"ENZYMATIC HYDROLYSIS OF CELLULOSIC WASTE"

A Major Project Report

Submitted In Partial Fulfillment of the Requirements

For The Degree Of

MASTER OF TECHNOLOGY

IN

CHEMICAL ENGINEERING

(ENVIRONMENTAL PROCESS DESIGN)

BY

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Certificate

This is to certify that the Major Project entitled *"**Enzymatic Hydrolysis of Cellulosic Waste"*** submitted by Biraju Sanghavi (05MCH011), towards the partial fulfillment of the requirements for the award of Degree of Master of Technology in Chemical Engineering (Environmental Process Design) of Nirma University of Science and Technology is the record of work carried out by her under my supervision and guidance. The work submitted has in my opinion reached a level required for being accepted for examination. The results embodied in this major project work to the best of my knowledge have not been submitted to any other University or Institution for award of any degree or diploma.

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Abstract

In order to produce the Bioethanol from agricultural residues such as Wheat straw, Rice straw and Jawar straw, it is necessary to decompose the straw into soluble sugars. Enzymatic hydrolysis is one of the method in common use, while pretreatment is the effective way to increase the hydrolysis rate. The optimal conditions of pretreatment using Boiling water, Acid and Alkali enzymatic hydrolysis of straws were determined. The evaluation of boiling water, alkali and acid treatment was done with concentration effect and time effect on lignin removal. The enzymatic hydrolysis of evaluated pretreated wheat straw, Rice straw and Jawar straw by Trichoderma Reesei enzyme system was studied. At favorable conditions, temperature 50ºC, pH 4 and 30 RPM at which enzyme gives maximum activity and rate of hydrolysis was proved. The pretreatment effect on enzymatic hydrolysis is studied. The kinetics of enzymatic hydrolysis was determined and maximum rate was obtained. Study proves that there is sugar inhibit the reaction and rate of hydrolysis. The results of SEM, XRD analysis showed that the structure and the surface of the straw were changed and crystallinity decreased, through pretreatment that is in favour of the following enzymatic hydrolysis.

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Nomenclature

AFEX	Ammonia Fiber Explosion
CBH1-2	cellobiohydrolases
EG1–5	five endoglucanases
EC 3.2.1.21	ß-Glucosidase
EC 3.2.1.4	Exo-ß-Glucanase
EC 3.2.1.4	Endo-ß-Glucanase
ICEVs	internal combustion engine vehicles),
K_m	M. M constant mg/mL
LHW	Liquid Hot Water
r	Rate of hydrolysis mg/mL.hr
[S]	Substrate Concentration mg/mL
SEM	Scanning Electron Microscope
Vmax	Maximum rate mg/mL
XRD	X- Ray Diffractometer

Chapter No. 1

Introduction
Of
Enzymatic Hydrolysis

Chapter No. 1

Introduction

1.1 Introduction

The search for sustainable, renewable transportation fuels has recently received increased attention as a result of both the rapid rise in petroleum prices and increasing international concerns regarding greenhouse gas emissions. Additionally, crude oil remains the most economical base product for transportation fuel production. However, in the long term rising fuel prices and increasing environmental concerns, along with the depletion in crude oil reserves will see the search for alternative transportation fuels necessary.

Some alternative energy sources which have been the subject of extensive research include the utilisation of solar energy, the hydrogen fuel cell and the production of ethanol from biomass. Currently Brazil and the USA produce large amounts of ethanol from sugar cane and corn respectively. In both countries the surplus of a readily fermented food product and a slump in its sale price contributed to the development of a bioethanol fuel program. Additionally the independence from oil imports was and still is viewed as a major benefit of bio-ethanol production. As the world searches for fuel alternatives to gasoline, a clear candidate to lead the way has emerged, Ethanol. Ethanol production for use as a fuel additive (or to be used directly as a fuel source) has grown in popularity due to governmental regulations and in some cases economic incentives. Both regulations and incentives seem to be based on environmental concerns as well as a desire to reduce the oil dependency of our industries and our everyday lives.

To sustainably produce ethanol in sufficient volumes to meet long term transportation needs the ethanol would probably have to be fermented from the sugars in lignocellulosic materials such as trees, paper waste (municipal solid waste) and crop

residues. In this process the three fractions in lignocellulose, the cellulose, hemicellulose and lignin are separated by pretreatment and hydrolysis operations. The sugars (present in the cellulose and hemicellulose fractions) can then be fermented to produce bioethanol by chemical method or enzymatically.

Of all Biofuels, ethanol is already produced on a fair scale about 14–26 Metric tonnes worldwide, and is easily applicable in present day internal combustion engine vehicles (ICEVs), as mixing with gasoline is possible. Ethanol is already commonly used in a10% ethanol/90% gasoline blend. Adapted ICEVs can use a blend of 85% ethanol/15% gasoline or even 95% ethanol (E95)

In a recent study, Scientists have pointed out three technologies having the potential of reducing net emissions by motor vehicles: batteries, fuel cells, and ethanol. Each of these technologies has their own advantages and drawbacks and none is in reality a "perfect put-in substitute" for the present worldwide fossil fuel model

1.2 Scope

1.2.1 Worldwide Bioethanol Production Scenario

Today, bioethanol can be produced using sugarcane in countries like Brazil, corn in the USA and China, and beets in the EU and China

The USA has been using ethanol as fuel since at least 1908. Production has increased enormously since the oil supply disruptions in 1980 decade. Ethanol production grew from 665 million liters in 1980 to 7 billion liters in 2000.

Annually, Brazil produces about 13 billion liters of ethanol at the lowest cost in the world (US$200/m3). It is possible to reduce costs even more, by about 13 percent in the next six years.

China is increasing ethanol share in gasoline to the 10 percent level. Current annual ethanol production of 2.7 billion liters should reach 4 billion liters as a consequence. Most of its ethanol is produced from sugar crops, corn and sugar beet.

In 2000-01 Australian gasoline consumption rose through 18,000 Million liters per annum (Mlpa) and reached 18,873 Mlpa for 2002-03. If the growth rate averaged 2% pa, the demand would have reached 20,000 Mlpa by 2006. This growth could be provided by blending 10% ethanol into gasoline rather than importing more gasoline. Under such a scenario the market potential for bioethanol blended into gasoline is in the order of 2,000 Mlpa.

France is producing ethanol from plants containing sucrose, beet for instance, or starch, such as cereals and potatoes. The resulting ethanol can be added to fuels in a pure form or as ETBE, a chemical composition obtained from the reaction of ethanol (45 percent) and isobutylene (55 percent).

The Swedish Government has spent over US$4 million in the last three years to demonstrate the advantages of renewable fuels. By 2010, Sweden will have 15 percent of its transportation fleet using fuels produced from biomass. The Swedish Foundation for the Development of Ethanol – founded over 15 years ago – is working on different projects for producing ethanol from residues originated in the paper and forestry industries. Spain, a major ethanol producer in Europe, is increasing its participation by buying new ethanol plants in the USA.

Japan is also developing an important program to add ethanol to diesel. It has formed a group denominated Global Alliance with countries such as Canada, Australia, Thailand and Guatemala, having as objective the trade of fuel ethanol

Other countries, such as Argentina, Colombia, and Mexico, among others, are introducing smaller scale fuel ethanol programs.

In India Ethanol is produced less than one million tonnes per year but its potential is up to 10 million tonnes per year from 1.2 million hectares of intensive cultivation with required inputs.

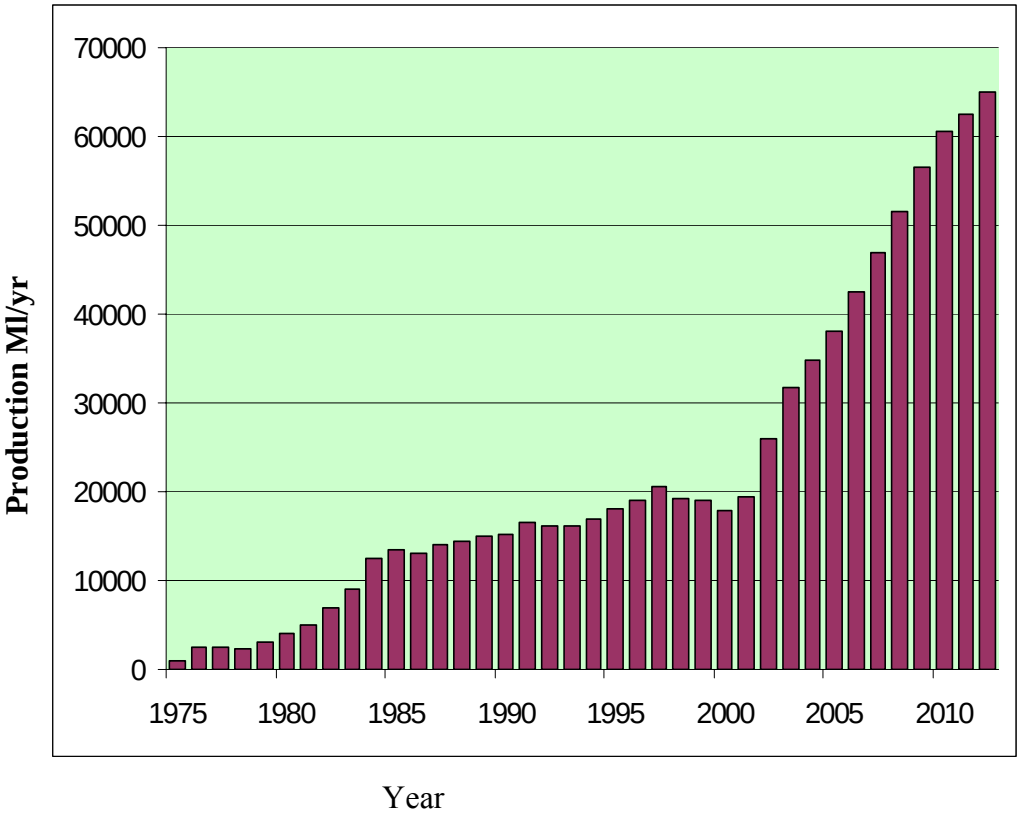

Figure 1.1: World Ethanol Production over the years [12]]

Table 1.1 : Worldwide Bioethanol production potential from wasted crops [10]

Eedstock (FromWaste Crops) Giga Liters	Africa	Asia	Europe	North America	Central America	Oceania	South America	Subtotal
Corn	2.17	6.82	1.09	0.21	1.21	0.01	2.87	14.4
Barley	0.12	0.83	1.35	0.005	0.001	0.13	0.03	2.46
Oat	0.002	0.004	0.30	0.01	0.0004	0.001	0.03	0.38
Rice	0.71	14.4	0.02	0.63	0.05	0.02	0.93	16.8
Wheat	0.55	6.78	2.70	0.02	0.16	0.54	0.60	11.3
Sorghum	1.55	0.37	0.003	-	0.09	0.0004	0.12	2.14
Sugar cane	0.23	0.82	-	-	0.18	0.0001	0.37	1.59
Jawar	-	-	-	-	-	-	-	-
Subtotal	5.33	30.1	5.45	0.87	1.70	0.70	4.95	49.1

Table 1.2: worldwide bioethanol production potential from Lignocellulosic Biomass [10]

Feedstock (From Lignocellulosic Biomass) Giga Liters	Africa	Asia	Europe	North America	Central America	Oceania	South America	Subtotal
Corn stover	-	9.75	8.23	38.4	-	0.07	2.07	58.6
Barley straw	-	0.61	13.7	3.06	0.05	0.60	0.09	18.1
Oat straw	-	0.07	1.79	0.73	0.009	0.12	0.06	2.78
Rice straw	5.86	186.8	1.10	3.06	0.77	0.47	6.58	204.6
Wheat straw	1.57	42.6	38.9	14.7	0.82	2.51	2.87	103.8
Sorghum straw	-	-	0.10	1.89	0.31	0.09	0.41	2.79
Bagasse	3.33	21.3	0.004	1.31	5.46	1.84	18.1	51.3
Jawar straw	-	-	-	-	-	-	-	-
Subtotal	10.8	261.0	63.8	63.2	7.42	5.70	30.2	442.0

Table 1.3: worldwide generation of wasted crops [10]

Wasted Crop Trillion gallons	Africa	Asia	Europe	North America	Central America	Oceania	South America	Subtotal
Corn	3.12	9.82	1.57	0.30	1.74	0.01	4.13	20.70
Barley	0.17	1.23	0.01	0.01	0.01	0.19	0.04	3.66
Oat	0.004	0.06	0.43	0.01	0.001	0.001	0.05	0.55
Rice	1.08	21.86	0.02	0.96	0.08	0.02	1.41	25.44
Wheat	0.83	10.28	4.09	0.02	0.24	0.82	0.91	17.20
Sorghum	2.27	0.54	0.004	0.00	0.13	0.001	0.18	3.12
Sugar cane	0.46	1.64	0.00	0.00	0.36	0.00	0.74	3.20
Jawar	-	-	-	-	-	-	-	-
Subtotal	7.94	45.43	8.13	1.30	2.56	1.05	7.45	73.86

Table 1.4: worldwide generation of lignocellulosic biomass [10]

Lignocellulosic Biomass Trillion gallons	Africa	Asia	Europe	North America	Central America	Oceania	South America	Subtotal
Corn stover	0.00	33.90	28.61	133.66	0.00	0.24	7.20	203.62
Barley straw	0.00	1.97	44.24	9.85	0.16	1.93	0.29	58.45
Oat straw	0.00	0.27	6.83	2.80	0.03	0.47	0.21	10.62
Rice straw	20.93	667.59	3.92	10.95	2.77	1.68	23.51	731.34
Wheat straw	5.34	145.20	132.59	50.05	2.79	8.57	9.80	354.35
Sorghum straw	0.00	0.00	0.35	6.97	1.16	0.32	1.52	10.32
Bagasse	11.73	74.88	0.01	4.62	19.23	6.49	63.77	180.73
Jawar straw	-	-	-	-	-	-	-	-
Subtotal	38.0	923.82	216.56	218.90	26.14	19.70	106.30	1549.49

Table 1.5: Current and Future requirement of Ethanol for blending in India [5]

Year	Ethanol Requirement for blending Million Tonnes		
	@ 5 %	@ 10 %	@ 20 %
2001 – 02	0.35	0.70	1.40
2002 – 03	0.38	0.76	1.52
2003 – 04	0.41	0.82	1.64
2004 – 05	0.44	0.88	1.76
2005 – 06	0.47	0.94	1.88
2006 – 07	0.50	1.00	2.00

Table 1.6: Major Countries and their bioethanol production and usage [9]

Country	Volume (Million hectoliters)	Feedstock	Fuel type with Ethanol in % blend
Brazil	125	Sugar cane	Hydrous Alcohol (95 %) Anhydrous gasoline (20 -25 %)
U.S.A.	76	Corn (90 %), wheat, side streams food industry	E 10 or Gasohol (10 %) Reformulated gasoline (5.7 %) E 85 (85 %)
China	2.5	Grains	Unknown
Canada	2.35	Wheat, corn	E 85, E 10
Spain	2.26	Wheat, Barley, Wine	ETBE (up to 4 %)
Australia	1.3	Sugar cane, Wheat	E 10
France	1.19	Sugar beets, Wheat	ETBE (3.7 %)
Sweden	0.5	Barely	E 85, E 5 (5 %)

1.3 Objective of the Study.

The study is basically divided into two parts first is pretreatment and the second Enzymatic Hydrolysis. Here Study is carried out for three cellulosic waste materials,

(A) Wheat straw,

(B) Rice straw,

(C) Jawar straw

- To evaluate the effects of various factors like concentration of chemical, Time of treatment on various pretreatments like Boiling water, Alkali and Acid treatments for maximum delignification which enhance the rate of enzymatic. Out of which economic and effective option of treatment to select.

- The effect of pretreatment on Structure and crystallinity of Biomass materials to analyze

- To evaluate the effects of various factors like Temperature, pH, Agitation, Particle size, Substrate concentration on conversion and rate of enzymatic hydrolysis

- To evolve a kinetic model by fitting the rate data on hydrolysis for reaction and inhibition

Chapter No. 2

Literature Review

Chapter No. 2

Literature Review

2.1 Biomass Basics

2.1.1 Biomass Facts [28]

- Only 3% is used by humankind.
- Approximately 350 million tons of biomass waste is disposed each year. Total plant matter utilization in industrial products is 87.5 million tons.
- Only 6 million tons of plant matter is used to produce other than paper products, mostly by textile and coating industries

2.1.2 Biomass Direct Applications [28]

- Wood and agri fibre filled plastics
- Chemically modified wood
  Acetylation, etherification, silanation, urethane treatments
- Engineered materials
  Filters, oil absorbing, Garden, play ground surface covers
- White rot fungi cultivation

2.1.3 Biomass Structure

Lignocellulosic biomass is principally composed of the compounds cellulose, hemicellulose, and lignin. Cellulose, a primary component of most plant-cell walls, is made up of long chains of the 6-carbon sugar, glucose, arranged in bundles. In the plant-cell wall, the cellulose molecules are interlinked by another molecule, hemicellulose. The hemicellulose is primarily composed of the 5-carbon sugars and xylose. Another

compound called lignin is also present in significant amounts and gives the plant its structural strength. A typical plant cell wall is illustrated in Figure 2

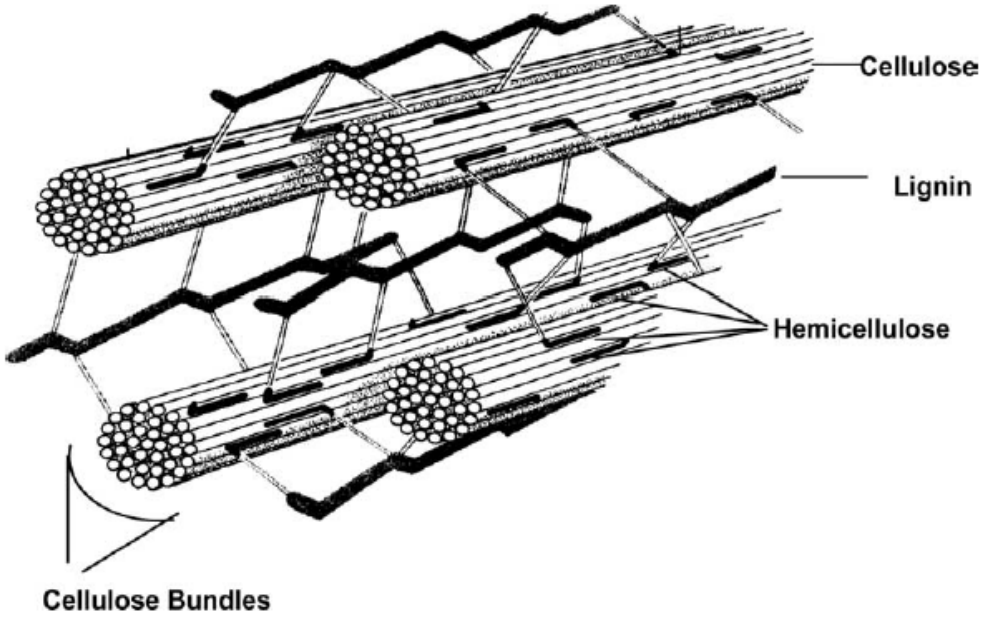

Figure 2.1: Typical plant cell wall arrangement in biomass material [3]

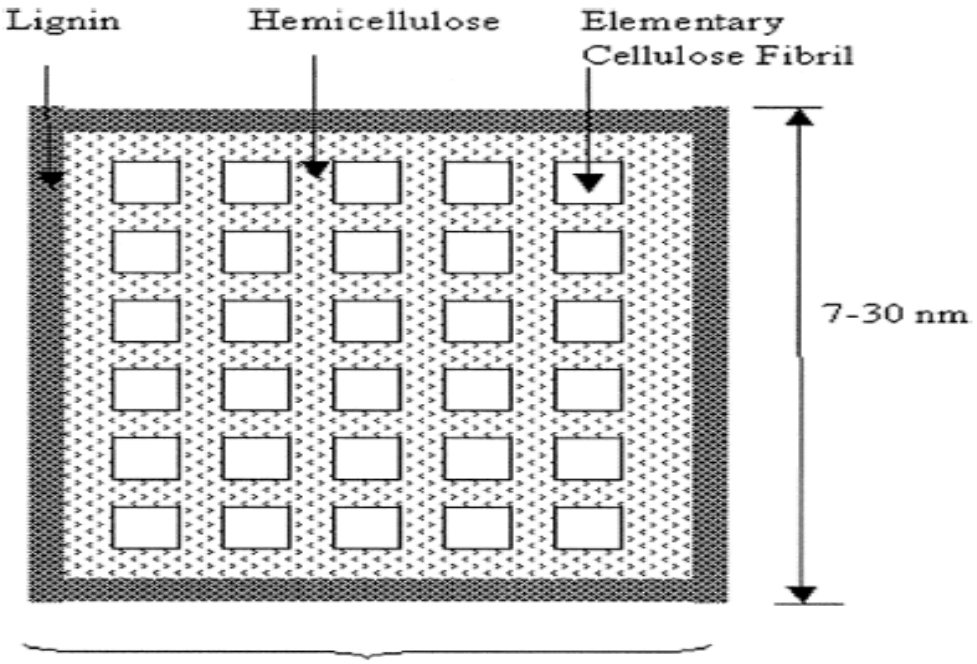

Figure 2.2: lignocellulose origanization into elementary fibrils and microfibrils [12]

2.1.4 Biomass composition

Lignocellulose biomass materials mainly consist of Cellulose, Hemicellulose, and Lignin. Other component like pectin, Ash and moisture are in small amount. The composition of various lignocellulosic biomass materials are given in table 7 here. Cellulose, hemicellulose and lignin details are described further. [1]

Table 2.1: composition of various Lignocellulosic Biomass materials

Lignocellulosic Biomass	Moisture %	Ash Content %	Lignin %	Hemicellulose %	Cellulose %
Bagasse	4.0	1.8	25.8	16.0	52.3
Bajra straw	9.6	9.0	24.5	28.2	27.6
Jawar straw	12.5	11.2	20.7	25.9	28.7
Maize straw	3.5	4.9	25.6	33.3	26.7
Rice straw	7.0	15.3	12.1	36.5	27.7
Wheat straw	8.7	7.7	21.6	28.0	33.1

2.1.4.1 Cellulose

Cellulose (40–60% of the dry biomass) is a linear polymer of cellobiose (glucose–glucose dimer); the orientation of the linkages and additional hydrogen bonding make the polymer rigid and difficult to break. In hydrolysis the polysaccharide is broken down to free sugar molecules by the addition of water. This is also called saccharification. The product, glucose, is a six-carbon sugar or hexose. [1, 16]

2.1.4.2 Hemicellulose

Hemicellulose (20–40%) consists of short highly branched chains of various sugars: mainly xylose (five carbon), and further arabinose (five carbon), galactose, glucose and mannose (all six-carbon). It also contains smaller amounts of non-sugars such as acetyl groups. Hemicellulose, because of its branched, amorphous nature, is relatively easy to hydrolyse. [1, 16]

2.1.4.3 Lignin

Lignin (10–25%) is present in all Lignocellulosic biomass. Therefore, any ethanol production process will have lignin as a residue. It is a large complex polymer of phenyl propane and methoxy groups, a non-carbohydrate polyphenolic substance that encrusts the cell walls and cements the cells together. It is degradable by only few organisms, into higher value products such as organic acids, phenols and vanillin. Via chemical processes valuable fuel additives may be produced. These by-products can significantly enhance the competitiveness of ethanol technology.

2.2 Hydrolysis

2.2.1 Role of hydrolysis

Hydrolysis is an essential element in the waste to food and energy concepts, because it is through hydrolysis that the cellulose and carbohydrates in wastes are split into their constituent sugars. For example, the cellulose molecule may consist of more than 5,000 glucose units. The carbon in the glucose and other simple sugars is readily available to most microorganisms. Without the intervention of hydrolysis, the carbon in the cellulose and the complex carbohydrates are not available to microorganisms, particularly to those associated with single-cell protein or with ethanol fermentation. (The term “complex carbohydrates” will be referred to as “carbohydrates”.) It is through hydrolysis that the carbons in the glucose units that make up cellulose, and in the simple sugars that make up other carbohydrate molecules, are rendered available to yeasts and any other microorganisms that may be responsible for fermentation. (“Hydrolysis” often is termed “saccharification” when used in reference to the concept.) [3, 16]

2.2.2 Factors Influencing Hydrolysis

The following factors are influencing hydrolysis process. The details of each and how they affect the process are given further.

2.2.2.1 Ratio of Crystalline to Para Crystalline (amorphous) Cellulose

An especially influential factor in the hydrolysis of cellulosic waste is the ratio of crystalline to Para crystalline (amorphous) cellulose. The ratio has a major bearing on the practicality of using a particular waste as a feedstock to the process. The crystalline region of cellulose molecules is marked by a very closely packed structure and, hence, strong internal forces of attraction. On the other hand, the Para crystalline region is more randomly oriented. The high degree of order in the crystalline region renders the region more resistant than the amorphous (Para crystalline) region to hydrolysis. Therefore, the higher the ratio of crystalline to Para crystalline cellulose in a waste, the more difficult it is to hydrolyze the waste.

2.2.2.2 The Surface-to-Mass Ratio of The Waste Particles

The surface-to-mass ratio of the waste particles exerts an important impact on hydrolysis, in that the smaller the particle, the more rapid is the physical or biological hydrolytic reaction.

2.2.2.3 The Partial or Complete masking of the Cellulose Molecules

Another rate-related factor is the partial or complete masking of the cellulose molecules by lignin or some other resistant substance. The masking inhibits access of the hydrolytic mechanisms to the cellulose.

2.2.3 Classification of Methods of Hydrolysis

The various methods of hydrolysis can be classified into three classes on the basis of the mechanism or process of splitting, i.e., disrupting cellulose and carbohydrate molecules. The classes are

(I) Chemical (Acid)

(II) Physical-chemical

(III) Enzymatic

The terms "chemical hydrolysis" and "acid hydrolysis" are often used synonymously. Even though physical disruption does not fully fit the classic definition of "hydrolysis", it includes physical and physical-chemical disruption. Enzymatic hydrolysis is mostly biological in nature. Yet another class could be formed by integrating enzymatic hydrolysis with chemical hydrolysis.

2.2.3.1 Chemical (Acid) Hydrolysis

Basically, acid hydrolysis is a process in which the cellulosic fraction of a waste is suspended in an acidified aqueous medium that is maintained under pressure at an elevated temperature.

Factors of particular significance to acid hydrolysis are liquids-to-solids ratio, acid concentration, and temperature. The rate of acid hydrolysis increases with increase in liquids-to-solids ratio.

Minimum particle size is determined by economic practicality, because energy and monetary costs of size reduction increase almost exponentially when the intended particle size is less than 5 cm. The permissible upper limit of the liquids-to-solids ratio also is determined by economic practicality. The consensus apparently places the upper ratio at 10 parts liquid to 1 part solids.

Cost of acid, percentage of acid recovery, and rate of acceleration of corrosion establish the maximum permissible acid concentration. For sulphuric acid, the concentration would be about 0.5% in most situations. Yield of sugar is highest at the higher temperature levels and acid concentrations.

2.2.3.2 Enzymatic Hydrolysis

Hydrolysis of cellulosic and carbonaceous wastes is accomplished through the agency of the enzyme, cellulase. The process is essentially biological in that the hydrolytic enzyme is produced by microorganisms genetically capable of synthesizing it.

Cellulase is an enzyme that specifically splits cellulose molecules into their constitutive sugars (hexoses and pentoses).

2.3 Hydrolysis of Lignocellulosic Biomass,

2.3.1 Lignocellulosic material Hydrolysis

Since cellulose and hemicellulose are principally composed of tightly bonded sugars, the bonds need to be broken before fermentation to ethanol can proceed. Cellulose and hemicellulose must be broken down into the simple sugars glucose and xylose respectively. This process, as illustrated in Figure 4 for cellulose conversion, is known as hydrolysis.

There are various methods of completing the hydrolysis of the raw material into sugars. These methods include:

- Simultaneous saccharification and fermentation.
- Concentrated acid hydrolysis, neutralization and fermentation.
- Enzymatic Hydrolysis followed by fermentation

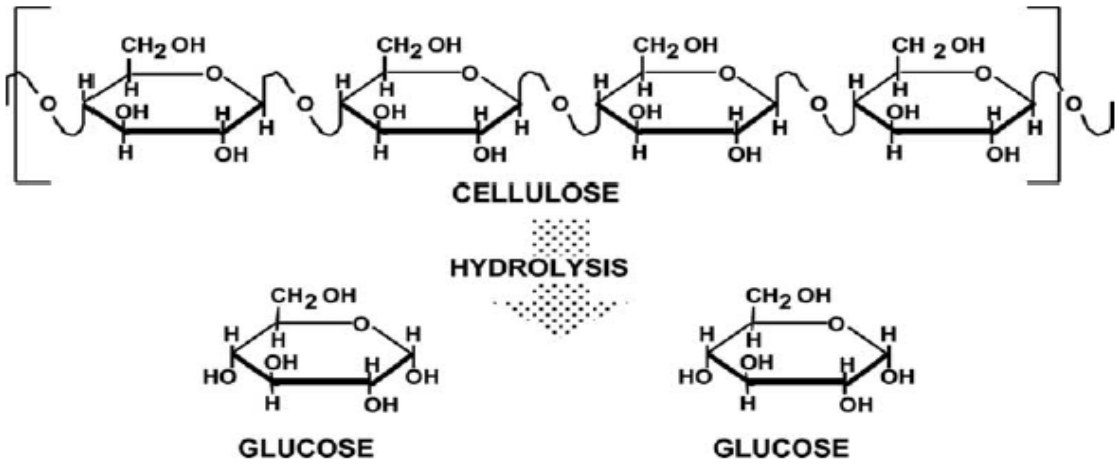

Figure 2.3: Cellulose Hydrolysis [3]

2.3.2 Fermentation

Once the cellulose and hemicellulose have been broken down to simple sugars, fermentation can then take place as illustrated in Figure 5. One molecule of glucose produces 2 molecules of ethanol and 2 molecules of carbon dioxide.

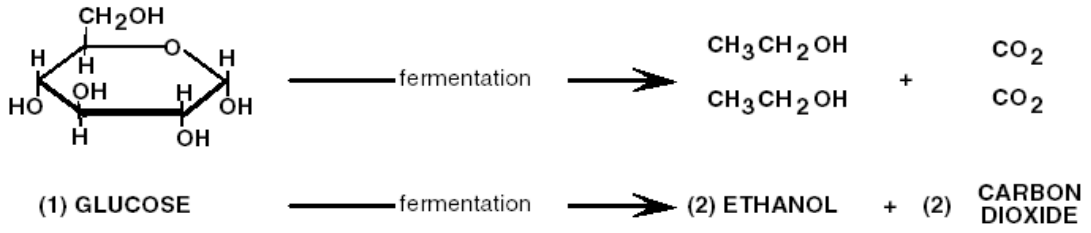

Figure 2.4: Glucose Fermentation [3]

2.3.3 History and Development

In the South Pacific during World War II, a fungus broke down cotton clothing and tents. This fungus, Trichoderma reesei, in fact produced cellulase enzymes, which hydrolyses cellulose. The first application of these enzymes for wood hydrolysis in an ethanol process was to simply replace the cellulose acid hydrolysis step with a cellulase enzyme hydrolysis step.

To improve the yield and rate of the enzymatic hydrolysis, research focuses both on enhancing enzyme activity in distinctive hydrolysis and fermentation process steps, as well as combining the different steps in fewer reactors Intermediate and end products of the hydrolysis, cellobiose and glucose, inhibit the cellulase activity. This can be avoided by supplying extra enzymes during the reaction, or by taking away the product by ultra filtration or by simultaneous fermentation in the same reactor. Enzymes can be recovered and recycled, so that the enzyme concentration can be higher against lower enzyme cost, although the enzyme quality decreases gradually. Where chemical pre-treatment precedes enzymatic hydrolysis, poisonous materials to the enzymes need to be removed

2.3.4 Advantages

- Very mild process conditions give potentially high yields
- Maintenance costs are low compared to acid or alkaline hydrolysis (no corrosion problem).
- The process is compatible with many pre-treatment options, although purely physical methods are typically not adequate.
- Enzymatic hydrolysis as key to cost-effective ethanol production in the long run. Although acid processes are technically more mature, enzymatic processes have comparable projected costs and the potential of cost reductions as technology improves.

2.3.5 Factors Influencing Process

- Hydrolysis is negatively influenced by structural features such as
    - crystallinity,
    - degree of cellulose polymerisation,
    - lignin content,
    - Surface area.
- A low substrate concentration gives low yield and rate, and a high cellulase dosage may increase the costs disproportional. However, the substrate/enzyme ratio should not be too high (inhibition).

- Hydrolysis can be enhanced by
    - adding certain surfactants (to facilitate desorption of cellulase after reaction),
    - by using mixes of cellulase from different organisms,
    - By adding other enzymes (e.g. pectinase).

Nearly complete saccharification of steam-exploded chips is possible.

2.3.6 Comparison of Acid Hydrolysis and Enzymatic Hydrolysis

Acid hydrolysis has been practiced and understood for half a century and analyses of R&D-driven improvements project only modest cost improvements. The dilute acid process has a low sugar yield (50–70% of the theoretical maximum).

The enzymatic hydrolysis has currently high yields (75–85%) and improvements are still projected (85–95%), as the research field is only a decade young.

Table 2.2: Comparison of process conditions and performance of three cellulose hydrolysis processes

	Consumables	Temp (ºC)	Time	Glucose Yield	Available
Dilute acid	< 1 % H_2SO_4	215	3 min	50 – 70 %	Now
Conc. Acid	30 – 70 % H_2SO_4	40	2 – 6 hr	90 %	Now
Enzymatic	Cellulase	70	1.5 days	75 % ↓ 95 %	Now ↓ 2020

2.4 Basics of Cellulases

2.4.1 Types of Cellulases

2.4.1.1 Constitutive vs. Induced

- **Constitutive**

  The presence of cellulase is continuous in some microbes and is continuously synthesised.

- **Induced**

  It is not continuously present in certain other microbes, and its synthesis must be triggered, i.e., induced by an external stimulus, usually the presence of cellubiose or other reducing agent.

2.4.1.2 Extracellular vs. intracellular

The microbial origin of cellulase necessitates a two-stage process in enzymatic hydrolysis. In stage-1, cellulases are produced and harvested. In stage-2, the harvested enzymes are introduced into a waste. In the waste, the enzymes split, i.e., hydrolyse cellulose and carbohydrate molecules into fermentable sugars, which are then harvested. Generally, the harvested sugars are used as the carbon source in ethanol fermentation.

- **Extracellular**

  Harvesting is facilitated by the fact that the cellulases involved in enzymatic hydrolysis are synthesised extracellularly by the cellulolytic microorganisms that produce them. Because they are Extracellular, the cellulases are in the culture medium. If necessary, they can be extracted from the medium.

- **Intracellular**

  Some cellulolytic bacteria synthesis their enzymes intracellularly. For example, the cytophage have their enzymes system bound in the cell wall or membrane. With such an arrangement, hydrolysis depends upon the existence of a close contact between the cellulose and the cell wall or membrane. Access to the bound enzyme would necessarily be by way of disrupting the individual microbes. Obtaining a cell-free enzyme extract would be an expensive operation.

2.4.1.3 Enzymatic Systems

Various cellulolytic enzyme (cellulase) systems can be divided into two groups

- **C1**

  C1 groups are effective on highly crystalline forms of cellulose (e.g., cotton fibre). They split crystalline cellulose into linear anhydroglucose.

- **CX.**

  Anhydrous glucose chains are then split into soluble carbohydrates by CX enzymes.

This sequence has an important bearing on rate of hydrolysis of cellulosic waste because the first step in the hydrolysis must be the splitting of crystalline, i.e., resistant forms into simple forms that are accessible by a wider array of enzymes. Thus, the higher the concentration of C1 enzymes and hence the greater the concentration of microbes that synthesise them, the faster is the rate of hydrolysis.

2.4.2 Mechanism of Cellulose degradation by Cellulases

A cellulosic enzyme system consists of three major components: Endo-ß-Glucanase (EC 3.2.1.4), Exo-ß-Glucanase (EC 3.2.1.4) and ß-Glucosidase (EC 3.2.1.21). The mode of action of each of these being:

- Endo-P-Glucanase, 1, 4-ß-D-Glucan glucanohydrolase, CMCase, Cx: "random" scission of cellulose chains yielding glucose and cello-oligo saccharides.

- Exo-P-Glucanase, 1, 4-ß - D-Glucan cellobiohydrolase, Avicelase, C1: exo-attack on the non-reducing end of cellulase with cellobiose as the primary structure.

- ß-Glucosidase, cellobiase: hydrolysis of cellobiose to glucose.

Enzymatic hydrolysis of cellulose is performed by several different enzymes, cellulases. The rate of the hydrolysis reactions are influenced greatly by characteristics of the raw material such as crystallinity, degree of polymerization and accessibility.

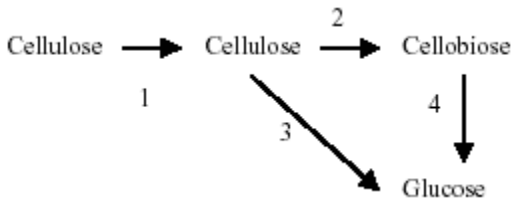

Figure 2.5 : The mode of action of Endoglucanase (1), cellobiohydrolases (2), Glucohydrolases (3) and β-Glucosidase (4)

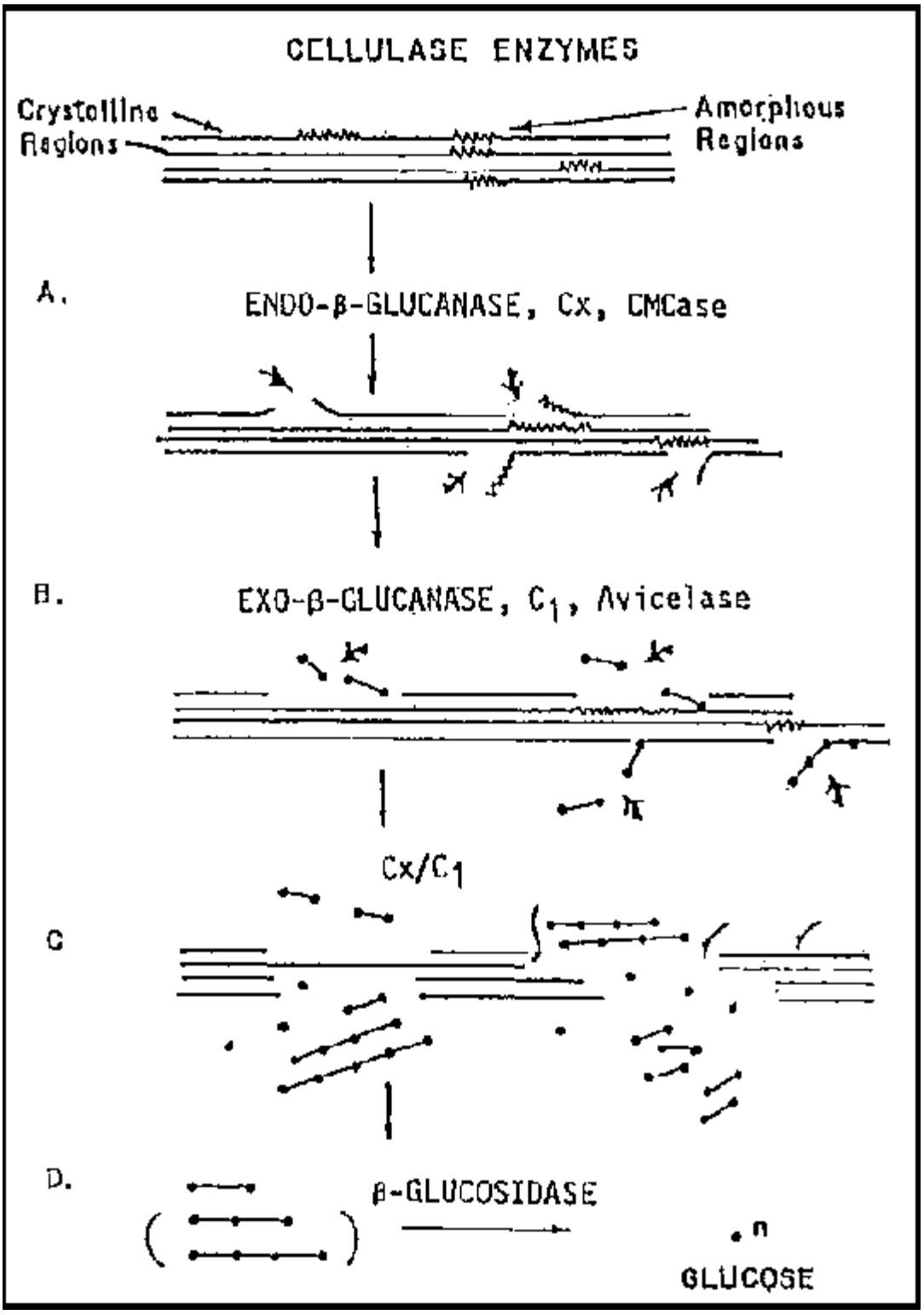

Figure 2.6: Cellulase action on biomass

2.4.3 Cellulase Producing Microorganisms

Table 2.3: Cellulase producing microorganisms

Fungi	Bacteria	Actinomycetes
Aspergillus acculeatus	Clostridium Thermocellum	Streptomyces sp.
Aspergillus niger	Ruminococcus albus	Thermoactinomyces sp.
Fusarium solani	Streptomyces sp.	Thermomonospora curvata
F. moniliferma		
Irpex lacteus		
Penicillium funmiculosum		
Phanerochaete chrysosporium		
Sclerotium rolfsii		
Sporotrichum cellulophilum		
Talaromyces emersonii		
Thielavia terrestris		
Trichoderma koningii		
Trichoderma reesei		
Trichoderma viride		

2.4.4 Screening of cellulase-producing microorganisms

Although a large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell-free enzymes capable of completely hydrolysing crystalline cellulose in vitro. Fungi are the main cellulase-producing microorganisms, though a few bacteria and actinomycetes have also been recently reported to yield cellulase activity. Microorganisms of the genera Trichoderma and Aspergillus are thought to be cellulase producers, and crude enzymes produced by these microorganisms are commercially available for agricultural use. Microorganisms of the genus Trichoderma produce relatively large quantities of endo-ß-glucanase and exo-ß-glucanase, but only low levels of ß-glucosidase, while those of the

genus Aspergillus produce relatively large quantities of endo-ß-glucanase and ß-glucosidase with low levels of exo-ß- glucanase production.

Successful utilization of cellulosic materials as renewable carbon sources is dependent on the development of economically feasible process technologies for cellulase production, and for the enzymatic hydrolysis of cellulosic materials to low molecular weight products such as hexoses and pentoses. It is evident that cellulase production was the most expensive step during ethanol production from cellulosic biomass, in that it accounted for approximately 40% of the total cost. Significant cost reduction is required in order to enhance the commercial viability of cellulase production technology.

Reese et al. 1950 proposed that exo-ß-glucanase causes a disruption in cellulose hydrogen bonding, followed by hydrolysis of the accessible cellulose with endo-ß-gucanase. Although cellulase isolation techniques have not been fully developed, the hypothesis depicted. According to this hypothesis, in a synergistic sequence of events, endo-ß-glucanase acts randomly on the cellulose chain, while exo-ß-glucanase acts on exposed chain ends by splitting off cellobiose or glucose. Cellobiose is subsequently hydrolysed by p-glucosidase to glucose. This hypothesis is however the opposite of that proposed by Reese et al., and indicates that three, rather that two enzymes are essential for the decomposition of cellulosic biomass.

2.4.5 Trichoderma reesei Cellulase System

Cellulases of the genus Trichoderma have received intensive attention due in significant part to the high levels of cellulase secreted. Trichoderma viride is a valid species aggregate, which is used for all unknown Trichoderma species while all T. reesei are developed from a single isolate named in recognition of the pioneering contributions of Elwin Reese.

Most commercial cellulases are produced from Trichoderma spp. with a few also produced by Aspergillus Niger. The T. reesei cellulase mixture consists of many catalytically active proteins. At least two cellobiohydrolases (CBH1-2), five endoglucanases (EG1–5), h-glucosidases, and hemicellulases have been identified by 2D electrophoresis.

CBH1, CBH2, and EG2 are the three main components of the T. reesei cellulase system,

2.5 Pre treatment Methods

2.5.1 Role of Pre Treatment Methods

Ideally, pre-treatment at a reasonable cost decreases cellulose crystallinity, disrupts the physical structure of lignin, and curtails cellulose polymerisation. Therefore pre treated biomass can be more accessible by Enzyme or Acid, whatever hydrolysis method selected.

Delignification, Hemicellulose hydrolysis, Size reduction, hydrolysis, explosion, biological methods are various examples of pre treatments.

2.5.2 Types of Pre Treatments

The various proposed forms of pre-treatment may involve one or all of following three major steps: particle size reduction, heating, and perhaps, chemical treatment.

Most pre-treatment methods are based on the assumption that the cellulosic waste has been separated from the municipal solid waste stream and that all contaminants have been removed to the maximum extent permitted by economic feasibility.

2.5.2.1 Chemical Pre Treatments

2.5.2.1.1 Acid Catalyzed Hydrolysis Pretreatment

Acid catalyzed hydrolysis uses dilute sulphuric, hydrochloric, or nitric acids. Of all chemical pretreatments, historically dilute sulphuric acid (0.5–1.5%, Temperature above 160ºC) has been most favoured for industrial application, because it achieves reasonably high sugar yields from hemicellulose at least xylose yields of 75–90%. The acid will have to be removed / neutralized before fermentation, yielding a large amount of gypsum. This is usually done after the cellulose hydrolysis. A concentrated acid based process also exists but is ranked to be very expensive.

2.5.2.1.2 Alkaline Pre Treatment

Alkaline pre-treatment uses bases like sodium hydroxide or calcium hydroxide. All lignin and part of the hemicellulose are removed, and the reactivity of cellulose for later hydrolysis is sufficiently increased. Reactor costs are lower than those for acid technologies. However, the use of these more expensive salts in high concentrations raises environmental concerns and may lead to prohibitive recycling, wastewater treatment and residual handling costs. Alkaline-based methods are generally more effective at solubilising a greater fraction of lignin while leaving behind much of the hemicellulose in an insoluble, polymeric form.

2.5.2.2 Physical. Uncatalysed Pre Treatment Methods

2.5.2.2.1 Steam Explosion

Steam explosion is one of the most promising methods to make biomass more accessible to cellulase attack. The material is heated using high-pressure steam (20–50 bar, 210–290 ºC) for few minutes; these reactions are then stopped by sudden decompression to atmospheric pressure. Most steam treatments yield high hemicellulose solubility and low lignin solubility. Studies conducted without added catalyst report xylose-sugars recoveries between 45% and 65%. To make it a viable option for the long term, the overall yield has to be increased and the costs have to be decreased.

2.5.2.2.2 Liquid Hot Water Pre Treatment

The LHW process uses compressed, hot liquid water (at pressure above saturation point) to hydrolyse the hemicellulose. Xylose recovery is high (88–98%), and no acid or chemical catalyst is needed in this process, which makes it economically interesting and environmentally attractive. Development of the LHW process is still in laboratory stage.

2.5.2.2.3 Ultrasonication Pretreatment

A desired amount of Carboxy methyl cellulose (substrate) powder was suspended in 0.2M acetate buffer, pH 4.8, and the suspension was pretreated by ultrasonic irradiation at 323 K, delivered by immersing the radiating tip of a Branson Sonifier 450 directly in the solution. The enzyme solution was then added to begin the reaction, which was also carried out at 323 K with stirring. Pretreatment of substrate in suspension by ultrasonic irradiation improves the reaction rate. [6]

2.5.2.3 Biological Pre Treatments

Biological pre treatments use fungi to solubilise the lignin Biodelignification is the biological degradation of lignin by microorganisms. It is mentioned in 1984 as possibly useful in the future, although at that time it was an expensive process, with low yields after long reaction time, and the microorganisms were poisoned by lignin derivatives. Biological pretreatment has the advantages of low energy use and mild environmental conditions. However, the very low hydrolysis rate is impedes implementation. Sometimes biological treatments are sometimes used in combination with chemical treatments.

2.5.2.4 Combinations of Several Pre Treatment Processes

2.5.2.4.1 Combine physical and chemical elements.

Addition of dilute acid in steam explosion can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds, and lead to more complete removal of hemicellulose. It is possible to recover around 70% potential xylose as monomer. Acid catalysed steam explosion is one of the most cost-effective processes for hardwood and agricultural residues, but it is less effective for softwoods. Limitations include destruction of a portion of the xylan fraction, incomplete disruption of the biomass structure, and generation of compounds that may inhibit microorganisms uses in downstream processes. The necessary water wash decreases the overall sugar yields.

2.5.2.4.2 Ammonia Fiber Explosion (AFEX)

It involves liquid ammonia and steam-explosion. The process conserves the protein part; this high-value co product is important for compensating the high process costs of the process. This is not interesting for the present research, in which low protein feedstock prevails. Also, although AFEX enhances hydrolysis of (hemi)cellulose from grass, the effect on biomass that contains more lignin (soft and hardwood) is meager.

2.5.2.4.3 CO_2 Explosion

Explosion pretreatments of the cellulosic materials by supercritical carbon dioxide were performed in a static type of apparatus as shown in Figure 2.7. After a sample of Avicel with addition of a certain content of buffer solution (0.05 M potassium dihydrogen phosphate buffer solution, pH 4.7) was placed in the reactor at the beginning of an experiment, the reactor was then enclosed and immersed in the thermostatic bath at a constant temperature of interest (35-80 °C). When the temperature was equilibrated and all tubing connections were secured, carbon dioxide was injected from a Ruska pump into the reactor at the experimental pressure (1000-4000 psi). Before entering the Ruska pump, carbon dioxide in a coil was placed in a ice bath which is helpful to easily obtain a higher carbon dioxide pressure. The sample was agitated by a magnetic stirrer and subjected to the carbon dioxide pressure for a controlled length of time to let the carbon dioxide molecules penetrate the micropores in the cellulosic structure. After the sample

was exposed to carbon dioxide for a designated length of time, a quick pressure release was done by opening a valve attached to the reactor. [13, 4]

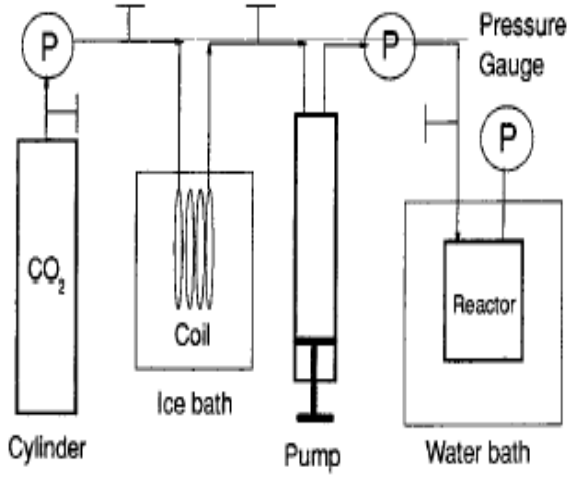

Figure 2.7: Supercritical carbon dioxide explosion apparatus [13]

2.5.3 Comparison of Treatment :

Table 2.4: comparison of types of pretreatments [9]

Method	Operations (Factors) causing changes in the substrate structure	Kind of changes
Physical	Milling and grinding (ball, vibro energy, two roll, pressure, hammer); Irradiation (electron beam, γ rays, microwaves); High temp (pyrolysis, steam explosion)	Increase in specific surface area and size of pores, decrease of the degrees of polymerization of cellulose and its crystallinity, hydrolysis of hemicelluloses, partial depolymerization of lignin
Chemical	Alkalis, Acids, Gases, Oxidizers, Reducers, Organic Solvents	Delignification, decrease of the degree of polymerization and crystallinity of cellulose associated with its swelling, porosity growth
Biological	White-rot fungi (Pleurotus, Pycnoporus, Ischnoderma, Phlebia etc.)	Delignification and reduction in degree of polymerization of cellulose and hemicellulose
Combined	Alkali pulping associated with steam explosion, grinding followed by alkaline or acid treatment	Degradation of hemicelluloses, Delignification, Increase of the specific surface area and size pores

Chapter No. 3

Pre Treatment Experiments And Evaluation

Chapter No. 3

Pre Treatment Experiments and Evaluation

3.1 Experimental Methods

3.1.1 Analysis of Substrates

Substrates selected for the study are Wheat straw, Rice straw and Jawar straw. Which are collected from local farmers near by Ahmedabad city. Then they had crushed and grinded for size reduction.

The practical analysis of all three substrates (wheat straw, Rice straw and Jawar straw) had been carried out. The analysis method is given in appendix II. The analysis of each substrates are given in Table 3.1

3.1.2 Boiling Water Treatment:

Procedure

25 gms of substrate (250 micron size particles) was boiled in 950 ml of distilled water, till volume reduced to 400 ml, Then it was washed with fresh water and filtered with nylon cloth and dried at 80 ºC for 3 hours in hot air drier. To evaluate this procedure with time, here substrate is heated at boiling temperature of water for different time period then, % lignin removed was measured. This treatment was applied to all three substrates (wheat straw, Rice Straw, Jawar straw) which have been used here.

This method is evaluated with respect to time and Experimental results are show in table 3.2 and figure 3.1

3.1.3 Alkali (NaOH) Treatment:

Procedure

The substrate with particle size 250 micron was treated with 1% sodium hydroxide solution at 100 ºC for one hour using 1:20 ratio of substrate to sodium hydroxide solution. After that is was washed with fresh water and filtered with nylon cloth then it was dried at 80 ºC in hot air dryer for three hr.

This method is applied to all three substrates (Wheat straw, Rice straw, Jawar straw) which have been used here.

This method is evaluated here with respect to sodium hydroxide concentration and time basis to obtain maximum lignin removal. Experimental results are show in table 3.3, 3.4 and figure 3.2, 3.3

3.1.4 Phosphoric Acid Treatment:

Procedure

The substrate with particle size of 250 micron was treated with 2% phosphoric acid (v/v) at 80 ºC for one hour, keeping the solid to liquid ratio 1:20. After that it was washed with fresh water and filtered with nylon cloth followed by drying at 80 ºC in hot air dryer for three hours period.

This method is applied to all three substrates (Wheat straw, Rice straw, Jawar straw) which have been used here.

This method is evaluated here with respect to phosphoric acid concentration and time basis to obtain maximum lignin removal. Experimental results are show in table 3.5, 3.6 and figure 3.4, 3.5

3.1.5 Sulfuric Acid Treatment:

Procedure

The substrate with particle size of 250 micron was treated with 2% Sulfuric acid (v/v) at 80 ºC for one hour, keeping the solid to liquid ratio 1:20. After that it was washed with fresh water and filtered with nylon cloth followed by drying at 80 ºC in hot air dryer for three hours period.

This method is applied to all three substrates (Wheat straw, Rice straw, Jawar straw) which have been used here.

This method is evaluated here with respect to Sulfuric acid concentration and time basis to obtain maximum lignin removal. Experimental results are show in table 3.6, 3.7 and figure 3.5, 3.6

3.1.6 Hydrochloric Acid Treatment:

Procedure

The substrate with particle size of 250 micron was treated with 2% Hydrochloric acid (v/v) at 80 ºC for one hour, keeping the solid to liquid ratio 1:20. After that it was washed with fresh water and filtered with nylon cloth followed by drying at 80 ºC in hot air dryer for three hours period.

This method is applied to all three substrates (Wheat straw, Rice straw, Jawar straw) which have been used here.

This method is evaluated here with respect to Hydrochloric acid concentration and time basis to obtain maximum lignin removal. Experimental results are show in table 3.8, 3.9 and figure 3.7, 3.8

3.2 Results and Discussion

Table 3.1: Practical Analysis of Biomass Material (Wheat straw, Rice straw, Jawar straw)

	Wheat Straw	Rice Straw	Jawar Straw
Cellulose (%)	31.8	26.6	29.3
Hemicellulose (%)	33.5	37.2	26.2
Lignin (%)	19.8	13.7	21.5
Ash content (%)	8.3	14.8	12.3
Moisture (%)	6.6	7.7	10.7

Table 3.2: Evaluation of boiling water treatment with time

(Particle size 250 micron, boiling temperature)

Sample No.	Time hr	% Delignification		
		wheat straw	Rice straw	Jawar straw
1	0.5	11.1	14.3	8.4
2	1	15.3	16.8	11.6
3	1.5	19.7	22.9	17.2
4	2	24.1	27.2	22.8
5	2.5	26.7	29.3	23.4

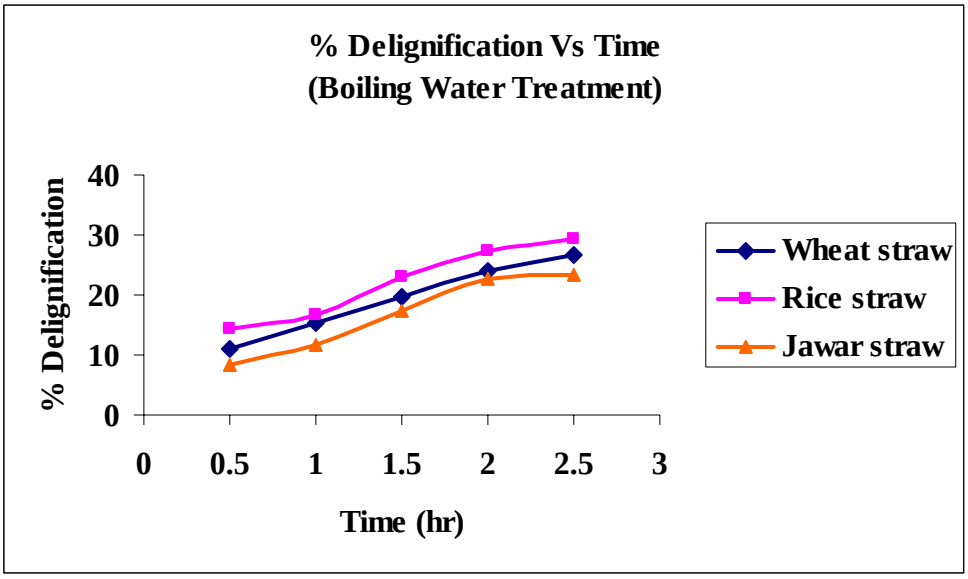

Figure 3.1: % Delignification Vs Time plot for Boiling water treatment

Table 3.3: Evaluation of Alkali(NaOH) treatment with NaOH Concentration.
(Particle size 250 microns, Temp. – 100ºC, Time- 1 hour)

Sample No.	NaOH Alkali Conc.	% Delignification		
		wheat straw	Rice straw	Jawar straw
1	1	20.3	23.1	18.3
2	2	27.4	39.2	26.9
3	4	34.5	43.5	32.1
4	5	44.7	48.9	41.2
5	6	46.1	51.7	47.7
6	10	62.3	58.7	64.4
7	15	71.3	62.5	78.1
8	20	73.2	64.2	81.3

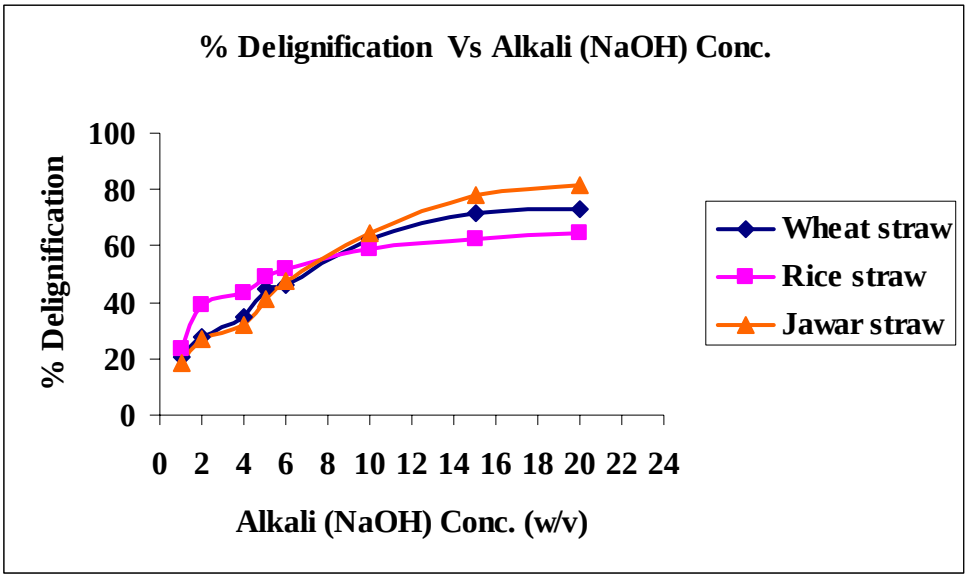

Figure 3.2: %Delignification Vs Alkali (NaOH) Concentration plot

Table 3.4: Evaluation of Alkali (NaOH) treatment with time
(Particle size 250 microns, Temp. – 100ºC, NaOH Concentration 10%)

Sample No.	Time (hr)	% Delignification		
		wheat straw	Rice straw	Jawar straw
1	0.5	44.7	48.3	39.2
2	1	62.3	58.7	64.4
3	1.5	67.4	71.7	66.1
4	2	76.2	79.5	73.5
5	2.5	79.8	81.9	76.3
6	3	83.7	84.6	79.7

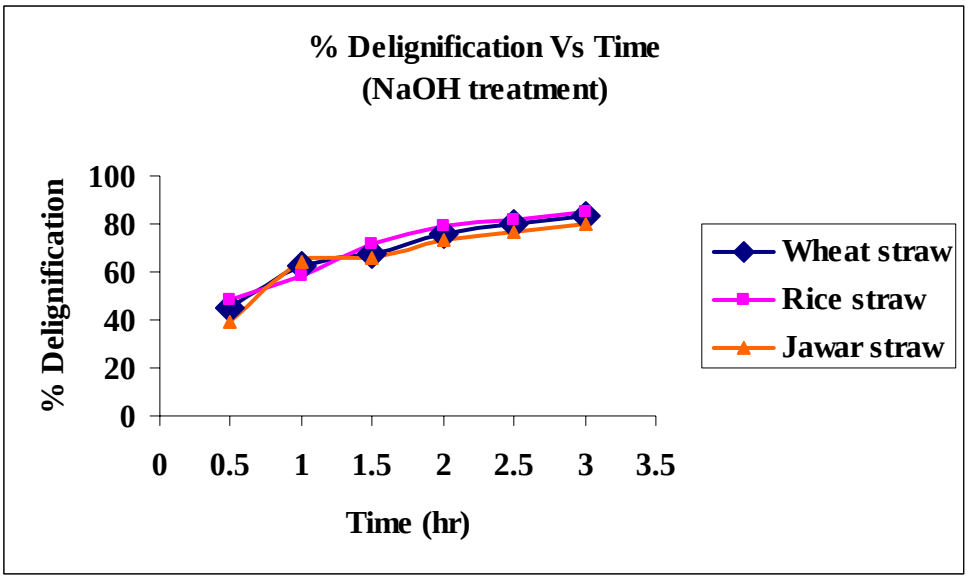

Figure 3.3: % Delignification Vs Time plot for Alkali (NaOH) Treatment

Table 3.5: Evaluation of Phosphoric Acid Treatment with Acid concentration (Particle size 250 microns, Temp. – 80ºC, Time- 1 hour)

Sample No.	Phosphoric Acid Conc.	% Delignification		
		wheat straw	Rice straw	Jawar straw
1	2	67.4	71.2	62.4
2	4	73.3	74.4	63.7
3	5	74.7	76.3	64.3
4	6	77.8	79.6	65.4
5	8	79.4	83.1	67.1

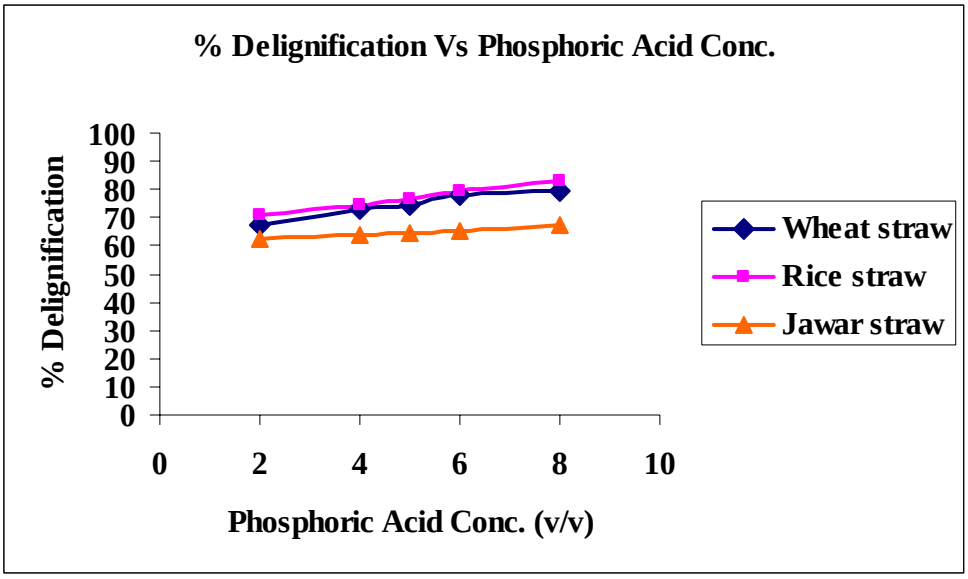

Figure 3.4: % Delignification Vs Phosphoric Acid Concentration plot

Table 3.6: Evaluation of Phosphoric Acid Treatment with time

(Particle size 250 microns, Temp. – 80ºC, Acid Concentration – 2%)

Sample No.	Time hr	% Delignification		
		wheat straw	Rice straw	Jawar straw
1	0.5	56.2	62.9	49.5
2	1	73.3	74.4	63.7
3	1.5	78.2	79.7	69.2
4	2	80.9	82.4	73.1
5	2.5	81.5	84.5	75.2

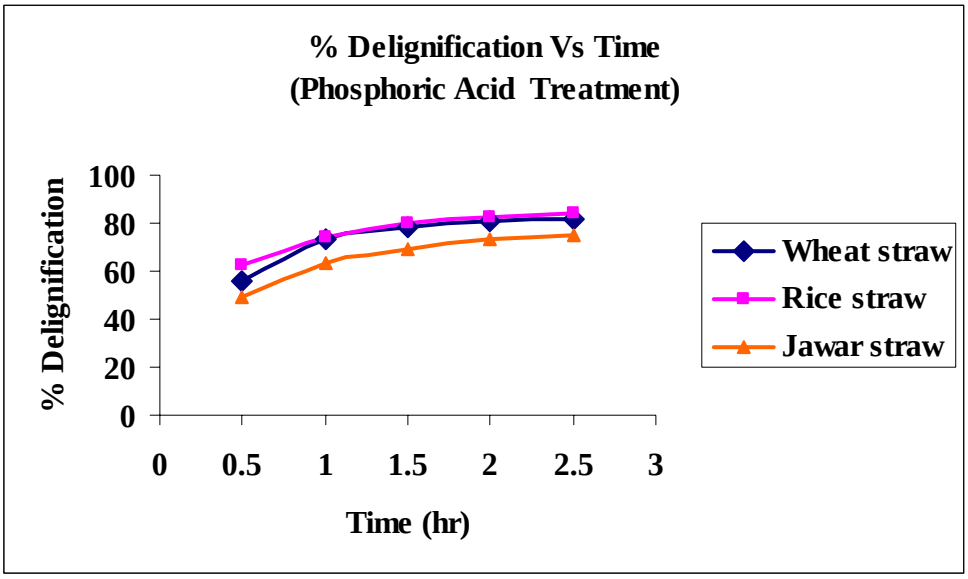

Figure 3.5: % Delignification Vs Time plot for Phosphoric Acid treatment

Table 3.7: Evaluation of Sulfuric acid treatment with Acid Concentration (Particle size 250 microns, Temp. – 80ºC, Time – 1 hr)

Sample No.	Sulfuric Acid Conc.	% Delignification		
		wheat straw	Rice straw	Jawar straw
1	2	72.2	78.8	65.1
2	4	74.1	83.6	73.5
3	5	76.2	84.4	73.7
4	6	79.9	84.4	76.6
5	8	84.5	87.7	79.5

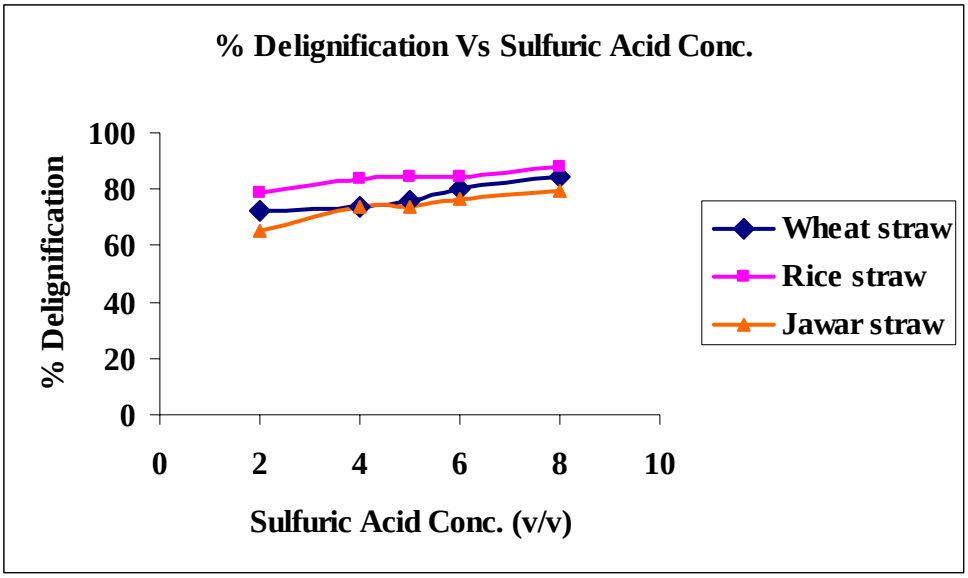

Figure 3.6: % Delignification Vs Sulfuric Acid Concentration plot

Table 3.8: Evaluation of Sulfuric Acid Treatment with time
(Particle size 250 microns, Temp. – 80ºC, Acid Concentration – 2%)

Sample No.	Time hr	% Delignification		
		wheat straw	Rice straw	Jawar straw
1	0.5	61.5	64.8	60.4
2	1	74.1	83.6	73.5
3	1.5	75.3	83.6	74.3
4	2	77.7	84.7	76.4
5	2.5	78.1	84.4	77.5

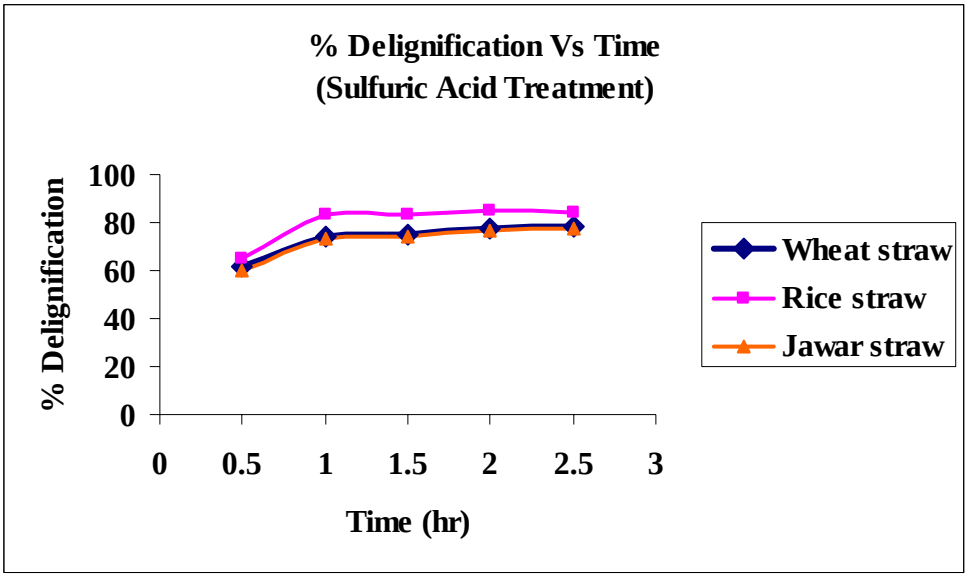

Figure 3.7: % Delignification Vs Time plot for Sulfuric acid treatment

Table 3.9: Evaluation of Hydrochloric acid treatment with Acid Concentration (Particle size 250 microns, Temp. – 80ºC, Time – 1 hr)

Sample No.	Hydrochloric Acid Conc.	% Delignification		
		wheat straw	Rice straw	Jawar straw
1	2	52.6	56.8	49.5
2	4	61.4	63.2	57.7
3	5	63.3	65.4	59.3
4	6	66.1	69.5	61.1
5	8	70.3	76.8	67.7

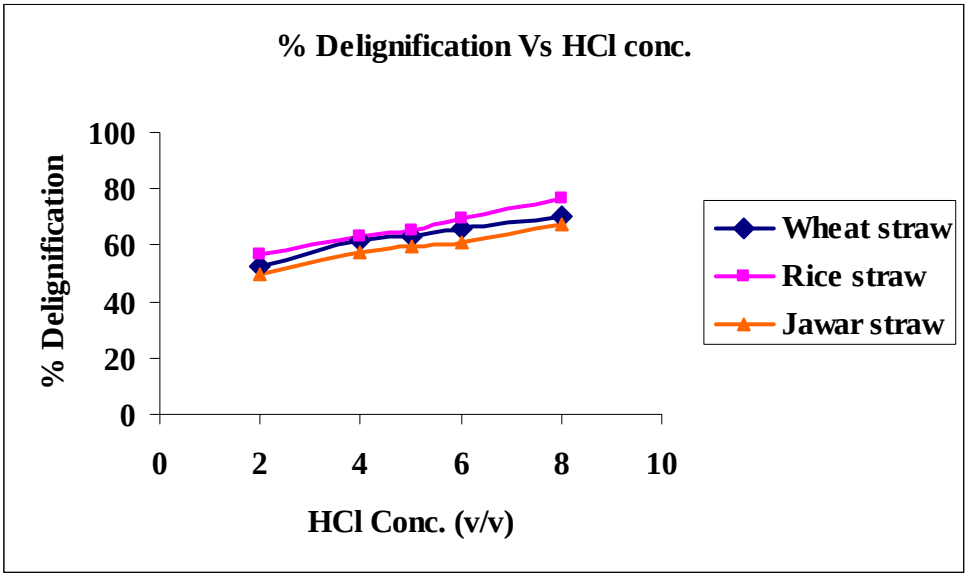

Figure 3.8: % Delignification Vs Hydrochloric Acid Concentration plot

Table 3.10: Evaluation of hydrochloric Acid Treatment with time
(Particle size 250 microns, Temp. – 80ºC, Acid Concentration – 2%)

Sample No.	Time hr	% Delignification		
		wheat straw	Rice straw	Jawar straw
1	0.5	47.5	50.3	42.8
2	1	61.4	63.2	57.7
3	1.5	64.1	68.1	62.2
4	2	71.2	74.6	69.8
5	2.5	78.9	81.7	76.7

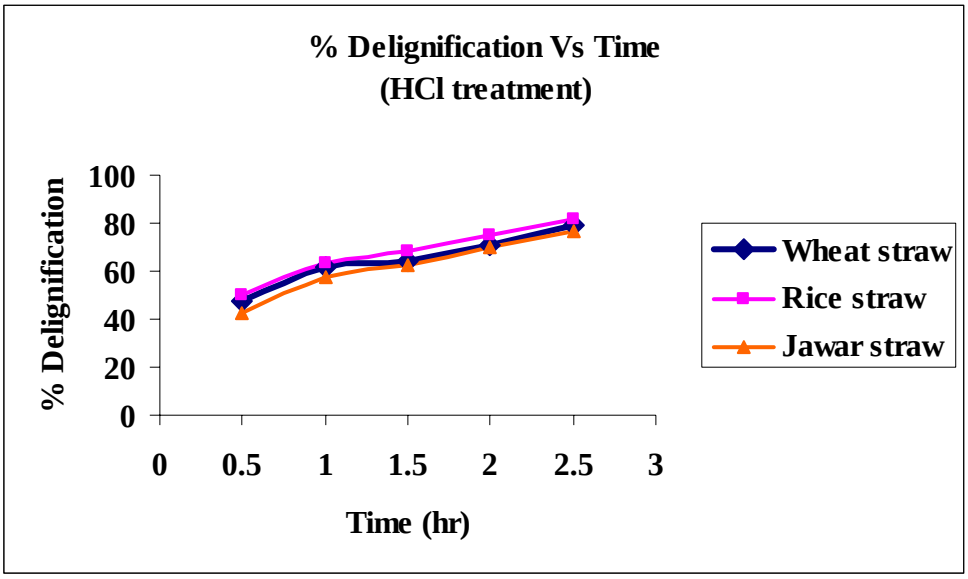

Figure 3.9: % Delignification Vs Time plot for Hydrochloric Acid treatment

Table 3.11 : Comparison of results of Acid treatments at same concentration
(Particle size 250 microns, Temp. – 80ºC, Time – 1 hr)

Sr No.	Chemical Conc. (%)	Type of Treatment	% Delignification		
			Wheat straw	Rice straw	Jawar straw
3	2	Acid (Phosphoric)	67.4	71.2	62.4
4	2	Acid (Sulfuric)	72.2	78.8	65.1
5	2	Acid (Hydrochloric)	52.6	56.8	49.5

Table 3.12: Comparison of results of all treatments at time of treatment
(Particle size 250 microns, Time – 1 hr)

Sr No.	Time (Hour)	Type of Treatment	% Delignification		
			Wheat straw	Rice straw	Jawar straw
1	1	Boiling Water	15.3	16.8	11.6
2	1	Alkali (NaOH)	62.3	58.7	64.4
3	1	Acid (Phosphoric)	73.3	74.4	63.7
4	1	Acid (Sulfuric)	74.1	83.6	73.5
5	1	Acid (Hydrochloric)	61.4	63.2	57.7

Results and Discussion:

Table 3.1 shows the practical analysis of selected lignocellulosic Biomass materials those are Wheat straw, Rice straw, and Jawar straw. Actually Biomass composition differs from place to place. They are not exactly same for one material any where. For example reported average cellulose content in wheat straw is 33% and here is 31.8%.

Pretreatments yielded good delignification but because of their cost and hazards in operation, they are not useful methods of treatment. Table 3.2 and Figure 3.1 show the time effect of lignin removal by boiling hot water treatment for all three substrates and graph shows that after 2 hr of treatment there is slice increase in delignification compared to 0 to 2 hour lignin removal. Hence beyond that limit there is no significant lignin removal.

Similarly Table 3.3 and graph 3.2 shows the NaOH concentration effect on lignin removal. Delignification increases linearly with concentration of NaOH up to 15% concentration beyond that no significant increase in lignin removal. For all three substrate similar kind of pattern of plot has been obtained. There is no need to go beyond 10% concentration of NaOH. The possible cause of good delignification with alkali is due to soponification of intermolecular ester bonds, which is turn cause swellin of cellulose molecule. The second reason is to fact that solubility of lignin in alkali at higher temperature is higher. The timely effect on lignin removal also carried out and results are show in table 14 and figure 11 they show that for all three substrate beyond 1.5 hr time period there is no significant lignin removal.

Table 3.5 and figure 3.4 shows the effect of concentration of phosphoric acid of treatment on delignification for phosphoric acid low concentration range for all three substrates. Results show that at 2% concentration. It can be achieved 60 to 70% lignin removal after that there is no sharp increase in delignification therefore no need to go beyond 2% concentration. Similar kind of patterns has been obtained for other two types

of acid treatments, sulfuric acid treatment and hydrochloric acid treatment. Therefore for acid concentration effect this study shows that at lower concentration (2%) desirable lignin removal have been achieved there is no need to go for further concentration.

Chapter No. 4

Enzymatic Hydrolysis Experiments And Evaluation

Chapter No. 4

Enzymatic Hydrolysis Experiments and Evaluation

4.1 Enzyme System:

Culture of Trichoderma Reesei was used as cellulase enzyme in this study. The cellulolytic fungal culture used in the present study was procured from, Institute of Microbial Technology Chandigarh. It was isolated and identified as Trichoderma Reesei earlier by National Centre for Industrial Microorganism, National Chemical Laboratory Pune.

4.2 Experimental Methods

4.2.1 Slant Preparation

In one flask (205 ml) 200 ml basal medium in addition agar agar powder is added and culture is submerged into the flask under sterilized condition. It stored at 4 °C

4.2.2 Growth Medium Preparation

The basal medium used for growth of submerged culture had following composition

KH_2PO_4	2	(gm/liter)
$MgSO_4.7H_2O$	0.3	(gm/liter)
$CaCl_2.2H_2O$	0.3	(gm/liter)
$(NH_4)_2SO_4$	1.4	(gm/liter)
$FeSO_4.7H_2O$	5	(mg/liter)
$MnSO_4.H_2O$	1.6	(mg/liter)
$ZnSO_4.7H_2O$	1.4	(mg/liter)

CoCl2.6H2O	2.0	(mg/liter)
Pepton	0.1%	(1 gm)
Tween 80	0.1%	(1 mL)

4.2.3 Culture Growth and Cultivation

5 flasks of (250 ml) were taken with 200 ml basal medium and culture was submerged into the each flask under the sterilized condition. Then flasks were put into the shaking water bath at 28 °C and culture was run for 8 days

4.2.4 Cellulase Production

To induce the cellulase production there should have to add carbon source into the growing culture.

Procedure

5 flasks were taken with 200 ml basal medium then culture was submerged into the each flask under the sterilized condition. Then flasks were kept at 28ºC in constant temperature shaking water bath. Wheat Straw Substrate was also added to each flask. pH 4 was maintained by 2N HCl and 2N NaOH solution.

4.2.5 Hydrolysis Rate Measurement

Rate of Hydrolysis was analyzed by reducing sugar measurement by Dinitrosalicylic Acid Method described by Millers et al.

4.2.6 Effect of Pre Treatment on Hydrolysis Rate

This study was carrying out to prove the need of pretreatment before enzymatic hydrolysis. Here two 250mL flasks are taken with same quantity of 10% NaOH solution

pretreated and untreated Wheat straw as substrate. Then rate of hydrolysis is measured by measurement of reducing sugars by DNS method in regular time periods (1 day). Then similarly sugar producing in untreated samples also had been measured. The Experimental results have shown in table 4.10 and graph 4.10

4.2.7 Effect of type of Pretreatment on Hydrolysis Rate

These experiments have been studied with 5 gm of substrate (wheat straw) treated with different treatment taken in 250mL flask and addition of constant amount of Enzyme filtrate to each flask for 1 day of period. After that sugar produced is measured by DNS method as described in Appendix I.

4.2.8 Effect of Lignin Removal on Hydrolysis Rate

Here Substrates, of particle size 250 micron and NaOH treated with different concentration and time are taken for study. They had known % delignification. In each flask 3gm of substrate in addition of same amount of culture filtrate (200mL) taken and kept in boiling water shaking bath for 1 day period of study at 50ºC and 4 pH

4.2.9 Effect of Substrate Concentration on hydrolysis Rate

10%NaOH treated wheat straw samples with different concentration taken in four different flasks with constant amount of enzyme filtrate taken for the study and rate of enzymatic hydrolysis is measured by sugar produced after the same time period 1 day.

4.2.10 Effect of Temperature on Hydrolysis Rate

Wheat straw substrate with 250 micron particle size and 200mL culture filtrate were taken and kept at boiling water bath at 4 pH and 25 RPM condition for constant 1 day hydrolysis. Then sample with different temperature made and sugar produced measured by DNS method.

4.2.11 Effect of pH on Hydrolysis Rate

Wheat straw substrate with 250 micron particle size and 200mL culture filtrate were taken and kept at boiling water bath at 50ºC and 25 RPM condition for 1 day constant period. Then sample with different pH made and sugar produced measured by DNS method.

4.2.12 Effect of agitation on hydrolysis Rate

Wheat straw substrate with 250 micron particle size and 200mL culture filtrate were taken and kept at boiling water bath at 50ºC and 4 pH condition with constant reaction time 1 day. Then samples at different RPM rotated made and sugar produced measured by DNS method.

4.2.13 Effect of Particle size of the substrate on hydrolysis Rate

Wheat straw substrate with four different particle size and 200mL culture filtrate were taken and kept at boiling water bath at 4 pH and 50ºC and 25 RPM condition for constant time period (1 day) of treatment then produced measured by DNS method.

Need of Pretreatment

4.3 Results and Discussion

4.3.1 Effect of Substrate Concentration on Hydrolysis Rate

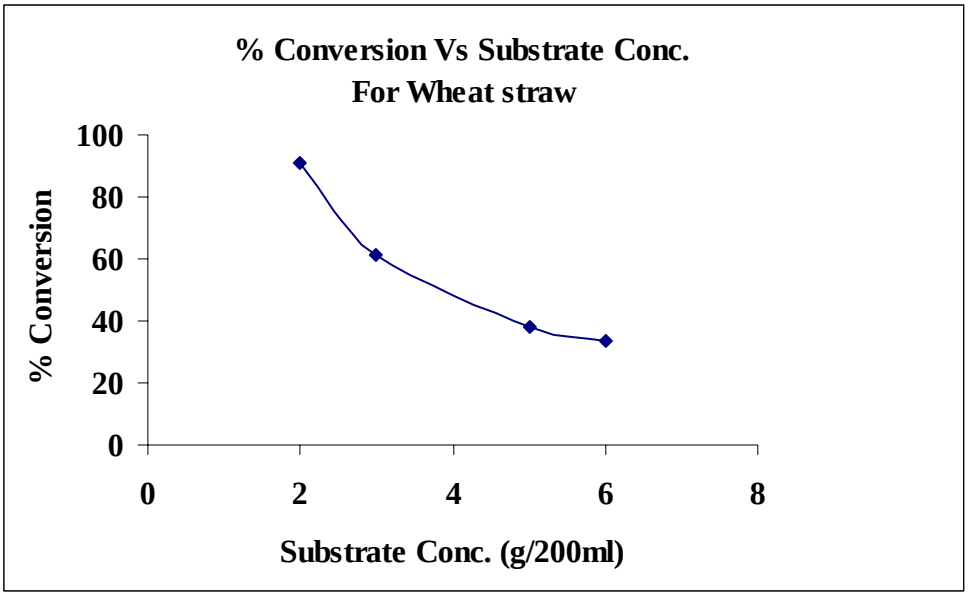

Figure 4.1: % Conversion Vs Substrate Concentration plot

Table 4.1 : Substrate Concentration effect on conversion

(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Sample No. (Wheat Straw)	Substrate Conc. (g/200ml)	Cellulose content (mg)	Sugar produced (mg/ml)	% Conversion
1	2	660	3.03	91.8
2	3	999	3.06	61.2
3	5	1650	3.13	37.9
4	6	1980	3.33	33.6

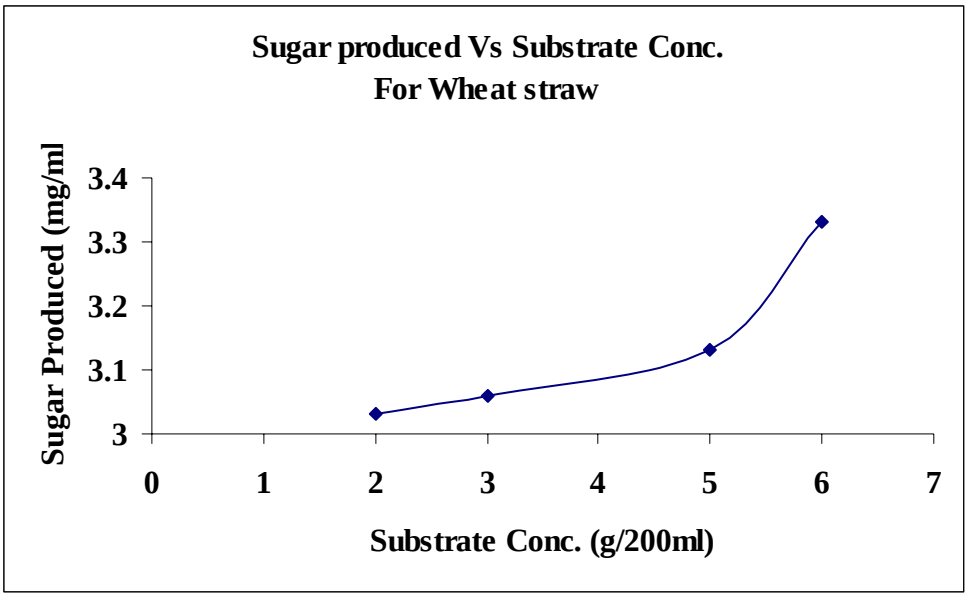

Figure 4.2: Sugar produced Vs Substrate Concentration plot.

Table 23 and Figure 19 show the experimental results to study the effect of saccharification of the substrate. This study showed that as substrate concentration increases the conversion of cellulose to sugar is decreases. And sugar produce is increases.

4.3.2 Effect of Type of Treatment on Hydrolysis Rate

Table 4.2: effect of different treatment on Rate and conversion

(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Sample No. (Wheat straw)	Type of Treatment	% Delignification	Sugar produced (mg/ml)	% Conversion
*	No Treatment	-	1.45	17.5
1	Boiling Water treated	26.7	1.76	21.4
2	Alkali (NaOH) (10%)	62.3	3.11	37.7
3	Phosphoric Acid (4%)	73.3	4.66	56.5
4	Sulfuric Acid (2%)	72.2	4.35	52.8
5	Hydrochloric Acid (4%)	61.4	2.73	33.1

The effects of different type of pretreatments are studied here. And why treatment is necessary. That can be understood by the above results. The experimental results are shown in table 4.32. It shows that without treatment there is 1.45 mg/mL sugar has been produced in and only 17% conversion has been achieved. In Boiling water treatment where much lignin not removed gives 17.5% conversion while other type of methods whose lignin removal capacities are high shows higher conversion. These experiments clearly established the need of pretreatment before enzymatic hydrolysis.

4.3.3 Effect of Lignin Removal on Hydrolysis Rate

Table 4.3: effect of % Delignification on Rate and conversion for Wheat straw

(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Sample No. (Wheat Straw)	% Delignification	Sugar produced (mg/ml)	% Conversion
1	20.3	2.10	25.4
2	34.5	2.46	29.8
3	46.1	2.73	33
4	62.3	3.11	37.7
5	71.3	4.35	52.8
6	73.2	4.92	59.7

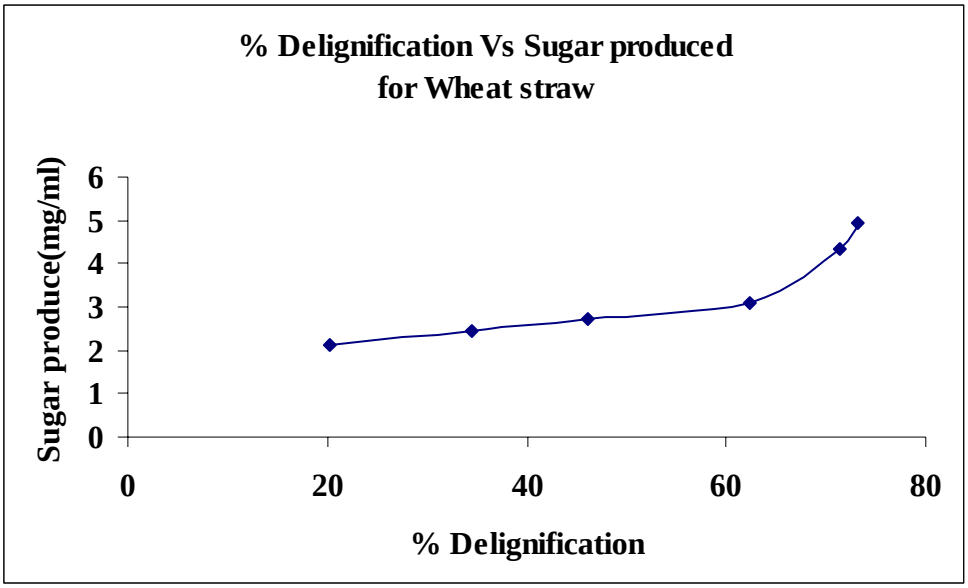

Figure 4.3: Sugar produced Vs % Delignification plot for wheat straw

Table 4.4: effect of % Delignification on Rate and conversion for Rice straw

(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Sample No. (Rice Straw)	% Delignification	Sugar produced (mg/ml)	% Conversion
1	23.1	2.27	27.6
2	39.2	2.84	34.5
3	48.9	3.39	41.2
4	58.7	3.81	46.3
5	62.5	3.91	47.4
6	64.2	4.22	51.2

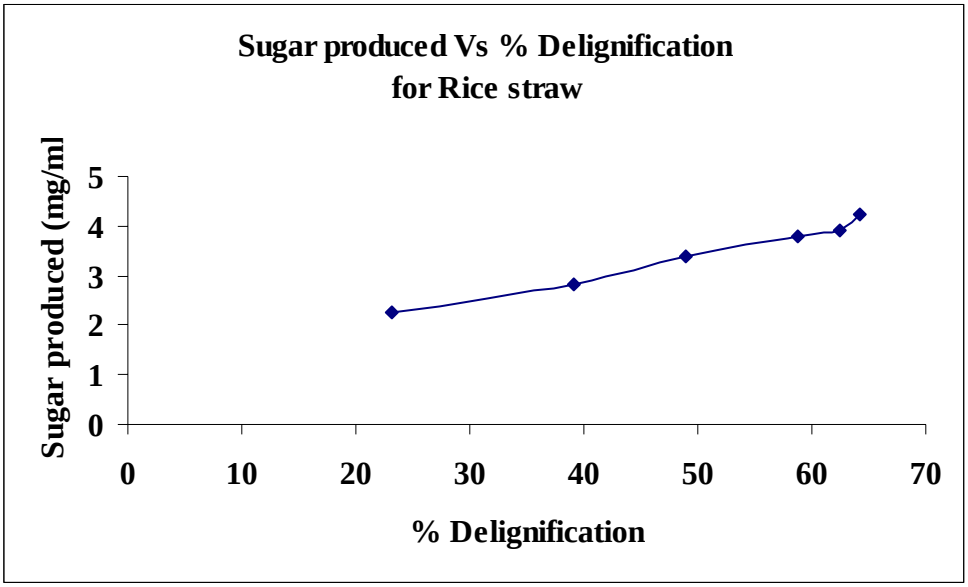

Figure 4.4: Sugar produced Vs % Delignification plot for rice straw

Table 4.5: effect of % Delignification on Rate and conversion for Jawar straw

(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Sample No. (Jawar Straw)	% Delignification	Sugar produced (mg/ml)	% Conversion
1	18.3	1.78	21.6
2	26.9	2.33	28.3
3	41.2	2.92	35.4
4	47.7	3.39	41.2
5	64.4	4.33	52.6
6	78.1	4.76	57.8

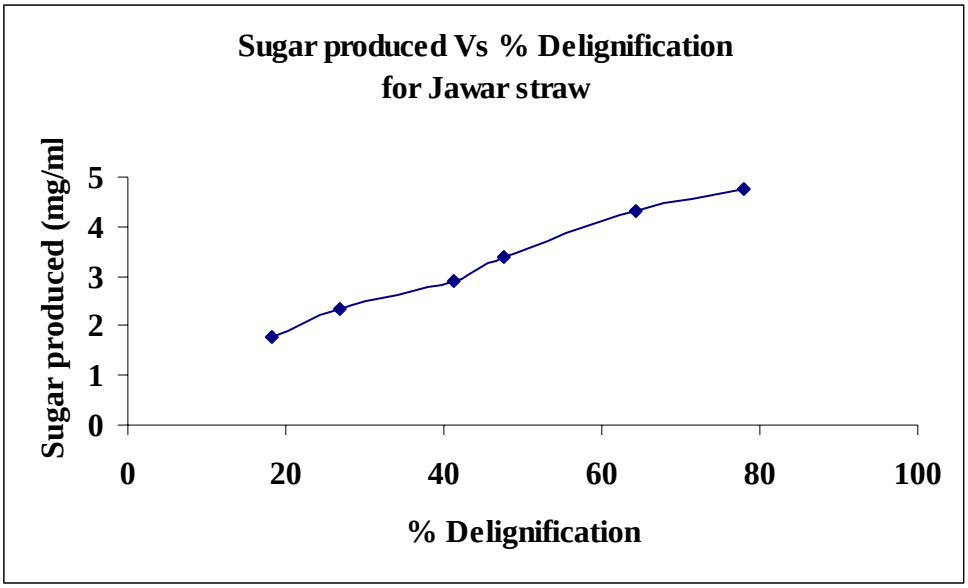

Figure 4.5: Sugar produced Vs % Delignification plot for Jawar straw

The effectiveness of pre treatment on hydrolysis is due to the removal of lignin which creates non crystalline and porous structure for better accessibility of enzyme to the substrate. Tables 4.3, 4.4, 4.5 and figures 4.3 4.4 4.4 show the effect of %Delignification on the rate and conversion of the enzymatic hydrolysis. Here this study is carried out for all three substrates (Wheat straw, Rice Straw, and Jawar Straw).

Result show that in wheat straw rate is gradually increased with % removal of lignin and in Rice straw and Jawar straw there is sharp increase in rate with %removal of lignin. This shows that wheat straw crystalline structure is more rigid and crystalline compared to other two substrate. And enzyme can more access the surface area in rice and Jawar straw compared to wheat straw.

4.3.4 Effect of Agitation on Hydrolysis Rate

Table 4.6: effect of Agitator speed on Rate of hydrolysis for Wheat straw
(Particle size 250 microns, Temperature – ambiant, pH – 4,)

Agitator Speed RPM	Sugar produced (mg/ml)
0	3.11
30	3.83
60	3.98
90	4.05
120	4.10

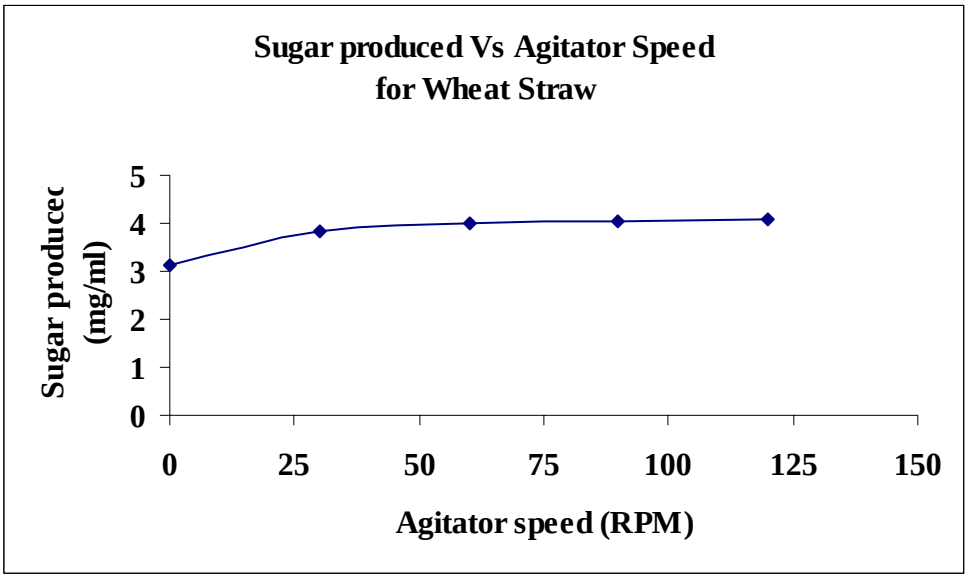

Figure 4.6: Sugar produced Vs Agitator speed plot for Wheat straw

Effect of agitation on production of reducing sugar was studied in the range of 0 to 120 RPM. Ass can be seen from figure 4.6 and table 4.6, beyond 30 RPM very little further increase in conversion was noticed. The purpose of agitation is to merely suspend the cellulose particles freely in the solution, as there is no interphase mass transfer involved.

4.3.5 Effect of Temperature on Hydrolysis Rate

Table 4.7: effect of Temperature on Rate of hydrolysis for Wheat straw

(Particle size 250 microns, pH – 4, RPM - 30)

Temperature (°C)	Sugar produced (mg/ml)
25	2.12
30	2.66
35	2.78
40	2.96
45	2.98
50	3.11
55	2.85

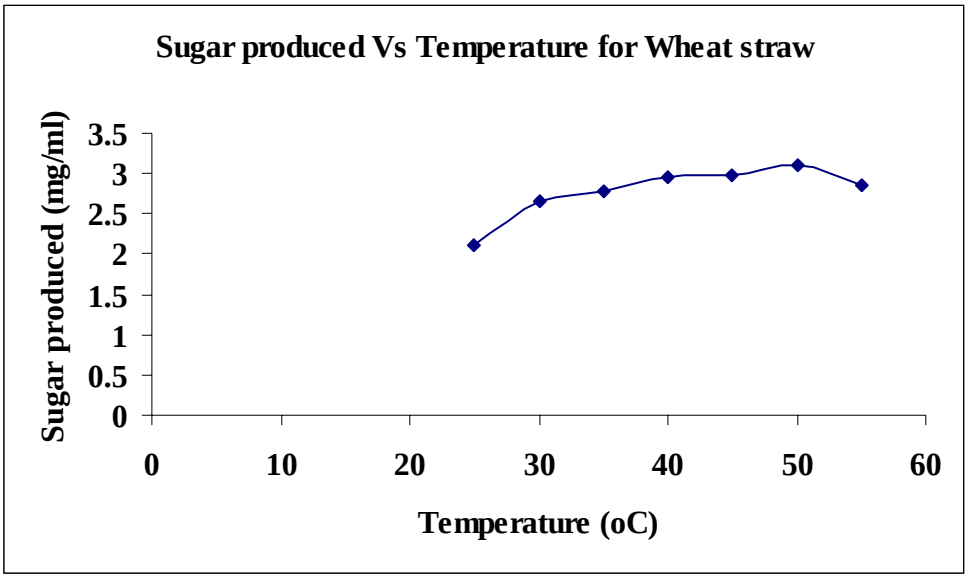

Figure 4.7: Sugar produced Vs Temperature plot for Wheat straw

The rate of enzyme catalyzed reaction increase with temperature up to a certain limit. Above a certain temperature the activity decreases with temperature because of enzyme denaturation. Therefore rate of hydrolysis is decreases

There are two parts

(I) Temperature activation

The rate varies as per the Arrhenius equation

$r = K_2[E]$

$K_2 = Ae^{-Ea/RT}$

E_a = activation energy (kcal/mol)

[E]= Active enzyme concentration (mol/L)

(II) Thermal denaturation

Kinetics of thermal denaturation can be expressed as follows:

[E] gives E_{de}

$-d[E]/dt = k_{de}[E]$

$[E] = [E]_0 e^{-kde}$

Variation in temperature may affect both K_m and V_{max} values. In general, the reaction velocity increases with increase in the temperature up to a maximum and then declines resulting in a bell shaped curve. As shown in figure 4.7 and table 4.7 rate of hydrolysis increases up to 50ºC then it is decreases.

4.3.6 Effect of pH on Hydrolysis Rate

Table 4.8: effect of pH on Rate of hydrolysis for Wheat straw
(Particle size 250 microns, Temperature – ambiant, RPM - 30)

pH	Sugar produced (mg/ml)
3.0	1.46
3.5	2.07
4.0	3.11
4.5	2.75
5.0	2.70
5.5	2.43
6.0	1.76

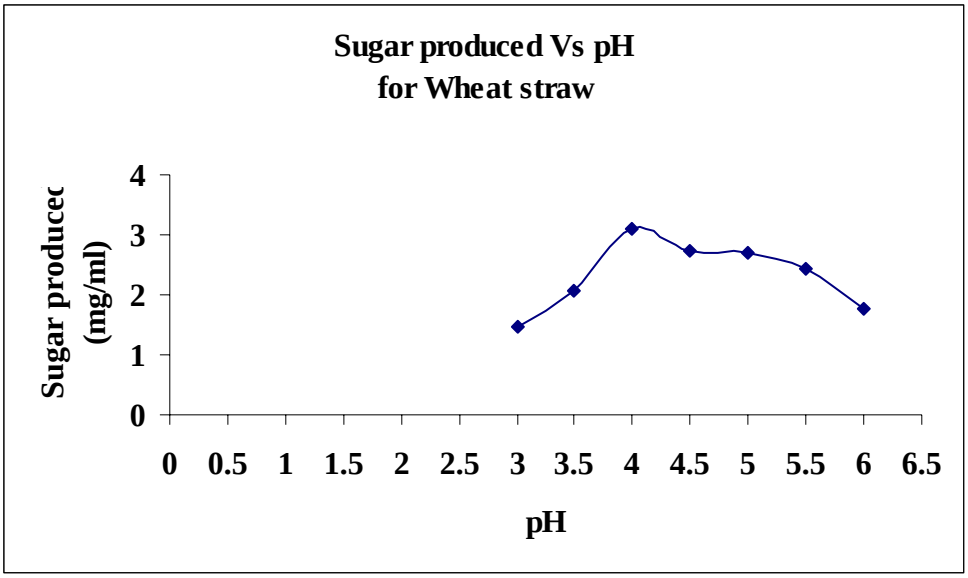

Figure 4.8: Sugar produced Vs pH plot for Wheat straw

Certain enzymes have ionic groups on their active sites and these ionic groups must be in a suitable form (acid or Base form) for the enzymes to function. Variations pH values of the medium results in changes in the ionic form of the active site, and changes in the activity of an enzyme and hence the reaction rates. The changes in the pH values may

also alter the three dimensional shape of an enzyme. For these reason enzyme is active in certain range. pH my affect the maximum rate V_{max}, K_m and enzyme activity.

Figure 4.8 show the rate of hydrolysis change with pH and it is maximum at 4 pH then it is decreases. Therefore enzyme is active in this region.

4.3.7 Effect of Particle Size on Hydrolysis Rate

Table 4.9: effect of Particle size on Rate of hydrolysis for Wheat straw

(Temperature – ambiant, pH – 4, RPM - 30)

Particle size (micron)	Sugar produced (mg/ml)
75	3.48
250	3.11
500	2.02
710	1.51

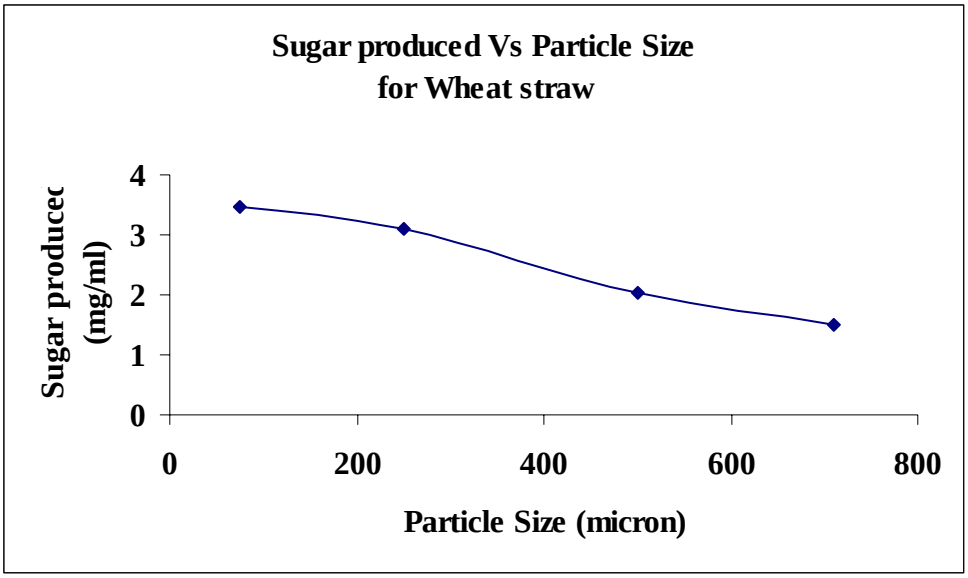

Figure 4.9: Sugar produced Vs Particle size plot for Wheat straw

Experimental results are shown in Table 4.9 and Figure 4.9. There are four particle sizes are chosen for study. Result shows that as particle size increases the rate of hydrolysis decreases because of reduction of available surface area. In small particle size there is large surface area and enzyme is more possibly allowed for reaction.

4.3.8 Effect of treatment with time on Hydrolysis Rate

Table 4.10: effect of time on Rate of hydrolysis for Wheat straw
(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Time Days	Treated Wheat straw Sugar produced (mg/ml)	Untreated Wheat straw Sugar produced (mg/ml)
0	0	0
1	0.45	0.21
2	0.93	0.39
3	1.12	0.51
4	1.43	0.73
5	1.82	0.95
6	2.14	1.15
7	2.80	1.32
8	3.11	1.45

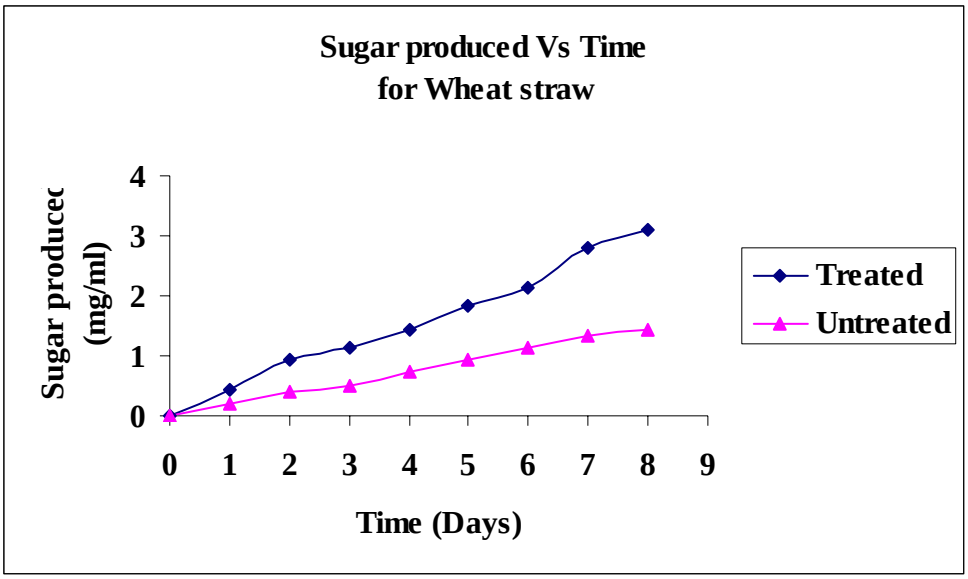

Figure 4.10: Sugar produced Vs Time plot for Wheat straw

Treated substrates gave the almost double production of sugar and graph (Figure 4.10) shows that how treated substrate is produced sugars quantitavely as compared to untreated substrate. Untreated substrate gives very less amount of sugar due to non removal of lignin.

Chapter No. 5

Kinetics

Of

Enzymatic Hydrolysis

Chapter No. 5

Kinetics of Enzymatic Hydrolysis

5.1 The The Lineweaver – Burk Method for Model Fitting

The evaluate of kinetic parameters basically involves the application of the transformation of the Michaelis-Menten Equation (MME) the transformation involve the rearrangement of the MME by using the following method.

It is also called the double reciprocal method and is comparatively simple originally; we have the MME as follows:

$$r = \frac{V_{max}\,[S]}{K_m + [S]}$$

Writing reciprocal for it is as

$$\frac{1}{r} = \frac{K_m + [S]}{V_{max}\,[S]}$$

$$\frac{1}{r} = \frac{K_m}{V_{max}\,[S]} + \frac{1}{V_{max}}$$

Where, r = Rate of hydrolysis mg/mL.hr
K_m = M. M constant mg/mL
V_{max} = Maximum rate mg/mL
[S] = Substrate Concentration mg/mL

Now, plot a graph of 1/r vs 1/[S]

Now Intercept C = $1/V_{max}$

Slope m = K_m/V_{max}

And plot cut the x axes on left hand plane that intercept on x axis is = $-1/K_m$

A double reciprocal plot give a good estimate on V_{max} but not necessarily on K_m data points at low substrate concentrations influence the slope and intercept more than those at high substrate concentrations.

5.2 Experimental Procedure

This study was done by using 10% NaOH treated three type of substrates (wheat straw, rice straw, Jawar straw) 150 micron size particle powder and culture filtrate from Trichoderma Reesei at a temperature of 50ºC. in test tube 10 ml culture filtrate and substrate with different concentrations taken and kept in boiling water bath for 1 hour at 4 pH and 50ºC The sugar produced was measured by DNS method using glucose as standard. The initial rate of hydrolysis calculated as milligrams produced / 10mL /hr. This was repeated by adding glucose in same concentration of 20mg/10mL before starting to study inhibition.

5.3 Results and Discussion

Table 5.1: The Lineweaver-Burk Method for Wheat straw without sugar addition
(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Substrate (WS) Conc. S (g/10ml)	1/S	Rate of Sugar produced r(mg/ml.hr)	1/r
0.1	10	0.9577	1.0441683
0.15	6.66	0.9624	1.039069
0.2	5	0.9639	1.037452
0.25	4	0.9655	1.0357328
0.3	3.33	0.9702	1.0307153
0.35	2.85	0.9748	1.0258515

Table 5.2: The Lineweaver-Burk Method for wheat straw with sugar addition
(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Substrate Conc. (WS + 0.2 mg/ml Sugar) S (g/10ml)	1/S	Rate of Sugar produced r(mg/ml.hr)	1/r
0.1	10	0.7358	1.35906
0.15	6.66	0.7436	1.34481
0.2	5	0.7530	1.32802
0.25	4	0.7546	1.32521
0.3	3.33	0.7639	1.30907
0.35	2.85	0.7686	1.30107

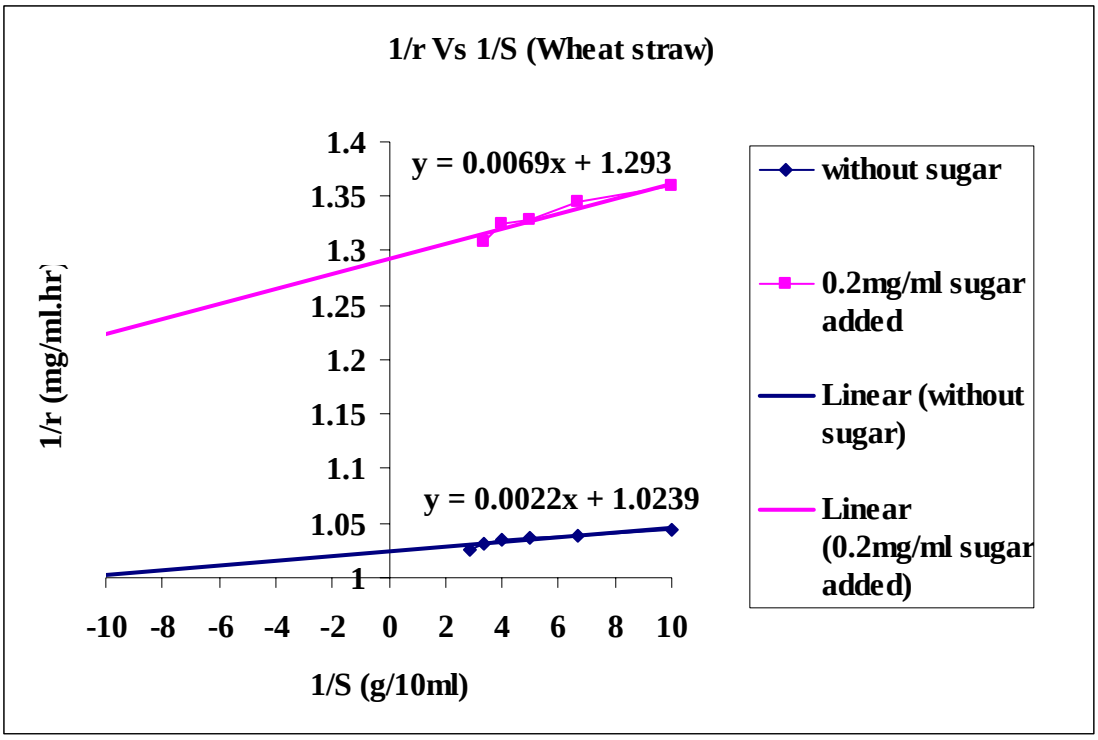

Figure 5.1: The Lineweaver-Burk Method for wheat straw

Calculation

Without Inhibitor (Without glucose addition)

Slope m = 0.0022 = K_m/V_{max}

Intercept C = 1.0239 = $1/V_{max}$

V_{max} = 0.976 mg/mL.hr

Intercept on X axis = $-1/K_m$ = -467.28

K_m = 0.00214 mg/mL

With Inhibitor (With glucose addition)

Slope m = 0.0069 = K_m/V_{max}

Intercept C = 1.293 = $1/V_{max}$

V_{max} = 0.77 mg/mL.hr

Intercept on X axis = $-1/K_m$ = -187.39

K_m = 0.00533 mg/mL

Table 5.3: The Lineweaver-Burk Method for Rice straw without sugar addition (Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Substrate (RS) Conc. S (g/10ml)	1/S	Rate of Sugar produced r(mg/ml.hr)	1/r
0.1	10	0.9951	1.00492
0.15	6.66	0.9998	1.00020
0.2	5	1.0138	1.98638
0.25	4	1.0154	1.98483
0.3	3.33	1.0170	1.98328
0.35	2.85	1.020	1.98039

Table 5.4: The Lineweaver-Burk Method for Rice straw with sugar addition
(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Substrate (RS + 0.2 mg/ml Sugar) Conc. S (g/10ml)	1/S	Rate of Sugar produced r(mg/ml.hr)	1/r
0.1	10	0.8029	1.24549
0.15	6.66	0.8170	1.22399
0.2	5	0.8263	1.21021
0.25	4	0.8357	1.1966
0.3	3.33	0.8497	1.17689
0.35	2.85	0.8669	1.15354
0.4	2.5	0.8887	1.12524

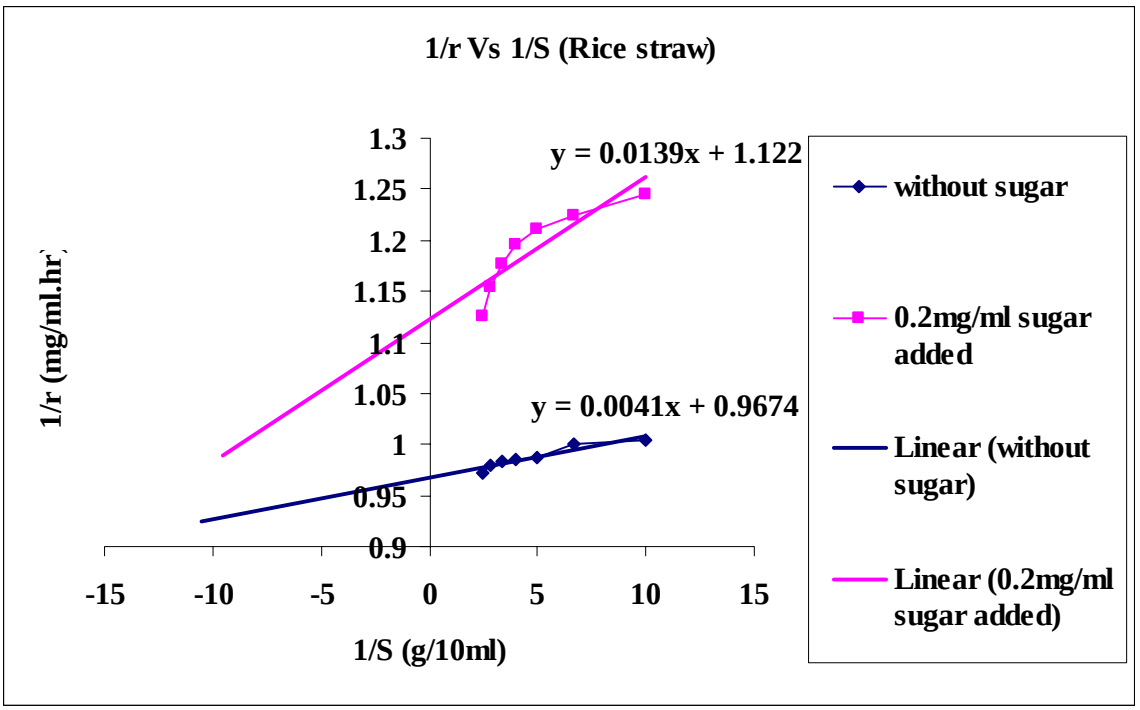

Figure 5.2: The Lineweaver-Burk Method for Rice straw

Calculation

Without Inhibitor (Without glucose addition)

Slope m = 0.0041 = K_m/V_{max}

Intercept C = 0.9674 = $1/V_{max}$

V_{max} = 1.033 mg/mL.hr

Intercept on X axis = $-1/K_m$ = -235.95

K_m = 0.00423 mg/mL

With Inhibitor (With glucose addition)

Slope m = 0.0139 = K_m/V_{max}

Intercept C = 1.122 = $1/V_{max}$

V_{max} = 0.891 mg/mL.hr

Intercept on X axis = $-1/K_m$ = -80.71

K_m = 0.0123 mg/mL

Table 5.5: The Lineweaver-Burk Method for Jawar straw without sugar addition
(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Substrate (JS) Conc. S (g/10ml)	1/S	Rate of Sugar produced r(mg/ml.hr)	1/r
0.1	10	0.9740	1.026694
0.15	6.66	0.9904	1.009693
0.2	5	0.9951	1.0049241
0.25	4	1.0123	1.987849
0.3	3.33	1.0138	1.986878
0.35	2.85	1.0185	1.981836
0.4	2.5	1.020	1.980392

Table 5.6: The Lineweaver-Burk Method for Rice straw without sugar addition
(Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Substrate Conc. (JS + 0.2 mg/ml Sugar) S (g/10ml)	1/S	Rate of Sugar produced r(mg/ml.hr)	1/r
0.1	10	0.7951	1.257703
0.15	6.66	0.8029	1.245485
0.2	5	0.8060	1.240694
0.25	4	0.8076	1.238236
0.3	3.33	0.8092	1.235788
0.35	2.85	0.8170	1.223990

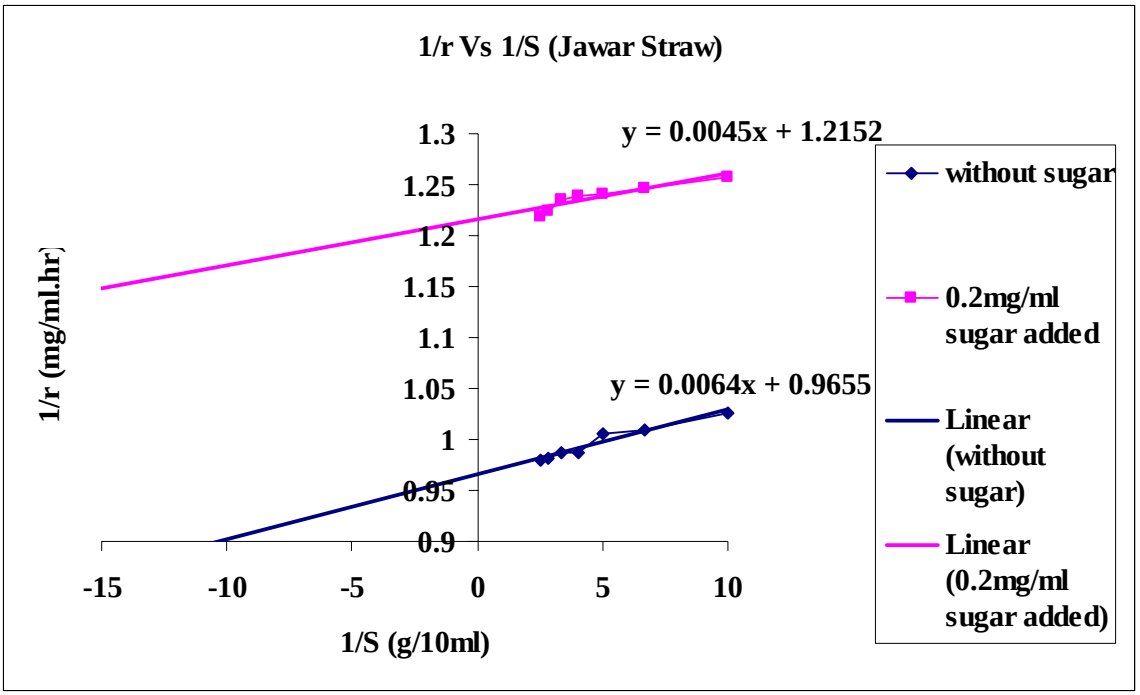

Figure 5.3: The Lineweaver-Burk Method for Jawar straw

Calculation

Without Inhibitor (Without glucose addition)

Slope m = 0.0064 = K_m/V_{max}

Intercept C = 0.9655 = $1/V_{max}$

$\mathbf{V_{max}}$ **= 1.0357 mg/mL.hr**

Intercept on X axis = $-1/K_m$ = -150.85

$\mathbf{K_m}$ **= 0.00662 mg/mL**

With Inhibitor (With glucose addition)

Slope m = 0.0045 = K_m/V_{max}

Intercept C = 1.2152 = $1/V_{max}$

V_{max} = 0.822 mg/mL.hr

Intercept on X axis = $-1/K_m$ = -250.04

K_m = 0.0034 mg/mL

Summary of Kinetic Parameters for each straw:

Table 5.7: Results comparison of The Lineweaver-Burk Method for three substrates (Particle size 250 microns, Temperature – ambiant, pH – 4, RPM - 30)

Parameter	Wheat Straw	Rice Straw	Jawar Straw
Without Inhibition			
V_{max} (mg/mL.hr)	0.976	1.033	1.0357
K_m (mg/mL)	0.00214	0.00423	0.00662
With Inhibition			
V_{max} (mg/mL.hr)	0.77	0.891	0.822
K_m(mg/mL)	0.00533	0.0123	0.0034

As we can see from the table 39 that as glucose added to inhibit the reaction therefore Maximum rate of hydrolysis is decreased in inhibition that shows the product glucose is inhibiting the reaction.

Chapter No. 6

Biomass Material Structure Analysis

Chapter No. 6

Biomass Materials Structure Analysis

6.1 Scanning Electron Microscope Results

6.1.1 Untreated Wheat straw

Figure 31 SEM image of untreated Wheat straw

6.1.2 10% Alkali (NaOH) Treated Wheat straw

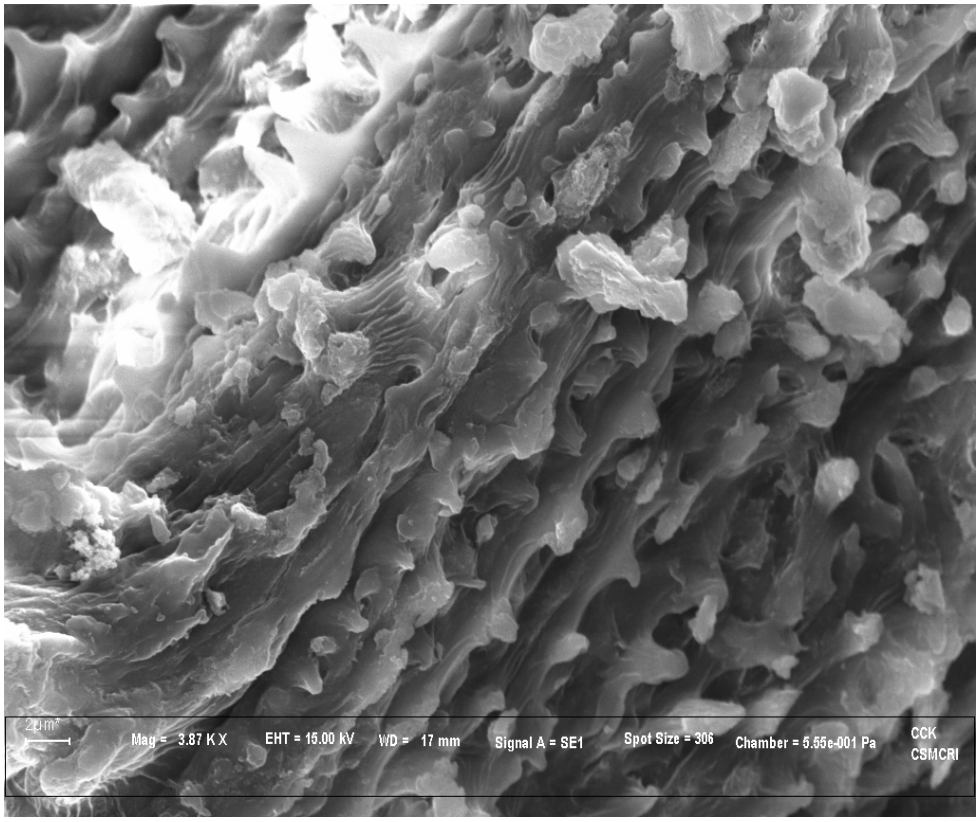

Figure 32 SEM image of 10% NaOH treated Wheat straw

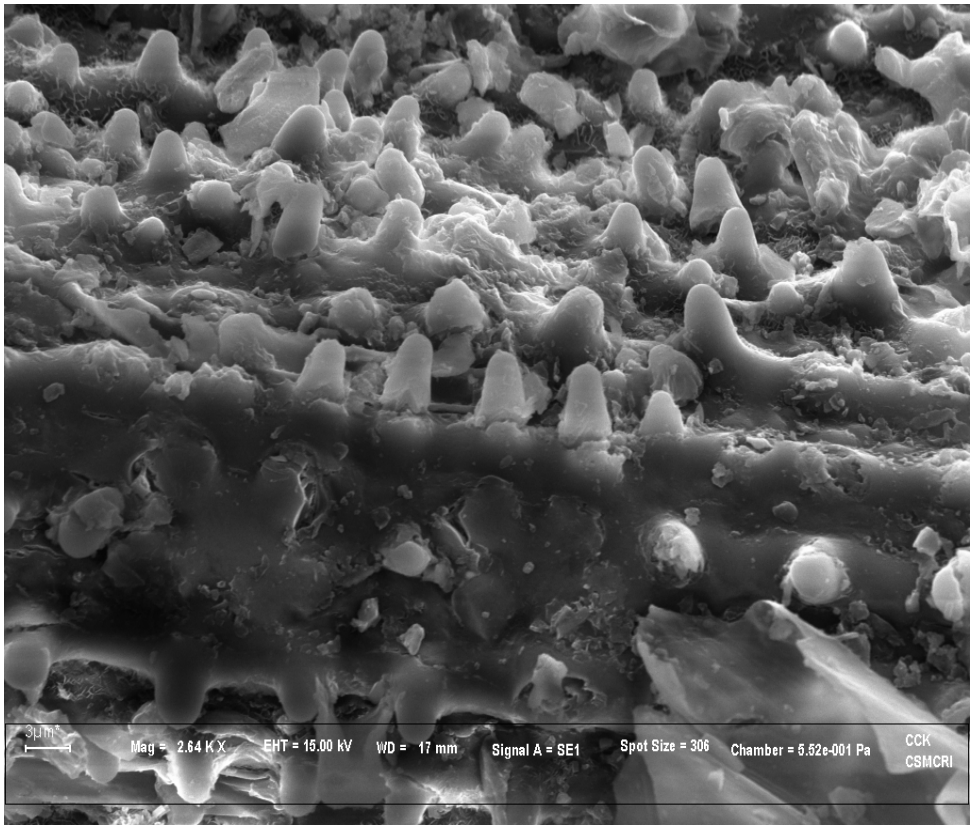

Figure 33 SEM image of 10% NaOH treated Wheat straw

6.1.4 10% Alkali (NaOH) treated Rice straw

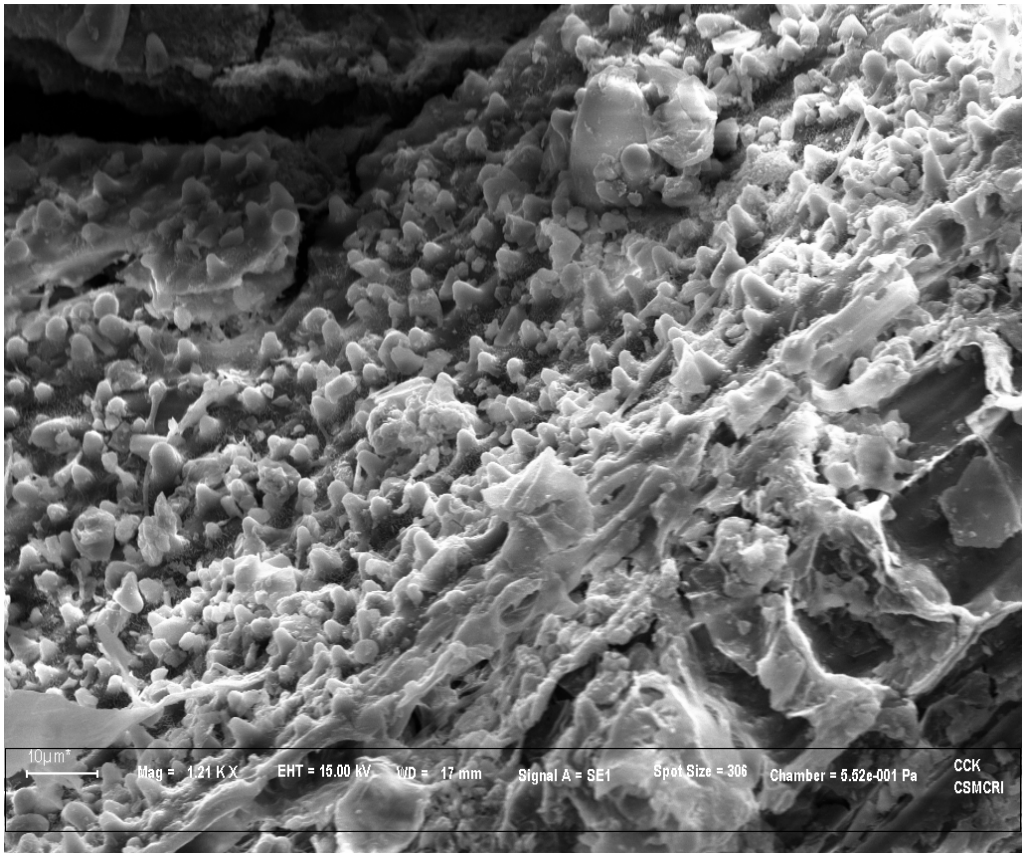

Figure 34 SEM image of 10% NaOH treated Rice straw

6.2 X – Ray Diffractometer analysis Results

6.2.1 Untreated Wheat straw

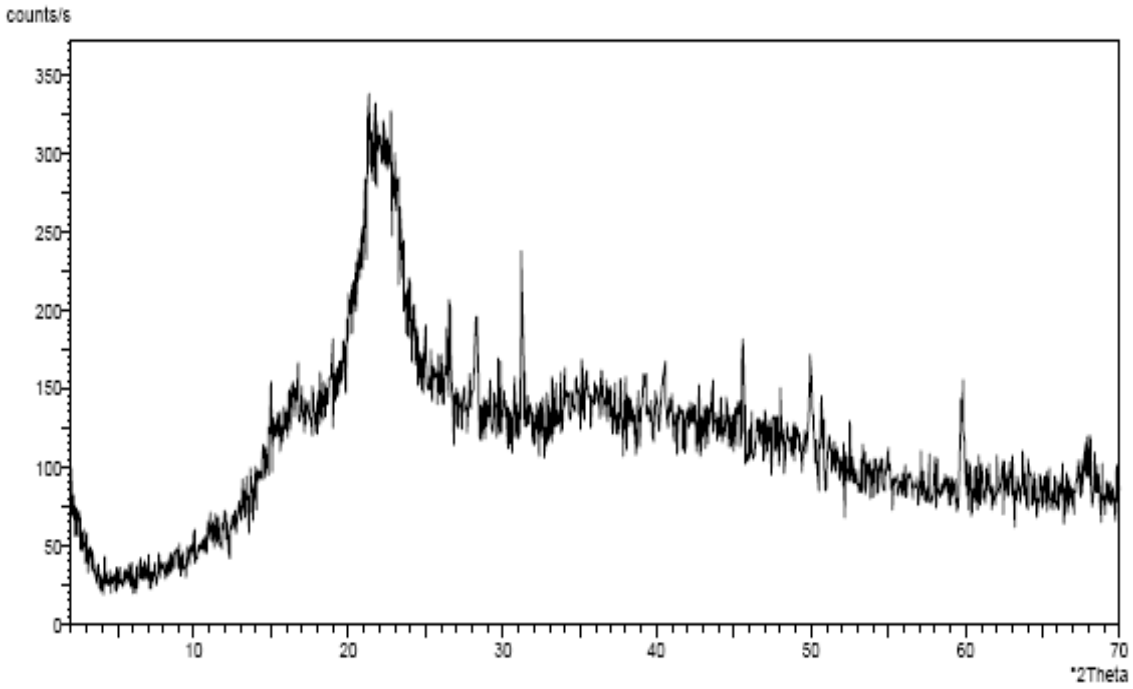

Figure 35 XRD of untreated wheat straw

6.2.2 5% Alkali (NaOH) treated Wheat straw

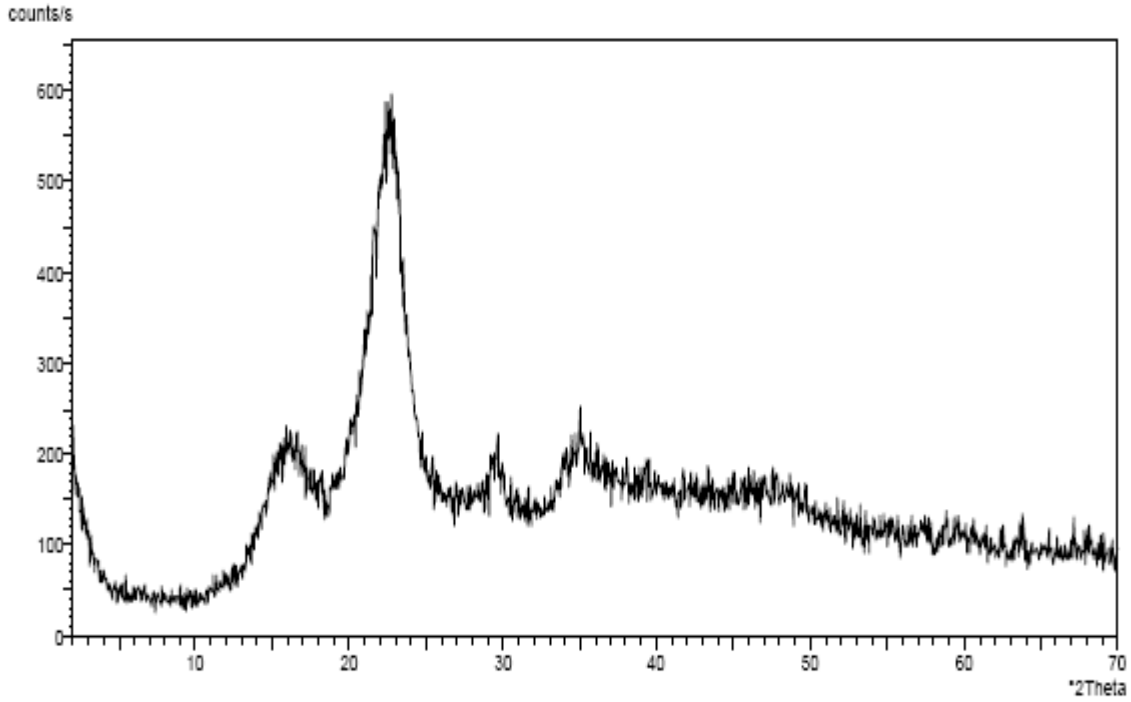

Figure 36 XRD of 5% NaOH treated wheat straw

6.2.3 10% Alkali (NaOH) treated Wheat straw

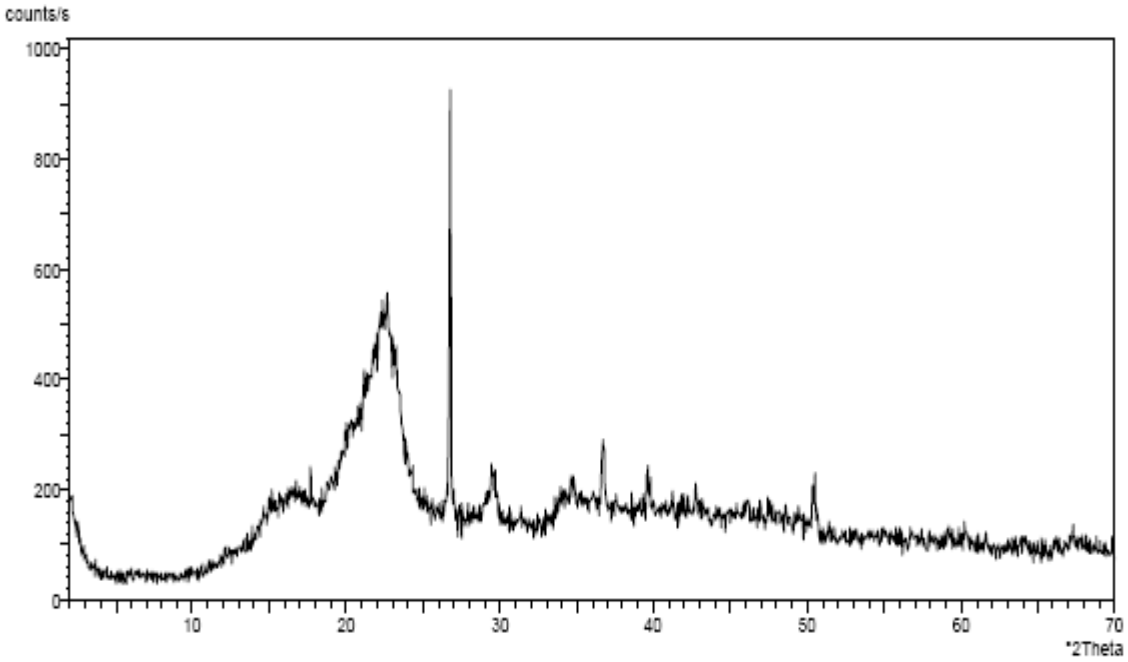

Figure 37 XRD of 10% NaOH treated wheat straw

6.2.4 Untreated Rice straw

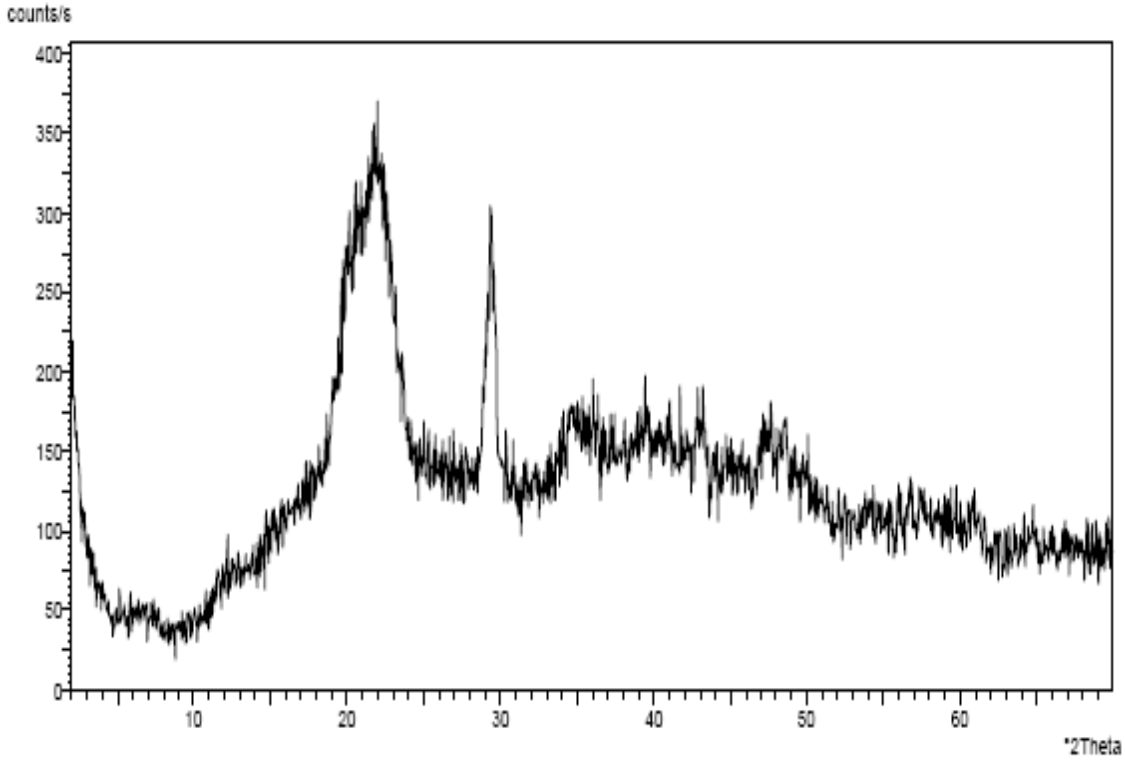

Figure 38 XRD of untreated rice straw

6.2.5 10% Alkali (NaOH) treated Rice straw

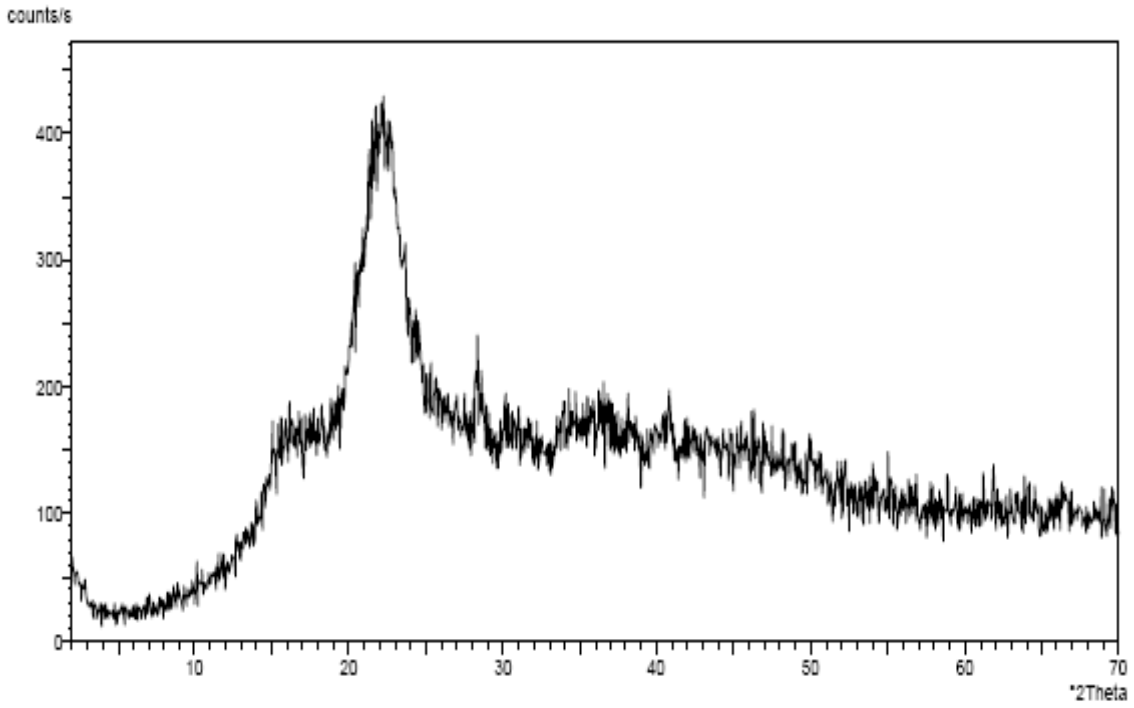

Figure 39 XRD of 10% NaOH treated rice straw

6.3 Results and Discussion

6.3.1 SEM

Because a large fraction of hemicellulose and lignin was removed by pretreatment, there was some physical changes in the straw. For this reason, SEM pictures of pretreated and untreated Wheat straw and treated rice straw (Figure 34) were produced. The untreated wheat straw (Figure 31) exhibited rigid and highly ordered fibrils, while the fibers of pretreated samples (Figure 32) by NaOH were distorted. The microfibrils were also separated from the initial connected structure and fully exposed, thus increasing the external surface area and the porosity.

The SEM pictures indicated that the straw structure was deformed and fibers exposed by the treatment.

6.3.2 XRD

The chemical composition was not the sole factor influencing the enzymatic hydrolysis. Physical properties and cellulose microstructure were among the potential factors influencing enzymatic hydrolysis. One frequently cited property was wheat straw and rice straw crystallinity. The XRD of pretreated and untreated wheat straw and rice straw and alpha cellulose was measured showing that crystallinity of Wheat straw and rice straw actually decreased with increase in concentration of NaOH in treatment. The content of alpha cellulose increased due to partly removal of lignin and hemicellulose.
we can observe the peak of Figure 34 to 39.

The XRD spectra showed decrease of crystallinity with increase of chemical concentration in pretreatment, in favour of following enzymatic hydrolysis.

Chapter No. 7

Conclusion And Future Scope

Chapter No. 7
Conclusion and Future Scope

7.1 Conclusion

Studies on delignification indicated that each method has its own advantages and limitations. Though boiling water treatment is economical and non hazardous, But it gives low delignification. The alkali treatment gives good results of delignification but at higher concentrations range. As per evaluation of that treatment 10% NaOH treatment to all three straws gives good result of delignification. The acid treatment gives good results. Out of three acids, Phosphoric acid, Sulfuric Acid, and hydrochloric acid if we compare the results of three, sulfuric acid give good performance and delignification. From time of treatment study one hour is sufficient for treatment.

From the results of the studies on hydrolysis of wheat straw using enzyme from culture Trichoderma reesei it can be concluded that the compared to coarse particles, fine particles yield higher conversion to sugars.

Pretreatment before hydrolysis is essential, as the untreated biomass materials gave poor yield and sugar formation. The yield of sugar formation is related to the extent of delignification by pretreatment. Experimental results show that as delignification increases there is increase in sugar formation

The optimum pH condition is 4.0 and at temperature 50ºC and 30 RPM there is maximum sugar formation high enzyme activity.

The kinetic study shows that value of maximum rate is decreases with addition of sugar and induction of inhibitor. It shows that the sugar produced in reaction is inhibit the reaction as a result of decrease in sugar formation.

The physical change due to 10%Alkali (NaOH) treatment to Wheat straw and Rice straw clearly can be seen in SEM Pictures of treated and untreated straw samples. After removal of lignin and hemicellulose the there is increase in crystallinity. It can be prove with XRD graphs in which alpha cellulose peak gradually increase with increase in concentration of NaOH.

7.2 Future Scope

Future work will focus on increasing efficiency of the enzyme and therefore it can help in increase in conversion and yield of sugar. From literature survey and experiments it is evident that the maximum conversion of cellulose to ethanol can be obtained from Trichoderma reesei is range of 50 to 60%. Thus an important opportunity is to enhance the yields and reduce the cost of pretreatment which will enhance the possibility of cheaper bioethanol fuel.

It is evident that In addition of positively charge colloidal materials to the growth medium there can be enhance the cellulase production from Trichoderma reesei. Such kind of research will be help to increase the efficiency of the enzyme.

Future outlook also depends on the advanced and economical pretreatment methods which give high delignification ultimate increase the rate of hydrolysis.

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2 Janusz Szczodrak and Jan Fiedurek; Technology for Conversion of Lignocellulosic Biomass to Ethanol; Biomass and Bioenergy Vol. 10, Nos S/6, pp. 367-375, 1996

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9 Rick Elander; ppt on Ethanol From CellulosicMaterials; U.S. DOE Fuel Ethanol Workshop Honolulu, HI November 14, 2002

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PPTs

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Appendices

Appendix I

Estimation of Reducing Sugar Measurement by DNS Method

Estimation of Reducing Sugar by Dinitrosalicylic Acid (DNS) Method

Sugars with reducing property (arising out of the presence of a potential aldehyde or keto group) are called reducing sugars. Some of the reducing sugars are glucose, galactose, lactose and maltose. The Dinitrosalicylic acid is one of the classical and widely used methods for the quantitative determination of reducing sugars. DNS method is simple, sensitive and adoptalble during handling of a large number of samples at a time.

Materials:

- Dinitrosalicylic Acid Reagent (DNS Reagent):

Dissolve by stirring 1g Dinitrosalicylic acid, 200mg crystalline phenol and 50mg sodium sulphite in 100mL 1% NaOH. Store at 4ºC. Since the reagent deteriorates due to sodium sulphite, if long storage is required, sodium sulphite may be added at the time of use.

- 40% Rochelle salt solution (Potassium sodium tartrate).

Procedure:

1. Pipette out 0.5 to 3mL of the extract in test tubes and equalize the volume to 3mL with water in all the tubes.
2. Add 3mL of DNS reagent.
3. Heat the contents in a boiling water bath for 5min.
4. When the contents of the tubes are still warm, add 1mL of 40% Rochelle salt solution.
5. Cool and read the intensity of dark red colour at 510nm.
6. Run a series of standards using glucose (0 to 10mg/ml) and plot a graph.

Calculation

Calculate the amount of reducing sugars present in the sample using the standard graph.

Reference:

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Appendix II

Biomass Composition Analysis Method

Principle:

Refluxing the sample material with acid detergent solution removes the water-solubles and materials other than the fibrous component. The left-out material is weighed after filtration, dried, treated with 72% H_2SO_4 and filtered, dried and ashed. The loss of weight on ignition gives the acid detergent lignin.

Materials

- Acid Detergent Solution (ADS)
  Dissolve 20g cetyl trimethyl ammonium bromide in one liter of 1N sulphuric acid.
- 72% H_2SO_4 (v/v)
- Acetone
- Round Bottom Flask and Refluxing set
- Muffle Furnace
- Sintered Glass Crucible – G2]

Procedure

1. 1g substrate + 100mL ADS then heat to boil for 5 to 10min.
2. After boiling starts, Reflux for 1h.
3. Filtered the content and wash with hot water twice.
4. Wash with acetone and breakup lumps. Repeat the acetone washing until filtrate will colourless.
5. Dry at 100ºC for 3h.
6. Weigh after cooling It is Acid Detergent Fibre (ADF) and weigh of it is W
7. With ADF add 25-50 mL of 72% H_2SO_4 and 1g Asbestos and stirring for 3h.

8. dilute the acid with distilled water and filter with pre weighed Whatman filterpaper No. 1 then wash the residue several times. Dry filter paper at 100ºC and weigh after cooling. Say it W_1 (After subtracting the weigh of filter paper)
9. Then put the matter on pre weighed silica crucible in furnace at 550ºC for 3h. cool the crucible and weigh. Say it W_2 (after subtracting the weigh of crucible and fitler paper weigh). W_2 is ash content.
10. For making blank sample 1g of asbestos + 72% H_2SO_4 then stirring for 3h and dilute and washing with filter paper then drying at 100ºC and weigh after cooling say it W_{1b} then put it into furnace cool and weigh say it W_{2b}

Calculation

Acid Detergent Lignin (ADL)

$$ADL = (W_1 - W_{1b}) - (W_2 - W_{2b})$$

$$\%ADL = \frac{(W_1 - W_{1b}) - (W_2 - W_{2b})}{\text{Weight of Sample}} \times 100$$

$$\text{Lignin Remained} = W_1 - \text{Ash}(W_2)$$

$$\text{Cellulose} = W - W_1$$

Hemicellulose = Weight of sample – Cellulose – Lignin – Ash – Moisture

Reference:

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2 Updegroff, D M. Anal Biochem 1969; 32:420

Appendix III

X – Ray Diffraction Analysis Results

Sheet No.	Type of Samples
1.	Sample Analysis of Untreated Wheat straw
2.	Sample Analysis of 5% NaOH treated Wheat straw
3.	Sample Analysis of 10% NaOH treated Wheat straw
4.	Sample Analysis of Untreated Rice straw
5.	Sample Analysis of 10% NaOH treated Rice straw

Description:
Untreated wheat straw 7-5-07

Original scan: XRD-1 Date: 5/7/07 13:45
Description of scan:
Untreated wheat straw 7-5-07

Used wavelength: K-Alpha1

K-Alpha1 wavelength (Å): 1.54056
K-Alpha2 wavelength (Å): 1.54439
K-Alpha2/K-Alpha1 intensity ratio : 0.50000
K-Alpha wavelength (Å): 1.54056
K-Beta wavelength (Å): 1.39222

Peak search parameter set: **As Measured Intensities**
Set created: 2/21/03 13:11
Peak positions defined by: Minimum of 2nd derivative
Minimum peak tip width (°2Theta): 0.00
Minimum peak tip width (°2Theta): 1.00
Peak base width (°2Theta): 2.00
Minimum significance: 0.60

d-spacing (Å)	Relative Intensity (%)	Angle (°2Theta)	Peak Height (counts/s)	Background (counts/s)	Tip Width (°2Theta)	Significance
4.16123	71.20	21.33493	79.33	240.99	0.30000	0.65
3.34931	46.85	26.59202	52.20	144.12	0.15000	0.80
3.14883	54.63	28.31922	60.86	135.76	0.15000	1.28
2.86128	100.00	31.23425	111.42	126.23	0.15000	0.76
2.22648	29.12	40.48129	32.45	129.20	0.30000	0.96
1.98772	40.88	45.60045	45.54	117.71	0.15000	1.16
1.82478	56.45	49.93722	62.89	107.83	0.30000	2.00
1.79907	22.97	50.70114	25.59	104.16	0.20000	0.66
1.54598	38.95	59.76806	43.40	85.20	0.30000	2.11
1.50492	16.80	61.57312	18.72	83.45	0.35000	0.75
1.38270	15.56	67.70902	17.34	81.83	0.80000	0.82

Description:
5% NaOH treated wheat straw 7-5-07

Original scan: XRD-2 Date: 5/7/07 14:13
Description of scan:
5% NaOH treated wheat straw 7-5-07

Used wavelength: K-Alpha1

K-Alpha1 wavelength (Å): 1.54056
K-Alpha2 wavelength (Å): 1.54439
K-Alpha2/K-Alpha1 intensity ratio : 0.50000
K-Alpha wavelength (Å): 1.54056
K-Beta wavelength (Å): 1.39222

Peak search parameter set: **As Measured Intensities**
Set created: 2/21/03 13:11
Peak positions defined by: Minimum of 2nd derivative
Minimum peak tip width (°2Theta): 0.00
Minimum peak tip width (°2Theta): 1.00
Peak base width (°2Theta): 2.00
Minimum significance: 0.60

d-spacing (Å)	Relative Intensity (%)	Angle (°2Theta)	Peak Height (counts/s)	Background (counts/s)	Tip Width (°2Theta)	Significance
3.92889	100.00	22.61279	230.99	327.72	0.60000	1.41
3.02048	18.49	29.54946	42.70	149.33	0.70000	1.92
2.55431	23.65	35.10271	54.64	170.28	0.30000	0.64
1.60295	6.92	57.44131	15.99	102.59	0.60000	0.61
1.46092	7.60	63.64038	17.55	91.95	0.60000	1.08

Description:
7-5-07

Original scan: XRD-3 Date: 5/7/07 14:39
Description of scan:
7-5-07

Used wavelength: K-Alpha1

K-Alpha1 wavelength (Å): 1.54056
K-Alpha2 wavelength (Å): 1.54439
K-Alpha2/K-Alpha1 intensity ratio : 0.50000
K-Alpha wavelength (Å): 1.54056
K-Beta wavelength (Å): 1.39222

Peak search parameter set: **As Measured Intensities**
Set created: 2/21/03 13:11
Peak positions defined by: Minimum of 2nd derivative
Minimum peak tip width (°2Theta): 0.00
Minimum peak tip width (°2Theta): 1.00
Peak base width (°2Theta): 2.00
Minimum significance: 0.60

d-spacing (Å)	Relative Intensity (%)	Angle (°2Theta)	Peak Height (counts/s)	Background (counts/s)	Tip Width (°2Theta)	Significance
5.00264	6.61	17.71467	44.79	177.62	0.15000	0.78
4.40780	18.86	20.12868	127.84	179.49	0.60000	0.86
3.91657	48.94	22.68487	331.78	189.40	0.60000	1.55
3.81636	33.50	23.28871	227.11	191.75	0.40000	0.71
3.32881	100.00	26.75876	677.96	152.81	0.15000	3.61
3.01768	11.68	29.57750	79.16	144.11	0.30000	0.68
2.58061	9.39	34.73355	63.63	148.44	0.20000	0.60
2.44614	19.64	36.70902	133.12	152.88	0.15000	0.89
2.27426	8.48	39.59478	57.48	159.36	0.15000	0.79
1.80834	7.40	50.42298	50.15	123.86	0.10000	0.64
1.41074	1.92	66.18736	12.99	90.26	0.40000	0.61
1.39118	3.42	67.24105	23.18	92.18	0.60000	0.72

Description:
Untreated Rice Straw 7-5-07

Original scan: XRD-4 Date: 5/7/07 15:08
Description of scan:
Untreated Rice Straw 7-5-07

Used wavelength: K-Alpha1

K-Alpha1 wavelength (Å): 1.54056
K-Alpha2 wavelength (Å): 1.54439
K-Alpha2/K-Alpha1 intensity ratio : 0.50000
K-Alpha wavelength (Å): 1.54056
K-Beta wavelength (Å): 1.39222

Peak search parameter set: **As Measured Intensities**
Set created: 2/21/03 13:11
Peak positions defined by: Minimum of 2nd derivative
Minimum peak tip width (°2Theta): 0.00
Minimum peak tip width (°2Theta): 1.00
Peak base width (°2Theta): 2.00
Minimum significance: 0.60

d-spacing (Å)	Relative Intensity (%)	Angle (°2Theta)	Peak Height (counts/s)	Background (counts/s)	Tip Width (°2Theta)	Significance
5.82233	69.71	15.20477	26.46	119.10	1.00000	0.90
3.14876	97.17	28.31987	36.88	161.10	0.15000	0.69
2.32786	24.93	38.64635	9.46	151.84	0.70000	0.72
2.20707	100.00	40.85295	37.95	149.10	0.40000	0.64
1.63817	38.29	56.09543	14.53	103.09	0.40000	0.72

Description:
7-5-07

Original scan: XRD-5 Date: 5/7/07 15:36
Description of scan:
7-5-07

Used wavelength: K-Alpha1

K-Alpha1 wavelength (Å): 1.54056
K-Alpha2 wavelength (Å): 1.54439
K-Alpha2/K-Alpha1 intensity ratio : 0.50000
K-Alpha wavelength (Å): 1.54056
K-Beta wavelength (Å): 1.39222

Peak search parameter set: **As Measured Intensities**
Set created: 2/21/03 13:11
Peak positions defined by: Minimum of 2nd derivative
Minimum peak tip width (°2Theta): 0.00
Minimum peak tip width (°2Theta): 1.00
Peak base width (°2Theta): 2.00
Minimum significance: 0.60

d-spacing (Å)	Relative Intensity (%)	Angle (°2Theta)	Peak Height (counts/s)	Background (counts/s)	Tip Width (°2Theta)	Significance
4.46196	62.16	19.88184	105.41	152.82	1.00000	1.13
4.04531	94.83	21.95380	160.81	172.34	0.80000	1.05
3.03955	100.00	29.35989	169.57	132.59	0.15000	0.79
2.57510	12.68	34.81024	21.50	149.22	1.00000	0.78
2.09873	14.81	43.06424	25.11	140.43	0.40000	1.27
1.92151	18.02	47.26553	30.55	129.50	0.60000	1.06
1.87239	22.95	48.58422	38.92	128.44	0.40000	0.65
1.52061	10.84	60.87012	18.39	93.94	0.80000	0.63