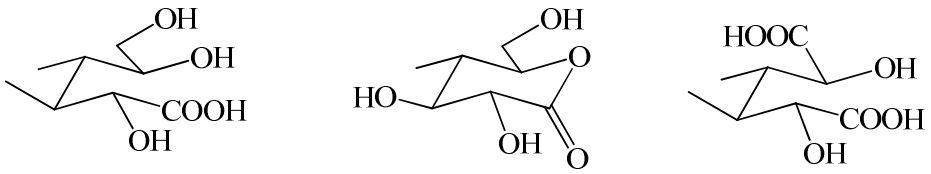


Figure 1.12: β-elimination of keto cellulose

1.16.5. Reducing end group oxidation

Oxidation of primary hydroxyl leads to glucuronic moieties and a number of methods available in literature for oxidizing primary hydroxyl to carboxyl (Jetten., 2000; De Nooy et al., 1995). For secondary hydroxyl oxidation of cellulose such as 2, 3- dicarboxycelluose can be obtained from 2, 3- dialdehyde cellulose. 2, 3-dialdehyde cellulose is first converted into sodium salt of dicarboxycelluose by sodium chlorite reaction and subsequent immersion in aqueous acidic medium leads to 2, 3-dicarboxycelluose (Chavan et al., 2002). Oxidation of reducing end group leads to gluconic acid end group as shown in figure 1.13 (Structure 1, 2,

and 3). In such oxidation ring character of pyran ring of anhydroglucose units (AGU) is destroyed and a new open chain structure is formed. A large amount of work is on-going to develop methodologies which exclusively oxidize reducing end group of cellulose for various synthetic applications such as grafting on synthetic polymers to make them biodegradable or to make hybrid polymers from combination of natural and synthetic polymers. Cellulose from *Acetobacter Xylinum* contains carbonyl group which is equal to the number of reducing group. (Klemn et al., 2001). The reducing end group in the cellulose is the only known naturally occurring carbonyl group in cellulose.



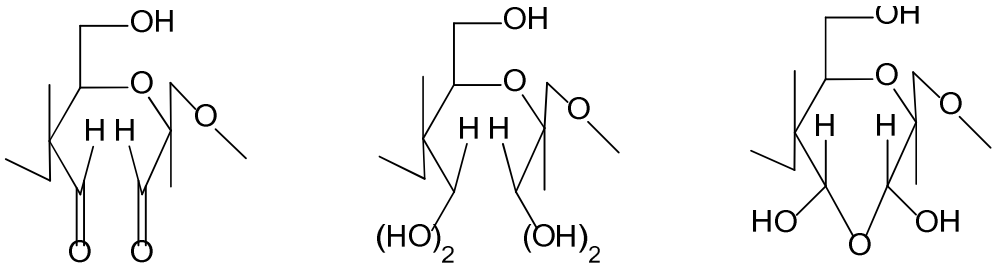
1 2 3

Figure 1.13: Structure of reducing end group of cellulose containing carboxyl group

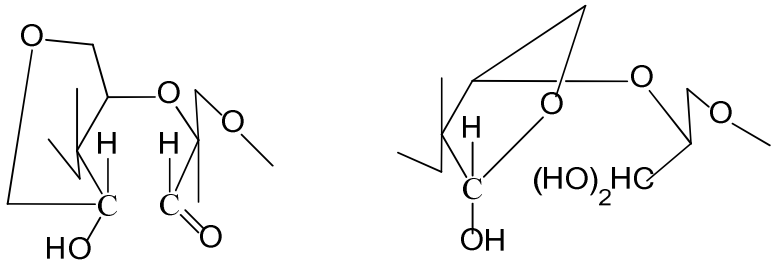
1.17. Determination of degree of oxidation

Quantitatively determination of total oxidized groups on cellulose chain and/or within anhydroglucose units (AGU) unit is a very challenging task because of its limited solubility, semi-crystalline structure and degradation of cellulose under harsh chemical conditions. In oxidiesd cellulose, oxidized groups, i. e., carbonyl/carboxyl groups are present most frequently in hydrated form, tautomric enol form, hemiacetal form etc. as shown in figure 1.14 besides the usual double bond of carbonyl/carboxyl groups which was demonstrate by various oxidized

model compound by NMR (Rohrling, 2002). This makes quantitatively determination of oxidised functionality very difficult.



Free aldehyde Hydrated aldehyde Hemialdol



Hemiacetal Mester form

Figure 1.14: Various forms of 2, 3-dialdehyde cellulose

In oxidized cellulose mechanism of hemiacetalisation and hydration states of aldehyde and keto group is largely unknown. Carbonyl groups in oxidized cellulose are hard to detect in room temperature by spectroscopic method such as FTIR spectroscopy. Similar facts were observed by Rowen (1951) even for high degrees of periodate oxidization cellulose. This is primarily due to hydration and acetalization effects, other possibilities are strong cross-linking by the formation of hemiacetals with neighboring hydroxyl group, such intra and inter molecular cross-linking (Morooka et al., 1989).

Several techniques are reported for determination of aldehyde and keto groups in oligo and polysaccharides (Potthast et al., 2003), but a majority of these techniques estimated the average number of carbonyl groups. Conventionally, copper number is the oldest method for determination of oxidized functionality of cellulose and still used in the paper industry for process control. In this method reducing power of cellulose is measured by reaction with alkaline Cu^{II} salt (blue) and the formed Cu^{I} ion (red) which can be titrated after re-oxidation (TAPPI Method T-430 om-94). Although this is a simple method, it gives only relative data and its mechanism is poorly defined. In another method, carbonyl groups can be reacted with hydroxyl amine (Cyrot, 1957) to corresponding oximes by Schiff base formation. Similarly imination can be achieved by reaction between carbonyl group with hydrazines or amines and the percentage of nitrogen can then be measured by a Kjeldahl procedure or elemental analysis. However, these methods are seldom of practical value due to non-quantitative Schiff base reactions, particularly with 2, 3-dialdehyde cellulose. A reaction of carbonyl groups with cyanide can also be taken for the formation of cyanohydrins which is used as a measure of oxidized functionalities (Lewin, 1972). Excess cyanide is determined by titration with $AgNO_3$. This cyanide method often yields too large values due to adsorption phenomena; toxicity of the reagent is another reason for its unpopularity.

Determination of dialdehyde functionality in periodate oxidized cellulose can also be based on consumption of periodate. Periodate consumed during reaction corresponds to the amount of dialdehyde group formed. A simple iodometric titration is used for this purpose (Scott, 1939). Extent of oxidation can also be done by hydroxyl ions determination by the Cannizzaro reaction (Pommering et al., 1992). In the reduction method, consumption of sodium borohydride upon reduction of carbonyl groups can also be used for its quantification; sodium

borohydride is quantified as the amount of hydrogen formed after the reaction with acid (Tihlarik and Pasteka, 1991). The β-elimination reaction of 2, 3-dialdehyde celluloses in combination with degree of polymerization by viscometry is used to roughly determine the extent of oxidation (Calvini et al., 2004). However, this viscometric technique detects only the oxidized groups randomly displaced along the chains, while it is insensitive to the overall content of dialdehyde groups. Determination of carboxyl function on oxidized cellulose can also be done via formation of salt with different cation and measurement of its anion exchange capacity which would be the same as carboxyl group presents. Cellulose carboxyl can also be determined by decarboxylation and measurement of freed carbon dioxide (Chai et al., 2003). Similarly, Clode and Horton (1971) proposed a method for quantification of C-6 aldehyde groups in substituted C-6 aldehyde cellulose derivative by reduction with $NaBD_4$ and then hydrolyzed products were analyzed by Mass spectroscopy.

Recently a variety of fluorescence labels have been introduced for detecting carbonyl groups and reducing ends in oligosaccharides e.g. 2-aminobenzamide or 9-fluorenyl-methoxycarbonylhydrazine. These fluorescence labels are often hydrazines or substituted hydroxylamines which bear a hydrophobic fluorescent group and can be detected at the picomolar level. These labels are connected directly via imine formation without the reduction step. The reducing power of aldehyde groups can be used to convert TTC (2, 3, 5 triphenyltetrazolium chloride) into triphenylformazane a red dye, which can be quantified spectrophotometrically and could be used to differentiate between aldehyde and keto groups in cellulose. By combining carbonyl detection with size exclusion chromatography (SEC) followed by multi-angle laser light scattering (MALLS) the distribution of carbonyl groups in oxidized cellulose can be obtained (Kostic et al., 2006). Fluorescent label

carbazole carbonyl oxyamine (CCOA) have been used in combination with SEC-MALLS. This method has been developed for measuring small amounts of carbonyl group in cellulose as a function of molecular weight (Potthast et al., 2003, Rohrling et al., 2002). The CCOA label has the advantage of being an O-substituted hydroxylamine which has an increased reactivity towards carbonyl groups compared to a hydrazine and thus reduction of the double bond formed upon imine formation is not necessary. This label is however, synthesized especially for the detection of carbonyl groups in cellulose in homogeneous condition particularly in N, N-dimethylacetamide (DMAc)/LiCl system and is not yet commercially available. The CCOA label contains a fluorophore and a flexible spacer and it generally gives neat and quantitative conversion of carbonyl functions into the corresponding *O*-substituted oximes. The reactive anchor group is an oxyamine. Oxyamine showed a higher reactivity towards carbonyl groups as compared to hydroxyl amines (Houdier et al., 1999). In addition to chemical methods FTIR, NMR and ESCA technique also applied for quantitative determination of carbonyl group in oxidized cellulose (Nishiyama et al., 2003; Sawatari and Nakamura, 2003). Table 1.4 summarizes the methods used for detection of carbonyl/carboxyl functionality in oxidized cellulose.

Thus, aldehyde, carboxyl, imines, hydrazones functionalities can be introduced on cellulose chain along with hydroxyl functionality which already presents by oxidation of cellulose. The detail characterization of these derivatives is important for understanding the structure, morphology, and establishing structure-property relationships in thermal, biodegradation, anti-microbial and saccharification properties and so on. The introduction of multi-functionality on cellulose can increase the range of applications of these materials.

Table 1.4: Chemical methods for carbonyl/carboxyl determination in oxidized cellulose.

Methods	Reactions	Detection methods	References
Fluorescence Label	Oximation, Imination	MALLS,GPC	Kostic et al., 2006
Copper No	Reducing power of copper	Titration	TAPPI Method T-430 om-94,1994
Hydroxyl amine	Oximation	Elemental analysis, photometric, titration	Rehder et al.,1965
Sodium bicarbonate/NaCl Zinc acetate	Ion exchange neutralization	Titration, Gravimetric	TAPPI Method T-237 cm-98 1998
Crystal violet base	Acid-base reaction in benzene	Photometric	Rebek et al.,1960
HCI	Neutralization	Conductometric	Saake, 1992
$NaBH_4$	Reduction to the alcohol	Titration	Tihlarik et al.,1991; Strole, 1956
Cyanide	Formation of cyanohydrins	Titration	Lewin,1972
TTC-reaction	Formation of a red dye from reducing ends	Photometric	Strlic and Pihlar, 1997 Szabolcs, 1961
Iodometric	Excess iodine measurement	Titration	Scott,1939

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Chapter 2

"Studies on extraction of pure cellulose from bagasse and its application in producing cellulose acetates with novel application of residual hemicellulose as plasticizer

2.1. Introduction

It is increasingly being realized that agricultural commodities are the key raw materials available to mankind for the sustainable production of numerous industrial and non-food consumer products such as fuels, paints, detergents, biodegradable polymers, textile fibers, fiber composites, and various other commodity and specialty chemicals. Research seeks to better understand properties of agricultural materials related to their quality, value, and processing characteristics and to develop innovative products and processes for conversion of agricultural materials to non-food products. While a question of ethics pervades the use of agricultural food materials for the production of chemicals, polymers and materials, the use of waste agricultural residues, produced in millions of tons quantities annually as waste, are not subject to these debates. Further, there is indeed a pressing need to value-add to these waste agricultural materials, as they are the most abundant source of organic raw materials available today. Even more importantly, the use of agricultural wastes is generally expected to ensure minimum carbon footprints in their utilization. Agricultural wastes are also relatively free from wide price fluctuations, and this is another major advantage of using this as a feedstock for value-added products, and justifies research and development efforts. For example, few commercial uses for the excess bagasse have been developed and its accumulation presents a waste problem for the sugar industry. The same is the case with wheat and rice straw, cereal straws, cotton stalks, etc.

One area where tremendous amount of research has been devoted pertains to the fractionation of agricultural biomass into its constituents, i.e., cellulose, hemicellulose, and lignin in what is best described as a bio-refinery. Each of these

fractions can then be separately value-added by making industrially important derivatives. For example, cellulose can be converted cellulose esters, which have wide ranging applications as biodegradable plastics, textile fibers, films, etc. Extensive work has been reported in published literature on the synthesis of cellulose acetates form cotton linters and wood cellulose. In recent times, lignocellulosic agricultural materials as a source of cellulose are also being investigated with renewed vigor, considering the cost differential between the different raw materials.

Biswas et al. (2006) have synthesized cellulose acetates from other agricultural residues like corn stalk, rice hulls and wheat straws. Filho et al. (2000) have synthesized cellulose triacetate from sugarcane bagasse and reported water flux through membranes prepared from these materials. More recently, Cerqueira et al. (2007) also reported the optimization of bagasse acetylation by varying acetic acid anhydride and catalyst volume and reaction and activation times. The effect of these variations on intrinsic viscosities and molecular weight of acetates have been reported. The same group reported that the lignin content of their bagasse cellulose was 3.84% (Viera et al, 2007). In this chapter we have shown that this level of purity is far less than the bagasse cellulose prepared by us, wherein we obtained a lignin content of only 0.2%. This low lignin content enabled us to evaluate accurately the role of residual hemicellulose present in the extracted cellulose as a plasticizer for cellulose ester films, thereby obviating the need for adding external plasticizers.

2. 2. Experimental

2.2.1. Preparation of bagasse cellulose

Our laboratory has developed a proprietary process for the extraction of α-cellulose from sugarcane bagasse (For determination of α-cellulose, see Appendix-1) and the process is under patenting (Varma, 2008). This bagasse cellulose contained ~94% α-cellulose, ~ 5% hemicellulose, ~0.2% lignin, and ~1% ash, and was found to be suitable for the production of cellulose esters and ethers.

2.2.2. Preparation of cellulose ester

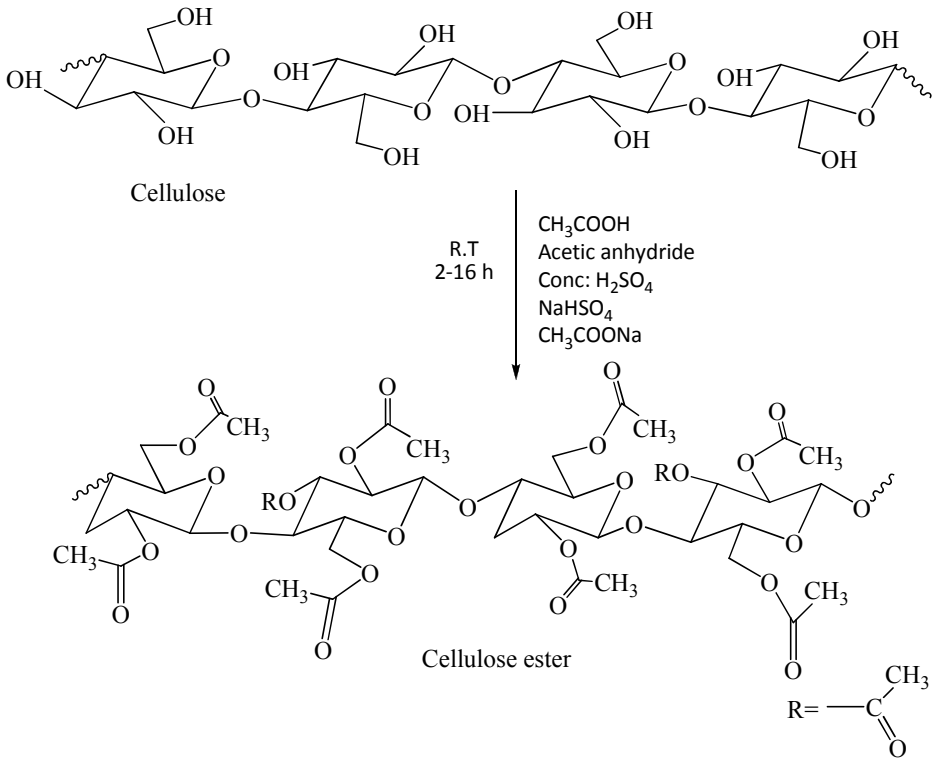

Figure 2.1: Reaction scheme of cellulose acetate synthesis

Acetylation of bagasse cellulose was conducted using a process described elsewhere. (Kuo & Bogan, 1997, and Ozaki et al., 2004). Cellulose was dried in vacuum oven (30 mm) at 60°C for 12 hours. 20 g of this cellulose was taken in a three neck round bottom flask fitted with an overhead stirrer. To this was added 350 mL of glacial acetic acid. After the addition of acetic acid the reaction mixture was kept at 0 – 5 °C for one hour; to this was added the acetylating mixture (135 ml acetic anhydride + 0.75 g sodium bisulphate + 0.5 ml sulfuric acid) over a period of half an hour, all the time maintaining the temperature from 0-5 °C in an ice-water bath. After one hour the cold water from the bath was removed and the flask allowed come to room temperature of ~ 30 °C; this process took about three hours. At this stage the reaction mixture started to become viscous. In successive experiments, the room temperature reaction was carried out for 2, 4, 6, 8, 10, and 18 hours respectively. In each case, one hour before the completion of the reaction time, a solution of 0.5 g sodium acetate in 10 ml glacial acetic acid was added to the reaction mixture and the stirring was continued for one hour to remove the sulfate group substituent on the cellulose molecule.

The reaction mixture was now slowly poured into ~ 3 liters of distilled water with constant stirring. Once the precipitation process was completed the product was filtered and washed with distilled water. The washings was continued till the product was free of acetic acid, it was also monitored by pH measurements until neutral pH was reached. Finally the product was washed with acetone, filtered and dried in vacuum oven (30 mm pressure) at 60°C for 6 hrs.

2.2.3. Casting of cellulose acetate films

Films with dimensions of 200mm X 200mm X 0.01mm were prepared as follows,

5.75 gm of cellulose acetate (CA) was dissolved in 200 ml chloroform. This solution was stirred for 4-5 hours, and then filtered to remove any insoluble matter. A glass tray with dimensions of 21cm X 21cm was used for making the films. The tray was cleaned thoroughly, dried and kept in the oven set at 40° C. The level of the tray was adjusted using leveling screws. The CA solution was slowly poured into the tray from one corner of the tray. The oven was closed and left undisturbed at 40°C for 8 hours. The film was exposed to 75°C for 1 hour. The thickness of the film was measured using a micrometer. Plasticized films of CA were made by adding 15 phr (i.e., parts per hundred of resin) of plasticizer (equal mixture of diethyl phthalate and triphenyl phosphate) during the dissolution stage. The thickness of the films was ~0.01 – 0.012 mm.

2.4. Methods

2.4.1. Determination of Degree of Substitution (DS) according to (ASTM D 871-96)

0.5 g of cellulose acetate samples were weighed accurately and transferred to a 250 ml flask; to each sample 20 mL of 75% aqueous ethanol was added and then heated for 30 minutes at 60°C. Then 20 ml of 0.49 N NaOH solution was added to each sample and again heated at 60°C for 15 ml min. The same procedure was also done for a control system (not containing CA). The flasks were stoppered and allowed to stand at room temperature for 72 hrs.

The excess alkali in the sample and control was titrated with HCl (0.5 N) using phenolphthalein as indicator. An excess of acid was added (1 mL) and the alkali was allowed to diffuse from the regenerated cellulose overnight. The disappearance of the pink color indicated complete neutralization of the alkali. The small excess of acid was then back titrated with sodium hydroxide to a phenolphthalein end point (i.e., until the solution had acquired faint pink color).

2.4.2. FTIR

A Perkin Elmer Spectrum One instrument was used. Spectra of CA in chloroform were recorded using KBr windows. Cellulose IR were recorded in KBr.

2.4.3. Thermogravimetry (TGA)

Thermal stability of CA was studied using TGA (Perkin Elmer TGA 7) with a heating rate of 10°C/min under nitrogen atmosphere.

2.4.4. Differential Scanning Chromatography (DSC)

TA Instruments DSC Model Q100 was used for measurement of glass transition temperatures. 3-4 mg of the sample was crimped in aluminum pan and loaded along with reference pan without sample. The heating rate was 10 ^{0}/min., and heating was carried out up to 300 °C.

2.4.5. Gel Permeation Chromatography (GPC)

GPC studies were carried out using a Dionex GPC with RI detector, at room temperature. Shodex KF 803, 804 and 805 columns were used with HPLC grade chloroform as an eluent. The flow rate was 1 mL/min. A polystyrene calibration curve (20 samples of weight average molecular weights from 164 to 1.5 million) was used to estimate the molecular weights and polydispersity of CA samples. Sample preparation: 15-20 mg of CA was dissolved in 5 mL of chloroform and 20 uL was injected.

2.4.6. Wide-angle X ray Crystallography (WAXRD)

WAXRD experiments were carried out with the sample in film form having a thickness of ~0.01 mm using a Rigaku, Japan, WAXRD instrument. The scanning speed was 4°/ min, with a radiation of CuK- α. The samples were scanned from 2 θ values of 5° to 40°.

2.4.7. High pressure ion chromatography (HPIC)

Dionex HPIC consisting of quaternary gradient pump, electrochemical detector and chromeleon software was used for the detection of carbohydrates detached from cellulose during the course of acetylation. A Dionex Carbopac PA 10 column was used for this purpose. The eluent used was 20 mM NaOH solution.

2.4.8. Viscosity measurements

Dilute solution viscosities were measured in 1% (w/v) solution of CA in dimethyl sulfoxide (DMSO) and chloroform using an Ubbelohde capillary viscometer at room temperature.

2.4.9. Mechanical properties

Tensile strength and modulus were determined on an Instron UTM instrument. The procedure of ASTM D 882 –95a was used for this purpose. The load cell used was 1 KN with 5-mm/min crosshead speed. The test specimens were prepared from 100 micron thick film by cutting the films with a sharp knife. The dimensions were 150 mm length and 20mm width.

2.5. Results and discussions

The properties of the cellulose acetates synthesized under the same reaction conditions but with different reaction times (2 h – 18 h) are shown in Table 2.1. After 2 h the reaction product was still insoluble in anhydrous DMSO, and its DS was not measured. After 4 h reaction time, the product was soluble in DMSO and its DS was found to be 2.65, reaching the maximum value of 3.0 after 6 h. Thereafter, as the reaction time increased, the DS was found to be steady in the range of 2.8 – 2.9. Apparently, an equilibrium stage is reached. The intrinsic

viscosities are seen to decrease as the reaction time increases, due to chain degradation under the acidic conditions.

Saka Shiro (1998) reported that the presence of hemicellulose in cellulose acetate gives false viscosity and turbidity to the solution. However, our 4 h and 6 h samples show no turbidity in solution even though they contain hemicellulose acetate fraction.

Figure 2.2 shows FTIR spectra of various CA samples. All the samples show similar spectral features. The FTIR shows the reduction of OH frequency and appearance of C=O band at 1747 cm^{-1} (Heinze, T., 2004; Rajini, R., 2001). Complete disappearance of the OH peak is not seen even for the sample with DS 3.0, perhaps due to adhering moisture. The absence of band at 1760-1840 cm^{-1} and 1700 cm^{-1} confirms the absence of acetic acid and acetic anhydride.

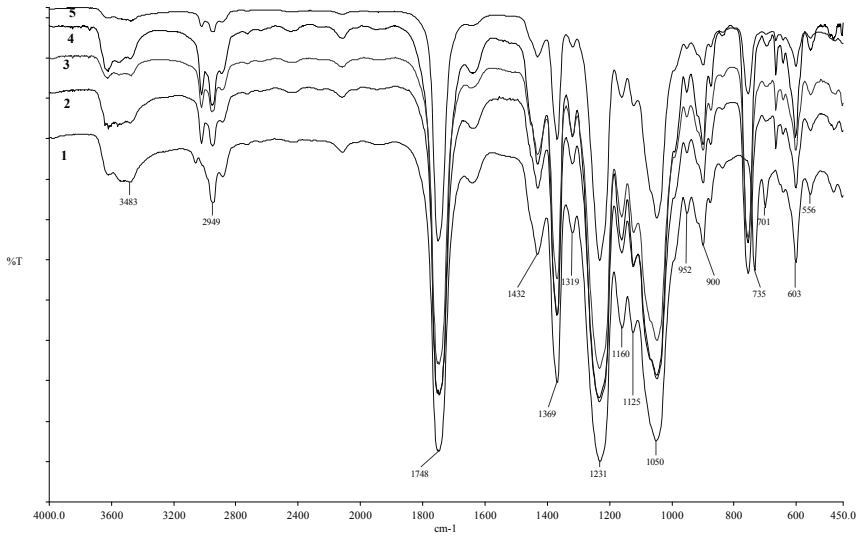

Figure 2.2: FTIR of various cellulose acetate samples 1) 4 hrs reaction, 2) 6 hrs reaction 3) 8 hrs reaction, 4) 10 hrs reaction, 5) 18 hrs reaction

Table 2.1: Properties of various cellulose acetates

CA Sample	DS	Intrinsic Viscosity dl/gm	MW	Mn	Mw/Mn	Tg °C	Peak Temp °C	TS (MPa) (Avg of 5 specimen)	TM (MPa) (Avg of 5 specimen)
CA 18 hrs	2.9	2.3	215804	125601	1.71	148	378	Could not be tested	
CA 10 hrs	2.8	2.5	191494	106497	1.8	136	383	Could not be tested	
CA 8 hrs	2.8	2.6	195165	68611	2.84	156	372	Could not be tested	
CA 6 hrs	3.0	2.8	164532	77768	2.11	139	367	40	2791
CA 4 hrs	2.6	3.1	129849	17066	7.62	146	365	61	2913
CA 2 hrs	-	Insoluble	-	-	-	-	-	-	-

Figure 2.3 shows the DSC scans of various CA samples. The endotherm around 100 °C is attributed to evaporation of water. The 4 h and 6 h samples have two endotherms at 215°C and 285°C, which could be due to outflow of xylan acetate present in these samples. The endotherm at 285 °C in 4 h and 6 h could be due to the triacetate fusion process which has shifted to lower temperature (Shiro Saka & Ohmae, 1996; Filho, et al., 2000). The intensity of this transition gets reduced with increased reaction time, since these endotherms are not seen in the DSC scans of 8 h–18 h samples, which is due to the removal of xylan acetate during the course of the reaction. This was confirmed by HPIC studies (see later discussion). The 8 h to 18 h CA samples shows an exotherm around 200 °C.

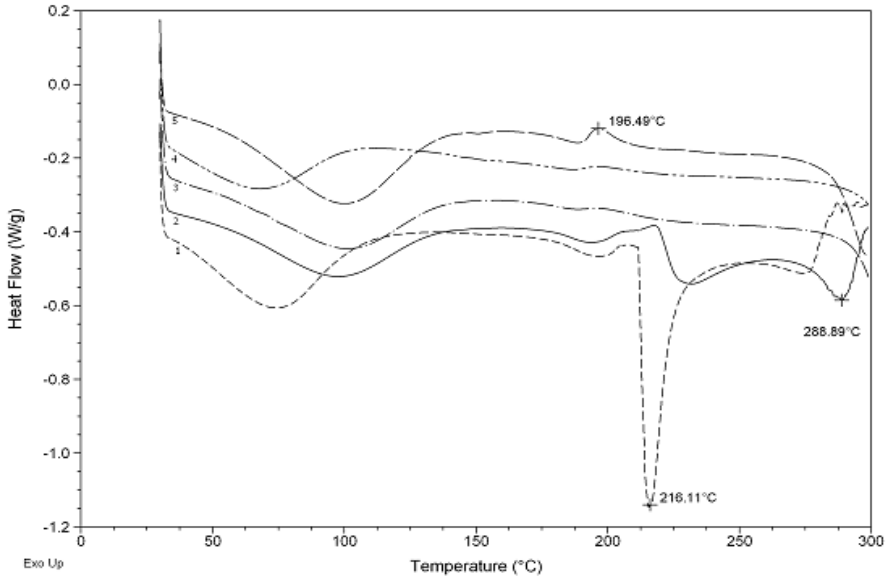

Figure 2.3: DSC of cellulose acetate samples: 1) 4 hrs reaction, 2) 6 hrs reaction, 3) 8 hrs reaction 4) 10 hrs reaction, 5) 18 hrs reaction time

In previous studies this exotherm was attributed to crystallization of a fraction of the CA during the scan (Filho, et al., 2000). This exotherm is absent in the 4 h and 6 h samples. The 8 h-18 h reaction time samples show an endotherm above 290°C. This endotherm resembles the previously reported data (Filho, et.al, 2000;

Zugenmaier, 2004), indicating that crystallinity increases with reaction time at 300 °C. This clearly shows the effect of reaction time on the thermal properties of cellulose acetates. The change in crystalline nature of the samples exposed to long durations (48 h), as reported by Filho (2000, 2008), has thus been confirmed by our observations. We have obtained the same product in 8 h-18 hrs as compared to 48 hrs reported by Filho (2000), by our synthetic procedure.

2.5.1. TG Analysis

Figure 2.4 shows the TGA curves of various cellulose samples. The thermal degradation of cellulose esters is known to take place in various steps such as removal of water and solvents (between 50-150 °C), deacetylation at 320 °C and thermal pyrolysis of the cellulose skeleton at 370 °C (Hung Mei Rong and Li, Xin-Gui 1998).

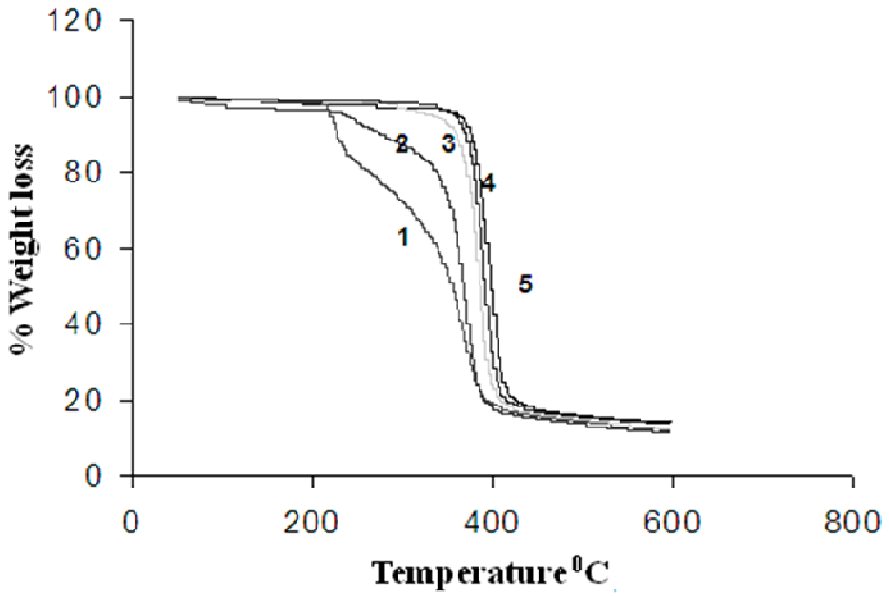

Figure 2.4: TGA curves of various cellulose acetate: 1) 4 hrs, 2) 6 hrs, 3) 8 hrs 4) 10 hrs, 5) 18 hrs reaction time

The thermal stability of CA increases with acetylation reaction time and reaches a maximum at 8 h reaction time. The thermal stability also depends on molecular weight and crystallinity of the polymer. Generally lower the molecular weight or lower the crystallinity, the easier the degradation of the polymer. We also have observed a similar pattern in our samples.

The 4 h samples show two weight loss regions, one at 221 °C and the other at 365 °C, the first one at 221 °C diminishes with time of acetylation and completely disappears after 8 hrs. This transition could be due to the xylan acetate degradation (Saka, Shiro and Ohmae, Kosuke 1996). This means higher the content of hemicellulose in the pulp lower will be the thermal stability of the CA (Amine Abou-State et al., 1984). This transition disappears when the reaction times are increased, which is due to removal of the hemicellulose acetate due to hydrolysis and dissolution during the reaction workup. The onset temperatures of 4 h and 6 h show two onset of degradation temperatures due to the presence of acetylated cellulose and acetylated hemicellulose, while the 10 h and 18 h samples show a single onset of degradation temperature (as only cellulose acetate is present). This was further confirmed by the mechanical property measurements (see later discussions on mechanical properties).

2.5.2. Gel Permeation Chromatography and High Pressure Ion Chromatography

As indicated in table 2.1 the MWD of the samples decreased with acetylation reaction time. This may be due to the detachment of xylan acetate from the molecule during the reaction. This is also reflected in the thermogravimetric analysis at 221°C, which is the appearance of xylan diacetate peak (Saka Shiro and Takanashi, Keiko 1998). The elution time for these samples is between 18-29

minutes. The samples having higher MWD values show a greater fraction of low molecular weight product. With increased reaction time (8 h – 18 h) the xylan acetate fraction gets detached from the cellulose and washed away during reaction work up, as explained earlier. This was also confirmed by High-pressure ion chromatography.

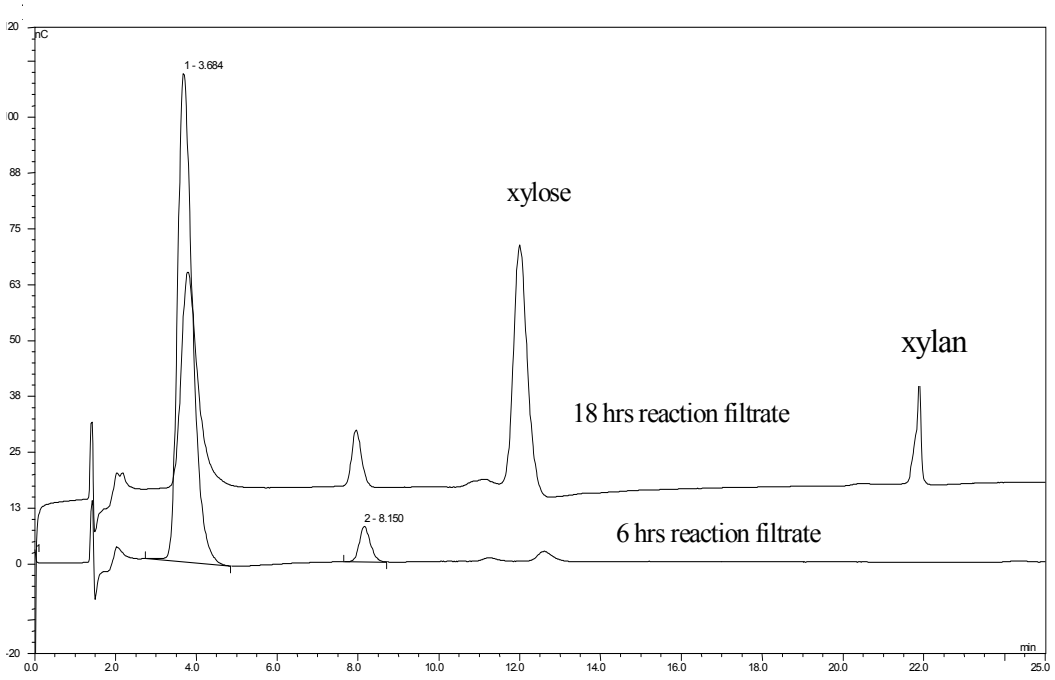

Figure 2.5: HPIC chromatograph of the filtrate, showing the presence of xylose and xylan: Top: 18 hrs reaction and bottom: 6 hrs reaction

The acetylation mixture of two reactions (6 h and 18 h) was precipitated in 500 ml of water; 10 ml filtrate was diluted to 100 ml and injected into the HPIC column. The remaining filtrate was extracted with chloroform and concentrated to get dry solid. Figure 2.5 shows the HPIC graphs of the two filtrates. It can be seen from the HPIC that the 6 h sample shows a very small amount of xylose and no detection of xylan, while the 18 h filtrate shows considerable amount of xylose

and also xylan. Tanghe, et al., (1970) reported the presence of a pre-hump attributed to the xylan acetate and showed the separation of this pre-hump; however in our samples we did not observe the pre-hump, instead we observed a broad molecular weight distribution which narrowed down when the acetylation time was increased.

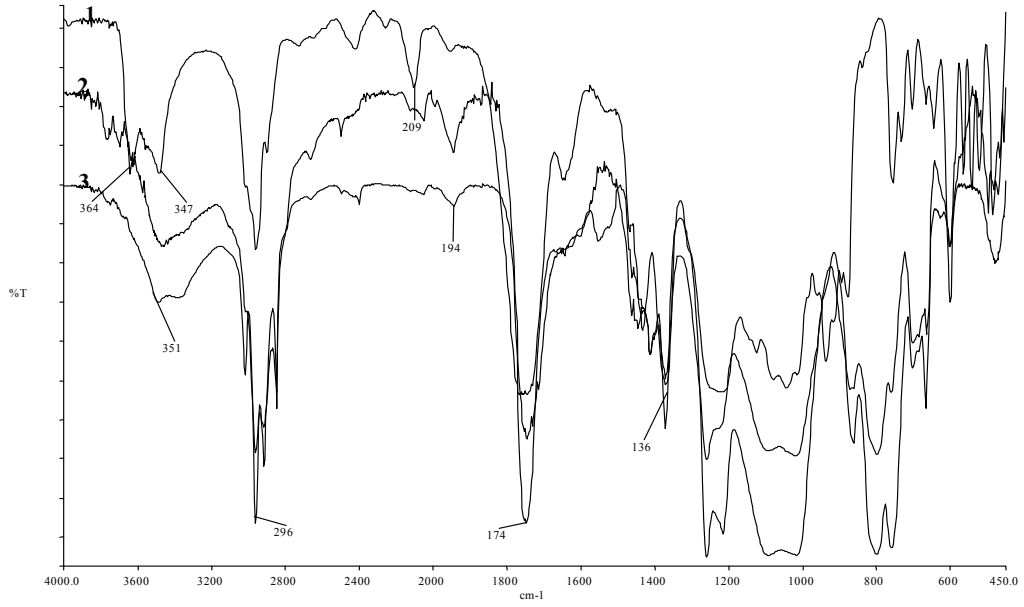

Figure 2.6: FTIR of the 1) Xylose acetate, 2) 6 hrs reaction filtrate, 3) 18 hrs reaction filtrate

The GPC curve of the samples reacted for more than 8 h showed lowest the MWD. This means during the acetylation reaction after 8 h the hemicellulose is almost completely removed during the workup. It is seen from figure 2.6 that the FTIR spectrum of the chloroform extracted solids from the reaction workup filtrate are very similar to the FTIR spectra of pure xylan acetate, thus confirming the xylan acetate separation during reaction.

2.5.3. X-ray diffraction study

The XRD study of the heterogeneous acetylation reaction of cellulose studied by Doyle & Pethrick (1987) indicated the presence of crystallinity in the triacetate. The changes in structure at various levels of organization, which occur during acetylation of cellulose linters using heterogeneous conditions, have been reported in their paper.

We also found changes in the crystalline nature of CA's of different reaction times. Figure 2.7 and 2.8 shows the WAXRD of CA samples. Filho, et al. (2000) has reported a 2θ of ~20. However, all our CA samples show a strong intensity peak at 2θ of 8.2^0, and other weak diffractions at 2θ of 11.9, 16.8 and 21.5. This value corresponds to the structure of cellulose triacetate (Kono, H., 1999). Fig: 2.7 indicate clearly that 4 h and 6 h samples have very similar crystallinity. However 8, 10 and 18 h sample also have similar crystallinity and are brittle in nature (Figure 2.8).

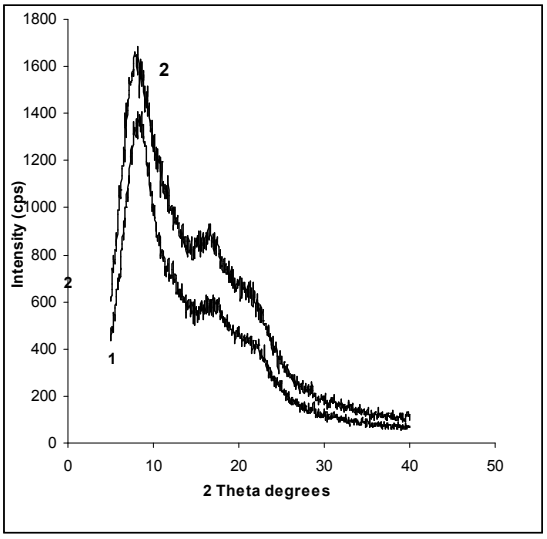

Figure 2.7: XRD curves of cellulose acetate:

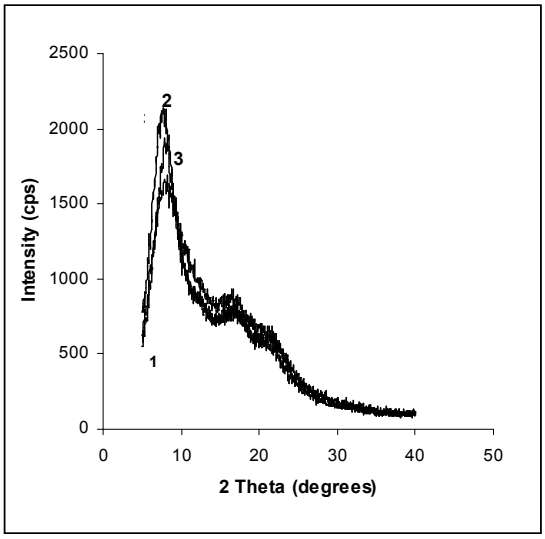

Figure 2.8: XRD curves of cellulose acetate: 1) 4 hrs reaction and 2) 6 hrs reaction 1) 8 hrs, 2) 10 hrs and 3) 18 hrs reaction

2.5.4. Mechanical characterization

The tensile properties of CA films were measured (ASTM D 882-95a) on 0.010-0.012 mm thick film. Five specimen of each sample were tested. Table 2.1. shows the tensile strength and modulus of various CA samples. The 8h-18 h samples could not be cut into notch free specimens; hence they could not be tested without plasticizer. These samples showed tensile strength of 14 MPa after addition of 15 % plasticizer (equal mixture of diethyl phthalate and triphenyl phosphate). The 4 h and 6 h samples have good tensile properties without addition of external plasticizer. The tensile strength of these samples is three to four times higher than 8 h -18 h samples in which the hemicellulose content is absent. This confirms the presence of hemicellulose acetate in the 4 h and 6 h samples of CA, which is shown to function like a plastizer.

Tensile strengths of commercial cellulose acetate films is about 100-140 MPa (Peter Zugenmaier, 2004) which are higher than our values. This can be due to the different origin of cellulose, its molecular weight, and purity, in addition to different methods of film preparation. Thus, we conclude that the higher strength cellulose acetate materials have less thermal stability due to the presence of xylan acetate; however these xylan acetates are responsible for better mechanical properties as they function as plasticizers.

2.6. Conclusions

From the above studies, we conclude that cellulose acetate reaches a DS of 3 in 6 h of acetylation time by the synthetic procedure reported in this chapter. Increased reaction time improves the solubility in chloroform, due to removal of xylan acetate from the triacetate. This was confirmed by HPIC analysis, DSC, TGA and

GPC and intrinsic viscosity. However, films made from these higher reaction time samples (samples with 8 h and above reaction time) could not be tested for mechanical properties as they could not give notch free specimen. For the 4 h and 6 h samples of CA, the xylan acetate is not fully removed. This residual xylan acetate functioned as a plasticizer for the CA samples; therefore they exhibited good mechanical properties without addition of external plasticizer. Xylan acetate is also a biodegradable material, so this can be an added advantage of this plasticizer.

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Appendix-1

Determination of Alpha-, beta- and gamma-cellulose (Adapted from TAPPI-T 203 om-99 method).

This method for determination of alpha-, beta- and gamma-cellulose can be applied to bleached or delignified pulps only. Unbleached and semi-bleached pulps must delignify before testing. Cellulose extracted consecutively with 17.5% and 9.45% sodium hydroxide solutions at 25°C. The soluble fraction, consisting of beta- and gamma-celluloses, is determined volumetrically by oxidation with potassium dichromate, and the alpha-cellulose, as an insoluble fraction, is derived by difference

In general, the alpha-cellulose indicates undegraded higher-molecular-weight cellulose content in pulp, the beta-cellulose indicates that of undegraded cellulose, and the gamma-cellulose consists mainly of hemicellulose

Reagents

I) Sodium hydroxide solution, 17.5% NaOH by weight II) Potassium dichromate solution, 0.5 N III) Ferrous ammonium sulfate solution 0.1 N. IV) Sulfuric acid. 3N and conc. sulphuric acid V) Phenanthroline-ferrous sulfate (ferroin) indicator

Procedure

1) 0.5 gm of oven dried sample was taken in 500 ml capacity of beaker and 100 ml of 17.5% NaOH was added. Temperature of reaction was adjusted to 25^0C.
2) The sample was stirred till it completely dispersed.
3) After a period of 30 minutes from the addition of 17.5% NaOH, 100 ml of distilled water was added and reaction mixture stirred for another 30 minutes at 25^0 C.
4) After completion of 60 minutes, suspension of centrifuged. Filtrate and residues and were collected.

α- cellulose determination

I. In 250 ml capacity of conical flask, 25ml of above filtrate was taken with the help of pipette and to it, was added 20 ml of 0.5 N of potassium dichromate solution.
II. To above mixture, 50 ml of conc., H_2SO_4 was added dropwise.
III. This solution was allowed to remain hot for 15 minutes and then 50 ml of distilled water was added so that reaction mixture comes to room temperature.
IV. To this solution, 2-4 drops of ferroin indicator was added and titrated against 0.1 N ferrous ammonium sulfate solutions till red colour of mixture was obtained.
V. Blanks titration was made by substituting filtrate with 12.5 ml of 17.5 % NaOH and 12.5 ml of distilled water.

β- and γ-cellulose determination

I. 50 ml of filtrate was taken with the help of pipette in to 1000 ml graduated cylinder and to it 50 ml of 3N H_2SO_4 was added.
II. This cylinder was then kept in water bath and heated to 70-90 ^{0}C for few minutes to coagulate the β-cellulose.
III. The precipitated was allowed to settle down for overnight and then centrifuged to obtained clear solution.
IV. To 50 ml of this clear solution, 10 ml of 0.5N $K_2Cr_2O_7$ solution and then 9o ml of conc. H_2SO_4 was added portionwise.
V. This solution was allowed to remain hot for 15 minutes and then 50 ml of distilled water was added so that reaction mixture comes to room temperature.
VI. To this solution, 2-4 drops of ferroin indicator was added and titrated against 0.1 N ferrous ammonium sulfate solutions till red colour of mixture was obtained.
VII. Blanks titration was made by substituting filtrate with 12.5 ml of 17.5 % NaOH and 12.5 ml of distilled water and 25 ml of 3 N H_2SO_4.

Calculations:

1. α- cellulose:

$$\alpha\text{- Cellulose \%} = \frac{100-6.85\ (V_2-V_1)\ X\ N\ X\ 20}{A\ X\ W}$$

Where,

V_1= titration of filtrate, ml

V_2= blank titration

N = Exact normality of FAS solution

A =Volume of filtrate used in the oxidation, ml

W=Weight of oven dry sample, gm

2. γ-cellulose:

$$\gamma\text{- Cellulose \%} = \frac{[6.85\ (V_4-V_3)\ X\ N\ X\ 20]}{25\ X\ W}$$

Where,

V_3= titration of solution after precipitation of β-cellulose, ml

V_4= blank titration

3. β-cellulose:

$$\beta\text{-Cellulose, \%} = 100-(\alpha\text{- cellulose, \%} + \gamma\text{-cellulose, \%})$$

Chapter 3

"Studies on production of hydrolytic enzymes from bagasse polysaccharides"

3.1. Introduction

In the previous chapter we have explored the application of bagasse derived highly pure cellulose. In this chapter, we have prepared bagasse cellulose with varying degrees of purity and explored their application in the production of cellulase and xylanase enzymes. These enzymes are expected to be useful in industrial application for hydrolysis of lignocellulosic substrates to produce sugars which can then be fermented or chemically converted to several chemicals as well as biofuel like ethanol or butanol.

Published literature shows that enzymatic hydrolysis of cellulosic material by cellulase enzymes is the most promising approach to get high product yields vital to economic success (Hinman, Schell, Riley, Bergeron, & Walter, 1992; Lynd, Elander, & Wyman, 1996). The high cost of cellulase enzyme production hinders the application of these enzymes to bioethanol production (Himmel, Ruth, & Wyman, 1999; Wooley, Ruth, Glassner, & Sheehan, 1999). The use of such waste and low cost cellulosic biomass would significantly reduce the cost of enzyme production provided cellulase productivities are comparable to those obtained in presence of other carbon sources like lactose (Kadam, 1996). Such high cellulase productivities can potentially be achieved by the use of chemically pretreated biomass as carbon sources.

Lynd (1996) has summarized the prerequisites for an ideal lignocellulose pretreatment, i.e., it should: a) produce reactive fibers; b) yield pentoses in non-degraded form; c) not release the compounds that significantly inhibit fermentation; d) work in reactors of reasonable size with moderate cost; e) produce no solid residues; f) have a high degree of simplicity, and g) be effective at low moisture contents. A number of pretreatment options are available such as acid

pretreatment, alkaline treatment, steam explosion, wet oxidation, organic solvent pretreatment, hot water etc, and are described in detailed in earlier chapter.

The present study deals with the evaluation of cellulase enzyme production using bagasse after different chemical pretreatments. This study will also assess whether this route could enhance performance on inexpensive biomass like bagasse and reduce the cost of enzyme production using cellulolytic strains, *Penicillium janthinellum* NCIM 1171 and *Trichoderma viride* NCIM 1051.

3. 2. Materials and methods

3.2.1. Chemicals

Cellulose powder 123 (CP-123) was obtained from Carl Schleicher and schull co. Dassel, FRG. p-Nitrophenyl β-D-glucopyranoside (pNPG), Carboxymethylcellulose (CMC), Xylan from oat spelts, 3, 5-dinitrosalysilic acid were obtained from Sigma-Aldrich co. st. Louis, mo. USA. The sugar cane bagasse samples prepared using different chemical pretreatments (Table 3.1) were used.

3.2.2. Microorganism and culture media

Penicillium janthinellum NCIM 1171 and *Trichoderma viride* NCIM 1051 were obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune, India. These cultures were maintained on Potato Dextrose Agar (PDA) and sub cultured once in every three months. PDA contained (g/L) extract from 200 gm of potatoes, glucose, 20.0 g; yeast extract, 1.0 g; and agar, 20.0 g. Production medium described by Mandels & Weber (1969) was used for enzyme production.

3.2.3. Enzyme production

Shake flask experiments were carried out in 250 mL Erlenmeyer flask with 70 mL of production medium containing 1% (w/v) cellulose-123 powder or pretreated sugar cane bagasse samples with different kappa numbers. The flasks were inoculated with spores (approximately 10^7) from 10-day-old culture grown on PDA slant and incubated at 30˚C with shaking at 180 rpm. The samples were removed at various time intervals and centrifuged at 3000 rpm for 10 min. The supernatant was analyzed for extra cellular enzyme activities and soluble protein.

3.2.4. Enzyme assay

Exoglucanase (FPase; Exo-1,4-β-D-glucanase, E.C.3.2.1.91) activity was determined according to Mandels et al., (1974). Assay mixture (2 mL) consisted of 1.9 mL citrate buffer (50mM, pH 4.5), filter paper Whatman No.1 (50mg, 1x3 cm^2) and 0.1mL suitably diluted enzyme. The reaction mixture was incubated at 50^0C for 60 min.

Endoglucanase (CMCase, Endo-1-4-β-D-glucanase; E.C. 3.2.1.4) activity was determined according to Mandels et al., (1974) with slight modification. The total reaction of 1 ml contained a 0.5 mL sample of suitably diluted enzyme and 0.5 mL of 1% (w/v) CMC solution in citrate buffer (50 mM, pH 4.5) and incubated at 50^0 C for 30 min.

Xylanase (1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.8) activity was determined under similar conditions as described above, except that 1% xylan solution was used as substrate in place of CMC.

β-Glucosidase (β-D-glucoside glucohydrolase; E.C.3.2.1.21) activity was estimated according to the method described by Eberhart (1961) using pNPG as substrate. The total of assay mixture (1 mL) consisting of 0.9 mL of pNPG (1 mg/mL) and 0.1

mL of suitably diluted enzyme was incubated at 50^{0}C for 30 min. The p-nitrophenol liberated was measured at 410 nm after developing the color with 2 mL of sodium carbonate (2%).

Table 3.1: Different bagasse samples with their kappa numbers and lignin contents, generated by different chemical treatments.

Sample	Treatment	Condition	Kappa No.	Lignin (%) w/w
I	a) NaOH, b) $NaClO_2$, c) H_2O_2 bleach	a) 42.85% NaOH, 70-75^{0}C, 6 h b) Pulp: $NaClO_2$ 1:0.5; pH=3.7, 70^{0}C, 4 h c) 10% H_2O_2, pH=10.5, 70^{0}C, 4 h	0.8	0.1
II	Bagasse Kraft pulp, unbleached	Standard Kraft pulping	12.4	1.9
III	$NaClO_2$ bleaching	Bagasse: $NaClO_2$=1:0.5,4 h, 79^{0}C	14	2.1
IV	$NaClO_2$ bleaching	Bagasse: $NaClO_2$= 1:0.25, 4 h, 70^{0}C	22.9	3.4
V	NaOH	20% NaOH, 4 h, 25^{0}C	54.7	--
VI	H_2O_2	10% H_2O_2, pH=10, 4 h, 70^{0}C	66	--
VII	Untreated sugarcane bagasse	-	79	--

3.2.5. Enzyme units

One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate one μmol of glucose, xylose or p-nitrophenol from the appropriate substrates per min per mL of crude filtrate under assay condition.

3.3. Analytical method

Protein was estimated according to the method of Lowry et al., (1951) using bovine serum albumin as a standard. The reducing sugar was estimated as either xylose or glucose equivalent by dinitrosalicylic acid (DNS) method (Fischer, & Stein, 1961). The kappa number of the samples was determined by TAPPI-T 236 om-99 method (*see appendix-2*) and the lignin content was reconfirmed by multiplying the kappa number with 0.15 factor.

3.4. Result and discussion

All experiments were carried out using holocelluloses derived from different treatments of bagasse introduced individually into the production medium. We also performed fermentation experiments with two control media, one containing untreated bagasse and the second containing pure CP - 123 as standard. The enzyme activities were determined up to 15 days and the values are presented in Table 3.2 and 3.3. We also used two different cultures, namely *Trichoderma viride* NCIM 1051 and *Penicillium janthinellum* NCIM 1171.

The standard medium with pure cellulose powder gave highest FPase and CMCase activities, indicating the more efficient utilization of pure cellulose than the native and treated bagasse. Bagasse sample IV was generated by selective removal of major part of lignin constituent keeping the other constituents (cellulose and hemicellulose) predominantly intact. The kappa number 22.9 of bagasse sample works out to a lignin content of 3.4% (Table 3.1). Apparently, the removal of lignin

leads to a more porous and accessible material for microbial attack, which helps in production of significantly, enhanced levels of xylanase and β-glucosidase enzymes. FPase activities produced by both strains in a medium containing bagasse sample IV are more or less the same as compared to those obtained in pure cellulose containing medium. However, xylanase and β-glucosidase activities produced by both *Trichoderma viride* and *Penicillium janthinellum* are higher when bagasse samples (both treated and untreated) were used as substrates in the medium. The bagasse samples IV yielded 4-fold increase of xylanase (130 IU/ml) and β-glucosidase (2.3 IU/ml) activities in case of *Penicillium janthinellum* NCIM 1171 when compared with the activities produced in presence of pure cellulose powder.

The untreated bagasse sample gave lower cellulase (FPase & CMCase) activities. This could be attributed to the much lower accessibility of microorganisms towards the cellulose and hemicellulose in the polymer matrix. Samples I & II (see Table 3.1) consists of alkali treated bagasse. This treatment leads to significant loss of both lignin and hemicellulose. This is reflected in the lower enzyme activities produced by use of these materials in the medium as carbon source. The difference in cellulase activities may be due to variation in the amounts of utilizable amorphous cellulose present in treated samples. This variation of amorphous cellulose in treated samples could be attributed to capability of each treatment to disrupt the hydrogen bonding of the crystalline form of cellulose (Ball, Godden, Helvenstein, Pennincks, & McCarthy, 1990). These observations led to the conclusion that physical structure of bagasse cellulose and enzyme activities are important factors for determining the efficiency of cellulose conversion to useful products (Azzam, 1987; Broda, 1992).

Table 3.2: Enzyme production by *Penicillium janthinellum* NCIM 1171 in medium containing 1% w/v bagasse samples or CP-123

Sample No.	Kappa No.	Enzyme Activity** (IU/ml)			
		Filter Paper (FPase)	β-Glucosidase	Endoglucanase (CMCase)	Xylanase
I	0.8	0.3	1.2	6.2	34.8
II	12.4	0.24	1.3	5.7	40.0
III	14	0.38	1.7	8.0	85.0
IV	22.9	0.48	2.3	7.1	130.0
V	54.7	0.33	1.0	6.7	53.0
VI	66	0.31	1.1	6.5	40.0
VII	79	0.27	0.61	5.7	68.0
VIII	CP -123	0.55	0.58	21.5	28.1

** All enzyme activities were determined after 8 days of incubation. The values are the average of three independent experiments with 5% - 8% variation.

Table 3.3: Enzyme production by *Trichoderma viride* NCIM 1051 the medium containing 1% w/v bagasse samples or CP-123

Sample No.	Kappa No.	Enzyme Activities** (IU/ml)			
		Filter Paper (FPase)	β-Glucosidase	Endoglucanase (CMCase)	Xylanase
I	0.8	0.41	0.19	7.7	13.1
II	12.4	0.26	0.18	5.4	14.2
III	14	0.81	0.26	10.7	58.2
IV	22.9	0.88	0.33	21.8	69.9
V	54.7	0.80	0.29	12.9	64.6
VI	66	0.62	0.24	9.4	81.4
VII	79	0.28	0.20	4.2	69.7
VIII	CP - 123	1.0	0.06	33.8	41.9

** All enzyme activities were determined after 10 days of incubation. The values are the average of three independent experiments with 6% - 8% variation.

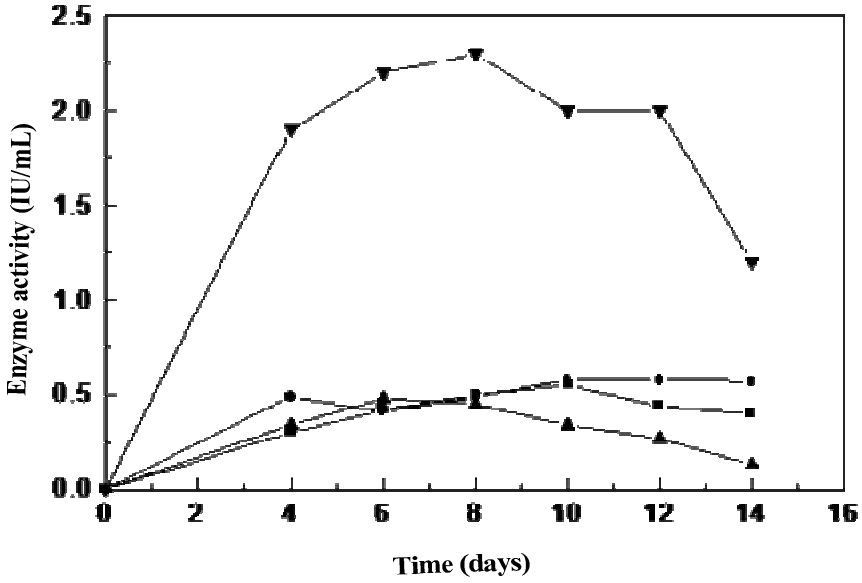

Figure 3.1: Enzyme activity profile during growth of *Penicillium janthinellum* NCIM 1171 in 1% CP (FPase,-■-; β-glucosidase,-●-) and in 1% BS – IV (FPase,-▲-; β-glucosidase,-▼-)

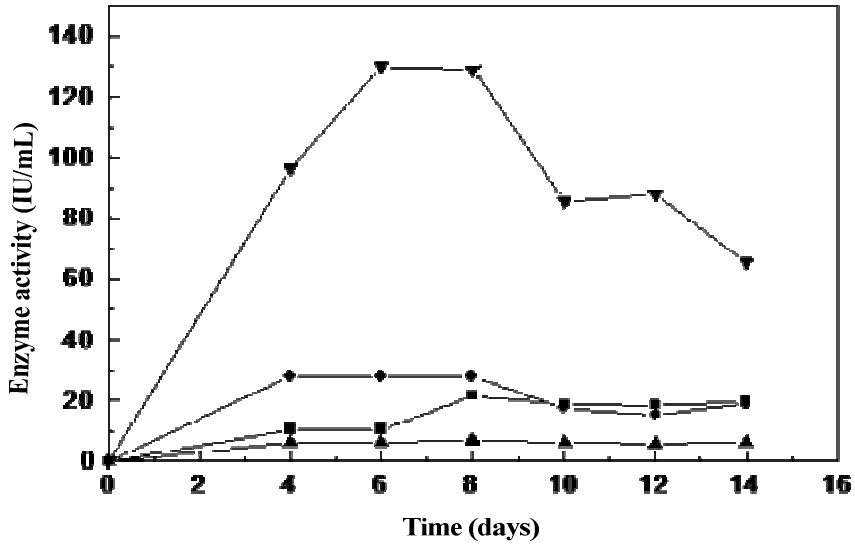

Figure 3.2: Enzyme activity profile during growth of *Penicillium janthinellum* NCIM 1171 in 1% CP (CMCase,-■-; xylanase,-●-;) and in 1% BS - IV (CMCase,-▲-; xylanase,-▼-).

We continued to use bagasse sample IV for further studies and this sample is hereafter denoted as BS–IV. The ratio of cellulase to cellulose substrate is one of the factors, which affects the effectiveness of cellulose hydrolysis. We carried out the fermentation experiments incorporating different concentrations (1.0%, 2.5%, 4.0%) of BS-IV for enzyme production and observed that BS-IV at 1% concentration gave highest enzyme activities.

The results in Figure 3.1 and 3.2 shows the activity profiles of enzymes produced by *Penicillium janthinellum* NCIM 1171 during growth cellulose powder 123 and BS-IV incorporated in the production medium individually. The extra-cellular enzymes were released in the medium during exponential phase and maximum activities were observed at the stationary phase. Xylanase and β-glucosidase production peaked on 6^{th} and 8^{th} day respectively in a medium containing BS–IV. Figure 3.3 and 3.4 show the activity profiles of *Trichoderma viride* NCIM 1051 during growth in a medium containing cellulose powder or BS-IV. All enzyme activities reached maximum levels on 10^{th} day of incubation irrespective of substrate used in the medium. *Trichoderma viride NCIM* 1051 produced highest CMCase & Fpase activities while *Penicillium janthinellum NCIM 1171* produced highest xylanase and β-glucosidase activities. Kansoh et al (1999) reported that cellulase production by *Trichoderma viride* C-30 was delayed when the organism was grown in medium with treated bagasse samples. This lag in enzyme production was attributed to the presence of lignin in the samples. Such lag in enzyme production was not observed when both the organisms were grown in a medium containing BS-IV. The results in Figure 3.5 reveal the protein production profile during growth of fungal strains in a medium containing CP-123 and BS–IV at 1% concentration. It appeared that the extra-cellular protein production was slightly lower in a medium with BS–IV compared to that with pure CP-123.

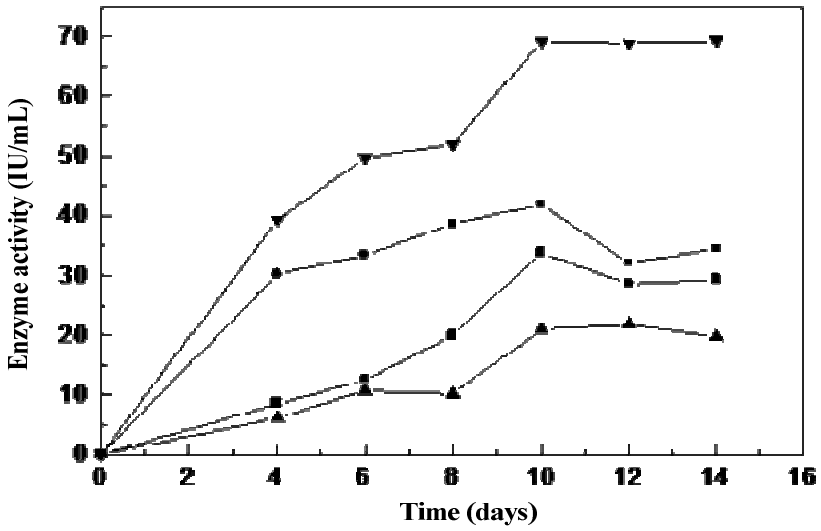

Figure 3.3: Enzyme activity profile during growth of *Trichoderma viride* NCIM 1051 in 1% CP-123 (CMCase,-■-; xylanase,-●-;) and in 1% BS-IV (CMCase,-▲-; xylanase,-▼-).

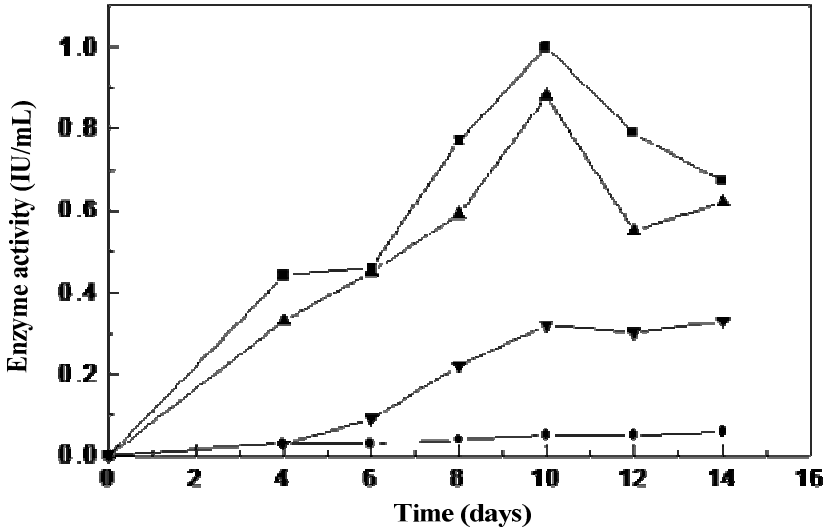

Figure 3.4: Enzyme activity profile during growth of *Trichoderma viride* NCIM 1051 in 1% CP-123 (FPase,-■-; β-glucosidase,-●- ;) and in 1% BS-IV (FPase,-▲-; β-glucosidase,-▼-).

Based on the activity measurements, *Penicillium janthinellum* NCIM 1171 produced enzymes except CMCase with much higher productivities in presence of BS–IV than with pure CP-123 (Table 3.4). The xylanase was produced by *P. janthinellum* NCIM 1171 with productivity of 902 U/h/L, which is three times than the xylanase productivity (353 U/h/L) of *P. janthinellum* CRC87M-115 isolated from decaying wood (Palma, Milagres, Prata, & de Mancilha, 1996). Xylanase and β-glucosidase enzymes were produced with enhanced productivities when *Trichoderma viride* NCIM 1051 was grown on BS-IV.

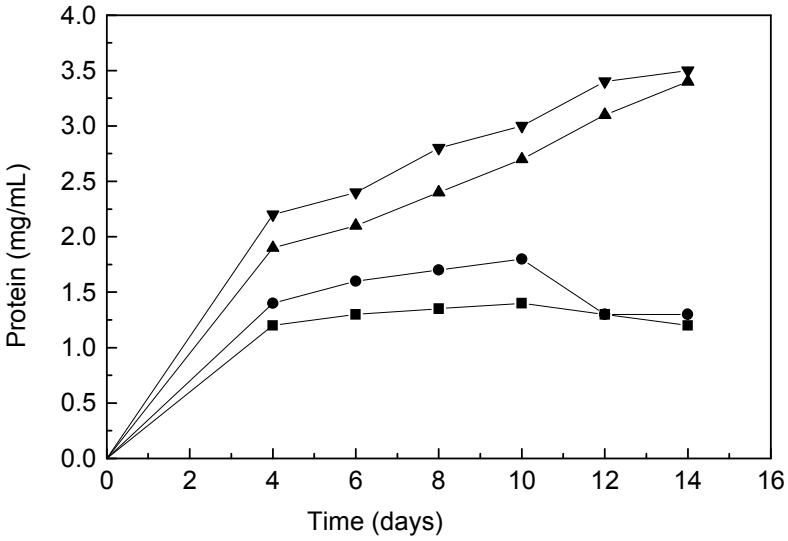

Figure 3.5: Extracellular protein profile during growth of *Trichoderma viride* NCIM 1051 (in 1% CP–123, -▼-; 1% BS–IV, -▲-) and *Penicillium janthinellum* NCIM 1171 (in 1% CP–123, -●-; 1% BS–IV, -■-)

Table 3. 4: Comparison of enzyme productivities during growth of fungal strains on bagasse samples and CP-123

Microorganism	Substrate	Enzyme Productivity (IU/h/L)			
		FPase	CMCase	β-Glucosidase	Xylanase
Penicillium janthinellum NCIM 1171	CP-123	2.30	111.80	2.40	291.40
	BS-IV	5.00	36.80	11.80	902.30
Trichoderma viride NCIM 1051	CP-123	4.10	140.70	0.17	174.30
	BS-IV	3.60	75.50	0.91	287.20

3.5. Conclusions

From the results, we conclude that, bagasse treated with $NaClO_2$ generates bagasse sample IV with kappa number 22.9 (lignin content 3.4% w/w, no changes in hemicellulose and cellulose contents) which appears to be an excellent source of carbon for cellulase production by *Penicillium janthinellum* NCIM 1171 and *Trichoderma viride* NCIM 1051. The incorporation of this sample in production medium resulted in the production of enhanced levels of xylanase and β-glucosidase enzymes by both *Penicillium janthinellum* and *Trichoderma viride* along with substantial levels (but not equal to the values obtained with CP-123) of both FPase and CMCase activities. The productivities of all enzymes except CMCase were higher in media with bagasse sample IV than with pure CP-123. However, *Trichoderma viride NCIM 1051* produced both CMCase and FPase with comparatively less productivity. The xylanase productivity was three time than the values obtained with CP-123 in case of *P. janthinellum NCIM 1171.* The use of such enzyme preparations in ligno-cellulose hydrolysis will lead to efficient conversion of cellulose materials to other important products.

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3.7. Appendix-2

Kappa number determination of delignified cellulose (Adapted from TAPPI-T 236 om-99 method)

The Kappa number is the number of milliliters of tenth-normal potassium permanganate solution consumed per gram of moisture-free pulp under conditions specified in this standard. The results are corrected to be equivalent to a 50% consumption of the permanganate in contact with the specimen. This method is generally used for the determination of the relative hardness, bleachability, or degree of delignification of pulp.

Relationship with lignin: The kappa number gives essentially a straight-line relationship with lignin. The percentage of lignin approximately equals K x 0.15.

Reagent: I) Potassium permanganate solution, 0.1 N II) sodium thiosulfate solution, 0.2 N III) potassium iodide solution, 1.0 N IV) sulfuric acid, 4 N V) starch indicator solution, 0.2%

Procedure:

Disintegrate the test sample (1 gm –oven dried) in 500-ml of distilled water until free from fiber clots and undispersed fiber bundles. Avoid extensive cutting of the fibers are separated.

Transfer the disintegrated sample to the two liter of beaker and add with enough distilled water to bring the total volume to 795 ml.

Adjust the temperature to 25^0C. Place the beaker and contents in the constant temperature bath and ensure that the temperature stays at 25 ^{0}C during the entire reaction. Continuously stir the suspension.

Pipette out 100 ml of 0.1 N $KMnO_4$ and 100 ml 4 N H_2SO_4 into a 250-ml beaker and bring this mixture to 25^0C. Add to it quickly the disintegrated sample and simultaneously start the stopwatch. Rinse out the 250 ml beaker, using about 5 ml of distilled water, and add to the reaction mixture. Its final volume should be 1000 ml. At the end of exactly 10.0 minutes, stop the reaction by adding 20 ml. of the 1.0 N KI from a graduated cylinder.

Immediately after mixing, but without filtering out the fibers, titrate the free iodine in the suspension with 0.2 N $Na_2S_2O_3$, adding a few drops of the indicator toward the end of the reaction.

Make a blank determination using exactly the same procedure, but without the pulp. In this case the mixture may be titrated with the $Na_2S_2O_3$ immediately.

Observation:

Burette - 0.2 N $Na_2S_2O_3$ solution

Indicator- starch solution

End point- reddish-brown to colorless.

blue to colorless (after addition of starch indicator)

Burette reading for sample- 'a' ml.

Burette reading for blank- 'b' ml.

Calculations:

Kappa Number $(K) = \frac{\rho \times f}{W}$

and

$$\rho = (b-a)\ N/0.1$$

Where,

f = Factor for correction to a 50% permanganate consumption, dependent on the

value of ρ.

W= weight of moisture free sample in gm

ρ = amount of 0.1 N permanganate actually consumed by the test sample, ml

b = amount of thiosulfate consumed in blank titration (without adding sample), ml

a = amount of thiosufate consumed by the test sample, ml

N = normality of thiosulfate solution

Chapter 4

"Studies on enzymatic hydrolysis of delignified bagasse polysaccharides"

4.1. Introduction

As mentioned in chapter three, production of cellulase and xylanase enzymes using variously treated bagasse substrates is likely to produce enzyme strains that would efficiently hydrolyze bagasse derived polysaccharides. In this chapter we investigate whether this was indeed the case. Lignocellulosic biomass such as agricultural crop residues provide a low cost feedstock for biological production of fuels and chemicals, and offer economic, environmental and strategic advantages (Wyman, C. E., ed. 1996). These materials generally contain 75-80% of cellulose and hemicellulose, which cannot be easily converted to simple monomeric sugars due to their recalcitrant nature. To convert this lignocellulosic biomass into useable energy that is economical and environmentally friendly, both cellulose and hemicellulose must be used. With the advent of modern genetics and other tools the cost of producing sugars from these recalcitrant fractions and converting them into products like ethanol has been significantly reduced. However additional cost reductions are desirable to achieve competitiveness *vis a vis* the existing conventional fuels. In a previous chapter we have shown that high cellulase productivities can potentially be achieved by the use of chemically pretreated biomass as carbon sources.

Thus the present study deals with the evaluation of delignified sugarcane bagasse as a source for the production of sugars (glucose, xylose, arabinose) using enzymes that were produced by treating delignified bagasse polysaccharides with a mutant of *Penicillium janthinellum* NCIM 1171 obtained from our own laboratory. The result of this study shows the high potential of agricultural waste biomass as important sources of sugars for fermentation to biofuels like ethanol and butanol.

4.2. Experimental and Methods

4.2.1 Chemicals

Cellulose-123 powder was obtained from Carl Schleicher and schull co. Dassel, FRG. p-Nitro phenyl β-D-glucopyranoside, carboxymethylcellulose (CMC), xylan (oat spelts), 3,5-dinitrosalysilic acid and Sigmacell were obtained from Sigma-Aldrich Co. St. Louis, Mo. USA. Sodium azide was obtained from S. D. Fine Chemical (India). Avicel PH-101 obtained from Fluka chemie GmbH.

4.2.2. Delignified bagasse sample

Sugarcane bagasse was obtained from Tamil Nadu Pulp and Paper Mills, Chennai, India. The bagasse was made into 100 mesh fine powder by use of a laboratory blender at 3000 rpm. The delignification of bagasse was generally done using different quantities of sodium chlorite at pH 3.8 at 70 °C (samples D, E, F), hydrogen peroxide treatment (sample B), and alkali (sample C). Samples G and H were prepared by a more elaborate steam explosion treatment followed by bleaching sequences. The details are shown in Table 4.1.

4.2.3. Microorganism and culture media

Mutant of *Penicillium janthinellum* NCIM 1171 obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory, Pune, India. This culture was maintained on Potato Dextrose Agar (PDA) and sub cultured once in every three months. PDA contained (g/L) extract from 200 gms of potatoes, glucose, 20.0 g; yeast extract, 1.0 g; and agar, 20.0 g. Production medium described by Mandels & Weber was used for enzyme production (Mandels, M.,& Weber, J. 1969)

4.2.4. Enzyme production and enzyme assay: Details of these methods are given in chapter 3.

4.2.5. Enzyme Units

One unit (IU) of enzyme activity was defined as the amount of enzyme required to liberate one μmol of glucose, xylose or p-nitrophenol from pNPG from the appropriate substrates per min per ml of crude filtrate under assay condition.

4.3. Enzymatic hydrolysis

The hydrolysis of the samples were carried out in 100 ml flask containing 50 ml citrate buffer (pH 4.5, 50mM), 0.5 gm different bagasse samples or other cellulosic materials (Avicel, Sigmacell and cotton), 5mg sodium azide and crude enzyme preparation. This mixture was incubated at 30^0 C or 50^0 C on shaker at 150 rpm. The samples were analyzed for the reducing sugars after suitable time intervals. The enzyme preparation contains 3.5 IU/ml FPA, 4.0 IU/ml β-Glucosidase, 75 IU/ml CMCase and 80 IU/ml xylanase. This preparation which is denoted further as 'x' concentration of enzyme. Similarly 2x and 3x denote multiples of the concentrations.

4.4. Analytical method

The reducing sugar was estimated as either xylose or glucose equivalent by dinitrosalicylic acid (DNS) method (Ulmann's Encyclopedia of Industrial Chemistry, 2002). The kappa number of the samples was determined by TAPPI (T 236 om-99 method and the lignin content was obtained by multiplying the kappa number with 0.15 factor (This factor is generally applied to only samples with low lignin contents say up to kappa no. 20 or so). High Pressure Ion Chromatography

(HPIC): A Dionex HPIC consisting of GS 50 quaternary gradient pump, ED-50 Electrochemical Detector, Rheodyne injector and chromeleon software was used. The column was a CarboPack PA-10, 4 x 250mm (Analytical) column with a CarboPack PA-10 guard column. The mobile phase was 20mM NaOH at a flow rate of 1 ml/min. The injection volume was 25 microlitre, and the observed pressure drop was 2700 psi

4.5. Results and Discussion

Figure 4.1 presents the data on enzymatic hydrolysis of delignified bagasse with Kappa no. 0.8 (lignin content 0.12%). The purpose behind using different concentrations of enzymes and different temperatures was to establish optimum conditions for the rapid hydrolysis of the bagasse sample. This would throw light on the economic feasibility of the method being developed. Figure 4.1 show that 3x enzyme activities at 50 °C achieved approximately 90% hydrolysis.

In the case of Avicel, 3x concentration at 30^0C seems to be optimum for maximum hydrolysis of Avicel. We get approximately 55% hydrolysis within 60 hrs; the slower rate could be due to its microcrystalline structure.

One would have expected sample G (Table 4.1, Kappa no. 1.2) to be less efficient than sample H with Kappa number 0.8, but we actually found it to be slightly superior. It appears that residual lignin play a part in binding the enzymes, leading to higher local concentration of enzymes, and consequently higher hydrolysis rates. This fact has also been noted in prior literature (Palonen, et al., 2004). This data is shown in Figure 4.2 where over 95% hydrolysis occurs within 48 hours at 50 °C. Indeed, in this case the initial rate of hydrolysis was such that about 90% of the hydrolysis occurred in less than 20 hours.

However, as the lignin content increases beyond a certain limit, it begins to have an adverse effect on the rate of hydrolysis, as now it not only acts as a phenolic (acidic) inhibitor, but also its cross linked structure provides a steric barrier to the enzymes to access the polysaccharide components. This is seen in Figure 4.3 for sample F (Kappa no. 16.8) where the total hydrolysis was limited to about 67% of the weight of the polysaccharide taken. As the lignin content raises further, the extent of hydrolysis decreases further, as shown in table 4.1. Ultimately, for untreated raw bagasse, with a Kappa no. of 79.6, only 9% hydrolysis was achievable. For pure microcrystalline cellulose (Avicel and Sigmacell) the hydrolysis extent was limited to 61.5 and 66.6% respectively, in spite of having no lignin content. This fact is attributable to their strongly crystalline nature, which prevents easy access to enzymes. Thus, the performance of Avicel (Figure 4.4) and Sigmacell was comparable to bagasse polysaccharides with Kappa nos. in the range 16-26. Considering the inexpensive nature of the bagasse polysaccharides, there is no doubt about their commercial potential for producing sugars like glucose, xylose, and arabinose. It is pertinent to point out here that the xylose content of bagasse xylan is 75-80% and that of arabinose, a relatively rare and expensive sugar, is about 10%. Both these major constituents of bagasse xylan were hydrolyzed easily by the enzymes produced and used by us. This is clearly shown by the HPIC data (Figure 4.5)

4.6. Conclusions

In conclusion, we have shown the vast techno-economical potential for using waste agricultural biomass like sugarcane bagasse for producing sugars, which can be used for producing biofuels like ethanol. Rare sugars like arabinose can also be produced by this method. This methodology is generic, and will be equally

applicable to other biomass like wheat, rice and cereal straws, which are produced annually in most countries in abundant quantities. Such technologies have great implication for using “green raw materials” and producing “green products”, much needed today.

Table 4.1: Total reducing sugars obtained by enzymatic hydrolysis of 0.5 g (1%) of each bagasse and cellulose samples.

Samples	Kappa No.	Total reducing sugars (mg) after first enzymatic hydrolysis using 0.5 g bagasse	Total reducing sugars from residue of first enzymatic hydrolysis (mg)	Total sugar obtained from 0.5 g of bagasse samples (mg)	% hydrolysis
A	79.4	34.5	10.5	45.0	9.0
B	66.6	97.5	18.7	116.2	23.2
C	54.7	216.6	26.3	242.9	48.5
D	26.0	320.0	5.8	325.8	65.1
E	17.8	281.6	8.0	289.6	57.9
F	16.8	338.3	0.0	338.3	67.6
G	1.2	402.9	70.1	473.0	94.6
H	0.8	365.0	55.5	420.5	84.1
I	Cotton	16.7	40.4	57.1	11.4
J	Avicell	143.5	164.1	307.6	61.5
K	Sigmacell	143.5	179.7	333.2	66.6

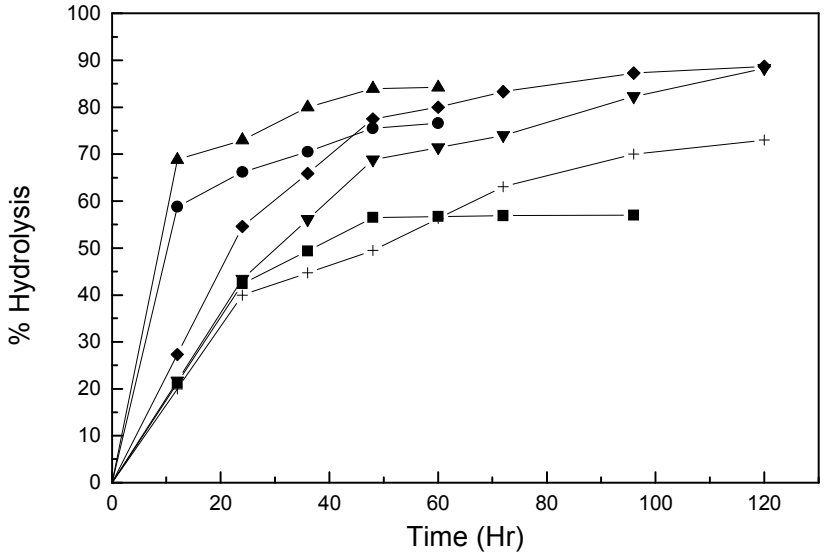

Figure 4.1: Hydrolysis profile of sample H (Kappa No. 0.8) at 30 ^{0}C (x, -+-; 2x, -▼-; 3x, -♦-) and 50 ^{0}C (x, -■-; 2x, -●-; 3x, -▲-). (x, 2x, and 3x denote the concentrations of cellulase and xylanase enzymes, mentioned in the experimental part).

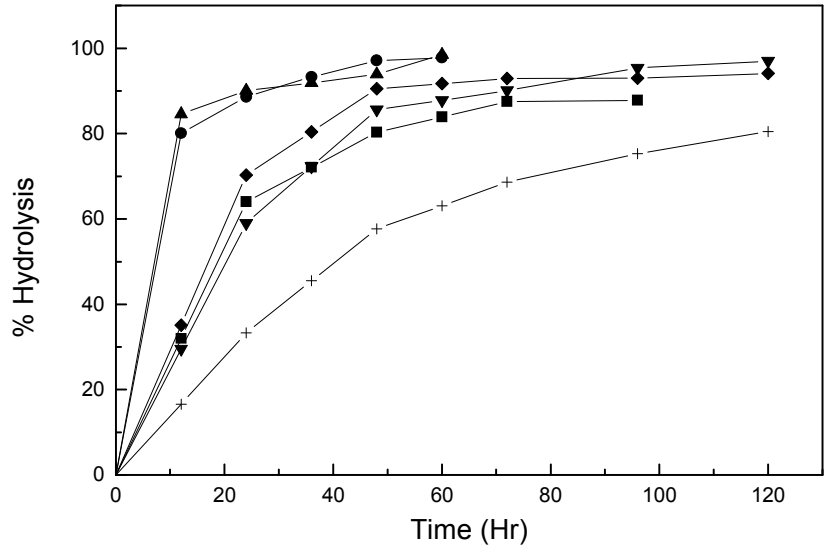

Figure 4.2: Hydrolysis profile of sample G (Kappa No. 1.2) at 30 ^{0}C (x, -+-; 2x, -▼-; 3x, -♦-) and 50 ^{0}C (x, -■-; 2x, -●-; 3x, -▲-) (x, 2x, and 3x denote the concentrations of cellulase and xylanase enzymes, mentioned in the experimental part).

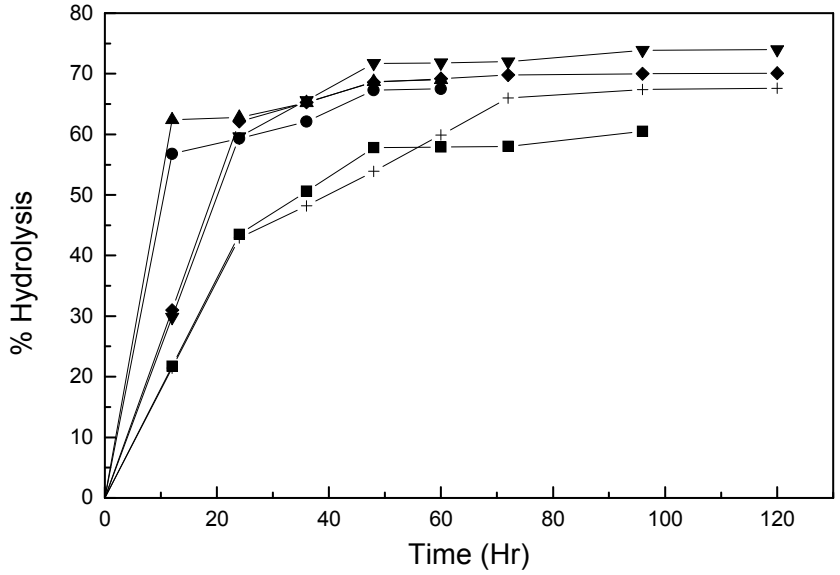

Figure 4.3: Hydrolysis profile of sample F (Kappa No. 16.8) at 30 ^{0}C (x, -+-; 2x, -▼-; 3x, -♦-) and 50 ^{0}C (x, -■-; 2x, -●-; 3x, -▲-), (x, 2x, and 3x denote the concentrations of cellulase and xylanase enzymes, mentioned in the experimental part)

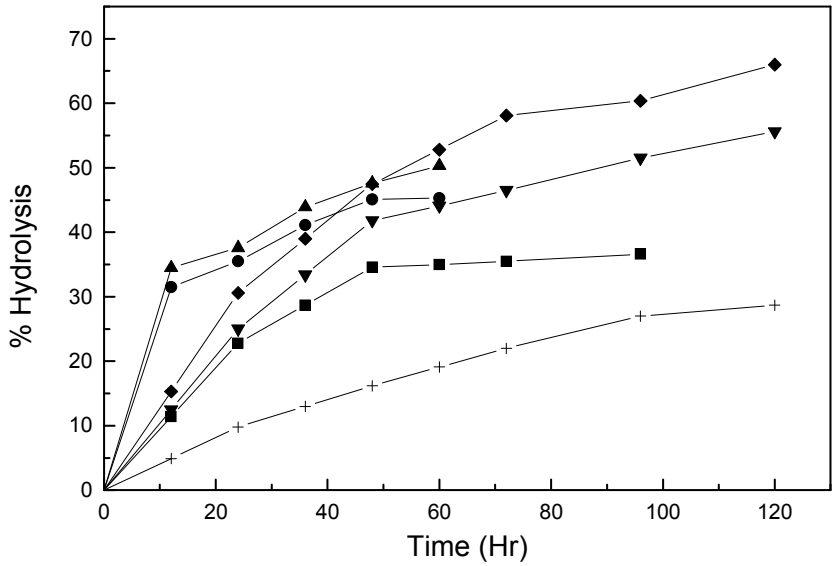

Figure 4.4: Hydrolysis profile of Avicel at 30 ^{0}C (x, -+-; 2x, -▼-; 3x, -♦-) and 50 ^{0}C (x, -■-; 2x, -●-; 3x, -▲-), (x, 2x, and 3x denote the concentrations of cellulase and xylanase enzymes, mentioned in the experimental part)

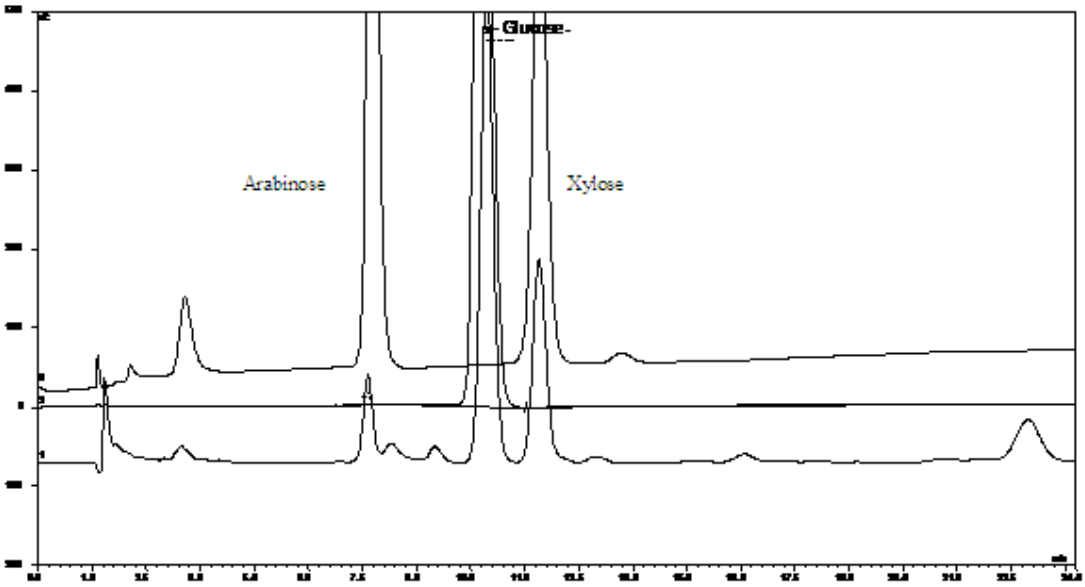

Figure 4.5: HPIC analysis of the sugars produced by the hydrolysis of sample G, showing standard curves for glucose, xylose and arabinose (upper spectrum) and that produced by enzymatic hydrolysis (lower spectrum)

4.6. References

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Chapter 5

"Synthesis and structural properties of dialdehyde cellulose and its derivatives"

5.1. Introduction

Cellulose is a homopolymer in which anhydroglucose unit are linked by β-1, 4-glycosidic bonds. Due to availability of three reactive hydroxyl functional groups in each anhydroglucose unit as well as semi-crystalline polymeric character of cellulose, a great variety of chemical modifications and morphologies are possible. There are several methods of oxidizing cellulose, as described in earlier chapter. Oxidised celluloses have been extensively investigated as it leads to products having several new properties and interesting applications such as biological active fibers (Svjetlana et al., 2009), tissue engineering scaffold (Verma et al., 2008), antimicrobial cellulose (Hou et al., 2008) etc. Other important applications are separation of mercury from waste water (El-Menshary et al., 2008), adhesion-prevention (Jelinkova et al., 2002), curing agents in an epoxy resins (Chavan, 1995), antitumor (Tokunaga et al., 1998) and wound healing (Finn et al., 1992).

However, periodate oxidation is a highly specific reaction for converting cellulose to 2, 3-dialdehyde cellulose without significant side reactions thereby providing a new polymer having aldehyde as well as hydroxyl functional groups. Therefore in this study, we have selected sodium periodate as the oxidant for cellulose. Further oxidation of the 2, 3-dialdehyde cellulose using aqueous hypochlorite leads to the formation of carboxyl functions on the polymers chain. Such diverse functional groups on the cellulose polymers chain increases the range of new derivatives that can be synthesized (Chavan, 1995., Varma, 1984, 1985).

The present study describes in detail the synthesis of controlled partial oxidation of cellulose to develop a series of dialdehyde cellulose having different extents of aldehyde groups (different levels of oxidation) on the cellulose chain. This dialdehyde cellulose has been used as a precursor for further modification to

carboxy, carboxylate and Schiff bases. These derivatives were extensively characterized to establish their chemical structures, morphologies and thermal properties to establish structure-property relationships. Literature on Schiff's bases of 2, 3-dialdehyde cellulose, particularly for low degree of oxidized celluloses is very scanty, perhaps due to low reactivity of 2, 3-dialdehyde cellulose. Therefore we decided to investigate a series of Schiff bases of 2, 3–dialdehyde cellulose, with a view to understanding the reactivity of the aldehyde groups of 2, 3-dialdehyde cellulose towards such reactions, and also the spectral feature, morphologies, thermal properties etc. (Studies on saccharification of these derivatives, surface morphology by SEM analysis and fluorescence visualization by confocal laser scanning microscope of these derivatives are explained in the subsequent chapter). These new series of Schiff bases of cellulose obtained in our study provides an exciting range of functionalized cellulosic polymers with new properties.

5.2. Experimental

5.2.1. Material

Cotton linter cellulose was supplied by Reliance cellulose product limited, Secundrabad, India. It contained > 95% alpha cellulose. A known weight of cellulose was dispersed in distilled water and stirred at room temperature with the help of rotatary shaft for about two and filtered through a nylon cloth placed on a Buchner funnel. The cellulose was then washed with methanol, filtered and dried in vacuum at 60^0 C until constant weight was achieved. This sample was used for synthesis of 2, 3-dialdehyde cellulose of various levels of oxidation.

5.2.2. Chemicals

Sodium metaperiodate (99.5%), sodium thiosulphate (99.5%), sodium bicarbonate (99.5%) were products of S.D. Fine Chemicals. India. Soluble starch (Merck), sodium hydroxide, methanol GR grade, potassium iodide and potassium dichromate were procured from Rankem, India. All chemicals were used without further purification.

5.2.3. Synthesis of 2, 3-dialdehyde cellulose

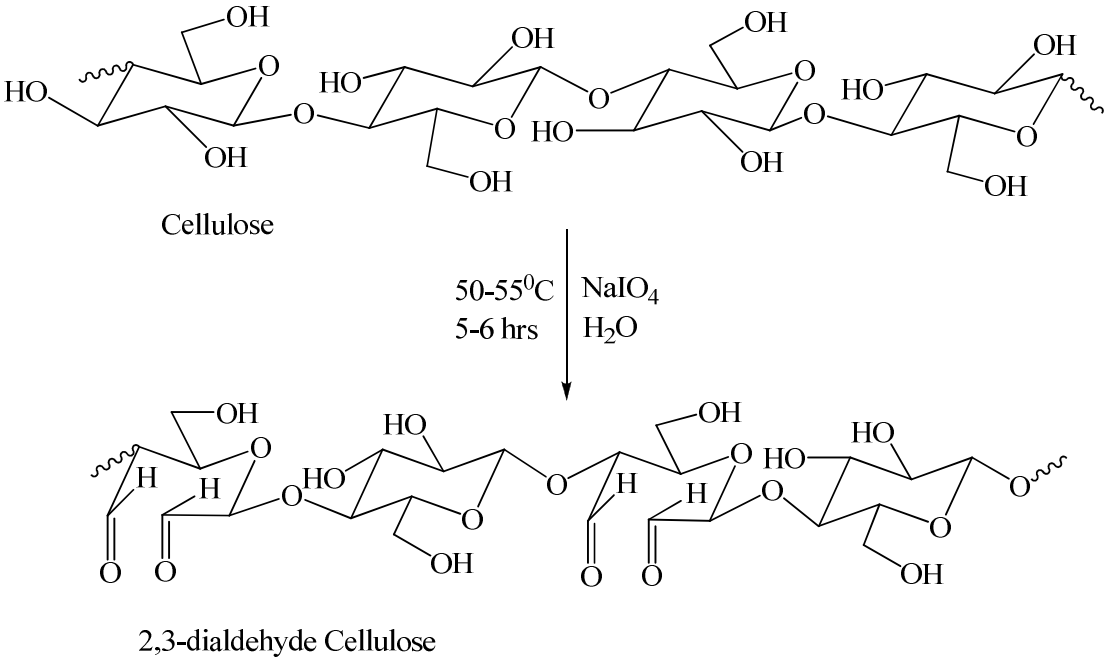


Figure 5.1: Synthesis of 2, 3-dialdehyde cellulose (DAC, partial oxidation))

5.3.3. Methods

FTIR

Fourier-Transform Infrared Spectroscopy (FTIR) Spectrum One was used to record FTIR spectra, between 450 to 4000 cm^{-1} with accumulation of 20 scan and resolution of 4 cm^{-1}.

NMR

A Bruker AV-300 NMR Spectrometer was used to obtain solid state ^{13}C NMR with CP-MAS operating at 75.47 MHz, it was equipped with 7.05 T superconducting magnet, having 4 mm Bruker probe. The samples were packed in 4 mm zirconia rotor and spun at 10 KHz.

WAXRD

Wide Angle X-Ray Diffraction (WAXRD) analysis was carried by using powder XRD Xpert-1217 diffractometer. The scanning speed was 4^{o}/ min, with a radiation of CuK- α. The samples were scanned from 2 θ values of 5^{o} to 40^{o}.

Thermogravimetry (TGA)

Thermal stability of partially oxidized cellulose was studied using TGA (Perkin Elmer TGA 7) with a heating rate of 10^{o}C/min under nitrogen atmosphere.

5.4. General method for synthesis of 2, 3-dialdehyde cellulose (DAC)

In a two neck three liter round bottom flask equipped with mechanically rotatary shaft, a known weight of cellulose was taken. This flask was wrapped with aluminum foil to prevent exposure to light. To this flask were added quantities of sodium metaperiodate as shown in table-5.1 (1.3 times more than the theoretical amount for each degree of oxidation). Sodium metaperiodate was dissolved slowly in two liters of distilled water and the pH of this solution was measured to be

between 3.2 - 3.5. Nitrogen gas was purged in the reaction mixture. The reaction was carried out in the dark under nitrogen atmosphere to avoid decomposition of sodium metaperiodate and photo-oxidation. Temperature of the reaction mixture was maintained between 50-55^{0}C using a water bath for various time intervals, after which the reaction mixture was cooled to room temperature. Oxidized cellulose was filtered off, washed thoroughly with distilled water several times until the filtrate became neutral. The final washing was done by methanol and the product was dried under vacuum at 60^{0}C and characterized by FTIR, CP-MAS ^{13}C NMR, WAXRD and TGA. Quantification of sodium metaperiodate consumed in the reaction was carried out by sodium thiosulphate method (Scott, 1939), described below.

Table 5.1: Quantities of reagent used for synthesis of 2, 3-dialdehyde cellulose (DAC)

Cellulose gm	Sodium metaperiodate, gm	Oxidation %	Weight of DAC gm
50	4.2	5	49.2
50	12.9	15	48
50	21.5	25	46.3
50	43	50	43

5.4.1. Determination of periodate consumption by thiosulphate method

5.4.2. Preparation of 0.1 N sodium thiosulphate ($Na_2S_2O_3$) solutions:

24.817 gm (0.1mole) of sodium thiosulphate was dissolved in 100 ml of distilled water and then diluted to 1000 ml in a standard volumetric flask

5.4.2.1. *Standardization of 0.1 N sodium thiosulphate ($Na_2S_2O_3$) solution*

Standardization of sodium thiosulphate ($Na_2S_2O_3$) solution was carried according to well established method (Vogel, 1961). Potassium dichromate 0.1032 gm (0.00035 mole) was accurately weighed and transfer to a conical flask containing 100 ml of distilled water. After potassium dichromate was completely dissolved, it was acidified with 5 ml of conc. HCL (35%). To it was quickly added 20 ml of 10% aqueous potassium iodide solution followed by 50 ml of distilled water. The flask was securely closed and kept in the dark for 20 minutes. Subsequently, it was titrated against approximately 0.1 N sodium thiosulphate ($Na_2S_2O_3$) solution. During titration the solution turned brown to pale yellow. At this point starch indicator was added and the solution became bluish green colored. Drop wise addition of 0.1 sodium thiosulphate ($Na_2S_2O_3$) solution was continued. The change of colour of the dichromate solution from greenish blue to light green was taken as the end point of the titration.

5.4.2.2. *Preparation of 10% potassium iodide (KI) solution*

100 gm of potassium iodide was weighed accurately and dissolved in 100 ml of distilled water. After complete dissolution of potassium iodide, this solution was diluted to 1000 ml in standard volumetric flask.

5.4.2.3. *Saturated solution of sodium bicarbonate*

In 50 ml of distilled water sodium bicarbonate was added with constant string till no further sodium bicarbonate was dissolved. Excess sodium bicarbonate was

added and solution was stirred about 5 minute. Insoluble bicarbonate was allowed to settle down and clear solution was collected by decantation.

5.4.2.4. Determination of periodate consumption

$$NaIO_4 + 7KI + 8HCl \longrightarrow NaCl + 7KCl + 4H_2O + 4I_2 \uparrow$$

In 1 ml of reaction mixture was added to 20 ml distilled water containing 10 ml cold saturated solution of sodium bicarbonate and 20 ml of 10% KI solution. The solution was made acidic by addition of 5 ml concentrate HCl (35%) and kept in the dark for 20 minutes. The librated iodine was titrated with standard 0.1 N $Na_2S_2O_3$ solution using starch as indicator. The end point was blue to colorless.

1ml 1N $Na_2S_2O_3$ = 0.02675 gm $NaIO_4$ gm

1ml 0.1N $Na_2S_2O_3$ = 0.002675 gm $NaIO_4$ gm

$$\text{gm periodate} = \frac{\text{burette reading X 0.002675 X total volume}}{\text{ml of volume taken from reaction mixture}}$$

Representative calculation for 50 gm of cellulose,

Cellulose molecular weight=162

Sodium metaperiodate molecular weight=213.89

For 162 gm of cellulose 213.89 gm of sodium metaperiodate will give 100% oxidation

For 50 gm of cellulose 66.01 gm of sodium metaperiodate will give 100% oxidation

66.01 gm of sodium metaperiodate will give 100% oxidation

3.3 gm of sodium metaperiodate will give 5% oxidation (Theoretical amount for 50 gm cellulose)

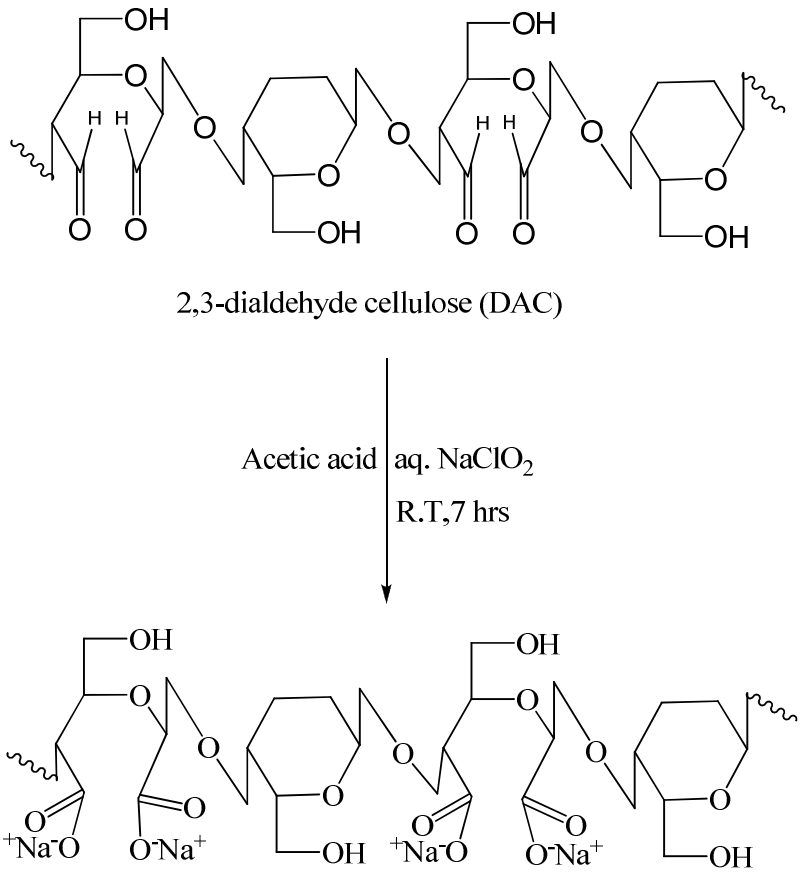
4.2 gm of sodium metaperiodate was used in the reaction (1.3 time more than theoretical amount, see table 5.1)

3.23 gm consumed during the reaction (calculated by titration)

66.01 gm of sodium metaperiodate will give 100% oxidation

3.23 gm of sodium metaperiodate will give 4.9 % oxidation which is taken as 5 % oxidation.

5.5. Synthesis of sodium 2, 3-dicarboxycelluloses (NaDCC)



2,3-dialdehyde cellulose (DAC)

Sodium 2,3-dicarboxy cellulose (NaDCC)

Figure 5.2: Synthesis of sodium 2, 3-dicarboxy cellulose (NaDCC)

Material : 2, 3-dialdehyde cellulose (5, 15, 25, and 50% based on glucose monomer unit) was synthesized as described earlier.

Chemicals: Sodium chlorites of 90% purity, potassium iodide (Rankem, India Ltd) glacial acetic acid (99.5% Merck) were used without further purification. However an assay of sodium chlorite was checked before use.

5.5.1. *Assay of sodium chlorite ($NaClO_2$)*

Assay of sodium chlorite ($NaClO_2$) was carried out as per reported method (Snell and Etter, 1970). According to this method 0.1 gm of $NaClO_2$ is dissolved in 50 ml of distilled water in stoppered conical flask. After complete dissolution of sodium chlorite, 20 ml of 10% potassium iodide (KI) and 5 ml of glacial acetic acid are added. The flask is stoppred and kept in the dark for about 15 minutes, after which the liberate iodine was titrated with 0.1N sodium thiosulphate ($Na_2S_2O_3$) solution. When pale yellow colored was observed, 2 ml of 0.1% starch solution was added. At this stage the reaction mixture turns black bluish in color. Sodium thisulphate was added drop wise till the solution become colorless.

1 ml of 0.1 mol sodium thisulpahte = 2.2610 mg of $NaClO_2$

$$\% NaClO_2 = \frac{V \times N \times 2.2610}{W}$$

Where,

V = Volume of $Na_2S_2O_3$ Solution

N = Normality of $Na_2S_2O_3$ Solution

W = Weight of sample taken

5.5.2. General procedure for synthesis of sodium 2, 3-dicarboxy celluloses (NaDCC)

For synthesis of sodium 2, 3-dicarboxy celluloses (NaDCC) with different degree of oxidation (i e. ~5, 15, 25, 50 and 75%) the required quantities of 2,3-dialdehyde cellulose was dispersed in distilled water. To dialdehyde cellulose, sodium chlorite solution and required amount of acetic acid dissolved in distilled water and added slowly with constant string. The temperatures of reaction mixture were maintained at 25^0-30^0C. Details of quantities of sodium chlorite and acetic acid used are given in table 5.2.

Table 5.2: Quantities of reagent required for synthesis of sodium 2, 3-dicarboxycelluose (NaDCC)

Wt of DAC gm	Oxidation %	Wt.of $NaClO_2$ gm	Acetic acid ml	Wt. of NaDCC gm
10	5	1.11	0.35	9.5
10	15	3.35	1.11	9.1
10	25	5.55	1.85	8.8
10	50	11.17	3.70	8.1
5	75	10.0	3.35	3.5

The start of oxidation was indicated by the formation of yellow coloration and evolution of chlorine dioxide (ClO_2) gas. The reaction was continued for 7 hrs until there was no further evolution of gas. Nitrogen gas was bubbled through the reaction mixture to remove the dissolved gases from the reaction. The pH of the reaction mixture was adjusted to 8.5-9 by addition of 10N NaOH solution. The product was settled at the bottom and was separated by decantation. The product was washed several times with distilled water until the filtrate was neutral. It was

dried under vacuum at 60 ^{0}C and characterized by FTIR CP-MAS ^{13}C NMR and TGA. Sodium detection was done by Energy-dispersive X-ray spectroscopy (EDAX). These products of different level of oxidations are insoluble in common organic solvents.

5.5.3. Synthesis of 2, 3-dicarboxy cellulose (DCC)

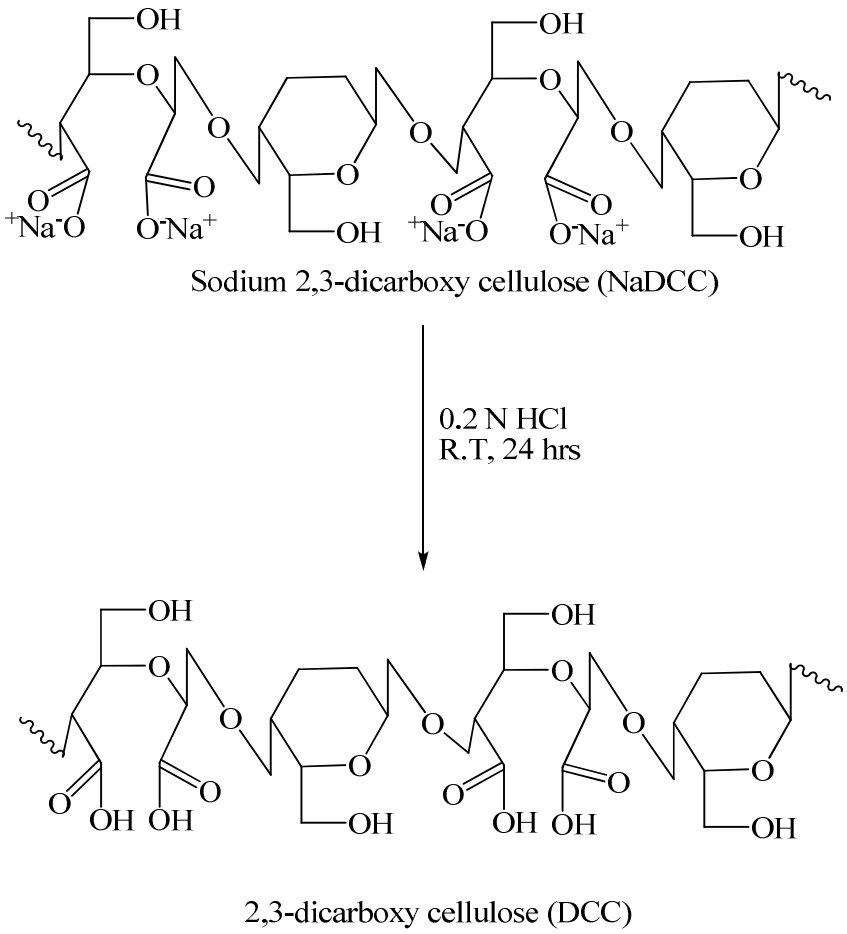


Figure 5.3: Synthesis of 2, 3-dicarboxy cellulose (DCC)

Materials: Sodium 2, 3- dicarboxy cellulose (5, 15, 25, and 50% based on glucose monomer unit) was synthesized as per described method.

Chemicals: Sodium hydroxide (99.5% Merck), Hydrochloric acid 35%, potassium hydrogen phthalate (99.5%, Rankem, India Ltd), phenolphthalein indicator (pH range 8-10), bromocresol purple indicator (pH 5.2-6.8) were used without further purification.

5.5.3.1. General procedure for synthesis of 2, 3-dicarboxycelluloses (DCC)

For synthesis of 2, 3-dicarboxycelluloses (DCC) with different degree of oxidation (i e., 5, 15, 25 and 50%), required quantities of sodium 2, 3-dicarboxy cellulose was dispersed in distilled water and 0.2 N HCl was added with string till the pH reached 3.5. This solution was kept in freezer at 1 ^{0}C for about half an hour with stirring at regular intervals. After this the aqueous fraction was decanted off and 30 ml distilled water was added and kept at 5 ^{0}C for 24 hours. Details of quantities of reagents are given in Table 5.3. After a fixed time the solution was decanted off and the product washed several time until the filtrate was neutral. It was dried in vacuum at 60 ^{0}C and characterized by FTIR, CP-MASS ^{13}C NMR, WAXRD and TGA. These products of different levels of oxidation are insoluble in common organic solvents.

Table 5.3: Quantities of reagent required for synthesis of 2, 3-dicarboxycelluose (DCC)

NaDCC %	Wt. of NaDCC gm	0.2 N HCl ml	Wt of DCC gm
5	10	25	9.5
15	10	60	9.0
25	10	120	8.8
50	10	200	8.0

5.5.3.2. Carboxy group determination

Carboxy group was determined by alkali titration method, as described below,

5.5.3.3. Preparation of 0.1 N sodium hydroxide solution (0.1 N NaOH)

4 gm of sodium hydroxide was taken in 1000 ml volumetric flask and initially dissolved in 100 ml of distilled water. After complete dissolution of alkali this solution was diluted to 1000 ml in a volumetric flask by distilled water

5.5.3.4. Standardization of 0.1 N NaOH solutions: Potassium hydrogen phthalate (0.25 g) was dissolved in 100 ml of distilled water and a drop of phenolphthalein indicator was added. The solution was titrated with the prepared 0.1 N NaOH solution, till end point (pink color) was obtained.

Calculation:

$$\text{Normality of 0.1 N NaOH} = \frac{\text{Wt of potassium hydrogen phthalate X factor}}{\text{burette reading}}$$

$$\frac{\text{Wt of potassium hydrogen phthalate X factor}}{\text{burette reading}}$$

$$\text{Factor} = \frac{1000}{\text{Eq.wt of potassium hydrogen phthalate}}$$

5.5.3.5. Preparation of 0.1 N hydrochloric acid solution (0.1 N HCl)

8.9 ml conc. HCl (35%) was taken in 1000 ml capacity of volumetric flask and diluted to 1000 ml by distilled water.

5.5.3.6. Standardization of 0.1 HCl solution

To 10 ml of 0.1 N hydrochloric acid solution 50 ml of distilled water was added and this solution was titrated with standardized 0.1N NaOH solution using phenolphthalein indicator till colorless to pink endpoint was obtained.

5.5.3.7. Quantification of carboxy group of 2, 3-dicarboxycellulose

In a 40 ml of 0.1 N NaOH solution 0.1 gm of 2, 3-dicarboxycellulose was added. To it was added 1 gm of sodium chloride and 5 ml of distilled water. This mixture was kept at room temperature for about 18 hours. It was then titrated with 0.1N HCl by using bromocresol purple indicator. The end point was indicated by a change in color from violet to yellow. Similarly blank solution was made without adding 2, 3-dicarboxycellulose.

Calculation:

$$\%, 2, 3 -\text{dicarboxycelluose} = \frac{R \times N \times W \times 100}{\text{Wt of DCC} \times 1000 \times 2}$$

$$= \frac{R \times N \times 9.6}{\text{Wt of DCC}}$$

Where,

R = blank

N = normality of 0.1 N HCl

W= mol. wt of DCC (192)

5.6. Synthesis of Schiff bases from 2, 3-dialdehyde cellulose

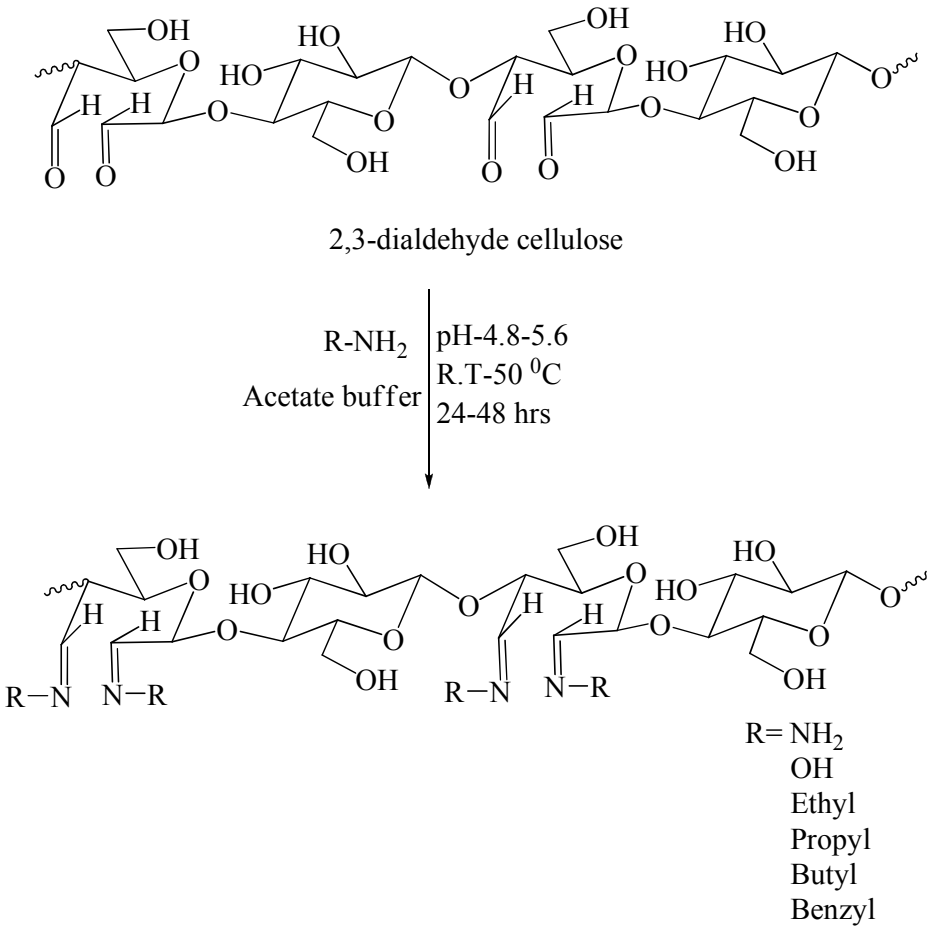


Figure 5.4: Synthesis of Schiff bases from 2, 3-dialdehyde cellulose

Materials: 2, 3- dialdehyde cellulose (5, 15, 25 and 50% based on glucose monomer unit) synthesized as described earlier, were used for these experiments.

Chemicals: Anhydrous hydrazone, ethyl amine (70%), propyl amine, butyl amine, benzyl amine, acetic acid, sodium acetate (Aldrich, India Ltd) and hydroxyl amine hydrochloride, (Rankem India, Ltd) were used without further purification.

5.6.1. Acetate buffer: Acetate buffer was made according to a procedure given in the chapter "General method for handling protein and enzymes (In the book Buffer: Principle and Practice by Vincent S. Stoll and John S. Blanchard, Section 4).

Stock Solutions

5.6.1.1. 0.2 M acetic acid solution: 11.5 ml of acetic acid was taken in a 1000 ml capacity of standard volumetric flask and diluted up to the mark by distilled water.

5.4.1.2. 0.2 M sodium acetate solution: 16.4 gm of sodium acetate was dissolved in 100 ml of distilled water and this solution was then diluted to 1000 ml in a standard volumetric flask by distilled water. Desired pH of acetate buffer was obtained by taking given amount of acetic acid and sodium acetate and diluted to 100 ml in a standard volumetric flask.

5.6.2. General procedure synthesis of Schiff bases of 2, 3-dialdehyde cellulose

To a 250 ml round bottom flask 100 ml of 0.2 M acetate buffer was taken, pH of this buffer was adjusted between 4.8- 5.6. To this buffer known amounts of hydrazine, oxime and amines was added slowly with constant stirring. The known quantity of oven dried 2, 3- dialdehyde cellulose (DAC) was added portion wise over a period of 10 minutes. These reaction mixtures were then stirred from room temperature to 55 ^{0}C for different reagents (See table 5.4-5.9 for details) for various time intervals.

Reaction mixtures slowly turn faint yellow to pale yellow in colour (deeper colour for higher oxidation levels of DAC). After a fixed time interval the reaction mixtures were allowed to settle down. The insoluble solid settled at the bottom was separated by centrifugation. The filtrates were drained and solid products were washed several times with distilled water till neutral filtrate was obtained. The products were dried under vacuum at 60 ^{0}C for 24 hrs and characterized by FTIR,

CP-MASS ^{13}C NMR, WAXRD and TGA. Nitrogen contents were determined by elemental analysis (Elemental analysis by semi-micro analysis is inaccurate for nitrogen contents below 1%. Therefore Energy-dispersive X-ray spectroscopy (EDAX) was used to confirm the presence of nitrogen in such cases, even though not quantitatively). All products obtained from different level of oxidiesd 2,3-dialdehyde cellulose are insoluble in common organic solvents.

Table 5.4: Quantities of hydroxyl amine hyrdrochloride required for synthesis of 2, 3- dioxime cellulose

DAC gm	Oxidation %	Quantities of reagents	Wt. of products gm	'N' content calculated %	'N' content obtained %
2	5	0.12	1.9	0.7	0.6
2	15	0.38	1.7	2.2	1.8
2	25	0.66	1.6	3.7	3.4
2	50	1.2	1.1	7.3	7.1

Table 5.5: Quantities of hydrazone required for synthesis of 2, 3-dihydrazone cellulose

DAC gm	Oxidation %	Quantities of reagents	Wt. of products gm	'N' content calculated %	'N' content obtained %
2	5	0.2	2.2	1.4	1.2
2	15	0.6	2.0	4.4	3.4
2	25	1.0	1.8	7.4	5.4
2	50	2.0	1.4	14.8	10.5

Table 5.6: Quantities of ethyl amine (70%) required for synthesis of 2, 3-diethylimine cellulose

DAC gm	Oxidation %	Quantities of reagents	Wt. of products gm	'N' content calculated %	'N' content obtained %
2	5	0.6	2.4	0.65	!!
2	15	1.7	2.2	1.9	1.1
2	25	2.8	1.8	3.2	2.2
2	50	4.0	1.5	6.5	4.8

!! - Detected by Energy-dispersive X-ray spectroscopy (EDAX see appendix-4)

Table 5.7: Quantities of propyl amine required for synthesis of 2, 3-dipropylimine cellulose

DAC gm	Oxidation %	Quantities of reagents	Wt. of products gm	'N' content calculated %	'N' content obtained %
2	5	0.6	2.6	0.5	!!
2	15	2.2	2.4	1.7	1.2
2	25	3.0	2.3	2.8	2.3
2	50	7.7	1.9	5.7	5.0

!! - Detected by Energy-dispersive X-ray spectroscopy (EDAX see appendix-4)

Table 5.8: Quantities of butyl amine required for synthesis of 2, 3-dibutylimine cellulose

DAC gm	Oxidation %	Quantities of reagents	Wt. of products gm	'N' content calculated %	'N' content obtained %
2	5	0.6	3.0	0.5	!!
2	15	1.8	2.8	1.5	1.1
2	25	3.0	2.5	2.6	2.2
2	50	6.6	1.8	5.2	4.5

!!- Detected by Energy-dispersive X-ray spectroscopy (EDAX see appendix-4)

Table 5.9: Quantities of benzyl amine required for synthesis of 2, 3-dibenzylimine cellulose

DAC gm	Oxidation %	Quantities of reagents	Wt. of products gm	'N' content calculated %	'N' content obtained %
2	5	0.7	3.5	0.5	!!
2	15	2.0	3.1	1.2	1.0
2	25	3.5	2.7	2.0	1.7
2	50	7.0	2.2	4.1	3.9

!!- Detected by Energy-dispersive X-ray spectroscopy (EDAX see appendix-4)

5.7. Results and discussion

Oxidation of cellulose takes place by the well known Malaprade reaction in which 2, 3 hydroxyl groups are rapidly oxidized by the periodate ion. It is reported that periodate functions best in water which makes glycol-cleavage oxidation possible with all types of carbohydrates and their derivatives (Bobbitt, 1956).

5.7.1. Discussions on characterizations by FTIR

In 2, 3-dialdehyde cellulose, the aldehyde group tends to react with hydroxyl groups of cellulose in the presence of moisture to form acetals and hemiacetal (figure.5.5) therefore removal of moisture from the cellulose is needed prior to FTIR measurements to push the equilibrium reaction to the free aldehyde side. This was done by keeping the samples pellets under the vacuum oven at 80 °C for 24 hrs. Before the FTIR measurement, the maximum air exposure of the samples was only about 10 seconds. It is generally observed from FTIR spectra of partially oxidized cellulose that the carbonyl peaks are undetectable. However, after careful drying under vacuum, FTIR spectra of oxidized cellulose exhibit two characteristic peak at 1735 and 880 cm^{-1}. 1735 cm^{-1} is characteristic peak of carbonyl group stretching and 880 cm^{-1} assigned to the hemiacetal linkage in the dialdehyde cellulose (Calvini et al., 2006). The absence of free aldehyde group in 2, 3-dialdehyde cellulose was well studied by various authors (Potthast, 2005; Fan, 2001; Spedding, 1960; Higging and McKenzie, 1958). This indicates that the moisture content as well as inter and intra molecular hydrogen bonding in the samples made such analysis difficult. It is reported that not all of the carbonyl functions of the dialdehyde cellulose are free, they may form cyclic hemiacetal linkages with primary hydroxyl groups or hemialdol linkages with water or one or both carbonyl groups may be hydrated. All possible forms of 2, 3-dialdehyde structure of

cellulose are depicted in figure 5.5 (Fan, 2001). However, the aldehyde functional groups of 2, 3-dialdehyde cellulose does reacts as aldehyde, even it sluggish, in whatever forms it may exist; the reactivity of these aldehyde groups is highly pH dependant. These hemiacetal and hemialdol linkages are fairly weak and are readily broken to liberate the carbonyl groups to react with other functional groups (Radley, 1976). The concentration of free aldehyde groups in partially periodate-oxidized cellulose is thus greatly affected by moisture content and therefore careful drying and then using FTIR are essential to detect the carbonyl group of aldehydes in partially oxidized samples. It is obvious that the intensity of carbonyl peaks increases with oxidation percentage. After converting these aldehyde groups into other functional groups like acid, salt of acid, hydrazone, oximes and imines, similar facts were observed and these functionalities cannot be detected at normal condition because of masking of these groups by hydrogen bonding. These functionalities could be detected after careful drying and then measuring FTIR spectra. Further, after formation of Schiff bases, the carbonyl groups of 2, 3-dialdehyde cellulose could not be detected by FTIR even after through drying as explained above. This is another proof of the occurrence of the reaction between the aldehyde with oxime, hydrazone and amines. These spectra are presented in the appendix-3 of this chapter.

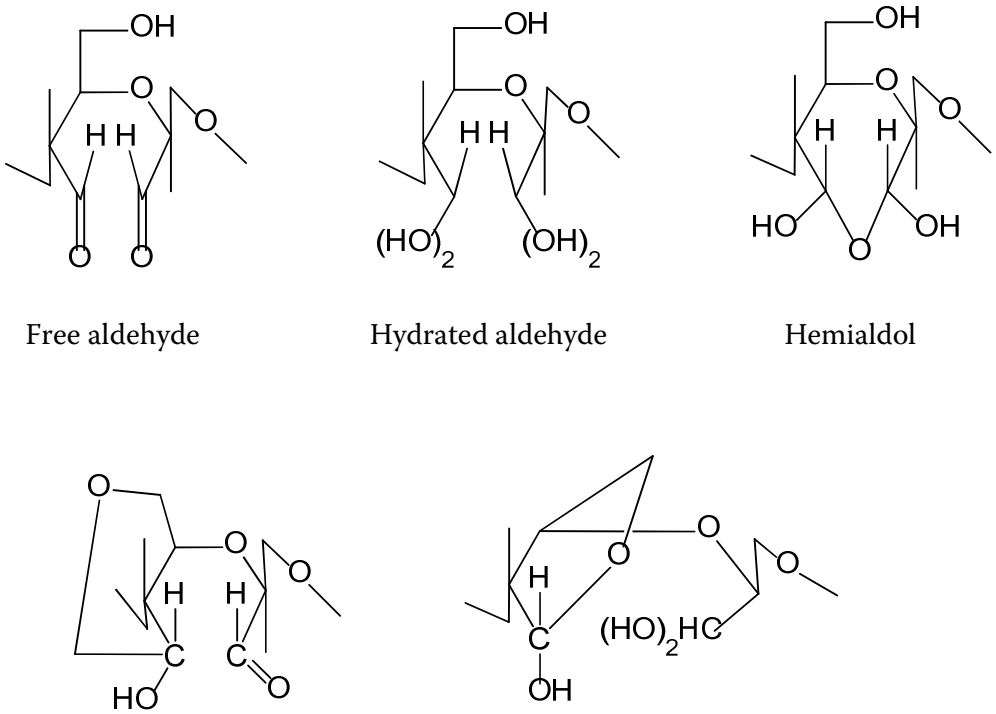


Figure 5.5: Structural forms of 2, 3-dialdehyde cellulose

5.7.2. Discussions on characterizations by CP-MAS ^{13}C NMR

Oxidation affects the crystallinity of the cellulose molecule. We attempted to detect these morphological changes by a study of CP-MAS ^{13}C NMR of partially oxidized cellulose and their derivatives. Beginning with cellulose (Figure 5.6), upfield part of the spectrum (the region between 60-70 ppm) is assigned to C-6 of primary hydroxyl group. The next cluster of resonance between 70-80 ppm is attributed to C-2, C-3 and C-5 of pyranose ring. The region between 81-95 ppm is associated with C-4 and that between 102-108 for C-1 the anomeric carbon (Attala and VanderHart, 1999). A detailed description of CP-MAS ^{13}C NMR study of native cellulose, regenerated cellulose, low DP (degree of polymerization) regenerated cellulose and bacterial cellulose has been reported by Attala and

VanderHart (1999). Our studies have gone beyond this work by exploring several series of cellulose derivatives with incremental changes in chemical structures, for e. g. by oxidation of cellulose to 2, 3 –dialdehyde cellulose by 5%, 15%. 25% and 50% oxidation, followed by conversation to sodium salt of 2, 3-dicarboxy cellulose (NaDCC), 2,3-dicarboxy cellulose (DCC), 2,3-dioxine cellulose, 2,3-dihydrazone cellulose and 2,3-diimine cellulose (2,3-diethylimine cellulose, 2,3-dipropylimine cellulose, 2,3-dibutylimine cellulose and 2,3-dibenzylimime cellulose). In 2,3-dialdehyde cellulose we found that there are no significant chemical shifts. Even at high oxidation levels no carboxyl peak at 180-190 ppm was observed. At lower oxidation (~5%) splitting resonance of C-2, C-3 and C-5 remains intact and can be clearly seen in the spectra (Figure 5.7). From 15% oxidation onwards gradual broadening of peaks due to merging are observed in the C-2, C-3 and C-5 regions and clearly seen in the spectra (Figure 5.8-5.10). The sharp spectra of cellulose are due to its high degree of crystallinity and continued decreases in crystallinity by oxidation leads to broad spectra. This is most clearly seen in 75% oxidation level of cellulose (Figure 5.11) synthesized especially to prove this point). These facts were also supported by WAXRD analysis (see later paragraph).

In sodium salt of 2, 3-dicarboxy cellulose (NaDCC), lower oxidation derivatives shows similar spectra as that of 2, 3-dialdehyde cellulose (DAC). This is because of at lower level of oxidation, only surface groups and amorphous region may get oxidized. However, from 50% oxidation onwards the carbonyl peak starts appearing at 176 ppm and can be seen in the spectra figure 5.15. (In oxidation level more than 50% a sharp carbonyl peak is observed and can be seen in figure 5.16 which represent 75% oxidized NADCC synthesized especially to prove this point). In case of 2, 3-dicarboxy cellulose (DCC), splitting resonance of C-2, C-3 and C-5 remains intact at all levels of oxidation and exhibits similar spectral pattern as that

of cellulose (Figure 5.18-5.21). This could be due to increase in hydrogen bonding and regaining crystallinity to some extent by the molecules (These facts also supported by SEM and confocal analysis, see chapter VI). Varma et al (1997) studied the CP-MAS ^{13}C NMR of DAC, DCC and NaDCC synthesized from wood cellulose and observed that the formation of upfield shoulders on C-6, C-4 and C-1 with increasing oxidation levels. Similar facts were observed in these cases of high molecular weight cellulose of cotton linters (Figure 5.8-5.16).

The oxime derivatives also shows similar spectral pattern that of DCC as splitting resonance of C-2, C-3 and C-5 remains intact in all levels of oxidation (See appendix-5). However, the broad C=N peak observed in 50% oxidized sample is shown in figure 5.22. In hydrazone derivatives splitting resonance of C-2, C-3 and C-5 starts from 5% oxidation onwards with gradual broadening of peaks as oxidation level increases, are observed in this regions. The C-1 peak also appears as broad due to merging and is clearly seen in the spectra (see appendix-5). The C=N peaks could not be observed up to 50% of oxidized samples, however, this can be seen as a broad peak in more higher level of (~75%) oxidation synthesized especially to detect this peak (Figure 5.23).

The CP-MAS ^{13}C NMR shows that for the imine derivatives (2,3-diethylimine cellulose, 2,3-dipropylimine cellulose, 2,3-dibutylimine cellulose and 2,3-dibenzylimime cellulose) spectral line broadening occurs for all levels chemical modification(5%, 15%, 25%, 50% all spectra given in appendix-5). For other derivatives described in this chapter, spectral line broadening is observed only for higher levels of chemical modification. In imine derivatives, specially alkylimine derivatives there are no imine peaks observed in all levels of oxidation and only gradual broadening of peaks observed (see appendix-5) which indicates that these groups are not in a free form. However, benzylimine derivatives shows imine peak

at 130 ppm at all level of oxidation (fig.5.24). Since increasing oxidation was shown to leads to a continual decrease in the degree of crystallinity, broadening of peaks represents increase in the amorphous content of the samples. Thus solid state CP-MAS ^{13}C-NMR spectroscopy can also be used to study and monitor morphological changes in the cellulose.

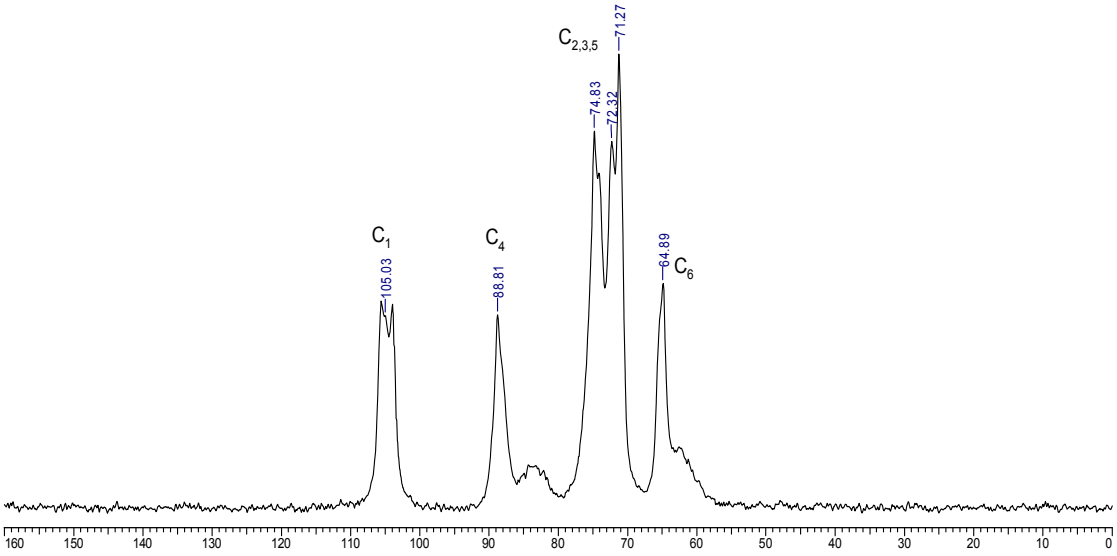


Figure 5.6: CP-MAS ^{13}C NMR of cellulose

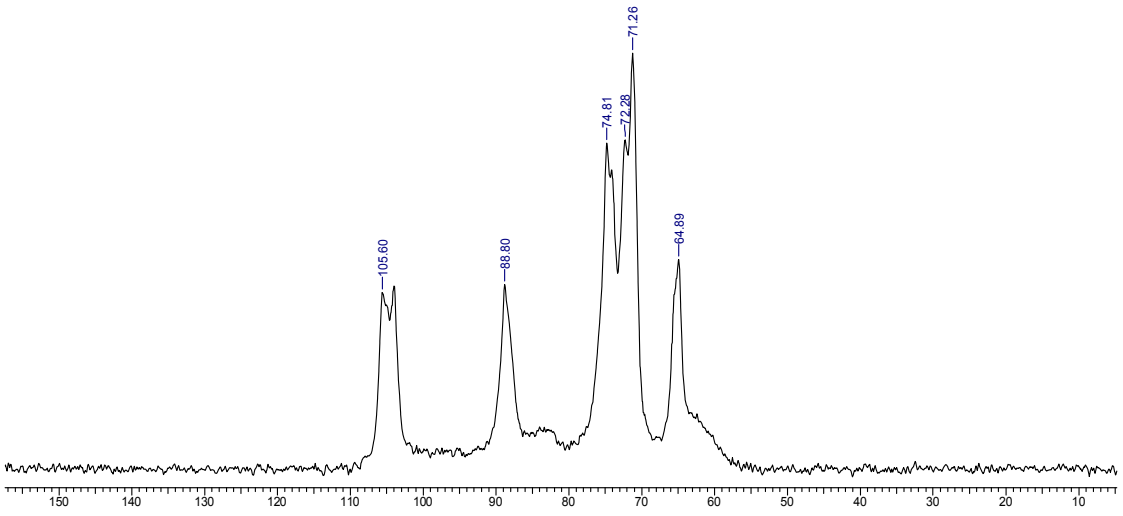


Figure 5.7: CP- MAS ^{13}C NMR of 2, 3-dialdehyde cellulose-5 (DAC-5)

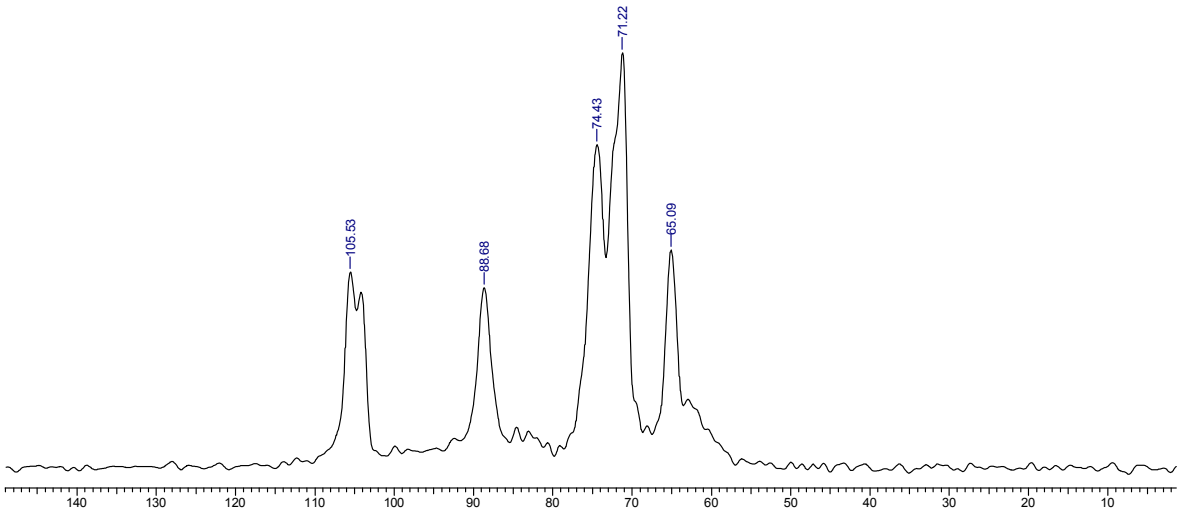


Figure 5.8: CP-MAS ^{13}C NMR of 2, 3-dialdehyde cellulose-15 (DAC-15)

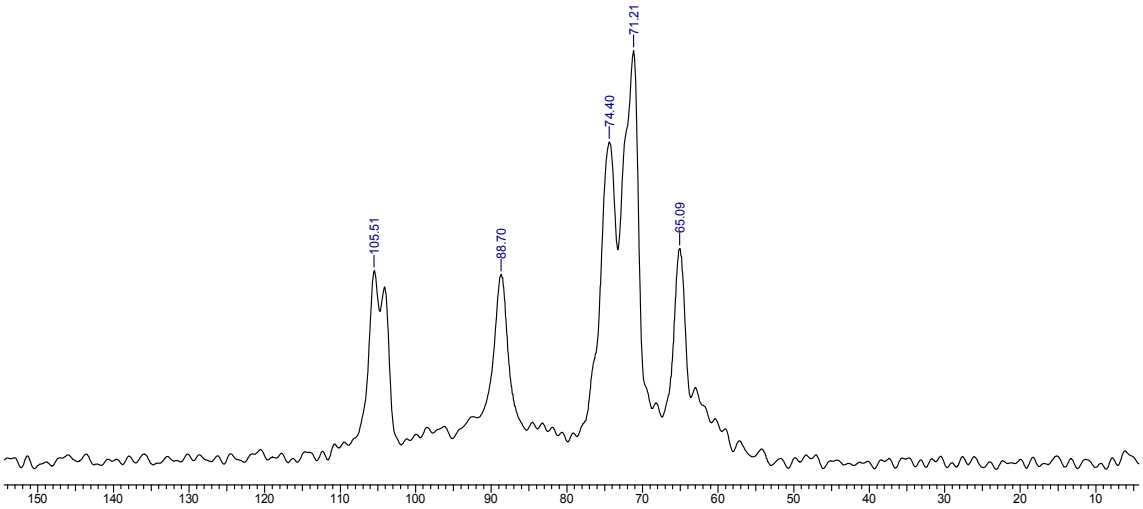


Figure 5.9: CP-MAS ^{13}C NMR of 2, 3-dialdehyde cellulose-15 (DAC-25)

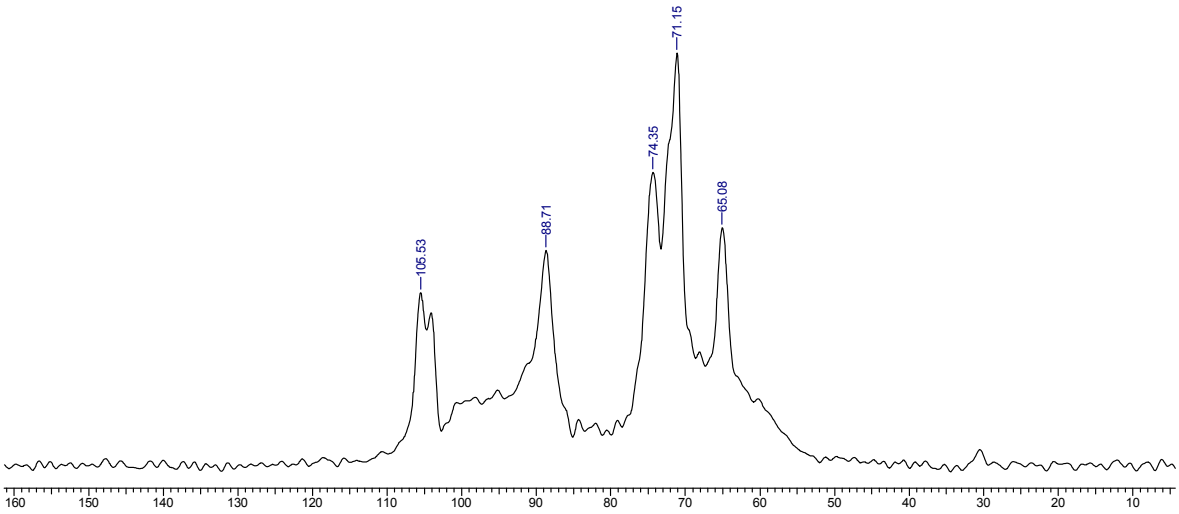


Figure 5.10: CP-MAS ^{13}C NMR of 2, 3-dialdehyde cellulose-50 (DAC-50)

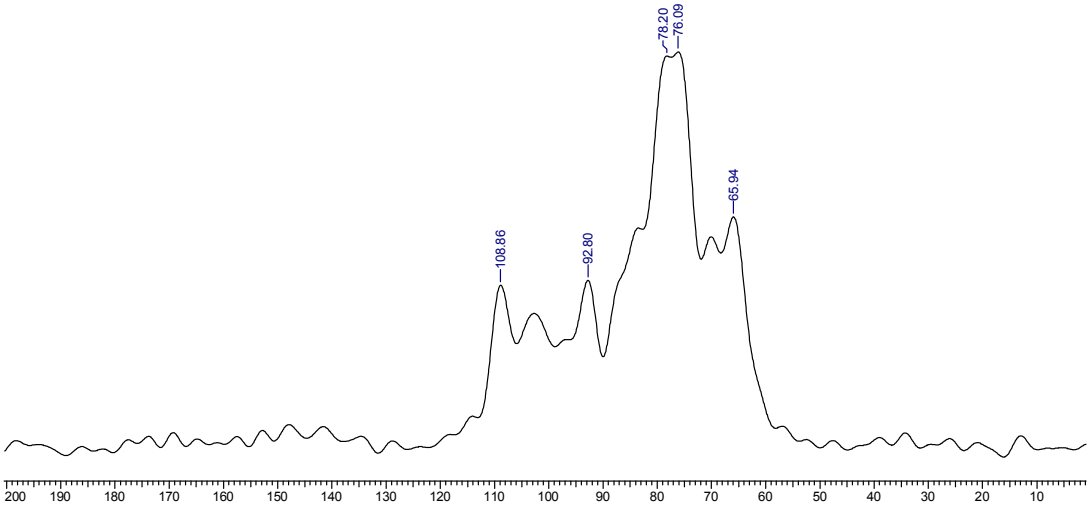


Figure 5.11: CP-MAS ^{13}C NMR of 2, 3-dialdehyde cellulose-75 (DAC-75)

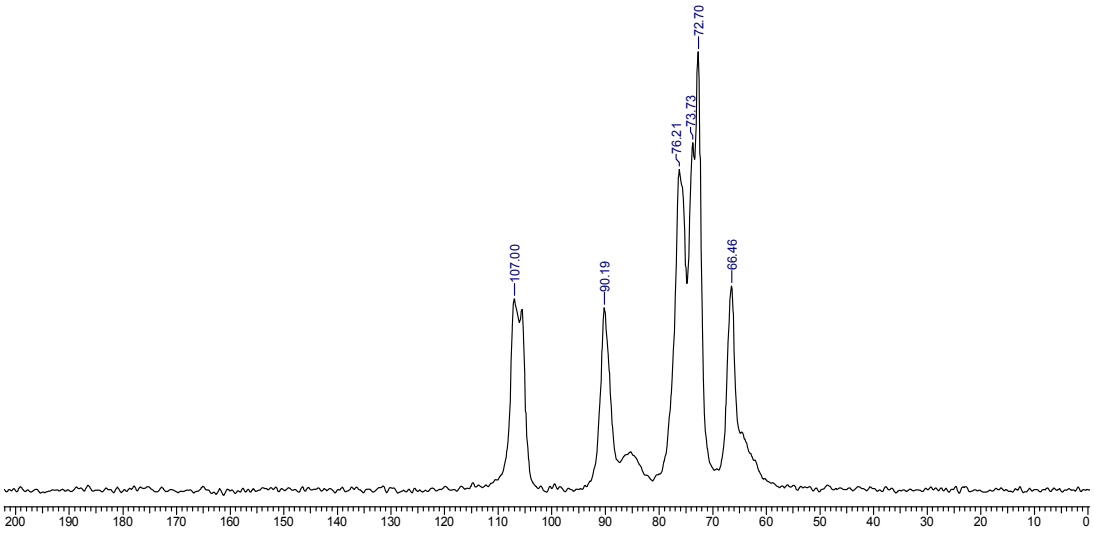


Figure 5.12: CP-MAS ^{13}C NMR of sodium 2, 3- dicarboxy cellulose-5

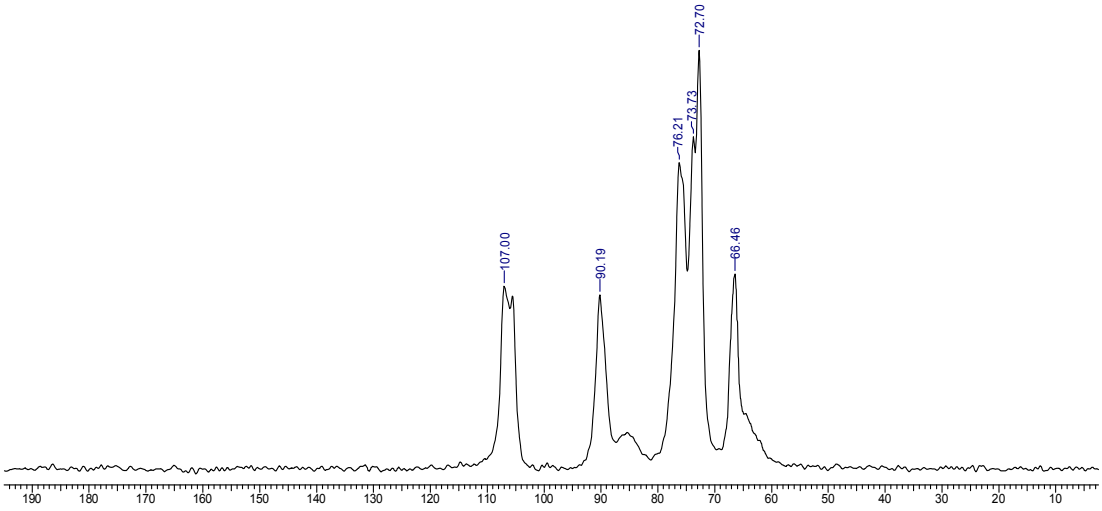


Figure 5.13: CP-MAS ^{13}C NMR of sodium 2, 3- dicarboxy cellulose-15

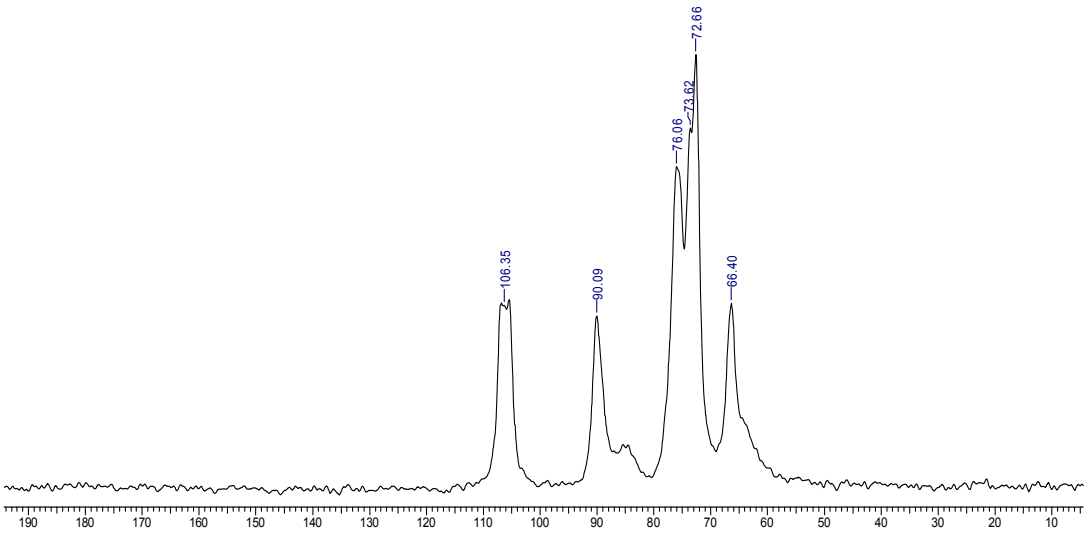


Figure 5.14: CP-MAS ^{13}C NMR of sodium 2, 3- dicarboxy cellulose-25

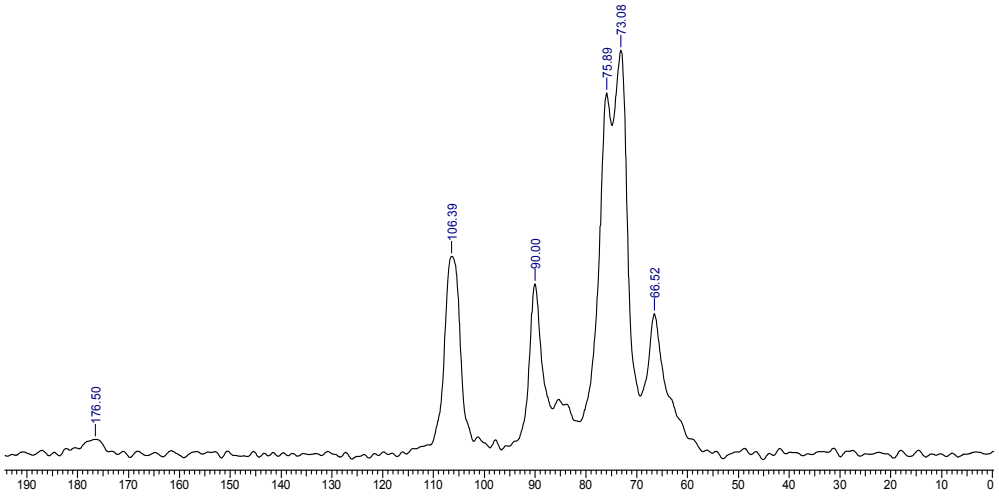


Figure 5.15: CP-MAS ^{13}C NMR of sodium 2, 3-dialdehyde cellulose-50

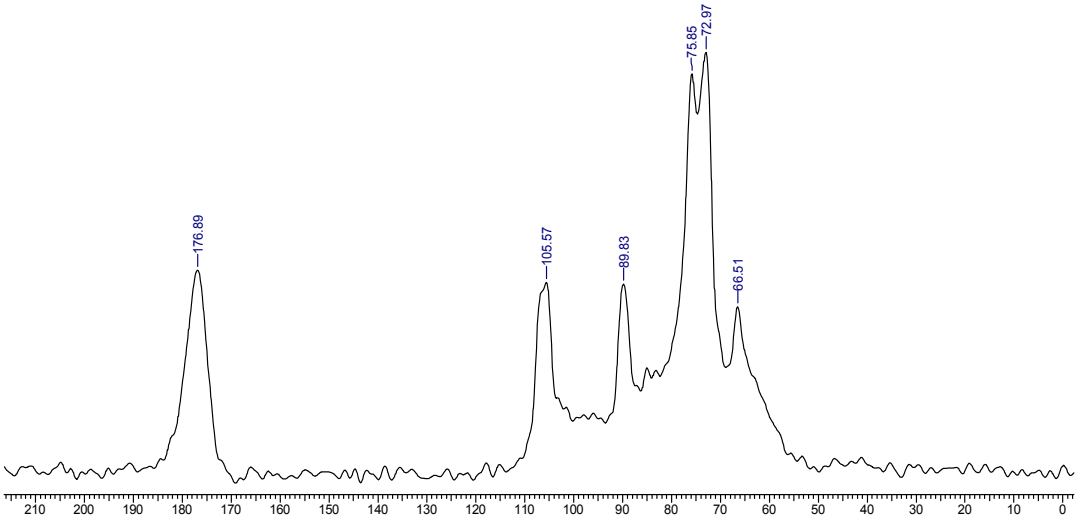


Figure 5.16: CP-MAS ^{13}C NMR of sodium 2, 3-dialdehyde cellulose-75

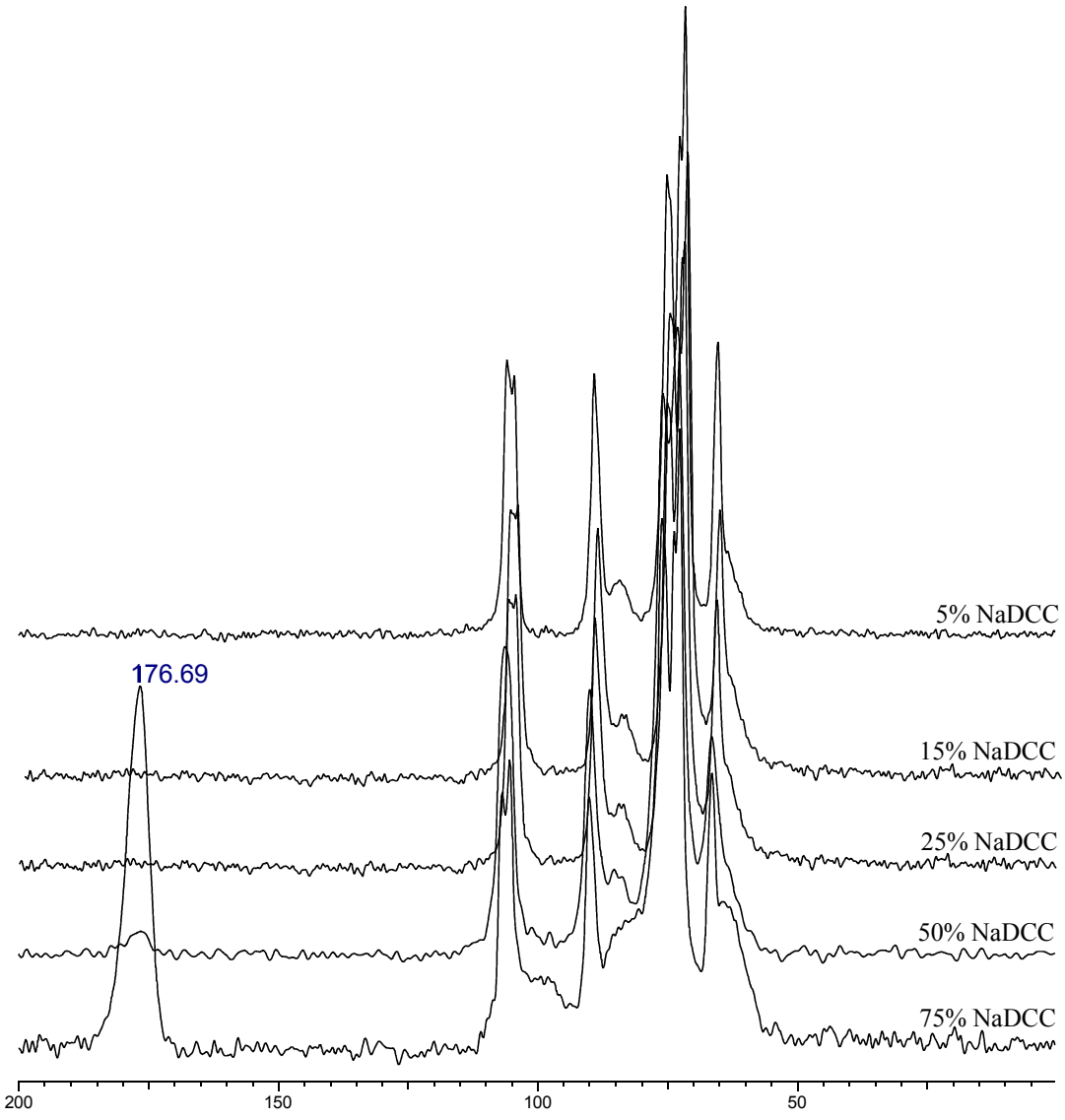


Figure 5.17: Overlapping of CP- MAS ^{13}C NMR of sodium 2, 3- dicarboxy cellulose (NaDCC)

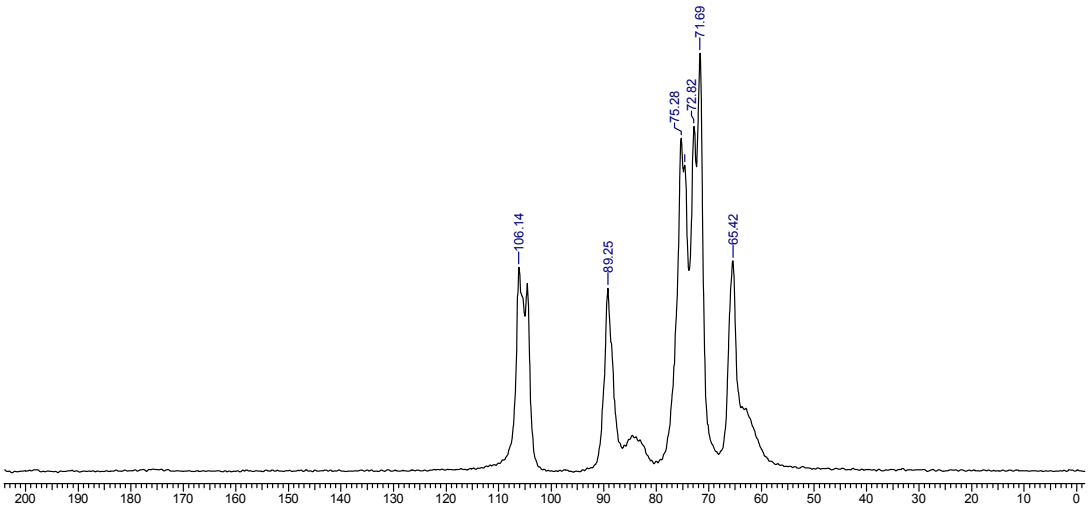


Figure 5.18: CP-MAS ^{13}C NMR of 2, 3-dicarboxy cellulose (DCC-5)

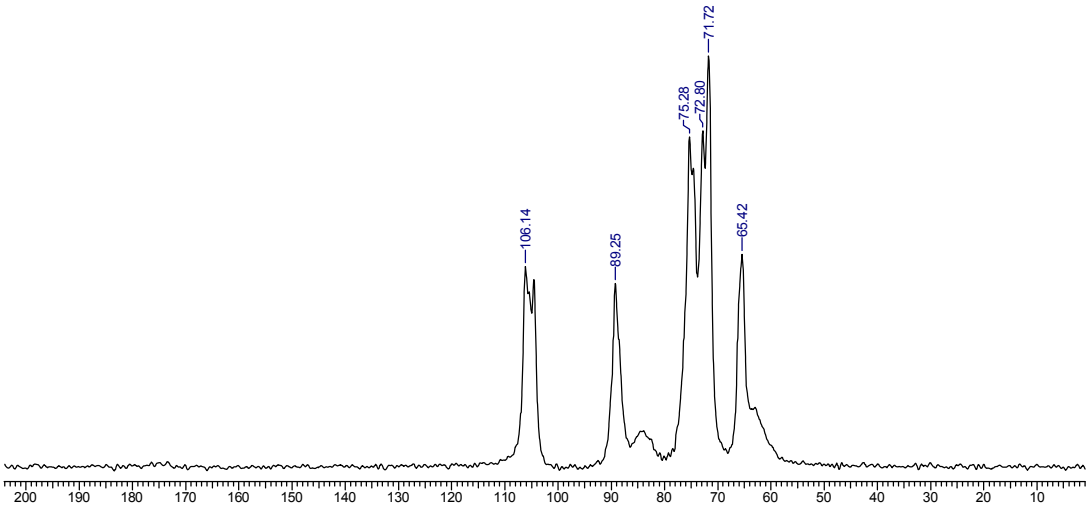


Figure 5.19: CP-MAS ^{13}C NMR of 2, 3-dicarboxy cellulose (DCC-15)

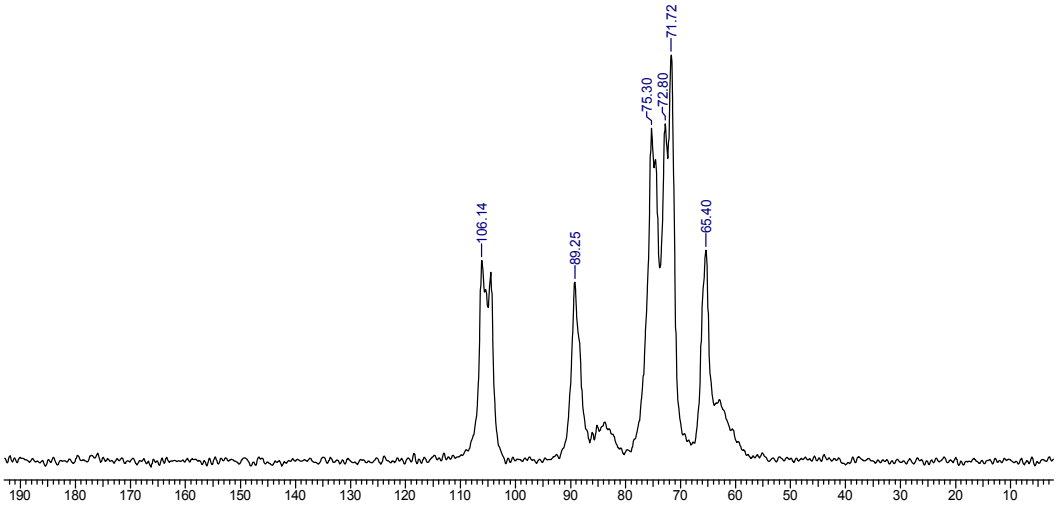


Figure 5.20: CP-MAS ^{13}C NMR of 2, 3-dicarboxy cellulose (DCC-25)

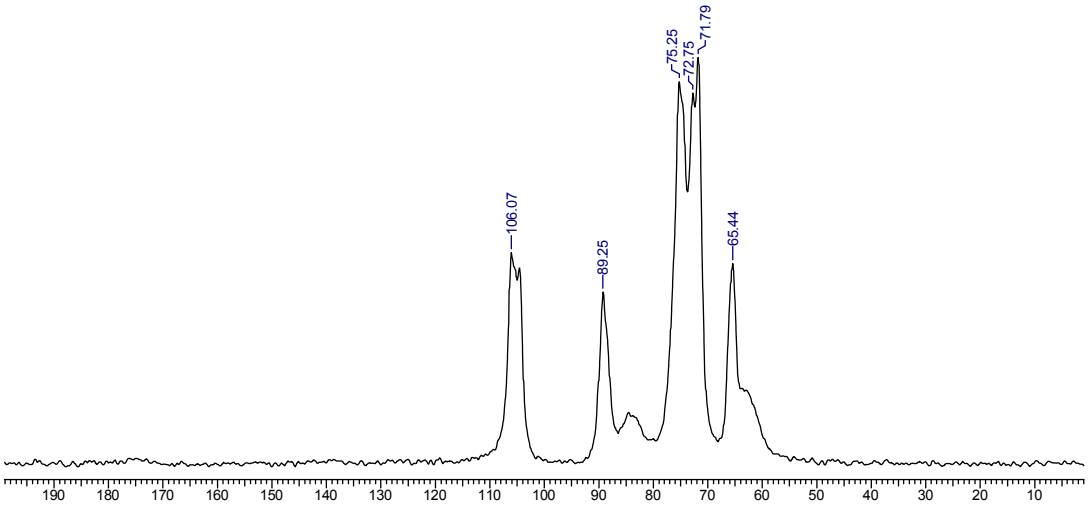


Figure 5.21: CP-MAS ^{13}C NMR of 2, 3-dicarboxy cellulose (DCC-50)

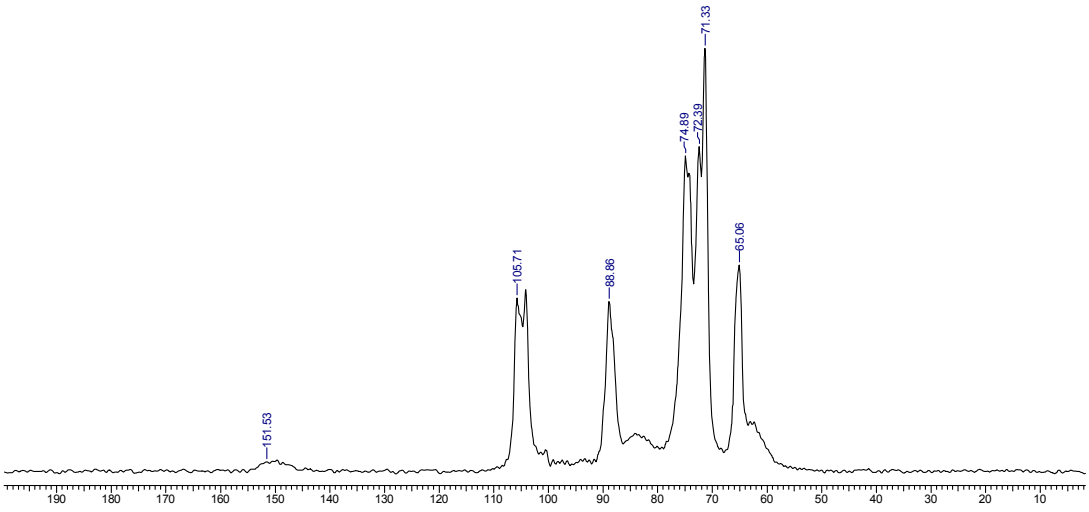


Figure 5.22: CP-MAS ^{13}C NMR of 2, 3-dioxime cellulose-50

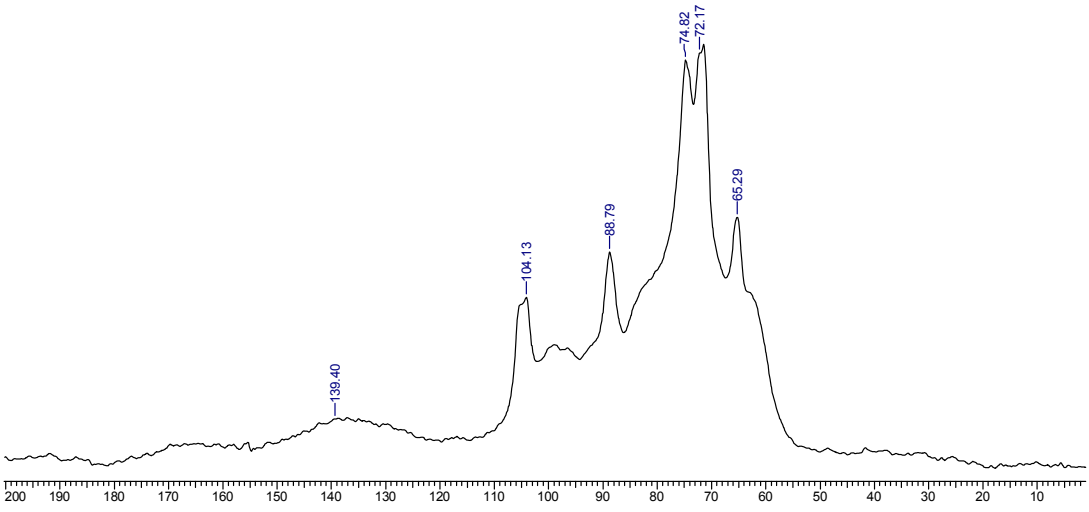


Figure 5.23: CP-MAS ^{13}C NMR of 2, 3-dihydrazone cellulose-75

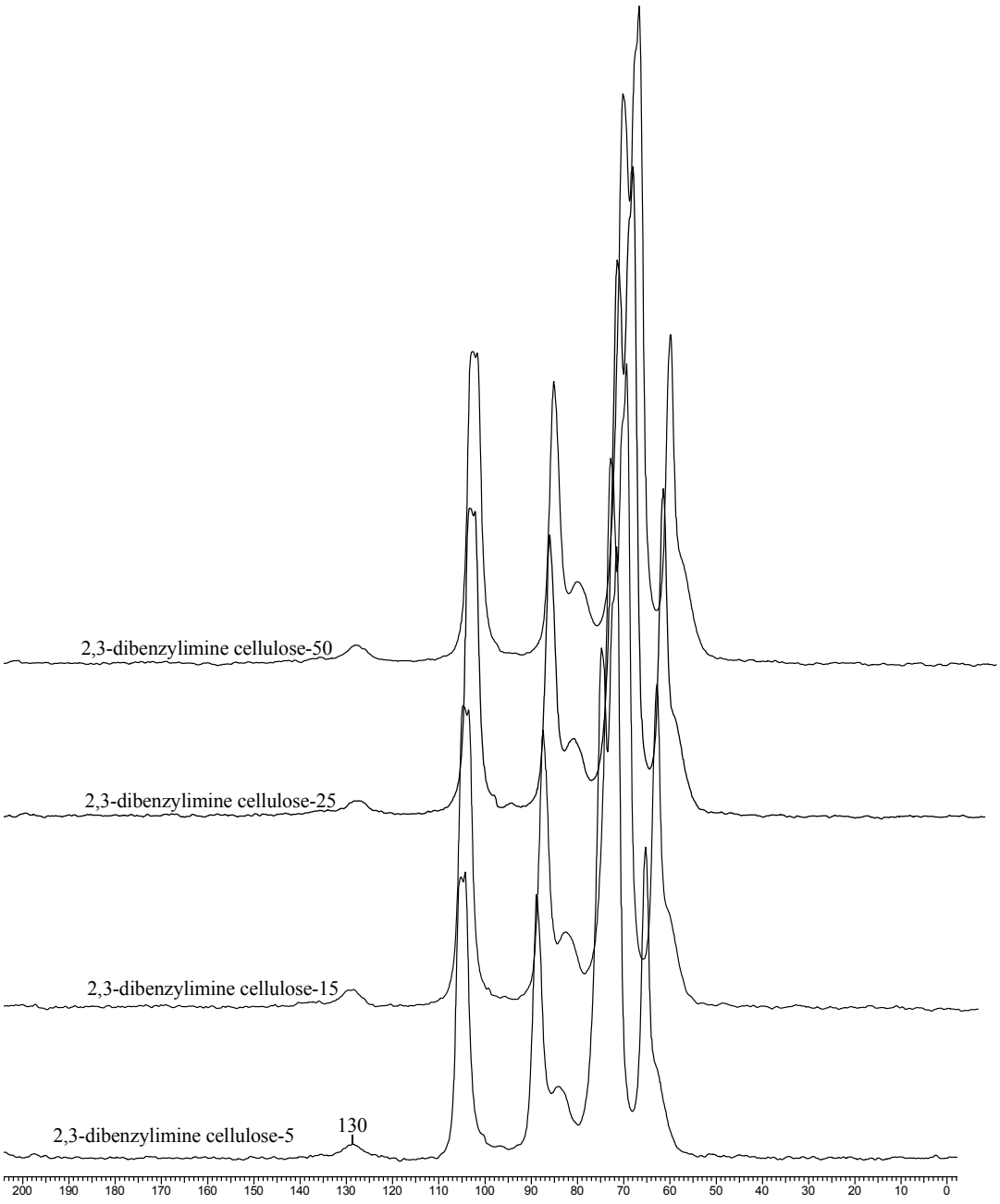


Figure 5.24: Overlapping of CP-MAS ^{13}C NMR of 2, 3- dibenzylimine cellulose with various level of oxidation

5.7.3. Discussions on WAXRD analysis of partially oxidized cellulose and its derivatives

WAXRD analysis shows that X-ray diffraction peak at 2θ = 22.9° for the oxidized cellulose samples decreases almost proportionally to the degree of oxidation of the starting cellulose (Figure 5.25). For 2, 3-dicarboxy cellulose (DCC) similar trend is observed (Figure 5.26). Interestingly in other derivatives like hydrazone, oxime and imines (2, 3-diethylimine cellulose, 2, 3-dipropylimine cellulose, 2, 3-dibutylimine cellulose and 2, 3-dibenzylimine cellulose) decrease of crystallinity are almost proportional to the degree of oxidation of the starting cellulose (see appendix-6 at the end of this chapter). Oxidation of cellulose causes change in the structure and crystallinity of the resulting molecules which affect its chemical and physical properties. Earlier studies have also shown that X-ray diffractogram of cellulose becomes more diffuse as the degree of oxidation increases (Davidson 1914). Mc-Burney (1954) had shown that oxidation most likely occurs in the amorphous region by analyzing oxidized cellulose sample with X-ray diffraction. These results also parallel of Chavan et al (1995).

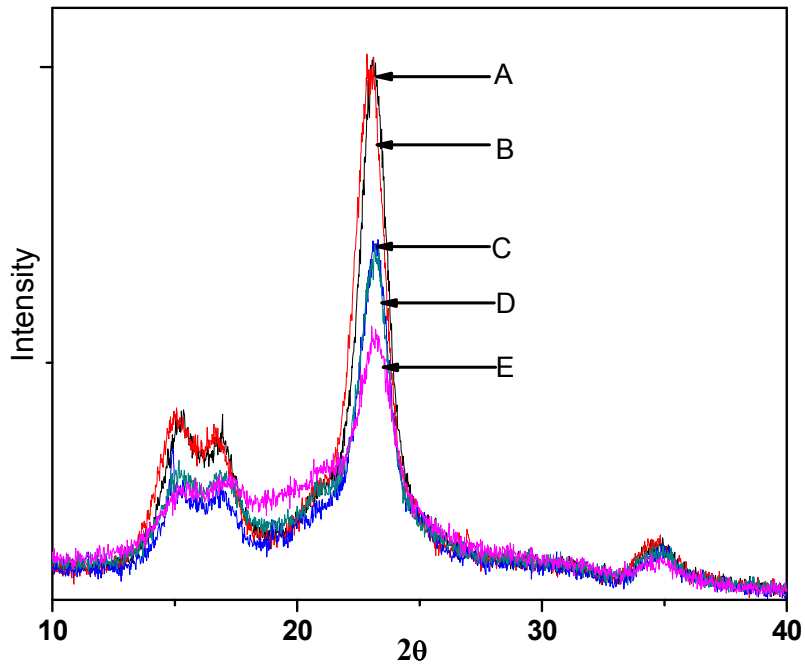


Figure 5.25: WAXRD of cellulose and 2, 3-dialdehyde cellulose (DAC) with various level of oxidation (A-Cellulose, B-DAC-5, C-DAC-15, D-DAC-25, and E-DAC-50)

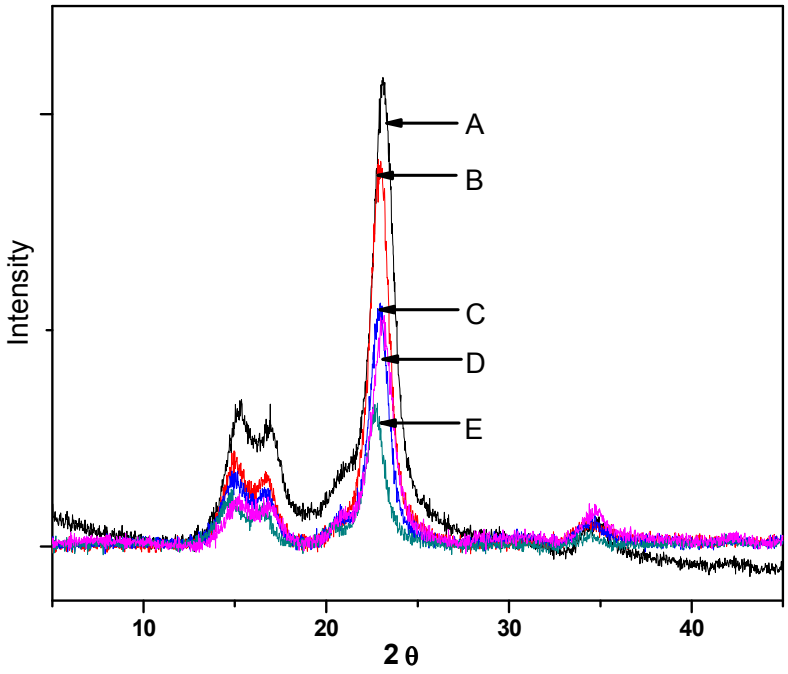


Figure 5.26: WAXRD of cellulose and 2, 3-dicarboxy cellulose (DCC) with various levels of oxidation (A-Cellulose, B-DCC-5, C- DCC -15, D- DCC -25, and E- DCC 50)

5.7.4. Discussion on thermal analysis of partial oxidized cellulose and its derivatives

TGA curves of cellulose and partially oxidized dialdehyde cellulose with various oxidation levels shows that thermal stability depends on the chemical structure, molecular weight and crystallinity of the polymer. Oxidised cellulose have higher amorphous contents (as shown by WAXRD) and lower molecular weight than the parent cellulose. Therefore, higher the degree of oxidation earlier the onset of degradation (Figure 5.27-5.29 and appendix-7 of this chapter).

Rapid decomposition in the temperature range from 310 to 420 °C is observed for the initial cellulose sample. The oxidized sample exhibits a lower onset temperature for decomposition as carbonyl group catalyzed the thermal degradation and lowers the initial degradation temperature. Table 5.10 gives representative thermal analysis of cellulose and 2, 3-dialdehyde cellulose. Other derivatives also exhibit the similar pattern of thermal stability. These are listed in appendix-7 at the end of this chapter. These samples have high residual mass after the decomposition, perhaps due to increased crosslinking reaction.

Table 5.10: Thermal analysis of cellulose and 2, 3-dialdejyde cellulose

Sample	**T_{onset} (^{0}C)**	**T_{25} (^{0}C)**	**T_{50} (^{0}C)**	**T_{75} (^{0}C)**	**T_{max} ((^{0}C))**	**Residue (Wt %)**
Cellulose	310	367	384	397	414	9.0
DAC-5	250	350	363	378	396	12.8
DAC-15	232	344	360	370	382	15.5
DAC-25	225	337	354	365	375	15.2
DAC-50	185	310	346	360	370	19.2

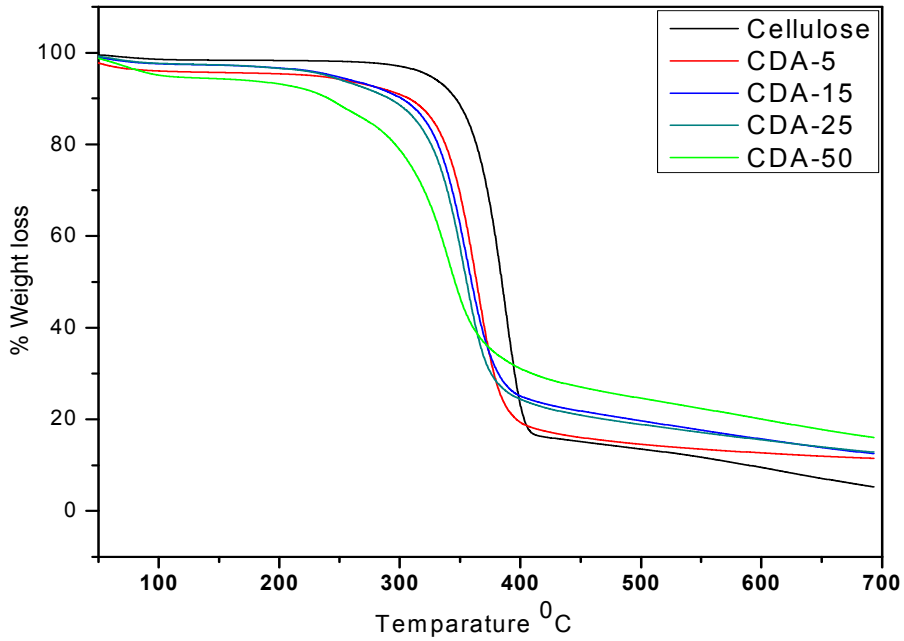


Figure 5.27: TGA of cellulose and 2, 3- dialdehyde cellulose (DAC) with various level of oxidation

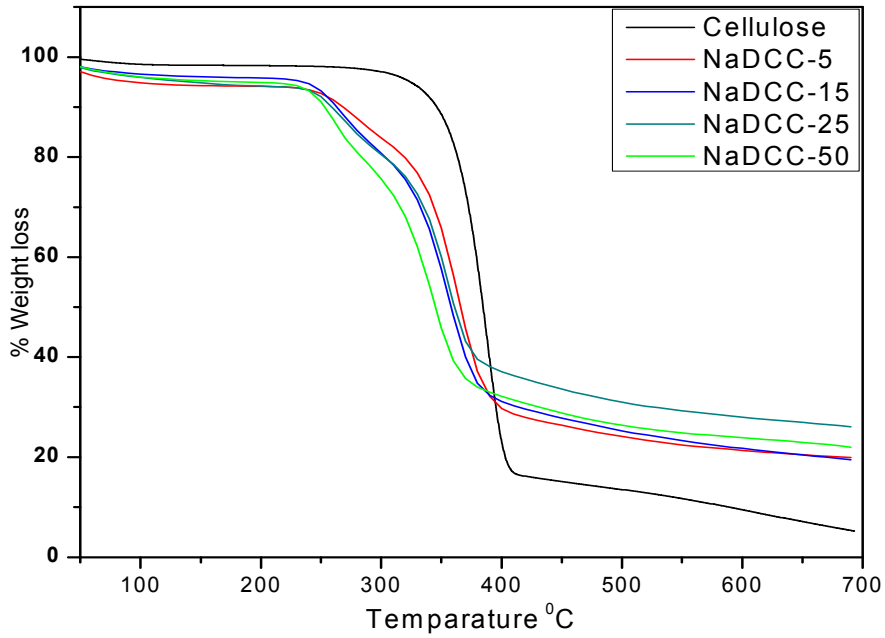


Figure.5.28: TGA of cellulose and sodium 2, 3-dicarboxy cellulose (NaDCC) with various level of oxidation

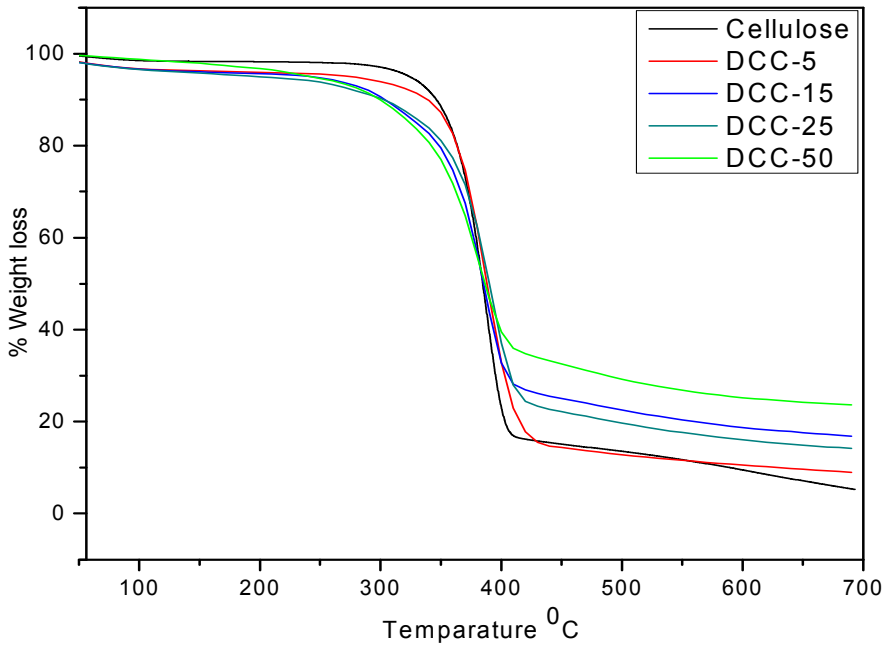


Figure 5.29: TGA of cellulose and 2, 3-dicarboxy cellulose (DCC) with various level of oxidation

5.8. Conclusions

Partial oxidation of cellulose was carried out to in order to develop a series of dialdehyde cellulose having different extents of aldehyde groups (different levels of oxidation) on the same cellulose chain. This 2,3-dialdehyde cellulose was further transformed to carboxy, carboxylate and Schiff bases (2,3-dioxime cellulose, 2,3-dihydrazone cellulose, 2,3-diethylimine cellulose, 2,3-dipropylimine cellulose, 2,3-dibutylimine cellulose and 2,3-dibenzylimime cellulose). These derivatives were exhaustively characterized by FTIR, ^{13}C CP-MAS spectroscopy, thermal analysis and wide angle X-ray diffraction. Thus several new series of functionalized co-polymers of cellulose have been prepared, which can serve as bases for developing new applications for cellulose, such as biodegradable polymers, anti-microbial coatings, and so on. These new bio-derived polymers may ultimately serve as replacements for some petroleum derived polymers.

5.9. References

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5.10. Appendix-3

FTIR spectral data of cellulose, 2, 3-dialdehyde cellulose and its derivatives with various levels of oxidation (Numbers 5, 15, 25 and 50 denotes the % of oxidation).

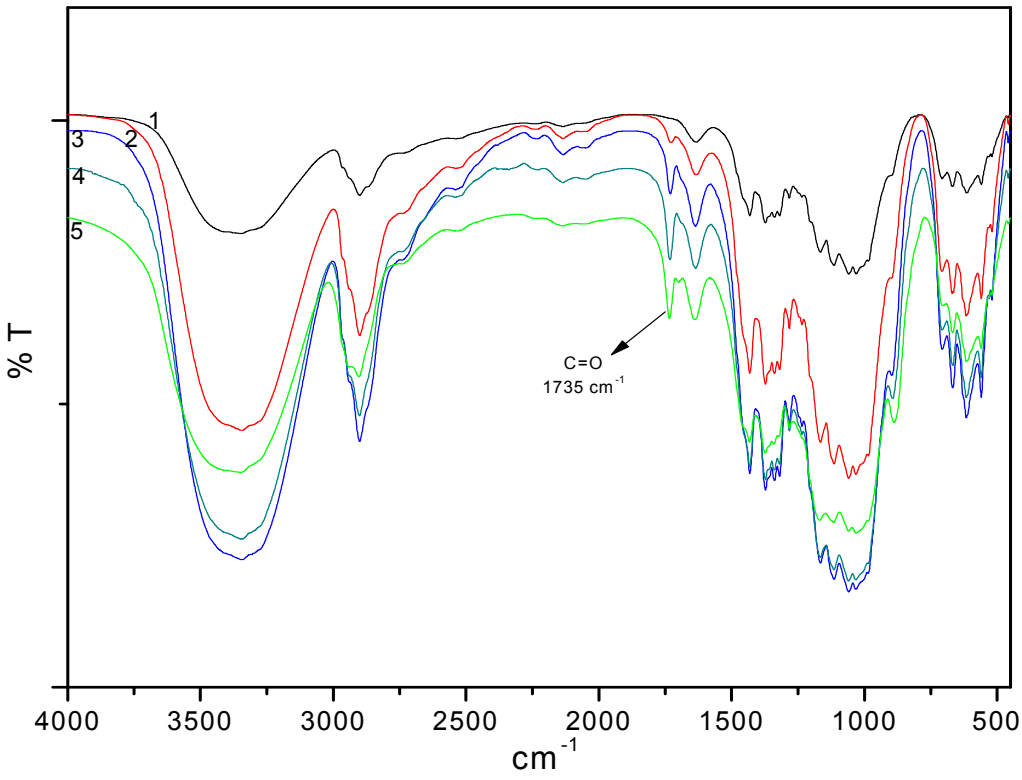


Figure 5.30: Overlapping of FTIR spectra of cellulose and 2, 3-dialdehyde cellulose (DAC) with various level of oxidation showing carbonyl peak at 1735 cm^{-1} in dialdehyde cellulose 1) Cellulose 2) DAC-5 3) DAC-15 4) DAC-25 and 5) DAC-50

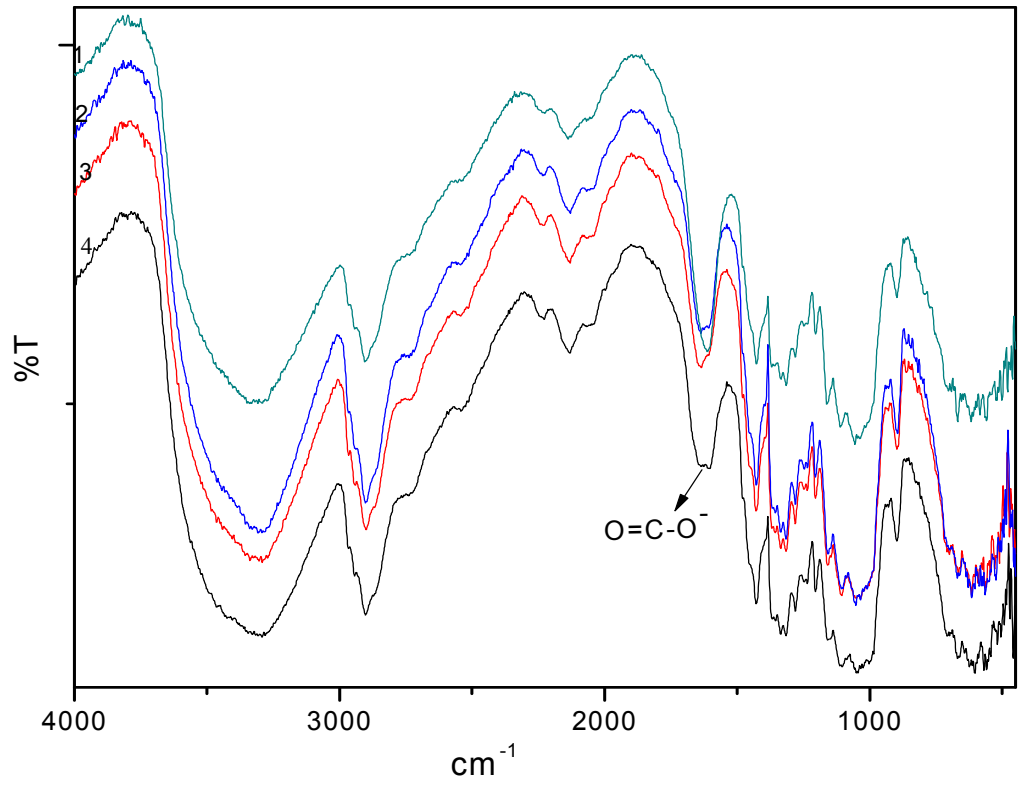
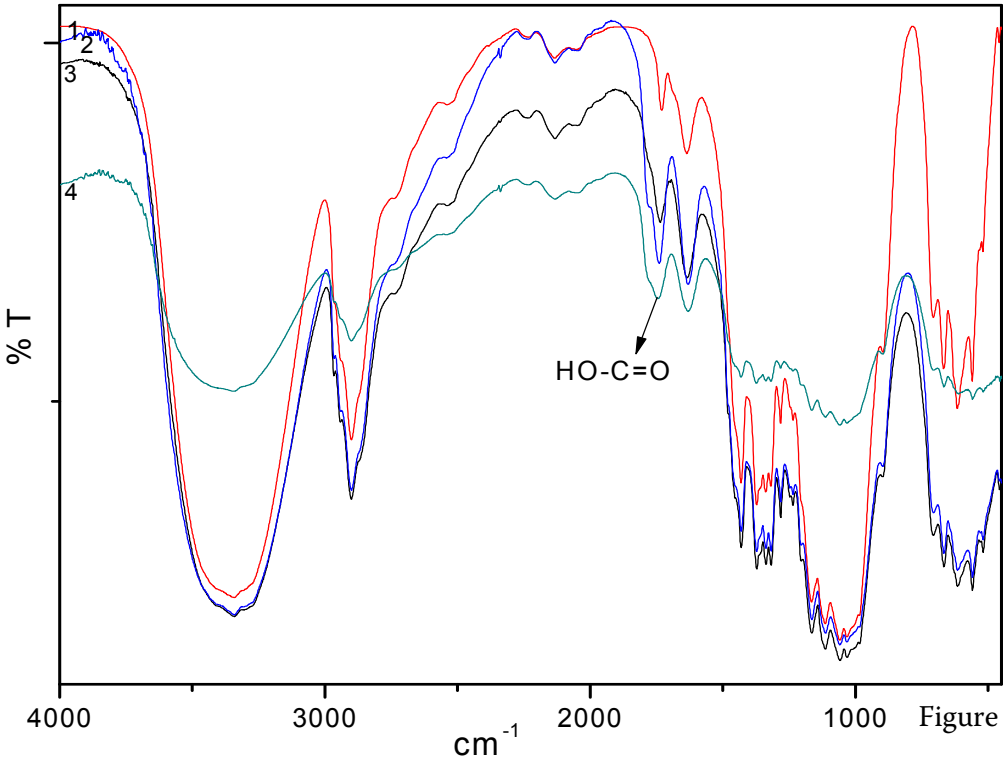


Figure 5.31: Overlapping of FTIR spectra of sodium 2,3-dicarboxy cellulose (Na DCC) showing disappearance of carbonyl peak (1735 cm^{-1}) and appearance of carboxylate peak at 1625 cm^{-1}1) NaDCC-5 2) NaDCC-15 3) NaDCC-25 4) NaDCC-50



Figure

5.32: Overlapping of FTIR spectra of 2, 3-dicarboxy cellulose (DCC) with different level of oxidation showing carboxyl peak at 1720 cm^{-1} 1) DCC-5 2) DCC-15 3) DCC-25 4) DCC-50

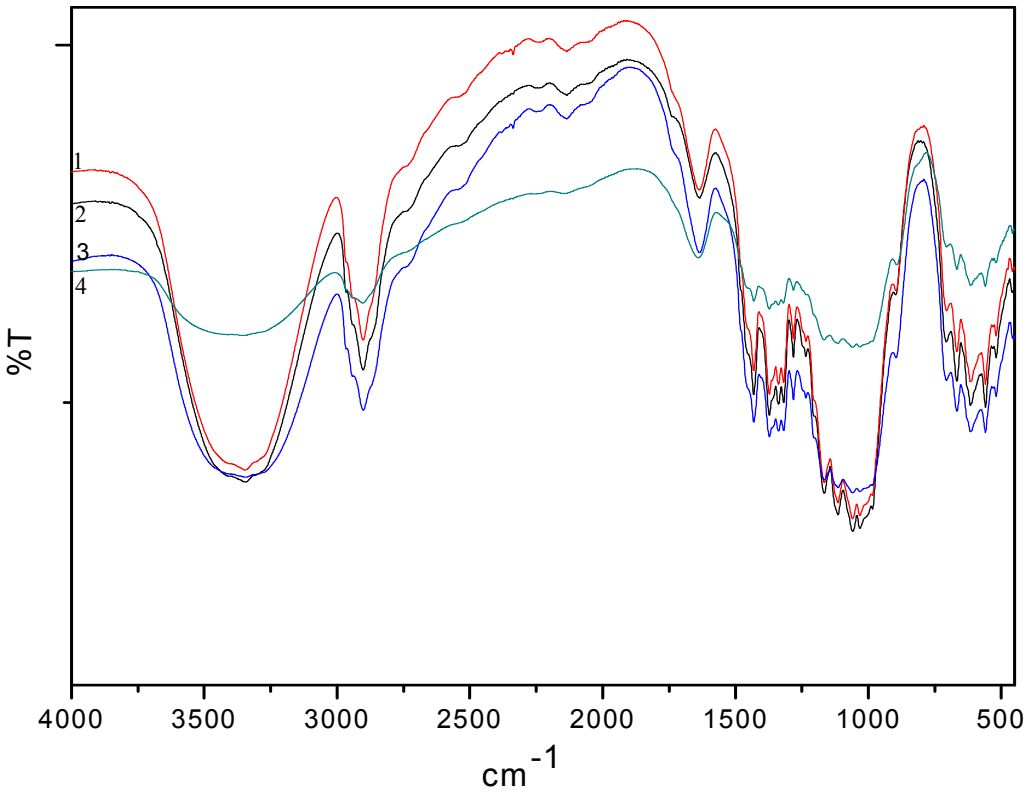


Figure 5.33: Overlapping of FTIR spectra of 2, 3-dihydrazone cellulose showing disappearance of C=O (1735 cm^{-1}) peak and appearance of C=N peak at 1640 cm^{-1} 1) 2, 3-dihydrazone cellulose-5 2) 2, 3-dihydrazone cellulose-15 3) 2, 3-dihydrazone cellulose-25 4) 2, 3-dihydrazone cellulose-50

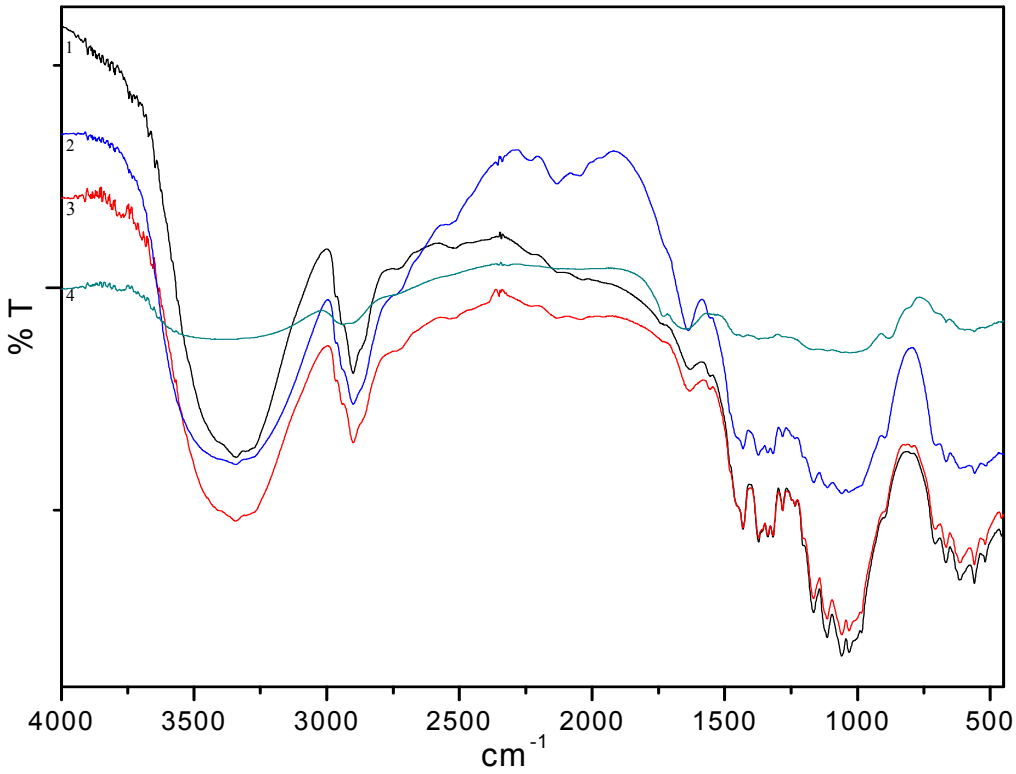


Figure 5.34: Overlapping of FTIR spectra of 2, 3-dioxime cellulose showing disappearance of C=O (1735 cm^{-1}) peak and appearance of C=N peak at 1643 cm^{-1} 1) 2, 3-dioxime cellulose-5 2) 2, 3-dioxime cellulose-15 3) 2, 3-dioxime cellulose-25 4) 2, 3-dioxime cellulose-50

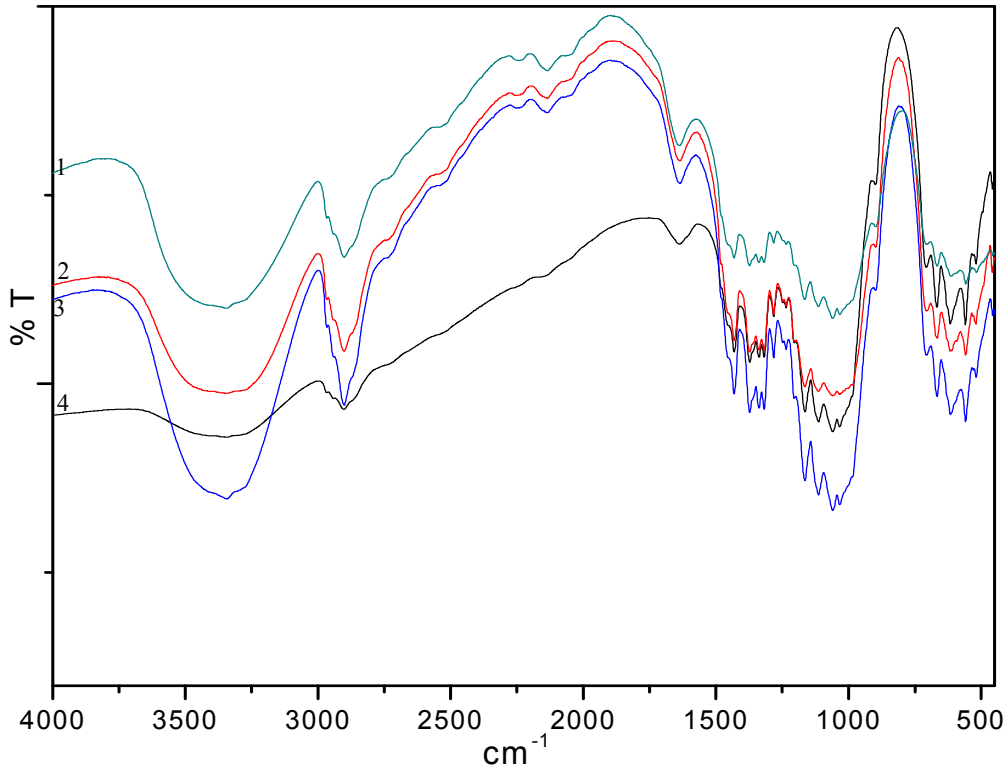


Figure 5.35: Overlapping of FTIR spectra of 2, 3-diethylimine cellulose showing disappearance of C=O (1735cm^{-1}) peak and appearance of C=N peak at 1635 cm^{-1}1) 2, 3-diethylimine cellulose-5 2) 2, 3-diethylimine cellulose-15 3) 2, 3-diethylimine cellulose-25 4) 2, 3-diethylimine cellulose-50

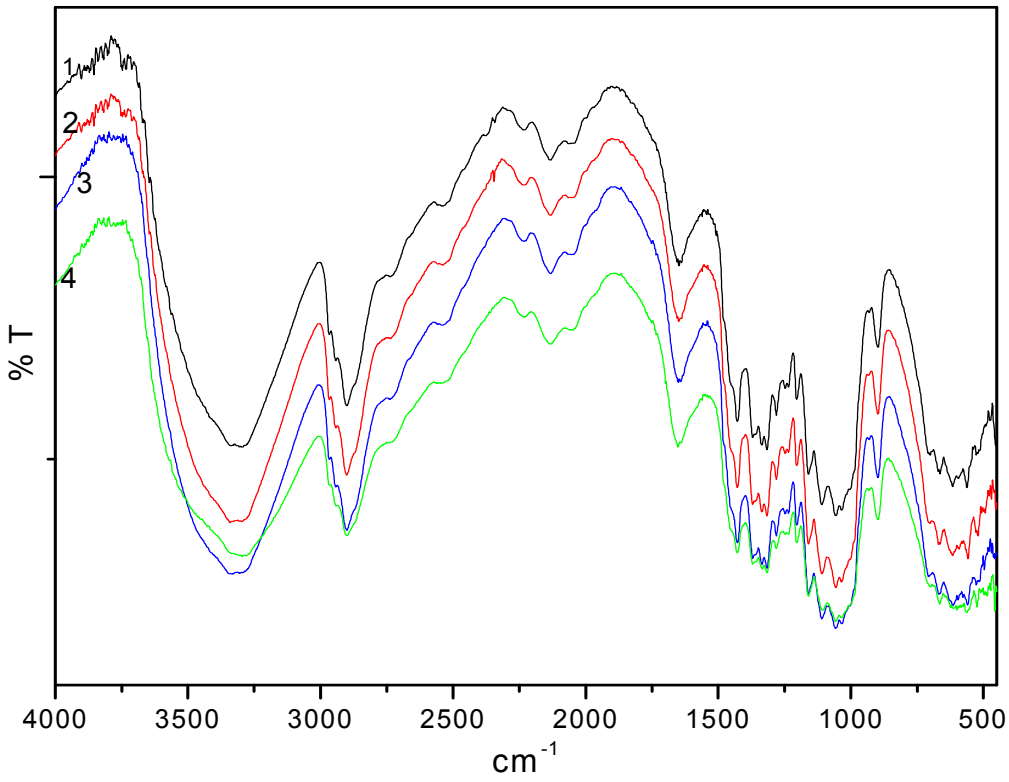


Figure 5.36: Overlapping of spectra of 2, 3-dipropylimine cellulose showing disappearance of C=O (1735cm^{-1}) peak and appearance of C=N peak at 1650 cm^{-1} 1) 2, 3-dipropylimine cellulose-5 2) 2, 3-dipropylimine cellulose-15 3) 2, 3-dipropylimine cellulose-25 4) dipropylimine cellulose-50

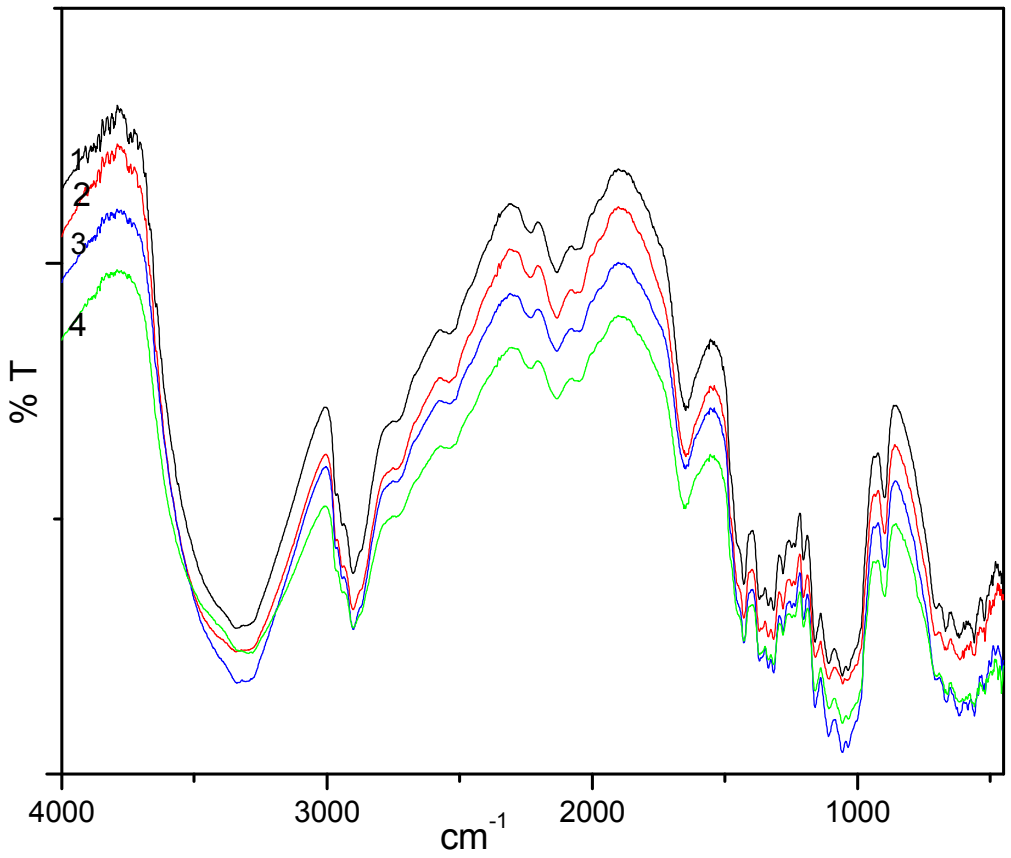


Figure 5.37: Overlapping of FTIR spectra of 2, 3-dibutylimine cellulose showing disappearance of C=O (1735cm^{-1}) peak and appearance of C=N peak at 1647 cm^{-1} 1) 2, 3-dibutylimine cellulose-5 2) 2, 3-dibutylimine cellulose-15 3) 2, 3-dibutylimine cellulose-25 4) 2, 3-dibutylimine cellulose-50

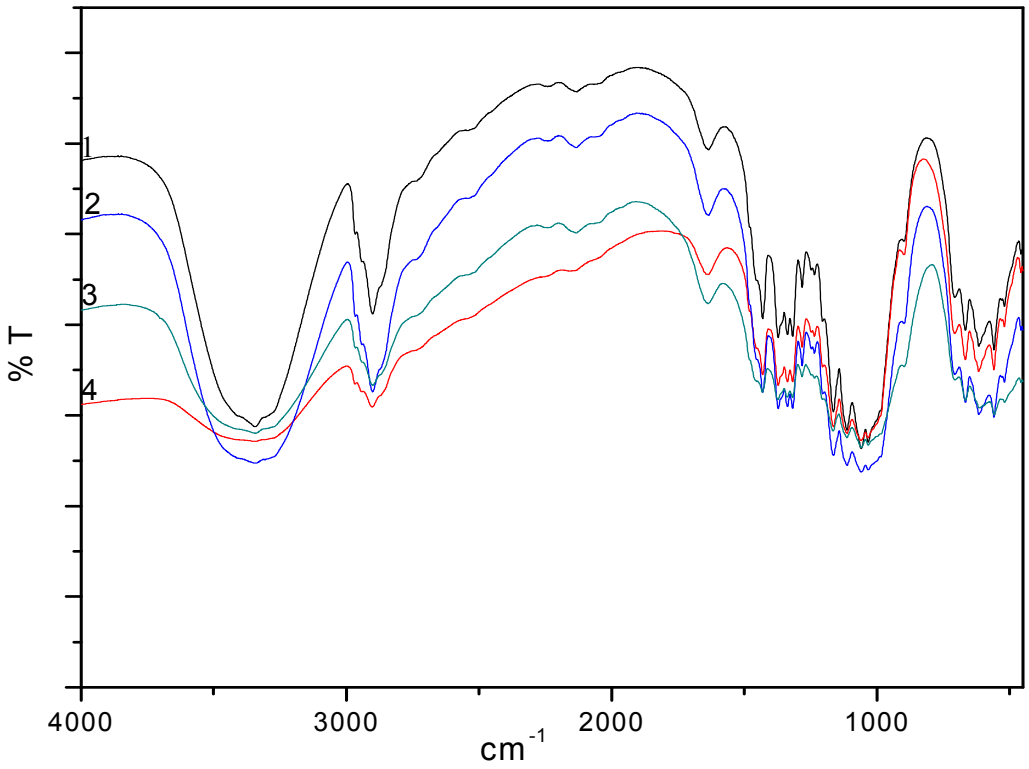


Figure 5.38: Overlapping of FTIR spectra of 2, 3-dibenzylimine cellulose showing disappearance of C=O (1735cm^{-1}) peak and appearance of C=N peak at 1639 cm^{-1} 1) 2, 3-dibenzylimine cellulose-5 2) 2, 3-dibenzylimine cellulose-15 3) 2, 3-dibenzylimine cellulose-25 4) 2, 3-dibenzylimine cellulose-50

5.11. Appendix-4

Energy Dispersive X-ray (EDAX) spectra of 2, 3-dialdehyde cellulosic derivatives, (All derivatives were synthesized from 5% oxidation level of 2, 3-dialdehyde cellulose).

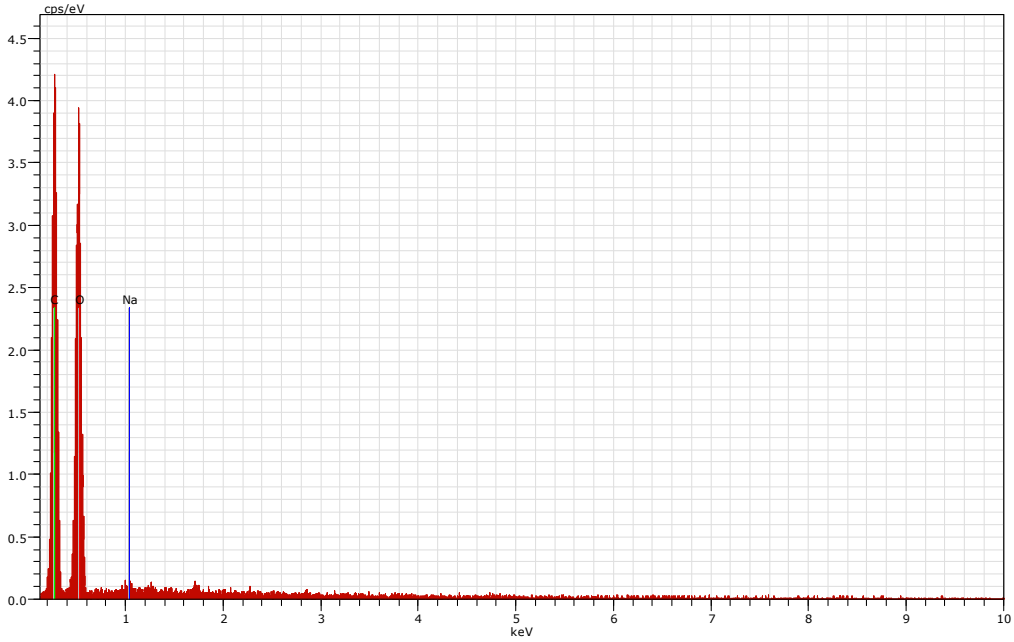


Figure 5.39: EDAX spectrum of sodium 2, 3-dialdehyde cellulose-5 (NaDCC-5) showing sodium peak

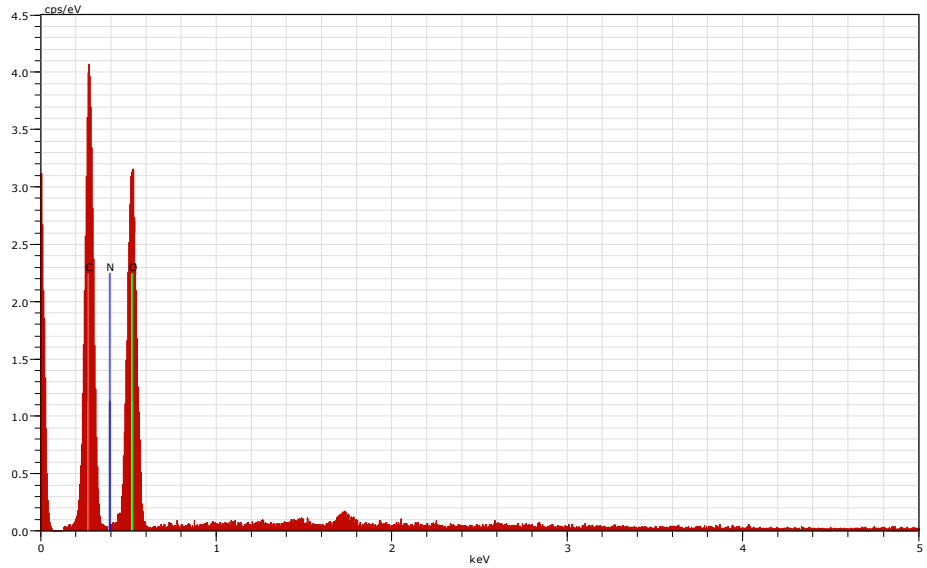


Figure 5.40: EDAX spectrum of 2, 3-diethylimine cellulose-5 showing nitrogen peak

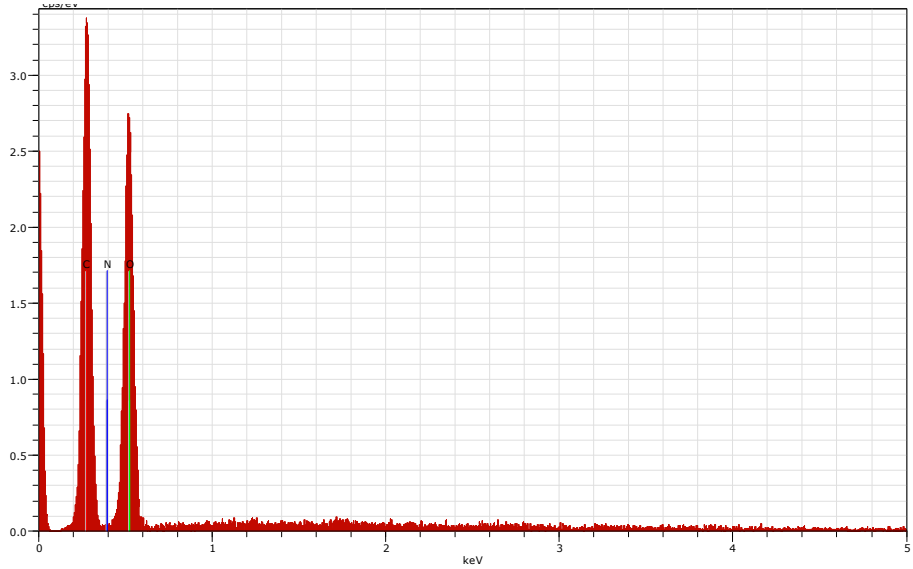


Figure 5.41: EDAX spectrum of 2, 3-dipropylimine cellulose-5 showing nitrogen peak

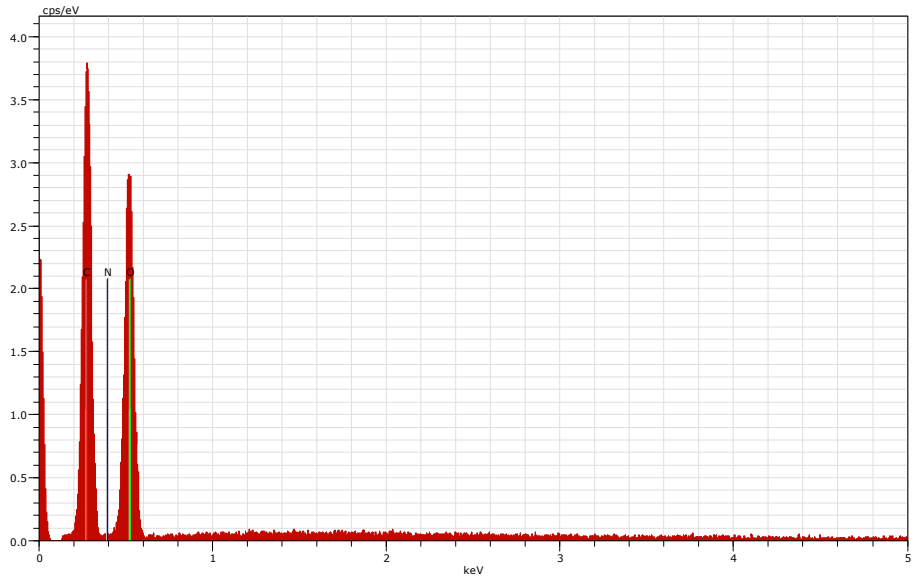


Figure 5.42: EDAX spectrum of 2, 3-dibutylimine cellulose-5 showing nitrogen peak

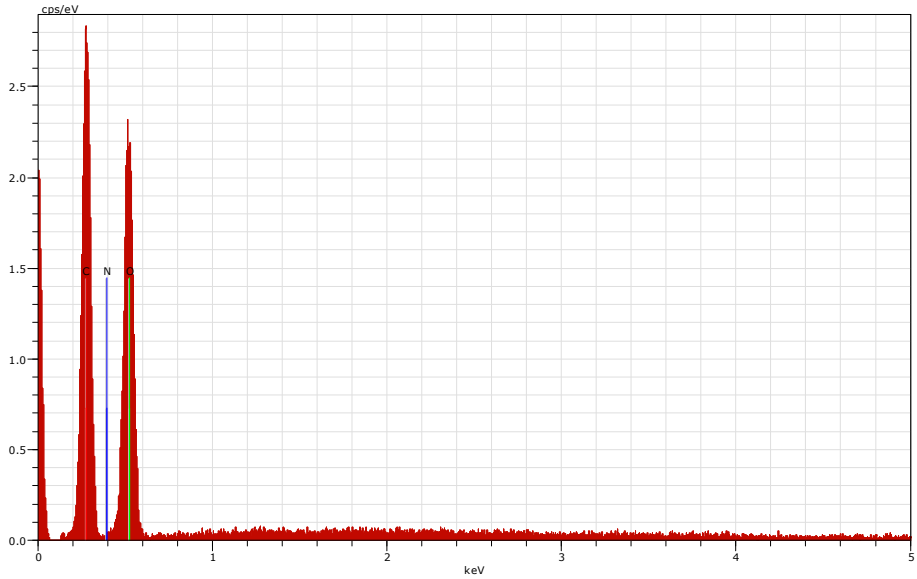


Figure 5.43: EDAX spectrum of 2, 3-dibenzylimine cellulose-5 showing nitrogen peak

5.12. Appendix-5

CP-MAS ^{13}C NMR spectra of Schiff bases of 2, 3- dialdehyde cellulose with various levels of oxidation. (Numbers 5, 15, 25 and 50 denots the levels of oxidation (%) of 2,3-dialdehyde cellulose).

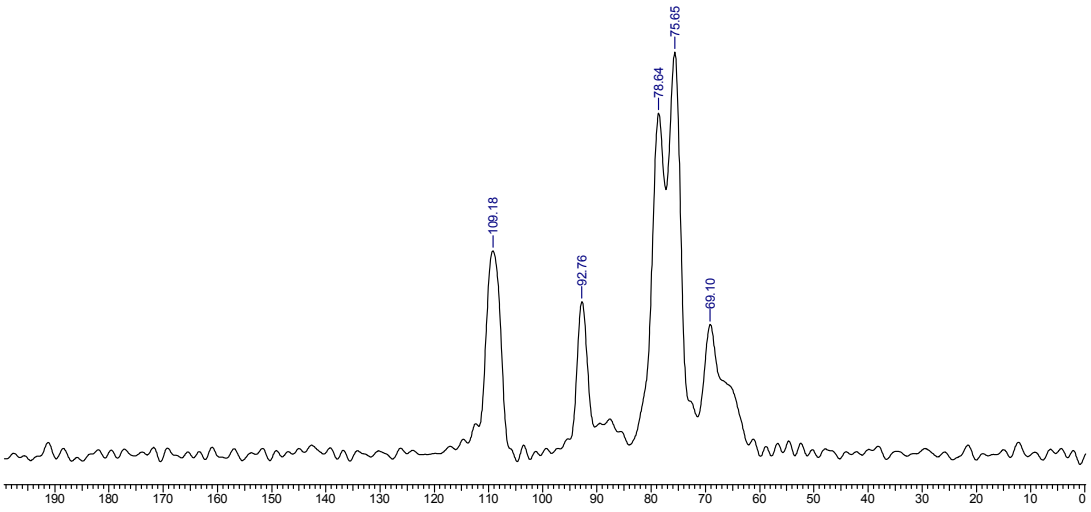


Figure 5.44: CP-MAS ^{13}C NMR of 2, 3-dihydrazone cellulose-5

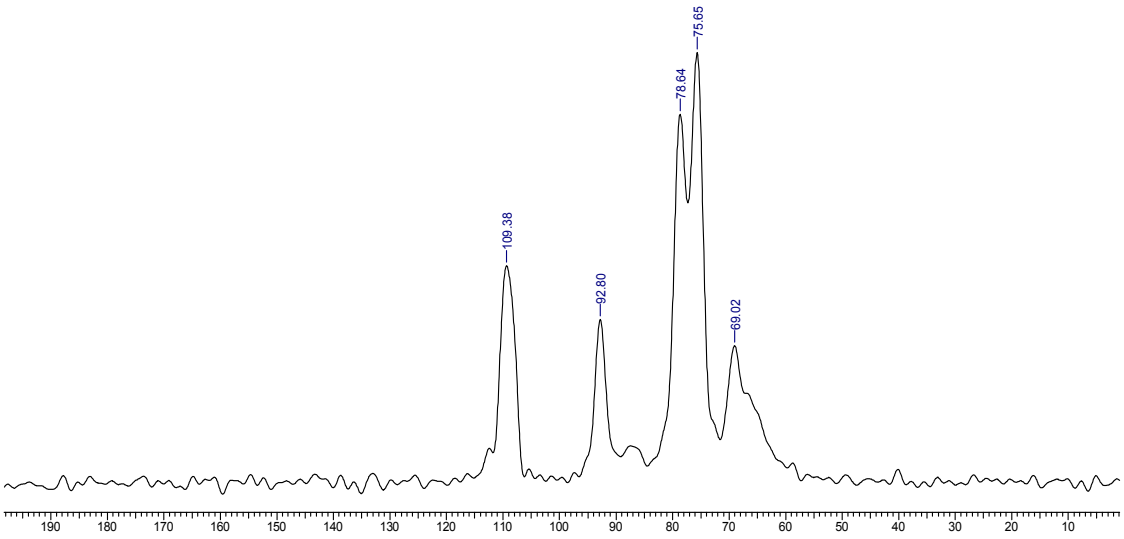


Figure 5.45: CP-MAS ^{13}C NMR of 2, 3-dihydrazone cellulose-15

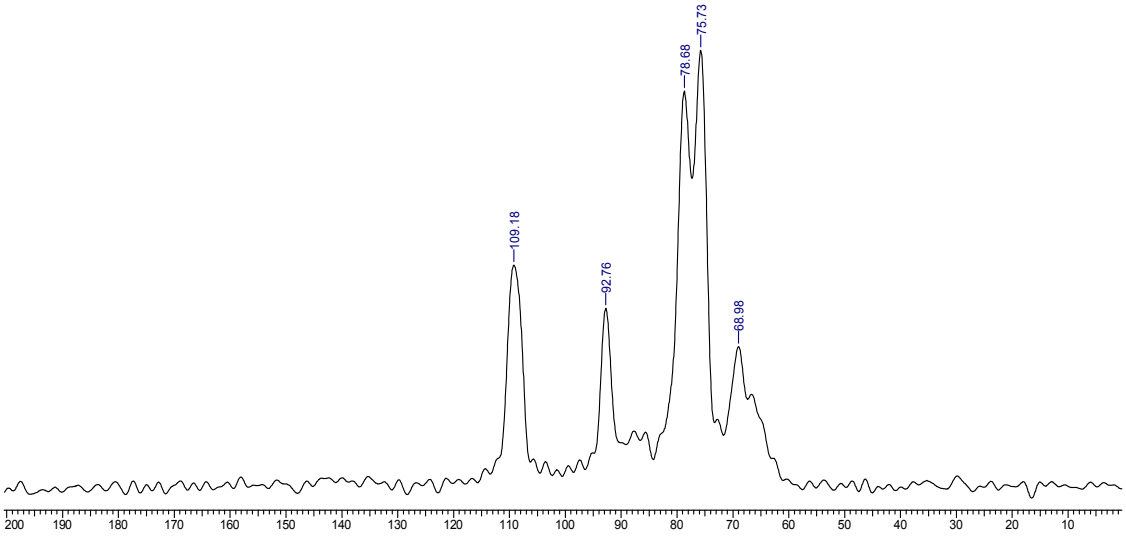


Figure 5.46: CP-MAS ^{13}C NMR of 2, 3-dihydrazone cellulose-25

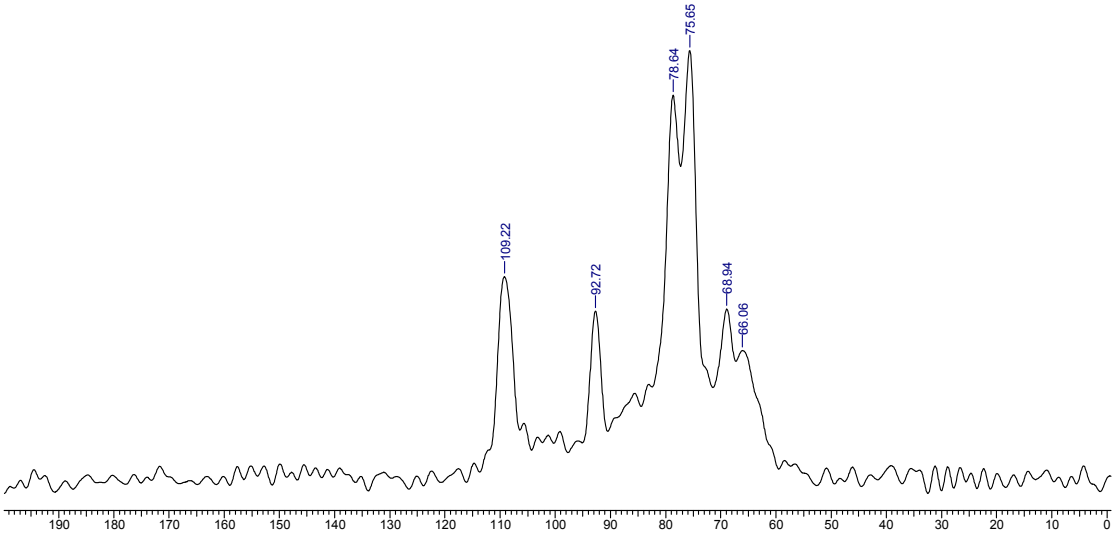


Figure 5.47: CP-MAS ^{13}C NMR of 2, 3-dihydrazone cellulose-50

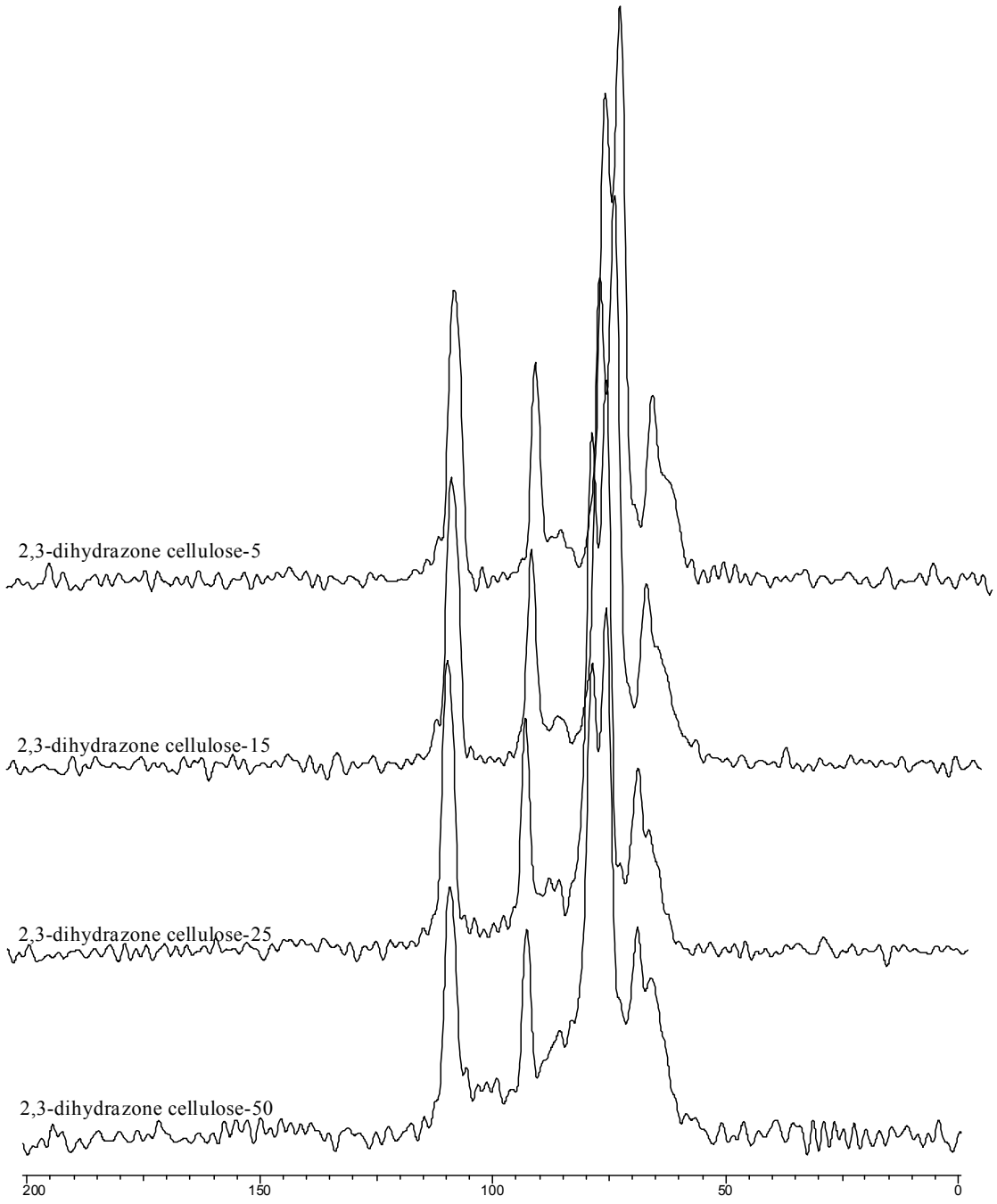


Figure 5.48: Overlapping of CP-MAS ^{13}C NMR of 2, 3- dihydrazone cellulose of various levels of oxidation

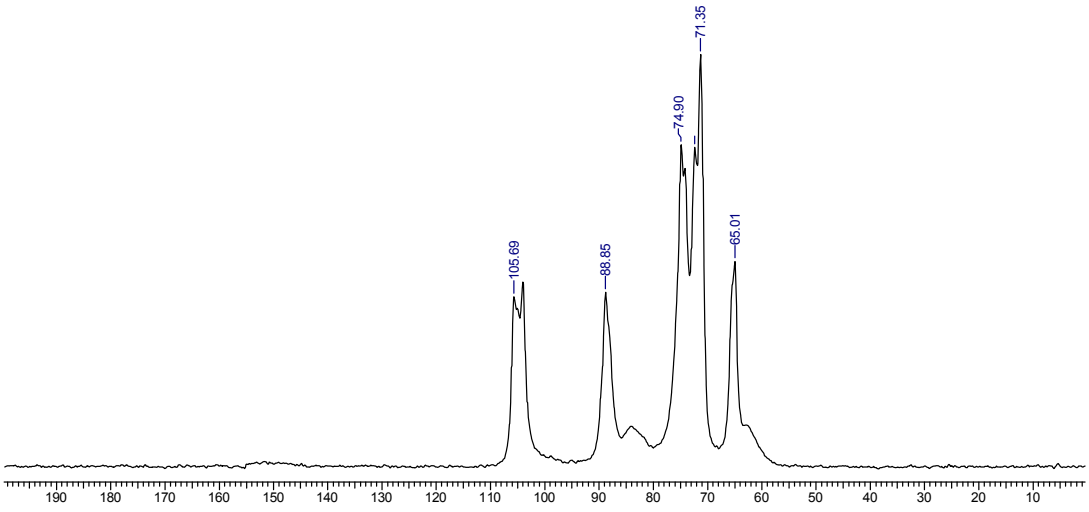


Figure 5.49: CP-MAS ^{13}C NMR of 2, 3- dioxime cellulose-5

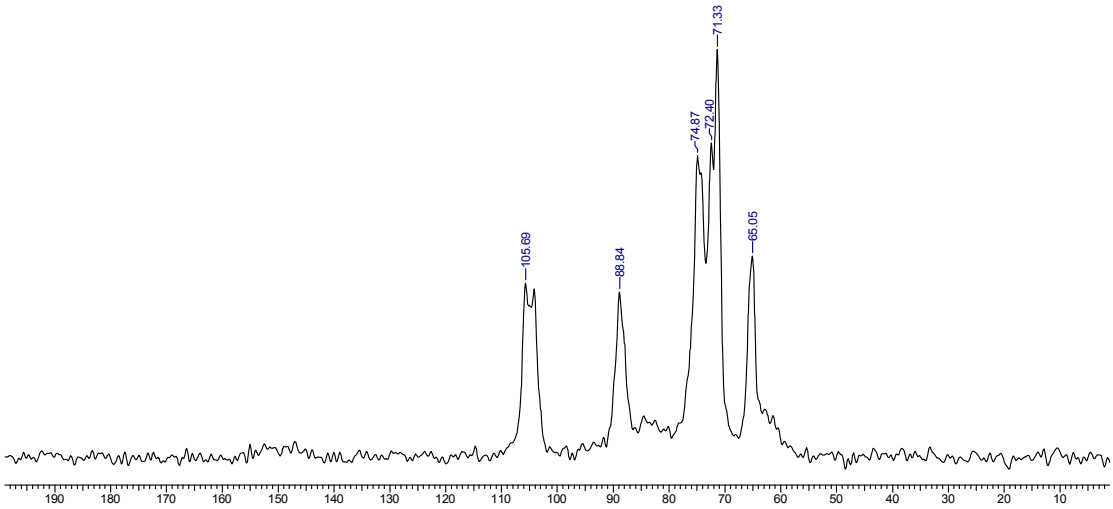


Figure 5.50: CP-MAS ^{13}C NMR of 2, 3- dioxime cellulose-15

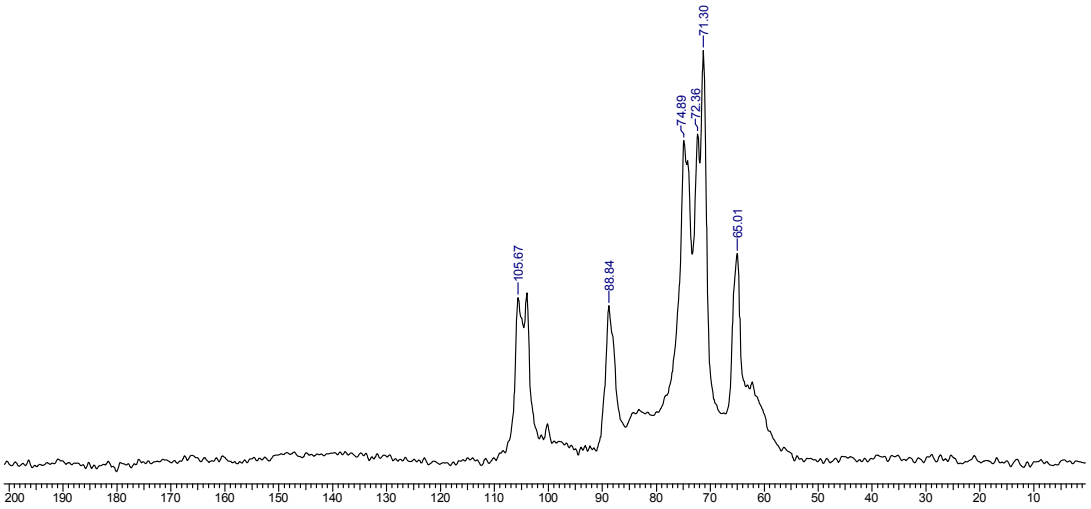


Figure 5.51: CP/MAS ^{13}C NMR spectra of 2, 3- dioxime cellulose-25

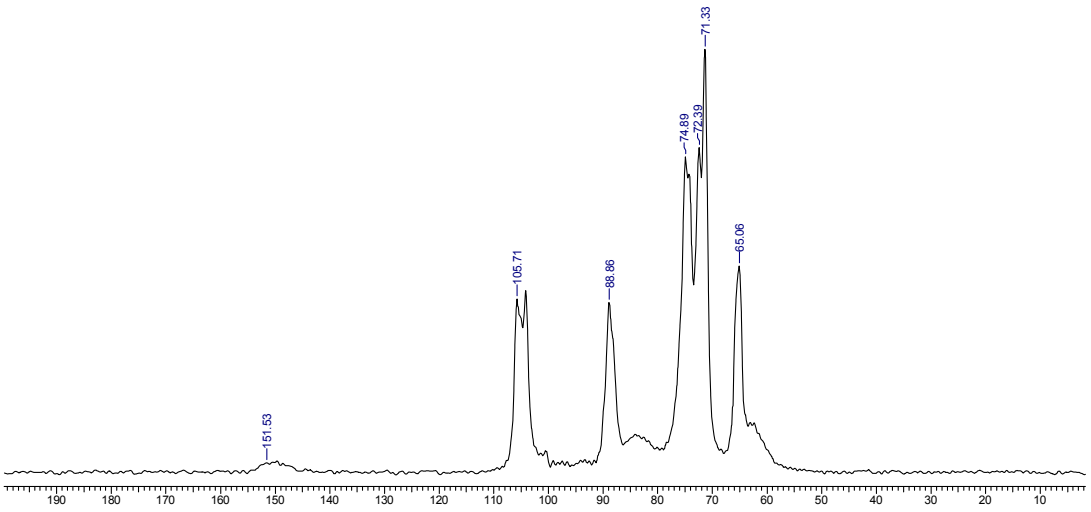


Figure 5.52: CP/MAS ^{13}C NMR spectra of 2, 3- dioxime cellulose-50

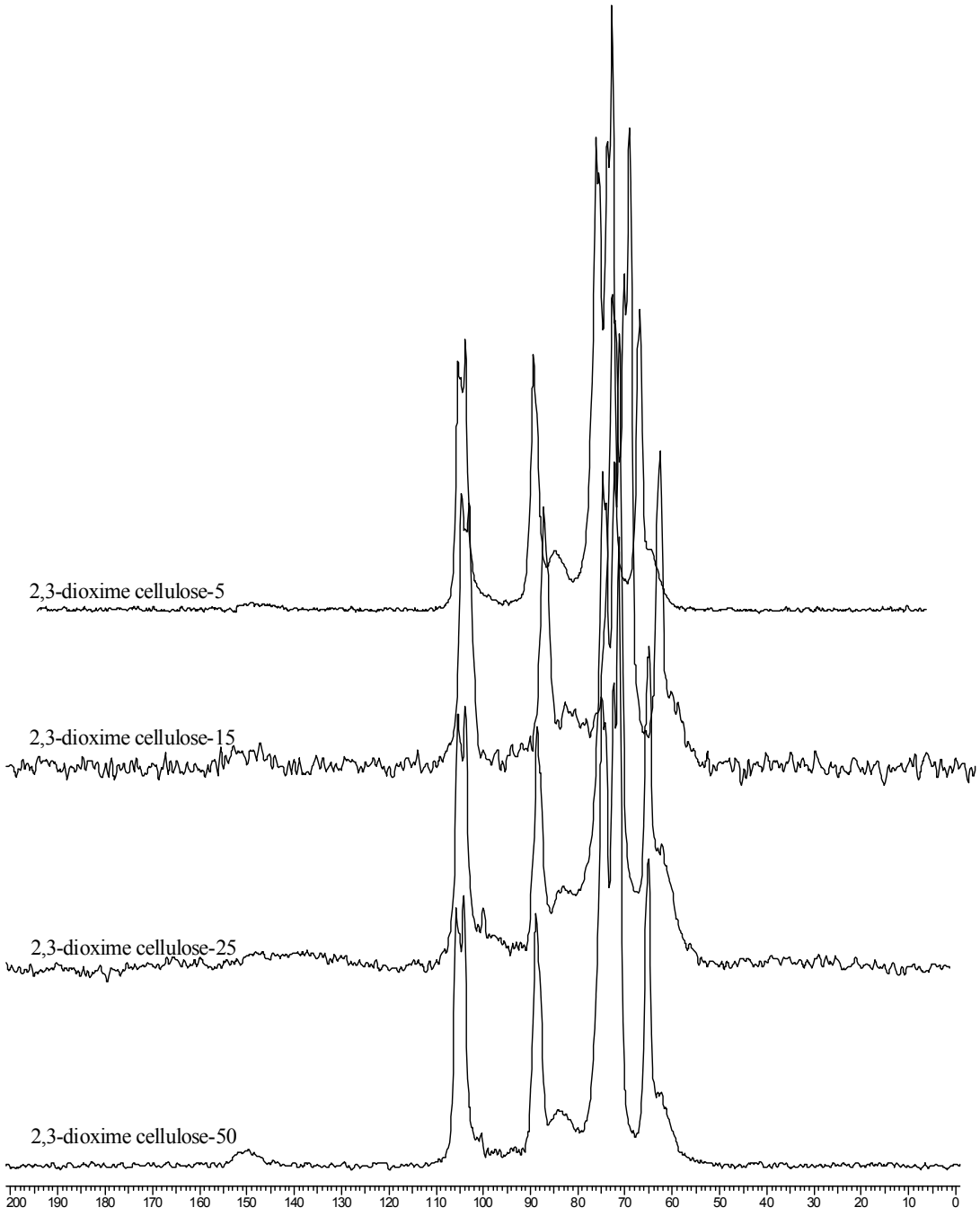


Figure 5.53: Overlapping of CP-MAS ^{13}C NMR of 2, 3- dioxime cellulose of various levels of oxidation

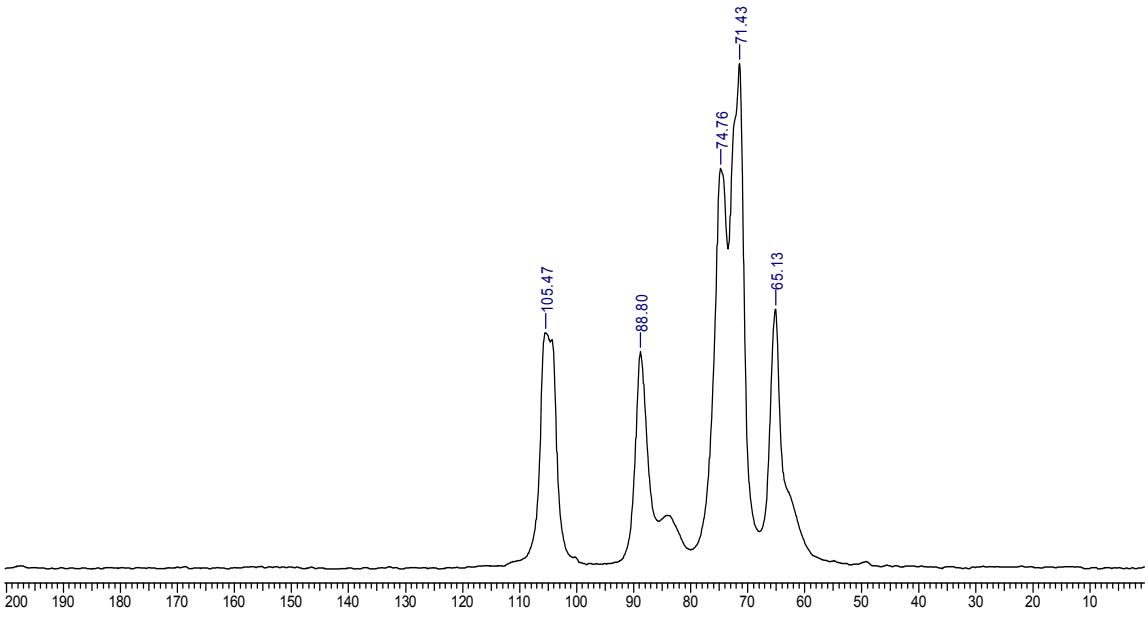


Figure 5.54: CP-MAS ^{13}C NMR of 2, 3-diethylimine cellulose – 5

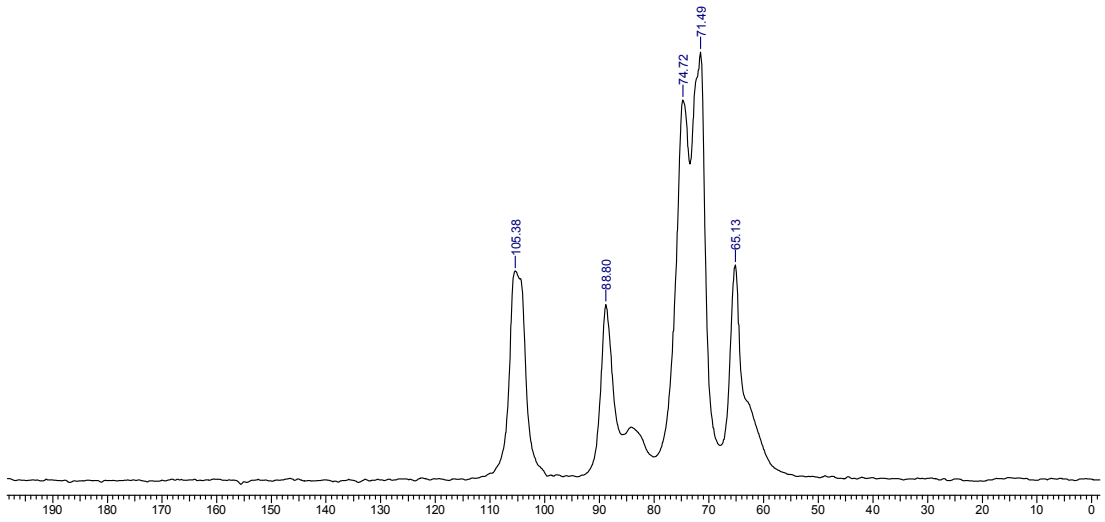


Figure 5.55: CP-MAS ^{13}C NMR of 2, 3-diethylimine cellulose -15

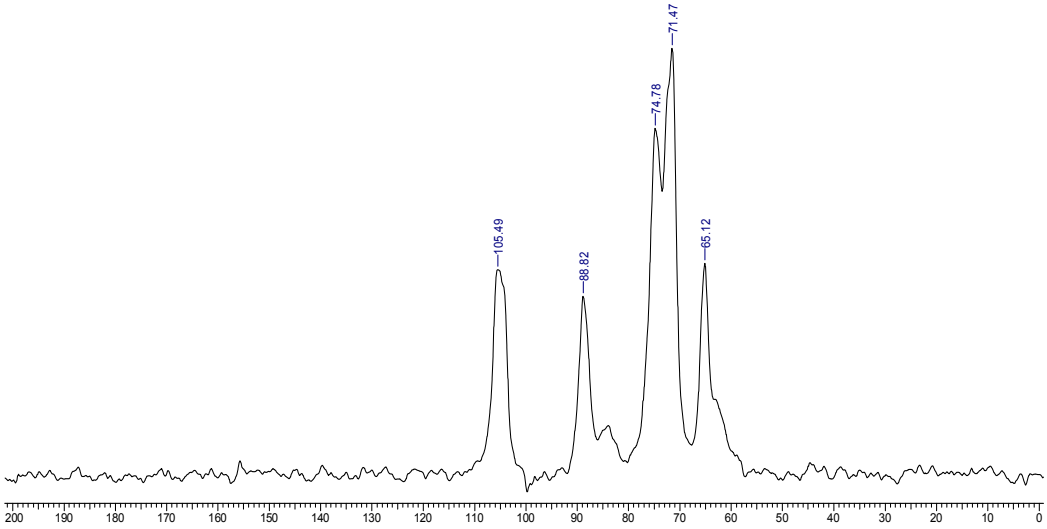


Figure 5.56: CP-MAS ^{13}C NMR of 2, 3-diethylimine cellulose -25

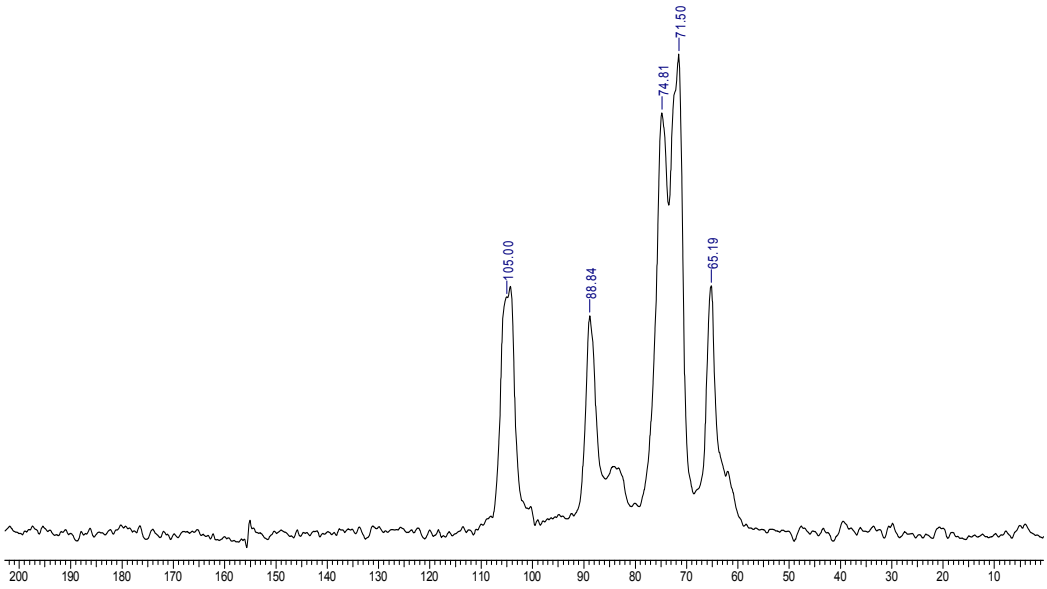


Figure 5.57: CP-MAS ^{13}C NMR of 2, 3-diethylimine cellulose-50

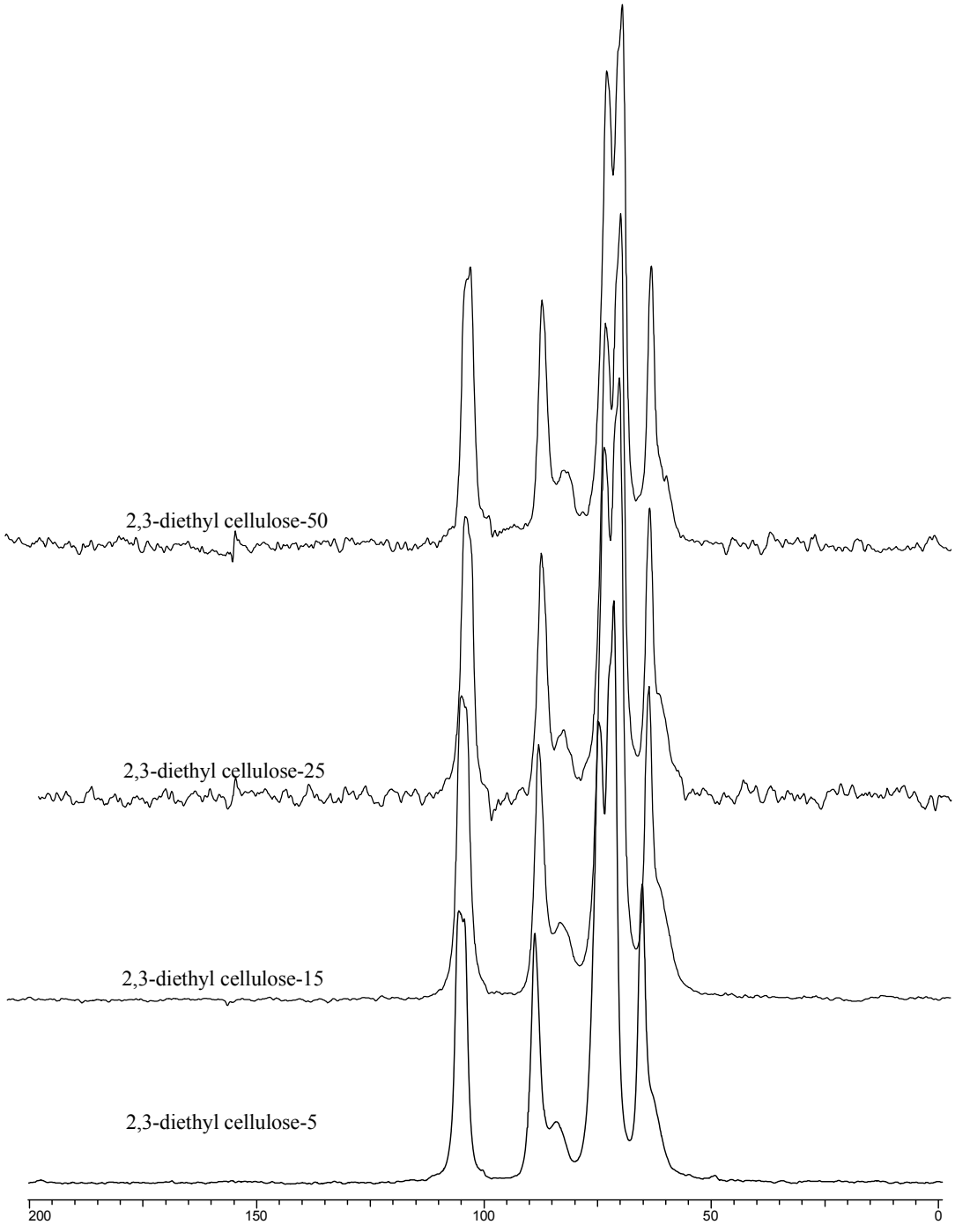


Figure 5.58: Overlapping of CP-MAS ^{13}C NMR of 2, 3-diethylimine cellulose with various levels of oxidation

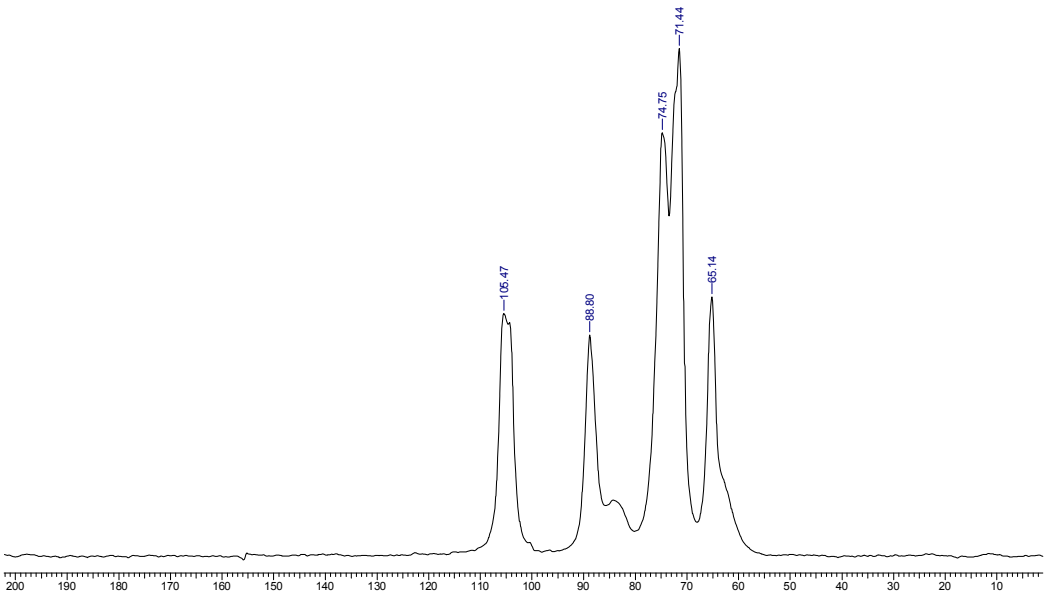


Figure 5.59: CP-MAS ^{13}C NMR of 2, 3-dipropylimine cellulose -5

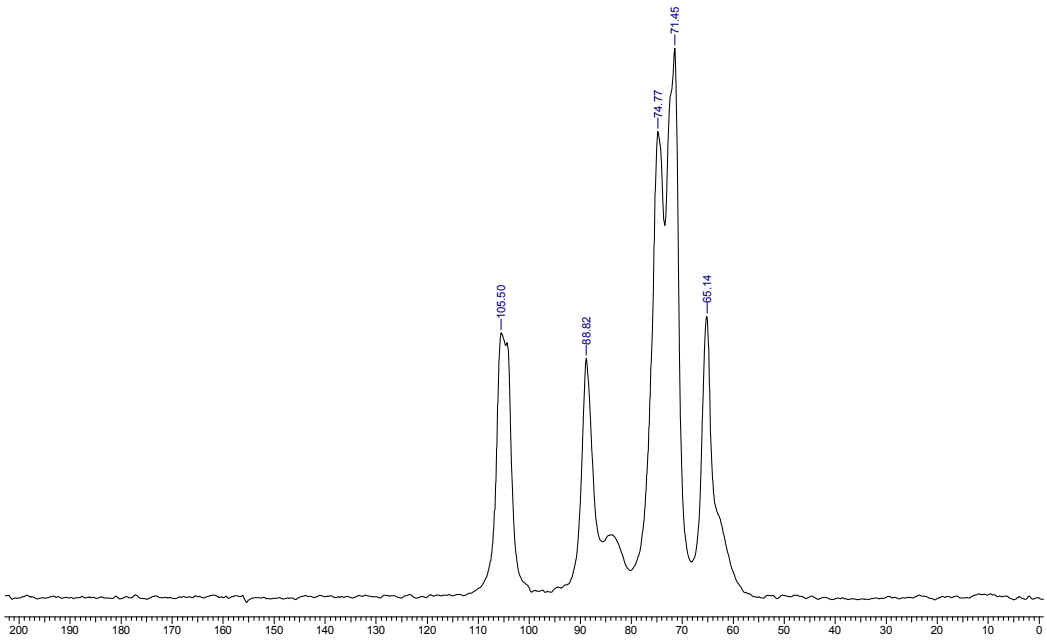


Figure 5.60: CP-MAS ^{13}C NMR of 2, 3-dipropylimine cellulose -15

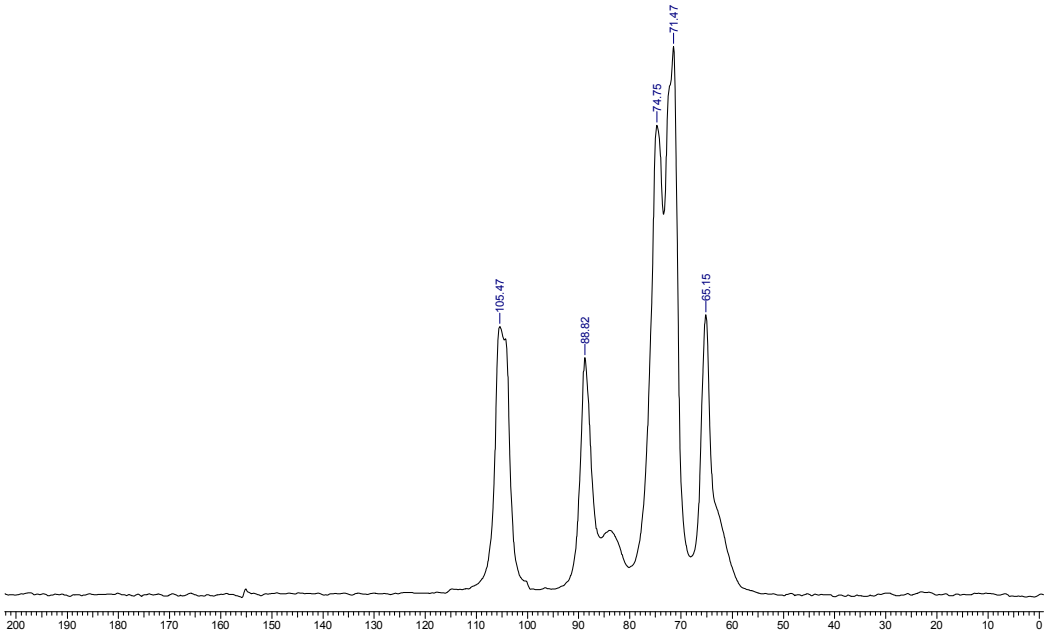


Figure 5.61: CP-MAS ^{13}C NMR spectra of 2, 3-dipropylimine cellulose–25

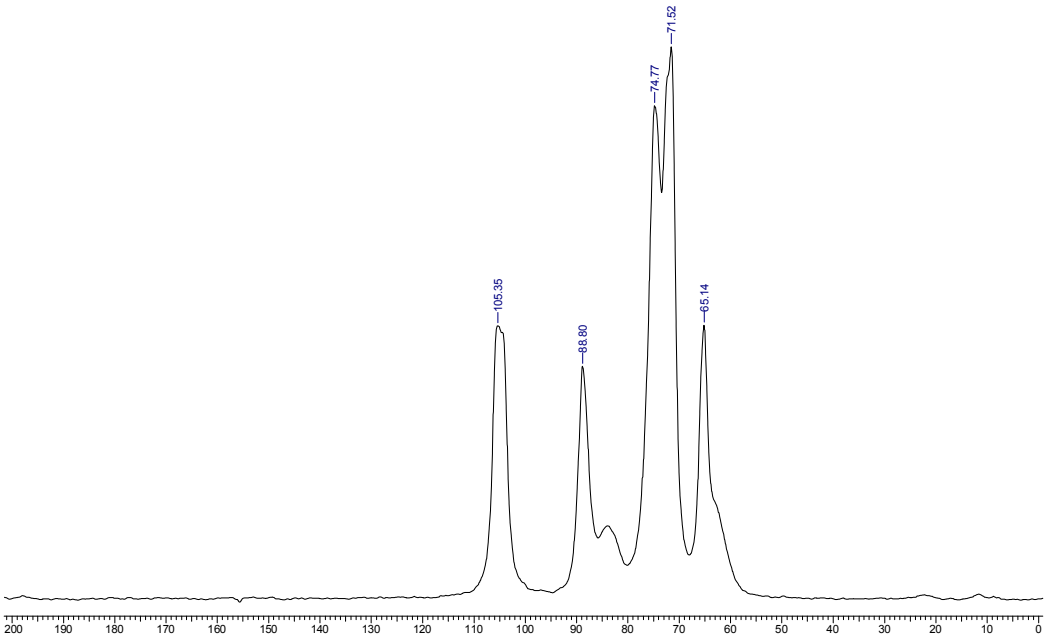


Figure 5.62: CP-MAS ^{13}C NMR of 2, 3-dipropylimine cellulose -50

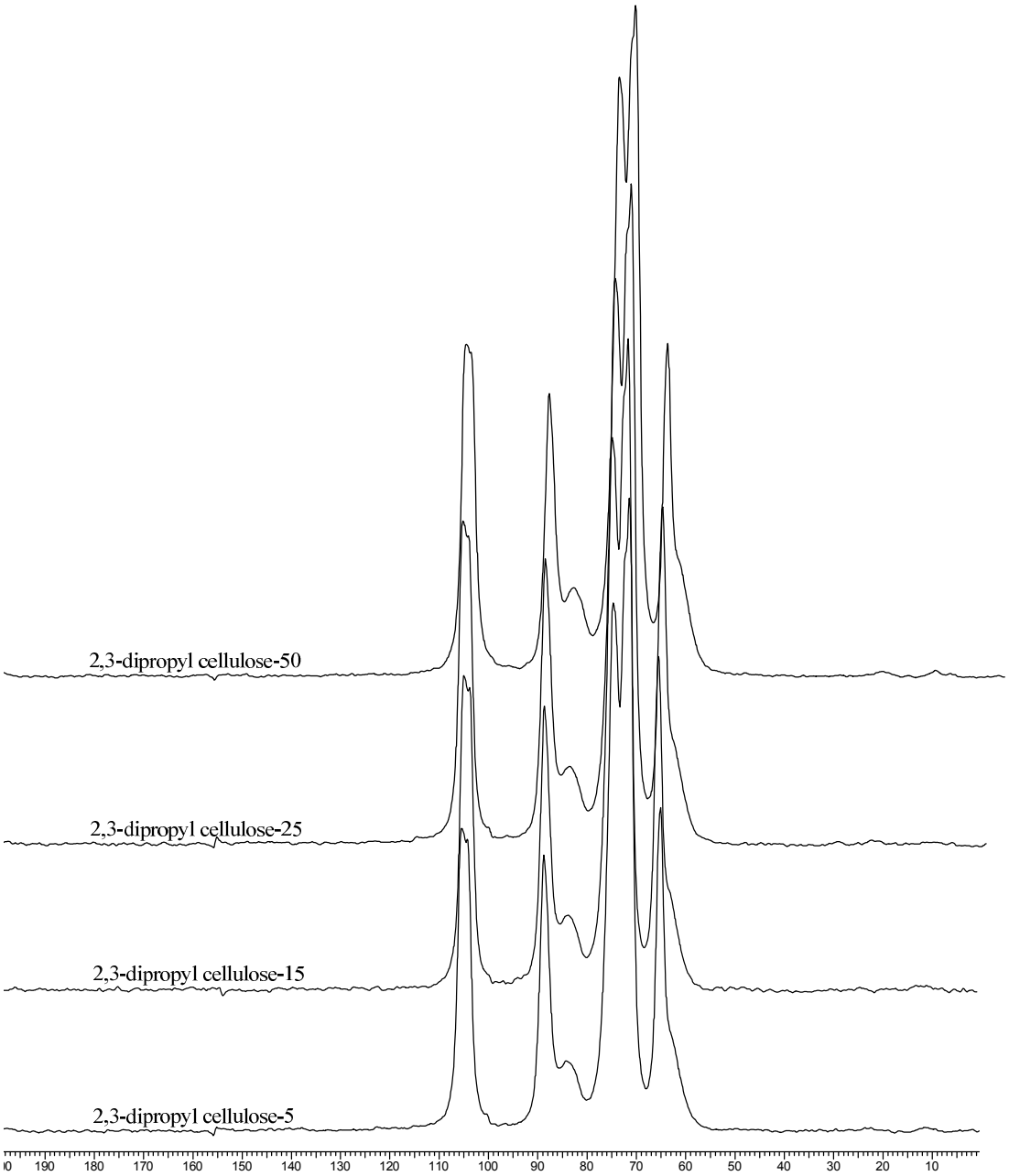


Figure 5.63: Overlapping of CP-MAS ^{13}C NMR of 2, 3-dipropylimine cellulose with various level of oxidation

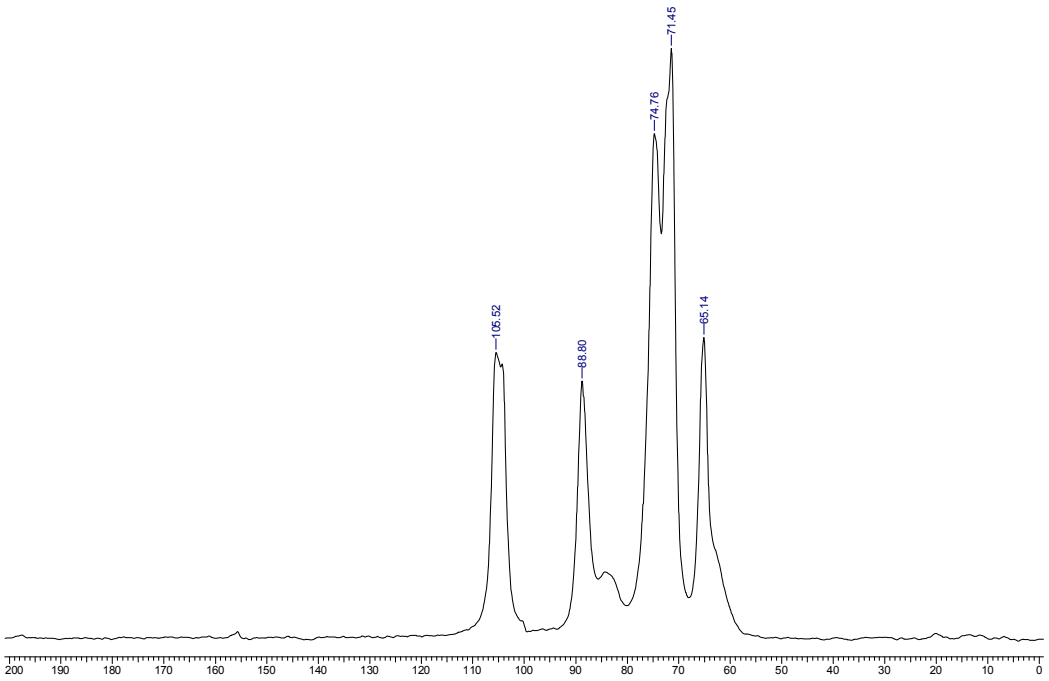


Figure 5.64: CP-MAS ^{13}C NMR of 2, 3-dibutylimine cellulose-5

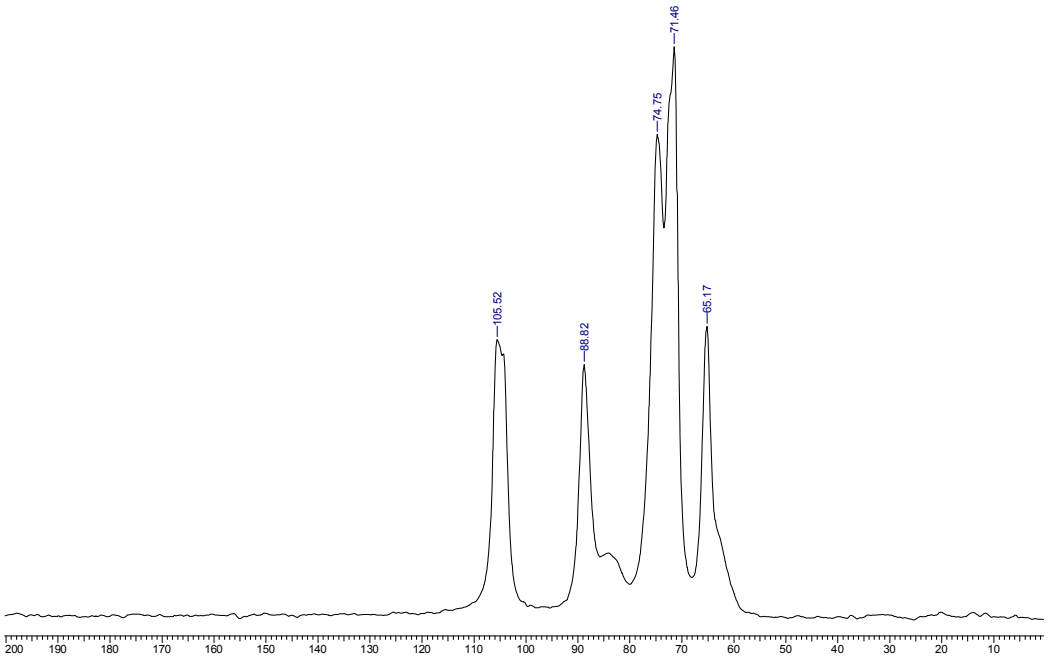


Figure 5.65: CP-MAS ^{13}C NMR of 2, 3-dibutylimine cellulose-15

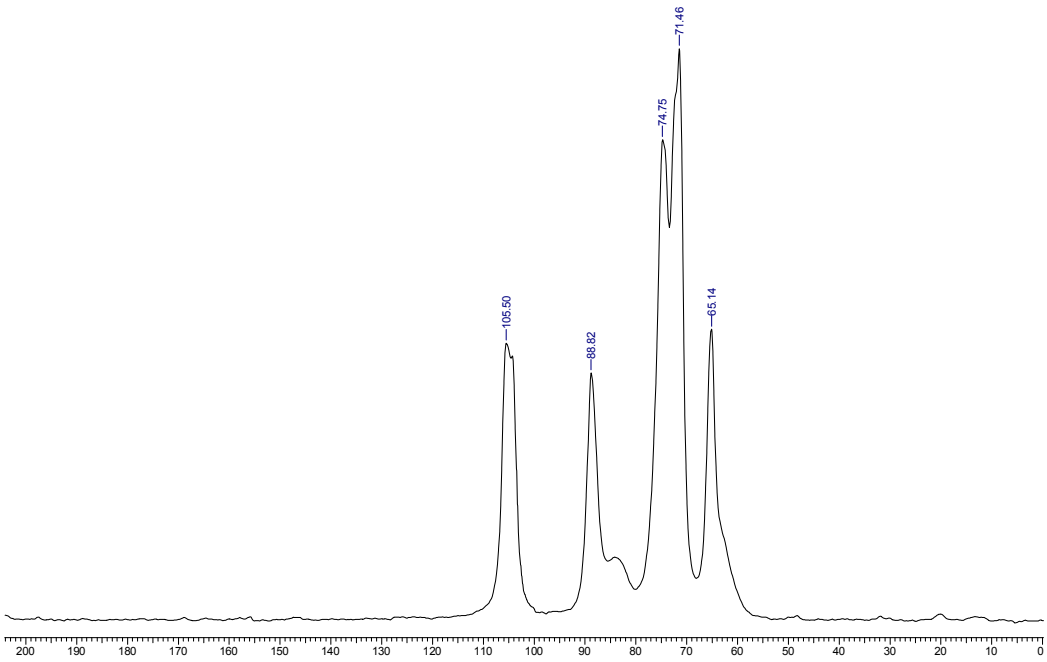


Figure 5.66: CP-MAS ^{13}C NMR of 2, 3-dibutylimine cellulose-25

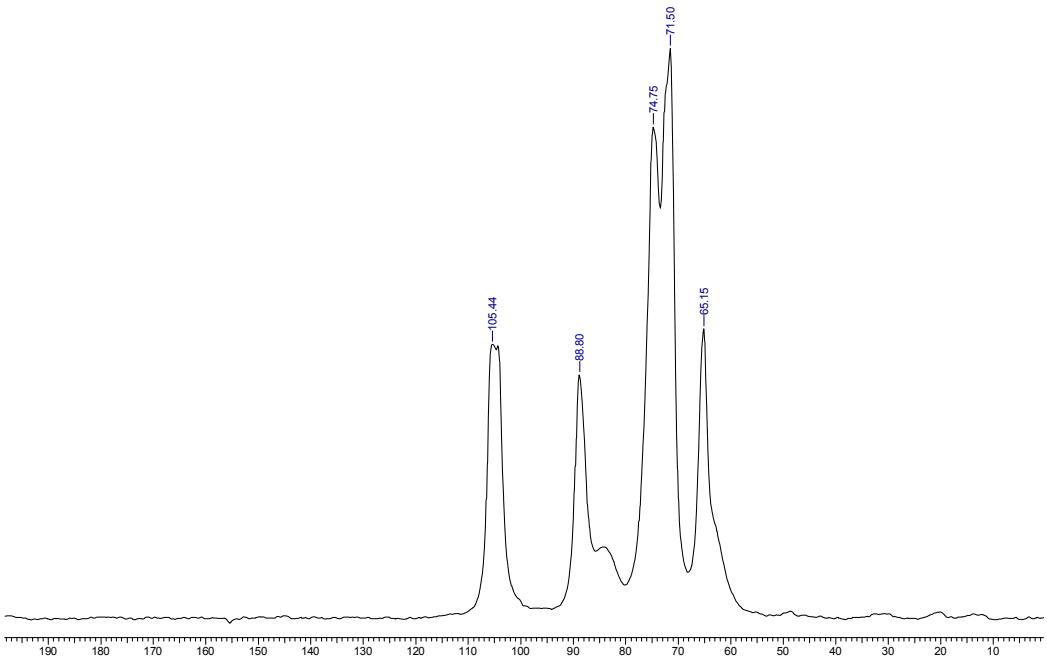


Figure 5.67: CP-MAS ^{13}C NMR of 2, 3-dibutylimine cellulose-50

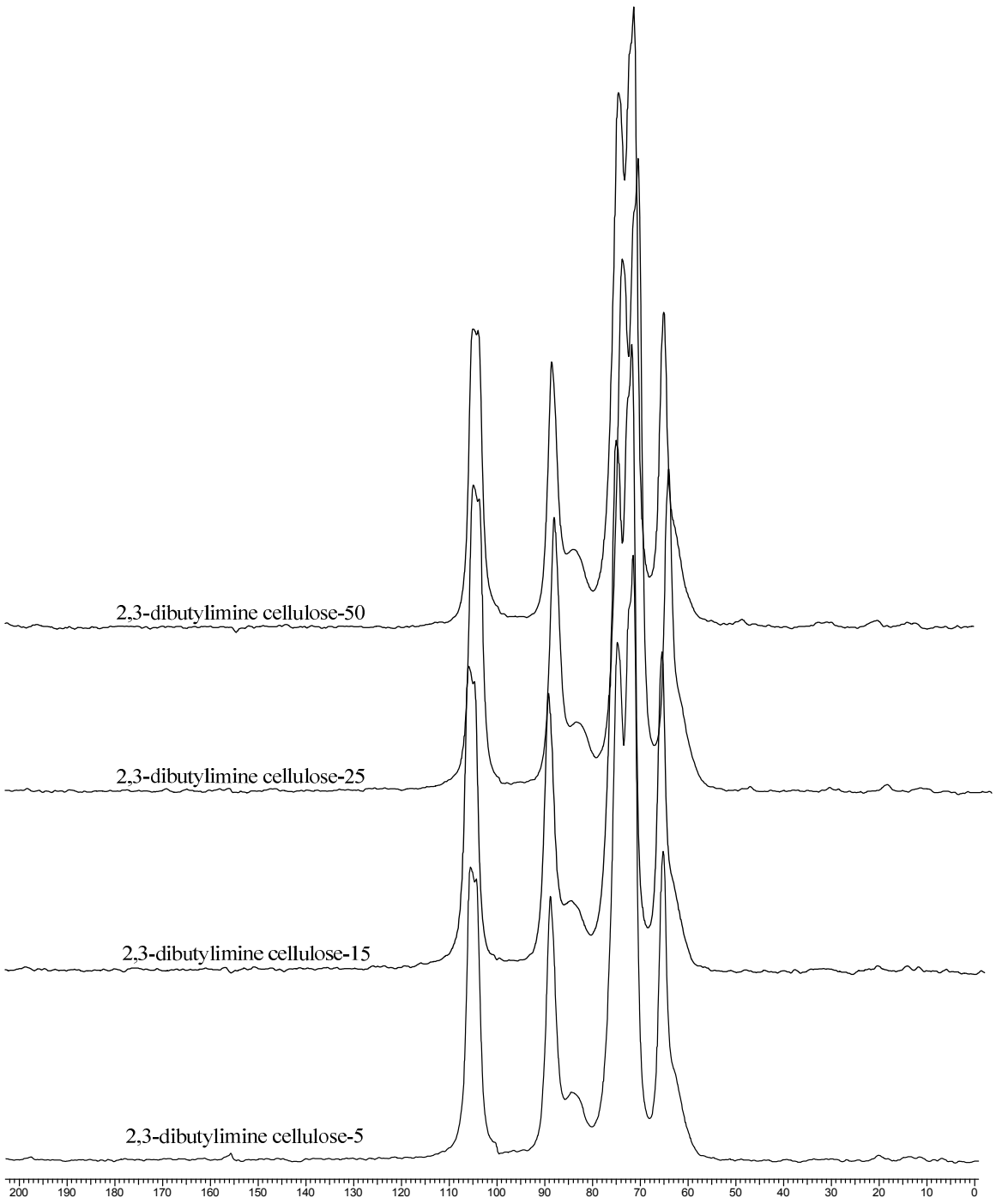


Figure 5.68: Overlapping of CP-MAS ^{13}C NMR of 2, 3-dibutylimine cellulose with various level of oxidation

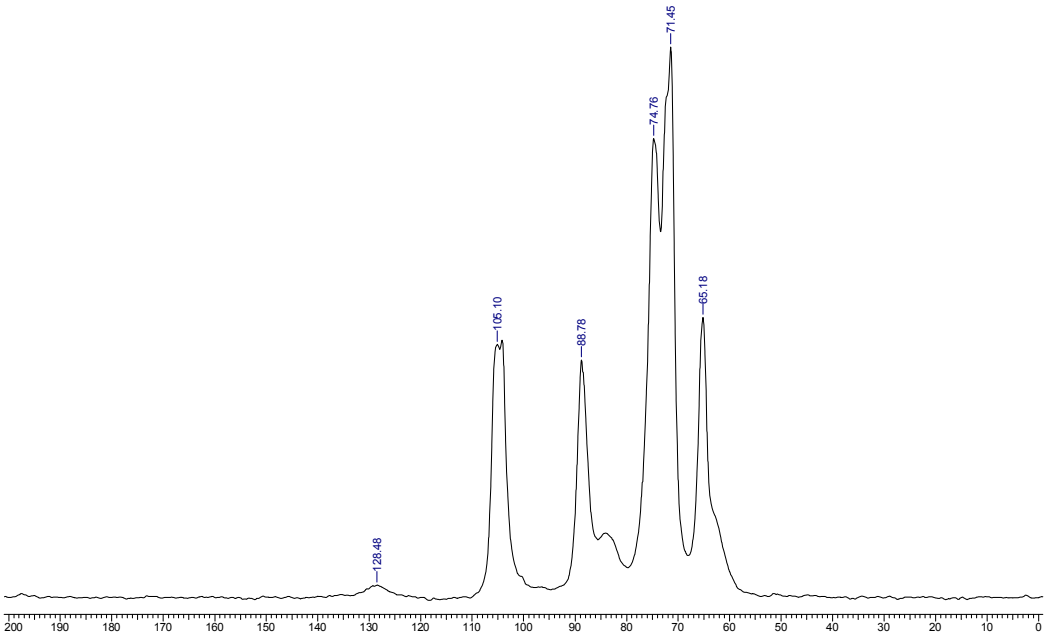


Figure 5.69: CP-MAS ^{13}C NMR of 2, 3-dibenzylimine cellulose-5

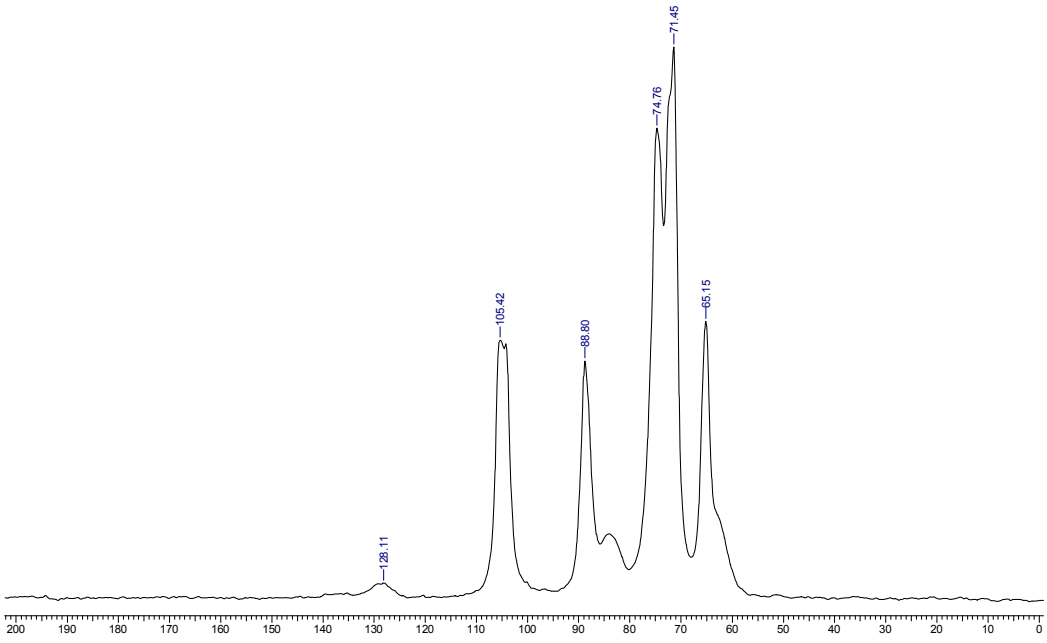


Figure 5.70: CP-MAS ^{13}C NMR of 2, 3-dibenzylimine cellulose-15

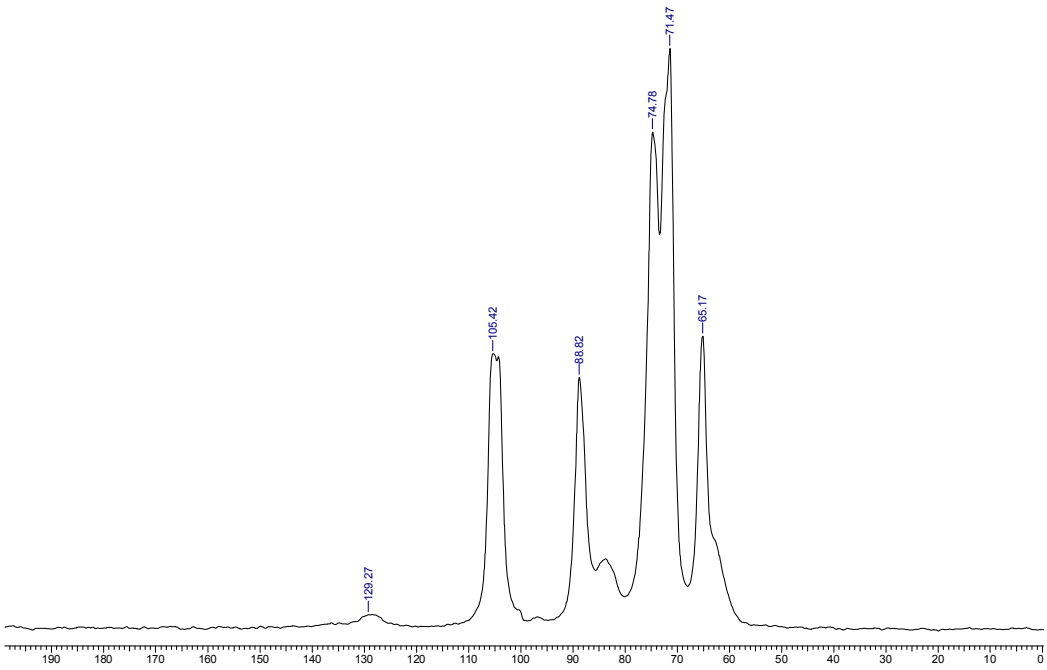


Figure 5.71: CP-MAS ^{13}C NMR of 2, 3-dibenzylimine cellulose-25

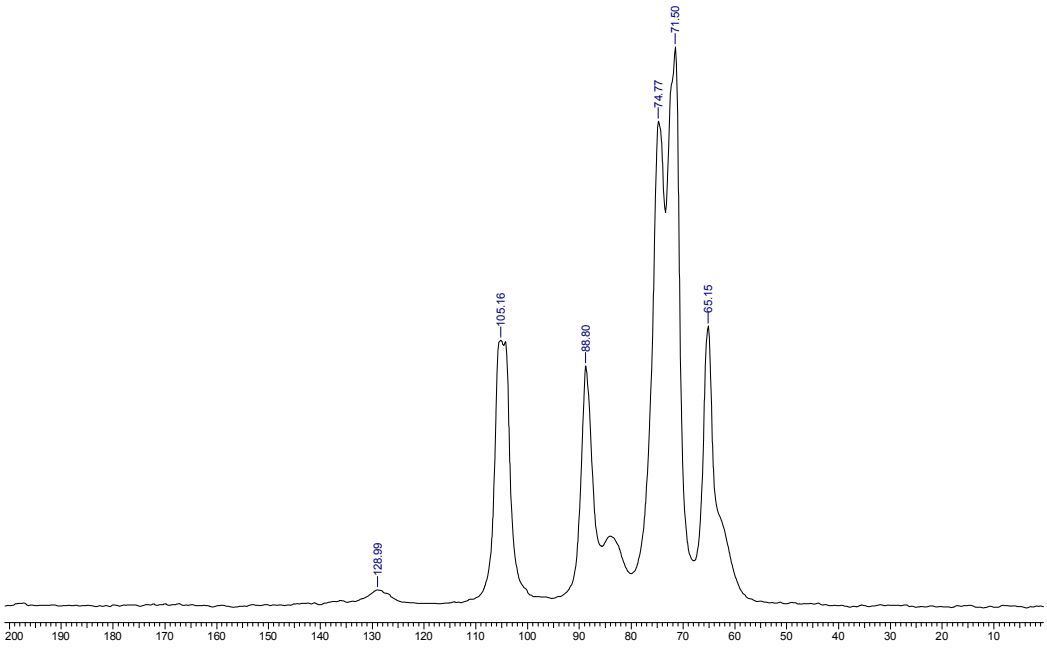


Figure 5.72: CP-MAS ^{13}C NMR of 2, 3-dibenzylimine cellulose-50

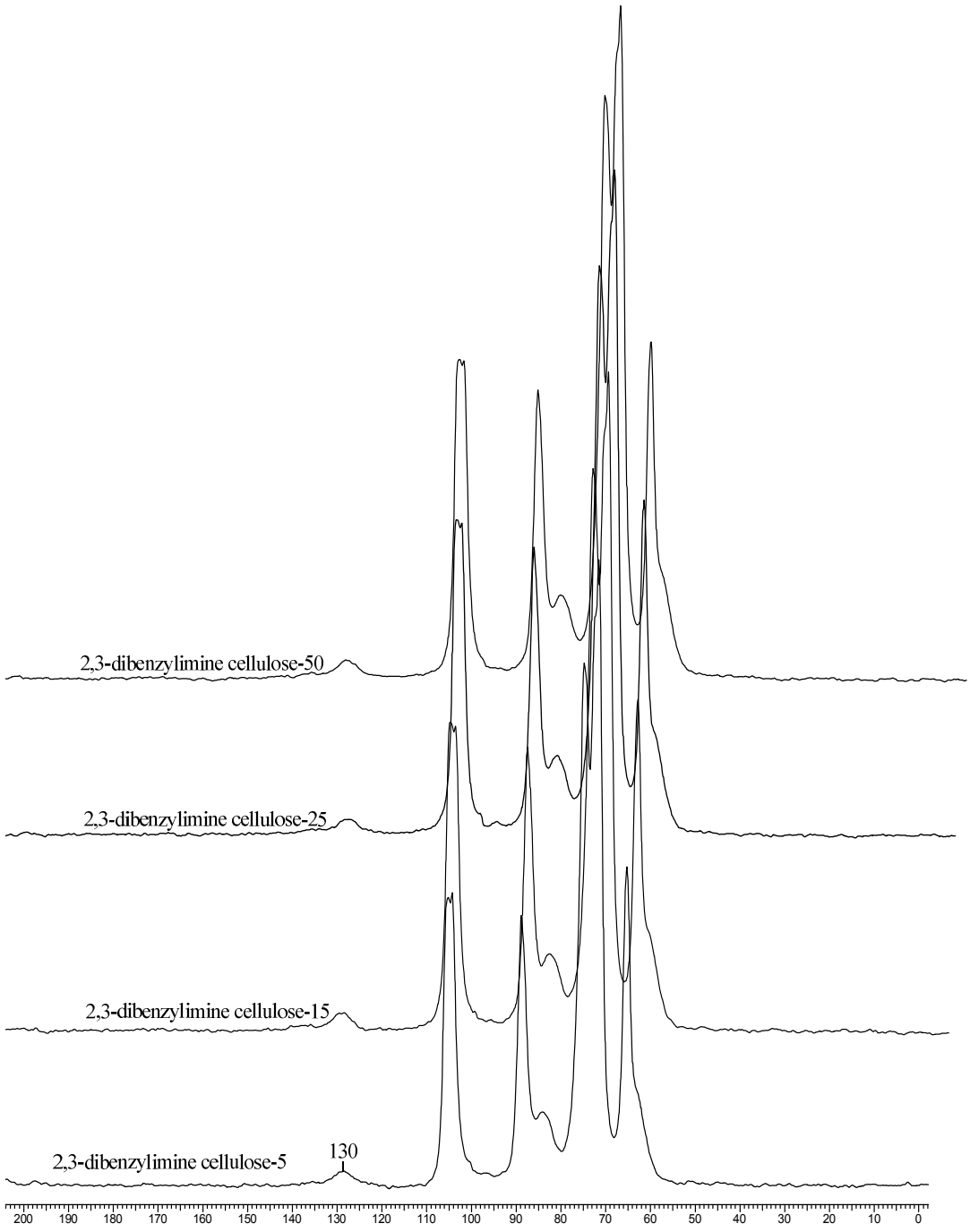


Figure 5.73: Overlapping of CP-MAS ^{13}C NMR of 2, 3- dibenzylimine cellulose with various levels of oxidation

5.13. Appendix-6

WAXRD diffractogram of cellulose and Schiff bases of 2, 3- dialdehyde cellulose with various levels of oxidation, (Numbers 5, 15, 25 and 50 denots the levels of oxidation (%) of 2,3-dialdehyde cellulose).

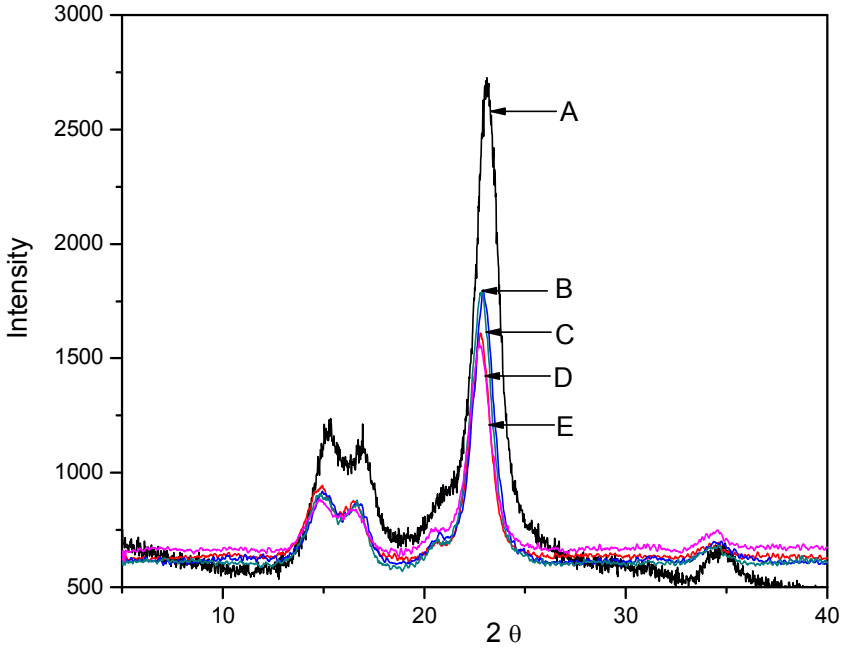


Figure 5.74: WAXRD of cellulose and 2, 3-dihydrazone cellulose with various levels of oxidation (**A**-cellulose, **B**-2,3-dihydrazone cellulose -5, **C**- 2,3-dihydrazone cellulose -15, **D**- 2,3-dihydrazone cellulose -25, **E**- 2,3-dihydrazone cellulose -50)

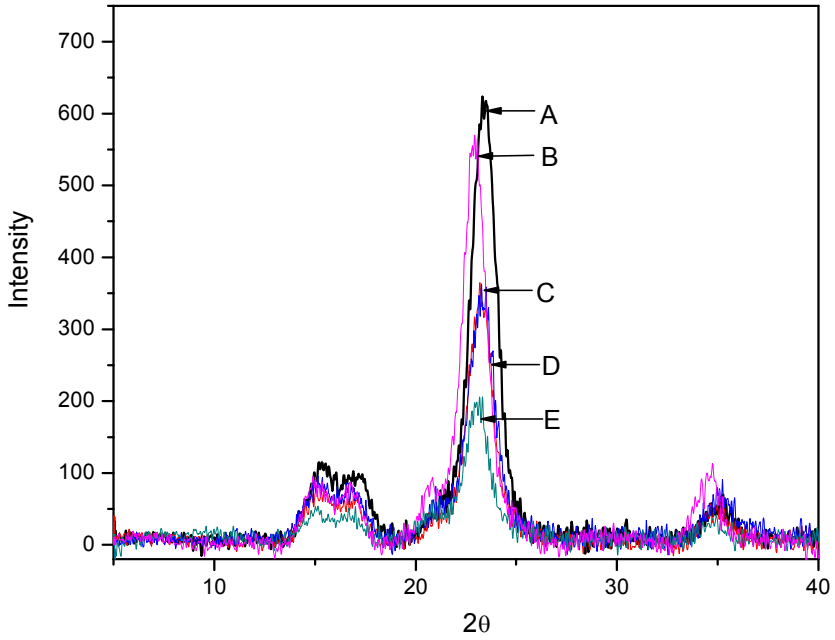


Figure 5.75: WAXRD of cellulose and 2, 3-dioxime cellulose with various levels of oxidation (**A**-Cellulose, **B**-2,3-dioxime cellulose-5, **C**-2,3-dioxime cellulose-15, **D**-2,3-dioxime cellulose-25, **E**-2,3-dioxime cellulose-50)

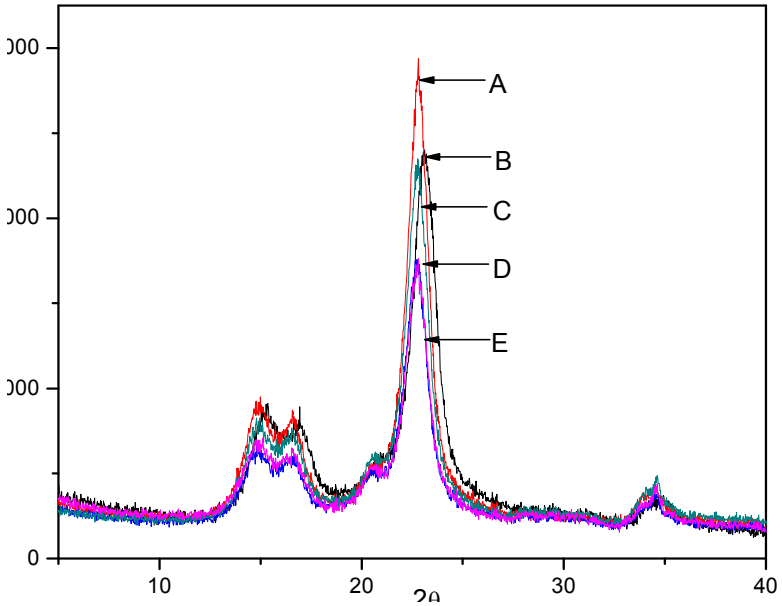


Figure 5.76: WAXRD of cellulose and 2, 3-diethylimine cellulose with various levels of oxidation (**A**-Cellulose, **B**-2,3-diethylimine cellulose-5, **C**-2,3-diethylimine cellulose-15, **D**-2,3-diethylimine cellulose-25, **E**-2,3- diethylimine cellulose-50)

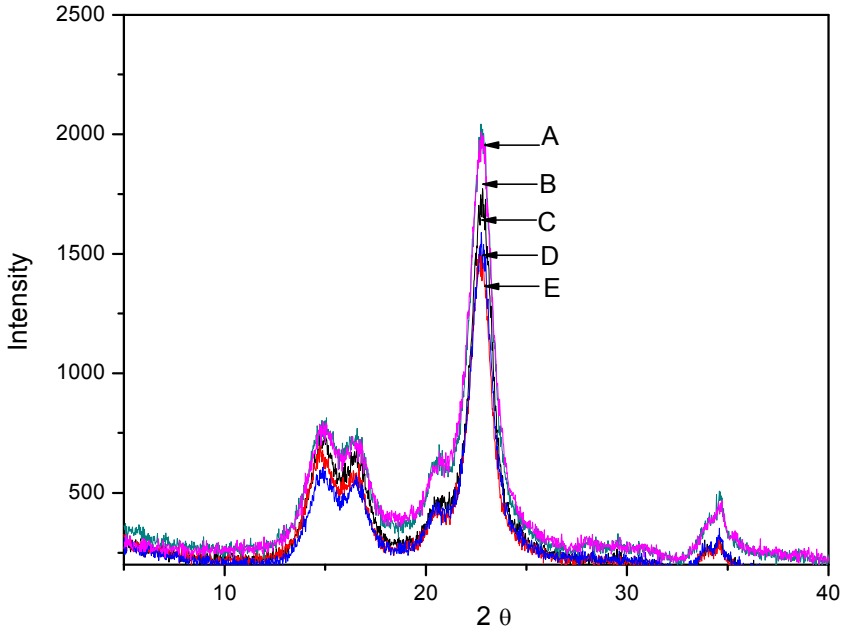


Figure 5.77: WAXRD of cellulose and 2, 3- dipropylimine cellulose with various levels of oxidation (**A**-Cellulose, **B**-2,3-dipropylimine cellulose-5, **C**-2,3-dipropylimine cellulose-15, **D**-2,3-dipropylimine cellulose-25, **E**-2,3-dipropylimine cellulose

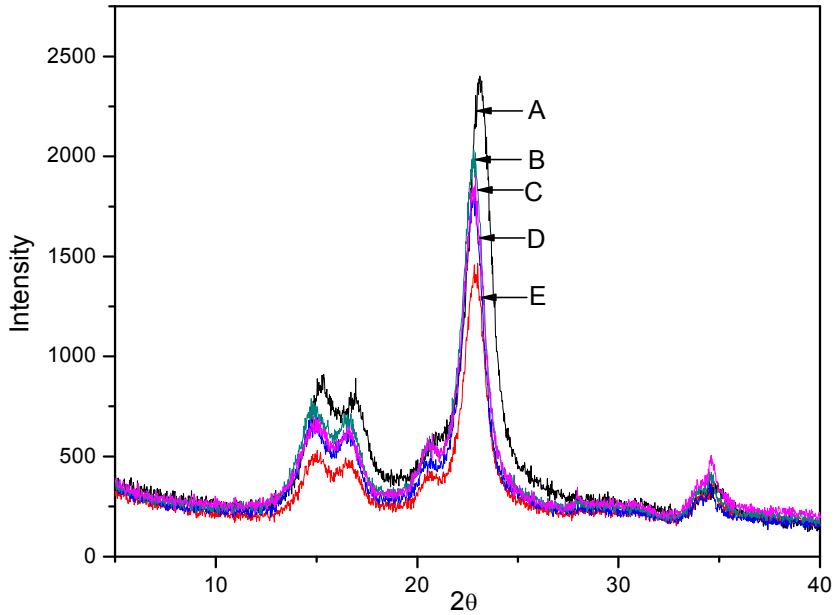


Figure 5.78: WAXRD of cellulose and 2, 3- dibutylimine cellulose with various levels ofoxidation (**A**-Cellulose, **B**-2,3-dibutylimine cellulose-5, **C**-2,3- dibutylimine cellulose-15, **D**- 2,3-dibutylimine cellulose-25, **E**-2,3-dibutylimine cellulose-50)

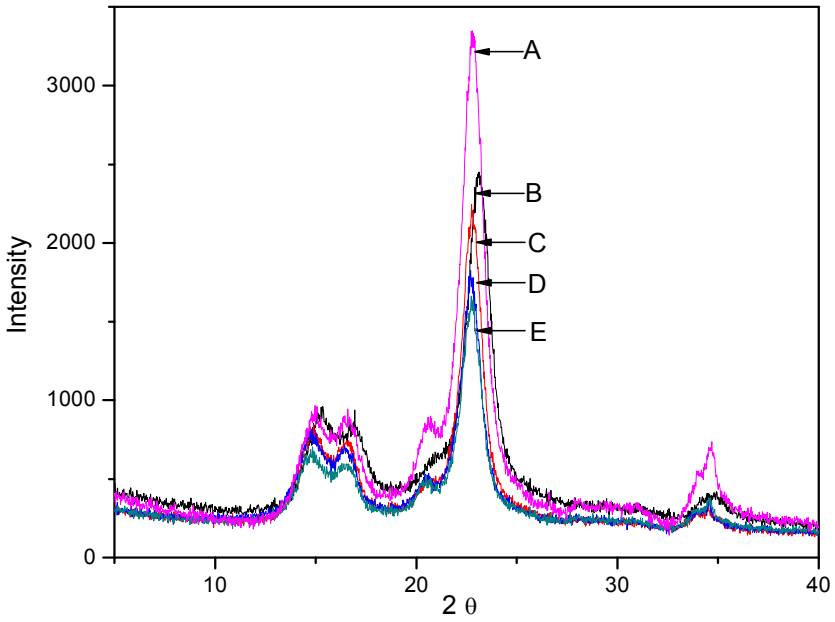


Figure 5.79: WAXRD of cellulose and 2, 3- dibenzylylimine cellulose with various levels of oxidation (**A**-Cellulose, **B**-2,3-dibenzylylimine cellulose-5, **C**-2,3-dibenzyl-imine cellulose-15 **D**-2,3-dibenzylimine cellulose-25 **E**-2,3-dibrnzylimine cellulose-50)

5.14. Appendix-7

TGA graph of cellulose and Schiff bases of 2, 3- dialdehyde cellulose with various levels of oxidation. (Numbers 5, 15, 25 and 50 denots the levels of oxidation (%) of 2,3-dialdehyde cellulose).

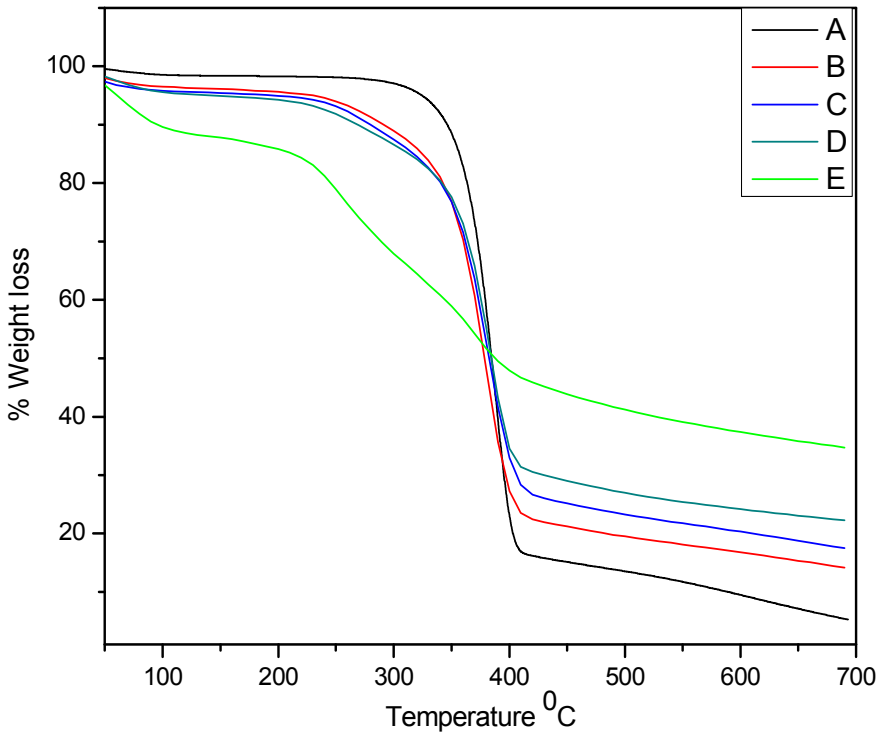


Figure 5.80: TGA of cellulose and 2, 3-dihydrazone cellulose with various levels of oxidation. (**A**-Cellulose, **B**-2,3-dihydrazone cellulose-5, **C**-2,3-dihydrazone cellulose -15, **D**- 2,3-dihydrazone cellulose -25, **E**- 2,3-dihydrazone cellulose -50)

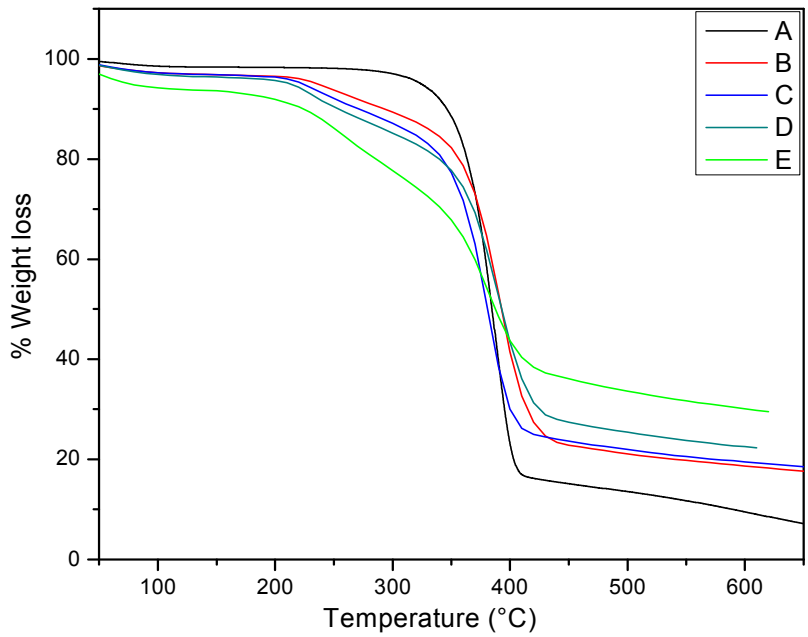


Figure 5.81: TGA of cellulose and 2, 3-dioxime cellulose with various levels of oxidation. (**A**-Cellulose, **B**-2,3-dioxime cellulose-5, **C**-2,3-dioxime cellulose-15, **D**-2,3-dioxime cellulose -25, **E**-2,3-dioxime cellulose -50)

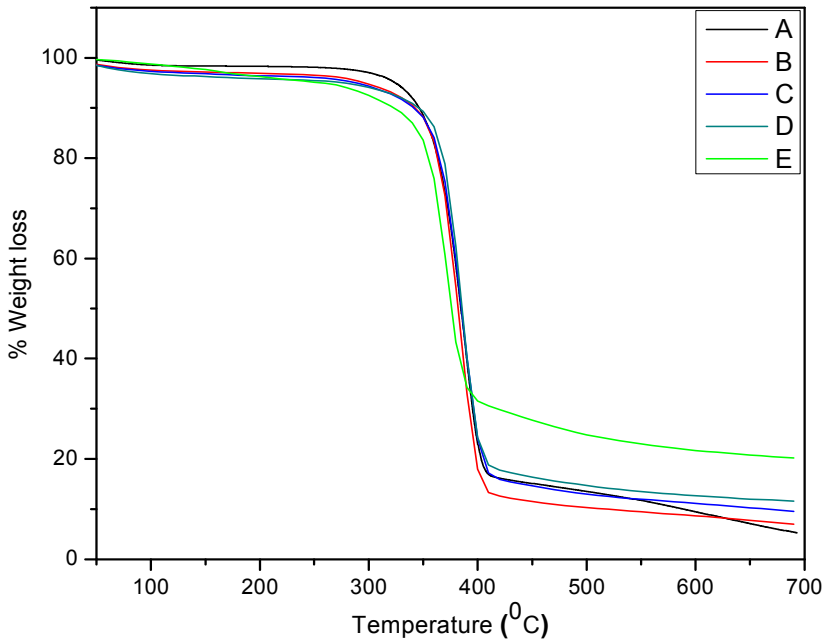


Figure 5.82: TGA of cellulose and 2, 3-diethylimine cellulose with various levels of oxidation. (**A**-Cellulose, **B**-2,3-diethylimine cellulose-5, **C**-2,3-diethylimine cellulose-15, **D**-2,3- diethylimine cellulose-25, **E**-2,3- diethylimine cellulose-50)

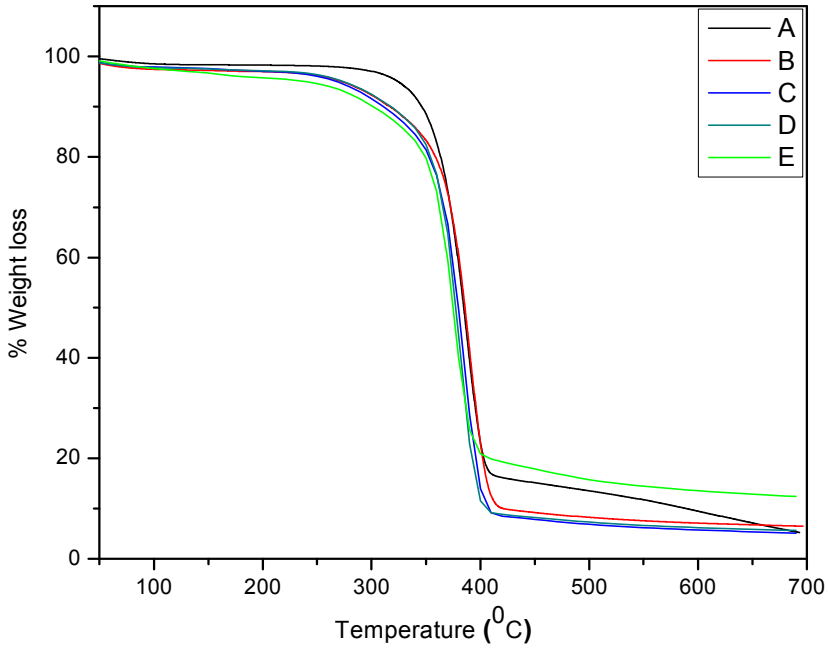


Figure 5.83: TGA of cellulose and 2, 3-dipropylimine cellulose with various levels of oxidation. (**A**-Cellulose, **B**-2,3-dipropylimine cellulose-5, **C**-2,3-dipropylimine cellulose-15, **D**-2, 3-dipropylimine cellulose-25, **E**-2, 3-dipropylimine cellulose-50)

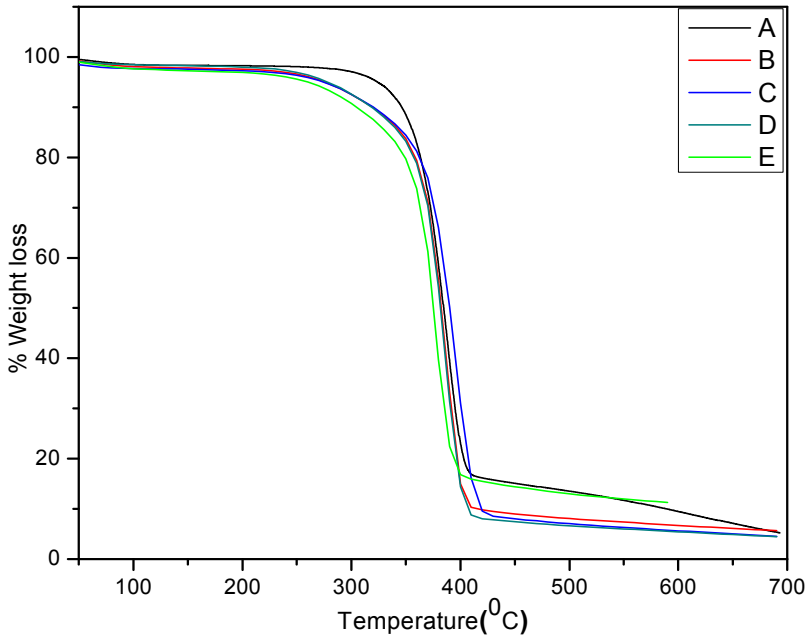


Figure 5.84: TGA of cellulose and 2, 3-dibutylimine cellulose with various levels of oxidation. (**A**-Cellulose, **B**-2,3-dibutylimine cellulose-5, **C**-2,3-dibutylimine cellulose-15, **D**-2, 3-dibutylimine cellulose-25, **E**-2, 3-dibutylimine cellulose-50)

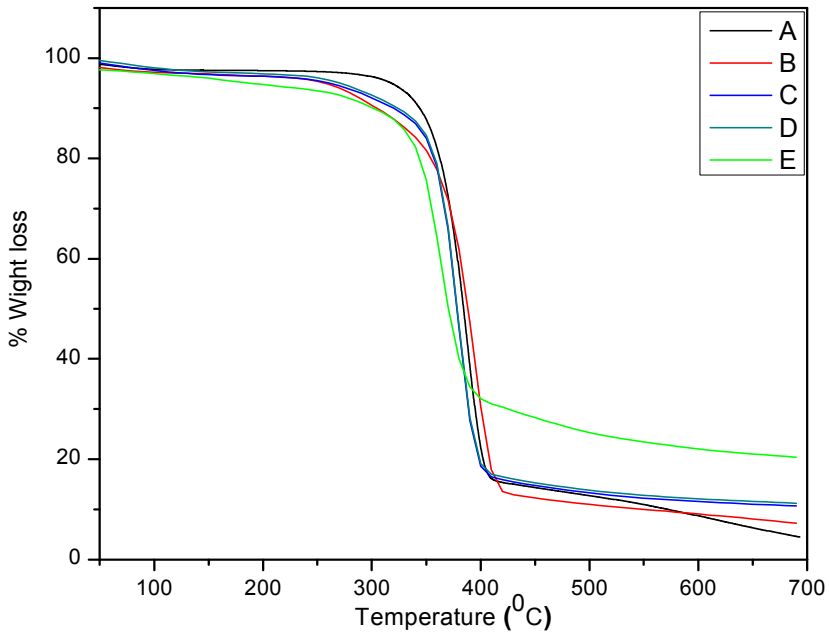


Figure 5.85: TGA of cellulose and 2, 3-dibenzylimine cellulose with various levels of oxidation.(**A**-Cellulose, **B**-2,3-dibenzylimine cellulose-5, **C**-2, 3-dibenzylimine cellulose-15, **D**-2, 3-dibenzylimine cellulose-25, **E**-2, 3-dibenzylimine cellulose-50)

Chapter 6

"Study of hydrolytic behavior and morphology of dialdehyde cellulose and its derivatives"

6.1. Introduction

Cellulose is the most abundant bio-renewable source and holds huge potential as an alternative to fossil fuels for sustainable production of fuels and chemicals (Barti -eri et al., 2008). Conversion of lignocellulose into biofuels (particularly ethanol and butanol) and useful chemicals has attracted increasing attentions in recent years. Although biofuels possess several advantages over conventional fossil fuels, lignocellulosic biofuel has not yet been produced on a commercial scale because of the low yields and high production cost. The lignocellulosic biofuel process can be made cost-competitive by making cost-effective pretreatment and hydrolysis strategy. The effective conversion of lignocellulosic to sugars and recovery of the sugars is regarded as holding the key to the success of this technology for a sustainable future.

The hydrolysis of cellulose by mineral acids is strongly affected by the acid concentration and temperature. However, recoveries of the acid or disposal of the neutralized acid, along with corrosion problems, pose significant challenges to this methodology. If enzymes are to be used for hydrolysis of cellulose, various factors play important roles such as physical properties of the substrate, composition of substrate, crystallinity of cellulose, degree of polymerization, enzyme synergy, bulk and pore diffusion, and kinetics (Zhang & Lynd, 2004). Other workers have reported factors such as available surface area, particle size, pore size of the substrate and lignin content as limiting the enzymatic hydrolysis of cellulosic materials (Chang and Holtzapple, 2000; Koullas et al., 1992; Laureano-Perez et al., 2005; Thompson et al., 1992; Puri, 1984).

The objective of the current work is to study how oxidative modifications and presence of various functionalities on the cellulose chain affect the enzymatic hydrolysis of cellulose. Thus we prepared a series of periodate oxidized cellulose to

obtain 2, 3-dialdehyde cellulose in which the pyranose ring of cellulose selectively breaks at C-2 and C-3 positions. This 2, 3-dialdehyde cellulose was then further modified to sodium 2, 3-dicarboxy cellulose, 2, 3- dicarboxycellulose and Schiff bases (2, 3-dioxime cellulose, 2, 3-dihydrazone cellulose, 2, 3-diethylimine cellulose, 2, 3-dipropylimine cellulose, 2, 3-dibutylimine cellulose and 2, 3-dibenzylimime cellulose). The hydrolysis behavior of these derivatives was studied by hydrolytic enzymes. It was observed that depending on the chemical functionality on the cellulose backbone, cellulose may or may not hydrolyze efficiently. The effects of oxidation of cellulose and subsequent transformation to Schiff bases on cellulose surface were investigated by fluorescence visualization by confocal laser scanning microscopy and scanning electron microscopy for changes in morphology of the different cellulosic derivatives.

6.2. Experimental

6.2.1. Materials

Cotton linter cellulose was supplied by Reliance Cellulose Products limited, Secundrabad, India. It contained > 95% alpha cellulose. Similarly cellulose from sugarcane bagasse obtained by a proprietary process developed by our laboratory was used (Varma, WO PCT/IN08/00569, 2008).

6.2.2. Chemicals

Sodium metaperiodate (99.5%), sodium thiosulphate (99.5%), sodium bicarbonate (99.5%) was a product of S. D. Fine Chemicals, India. Soluble starch (Merck), sodium hydroxide, methanol GR grade, potassium iodide, potassium dichromate, 3, 5-dinitrosalysilic acid (Rankem India, Ltd.) were procured and used without further purification.

6.3. Methods

6.3.1. Oxidation of cellulose to 2, 3-dialdehyde cellulose with different levels of oxidation

In a two neck three liter round bottom flask equipped with mechanically rotatary shaft, a known weight of cellulose was taken. This flask was wrapped with aluminum foil to prevent exposure to light. To this flask were added quantities of sodium metaperiodate (as shown in table-5.1 of previous chapter). Sodium metaperiodate was dissolved slowly in two liters of distilled water and the pH of this solution was measured to be between 3.2 - 3.5. Nitrogen gas was purged in the reaction mixture. The reaction was carried out in the dark under nitrogen atmosphere to avoid decomposition of sodium metaperiodate and photo-oxidation. Temperature of the reaction mixture was maintained between 50-55^{0}C using a water bath for various time intervals, after which the reaction mixture was cooled to room temperature. Oxidized cellulose was filtered off, washed thoroughly with distilled water several times until the filtrate became neutral. The final washing was done by methanol and the product was dried under vacuum at 60^{0}C and characterized by FTIR, CP/MAS ^{13}C NMR, WAXRD and TGA. (Details of characterization are given in chapter 5). Quantification of sodium metaperiodate consumed in the reaction was carried out by sodium thiosulphate method (Scott, 1939).

6.3.2. General procedure for synthesis of sodium 2, 3-dicarboxy celluloses (NaDCC)

For synthesis of sodium 2, 3-dicarboxy celluloses (NaDCC) with different degree of oxidation (i e. ~5 %, 15 %, 25 %, and 50%) the required quantities of dialdehyde cellulose was dispersed in distilled water. To dialdehyde cellulose, sodium chlorite solution and required amount of acetic acid dissolved in distilled water and added slowly with constant string. The temperatures of reaction mixture were maintained

at 25^0-30^0C. (Details of quantities of sodium chlorite and acetic acid used are given in table 5.2 of chapter 5). The start of oxidation was indicated by the formation of yellow coloration and evolution of chlorine dioxide (ClO_2) gas. The reaction was continued for 7 hrs until there was no further evolution of gas. Nitrogen gas was bubbled through the reaction mixture to remove the dissolved gases from the reaction. The pH of the reaction mixture was adjusted to 8.5-9 by addition of 10 N NaOH solution. The product was settled at the bottom and was separated by decantation. The product was washed several times with distilled water until the filtrate was neutral. It was dried under vacuum at 60 ^{0}C and characterized by FTIR CP/MAS ^{13}C NMR and TGA. Sodium detection was done by Energy-dispersive X-ray spectroscopy (Details of characterization are given in chapter 5). These products of different level of oxidations are insoluble in common organic solvents.

6.3.3. General methods for synthesis of 2, 3-dicarboxycelluloses (DCC)

For synthesis of 2, 3-dicarboxycelluloses (DCC) with different degree of oxidation (i e., 5, 15, 25 and 50%), required quantities of sodium 2, 3-dicarboxy cellulose was dispersed in distilled water and 0.2 N HCl was added with string till the pH reached 3.5. This solution was kept in freezer at 1 ^{0}C for about half an hour with stirring at regular intervals. After this the aqueous fraction was decanted off and 30 ml distilled water was added and kept at 5 ^{0}C for 24 hours (Details of quantities of reagents are given in Table 5.3. of chapter 5). After a fixed time the solution was decanted off and the product washed several time until the filtrate was neutral. It was dried in vacuum at 60 ^{0}C and characterized by FTIR, CP/MASS ^{13}C NMR, WAXRD and TGA (Details of characterization are given in chapter 5). These products of different levels of oxidation are insoluble in common organic solvents.

6.3.4. General methods synthesis of Schiff bases of 2, 3-dialdehyde cellulose

To a 250 ml round bottom flask 100 ml of 0.2 M acetate buffer was taken, pH of this buffer was adjusted between 4.8- 5.6. To this buffer known amounts of hydrazine, oxime and amines was added slowly with constant stirring. The known quantity of oven dried 2, 3- dialdehyde cellulose (DAC) was added portion wise over a period of 10 minutes. These reaction mixtures were then stirred from room temperature to 55 ^{0}C for different reagents for various time intervals (See table 5.4-5.9 of chapter 5 for details). Reaction mixtures slowly turn faint yellow to pale yellow in colour (deeper colour for higher oxidation levels of DAC). After a fixed time interval the reaction mixtures were allowed to settle down. The insoluble solid settled at the bottom was separated by centrifugation. The filtrates were drained and solid products were washed several times with distilled water till neutral filtrate was obtained. The products were dried under vacuum at 60 ^{0}C for 24 hrs and characterized by FTIR, CP/MASS ^{13}C NMR, WAXRD and TGA. Nitrogen contents were determined by elemental analysis as well as by EDAX for low level of oxidation (Details of characterization are given in chapter 5). All products obtained from different level of oxidiesd dialdehyde cellulose are insoluble in common organic solvents.

6.3.5. Enzymatic hydrolysis

The hydrolysis of the samples was carried out in 25 ml flask containing 9 ml citrate buffer (pH 4.5, 50mM) and 1 ml of cellulase enzyme preparation obtained from mutant of *Penicillium janthinellum* NCIM 1171. The flasks were incubated at 50^0 C with shaking at 200 rpm. The samples were analyzed for the reducing sugars after 12, 24, 36 and 48 hrs by dinitrosalicylic acid methods. 1 ml of enzyme preparation contains 5 unit of FPA, 15 units of β–glucosidase and 200 units of CMCase.

6.3.5.1. Hydrolysis of cellulose (cotton linters) in the presence of physically added reagent.

100 mg of cotton linters were hydrolyzed in 25 ml conical flask containing 9 ml of Citrate buffer (pH 4.5, 50 mM), 1ml enzyme preparation and 7.0 mg and 18 mg of anhydrous NH_2-NH_2 reagent separately. 1 ml of enzyme preparation contains 5 unit of FPA, 15 units of β–glucosidase and 200 units of CMCase. The pH adjusted to 4.5 to 5.0. The flasks were incubated at 50^0C with shaking. The 1 ml of hydrolyzed samples was removed after suitable time interval for the analysis of reducing sugars.

6.3.5.2. Hydrolysis of cellulose (cotton linters) in the presence of 2, 3-dihydrazone cellulose.

50 mg of cotton linters, 50 mg of 2,3-dihydrazone cellulose and a mixture of 25 mg of cotton linters and 25 mg of 2,3-dihydrazone cellulose were hydrolyzed in 25 ml conical flask containing 4.5 ml of Citrate buffer (pH 4.5, 50 mM), 0.5 ml enzyme preparation. The pH adjusted to 4.5 to 5.0. The flasks were incubated at 50 ^{0}C with shaking. The 0.5 ml of hydrolyzed samples was removed after suitable time interval for the analysis of reducing sugars. 1 ml of enzyme preparation contains 5 unit of FPA, 15 units of β–glucosidase and 200 units of CMCase.

***6.3.6**. Antimicrobial activity*

10 mg sample, 10 mL nutrient broth and 10% inoculum were incubated at 37 ^{0}C with 200 rpm for 24 hrs. Samples were withdrawn at regular interval of 6h and absorbances were taken at 600 nm against the control.

6.3.7. Scanning electron microscopy

Surface morphology of modified cellulose was studied by using scanning electron microscope (Model Leica Cambridge stereoscan 440 SEM Cambridge, UK). Sample specimens were coated with gold in an automatic sputter coater (Polaron

equipment, scanning microscope coating unit E5000, UK). Accelerating potential was 20 kV. Photograph of representative areas of the sample were taken at 500 and 50000 magnifications.

6.3.7. Confocal laser scanning microscopy

The confocal microscope images were obtained on LSM 510 meta (Carl Zeiss) using a 63X (N.A. =1.4) oil immersion objective and excitation wavelengths of 405 nm, 488 and 543 nm (He-Ne laser) with band pass BP 420-480, BP 505-530, BP 550-690 laser filters. All images were captured after setting their thresholds below saturation. Laser power and other parameters were kept identical for all samples.

6.4. Result and discussion

Complete enzymatic hydrolysis of cellulose to glucose requires a cocktail of endoglucanase, exoglucanse and β-glucosidase enzymes. During the hydrolysis of cellulose, the endoglucanases attack the cellulose in a random manner creating new reducing ends. This reaction is followed by the hydrolysis with exoglucanases, which attack the cellulose from either end, forming cellobiose. Finally, the β-glucosidase completes the hydrolytic process through the formation of glucose from cellobiose. It is considered that all three enzymes work in a synergistic manner for hydrolysis of both cellulose and modified cellulose (Mansfield et al., 1999).

In this study we present the results of our studies on hydrolysis of pure cellulose and its chemical modifications containing aldehyde, carboxylate, carboxy and schiff bases. The data are presented in Tables 6.1 and 6.2. Table 6.1 represents the enzymatic hydrolysis behavior of cotton linter cellulose which is 15% oxidized to an extent of 15% of the glucose monomer units to 2, 3-dialdehyde cellulose (DAC-15). This oxidized cellulose is then converted to Na salt of dicarboxy

cellulose and then to dicarboxy cellulose. It was observed that dialdehyde cellulose appears to hydrolyze at a slightly lower rate than the parent cellulose.

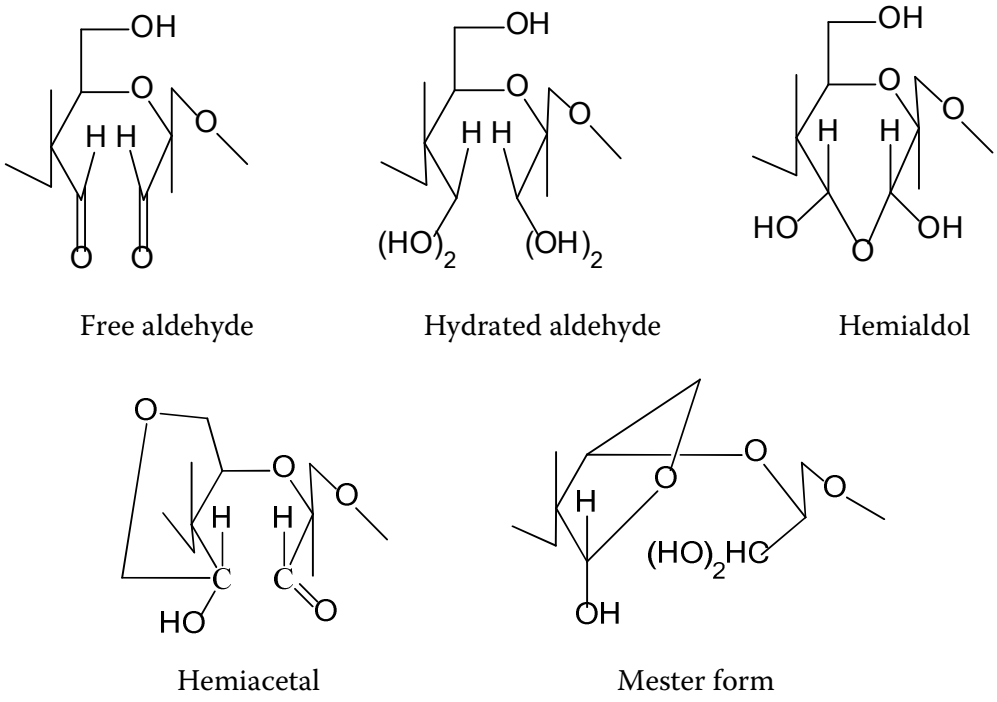
Table: 6.1: Enzymetic hydrolysis of oxidized cellulose with same level of oxidation (15% oxidation level)

Sr. No.	Samples	Hydrolysis (%)			
		12 hrs	24 hrs	36 hrs	48 hrs
A	**Cellulose(Cotton linter)**	6.6	11.1	16.3	20.5
1	2,3-dialdehyde cellulose-15	4	6.6	13.8	17
2	Sodium 2,3-dicarboxy cellulose-15	19.5	29	40.5	48
3	2,3-dicarboxy cellulose-15	17	27	38.6	42
4	2,3-dioxime cellulose-15	4.2	7.5	10.4	12.3
5	2,3-diethylimine cellulose-15	16	25.5	30	37.3
6	2,3-dipropylimine cellulose-15	20	25	28	34.3
7	2,3-dibutylimine cellulose-15	25	31.8	35.4	38.8
8	2,3-benzylimine cellulose-15	16.3	27.5	32	36.1
9	2,3-dihydrazone cellulose-15	N. D.			
B	**Cellulose(bagasse)**	25.3	35	36.5	40.0
1	2,3-dialdehyde cellulose-15	8.8	11	24.5	30.0
2	Sodium 2,3-dicarboxy cellulose-15	38	50.5	65.5	75.0
3	2,3-dicarboxy cellulose-15	28.7	39	45.8	60.5

N. D. : Not detectable

Since oxidation causes decrease in crystallinity of cellulose, it is expected that oxidized cellulose should hydrolyze at a rate faster than native cellulose. It appears that in dialdehyde cellulose, aldehyde substituent hinders the enzymes perhaps due

to hydrogen bonding of aldehyde with other hydroxyl in cellulose as well as formation of hemiacetal and hemialdol like structure as shown in figure 6.1.



Free aldehyde Hydrated aldehyde Hemialdol

Hemiacetal Mester form

Figure 6.1: Structural form of 2, 3-dialdehyde cellulose

Similar observation was made by Feng et al, (2009) who conducted similar studies on cupric ion and hypochlorite oxidized cellulose (C-6 oxidation) and observed profound decrease in hydrolysis rate mainly due to interference of functional groups. However, as shown in Table 6.1. NaDCC-15 (15% oxidised sodium 2, 3-dicarboxycellulsoe) and DCC-15 (15% oxidised 2, 3-dicarboxycellulsoe) the observed hydrolysis rate was significantly greater than the parent DAC-15 and cotton linter cellulose (the hydrolysis profile is shown in figure 6.16, see appendix-8). Thus while cellulose and DAC-15 hydrolyzed only to ~20% in 48 hrs, NaDCC-15 and DCC-15 hydrolysed to an extent of 42-48% in the same time. This enhanced

rate could be due to decrease in crystallinity and enhanced binding of the enzymes to the carboxylate/carboxy functional group of NaDCC and DCC.

Similar hydrolysis was conducted on bagasse derived cellulose, the preparation of which is described in chapter 2. Bagasse cellulose was also oxidized ~15 % to make 2, 3-dialdehyde cellulose and then converted to sodium salt of 2, 3-dicarboxy cellulose and 2, 3- dicarboxy cellulose. Here also, a similar hydrolytic pattern was seen as for cotton cellulose and its derivatives but the extent of hydrolysis was much greater, going upto > 70% for NaDCC-15. The reason for this enhancement can be attributed to lower crystallinity and lower degree of polymerization (DP) of bagasse cellulose as compared to cotton cellulose (figure 6.17 in appendix-8 show the hydrolysis profile of these derivatives).

Enzymatic hydrolysis studies were also carried out on Schiff bases of 2, 3-dialdehyde cellulose such as oxime, hydrazone and imines as described earlier. Table 6.2 compiles the details of enzymatic hydrolysis of all cellulosic derivatives. It was observed that 2, 3-dialdehyde cellulose with low levels of oxidation hinders the enzymatic hydrolysis (discussed earlier) but higher level of oxidation hydrolyses faster that the native cellulose. This could be due extensive molecular weight degradation of this derivative which helps the enzyme for easy access for hydrolysis. Thus 2, 3-dialdehyde cellulose-50 (50% oxidation level) hydrolyses upto 35.7% in 48 hrs which is more than the cellulose. In case of sodium 2, 3-dicarboxy cellulose hydrolysis was faster than cellulose and dialdehyde cellulose as discussed earlier. A similar effect was observed for 2, 3-dicarboxy cellulose. This enhanced rate could be due to decrease in crystallinity and enhanced binding of the enzymes to the carboxylate/carboxy functional groups as well as molecular weight degradation of NaDCC and DCC. Thus highest level of oxidised NaDCC and DCC

hydrolyses upto 70 and 60% respectively within 48 hrs which is 3-4 times more than cellulose.

Table 6.2: Enzymatic hydrolysis of oxidized cellulose with various level of oxidation

Sr. No.	Modified 2,3-dialdehyde cellulose	Hydrolysis (%)			
		12 hrs	24 hrs	36 hrs	48 hrs
1	Cellulose(Cotton linter)	6.6	11.1	16.3	20.5
2	2,3-dialdehyde cellulose-5	2.8	3.2	4.6	5.6
3	2,3-dialdehyde cellulose-15	4	6.6	13.8	17.0
4	2,3-dialdehyde cellulose-25	7.2	9.1	13.8	16.6
5	2,3-dialdehyde cellulose-50	14.5	26.8	32.1	35.7
6	Sodium 2,3- dicarboxy cellulose-5	10.7	20	31.6	35.1
7	Sodium 2,3-dicarboxy cellulose-15	19.5	29	40.5	48.6
8	Sodium 2,3-dicarboxy cellulose-25	25	35.5	48.7	56.4
9	Sodium 2,3-dicarboxy cellulose-50	48	55.6	63.9	72.3
10	2,3- dicarboxy cellulose-5	8.9	18.2	28	30.3
11	2,3- dicarboxy cellulose-15	17	27	38.6	42.7
12	2,3- dicarboxy cellulose-25	20	31.2	42.6	50.4
13	2,3- dicarboxy cellulose-50	42	50.2	59.9	61.5
14	2,3-dioxime cellulose-5	3	4.7	6.1	7.8
15	2,3-dioxime cellulose-15	4.2	7.5	10.4	12.3
16	2,3-dioxime cellulose-25	4.5	6.3	10.2	14.4
17	2,3-dioxime cellulose-50	5.5	8.3	13.3	16.3
18	2,3-diethylimine cellulose-5	15	24.1	28	34.6
19	2,3-diethylimine cellulose-15	16	25.5	30	37.3
20	2,3-diethylimine cellulose-25	17.2	25	33.4	40.0
21	2,3-diethylimine cellulose-50	20	38	60.5	75.3
22	2,3-dipropylimine cellulose-5	17	24.2	26.3	31.1
23	2,3-dipropylimine cellulose-15	18	25	28	34.3
24	2,3-dipropylimine cellulose-25	20.2	25.5	30	35.9
24	2,3-dipropylimine cellulose-50	25.7	40	45.9	51.5

26	2,3-dibutylimine cellulose-5	22	28.3	32.6	35.7
27	2,3-dibutylimine cellulose-15	25	31.8	35.4	38.8
28	2,3-dibutylimine cellulose-25	28.5	36.6	45.3	50.2
29	2,3-dibutyllimine cellulose-50	33.3	41	48.7	56.6
30	2,3-benzylimine cellulose-5	11.3	21	23.1	25.7
31	2,3-benzylimine cellulose-15	14.0	19.8	24.3	27.2
32	2,3-benzylimine cellulose-25	15.6	20.4	25.0	30.6
33	2,3-benzylimine cellulose-50	16.3	22.6	26.7	36.6
34	2,3-dihydrazone cellulose-5	N. D.			
36	2,3-dihydrazone cellulose-15				
37	2,3-dihydrazone cellulose-25				
38	2,3-dihydrazone cellulose-50				

N.D. : Not detectable

The oxime derivatives i.e. 2, 3-dioxime cellulose derivative hydrolyzes up to 5.5% in initial 12 hrs and maximum hydrolysis reached was 16.3% in 48 hrs for the highest level of oxidised dioxime. This is less than the cotton cellulose. This may be due to oxime substituent creating inter- and intra-molecular hydrogen bonds (similar to the dialdehyde cellulose case) which cause the slow enzymatic hydrolysis, even though the crystallinity of this derivative is less than the initial cellulose (see previous chapter).

In case of 2, 3-diethylimine cellulose, all derivatives hydrolyse faster than the native cellulose perhaps due to molecular weight degradation. Upto 75% hydrolysis within 48 hrs was observed for 2, 3-diethyinine cellulose-50. Similar facts were observed for 2, 3-dipropylimine and 2,3-dibutylimine cellulose where maximum hydrolysis reaches upto 51.5 and 56.6 % respectively for highest level of oxidation. While 2, 3-dibenzylimine cellulose hydrolyses little slower than alkylimine derivatives because of steric effect of phenyl ring which may hinder the easy access of enzyme for

hydrolysis. Thus only 36.6% hydrolysis was observed in 48 hrs for highest level of oxidised 2, 3-dibenzylimine derivatives.

Interestingly, 2, 3-dihydrazone cellulose derivatives with all level of oxidation show resistance towards enzymatic hydrolysis. To confirm this fact, two sets of hydrolysis experiment were carried out. In first the experiment, the reagent (anhydrous hydrazine) from which this derivative was synthesized, was physically mixed with cotton linter to study whether this is due to residual reagent attached physically to the cellulose. In the second set of experiment, 2, 3-dihydrazone cellulose was directly added to pure cellulose to study the hydrolysis behavior of cellulose to study whether this derivative hydrolyses or prevents hydrolysis of pure cellulose. The details of hydrolysis of these experiments are presented in Table 6.3.

Table 6.3: Enzymatic hydrolysis of cellulose in the presence of physically added reagent and 2, 3-dihydrazone cellulose

Sr.No.	Substrates	Hydrolysis (%)			
		24 hrs	48 hrs	72 hrs	98 hrs
1	Cellulose without any reagent (50 mg)	2.96	13.7	16.6	30.80
2	Cellulose (50 mg) + NH_2-NH_2 (7 mg)	3.00	-	-	8.33
3	Cellulose (50 mg) + NH_2-NH_2 (18 mg)	2.16	6.00	6.60	7.66
4	2 ,3-dihydrazone cellulose-50 (50 mg)	2.60	5.16	6.33	7.83
5.	Cellulose (25 mg) + 2 ,3-dihydrazone Cellulose (25 mg)	5.50	8.90	18.33	31.50

It was observed that cellulose without added reagent hydrolyses to a maximum of 30.8% in 98 hrs. However, added hydrazine inhibits hydrolysis of cellulose, and only ~ 8 % hydrolysis was observed in 98 hrs. Polymeric 2, 3-dihydrazone cellulose

(50% modified) also did not hydrolyse significantly, reaching only 7.83 % in 98 hrs. On the other hand, cellulose with added 2, 3-dihydrazone cellulose (50:50 mixtures) hydrolyses almost equally with that of control (pure cellulose) indicating that the presence of 2, 3-dihydrozone cellulose in the mixture does not affects the hydrolysis of pure cellulose. This result indicates that prevention of hydrolysis by 2, 3-dihydrozone cellulose is not a physical phenomenon (such as inactivation of enzymes) but a property of the material itself. This data led us to test this sample for the bactericidal activity. Table 6.4 shows the decrease in optical density of 2, 3-dihydrazone cellulose compared to the control (microorganism broth without samples) which reflects its antimicrobial activity. It is well established that optical density (OD) is directly proportional to the bacterial cell concentration (Cen Lian et al., 2003); hence decreasing value of OD gives the bactericidal property of materials.

Table 6.4: Effect of 2,3-dihydrazone cellulose on the growth of *E.coli.*

Time (hrs)	Absorbances (600 nm)				
	DHC-5	DHC-15	DHC-50	DHC-75	Control
6	0.2187	0.2659	0.2867	0.2550	0.2433
12	0.5813	0.5036	0.4865	0.5319	0.5571
24	0.7454	0.7728	0.7752	0.7861	0.7586
48	0.9153	0.9481	1.0286	1.0681	1.1261
Inhibition % [a] after 24 hrs	18.7	15.8	8.6	5.1	--

DHC: 2, 3-dihydrazone cellulose (number indicates the level of oxidation as explained in chapter 5).

[a] calculated by the difference between the number of colonies from the bacteria with samples and that of from bacteria in control.

Thus from Table 6.4 above it is observed that 2,3-dihydrazone cellulose shows 5-19% of inhibition of *E.coli*. It has indeed been reported that Schiff base made by reaction of dialdehyde cellulose with chitosan shows antimicrobial properties (Svjetlana et al., 2009). Similarly it is also known that several other hydrazones derivatives show antimicrobial properties (Sevim Rollas and Guniz Kucukguzel, 2007). Therefore, it can be concluded that the 2, 3-dihydrazone cellulose is a polymer showing antimicrobial properties, and it may be possible to develop applications for this polymer in antimicrobial polymer coatings.

6.5. Morphological studies of cellulose, 2-3-dialdehyde cellulose and its derivatives:

To further explore various effects of oxidation and derivatization of oxidised cellulose all these derivatives were subjected to scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) analysis. Figure 6.2 shows the SEM images of the starting cellulose polymer. The SEM image clearly shows elongated shape of cellulose fibers; the SEM image of individual fiber (at higher magnifications, 6.2b) shows that the surface of cellulose to be smooth in appearance.

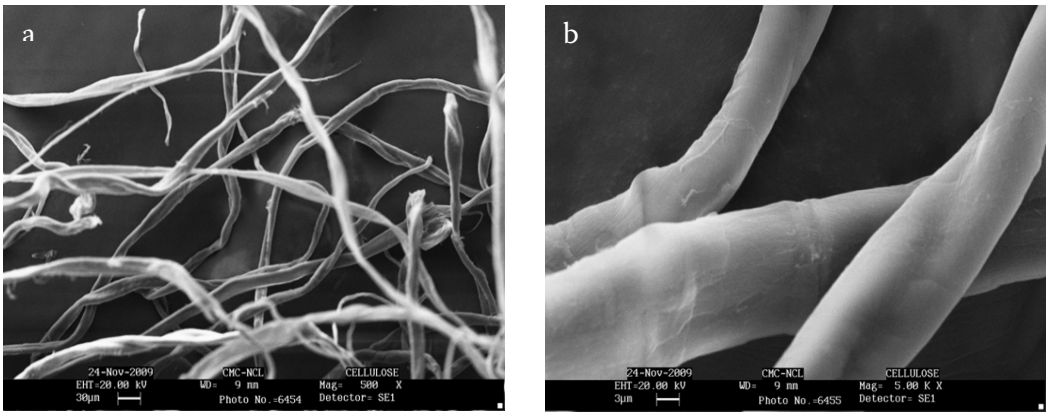


Figure 6.2: SEM images of cellulose. The cellulose appears as long fibers with very smooth surface before oxidation

Figure 6.3 shows the SEM images of oxidised cellulose i.e., 2, 3-dialdehyde cellulose-50 (50% oxidation level) which was made from oxidation of cotton cellulose. It is seen that the fibres form of cellulose is intact but the length of fiber are reduced due to breaking of fibers by oxidation while some fibers remains unreacted having close resemblance to the cellulose fibers. Individual fiber shows roughness on the surface of oxidiesd cellulose (seen at higher magnification, figure 6.3b).

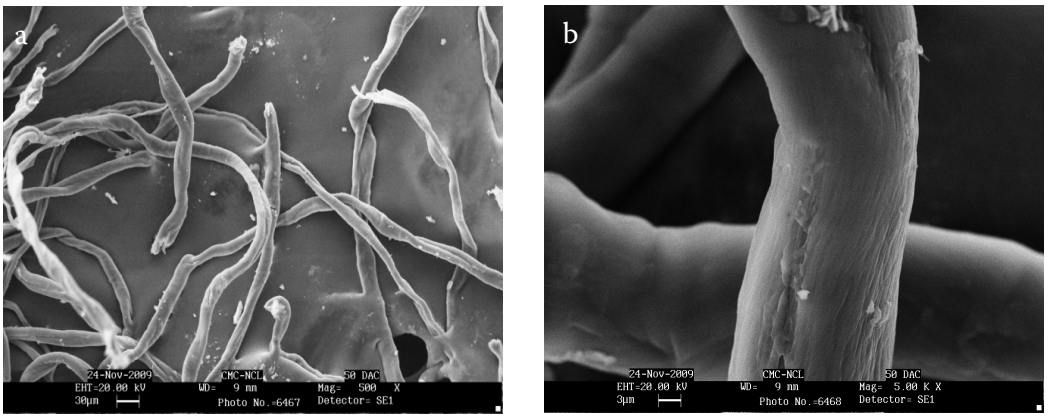


Figure 6.3: SEM images of 2, 3-dialdehyde cellulose. Cellulose fibers break down and roughness of surface observed upon oxidation.

When 2, 3-dialdehyde cellulose was converted to sodium 2, 3-dicarboxy cellulose (NaDCC-50), greater clustering of individual fibers is observed in NaDCC (Figure 6.4.). Reduction of particle size was also supported by confocal laser analysis and can be clearly seen in CLSM images of this derivative (figure 6.5), whereas discrete fibers are seen in case of DCC-50 (figure 6.6) as well as fall in particle size was observed and shown in CLSM image (Figure 6.7).

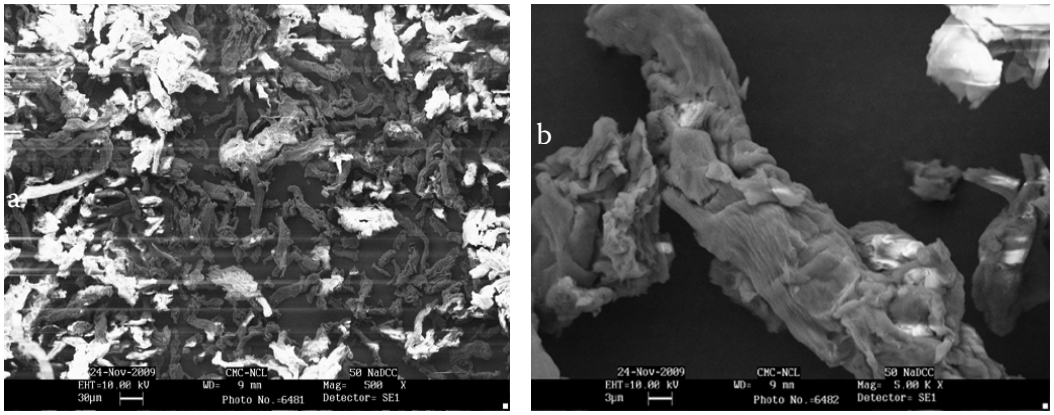


Figure 6.4: SEM images of sodium 2, 3-dialdehyde cellulose (NaDCC-50). This derivative shows clustering of cellulose fibers and more cracking on surface.

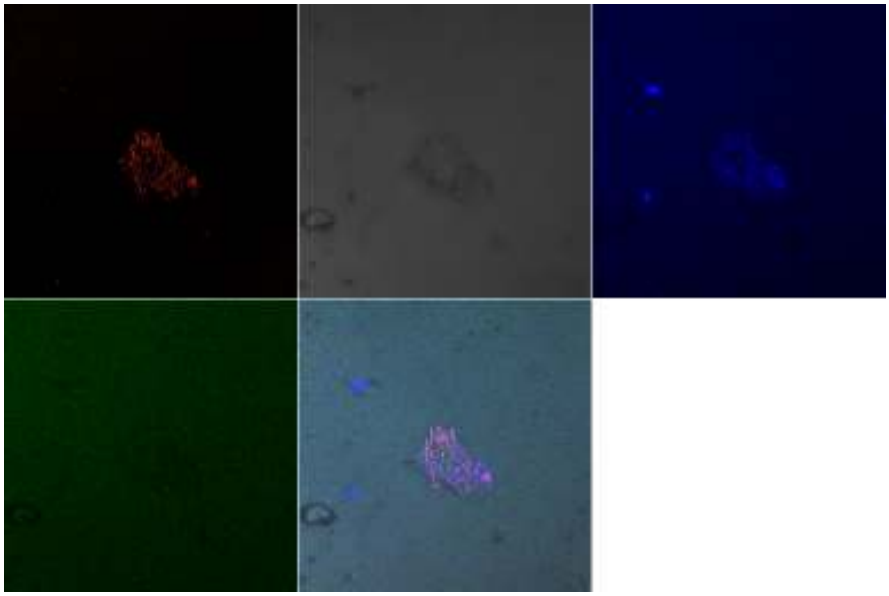


Figure 6.5: CLSM image of sodium 2, 3-dialdehyde cellulose (NaDCC-50) showing small particles of NaDCC.

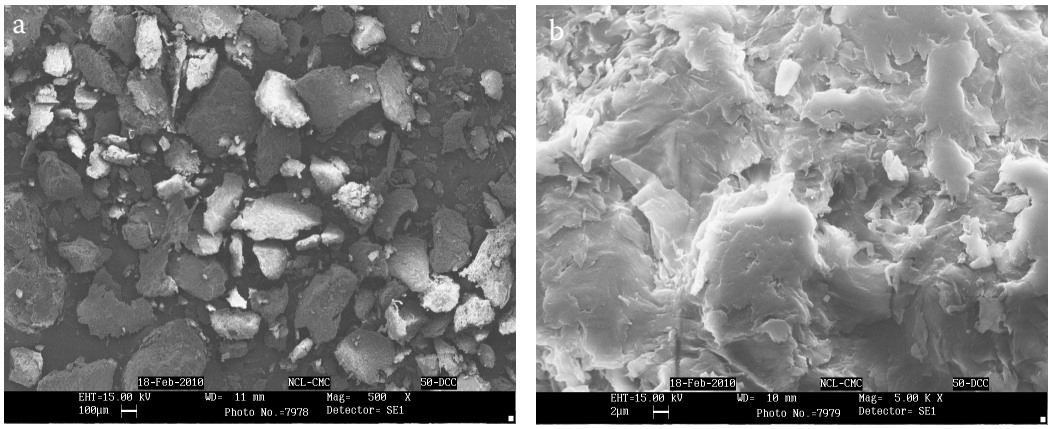


Figure 6.6: SEM images of 2, 3-dicarboxy cellulose (DCC-50)
This derivative show discrete fibers with large roughness on the surface

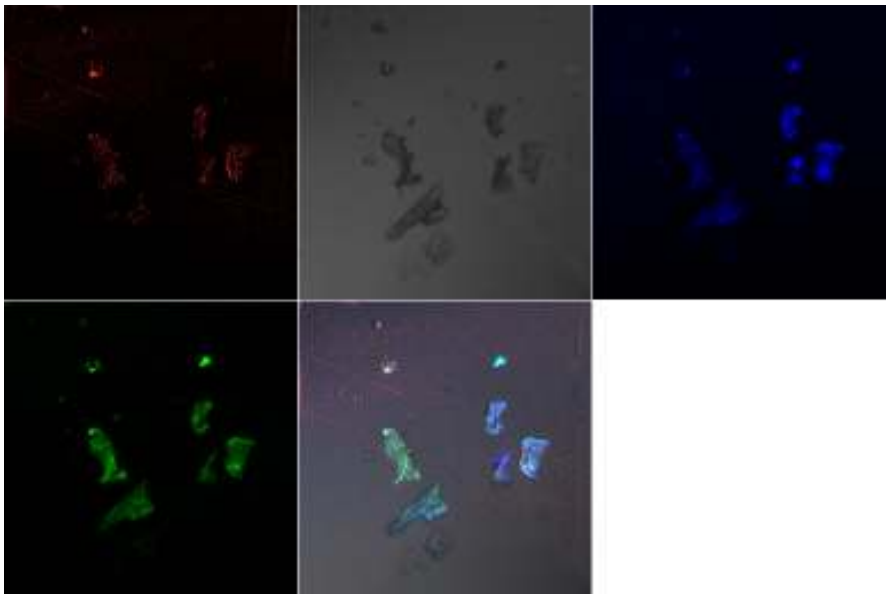


Figure 6.7: CLSM image of sodium 2, 3-dialdehyde cellulose (DCC-50) showing small crystalline particles of DCC.

On the other hand, oxime and hydrazone derivatives i.e., 2, 3-dioxime cellulose-50 and 2, 3-dihydrazone-50 cellulose shows cellulose fibers having images similar to 2.3-dialdehyde cellulose-50 (DAC-50) and can be seen in SEM images (Figure 6.8 &

6.10). However, the CLSM images (Figure 6.9 & 6.11) show that individual molecules have been fractured which have lower D.P. than the parent molecules.

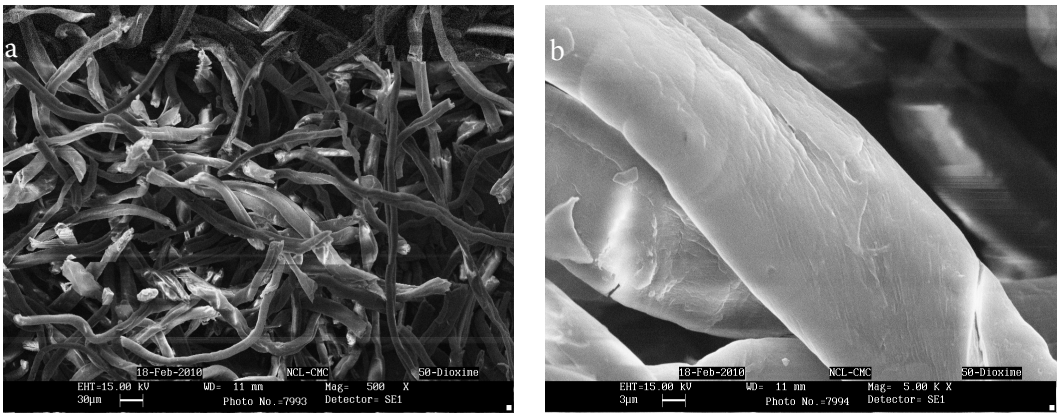


Figure 6.8: SEM images of 2, 3-dioxime cellulose. This derivative shows clustering of cellulosic fibers.

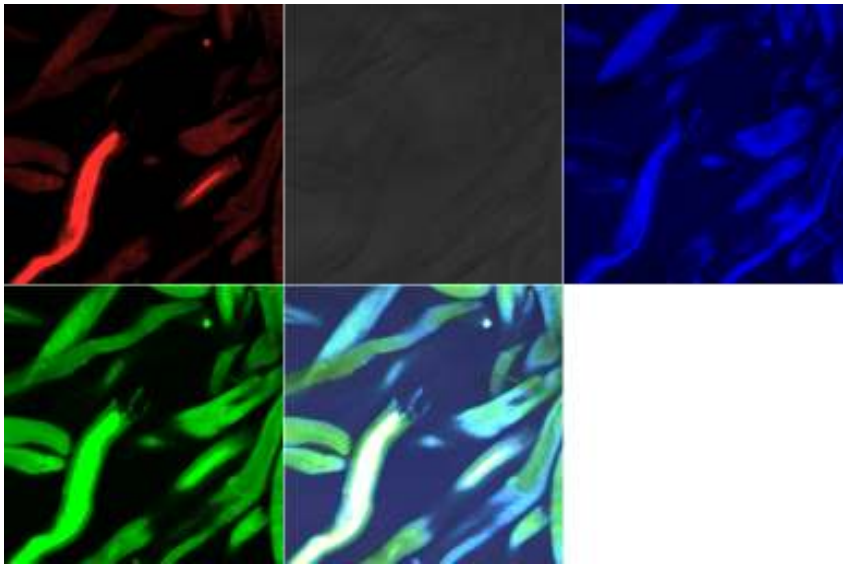


Figure 6.9: CLSM image of 2, 3-dioxime cellulose showing breaking of cellulose fibers

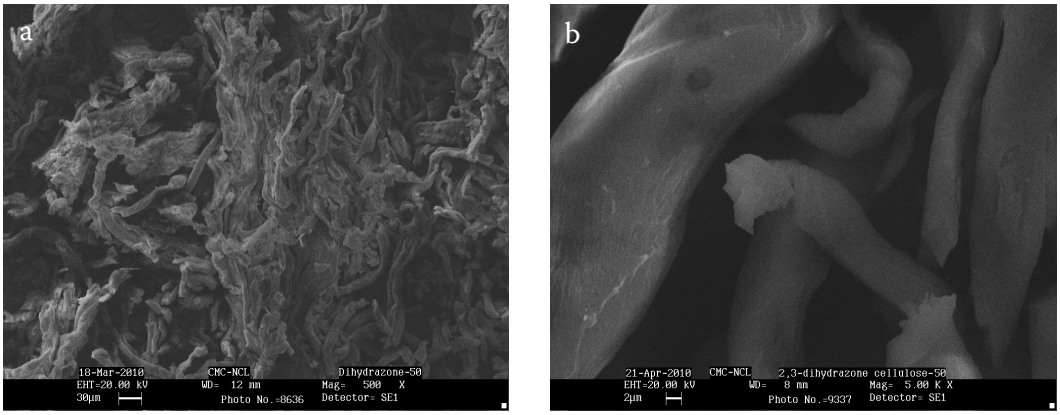


Figure 6.10: SEM images of 2, 3-dihydrazone cellulose. This derivative shows greater clustering of cellulose fibers

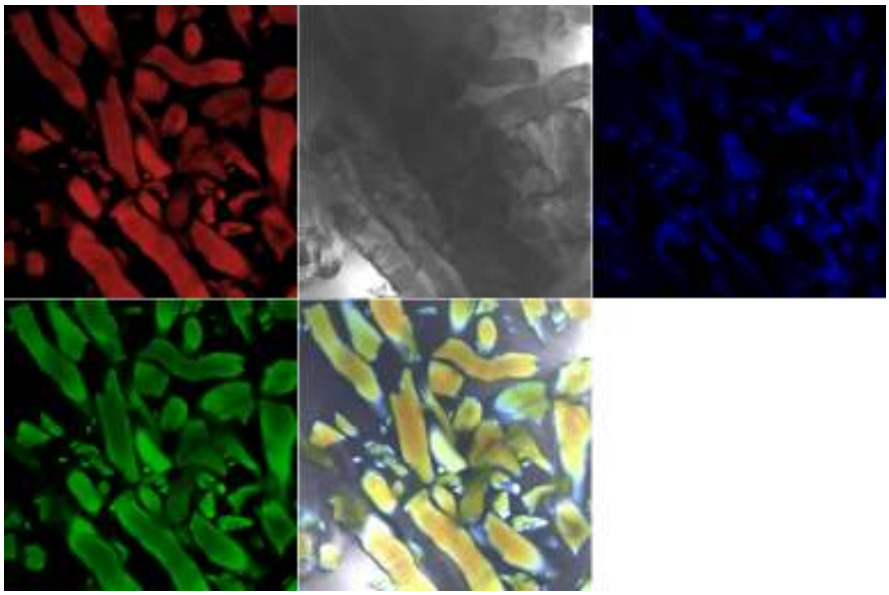


Figure 6.11: CLSM image of 2, 3-dihydrazone cellulose showing extensive breaking of fibers

For other derivatives such as 2,3-diethylimine cellulose, 2,3-dibutylimine cellulose and 2,3-dibenzylimine cellulose, figure 6.12, 6.14 and 6.15 the SEM images show discrete fibers similar to 2,3-dicarboxy cellulose (DCC), while the propylimine derivative (figure 6.13) shows clusters of fibers. These facts are also supported by CLSM analysis (***see appendix-9 of this chapter***). In general we can say that derivatization of 2, 3- dialdehyde cellulose in various reaction conditions leads to fall in fiber size and in some cases causes greater clustering of fiber. Individual fibers of all derivatives under higher magnification show much more roughness on the surface than the starting cellulose. Thus SEM helps in studying the morphology of the cellulose fiber and gives new insight into the physical form of oxidized cellulose and its derivatives.

More surprisingly it was observed that the introduction of COONa, COOH and C=N< functionality on 2, 3-dialdehyde cellulose leads to fluorescence emitting cellulosic derivatives (***see appendix-9 for CLSM images***). Except starting cellulose and 2, 3-dialdehyde cellulose all other derivatives show high fluorescence in red, green and blue wave length. More detailed study of this behavior is under investigation and it can open new applications of cellulose derivatives in biotechnology, such as biological imaging, as fluorescence emitting cellulosic nano-particles, sensor technology and fluorescence emitting biodegradable materials for biomedical applications.

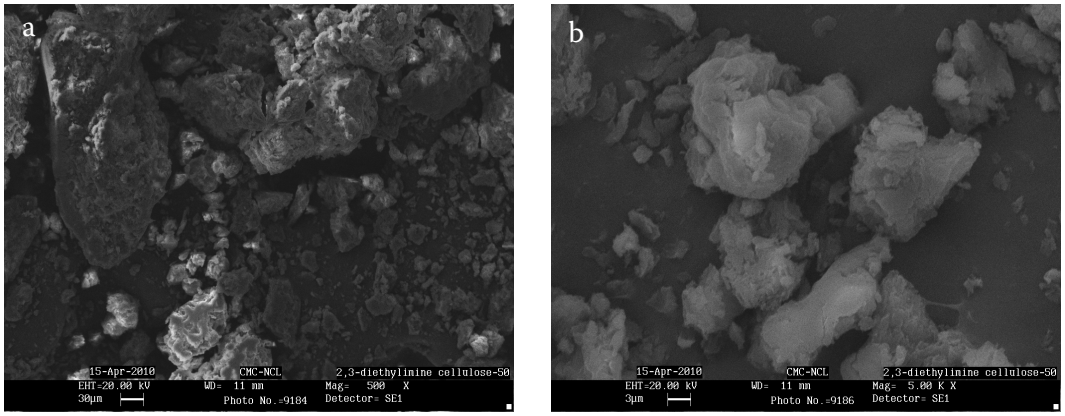


Figure 6.12: SEM images of 2, 3-diethylimine cellulose. These images shows large reduction in fiber size

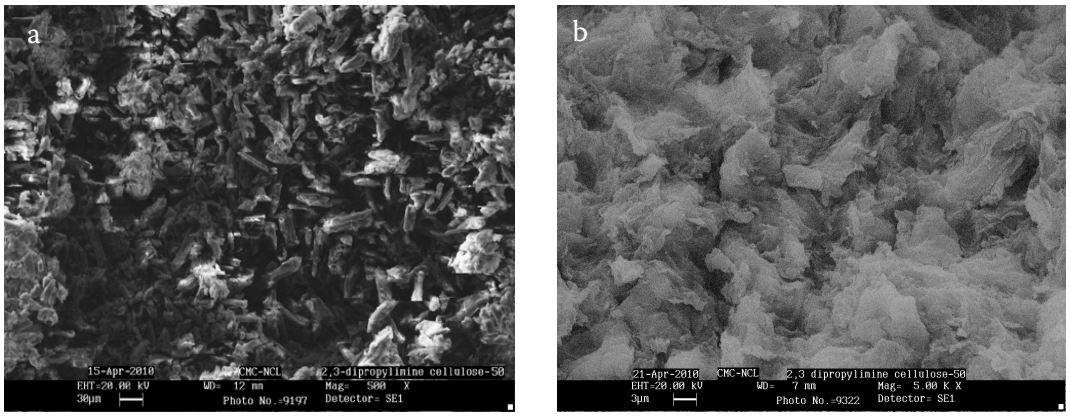


Figure 6.13: SEM images of 2, 3-dipropylimine cellulose. This derivative shows agglomeration of cellulosic fibers with large trenches on the surface

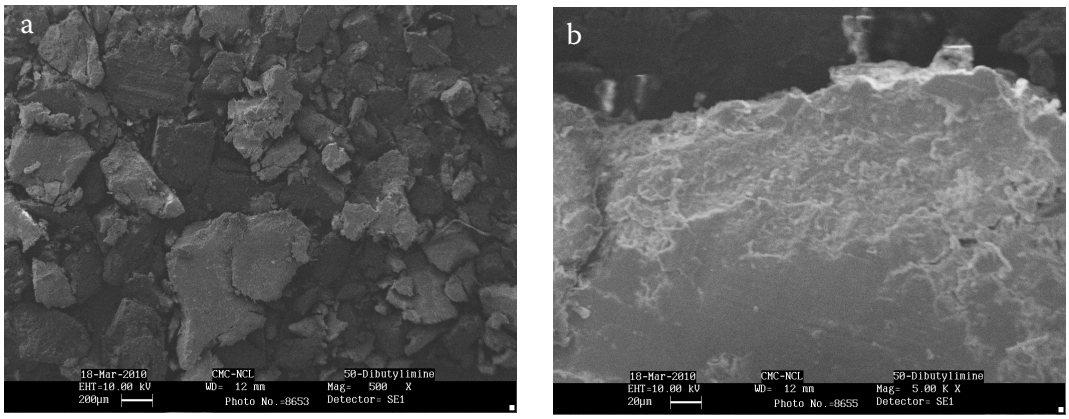


Figure 6.14: SEM images of 2, 3-dibutylimine cellulose. This derivative shows discrete fibers with large damage on the surface

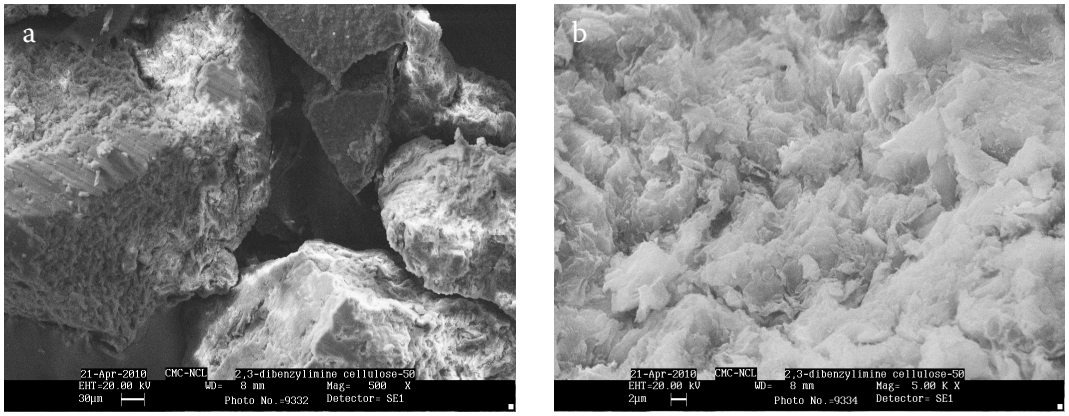


Figure 6.15: SEM images of 2, 3-dibenzylimine cellulose. This derivative shows discrete fibers with large trenches on the surface

6.6. References

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6.7. Appendix-8: Enzymatic hydrolysis profile of cellulose, 2, 3-dialdehyde cellulose and its derivatives

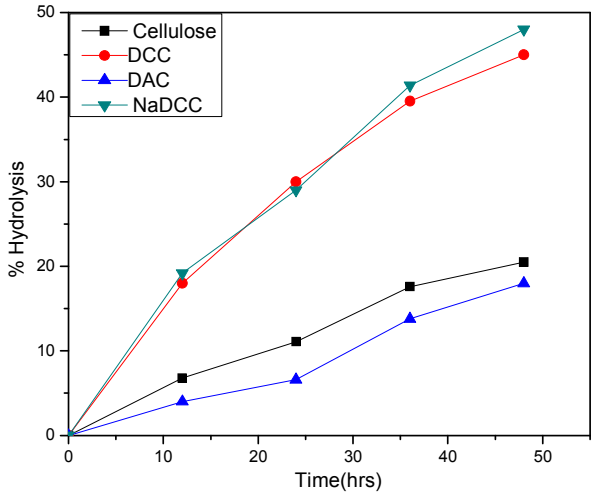

Figure: 6.16: Enzymatic hydrolysis profile of cellulose (cotton linters), 2, 3-dialdehyde cellulose (DAC-15), Sodium 2, 3-dicarboxy cellulose (NaDCC-15) and 2, 3-dicarboxy cellulose (DCC-15).

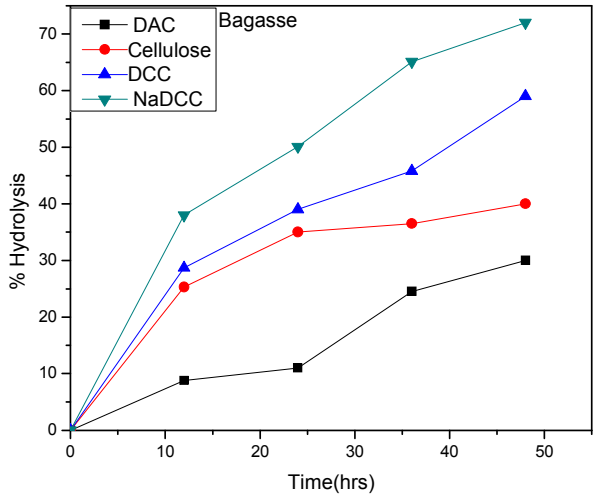

Figure 6.17: Enzymatic hydrolysis profile of cellulose (bagasse), 2, 3-dialdehyde cellulose (DAC-15), Sodium 2, 3-dicarboxy cellulose (NaDCC-15) and 2, 3-dicarboxy cellulose (DCC-15).

6.8. Appendix-9

Confocal Laser Scanning Microscope (CLSM) images of cellulose, 2, 3-dialdehyde cellulose (50% level of oxidation) and its derivatives.

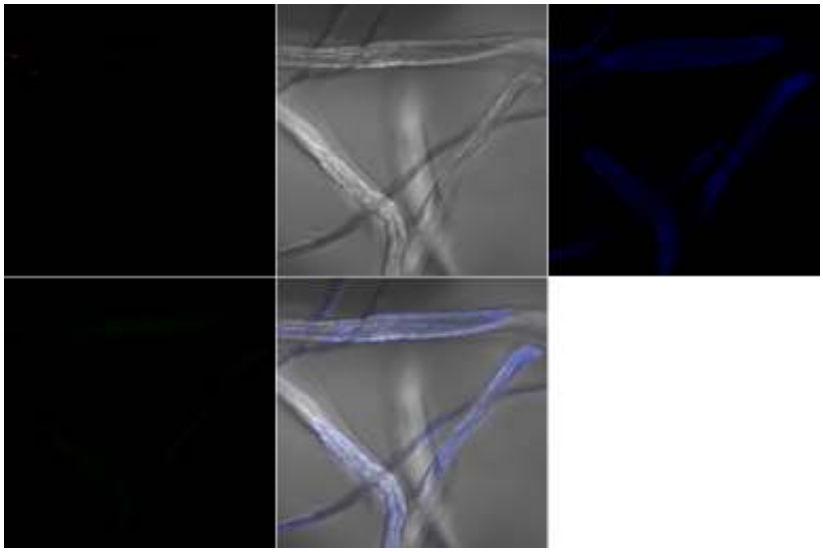

Figure 6.18: Cellulose does not show any fluorescence

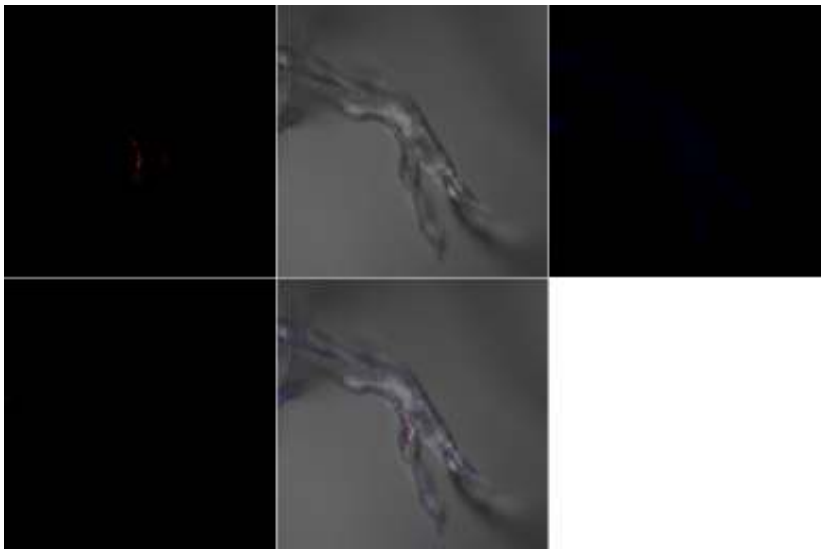

Figure 6.19: No fluorescence emission by 2, 3-dialdehyde cellulose (DAC-50)

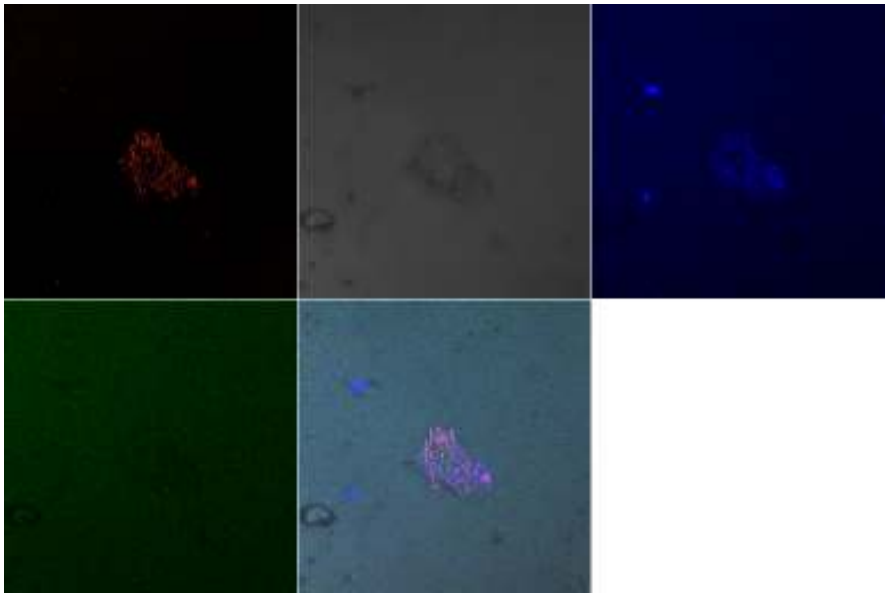

Figure 6.20: 2, 3-sodium salt of dicarboxy cellulose (NaDCC-50) shows medium fluorescence.

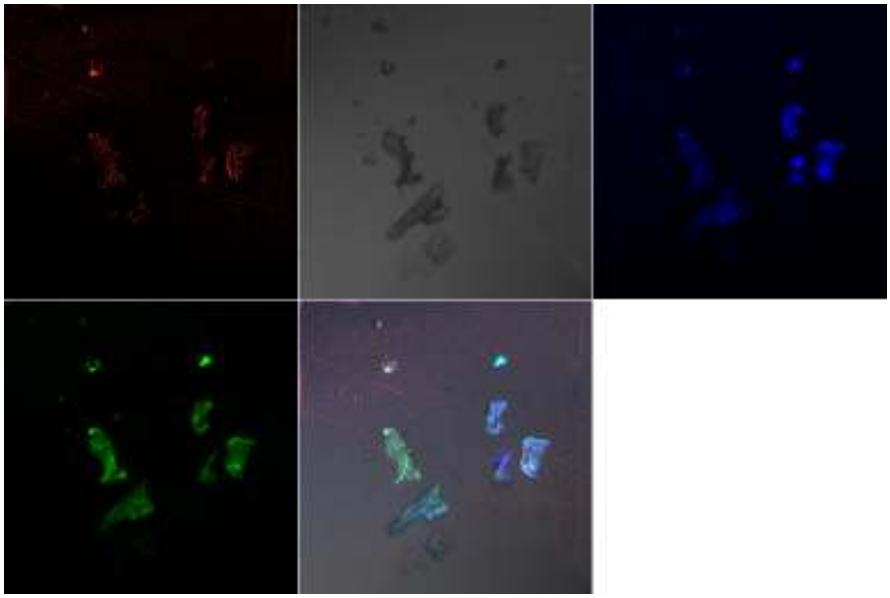

Figure 6.21: 2, 3-dicarboxy cellulose (DCC-50) shows medium fluorescence.

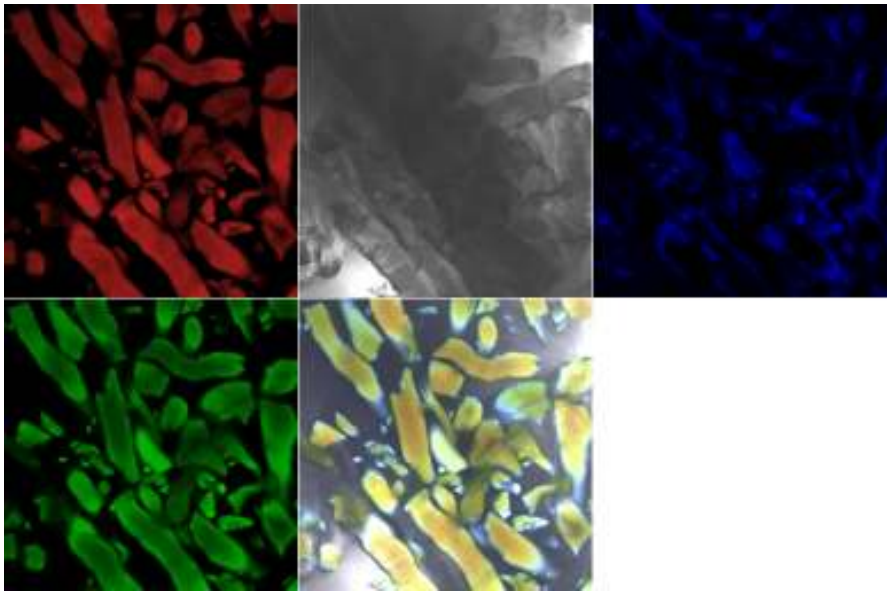

Figure 6.22: 2, 3-dihydrazone-50 cellulose shows high fluorescence.

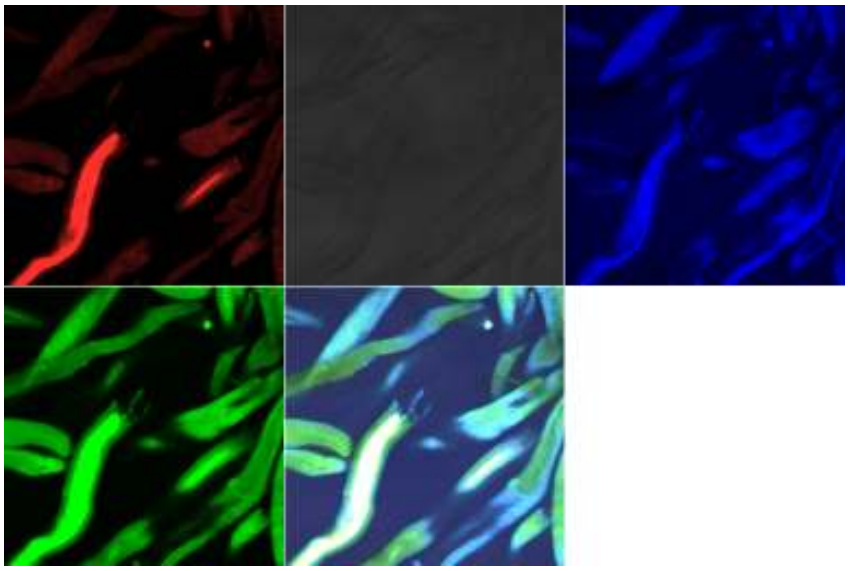

Figure 6.23: 2, 3-dioxime cellulose-50 shows high fluorescence.

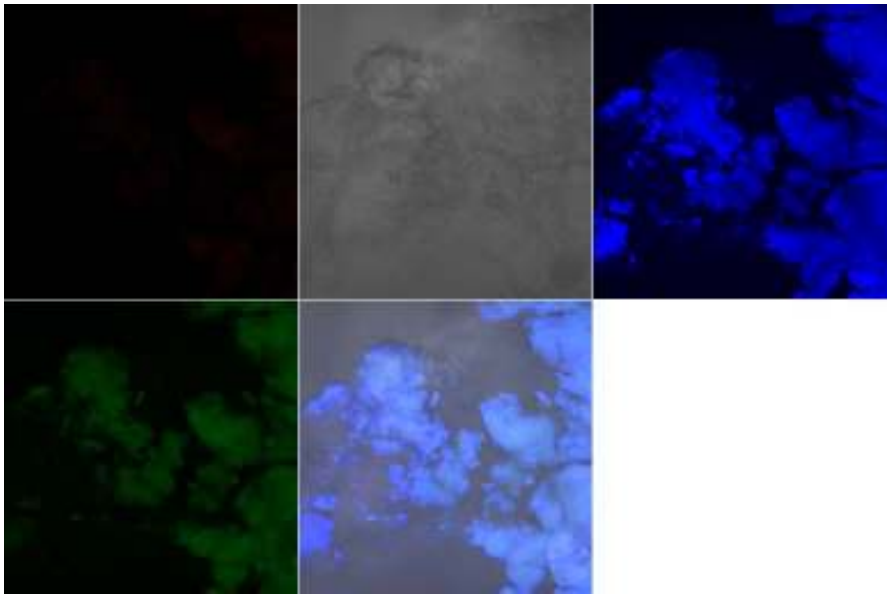

Figure 6.24: 2, 3-diethylimine-50 cellulose shows fluorescence in red, green and blue wavelength

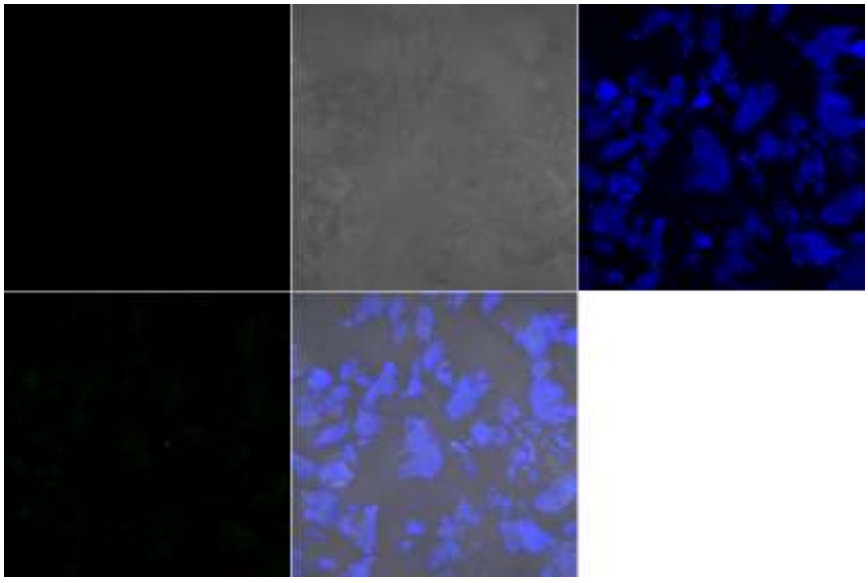

Figure 6.25: 2, 3-dipropylimine cellulose-50 shows fluorescence in red, green and blue wavelength

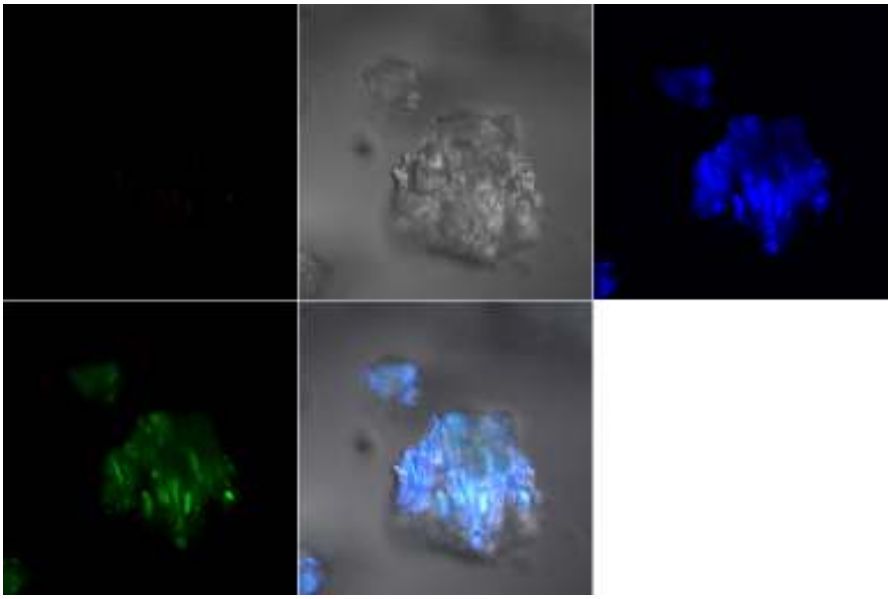

Figure 6.26: 2, 3-dibutylimine cellulose-50 shows fluorescence in red, green and blue wavelength

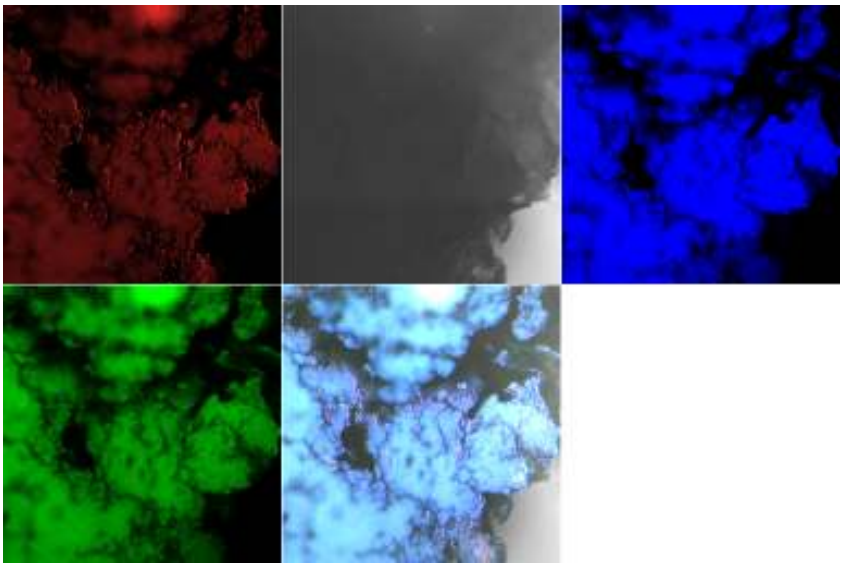

Figure 6.27: 2, 3-dibenzylimine cellulose-50 shows fluorescence in red, green and blue wavelength

Chapter 7

Conclusion and suggestion for future work

The work presented in this thesis is focused on the study of chemistry of fractionation of plant biomass into its polymeric components using the concept of "biorefinery" to develop "second generation" biobased materials utilizing agricultural residues as sources for industrially useful polymers like cellulose. In the present thesis sugarcane bagasse was chosen as the biomass material from which the polymeric components cellulose, hemicelluloses and lignin were isolated in a step-wise manner, and then used as organic raw feedstock to produce new polymers with new properties. The overall conclusions from the present work are summarized below.

A detailed literature search was done on the different pretreatment methods employed for the fractionation of lignocellulosic biomass in to its chemical constituent polymers. The advantages and the disadvantages associated with each method have been described. The potential industrial applications of these lignocellulosic polymers have also been listed. It is clearly seen that obtaining pure cellulose from agricultural biomass as a key raw material to prepare a series of new polymers is a prime objective of several research laboratories around the world. Therefore, the main objective of this work was to isolate pure grade of cellulose from agricultural residue and utilize them for synthesis of new series of polymers based on oxidation reactions and Schiff base reaction and their potential for some application was demonstrated. In a related aspect, pretreated lignocellulose was used in the preparation of cellulase and xylanase enzymes of industrial importance.

The work presented demonstrates that high alpha cellulose could be obtained from waste bagasse. The fractionated cellulose contained ~ 94 % cellulose. It is also shown

that this cellulose contains about 5% residual hemicellulose. This cellulose was acetylated under heterogeneous conditions to obtain cellulose acetates. The novel feature of this study was the utilization of the hemicellulose content (5%) of bagasse cellulose as an internal plasticizer. Through kinetic experimentation, it was demonstrated that the residual hemicellulose need not be considered as an impurity; rather it can be used in acetylated form as a plasticizer as well as a biodegradable additive for cellulose acetates made from cellulose produced from sugarcane bagasse. These cellulose acetates were extensively characterized using FTIR, TGA, DSC, GPC, HPIC, WAXRD, and viscometry. Our results therefore show lignocellulosic agricultural wastes can be utilized to produce high value polymers.

In order to show the usefulness of pretreatment methodologies to obtain different grades of cellulose for applications in enzyme production, the bagasse was chemically treated to generate different bagasse samples with varying quantities of lignin and hemicelluoses, keeping the cellulose fraction intact in all cases. It is concluded that, bagasse treated with $NaClO_2$ generates bagasse sample having kappa number 22.9 (lignin content 3.4% w/w) is an excellent source of carbon for cellulase production by *Penicillium janthinellum* NCIM 1171 and *Trichoderma viride* NCIM 1051. Higher xylanase and β-glucosidase activities were observed in the medium with bagasse samples as compared to the values obtained with pure cellulose powder 123 (CP-123). Amongst all bagasse samples, sample having kappa number 22.9 gave the highest yields of xylanase (130 IU/ml) and β-glucosidase activities (2.3 IU/ml) in case of *Penicillium janthinellum.*

Similarly, the same pretreated bagasse polysaccharides were explored for hydrolysis of the polymeric components cellulose and hemicellulose into simple sugars like glucose, xylose and arabinose by using same cellulase and xylanase enzymes produced earlier (using pretreated bagasse as a carbon source with *Penicillium janthinellum* NCIM 1171 and *Trichoderma viride* NCIM 1051). It was found that while the maximum hydrolysis of Avicel (pure cellulose) was 70%, some of the bagasse polysaccharides were as high as 95%. The products of hydrolysis were glucose, xylose, and arabinose, as confirmed by High Pressure Ion Chromatography (HPIC). It is interesting to note that arabinose, which constitutes about 10% of the weight of bagasse xylan, could also be hydrolyzed easily by the enzymes. Also, the initial rates of hydrolysis was found to be much higher for the bagasse polysaccharides, and in some cases about 90% of the hydrolysis occurred within 20 hours. Amongst all bagasse samples, the sample with kappa number 1.2 (lignin content 0.18% w/w) gave the highest degree of hydrolysis at 50 ^{0}C. Even the bagasse polysaccharide with kappa number 16.8 (lignin content 2.5% w/w) underwent greater extent of hydrolysis than Avicel. Apparently, the partially delignified bagasse medium appears to be a facile medium for the combined hydrolytic action of the cellulase and xylanase enzymes. These sugars are valuable platform chemicals to produce a variety of fuels (ethanol, butanol) and other chemicals which so far are produced only from petroleum sources.

Pure cellulose has application in synthesis of new polymers. Synthesis of partially oxidized 2,3-dialdehyde cellulose (DAC) from highly pure cellulose were carried out to develop a series of dialdehyde cellulose having different extents of aldehyde groups (different levels of oxidation) on the same cellulose chain. This dialdehyde cellulose

further modified to carboxy, carboxylate and Schiff's bases (2, 3-dioxime cellulose, 2, 3-dihydrazone cellulose, 2, 3-diethylimine cellulose 2,3-dipropylimine cellulose, 2,3-dibutylimine cellulose and 2,3-dibenzylimine cellulose). Thus, we were successful in synthesizing a complete series of oxidized celluloses and further using these oxidised celluloses to prepare a series of dicarboxy cellulose and Schiff bases (2, 3-dioxime cellulose, 2, 3-dihydrazone cellulose, 2,3-diethylimine cellulose 2,3-dipropylimine cellulose, 2,3-dibutylimine cellulose and 2,3-dibenzylimine cellulose). These derivatives were exhaustively characterized by FTIR, ^{13}C CP-MAS NMR spectroscopy, thermal analysis and wide angle x-ray diffraction and their structure-property relationships were established. This has made available sets of new functionalized polymers that can be investigate for new applications.

Finally it is shown here that all new polymers synthesized (series of oxidised celluloses and their Schiff bases (2, 3-dioxime cellulose, 2, 3-dihydrazone cellulose, 2, 3-diethylimine cellulose 2, 3-dipropylimine cellulose, 2, 3-dibutylimine cellulose and 2, 3-dibenzylimine cellulose) can be hydrolysed by enzymes to produce sugar as well as other chemicals like open chain Schiff bases, etc. Similarly slight modification of cellulose by various chemical functionalities shows fluctuation in hydrolysis behaviors. It was observed that dialdehyde cellulose hydrolyzes at a slower rate than native cellulose, 2, 3-dihydrazone cellulose derivatives dose not hydrolyses and shows antimicrobial property and sodium salt of dicarboxy cellulose (50% oxidation), dicarboxy cellulose (50% oxidation) and Schiff bases of higher level of oxidation(50% oxidation) hydrolyses faster than native cellulose.

Morphologies of oxidized cellulose and other oxidized cellulosic derivatives show that cellulose fibrils break down upon oxidation and subsequent clustering occurs by further derivatization. Thus scanning electron microscopy provided the complete morphological picture of dialdehyde cellulose and its derivatives. More interestingly it was observed that the introduction of COONa, COOH and C=N functionality on dialdehyde cellulose leads to fluorescence emitting polymers. Modified cellulosic derivatives showed high fluorescence at red, green and blue wave length; this can open new applications of cellulosic derivatives in biotechnology as biological imaging, nanotechnology as fluorescence emitting nano particles, sensor technology and fluorescence emitting biodegradable materials for biomedical applications.

Suggestions for future work

Extraction of high alpha-cellulose and other polymeric components should be tried out from other lignocellulosic waste biomass such as jute, wheat, rice and cereal straws, etc to understand the effects of specific constituents present in each biomass such as pectins, composition of hemicellulose, silica etc.

In our study, we acetylated the cellulose in heterogeneous condition to obtained high degree of substitution. It would be interesting to study acylation of bagasse cellulose in homogeneous condition such as in ionic liquids, to better control on degree of substitution.

To understand better the oxidation of cellulose and their chemical structures, model compounds such as cellobiose, cellotriose, and higher oligomers is highly desirable.

Oxidative modification in homogeneous media such as ionic liquids can give better insight into the structure of oxidized cellulose and its derivatives.

Study on nano particle of cellulose (synthesis and properties) will yield great insights into chemical and biochemical properties of the molecule.

It would be interesting to study the antimicrobial properties of all oxidized cellulosic derivatives.

Soluble derivatives should be synthesized from dialdehyde cellulose and fluorescent emitting properties should be checked.

Publications:

1. Utilization of sugarcane bagasse cellulose for producing cellulose acetate: Novel use residual hemicellulose as a plasticizer

   H. Shaikh. K. V. Pandhare., G. Nair. A. J. Varma.

   Carbohydrate Polymers, 76, (1), 23-29, 2009.

   (*Most accessed article, placed at 10 th position among top 25).*

2. Enzymatic hydrolysis of delignified bagasse polysaccharides
   M. G. Adsul, J. E. Ghule, R. Singh, **H.Shaikh**, K. B. Bastawde, D. V. Gokhale and A.J.Varma**.**
   Carbohydrate Polymers, 62, (1), 6-10, 2005.
   (Most accessed article, placed at 13 th position among top 25).

3. Polysaccharides from bagasse: Applications in cellulase and xylanase production
   M. G Adsul, J. E. Ghule, R. Singh, **H. Shaikh**, K. B. Bastawde, D. V. Gokhale., and A. J. Varma.
   Carbohydrate Polymers, 57, (1), 67-72, 2004.
   (*Most accessed article, placed at 5 th position among top 25).*

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