PROFILING OF SELECTED GRASS SPECIES FOR BIOETHANOL PRODUCTION

A Thesis

Submitted in Partial Fulfilment of the Requirements for the Award of the Degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

This is to declare that the matter embodied in this thesis entitled **"Profiling of Selected Grass Species for Bioethanol Production**" is the result of investigations carried out by me under the supervision of **Prof. Utpal Bora** and **Prof. Pinakeswar Mahanta** and is submitted to the Indian Institute of Technology Guwahati, Guwahati-781039, Assam, India for the award of degree of *Doctor of Philosophy in Energy*. This work has not been submitted elsewhere for any degree or diploma of any institute or university to the best of my knowledge and belief.

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CERTIFICATE

This is to certify that the work described in this thesis entitled "**Profiling of Selected Grass Species for Bioethanol Production"** by **Mr. Yengkhom Disco Singh (Roll No.:11615103)** for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision in the Center for Energy, Indian Institute of Technology Guwahati-781039, Assam, India and this work has not been submitted elsewhere for a degree.

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Dedicated to Almighty, Ema, Baba, Panda Sir, Panda Mam, My family and all teachers

....Disco

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March, 2017 ***Yengkhom Disco Singh***

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SYNOPSIS

Due to the long term availability, low cost and easily available of lignocellulosic biomass (LCB) such as agricultural residues and grasses tends to be a sustainable feedstock for production of biofuel and bio-based products. The LCB to biofuel conversion process involves series of significant steps such as pretreatment, hydrolysis and fermentation. However, the biochemical conversion process faces various technical challenges of being contamination with phenolic compounds, aromatics, aliphatic acids, furan aldehydes, inorganic ions, acetic acid, formic acid, levulinic acid, 5-hydroxymethyl-2-furaldehyde (HMF), vanillin, syringaldehyde, conferyl aldehyde and bioalcohol or other fermentation products. Despite of the existing problems and challenges, LCB is considered as one of the most sustainable potential feedstock used for production of biofuel. The feedstock such as *Saccharum spontaneum*, *Phragmites karka*, and switch grass has been extensively explored by many researchers for its bioethanol production. To find a new raw material, that can be utilized as feedstock for biofuel production is another challenged in the bioethanol production. In this regard, we aim to explore the LCB found in Kamrup district of Assam and Thoubal district of Manipur of North-East India for biofuel production. The characterization of lignocellulosic biomass gives prior information about the biomass for its fuel content before going through pretreatment and enzymatic saccharification.

Although many advances have been made in the field of biochemical conversion of biomass to biofuel, there are some sort of technical constraints, which limit the existing protocol especially in pretreatment technology. The challenges such as lack of a common pretreatment method for all biomass as well as accumulation of lignin and other hydrolysate inhibitors, which make the barrier in sugar conversion, can be counted.

Further, there is much scope for lignocellulosic biomass in terms of sustainability and process integrity. The high-throughput techniques have been developed and aiming in mass production of bioethanol for future alternate fuels. The concept of bio-refinery system have been developed to produce fuel, power, heat and value added chemicals without any waste residue. The bio-refinery system is an analogous to today's petroleum refinery, which produce multiple fuels and products from petroleum.

In the present study, 29 species of lignocellulosic biomasses were collected and characterized to check their energy content. The characterization was done based on the proximate, ultimate, and compositional (carbohydrate) analysis of the lignocellulosic biomass. Based on

the physico-chemical characterization and energy content of the biomass, the best potential candidate was considered as *Eragrostis airoides*. Further, this biomass was pretreated, saccharified and fermented to produce ethanol. Physico-chemical pretreatment condition of different concentration of acid/alkali autoclaved was performed. The enzymatic hydrolysis was carried out with *Trichoderma reesei* strain procured from IMTECH Chandigarh. Among the pretreatment conditions employed, the maximum ethanol yield (17.56 g/L) was obtained in 5 % acid + autoclave for *Saccharomyces cerevisiae* and for Hamei treatment was 18.66 g/L. However, the poor yield was obtained in alkali treatment.

The thesis is organized into six (6) chapters as described herein.

Chapter 1 describes the biomass energy and its usefulness in details. It also described about the status of used of biomass energy in India and around the world according to IEA 2014 and 2015. The Indian scenario for bioethanol production is far behind the targeted blending of 20% according to National biofuel policy, 2009. It also described about the current production of ethanol in India which is totally depend on sugar molasses. The current production of ethanol for India is totally depend on the sustainable production of sugar molasses. The optimal supply of sugar does not correspond with the demand. Therefore, there is always mismatch production of ethanol from the sugar mill. The chapter is aimed at explaining the target problem, evolving the rationale behind the thesis and formulation of objectives for the study to address some of the existing lacunae in the field.

Chapter 2 discussed a systematic review of literature to understand the various existing technologies in other to devise an effective plan of work to accomplish the chosen objectives of the research work. The chapter discussed in detail of the feedstock for bioethanol production, composition and structure of lignocellulosic biomass, ethanol production from biomass, pretreatment technologies, enzymatic hydrolysis and fermentation. The chapter also involves the review of the studies considering following major aspects of cellulosic ethanol production:

(a) *Biomass composition:* The main components of the lignocellulosic biomass are cellulose, hemicellulose, lignin, and other inorganic materials. The carbohydrate component (cellulose and hemicellulose) is utilized to convert into bioethanol by using the microbial fermentation.

(b) *Pretreatment of lignocellulosic biomass:* The review also covered the various pretreatment technologies and their impact on the lignocellulosic biomass. These include acid, alkali, enzymes and biological pretreatment methods.

(c) *Enzymatic hydrolysis of cellulosic biomass:* One of the main components in conversion of lignocellulosic biomass to bioethanol is enzymatic hydrolysis where various enzymes likes cellulases plays a major role. The economy of the ethanol production depends on the potential of efficiency of the enzymatic hydrolysis and pretreatment technologies.

(d) *Fermentation for ethanol production:* The chapter includes different modes of ethanol fermentation.

Chapter 3 describes the collection and identification of plant species of Kamrup district of Assam and Thoubal district of Manipur of North-East India for its bioethanol production. 29 species of lignocellulosic biomass (LCB) were collected.

Chapter 4 discussed the physical, chemical and thermochemical characterization of biomass sample collected from Kamrup district of Assam and Thoubal district of Manipur of North-East India. The thermochemical characterization was performed on each sample by proximate, ultimate and compositional analysis.

Chapter 5 describes the conversion of lignocellulosic biomass (*Eragrostis airoides)* to bioethanol by microbial fermentation. The enzymatic saccharification and microbial fermentation was carried out. Physico-chemical pretreatment condition of different concentration of acid and alkali was performed in order to determine their effect on the enzymatic hydrolysis and the fermentability of the cellulosic portion of the lignocellulosic biomass.

Chapter 6 described the conclusion and scope of lignocellulosic biomass for bioethanol production. The cellulosic-based biofuel is a potential alternative to first generation of biofuels. One of the most costly steps in bioconversion process of lignocellulosic biomass is the pretreatment condition and presence of high recalcitrance. Although the lignocellulosic materials are versatile, it is very difficult to have a general pretreatment method. However, efforts are going on to design a common pretreatment methods that would be effective against a wide range of cellulosic material.

NOMENCLATURE

amu	**:**	Atomic mass unit
kJ	**:**	Kilojoule
MJ	**:**	Megajoule
kt	**:**	Kilo ton
mt	**:**	Mega ton
ha	**:**	Hectare
CO_2	**:**	Carbon dioxide
O_2	**:**	Oxygen
t	**:**	Time
H/C	**:**	Hydrogen to Carbon ratio
O/C	**:**	Oxygen to Carbon ratio
W	**:**	Weight of the sample
V	**:**	Enzyme hydrolysis rate
Glu_t	**:**	Concentration of glucose at time t
Glu_o	**:**	Initial glucose concentration at time

LIST OF ABBREVIATIONS

AFEX	**:**	Ammonia fiber explosion
ADF	**:**	Acid detergent fiber
ADL	**:**	Acid detergent lignin
B	**:**	Biomass
BSI	**:**	Botanical survey of India
CBH	**:**	Cellobiohydrolases
CBM	**:**	Cellulose binding molecule
CBP	**:**	Consolidated bioprocessing
CrI	**:**	Crystalline index
DM	**:**	Dry-Matter
DNS	**:**	Dinitrosalicylic acid
DTG	**:**	Derivative thermograph
ED	**:**	Endoglucanases
IEA	**:**	International Environmental Agency
EDX	**:**	Energy-dispersive X-ray spectroscopy
EH	**:**	Enzymatic hydrolysis
FPU	**:**	Filter Paper Unit
FSI	**:**	Forest survey of India
FC	**:**	Fixed carbon
FTIR	**:**	Fourier Transform Infrared Spectroscopy
GHG	**:**	Green House gases
GAIN	**:**	Global Agricultural Information Network
GC	**:**	Gas chromatography
GIS	**:**	Geographical information system
HPLC	**:**	High-Performance Liquid Chromatography
HHV	**:**	Higher heating values
HMF	**:**	Hydroxy methyl furfural
Kg	**:**	Kilogram
LCB	**:**	Lignocellulosic biomass

LHV	**:**	Lower heating values
MJ	**:**	Megajoules
MTCC	**:**	Microbial type culture collection
NREL	**:**	National Renewable Energy Laboratory
NE	**:**	North East
NDF	**:**	Neutral detergent fiber
OPEC	**:**	Organization of the petroleum Exporting countries
SEM	**:**	Scanning Electron Microscope
SHF	**:**	Separate Hydrolysis and Fermentation
SSCF	**:**	Simultaneous Saccharification and co-Fermentation
SSF	**:**	Simultaneous Saccharification and Fermentation
TPES		Total primary energy supply
TFS	**:**	Total fermentable sugar
TGA	**:**	Thermogravimetric analysis
TRS	**:**	Total reducing sugar
UNFAO		United Nations Food and Agriculture Organization
VM	**:**	Volatile matter
XRD	**:**	X-Ray Diffraction Spectroscopy

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Chapter 1

Introduction

1.1 BACKGROUND

Biomass is a general term, which includes plant biomass (phytomass), animal biomass (zoomass) and microbial biomass. It is an organic matter derived from living organism. Biomass contains stored energy, which can be used as a source of energy. In essence, solar energy is trapped by the biomass (plants) through the process called photosynthesis. In the process of photosynthesis, light energy is converted into chemical energy that can be later released to fuel. This chemical energy is fixed or stored in the form of carbohydrate molecules, such as glucose, which is synthesized from carbon dioxide and water molecule in the plant leaf. The following equation summarizes the overall process of photosynthesis.

$$6CO_2 + 12\,H_2O + \text{Light} \longrightarrow C_6H_{12}O_6 + 6H_2O + 6O_2 \qquad (1.1)$$

The energy derived from biomass is considered as bioenergy or biomass energy. The products of bioenergy are in the form of solid, liquid or gas. As an energy source, biomass can be used either directly via combustion to produce heat, or indirectly after converting it to various forms of biofuel. When the biomass is converted to liquid fuels, it is called biofuels and use in cars, trucks, and tractors etc. Biofuel can be produced directly or indirectly from biomass through the thermal, chemical and biochemical processes. Mostly, it includes bioethanol, biodiesel, and biobutanol.

Biomass energy has many advantages over the traditional fossil-based fuels. Most importantly, the issues of global warming due to the accumulation of carbon dioxide in the atmosphere can be discussed. Burning of biomass or use as types of solid, liquid and gaseous fuels (for example charcoal, ethanol, methane) releases carbon dioxide into the atmosphere. However, plants also take carbon dioxide out of the atmosphere, so there is no net addition of CO_2 (Tasneem et al., 2010).

The use of biomass in the solid form has been pondering ever since man discovered fire. Wood was the first form of biomass-derived energy (biofuel) that was used by our ancestor and are still used today for cooking and heating in many communities mostly in developing countries. Since then, with the exploration of the fossil fuel like gas, coal, and oil, the production and use of biomass-derived fuel decreases and there was a time where science shifted to fossil fuels research. However, biofuel had been used even before the discovery of the fossil fuels in the automotive industry since its inception.

In the mid-1700s and early 1800s, the use of Whale oil as fuel for lighting the houses was quite common. Consequently, the Whale populations were declined and oil price went up. Around the mid-1800s, the fossil fuel production started and whaling industry ran out of customers. Similarly, after the 1900s, wood fuels were fully replaced by fossil fuels.

The US first patent for alcohol as lamp fuel was awarded to S.Casey in 1834 (Kovarik, 1998). Later in the 1860s, the first combustion engine "Otto cycle" was invented by German engineer Nikolaus August Otto which ran on ethanol, a fermentation product of yeasts. Furthermore, in the 1880s, Henry Ford's first prototype automobile called "Quadricycle" operated by ethanol came up. He told: "The fuel of the future is going to come from fruit like that sumach out by the road, or from apples, weeds, sawdust--almost anything. There is fuel in every bit of vegetable matter that can be fermented. There's enough alcohol in one year's yield of an acre of potatoes to drive the machinery necessary to cultivate the fields for a hundred years." (Ford, 1925). However, with the exploration of huge supplies of crude oil in some part of Texas and Pensylvania, the use of petroleum became cheap and this leads to the reduction of use of biofuels.

During the World War II, vegetable oils and alcohols were used as supplementary emergency fuels as a result; the demand for biofuels was increased. It was during this period that various other fuels were introduced blending with alcohol. After the war, gasoline dominated the market because of the cheap oil production from the Middle East. A serious fuel crisis again hit back the various countries during the 1973-74 and 1979-80 because of the geopolitical conflict. Meantime, the organization of the petroleum Exporting countries (OPEC) cut down the oil export to the non-OPEC nations. The constant shortage of fuel leads to attract many countries to take attention on energy crisis and renewable used of biofuels. The main reasons for the people shifting towards biofuel research were the rising prices of oil, emission of harmful gases from fossil fuels and interest like rural development. Brazil became one nation that revived the bioethanol industry permanently by using the sugar cane as raw material. The Brazilian bioethanol production increased from 600 million liters (160 million gallons) in 1975 to 13.7 billion liters (3.6 billion gallons) in 1997, by far the highest in the world (International Energy Agency, 2004). Countries like the United States and Brazil produced mainly bioethanol, whereas, European nations such as Germany, France, Spain, and Italy produced mainly biodiesel. However, it can be said that first generation of bioethanol and biodiesel are produced at a large scale. In 2009, Brazil and United states contribute around

85% of total bioethanol production of the World, whereas European countries produced about 85% of all biodiesel (European Biodiesel Board, 2011). The demand for energy increased with the change in the lifestyle of our society. In 2013, world total primary energy supply (TPES) was 13555 million tonnes of oil. The renewable energy sources contribute around 13.5%. Since 1990, the world renewable energy sources have grown at an average of 2.2%, which is slightly higher than TPES (1.9%). The Solar photovoltaic energy and wind power have been especially high at a growth rate of 46.6% and 24.8% **(Fig.1.1)**. The energy like liquid biofuel, biogas and solid biofuel is growing at rate 10.2%, 13.9% and 1.4% respectively.

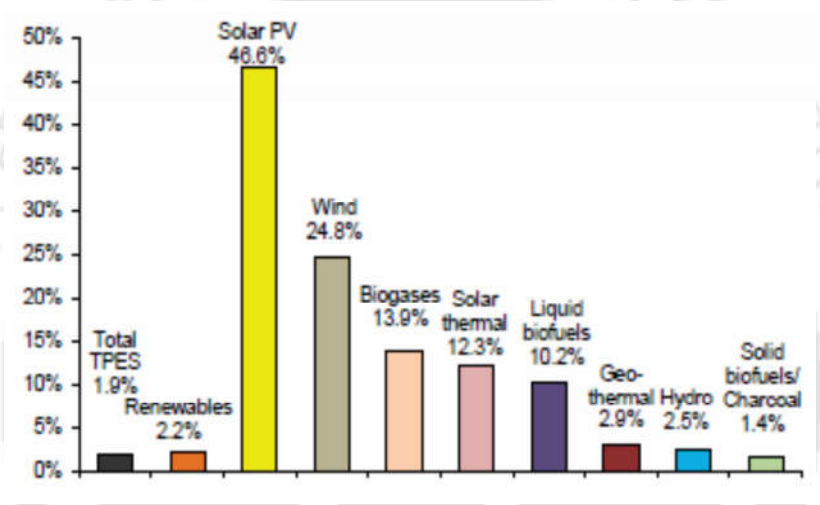


Fig. 1.1 Annual growth rate of world renewable energy supply, 1990-2013 (IEA, Renewable information, 2015).

One of the most important used of fossil fuels is in the transport sector. Theoretically, the option of replacing the fossil fuel by biofuel or other alternate fuel in road transportation exist, however, for various reasons none of the potential candidates and technologies is suitable for the niche market. There are various barriers to uptake the alternate fuel and vehicle technology, including cost-competitiveness, and environmental performance relative to oil. The alternative fuel cannot be directly imposed to the existing phenomena because; it needs to develop a distribution network and applications, requires dedicated infrastructure and government support of one form or other.

1.2 INDIA'S ENERGY OUTLOOK

India contributes around 18% of the world population next to China. It uses only 6% of the world's primary energy. Since 2000, India's energy consumption has almost doubled and the potential for further rapid growth is enormous. However, this is slower than the rate of economic growth over the same period. This is due to the shifting away from bioenergy consumption in the residential sector and the rising importance of service sector. As a consequence of this, it took 12% less energy to create a unit of Indian gross domestic product (GDP). It is suggested that India will emerge as major energy driving force in global trends with all modern fuels and technologies playing apart (India energy outlook, 2015).

The biomass-derived energy is the major source of energy that needs to develop in large scale in other to fulfil the increasing energy demand. In rural areas of India, this primary fuel is the rural energy economy of which 840 million people are using as cooking fuel until today. The bioenergy roughly accounts a quarter of India's energy consumption. The production of biofuels needs to be scale up and possible technologies could be implemented.

The biofuel like bioethanol is produced mainly from molasses (a by-product of sugarcane industry). The demand for bioenergy as a household use in the form of solid biomass for cooking has decreased **(Fig.1.2).**

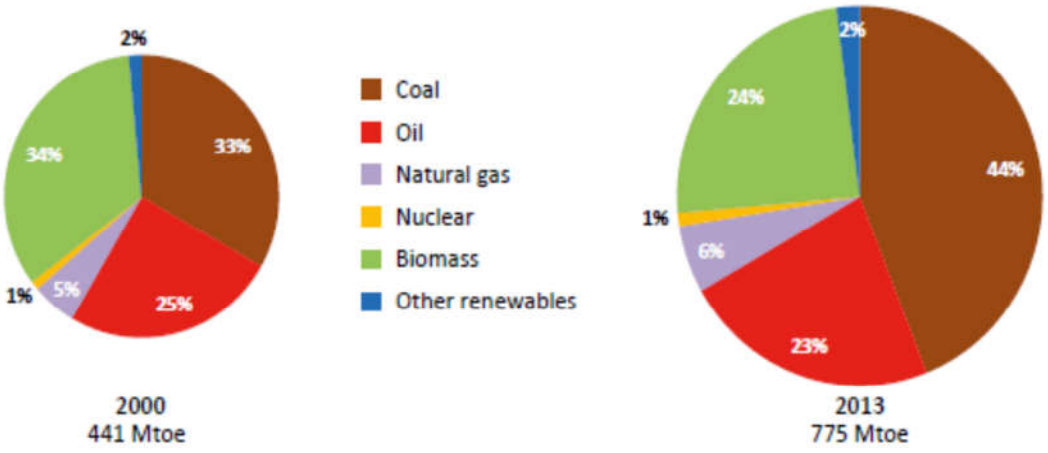


Fig.1.2 Primary energy demand in 2000 and 2013 (India energy outlook, 2015).

From the year 2000 to 2013, the total primary energy demand rises from 441 Mtoe to 775 Mtoe. Coal accounting 33% to 44% is due to the expansion of coal-fired power generation fleet. The demand for bioenergy has grown in absolute term, but the primary energy demand

showing dropping of the almost ten percentage point since 2000, this is due to the households used are shifting to other fuels for cooking possibly liquefied petroleum gas (LPG). However, bioenergy demand rises by 11% over the projection period to 2040. According to United Nations Food and Agriculture Organization (UNFAO, 2015), the total area covered by forest in India has actually increased in recent years including the North-East India. There is no overall scarcity of biomass for rural household uses. It can be mentioned that there is surplus 250 million metric tonnes per annum (MMTPA) production of lignocellulosic biomass after fulfilling all the conventional uses like cattle, feed, and domestic fuel (Biomass Atlas statistic, Indian Institute of Science Bangalore).

The geographical space available for agricultural land in India is around 57%, pasture 3%, 8% of woodlands and remainder being forest areas or not suitable for cultivation (mountains, deserts, built-up areas). The primary energy demand of India in new policy scenario suggested (IEA, India energy outlook 2015) that in all sector bioenergy is predicted to increase except the building sector. There is slow growth of the biofuel energy demand in the transport sector. By considering the current increased in the need of fuel and harmful effect caused by using of fossil fuels, bioenergy needs to play the major role.

According to India's National biofuel policy 2009, the targeted blending of bioethanol with petrol is up to 20% by the end of the fifth plan (2017). In 2013, the actual level of blending was below 1% with bioethanol making it more constraints on supply. The policy encourages the use of renewable energy resources as an alternate fuel in the transport sector. In a bid to renew its focus, on November 22, 2012, the government of India recommended 5% blending of ethanol with gasoline. This 5% blending of ethanol in gasoline was partially successful when more sugar production occurred in a year.

The Global Agricultural Information Network (GAIN, 2014, 2015) reported that in India, there are more than 330 ethanol distilleries with annual production capacity of over 4 billion liters of rectified spirit (alcohol) and 1.5 billion liters of fuel ethanol per year. The current ethanol production level is 2.2 billion liters and blending rate of 2.8 percent was achieved in 2015 **(Table 1.1).** Technically, the current capacity for ethanol production is sufficient to meet around 8% of blending with gasoline only. This implies to look for exploration of other raw material like lignocellulosic biomass to achieve the 20% blending target by 2017 rather importing. It is suggested that, both the private and public sector claim to be successful in

Table 1.1 India: Ethanol Used as Fuel and Other Industrial Chemicals (Million Liters)

Calendar Year	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Beginning Stocks	734	1,374	1,642	1,240	1,021	627	624	468	377	166
Production	2,398	2,150	1,073	1,522	1,681	2,154	2,057	2,002	2,219	2,186
Imports	15	70	320	92	39	34	33	107	120	300
Exports	23	12	14	53	119	177	234	175	200	140
Consumption	1,750	1,940	1,780	1,780	1,995	2,015	2,012	2,025	2350	2350
Fuel Consumption	200	280	100	50	365	305	382	350	800	900
Ending Stocks	1,374	1,642	1,240	1,021	627	624	468	377	166	162
Production Capacity										
No. of Refineries	115	115	115	115	115	115	115	115	115	115
Nameplate Capacity	1,500	1,500	1,500	1,500	1,500	2,000	2,000	2,000	2,000	2,000
Capacity Use (%)	160	143	72	101	112	108	103	100	111	109
Feedstock Use (1,000 MT)										
Feedstock A	9,992	8,958	4,469	6,342	7,004	8,975	8,573	8,343	9,246	9,108
Market Penetration										
Fuel Ethanol	200	280	100	50	365	305	382	350	800	900
Gasoline	14,189	15,368	17,606	19,563	20,716	21,842	23,749	25,848	28,252	30,879
Blend Rate (%)	1.4	1.8	0.6	0.3	1.8	1.4	1.6	1.4	2.8	2.9

Source: FAS/New Delhi Estimates based on information from trade sources

customizing technology (low-cost) for the bioethanol production by using the lignocellulosic biomass. However, scaling up of such projects on large scale is yet to be seen.

The main substrate for production of ethanol in India is sugarcane molasses a by-product of the sugar industry. The current production of ethanol for India is totally depending on the sustainable production of sugar molasses. The optimal supply of sugar does not match with the demand. Therefore, there is always mismatch production of ethanol from the sugar mill. The obvious consequences of these are large importing of ethanol to India. A potential solution to boost the ethanol production on sustainable development is to make use of alternative feedstock. Researchers have paid attention to use lignocellulosic biomass as a potential alternative for ethanol production (Venkatesh 2014, Kataria et al. 2011, Anuj et al. 2009).

North Eastern region of India is unique in terms of availability of huge amount of lignocellulosic biomass (LCB). Present study includes grass species of two states of North East India i.e Assam and Manipur. The species were identified and collected randomly from Kamrup distric of the state Assam as well as Thoubal district of the state Manipur. Selection of these two districts for collection of the grass was done based on the topographical stratum and ecotype of the grass family found to these regions. Many of the biomass species of the region is still remains unexplored. Hardly a few LCB have been explored for bioethanol production from this region of the country. Plant biomass such as *Lantana camara*, *Saccharum sponteneum, Parthenium hysterophorus,* and *Echhornia sp.* of the region have been tested for ethanol production to meet the future energy demand as reported by Mohan et al. 2016. However, the production of bioethanol from these LCBs were reported to be insignificant. Hence, there is a need for alternate feedstock for ethanol production. Lignocellulosic biomass such as grasses are one of the dominating plant biomass growing profoundly in hilly terrain areas of this region and these biomasses can be harnessed to produce bioethanol.

1.3 AIM AND OBJECTIVE OF THE PRESENT WORK

The aim of the present investigation is to explore the potential lignocellulosic biomass of Kamrup and Thoubal districts of the states Assam and Manipur of the North Eastern region of India for bioethanol production. These include collection, identification, characterization, and conversion to bioethanol.

The following objectives are addressed in the present investigation

- Collection and identification of different plant species of Kamrup and Thoubal districts of Assam and Manipur states to use as potential feedstock in bioethanol production.
- Screening and characterization of the collected plant biomass to check for fuel efficacy
- Conversion of the best potential biomass to bioethanol by pretreatment, enzymatic hydrolysis and microbial fermentation.

1.4 OUTLINE OF THE THESIS

This thesis comprises of six chapters. Chapter 1 introduces the brief idea about the fossil fuels, current scenario (around the world and India) and need of second generation of biofuels. It also discussed the amount of bioethanol that India needs for future blending and different options of raw materials for biofuel production other than sugarcane molasses. Chapter 2 described the review of literature. The review covered the different aspect of biomass, feedstock for bioethanol production, structural and compositional analysis of biomass, different techniques of pretreatment technology, enzymatic saccharification, separate hydrolysis and fermentation (SHF) and about the biodiversity of North-East India. Chapter 3 discussed the collection and identification of different plant species of Northeastern region of India (Kamrup and Thoubal districts) to check for viability of feedstock for bioethanol production. The different plant species were collected and herbarium specimens were made. The collected plant biomass were systematically identified and voucher number of each deposited and identified specimen was maintained at Center for Energy, Indian Institute of Technology Guwahati, Assam, India. Chapter 4 studied the physico-chemical characterization of lignocellulosic biomass for bioethanol production. The characterization includes proximate, ultimate, and compositional analysis in other to check fuel efficacy. The best potential candidate was chosen for further conversion to bioethanol. Chapter 5 discussed the conversion of lignocellulosic biomass (*Eragrostis airoides)* to bioethanol by using the standard yeast (*Saccharomyces cerevisiae*) and traditional Hamei and comparisons of the product and process. Chapter 6 discussed the summary and future prospects of the thesis.

Chapter 2

Review of Literature

2.1 INTRODUCTION

Biomass energy or 'Bioenergy' is one of the most challenging renewable energy in modern fuels. The challenge is to ensure that biomass energy is produced in large scale and compensate the increased price of food. In other words, bioenergy should do a better job than fossil fuels in terms of its production, efficiency, and availability. Modern bioenergy resources include liquid biofuels, biomass-fired electricity, or methane from animal wastes. It also includes cellulosic ethanol and Fischer-Tropsch fuels (Naik et al. 2010).

The world is facing serious energy problem due to depletion of fossil fuels. At the same time, the demand and need for energy in the transport sector have risen vigorously. One viable solution to this problem is making used out of alternate and renewable resources. Many countries have implemented policies for bioethanol production in other to replace the petrol-based engines as described in previous chapter 1. In this chapter, an attempt has been made to understand the overview of research activities in the area of bioethanol production from lignocellulosic biomass. The review focused on the different technologies that are involved in the synthesis of bioethanol.

According to the International Energy Agency (IEA), the global demand for biofuel would rise significantly by 2050. The increasing global warming and its impact on the environment due to CO_2 emission can be minimized by using the biofuel and bioenergy (International Energy Agency, 2010, Chum et al. 2011). One of the most promising biofuels that we have is ethanol produced from lignocellulosic biomass by fermentation. Lignocellulosic biomass (LCB) such as crop residues, grasses, sawdust, wood chip, oil palm, fruit bunch, trunk etc. are the potential feedstocks for production of bioethanol (Nicolas et al. 2011). These raw materials are easily available in local areas throughout the world. It is rather to say that ethanol production would be a more rural-friendly occupation.

Bioethanol is a clean and renewable fuel (Ping et al. 2013). It is one of the most important products of industrial biotechnology. The main raw materials used for the production of ethanol in industry scale are sugar-based such as sugarcane, molasses, and starch-based materials such as wheat and corn. Based on such raw material, bioethanol may be divided into first, second and third-generation (Nigam et al. 2011). The second generation of biofuel is more promising in terms of efficiency and low cost of raw material as compared to the first and third generation of biofuel (discussed in chapter 3). The second generation of biofuel is mainly depended on the lignocellulosic biomass. It seems that those countries, which have

significant agronomic-based economy or agricultural work for their livelihood, have greater advantages to incorporate currently available technology for fuel ethanol production. Bioethanol production will not be signed without proper improvement in the process as well as lowering energy consumption. During the last two decades, technology for bioethanol production from different feedstock has been developing much higher and large-scale production became reality.

2.1.1 FEEDSTOCK FOR BIOETHANOL PRODUCTION

Currently, the commercial production of bioethanol depends on sugar and starch-based feedstock. However, this ethanol production is not sufficient with the material cost and the demand that we need in today's scenario as stated earlier. Here comes the second-generation ethanol produced from lignocellulosic biomass such as agricultural waste, forest residues, grasses, weeds, industrial and municipal solid wastes etc. The list of the feedstock used in bioethanol production with the key pretreatment method applied is given in **Table 2.3.**

Lignocellulosic biomass derived carbohydrate is the most abundant biopolymer on the Earth and it comprises about 50% of the world total biomass. Various attempts have been made through the world for bioethanol production from different sources of feedstock, however, the complex nature of the biomass make limiting factor for processing as feedstock. These complexities of the biomass vary according to the type of plant species and thus need different technologies to develop. Among various feedstocks, biomass with minimum cost, no associated with food crops, but highly potential for energy production such as grasses are of great interest.

2.1.2 COMPOSITION AND STRUCTURE OF LIGNOCELLULOSIC BIOMASS (LCB)

Lignocellulose is the most abundant renewable biomass with a worldwide annual production of 1x1010 MT (Sánchez et al. 2008). The term "Lignocellulosic biomass' is use to refer to the higher plants such as hardwood or softwood. The main components of LCB are cellulose, hemicelluloses, lignin and inorganic materials (Tasneem et al. 2010). Cellulose is the main component in lignocellulosic material followed by hemicelluloses and then lignin. Cellulose and hemicelluloses are polysaccharides, which are tightly bound to lignin by covalent cross-linkages or non-covalent forces (Parez-Garcia et al. 2005) whereas, lignin is an aromatic polymer made up of phenylpropanoid precursors. Apart from the three basic

chemical compounds (cellulose, hemicellulose and lignin), lignocellulosic biomass content water, proteins, minerals and other compounds.

The organic component (cellulose, hemicellulose and lignin) of biomass plays a major role in processing and producing biofuels. Cellulose is a major structural component of cell walls. It provides mechanical strength and contributes major fuel. The solar energy is absorbed through the process of photosynthesis and stored as cellulose or hemicellulose (Raven et al.1992) as described in chapter 1.

It has been estimated that around 7.5x 10 tonne of cellulose are consumed and regenerated every year in the wold (Kirk-Otmer 2001). This is the main reasons why cellulose is considered as the most abundant organic compound on the Earth. The composition and proportion of these compounds vary from species to species depending upon plant cell wall structure (Prasad et al. 2007, John et al. 2006). There is a significant variation of lignin, hemicellulose and cellulose content of lignocellulosic biomass depending on whether it is derived from hardwood, softwood or grasses. **Table 2.1** summarised the composition of lignocellulosic biomass of different sources.

Table 2.1 Composition of lignocellulosic biomass from several sources (Zahid et al. 2014)

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Paper	85–99	0	0–15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15–20	80–85	0
Cotton seed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Waste papers from chemical pulps	60–70	10–20	5–10
Primary wastewater solids	8–15	NA	24–29

Swine waste	6.0	28	NA
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switchgrass	45	31.4	12.0
Cane bagasse	36	28	20
Cane straw	36	21	16
Maize straw	36	28	29
Corn straw	39	36	10
Barley straw	44	27	7
Rice straw	33	26	7
Oat straw	41	16	11
Cotton straw	42	12	15
Rice shell	36.1	19.7	19.4
Barley bran	23	32.7	21
Pine tree	44	26	29
Card board	47	25	12
Sweet sorghum	45	27	21

2.1.2.1 STRUCTURAL FEATURES OF CELLULOSE

Cellulose is the major component of plant cell wall. It is made of high molecular weight linear condensation of β-1, 4-polyacetal of cellobiose (4-O-β-D-glucopyranosyl-D-glucose) **(Fig.2.1).** One molecule of cellobiose consists of two molecules of glucose. Therefore, cellulose is usually considered as a polymer of glucose **(Fig.2.2).** The chemical formula of cellulose polymer (s) is $(C_6H_{10}O_5)n$ and typically it contains 100-20,000 β (1→4) linked D-glucose molecules (Morohoshi 1991; Delmer et al. 1995; O'Sullivan 1997; Zhang et al. 2004; Taherzadeh et al. 2007; Mohnen et al. 2008). In a cellulose molecule (**Fig.2.1)**, hydroxides are eventually distributed on both sides of the monomers. This allows the formation of hydrogen bonds in between the cellulose molecules **(Fig.2.2).** The hydrogen bonds in turn results in the formation of long chain polymer comprise of several repeating units of monomers (Faulon et al. 1994).

In a cellulose fiber, smaller unit of cellulose polymer (36) are parallel link up with hydrogen bonds and Van der waal's forces (Zhao et al. 2012) to form elementary fibrils.

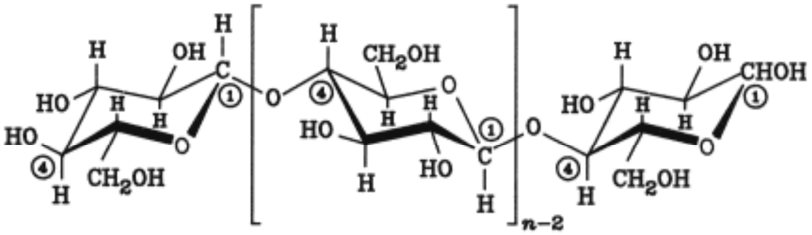


Fig.2.1 Structure of single cellulose molecule (Harmsen et al. 2010)

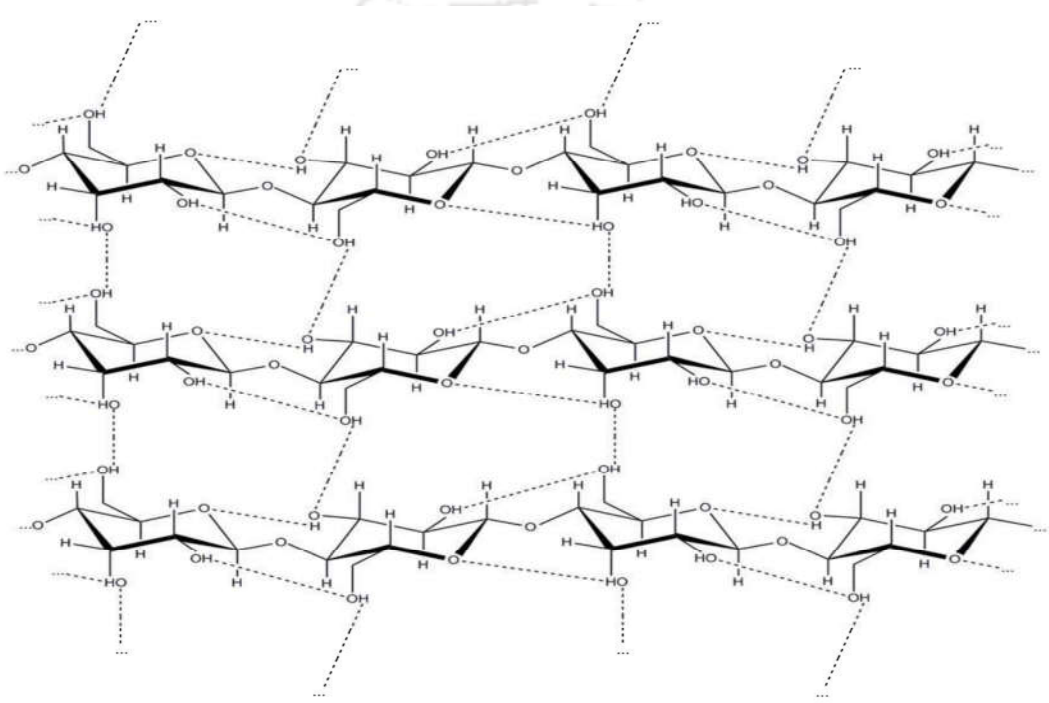


Fig.2.2 Structure of cellulose. In parenthesis, cellobiose, a disaccharide of two glucose residues linked through β (1→4) glycosidic bonds, the fundamental building blocks and the repeating structural unit of cellulose (Klemm et al. 1998).

These elementary fibrils are in crystalline structure with straight, stable supramolecular fibers having great tensile strength and low accessibility (Percival et al. 2006). The elementary fibrils are attached with hemicellulose, pectin and lignin to form the cellulose microfibrils (Ha et al. 1998). This microfibril provides mechanical strength and chemically stable to the plant cell (Harmsen et al. 2010). Several microfibrils combined to form macrofibrils **(Fig.2.3)** (Delmer et al. 1995).

Cellulose molecules have usually two regions. One, with crystalline, highly ordered, less degradable, highly insoluble in water and organic solvents. Second, with a non-crystalline

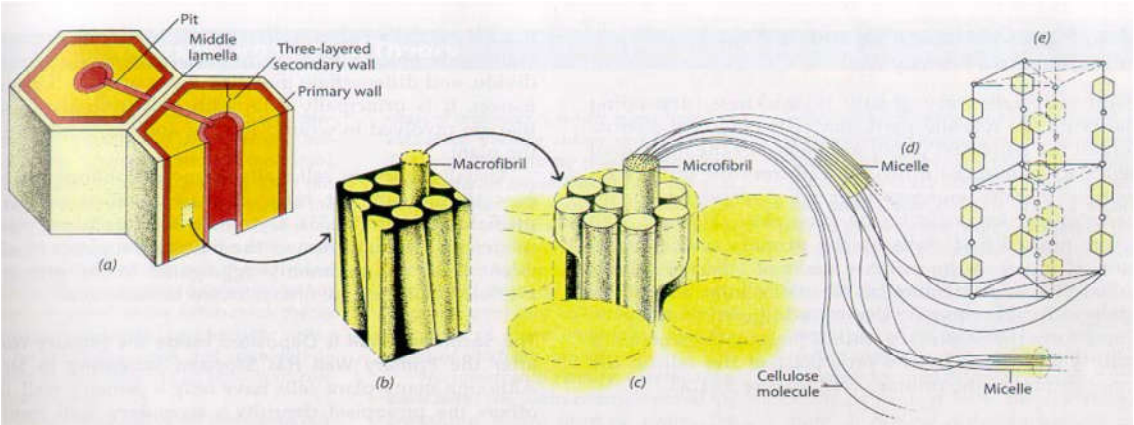


Fig.2.3 Cellulose fibre formation (Micro- and macrofibrils) (Harmsen *et al.* 2010).

portion, amorphous, less ordered, easily degradable and soluble in water (Zhang et al. 2004; Taherzadeh et al. 2008). Cellulose is also soluble in concentrated acids, and alkali (Krassig et al. 2002). However, the solubility of the polymer is strongly related to the degree of polymerisation and its hydrolysis rate (Harmsen et al. 2010). The degree of polymerisation (DP) is the number of glucose units that make one polymer chain. It can extend up to 17000 units. The cellulose becomes soluble at higher temperatures, as the energy provided enough potential to break the hydrogen bond that holds the crystalline structure of the molecule and dissociated into a smaller unit.

2.1.2.2 STRUCTURAL FEATURES OF HEMICELLULOSE

The term hemicellulose is a collective term. It is used to describe the family of polysaccharides chain such as arabinose, xylose, mannose, galactose etc. Chemically hemicellulose $(C_5H_8O_4)n$ are heterogeneously branched biopolymers with different pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α –D-galactose) and some uronic acids (α-D-glucuronic, α-D-4-0-methyle-galacturonic) (Girio et al. 2010).

It is mainly composed of five carbon sugar monomers, xylose, and lesser extent six carbon sugar monomers such as glucose. The molecule is linked by 1->4 linkages of xylopyranosyl units with α-(4-O)-methyl-D-glucuronopyranosyl units attached to anhydroxylose units. This results in branching of the polymer chain.

Hemicellulose does not have a crystalline structure, instead, it has highly branched structure with acetyl groups connected to the polymer chain **(Fig.2.4).** Hemicellulose possesses a high

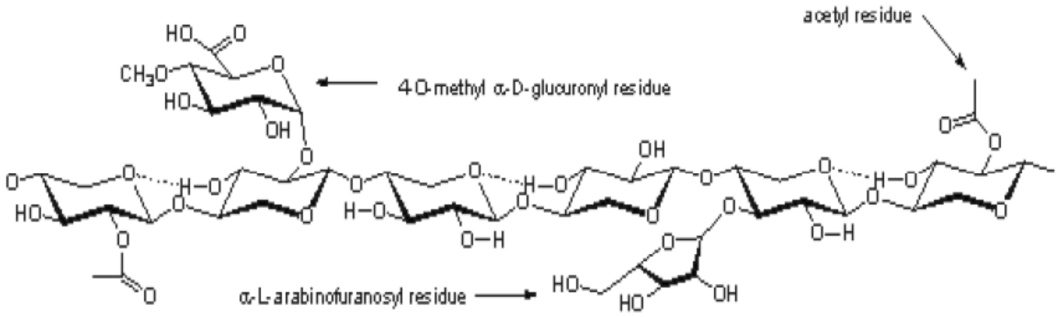


Fig. 2.4 A schematic representation of the hemicellulose backbone of arborescent plants (Kirk-Otmer 2001)

degree of polydispersity, polydiversity and polymolecularity (a broad range of size, shape differences). It is insoluble in water at low temperature (Thermo wood handbook, 2003), however readily hydrolysed by weak acids, bases, enzymes and high temperature (lower than of cellulose). However, the presence of acid in the water highly improves the solubility of hemicellulose.

Due to its high branched structure, it has a lower degree of polymerization, typically around 80-200 (Sjöström 1993). Hemicelluloses are bound to cellulose fibers by hydrogen bonds and providing the backbone of the cell wall structure (Mosier et al. 2005). The structural composition of hemicellulose is not utmost similar to softwood plants and grasses. The main part of hemicellulose present in softwood is typically built up by linear, or slightly branched, galactoglucomannas (Fengel and Wegener 1989; Sjöström 1993), whereas in straw and grass, the main dominant component of hemicelluloses are arabino-glucoronoxylan, glucorono-arabinoxylan and arabinoxylan (Gírio et al.2010). As compared to cellulose fibers, they are easy to hydrolyse because of their branched structure and amorphous. In the fermentation process, hemicelluloses are more relatively sensible to temperature, retention time and hence must be controlled to avoid the formation of furfurals and hydroxymethyl furfurals, which inhibits fermentation (Sohrab et.al, 2013).

2.1.2.3 STRUCTURAL FEATURES OF LIGNIN

Lignin is the second most abundant polymer present in the plant after the carbohydrates (Ragauskas et al. 2014; Calvo-Flores et.al. 2010). Lignin $(C_{31}H_{34}O_{11})n$ is the stuff that makes the biomass woody in nature. It is a giant polymer molecule with both aliphatic and aromatic

portions synthesized from phenylpropapoid precursors. Lignin is highly branched irregular amorphous complex polymer with predominantly constituting of three phenylpropane units as the basic building blocks: p-coumaryl alcohol, coniferyl alcohol and sinaply alcohol **(Fig. 2.5).** These three major component give rise to a random sequence of p-hydroxyphenyl (H-lignin), guaicyl (G-lignin) and syringyl (S-lignin) subunits in the polymer (Ralph et al. 2004, Guo et al. 2014) **(Fig. 2.6).** The composition of these subunits (H,G and S-lignin) vary depending on the source of lignocellulosic biomass (Guo et al.2014). However, in the softwood plants, the major component is G units with the remaining being H units.

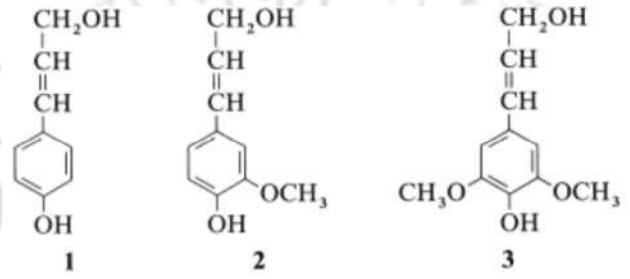


Fig. 2.5 P-coumaryl- , coniferyl- and sinapyl alcohol: dominant building blocks of the three-dimensional polymer lignin (Kirk-Otmer 2001)

In the hardwood plants, it has been identified that G and S monolignols with trace amounts of H units are major components and in herbaceous plants, all three G,S and H units contain significant amount but in different ratios (Buranov et al. 2008; Chundawat et al. 2011). Lignin is covalent bond to hemicelluloses and also cellulose by other bonds such as ether, ester and glycosidic bonds (Ralph et al. 2004). These bonds provided the plant cell with a stuff fiber. Moreover, lignin plays a crucial part for transportation of water, nutrients and metabolites in the plant vessels due to its hydrophobicity nature (Sorek et al. 2014). In a plant cell, lignin also works as a strengthening agent, binding substances for cells, fibers, vessels and gluing the cellulose fibers together, filling the gap between cellulose, hemicellulose and other components (Sticklen 2008).

2.1.3 CARBOHYDRATES (SACCHARIDES)

The term carbohydrate or saccharides are generated from carbon and hydrate; though some also contain nitrogen, phosphorus, or sulphur. Chemically, carbohydrates are a biological molecule which is composed of carbon, along with hydrogen and oxygen in the same ratio as that found in water (H_2O).

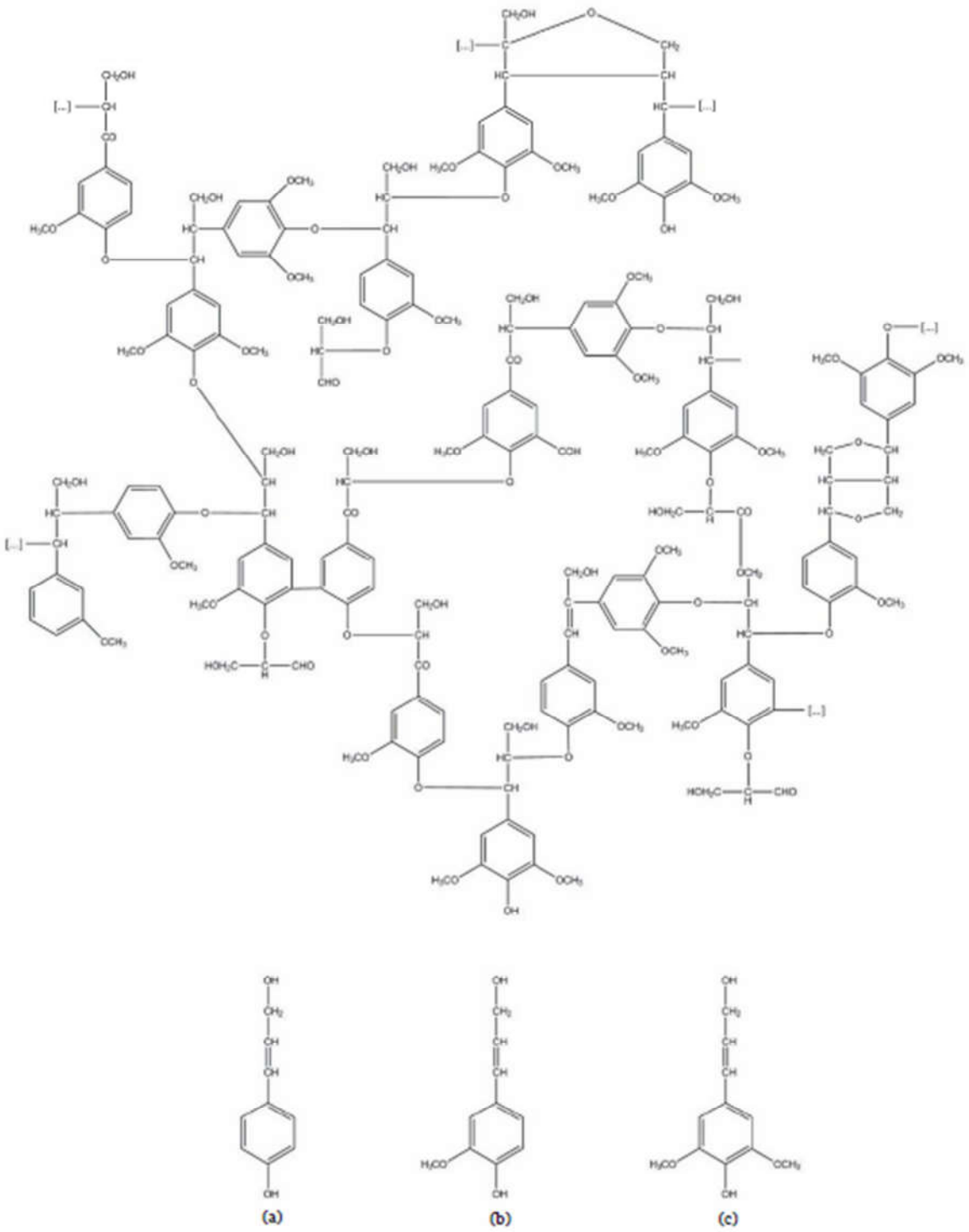


Fig.2.6 Schematic representation of lignin and the three major monolignol precursors a) p-coumaryl alcohol b) coniferyl alcohol and c) sinapyl alcohol (Ralph et al. 2004, Guo et al. 2014).

They have the general empirical formula $(CH_2O)n$. Carbohydrates are the diverse group of compounds that are ubiquitous in nature. It is suggested that more than 75% of the dry weight of the plant biomass is carbohydrate in nature particularly in the form of cellulose, hemicellulose and lignin (Morrison et al. 1992, Pigman et al. 1980). Carbohydrates are generally cyclic or straight form with polyhydroxy aldehydes or ketones as functional groups.

2.1.4 CLASSIFICATION OF CARBOHYDRATES

Carbohydrates are divided into four major classes: monosaccharides, disaccharides, oligosaccharides, and polysaccharides **(Fig.2.7).** In general, the monosaccharides and disaccharides are referred as simple sugars having lower molecular weight carbohydrates, consist of a single aldehyde or ketone unit (Cox et al. 2000).

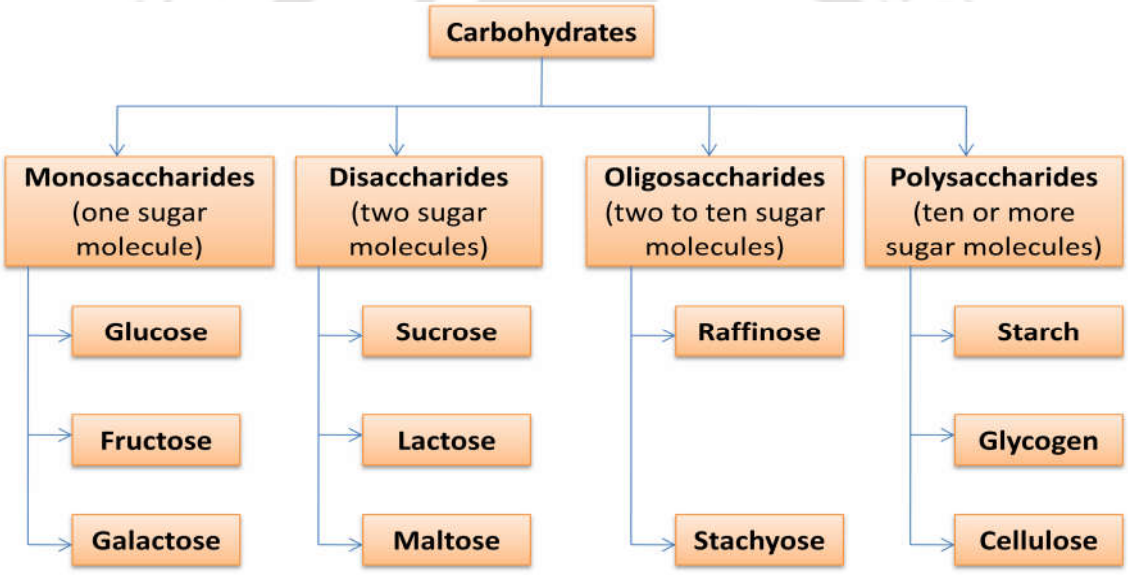


Fig. 2.7 Classification of carbohydrates.

2.1.4.1 MONOSACCHARIDES

Monosaccharides are the simplest and smallest unit of the carbohydrates (mono = one, saccharide = sugar) from which disaccharides, oligosaccharides, and polysaccharides are constructed. Monosaccharides are either aldehydes or ketones, with one or more hydroxyl groups. For example, the six-carbon monosaccharides glucose (an aldohexose) and fructose (a keto hexose) have five hydroxyl groups **(Fig.2.8).** Further, the two main classes of monosaccharides (aldoses and ketoses groups) can be divided into sub-groups on the basis of their carbon atoms molecules such as trioses, tetroses, pentoses, hexoses, heptoses, octoses

etc. Glucose and fructose are specific examples of an aldose and ketose **(Fig.2.8).** Glucose (glyceraldehydes derivatives) is one of the most abundant monosaccharides occurring in nature (deMan 1999).

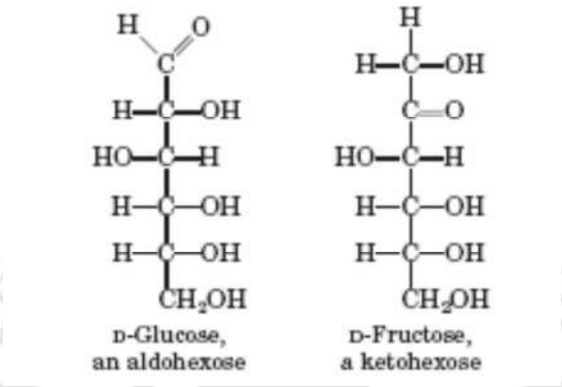


Fig. 2.8 Monosaccharides

2.1.4.2 DISACCHARIDES

A disaccharide is a carbohydrate; consist of two monosaccharides joined by a glycosidic bond. These two monosaccharides can be homo or hetero **(Fig.2.9).** The three most abundant disaccharides are sucrose, lactose, and maltose. Sucrose and lactose are heterosaccharides and maltose is homosaccharide.

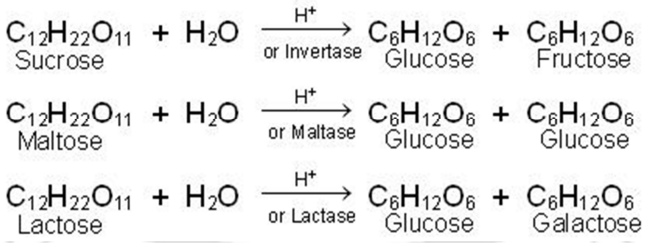


Fig.2.9 Hydrolysis of disaccharides into monomers

2.1.4.3 OLIGOSACCHARIDES

Oligosaccharides are carbohydrates having 2-10 monosaccharide unit covalently bonded to each other by glycosidic bonds (Seema et al. 2011). They are classified according to the number of sugar units as a disaccharide, trisaccharide and so on.

2.1.4.4 POLYSACCHARIDES

Polysaccharides are carbohydrate polymers with a complex structure made up of more than ten monosaccharides joined together by glycosidic bonds (Morrison et al. 1992, Pigman et al. 1980, David et.al. 2006). They are, therefore, very large, often branched biomacromolecules. The predominant monosaccharide found in polysaccharide is D-glucose. Polysaccharides are usually divided into two categories: homopolysaccharide (polysaccharides of the same type of monosaccharides) and heteropolysaccharides (polysaccharides of different monosaccharides). Homopolysaccharides includes starch, cellulose, glycogen, and insulin etc., whereas heteropolysaccharides consist of xylan, glycosaminoglycans, proteoglycans, glycoproteins, agar and gum arabic.

2.1.4.4 REDUCING SUGARS

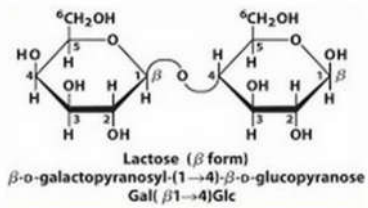
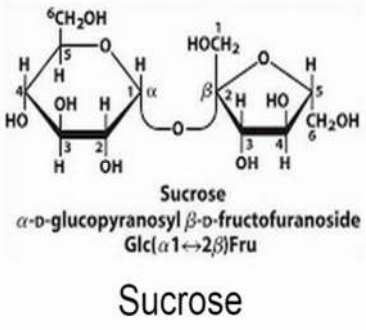
A reducing sugar is any type of sugar that is capable of acting as the reducing agent due to the presence of a free aldehyde or ketone group. For example, all the free monosaccharides having free aldehyde or hydroxyl ketonic group are capable of being oxidised (Cox et al. 2000). After being oxidised they cause a reduction of the other substances. The basic difference between reducing and non-reducing sugars is given below in **Table 2.2.**

2.2 THE ETHANOL PRODUCTION – A BRIEF OVERVIEW

Ethanol is currently produced in great amounts from corn-derived starch in the United States and sugarcane-derived sucrose in the Brazil. However, these dependent raw materials (first generation) could not supply enough bioethanol for transportation in lower middle-income countries. The ultimate significant raw material with high potential of sustainable production of ethanol is LCB. Moreover, ethanol production from LCB becomes major outlook. Many steps are involved in the production of ethanol from LCB, however, some of the main steps that can be considered are; Conversion of cellulose and hemicellulose to soluble sugars by depolymerization process, Sugar fermentation as well as fermentation of inhibitory compounds and cost effective use of lignin where lignin content can be minimized. The polysaccharides obtained from LCB could be hydrolyzed into simple monomeric sugar by acid or enzyme treatment. It has been reported that enzymatic hydrolysis are generally more preferred than acid hydrolysis in terms of its environmentally friendly. The monomeric sugars obtained from hydrolysis is used in fermentation to produce ethanol by using microbes like bacteria, yeast, filamentous fungi or genetically modified microorganisms (Yi et

al.2009). The final product ethanol is purified by the distillation process. The remaining valuable co-products have many industrial applications and new technologies are under development to convert it into higher value products (Fernando et al. 2006). A brief schematic diagram of bioethanol conversion from biomass is shown in **Fig.2.10.**

Table 2.2 Difference between reducing and non-reducing sugars (Cox et al. 2000)

Sl.No.	Reducing sugars	Non-reducing sugars
1	Possess a free aldehyde(-CHO) or ketone (-C=0) Group.	A free aldehyde or ketonic group is lacking.
2	Can reduce the Cu^{2+} cupric ions (blue)in Fehling's or Benedict's Solution to Cu^{+} cuprous ions (reddish) that precipitate out as Cu_2O(cuprous oxide).	No such reaction.
3	Maltose. lactose, melibiose, gentiobiose, cellobiose, mannotriose, rhamnotriose.  Lactose	Sucrose, trehalose,raffinose, gentiarose, melezitose  Sucrose

The technologies of biomass conversion to biofuel can be described in two distinct pathways: thermochemical and biochemical processes (Demirbas 2007; Kamm et al. 2005; Menon et al. 2012; Nanda et al. 2014). The thermochemical conversion also called as the syngas process, involves controlled heating or oxidation of biomass (Demirbas 2004; Goyal et al. 2008; Tanger et al. 2013). The biochemical conversion involves the production of fermentable sugars and their conversion to liquid fuels or gas by microbial population (Cuellar et al. 2014). Both thermochemical and biochemical technologies are competitive in the calculation of their energy conversion efficiencies (Mousdale 2008; Foust et al. 2009).

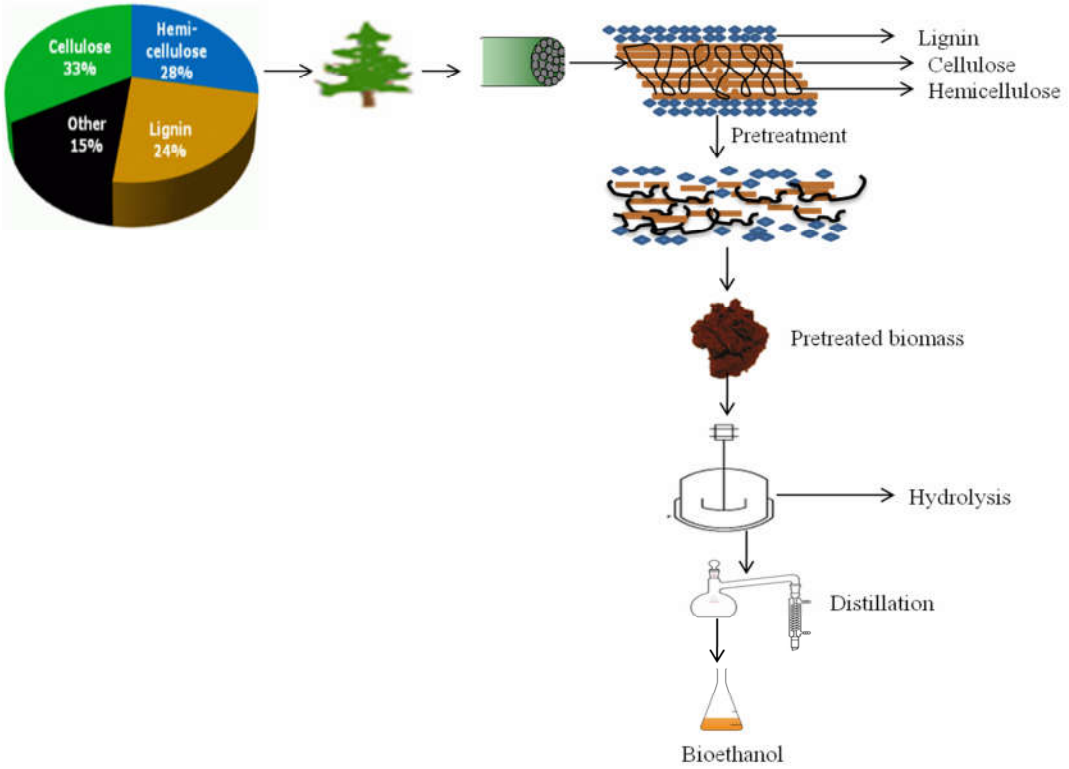


Fig.2.10 Schematic representation of LCB conversion to bioethanol

The overall economies of these two processes are similar. However, the comparative life cycle assessment suggested that biochemical conversion shows better performance than thermochemical conversion regarding greenhouse gases emissions (Mu et al. 2010). It is suggested that the combination of technologies will enhance the effective biomass conversion (Tanger et al. 2013). Indeed, there should be a bilateral role for both the technologies in advanced biofuel production. As far as the fuel alcohol is concerned, biochemical conversions routes appear to be well suited and accepted, whereas, for the hydrocarbon fuels, thermochemical conversion routes tend to be more favourable (Foust et al. 2009).

2.2.1 THERMOCHEMICAL CONVERSION OF BIOMASS TO BIOFUEL

In the thermochemical conversion of biomass to biofuel, the thermal and pressure gradient is applied to the biomass to convert it into various fuels and chemicals products. Combustion, gasification, pyrolysis, solvolysis and extraction are methods which are referred to as thermochemical conversion technologies of biomass, which can produce heat, electricity, gaseous or liquid fuels (Butler et al. 2011; Wang et al. 2008; Brar et al. 2012; Bridgwater 2012; Solantausta et al. 2012; Tanger et al. 2013) **(Fig. 2.11).** The key parameters on which

the thermochemical conversion of biomass depends on are temperature, pressure, feed rate, time of heating, a particle size of the biomass, and quenching processes that are applied. The major problem of thermochemical conversion is high-energy consumption, which led to the less efficient utilization of biomass.

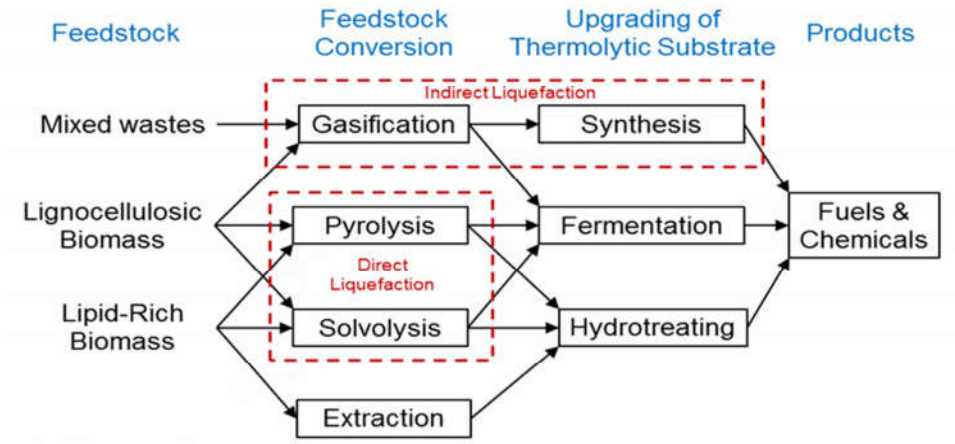


Fig. 2.11 Thermochemical processing uses heat and pressure to convert various types of feedstocks into fuels and chemicals. (Robert et al. 2014)

2.2.2 BIOCHEMICAL CONVERSION OF BIOMASS TO BIOFUEL

The biochemical conversion involves hydrolysis of lignocellulosic biomass (polysaccharides) into simple sugars (glucose, xylose, mannose etc.) by the chemicals or enzymes and their further conversion into fuel ethanol by microbial fermentation process (Balat 2011) **(Fig.2.12).** The compact rigid structure of biomass and presence of recalcitrant during the fermentation process is a major challenge in biochemical conversion of biomass to biofuel. The lignocelluloses recalcitrance blocks the decomposition of structural carbohydrate from the microbial activity during the fermentation (Himmel et al. 2007).

Unlike sugar extraction and ethanol production from 1st generation of feedstock, lignocellulosic biomass requires three basic steps: pretreatment, hydrolysis, and fermentation. In addition to these core three process, product recovery through the distillation process is also needed. The ethanol production by degrading the cellulose to glucose by means of enzymatic process is regarded as the most attractive and efficient way (Galbe et al. 2007). However, due to the rigid, compact and structural characteristics of biomass, pretreatment technology is essential to efficiently conversion of cellulose to ethanol. The pretreatment

technology allows to open up the cellulose, hemicellulose and lignin portion, making the substrate more reliable for enzymatic hydrolysis (Alvira et al. 2010; Yang et al. 2011).

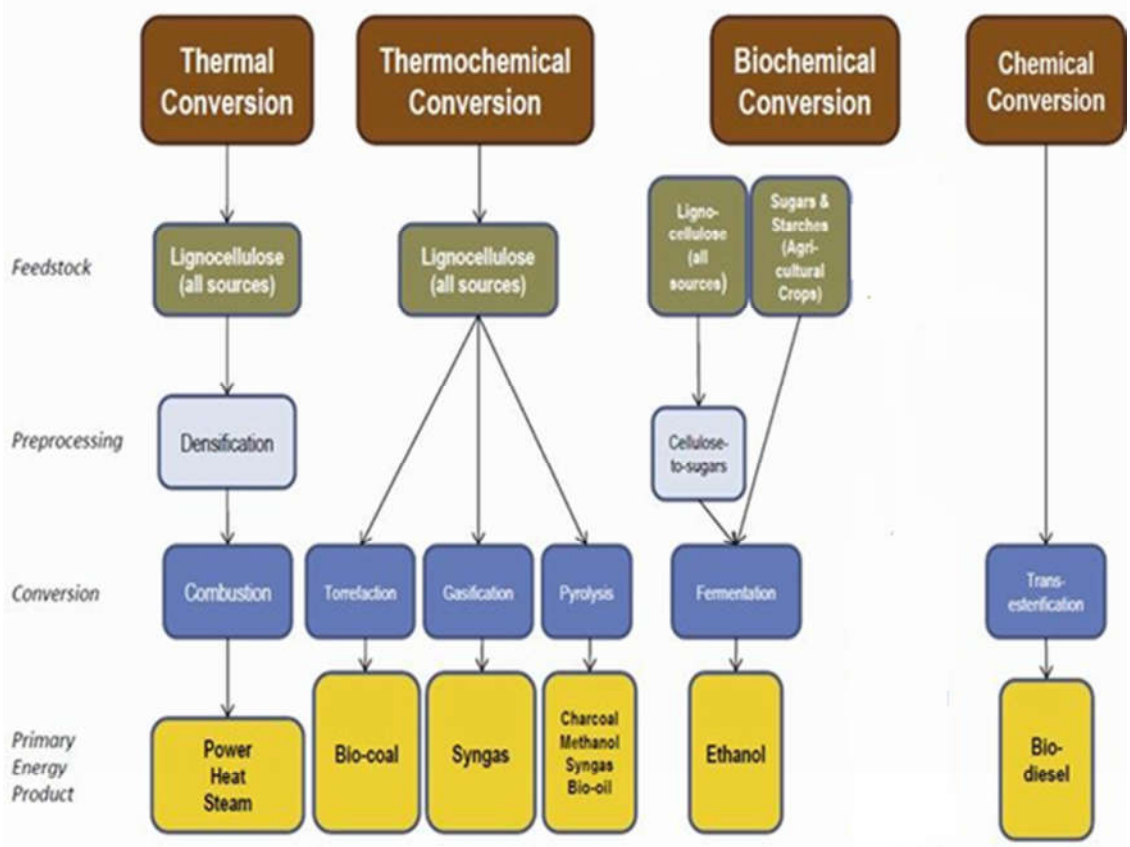


Fig.2.12 Bioenergy Conversion Technologies (Source: Wisconsin Grasslands Bioenergy Network, Biomass Conversion)

2.3 OVERVIEW OF PRETREATMENT ROUTES

2.3.1 INTRODUCTION

One of the most common and important steps in biochemical conversion of biomass into ethanol is pretreatment technology. The pretreatment technology helps in loose the strong bond held between the cellulose fibers. It also induces breaking of the bonds that bind with lignin, cellulose, and hemicelluloses. Pretreatment process is necessary to achieve higher enzymatic degradation in the production of ethanol from LCB. It is essential because: (1) It allows to attack by enzymes (2) avoiding the formation of inhibitory compounds (3) hemicellulose and cellulose recovery (4) size reduction and reducing the cost of materials for construction of fermentation reactors (5) increases pore size (**Fig.2.13).** Pretreatment has been

recognised as one of the most expensive processing steps in cellulosic biomass-to-fermentable sugars conversion and several recent review articles provide a general overview of the field (Alvira et al. 2010; Carvalheiro et al., 2008; Hendriks et al. 2008).

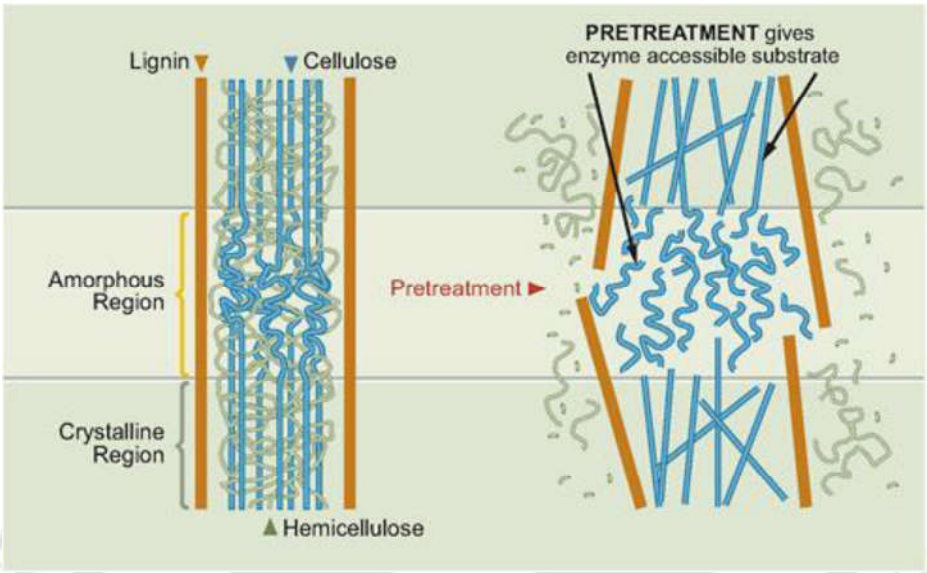


Fig.2.13 Schematic representation of pretreatment to disrupt the physical structure of biomass (Mosier et al. 2005)

The cellulose portion of the biomass is affected by mostly two factors: direct and indirect factors (Zhao et al. 2012a). The direct factors include the enzyme accessible surface areas, whereas indirect factors referred to biomass structure relevant characteristics such as pore size, particle size, chemical composition, and cellulose crystallinity etc. (Zhao et al. 2012a). Pretreatment is actually the process, with an objective of removing the recalcitrant barriers of biomass by altering the indirect factors and improving the direct factors thus enhancing the cellulose accessibility to the enzymes (Mosier et al. 2005; García et al. 2011).

There are several methods for pretreatment technologies of LCB prior to enzymatic hydrolysis. These methods could be classified into "Physical pretreatment", "Physico-chemical pretreatment", "Chemical pretreatment", and "Biological pretreatment" (Schell et al. 1996, Berlin et al. 2006, Karimi et al. 2006, Fan et al. 1982). Schematic outline of pretreatment technologies is given in **Fig.2.14.** In the past several decades, a large number of pre-treatment techniques have been developed and comprehensively reviewed (Hsu 1996, Chandra et al. 2007, Mosier et al. 2005, Kilpeläinen et al. 2007, Galbe and Zacchi 2007, Carvalheiro et al. 2008, Taherzadeh and Karimi 2008, Yang and Wyman 2008,

Hendriks et al. 2009, Kumar et al. 2009, Alvira et al. 2010, Harmsen et al. 2010, Mäki-Arvela et al. 2010, Pedersen et al. 2010, Brodeur et al. 2011, and Zhang et al. 2011).

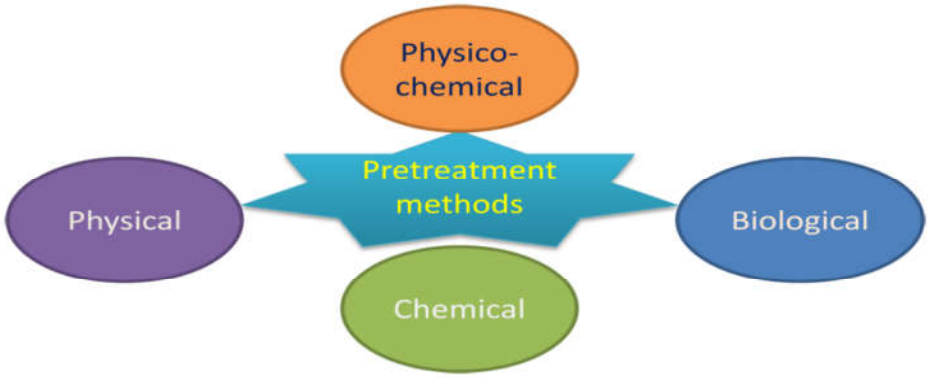


Fig.2.14 Pretreatment methods for LCB before enzyme hydrolysis.

However, none of the techniques is suitable for all types of biomass. Every technique has certain pros and cons. The overall advantages and disadvantages of different pretreatment methods for lignocellulosic biomass are given in **Table 2.3.**

In an ideal case, the use of pretreatment methods leads to a limited formation of degradation products that inhibit enzymatic hydrolysis and fermentation. However, these are actually the most challenging steps in the bioconversion process that is why; pretreatment research has been mainly focused on developing methods that supports subsequent enzymatic hydrolysis, which should result in higher sugar yields with lower enzyme dosage and shorter reaction times (Alvira et al. 2010). In the following sections, the most common pretreatment techniques of biomass are discussed.

2.3.2 MECHANICAL AND PHYSICAL PRETREATMENT

Physical pretreatment is the process of applying mechanical force such as ball milling, two-roll milling, hammer milling, colloid milling, vibrato energy milling **(**Taherzadeh et al. 2008) chipping, grinding (Sun et al. 2002) irradiation by gamma rays, electron beam or microwaves etc. to the LCB to reduce its size. The size reduction of biomass is often needed to make the biomass easier to hydrolyze. The energy provided (e.g. irradiation) to the lignocellulosic biomass would break down the hydrogen bonds of the substrate which is expected to result in improving the subsequent polysaccharides hydrolysis (Harmsen et al. 2010). The individual treatment of mechanical or physical would produce inefficient hydrolysis of the substrate

(Agbor et al. 2011). Therefore, physical and mechanical treatments are used in the feedstock preparation prior to further pretreatment processes. These process provides more surface areas and pore size, densification of feedstock, decrease degree of a polymerization of cellulose molecules and most importantly de-crystallisation.

2.3.2.1 MILLING

There are several types of milling pretreatment method used in bioethanol production. Ball milling, two-roll milling, hammer milling, colloid milling and disk milling are commonly used pretreatment methods. This method enforces to reduce the size of the biomass by applying physical forces. The size of the particle obtained from milling pretreatment depends on the methods enforced. However, 10-30 mm and 0.2-2 mm of particle size are achieved in chipping (Sun et al. 2002). The milling pretreatment has some drawbacks, which include high-energy consumption and a high cost of investment. Another disadvantage of milling is its inability to remove lignin from the LCB thereby restricting the enzyme activity (Berlin et al. 2006). To overcome such drawbacks, another milling called wet disk millings are adopted.

2.3.2.2 IRRADIATION

Irradiation is another physical pretreatment technology where a ray of light is allowed to impose on LCB. The irradiation helps in degrading the cellulose component due to dissociation of glycosidic bonds of the cellulose molecules. It includes gamma rays, electron beam and microwaves irradiation techniques. A high beam irradiation, above 100 MR, can disintegrate glucose ring structure (Kumakura et al. 1983). By using this technique, a glucose yield of pretreated bagasse became double as compared to untreated one prior to its enzymatic hydrolysis (Kumakura et al. 1983). It helps in improving enzymatic hydrolysis of LCB in the conversion of cellulose to glucose (Kumakura et al. 1984, Mamar et al. 1990). However, some of the irradiation techniques are quite expensive and not applicable for industrial application.

2.3.2.3 MICROWAVE-MEDIATED PRETREATMENT

Microwave-mediated pretreatment is a combination of the thermal effect of microwave and chemical reagents. Mostly, the chemical used in this process are acids and alkali. Microwaves are electromagnetic waves spanning a frequency range of 300 (3×108 cycles/s) to 300 GHz (3×1011 cycles/s) (Shi et al. 2011). It is based on the principle that the heat wave produced from the microwaves interacts with the polar molecules and ions, and result

in both thermal and non-thermal effects that drive physical, chemical and biological reactions (Sridar 1998). The thermal effect in microwave is due to the dielectric heating of the system. This process is rapid and volumetric. The microwave irradiations have been commonly used in treating with the biomass because it has high heating efficiency and can enhance the effect of reaction time. The techniques have been significantly using in the production of ethanol from LCB. The technique helps in improving the ethanol production more efficiently. It is one of the efficient and modified ways for easily treating the sample in domestic level (Renu et al. 2014)**.**

In this method, the parameters of microwave conditions are very important in dissociating the cellulose fiber from lignin. In pretreatment of rice straw by this process increases the saccharification by removing lignin and hemicellulose in large amount. Maximum reducing sugar obtained from the process was found to be 1334.79 µg/mL (Renu et al. 2014)**.** Some studies have shown that microwave irradiation could change the ultrastructure of cellulose (Xiong et al. 2000), degrade lignin and hemicellulose portion of biomass, and enhance the enzyme susceptibility (Hu et a.l 2008, Xiong et al. 2000, Azuma et al. 1984). The use of microwave irradiation pretreatment method helps significantly in increasing the conversion rate of starch materials to glucose (Palav et al. 2007, Zhu et al. 2006). However, a combination of microwave treatment with either acid or alkali or combined acid/alkali might be an alternative pretreatment method for high production of biofuel (Zhu et al. 2006, Hu et al. 2008, Binod et al. 2012). The microwave-based technologies are considered for its less energy consumption, uniform distribution of the molecules, ability to start and stop the process when required (Datta 2001, Hu et al. 2008).

2.3.2.4 EXTRUSION

It is a promising physical pretreatment technology for biomass where LCB were subjected to heating, mixing and shearing through an extruder. During this technique, physical and chemical modification of cellulose fibers took place thereby causing defibrillation, fibrillation and shortening of the fibers (Karunanithy et al. 2011, Alvira et al. 2010). This technique is highly acceptable due to its high shear, rapid mixing, no furfural formation, easy scale up and no washing and conditioning **(**Karunanithy et al. 2011). Pretreatment of soybean hulls by this technique with screw speed 350 rpm, barrel temperature of 80 ^{0} C, barrel moisture 40% could achieve 94.8% glucose conversion and glucose yield of 0.37 g/g biomass (Yoo et al. 2011).

2.3.3 CHEMICAL PRETREATMENT OF LCB

Chemical pretreatment technology is a type of pretreatment method where a chemical agent is used to degrade the biomass subsequently in other to produce bioethanol by the fermentation process. Chemical pretreatment has become one of the most promising methods for removing the lignin from the polysaccharide components of biomass and for decreasing the degree of polymerisation (DP) (Mtui 2009, Agbor et al. 2011). Different chemicals agents are used to treat the biomass samples. The chemicals such as oxidizing agents, alkali, acids, and salts can be used to degrade lignin, hemicellulose and cellulose from the lignocellulosic biomass. An investigation on pulp and paper in treatment with chemicals efficiently removed the lignin portion (Zheng et al. 2009).

2.3.3.1 ACID HYDROLYSIS

Acid pretreatment is one of the most effective and traditionally used methods for treating the biomass. In this method, dried powdered biomass is treated with the acidic solution under specific temperature for a particular period. The treated biomass is subjected for filtration to separate out the liquid from the unhydrolyzed debris. There are different acids, which can be used in the pretreatment of LCB. Inorganic mineral acids such as H_2SO_4 (Shuai et al. 2010; Digman et al. 2010; Wyman et al. 2009), HCl (Wang et al. 2010), H_3PO_4 (Marzialetti et al. 2008), and HNO_3 (Himmel et al. 1997) etc. are used as catalyst to degrade the biomass sample. The organic acid such as fumaric acid and maleic acid are also used in pretreatment (Kootstra et al. 2009). However, most commonly used acid is H_2SO_4 because of its ability to treat with different biomass example (Sun et al. 2002). It was reported that hydrochloric acid and nitric acid have better cellulose to sugar conversion rate than sulphuric acid (Tutt et al. 2012). However, both acids are more expensive than sulphuric acid. The acid treatment of LCB helps in degrading hemicellulose portion faster than cellulose molecules due to a structural configuration (Samuel et al. 2010, Foston et al. 2010). The hydrolysis of cellulose portion in acidic medium is depicted in **Fig.2.15.**

2.3.3.2 DILUTE ACID PRETREATMENT (DAP)

Industrially made acids can be diluted and used in the pretreatment of biomass for ethanol production. These acids include H_2SO_4, HNO_3, and HCL. Different concentration of acids

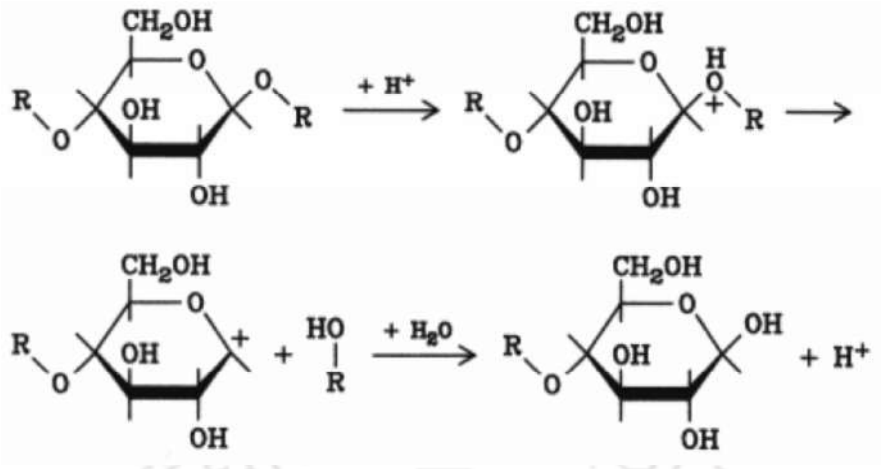


Fig.2.15 Hydrolysis of cellulose in acidic media (Krassig et al. 2002)

could be utilized for treating with biomass however, 0.2-5 % w/w is used for treating the LCB under high pressure and temperature at 120-210 ^{0}C for short period of time (Tao et al. 2011). This treatment hydrolyzed hemicelluloses and amorphous cellulose into monomer sugars and soluble oligomers into the hydrolysate and leaving most of the cellulose and lignin in the solid phase. DAP is effective in terms of low acid consumption and process severity. Another advantage is that low acid concentration releases essential nutrients such as sulphur and phosphorus that enhance downstream fermentation of sugars to ethanol (Zhang et al. 2012). Sometimes two stages processes could be used where both dilute and concentrated acid treatment have to perform. In such case, most of the hemicelluloses solubilized in dilute acid and celluloses hydrolysed in concentrated acid (Kazi et al. 2010, Talebnia et al. 2010).

The rate of cellulose conversion to glucose depends on the severity of the acid concentration. Pretreatment of grass, newspaper, potato peeling with the acids such as H_2SO_4, HNO_3 or HCL found that glucose conversion was high when the acid concentration was high (Li et al. 2007). In pretreatment of rye straw with different acids, dilute HNO_3 pretreatment was found to be more effective in the conversion of glucose than dilute H_2SO_4 (Tutt et al. 2012).

Dilute H_3PO_4 acid can be used in treating potato peels, where 82.5% of sugar yield was achieved (Leninhan et al. 2010) and in corn stover, 85% of glucose, 91.4% of xylose was yielded (Avci et al. 2013). Not only this, some people had tried to combine acids to improve process performance. HCL and H_2SO_4 were mixed in pretreating of sweet sorghum bagasse, but the performance was not effective (Heredia-Olea et al. 2012). However, a combination of H_2SO_4 and H_3PO_4 used in pretreating of oil palm empty fruit recorded high yield of xylose

(Zhang et al. 2012) as compared to single acid treatment.

2.3.3.3 CONCENTRATED ACID PRETREATMENT

The efficiency of the pretreatment of LCB depends on process adapted and affected by acid/biomass ratio, process temperature, time and concentration of the acid. Acid pretreatment can be effectively used in two ways with respect to temperature; high temperature and low acid concentration (dilute acid pre-treatment) or low temperature and high acid concentration (Concentrated acid pretreatment). Enzymatic hydrolysis of LCB could be improved with treatment of acids at high temperature. Among the acids, sulphuric acid is most widely used while other acids such as HCL, HNO_3 (Taherzadeh et al. 2007) and H_3PO_4 are also used in pretreating the biomass. Concentrated acids such as sulphuric (65-86% w/v), hydrochloric (41%) and phosphoric (85% w/w) were generally used in pretreating the dried LCB (5-10% moisture) at low temperature (30-60 ^oC).

2.3.3.4 ALKALI PRETREATMENT

In alkali pretreatment, LCB are mixed with alkali solutions such as NaOH, $Ca(OH)_2$, Potassium hydroxide, ammonia (Xu et al. 2012) and sodium bicarbonate (Na_2CO_3) (Vaccarino et al. 1987) at specific temperature and pressure to remove lignin and to degrade ester and glycosidic side chains (Sarkar et al. 2012) (**Fig.2.16).**

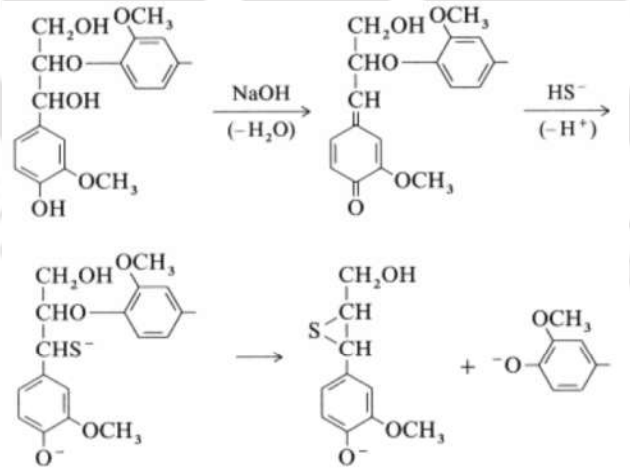


Fig.2.16 Cleavage of ether bond of lignin in alkaline solution (Lin et al. 2002)

Soaking of biomass sample in aqueous solution of alkali helps in increasing the internal surface area due to swelling, decreasing the DP and crystallinity, breaks the bond between the lignin and polysaccharides, disrupt lignin structure (Fan et al. 1987). The mechanism of alkali

treatment not only breaks lignin and polysaccharide bonds, but also hydrolyzed bond of ester (Sun et al. 2002), removed acetyl and various uronic acid substitution of hemicellulose (Chang et al. 2000), thus leaving the biomass enriched with cellulose. Alkali treatment helps in extraction of a hemicellulose part from the polysaccharide and efficiently increases the accessibility of enzyme to the cellulose. In most of the treatment process, a high concentration of base is required with relatively low temperature and longer time. In contrast, low alkali concentration (4% w/w) at high temperature and pressure could be used with high retention times (Yoo et al. 2012). After the treatment, two formed of by-products are obtained, a wet solid fraction (Cellulose) and a liquid fraction (hemicellulose, lignin, inorganic chemicals). The solids portions could be separated by simple filtration and washed with warm water. Washing helps in removing enzyme inhibitors and other chemicals. So in many cases, the bases- NaOH and $Ca(OH)_2$ have been extensively used for pretreatment of LCB in industries and research field.

2.3.3.5 SODIUM HYDROXIDE (NaOH) PRETREATMENT

Sodium hydroxide is used widely as an agent for treating the biomass of agricultural residues and soft wood. As compared with acid or oxidative reagents, alkali pretreatment appears to be the most effective technique for breaking the esters bonds in between lignin, hemicellulose, and cellulose (Gaspar et al. 2007). Dilute NaOH treatment loosens the biomass structure, increases the internal surface area, decreases the degree of polymerization and crystallinity, and disrupts the lignin structure (Bougrier et al. 2006).

The treatment of biomass with NaOH is totally depends on the temperature, concentration, and pH of the solution. Increasing pH from 10 to 13 increased the removal of lignin from 40 to 80% w/w in the wheat straw at 140 ^{0}C (Pedersen et al. 2011). It is suggested that alkali treatment has more capability to delignify than acid hence NaOH pretreatment appears to improve enzymatic hydrolysis, sugar yield and biomass utilisation rate (Ioelovich et al. 2012). For example, pretreatment for enzymatic conversion of cotton stalk using various agents like sulphuric acid, sodium hydroxide, hydrogen peroxide and ozone treatment, the highest level of delignification (65% with 2% NaOH, 90 min, 121 ^{0} C) was achieved in sodium hydroxide treatment with 60.8% cellulose conversion rate (Silverstein et al. 2007).

2.3.3.6 LIME PRETREATMENT

Lime (quick lime and slaked lime) has been extensively used as pretreatment agent due to its low cost, easily available, safety in handling, and easy recovery. Lime pretreatment has been considerably applied in many feedstocks with encouraging results. This pretreatment process is usually applied to biomass having low lignin content such as grasses (Garlock et al. 2011) sugarcane bargasse (Rabelo et al. 2008) and soft wood. Lime pretreatment improves hydrolysis rates of biomass promoting cellulose accessibility to enzymes (Kumar et al. 2009). In general, LCB is pretreated with lime water at 130 ^{0}C in the presence of oxygen to enhance delignification (Tao et al. 2011, Falls et al. 2011). For example, Corn stover was pretreated in non-oxidative condition with an excess supply of calcium hydroxide at 55 ^{0}C for 4 weeks with aeration. A total of 91.3% glucose and 51.8% xylose was yielded (Kim et al. 2005). Similarly, rice hulls were pretreated with different concentration of lime mixed with water for ethanol production (Saha et al. 2008). A total yield of sugar (12.6%) was achieved at 100 mg lime/g rice hulls in 1 hr. It was also found that lime pretreatment enhancing enzymatic saccharification without generating furfural and 5-hydroxymethylfurfural (HMF) in the hydrolysate. Despite the merits of lime pretreatment, it has many drawbacks such as longer reaction time as compared to NaOH treatment, lesser dissolving rate of water thus requires more volume of water to hold the process.

2.3.3.7 ORGANOSOLV PRETREATMENT

This pretreatment process involves extraction of lignin from plant biomass by addition of organic solvent mixture in presence of water with /without a catalyst – such as an acid (HCl, H_2SO_4), base (NaOH), or salt ($MgCl_2$, $Fe_2(SO_4)$ under certain conditions like temperature and pressure (Sun et al. 2002, Alriols et al. 2009). The most commonly used solvent are ethanol, methanol, acetone, and ethylene glycol (Ichwan et al. 2011). These solvents hydrolysed the internal bond between lignin to hemicellulose and glycosidic bonds of hemicellulose and cellulose. The maximum temperatures used in the process is at 200 ^{0}C and lower temperature depends on the type of biomass as well as the type of catalyst used. The technique involved pretreatment of biomass and separation of pretreated residues by filtration and washing with distilled water. From such pretreatment processes, usually, three main fractions could be produced- a high purity lignin, a hemicellulose syrup, and relatively pure cellulose. However, variation in the reaction time, temperature, concentration of solvent and dose of the catalyst

affects the physical characteristics (crystallinity, a degree of polymerisation and length of fibre) of the pretreated residues.

Using of sulphuric acid as a catalyst in organosolv pretreatment process has been extensively showing a good yield of sugar despites its corrosive nature. This catalyst degraded the monosaccharide into furfural and 5- HMF (Chum et al. 1985). On the treatment of pine with various catalysts, the most efficient catalyst was observed with acid treatment which produced maximum ethanol yield (Park et al. 2010). Pretreatment of wheat straw using glycerol based autocatalytic organosolv showed 98% cellulose retention (Sun et al. 2008). Likewise, organosolv pretreatment was also seen in using horticultural waste (HW) for bioethanol production (Geng et al. 2012). A modified approached of an organosolv method was introduced in HW and Japanese cypress (*Chamaecyparis obtusa*) where, used of ethanol under mild condition followed by H_2O_2 post-treatment (Geng et al. 2012, Hideno et al. 2013). A total of 11.69g/L ethanol was produced from HW hydrolysate after 8 hr of enzymatic hydrolysis and fermentation using *Saccharomyces cerevisiae*.

2.3.3.8 WET OXIDATION (WO)

This technique is one of the most common platforms for both ethanol and biogas production. In this technique, biomass is allowed to undergo oxidation in the presence of aqueous solutions with conditions like acid, neutral, and alkali provided with oxygen at an elevated temperature of 125-315 ^{0}C, 0.5- 5 MPa (Talebnia et al. 2010, Schutt et al. 2004). The process is exothermic, and therefore, it is self-supporting with respect to heat once the reaction starts (Schmidt et al. 1998). The pretreatment process involved the formation of acids from a hydrolytic process and the oxidative reaction. Due to this, cellulose, hemicellulose, and lignin undergo cleavage. Interestingly, hemicelluloses were cleaved to monomeric sugars; whereas lignins undergo oxidation and cellulose degraded to sugars (Schultz et al. 1984). The pretreated suspension was filtered to separate out the cellulose and hemicellulose substrate. The solid component was washed with de-ionized water before going through enzymatic hydrolysis. Hemicellulose portion gets oxidised into intermediates such as carboxylic acids and then cleavage into phenolic compounds, acetaldehydes, and alcohol and then finally degraded into CO_2 and H_2O (Palonen et al. 2004). One of the main problems of WO pretreatment is about the heat generation, which is very rapid and high because of the fast rate of reaction, hence difficult to control the reactor temperature (Garrote et al. 1999).

Alkaline WO pretreatment helps in reducing the inhibitors of enzymes such as furfural and HMF as compared to acidic and neutral conditions (Mart´ın et al. 2006, Thomsen et al. 1999). However, the presence of high concentration of phenolic compounds in the biomass is much more toxic than HMF and furfural (Harmsen et al. 2010). It is because phenolic compounds causing partition and loss of integrity of the cell membrane of the fermenting microorganism. Feedstock such as spruce (Rovio et al. 2012, Palonen et al. 2004) wheat straw (Klinke et al. 2003) rape straw (Arvaniti et al. 2012) and rice husk (Banerjee et al. 2009) have been successfully treated with WO and achieved good hydrolysis rate and fermentation yield.

2.3.3.9 SULPHITE PRETREATMENT

This pretreatment process is usually used in pulp and paper industry. In this method, ground biomass is mixed with sulphite (Na_2SO_3, $NaHSO_3$, etc.) solution 1-10% w/w in acidic, basic, or neutral condition at 80-200 ^{0}C and reaction time of 30-180 min. The pretreated solids were separated by filtration and washed with distilled water to remove unwanted inhibitors. This dried pretreated solid further proceed for enzymatic saccharification. In the entire process, the biomass is allowed to degrade partially and lignin tied up with sulphite to form sulphonates lignin. This technique helps in enhancing glucose yields. Later on, sulphite forms a sulphonic acid that helps in improving the hydrophilicity of the pretreated substrates (Bu et al. 2012). Sometimes, formaldehyde is added in the process to enhance removal of lignin by the formation of sulpho-methyl groups to produce the high yield of sugar (Jin et al. 2013). While treating with corn stover in alkaline $NaHSO_3$ at 140 ^{0}C, a total of 78.2% of sugar was yielded with 92% lignin removal after enzymatic hydrolysis which was higher than other alkali based methods in similar conditions (Qiang et al. 2012).

Sulphite pretreatment technique is a new emerging promising technique. This technique can be applied in many feedstocks with good results. For example, on corn cob pretreatment, at 156 ^{0}C, 1.4h, 7.1% charge and solid loading of 1:7.6 w/w, 79.3% of total glucan could be converted into glucose and cellobiose of 72.2% theoretical ethanol yield was produced (Cheng et al. 2011). However, the residue of corn cob in treating with sulphite can produce the highest glucose of 81.2% (Bu et al. 2012). In a proprietary process, known as Sulfite pretreatment to overcome recalcitrance of lignocelluloses (SPORL) is another emerging method for pretreatment technology.

2.3.3.10 SULFITE PRETREATMENT TO OVERCOME RECALCITRANCE OF LIGNOCELLULOSES (SPORL)

In this pretreatment process, biomass is pretreated in a dilute solution of sulphuric acid and sodium bisulphite ($NaHSO_3$). The residual solids are separated out from hydrolysate. The lignin portion is removed by sulphonating. SPORL (Sulfite pretreatment to overcome recalcitrance of lignocellulose) pretreatment is a good technology for removal of the recalcitrance of sulfite lignin by 32% in spruce wood (Shuai et al. 2010) and this technique can be applied to biomass having high lignin content (Tian et al. 2010, Zhu et al. 2010). Using SPORL pretreatment technology, it can reduce inhibitors of enzymes up to 65% and increases in sugar yield from 57% to 88% in softwood (Shuai et al. 2010, Tian et al. 2010, Zhu et al. 2010). In lodgepole pine pretreatment (180 ^{0}C, 25 min, liquor/wood=3:1/vw), 276 L/tonne ethanol was yielded by SPORL process (Zhu et al. 2010). In Switch grass, treatment with SPORL can digest the raw material more vigorously than other dilute acid and alkali treatment processes (Zhang et al. 2013).

On pretreatment of agave stalk with SPORL, high yield of sugar and lower inhibitors were found as compared to dilute acid and NaOH treatment (Yang et al. 2012). Moreover, this process was also found to be more efficient in sugar recovery than organsolv and steam explosion pretreatments (Zhu et al. 2010). The process has advantages such as high sugar yield, lignin removal, and biomass recovery. The drawbacks include sugar degradation, a large volume of water required in washing the pretreatment solids, a high cost of recovering pretreatment chemicals etc.

2.3.4 PHYSICO-CHEMICAL PRETREATMENT

A variety of pretreatment methods is being developed using the chemical reactions to disrupt the cellulose crystalline structure of lignocelluloses biomass. However, a combined step of physical and chemical treatment can be performed. Therefore, these pretreatment methods are two stage processes where both chemical and physical treatment combined to enable the ethanol production (Chandra et al. 2007). This method comprises a large group of various processes. In this section, we have mentioned only the important processes which are useful for the LCB conversion.

2.3.4.1 STEAM EXPLOSION (AUTOHYDROLYSIS)

This pretreatment method is substantially used in both ethanol and biogas production. It is one of the most commonly used methods for LCB pretreatment (McMillan et al. 1994). This pretreatment method allows to open the biomass fibers and makes the biomass polymers to easily accessible by enzymes in hydrolysis, fermentation, or densification processes. It is also a common pretreatment process for the production of solid biofuel pellets to increase the calorific value. In most of the cases, biomass is treated with high-pressure saturated steam, and then sudden released of pressure makes the materials to undergo an explosive decompression. This allows to degrading hemicelluloses into sugars and lignin transformation due to high temperature, thereby increasing the potential of cellulose hydrolysis in later subsequent steps. In the process, the temperature is set in the range of around 160-260 ^{0}C, pressure 0.69-4.83 MPa, 30 sec to 20 min before materials are exposed to atmospheric pressure (Sun et al. 2002). The steam mixture is held for a certain period to hydrolyze the hemicellulose into sugars, and the process is terminated by an explosive decompression.

In poplar chips pretreated by the steam explosion, a total of 90% efficiency of enzymatic hydrolysis was achieved in 24 hr as compared to untreated chip, which produces nearly 15% hydrolysis efficiency (Grouse et al. 1986). Steam explosion method was also applied in sunflower stalk at 180-230 ^{0}C before enzymatic hydrolysis (Ruiz et al. 2008). The highest glucose yield was obtained at 220 ^{0}C and hemicellulose recovery at 210 ^{0}C. In another report, poplar and eucalyptus chips were pretreated at 210 ^{0}C for 4 min, wheat straw at 190 ^{0} C for 8 min, *Brassica carinata* residue at 210 ^{0}C at 8 min and sweet sorghum bagasse at 210 ^{0} C for 2 min (Ballesteros et al. 2004). In all cases, hemicellulose sugars were extensively solubilised and found that there was decreased of xylose content from 75-90%. This technique seems to be cost effective and satisfied most of the requirement in pretreatment process. Therefore, many researchers have tested this method on lab and pilot scale related to industrial-scale production. For example, Masonite plants were set up for large-scale production (Chum et al. 1985).

2.3.4.2 CATALYZED STEAM-EXPLOSION

A steam explosion can be discussed in many ways with the addition of various chemical agents. The chemical agents such as SO_2, H_2SO_4, CO_2, oxalic acid, etc. are used as a catalytic agent to impregnate the biomass. Use of such catalytic agents prior to steam-

explosion has a greater impact on complete hemicellulose removal with better enzymatic digestion and less formation of inhibitory compounds (Morjanoff et al. 1987). For example, SO_2 impregnated was used in pretreating agricultural residues (Zhu et al. 2010). Moreover, SO_2 impregnated biomass is relatively much easy to hydrolyze and ferment the hydrolysate than that of dilute acid impregnated because the later produces inhibitory compounds (Soderstrom et al. 2005).

2.3.4.3 AMMONIUM FIBER/FREEZE EXPLOSION (AFEX)

It is one of the alkaline physico-chemical pretreatment processes. In this treatment, biomass is exposed to hot liquid ammonia at 90- 100 ^{0} C for 30 min under high-pressure and then the sudden release of pressure disrupts the structure of LCB leading to increasing digestibility and simultaneously delignifying the biomass (Mosier et al. 2005). This process can modify or effectively reduce lignin content in biomass without disturbing hemicellulose and cellulose fractions. The optimum condition for pretreatment of LCB by AFEX process varies according to nature of the material. For example, in Switch grass, the optimum conditions of pretreatment were 100 ^{0}C, ammonia loading of 1:1 kg of ammonia per kg of dry matter and 5 min retention time (Alizadeh et al. 2005). It was also observed that AFEX pretreatment is very much effective in fiber digestion and produced a low level of inhibitors than acid pretreatment (Balan et al. 2009, Lau et al. 2009) and it is a highly effective method for pretreatment of grasses. Further studies on AFEX pretreatment technology found that compounds like 4-hydroxybenzaldehyde, lactate and acetate inhibit fermentation of *E.coli* KO11 strain (Gollapalli et al. 2002). That is why this technology is difficult to have one general method for a different type of biomass pretreatment. However, AFEX treatment of agricultural residues showed less efficiency than that treatment with wet oxidation and liquid hot water (LHW) (Varga et al. 2002, Van et al. 1996, Chundawat et al. 2007).

One of the major advantages of AFEX process is that there is no formation of inhibitory by-products such as furans (Chundawat et al. 2007). However, AFEX pretreatment does not significantly solubilize hemicellulose as other pretreatment processes do. The ammonia present in the process must be recycled after pretreatment to reduce cost and mitigation of environment (Eggeman et al. 2005, Sun et al. 2002).

2.3.5 BIOLOGICAL PRETREATMENT

These pretreatment methods employed wood degrading microorganism such as white, brown, soft-rot fungi and bacteria for the production of bioethanol from biomass. The biological pretreatment by white rot fungi is a co-oxidative process where a carbon source is required. The required carbon is supplied from cellulose and hemicelluloses present in the biomass. The employed microorganism modifies the chemical composition of LCB so that enzyme can easily digest the biomass (Kurakake et al. 2007, Lee et al. 2007, Singh et al. 2008). Different types of fungi can be employed according to the target substrate requirement. In general, brown and soft rots are used to attack the cellulose component of biomass while white rot fungi usually degraded lignin (Schurz 1978). Among the fungi used in the biological pretreatment of biomass, White rot fungi are the most promising one (Ander et al. 1978). In one of the treatment, using four different types of white rot fungi on wood chips for 30 days, it was found that *Trametes versicolor* MrP1 could hydrolyzed maximum substrate up to 45% and glucose conversion rate of 35% (Hwang et al. 2008). In another treatment of Japanese red pine *Pinus densiflora* with three species of white rot fungi such as *Ceriporia lacerata*, *Stereum hirsutum,* and *Polyporus brumalis,* the most effective for lignin degradation was observed in *Stereum hirsutum (*Lee et al. 2007).

Selection of the type of biomass and choosing the species to use for pretreatment is an important aspect of biological pretreatment method. The pretreatment of biomass like corn stover with *Cyathus stercoreus* found that there is an improvement of 3 to 5 fold in enzymatic digestibility (Keller et al. 2003). While in treating of bamboo with different 35 isolate species of white-rot fungi, lignin degradation was more active in 3 species namely *Echinodontium taxodii* 2538, *Trametes versicolor G*20 and *Coriolus versicolor B*1 (Zhang et al. 2007).

Biological pretreatment has potential advantages over physical/chemical pretreatments. It is a promising technique and can overcome the chemical treatment method likes, low energy input, mild environment conditions, environment-friendly etc. (Sun et al. 2002). However, the characters like slow rate, a large amount of space required, consumption of hemicellulose and cellulose by microorganism shows disadvantages of using biological pretreatment. Therefore, bio-pretreatment faces techno-economic challenges, which led to less attractive in commercialization *(*Lee et al. 2007, Singh et al. 2008).

Each method of pretreatment has its own characteristics and a large impact in the overall lignocellulose conversion process. Each pretreatment method offers distinct advantages and disadvantages **(Table 2.4).** The ideal pretreatment methods are cost effective, less degradation of carbohydrate, and low formation of inhibitory compounds (Sun et al. 2002). Among the various pretreatment methods, the thermo-chemical method such as lime treatment, dilute acid treatment, ammonia treatment, steam explosion are the most cost-effective and promising pretreatment methods (Mosier et al. 2005).

Table 2.3 Pretreatment methods of lignocellulosic biomass for fuel ethanol production (Jitendra et al. 2005)

Methods	Procedure/agents	Remarks	Examples of pretreated materials	References
I. ***Physical methods***				
Mechanical size reduction	Chipping, grinding, milling	Milling: vibratory ball mill Wiley mill (final size: 0.2–2 mm), knife or hammer mill (final size: 3–6 mm)	Hardwood, straw, corn stover, timothy, alfalfa, cane and sweet sorghum bagasse	(Sun et al. 2002)
Pyrolysis	T > 300 °C, then cooling and condensing	Formation of volatile products and char Residues; produce 80–85 % reducing sugars (>50 % glucose); can be carried out under vacuum	Wood, Waste cotton, corn stover	(Khiyami et al. 2005)
II. *Physico–chemical methods*				
Steam explosion	Saturated steam at 160–290 °C, p = 0.69–4.85 MPa for several sec or min, then decompression until atm.	It can handle high solid loads; size reduction with lower energy input, compared to comminution, 80–100 % hemicellulose hydrolysis, Inhibitors formation; addition of H_2SO_4 , SO , or	Poplar, aspen, eucalyptus softwood (Douglas fir) bagasse, corn stalk, wheat straw, rice straw, barley	(Ballesteros et al. 2004, Hamelinck et al. 2005, Lynd et al. 2002, Soderstrom et

	pressure	CO improves efficiency of further enzymatic hydrolysis; cellulose depolymerization	straw, sweet sorghum bagasse, *Brassica carinata* residue, olive stones, Timothy grass, alfalfa, reed canary grass	al. 2005)
Liquid hot water (LHW)	Pressurized hot water, $p > 5$ MPa, $T = 170–230$ °C, 1–46 min; solids load <20 %	Lignin is not solubilized, but redistributed; 80-100 % hemicellulose hydrolysis, 88–98 %xylose recovery; low or no formation of inhibitors; cellulose conversion >90 %; partial solubilization of lignin (20–50 %)	Bagasse, corn stover, olive pulp, Alfalfa fiber	Ballesteros et al. 2002, Koegel et al. 1999, Lynd et al. 2002)
Ammonia fiber explosion (AFEX)	1–2 kg ammonia/kg dry biomass, 90 °C, 30 min $p = 1.12–1.36$ MPa	Ammonia recovery is required 0–60 % hemicellulose hydrolysis; no inhibitor formation; further cellulose conversion can be >90 %, for high-lignin biomass (<50 %); 10–20 % lignin solubilization	Aspen wood chips bagasse, wheat straw, barley straw, rice hulls, corn stover, switchgrass, coastal bermudagrass, alfalfa newsprint	Sun et al. 2002, Lynd et al. 2002)
CO_2 explosion	4 kg CO_2/kg fiber, $p = 5.62$ MPa	No inhibitors formation Further cellulose conversion can be >75 %	MSW Bagasse Alfalfa recycled paper	(Sun et al. 2002)
Ozonolysis	Ozone, room temperature and pressure	No inhibitors formation further cellulose conversion can be >57 % lignin degradation	Poplar, sawdust, pine, bagasse, wheat straw, cotton straw, green hay, peanut	(Sun et al. 2002)

Dilute-acid hydrolysis	0.75–5 % H_2SO_4 , HCl, or HNO3, p = 1 MPa; continuous process for low solids loads (5–10 wt% substrate/mixture); T = 160–200 °C; batch process for high solids loads (10–40 % substrate/mixture); T = 120–160 °C	pH neutralization is required that generates gypsum as a residue; 80-100 % hemicellulose hydrolysis, 75-90 % xylose recovery; high temperature favors further cellulose hydrolysis lignin is not solubilized, but it is redistributed	Poplar wood bagasse, corn stover, wheat straw, rye straw, rice hulls, switchgrass, Bermudagrass	Hamelinck et al. 2005, Lynd et al. 2002, Sun et al.2002, Wooley et al. 1999)
Concentrated-acid hydrolysis	10–30 % H_2SO_4 , 170–190 °C, 1:1, solid–liquid ratio	Acid recovery is required; residence time greater compared to dilute-acid hydrolysis; peracetic acid provokes lignin oxidation	Poplar sawdust, bagasse	(Cuzens et al. 1997)
Alkaline hydrolysis	Dilute NaOH, 24h, 60 ^{0}C; Ca(OH) 2, 4 h, 120 °C; it can be complemented by adding H_2O_2 (0.5–2.15 vol.%) at lower temperature (35 °C)	Reactor costs are lower compared to acid pretreatment >50 % hemicellulose hydrolysis, 60–75 % xylose; recovery low inhibitors formation; cellulose swelling; further cellulose conversion can be >65 %; 24–55 % lignin removal for hardwood, lower f or softwood	Hardwood bagasse, corn stover, straws with low lignin content (10-18%), cane leaves	(Hamelinck et al. 2005, Kaar et al. 2000, Lynd et al. 2002, Saha et al. 2008, Sun et. al. 2002)
Organosolv process	Organic solvents (methanol, ethanol, acetone, ethylene	Solvent recovery required; almost total hydrolysis of hemicellulose; high yield of xylose	Poplar wood mixed softwood (spruce, pine, Douglas fir)	(Lynd et al. 2002, Pan et al. 2005, Sun et al.

	glycol, triethylene glycol) or their mixture with 1 % of H_2SO_4 or HCl; 185–98 °C, 30–60 min, pH = 2.0–3.4	almost total lignin solubilization and breakdown of internal lignin and hemicellulose bonds		2002)
III. *Biological methods*				
Fungal pretreatment	Brown-, white- and soft-rot fungi; Cellulase and hemicellulase production by solid-state fermentation of biomass	Fungi produces cellulases, hemicellulases, and lignin-degrading enzymes: ligninases, lignin. peroxidases, polyphenoloxidases, laccase and quinone-reducing enzymes; very slow process	Corn stover, wheat straw	(Sun et al. 2002)
Bioorganosolv pretreatment	*Ceriporiopsis subvermispora* for 2–8 weeks followed by ethanolysis at 140–200 °C for 2 h	Fungi decompose the lignin network ethanol action allows hemicellulose hydrolysis biological pretreatment can save 15 % of the electricity needed for ethanolysis ethanol can be reused; environmentally friendly process	Beech wood	Itoh et al. (2003)

Table 2.4 Advantages and disadvantages of different pretreatment methods for lignocellulosic biomass (Kumar et al. 2009; Alvira et al. 2010; Brodeur et al. 2011; Nanda et al. 2014, Rasool et al. 2014, Hendriks et al. 2009)

Pretreatment method	**Advantages**	**Disadvantages**
Mechanical comminution	Reduces cellulose crystallinity	Power consumption is usually high
Alkali	Efficient removal of lignin Low inhibitor formation	High cost of alkali Alters lignin structure
Acid (dilute)	Solubilizes hemicellulose Low acid consumption Short processing time Acid recovery is not required	High pressure and temperature are needed Cellulose hydrolysis is not effective Formation of inhibitors Equipment corrosion
AFEX	Highly effective for agricultural residues Cellulose becomes more accessible Reduce the lignin content Low formation of inhibitors	High cost of ammonia Ammonia recycling is needed Alters lignin structure Less effective for the lignin rich materials e.g. softwoods
ARP	Removes most of the lignin Cellulose rich pulps are obtained after pretreatment Most suitable to herbaceouses	High energy costs and liquid loading Less satisfactory for softwoods
CO2 explosion	Accessible surface area is increased Cost effective Does not cause formation of byproducts	Lignin or hemicelluloses are unaffected
Green solvents (ILs)	Lignin and hemicellulose hydrolysis Ability to dissolve high loadings of different type of lignocelluloses Mild processing conditions	Potentially high solvent costs Need for solvent recovery and recycle Unknown eco-toxicology of many formulations
LHW	Separation of nearly pure	High energy/water input

	hemicellulose from rest of feedstock No need for catalyst Hydrolysis of hemicellulose	Solid mass left over will need to be dealt with (cellulose/lignin)
Ozonolysis	Reduces lignin content Does not produce byproducts	Large amount of ozone is required Expensive
Organosolv	Hydrolyzes lignin and hemicelluloses	Solvents recovery and recycle is needed Requires high energy High cost
SCF	Low degradation of sugars Cost effective Increases cellulose accessible area	High pressure requirements Lignin and hemicelluloses are unaffected
Steam	Cost effective Lignin transformation Solubilization of hemicelluloses	Hemicellulose degradation Acid catalyst is needed to improve the process performance Formation of byproducts
Biological	Degrades lignin and hemicelluloses Low energy requirement	Hydrolysis is very slow
Wet oxidation	Increases accessible surface area Removes lignin and hemicellulose to an extent	Expensive
SPORL	Slight degradation of cellulose Nearly complet solubilisation	Possible need great capital investment
Thermal	High efficiency in improving organic matter solubilsation	High energy consumption
Milling	Decreases in cellulose cyrtallinity	High energy consumption

AFEX: Ammonia Fiber Explosion; ARP: Ammonia Recycle Percolation; IL: Ionic liquid; LHW: Liquid hot water; SCF: Supercritical fluid; SPORL: sulfite pretreatment to overcome recalcitrance of lignocelluloses.

2.3.6 INHIBITORY COMPOUNDS

The major drawback of any pretreatment methods is the formation of inhibitory compounds in the hydrolysate that inhibits the activity of yeast and enzyme. These inhibitory compounds are the by-product formation from the degradation of cellulose, hemicellulose, and lignin. They are highly toxic to the hydrolytic enzymes and fermenting microorganism and thus lead to decrease ethanol yield. The nature and concentration of inhibitory substances vary with the type of biomass sample, the amount of solids in the reactor, and conditions of pretreatment applied. These substances include phenolic compounds, aromatics, aliphatic acids, furan aldehydes, inorganic ions, acetic acid, formic acid, levulinic acid, 5-hydroxymethyl-2-furaldehyde (HMF), vanillin, syringaldehyde, conferyl aldehyde and bioalcohol or other fermentation products (Leif et al. 2013, Parawira et al. 2011). The formation of inhibitor substances in the hydrolysate is given in **Fig.2.17.**

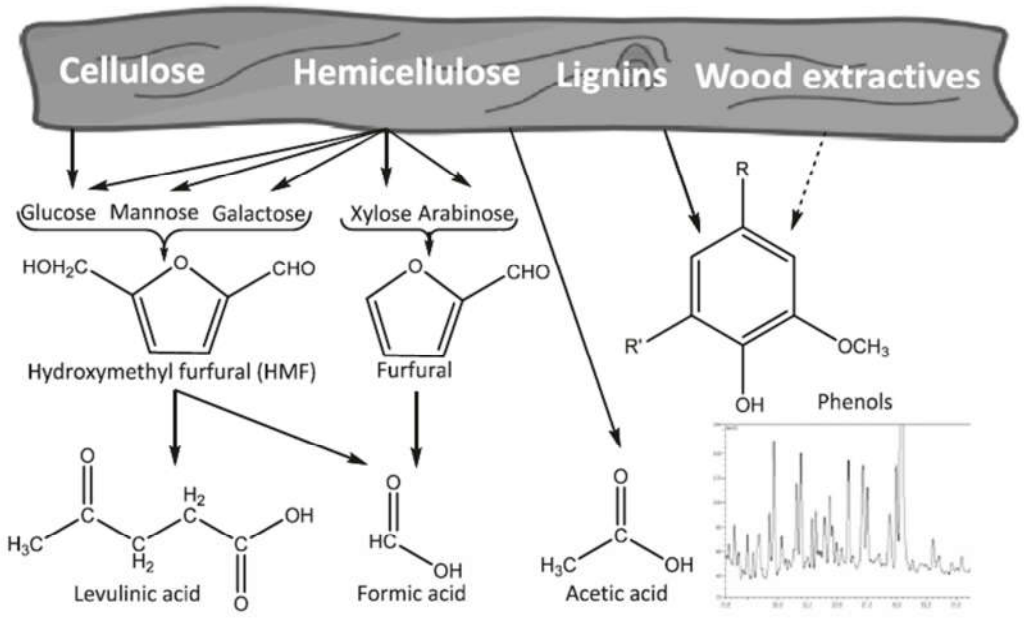


Fig.2.17 Formation of inhibitors. Scheme indicating main routes of formation of inhibitors. Furan aldehydes and aliphatic acids are carbohydrate degradation products, while lignin is the main source of phenolic compounds, as indicated by guaiacyl (4-hydroxy-3-methoxyphenyl) and syringyl (4-hydroxy-3,5-dimethoxyphenyl) moieties found in many phenolics. The peaks representing phenolic compounds found in a hydrolysate by Liquid chromatography-mass spectrometry (LC-MS) (Leif et al. 2013).

2.4 HYDROLYSIS

The hydrolysis of polysaccharides component of lignocelluloses is generally complying with either acid or enzyme as catalyst. However, the combination of both the acid and enzyme is the most preferred approach to achieving high sugar yield. The advantages and disadvantages of using of acids and enzymes are mentioned in **Table 2.5.**

Table 2.5 Acid and enzyme hydrolysis advantages and disadvantages (Kumar et al. 2009, Bhat 2000)

Hydrolysis process	**Advantages**	**Disadvantages**
Concentrated acid	Mild reaction conditions ($T<50^oC$))	Acid consumption is high
	High sugar yield	Longer reaction time (2–6 h)
	Pretreatment is not required	Sever corrosion of equipment
		Acid recovery is needed
Enzymatic	Mild reaction conditions	Enzyme cost is high, at present
	(~pH 5.5, T 50^oC)	Hydrolysis is usually performed for a long time, several days
	High sugar yield	
	No formation of inhibitors	Pretreatment is required

2.4.1 ACID HYDROLYSIS

It is performed in treating the biomass with mineral acids such as H_2SO_4, and HCl at relatively low temperatures (T<50 ^{O}C) with a high acid concentration of 30-70 wt% (Kumar et al. 2009a, Goldstein et al. 1983). The biomass is thus hydrolyzed and sugars are released into the hydrolysate and leaving mostly the solid lignin phase. The concentrated acid treatment with biomass is usually more significant than dilute acid treatment. Also, when dilute acid treatment is conducted, subsequently enzymatic hydrolysis is needed, while this is not required in case of concentrated acid hydrolysis. However, both the acid concentration treatment offers good performance in terms of sugars recovery.

2.4.2 ENZYMATIC HYDROLYSIS

After pretreatment of biomass, the integral part of cellulose and hemicellulose is still remained in polymeric/oligomeric form. To release the cellulose/hemicellulose into monomeric sugars, a set of enzymes, mainly cellulases, are needed. Enzymatic hydrolysis is considered as the most attractive treatment over the concentrated acids **(Table 2.5).** As we know that, lignocelluloses biomass has complex structure; therefore, multiple enzymes are often needed for the degradation of its carbohydrates polymer (cellulose and hemicellulose). For instance, degradation of cellulose required a mixture of cellulase enzymes and for hemicellulose, different hemicellulases such as xylanases, mannoses etc. are required (Bhat 2000).

The main advantages of enzymatic hydrolysis include high sugar yield, moderate temperatures for reaction, no corrosion, and low formation of by-products. However, the major bottleneck of using enzymatic hydrolysis includes the high cost of enzyme and low rate of reaction. Although, many experts considered enzymatic hydrolysis as the most cost-effective process in the long run (Hamelinck et al., 2005) and it is thought to be the key process to achieve an economically viable ethanol production (Horn et al. 2010). There are several factors, which involved in the enzymatic hydrolysis of cellulose. Specifically, type of pretreatment employed, particle size, lignin content, substrate concentration, enzyme activity and loading, synergism and hydrolysis condition (pH, temperature etc.) (Klyosov 1986; Gregg et al. 1996; Chang et al. 2000; Sun et al. 2002; Jørgensen et al. 2007; Zhu et al. 2008; Kristensen et al. 2009; Alvira et al. 2010; Binod et al. 2011; Yang et al. 2011; Zhao et al. 2012b; Guo et al. 2014). Even though the individual impact of these factors on determining

the efficiency of enzymatic hydrolysis has not been fully resolved, many of these factors are found to be interrelated during the saccharification process.

2.4.2.1 CELLULASE COCKTAIL

Most of the cellulase-producing micro-organism are from fungal families such as *Trichoderma* and *Aspergillus.* They are used in industrial cellulase production due to their high secretion level of cellulases (Zhang et al. 2004). The cellulase systems comprised of three main classes of enzymes, i.e. endoglucanase (EGs), exoglucanase also known as cellobiohydrolase (CBHs), and β-glucosidase (BGs), having a synergetic effect on the hydrolysis of cellulose (Van et al. 2012). In the primary hydrolysis, EGs breaks the low crystallinity regions of cellulose fibre and forms new free chain-ends whereas, CHBs further cleave the cellulose chains from free ends to release cellobiose units. This process releases soluble sugars into the liquid phase (Binod et al. 2011). Secondary hydrolysis occurs in the liquid phase by BGs where hydrolysis of cellobiose units into glucose molecules took place (Percival et al. 2006, Binod et al. 2011).

2.4.2.1.1 *TRICHODERMA REESEI*

Cellulases are the class of enzymes, which are able to hydrolyze the long cellulose chain. The fungi such as *Aspergillus, Bacillus, Humicola, Phanerochaete* and *Trichoderma* are a good source for producing cellulose catalytic enzymes (Sukumaran et al. 2005). Among the cellulases producing fungi, *Trichoderma reesei* is widely used enzyme in industry scale for producing the wide range of commercial enzymes including cellulases and hemicellulases (Sheehan et al. 19999). *Trichoderma reesei* has the advantage of being non-toxic and non-pathogenic in used, which is important in large-scale production (Nevalainen et al. 1998). *Trichoderma reesei* excretes at least two different cellobiohydrolases (CBH1-2), five endoglucanases (EG1-5), β-glucosidases and hemicellulases (Vinzanta et al. 2001). The main enzymes (EG1-2 and CBH1-2) accounts 90% of total excretes cellulases in *T.resei.* The CBH cuts the cellobiose units from ending chain. A typical CBH enzyme consists of 3 different domain; cellulose binding molecule (CBM), hydrolytic domain containing active site and a linker giving the enzyme its flexibility (**Fig.2.18).** CBM binds the enzyme to the cellulose chain in other to keep the catalytic domain close to its substrate (**Fig.2.18).** It has also been found that CBMs help in breaking down of the intermolecular bond of the cellulose, disrupting the crystalline structure and helping the hydrolytic domain to access more surface areas (Hall et al. 2001).

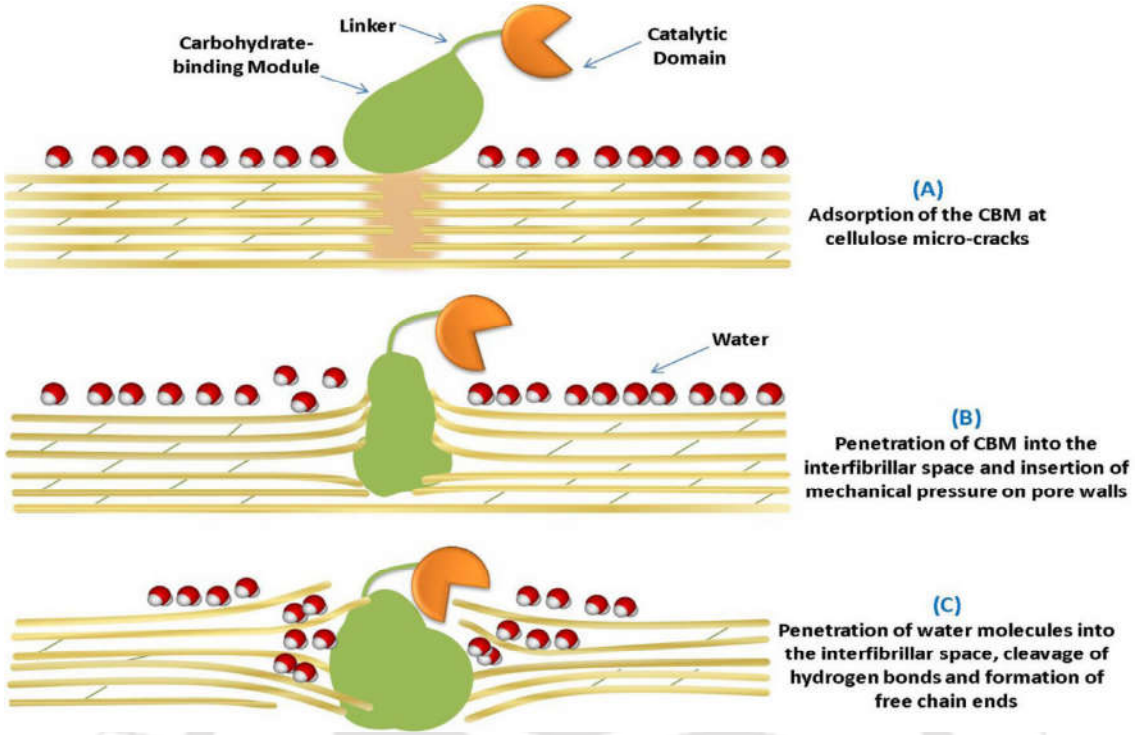


Fig.2.18 Schematic representation of amorphogenesis of cellulose fibers mediated by the carbohydrate-binding module (CBM) of cellobiohydrolase I (CBHI) (Steghallian et al. 2000).

2.4.3 FERMENTATION

Fermentation is a metabolic process of conversion of monomeric sugars into ethanol by the microorganism. Different organisms such as bacteria, yeast and fungi can be used for the conversion, however, the most frequently, and industrially used organism are the robust yeast (*Saccharomyces cerevisiae)* (Galbe et al. 2002). French microbiologist, Louis Pasteur is often remembered for his insights into fermentation and its microbial causes. The science of fermentation is known as zymology. Under the anaerobic conditions, *Saccharomyces cerevisiae* produces ethanol from the glucose (**Fig.2.19).** Theoretically, the conversion rate of glucose to ethanol is 0.5 g EtOH/g glucose. However, the fermentation efficiency of the yeast is generally achieved up to 90% and hence, the maximum conversion of 0.46 EtOH/g glucose is advisable (Öhgren et al. 2007). One major drawback of using *Saccharomyces cerevisiae* is that it cannot ferment the pentoses. Studies have therefore been performed on *Saccharomyces cerevisiae* to genetically modify to become both pentose and glucose fermenting yeast. The efficiency of the fermenting process depends on several factors such as pretreated method, a

hydrolysis method, choice of microorganism, pH, temperature, substrates and ethanol concentration.

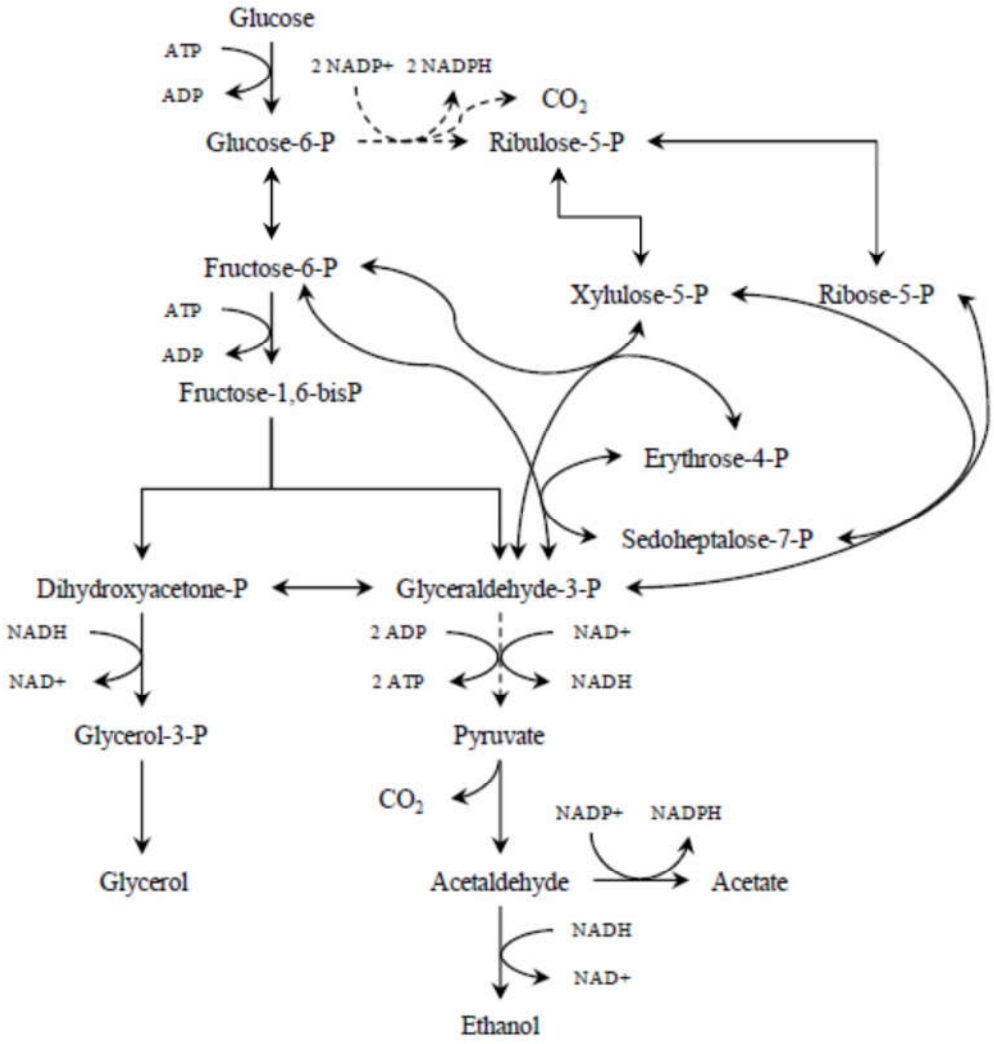


Fig.2.19 The central carbon metabolism of *S. cerevisiae* during fermentation. (Linda et al. 2016)

In general, the fermentation is carried out at pH 5.0 and a temperature of maximum 37 C (Alfani et al. 2000). The inhibitors, generated from the upstream processes, also affect the efficiency of the ethanol production. In addition, ethanol, the product itself, has an inhibiting effect on the fermenting microorganism, thus limits the conversion rate of glucose to ethanol (Olsson et al. 1996).

2.4.3.1 SEPARATED HYDROLYSIS AND FERMENTATION (SHF)

The enzymatic hydrolysis step is often in close collaboration with the following steps in the ethanol production from LCB. These processes may be in several ways; separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP) (**Fig.2.20)**. Each process has its own pros and cons (Galbe et al. 2002).

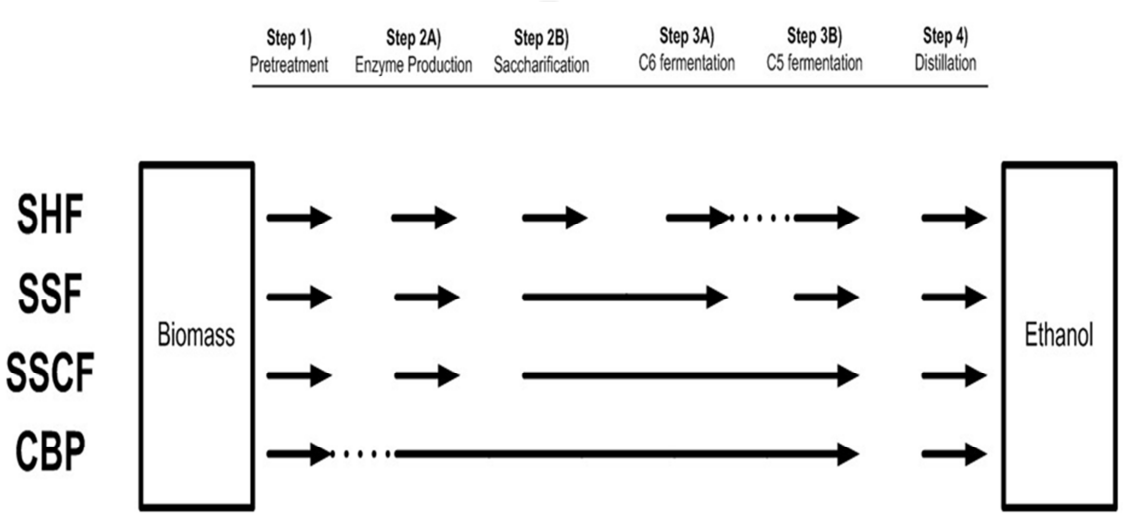


Fig.2.20 Process steps of bioethanol production processes. SHF—separate hydrolysis and fermentation; SSF—simultaneous saccharification and fermentation; SSCF—simultaneous saccharification and co-fermentation; CBP—consolidated bioprocessing; C6—hexose; and C5—pentose (Sean et al. 2015).

This thesis will focus on SHF. It is the most ancient concept, where the hydrolysis and fermentation of lignocellulosic biomass took place at two stages. The hydrolysis of biopolymers to sugars and fermentation of sugars to ethanol are conducted separately **(Fig.2.21).** The main advantage of this method is that the two processes (hydrolysis and fermentation) can be performed at their own individually optimal conditions. Another advantage with SHF is the possibility to run the fermentation process in a continuous mode with cell cycling (Galbe et al. 2002). The main limitation of SHF is the accumulation of glucose during enzymatic hydrolysis that can inhibit the cellulase activity (Chang et al. 2011).

After pretreatment, the slurry can be filtered to separate out the pre-hydrolysate and the solids. The sugars, mainly the pentoses that have been released from the hemicellulose during

the pretreatment will be removed and solid part (substrate) having cellulose and lignin is supplied to the enzymatic hydrolysis. Sometimes, the solid material obtained after the pretreatment was washed with water to remove the toxic substances (Lu et al. 2010). However, these methods require extra processing steps and increase the water consumption. This can be avoided by utilizing the whole slurry for enzymatic hydrolysis without further washing (Horn et al. 2010).

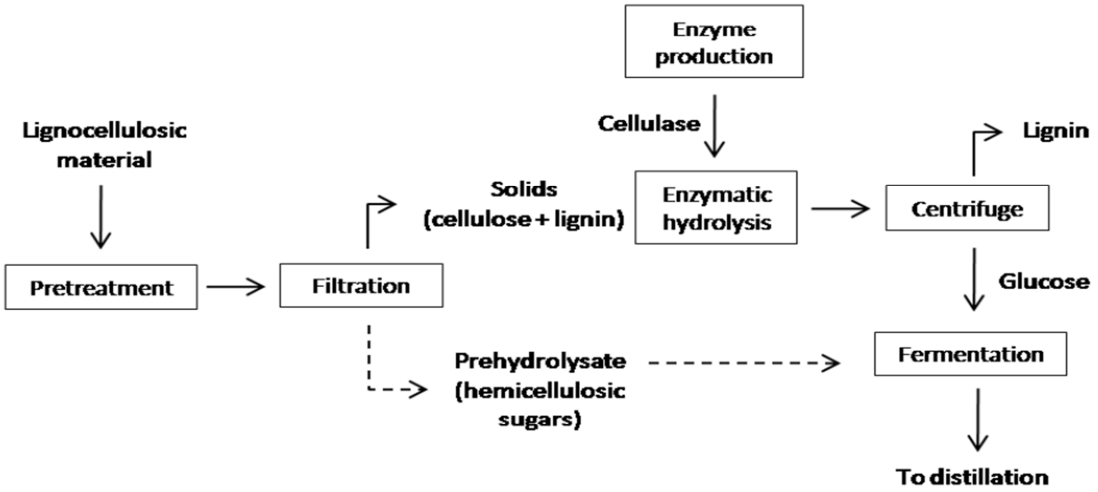


Fig 2.21 Flow sheet showing a schematic picture of a possible bioethanol process using separate hydrolysis and fermentation (SHF).

2.5 THE NORTH EAST INDIA

The North Eastern (NE) region of India is situated geographically between 22° N' and 29° 5' N latitude and 88° 00' E and 97° 30' E longitudes. It covers nearly 2,62,379 sq.km area. The region comprises seven states (Arunachal Pradesh, Assam, Meghalaya, Manipur, Tripura, Mizoram, Nagaland) which are called as seven sisters of India with extending state Sikkim (**Fig.2.22)** (Ministry of Environment & Forest, Govt. of India, 2010). This region is officially recognized as a special category of Indian states. The region contributes around 7.9% of the country's total geographical area. The Northeast region of India contributes more than one-third of the India's total biodiversity. The region harbours one of the most important biodiversity hotspot (Indo-Myanmar biodiversity hotspot) of India out of 25 global biodiversity hotspots recognized. The region is known for its richest biological values having different vegetation types. The NE region is unique in providing the habitats of different features of biota with a high endemic species. The region is also the abode of different 225

tribes of India, out of 450 tribes in the country with different culture and customs of which have an important role in understanding the biodiversity conservation and management.

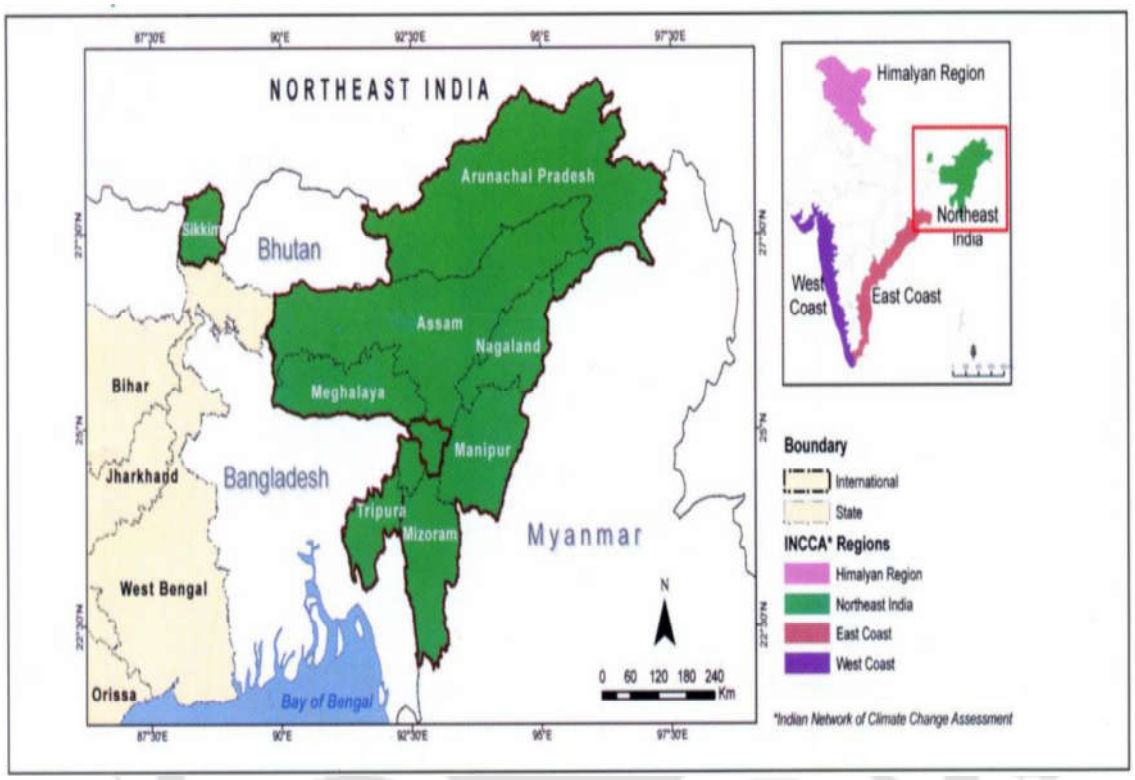


Fig.2.22 The North Eastern Region and its constituent states in India (Ministry of Environment & Forest, GoI, 2010).

2.5.1 FOREST RESOURCE OF NORTH EAST INDIA

The majority of the region is covered by forest areas (54%) (Kushwaha 2006). The total land area, which is under uncultivated land, is 1376 thousand hectare (sum of all states) **(Table 2.6).** Among the states, Assam recorded maximum land area comprises of totals 445 out of 1376 thousand hectares. The North Eastern region of India is famous for having the rainforests. Most of the state's area is covered by forest more than 60% of their geographical area. The terrestrial natural ecosystem of this region including the Assam are mainly forest ecosystems covering both the hills and plains, besides the grassland ecosystem dominated by both grasses and forest mainly of secondary nature.

The details of the land cover under the forest areas are given in **Table 2.7.** The table shows that Assam (34.21%) and Meghalaya (42.34%) are having relatively less forest area as

compared to other states. Sikkim (82.31%) has highest majority land area under the forest covered.

Table 2.6 Land Use Classification of different NE States (thousand ha) (Ministry of Agriculture, GoI, 2006-07)

State	Geographical area	Reporting area for land utilization statistics	Forest	Not available for cultivation			Other uncultivated land excluding fallow land			
				Area put to non agricultural uses	Barren& uncultur-able land	Total	Permanent pastures & other grazing lands	Land under Misc. Tree Crops & groves not inc. in net area sown	Culturable waste land	Total
Sikkim	710	728	319	143	107	250	4	5	2	12
Arunachal Pradesh	8374	5547	5154		32	32		46	28	74
Manipur	2233	1950	1693	26	1	27	1	66	1	8
Meghalaya	2243	2227	942	90	137	227		158	453	611
Mizoram	2108	1951	1594	125	9	134	5	10	5	21
Nagaland	1658	1582	863	75		75		120	56	177
Tripura	1049	1049	606	131	3	134		27	1	28
Assam	7844	7850	1933	1081	1453	2533	160	209	77	445

There are usually six types of forest found in this region viz., tropical moist deciduous forests, tropical semi-evergreen forests, tropical wet evergreen forests, subtropical forests, temperate forests and alpine forests. Among the different vegetation type found in India, six types are found in this region resulting in great biodiversity significance in flora and fauna (Hegde 2000).

2.5.2 FLORA AND GRASSLAND TYPE OF NORTH EAST INDIA

The region contributes considerably in the form of wild relatives of several crop plants and domesticated animals. This region forms the richest reservoir of genetic variability of many groups of crop plants. The following are the highlights figure of biodiversity significance of this region (Hegde 2003).

Table 2.7 Recorded Forest Area in NE states (2007) (State Forest Report (2001, 2005, 2009) FSI, Dehradun

State	Recorded Forest Area (sq.km)				% of Forest Area to G.A.
	Reserved forest	Protected Forest	Un-classed Forest	Total Forest Area	
Sikkim	5452	389	--	5841	82.31
Arunachal Pradesh	10546	9528	31466	51540	61.65
Manipur	1467	4171	11780	17418	78.01
Meghalaya	1113	12	8371	9496	42.34
Mizoram	7909	3568	5240	16717	79.30
Nagaland	86	508	8628	9222	55.62
Tripura	4175	2	2117	6294	60.02
Assam	17864	--	8968	26832	34.21

1. Out of 9 important vegetation types of India, 6 are found in the North East India
2. 40 out of 54 species of gymnosperms
3. 500 out of 1012 species of Pteridophytes
4. 825 out of 1145 species of orchids
5. 80 out of 90 species of rhododendrons
6. 60 out of 110 species of bamboo
7. 25 out of 56 species of canes

Nearly, 50% of the total flowering plants recorded in India are found in the North East India. The region is also known as 'Cradle of flowering plants' (Takhtajan 1969). From the point of biodiversity, the North East India is very rich and harbours the largest number of endemic species than anywhere in the country (Mackinnon et al. 1986). The state wise flowering species distribution is presented in **Table 2.8.**

The region being remote and inaccessible in communication most cases has not been explored completely in terms of plants and animals and holds great potential for new plant discoveries. During the year 2010, 24 plant species of different genera were discovered by the different plant taxonomist of NE region of India (**Table 2.9).**

Table 2.8 State wise flowering species of North East India (Sudipto et al. 2006)

State Species richness (flowering plants)	State Species richness (flowering plants)
Arunachal Pradesh	5000
Sikkim	± 4500
Meghalaya	± 3500
Assam	± 3010
Manipur	± 2500
Nagaland	± 2250
Mizoram	± 2200
Tripura	± 1600

Table 2.9 New plant discoveries during the year 2010 from NE region of India (Botanical Survey of India (2011): Plant Discoveries 2010.)

S.N.	**New species/var./sub spp.**	**Family**	**NE State from where discovered**
Angiosperms			
New Genera			
1.	Larsenianthus (4 spp)	Zingiberaceae	NE India
2.	Stapletonia (1 sp)	Poaceae	Arunachal Pradesh
New Species/varieties/sub species			
1	Bambusa dampaeana	Poaceae	Mizoram
2	Boehmeria listeri	Urticaceae	Arunachal Pradesh
3	Boehmeria manipurensis	Urticaceae	Manipur
4	Cephalostachyum longwanum	Poaceae	Nagaland
5	Coelogyne pendula	Orchidaceae	Mizoram
6	Dendrocalamus manipureanus	Poaceae	Manipur

7	Epigeneium arunachalense	Orchidaceae	Arunachal Pradesh
8	Heteropanax dhruvii	Araliaceae	Arunachal Pradesh
9	Larsenianthus arunachalense	Zingiberaceae	Arunachal Pradesh
10	Larsenianthus assamensis	Zingiberaceae	Assam
11	Dendrobium falconeri var. senapatianum	Orchidaceae	Manipur
12	Morus macroura var. laxiflora	Moraceae	Arunachal Pradesh
13	Tibetoseris depressa sub sp gauri	Asteraceae	Sikkim
Ferns and Fern allies			
14	Pteris mawsmaiensis	Preridaceae	Meghalaya
Bryophytes			
15	Leptolejenea mirikana	Lejeunaeceae	West Bengal Hills
16	Leptolejenea udarii	Lejeunaeceae	Sikkim
17	Notoscyphus darjeelingensis	Jungermanniaceae	Sikkim
Lichens			
18	Leirreuma subpatellum	Graphidaceae	Arunachal Pradesh
19	Pyrenula darjeelingensis	Pyrenulaceae	
Fungi			
20	Russula khanchanjungae	Russulaceae	Sikkim

21	Russula tsokae	Russulaceae	Sikkim
22	Phallus calongei	Phallaceae	Sikkim
23	Russula griseocarnosa	Russalaceae	Sikkim
Algae			
24	Stigeoclonium iyengarii	Chaetophoraceae	Sikkim

2.5.2.1 GRASSES OF NORTH EAST INDIA

Grasses covered the one-fifth of the Earth's land surface area. It occurs virtually in every terrestrial habitat (Shantz 1954). According to the Ecological Society of America, grassland is a community dominated by grasses or grass-like plants. The grassland occurs in temperate zones where rainfall varies from 25 to 80 cm, while in tropics region, they may be found rainfall up to 150 cm. The favourable conditions for the development of stable and profound grassland are frequent rainfall and warmth weather during the growing season. This may the one reason why North East India is profoundly covered with grasses (discussed later).

The grasses and their values have been recognized since time immemorial, as the present day cereal crops are cultivated. Use of grasses as food or as fodder has led to extensive breeding and improvement programmed especially in the pasture land. In India, the concept of pasture management has not been properly developed, despite the fact that India has one of the largest livestock populations in the world. There is no sound management plan for the development of pastureland and its protection. We have not even fully documented the values and aspect of these grasslands in terms of resources and biodiversity.

The grass family which comes under the Poaceae family contributes approximately 11,000 species of 700 genera (Chen et al. 2006). In India, there are about 13,00 grass species distributed in 25 tribes and 263 genera out of 17,500 known flowering plants (Nair et al. 2012). As far the North-East India is concerned, many researchers have worked out in the grass species with their economical viable (Bor 1940, Shukla 1996, Barooah et al. 2003, Chowdhury 2005). There is a report of 40 species of bamboos belonging to 10 genera from Assam state (Barooah et al. 2003). In addition to this, 303 species of grasses have been introduced within the boundary of Assam (Chowdhury 2005).

The grasses of India are divided into eight types (Whyte 1957) (**Table 2.10)**. Accordingly, further research on grasses classified it into three types (Champion & Seth 1968). However, the Indian Council of Agricultural Research classified the grass cover of India into five major types (Dibadghao & Shankarnarayan 1973) (**Fig.2.23).**

1. *SEHIMA-DICHANTHIUM* TYPE: These are found in the Central Indian plateau, Choto Nagpur plateau and Aravalli ranges, covering an area of about 17,40,000 km². In this region, both the perennial grasses (24 species) and annual grasses (89 species) with 129 species of dicots, including 56 legumes are found. This region is well protected and includes a large number of wildlife sanctuaries and national park.

Table 2.10 Classification of Indian grassland by Whyte 1957.

	Major grassland types	**Environment**	**Regions of distribution types**
1.	*Sehima-Dichanthium*	Black soil	Hyderabad, Mumbai, Andra Pradesh, Tamil Nadu
2.	*Dichanthium-Cenchrus*	Sandy loam soil	Punjab, Delhi, Rajasthan, Gujarat, Bihar
3.	*Phragmites-Saccharum*	Marshy areas	Uttaranchal, Bihar, Bengal, Assam, Sunderban
4.	*Bothriochloa*	High rainfall and low lying areas	Lonavala tract of Mahastra
5.	*Cymbopogon*	Low hills	Western Ghats, Satpura, Aravalli ranges, Orissa
6.	*Arundinella*	High mountains	Western Ghats, Nilgiris, Himalayas, Ounjab, Himachal Pradesh
7.	*Deyeuxia-Arundinella*	Mixed temperate climate	Himalayas, Kashmir, Bengal, Assam, Uttaranchal
8.	*Deschampsia-Deyeuxia*	Temperate-alpine climate	Himalayas, Kashmir above 2500 metre.

***2. DICHANTHIUM-CENCHRUS-LASIURUS* TYPE**: These are found in the northern parts of Delhi, Aravalli ranges, parts of Punjab, almost whole Rajasthan, and Gujarat, and southern Uttar Pradesh, spread over an area of about 436,000 km². There are 11 perennial grass species, 43

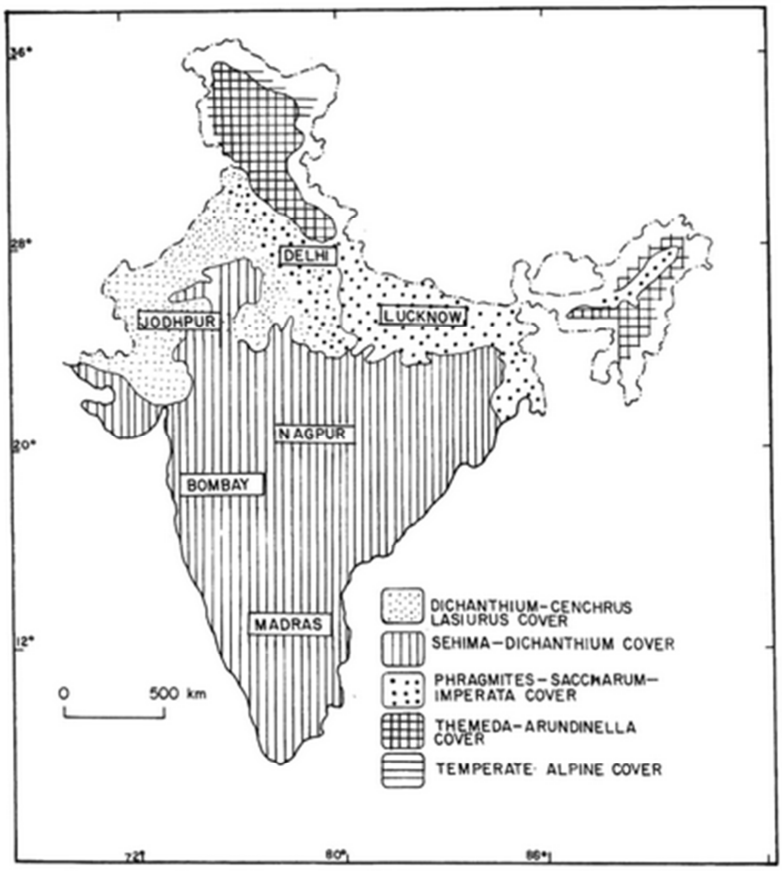


Fig.2.23 The grass cover of India (Dabadghao et al. 1973)

annual grass species, and 45 dicots including 19 legumes. This area has many protected areas, mainly in the hilly regions. These grasslands are extremely important for the survival of certain bird species.

***4. PHRAGMITES-SACCHARUM-IMPERATA* TYPE**: These types of grasslands cover about 2,800,000 km² in the Gangetic Plains, the Brahmaputra Valley and the plains of Punjab and Haryana. The elevation of this region is between 300 to 500 m. There are 10 perennial kinds of grasses, 26 annual kinds of grasses, and 56 herbaceous species, including 16 legumes. The Gangetic plain is one of the most thickly populated regions in the world so original grassland type is almost gone. Some wet grasslands survive in protected areas of the

terai region and the Brahmaputra floodplains. These wet grasslands harbour many globally threatened wildlife species.

***4. THEMEDA-ARUNDINELLA* TYPE**: These grasslands cover about 230,000 km² and include the states of Assam, Himachal Pradesh, Jammu and Kashmir, Manipur, Uttar Pradesh and West Bengal. The elevations range between 350 and 1200 m. There are 37 major perennial grass species, 32 annual grass species, and 34 dicots including 9 legumes.

5. *TEMPERATE AND ALPINE COVER*: These are spread across altitudes higher than 2100 m and include the temperate and cold desert areas of Himachal Pradesh, Jammu and Kashmir, Uttar Pradesh, West Bengal and the north-eastern states. There are 47 perennial kinds of grasses, 5 annual kinds of grasses and 68 dicots, including 6 legumes. These high altitude grasslands harbour wildlife not generally found in other parts of the country. Depending upon the biotic influences and local variations in topography and soil structures, these five broad categories can still be subdivided into several grass associations.

It is considered that the grasses are the most evolved species of plants. They have certain advantages of being short life cycle and high yield of biomass, which is a major criteria for a feedstock. Unlike trees, when cut, they sprout back quickly. They are capable of producing a huge amount of biomass. They are also supporting to retain the water and hydrology of an area. Some of the selected grasses have been studied for their ash content, phosphorus and calcium content along with some other shrubs (Tejwani 1994) (**Table 2.11)**

Table 2.11 Total ash, calcium and phosphorus content of some of the shrubs including grasses (Tejwani 1994)

Botanical Name	**Percent on dry matter basis**		
	Total ash	**CaO**	**P_2O_5**
Echinochloa colonum (syn. Panicum colonum)	7.97	0.39	0.09
Elionesus hirsutus (Post flowering)	11.00	1.09	0.06
Erianthus revennae (Flowering)	6.94	0.31	0.06
Eulaliopsis binata (Pre-flowering)	7.69	0.36	0.60
Heteropogon contortus			
Flowering	8.76-11.06	0.30-0.34	0.09-0.13
Young	9.22	-	-

Prime	9.22	-	-
Ripe	9.73	-	-
Pre-flowering	13.31	0.34	0.13
Seeding	6.58-10.11	0.28-0.45	0.08-0.10
Hymenachne interrupta	2.90	0.15	0.20
Imperata cylindrical			
Pre-flowering	7.84	0.33	0.10
Young	6.32	0.50	0.15
Ipmoea pestigridis	11.88	1.84	0.34
Ischaemum rugosum	9.63	0.49	0.27
Iseilema laxum (Pre-flowering)	6.78-11.22	0.31-0.59	0.10-0.37
Leersia hexandra	14.92	0.14	0.14
Melinis minutiflora			
Pre-flowering	8.83	0.54	0.31
Flowering	10.14	0.37	0.29
Rhynchelytrum Repens	7.99	0.34	0.11
Sehima nervosum	8.88	0.51	0.12
Sporobolus marginatus	8.84	0.21	0.14
Themeda anathera	7.20	0.43	0.01
Themeda arundinaca (Flowering)	4.58	0.46	0.14
Apluda mutica (syn. A. aristata)	8.13-14.30	0.34	0.10
Arundo donax	12.40	0.65	0.05
Cenchrus ciliaris	10.18	0.26	0.18
Chloris barbata (Pre-flowering)	11.93	0.74	0.15
Chloris gayana			
Young	10.40	0.42	0.20
Prime	11.08	0.30	0.10
Ripe	9.08	-	-
Chrsopogon fulvus (Sny. C. montanus)	9.73	0.57	0.24
Cynodon dactylon	10.07-14.97	0.35-0.51	0.19-0.26
Cynodon plectostachyus (Pre-flowering)	6.78	0.31	0.37

2.6 SUMMARY OF THE LITERATURE REVIEW

Since 1970s energy crisis, people have given importance to sustainable and alternative energy production, which comprises the lignocellulosic material as one of the important sources for bioethanol production. A large-scale production of bioethanol for blending as well as to compensate the petrol-based engine is still in progressive level. Various raw materials including grasses, hardwood, and lignocellulosic biomass have been extensively used to produce bioethanol. However, a much research is needed on an exploration of new raw materials for bioethanol production at present scenario. In overall bioconversion of biomass to bioethanol, pretreatment method plays a major role to produce more ethanol and easy conversion of complex sugars to monomers. Based on the pretreatment, a large amount of published prospective have been surveyed and found that different techniques are used to apply and no single pretreatment technology is sufficient to produce a high yield of ethanol. The contamination of lignin in the cellulosic component is another hurdle in the conversion process. In respect, of different lignocellulosic biomass and techniques used for the conversion process and based on the weight of published, the grasses of indigenous materials has most comprehensive promising materials and can be used as feedstock for ethanol production.

Chapter 3

Collection and Identification of different plant species to investigate the feasibility of production of bioethanol

3.1 INTRODUCTION

India is bestowed with a unique diversity of cultural and natural vegetation. A vegetation may be defined as an assemblage of plants growing together in a particular area and characterized by the species component having the structural and functional characters (Goldsmith et al. 1986). Vegetation is affected by many factors such as climate, geology, edaphic, biotic, soil types, latitude and topography of an area. These, in turn, influenced the distributions of plants and animals in the vegetation (Mueller et al. 1974). There are many areas in the Indian Himalayan regions with full of flora and fauna having an importance of bioresource and bioenergy production, which remain unexplored. The assessment of plant diversity normally provides a basis for devising suitable strategies for conservation of plant resources and keeping this basic idea in mind, the present work was carried to explore new biomass feedstock suitable for biofuel production from Kamrup district of Assam and Thoubal district of Manipur of North East India.

Before a biomass is explored as feedstock, one has to properly collected, identified, observed and documented the species. It is suggested that at the earlier time, only the natural lovers and herbalists used to observe and recorded the various facts of plant's life. Subsequently, it was passed on to one generation to another. It was Theophrastus (372-282 BC) the great Greek philosopher, later gave scientific footing to this knowledge of plants. The continual observations of plant resources further kept in addition to the plant repository of facts and figure. Linnaeus prepared a binomial system of nomenclature of 265 Indian plant species in his Species Plantarum during 1753. Scientifically, it is necessary to identify, classify and documented a new plant species. Survey, identification, inventorization and documentation of plant species is important on the fact that, this act as a source of information to assist planning and operation of any germplasm activities.

Plants are of fundamental importance to life on Earth. They provide breathable oxygen, food, fuel, medicine and much more besides. Plants also help to regulate the climate change, provide habitats and food for insects. A good understanding of plants is the core area to improve the agricultural productivity and sustainability, for biomass yield. With changing the climate and growing human population, there is a threat to many ecosystems. It is, therefore, very important to identify new or rare species in the context of a geographical extent as part of wider biodiversity (Govaerts 2001).

The different parts of plants exhibit significant important as they can distinguish between different plant species. Botanists collect the plant specimens and preserve them in archives in the form of herbaria. The collection of herbarium specimen can fill up the lack of long-term phenological observations (Delisle et al. 2003). The phenological observations provide one of the best biological indicators of climate change (Schwartz 2000). Therefore, herbarium collection is an important aspect of plant science and can be seen as major, structured repositories of expert knowledge.

3.1.1 GENERATIONS OF BIOFUELS

Biofuels are energy sources made from biomass. The biomass energy derived biofuels have been around for a long time, however, petroleum and coal have been used primarily due to high energy value, and low prices (Discussed in Chapter 1). As we know that fossil fuel products are gradually increasing prices, dwindling reserves, and causing harmful effects on the environment. The use of fossil fuels and its impact is a matter of concerned. Therefore, a new alternate source of energy is highly important to discuss that it can substantially replenish the existing fuels. Biofuels are renewable resources which are promising alternate fuel for fossil fuels.

There are three types of biofuels at present: 1st, 2nd, and 3rd generation of biofuels. They are characterized by their source of raw materials, limitations and their technological progress. The 1st generation of biofuel usually comes from the raw materials such as sugarcane, wheat, maize etc. The main drawback of the 1st generation of biofuel is that the biomass source is also a food source. The problem arose when there is not enough amount of food to feed the vast population. The 2nd generation biofuels usually come from non-food biomass, especially lignocellulosic biomass. However, they compete with the food production land. There rises another biofuel type, 3rd generation of biofuel. This 3rd generation of biofuel presents the best option because they don't compete with the food crops. However, the big challenge lies in making them economically viable.

1ST GENERATION OF BIOFUEL

The first generation of biofuel is also called as conventional biofuel, because they are made from sugar, or starch rich food crops. They are produced through the well-developed process called fermentation, and distillation. These processes have been used for hundreds of years, where sugars or starches are fermented to produce primarily alcohol, and smaller quantities

like butanol and propanol. A benefit of ethanol is that it burns cleaner than gasoline and therefore produces fewer greenhouse gases. The raw materials used in first generation of biofuel production are sugarcane, maize, millets, corn, rice etc.

The first generation of biofuel has several disadvantages. They pose a threat to food price hike. The biomass used for first generation of biofuels requires lots of lands to grow and contribute negative impact on biodiversity. In terms of cost effectiveness, they are not economically viable.

2ND GENERATION OF BIOFUEL

These biofuels sources are not competing with food crops. They rely on the biomass which is not suitable to be used as food. The biomass source for 2nd generation of biofuel includes lignocellulosic biomass, food waste, municipal waste, agricultural residues, forest residues, organic waste, woods etc. These biofuels are produced from biochemical conversion/thermochemical conversion of biomass. The process needs pretreatment of the biomass. In this thesis, we discussed extensively the pretreatment methods (chapter 2). The main motive behind for highlighting different biofuel generation is that the selected biomass collected from North East India falls in second generation of biofuels.

2nd generation biofuels address many advantages associated with 1st generation biofuels. They don't compete with food crops, generate higher energy yields per acre, and utilized the land not fit for cultivation. However, second generation biofuel has certain drawbacks which include, more process elaborate, requires pretreatment to release the sugars molecules, efficient enzymes and chemicals consumptions, more energy and materials are needed.

3RD GENERATION OF BIOFUEL

Third generation of biofuel use specially engineered crops such as algae as the energy source. Usually, this source of biofuels is mean to be produced biodiesel by harvesting the oil from such sources. The challenge is to find out the energy source that will provide sustainable energy resources. At the same time, this energy source must be dependable, renewable and non-contributing to climate change. This third generation of biofuel show a hope, however, a plenty of research is needed. It is important from societal and environmental views to keep investigating for the sustainable development of these technologies.

Kamrup districts of Assam and Thoubal district of Manipur of North East India was chosen as the site of interest for collecting the plant samples. As we know that, this region's flora needs to explore and many unexplored plants, animal and microbes remained lying inside the thick forest. The hilly terrain areas of this region have plenty of lignocellulosic biomass growing profoundly in the natural condition and one can easily explore such resources having varieties of plants mostly grasses. This led to an idea of exploring such new native plants for production of biofuel.

3.2 MATERIALS AND METHODS

A herbarium is defined as a collection of plants that usually have been dried, pressed, preserved on sheets and arranged accordingly to any accepted system of classification for future reference and study. From the sheet of a herbarium, information about the plants can be determined. The collection of the botanical material involves two things - gathering the biomass sample and recording the information. The field notes were taken on the spot of collection site and collection numbers of the plants were assigned respectively. The following steps are considered in collecting, pressing and drying of the herbarium.

3.2.1 COLLECTION OF SPECIMENS

The collection of plant material was done randomly based on ensuring healthy plants, with typical average leave, flowers. A complete specimen possesses all parts including root system, flowers and fruits. Photograph of the plant material at the field site of Kamrup districts of Assam and Thoubal district of Manipur was taken along with field note consist of habitat, a label with the name and date of collection. While collecting the sample, the following criteria were recorded: name of the plant, date of collection, the name of collector, site of collection, the original source of plant etc. Different types of tools were used in making the herbarium specimen (**Fig. 3.1).** To avoid the mechanical damage during the transportation and preservation, at least 5-9 specimens of a plant was collected. The collected specimen was handled carefully and kept in the specimen box to prevent willing.

- Plastic bags
- Tape measure
- Label sticky notes
- Pencils
- Camera

3.2.2 PRESSING AND DRYING

The specimens were spread out between the folds of old newspapers or blotting sheets avoiding overlapping of the parts. The larger specimen was folded in N or W or Z shapes. The unwanted materials were removed from the plants. A pressure was made by using the pair of hardboard to the same size as the drying paper. Arranged the plant material to retain the character of the plant. Removed leaves and flowers of congested specimens to reduce the bulk without losing the character of the plant. Covered the sample with two further sheets of blotting paper and corrugated card. Covered the top board and placed bricks applying pressure evenly throughout. The sample was inspected regularly - at least once a week.

- Two pieces of hardboard/plywood measuring 40 X 30 cm (16 X 12 inch)
- Sheets of blotting paper
- Sheets of corrugated card
- Foam sheet
- Bricks/telephone directories/weighty books/straps

3.2.3 MOUNTING

The dried specimens were mounted on the herbarium sheets of standard size (41 X 27 cm). It was done with the help of glue, adhesive or cello-tape. Attached the specimen to the paper using a combination of neutral-pH PVA adhesive. The mounting was labelled including plant name and author, plant family, description, location, date, collector and any other relevant details. The label was kept on the bottom right-hand corner.

- Acid free paper measuring 42 X 27 cm
- Tweezers, Scissors
- Neutral-pH PVA adhesive/Fevicol

3.2.4 PRESERVATION AND STORAGE

The mounted specimens were sprayed with fungicides with 2% solution of mercuric chloride. Type specimens were generally stored in separate and safe places.

- Plastic bags
- Boxes

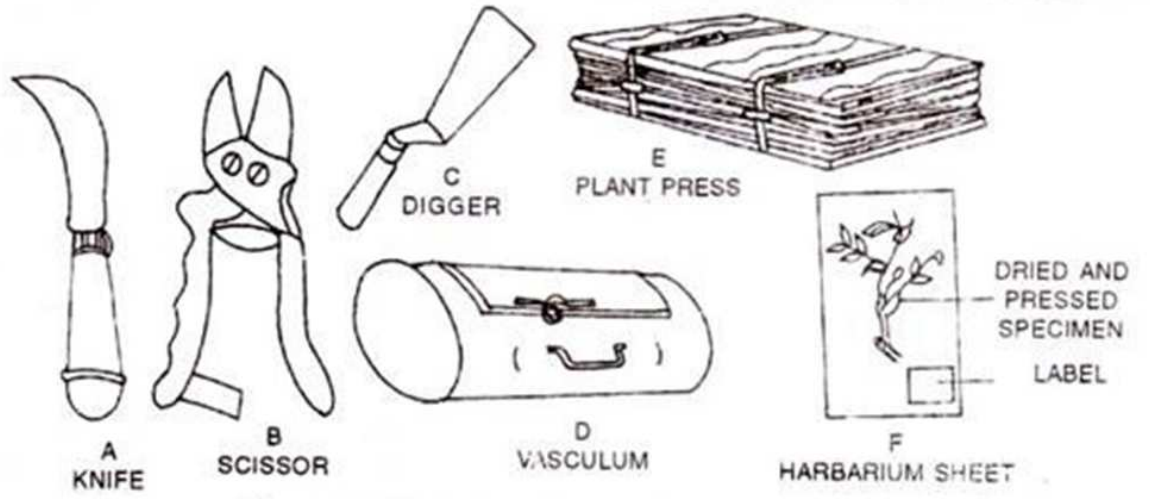


Fig.3.1 The instrument used in making herbarium

3.2.5 HERBARIUM DEPOSITION AND IDENTIFICATION

The biomass was obtained from the two states (Assam and Manipur) of North-Eastern India. The latitude and longitude of the collection sites were recorded. The living photographs were taken for each biomass sample. The feed-stocks were collected between 3 and 5kg in weight, and visible sands, soil, or other contaminants were removed manually. The experimental data presented are the average of three readings.

The herbarium sheet provided by Gauwahati University was procured from local market. The simple tools like scissor, knife, field notebook, high-resolution camera, GIS tool, gloves, glue, and polyethene pack, etc. were used for collection and preparation of the sample. Two sets of each herbarium specimens were submitted to Herbarium Unit, Department of Botany, Gauwahati University, Assam, India for systematic identification.

3.2.1.1 MOLECULAR IDENTIFICATION OF PLANT SPECIES

Molecular identification of plant species through the DNA barcoding technology is a novel method which is based on the optical representation of data to identify a species by matching the short sequences tags in the gene regions and which is very rapid and accurate (Paul et al., 2005, Elena et al., 2011). It is very different from molecular phylogeny as it deals with classification, however, DNA barcode discussed the identifying an unknown species in terms of a known classification (Kress et al., 2005). The idea of being able to differentiate from one species to another species using the short gene sequence strike to draw the attention among many taxonomists, genetics and evolutionary biologists (Paul et al., 2003). It was suggested

that 0.01% of the estimated 10-15 million species could hardly identify by the taxonomist (Hammond 1992, Hawksworth et al., 1995). There arises some limitation on morphological diagnosis to identify a species. First, phenotypic and genetic variability used for recognition of species is not susceptible. Secondly, the morphologically cryptic taxa are common in many groups (Knowlton 1993, Jarman et al. 2000). Cryptic species are distinct species of some groups but morphologically similar species hence classified as a single species (Markus et al., 2007). Third, biological keys are effective only for few gender and life stage and hence many individuals can't be identified. Lastly, the interaction with modern techniques relies on keys, which occurs misdiagnosis. That's why DNA barcoding will play a dramatic role in taxonomic screening tool because of its ability to reveal the genetic discontinuities that make the distinction among species (Janzen et al. 2005).

As we know that in animals and other species like fungi, researcher agreed that mitochondrial gene encoding cytochrome c oxidase subunit 1 (CO1) is a favourable region for use as DNA bar code. However, the mitochondrial genes have limited used fullness in plant barcode because of low amounts of variation in the genes as well as a variable structure of the mitochondrial genome. Thus to get a candidate gene for DNA barcode, nuclear and plastid genome has been an important focus. Single copy genes in the nuclear genome and their introns have been excluded for barcode candidates because of lack of universal primers for their amplification. However internal transcribed spacer (ITS) of nuclear ribosomal DNA is one of the targeted novel barcode regions in plants.

3.2.1.2 EXTRACTION OF PLANT DNA

The CTAB DNA isolation protocol was optimized for genomic DNA isolation. **CTAB Buffer (**100 ml 1 M Tris HCl pH 8.0**,** 280 ml 5 M NaCl, 40 ml of 0.5 M EDTA**,** 20 g of CTAB (cetyltrimethyl ammonium bromide)**,** was prepared to total volume of 1 L with doubled distilled water. 300 mg of leave sample was ground with liquid nitrogen in mortar pestle to disrupt the cell wall. 6 ml of CTAB buffer was added in a falcon tube with the ground sample. Added 20 µl of β-Mercaptoethanol and it was vortex. The sample was taken for water bath at 65 ^{0}C, 1:30 hr. After water bath, 6 ml of chloroform: isoamyl alcohol (24:1) was added. Then it was vortex and centrifuge at 10000 rpm, 4 ^{0}C, 10 min. Slowly, took the upper layer in an eppendorf and added 1 ml of chloroform: isoamyl alcohol and again centrifuge at 10000 rpm, 4 ^{0}C, 10 min. 5M sodium acetate of 500 µl was added with isopropanol of double the volume of the solution. It was allowed to precipitate overnight in –

20 ^{0}C. After overnight precipitation, the solution was centrifuged at 10000 rpm, 4 ^{0}C, 15 min. Discard the supernatant and 70% ice cold ethanol was added. It was centrifuged at 10000 rpm, 4 ^{0}C, 10 min. Discard the supernatant and pellet was allowed to dry. Elute the DNA with TAE buffer (50 µl). The presence of DNA was checked by gel electrophoresis.

3.3 RESULTS

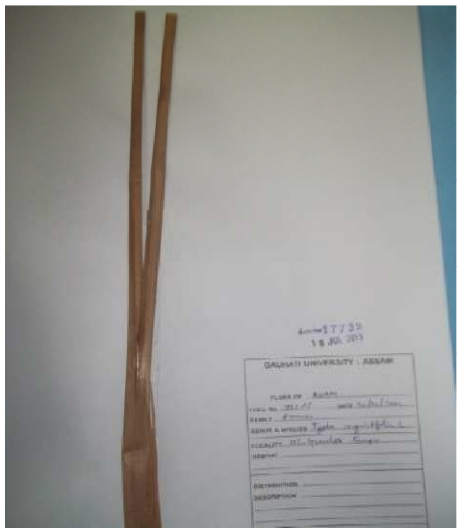
29 specimens were collected and deposited to Herbarium Unit, Department of Botany, Gauwahati University, Assam, India. One of the each duplicate copy of the herbarium sheet was retained at Indian Institute of Technology Guwahati, India for future references **(Fig.3.2.)**. The voucher number of each specimen was provided along with the systematic classification **(Table 3.1).** From the field surveyed, it was known that most of the collected grasses were profoundly grown in hilly terrain regions of this part. The GIS location was specifically provided.

Table 3.1 Biomass specimen collected from Assam (Kamrup) and Manipur (Thoubal) of North-East India for biofuel production

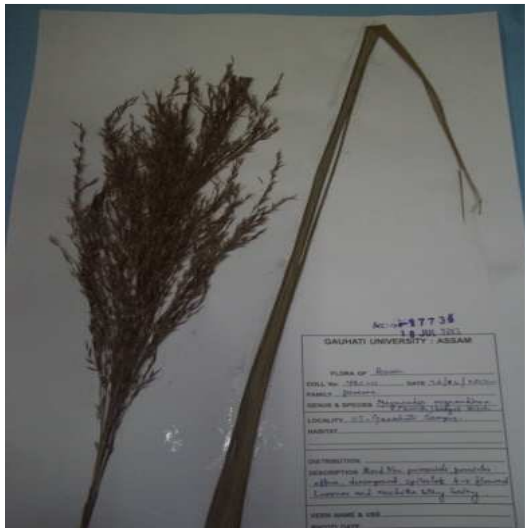
Sl No.	Name of species	Family	Latitude and Longitude	Voucher No.
1	*Phragmitis karka* (Retz.)	Poaceae	26° 11' 10.1538" 91° 41' 40.2138"	17742
2	*Thysanolaena agrostis* Nees	Poaceae	26° 11' 11.8788" 91° 41' 35.214"	17740
3	*Typha angustifolia* L.	Typhaceae	26° 11' 7.155" 91° 41' 47.1084"	17739
4	*Dichanthium assimile* (Steud.) Deshp	Poaceae	24° 28' 35.6298" 94° 1' 17.7306"	17737
5	*Imperata cylindrica* (L.) Beauv	Poaceae	24° 29' 34.0938" 93° 59' 57.2238"	17736
6	*Neyraudia reynaudiana* (Kunth) Keng ex A.S.Hitchc.	Poaceae	26° 11' 14.7222" 91° 41' 57.6774"	17735
7	*Cymbopogon exertus* A.Camus	Poaceae	24° 28' 35.5944" 94° 1' 17.025"	17733

8	*Erianthus fultus* Nees.	Poaceae	26° 11' 16.9296" 91° 41' 50.0316"	17725
9	*Sclerostachya fusca* (Roxb.) A. Camus	Poaceae	26° 11' 14.8698" 91° 42' 2.7678"	17730
10	*Arundo donax* L.	Poaceae	26° 11' 15.5256" 91° 41' 44.3286"	17743
11	*Saccharum sponteneum* L.	Poaceae	26° 11' 1.194" 91° 41' 44.3286"	17741
12	*Cymbopogon longifolium*	Poaceae	26° 11' 21.1236" 91° 41' 41.895"	17734
13	*Arundinella khasiana* Nees ex Steud	Poaceae	26° 11' 20.0826" 91° 41' 37.086"	17731
14	*Setaria glauca*	Poaceae	24° 29' 47.3892" 94° 0' 7.578"	17732
15	*Cynodon dactylon* (L.) Pers	Poaceae	26°11'01.5"N 91°41'46.0"E	17822
16	*Eragrostis airoides*	Poaceae	26°11'12.9"N 91°42'00.3"E	17815
17	*Coelorachis striata* A camus.	Poaceae	26°11'41.9"N 91°41'40.6"E	17826
18	*Echinochloa colonum* (L). Link.	Poaceae	24°28'55.4"N 93°59'01.8"E	17823
19	*Chrysopogon aciculatus*	Poaceae	26°14'04.0"N 91°57'23.6"E	17824
20	*Eleusine indica* L.	Poaceae	24°29'48.9"N 94°00'27.9"E	17827
21	*Fimbristylis dichotoma* Vahl.	Cyperaceae	24°29'23.5"N 93°59'14.3"E	17830

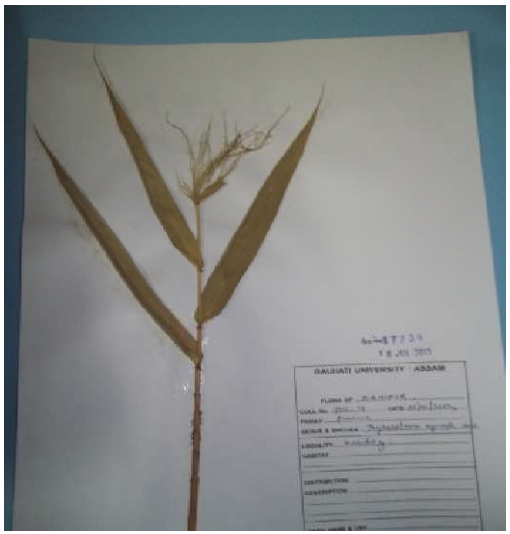
22	*Cyperus exaltatus* Retz.	Cyperaceae	24°29'33.6"N 93°59'13.9"E	17831
23	*Cyrtococcum oxyphyllum* (Steud.) Stapf	Poaceae	24°29'28.5"N 93°59'14.6"E	17821
24	*Paspalum scrobiculatum* L.	Poaceae	24°29'27.9"N 93°59'14.4"E	17818
25	*Coix lacryma-jobi* L.	Poaceae	24°29'17.8"N 93°59'08.8"E	17816
26	*Meriscus sp.*	Poaceae	24°29'30.6"N 93°59'12.3"E	17832
27	*Echinochloa stagnina* (Retz.) P.Beauv.	Poaceae	24°29'23.3"N 93°59'14.3"E	17828
28	*Thysanolena maxima* kuntz	Poaceae	26°11'15.8"N 91°41'45.2"E	17817
29	*Cyperus Flavidus* Retz.	Cyperaceae	24°29'06.8"N 93°59'07.1"E	17829



Typha angustifolia

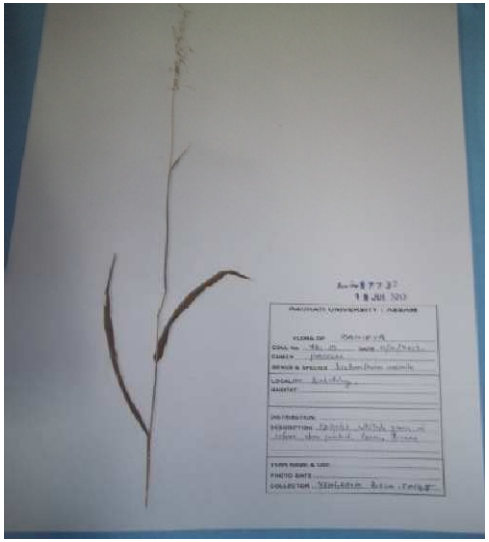


Neyraudia reynaudiana



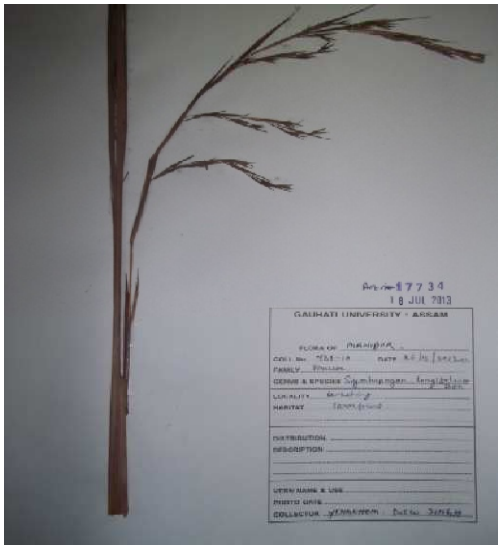
Thysanolaena agrostis

Phragmitis karka



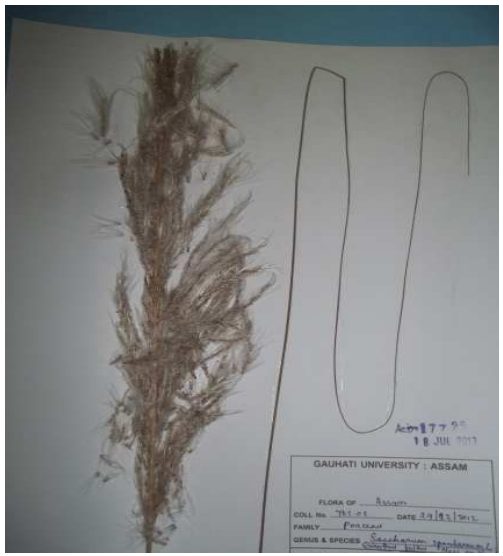
Dichanthium assimile
TH-1834_11615103

Sclerostachya fusca



Cymbopogon longifolium

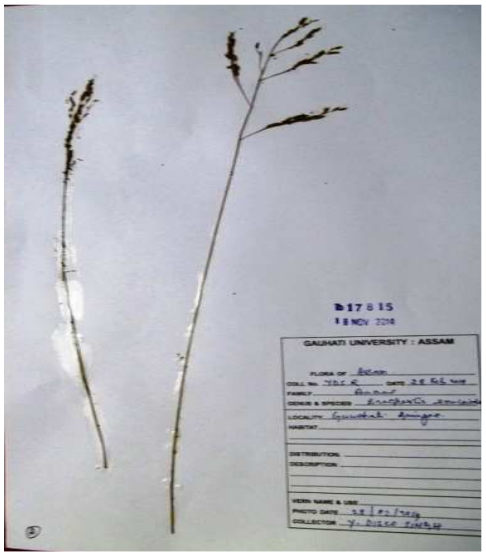
Cyperus Flavidus



Erianthus fultus

Imperata cylindrica

Setaria glauca



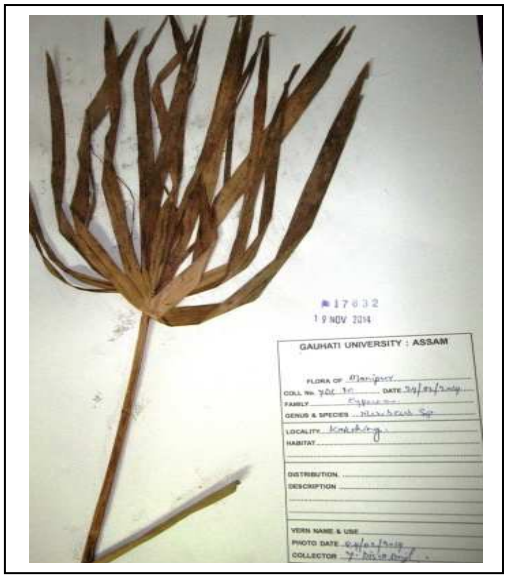
Eragrostis airoides

Cynodon dactylon

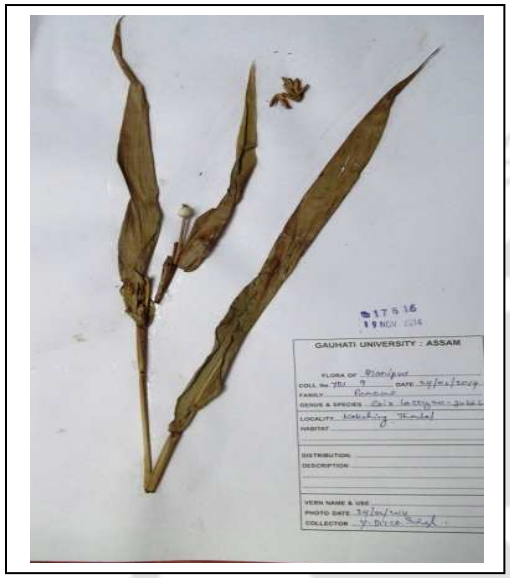
Coelorachis striata

Echinochloa colonum

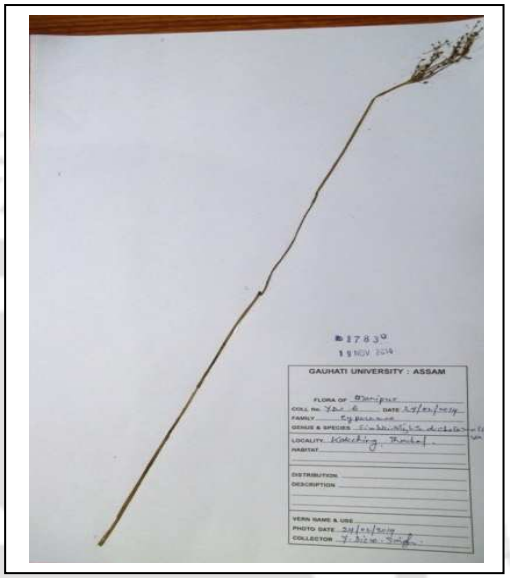
Chrysopogon aciculatus



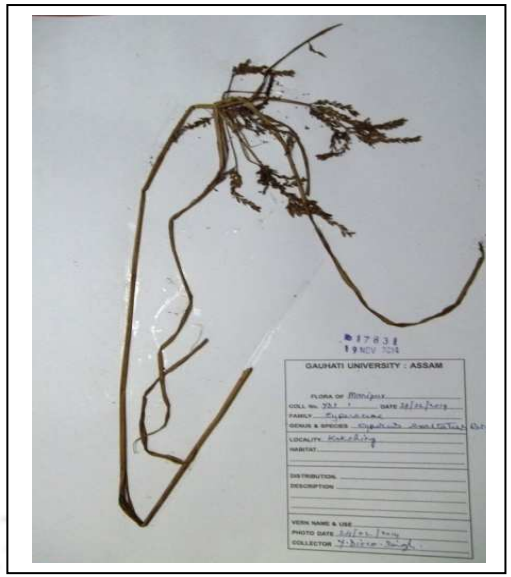
Merisceus



Coix lacryma-jobi

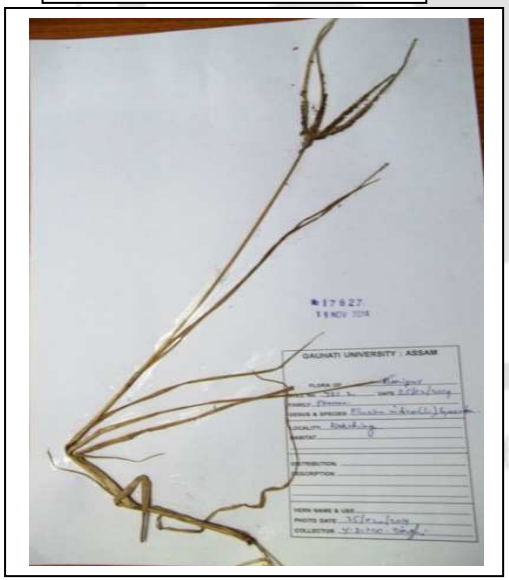


Fimbristylis dichotoma

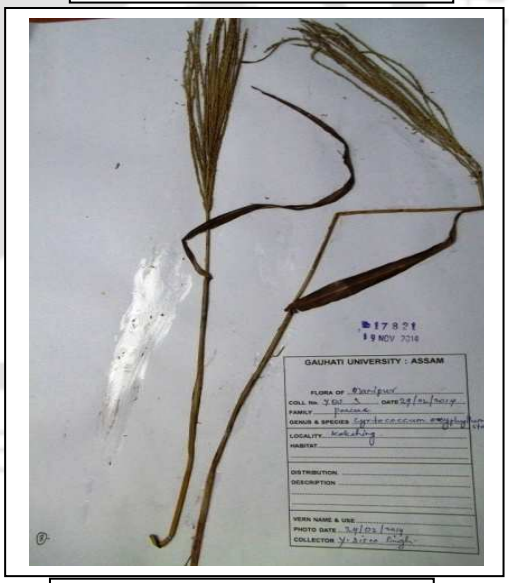


Cyperus exaltatus

Echinochloa stagnina

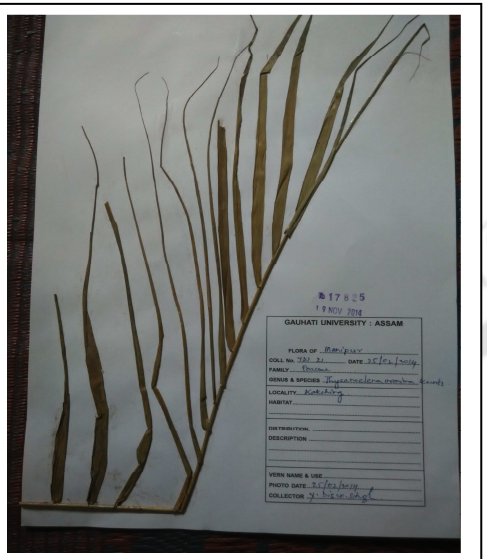


Eleusine indica

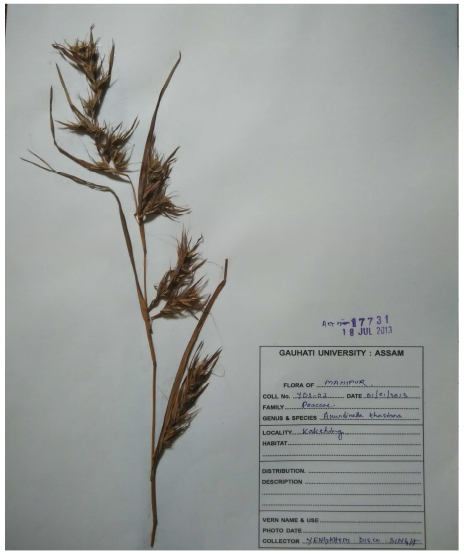


Cyrtococcum oxyphyllum

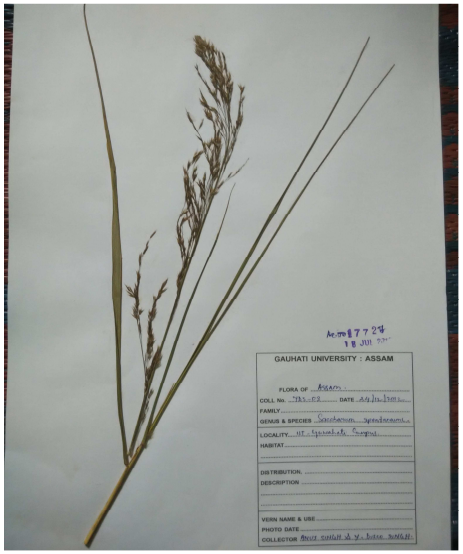
Paspalum scrobiculatum



Thysanolena maxima



Arundinella khasiana



Saccharum sponteneum

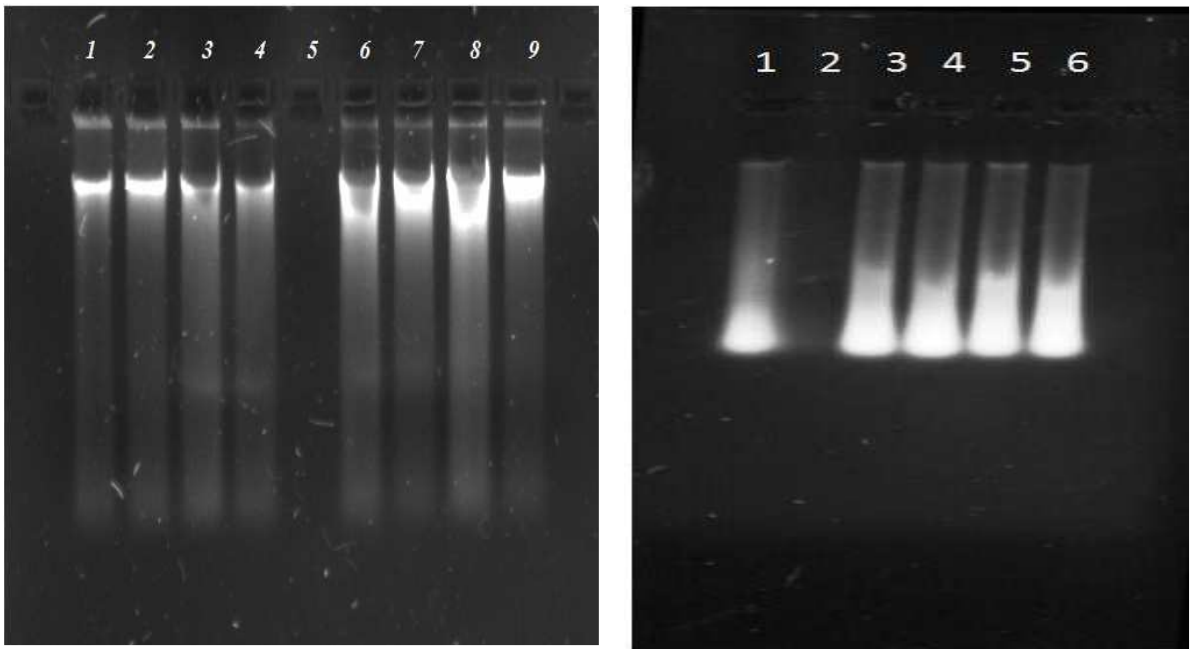
Cymbopogon exertus

Arundo donax

Fig.3.2. Herbarium specimen of different biomass sample collected from North-East India maintained at Indian Institute of Technology, Guwahati Assam

3.3.1 MOLECULAR IDENTIFICATION

The DNA was run in 0.8% of agarose gel electrophoresis with TAE buffer. The bands show the presence of DNA in the sample. The isolated genomic DNA of the plant samples were



Lane 1-4: SO

Lane 6-9: ST

Lane 5: Nil

Lane 1-6: SA

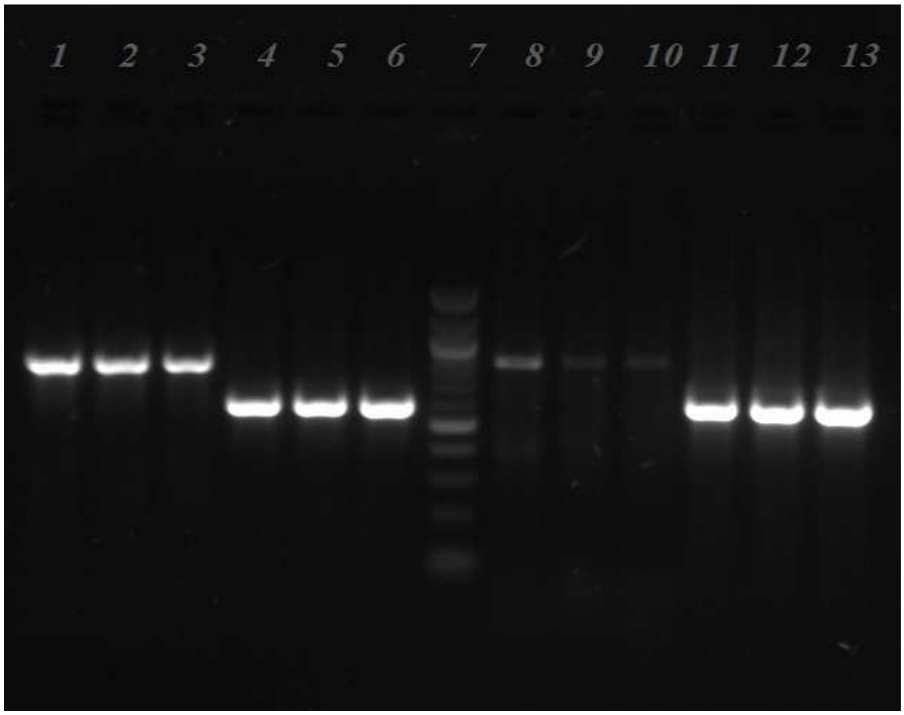
Lane 2: Nil

SA= Neyraudia reynaudiana voucher 17735, OR=Chrysopogon aciculatus voucher 17824,

ST= Thysanolaena latifolia voucher 17738, OM= Thysanolaena latifolia voucher 17738 maturase K (matK) gene

Fig.3.3 0.8% agarose gel electrophoresis of the isolated genomic DNA

in **Fig.3.3.** showing agarose gel electrophoresis of 0.8%. The PCR product agarose gel image is shown in **Fig.3.4, 3.5** and gel extraction image in **Fig.3.6.** A nanograph was generated and given in **Fig.3.7.** showing the different concentration of the DNA. The electrophoregram of the amplified DNA sequenced are given in **Fig. 3.8, 3.9, 3.10,** and **3.11** respectively.



Lane 1-3: matK SO

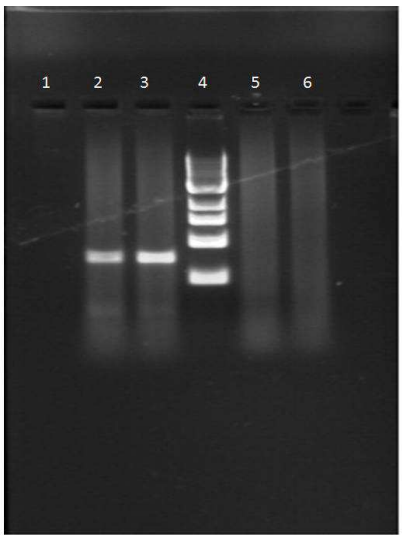
Lane 4-6: rbcl SO

Lane 7: 100 bp ladder

Lane 8-10: matK ST

Lane 11-13: rbcl ST

Fig. 3.4 1.5 % agarose gel electrophoresis of PCR product



Lane 1-Blank

Lane 2-3- SA rbcl

Lane 4- Ladder

Lane 5-6- SA matK

Fig. 3.5 1.5% agarose Gel PCR product of Sample A

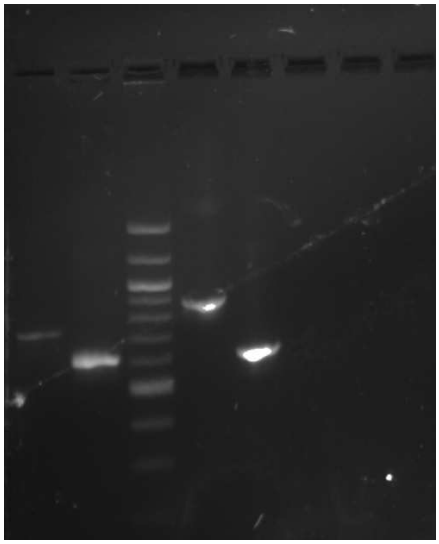
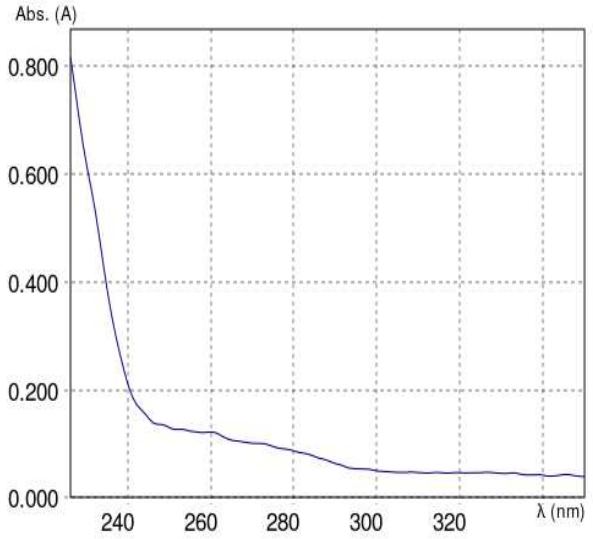
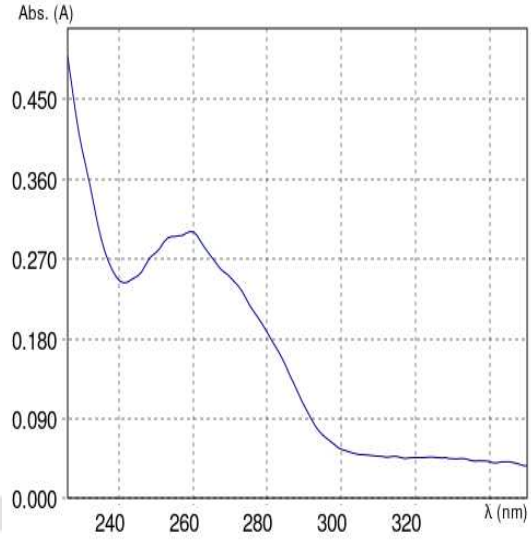


Fig. 3.6 1.5% agarose Gel extraction of Sample A, T, OM and OR DNA

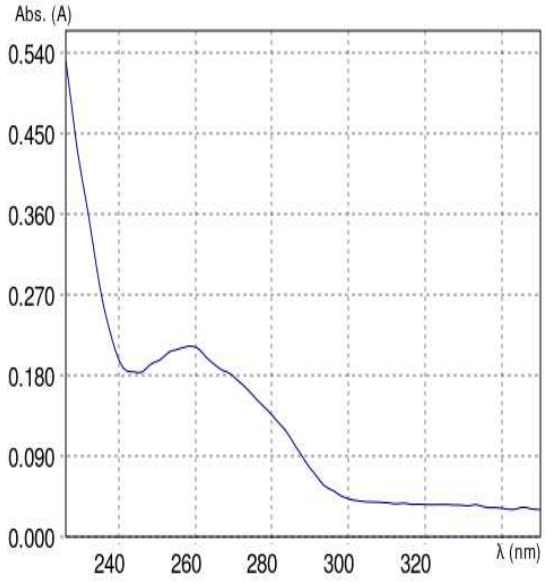
The PCR reaction volume was 50 µl, which contained 25 µl Ready mixed, primers (forward 1.5 µl, 1.5 reverse), water 17 µl, and DNA template 5 µl. The PCR thermal profile for initial denaturation at 95°C for 1 min, second denaturation at 95°C for 30 sec, annealing at 48.1°C for 30 sec, elongation at 68°C for 1 min for 35 cycles and final extension at 68°C for 5 min.



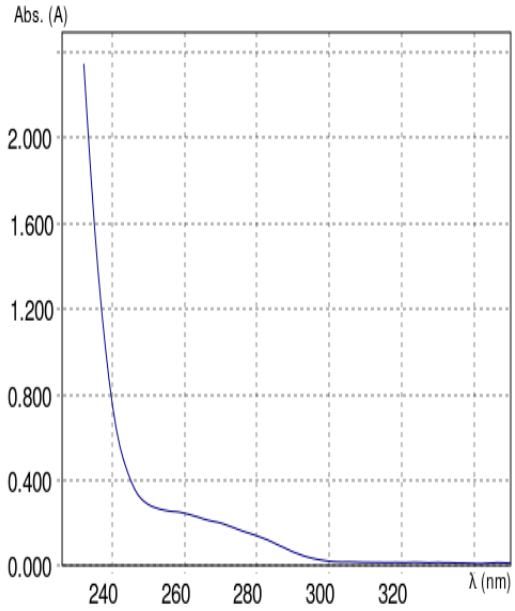
Sample A= 60.415 ng/µL



Sample T= 149.864 ng/µL



Sample Om= 105.594 ng/µL



Sample Or= 121.205 ng/µL

Fig.5.7 Nanograph of the DNA sample

>A_RBCL.1F_17730-1_6982,Trimmed Sequence(689bp)
TGTTAAGATTATAAATTGACTTACTACACCCCGGAATACGAAACCAAGGATACTGATATCTTG
GCAGCATTCCGAGTAACTCCTCAGCCCGGGGTTCCGCCTGAAGAAGCAGGGGCTGCAGTAGC
TGCGGAATCTTCTACTGGTACATGGACAACTGTTTGGACTGATGGACTTACCAGTCTTGATCG
TTACAAAGGACGATGCTATCACATCGAACCCGTTCCTGGGGAAGAGGATCAATATATCTGTT
ATGTAGCTTATCCATTAGATCTATTTGAAGAGGGTTCTGTTACTAACATGTTTACTTCCATTGT
GGGTAACGTATTTGGTTTCAAAGCCCTACGTGCTCTACGTTTGGAGGATCTACGAATTCCTCC
TGCTTATGCAAAAACTTTCCAAGGTCCGCCTCATGGTATCCAAGTCGAAAGGGATAAGTTGA
ACAAGTATGGTCGTCCTTTATTGGGATGTACTATTAAACCAAAATTGGGATTATCCGCAAAAA
ATTACGGTAGAGCATGTTATGAGTGTCTACGCGGTGGACTTGATTTTACCAAAGATGATGAAA
ACGTAAACTCACAACCATTTATGCGCTGGAGAGACCGTTTTGTCTTTTGTGCCGAAGCAATTT
ATAAAGCACAAGCCGAAACCGGCGAAATCAAGGGGCATTACTTGAATGCTACTGCAGGTAC

>A_RBCL.724R_17730-2_6982,Trimmed Sequence(696bp)
TTCGCCGGTTTCGGCTTGTGCTTTATAAATTGCTTCGGCACAAAAGACAAAACGGTCTCTCCA
GCGCATAAATGGTTGTGAGTTTACGTTTTCATCATCTTTGGTAAAATCAAGTCCACCGCGTAG
ACACTCATAACATGCTCTACCGTAATTTTTTGCGGATAATCCCAATTTTGGTTTAATAGTACAT
CCCAATAAAGGACGACCATACTTGTTCAACTTATCCCTTTCGACTTGGATACCATGAGGCGGA
CCTTGGAAAGTTTTTGCATAAGCAGGAGGAATTCGTAGATCCTCCAAACGTAGAGCACGTAG
GGCTTTGAAACCAAATACGTTACCCACAATGGAAGTAAACATGTTAGTAACAGAACCCTCTT
CAAATAGATCTAATGGATAAGCTACATAACAGATATATTGATCCTCTTCCCCAGGAACGGGTT
CGATGTGATAGCATCGTCCTTTGTAACGATCAAGACTGGTAAGTCCATCAGTCCAAACAGTTG
TCCATGTACCAGTAGAAGATTCCGCAGCTACTGCAGCCCCTGCTTCTTCAGGCGGAACCCCGG
GCTGAGGAGTTACTCGGAATGCTGCCAAGATATCAGTATCCTTGGTTTCGTATTCCGGGGTGT
AGTAAGTCAATTTATAATCTTTAACACCAGCTTGAAATCCAACACCTGCTTTAGTTTCTGTTTG
TGG

Fig.3.8 Electrophoregram data of sample A. (rbcl primer amplified gene).

>OR_RBCL.LAF_17730-7_6982,Raw Sequence(581 bp)
TAGGGTCTGCATTAGCTGGTGTTAGGATTATAAATTGACTTACTACACCCCGGAGTACGAAAC
CAAGGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAGCCCGGGGTTCCGCCTGAAGA
AGCAGGGGCTGCAGTAGCTGCGGAATCTTCTACTGGTACATGGACAACTGTTTGGACTGATG
GACTTACCAGTCTTGATCGTTACAAAGGACGATGCTATCACATCGAGCCCGTTCCTGGGGAGG
CAGATCAATATATCTGTTATGTAGCTTATCCATTAGACCTATTTGAAGAGGGTTCTGTTACTA
ACATGTTTACTTCCATTGTGGGTAACGTATTTGGTTTCAAAGCCCTACGCGCTCTACGTTTGGA
GGATCTACGAATTCCCCCTACTTATTCAAAAACTTTCCAAGGTCCGCCTCACGGTATCCAAGT
TGAAAGGGATAAGTTGAACAAGTATGGTCGTCCTTTCTTGGGATGTACTATTAAACCAAAATT
GGGATTATCCCCAAAAAATTATGTTAGAGCGTGTTATGATTGTCGGGGGGGGGGGGAGTGAA
AAAAAAAAAAAAAAT

>OR_RBCL.LAR_17730-8_6982,Raw Sequence(579 bp)
AGCATATAACGCTCTACATATTTTTTGCGGATAATCCCAATTTTGGTTTAATAGTACATCCCAA
GAAAGGACGACCATACTTGTTCAACTTATCCCTTTCAACTTGGATACCGTGAGGCGGACCTTG
GAAAGTTTTTGAATAAGTAGGGGGAATTCGTAGATCCTCCAAACGTAGAGCGCGTAGGGCTT
TGAAACCAAATACGTTACCCACAATGGAAGTAAACATGTTAGTAACAGAACCCTCTTCAAAT
AGGTCTAATGGATAAGCTACATAACAGATATATTGATCTGCCTCCCCAGGAACGGGCTCGAT
GTGATAGCATCGTCCTTTGTAACGATCAAGACTGGTAAGTCCATCAGTCCAAACAGTTGTCCA
TGTACCAGTAGAAGATTCCGCAGCTACTGCAGCCCCTGCTTCTTCAGGCGGAACCCCGGGCTG
AGGAGTTACTCGGAATGCTGCCAAGATATCAGTATCCTTGGTTTCGTACTCCGGGGTGTAGTA
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ATGACATAAAAAAA

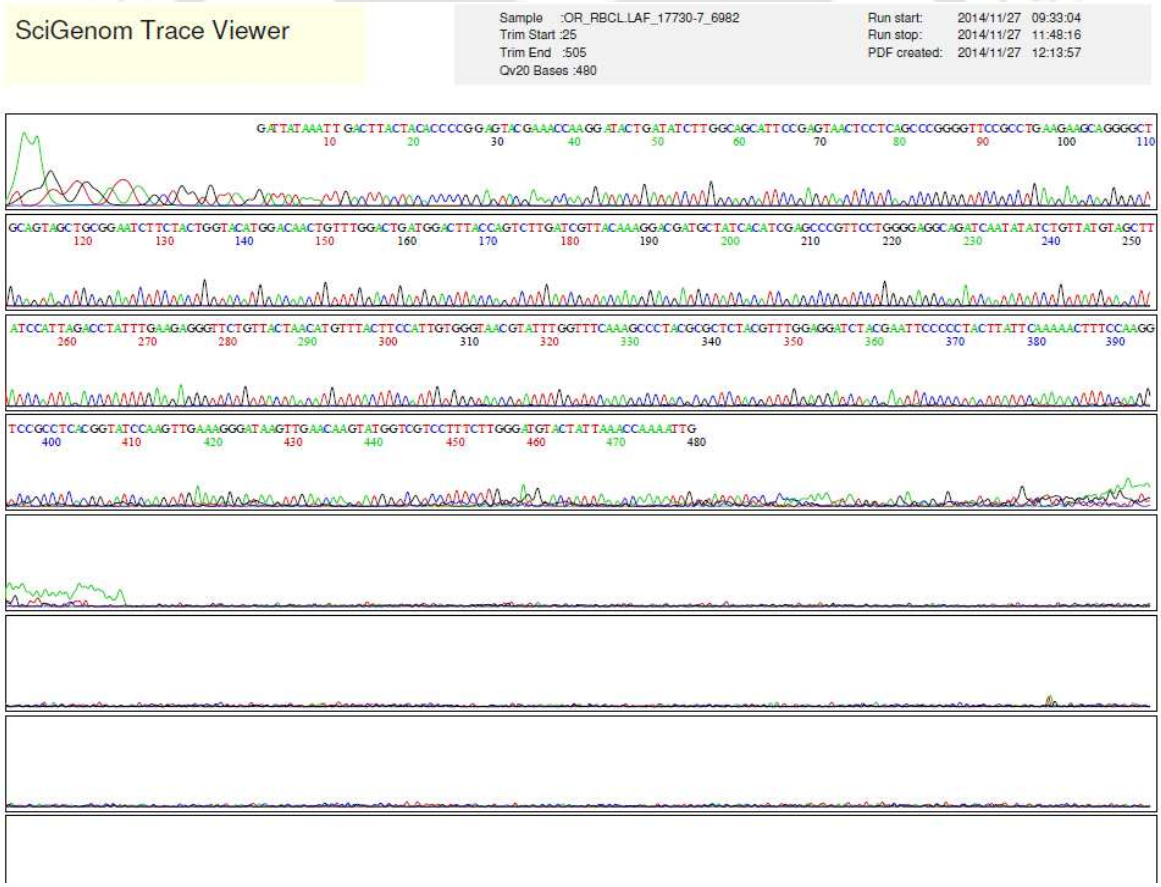


Fig.3.9 Electrophoregram data of sample O. (rbcl primer amplified gene).

>T_RBCL.LAF_17730-3_6982,Raw Sequence(575 bp)
ATGCATATGCATAAGCTGGTGTTAGGATTATAAATTGACTTACTACACCCCGGAGTACGAAAC
CAAGGATACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAGCTCGGGGTTCCGCCTGAAGA
AGCAGGAGCTGCAGTAGCTGCGGAATCTTCTACTGGTACATGGACAACTGTTTGGACTGATG
GACTTACCAGTCTTGATCGTTACAAAGGACGATGCTATCACATCGAGCCCGTTCCTGGGGACC
CAGATCAATATATCTGTTATGTAGCTTATCCATTAGACCTATTTGAAGAGGGTTCTGTTACTA
ACATGTTTACTTCCATTGTGGGTAACGTATTTGGTTTCAAAGCCTTACGCGCTCTACGTTTGGA
GGATCTACGAATTCCCCCTGCTTATGCAAAAACTTTCCAAGGTCCGCCTCACGGTATCCAAGT
TGAAAGGGATAAGTTGAACAAGTACGGCCGTCCTTTATTGGGATGTACTATTAAACCAAAAT
TGGGATTATCCGCAAAAAATTACGGTAGAGCGTGTTATGAGTGTCTACACGGTGGACATAAT
TTTACAAAAA

>T_RBCL.LAR_17730-4_6982,Raw Sequence(574 bp)
GCAATTACGCTCTACGTATTTTTTGCGGATAATCCCAATTTTGGTTTAATAGTACATCCCAATA
AAGGACGGCCGTACTTGTTCAACTTATCCCTTTCAACTTGGATACCGTGAGGCGGACCTTGGA
AAGTTTTTGCATAAGCAGGGGGAATTCGTAGATCCTCCAAACGTAGAGCGCGTAAGGCTTTG
AAACCAAATACGTTACCCACAATGGAAGTAAACATGTTAGTAACAGAACCCTCTTCAAATAG
GTCTAATGGATAAGCTACATAACAGATATATTGATCTGGGTCCCCAGGAACGGGCTCGATGT
GATAGCATCGTCCTTTGTAACGATCAAGACTGGTAAGTCCATCAGTCCAAACAGTTGTCCATG
TACCAGTAGAAGATTCCGCAGCTACTGCAGCTCCTGCTTCTTCAGGCGGAACCCCGAGCTGAG
GAGTTACTCGGAATGCTGCCAAGATATCAGTATCCTTGGTTTCGTACTCCGGGGTGTAGTAAG
TCAATTTATAATCCTTAACACCAGCTTTAAATCCAACACTTGCTTTAGTCACTGTTTGTGGGTG
ACATAAAA

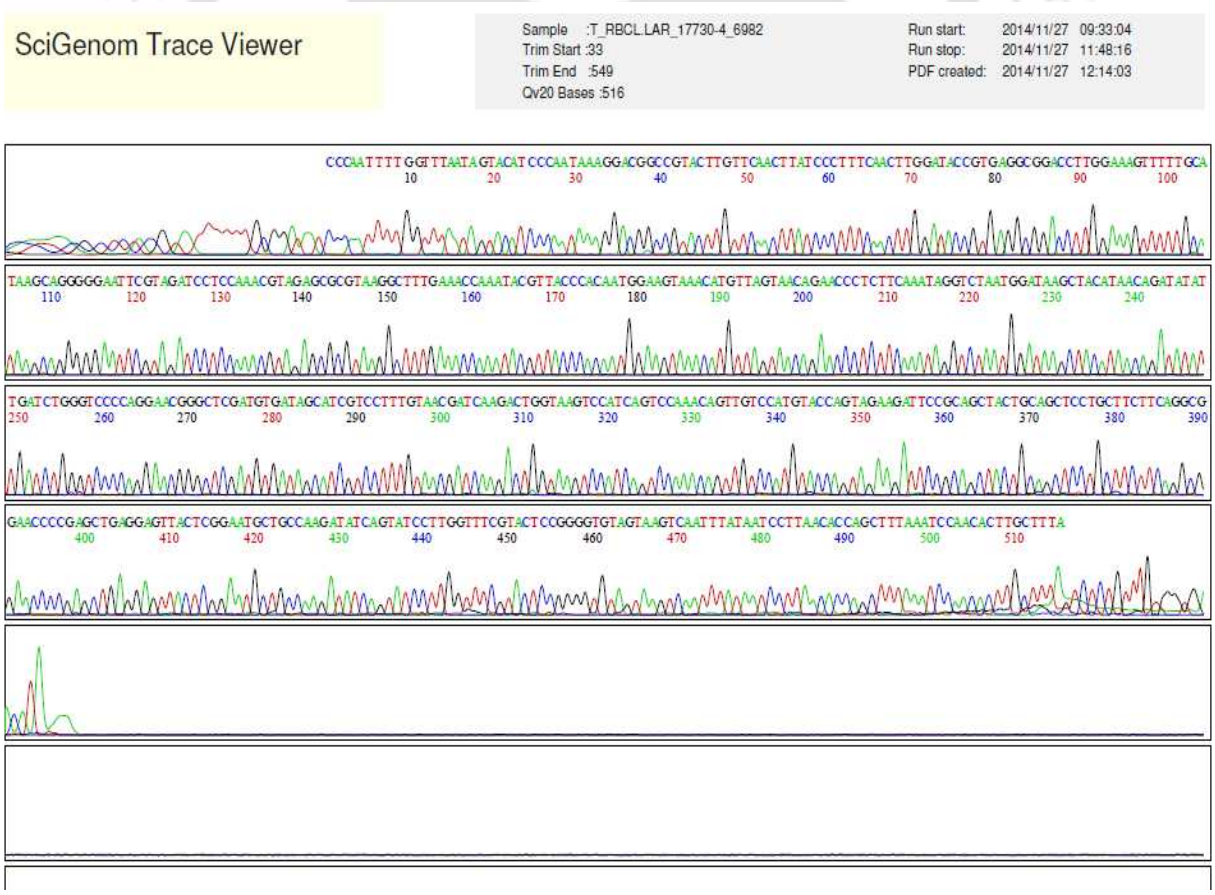


Fig.3.10 Electrophoregram data of sample T. (rbcl primer amplified gene).

>OM_MAT.K2.1F_17730-5_6984,Raw Sequence(822 bp)
ACCTTGATCGGATTCAGATATTCCATCTTTGCATTTATAGGCGATTCTTTCTCAACTATTATTC
GAATAGGAATAGTCTTATTGCTTCAATGAAATCGATTTTTCTTTTTTCAAAAGAAAATAAAAG
ACTATTTCGATTCCTATATAACTCTTATGTATCGGAATATGAATTTTTCTTGTTGTTTCTTCGTA
AACAATCTTCTTGCTTACGATTAACATCTTCTGGAACCTTTCTGGAACGAATCCACTTTTCTAG
GAAGATGGAACATTTTTGGGTAATGTACCCAGTTCGGAAAACCATATGGTTCTTTATGGATCC
TCTTATGCATTATGTTCGATATAAAGGAAAGGCGATTCTTGCATCAAAAGGAACTCTTCTTTT
GAAGAAGAAATGGAAATCTTACCTTGTCAATTTCTCACAATATTTTTTCTCTTTTTGGGCTCAA
CCACAAAGGATCCGTCTAAACCAATTAACAAACTCTTGCTTCGATTTTCTGGGGTACCTTTCA
AGTGTACCAATAAATACTTTGTTAGTAAGGAATCAAATGCTGGAGAATTCTTTTCTAATAGAT
ACTCAAATGAAAAAATTCGATACCACAGTCCCCACTACTCTCCTCATTGGATCCTTATCAAAA
GCTCAATTTTGTACTGGATCGGGGCATCCTATTAGTAAACCGGTTTGGACCGATTTATCATAT
TGGGATATTCTTGATCGCTTTGGTCTGATAGGTAGAAGTCTTTATCATTATCATAGTGGATCTT
CGAAAAAACAGACTTTGTATCGAGTAAGAGATAAAAATACAACTTTCTTGTGCTAGAGAA

>OM_MATK..5R_17730-6_6984,Raw Sequence(835 bp)
GGCTTTTACTCGATCAAGTCTGTTTTTTCGAGATCCACTATGATAATGAAAAAGATTTCTACCT
ATCCGACCAAAGCGATCAAGAATATCCCAATCTGATAAATCGGTCCAAACCGGTTTACTAAT
AGGATGCCCCGATCCAGTACAAAATTGAGCTTTTGATAAGGATCCAATGAGGAGAGTAGTGG
GGACTGTGGTATCGAATTTTTTCATTTGAGTATCTATTAGAAAAGAATTCTCCAGCATTTGATT
CCTTACTAACAAAGTATTTATTGGTACACTTGAAAGGTACCCCAGAAAATCGAAGCAAGAGT
TTGTTAATTGGTTTAGACGGATCCTTTGTGGTTGAGCCCAAAAAGAGAAAAAATATTGTGAGA
AATTGACAAGGTAAGATTTCCATTTCTTCTTCAAAAGAAGAGTTCCTTTTGATGCAAGAATCG
CCTTTCCTTTATATCGAACATAATGCATAAGAGGATCCATAAAGAACCATATGGTTTTCCGAA
CTGGGTACATTACCCAAAAATGTTCCATCTTCCTAGAAAAGTGGATTCGTTCCAGAAAGGTTC
CAGAAGATGTTAATCGTAAGCAAGAAGATTGTTTACGAAGAAACAACAAGAAAAATTCATAT
TCCGATACATAAAGAGTTATATAGGAATCGAAATAGTCTTTTATTTTCTTTTTGAAAAAAGAA
AAAAAAATTTCATTGAAGCAGAAGGACTATTCCTAGTCGAATTTGTAATTGAGTTAGAAAAA
TAATTCTAAGTAAAAGGGAAAGTAGGAAAAACCTGGAACTGGTTTTAAGGGTTATAAATTAA
AATCCCAAGGGGAAGGGAAT

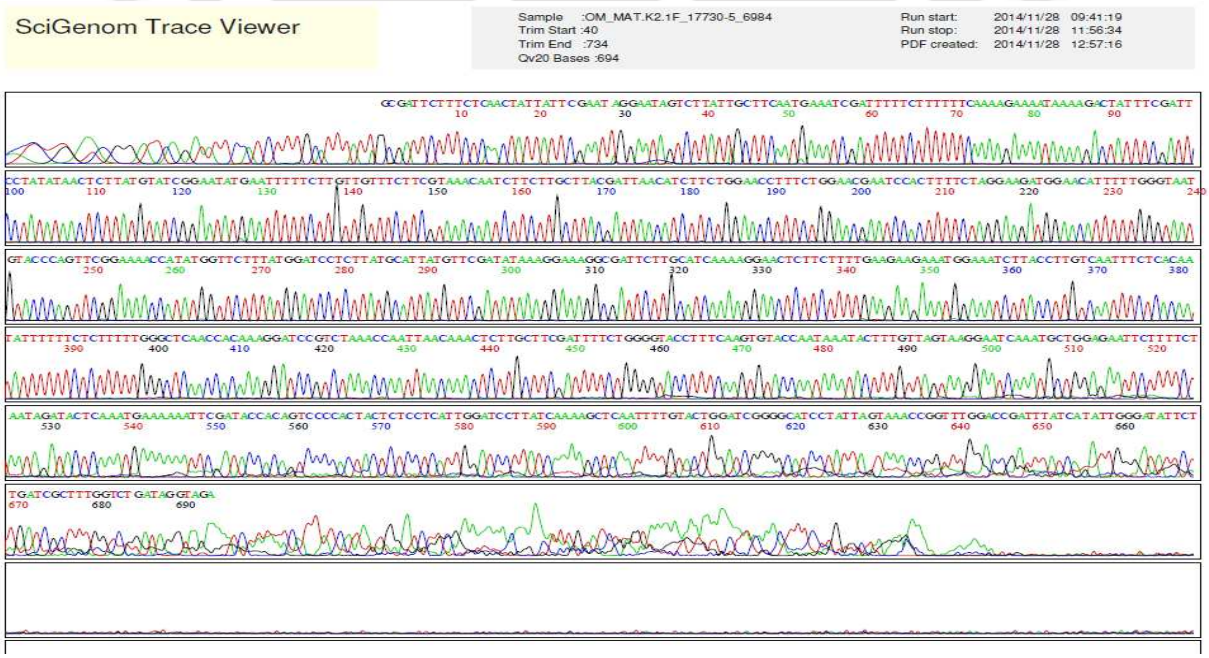


Fig.3.11 Electrophoregram data of sample OM. (matK primer amplified gene).

3.4 DISCUSSIONS

A herbarium is a collection of dried plants systematically named and arranged for ready reference and study. To make a herbarium specimen, the plant is collected, dried, pressed preserved and deposited in the herbarium unit. Usually, the collection of plants is made in duplicate or triplicate. Botanical collections are essential to understanding the floral diversity of North-East India. A good quality herbarium provides:

- a tool for plant identification;
- a data source for research on the taxonomy and distribution of plant groups and for writing handbooks and floras;
- a historical record of plant locations, and of a collector's contribution to the science of botany;
- a repository for voucher specimens related to published scientific reports;
- an educational resource for learning to recognize the plant species of an area; and
- accurate and permanent documentation of botanical information that adds credibility to data collected in vegetation surveys.

A collection of the botanical material involves two activities—gathering the specimens and recording the information. A proper identification of plant species is needed even if one have a relatively good knowledge of the local flora. The collected specimen provides a range of extensive knowledge of a plant's taxonomic or natural history. It may also show the ''plasticity'' or variations possible under different habitat conditions. A good-quality plant collection requires considerable advance preparation and a lot of effort.

RECORDING THE DATA

A data on the collected plant is very important to identify the species and used for future reference. Without the well-documented data of a particular plant species is of no use to the scientific community. This is the main reason why the collected plant species was properly recorded and field observations in a field notebook were maintained. The recorded data usually includes collection number, date, name(s) of collector(s), location (latitude and longitude or northing and easting) and habitat information (elevation, water depth (for aquatic plants), slope, aspect, soil, moisture regime, associated vegetation, and biogeoclimatic zone and/or subzone). The common subheadings of all specimens like a collector, slope, aspect,

associated vegetation, etc. were made at the top of the page, then list collection numbers with brief identifying information.

COLLECTION NUMBER

As soon as the specimen was bagged, a collection number was assigned in the field notebook. The collection number makes it easy to reference individual specimens or groups of specimens in a collection. Several numbering systems were used. Some taxonomists assign collection numbers to specimens sequentially throughout their careers (up to six digits may be used), and can, therefore, use a rotating number stamp directly on the pressing paper. This method allows researchers to identify when in a taxonomist's career the plant was collected, to track the specimen from a published account, or to calculate the total number of specimens collected.

Some collectors assign field numbers by year. The number consists of the last two digits of the collection year, a dash, then a sequential specimen number. For example, the first plant collected in 1991 would be 91-01, then, 91-02, and so on. This method is useful if the collector will return to the same area over a number of years, or for an organization that produces annual reports. It gives an immediate indication of when the specimen was collected. All the collected specimen were given collection number as YDS-1, YDS-2 etc.

DATE OF COLLECTION

To avoid confusion, the date of collection was written in full (August 7, 2013). Note that ''8/07/13'' could be interpreted as August 7, 2013, or July 8, 2013.

NAME(S) OF COLLECTOR(S)

The significant member of the collecting party was recorded. In the future, if one collector is unavailable to answer questions, the others may be contacted. First initial(s) plus the last name is sufficient.

LOCATION INFORMATION

The enough information was provided so that another person can follow the directions to the general area to observe more specimens of the same species. The Geographic Information Systems (GIS) was used in other to determine the latitude and longitude to the nearest 10 seconds.

HABITAT INFORMATION

Particularly, the collected species were terrestrial in nature. Therefore, in the herbarium, it was mentioned terrestrial habitation in all specimens.

COMMENTS

The specific characters of certain specimens were noted to help proper identification.

MOLECULAR IDENTIFICATION AND DATA ANALYSIS

The PCR amplified genes were sent for sequencing to the Scigenome, Bangalore, India. The sequenced data of the samples were found to be around 500 base paired to 800 base paired. The PCR amplification was done with the plant-specific primers such as rbcl and matK. After the sequencing of the DNA, the data were analysed using NCBI BLAST for sequenced similarity. In all four samples analysed, the similarity was found to be below 99%. Based on this fact, the samples were submitted to Genebank by using the offline submission tool Sequin. The submitted plant samples after published with voucher number assigned are:

- Neyraudia reynaudiana voucher 17735 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcl) gene, partial cds; chloroplast. **GeneBank (NCBI)** Accession number: KP404599,
- Thysanolaena latifolia voucher 17738 maturase K (matK) gene, partial cds; chloroplast. **GeneBank (NCBI)** Accession number: KP404600
- Thysanolaena latifolia voucher 17738 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit Thysanolaena agrostis (rbcl) gene, partial cds; chloroplast. **GeneBank (NCBI)** Accession number: KP404601,
- Chrysopogon aciculatus voucher 17824 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcl) gene, partial cds; chloroplast. **GeneBank (NCBI)** Accession number: KP404598,

The molecular investigation was done only to four samples. The work may be carried forward for future prospects and further analysis to construct a database source (described in chapter 6). However, the present study confined only to these four plant samples as a preliminary investigation. This small work gives an idea where plants can be identified at molecular level too in addition to the morphological identification.

3.5 CONCLUSIONS

The present study demonstrates the collection of different plant species of Kamrup district of Assam and Thoubal district of Manipur of North-East India and making the herbarium specimen for biofuel production. We have collected 29 species of lignocellulosic biomass from different parts of this region. The deposited herbarium specimens were included in the herbarium list of Guwahati University, herbarium unit, which is one of the statutory body of internationally recognised herbarium depositor. The extensive studies revealed that these plants are highly indigenous to this region and some of the plants are already explored by some other taxonomist (**Table 2.10, chapter 2).** Further the fact that these energy plants can be utilized for biofuel production. However, a series of characterisation (proximate, ultimate, composition, fuel criteria etc.) are required in other to call as feedstock. This characterisation will be discussed extensively in next chapter (**Chapter 4).** The study also demonstrate that many such species are lying unused in this regions and can be a good place for exploring new raw material which has a high potential for biofuel production.

Chapter 4

Screening, characterization and compositional analysis of lignocellulosic biomass

4.1 INTRODUCTION

In our environment, different types of renewable energy sources (biomass, biofuel, bioenergy, wind, solar etc.) are available. These sources are being utilized by every individual to fulfil their essential needs. It was observed that researchers use these as a resource to overcome the non-renewable energy deficiency. The depletion of fossil fuels, increasing global warming, and hike in the price of petroleum products directly affect our day to day life. The research on the biomass as the renewable source of energy can provide strong evidence to replace the fossil fuels. It can be said that the use of biomass as the renewable energy feedstock is extensively increasing and concept for replacing fossil fuels seems to be real. These renewable energy sources have become quickly popularized due to the favourable conditions like environmental friendly, less risk to environmental pollutions etc. In addition to this, they are eventually distributed throughout the Earth's surface and hence are considered as one of the most abundant resources on the Earth.

The major chemical components of lignocellulosic biomass are cellulose, hemicellulose and lignin as discussed in chapter 2. Cellulose and hemicellulose are polymers of repeating sugar units, whereas lignin is the complex polymer formed with phenylpropane units (Bobleter 1994, Monties et.al 1980). A difference in chemical composition of the biomass directly influenced the chemical reactivities. This is why, it is very important to find out the chemical components of biomass to foresee the efficiency of conversion (Ando et al. 2000, Loppinet-Serani et al. 2008). The accurate knowing of a chemical composition of lignocellulosic biomass would provide the better fuel converting efficiency and less effort. For this purpose, numerous chemical analysis methods (proximate, ultimate and compositional) are developed and have a high impact on fuel criteria analysis.

Before using the biomass as a feedstock for biofuel production, the biomass should satisfy some of the characteristics criteria from a fuel point of view. These factors include volatile content, moisture content, ash content, total solid, fixed carbon, elemental composition (hydrogen content, oxygen content, sulfur content, and nitrogen content), lignin and structural carbohydrate (hemicelluloses, cellulose). In all cases, a fundamental characterization of biomass as a feedstock is required in other to produce biofuel. The volatile concentration differs greatly according to the type of biomass, and even the same type of biomass can change in composition based on the climatic conditions and seasonal variations. Furthermore, the chemical composition of the biomass affects the conversion process in the biofuel

production. For example, a high concentration of ash and nitrogen reduces the hydrocarbon yield during thermochemical conversion (Sanderson et al. 1996). The composition of carbohydrate in the biomass determines the theoretical ethanol yield in the biochemical conversion, and can thereby have a significant role on the conversion process. From this information, one can notice that how much impact a biomass characterization has on biofuel production. From the knowledge of biomass composition and thermal behaviour in the conversion process, a mathematical model can be created to further understand the product, yield, economic analysis and more interestingly, new process intensification technology can be formed to make the biomass conversion more efficient.

Biomass is the world largest and most sustainable renewable energy on the Earth (mentioned in **Chapter 1**). More than 220 billion oven-dry ton (odt) per yr or 4500 EJ (1018J) of biomass is available (Anon 2004). The increasing capacity of energy consumption produced from biomass can reduce the fossil fuel utilization. The using of such biofuel will helps in reducing the greenhouse gases emission as compared to fossil fuels (Anon 2004). The lignocellulosic biomass refers to the agricultural residues, forest residues, energy crops etc. This LCB is one of the promising renewable energy feedstock for the production of biofuel. Currently, the lignocellulosic biomass is used to produce major biofuels such as bio-oil, bioethanol, synthesis gas, hydrogen, biobutanol and chemicals. Each of the biofuels has their own utilities. The bioethanol produced from lignocellulosic biomass is greatly used in the transport sector and has great potential for replacement of petrol-based fuels.

Ethanol is produced from the biomass by microbial fermentation (discussed in **chapter 5**) of sugars. The conversion of sugar to ethanol is not an easy step. The lignin components become the main obstacle in the conversion process. As it can be mentioned that, the cell wall is made up of polysaccharide which is usually made from cellulose, hemicellulose and lignin. The strong rigid nature of the cell wall is due to the presence of lignin. This cell wall has to break down in other to access the cellulose and hemicellulose component. Therefore, it needs to pre-treat the biomass sample before the enzymatic hydrolysis and fermentation. The characterization of biomass sample before pretreatment, enzymatic hydrolysis and fermentation will give prior information on feasibility, a degree of polymerization, types of pretreatment need to employ and efficient conversion based on the carbohydrate composition.

In this chapter, 29 different types of biomass are characterized using the thermo-chemical methods. The characterization of biomass sample includes proximate (moisture, volatile, total

solid, ash content), ultimate (elemental composition such as hydrogen, oxygen, carbon, sulphur) and compositional analysis (cellulose, hemicellulose and lignin). The selected 29 biomasses are abundantly available in North-East India. The selection of these biomasses is due to its ability to grow anywhere in this region. The basic objective of this study was to select the best potential candidate from different biomass collected from North-East India and evaluate the potential of these biomasses as feedstock for the production of biofuel based on their physical and chemical characterization.

4.2 MATERIALS AND METHODS

4.2.1 BIOMASS SAMPLE COLLECTION

The biomasses were collected from the two states (Assam and Manipur) of Northeast India. The latitude and longitude of the collection sites were recorded. The living photographs were taken for each biomass sample. The feedstock was collected between 3 to 5kg in weight, and visible sands, soil, or other contaminants were removed manually. The experimental data presented are the average of three readings. The general outline of the experimental methods is given in **Fig. 4.1.**

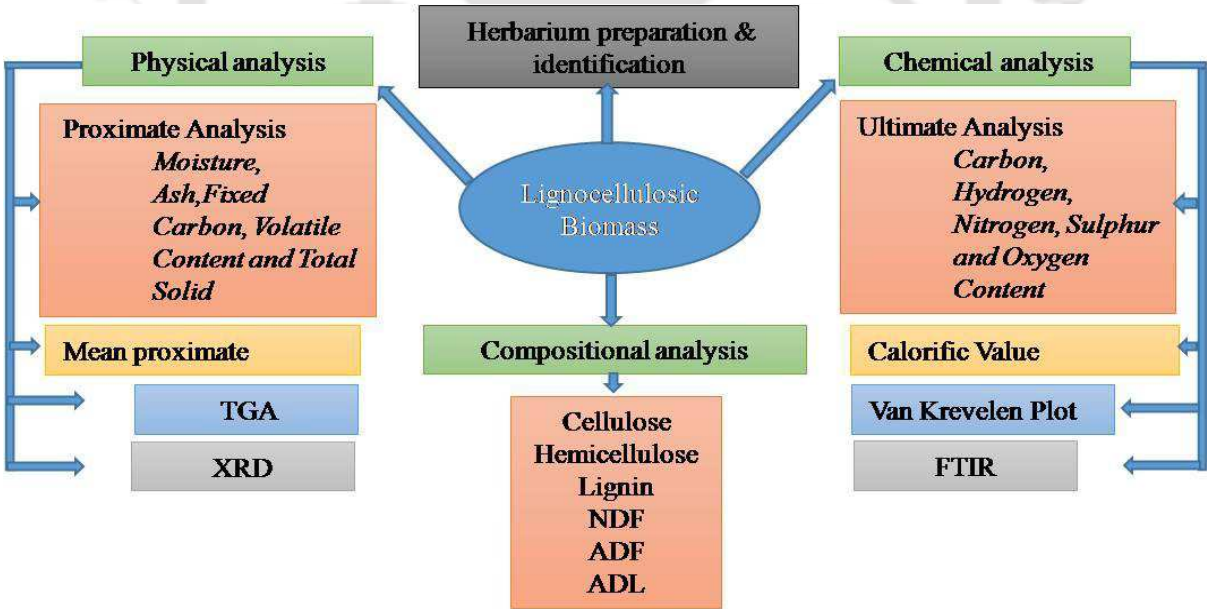


Fig. 4.1. The general outline of the experimental methods used for characterization of LCB collected from North-East India (Singh et al. 2017).

4.2.2 HERBARIUM SPECIMEN PREPARATION AND IDENTIFICATION

The herbarium sheet printed by Gauwahati University was procured from local market. The total inflorescence of the plant was collected carefully and removed all the visible impurities manually. The simple tools like scissor, knife, field notebook, high-resolution camera, GIS tool, gloves, glue, and polyethene pack, etc. were used for collection and preparation of the sample (discussed in **chapter 3).** The collected sample was washed under running tap water. It was pressed and treated with 0.03 % mercury chloride. The sample was washed with 70 % ethanol. The pasting was done on two sheets of herbarium for each specimen using glue and the size of the sample was adjusted by cutting the edges. The herbarium specimen was submitted to Department of Botany, Gauwahati University, Assam, India for identification. Based on the morphological characters and experience on observation, the specimens were identified and classified by the taxonomist. For each specimen, voucher number was assigned and one copy of each specimen was maintained at Indian Institute of Technology Guwahati, for future references.

4.2.3 PROCESSING AND SIZE REDUCTION

The feedstocks were sun-dried under ambient conditions over a period of 24hr. The samples were avoided over-exposure to sunlight. The dried samples were ground into powder to achieve a minimum particle size of 1mm. The ground biomass samples were sieved and stored in polythene having zip mouth.

4.2.4 PHYSICAL ANALYSIS

The powdered biomass samples were characterized physically by the parameters such as proximate analysis (moisture content, volatile content, ash content, fixed carbon content and total solid content), Thermogravimetric analysis (TGA), and X-Ray diffraction (XRD).

4.2.4.1 PROXIMATE ANALYSIS

The moisture percentage of the biomass sample was estimated by convection oven dry method (NREL protocol). The aluminium weighing dishes was placed at 105 ± 3ºC in a drying oven for a minimum of 4hr. Cool the dishes in desiccators. The weight was recorded. 1gm of powdered biomass sample was weighed on a pre-dried aluminium dish and placed in a convection oven at 105 ± 3ºC for 4hr. The dish containing the sample was removed and allow to cool down in a desiccator. Weigh the dish containing the over-dried sample to the nearest

0.1mg. Placed the sample back into the convection oven at the same temperature to record dry to constant weight. The moisture content was calculated as follows:

$$\% \text{ Moisture} = \left[1 - \frac{(\text{weight dried sample plus dish} - \text{weight dish})}{(\text{weight sample as received})}\right] \times 100 \quad (4.1)$$

For the total solid calculation,

$$\text{Total solid} = 100 - \text{moisture } \% \quad (4.2)$$

For the volatile matter, 1g of the oven-dried sample was taken and kept in a muffle furnace at 925±10 ^{0}C for 7 minutes. After heating, it was removed and allowed to cool in a desiccator. The difference in weight loss gives volatile content (ASTM 3175-89). The calculation is given as follows:

$$\text{Weight loss, } \% = [(A-B)/A] \times 100 \quad (4.3)$$

where: A= weight of sample, B = weight of sample after heating

$$\text{Volatile matter in sample, } \% = C - D$$

where: C = weight loss, %, D = moisture %

Ash content was determined by using muffle furnace. 1g of the oven-dried sample was taken in a crucible and heated at 575±10 ^{0}C for minimum 3hr. The crucible was carefully removed into a desiccator and cool down for a specific amount of time. The crucible containing ash was weighed to the nearest 0.1mg and recorded the weight. Place the sample back into the muffle furnace at the same temperature and ash to constant weight. The remaining weight gives ash percentage (NREL/TP-510-42622).

The fixed carbon (FC) content was calculated by as far the available empirical formula (Pazó et al. 2010).

$$FC = 100 - (\% \text{ of moisture} + \% \text{ of volatiles} + \% \text{ of ash}) \quad (4.4)$$

4.2.4.2 THERMOGRAVIMETRIC ANALYSIS (TGA)

TGA was performed on the collected LCB samples using the STA7200, Thermal analysis system, Hitachi. The alumina crucible was rinsed properly by using filter paper dipped in acetone. The alumina crucible was tired with another crucible kept mean for control and calibrated. 5-10 mg of powdered biomass sample was taken in the 150 µl alumina crucible

and kept next to each other so that the two crucibles should not touch each other. The devolatilisation characteristics were studied at 40-900 ^{0}C, a heating rate of 10 ^{0} C/min with a constant flow rate of pure nitrogen gas 40ml/min. During the thermal degradation of the biomass sample, the level of the nitrogen gas was checked regularly.

4.2.4.3 X-RAY DIFFRACTION (XRD)

The XRD analysis of the biomass samples was performed using Rigaku TT Rax diffractometer in conjunction with Cu-Ka radiation source that generated at 18kW and 250 mA with a scan speed of angle 2Θ range from 10 ^{0}- 40 ^{0} at a speed of 1 ^{0}/min. The crystalline indices of the biomass sample were calculated as follows (Cao 2005).

$$CrI = 100\ X\ [(\ I_{002} - I_{amorphous})\ /\ I_{002}] \quad (4.5)$$

Where I_{002} is the intensity of at 2Θ=20 for crystalline portion (cellulose) and Iamorphous is the peak at 2Θ= 16.6 for amorphous portion (cellulose, hemicellulose and lignin).

4.2.5 CHEMICAL ANALYSIS

The chemical analysis of the LCB was performed base on the ultimate analysis, calorific value and Fourier transform infrared spectroscopy (FTIR) techniques.

4.2.5.1 ULTIMATE ANALYSIS

The ultimate analysis of biomass samples was performed using CHNSO elemental analyser (Eurovector EA3000). The analyser was calibrated using 5 tin capsule packed with a 5L-cystine test. The powdered biomass sample of 0.1mg was taken in the tin capsule. It was heated at 980 ^{0} C with a constant flow of helium stream enriched with oxygen gas. The results were analysed using Callidus® software, which automatically provides the elemental composition of the biomass.

4.2.5.2 CALORIFIC VALUE

The calorific values of the biomass samples were determined using the elemental composition as follows (Demirbas 1997):

$$HHV = \{\ 33.5\ X\ \%\ C + 142.3\ X\ \%\ H - 15.4\ X\ \%\ O - 14.5\ X\ \%\ N\ \}\ X\ 10^{-2} \quad (4.6)$$

Where HHV represents the higher heating value, C, H, O, N represent the carbon, hydrogen, oxygen and nitrogen in a percentage basis.

4.2.5.3 VAN KREVELEN PLOT

The derivative plot of Van Krevelen Plot was drawn against the atomic ratio of Hydrogen (H): Carbon(C) to Oxygen (O): Carbon (C). This plot was drawn after the elemental composition was derived.

4.2.5.4 FTIR ANALYSIS

The functional groups present in the biomass were characterized using Fourier Transform Infra-Red (FTIR) spectroscopy (Model FTS 3500 GX) attached with DRS. The dry biomass sample of 10 g was mixed with 200 mg KBr and compressed to form pellets. The spectra were collected at a scan rate of 40 with a step size of $4cm^{-1}$ within the range of 400 to 4000 cm^{-1} wave numbers.

4.2.5 COMPOSITIONAL ANALYSIS

The determination of a polysaccharide fraction of the biomass samples was performed using the fibra plus automatic fibre estimation system, Pelican. For the neutral detergent fibre (NDF) estimation, 0.5-1 g of the powdered biomass sample was taken in a crucible. Added 100 ml of neutral detergent solution at room temperature into the crucible with 0.5 g of sodium sulphite. The crucible was heated to boiling at 400 ^{0} C and reflux 60 minutes from the onset of boiling. The mixer was filtered and washed with water and then acetone. The crucible was dried at 105 ^{0} C for 8 h and the weight was recorded. The NDF was calculated as follows (Van Soest et al. 1991):

$$NDF\ \% = \frac{(Wt\ of\ crucible + NDF) - Wt\ of\ crucible}{Wt\ of\ sample} \times 100 \quad (4.7)$$

For the acid detergent fibre (ADF) analysis, the similar protocol for NDF estimation was performed. However, the acid detergent solution was taken instead of a neutral detergent solution. The weight loss was calculated as follows:

$$ADF\ \% = \frac{(Wt\ of\ crucible + ADF) - Wt\ of\ crucible}{Wt\ of\ sample} \times 100 \quad (4.8)$$

The acid detergent lignin (ADL) was determined by preparing the ADF in a crucible by adding 72 % of H_2SO_4. The mixture was allowed to mix with constant stirring about 3h. It was filtered and washed with water twice. The crucible was kept in the conventional oven at 100 ^{0}C for 8h. The weight loss was recorded and the ashing was performed by keeping the same crucible in a muffle furnace at 500 ^{0}C for 7 min. This weight loss was recorded. The percentage of cellulose, hemicellulose and lignin were calculated as follows:

$$\text{Hemicellulose \%} = \text{NDF \%} - \text{ADF \%}$$

$$\text{Cellulose \%} = (Y\text{-}L/W) \text{ X } 100 \qquad (4.9)$$

$$\text{Lignin \%} = (L\text{-}A/W) \text{ X } 100$$

where, Y= weight of ADF+crucible, L= weight of crucible + lignin, A = weight of crucible + ash, W= weight of sample.

4.3 RESULTS

4.3.1 BIOMASS SAMPLE COLLECTION

29 species of plant biomass were chosen for the study. The collected biomasses are given in **Fig.3.1. (Chapter 3).** The location along with voucher number of each collected specimens are given in **Table 3 (Chapter 3).**

4.3.2 HERBARIUM PREPARATION AND IDENTIFICATION

The herbarium specimen for each biomass sample was submitted to Herbarium unit, Botany Department, Gauwahati University, Assam, India. One copy of each specimen was retained at Indian Institute of Technology Guwahati, Assam, India for future references. The identified herbarium specimen with voucher number is given in **Table 3.1 (Chapter 3)** and the specimen in **Fig.3.2 (Chapter 3).**

4.3.3 PROCESSING AND SIZE REDUCTION

The ground biomass of some selected samples are presented in **Fig. 4.2.** It was observed that the LCB exhibit different colours respective to the type of biomass.

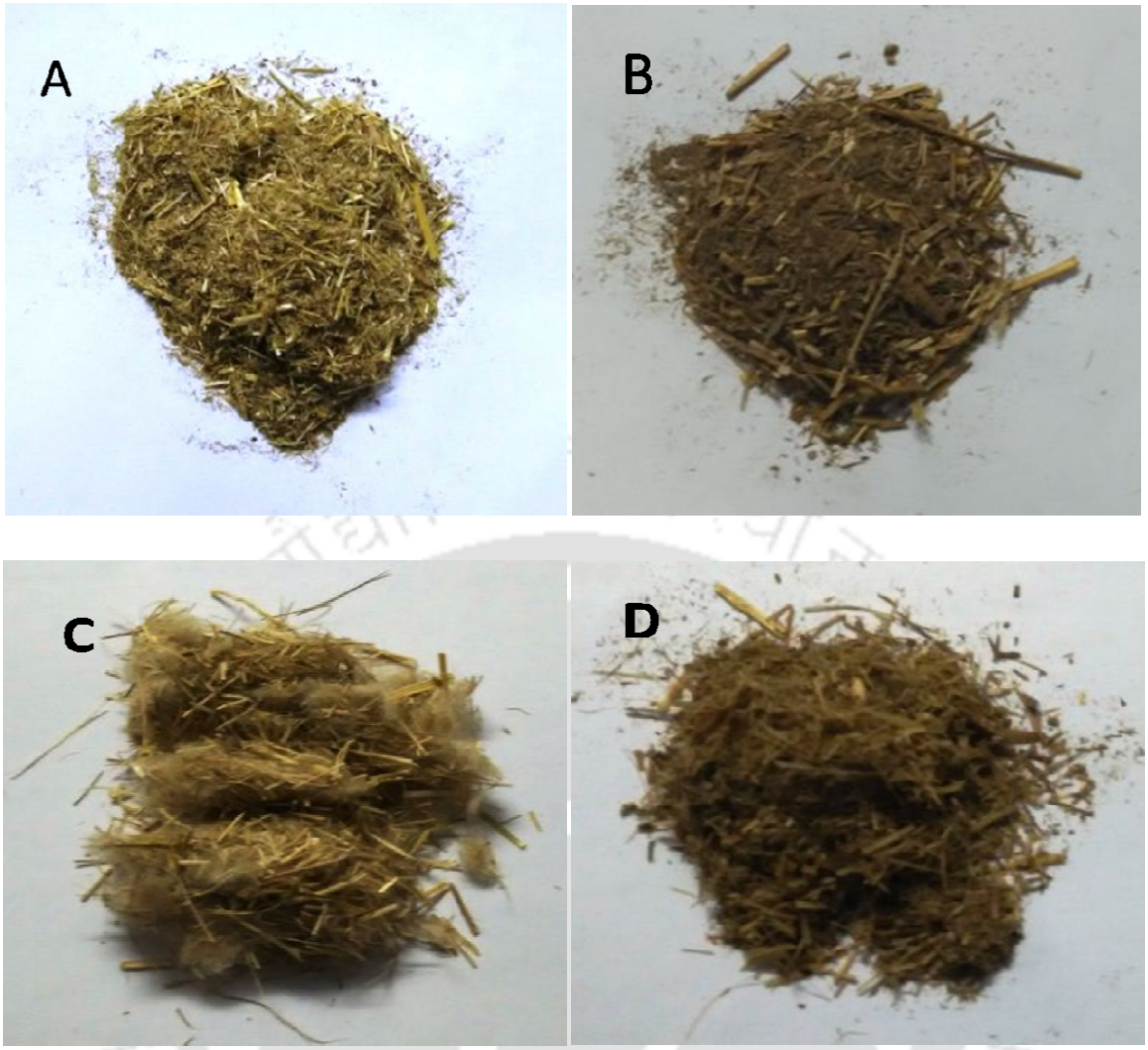


Fig. 4.2. Ground Lignocellulosic biomass: A- *Eragrostis airoides*, B- *Arundinella khasiana* Nees ex Steud, C- *Impereta cylindrica*, , D- *Echinochloa stagnina* (Retz.) P.Beauv.,

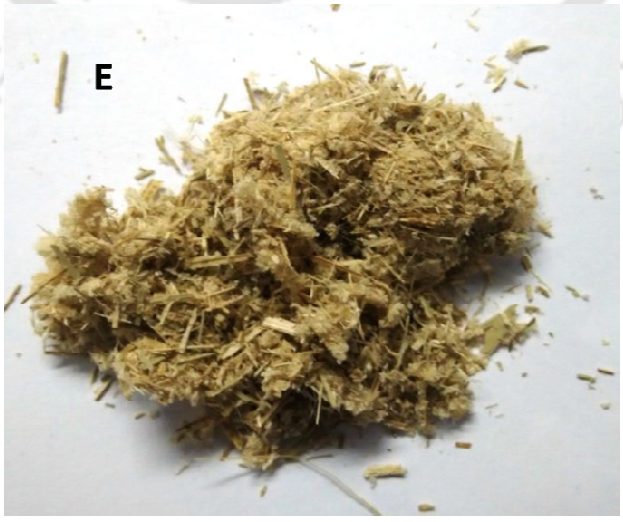


Fig. 4.2. Ground Lignocellulosic biomass: E- *Typha angustifolia* L.

4.3.4 PHYSICAL ANALYSIS

The physical analysis of the collected biomass sample based on the moisture content, volatile matter, ash, fixed carbon content and total solid content are given in proximate analysis.

4.3.4.1 PROXIMATE ANALYSIS

The biomass fuels are characterized and classified by the term proximate and ultimate analysis. The proximate analysis gives moisture content, volatile content, ash content, heating value and free carbon. The ultimate analysis gives the elemental composition of the biomass in weight percentage of carbon, hydrogen and oxygen as well as sulphur and nitrogen (if any). The elemental analysis is particularly important in evaluating the feedstock in terms of heating value and of potential technical problems like reactor slagging and pollution problems.

The data obtained from proximate analysis (moisture content, volatile matter, ash, fixed carbon content and total solid content) of the biomass samples are presented in **Table 4.1.**

Table 4.1. Proximate analysis of different lignocellulosic biomass

	Biomass sample	Moisture (%)	Total solid (%)	Volatile matter (%)	[a] Fixed carbon (%)	Ash (%)
1	*Impereta cylindrica*	8.34 ±0.16	91.452	84.5 ±0.31	0.42	6.74±0.20
2	*Eragrostis airoides*	8.35 ± 0.15	91.725	86.58±0.31	1.65	3.42±0.20
3	*Typha angustifolia*	13.38 ±0.60	86.049	80.25±0.20	2.91	3.46±0.26
4	*Arundinella khasiana*	10.02 ±0.21	89.63	80.46±0.18	1.22	8.30±0.13
5	*Echinochloa stagnina*	10.49 ±0.19	89.73	83.58±0.28	0.57	6.50±0.33
6	*Phragmites karka*	8.68 ± 0.16	91.1571	84.35±0.18	2.47	4.50±0.17
7	*Thysanolaena agrostis*	6.55 ± 0.30	93.0335	83.58±0.25	1.52	8.35±0.21
8	*Erianthus fultus*	8.56 ± 0.19	91.2613	80.47±0.23	3.48	7.49±0.15
9	*Sclerostachya fusca*	8.45 ± 0.22	91.2704	86.24±0.15	0.63	4.68±0.14
10	*Neyraudia reynaudiana*	8.53 ± 0.17	91.6168	85.76±1.06	3.17	2.54±0.12
11	*Coelorachis striata*	8.33 ± 0.15	91.8649	84.62±0.52	0.81	6.24±0.21
12	*Dichanthium assimile*	10.56 ±0.16	89.63	80.91±0.58	0.46	8.07±0.08
13	*Cymbopogon exertus*	8.88 ± 0.28	90.94	84.42±0.39	0.15	6.55±0.23

14	*Arundo donax*	9.76 ± 0.17	90.023	83.19 ±0.24	0.73	6.32±0.18
15	*Saccharum sponteneum*	7.52 ± 0.29	92.882	84.66 ±0.40	3.24	4.58±0.25
16	*Cymbopogon longifolium*	9.45 ± 0.08	90.437	84.50±0.24	1.54	4.51±0.26
17	*Setaria glauca*	8.44 ± 0.19	91.31	84.71±0.66	1.27	5.58±0.29
18	*Cynodon dactylon*	8.60 ± 0.23	91.725	86.51±0.27	1.35	3.54±0.28
19	*Echinochloa colonum*	8.40 ± 0.22	91.331	83.62±0.28	2.55	5.43±0.25
20	*Chrysopogon aciculatus*	7.51 ± 0.25	92.765	85.56±0.23	1.37	5.56±0.21
21	*Eleusine indica*	8.53 ± 0.19	91.438	82.56±0.41	2.5	6.41±0.25
22	*Fimbristylis dichotoma*	12.30 ±0.30	87.86	80.78±0.63	0.48	7.40±0.22
23	*Cyperus exaltatus*	9.42 ± 0.29	90.156	79.83±0.22	1.92	8.83±0.11
24	*Cyrtococcum oxyphyllum*	12.55 ±0.21	87.74	79.47±0.27	3.28	4.70±0.10
25	*Paspalum scrobiculatum*	7.59 ±0.25	92.28	82.92±0.19	1.98	7.51±0.28
26	*Coix lacryma-jobi*	9.30 ±0.25	90.89	79.72±0.50	0.09	11.07±0.16
27	*Meriscus sp.*	10.42 ±0.24	89.24	77.88±0.20	0.54	11.16±0.44
28	*Thysanolena maxima*	9.42 ±0.27	90.679	81.28±0.17	0.50	8.80±0.27
29	*Cyperus Flavidus*	8.57 ±0.28	91.75	82.72±0.58	1.29	7.42±0.19

[a] Fixed carbon content was calculated on the basis of *FC = 100 - (% of moisture + % of volatiles + % of ash)*

4.3.4.2 THERMOGRAVIMETRIC ANALYSIS (TGA) ANALYSIS

The thermal behaviour of the biomass samples were studied by measuring the rate of weight loss of the sample as function of the time and temperature (TGA). The observed rates are not only functions of time and temperature, but also of the size and the density of the sample. TGA offers an understanding of the dynamic measurements yield data equivalent to a standardised proximate analysis. In the test protocol, the biomass samples were heated up to 900 ^{0}C in a thermobalanced by applying heating rate of 10 Cel/Min in a nitrogen atmosphere.

The thermal degradation profile of the selected LCB is presented in **Fig. 4.3.** The degradation profile of the biomass sample can be seen at a different range of temperature. It can be

observed that three stages of biomass degradation took place (**Fig. 4.3.** (A). The DTG profile are depicted in **Fig. 4.3. (B).**

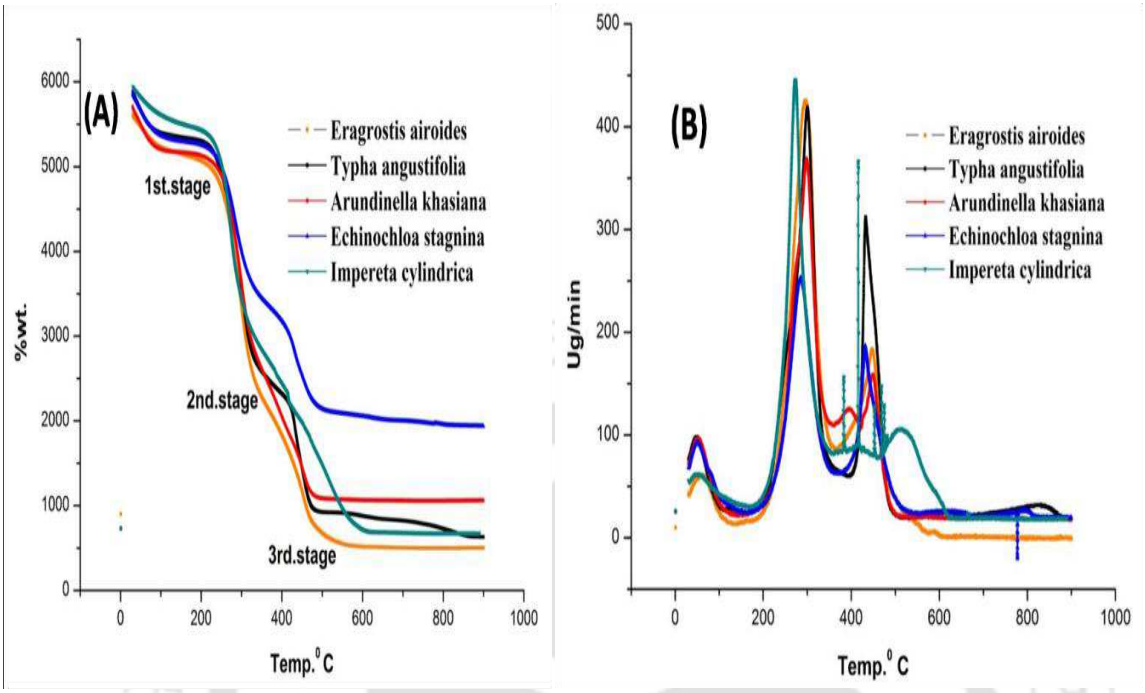


Fig. 4.3. The thermal degradation profile of the LCB: (A) TGA and (B) DTG thermograph.

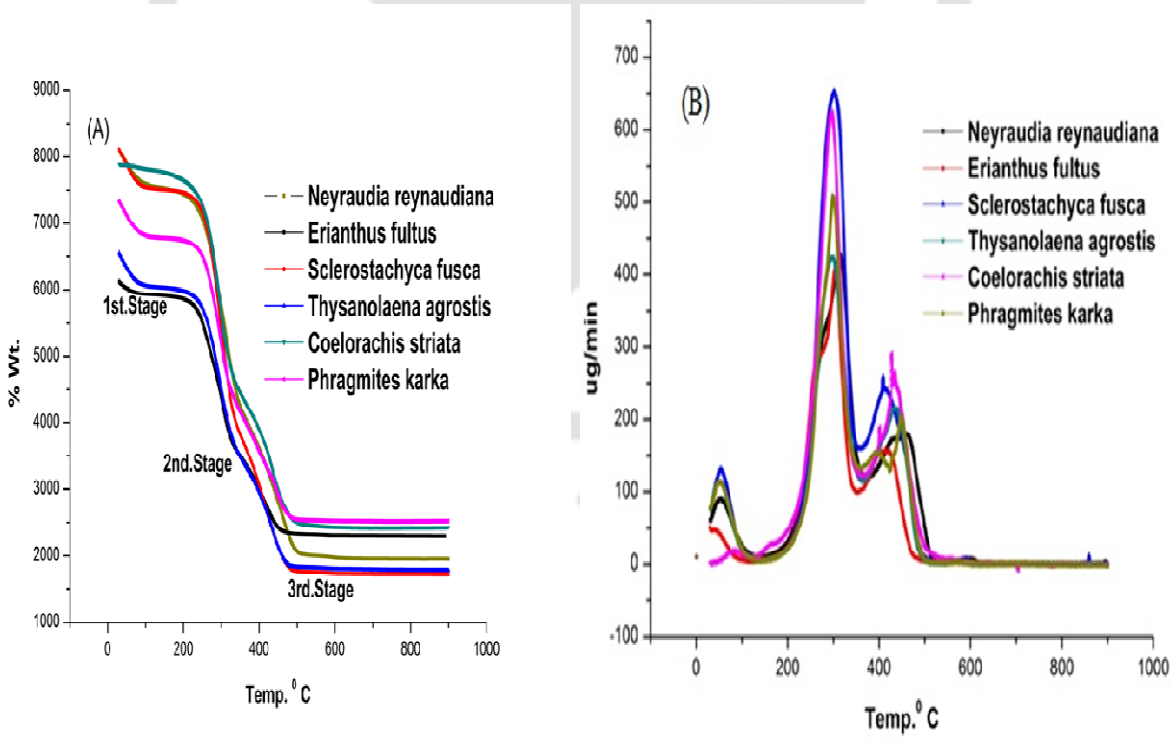


Fig. 4.3. The thermal degradation profile of the LCB: (A) TGA and (B) DTG thermograph.

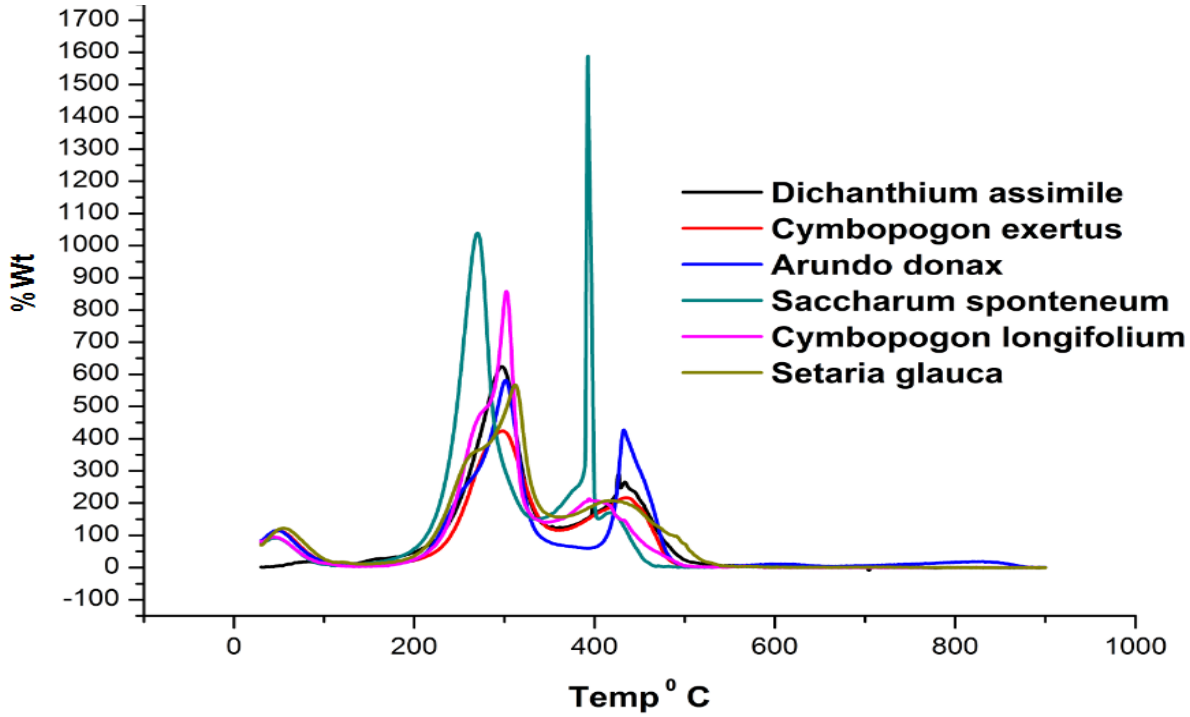


Fig. 4.3. The thermal degradation profile of the LCB

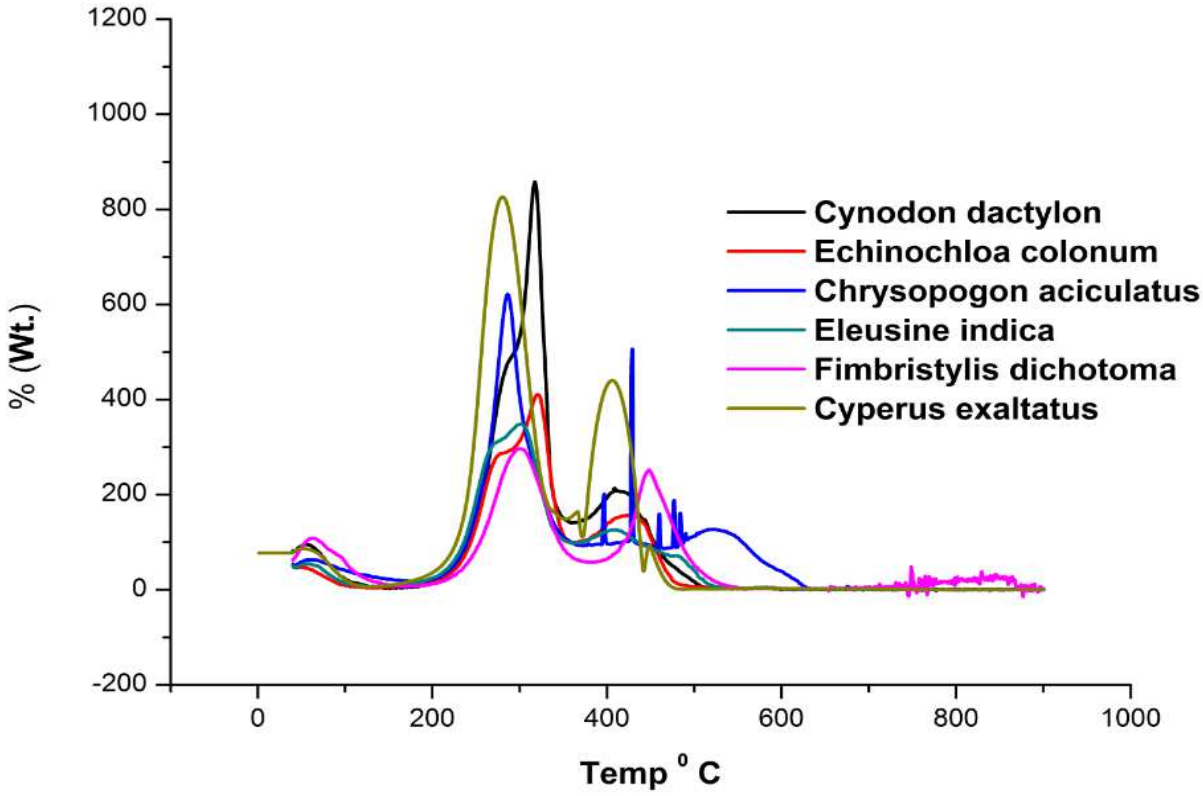


Fig. 4.3. The thermal degradation profile of the LCB

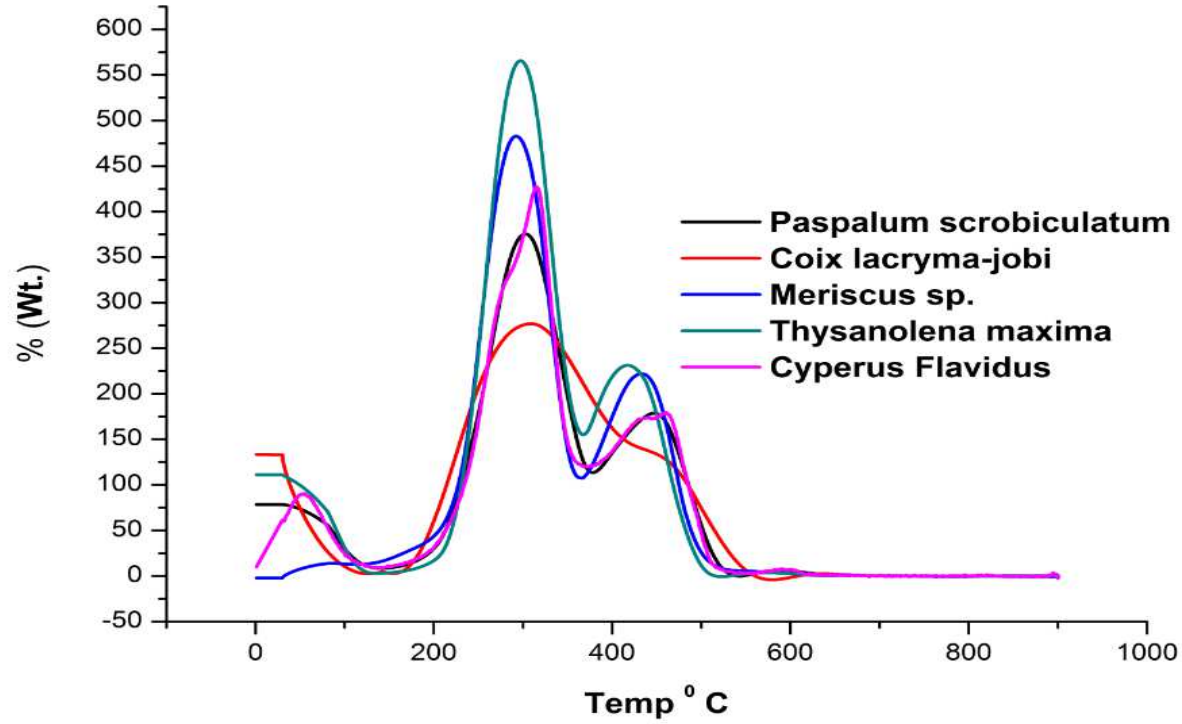


Fig. 4.3. The thermal degradation profile of the LCB

4.3.4.3 X-RAY DIFFRACTION (XRD) ANALYSIS

It is very difficult to measure the cellulose crystallinity of the entire biomass. Pre-treatment can alter the cellulose crystalline structure by disrupting the inter hydrogen bond of cellulose. There is a relation between the cellulose crystallinity and thermal degradation temperature. The thermal degradation activation energies decreases when the cellulose crystallinity is decrease and increases the rate of depolymerization. From the literature, we know that the biomass crystallinity depends on the presence of wax in the LCB. This wax is component of a high molecular mass hydrocarbons and fatty acid. Therefore, the crystallinity is highly related to the complex bonding structure of cellulose and wax. XRD pattern of the LCB is presented in **Fig. 4.4.** The amorphous cellulose and hemicellulose portion could be seen at 2Θ = 22.1◦, 22.0 283 or 2Θ= 16.6. The Crystalline index obtained from the peaks were presented in discussion section.

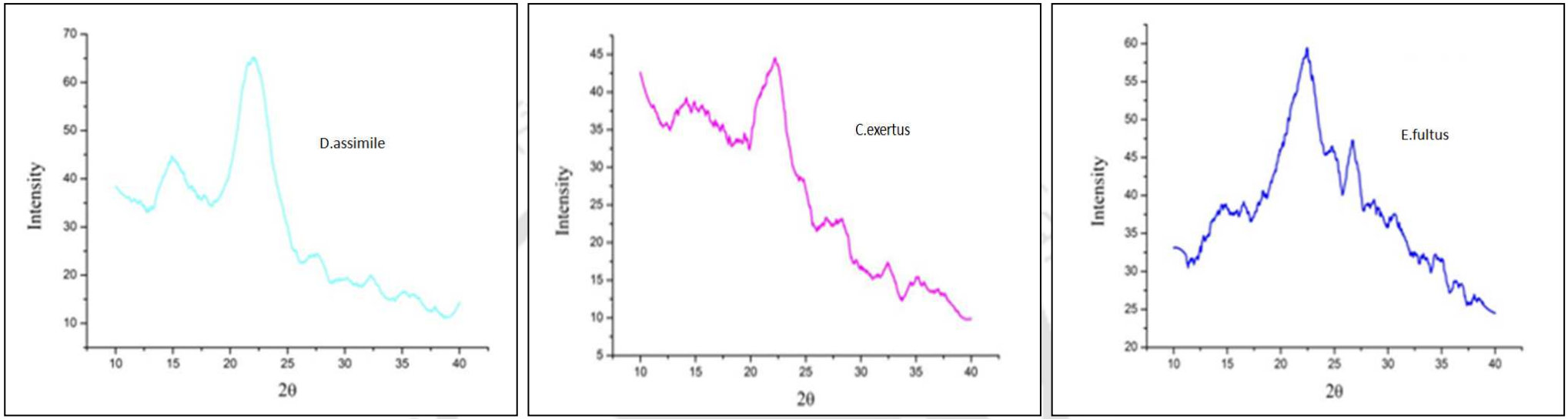
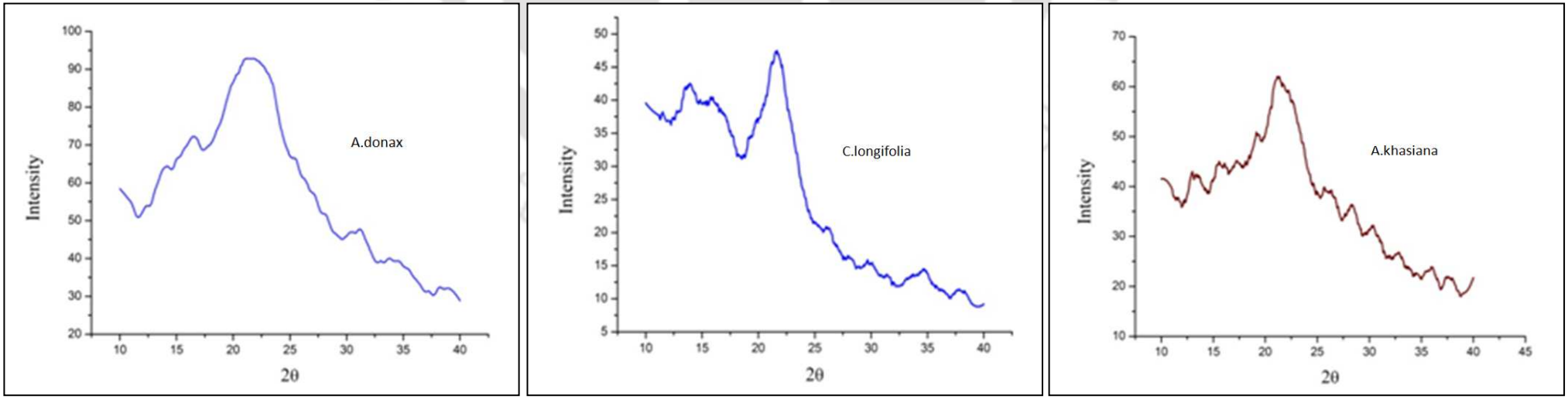


Fig. 4.4. XRD pattern for different types of LCB collected from North-East India



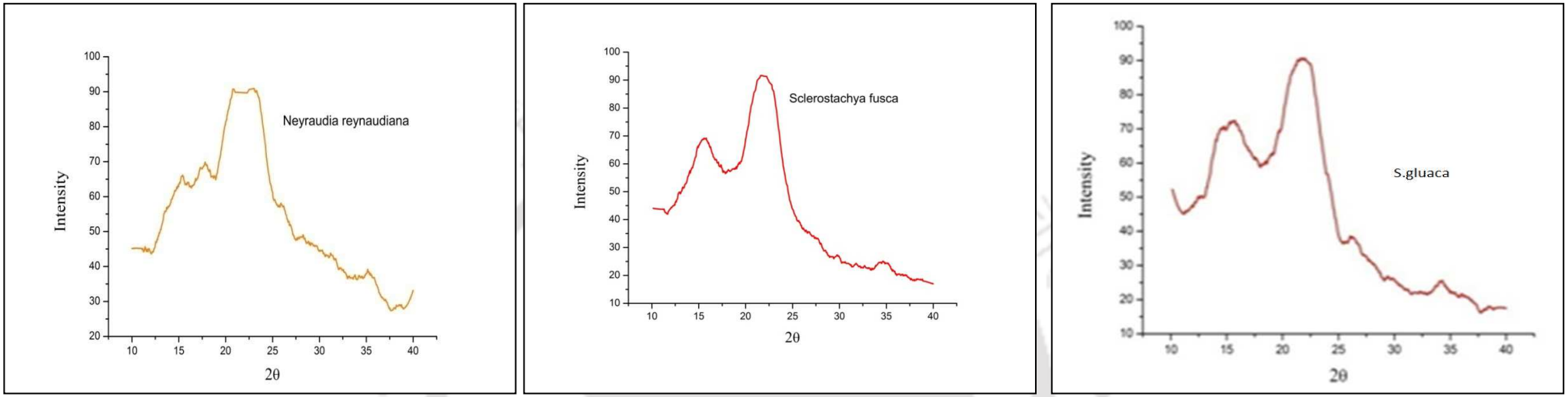
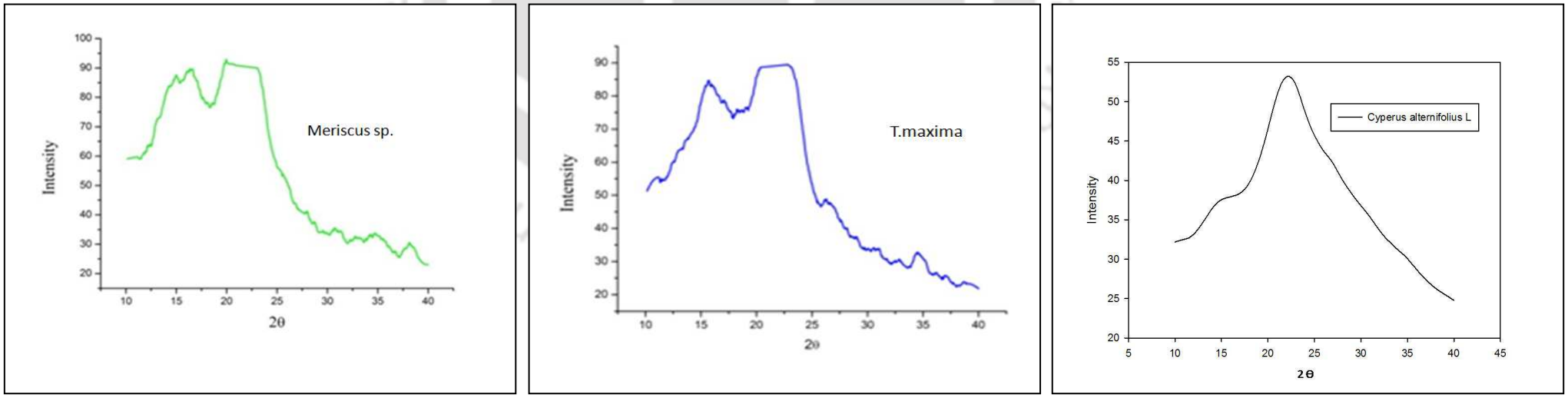


Fig. 4.4. XRD pattern for different types of LCB collected from North-East India



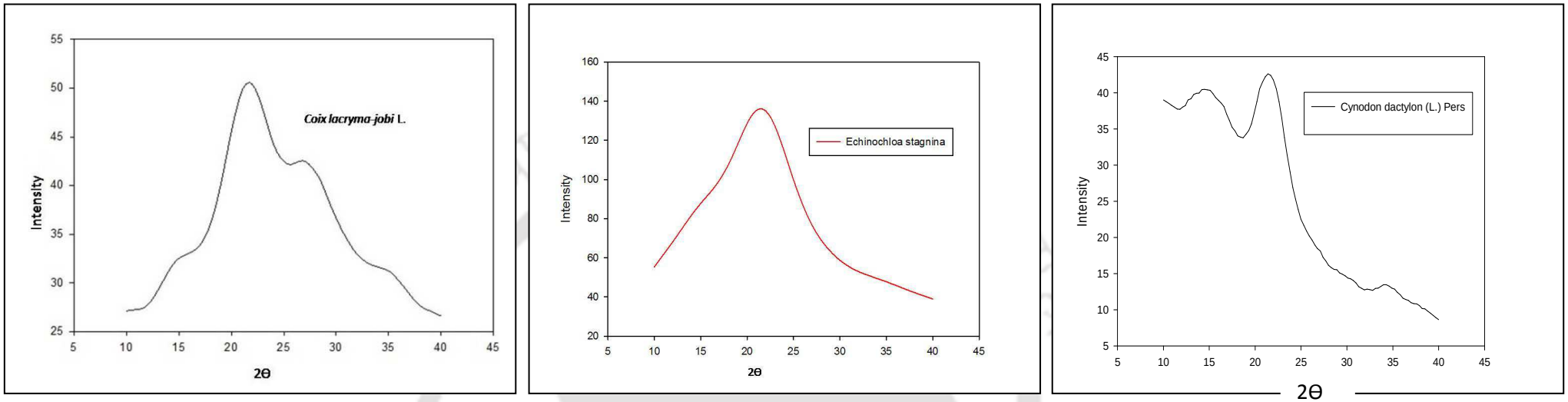
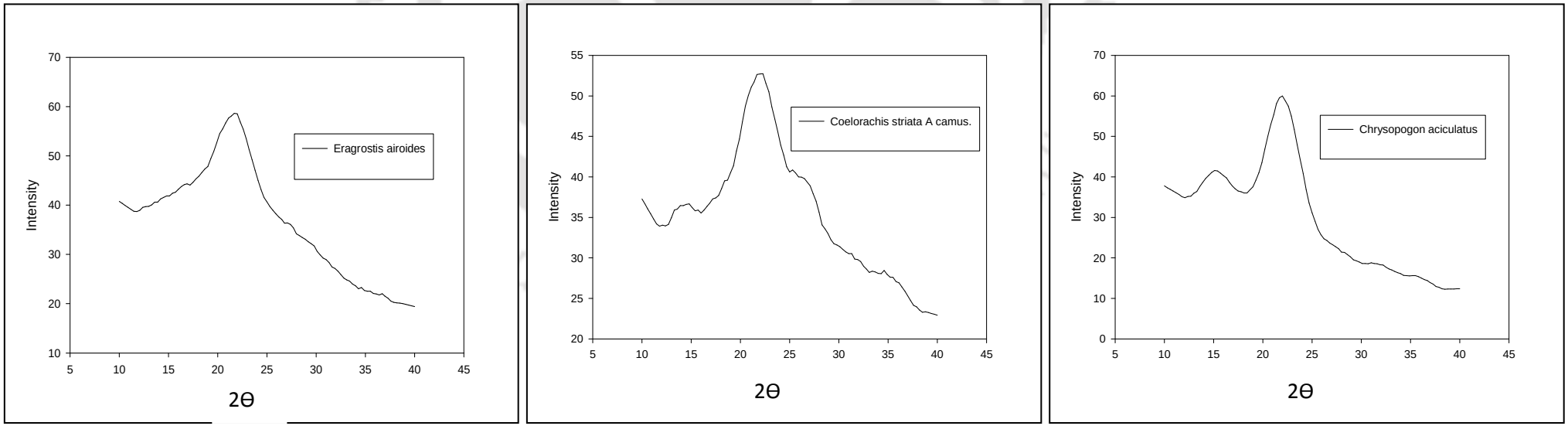
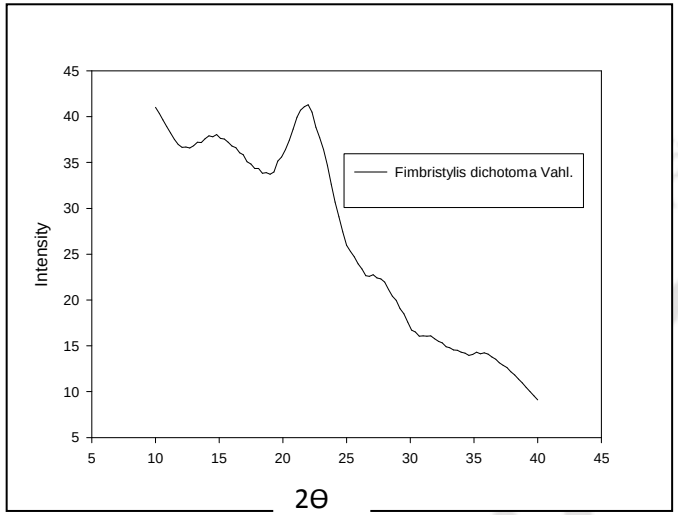
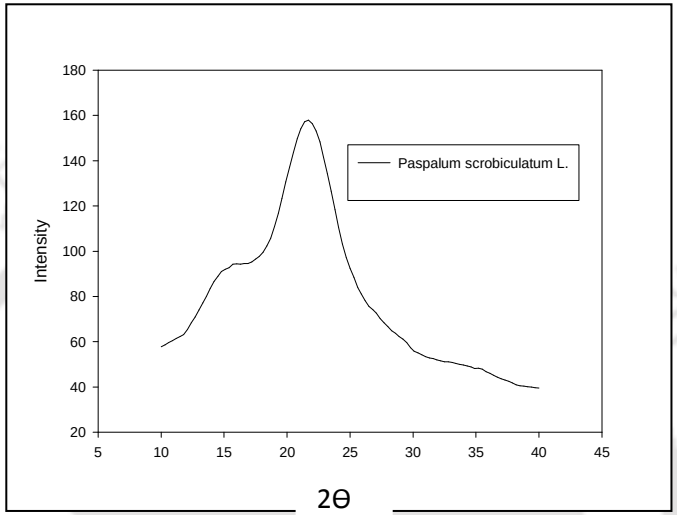
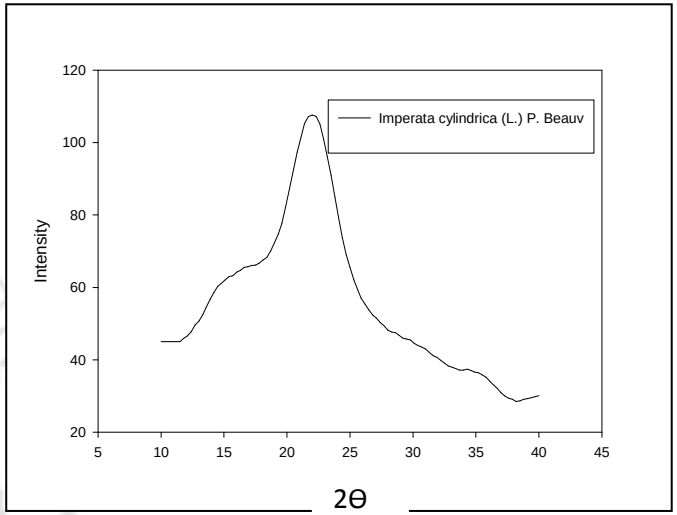


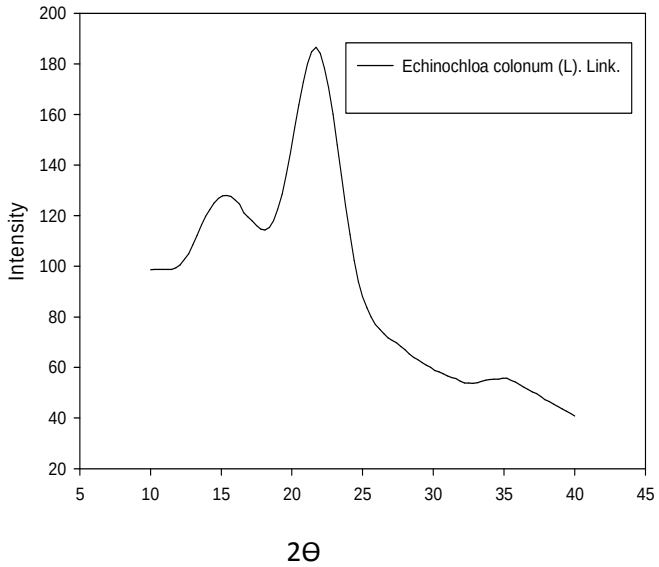
Fig. 4.4. XRD pattern for different types of LCB collected from North-East India

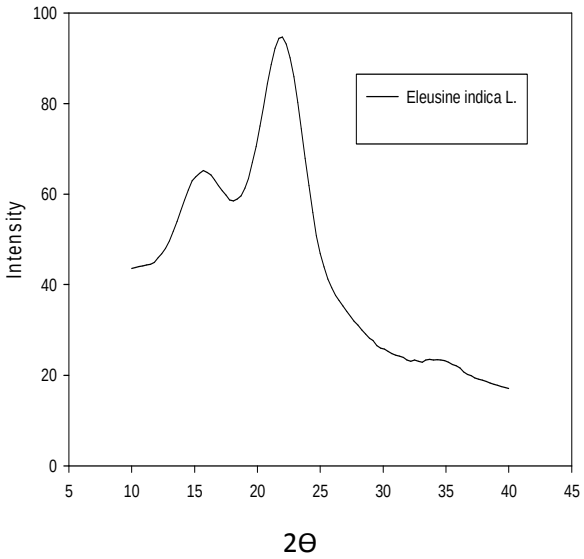


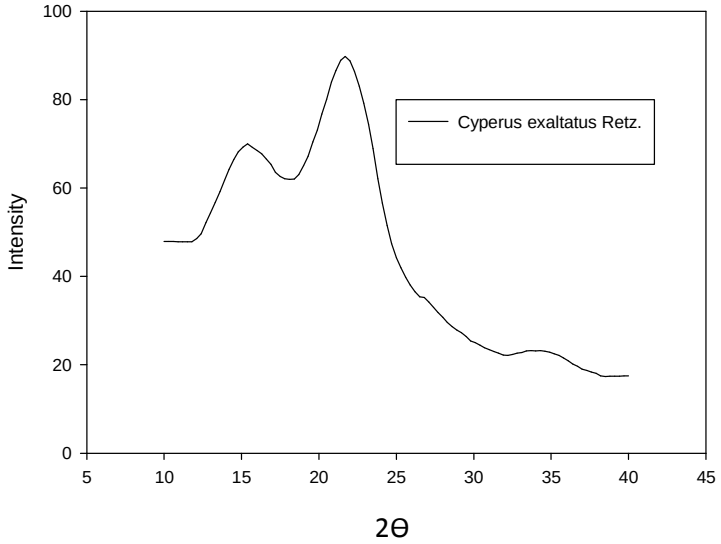












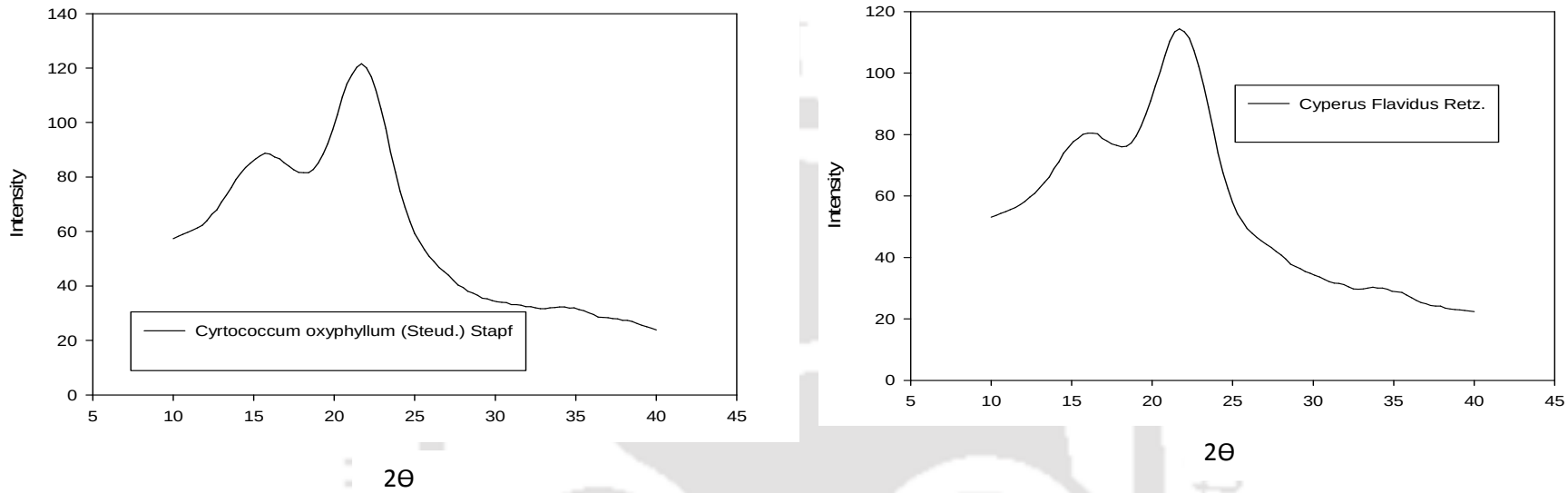


Fig. 4.4. XRD pattern for different types of LCB collected from North-East India

4.3.5 CHEMICAL ANALYSIS

4.3.5.1 ULTIMATE ANALYSIS

The ultimate analysis generally gives the elemental composition of the biomass in weight percentage of carbon, hydrogen and oxygen (the major components) as well as sulfur and nitrogen (if any). The elemental analysis was done based on dry matter of the biomass sample because otherwise moisture content would counted as additional hydrogen and oxygen. This helps in eliminating the confusion and give good representation of the fuel. A number of instruments have been developed to determine the elemental composition of the biomass. In some cases, C,H,N, and S, can be determined simultaneously. However, in this case, oxygen is determined by difference. The results are summarised in **Table 4.2.**

Table 4.2. Ultimate analysis of biomass sample

LCB	**Carbon (%)**	**Hydrogen (%)**	**Nitrogen (%)**	**Oxygen[b] (%)**
Neyraudia reynaudiana (Kunth) Keng ex A.S.Hitchc.	41.264	5.389	1.252	51.095
Typha angustifolia L.	52.895	5.844	1.217	40.044
Sclerostachya fusca (Roxb.) A. Camus	43.706	5.422	1.059	49.813
Thysanolaena agrostis Nees.	44.120	5.190	1.744	48.946
Imperata cylindrical (L) P.Beav	50.035	5.923	1.145	42.897
Cynodon dactylon	41.024	4.723	4.138	50.115
Cymbopogon longifolium	43.954	4.536	1.660	50.15
Coelorachis striata (Nees ex Steud.) A. Camus	45.954	5.536	1.660	46.850
Cyperus exaltatus retz	33.579	3.594	2.245	60.582

Fimbristylis dichatema (L) Vahl	41.264	4.389	1.252	53.095
Cymbopogon exertus	41.588	4.419	1.333	52.663
Merisceus sp.	35.220	4.152	3.663	56.965
Coix lacryma-jabi l	34.980	3.663	2.858	58.499
Echinochloa stagnina Beauv.ess.agrost	44.980	5.663	1.858	47.499
Thysanolena maxima kuntz	47.542	5.287	3.037	44.134
Setaria glauca	30.563	4.256	2.785	62.396
Eragrostis airoides	41.024	6.723	1.138	51.115
Arundinella khasiana Nees.ex.Steud	41.264	5.389	1.252	52.095
Echinochloa colonum (L). Link.	50.908	5.098	2.675	43.319
Chrysopogon aciculatus	42.786	4.780	3.467	48.967
Eleusine indica L.	49.284	3.960	2.775	43.981
Saccharum sponteneum L.	37.906	5.134	4.906	52.054
Erianthus fultus Nees.	46.650	5.568	1.632	46.150
Cyrtococcum oxyphyllum (Steud.) Stapf	31.786	4.120	1.451	62.643
Paspalum scrobiculatum L.	40.251	3.251	2.452	54.046
Arundo donax L.	39.254	4.124	2.664	53.958
Cyperus alternifolius L.	33.231	5.023	2.546	59.200
Dichanthium assimile (Steud.) Deshp.	41.245	4.263	3.542	50.950
Phragmite karka	44.043	5.141	1.524	49.292

Cyperus Flavidus Retz.	43.256	4.781	2.365	49.598

[b] % of oxygen calculated from the difference of C,H, and N.

4.3.5.2 CALORIFIC VALUES

Heating value is the amount of heat produced by a complete combustion of fuel and it is measured as a unit of energy per unit mass or volume of the substance (e.g., kcal/kg, kJ/kg, J/mol and Btu/m^3). Generally, the heating value of any biomass sample is expressed by the higher and lower heating values (HHV and LHV). The higher heating value or gross calorific value or gross energy or higher calorific value is defined as the amount of heat released when fuel is combusted and the products have returned to a temperature of 25°C. Here, the heat of condensation of water is included. The lower heating value is defined as the net calorific value obtained by subtracting the heat of vaporization of water (generated during combustion) from the higher heating value (Mericboyu et al. 1998).

The calorific value was calculated as the higher heating value. The results are summarised in **Fig.4.5.**

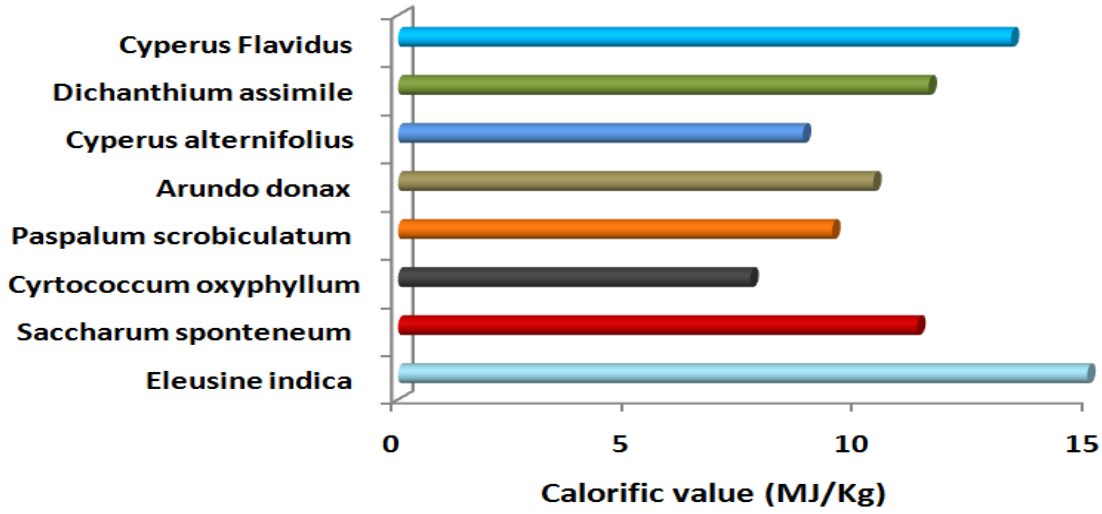


Fig. 4.5.(A) Calorific values of different biomass sample

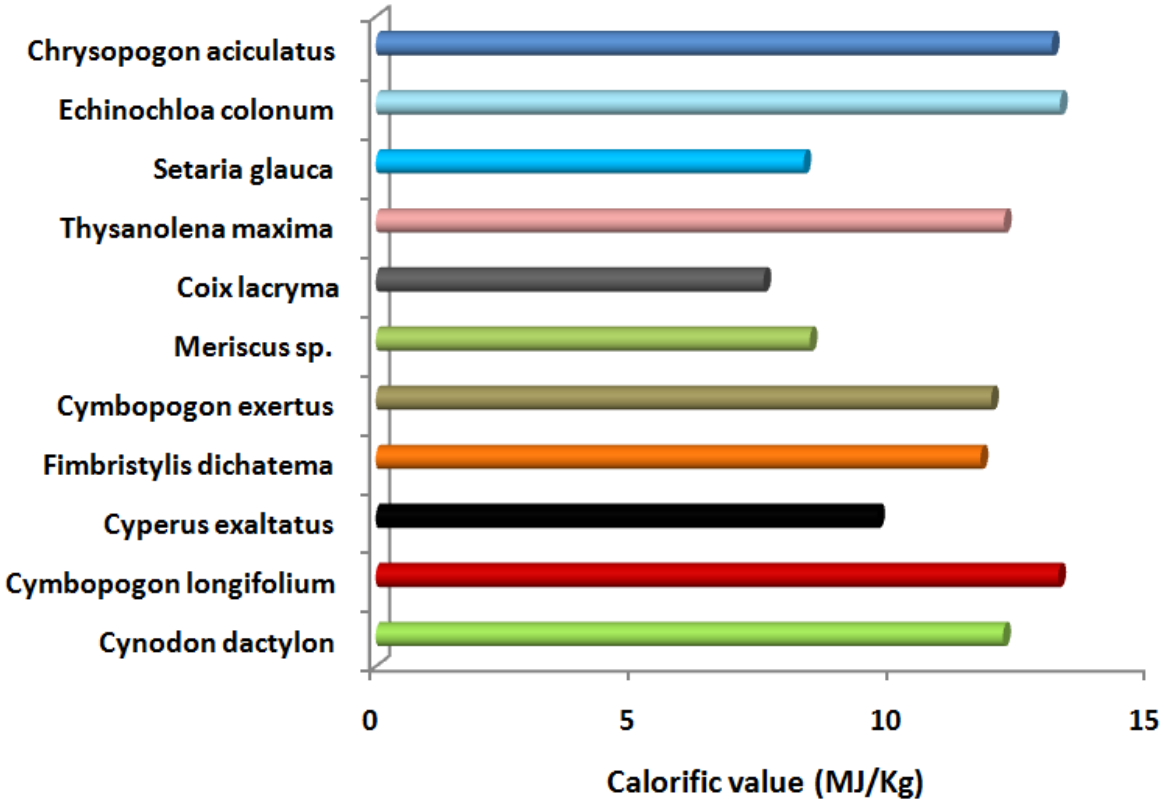


Fig. 4.5.(B) Calorific values of different biomass sample

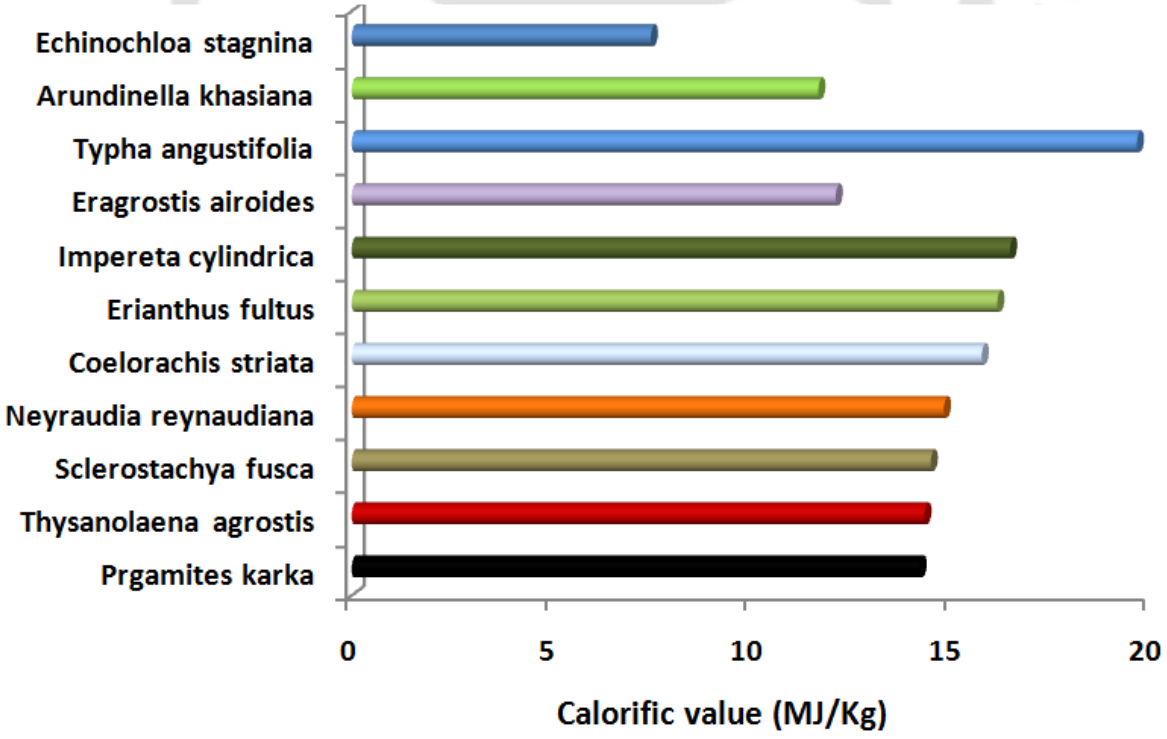


Fig. 4.5.(C) Calorific values of different biomass sample

4.3.5.3 VAN KREVELEN PLOT

Oxidation of the elements present in the biomass contributes in burning process. The important elements consist of carbon, hydrogen, oxygen, nitrogen and sulphur. In 1950, Van Krevelen tried to stratify the solid fuels such as biomass in a simple diagram to correlate between the elements by taking ratios such as H/C to O/C. A plot of the Van Krevelen diagram was presented in **Fig. 4.6.**

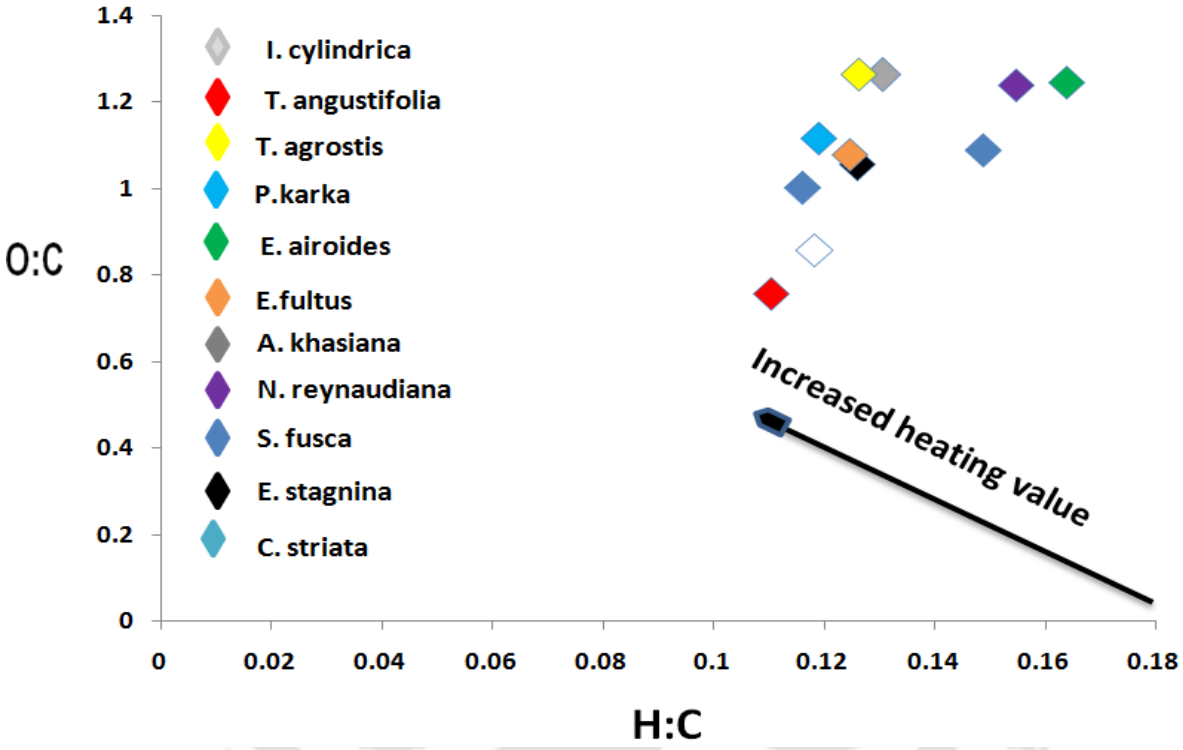


Fig. 4.6. (A) Van Krevelen plot against the H/C to O/C

4.3.5.4 FTIR ANALYSIS

Fourier Transform Infrared (FTIR) spectroscopy is a common technology used to study the functional groups present in the biomass and changes occurred due to the different pre-treatment methods. This technique measures the absorption of infrared radiation by the sample material versus wavelength. The infrared absorption bands identify molecular components and structures. The spectral provides the qualitative and semi-quantitative information about the presence and absence of a functional group of lignocellulosic biomass (Li et al. 2010). In the process, we have examined the FTIR spectra of biomass species collected from North-East India. The absorption bands were deconvoluted automatically,

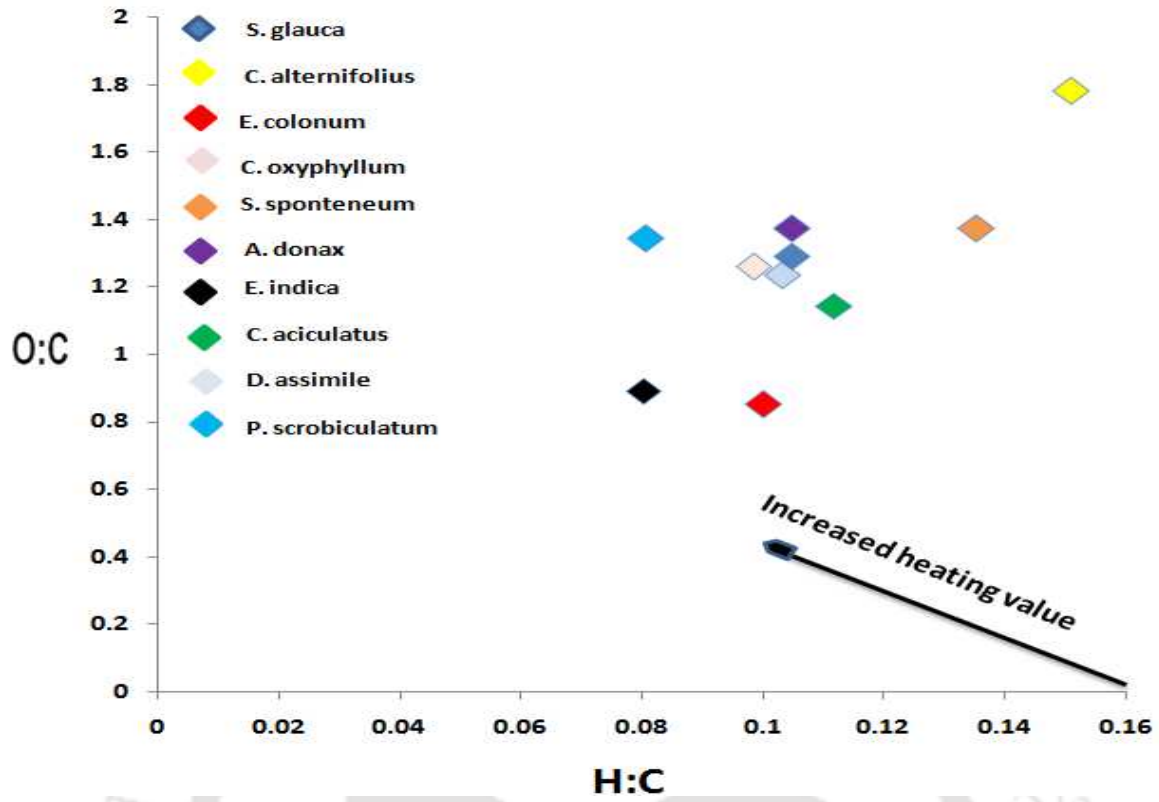


Fig. 4.6.(B) Van Krevelen plot against the H/C to O/C

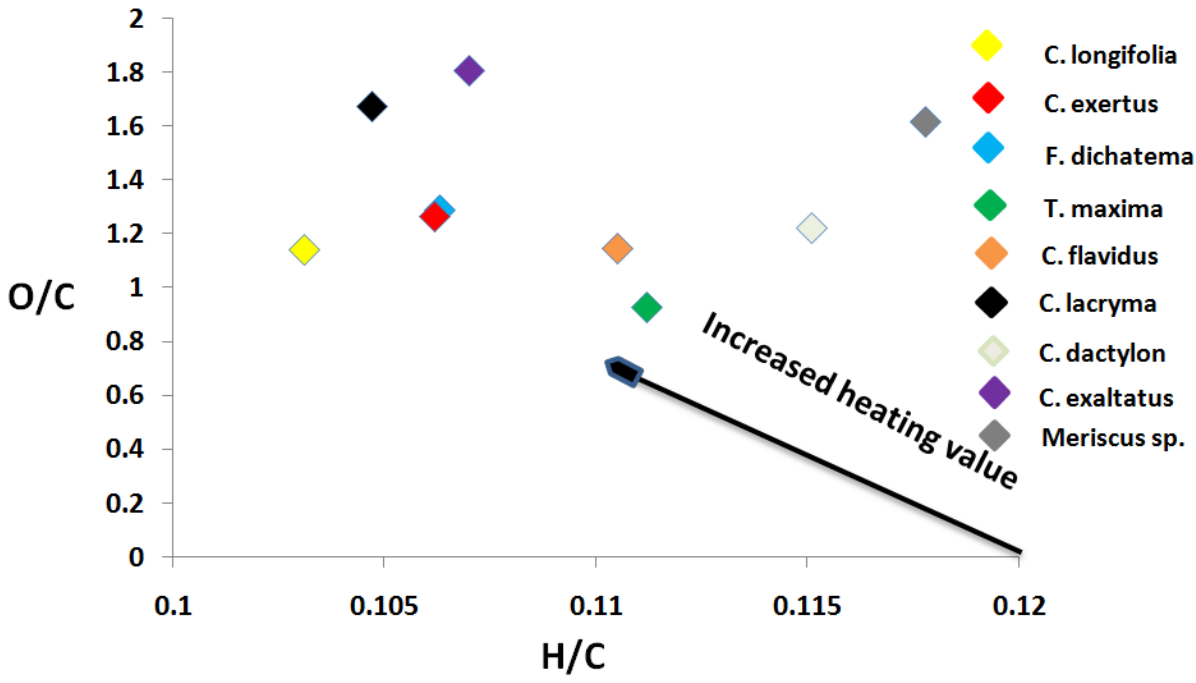


Fig. 4.6.(C) Van Krevelen plot against the H/C to O/C

generating the respective peaks readily available for multivariate analysis. The observations attained (**Fig. 4.7.)** were confirmed by referencing with the available literature.

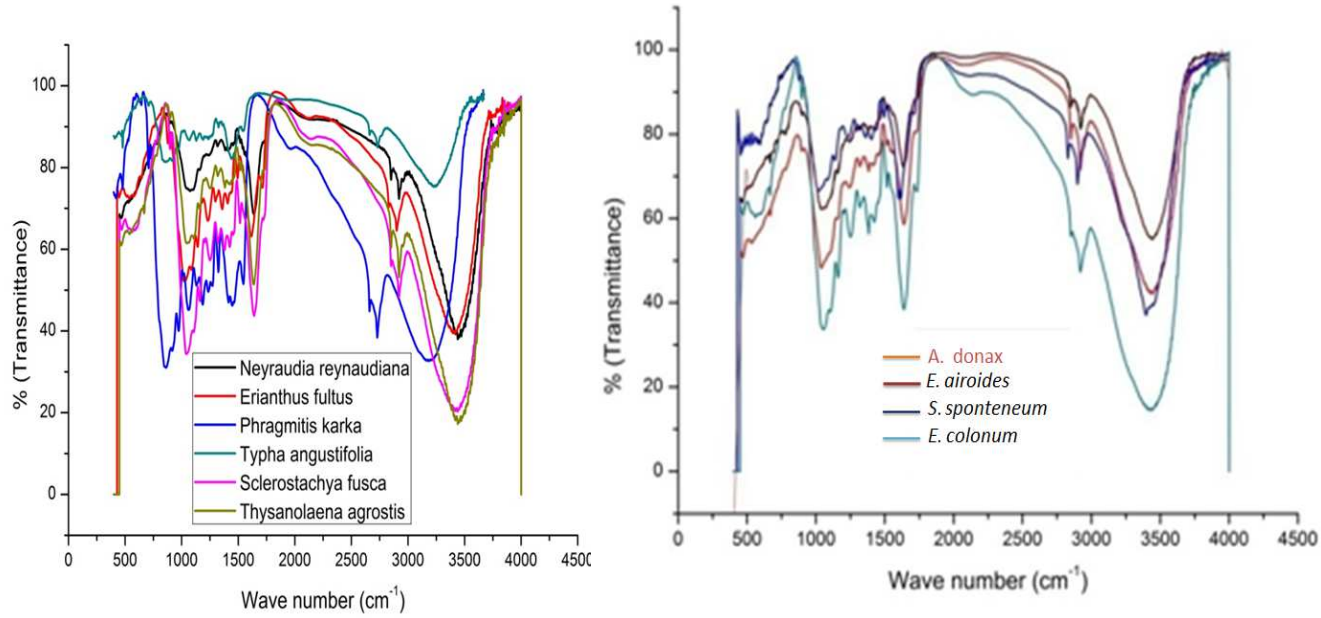


Fig. 4.7. FTIR spectra of different lignocellulosic biomass

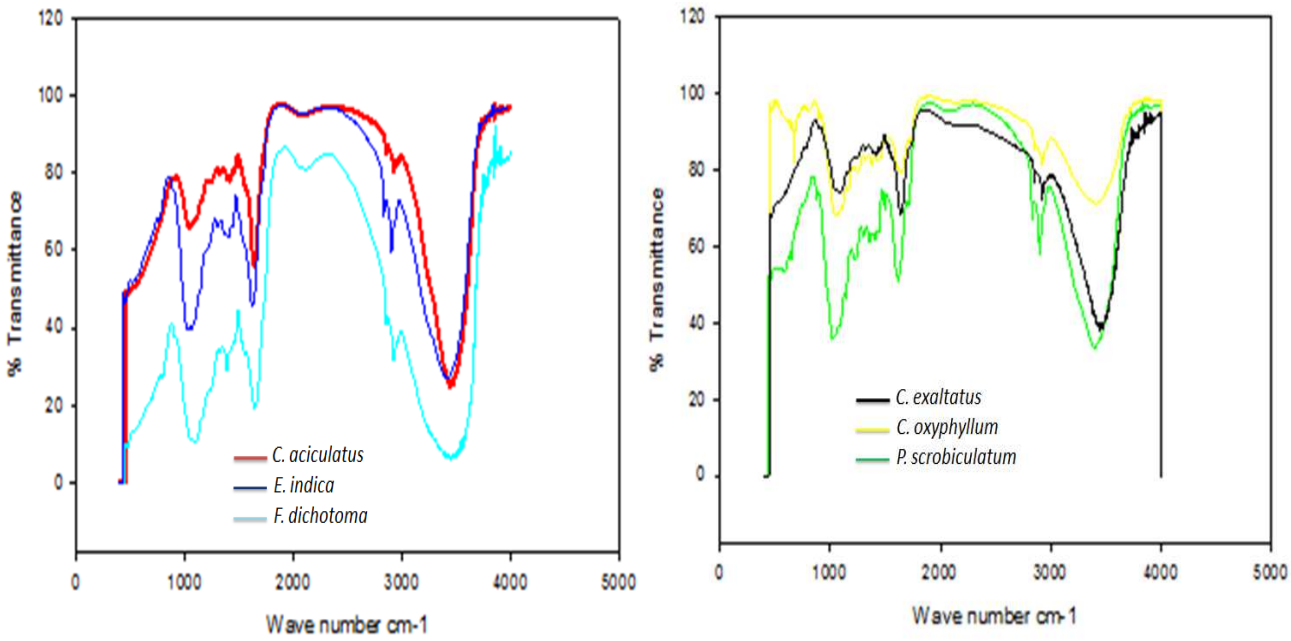


Fig. 4.7. FTIR spectra of different lignocellulosic biomass

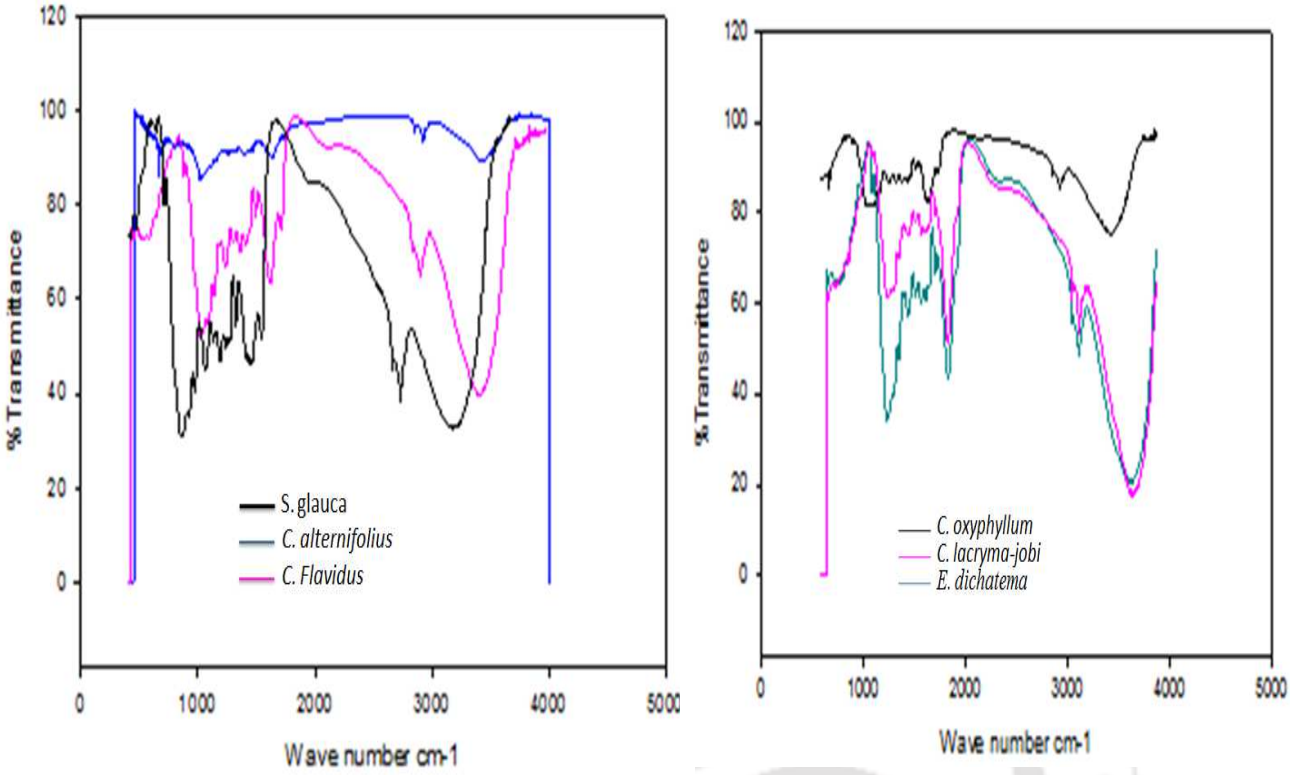
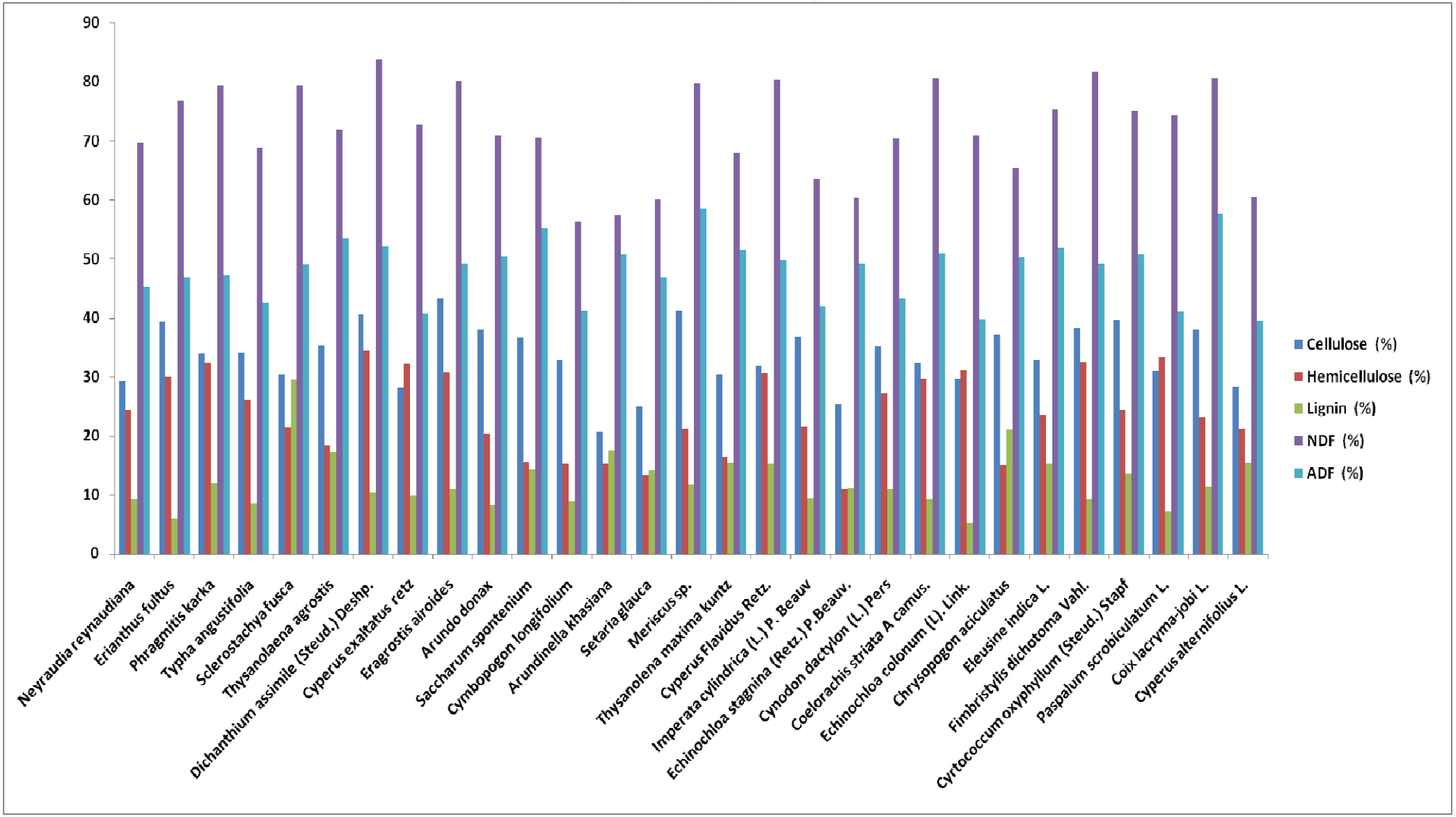


Fig. 4.7. FTIR spectra of different lignocellulosic biomass

4.3.6 COMPOSITIONAL ANALYSIS

The carbohydrates and lignin make up a major portion of the biomass samples. These components should be measured in other to know the fuel efficiency. These carbohydrates are the main energy source, which is further used to generate biofuel. The carbohydrate present in the biomass may be of structural or non-structural. The structural carbohydrates are bound in the matrix of the biomass hence, difficult to remove by a simple treatment, whereas non-structural carbohydrates can be easily removed by washing or extraction. The cellulose, hemicellulose, lignin, NDF and ADF of the biomass sample was determined and presented the percentage composition in **Fig. 4.8.**

Fig. 4.8. Compositional analysis of lignocellulosic biomass



4.4 DISCUSSIONS

Biomass is the most common form of renewable energy found abundantly throughout the world. The use of biomass as fuel has increased rapidly in last decade. Latterly, much attention has been focused on identifying suitable biomass species, which can provide high-energy outputs, to replace conventional fossil fuel energy sources. The type of biomass used for the production of biofuel and its efficiency depends on the fuel criteria. As we know that, phytomass is produced by the green plant through the process called photosynthesis. Typically, photosynthesis converts less than 1% of the available sunlight energy to chemical energy and stored in the plant cells. The chemical energy stored is in the form of carbohydrate. When the bonds between adjacent carbon, hydrogen and oxygen molecules are decomposed, it released energy. If the biomass is processed efficiently, either chemically or biologically, by extracting the energy stored in the chemical bonds and the subsequent energy product combined with oxygen, the carbon is oxidised to produce CO_2 and water. The process is cyclical, as the CO_2 is then available to produce new biomass.

Numerous crops have been proposed for commercial energy farming by many countries. The potential energy crops mainly include woody crops, grasses, herbaceous plants, starch and sugar crops, oilseeds crops etc. However, due to the lack of technology in converting the biofuel in an efficient way, these crops need to come up with many new technologies to produce biomass in large-scale.

In general, the characteristics of ideal energy crops are:

- high yield (maximum production of dry matter per hectare),
- low energy input to produce, low cost,
- composition with the least contaminants,
- low nutrient requirements.

We have collected 29 species of biomass belonging to the grass family. The collection of species was done randomly by considering the abundance and indigenous to the locality. The collection and type of biomass were highly important to discuss the aspect of that these LCB could be the best candidate for future biofuel production. We have explored 29 plant biomass which is totally new and no report have been found to check for biofuel production.

One of the most important factors for sample collection and herbarium documentation is to know the phenological changes occur due to global warming (Abraham et al. 2006). The documentation of the specimen provides the future references of the sample and local areas where it grows. That is the reason why we have collected the sample and submitted herbarium sheet to Botany department, Gauwahati University, Assam, India. One copy of the herbarium specimen was maintained at Center for Energy, Indian Institute of Technology Guwahati, for future references. These will helps in exploring the specimen and its characters if someone may require in the future (Detail discussed in chapter 3).

All the biomass used in the production of biofuel needs a typical degree of particle size reduction. Different techniques could apply to reduce the size until it reaches a small size enough to pass through the selected screen size. For the ethanol production from biomass, it usually verified at the range of ~1 to 6 mm. The characters such as moisture content, bulk density, true density and particle size are important for downstream processing of different kinds of biomass (Ebling et al. 1985). The comprehensive knowledge for biomass size reduction and size of the particle would allow for best selection of equipment types for the material, decreased operating costs, and aid in the predictable delivery of uniform quality biomass for processing and conversion.

Processing and size reduction of biomass prior to characterization is a very important aspect. The ground biomass sample was passed through the screen of sieve (1.18 mm). The particles unable to pass through sieve were regarded as oversized, while those passing through the sieve were used in the characterization. The particle size affects the thermochemical system of the sample (Bergman et al. 2005, Lu et al. 2010). We fixed our biomass sample to minimum particle size.

Proximate analysis of the biomass sample is one of the most important characterization methods where a biomass are allowed to pass a range of temperature, thereby biomass degraded at certain temperatures according to the composition of the structure. This consists of moisture, volatile matter, ash, and fixed carbon content. These values affect the combustion behaviour of the fuel (Garcia 2013).

The moisture content of a biomass sample expresses as the quantity of water per unit mass of the dry sample. We have followed the conventional oven drying method for moisture determination to constant weight at 105^{0} C. Prolonged drying more than 4hr was avoided as it can result in loss of volatile matter due to decomposition, distillation or oxidation of the

biomass sample. The moisture content may change during handling of the sample, as the dry biomass materials are hygroscopic in nature. High moisture content decreases the combustion yield of a biomass (Garcia 2013). Our findings show that moisture content was highest in biomass sample *Typha angustifolia* (13.38 ±0.60 %) and lowest in *Thysanolaena agrostis* (6.55 ±0.30 %). In most of the biomass, the moisture content was near to 8-10 % **(Table 4.1)**. The moisture percentage was calculated on the dry weight basis of the biomass sample. This is the main reason why our selected biomass shows less moisture content. Some of the biomass has very close similarity such as *Impereta cylindrical* (8.34 ±0.16%) and *Eragrostis airoides* (8.35 ± 0.15%).

Volatile matter refers to the part of the biomass that is released when the biomass is heated at 925±10 ^{0}C for 7 minutes. During this heating process, the biomass decomposed into gases and left out as char. Biomass typically has a high volatile matter than coal (less than 20 %). The presence of volatile matter in biomass influences the fuel reactivity (McKendry 2002). Volatile matter content in biomass varies in the interval of 79.47±0.27% – 86.58±0.31% (**Table 4.1.**). For example, the plant biomass like *Phragmites karka, Thysanolaena agrostis, Erianthus fultus, Sclerostachya fusca* has 84.35±0.18 %, 83.58±0.25 %, 80.47±0.23 %, and 86.24±0.15% respectively. The biomass like *Cymbopogon exertus* (84.42±0.39%), *Saccharum sponteneum* (84.66 ±0.40 %), *Cymbopogon longifolium* (84.50±0.24 %), *Setaria glauca* (84.71±0.66%), *Coelorachis striata* (84.62±0.52%), *Phragmites karka* (84.35±0.18 %), *Impereta cylindrical* (84.5 ±0.31 %) have very close similarities.

The ash content is one of the most studied characters of biomass. Ash consists of the mineral matter and inorganic matter (Vassilev et al. 2010). The solid residue of biomass fuel when produced by thermochemical conversion at 575±10 ^{0} C for 3hr is called ash. It is an integral part of plant material with a wide range of elements. Ash content affects the combustion rate in biomass sample. The percentage value varies according to the type of biomass. For example, 0.5 percent in wood, 5-10 percent in agricultural crops and 30-40 percent in rice husk. From our findings, the ash percentage was seen a maximum in *Meriscus sp.* (11.16±0.44%) and minimum in *Neyraudia reynaudiana* (2.54±0.12 %) (**Table 4.1.**). It can be observed that some biomass sample have very less ash content below the 5% such as *Eragrostis airoides, Typha angustifolia, Phragmites karka, Sclerostachya fusca, Neyraudia reynaudiana, Saccharum sponteneum, Cymbopogon longifolium, Cynodon dactylon,* and *Cyrtococcum oxyphyllum* respectively. The variation in ash content was visible in our selected biomass because the biomass has various types of inorganic elements.

Fixed carbon content is the biomass remaining after the release of volatile matter, excluding the ash and moisture content. This differs from the ultimate carbon content of the biomass sample in the fact that the carbon loss took place in the form of hydrocarbon with the volatile matter. It also related to ash, volatile and moisture content of the biomass. The volatile matter and fixed carbon influenced in the biological conversion process of the fuel (Vassilev et al. 2010). Woody biomass has much higher fixed carbon content as compared to LCB. The fixed carbon content varies in the interval of 0.09 % - 3.48 % (**Table 4.1.**). The total solid content was determined by subtracting the moisture percentage from the biomass. The maximum and minimum total solid content were seen in *Thysanolaena agrostis* (93.0335 %) and *Typha angustifolia* (86.049 %).

Thermogravimetric analysis (TGA) was conducted on the feedstock to understand the thermochemical profile at a different range of temperature. TGA is one of the most commonly used techniques in calculating the weight loss kinetics involved during biomass decomposition in the presence of nitrogen gas (Mani et al. 2010). It is also a powerful tool used in the determination of cellulose, hemicellulose and lignin composition of woody biomass as well as herbaceous biomass (Mothe et al. 2009, Greenhalfa et al. 2012). From our findings, it was observed that LCB went through three distinct phases (**Fig. 4.3. (A).** The first stage of decomposition occurs below temperature at 200 ^{0} C where moisture and volatile compounds (light volatiles) were removed. This stage is called drying period as the water molecules and light volatile molecules were liberated. In this stage, 10 % of weight loss of the sample took place as illustrated in **Fig. 4.3.** (A). A drastic change in the peak can be seen at 200-500 ^{0}C. This particular period is considered as the second stage. Here, the major devolatilsation of the biomass sample occurred. The slop in TG curves indicates that there is significant loss of biomass weight due to bond dissociation and decomposition of the biomass **Fig. 4.3.** (A). In this stage, volatile hydrocarbons, hemicellulose, cellulose and some part of lignin were liberated. That is why major loss of the weight of the biomass took place. In stage 3, the weight loss is not significant as in stage two. The lignin portion and remaining char material degrades slowly at 500 ^{0}C or beyond. The decomposition becomes stable when the temperature close to 900 ^{0} C.

Derivative Thermogravimetric (DTG) was obtained from the TGA curve. DTG gives an idea about the zone of reaction occurs in the multi-step reaction. The first peak was observed below 120 ^{0}C which indicate about the loss of water molecules from biomass (**Fig. 4.3. B**). The sharp peaks in between 200 ^{0}C to 350 ^{0}C denote removal of volatile compounds and

hemicellulose. Cellulose degradation was depicted in peak between 350 ^{0} C to 500 ^{0} C. Some of the biomass sample such as *Impereta cylindrical, cypersus exaltatus* shows multiple peaks at this particular temperature. This may be due to the presence of different types of cellulose component in the biomass. The peaks beyond 500 ^{0} C denotes lignin degradation. The thermal stability of lignin is due to the presence of the phenolic group in the structure. The degradation profile of biomass shows that hemicellulose degrades at low temperature followed by celluloses and lignin (Chen et al. 2011, Guo et al. 2000).

X-ray diffraction (XRD) is used to detect the presence of a crystalline structure of biomass sample in terms of its absorption peaks. From the XRD analysis, one could easily understand that cellulose exist in various crystalline forms. The crystalline nature of cellulose could resistant to microbial and enzymatic degradation, while amorphous cellulose would hydrolyze easily. The pretreatment of biomass by dilute acid or steam explosion altered the lignocellulosic biomass crystallinity as well as increased the crystalline index (CrI) (Zhang et al. 2004).

X-ray diffraction was performed on the biomass sample to describe the crystalline index in terms of absorption peaks. The XRD analysis revealed that cellulose exists in various crystalline forms. The crystalline index and pretreatment technology are related each other (Yi-Heng et al. 2004). The rate of depolymerization decreased with increased in the crystalline index. The crystallinity of a biomass sample also depends on the wax content and fatty acid components as stated earlier. From our findings, the XRD crystalline index (CrI) can be observed highest in *Cynodon dactylon* (L.) Pers (35.040) and lowest in *Cymbopogon longifolium* (7.777). A very close similarity can be observed in biomass *Typha angustifolia* and *Impereta cylindrical* **(Table 4.3).** The intensity of the peak decreased with increased in 2Ɵ (**Fig. 4.4).** The decrease in the degree of crystallinity is an indication of a decrease in the degree of polymerization (Driscoll et al. 2009, Hendriks et al. 2009, Gumuskaya et al. 2003). The increased in CrI is due to decrease in the amorphous region of the sample with respect to peak value (**Fig. 4.4).**

Ultimate analysis of the biomass sample was performed based on the elemental composition of the sample. The elemental composition of the biomass sample was derived through the elemental analyser. Mainly, we emphasised on carbon, hydrogen, oxygen and nitrogen content. These elements determined the fuel efficacy and are the major component of biomass sample.

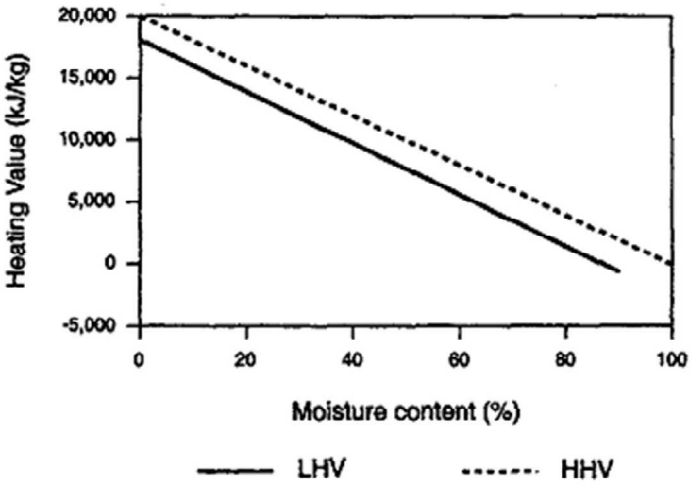
Table 4.3 Crystalline index of different lignocellulosic biomass

LCB	I_{002}	$I_{amorphous}$	CrI
Phragmite karka	68	45	33.82
Thysanolaena agrostis	48	40	16.66
Typha angustifolia L.	21.653	15.633	27.802
Dichanthium assimile (Steud.) Deshp.	44	37	15.909
Cyperus exaltatus retz	60	47	21.660
Erianthus fultus Nees.	97	70	27.83
Arundo donax L.	47	43	8.510
Saccharum sponteneum L.	62	50	19.350
Cymbopogon longifolium	90	83	7.777
Arundinella khasiana	21.170	16.796	20.661
Setaria glauca	90	73	18.888
Meriscus sp.	88	75	17.330
Neyraudia reynaudiana	93	65	30.10
Sclerostachya fusca	94	70	25.53
Cyperus Flavidus Retz.	21.75	16.16	25.700
Cynodon dactylon (L.) Pers	21.4	13.9	35.040
Eragrostis airoides	21.309	15.633	26.633
Coelorachis striata A camus.	96	83	13.54
Echinochloa colonum (L). Link.	21.41	15.15	29.230

Chrysopogon aciculatus	22	14.8	32.720
Eleusine indica L.	21.75	15.49	28.790
Fimbristylis dichotoma Vahl.	21.923	14.81	32.430
Cyperus exaltatus Retz.	21.59	15.49	28.250
Cyrtococcum oxyphyllum (Steud.) Stapf	21.75	15.7	27.810
Paspalum scrobiculatum L.	21.41	15.65	26.900
Coix lacryma-jobi L.	51	43	15.686
Cyperus alternifolius L.	54	44	18.518
Echinochloa stagnina (Retz.) P.Beauv.	21.924	17.886	18.418
Imperata cylindrica (L.) P. Beauv	21.997	15.838	27.999

The maximum carbon percentage was visible in *Typha angustifolia* (52.895 %). The minimum was recorded in biomass *Setaria glauca* (30.563 %). The hydrogen and nitrogen percentage was almost small as compared to carbon in all biomass. The oxygen percentage was determined by means of differences. The oxygen content was found highest in biomass *Cyrtococcum oxyphyllum* (62.643 %) and lowest in *Typha angustifolia* (40.044%).

The calorific value of a biomass sample is one of the most important characteristics of a fuel. It is useful in planning and control of the combustion plants. It refers to the amount of heat generated from the biomass (weight) during complete combustion in presence of oxygen. There are two types of calorific value (expressed in kcal/kg or MJ/kg): Higher heating value (HHV) and Lower heating value (LHV). The heating value of any fuel is the energy released per unit of mass per unit volume of the fuel when it completely burned (Joseph 2011). The higher heating value (HHV) refers to the condition where water is condensed out of the combustion products. HHV is called as gross calorific value. The high heating value can be determined experimentally in the laboratory with an adiabatic calorimeter. The lower heating value (LHV) refers to the condition where water vapours remain in the combustion product; i.e. the steam is not condensed out and thus latent heat does not account for.



Note: LHV=Lower heating value; HHV=higher heating value.

Fig. 4.9 Correlation between heating value and moisture content (Quaak et al. 1999).

In practice, the value is obtained by subtracting to the HHV the heat water condensation produced during combustion, using the following formula:

$$LHV = HHV - 51.14 \times Ht \quad (4.10)$$

where, HHV is the high heating value, Ht is the total hydrogen percentage.

A correlation between moisture content and heating value is given in **Fig.4.9.** Heating value decreases as increased in moisture content. We have calculated HHV based on the elemental composition of the biomass by available empirical formula (Demirbas 1997). The interval of 7.507-19.701 MJ/kg was seen in the selected biomass (**Fig. 4.5).**

Biomass energy is one of the alternative renewable energy that can be a replacement for the fossil fuels. The biomass fuel efficacy does not depend on the proximate and ultimate analysis, but also on the atomic ratio of H:C and O:C. A plot of Van Krevelen diagram was drawn based on the atomic ratio of hydrogen to carbon and oxygen to carbon. The correlation between hydrogen/carbon (H/C) and oxygen/carbon (O/C) ratios were used to locate the biomass sample in the Van Krevelen diagram (**Fig. 4.6.**). The lower the ratio, the higher is the energy content. The material with a relatively low O:C ratio has more energy density and higher HHV. This is because there is a more chemical energy in C-C bonds than in C-O bonds. This is the reason why our selected biomass *Typha angustifolia* has higher carbon percentage (52.895 %) (**Table 4.2**), hence higher heating value (19.6925 MJ/kg) (**Fig. 4.5.(C)**). From the Van Krevelen diagram (**Fig. 4.6. (A),** it is well illustrated that *Typha*

angustifolia has a high calorific value than other feedstocks, which was further explained by using the Demirba's formula for HHV. This diagram indicates about the co-relation between the calorific value and atomic ratio.

The FTIR spectrometer is based on the principal that when a material is irradiated with infrared radiation, the molecules gets excited to a higher vibrational state by the absorbed IR radiation. In this, the wavelengths that are absorbed by the samples show the characters of the molecular structure. The FTIR spectrometer used an interferometer to modulate the wavelength from a broadband infrared source. A detector is attached to measure the intensity of transmitted light as a function of wavelength. The FTIR spectra are usually presented as plots of intensity versus wave number (in cm^{-1}). Wave number is the reciprocal of the wavelength. The intensity can be plotted as the percentage of light transmittance or absorbance at each wave number.

The FTIR spectroscopy in conjunction with high-performance liquid chromatography was performed on dilute acid pretreated softwood and hardwood slurries to analyse the presence of glucose, mannose, xylose and acetic acid (Tucker et al. 2000). The structural constituent and chemical changes of lignocellulosic biomass were frequently investigated by using the FTIR spectrometer (Guo et al. 2008). Cellulose related bands are usually seen around 900, 1098, 1162, 1370, and 1430 cm^{-1}. Protein-related bands are seen at about 1549 and 1653 cm^{-1}. The hemicellulose-related bands were seen at 1732-1735 cm-1 (Liu et al. 2005, Gastaldi et al. 1998). There are two types of lignin in the woods and grass species—guacyl and syringyl rings. The guaicyl ring-related band and syringyl ring-related bands were seen at about 1516-1517 cm^{-1} and 1453-1456 cm^{-1}, respectively (Corredor et al. 2009).

The FTIR analysis was conducted on the biomass sample to know functional groups link with respective peaks. The FTIR peak indicate the mixed presence of structural carbohydrate (cellulose, hemicellulose and lignin). The respective spectral peak was assigned corresponding to their wave number (**Table 4.4).** The IR peaks in the range of 3200-3450 cm^{-1} indicates the presence of fiber due to axial deformation of OH (**Fig. 4.7)**. The absorption peak at 2,860, 2,928 cm^{-1} gives about the alkanes/aliphatic C-H stretching bonds. The presence of a carbonyl group like ester (C=O), ketones, and carboxylic acid is observed in the wave number 1745 cm^{-1}. The presence of an aromatic C-C ring stretching and the N-H amines group was observed in peak assigned at 1516-1616 cm^{-1}. The presence of cell wall polysaccharide (C-H) due to aliphatic stretching was observed at peak 1442 cm^{-1}. IR peaks at

1097 cm^{-1} depict asymmetric deformation of C-O-C of the cellulose and hemicellulose portion (**Fig. 4.7)**. Sugars like arabinan, galactan and B-D fructose were observed at lower peak value 480-815 cm^{-1}

Table 4.4 Determination of functional group of biomass by FTIR analysis.

Wave no. (cm-1)	Definition of the Spectral Assignments
3200–3400.	Presence of fibre in the sample which is attributed to the axial deformation of OH group.
2862	CH_2 asymmetric stretching: mainly lipids with a little contribution from proteins, carbohydrates, and nucleic acids
1710–1740	The presence of carbonyl group like ester (C=O) is observed in the range of which gives an evidence of hemicelluloses in the samples.
1733	Saturated ester C=O stretch: phospholipids, cholesterol esters, hemicellulose, and pectin
1601	C=O aromatic stretching: lignin
1452	C-H: cell wall polysaccharides
1420	O-H bending: cell wall polysaccharides, alcohols, and carboxylic acids
1380	C-H bending of aliphatic CH_2: cell wall polysaccharides
1320	N-acetylglucosamine: chitin and chitosan
1160	Symmetric bonding of aliphatic CH2, OH, or C-O stretch of various groups: cell wall polysaccharides
1167.24	The connection with the asymmetric deformation of C-O-C of the cellulose and hemicelluloses which is related to the presence of C-OH stretching vibrations.
1145	Cellulose (β-1.4 glucan)
1073	Rhamnogalactorunan. b-galactan
1064	C-O stretching: cell wall polysaccharides (glucomannan)
1035	OH and C-OH stretching: cell wall polysaccharides (arabinan)
895	Arabinan
893	Galactan
873	B-D-Fructose

One of the most concerned parameters for biomass fuel analysis is its compositional analysis, which comprises cellulose, hemicellulose and lignin. LCB feedstocks are primarily composed of carbohydrate polymer and lower concentration of proteins, acids, salts and minerals. Cellulose and hemicellulose are polysaccharides that can be hydrolyzed to monomeric sugars and can be used in ethanol fermentation. The ethanol yield from biomass is directly related to cellulose, hemicellulose and other sugars concentration present in the feedstock. Lignin concentration in the biomass is an obstacle to entire process as it forms a barrier in microbe fermentation; however, it may be useful for other purposes. The content of carbohydrate polymers differs according to the type of feedstock. For example, Switchgrass has cellulose (37 %), hemicellulose (29 %) and lignin (19 %). In corn stover, it is about cellulose (38 %), hemicellulose (26%) and lignin (19 %). Similarly, in wheat straw, it is about cellulose (38 %), hemicellulose (29%) and lignin (15%) (Mckendry 2002, Zheng et al. 2014).

From our findings, the biomass *Eragrostis airoides* has maximum cellulose content. It contributes cellulose (43.17%), hemicellulose (30.89%), lignin (10.94%), NDF (80.09%), and ADF (49.2%). The minimum cellulose content was found in biomass *Arundinella khasiana.* It contributes cellulose (20.62%), hemicellulose (15.26%), lignin (17.30%), NDF (57.28%), and NDF (50.61%) (**Fig. 4.8)**. The highest percentage of hemicellulose was seen in biomass *Dichanthium assimile* (34.39%). In an overall, the hemicellulose and lignin percentage were very much low in comparison with cellulose percentage.

The NDF, ADF and ADL were determined based on the detergent fiber analysis (DFA). DFA directly detects the structural component of the biomass sample, which give cellulose, hemicellulose and lignin percentage. The highest NDF was recorded in *Dichanthium assimile* (83.84 %) and the lowest was in *Cymbopogon longifolium* (56.30 %). Whereas, ADF was highest in *Meriscus sp.* (58.52%) and lowest in *Cyperus alternifolius L.* (39.42 %) (**Fig. 4.8)**.

4.5 CONCLUSIONS

With the advancement of biorefinery technologies enabling the different plant biomass to convert into biofuel, many researchers set out to study, explore, search and improve the potential candidate of biomass for biofuel production. Many of these candidates include lignocellulosic biomass, C4 grasses and agricultural wastages. A high and stable yield of lignocellulosic biomass is required in other to produce biofuel in a sustainable way. A minimal input of nutrients and water with marginal soils, a high efficiency in production and cultivation of biomass is essential at current scenario of biofuel production.

In search of new potential raw materials for the future production of biofuel is as much important as the steps involved in the conversion process. The native species of a particular location will give a higher potential candidate for exploration of biofuel. The production of biofuel from lignocellulosic biomass is not an easy task. The contamination of lignin in the biomass creates a barrier in the conversion process. The characterisation of biomass sample prior to further processing will give the percentage present of lignin content, and hence made easier in the conversion process. The microstructure and properties of lignocellulosic biomass were found to influence the bioconversion rate and ethanol yield. The crystallinity, moisture content, volatile matter, elemental composition and morphology greatly influenced the hydrolysis of the biomass.

From the correlation analysis, it can be seen that there are no direct relations between fixed carbon content and the holocellulose, however, a good relation can be seen between the lignin content and fixed carbon content (Demirbas 2003). Fixed carbon content can be calculated from the lignin content by using the correlation formulas. The existing refinery technologies require different types of biomass from different sources. These selected grasses are likely to represent different sources of biomass supply as they have distinct characters and potential roles in biofuel production. Collectively, this lignocellulosic biomass especially grasses are likely to play a central role in the supply of feedstock for the cellulosic ethanol industry. Moreover, as these species are evolutionary closely related, advances in each of these crops will expedite improvements in the other crops.

The feedstock such as *Eragrostis airoides* and *Impereta cylindrica* has a high percentage of cellulose, this implies that they may be a potential candidate for biofuel production. The data derived from moisture content, volatile content, fixed carbon content and calorific values of the selected biomass contribute to fuel efficacy criteria. The future biofuel production will be localised at domestic level. In this regards, the characterization of the indigenous biomass (LCB) will have a strong positive note on bioenergy production. The need of new raw material for the production of biofuel in a sustainable way is relatively important in order to bring down biofuel to domestic level. We try to explore new raw materials, which are highly indigenous to Northeast India and can be used as feedstock for biofuel production. The collected species has great potential to produce biofuel for future used which was indicated by the data derived from proximate, ultimate and compositional analysis.

Chapter 5

Conversion of lignocellulosic biomass to bioethanol

5.1 INTRODUCTION

The use of fossil fuels and its adverse effects resulting in the global warming by emitting greenhouse gases became a matter of concern to the human nation. Several countries have already started working on progressive alternative renewable energy production in order to replace the conventional fossil fuels, especially in the transport sector. In this context, bioethanol from lignocellulosic biomass (LCB) is considered to be one of the important promising alternative liquid fuels (Venkatesh 2014).

At present, the dominant cost for fermentative production of fuel is the substrate source, which may be starch or sucrose. The attempt on low-cost substrate such as lignocellulosic biomass offers a great potential for reducing the production cost and increasing the use of ethanol as a fuel additive. The lignocellulosic biomass is one of the most abundant raw materials for biofuel production. Lignocellulosic is more complex substrate than starch. Usually, it is composed of carbohydrate polymer (cellulose and hemicellulose) and lignin. The industrial ethanol is produced petrochemically through the acid-catalyzed hydration of ethylene. The ethanol for use in alcoholic beverages and vast use as biofuel is produced by fermentation of biomass where certain species of yeast are used.

The biological conversion of cellulosic biomass to bioethanol offers the high yield of products and economic viable and the potential for very low costs. However, the conversion process itself is a challenging step. The biological process for converting the lignocellulosic biomass to ethanol requires: (1) pretreatment of biomass sample; (2) hydrolysis of biomass to release sugar molecules; (3) and fermentation of the sugars (hexose or pentose) to produce ethanol. The enzymatic hydrolysis that converts the lignocellulosic biomass to fermentable sugars is a complex step due to the substrate-related and enzyme-related effects and the interaction within the parameters. The enzymatic hydrolysis gives higher yield, higher selectivity, lower energy cost and milder operating conditions than chemical hydrolysis.

The use of lignocellulosic biomass as feedstock for bioethanol production has certain advantages over the fossil fuels. LCB have a high potential for ethanol production, they are abundant renewable resources, has a variety of source, do not compete with the first generation of biofuel, and could integrate as Integrated Biomass Utilization System (IBUS) (Larsen et al. 2008).

The traditional production of bioethanol involves fermentation of sugars derived from sugarcane or starch followed by ethanol recovery (Khanal 2008). Biofuels produced from these processes is termed as "first generation of biofuels". The first generation of biofuel has certain disadvantages like, it compete with the food crops. Second generation biofuels are also known as advanced biofuels. The main feedstock used in producing second generation of biofuels are not food crops. It is suggested that second generation of biofuels are produced from biomass in a more sustainable fashion, carbon negative or neutral. The second generation of biofuel (based on non-food crops) has a greater potential of sustainability as compared to former. LCB falls under the second generation of biofuel and showed potential conversion to bioethanol. At present, the production of biofuel is not cost effective because of the technical barriers. The use of second generation of biofuel from the lignocellulosic biomass shows a potential promising fuel for future generation.

Structurally, LCB is composed of cellulose, hemicellulose and lignin with a trace amount of inorganic materials. A cellulose portion of the LCB is the predominant natural biopolymer with great abundance, sustainability supply and relatively low cost (Lynd et al. 2002). LCB are particularly attractive as feedstock and could be utilized for production of ethanol in three-step process: firstly, it is pretreated with dilute acid to rupture the polymeric structure thereby increasing the enzyme susceptibility; secondly, the cellulose is converted to glucose sugars by enzymatic hydrolysis; and thirdly, conversion of the resulting hexose sugars into ethanol by fermentation.

The pretreatment technology is one of the main steps in biomass to bioethanol conversion. It is performed before the saccharification process. It helps in loosened the cellulose fibres from the matrix of lignin, thereby allowing the enzyme to hydrolyze it. The treatment also provides more enzyme susceptibility to the biomass. Various types of pretreatment (physical, chemical and/or biological or a combination of all) have been developed in other to yield more ethanol cost-effectively. However, not every kind of LCB can be pretreated in the same way in same conditions (Waleed et al. 2011). The pretreatment methods, conditions, will be different according to the type of biomass used. For example, steam explosion pretreatment of agro-residues like corn stover and rice straw had been tested and found that an additional acid hydrolysis step was needed for producing high sugars from the biomass, thus need to put more research in to determine the best possible pretreatment strategy that can satisfy the features of good pretreatment (Waleed et al. 2011).

There are mainly two approaches for saccharification; acid based and enzyme based. In acid based saccharification, the dried powdered biomass is treated with acid under specific temperature for a certain period. There are different acids that can be used in the pretreatment of LCB. For example, sulphuric acid and phosphoric acid are broadly used for treating LCB because of its efficient in hydrolyzing celluloses. The enzyme -based technology has certain advantages over the acid based treatment due to higher conversion efficiency, lack of inhibitory compounds, no need of recycling the acid, non-corrosive, non-toxic to the environment etc. (Harmsen et al. 2010). For example, several strains of fungal have been developed since then to enhance the cellulase production due to increase cost of the fermentation process. One such commercially available enzyme used in the production of cellulase is *Trichoderma reesei* (Toyama 1969).

India is the world's second largest producer of sugar but contributes relatively low ethanol (Aradhay 2013). It can be noted that India is home to an extraordinary variety of climate regions, having tropical, temperate and alpine regions. This indicates that India has a high potential of producing a variety of lignocellulosic biomass in terms of biodiversity and availability. India has great advantages of being as three biodiversity hotspot (The Western Ghats, The Eastern Himalayas and Indo-Burma), allow an easy adaptation of different species and exploration of new raw material as energy crops such as grasses. The North-East India is one of the national important regions with Indo-Burma biodiversity hotspot. The region has eight states with varieties of flora and fauna.

The present work focuses on the cellulosic bioethanol production from grass sp. *Eragrostis airoides* collected from North-East India by enzymatic hydrolysis and microbial fermentation. *Trichoderma reesei* was used as cellulase for sugar released from the biomass. The solid residue after pretreatment was utilized for enzymatic hydrolysis and then fermentation was carried out by using *Saccharomyces cerevisiae* and locally available Hamei.

5.2 MATERIALS AND METHODS

5.2.1 PLANT MATERIAL

Eragrostis airoides (L.) Wolf. was collected in the month of April 2013 from Assam, a gateway of North-East India. This grass was collected with the total inflorescence of handful amount and was handled carefully. Some of the twig containing inflorescence was separated out. The remaining biomass was dried in air. The dried sample was made a fine powder to a

particle size less than 3 mm. The ground material was used as feedstock for bioethanol production. The biomass sample was characterized based on the physical, chemical and elemental composition (Discussed in chapter 4). The biomass *Eragrostis airoides* contributes cellulose (43.17%), hemicellulose (30.89%), lignin (10.94%), NDF (80.09%), and ADF (49.2%). The twig containing inflorescence was washed thoroughly, pressed and dried, poisoned and pasted on the two sheet of the herbarium. One copy of the herbarium specimen was submitted to Herbarium unit of Botany Dept. of Guwahati University, Assam India for taxonomical identification. The voucher number obtained from the herbarium unit was recorded properly. Another copy of the same herbarium specimen was maintained at Indian Institute of Technology Guwahati (IITG), Assam, India for future references and documentation.

5.2.2 MICROORGANISMS AND MAINTENANCE

The yeast strain *Saccharomyces cerevisiae* (MTCC 170) was procured from IMTECH, Chandigarh India and used for the fermentation process. The microorganism was grown at 30 ^{0}C maintained at 4 ^{0}C on MGYP agar plates containing (g L^{-1}) glucose, 10; yeast extract, 3; malt extract, 3; peptone, 5; agar 20 (pH 5.0). The cellulase from *Trichoderma reesei* ATCC 26921 was procured from sigma and used for the enzymatic hydrolysis.

5.2.3 PRETREATMENTS

5.2.3.1 PHYSICO-CHEMICAL PRETREATMENTS

The biomass was treated with different concentration of chemicals (acids and alkali). The concentration of the chemicals was examined properly. In all experiment 10% (w/v) powdered biomass in aqueous solution was taken. After the treatment, the biomass was filtered using a muslin cloth and washed repeatedly with distilled water until the neutral pH of the washed water was achieved. The biomass residue was dried overnight in an oven dry at 60 ^{0}C. This solid residue was used for further enzymatic hydrolysis. The filtrate was analysed for total reducing sugar released during pretreatment. In this study, the physical and chemical methods of pretreatment were applied simultaneously. The autoclave pressure was released swiftly after the pretreatment.

5.2.3.1.1 ACID + AUTOCLAVING The biomass was subjected to different concentration of dilute sulphuric acid (1%, 3%, 5%, 7% v/v), at 121 ^{0} C, 15 psi for 20 min in a 250 mL Erlenmeyer flask.

5.2.3.1.2 ALKALI + AUTOCLAVING

The biomass was subjected to different concentration of sodium hydroxide (1%, 3%, 5%, 7% w/v) at 121 ^{0} C for 20 min in a 250 mL Erlenmeyer flask.

5.2.4 TOTAL REDUCING SUGARS (TRS)

The total reducing sugars produced from pretreatment and hydrolysis of cellulose was measured by the dinitrosalicylic acid (DNS) method (Gail 1959). The sugar containing biomass sample (after pretreatment and hydrolysis) were centrifuged at 11,000 x g for 10 minutes. The supernatant was collected and analysed for sugar content using UV- visible spectrophotometer by running the standard glucose solution. The readings were taken at 550 nm.

5.2.5 ENZYME HYDROLYSIS

The pretreated biomass was used as carbohydrate substrate for enzymatic hydrolysis. The commercially available cellulase from *Trichoderma reesei* ATCC 26921 was procured from Sigma and used in enzymatic saccharification. The pretreated biomass (10 gm) was dissolved in a 100 ml of sodium acetate buffer at pH 4.7 and it was autoclave at 121 ^{0}C, 15 psi, for 20 min. The enzyme was loaded to the sample after it was cool down. The mixture was incubated in a shaker water bath at 50 ^{0}C, 75 rpm for four days. The reaction time was calculated as 1, 2, 3 and 4 days. The reaction was stopped by placing the sample flask in boiling water for 5 min. The mixture was filtered and glucose concentration was measured.

The main activity of enzyme used *Trichoderma reseei* is to break down the cellulose material into monomers. The enzyme was taken as a measured of 20FPU for the reaction.

5.2.6 ENZYME HYDROLYSIS RATE (V)

The enzyme hydrolysis rate was calculated on the enzyme hydrolysis time. The **v** was plotted as the concentration of glucose released per hydrolysis time.

$$\mathbf{V = dS/dt = Glu_t — Glu_o \,/\, t -- t_o} \qquad (5.1)$$

Where, **V**= enzyme hydrolysis rate (mg/ml glucose per hour), $\mathbf{Glu_t}$ = concentration of glucose at time t (mg/ml), $\mathbf{Glu_o}$= initial glucose concentration at time = 0 h (mg/ml), t= hydrolysis time (h) and $\mathbf{t_o}$= 0 h

5.2.7 FERMENTATION

The fermentation was carried out in two ways: using *Saccharomyces cerevisiae* and locally made Hamei collected from Manipur to convert the sugar molecules into ethanol. A growth medium (containing (g L^{-1}) glucose, 10; yeast extract, 3; malt extract, 3; peptone, 5; (pH 5.0) was prepared and fresh colonies from agar plate were selected and used to inoculate 50 ml of the growth medium in 250 ml Erlenmeyer flasks. The culture was grown in shaker bath, harvested and centrifuged at 11,000 x g. Cells were resuspended in 2ml of deionized water.
The 1 L flask containing enzyme hydrolysed pretreated biomass sample, 5 g yeast extract, and *S.cerevisiae* was inoculated. The pH was maintained at 6.0 and fermentation was carried out at 35 ^{0}C, 75 rpm, 72 h. After 72h, the supernatants were collected and analysed for ethanol estimation by Gas chromatography (Shimadzu GC-14A Gas Chromatograph with a Restek RTX-5 capillary column and Fisher 1-butanol A383-1 as the internal standard).

5.2.8 ETHANOL ESTIMATION

The quantitative monitoring of the ethanol production in the fermentation process was carried out by using Gas chromatography (Shimadzu GC-14A Gas Chromatograph with a Restek RTX-5 capillary column and Fisher 1-butanol A383-1 as the internal standard). The column temperature was initially adjusted at 100 ^{0}C for 2 min; and further increased to 180 ^{0}C for 1 min, 220 ^{0}C with a rate of 5 ^{0}C min^{-1} for 5 min. The carrier gas (N_2) flow rate was maintained at 1 mL min^{-1}.

5.3 RESULTS

5.3.1 PLANT MATERIAL

The living photograph of the plant material taken from the collection site is shown in **Fig.5.1. (A).** The herbarium specimen maintained at Indian Institute of Technology Guwahati, India in the herbarium unit is shown in **Fig.5.2.** The plant biomass was taxonomically identified and the systematic position was depicted in **Table 5.1.** The ground biomass of particle size less than 3 mm is given in **Fig.5.1.(B).**

Fig.5.1. A. *Eragrostis airoides* plant, B. Biomass powder of *Eragrostis airoides*.

Fig.5.2. Herbarium specimen of *Eragrostis airoides* maintained at Indian Institute of Technology Guwahati.

Table 5.1. Systematic classification of Plant biomass (*Eragrostis airoides*).

Kingdom	Class	Order	Family	Genus	Species	Voucher number	Latitudes & Longitudes
Plantae	Liliopsida	Poales	Poaceae	Eragrostis	E. airoides	17815	26°11'12.9"N 91°42'00.3"E

5.3.2 PHYSICO-CHEMICAL PRETREATMENT

Chemical and physical pretreatment methods were applied simultaneously. Dilute acid (H_2SO_4) and alkali (NaOH) of different concentration was treated with biomass and autoclaved were performed. The total fermentable sugar (TFS) was calculated based on the sugar released during pretreatment and enzymatic hydrolysis. The maximum TFS (60.67 g/100 g of biomass) was found in pretreatment condition with 5 % acid + autoclave treatment (**Table 5.2.**). The minimum TFS (44.90 g/100 g of biomass) was seen in 1% alkali + autoclaving.

Table 5.2. Results of total fermentable sugar release (Pretreatment + Enzymatic hydrolysis)

Pretreatment (P)	TRS* (P, g/L)	TRS (P, mg/g)	TRS (EH*, g/L)	TRS (EH, mg/g)	TFS* yield g/100 g of B*
1% Acid+Autoclaving	37.15 ± 0.02	371.5 ± 0.02	14.56 ± 0.036	145.6 ± 0.036	51.71
3% Acid+Autoclaving	38.23 ± 0.02	382.3 ± 0.02	16.35 ± 0.036	163.5 ± 0.036	54.58
5% Acid+Autoclaving	40.16 ± 0.02	401.6 ± 0.02	20.51 ± 0.034	205.1 ± 0.034	60.67
7% Acid+Autoclaving	39.54 ± 0.03	395.4 ± 0.03	17.81 ± 0.035	178.1 ± 0.035	57.35
1% Alkali+Autoclaving	36.73 ± 0.03	367.3 ± 0.03	13.23 ± 0.036	132.3 ± 0.036	49.96
3% Alkali+Autoclaving	34.26 ± 0.03	342.6 ± 0.03	12.16 ± 0.040	121.6 ± 0.040	46.42
5% Alkali+Autoclaving	33.38 ± 0.02	333.8 ± 0.02	11.52 ± 0.030	115.2 ± 0.030	44.90
7% Alkali+Autoclaving	35.52 ± 0.03	355.2 ± 0.02	12.66 ± 0.04	126.6 ± 0.02	48.18

B= Biomass, TRS= Total reducing sugar, TFS*= Total fermentable sugar, *EH= Enzymatic hydrolysis (values correspond to mean ± SD of measurement performed in duplicate).

5.3.3 SUGAR ASSAY

The individual sugars (glucose, xylose and arabinose) released during the pretreatment technology was determined by HPLC. The maximum released of glucose concentration (g/L) was found in pretreatment condition with 5 % acid + autoclave (1.62 g/L) (**Fig.5.3.**). The minimum glucose released was found in pretreatment condition with 1 % alkali + autoclave (1.12 g/L). The pentose sugars like xylose and arabinose was also released during pretreatment technology and their values are quite high as compared to glucose. The possible reasons are discussed in the discussion section later.

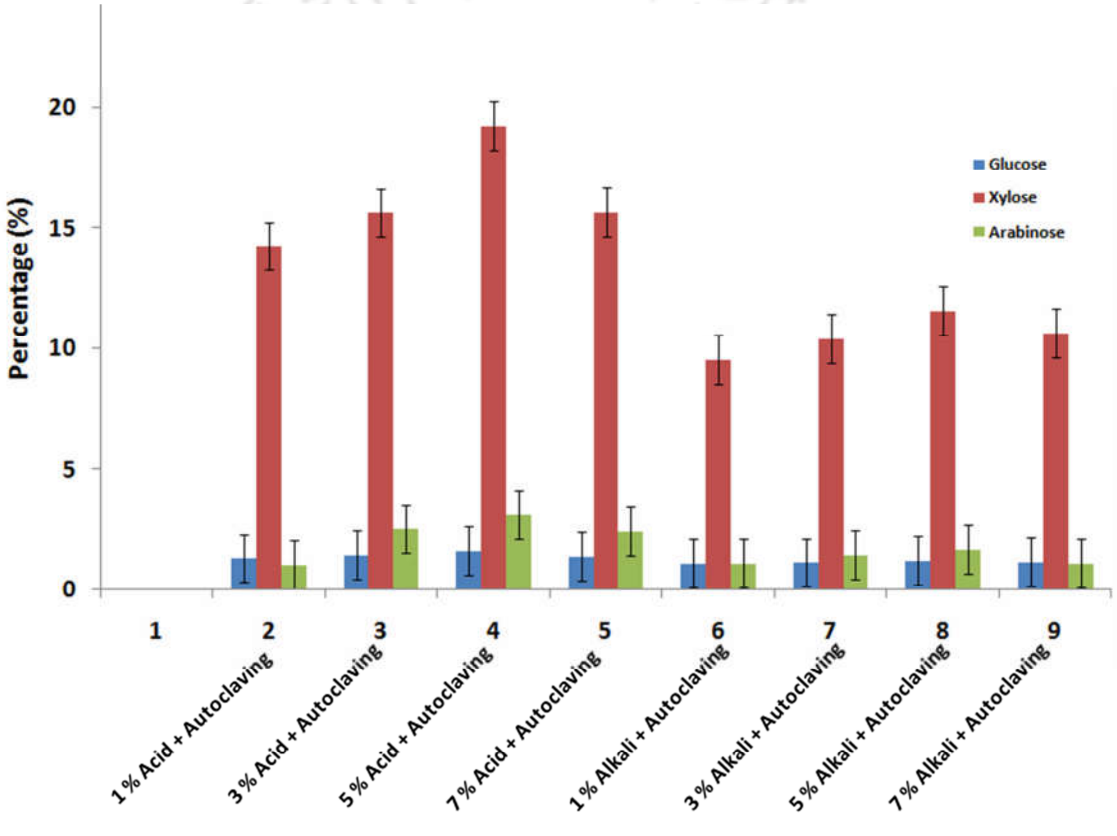


Fig.5.3. Individual sugar released during different pretreatment conditions of biomass *E. airoides*

The glucose concentration produced in different pretreatment condition was given in **Fig.5.4.** The maximum glucose concentration released was seen in pretreatment condition with 5% acid+autoclaving. The minimum glucose concentration was found in 1% Alkali+Autoclaving.

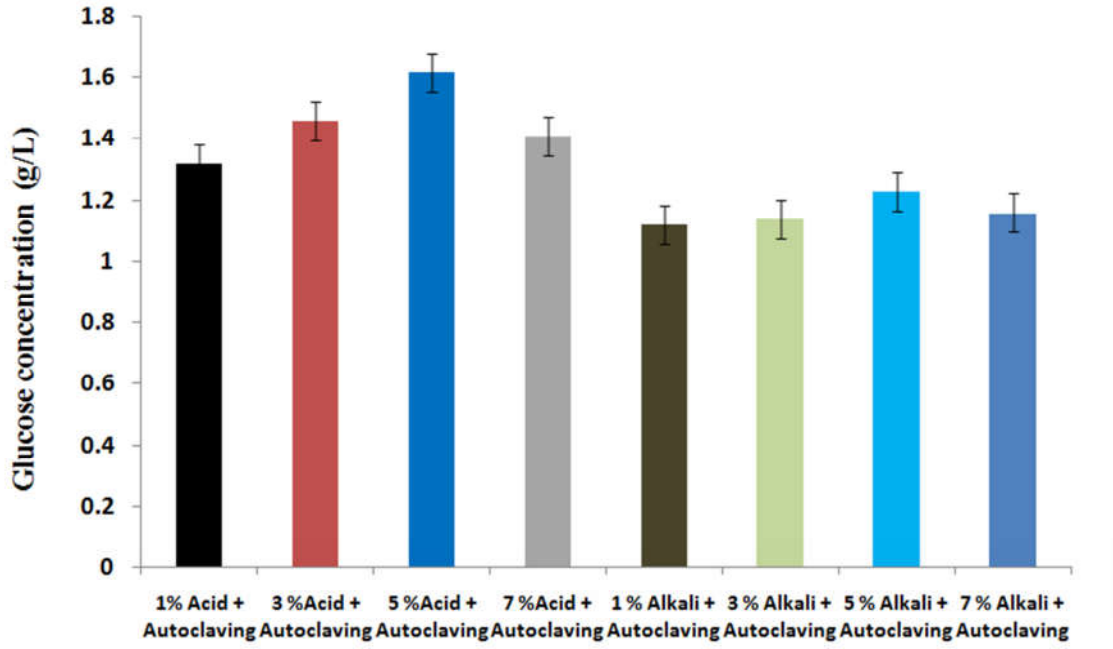


Fig.5.4. Effect of pretreatment on glucose concentration

5.3.4 ENZYMATIC HYDROLYSIS

The enzymatic saccharification was carried out in different pretreatment conditions by using *T. reesei*. The saccharification yield was determined and found that pretreatment condition in 5 % Acid + Autoclaving shows maximum yield (**Fig. 5.7.**). In all pretreatment conditions, the acid pretreated biomass showed higher saccharification than those treated with alkali (**Fig. 5.7.**). The rate of enzyme hydrolysis (mg/ml glucose per hour) on different pretreatment conditions of three days were calculated (**Table 5.3)** and given in **Fig. 5.5 and Fig.5.6.** It can be observed that the highest rate of enzyme hydrolysis was obtained in pretreatment condition with 5% Acid+ Autoclaving. As the time passed, the rate of enzyme hydrolysis decreased gradually.

5.3.5 ETHANOL PRODUCTION

The ethanol production from fermentation was checked. In *S.cerevisiae* treatment, the maximum ethanol yield (17.56 g/L) was obtained in pretreatment condition with 5 % Acid+Autoclave (**Fig. 5.10.**). The minimum ethanol yield (11.87 g/L) was obtained in pretreatment condition with 1 % Alkali + Autoclave (**Fig.5.10.)**. In Hamei treatment, the maximum ethanol yield (18.66 g/L) was obtained in pretreatment condition with 5 % Acid+Autoclave (**Fig. 5.11.**) and minimum (9.21 g/L) was found in pretreatment condition

with 1 % Alkali+Autoclave (**Fig.5.11.)**. Concentration of ethanol and sugar released in fermentation process of 5 % Acid+Autoclave pretreatment was depicted in **Fig. 5.8.** and **Fig. 5.9.** of *S.cerevisiae* and Hamei treatment respectively. In both the fermentation, acid treatment shows more ethanol yield as compared to alkali treatment (**Fig.5.10 and Fig.5.11).** More precisely, as the ethanol concentration increases, the level of the sugar in the system reduces simultaneously (**Fig. 5.9. and Fig. 5.8).**

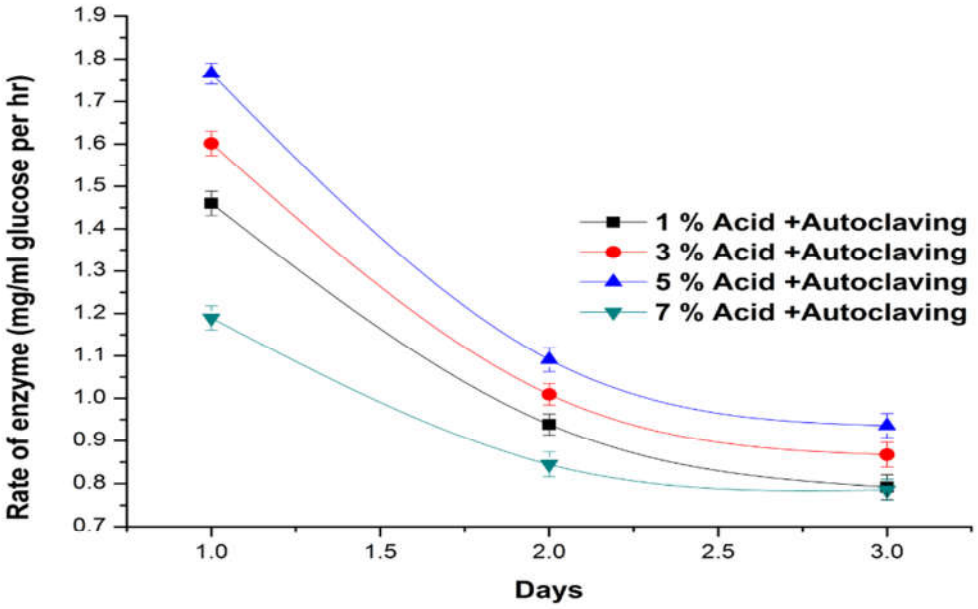


Fig. 5.5. Action of enzyme on acid pretreated biomass

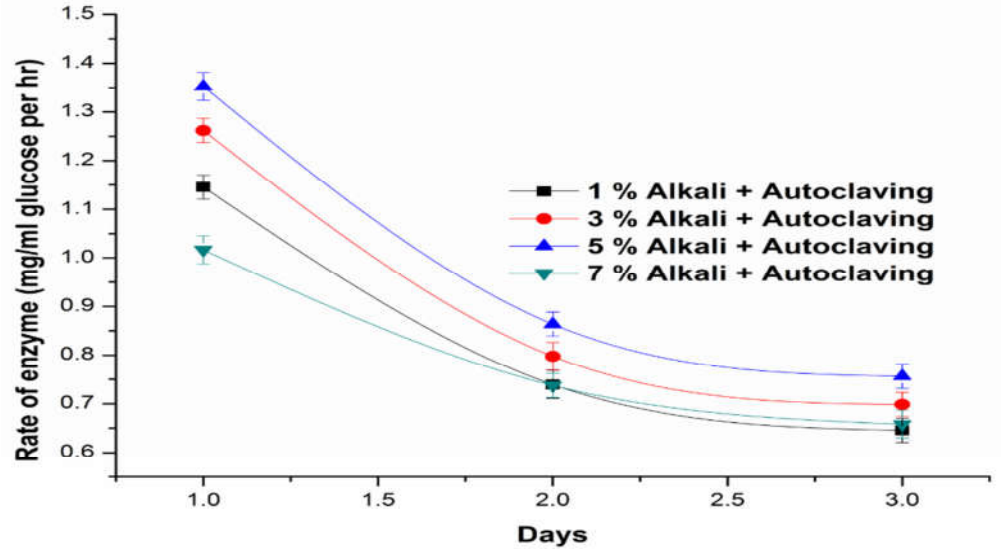


Fig. 5.6. Action of enzyme on alkali pretreated biomass

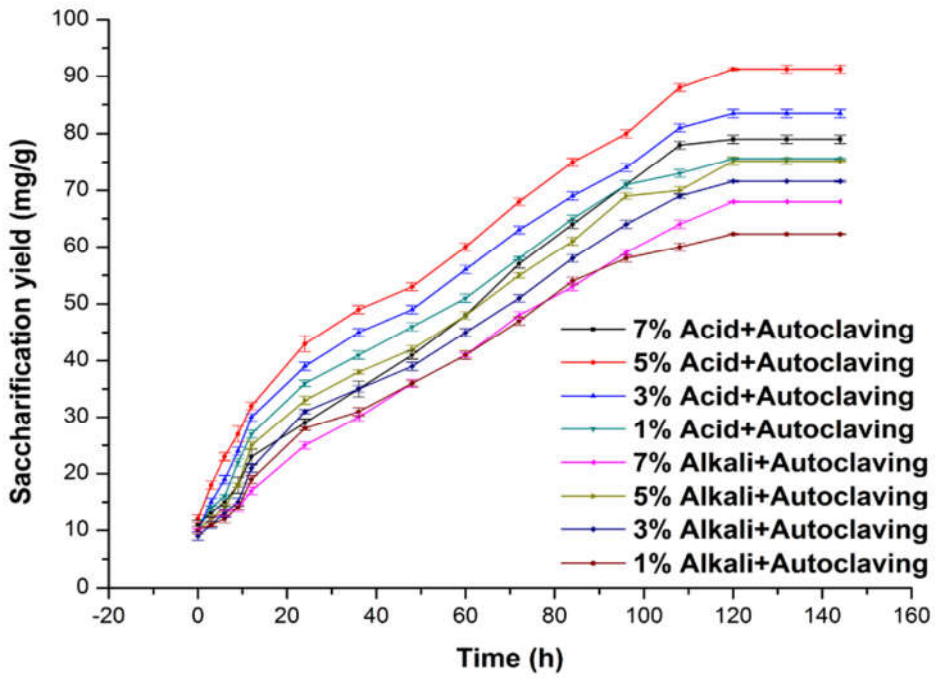


Fig. 5.7. Time profile Vs saccharification yield during enzymatic hydrolysis of biomass under different conditions.

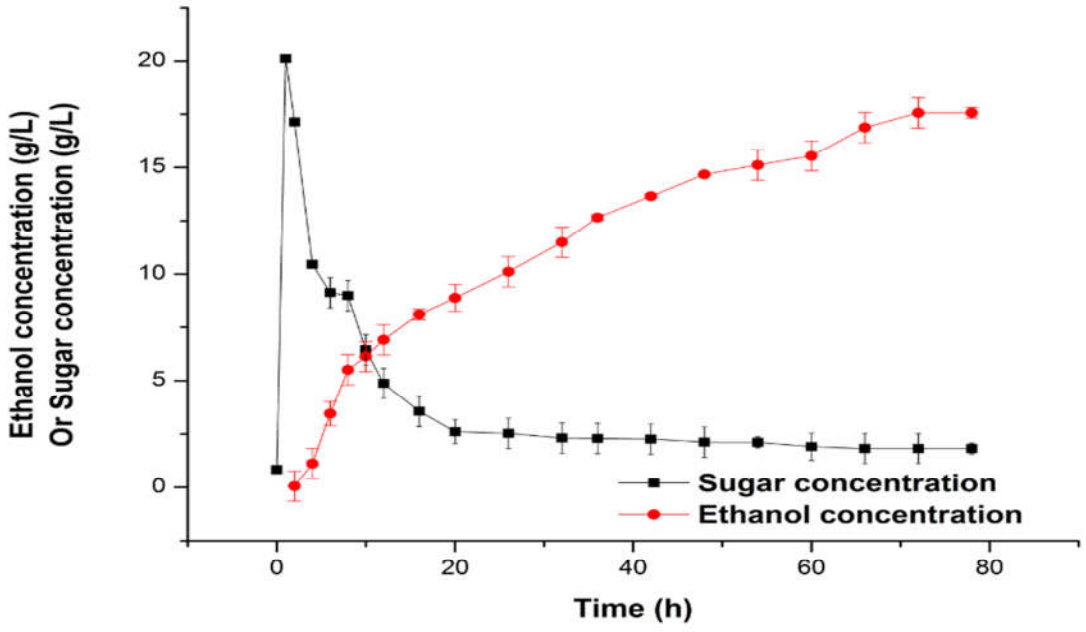


Fig. 5.8. Concentration of ethanol and sugar released in fermentation using *S.cerevisiae* on 5 % Acid+Autoclave pretreatment.

Table 5.3. Rate of enzyme hydrolysis for different pretreatment conditions of biomass material using *Trichoderma reesei.*

Pretreatment	Rate of enzyme hydrolysis														
	1st Day					2nd Day					3rd Day				
	Glu_t	Glu_o	t	t_o		Glu_t	Glu_o	t	t_o		Glu_t	Glu_o	t	t_o	
1% Acid+Autoclaving	36	0.96	24	0	1.4600	46	0.96	48	0	0.9383	58	0.96	72	0	0.7922
3% Acid+Autoclaving	39	0.56	24	0	1.6015	49	0.56	48	0	1.0091	63	0.56	72	0	0.8672
5% Acid+Autoclaving	43	0.62	24	0	1.7658	53	0.62	48	0	1.0912	68	0.62	72	0	0.9358
7% Acid+Autoclaving	29	0.45	24	0	1.1895	41	0.45	48	0	0.8447	57	0.45	72	0	0.7854
1% Alkali+Autoclaving	28	0.51	24	0	1.1454	36	0.51	48	0	0.7393	47	0.51	72	0	0.6456
3% Alkali+Autoclaving	31	0.71	24	0	1.2620	39	0.71	48	0	0.7977	51	0.71	72	0	0.6984
5% Alkali+Autoclaving	33	0.54	24	0	1.3525	42	0.54	48	0	0.8637	55	0.54	72	0	0.7563
7% Alkali+Autoclaving	25	0.60	24	0	1.0166	36	0.60	48	0	0.7375	48	0.60	72	0	0.6583

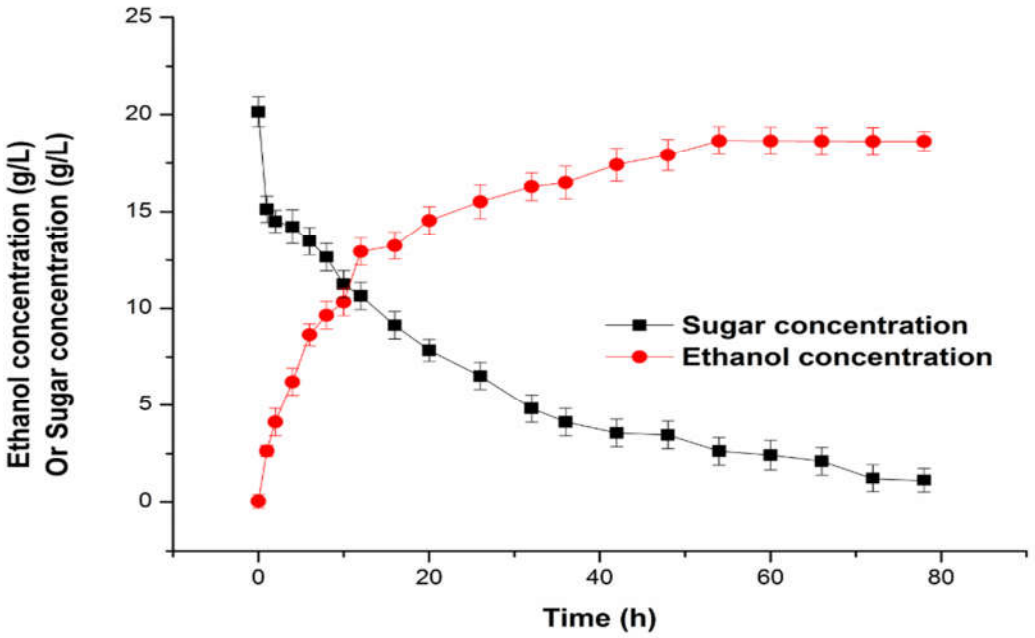


Fig.5.9. Concentration of ethanol and sugar released in fermentation using Hamei on 5 % Acid+Autoclave pretreatment.

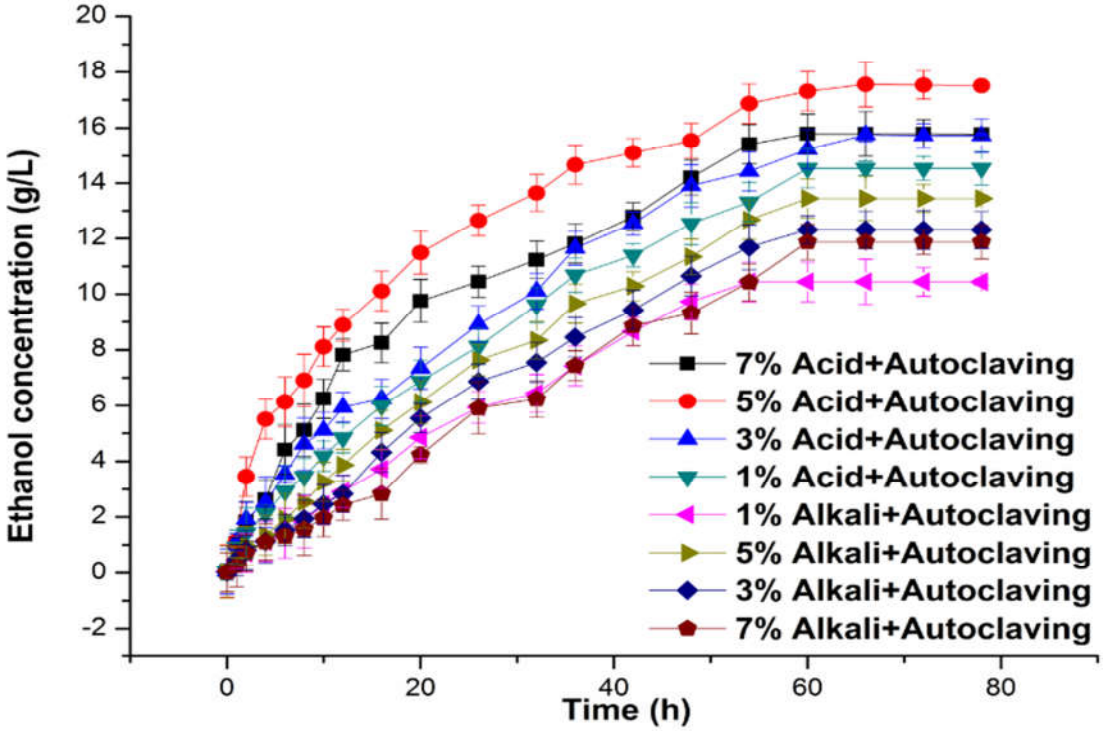


Fig.5.10. Ethanol concentration on different pretreatment conditions with *S. cerevisea*

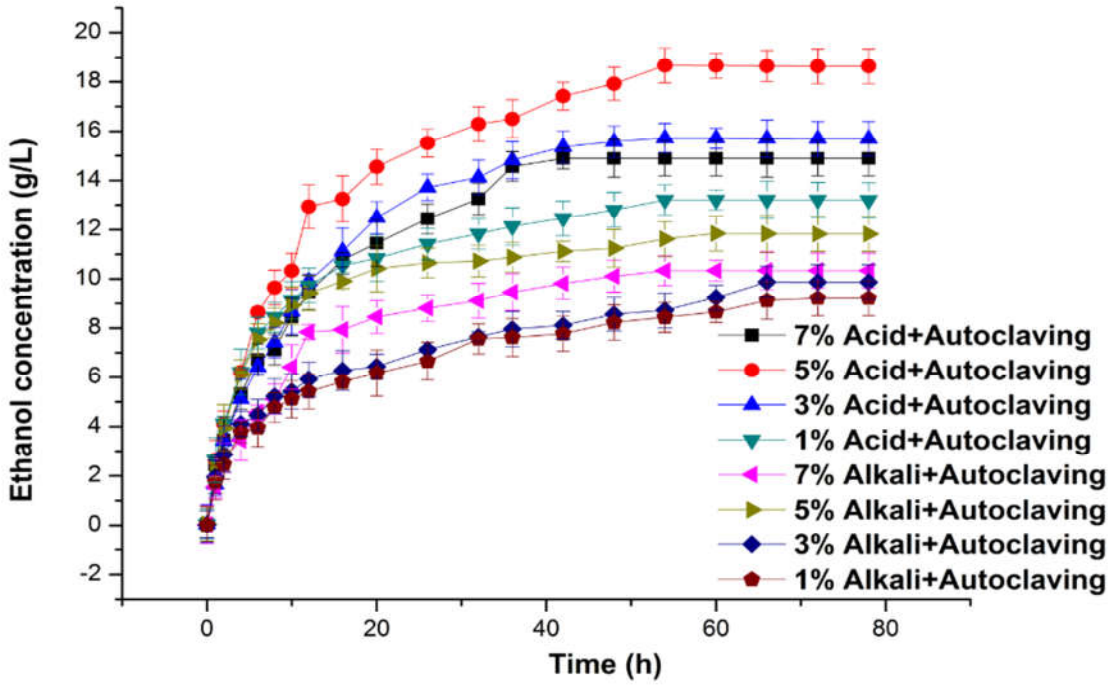


Fig.5.11. Ethanol concentration on different pretreatment condition with Hamei

5.4 DISCUSSIONS

The bioconversion of LCB into fermentable sugars for the production of bioethanol is a vibrant research area where enormous efforts are going on in terms of process simplicity and efficiency for higher yield. Although extensive studies have been carried out to meet the future challenges, there is no self-sufficient process or single step protocol for all the biomass to convert into bioethanol. The pretreatment methods and the hydrolysis process are the two crucial stages in the whole conversion process. These two stages mainly determined the overall efficiency process. The production of bioethanol from lignocellulosic feedstock such as grasses seems to be very attractive and sustainable. As we know that, using such biomass will drastically help in reducing the greenhouse gas emission. That is why it is very important to utilize LCB for ethanol production in larger scale cost effectively and in an environmentally sustainable way.

In India, the second generation of bioethanol is mainly confined to few plant species such as *Lantana, Saccharum spontaneum, Eichornia, Parthenium* sp. (Mohan et al. 2016). It is high

time to focus on an exploration of new raw material, which can be a potential candidate for bioethanol production. It can be noted that the production and utilization of feedstock for bioethanol should be derived from inedible parts of food crops or lignocellulosic biomass, in order to overcome the competition between food and fuel (Sakai et al. 2007).

The collection and identification of new raw material in bioethanol production is equally important. The North-East India was selected as the site of collection for LCB. Various plant species are available to this region. The hilly terrains and fallow lands of these regions are lying unused due to difficulties in the cultivation of food crops. However, the grass species could easily grow in such areas. The grasses such as *Saccharum spontaneum*, *Phragmites karka* have been used in the production of bioethanol by many researchers. Our attempt is to explore new feedstock material, easily available to this region with a high percentage of cellulose contain, and can be a potential candidate for biofuel generation. In search of the feedstock, we have collected 29 species **(described in chapter 3)** of which the biomass *Eragrostis airoides* seems to be a very potential candidate in terms of cellulose content, energy content and most importantly it is profoundly found to this region. The documentation in the form of herbarium specimen of such biomass was highly required in other to know the phenological changes occur due to global warming (Abraham et al. 2006). The documentation of the specimen provides the ecological habitat of the sample. That is the main reason why we have made herbarium specimen and submitted to the herbarium unit, which is well recognised by Botanical Survey of India (BSI). A brief morphological characters of the *Eragrostis airoides* is given in **Table 5.4.**

The pre-treatment of lignocellulosic biomass is a crucial step before hydrolysis. The objective of pre-treatment is to decrease the crystalline nature of cellulose thereby enhancing the enzymatic saccharification. The pretreatment was done in other to release the sugars easily. The efficient enzymatic conversion of cellulosic biomass into fermentable sugars depends on the effectiveness of the pretreatment condition. During the pretreatment, the impeding layers of carbohydrate are disrupted and the cellulose portion becomes easily accessible to enzymes (Gary et al. 2011). Numerous pretreatment strategies have been developed to enhance the enzymatic hydrolysis to yield more fermentable sugars. However, only small number of pretreatment methods have been reported as being potentially cost-effective. We have adopted physico-chemical pretreatment of dilute acid and alkali treatment along with autoclaving. The different concentration of acid and alkali was prepared (1%, 3%, 5% and 7% respectively).

Table 5.4. Morphological characters of *Eragrostis airoides*

Morphological characters	***Eragrostis airoides***
Habit	Perennial, Culms robust; 50-100 cm long. Lateral branches lacking.
Inflorescence	Inflorescence a panicle; embraced at base by subtending leaf
Fertile spikelets	Spikelets comprising (1–)2(–3) fertile florets; with a barren rhachilla extension, or with diminished florets at the apex.
Glumes	Glumes deciduous; similar; shorter than spikelet. Lower glume ovate; 1.7 mm long; 0.7–0.8 length of upper glume
Floret	Fertile lemma ovate; 1.1–1.3 mm long; membranous; grey; keeled; 3 -veined.
Flower	Lodicules 2; cuneate; fleshy. Anthers 3; 0.2–0.3 mm long.
Fruit	Lemma apex acute. Apical sterile florets resembling fertile though under developed.
Distribution	South America: Caribbean, Northern South America, Western South America, Brazil, and Southern South America, North-East India.

The parameters for autoclaving were kept constant for all pretreatment conditions. It was found that the most effective pretreatment condition was in 5 % acid+ autoclaving and also the maximum glucose (1.62 g/L) concentration was released.

From the HPLC analysis, it was found that pentose sugars like xylose and arabinose were released during the pretreatment methods (**Fig.5.3.).** This implies that the pretreatment condition adopted also degrading the hemicellulose (pentose sugars) present in the biomass. It was also seen that the TRS yield in enzymatic hydrolysis did not show much rise. This may be due to the consequences of some cellulose undergoing hydrolysis during acid + autoclave pretreatment which limits the TRS released in enzymatic hydrolysis (**Table 5.2.**).

The enzymatic hydrolysis of the grass biomass was carried out by cellulase enzyme (*Trichoderma reesei*). Unlike chemical hydrolysis, enzymatic hydrolysis is highly specific to the substrate, pH and temperature. The hydrolysis was conducted with mild conditions of a pH of 4.7 and temperature at 50 ^{0}C. The enzymatic hydrolysis using *T.reesei* was considered as because the hydrolysis does not corrode the apparatus in spite of taking several days over

the chemical hydrolysis, the enzymes are more specific to the substrate and highly depend on the reaction environment such as pH. Moreover, when the enzyme is saturated, it affects the process unless they are removed subsequently (Duff et al. 1996) which was seen in **Fig. 5.5** and **Fig. 5.6**. However, the use of enzymes in large-scale industry is limited and the process of hydrolysis took around 4 days.

It is well known that the acid pretreatment helps in improving the enzymatic hydrolysis of the cellulosic component; however, it has no effect on lignin composition (Yu et al. 2011). Results on the enzymatic hydrolysis of the pretreated grass (*Eragrostis airoides*) using the enzyme *Trichoderma reesei* shows that the best hydrolysis was obtained with the acid+autoclaving. The 5 % acid+autoclave pretreatment is more superior to other conditions as it produces saccharification yield of 91.3 mg/g and TRS of 40.16 g/L (**Fig. 5.7. and Table 5.2**). The TRS yield depends on the composition and molecular structure of the biomass as well as the condition applied for pretreatment and enzymatic hydrolysis (Cardona et al. 2014). The TRS yield obtained from another type of LCB could not be compared with the TRS obtained in these studies. For instance, a TRS yield of 36.15 mg gdb^{-1} was reported on Kans grass with 1 % biomass loading (Rashmi et al. 2011). A TRS yield of 118.1 mg/g was obtained on rice straw pretreated with 4 % NaOH (Cheng et al. 2010). For the Switchgrass, a TRS yield of 810 mg/g was achieved on pretreatment condition with 2 % NaOH (Zheng et al. 2006).

Enzymatic hydrolysis is a crucial step in the conversion of cellulosic biomass to biofuels. There are several steps involved in the process. The cellulose portion present in the biomass is separated out from the lignin mixture. Then, the cellulose is broken down into respective smaller monomers (sugars) and then these monomeric molecules are utilized further for ethanol production by fermenting microbes such as *Saccharomyces cerevisiae* (Zhang et al. 2004).

It was seen that the rate of enzyme hydrolysis decreases as the time passed (**Table 5.3.**). The rate of the enzyme hydrolysis was found maximum on the first day as compared to the 2nd and 3rd day (**Fig.5.6** and **Fig.5.7)**. This may be due to the formation of inhibitory substances in the hydrolysate as the time increased. The maximum enzymatic action was visible in 5 % acid+autoclaving. This may be due to the maximum alteration of cellulose crystallinity occur by the pretreatment condition which affecting the rate of enzyme hydrolysis (Waleed et al. 2011).

5.4.1 HAMEI-TRADITIONAL STARTER

Hamei is a natural starter, dry, round-to-flattened, solid ball like structure used in the traditional preparation of alcohol (Yu in Manipuri) (**Fig.5.14.)** by the people of Manipur from local rice**.** The Hamei cakes are prepared from crushed raw rice with bark/root of a plant called Yangli (*Albizia myriophylla*) (**Fig.5.12.)**. The preparation steps are given in **(Fig.5.13).** The desired state of fermentation is indicated by swelling of cakes and alcoholic flavour. In most of the cases, the hamei preparation is done during summer and dried cakes are stored yearlong (Jayeram et al. 2009).

Hamei is a consortium of microbiome consists of different species of yeast, molds and bacteria (Jayeram et al. 2009, Tamang et al. 2007). A total of 163 yeast species were isolated from Hamei (Jayeram et al. 2009). The microorganism such as *Molds: Mucor spp, Rhizopus spp, Yeast: Sacchromecyes cerevisiae, Pichia anomala, P. Guilliermondii, P. Fabianii, Trichosporon sp, Candida tropicalis, C. Parapsilosis, C. Montana, Pichia guilliermondi, Candida parapsilosis and Torulaspora delbrueckii, LAB: Pediococcus pentosaceus* and *lactobacillus brevis* (Tamang et al. 2007, Jeyeram et al. 2008) are present in Hamei. The most frequent yeast species associated with 'Hamei' were *S. cerevisiae* (32.5%), *P. anomala* (41.7%) and *Trichosporon sp.* (8%).

The Hamei is not only importance for the fermentation but also an economic importance of the state. People sell the Hamei in the market. A good quality of Hamei increases the production of alcohol. Different types of morphological shapes of the microbes were visible under the microscope. As the microbes were not isolated properly, a mixed organisms having different shapes and size were observed. Some are rounded, flagella, spiral, irregular and rode shape **(Fig. 5.17).** A colony morphology having true mycelia with balistoconidia could be seen in **Fig.5.18 (A).**

Saccharomyces cerevisiae is one of the most effective ethanol-producing microorganisms, which utilized the hexose sugars including glucose, mannose and galactose for ethanol production (Hiba et al. 2014, Cuevas et al. 2010, Lin et al. 2012). The main component of lignocellulosic hydrolysate is glucose (a hexose sugar). In the enzymatic hydrolysis, the cellulose portion is converted to glucose. *Saccharomyces cerevisiae* with high ethanol productivity then used up these glucose molecules to produce ethanol.

Fig.5.12. Raw Yangli (*Albizia myriophylla)* bark

Fig.5.13. Flowchart for Hamei preparation

Fig.5.14. Hamei

5.4.1.1 PHENOTYPIC CHARACTERIZATION

The cell morphology and phenotypic characteristics of Hamei were observed in a fluorescence microscope (ZEISS Axiocam 503, Germany) (**Fig.5.15, 5.16, 5.17, 5.18).**

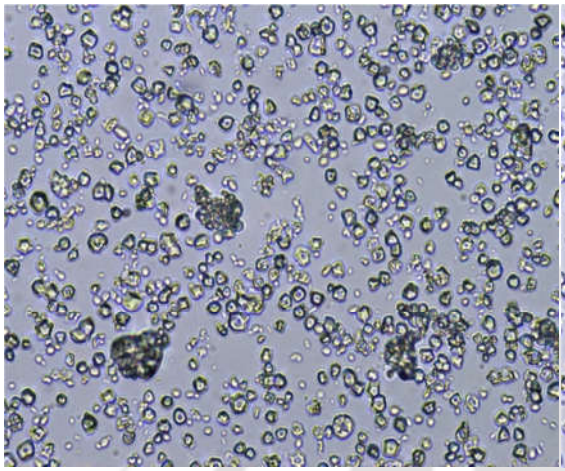


Fig. 5.15. Hamei morphology at 50 x Microscopic view

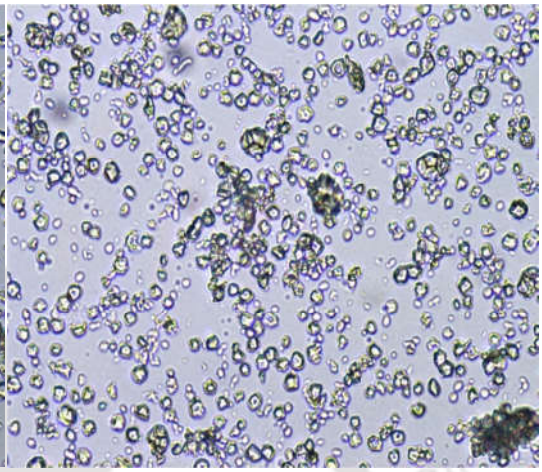


Fig. 5.16. Hamei morphology at 100 x Microscopic view

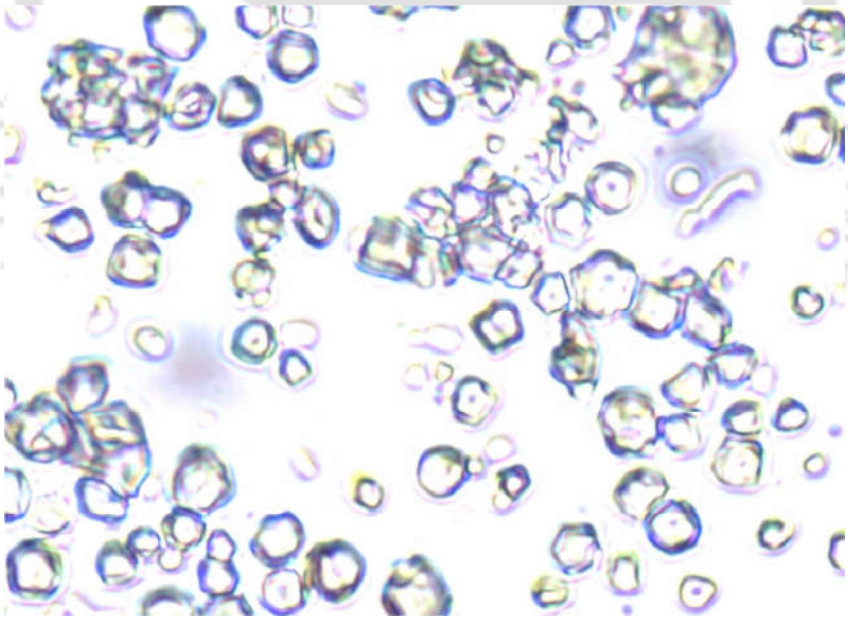


Fig. 5.17. Hamei morphology at 100 x Microscopic view citing branched structure

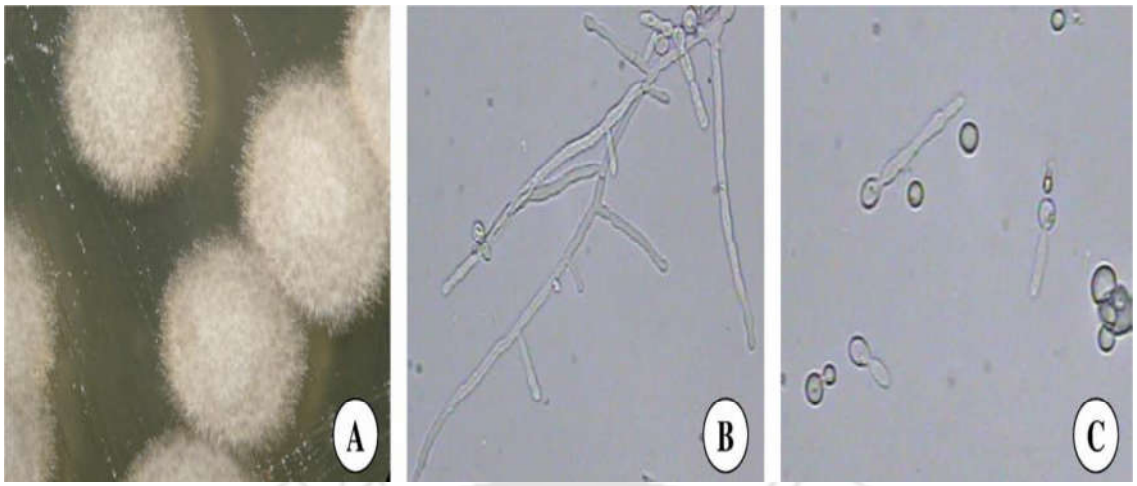


Fig. 5.18. Colony morphology (A), true mycelia with balistoconidia (B) and cells with germ tubes (C) of Trichosporon sp. H24Y9 (Jayeram et al. 2009).

It is reported that *Saccharomyces cerevisiae* is high tolerance to inhibitory compounds present in the hydrolysate of lignocellulosic biomass (Hahn-Hagerdal et al. 2007, Matsushika et al. 2009). However, this strain is unable to utilize the pentose sugars for the production of ethanol through fermentation. Some yeast strains have been developed to ferment xylose into ethanol but the rate and ethanol yield are considerably low as compared to their glucose fermentation (Lin et al. 2012, Matsushika et al. 2009).

5.4.1.2 COMPARATIVE ASSESSMENT OF RESULTS WITH PUBLISHED LITERATURE

Many lignocellulosic biomass such as wheat straw, rice straw, rice husk, sugarcane bagasse, grasses etc. used as feedstock for production of biofuel. However, these raw materials could not meet the demand of ethanol production. Therefore, a new raw material could substantially contribute ethanol production. As it can seen that, the selected grass *Eragrostis airoides* is completely a wild grass and capable of producing bioethanol in a promising way. The extensive comparision of the results of this study with published literature is given in **Table 5.5.** and **Table 5.6.** The method of pretreatment, enzymatic hydrolysis, mode of fermentation, sugar released, and ethanol production thereafter was compared. The biomass *Saccharum spontaneum* with *Saccharomyces cerevisiae* treatment could yield of 19.45 g/L ethanol (**Table 5.5.**). The highest ethanol yield (20 g/l) was obtained in Reed canary grass by yeast strain VTT-B-03339 (Kallioinen et al. 2012). However, our study on *E. airoides* using the *Saccharomyces cerevisiae* could produce ethanol yield of 17.56 g/L. On Hamei treatment,

the ethanol yield is a little bit higher i.e 18.66 g/L. The reason may be due to the presence of different microorganisms, which may directly or indirectly induce in the fermentation process.

Table 5.5 Comparison of separate hydrolysis and fermentation results from various lignocellulosic materials fermented by yeast strain.

Lignocellulose biomass	Organism	Mode of fermentation	Ethanol (g/L)	References
E. airoides	Saccharomyces cerevisiae	SHF	17.56	This study
E. airoides	Local yeast (Hamei)	SHF	18.66	This study
Saccharum spontaneum	Saccharomyces cerevisea VS3	SHF	19.45	(Anuj et al. 2009)
Parthenium hysterophorus	Saccharomyces cerevisea	SHF	10.93	(Shuchi et al. 2015)
Elephant grass	Saccharomyces cerevisiae	SSF	15.10	(Cardona et al. 2014)
Reed canary grass	Yeast strain VTT-B-03339	SHF	20.00	(Kallioinen et al. 2012).

Table 5.6 Comparative analysis of sugar release from pretreatment, enzymatic hydrolysis of lignocellulose biomass and *Eragrostis airoides.*

Lignocellulose biomass	Pretreatment	TRS on P (mg/g)	Enzymes	TRS on EH (mg/g)	Reference
Reed canary grass	2.5 % H_2SO_4 , 150 ^{0} C, 20 min	145.0	Celluclast	201.0	Dien et.al, 2006
Switchgrass	2.5 % H_2SO_4 , 150 ^{0} C, 20 min	267.0	Celluclast	197.0	Dien et.al, 2006
Kans grass	2 % H_2SO_4 , 120 ^{0} C, 1.5 h		Cellulases 20 FPU/g	69.08	Kataria et.al 2011

Elephant grass	water-washed steam-treated, 10 min at 190 ^{0} C	248.34	--	--	Angélica Luisi et. al, 2015
Elephant grass	2 wt.%. NaOH, 120 ^{0}C for 1 h	344.0	---	----	Cardona Eliana et.al 2014
Parthenium hysterophorus	1 % H_2SO_4 , at 121 ^{0} C, 15 psi for 20 min	285.3	Cellulase from Bacillus amyloliquefaciens	187.4	Shuchi et.al 2015
Eragrostis airoides	5 % H_2SO_4 , at 121 ^{0} C, 15 psi for 20 min	254.6	Cellulases 20 FPU/g	91.3	Present study
	3% % H_2SO_4 , at 121 ^{0} C, 15 psi for 20 min	212.3	Cellulases 20 FPU/g	83.5	
	1% % H_2SO_4 , at 121 ^{0} C, 15 psi for 20 min	191.5	Cellulases 20 FPU/g	75.6	

5.5 CONCLUSIONS

The exploitation of new raw materials for second generation of biofuel production is a challenging task. Identification and development of dedicated new lignocellulose feedstocks for bioethanol production is equally important as the process is concerned. However, we are still far from the ideal of high and efficient production of biofuel. It is important to stress here that a single crop is unlikely to attend a wide variety of agronomical and physiological requirements for used in biofuel production. A variety of crops especially lignocellulosic biomass is needed to explore more in other to produce biofuel in a sustainable way. The lignocellulosic biomass such as grasses are envisioned to be the key players in the future to supply the raw material for biofuel due to their productivity under diverse ecological

conditions and because they include both dual-purpose and biomass dedicated crops. Grasses are amongst the most promising plant biomass for biofuel production due to its high productivity resource, and great genetic diversity.

As for bioethanol production, less biomass feedstock has been discovered and utilized. There is need of exploring more suitable and potential candidate for bioethanol production. Therefore, in the present study, effort was done on a new raw material of grass biomass (*Eragrostis airoides)* for cellulosic ethanol production by using the *Saccharomyces cerevisiae* and locally available Hamei collected from Manipur. The enzymatic hydrolysis was carried out with *T. reesei* strain and physico-chemical pretreatment condition of different concentration of acid and alkali. The maximum glucose yield was obtained in the acid + autoclave as compared to alkali + autoclave. Among the pretreatment conditions employed, the maximum ethanol yield (17.56 g/L) was obtained in 5 % acid + autoclave for *Saccharomyces cerevisiae* and for Hamei treatment ethanol yield was 18.66 g/L. The results indicated that high ethanol concentration can be obtained from the 5 % acid + autoclave followed by 3 % acid + autoclave and 1 % acid + autoclave while for alkaline + autoclave pretreatment the ethanol yield was comparatively less. This can be explained by the fact that, pretreatment can affect the cellulosic portion of the lignocellulosic biomass differently and hence the enzymatic hydrolysis. To the best of our knowledge, the grass *Eragrostis airoides* is a viable feedstock for a source of bioethanol production.

Chapter 6

Conclusions and scope for future work

6.1 CONCLUSIONS

The present study demonstrates the exploration of new raw materials belonging to grass family (Poaceae) collected from Kamrup district of Assam and Thoubal district of Manipur of North-East India for its ethanol production. Although grasses are abundantly available to this region, hardly some species have been explored to check for its fuel contained. We hereby collected 29 species of LCB, which are highly indigenous to this region and characterized, based on the fuel criteria. The characterization was performed based on the proximate, ultimate and compositional (carbohydrate/fibre) analysis of the biomass sample. All the collected biomass samples show high energy content. Among the collected biomass species, *Eragrostis airoides* was found to be the most cellulosic biomass sample. This biomass sample was further pretreated physico-chemically and enzymatic hydrolysis was performed. The fermentation of the biomass sample was carried out by using the *Saccharomyces cerevisiae* and Hamei. Further, the performance of both the fermenting organisms was checked and concluded that Hamei treatment exhibit high yield of ethanol than *Saccharomyces cerevisiae.*

6.1.1 COLLECTION AND IDENTIFICATION OF DIFFERENT PLANT SPECIES TO INVESTIGATE THE FEASIBILITY OF PRODUCTION OF BIOETHANOL

The present investigation described the collection and identification of different plant species of Kamrup district of Assam and Thoubal district of Manipur of North-East India for its bioethanol production. 29 species of biomass sample mainly belongs to Poaceae were collected. The collection of plant materials were done based on the abundance and availability. The collected plant materials were clean, pressed, dried, poisoned, and pasted on the herbarium sheet. To ascertain the selected biomass sample, the collection number was assigned according to the field note. The curator of herbarium unit, Botany department, Guwahati University, identified the collected specimens. Two copies of each herbarium specimen were prepared and one copy of each of them was maintained at Center for Energy, Indian Institute of Technology Guwahati, for future reference. The specific voucher number assigned to each herbarium specimen was recorded.

6.1.2 SCREENING, CHARACTERIZATION AND COMPOSITIONAL ANALYSIS OF LIGNOCELLULOSIC BIOMASS

We report the physical, chemical and thermochemical characterization of biomass sample collected from Kamrup district of Assam and Thoubal district of Manipur of North-East India. The collected biomass samples were screened properly and categorized according to the family. Most of the collected species were belongs to Poaceae family. The thermochemical characterization was performed on each sample by proximate, ultimate and compositional analysis. The characterization involves series of high sophisticated instruments like X-ray diffraction, Fourier transform infrared spectroscopy, Thermogravimetric analysis, Fibra plus analyser etc. The moisture content, volatile content, fixed carbon content and ash content were analyzed by using the muffle furnace and followed the NREL protocol. For the heating values, the higher heating values were calculated according to the Demirbas derivative methods. The correlation between the hydrogen, carbon and oxygen atoms were depicted as Van diagrams. The cellulose, hemicellulose and lignin content were determined by Van Soest method. We determined the neutral detergent fibre, acid detergent fibre and acid detergent lignin. The percentage of this component gives about the cellulose, hemicellulose and lignin content of the biomass sample. The highest cellulose content was found in the biomass *Eragrostis airoides.* Based on the fact of characterization of the biomass samples, we used *Eragrostis airoides* as our substrate biomass for bioethanol production.

6.1.3 CONVERSION OF LIGNOCELLULOSIC BIOMASS TO BIOETHANOL

The present study deals with the conversion of lignocellulosic biomass (*Eragrostis airoides)* to bioethanol by microbial fermentation. The enzymatic saccharification and microbial fermentation of locally available *Eragrostis airoides* (L.) Wolf grass was studied. Physico-chemical pretreatment condition of different concentration of acid and alkali were performed in other to determine their effect on the enzymatic hydrolysis and the fermentability of the cellulosic portion of the lignocellulosic biomass. In the screening of the pretreatment methods, the 5 % acid + autoclave yielded the highest concentrations of total reducing sugar (TRS) (25.46 g/L). The pretreated biomass was efficiently hydrolysed by cellulase enzyme *Trichoderma reesei* ATCC 26921 (sigma). Furthermore, the maximum glucose concentration (1.62 g/L) was released in pretreatment condition 5 % acid + autoclave. The fermentation was carried out by using the *Saccharomyces cerevisiae* and Hamei. The conditions of the fermentation in both the treatment were remained constant. The maximum ethanol yield of

(17.56 g/L) *Saccharomyces cerevisiae* treatment was obtained in 5 % acid + autoclave whereas, 18.66 g/L of ethanol was produced Hamei treatment. The result indicated that high ethanol concentration can be obtained from the 5 % acid + autoclave followed by 7 % acid + autoclave, 3 % acid + autoclave and 1 % acid + autoclave while for alkaline + autoclave pretreatment the ethanol yield was comparatively less. This can be explained by the fact that, pretreatment can affect the cellulosic portion of the lignocellulosic biomass differently and hence the enzymatic hydrolysis. To the best of our knowledge, the grass *Eragrostis airoides* is a viable feedstock for a source of bioethanol production.

6.2 SCOPE FOR FUTURE WORK

Lignocellulose is the most abundant biomass available on Earth. It has attracted considerable attention as an alternate feedstock and energy resource because of the large quantities available and its renewable nature. The cellulosic-based biofuel is a potential alternative to the first generation of biofuels. One of the most costly steps in bioconversion process of lignocellulosic biomass is the pretreatment condition and presence of high recalcitrance. Although the lignocellulosic materials are versatile, it is very difficult to have a general pretreatment method. However, efforts are going on to design common pretreatment methods that would be effective against a wide range of cellulosic material.

Our attempt is to explore new raw materials based on the lignocellulosic biomass for bioethanol production collected from Kamrup district of Assam and Thoubal district of Manipur of North-East India. The collected biomass sample shows high potential for bioethanol production. The unexplored areas of these regions with high resources of feedstock could be a better place for exploring new raw materials in near future, which may have the promising candidate for bioethanol production. The possible future prospects of these raw materials and its applications are summarized as below:

1) The biomass samples like *Eragrostis airoides* could be used for the production of bioethanol. This biomass sample has the very short life cycle and high yield of biomass. The biomass itself grows in hilly terrains areas, follow lands and spreading throughout the North-East India. This could be a promising candidate for bioethanol production to best of our knowledge.
2) The biomass may proceed for tissue culture experiment where the gene expression could be checked for particular cellulosic components to enhance its biomass production.

3) The DNA barcoding could be conducted for further molecular identification of the samples.
4) The biotechnological tools may apply in other to produce in mass production of the sample. The genetical modification of the targeted gene for cellulosic production may be a good option.
5) More such new raw materials could be explored from these regions as the region's flora and fauna diversity are huge.
6) The advance techniques may be used in other to produce more ethanol from this biomass. The described pretreatment method can be changed and analysis by different pre-treatment conditions.
7) The specific strains of fermenting microbes may be used by using this biomass as substrates.
8) Process intensification can be conducted on the selected biomass.

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List of Publications

Journals

1. Yengkhom Disco Singh, Utpal Bora, Pinakeswar Mahanta, Physico-chemical characterization and thermal behaviour study of Lignocellulosic biomass obtained from a few wild grasses of Kamrup (Assam) and Thoubal (Manipur) for biofuel production, **International Journal of Advance Engineering and Research Development**, Volume 3, Issue 9, September -2016.
2. Yengkhom Disco Singh, Utpal Bora, Pinakeswar Mahanta, Comprehensive characterization of lignocellulosic biomass through proximate, ultimate and compositional analysis for bioenergy production, **Renewable Energy** 103 (2017) 490-500.
3. Yengkhom Disco Singh, Utpal Bora, Pinakeswar Mahanta, Conversion of Lignocellulosic biomass to bioethanol: An overview with a focus on pretreatment (**under review fuel**).

Conferences

4. Yengkhom Disco Singh, Utpal Bora, Pinakeswar Mahanta, Compositional analysis of lignocellulosic biomass collected from North-East India for biofuel production, International conference on recent advances in bioenergy research, **25-27 Feb, SSS NIRE, Kapurthala, 2016.**
5. Yengkhom Disco Singh, Utpal Bora, Pinakeswar Mahanta, Bio-refinery of indigenous lignocellulosic biomass: a ray to North-east India, Frontier energy research with industry academia partnership **20-21 march**, **IIT Guwahati, 2015.**
6. Yengkhom Disco Singh, Utpal Bora, Pinakeswar Mahanta, Biofuel- A fuel for today, tomorrow from biomass residue, "**Bioengineering 2012" (ISBE2012), IIT Guwahati, India. 2012.**

Sequence submitted to NCBI genebank

7. Yengkhom Disco Singh, Utpal Bora, Neyraudia reynaudiana voucher 17735 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcl) gene, partial cds; chloroplast. **GeneBank (NCBI)** Accession number: KP404599,

8. Yengkhom Disco Singh, Utpal Bora, Thysanolaena latifolia voucher 17738 maturase K (matK) gene, partial cds; chloroplast. **GeneBank (NCBI)** Accession number: KP404600
9. Yengkhom Disco Singh, Utpal Bora, Thysanolaena latifolia voucher 17738 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit Thysanolaena agrostis (rbcl) gene, partial cds; chloroplast. **GeneBank (NCBI)** Accession number: KP404601,
10. Yengkhom Disco Singh, Utpal Bora, Chrysopogon aciculatus voucher 17824 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcl) gene, partial cds; chloroplast. **GeneBank (NCBI)** Accession number: KP404598

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